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Common and Distinct Clinical Features in Adult Patients with Anti-Aminoacyl-tRNA Synthetase Antibodies: Heterogeneity within the Syndrome

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Abstract

Objective: To identify similarities and differences in the clinical features of adult Japanese patients with individual anti-aminoacyl-tRNA synthetase antibodies (anti-ARS Abs).

Methods: This was a retrospective analysis of 166 adult Japanese patients with anti-ARS Abs detected by immunoprecipitation assays. These patients had visited Kanazawa University Hospital or collaborating medical centers from 2003 to 2009.

Results: Anti-ARS Ab specificity included anti-Jo-1 (36%), anti-EJ (23%), anti-PL-7 (18%), anti-PL-12 (11%), anti-KS (8%), and anti-OJ (5%). These anti-ARS Abs were mutually exclusive, except for one serum Ab that had both anti-PL-7 and PL-12 reactivity. Myositis was closely associated with anti-Jo-1, anti-EJ, and anti-PL-7, while interstitial lung disease (ILD) was correlated with all 6 anti-ARS Abs. Dermatomyositis (DM)-specific skin manifestations (heliotrope rash and Gottron's sign) were frequently observed in patients with anti-Jo-1, anti-EJ, anti-PL-7, and anti-PL-12. Therefore, most clinical diagnoses were polymyositis or DM for anti-Jo-1, anti-EJ, and anti-PL-7; clinically amyopathic DM or ILD for anti-PL-12; and ILD for anti-KS and anti-OJ. Patients with anti-Jo-1, anti-EJ, and anti-PL-7 developed myositis later if they had ILD alone at the time of disease onset, and most patients with anti-ARS Abs eventually developed ILD if they did not have ILD at disease onset.

Conclusion: Patients with anti-ARS Abs are relatively homogeneous. However, the distribution and timing of myositis, ILD, and rashes differ among patients with individual anti-ARS Abs. Thus, identification of individual anti-ARS Abs is beneficial to define this rather homogeneous subset and to predict clinical outcomes within the "anti-synthetase syndrome."

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Introduction

The presence of autoantibodies (Abs) is one of the hallmarks of connective tissue diseases, such as systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and idiopathic inflammatory myopathy. In particular, a variety of serum Abs is found in patients with idiopathic inflammatory myopathies, including polymyositis (PM) and dermatomyositis (DM) [1,2]. It is clinically of considerable importance to identify Abs in patients with PM/DM, because each Ab is closely associated with certain clinical features [3]. For example, anti-Mi-2 is associated with classic DM without interstitial lung disease (ILD) or malignancy and with good response to treatment [4–6]; anti-155/140 is associated with malignancy-associated or juvenile DM [7–10]; and anti-CADM-140/MDA5 is associated with clinically amyopathic DM (CADM) and rapidly progressive-ILD (RP-ILD) that results in poor prognosis [11,12]. Abs reactive with aminoacyl-tRNA synthetases (ARS) are also representative Abs that are detected in patients with PM/DM. Eight anti-ARS Abs have been described: anti-histidyl (anti-Jo-1), anti-threonyl (anti-PL-7), anti-alanyl (anti-PL-12), anti-glycyl (anti-EJ), anti-isoleucyl (anti-OJ), anti-asparaginyl (anti-KS), anti-phenylalanyl (anti-Zo), and anti-tyrosyl (anti-Ha) tRNAs [13–20]. Based on a unique combination of clinical features commonly observed in patients with anti-ARS Abs, Targoff proposed a

disease entity termed "anti-synthetase syndrome," which is characterized by myositis, ILD, fever, Raynaud's phenomenon, arthritis, and mechanic's hands [21]. Although anti-synthetase syndrome has common clinical manifestations, further observations have distinguished some differences in clinical features associated with individual anti-ARS Abs [22]. For example, it has been reported that anti-Jo-1 Abs are closely associated with myositis [14,17], whereas patients with anti-KS are more likely to have ILD without clinical evidence of myositis [18,23]. On the other hand, Sato *et al* previously reported that the presence of anti-PL-7 is closely associated with PM/DM-SSc overlap as well as ILD in Japanese patients [24].

This is a large comprehensive study to focus on the clinical and laboratory features in adult patients with anti-ARS Abs for the investigation of similarities and differences in these anti-ARS Abs. The results of this study indicate that anti-ARS Abs share several clinical features, but also have some considerable differences. Thus, identification of each anti-ARS Ab is beneficial to define this rather homogeneous subset of patients and to predict clinical outcomes.

Patients and Methods

Ethics Statement

Ethical approval for the study was obtained from the individual institutional review boards (Kanazawa University, Keio University, Nagasaki University, St. Marianna University, Social Insurance Chukyo Hospital, and Ogaki Municipal Hospital) and all sera were collected after the subjects gave their written informed consent.

Patients and Sera

Serum samples were obtained from Japanese patients with autoimmune diseases or related disorders who had visited Kanazawa University Hospital or collaborating medical centers from 2003 to 2009. In total, 3164 samples (from 478 patients with DM/PM, 498 with SSc, 183 with ILD alone, 376 with SLE, 102 with mixed connective tissue disease, 398 with Sjogren's syndrome, and 1129 with rheumatoid arthritis) were screened by immunoprecipitation (IP) assay for the detection of antinuclear or anticytoplasmic antibodies. These patients were referred mainly by rheumatologists, dermatologists, or pulmonologists. PM and classic DM were defined by fulfillment of the Bohan and Peter criteria for definite or probable diagnoses [25]. DM was distinguished from PM based on the presence of heliotrope rash or Gottron's lesions (Gottron's papules and/or Gottron's sign). The diagnosis of CADM was based on the criteria proposed by Sontheimer [26], as follows: clinical skin manifestations typical of DM but minimal or no clinical features of myositis for >2 years after the onset of skin manifestations. All patients with SLE or SSc fulfilled the American College of Rheumatology criteria [27,28]. PM/DM-overlap was diagnosed by the coexistence of SLE and/or SSc in addition to PM or DM. "ILD alone" was defined by the presence of ILD without fulfillment of any of the criteria for PM, DM, CADM, SLE, or SSc. Patients with ILD alone were examined for potential coexistence of myositis by evaluating muscle weakness and serum muscle enzyme levels including creatine kinase (CK) and aldolase during follow-up, while those without ILD were examined for potential coexistence of ILD by examining dyspneic symptoms and chest radiograph and/or high-resolution computed tomography (HRCT) at every 3 to 6 months.

Clinical information was collected retrospectively for all patients with anti-ARS Abs by reviewing their clinical charts. Initial manifestations were defined as the clinical presentation at the first clinic visit. Patients who had at least one of the following symptoms: symmetrical proximal muscle weakness, muscle pain, or elevated levels of myogenic enzymes, underwent electromyogram, MRI, and/or muscle biopsy for confirmation of the presence of myositis. Patients were diagnosed with myositis if at least one of these confirmatory examinations showed findings compatible with inflammatory myopathy: a myogenic pattern on electromyogram [25], muscular edema on T2-weighted images with fat suppression on MRI [29], or necrosis, regeneration, and some atrophy of muscle fibers and inflammatory cell infiltration on muscle biopsy [25]. Patients were diagnosed as having ILD according to the images on chest HRCT. RP-ILD was defined as progressive dyspnea and progressive hypoxemia with a worsening of interstitial changes on the chest images within 1 month from the onset of respiratory manifestations [11]. Internal and hematologic malignancies in anti-ARS-positive patients was defined if the malignant disease was diagnosed concurrently with or within 3 years after diagnosis of anti-synthetase syndrome or if a preceding malignant disease occurred within 3 years before diagnosis of anti-synthetase syndrome [4]. Sjögren's syndrome was defined in accordance with the revised European criteria [30].

IP Assays

Protein IP assays were carried out with extracts of the leukemia cell line, K562 [11]. A total of 10 μl of the patient's serum was bound to 2 mg protein-A Sepharose beads (Amersham Biosciences, Piscataway, NJ) in 500 μl of IP buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1% Nonidet P-40), incubated for 2 h at 4°C, and then washed five times with IP buffer. Ab-coated Sepharose beads were mixed with 100 μl ^{35}S-methionine-labelled K562 cell extracts derived from 10^6 cells and rotated at 4°C for 2 h. After five washes, the beads were resuspended in sodium dodecyl sulphate (SDS) sample buffer and the polypeptides were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. For the analysis of RNA, immunoprecipitated RNA was detected in 8% urea-PAGE from a cell extract obtained from 3×10^6 non-radiolabeled K562 cells by phenol/chloroform, visualized by silver staining [31]. Each anti-ARS Ab was considered positive if serum samples produced precipitin lines with immunological identity to reference sera by both protein and RNA IP [32]. Anti-Ro Ab and anti-La Ab were detected by IP assays as well. Serum was considered positive for anti-Ro Ab if at least one of the Y1–Y5 RNAs was detected by RNA IP and the 60 kDa protein was detected by protein IP; serum was considered positive for anti-La Ab if RNAs contained in the 7S and 5.8S lesions were detected by RNA IP and the 48 kDa protein was detected by protein IP.

Immunofluorescence

Indirect immunofluorescence tests were carried out with slides of monolayer HEp-2 cells (Medical & Biological Laboratories [MBL], Nagoya, Japan) as substrate [33]. Anticentromere antibody was considered positive if serum diluted at 1:40 produced a characteristic staining pattern on HEp-2 cells as well as on commercially prepared HeLa cell chromosomal spreads (MBL) [34].

Statistical Analysis

Frequencies among all six anti-ARS-positive subgroups were compared with a chi-square test. If the overall P value was less than 0.05, pairwise comparisons were performed with a chi-square test with Yates' correction where appropriate. Continuous variables confirmed to be normally distributed were shown as mean and SD, and their comparisons among groups were carried

out with an ANOVA. All statistical analyses were performed with StatView software.

