Immunological Medicine

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/timm20

Anti-aminoacyl tRNA synthetase antibodies showing the discrepancy between enzyme-linked immunosorbent assay and RNA-immunoprecipitation

Tsuneo Sasai, Yuki Ishikawa, Ran Nakashima, Takuya Isayama, Kiminobu Tanizawa, Tomohiro Handa, Mirei Shirakashi, Ryosuke Hiwa, Hideaki Tsuji, Koji Kitagori, Shuji Akizuki, Hajime Yoshifuji, Tsuneyo Mimori & Akio Morinobu

To cite this article: Tsuneo Sasai, Yuki Ishikawa, Ran Nakashima, Takuya Isayama, Kiminobu Tanizawa, Tomohiro Handa, Mirei Shirakashi, Ryosuke Hiwa, Hideaki Tsuji, Koji Kitagori, Shuji Akizuki, Hajime Yoshifuji, Tsuneyo Mimori & Akio Morinobu (2024) Anti-aminoacyl tRNA synthetase antibodies showing the discrepancy between enzyme-linked immunosorbent assay and RNA-immunoprecipitation, Immunological Medicine, 47:3, 166-175, DOI: 10.1080/25785826.2024.2328918

To link to this article: https://doi.org/10.1080/25785826.2024.2328918

IMMUNOLOGICAL MEDICINE
2024, VOL. 47, NO. 3, 166–175
https://doi.org/10.1080/25785826.2024.2328918

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

OPEN ACCESS Check for updates

Anti-aminoacyl tRNA synthetase antibodies showing the discrepancy between enzyme-linked immunosorbent assay and RNA-immunoprecipitation

Tsuneo Sasai[a], Yuki Ishikawa[b], Ran Nakashima[a], Takuya Isayama[c], Kiminobu Tanizawa[d], Tomohiro Handa[e], Mirei Shirakashi[a], Ryosuke Hiwa[a], Hideaki Tsuji[a], Koji Kitagori[a], Shuji Akizuki[a], Hajime Yoshifuji[a], Tsuneyo Mimori[f] and Akio Morinobu[a]

[a]Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan; [b]Laboratory for Statistical and Translational Genetics, Center for Integrative Medical Sciences, RIKEN, Yokohama, Japan; [c]Medical & Biological Laboratories Co., Ltd, Nagoya, Japan; [d]Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [e]Department of Advanced Medicine for Respiratory Failure, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [f]Takeda Clinic for Rheumatic Diseases, Kyoto, Japan

ABSTRACT

Anti-aminoacyl-tRNA synthetase (ARS) antibodies are myositis-specific antibodies associated with anti-synthetase syndrome (ASSD). Some patients are positive for anti-ARS antibodies on enzyme-linked immunosorbent assay (ELISA) but negative on RNA-immunoprecipitation (RNA-IP) (the gold standard method). Whether these patients should be considered truly positive for anti-ARS antibodies remains unclear. Therefore, we investigated the clinical characteristics of these patients and verified the authenticity of their anti-ARS positivity. Patients who were positive for anti-ARS antibodies on ELISA were divided into the non-discrepant (positive on RNA-IP, $n=52$) and discrepant (negative on RNA-IP, $n=8$) groups. Patient clinical characteristics were compared between the groups. For each positive individual, the authenticity of anti-ARS antibody positivity on ELISA was cross-examined using protein-IP and western blotting. All patients in the discrepant group had lung involvement, including five (63%) with interstitial lung disease. The overall survival time was significantly lower in the discrepant group than in the non-discrepant group ($p<0.05$). Validation tests confirmed the presence of anti-ARS antibodies in the sera of the discrepant group but indicated different reactivity from typical anti-ARS antibodies. In conclusion, some anti-ARS antibodies are detected by ELISA but not RNA-IP. Such anti-ARS antibody discrepancies need further elucidation to attain validation of the diagnostic process in ASSD.

ARTICLE HISTORY
Received 7 November 2023
Accepted 6 March 2024

KEYWORDS
Anti-aminoacyl tRNA synthetase antibody; anti-synthetase syndrome; dermatomyositis; interstitial lung disease

1. Introduction

Anti-aminoacyl-tRNA synthetase (ARS) antibodies are myositis-specific autoantibodies and comprise the following six main types: anti-histidyl-tRNA synthetase (Jo-1), threonyl-tRNA synthetase (PL-7), alanyl-tRNA synthetase (PL-12), glycyl-tRNA synthetase (EJ), isoleucyl-tRNA synthetase (OJ), and asparaginyl-tRNA synthetase (KS) antibodies [1]. The corresponding autoantigen is aminoacyl-tRNA synthetase, which catalyzes protein synthesis *in vivo*. Patients with anti-ARS antibodies frequently develop anti-synthetase syndrome (ASSD), which is characterized by myositis, interstitial lung disease (ILD), polyarthritis, Raynaud's phenomenon, fever, and mechanic's hands [2,3]. ILD is the most frequent and often the initial manifestation of ASSD [2,3].

The clinical diagnosis of ASSD is sometimes difficult, given that the clinical characteristics can vary. Therefore, the detection of anti-ARS antibodies is useful for the diagnosis of ASSD. RNA-immunoprecipitation (RNA-IP) is the gold standard method for screening for anti-ARS antibodies [4–6]. However, this IP method requires specialized skills and can only be performed at a limited number of laboratories. Enzyme-linked immunosorbent assay (ELISA) has recently become widely utilized for the detection of anti-ARS antibodies (MBL Co. Ltd., Nagoya, Japan) [7].

Some patients have discrepant anti-ARS antibody positivity: ELISA shows positive findings for anti-ARS antibodies; however, RNA-IP shows negative findings. It is unclear whether these patients should be considered truly positive for anti-ARS antibodies. This study aimed to examine the clinical significance

CONTACT Ran Nakashima ranran@kuhp.kyoto-u.ac.jp
Supplemental data for this article can be accessed online at https://doi.org/10.1080/25785826.2024.2328918.

of discrepant anti-ARS antibody positivity by comparing the clinical characteristics of patients with and without discrepant anti-ARS antibody positivity and verifying the positivity using additional detection methods.

2. Materials and methods

2.1. Patients

In this retrospective study, the medical records of patients who were screened for anti-ARS antibodies by ELISA at Kyoto University Hospital between 2014 and 2017 were reviewed. All samples were obtained from adult Japanese patients to investigate connective tissue diseases or ILD. Patients with anti-ARS antibody positivity on ELISA who did not undergo anti-ARS antibody examination by RNA-IP were excluded from the study. The patients were divided into the discrepant (anti-ARS antibody positivity on ELISA but not RNA-IP) and non-discrepant (anti-ARS antibody positivity on ELISA and RNA-IP) groups (Figure 1). All patients provided written informed consent to participate in this study before sample collection. This study was conducted under the Declaration of Helsinki and approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (approval number: R1540).

The clinical features of ASSD and the comorbidity rates of malignancy between the discrepant and non-discrepant groups were compared. Lung disease characteristics (radiological patterns of ILD, responsiveness to initial treatment for ILD, recurrence rates of ILD, and patient prognosis) were also compared between the groups.

Patients who were not treated for lung disease were excluded from the evaluation of lung disease prognosis. The clinical course of the lung disease was evaluated in patients who underwent treatment for lung disease. The overall survival time and progression-free time of the patients were compared between the groups. Overall survival time was defined as the time from ILD diagnosis until death or final follow-up. Progression-free time was defined as the time from treatment initiation until disease progression, recurrence, or final follow-up. Progression or recurrence was defined as the exacerbation of radiological findings of ILD leading to augmentation or re-institution of treatment.

2.2. ELISA

Anti-ARS antibodies were measured using an ELISA kit (MESACUP Anti-ARS test, MBL Co. Ltd., Nagoya, Japan), thereby enabling the simultaneous evaluation of anti-ARS, anti-Jo-1, anti-PL-7, anti-PL-12, anti-KS, and anti-EJ antibodies. Anti-OJ antibodies were not detected using this assay. Five recombinant ARS antigens (GST-Jo-1, His-PL-12, His-EJ, GST-KS, and His-PL-7) were fixed as a solid phase in the ELISA. GST-Jo-1, His-PL-12, His-EJ, and GST-KS were expressed in *Escherichia coli*, and His-PL-7 was expressed in Hi-5 cells. A value of >25

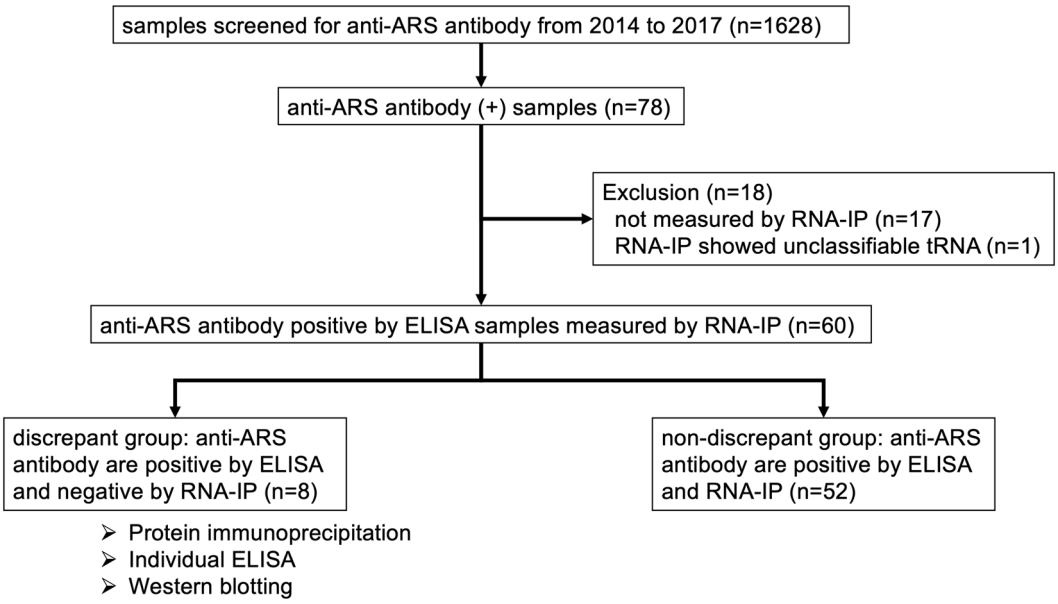

Figure 1. Patient flowchart. A total of 1,628 samples collected between 2014 and 2017 at Kyoto University Hospital were analyzed using an enzyme-linked immunosorbent assay (ELISA). Among the 78 samples that were positive for anti-aminoacyl-tRNA synthetase (ARS) antibodies, 60 with RNA-immunoprecipitation (RNA-IP) results were included. Eight patients showed positive findings on ELISA but negative findings on RNA-IP. The positivity of anti-ARS antibody in the discrepant group was validated by protein-IP, individual ELISA, and Western blotting.

was defined as positive according to manufacturer instructions. The kit did not provide information about the presence of each antibody [7].

2.3. ELISA detection of specific anti-ARS antibodies

ELISA was used to detect each ARS antibody separately. The antigen was purified recombinant ARS coated onto the wells of 96-well Maxisorp microtiter plates (Nalge Nunc International, Rochester, NY, USA). The PL-12, EJ, PL-7, KS, and Jo-1 antigens were diluted in phosphate-buffered saline (PBS) to a final concentration of 5 µL/mL, and 100-µL samples were incubated overnight at 4 °C. The plates were washed twice with PBS, and non-specific binding was blocked *via* an overnight incubation with PBS, 1% bovine serum albumin (BSA), and 5% sucrose at 4 °C. Sera from patients and healthy donors were diluted to 1:100 in PBS containing 0.15% Tween 20 (PBS-T), 1% casein enzymatic hydrolysate, and 0.2 mg/mL *E. coli* extract, and 100-µL samples were added to each well. After incubation for 30 min at room temperature (RT, 20–25 °C), the wells were washed with PBS-T four times. Subsequently, 100 µL of peroxidase-conjugated goat anti-human IgG (Code No. 208, MBL), diluted 1:5,000 in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxyphenylacetic acid (peroxidase stabilizer), was added to each well. The samples were incubated for 30 min at RT and then washed four times with PBS-T. Subsequently, the samples were incubated for 15 min at RT with 3, 3″, 5, 5″-tetramethylbenzidine substrate. The reaction was stopped using 100 µL of 0.25 N sulfuric acid before the absorbance was read at 450 nm. The absorbance of the healthy control serum samples was used as a reference, and the highest absorbance of the antigen reactions was used to identify the anti-ARS antibody. When the optical density value was greater than the mean plus three standard deviations of the ten healthy control samples, the sample was considered positive. The serum autoantibody was verified by comparing the absorption to the corresponding antigens.

2.4. Immunoprecipitation

The presence of anti-ARS antibodies was determined by RNA-IP, as described previously [8]. The immunoprecipitated RNA was resolved using urea-polyacrylamide gel electrophoresis and visualized using silver staining. Each anti-ARS antibody was identified based on its mobility and tRNA pattern compared with a standard serum. Protein-IP was performed using extracted protein from HeLa cells labeled with 35S methionine, as described previously [9,10]. Radiolabeled polypeptide components were analyzed using autoradiography.