Results

Detection of Anti-ARS Abs

Of 3164 samples screened by IP assays, anti-ARS Abs were detected in 166 patients (5.2%) (Figure 1). As shown in Figure 2, 6 anti-ARS specificities, including anti-Jo-1, anti-EJ, anti-PL-7, anti-PL-12, anti-KS, anti-OJ, were easily detectable and distinguishable by IP assays. Of 166 patients with anti-ARS Abs, anti-Jo-1 was found in 59 (36%) patients, anti-EJ was found in 38 (23%) patients, anti-PL-7 was found in 30 (18%) patients, anti-PL-12 was found in 19 (11%) patients, anti-KS was found in 13 (8%) patients, and anti-OJ was found in 8 (5%) patients. One patient with classic DM had antibodies reactive to both PL-7 and PL-12, and was excluded from the following analyses for clinical associations.

Coexistence of anti-ARS Abs and other autoimmune connective tissue disease-related Abs was examined (Table 1). Antibodies against Mi-2, 155/140, CADM-140/MDA5, MJ/NXP-2, topoisomerase I, centromere, U1RNP, Th/To, U3RNP, Sm and La/SS-B were rarely found in patients with anti-ARS Abs. In contrast, anti-Ro/SS-A Abs were found in 31 (19%) patients. These results were principally consistent with previous findings that myositis-specific Abs are relatively mutually exclusive, while myositis-associated Abs coexist with myositis-specific Abs [13,35].

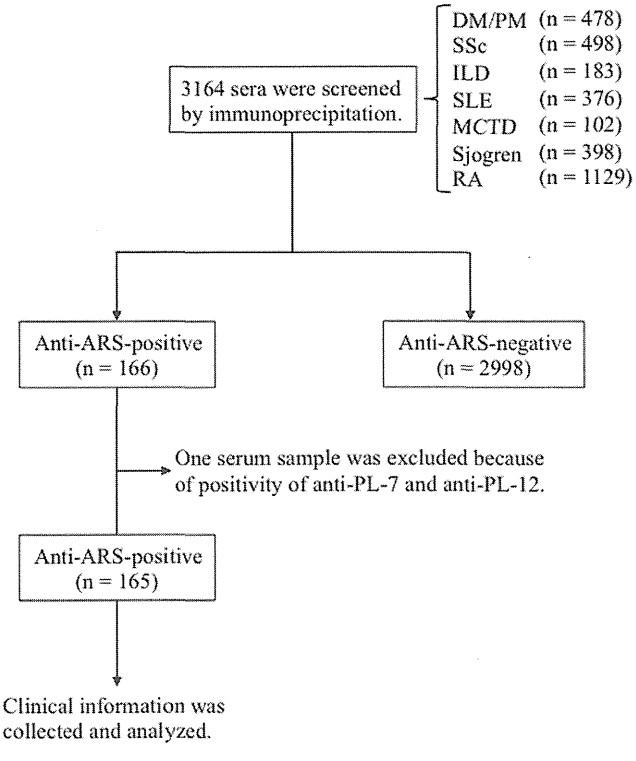

Figure 1. Enrollment and selection of patients. DM; dermatomyositis, PM; polymyositis, SSc; systemic sclerosis, ILD; interstitial lung disease, SLE; systemic lupus erythematosus, MCTD; mixed connective tissue disease, Sjogren; Sjogren's syndrome, RA; rheumatoid arthritis.
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Associations between Clinical Diagnoses and Anti-ARS Abs

The distributions of classic DM, CADM, PM, PM/DM-overlap, SLE, SSc, and ILD alone in patients with individual anti-ARS Abs are shown in Figure 3. More than half of the patients with anti-Jo-1, anti-EJ, or anti-PL-7 had apparent myositis, including classic DM, PM, and PM/DM-overlap. The proportion with ILD alone was different among patients with various anti-ARS Abs. In particular, 10 of 13 (77%) patients with anti-KS and 5 of 8 (63%) patients with anti-OJ were diagnosed with ILD alone. Some patients with anti-ARS Abs were diagnosed with SSc or SLE, but the frequency was relatively low. Thus, most patients with anti-ARS Abs were diagnosed as having classic DM, CADM, PM, PM/DM-overlap, or ILD alone, while the proportion of these diagnoses was different among the subgroups of each anti-ARS Ab.

Comparison of Clinical Features among Patients with Anti-ARS Abs

A total of 95 patients with anti-ARS Abs had myositis and were diagnosed as having classic DM, PM, or PM/DM-overlap. We first compared clinical features between patients with myositis in the presence and absence of anti-ARS Ab (n = 95 and 152, respectively). Anti-ARS-positive patients with myositis had higher frequencies of Raynaud's phenomenon ($P = 0.034$), ILD ($P < 0.0001$), and polyarthritis ($P = 0.0015$) compared with anti-ARS-negative patients with myositis. There was no difference in the frequency of fever between the two groups ($P = 0.87$).

Then, we compared the demographic features among anti-ARS-based subgroups, as shown in Table 2. No differences were found in age of onset or sex. We next compared muscle weakness and ILD among individual anti-ARS subgroups, both at the initial visit and during the entire follow-up period. Muscle weakness was found in 71 (43%) patients at the initial visit and 95 (58%) during the entire follow-up period, but the frequencies varied among anti-ARS-based subgroups (overall $P = 0.0011$ and $P < 0.0001$, respectively). Patients with anti-Jo-1, anti-EJ, and anti-PL-7 had a higher frequency of muscle weakness (59%, 39%, and 52%, respectively, at the initial visit and 78%, 55%, and 76%, respectively during the entire follow-up period) than those with anti-PL-12 (17% for both), anti-KS (7% for both), and anti-OJ (25% for both). In contrast, most patients had ILD at the initial visit, and almost all patients eventually suffered from ILD. While most of them had the chronic type of ILD, a total of 13 patients (8 with anti-Jo-1, 4 with anti-EJ, and 1 with anti-PL-7) developed RP-ILD at their first visit or during their clinical course. Thus, the frequency of muscle weakness varied among anti-ARS subgroups, while ILD was observed at equally high frequencies among these subgroups.

Fever, Raynaud's phenomenon, polyarthritis, and mechanic's hands during the entire follow-up period were compared among anti-ARS subgroups. The frequency of fever varied among anti-ARS-based subgroups (8–44%), but there was no statistical difference. Raynaud's phenomenon was found in 40 of 165 (24%) patients with anti-ARS Abs and more frequently observed in patients with anti-PL-12 and anti-PL-7 (overall $P = 0.044$). Polyarthritis was most common in patients with anti-Jo-1 (58%) and infrequently observed in patients with anti-OJ (13%) (overall $P = 0.0029$). Mechanic's hands, which are the representative skin manifestation in anti-synthetase syndrome, were observed in all anti-ARS Ab-based subgroups, but the frequency was highest in patients with anti-Jo-1 (56%) (overall $P = 0.031$). Collectively, Raynaud's phenomenon, polyarthritis, and mechanic's hands were

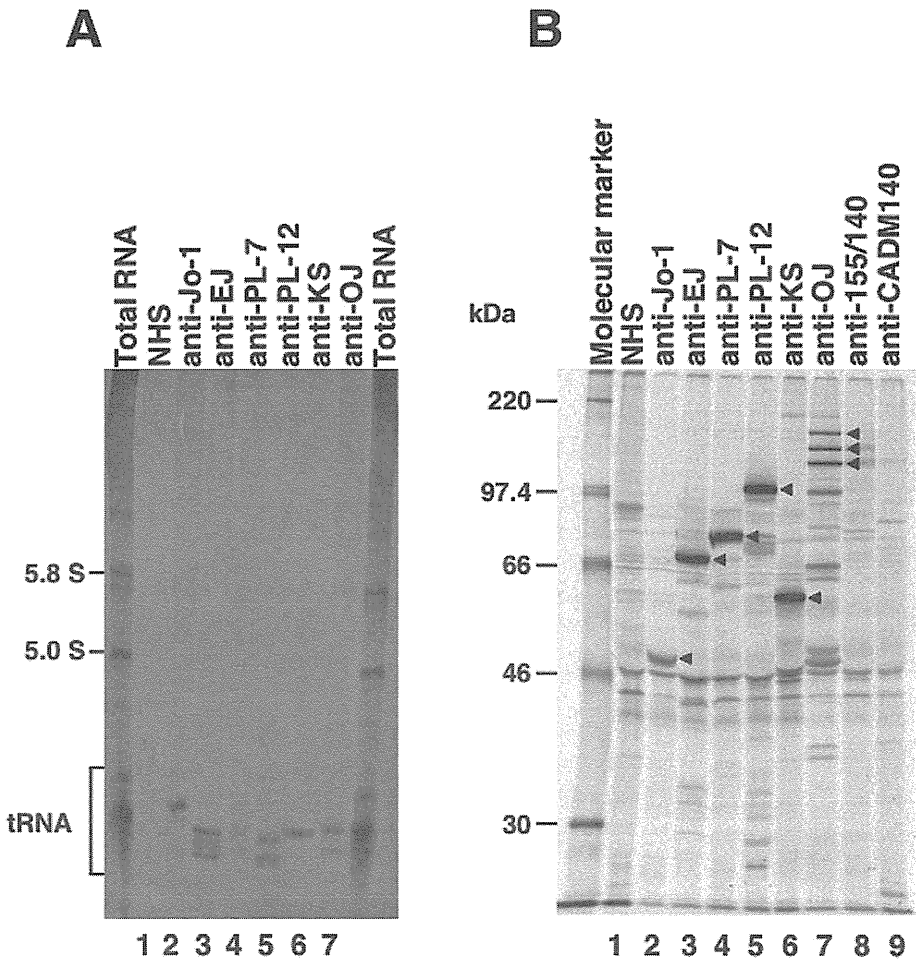

Figure 2. Representative immunoprecipitation assay for RNA with anti-aminoacyl-tRNA synthetase (anti-ARS) sera. A, Immunoprecipitation of histidyl-tRNA synthetase, glycyl-tRNA synthetase, threonyl-tRNA synthetase, alanyl-tRNA synthetase, asparaginyl-tRNA, and isoleucyl-tRNA synthetase by sera. K562 cell extracts were immunoprecipitated with sera, and RNA was extracted, electrophoresed on 8% urea-polyacrylamide gels, and visualized by silver staining. Total RNA, with the 5.8 and 5.0 S small ribosomal RNAs and the tRNA region indicated; Lane 1, normal health serum (NHS) indicated; Lanes 2–7: anti-ARS sera indicated, with antibodies to Jo-1 (histidyl-tRNA synthetase), EJ (glycyl-tRNA synthetase), PL-7 (threonyl-tRNA synthetase), PL-12 (alanyl-tRNA synthetase), KS (asparaginyl-tRNA synthetase), and OJ (isoleucyl-tRNA synthetase). **B,** Immunoprecipitation of ^{35}S-methionine-labeled K562 cell extracts was performed on anti-ARS sera and NHS, separated on 10% SDS-PAGE, and analyzed by autoradiography. Molecular weight markers include protein bands corresponding to 220, 97.4, 66, 46, and 30 kDa.
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observed in each anti-ARS Ab subgroup, but the frequencies were rather heterogeneous.