2.5. Western blotting

Purified recombinant ARS antigens were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane with minor modifications to the previously described method [11]. After blocking with 5% skim milk, the membrane was incubated for 60 min with serum (diluted to 1:100) and for an additional 60 min with a 1:10,000 dilution of goat anti-human IgG conjugated to peroxidase. Immunoreactive bands were detected using the V3 Western Workflow (Bio Rad Laboratories, Inc. Hercules, CA, USA).

2.6. Radiological evaluation of the lungs

All high-resolution computed tomography (HRCT) scans obtained within 6 months of blood sampling were reviewed by three observers (T.K., T.H., and K.T. with 22, 19, and 17 years of experience, respectively) who were blinded to the patient's clinical information. The HRCT pattern was categorized as usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP), organizing pneumonia (OP), NSIP with OP overlap, lymphocytic interstitial pneumonia, pleuroparenchymal fibroelastosis (PPFE), or unclassifiable [12–14]. Interobserver disagreements were resolved by consensus.

2.7. Statistical analysis

Fisher's exact test was used to compare the frequency of clinical features between the groups. Overall survival time and progression-free time were estimated using the Kaplan–Meier method and compared using the log-rank test. Data were censored on August 30, 2018. Patients who were lost to follow-up were censored at the date of last contact/follow-up. Patients who were alive on August 30, 2018, were censored for overall survival time. All statistical analyses were performed using R version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Patient characteristics

Among the 1,628 samples that were screened for anti-ARS antibodies using ELISA, 78 were positive. After excluding 17 samples not measured by RNA-IP,

61 samples (61 patients) were included in the study. However, one sample whose tRNA precipitated was excluded, as this result had not been reported previously. Among the 60 samples included in the final analysis, RNA-IP was negative for 8 (discrepant group) and positive for 52 (non-discrepant group) (Figure 2). The patients in the discrepant group did not have immunologic abnormalities such as immunodeficiency, infection with type-C hepatitis, or the requirement to be treated with immunosuppressants at the time of collecting their sera.

The proportion of male patients was significantly higher in the discrepant group than in the non-discrepant group (63% vs. 19%, $p < 0.05$) (Table 1). Patients in the discrepant group were less likely to have a dermatomyositis-specific rash (13% vs. 56%, $p = 0.052$), polyarthritis/polyarthralgia (13% vs. 56%, $p = 0.052$), mechanic's hands (0% vs. 44%, $p < 0.05$), and myositis (0% vs. 48%, $p < 0.05$) than patients in the non-discrepant group. All patients in the discrepant group had lung involvement, including 63% with ILD (Table 2 and Figure 3). Lung lesions in patients without ILD primarily consisted of bronchial lesions, and patient 9 had been treated with glucocorticoids for coexisting ground-glass opacity and consolidation. The frequency of malignancy within 3 years of anti-ARS antibody detection was not significantly different between the groups. The antinuclear antibody patterns, anti-SS-A antibody, Krebs von den Lungen 6, and the onset of ILD were not significantly different between the groups. More patients in the non-discrepant group were treated with immunosuppressive treatments, including glucocorticoids (GCs). The observational period was significantly shorter in the discrepant group than in the non-discrepant group ($p < 0.01$).

3.2. Characteristics of lung involvement in the discrepant group

The OP pattern was observed in two patients (25%), the NSIP with OP pattern in one patient (13%), the UIP pattern in one patient (13%), and the PPFE pattern in one patient (13%) (Table 2). Three patients had lung findings other than interstitial pneumonia and were not classified as any type of ILD (Figure 3).

3.3. Treatment and outcome

Among three patients in the discrepant group who were treated with GCs, two relapsed after a positive initial response. Three patients (38%) in the discrepant group died, including one who died of respiratory failure due to an exacerbation of unclassifiable ILD (Table 2).

The overall survival time of patients treated for lung disease was significantly lower in the discrepant group ($p < 0.05$) (Figure 4A). Among all patients, the overall survival time was lower in the discrepant group than in the non-discrepant group ($p < 0.001$) (Figure 5). The progression-free time was not significantly different between the groups (Figure 4B).

The antibody-absorption test was used to verify the presence of one or several anti-ARS antibodies in seven samples in the discrepant group (Table 3). Six of the eight samples were negative for antinuclear antibody (ANA), and one patient showed high titers of ANA that were positive for the anti-centromere antibody. Five samples in the discrepant group were verified using protein-IP (Figure 6A). However, two of the five samples were positive for a larger variety of anti-ARS antibodies than those detected using ELISA. Moreover, the precipitated peptide bands of all the discrepant group's samples were much thinner than those of the standard

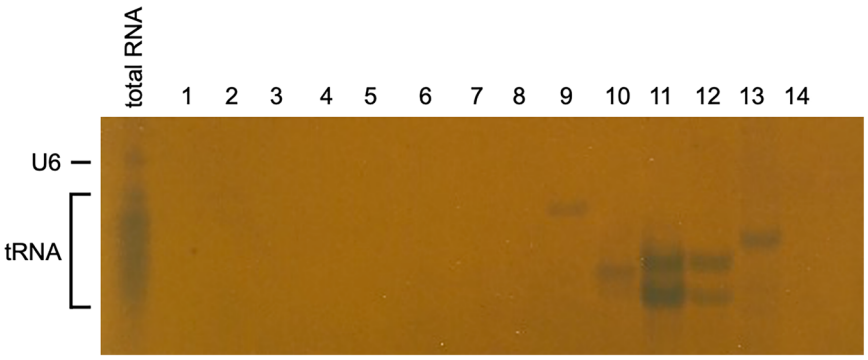

Figure 2. RNA-immunoprecipitation. No anti-aminoacyl-tRNA synthetase antibodies were detected in lanes 1–8 (lanes 1–8 correspond to patients 1–8, respectively) of HeLa cell extracts using RNA-immunoprecipitation. Anti-Jo-1, anti-PL-7, anti-PL-12, anti-EJ, and anti-KS antibodies are shown as positive controls in lanes 9–13, respectively. Lane 14 included serum from a healthy control patient (negative control). Lane T shows the electrophoresis of total RNA. Abbreviations: Jo-1, histidyl-tRNA synthetase; PL-7, threonyl-tRNA synthetase; PL-12, alanyl-tRNA synthetase; EJ, glycyl-tRNA synthetase; KS, asparaginyl-tRNA synthetase.

Table 1. Patient characteristics.

Subjects	Discrepant group (*N* = 8)		Non-discrepant group (*N* = 52)		p
Age (years)	64	± 11	58	± 13	0.23
Male sex	5	(63)	10	(19)	<0.05
Smoking history	4	(50)	22	(42)	0.72
Fever	0	(0)	18	(35)	0.091
Dermatomyositis-specific rash	1	(13)	29	(56)	0.052
Polyarthritis/polyarthralgia	1	(13)	29	(56)	0.052
Raynaud's phenomenon	3	(38)	18	(35)	1
Mechanic's hands	0	(0)	23	(44)	<0.05
Myositis	0	(0)	25	(48)	<0.05
Lung involvement	8	(100)	51	(98)	1
Interstitial lung disease	5	(63)	51	(98)	<0.01
Malignancy	2	(25)	7	(13)	0.59
KL-6, units/mL	1022.1±911.4		1135.2±1072.6		0.689
Antinuclear antibody					
<40 titers	5	(63)	24	(46)	0.47
40 titers	2	(25)	14	(27)	1
80 titers	0	(0)	7	(13)	0.58
160 titers	0	(0)	1	(2)	1
320 titers	0	(0)	4	(8)	1
>320 titers	1	(13)	2	(4)	0.35
Anti-SS-A	0	(0)	18	(35)	0.091
Anti-ARS test (ELISA)	50.3±19.3		155.7±40.8		<0.001
ILD onset					
Acute or subacute	1	(13)	13	(25)	0.67
Chronic or undetectable	7	(88)	39	(75)	0.67
Initial therapy					
Only PSL	2	(25)	20	(38)	0.70
PSL + Tac	0	(0)	16	(31)	0.095
PSL + CyA	1	(13)	5	(10)	1
PSL + AZA	0	(0)	1	(2)	1
PSL + Tac + IVCY	0	(0)	3	(6)	1
None	5	(63)	7	(13)	<0.01
Observation period (days)	458±522		2475±2110		<0.01

Data are provided as mean ± standard deviation or number (frequency). The results of the anti-ARS test indicate the titer of the index.

Acute onset, within 1 month; Subacute onset, 1–3 months; Chronic onset, >3 months.

p-values were generated using Fisher's exact test or the Mann–Whitney U test.

Abbreviations: KL-6, Krebs von den Lungen 6; ELISA, enzyme-linked immunosorbent assay; ILD, interstitial lung disease; ARS, aminoacyl tRNA synthetase; PSL, prednisolone; Tac, tacrolimus; CyA, cyclosporin A; AZA, azathioprine; IVCY, intravenous cyclophosphamide.

Table 2. Lung involvement in the discrepant group.

Patient	ILD	Radiological pattern	Response	Recurrence	Outcome (cause)
1	+	PPFE	Untreated	–	Alive (Hospital transfer)
2	+	OP	Untreated	–	Alive (Hospital transfer)
3	–	Unclassifiable	Untreated	–	Alive (Hospital transfer)
4	–	Unclassifiable	Untreated	–	Dead (pneumonia)
5	+	NSIP with OP	+	+	Alive
6	+	UIP	Untreated	–	Dead (lung cancer)
7	+	OP	+	–	Alive
8	–	Unclassifiable	+	+	Dead (respiratory failure)

Unclassifiable refers to dominant findings other than interstitial pneumonia that cannot be classified as a type of ILD. Abbreviations: ILD, interstitial lung disease; NSIP, non-specific interstitial pneumonia; OP, organizing pneumonia; PPFE, pleuroparenchymal fibroelastosis; UIP, usual interstitial pneumonia.

serum, suggesting that the antibodies in the former had a weak affinity for the ARS antigens or did not react with the original ARS proteins produced by eukaryotic cells. One sample with multiple precipitants on protein-IP reacted with all the recombinant ARS antigens on western blotting. Another sample reacted with PL-12, consistent with the ELISA results, and one sample reacted with Jo-1, which was partially compatible with the ELISA results (Figure 6B).

4. Discussion

In this study, patients in the discrepant group were less likely to have classical features of ASSD other than lung involvement than those in the non-discrepant group. Various radiological patterns of lung involvement were observed in the discrepant group. Patients with lung involvement in the discrepant group had a favorable initial response to GC treatment, though the recurrence rates were high, which is consistent with the recurrent rates of patients with ASSD [15–17]. The overall survival time was lower in the discrepant group than in the non-discrepant group, possibly due to the lower frequency of immunosuppressive treatments or different treatment regimens used in this study.

In some patients, anti-ARS antibodies are detected using ELISA but not using RNA-IP. A previous study reported the sensitivity and specificity of the anti-ARS-detecting ELISA system as 97.1% and 99.8%, respectively, and the false positive rate as less than 0.2% [7]. However, the false positive rate of ELISA was 13.3% (8/60) in our study when the RNA-IP results were used as the gold standard. The high false positive rate in this study may be due to the differences in patient characteristics between the two studies, as patients with connective tissue diseases, particularly myositis, were more frequent than in the previously reported study [7]. However, anti-ARS antibody screening is conducted more frequently in patients with various types of ILD than in patients with suspected myositis in the clinic. Therefore, the rate of discrepancy may depend on the patient population.

The presence of anti-ARS antibodies is important for the diagnosis of ASSD. RNA-IP is the gold standard for detecting anti-ARS antibodies, although various commercial assay systems have been developed and are available for use in daily practice or research. Tansley et al. surveyed members of the International Myositis Assessment and Clinical Studies group and found that ELISA was the most popular method for detecting myositis-specific-autoantibodies (MSAs) and was used by 46% of the 111 respondents from institutions in the USA/Canada, Europe, or Asia [18]. In addition, commercial line blot assays were used more frequently than IP. Respondents cited technical difficulties, labor