We then compared heliotrope rash and Gottron's signs, which are the representative skin manifestations in DM. Heliotrope rash was found in 26 of 165 (16%) patients with anti-ARS Abs (overall $P = 0.0019$) and Gottron's sign (elbow and/or knee) was found in 51 (31%) (overall $P = 0.043$). These manifestations were predominantly found in patients with anti-EJ, anti-PL-7, and anti-PL-12.

With regard to laboratory findings, CK levels were lower in patients with anti-PL-12 and anti-KS (overall $P = 0.024$), and lactate dehydrogenase (LDH) was lowest in patients with anti-KS (overall $P = 0.019$). It is likely that these results were associated with the frequencies of muscle involvement. KL-6 and pulmonary surfactant protein D (SP-D) levels are associated with the activity and severity of ILD [36,37]. While elevations of both KL-6 and SP-D were observed in all anti-ARS-based subgroups, no significant differences were observed in serum KL-6 and SP-D levels.

As an association of malignancy with PM/DM has been reported, we examined the frequency of malignancies in patients with anti-ARS Abs (Table 2). Malignancies were observed in 19 (12%) of 165 patients with anti-ARS Abs, and 1 of those had a double malignancy. A summary of the malignancies is listed in Table 3. There were 4 patients with colon cancer, 4 with gastric cancer or carcinoid, 3 with breast cancer, 3 with lung cancer, and single cases of prostate cancer, nasopharyngeal cancer, uterine corpus cancer, thyroid cancer, ovarian cancer, and non-Hodgkin lymphoma. There was no trend in the prevalence of malignancy or the type of malignancy among anti-ARS-based subgroups. Seven of 19 patients with malignancy simultaneously developed PM/DM or ILD, while 7 of 19 had malignancy prior to the development of PM/DM or ILD, and 5 of 19 developed malignancy after the diagnosis of PM/DM or ILD.

Table 1. Coexistence of other autoantibodies in patients with anti-aminoacyl-tRNA synthetase antibodies.*

	Anti-Jo-1 (n = 59)	Anti-EJ (n = 38)	Anti-PL-7 (n = 29)	Anti-PL-12 (n = 18)	Anti-KS (n = 13)	Anti-OJ (n = 8)	Anti-PL-7/ PL-12 (n = 1)
Anti-Mi-2	0	0	0	0	0	0	0
Anti-155/140	0	0	0	0	0	0	0
Anti-CADM-140/MDA5	0	0	0	0	0	0	0
Anti-MJ/NXP-2	0	0	0	0	0	0	0
Anti-topoisomerase I	0	1	0	0	0	0	0
Anti-centromere	1	0	0	1	2	0	0
Anti-U1RNP	0	0	1	1	0	0	0
Anti-Th/To	0	0	0	1	0	0	0
Anti-U3RNP	1	0	0	0	0	0	0
Anti-Sm	0	0	1	0	0	0	0
Anti-Ro/SS-A	9	9	8	4	1	0	0
Anti-La/SS-B	0	2	2	0	0	0	0

*Values are the number of patients.
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Causes of Death

Sixteen (10%) of 165 anti-ARS-positive patients died during the follow-up period (Table 4). Causes of death included ILD in 8, malignancy in 3, infection in 2, and one each of myocardial infarction, rupture of an abdominal aortic aneurysm, and hypertrophic cardiomyopathy.

Timing of Development of ILD and Myositis in Patients with Anti-ARS Abs

Initial manifestations in patients with anti-ARS Abs are summarized in Table 5. At initial presentation, the combination of manifestations, including DM rashes, myositis, and ILD, varied among patients with anti-ARS Abs. The frequency of ILD alone at

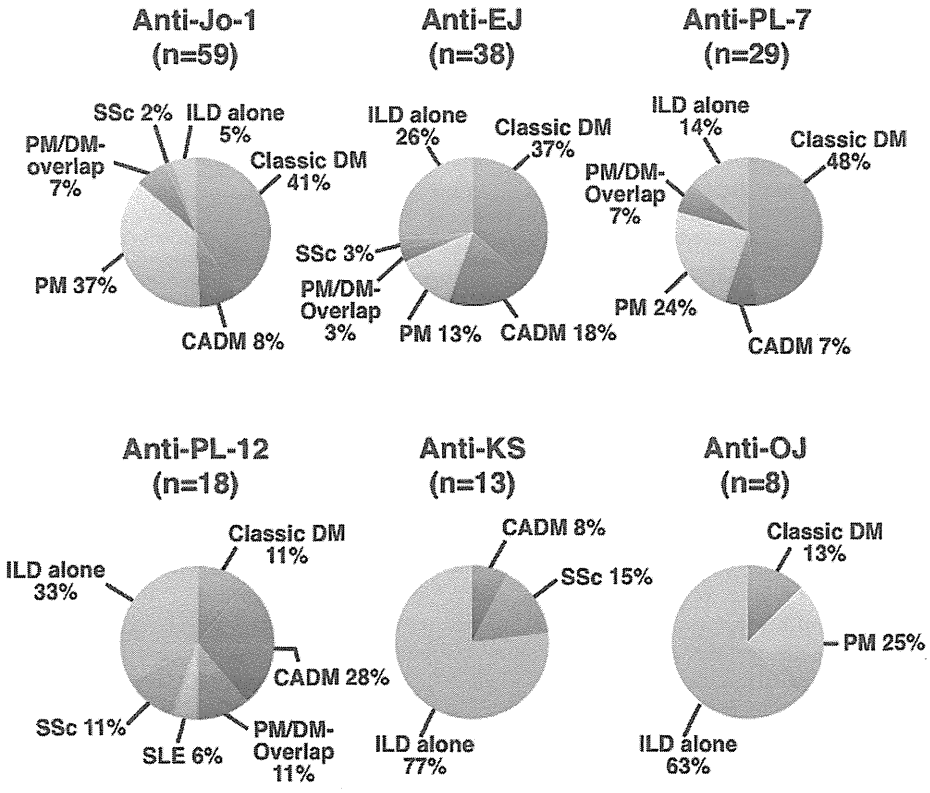

Figure 3. Prevalence of dermatomysitis (DM), clinically amyopathic DM (CADM), polymyositis (PM), PM/DM-overlap, systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and interstitial lung disease (ILD) alone, in each subgroup of anti-synthetase syndrome.
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Table 2. Comparison of clinical features in 165 adult Japanese patients with anti-aminoacyl-tRNA synthetase antibodies.*

	Anti-Jo-1 (n = 59)	Anti-EJ (n = 38)	Anti-PL-7 (n = 29)	Anti-PL-12 (n = 18)	Anti-KS (n = 13)	Anti-OJ (n = 8)	Overall *P*
Age at onset, median (range), y	53 (22–76)	53 (18–78)	53 (25–79)	48 (20–75)	54 (39–67)	57 (32–79)	0.61
No. of females/no. of males	43/16	32/6	26/3	16/2	7/6	6/2	0.077
Clinical features (at initial visit)							
Interstitial lung disease	71	84	76	89	100	100	0.077
Muscle weakness	59	39	52	17	7	25	0.0011[a]
Clinical features (entire follow-up period)							
Fever	27	39	34	44	8	13	0.16
Raynaud's phenomenon	19	13	38	44	31	13	0.044[b]
Interstitial lung disease	90	97	93	94	100	100	0.56
Muscle weakness	78	55	76	17	7	25	<0.0001[c]
Polyarthritis	58	24	31	22	31	13	0.0029[d]
Erosive arthritis	12	5	0	17	23	0	0.16
Malignancy	15	3	7	17	15	25	0.22
Sjögren's syndrome	7	16	14	0	8	0	0.32
Skin manifestations							
Heliotrope rash	7	21	38	17	0	0	0.0019[e]
Gottron's sign (hand)	44	45	41	33	8	13	0.10
Gottron's sign (elbow and/or knee)	27	39	45	33	0	13	0.043[f]
Mechanic's hands	56	29	45	22	23	38	0.031[g]
Laboratory findings							
CK, IU/L, mean ± SD	2213±3168	1681±2967	1768±2096	250±306	143±84	881±1129	0.024[h]
LDH, IU/L, mean ± SD	595±5961	427±223	565±406	346±187	215±77	355±197	0.019[i]
KL-6, U/mL, mean ± SD	1335±2067 (n = 54)	1425±1030	1374±1444	1630±1650	1527±1404 (n = 12)	1307±877	0.99
SP-D, ng/mL, mean ± SD	206±229 (n = 39)	318±626 (n = 36)	229±275 (n = 25)	250±170 (n = 15)	185±129	123±53 (n = 6)	0.74