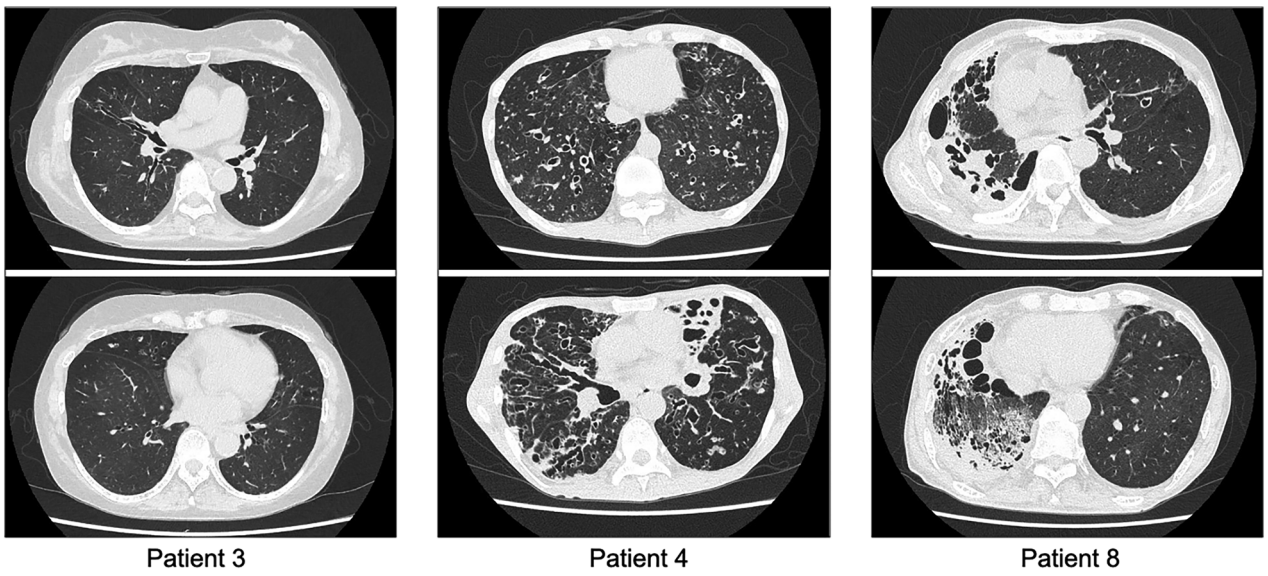

Figure 3. Representative image of lung disease. Three patients in the discrepant group had lung disease that was not classified as any type of interstitial lung disease. Lung lesion in patient 3 was primarily an airway lesion comprising the thickness of the bronchial wall and intrabronchial fluid. Lung lesion in patient 4 was primarily bronchiectasis. Lung lesion in patient 8 had ground-glass opacity with consolidation in the right lung, which appeared on the background of bronchiectasis and lung cysts.

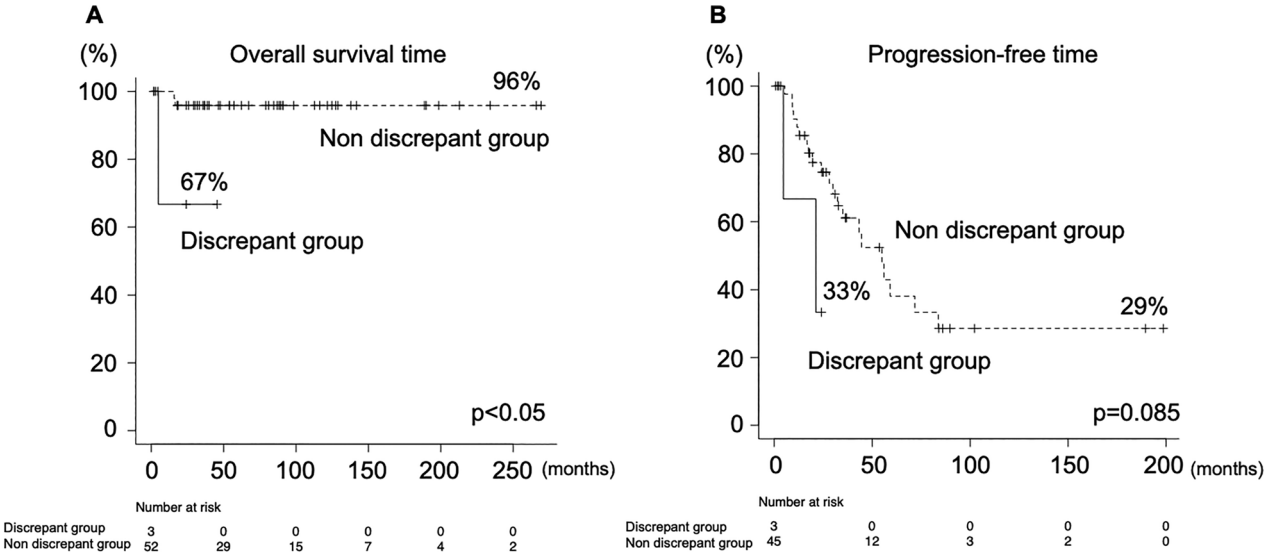

Figure 4. Kaplan–Meier analysis for overall survival time and progression-free time. (A) The overall survival time was significantly higher in the non-discrepant group than in the discrepant group (96% vs. 67%, $p < 0.05$). (B) The progression-free time was not significantly different between the groups (discrepant group: 33%, non-discrepant group: 29%; $p = 0.085$).

intensity, and time consumption as reasons to seek alternatives to IP. The reliability of various MSA detection systems remains unclear. Several studies have compared the reliability of line blot, ELISA, and particle-based multi-analyte technology (PMAT) with that of IP [19,20]. One study reported a false positive rate of 13.7% when the line blot method was used [18]. The performances of protein-IP and line blot assays were compared in another study, which reported a 3.7% false positive rate using PMAT and a 13.0% false positive rate using line blots [21]. Patients with false positive results were diagnosed with overlap syndrome, polymyositis, or dermatomyositis. However, little information regarding the frequency of each ASSD symptom or patient prognosis was provided in previous studies [21]. In a study comparing ELISA and RNA-IP, four patients (9%) had discrepant results: two patients with polymyositis, one patient with mixed connective tissue disease, and one patient with immune-mediated necrotizing myopathy [22]. This report mentioned mainly clinical phenotypes, and all four patients had ILD and myositis; none of them had fever, and two had mechanic's hands, arthritis, and Raynaud's phenomenon. The frequencies of ASSD symptoms in the aforementioned report are consistent with those

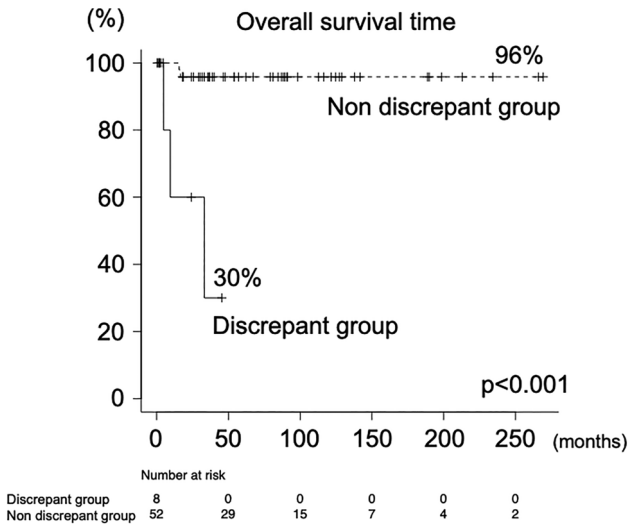

Figure 5. Kaplan–Meier analysis for overall survival time. The overall survival time was significantly lower among patients in the discrepant group who did not undergo treatment for lung involvement than among patients in the non-discrepant group (30% vs. 96%, $p < 0.001$).

Table 3. Antibody detection.

Patient	ANA	IgG (mg/dL)	ELISA	RNA-IP	Protein-IP	Western blotting	Individual ELISA	Absorbed by
1	1:1280 (Di/Sp)	N/A	59.6	–	Jo-1, EJ, KS, PL-7, PL-12	Jo-1, PL-7, PL-12, EJ	Jo-1, EJ	EJ
2	1:40 (Di)	775	73.3	–	Jo-1, EJ, KS, PL-12	–	Jo-1, EJ	Jo-1, EJ
3	1:<40	850	32.6	–	–	PL-12	PL-12	PL-12
4	1:<40	3158	29.5	–	–	–	PL-12, EJ, KS	PL-12
5	1:<40	710	58.5	–	PL-7	Jo-1	Jo-1, PL-7	PL-7
6	1:<40	N/A	29	–	–	–	PL-7	PL-7
7	1:<40	N/A	76.2	–	KS	–	KS	KS
8	40 (Ho/Sp)	N/A	43.4	–	PL-12	–	PL-12	–

Abbreviations: ANA, antinuclear antibody; IgG, immunoglobulin; ELISA, enzyme-linked immunosorbent assay; IP, immunoprecipitation; Di, discrete speckled; Sp, speckled; Ho, homogeneous; N/A, not available; Jo-1, histidyl-tRNA synthetase; PL-7, threonyl-tRNA synthetase; PL-12, alanyl-tRNA synthetase; EJ, glycyl-tRNA synthetase; KS, asparaginyl-tRNA synthetase.

of patients with ASSD detected using RNA-IP. In the current study, the clinical features of ASSD were different between the discrepant and non-discrepant groups. The frequency of mechanic's hands, myositis, fever, arthritis/arthralgia, and rash was lower in the discrepant group than in the non-discrepant group. In contrast, the frequency of Raynaud's phenomenon and lung involvement was not different between the two groups in this study. Therefore, the phenotype of patients in the discrepant group differed from the traditional ASSD phenotype, though these patients were likely to have lung involvement.

The radiological patterns of lung involvement in the discrepant group revealed airway lesions, NSIP, OP, and unclassifiable patterns. The most common radiological pattern of ASSD is NSIP, and the UIP, OP, and diffuse alveolar damage patterns are observed in less than 20% of patients with ASSD [23–25]. NSIP and NSIP with OP are characteristic HRCT patterns in patients with anti-ARS antibodies [26,27]. Although the lung lesions observed in the discrepant group in this study differed from those associated with classical ASSD, the ILD pattern in the discrepant group was similar to that of patients with classical ASSD.

Previous studies have suggested that patients with anti-ARS antibodies and ILD have a more favorable response to initial immunosuppressive treatments and a higher recurrence rate than patients with myositis and ILD who do not have anti-ARS antibodies [15,26,28]. In the current study, the response to immunosuppressive treatments was not comparable between the discrepant and non-discrepant groups due to the differences between the rates and intensity of treatments. However, all three patients in the discrepant group who received immunosuppressive treatment initially responded well, while two patients had recurrence during tapering, which is similar to the clinical course of ILD in patients with ASSD. The overall survival time was significantly lower in the discrepant group than in the non-discrepant group, which may also be due to the different frequency and intensity of immunosuppressive treatments in the two groups. Although statistical analysis showed a significant difference, the number of treated patients in the discrepant group was significantly small. We need more cases to clearly determine whether the clinical course and prognosis of the discrepant group are similar to those of the non-discrepant group.