*Unless noted otherwise, values are percentages of patients. NS: not significant; CK: creatine kinase; LDH: lactate dehydrogenase. One patient with DM who had antibodies reactive to both PL-7 and PL-12 was excluded from the analysis. Significant differences (overall $P<0.05$) were further analyzed by pairwise comparisons.
[a]$P<0.05$ between anti-PL-7 and anti-PL-12; $P<0.01$ between anti-Jo-1 and anti-PL-12, and between anti-KS and anti-Jo-1 or anti-PL-7;
[b]$P<0.05$ between anti-Jo-1 and anti-PL-7 or anti-PL-12, and between anti-EJ and anti-PL-7; $P<0.01$ between anti-EJ and anti-PL-12.
[c]$P<0.05$ between anti-EJ and anti-PL-12; $P<0.01$ between anti-Jo-1 and anti-PL-12, anti-KS or anti-OJ, between anti-EJ and anti-KS, and between anti-PL-7 and anti-PL-12, anti-KS or anti-OJ.
[d]$P<0.05$ between anti-Jo-1 and anti-PL-7, anti-KS or anti-OJ; $P<0.01$ between anti-Jo-1 and anti-EJ or anti-PL-12.
[e]$P<0.05$ between anti-Jo-1 and anti-EJ; $P<0.01$ between anti-PL-7 and anti-Jo-1 or anti-KS.
[f]$P<0.05$ between anti-KS and anti-EJ or anti-PL-12; $P<0.01$ between anti-PL-7 and anti-KS.
[g]$P<0.05$ between anti-Jo-1 and anti-PL-12 or anti-KS; $P<0.01$ between anti-Jo-1 and anti-EJ.
[h]$P<0.05$ between anti-EJ and anti-PL-12 or anti-KS; $P<0.01$ between anti-Jo-1 and anti-PL-12 or anti-KS, and between anti-PL-7 and anti-PL-12 or anti-KS.
[i]$P<0.05$ between anti-PL-7 and anti-PL-12; $P<0.01$ between anti-Jo-1 and anti-PL-12, and between anti-KS and anti-Jo-1, anti-EJ or anti-PL-7.
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presentation was different among groups stratified by anti-ARS Abs (overall $P=0.0001$). While some patients with anti-ARS Abs had 2 or more manifestations at initial diagnosis, others sequentially developed different manifestations, even when they were receiving therapy. Thus, we analyzed the timing of development of ILD and myositis. Figure 4A includes patients with ILD alone and DM rashes and ILD, and Figure 4B includes those with myositis alone and DM rashes and myositis at initial presentation. Patients with DM rashes alone, myositis and ILD, DM rashes, myositis, and ILD, and none of DM rashes, myositis, and ILD were excluded from this analysis. We assessed whether patients who had ILD alone at presentation developed myositis during follow-up (Figure 4A). As a result, 39%, 29%, and 64% of patients with anti-Jo-1, anti-EJ, and anti-PL-7, respectively, subsequently developed myositis. In contrast, none of the patients with anti-PL-12, anti-KS, and anti-OJ who had ILD alone at presentation developed myositis later in the course of the disease. The distribution of the frequencies for developing myositis among anti-ARS-based subgroups was statistically significant (overall $P=0.0008$). In contrast, when patients who had myositis without ILD at presentation were selected, nearly all of them developed ILD later in the course of the disease (Figure 4B). There was no difference in observation period among the 6 groups (Jo-1, 62±24; EJ, 56±27; PL-7, 50±27; PL-12, 53±27; KS, 70±20; and OJ, 62±32 months). In addition, there was no difference in initial treatment regimen among the 6 groups stratified by anti-ARS Abs (Table 6), although 38% of patients with anti-KS did not receive immunosuppressive therapy and this frequency was highest among the 6 groups (overall $P=0.0070$). Almost all patients with anti-ARS Abs who had ILD or myositis received immunosuppressive treatment, including corticosteroids alone or in combination with immunosuppressants. Accordingly, patients with anti-PL-12, anti-

Table 3. Summary of malignancy in patients with anti-aminoacyl-tRNA synthetase antibodies.

Anti-ARS	Age, y	Sex	Diagnosis	ILD	Type of malignancy	Onset
Anti-Jo-1	54	M	PM	−	Lung cancer	At same time
Anti-Jo-1	59	F	DM	+	Gastric cancer	Before DM
Anti-Jo-1	38	F	DM	+	Ovarian cancer	At same time
Anti-Jo-1	54	M	PM	+	Colon cancer	After PM
Anti-Jo-1	74	M	DM	+	Colon cancer	Before DM
Anti-Jo-1	42	F	DM	+	Breast cancer	Before DM
Anti-Jo-1	67	F	DM	+	Non-Hodgkin lymphoma	At same time
Anti-Jo-1	62	M	PM	−	Gastric cancer	After PM
Anti-Jo-1	57	F	DM	+	Thyroid cancer	At same time
Anti-EJ	43	F	DM	+	Nasopharyngeal cancer	At same time
Anti-PL-7	70	F	DM	+	Breast cancer	Before DM
Anti-PL-7	79	M	ILD	+	Gastric cancer	After ILD
Anti-PL-12	53	F	ILD	+	Lung+uterine corpus cancer	Before ILD
Anti-PL-12	66	M	ILD	+	Colon cancer	After ILD
Anti-PL-12	59	F	DM	+	Breast cancer	Before DM
Anti-KS	59	M	ILD	+	Lung cancer	After ILD
Anti-KS	66	M	ILD	+	Prostate cancer	Before ILD
Anti-OJ	71	F	DM	+	Gastric carcinoid	At same time
Anti-OJ	77	M	PM	+	Colon cancer	At same time

ILD: interstitial lung disease; PM: polymyositis; DM: dermatomyositis.
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Table 4. Cause of death in patients with anti-aminoacyl-tRNA synthetase antibodies.

Anti-ARS	Age, y	Sex	Diagnosis	ILD	Cause of death	Time after diagnosis (y)
Anti-Jo-1	64	F	DM	+	ILD	0.3
Anti-Jo-1	38	F	DM	+	Infection	3
Anti-Jo-1	36	F	DM	+	ILD	5.5
Anti-Jo-1	62	M	PM	−	Gastric cancer	5
Anti-EJ	65	F	DM	+	ILD	2.5
Anti-EJ	55	F	ILD	+	ILD	0.6
Anti-EJ	55	F	DM	+	ILD	4.25
Anti-EJ	53	F	SSc	+	Infection	6
Anti-EJ	50	F	DM	+	Myocardial infarction	5.25
Anti-PL-7	63	F	DM	+	ILD	1.8
Anti-PL-7	71	F	DM	+	ILD	3
Anti-PL-7	75	M	ILD	+	ILD	0.3
Anti-PL-12	53	F	ILD	+	Lung cancer	3
Anti-PL-12	74	F	DM	+	Rupture of an abdominal aortic aneurysm	0.6
Anti-PL-12	75	F	ILD	+	Hypertrophic cardiomyopathy	2
Anti-KS	59	M	ILD	+	Lung cancer	1.5

ILD: interstitial lung disease; DM: dermatomyositis; PM: polymyositis; SSc: systemic sclerosis.
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Table 5. Initial manifestations in patients with anti-aminoacyl-tRNA synthetase antibodies.*

	Anti-Jo-1 (n = 59)	Anti-EJ (n = 38)	Anti-PL-7 (n = 29)	Anti-PL-12 (n = 18)	Anti-KS (n = 13)	Anti-OJ (n = 8)	Overall *P*
DM rashes alone	2	0	14	11	8	0	0.14
Myositis alone	14	11	21	0	0	0	0.14
ILD alone	29	39	28	56	92	63	0.0001[a]
DM rashes and Myositis	10	5	4	6	0	0	0.45
DM rashes and ILD	19	16	10	11	0	0	0.46
Myositis and ILD	7	13	7	0	0	25	0.24
DM rashes, Myositis, and ILD	10	16	17	11	0	13	0.75
No DM rashes, Myositis, or ILD**	10	0	0	6	0	0	0.11

*Values are percentages of patients.
**These patients had polyarthritis at presentation. Significant differences (overall $P<0.05$) were further analyzed by pairwise comparisons.
[a]$P<0.05$ between anti-PL-12 and anti-Jo-1 or anti-KS; $P<0.01$ between anti-KS and anti-Jo-1, anti-EJ or anti-PL-7.
doi:10.1371/journal.pone.0060442.t005

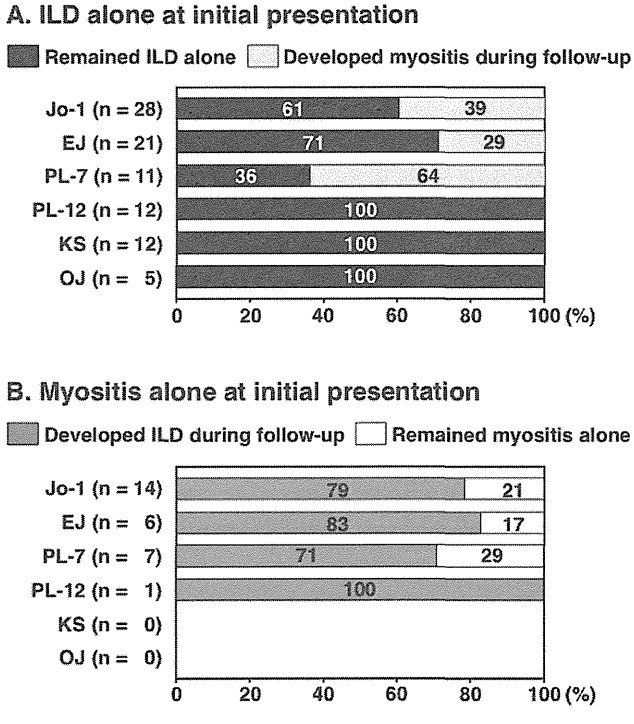

Figure 4. The clinical course of anti-synthetase syndrome patients who developed myositis or interstitial lung disease (ILD) with or without skin manifestations at disease onset. According to the clinical course, patients were classified into four types: remained with ILD alone, developed myositis during follow-up, developed ILD during follow-up, and remained with myositis alone. The clinical course of those who had ILD with or without skin manifestations, but without muscle involvement at their first assessment (A), and the clinical course of those who had myositis with or without skin manifestations, but without ILD at their first assessment (B).
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KS, or anti-OJ were less likely to develop myositis during follow-up than those with anti-Jo-1, anti-EJ, or anti-PL-7.

Discussion

This comprehensive report aimed to compare clinical features among anti-ARS-based subgroups on a large scale. As reported previously, more than one anti-ARS Ab did not coexist in general. While this study confirmed that ILD, myositis, Raynaud's phenomenon, polyarthritis, and mechanic's hands were common manifestations in anti-synthetase syndrome, the frequencies of each manifestation varied. That is, myositis was well associated with anti-Jo-1, anti-EJ, and anti-PL-7. Additionally, a substantial number of patients positive for anti-EJ or anti-PL-12 had CADM. Therefore, most of the clinical diagnoses were PM or DM for anti-Jo-1, anti-EJ, and anti-PL-7; CADM or ILD for anti-PL-12; and ILD for anti-KS and anti-OJ. Although patients with anti-ARS Abs share several common manifestations, it is likely that each of these Abs defines a clinically distinct phenotype and may serve as a predictor for clinical complications.