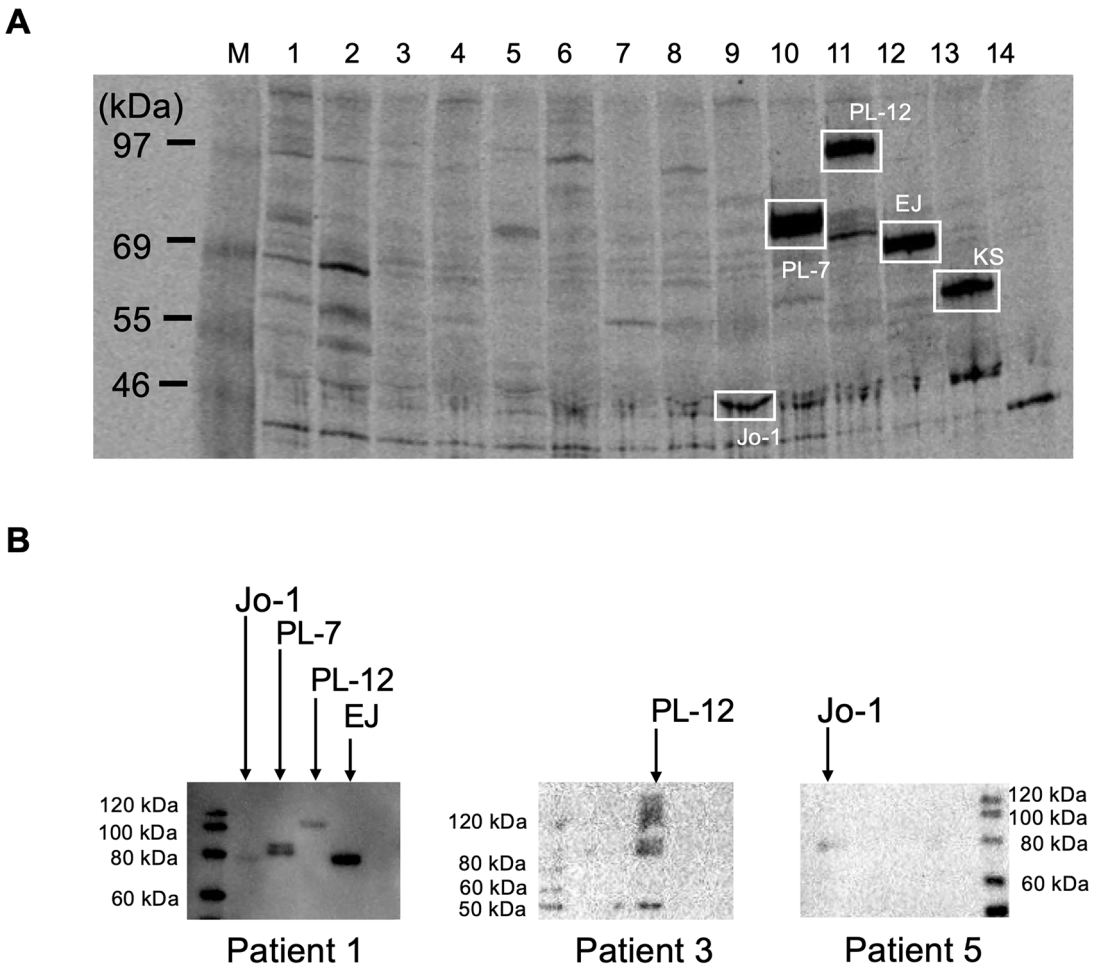

Figure 6. Protein-immunoprecipitation and western blotting. (A) Protein-immunoprecipitation. Using ^{35}S methionine-labeled HeLa cell extracts, autoantigens were precipitated. Lanes 1–8 correspond to patients 1–8, respectively. Lane 1 shows bands for Jo-1, EJ, KS, PL-7, and PL-12 antigens. Lane 2 shows the bands for Jo-1, EJ, KS, and PL-12 antigens. Lane 5 shows the bands for PL-7 antigen. Lane 7 shows the bands for KS antigen. Lane 8 shows the bands of PL-12 antigen. Anti-Jo-1, anti-PL-7, anti-PL-12, anti-EJ, and anti-KS antibodies are shown in lanes 9–13, respectively, as positive controls. Serum from a healthy control patient (negative control) is shown in lane 14. (B) Western blotting. Patient 1 shows the Jo-1, PL-7, PL-12, and EJ antigens. Patient 3 shows the PL-12 antigen. Patient 5 shows the Jo-1 antigen. Samples from the remaining patients did not react with any tested antigens. Abbreviations: Jo-1, histidyl-tRNA synthetase; EJ, glycyl-tRNA synthetase; KS, asparaginyl-tRNA synthetase; PL-7, threonyl-tRNA synthetase; PL-12, alanyl-tRNA synthetase; M, molecular weight marker.

Although the anti-ARS antibody titers were significantly lower in the discrepant group in this study, anti-ARS positivity in the discrepant group was confirmed using additional detection systems, suggesting that these results were not false positives. Patients who were positive for ANA sometimes have disease-specific autoantibodies [29]; however, most patients in the discrepant group were negative for ANA, and the possibility was low that these patients had specific autoantibodies other than anti-ARS antibodies. The discrepancy in the anti-ARS antibody results may be due to the antibody blocking the RNA binding site of ARS or the antibody reacting with denatured ARS (Figure S1). Considering the result in Table 3, serum from patients 1, 2, 5, 7, and 8 in the discrepant group weakly immunoprecipitated ARS antigens on protein-IP. These samples may have contained antibodies that blocked RNA from binding to ARS. Thin bands on protein-IP result from weak affinity of the ARS antigens or low titers of antibodies. In contrast, the other samples in the discrepant group did not immunoprecipitated ARS antigens on protein-IP. Autoantibodies such as anti-centromere antibody or anti-β2-Glycoprotein I antibody were reported to react with cryptic epitopes hidden in the three-dimensional structures [30,31]. Some samples in the discrepant group may have reacted with cryptic epitope due to the recombinant protein derived from E.coli or heat denaturation. Specifically, samples from patient 3 reacted with some ARS antigens on western blotting but not on protein-IP; these antibodies may have reacted with thermally denatured ARS. The sera of patients 4 and 6 reacted on ELISA only, which suggested those antibodies reacted with non-thermally denatured ARS.

This study has some limitations. First, it was a retrospective study, and the target population screened for anti-ARS antibodies using ELISA included a high proportion of patients with ILD. Second, the patient population was small. Third, the treatment and management of the patients were not standardized, resulting in differences in the frequency and intensity of immunosuppressive treatments. These differences may limit the accuracy of comparing the outcomes and clinical courses between the groups.

In conclusion, in some patients, anti-ARS antibodies are detected using ELISA but not RNA-IP. These discrepant findings are not due to false positives but may be due to antibodies blocking RNA-ARS binding or antibodies binding to denatured ARS. Patients with discrepant results may exhibit some ASSD characteristics, including lung involvement that can be treated with immunosuppressive medications. Future studies in larger patient populations should determine whether such patients should be diagnosed with ASSD.

Acknowledgments

We are grateful to Ms. Sze Ming Law for her technical assistance with immunoprecipitation using HeLa cells.

Disclosure statement

Ran Nakashima has received research grants from Takeda Pharmaceutical, collaboration fees from Medical & Biological Laboratories, and speaker fees from Asahi Kasei Pharma, Nihon Pharmaceutical, Boehringer Ingelheim, and Japan Blood Products Organization. Takuya Isayama is an employee of Medical & Biological Laboratories, Co., Ltd. Tomohiro Handa and Kiminobu Tanizawa have received research grants from FUJIFILM Corporation. Tomohiro Handa is an employee of the Collaborative Research Laboratory, which is funded by Teijin Pharma Co., Ltd.

Funding

This work was supported by Takeda Science Foundation.

Data availability statement

The data underlying this article cannot be shared publicly to protect the privacy of individuals who participated in this study. The data will be shared upon reasonable request to the corresponding author.

References

[1] Lundberg IE, Fujimoto M, Vencovsky J, et al. Idiopathic inflammatory myopathies. Nat Rev Dis Primers. 2021;7(1):86. doi: 10.1038/s41572-021-00321-x.

[2] Cavagna L, Trallero-Araguas E, Meloni F, et al. Influence of antisynthetase antibodies specificities on antisynthetase syndrome clinical spectrum time course. J Clin Med. 2019;8(11):2013. doi: 10.3390/jcm8112013.

[3] Lilleker JB, Vencovsky J, Wang G, et al. The EuroMyositis registry: an international collaborative tool to facilitate myositis research. Ann Rheum Dis. 2018;77(1):30–39. doi: 10.1136/annrheumdis-2017-211868.

[4] Mimori T. Autoantibodies in connective tissue diseases: clinical significance and analysis of target autoantigens. Intern Med. 1999;38(7):523–532. doi: 10.2169/internalmedicine.38.523.

[5] Love LA, Leff RL, Fraser DD, et al. A new approach to the classification of idiopathic inflammatory myopathy: myositis-specific autoantibodies define useful homogeneous patient groups. Medicine (Baltimore). 1991;70(6):360–374. doi: 10.1097/00005792-199111000-00002.

[6] Suzuki S, Yonekawa T, Kuwana M, et al. Clinical and histological findings associated with autoantibodies detected by RNA immunoprecipitation in inflammatory myopathies. J Neuroimmunol. 2014;274(1–2):202–208. doi: 10.1016/j.jneuroim.2014.07.006.

[7] Nakashima R, Imura Y, Hosono Y, et al. The multicenter study of a new assay for simultaneous detection of multiple anti-aminoacyl-tRNA synthetases in myositis and interstitial pneumonia. PLoS One. 2014;9(1):e85062. doi: 10.1371/journal.pone.0085062.