Since nearly all patients with anti-ARS Abs had ILD, this study confirms previous findings that anti-ARS Abs are a marker for ILD [38–42]. Most of the clinical diagnoses in patients with anti-ARS Abs were classic DM, CADM, PM or ILD alone in this study. This finding was also in accordance with previous reports that anti-ARS Abs were highly specific for a proportion of patients with PM, DM, or ILD [4,38,43–45]. However, classic DM, CADM, or PM was found predominantly in patient subgroups with anti-Jo-1, anti-EJ, and anti-PL-7, whereas two-thirds of patients with anti-PL-12 were diagnosed with CADM or ILD. In contrast, anti-KS and anti-OJ were associated with ILD alone. Therefore, it is likely that the clinical diagnosis varies among anti-ARS-based subgroups.

Regarding myositis, it appears that anti-ARS Abs are divided into myositis-related and non-myositis-related subgroups. Anti-Jo-1, anti-EJ, and anti-PL-7 belong to the myositis-related subgroup, since myositis was found in at least half of the patients with these anti-ARS Abs. These findings agreed with previous reports describing a relationship of myositis with anit-Jo-1 [46], anti-EJ [13,17,47,48], and anti-PL-7 [24,49]. In contrast, anti-PL-12, anti-KS, and anti-OJ were not well related to myositis in this study. These results also paralleled those of former reports that anti-KS is highly associated with ILD [32,48]. However, rates of myositis in anti-PL-12 and anti-OJ appear to be different from previous

Table 6. Initial treatment in patients with anti-aminoacyltransfer RNA synthetase antibodies.*

	Anti-Jo-1 (n = 59)	Anti-EJ (n = 38)	Anti-PL-7 (n = 29)	Anti-PL-12 (n = 18)	Anti-KS (n = 13)	Anti-OJ (n = 8)	Overall *P*
No immunosuppressive therapy	7 (4)	5 (2)	3 (1)	11 (2)	38 (5)	13 (1)	0.0070[a]
Initial treatment							
CS oral only	68 (40)	68 (26)	59 (17)	67 (12)	46 (6)	88 (7)	0.45
CS pulse+oral	8 (5)	16 (6)	21 (6)	6 (1)	8 (1)	0 (0)	0.36
CS (pulse and/or oral)+CsA	10 (6)	3 (1)	3 (1)	11 (2)	0 (0)	0 (0)	0.41
CS (pulse and/or oral)+Tac	2 (1)	0 (0)	3 (1)	0 (0)	0 (0)	0 (0)	0.81
CS (pulse and/or oral)+CY (oral and/or iv)	3 (3)	0 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0.82
CS (pulse and/or oral)+CsA or Tac+CY (oral and/or iv)	0 (0)	0 (0)	7 (2)	6 (1)	0 (0)	0 (0)	0.17
CS (pulse and/or oral)+MZR	0 (0)	3 (1)	3 (1)	0 (0)	0 (0)	0 (0)	0.69
CS (pulse and/or oral)+Buc	0 (0)	3 (1)	0 (0)	0 (0)	8 (1)	0 (0)	0.25

*Values are percentages of patients. Patient numbers are given in parenthesis. CS: corticosteroid; CsA: cyclosporine A; Tac: tacrolimus; CY: cyclophosphamide; iv: intravenous administration; MZR: mizoribine; Buc: bucillamine. Significant differences (overall $P<0.05$) were further analyzed by pairwise comparisons.
[a]$P<0.01$ between anti-KS and anti-Jo-1, anti-EJ or anti-PL-7.
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reports. Of a total of 47 cases with anti-PL-12, muscle weakness was observed in 27 (57%) patients [16,23,50]. Sato *et al* reported 7 Japanese patients with anti-OJ, in which muscle weakness was seen in 4 patients [51]. Thus, whether anti-PL-12 and anti-OJ are related to myositis remains controversial. Collectively, patients with anti-ARS Abs form a basically homogenous clinical entity, as previously reported; mutual comparisons in this study elucidated certain differences in clinical features among patients with specific anti-ARS Abs.

Regarding skin manifestations, this study revealed an interesting observation. The main clinical diagnoses in anti-Jo-1, anti-EJ, anti-PL-7, and anti-PL-12 were classic DM or CADM. This resulted from the higher frequencies of DM-specific skin manifestations in these patients, which included heliotrope rash and Gottron's signs. However, the distribution of skin manifestations varied among anti-ARS Abs. Only less than 10% of patients with anti-Jo-1 had heliotrope rash, while approximately 20–30% of those with anti-EJ, anti-PL-7, and anti-PL12 had this eruption. On the other hand, the frequency of anti-Jo-1-positive patients who had Gottron's sign was similar compared to those with anti-EJ, anti-PL-7, and anti-PL-12. Thus, the prevalence of DM-specific skin manifestations is not identical among different anti-ARS Abs, even though the main diagnosis is classic DM or CADM.

With respect to the onset of evident manifestations of myositis and ILD, these patients were divided into three groups: i) patients with myositis preceding ILD; ii) patients with ILD preceding myositis; and iii) patients with simultaneous onset of both conditions. We reported previously that the onset of anti-synthetase syndrome is acute, but that the development of myositis may lag behind the onset of ILD in anti-ARS-positive DM patients [38]. A similar finding was described in another report [44]. In this study, most patients with anti-ARS Abs who had myositis without ILD at the onset of disease developed ILD later. On the other hand, the rate of subsequent occurrence of myositis differed among the subsets of anti-ARS Abs when the patients had ILD without myositis as their initial manifestation. Thus, screening and identification of anti-ARS Abs is found to be beneficial in predicting the onset of ILD.

Other than ILD and myositis, previous reports described that arthritis, Raynaud's phenomenon, fever, and mechanic's hands are common clinical features in anti-synthetase syndrome [21,40,44]. There was no significant difference in the frequency of fever in this study. On the other hand, this study revealed some differences in the frequencies of polyarthritis, Raynaud's phenomenon, and mechanic's hands. While these three manifestations were observed with each anti-ARS Ab at a comparable rate, polyarthritis and mechanic's hands were most frequently found with anti-Jo-1, and Raynaud's phenomenon was most frequently found with anti-PL-12. Nonetheless, the differences in frequencies of these manifestations among anti-ARS subgroups were less evident than that with myositis.

We acknowledge several limitations of this study. First, it included a relatively small number of patients with anti-PL-12, anti-KS, or anti-OJ. Second, most facilities enrolled in this study were referral centers. This study had a higher frequency of DM and a relatively lower frequency of PM compared with other similar studies. This may be explained by the fact that our patients were mainly referred to us by rheumatologists, dermatologists, and pulmonologists, and only a few of them were referred by neurologists. Therefore, we cannot exclude selection bias. Third, the possibility cannot be ruled out that coexistence of anti-Ro/SS-A Abs influence the clinical feature of anti-ARS-positive patients with anti-Ro/SS-A Abs, as anti-Ro/SS-A Abs are considered as myositis-associated Abs and form the subgroup. In the analysis of clinical course, possibilities are raised that the short observation period and the differences in treatment potentially affected the results. Additionally, patients who visited to referral centers were examined for the existence of myositis and they were categorized by Bohan and Peter and Sontheimer criteria that are commonly used for diagnosis of myositis in a current condition. However, as clinical features of patients with anti-ARS Abs are largely heterogeneous, it appears difficult to stratify the patients by current criteria. It may be clinically useful to classify the anti-ARS-positive patients based on the type of anti-ARS Abs, not current criteria. It needs to consider the conformity of the classification of the patients with anti-ARS Abs with diagnosis criteria for myositis. Indeed, Connors *et al* have proposed the criteria for anti-ARS syndrome as follows [40]. First, patients must have positive serologic testing for anti-ARS Abs. Then, patients have one or more of the following conditions: Evidence of myositis by Bohan

and Peter criteria, evidence of ILD by American Thoracic Society criteria, evidence of arthritis by clinical examination, radiographic findings, or patient self-report, unexplained, persistent fever, Raynaud's phenomenon, and mechanic's hands. Therefore, more studies are needed for a better general understanding of the clinical characteristics of patients with anti-ARS Abs.

In summary, although anti-ARS Abs share common clinical features, each anti-ARS Ab appears to form some distinct clinical subset. However, the identification of anti-ARS Abs (except for anti-Jo-1) is limited only to certain facilities, as it requires a complicated technique. Establishment of a system routinely available to screen all anti-ARS Abs specificities is needed.

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Author Contributions

Conceived and designed the experiments: YH MF. Performed the experiments: YH MF. Analyzed the data: YH RY MF. Contributed reagents/materials/analysis tools: YH MF TM K. Kaji K. Komura MH M. Kodera EM KF MS HY SS KT M. Kuwana. Wrote the paper: YH MF M. Kuwana.

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ORIGINAL ARTICLE
Pulmonary Circulation

Double-Blind, Placebo-Controlled Clinical Trial With a Rho-Kinase Inhibitor in Pulmonary Arterial Hypertension

– A Pilot Efficacy Trial –

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Background: We have previously demonstrated that long-term inhibition of Rho-kinase ameliorates pulmonary arterial hypertension (PAH) in animal models. In the present study, we examined the clinical effects of mid-term oral treatment with an extended release formulation of AT-877 (fasudil hydrochloride), a specific Rho-kinase inhibitor (AT-877ER) on PAH.

Methods and Results: 23 PAH patients were treated with either placebo (10/2 females/males, 51±16 years, idiopathic PAH (IPAH) in 6, PAH associated with connective tissue disease (CTD-PAH) in 3, PAH with congenital heart disease (CHD-PAH) in 2, and portal PAH in 1) or AT-877ER (6/5 females/males, 47±14 years, IPAH in 2, CTD-PAH in 5, and CHD-PAH in 4); 3 patients were excluded. We performed a 6-min walk test and right heart catheterization in the remaining 20 patients, before and 3 months after the treatment (placebo n=11, AT-877ER n=9). Although there were no significant differences between the 2 groups for the 6-min walk distance, pulmonary hemodynamics tended to be improved in the AT-877ER group, especially the prevalence of improved cardiac index from baseline, which was significantly higher in the AT-877ER than in the placebo group. In the AT-877ER group, serum levels of hydroxyfasudil, an active metabolite of AT-877ER tended to correlate with improvements in the cardiac index and mean pulmonary artery pressure.

Conclusions: Mid-term treatment with oral AT-877ER showed additional improvement in pulmonary hemodynamics in patients with PAH. (*Circ J* 2013; **77:** 2619–2625)

Key Words: Pulmonary arterial hypertension; Rho-kinase inhibitor; Signal transduction

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Pulmonary arterial hypertension (PAH), defined as mean pulmonary arterial pressure (PAP) ≥25 mmHg at rest during right heart catheterization,[1–3] is a fatal disease caused by small pulmonary artery obstruction from vascular proliferation and remodeling.[4] PAH is characterized by elevated PAP and increased pulmonary vascular resistance (PVR), frequently leading to right-sided heart failure and death.[4–6] The pathological changes of the pulmonary arteries in PAH include endothelial injury, proliferation and hypercontraction of vascular smooth muscle cells (VSMC), and migration of inflammatory cells.[4,6,7] Anticoagulant agents, vasodilators and lung transplantation are currently used for the treatment of PAH, but more effective treatment needs to be developed.[6,8,9]

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In mid-1990s, 2 Japanese groups and 1 Singapore group independently identified Rho-kinase/ROK/ROCK as an effecter of the small GTP-binding protein Rho,[10–12] which plays an important role in various cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expressions.[13–15] We and others have demonstrated that Rho-kinase activation is substantially involved in the pathogenesis of cardiovascular diseases. First, the Rho-kinase pathway plays an important role in various cellular functions in response to various vasoactive substances.[14] Second, the so-called pleiotropic effects of statins, especially of high-doses of statins, may be mediated, at least in part, by their inhibitory effects on Rho, with a resultant inhibition of Rho-kinase.[14] Third, the effectiveness of AT877 (fasudil hydrochloride), a specific Rho-kinase inhibitor, for PAH has been demonstrated.[6,16–20]

In the present study, we examined the effects of mid-term oral treatment with an extended-release formulation of AT-877 (AT-877ER) in patients with PAH.

Methods

This study was a phase IIa clinical trial with fasudil for pulmonary arterial hypertension conducted by Asahi Kasei Pharma Corporation (Tokyo, Japan).

The ethics committees of all participating institutes approved the study protocol and all patients provided written informed consent. This report follows the recommendations of the 2010 Consolidated Standards of Reporting Trials Statement.

Study Patients

Patients with PAH were included when they had a baseline 6-min walk distance of ≥150 m with WHO functional class I–III. However, patients were excluded if they had received treatment with epoprostenol sodium, vardenafil hydrochloride hydrate, or tadalafil, or if they had changed dosages of bosentan, beraprost sodium, sildenafil citrate, warfarin potassium, calcium antagonists, cardiac glycosides or diuretics and/or doses of oxygen and nitrogen monoxide within 30 days, or had been started on any such regimens within 30 days prior to consent (for warfarin potassium, however, dosage modification as adjustment of the international normalized ratio of prothrombin was allowed). Further, patients were excluded if they had received concomitant treatment with bosentan and sildenafil citrate within 30 days prior to their informed consent. Patients with serum creatinine levels exceeding the upper limit of the study site's reference range were also excluded.

Study Design

From the viewpoint of feasibility, the sample size was planned as 30 patients in total (10 patients for WHO functional class I and 20 for functional classes II–III. The present study was designed as a 3-month, double-blind, randomized, placebo-controlled, multicenter trial in which 14 PAH centers in Japan participated. All patients were hospitalized 3–6 days before the first examination (day 1) and the last examination (week 12) (Figure S1). Administration of the study drug was started and ended during the hospitalization periods. Patients received either AT-877ER or placebo capsule twice daily (Asahi Kasei Pharma Corporation, Tokyo, Japan) for 12 weeks in a blind manner (Figure S1). The dosage of AT-877ER was increased every 3 days in a stepwise manner from 2 to 6 capsules/day (Figure S1). All patients were administered 2 capsules/day until day 4, when the dose was increased by the investigator's decision to 4 capsules/day. Until day 7, 4 capsules/day were given and the next doses were decided by investigators on day 7. Before increasing the study drug on days 4, 7, and 10, investigators checked the safety of the treatment in each subject and determined the subsequent treatment plan as follows. Whenever it was difficult to follow the intended regimen because of adverse effects or other reasons, the situation was required to be judged as "continuation at the dose level at the time of occurrence of the adverse drug reaction", "continuation with reduced dosage", or "discontinuation of study treatment". The treatment was randomized according to the 6-min walk distance, with drugs prescribed at baseline as stratifying factors, and used minimization with a randomized method. The 6-min walk distance was assessed before drug administration and at 4, 8 and 12 weeks of administration of the study drug. Cardiac catheterization was performed on the first and last days of the treatment protocol (Figure S1).

Diagnosis of Pulmonary Hypertension

PAH was defined as mean PAP ≥25 mmHg with pulmonary capillary wedge pressure (PCWP) ≤15 mmHg at rest.[1,2,6] Connective tissue disease and liver disease were diagnosed clinically and by blood tests. Congenital heart disease was diagnosed by echocardiography, and chronic thromboembolic pulmonary hypertension was diagnosed by ventilation-perfusion RI scans and computed tomography (CT).[6] Pulmonary function tests, arterial blood gases, chest X-ray and CT scan were used to diagnose lung disease and hypoxia. When the aforementioned abnormalities were ruled out, the patients were diagnosed as having idiopathic PAH (IPAH).[2,7] Heritable PAH was diagnosed as IPAH with a family history of PAH.[2,7,21]

Data Collection

Baseline demographic information (including age, sex, height and body weight), clinical diagnosis, comorbidities (connective tissue disease, liver disease, congenital heart disease, and thyroid dysfunction) and hemodynamic data from catheterization were recorded for each patient. Hemodynamic parameters examined included PCWP, PAP, right atrial pressure (RAP), cardiac output (CO), cardiac index (CI), systolic, diastolic and mean blood pressures, PVR, systolic vascular resistance, and mixed venous oxygen saturation. Blood data, including serum levels of creatinine and N-terminal pro-brain natriuretic peptide (NT-pro-BNP), plasma levels of BNP and other renal and liver functions, and urinary data were also collected.

To measure CO, both thermodilution and the Fick method were performed in 7 patients in the placebo group and in 4 in the AT-877ER group. In the present study, thermodilution data

Table 1. Baseline Characteristics of the Patients

	Placebo (n=12)	AT-877ER (n=11)
Age (years)	51.4±16.2	47.4±14.2
Female/Male (n)	10/2	6/5
Weight (kg)	51.0±7.9	62.9±12.6
WHO functional class (n)		
I	2	0
II	9	9
III	1	2
IV	0	0
Cause of PAH (n)		
Idiopathic	6	2
Connective tissue disease	3	5
Congenital heart disease	2	4
Portal hypertension	1	0
Background therapy		
Naive	2	0
Naive within 1 month of written informed consent	1	1
Beraprost alone	4	3
Bosentan	4	5
Sildenafil	1	2
Diuretics		
Without	8	5
With	4	6
Warfarin potassium		
Without	5	5
With	7	6
Oxygen therapy		
Without	5	3
With	7	8
Duration of PAH (years)		
<1	5	4
1–10	4	4
≥10	3	3
Pulmonary arterial pressure (mmHg)	47.2±14.3	40.5±17.2
Pulmonary vascular resistance (dyne·s^{-1}·cm^{-5})	865.7±476.9	687.9±550.3
Cardiac index (L·min^{-1}·m^{-2})	2.456±0.542	2.609±1.035
Arterial oxygen saturation (%)	95.56±2.58	94.63±2.30
Oxygen saturation of pulmonary artery (%)	70.18±6.36	70.30±6.18
6-min walk distance (m)	397.3±107.7	392.9±107.5
BNP (pg/ml)	119.3±140.5*	107.2±236.3
Creatinine (mg/dl)	0.65±0.11	0.77±0.18

Data are shown as mean±SD. *n=11.
BNP, B-type natriuretic peptide; PAH, pulmonary arterial hypertension.

acquired by the same technique during cardiac catheterization on the first and the last days of the treatment protocol took precedence over that acquired with the Fick method.

Blood samples were taken before oral administration of placebo or AT-877ER to measure the blood concentration of hydroxyfasudil, an active metabolite of fasudil, on the same day as cardiac catheterization was performed.[18]

Efficacy Endpoint

Efficacy was judged as a change in pulmonary hemodynamics and 6-min walk distance from baseline after 12 weeks of therapy.

Statistical Analysis

The analysis data set comprised all randomized patients who received at least 1 dose of the study medications. No estimation of missing data was performed. Demographics and baseline characteristics were summarized with descriptive statistics, including mean and standard deviation (SD) for continuous variables or counts and percentages for categorical variables. Changes from baseline for the hemodynamic parameters and 6-min walk distance were summarized with mean and SD. Student's t-test was performed for comparison of the AT-877ER and placebo treatment groups, and 2-sided 95% confidence intervals for the difference between treatment groups were calculated. Percent changes from baseline were also analyzed in a similar manner. Treatment comparison of the pro-

Table 2. Changes in Cardiac Hemodynamics and 6-min Walk Distance in the Placebo and AT-877ER Groups at Week 12

	Change from baseline		Difference between groups	
	Placebo (n=11)	AT-877ER (n=9)	Difference (95% CI)	P value*
Mean pulmonary arterial pressure (mmHg)	2.2±8.6 (11)	−0.6±2.9 (9)	−2.7 (−8.7 to 3.2)	0.3398
Pulmonary vascular resistance (dyne·s^{-1}·cm^{-5})	72.2±252.1 (11)	−31.8±137.6 (9)	−104.0 (−301.5 to 93.4)	0.2829
Cardiac index (L·min^{-1}·m^{-2})	0.09±0.397 (11)	0.368±0.496 (9)	0.278 (−0.141 to 0.697)	0.1805
Arterial oxygen saturation (%)	−0.48±3.61 (10)	−0.96±2.68 (9)	−0.48 (−3.58 to 2.63)	0.7507
Oxygen saturation of pulmonary artery (%)	−0.53±4.75 (11)	−2.10±3.48 (9)	−1.57 (−5.57 to 2.42)	0.4192
6-min walk distance (m)	31.3±47.9 (12)	18.9±32.3 (9)	−12.4 (−51.1 to 26.4)	0.5131

Data are shown as mean±SD. *t-test.
95% CI, 95% confidence interval.