[8] Sato T, Fujii T, Yokoyama T, et al. Anti-U1 RNP antibodies in cerebrospinal fluid are associated with Central neuropsychiatric manifestations in systemic lupus erythematosus and mixed connective tissue disease. Arthritis Rheum. 2010;62(12):3730–3740. doi: 10.1002/art.27700.

[9] Nakashima R, Imura Y, Kobayashi S, et al. The RIG-I-like receptor IFIH1/MDA5 is a dermatomyositis-specific autoantigen identified by the anti-CADM-140 antibody. Rheumatology (Oxford). 2010;49(3):433–440. doi: 10.1093/rheumatology/kep375.

[10] Mimori T, Hardin JA, Steitz JA. Characterization of the DNA-binding protein antigen Ku recognized by autoantibodies from patients with rheumatic disorders. J Biol Chem. 1986;261(5):2274–2278. doi: 10.1016/S0021-9258(17)35929-X.

[11] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA. 1979;76(9):4350–4354. doi: 10.1073/pnas.76.9.4350.

[12] Raghu G. Idiopathic pulmonary fibrosis: guidelines for diagnosis and clinical management have advanced from consensus-based in 2000 to evidence-based in 2011. Eur Respir J. 2011;37(4):743–746. doi: 10.1183/09031936.00017711.

[13] Fischer A, Antoniou KM, Brown KK, et al. An official European Respiratory Society/American Thoracic Society research statement: interstitial pneumonia with autoimmune features. Eur Respir J. 2015;46(4):976–987. doi: 10.1183/13993003.00150-2015.

[14] Reddy TL, Tominaga M, Hansell DM, et al. Pleuroparenchymal fibroelastosis: a spectrum of histopathological and imaging phenotypes. Eur Respir J. 2012;40(2):377–385. doi: 10.1183/09031936.00165111.

[15] Yoshifuji H, Fujii T, Kobayashi S, et al. Anti-aminoacyl-tRNA synthetase antibodies in clinical course prediction of interstitial lung disease complicated with idiopathic inflammatory myopathies. Autoimmunity. 2006;39(3):233–241. doi: 10.1080/08916930600622884.

[16] Hozumi H, Fujisawa T, Nakashima R, et al. Efficacy of glucocorticoids and calcineurin inhibitors for anti-aminoacyl-tRNA synthetase antibody-positive polymyositis/dermatomyositis-associated interstitial lung disease: a propensity score-matched analysis. J Rheumatol. 2019;46(5):509–517. doi: 10.3899/jrheum.180778.

[17] Hozumi H, Enomoto N, Kono M, et al. Prognostic significance of anti-aminoacyl-tRNA synthetase antibodies in polymyositis/dermatomyositis-associated interstitial lung disease: a retrospective case control study. PLoS One. 2015;10(3):e0120313. doi: 10.1371/journal.pone.0120313.

[18] Tansley SL, Snowball J, Pauling JD, et al. The promise, perceptions, and pitfalls of immunoassays for autoantibody testing in myositis. Arthritis Res Ther. 2020;22(1):117. doi: 10.1186/s13075-020-02210-2.

[19] Tansley SL, Li D, Betteridge ZE, et al. The reliability of immunoassays to detect autoantibodies in patients with myositis is dependent on autoantibody specificity. Rheumatology (Oxford). 2020;59(8):2109–2114. doi: 10.1093/rheumatology/keaa021.

[20] Cavazzana I, Richards M, Bentow C, et al. Evaluation of a novel particle-based assay for detection of autoantibodies in idiopathic inflammatory myopathies. J Immunol Methods. 2019;474:112661. doi: 10.1016/j.jim.2019.112661.

[21] Cavazzana I, Fredi M, Ceribelli A, et al. Testing for myositis specific autoantibodies: comparison between line blot and immunoprecipitation assays in 57 myositis sera. J Immunol Methods. 2016;433:1–5. doi: 10.1016/j.jim.2016.02.017.

[22] Shinoda K, Okumura M, Yamaguchi S, et al. A comparison of line blots, enzyme-linked immunosorbent, and RNA-immunoprecipitation assays of antisynthetase antibodies in serum samples from 44 patients. Intern Med. 2022;61(3):313–322. doi: 10.2169/internalmedicine.7824-21.

[23] Solomon J, Swigris JJ, Brown KK. Myositis-related interstitial lung disease and antisynthetase syndrome. J Bras Pneumol. 2011;37(1):100–109. doi: 10.1590/S1806-37132011000100015.

[24] Waseda Y, Johkoh T, Egashira R, et al. Antisynthetase syndrome: pulmonary computed tomography findings of adult patients with antibodies to aminoacyl-tRNA synthetases. Eur J Radiol. 2016;85(8):1421–1426. doi: 10.1016/j.ejrad.2016.05.012.

[25] Takato H, Waseda Y, Watanabe S, et al. Pulmonary manifestations of anti-ARS antibody positive interstitial pneumonia–with or without PM/DM. Respir Med. 2013;107(1):128–133. doi: 10.1016/j.rmed.2012.09.005.

[26] Hozumi H, Fujisawa T, Nakashima R, et al. Comprehensive assessment of myositis-specific autoantibodies in polymyositis/dermatomyositis-associated interstitial lung disease. Respir Med. 2016;121:91–99. doi: 10.1016/j.rmed.2016.10.019.

[27] Yamakawa H, Hagiwara E, Kitamura H, et al. Predictive factors for the long-term deterioration of pulmonary function in interstitial lung disease associated with anti-aminoacyl-tRNA synthetase antibodies. Respiration. 2018;96(3):210–221. doi: 10.1159/000488358.

[28] Sato S, Masui K, Nishina N, et al. Initial predictors of poor survival in myositis-associated interstitial lung disease: a multicentre cohort of 497 patients. Rheumatology (Oxford). 2018;57(7):1212–1221. doi: 10.1093/rheumatology/key060.

[29] Granito A, Muratori P, Muratori L, et al. Antinuclear antibodies giving the 'multiple nuclear dots' or the 'rim-like/membranous' patterns: diagnostic accuracy for primary biliary cirrhosis. Aliment Pharmacol Ther. 2006;24(11–12):1575–1583. doi: 10.1111/j.1365-2036.2006.03172.x.

[30] Mahler M, Mierau R, Schlumberger W, et al. A population of autoantibodies against a centromere-associated protein a major epitope motif cross-reacts with related cryptic epitopes on other nuclear autoantigens and on the Epstein-Barr nuclear antigen 1. J Mol Med (Berl). 2001;79(12):722–731. Epub 20010725 doi: 10.1007/s001090100258.

[31] Matsuura E, Igarashi Y, Yasuda T, et al. Anticardiolipin antibodies recognize beta 2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. J Exp Med. 1994;179(2):457–462. doi: 10.1084/jem.179.2.457.