Table 3. Categorical Counting for Cardiac Index

	Change from baseline >0		Difference between groups	
	Placebo (n=11) % (n/N)	AT-877ER (n=9) % (n/N)	Difference (95% CI)*	P value#
Cardiac index (L·min^{-1}·m^{-2})	45.5% (5/11)	88.9% (8/9)	43.4% (7.6 to 79.3)	0.0428

*No continuity correction; #Chi-square test.

portion of patients who showed improvement in their CI from baseline was performed using the chi-square test. No multiplicity adjustment was performed. All statistical analyses were performed using SAS (version 9.1.3 or later, SAS Institute, Cary, NC, USA).

Results

Patient Enrollment

Of the 34 patients included in this trial, 32 were enrolled for randomization of treatment with either AT-877ER or placebo (Figure S2); 2 patients failed to meet the inclusion criteria. After randomization, 23 of the 32 patients started receiving the study drug, because 5 patients in the AT-877ER group and 4 in the placebo group were excluded according to the inclusion/exclusion criteria (mean PAP <25 mmHg in 4 in the AT-877ER group and in 3 in the placebo group; PCWP >15 mmHg in 1 in the placebo group; serum creatinine level exceeding the upper limit in 1 in the AT-877ER group) (Figure S2). Of the 23 patients, 11 were randomized into the AT-877ER group and 12 as the placebo group (Figure S2), and of them, 9 patients in the AT-877ER group and 11 in the placebo group completed the treatment; 2 patients in the AT-877ER group discontinued the treatment because of the adverse events of renal impairment and heart failure death, respectively, and 1 in the placebo group because of an investigator's decision (Figure S2).

Baseline Patient Characteristics

There were 2 males and 10 females in the placebo group and 5 males and 6 females in the AT-877ER group (Table 1). Age, WHO functional class, type of PAH, combination treatment, and pulmonary hemodynamics are listed in Table 1. In the placebo group, 6 had IPAH, 3 had PAH associated with connective tissue disease (CTD-PAH), 2 had PAH with congenital heart disease (CHD-PAH), and 1 had portal hypertension PAH. In the AT-877ER group, there were 2 cases of IPAH, 5 of CTD-PAH, and 4 of CHD-PAH. There were 3 naive patients in the placebo group and 1 in the AT-877ER group (Table 1).

Tolerance of the Trial Drugs

In the placebo group, 1 patient received 2 capsules/day, 2 patients had 4 capsules/day, and 8 patients had 6 capsules/day, while in the AT-877ER group, 3 patients received 2 capsules/day, 3 patients had 4 capsules/day, and 3 patients had 6 capsules/day; 2 of these patients discontinued the treatment because of renal impairment and heart failure death, respectively, both on day 10. In the placebo group of 6 capsules/day, 1 patient discontinued the treatment on day 84 because of an investigator's decision.

Hemodynamic Parameters

Baseline mean PAP and PVR were lower and baseline CI was higher in the AT-877ER group than in the placebo group (Table 1). After the 3-month study period, mean PAP and PVR tended to be improved in the AT-877ER group compared with the placebo group (Table 2; Tables S1,S2). The incidence of a CI change from baseline was significantly improved in the AT-877ER group compared with the placebo group (Table 3).

Serum Levels of Hydroxyfasudil and Pulmonary Hemodynamics in the AT-877ER Group

In the AT-877ER group, serum levels of hydroxyfasudil, an active metabolite of fasudil, were dose-dependent of AT-877ER (Figure A). Further, serum levels of hydroxyfasudil tended to correlate with the improvements in CI (Figure B) and mean PAP (Figure C), but not of PVR (Figure D).

Safety

Adverse events occurred in all patients in the treatment and placebo groups (Table S3). The patient in the AT-877ER group who died had comorbid heart failure, and a causal relationship with the study drug was ruled out. Three patients experienced serious adverse events other than death (1 in the AT-877ER group and 2 in the placebo group). Pulmonary edema and pleural effusion occurred in 1 patient in the AT-877ER group with resultant death and a causal relationship with the study drug was not definite but possible. Idiopathic thrombocytopenic purpura and increased BNP occurred in 2 patients, respectively, in the placebo group.

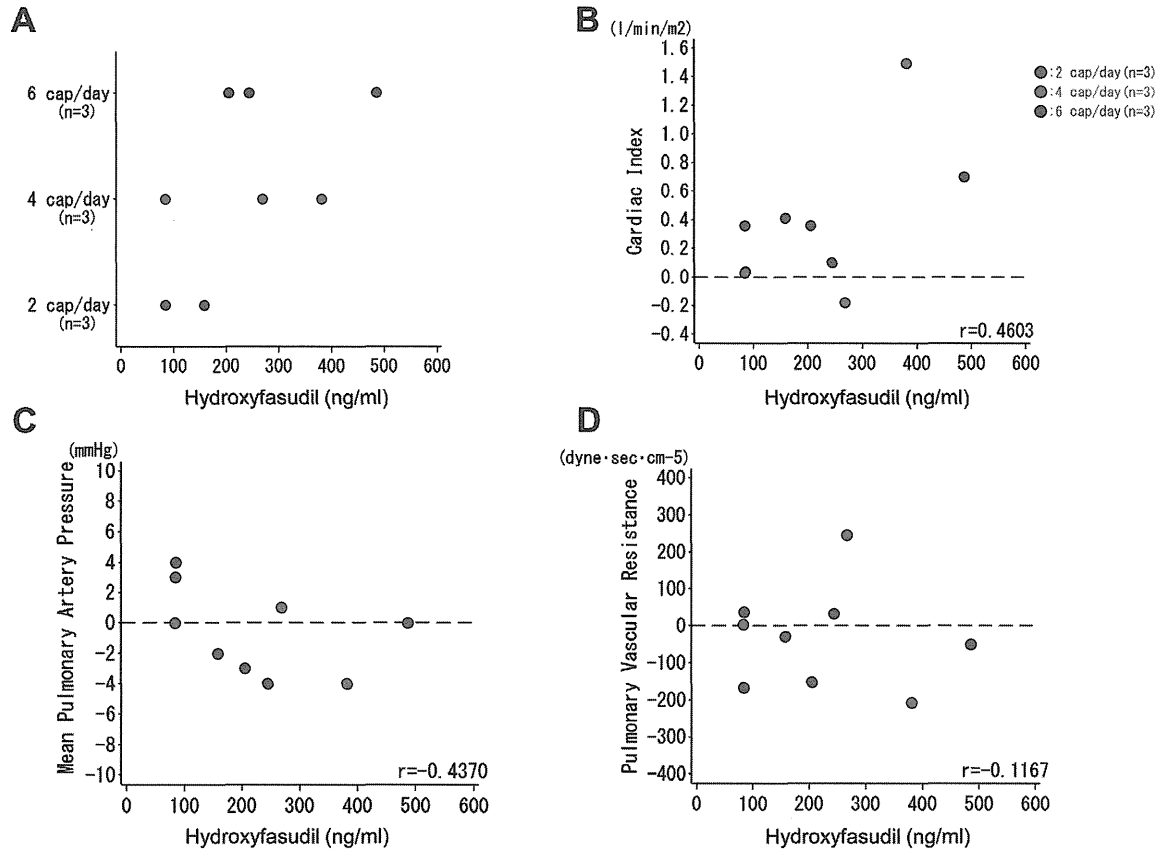

Figure. Serum levels of hydroxyfasudil and pulmonary hemodynamics. (**A**) Serum levels of hydroxyfasudil hydrochloride were dose-dependent of AT-877ER. (**B–D**) Serum levels of hydroxyfasudil tended to correlate with improvements in the cardiac index (**B**, r=–0.4370, P=0.2125) and mean pulmonary artery pressure (**C**, r=0.4603, P=0.2395), but not pulmonary vascular resistance (**D**, r=0.1167, P=0.7650). r, Spearman's rank correlation coefficient.

There were 3 discontinuations (2 in the AT-877ER group and 1 in the placebo group). The 2 discontinuations in the AT-877ER group included the patient who had cardiac failure, pulmonary edema and pleural effusion and eventually died, and 1 patient with renal impairment (increased BUN and creatinine levels, and positive proteinuria). The treatment was 3 capsules per dose for these patients, including the patient who discontinued the study because of renal impairment, which had recovered 13 days after discontinuation, and the patient in the placebo group who discontinued because of personal circumstances.

Discussion

The results of the present study showed that 3-month treatment with AT-877ER, a Rho-kinase inhibitor, significantly improved the CI in patients with PAH and that serum levels of AT-877ER tended to correlate with improvements in both CI and mean PAP. Importantly, all patients in the AT-877ER group had been maximally treated with pulmonary vasodilators, including 3 different vasodilators, beraprost, bosentan, and sildenafil.

Rho-Kinase and Inflammation

Inflammatory processes may be involved in the pathogenesis of PAH.[6,22] It has been demonstrated that Rho-kinase is up-regulated by inflammatory stimuli[14,23,24] and that Rho-kinase inhibition increases endothelial nitric oxide synthase (eNOS) expression and inhibits inflammatory cell migration and angiotensin II-induced upregulation of monocyte chemoattractant protein-1 and plasminogen activator inhibitor-1 in vivo or in vitro,[16] suggesting that the Rho-kinase pathway plays an important role in the pathogenesis of PAH.

Rho-Kinase Inhibitor

AT-877ER, fasudil, is a potent and selective inhibitor of Rho-kinase,[25] with its inhibitory effect on Rho-kinase being 100-fold and 1,000-fold more potent than on protein kinase C and myosin light chain kinase, respectively.[16] Several studies, including ours, have demonstrated in animal models that long-term inhibition of Rho-kinase with fasudil ameliorates monocrotaline-induced PAH and hypoxia-induced PAH.[6,17,19,26] Consistent with these findings, intravenous administration of fasudil also effectively reduces PVR in patients with PAH.[18]

Although beraprost sodium has no inhibitory effect on Rho-kinase, we have demonstrated that the combination of fasudil and beraprost is more effective than each monotherapy for ameliorating pulmonary hypertension in a rat model of monocrotaline-induced PAH.[27,28] Furthermore, it has been consistently demonstrated that IPAH patients under intravenous prostacyclin therapy show a favorable acute responses to

fasudil administration.[18,29]

Although inhibition of the ETA and ETB endothelin receptors is another effective strategy for the treatment of PAH,[30] endothelin and many other vasoactive substances (eg, serotonin, thrombin and platelet-derived growth factor) are involved in the pathogenesis of PAH, all of which could activate the Rho-kinase pathway.[14,16,24,31] Because Rho-kinase inhibitors could inhibit signal transductions initiated by all these vasoactive substances, it is highly possible that they exert more broadly beneficial effects than each single receptor antagonist.[14,16,24,31] Thus, the present clinical trial was designed to combine beraprost/sildenafil/bosentan with fasudil in order to develop additional and more beneficial treatment of PAH.

Enhanced Rho-Kinase Expression and Activity in PAH

The experimental studies using animal models have demonstrated that Rho-kinase activity in the pulmonary arteries is enhanced irrespective of etiology and that long-term treatment with Rho-kinase inhibitors ameliorates endothelial dysfunction and suppresses the hypercontraction and proliferation of VSMC and migration of inflammatory cells.[17,19,26] We and others have shown direct clinical evidence of Rho-kinase activation in patients with PAH, in whom Rho-kinase activity is enhanced in circulating neutrophils and the pulmonary arteries, resulting in hypercontraction of the pulmonary arteries[20] and thus supporting the previous findings in both animal models of PAH and patients with PAH.[14,17–19,26,29,32] Furthermore, we have demonstrated that endothelial vasodilator function is impaired and VSMC contraction is enhanced in the pulmonary arteries from patients with PAH,[20] and that inhibition of Rho-kinase abolishes hypercontraction of the VSMCs in the pulmonary arteries from IPAH patients,[20] which could explain the mechanism for the present findings.[18,29,32]

Study Limitations

Several limitations should be mentioned. First, the study group consisted of a small number of Japanese patients with PAH, demonstrating significant effects of AT-877ER on CO but not on pulmonary hemodynamics. Our calculation using the present results predicts that it would reach statistical significance for pulmonary hemodynamics if 100 patients could be recruited for each group. Thus, the present findings need to be confirmed in future studies with a large number of patients. Second, renal impairment occurred in some patients in the AT-877ER group, although a higher concentration of hydroxyfasudil seemed to be favorable for improving pulmonary hemodynamics. Thus, the appropriate dosage of AT-877ER remains to be determined in future trials. Third, the long-term effects of AT-877ER (ie, >3 months) remain to be examined in PAH patients in future clinical trials. Fourth, more male patients were enrolled in the AT-877ER group because there was no randomization by sex. The sex difference in Rho-kinase activity remains be examined in future studies with a larger number of patients. Fifth, the AT-877ER group had better pulmonary hemodynamics and more prevalence of congenital heart disease, which should be adjusted in future studies with a larger number of patients.

Conclusions

Treatment with AT-877ER, an oral form of Rho-kinase inhibitor, could be a new strategy in addition to the present medical treatment of PAH.

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YF: analysis and interpretation of data, drafting of the manuscript. NY, HM, MM, KU, AY, YK, MK, HW, YT, TA, SO, NY, TI: acquisition of data. TN: analysis and interpretation of data; HS: study conception, design, and final approval of the manuscript.

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Disclosures

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Patient Consent

Obtained.

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Supplementary Files

Supplementary File 1

Figure S1. Study design.

Figure S2. Numbers of patients enrolled in the present study who underwent screening and randomization.

Table S1. Cardiac hemodynamics changes and adverse events in the placebo and the AT-877ER groups

Table S2. %Changes in cardiac hemodynamics and 6-min walk distance in the placebo and AT-877ER groups at week 12

Table S3. Adverse events

Please find supplementary file(s);
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Long-term patient survival with idiopathic/heritable pulmonary arterial hypertension treated at a single center in Japan

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ABSTRACT

Aims: Idiopathic/heritable pulmonary arterial hypertension (I/HPAH) carries a poor prognosis despite the therapeutic options available. Patient survival from Western countries has been reported, but data from Asia are scarce.

Main methods: We retrospectively reviewed 56 patients with I/HPAH treated at a single referral center in Japan. Survival analyses were conducted using the Kaplan–Meier method with the log-rank test. Variables associated with survival were determined using a Cox proportional hazard model.

Key findings: There were 41 women (73%) and the mean age at the diagnosis was 32 ± 17 years. Mean survival time from the diagnosis was 14.9 ± 0.8 years (95% CI, 13.4–16.4 years), with 1-, 2-, 3-, 5- and 10-year survival rates of 98, 96, 96, 96 and 78%, respectively. In patients who underwent follow-up right-heart catheterization >3 months after initial catheterization, mean pulmonary arterial pressure (mPAP) was decreased significantly from 63 ± 15 to 35 ± 10 mm Hg with an improved cardiac index. Patients with high levels of brain natriuretic peptide (BNP) or low oxygen saturation at baseline showed worse survival. At follow-up, 98% of patients were on PAH-targeted drugs. WHO functional classes I and II, mPAP <42.5 mm Hg, cardiac index >2.5 L/min/m^2, BNP <52 pg/mL, and 6-min walk distance >347 m at follow-up were predictors of good prognosis in the univariate analysis.

Significance: The study revealed a long-term survival of Japanese patients with I/HPAH. Hemodynamic parameters improved significantly after treatment, which might be related to high prescription rates of PAH-targeted drugs. Multicenter studies are needed to reveal the prognostic factors for I/HPAH.

Introduction

Pulmonary arterial hypertension (PAH) has been reported to carry a poor prognosis despite the therapeutic options available. In the past two decades, several PAH-targeted drugs have become available. Each PAH-targeted drug has been reported to improve the prognosis or progression of PAH (Sitbon et al., 2002; McLaughlin et al., 2005; Rubin et al., 2011). A treatment algorithm that includes all of these treatment options is now shown in the guidelines for treatment of pulmonary hypertension (Galie et al., 2009). However, despite all the improvements in treatment, overall survival has been reported to be unsatisfactory (Humbert et al., 2010; Lee et al., 2012; Benza et al., 2012).

Although survival analyses of patients with PAH have been reported from Western countries, there is a shortage of data from Asia. A report from China demonstrated better survival of patients compared with previous reports despite the limited treatment options (Zhang et al., 2011). There is no report from Japan on the survival of patients treated with PAH-targeted drugs. To elucidate the survival of Japanese patients with idiopathic pulmonary arterial hypertension/heritable pulmonary arterial hypertension (I/HPAH), we conducted a retrospective study at a single center in Japan that deals with referrals for subjects with pulmonary hypertension.

Materials and methods

Patient selection

We undertook a retrospective review of medical charts on 56 consecutive patients with I/HPAH who received treatment at the National Hospital Organization Okayama Medical Center (Okayama, Japan) between October 1998 and December 2012. The study protocol was approved by the Institutional Review Board of our hospital. The diagnosis was based on detailed medical history, physical examination,

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and standardized diagnostic approach for PAH (Galie et al., 2009). An "incident case" was defined as a patient who was referred to our hospital in <30 days after diagnostic catheterization, or the initial diagnostic catheterization was conducted at our hospital. All other cases were considered as "prevalent cases".

Study protocol

Physical examination, laboratory measurements, 6-min walk test, and right-heart catheterization were undertaken before treatment was initiated. Examinations were conducted repeatedly according to physical status. The follow-up period for analyses of survival data ended in March 2013. The end-point for survival analyses was disease-related death.

Clinical outcomes

Follow-up data were collected when patients achieved the best values for the mean pulmonary arterial pressure (mPAP) with preserved cardiac index. WHO functional class, 6-min walk distance (6MWD), plasma levels of brain natriuretic peptide (BNP) and uric acid, hemodynamic parameters [mPAP, right atrial pressure (RAP), pulmonary capillary wedge pressure, mixed venous oxygen saturation, cardiac index, and pulmonary vascular resistance (PVR)], heart rate, and oxygen saturation (SpO_2) were compared between baseline and follow-up. In patients who did not undergo follow-up catheterization, the last available data (other than hemodynamic data) was evaluated.

Treatments

We also evaluated the treatment received by patients. For survivors, treatment data were collected when the follow-up data were collected as described above. For non-survivors, treatment data were collected at the time when patients received maximum treatment. With regard to intravenous prostacyclin, all patients received epoprostenol except for one patient who received treprostinil. We evaluated the maximum doses of epoprostenol.

Statistical analyses

Results are expressed as the mean ± standard deviation, unless otherwise specified. Continuous variables were compared using *t*-tests. The χ^2 test was used to assess the significance of differences between categorical variables. WHO functional class is expressed as the median and number of patients in each class, and changes in WHO functional class were evaluated using the Wilcoxon signed rank test. Survival analyses were conducted using the Kaplan–Meier method. Differences between survival curves were assessed using the log-rank test. A Cox proportional hazard model was conducted to determine the variables associated with increased mortality. The hazard ratio (HR) and 95% confidence interval (CI) were defined. To confirm their predictive value, variables with $P < 0.1$ were tested in a multivariate model. Receiver operating characteristic (ROC) curves were constructed to determine an optimal cutoff value for 6MWD, BNP, mPAP, RAP, cardiac index, and SpO_2. All analyses were undertaken with IBM SPSS Statistics 20 (IBM, Armonk, NY, USA). Statistical significance was defined as $P < 0.05$.

Results

Baseline characteristics

There were 41 women (73%) and 15 men (27%) in the study. The mean age was 32 ± 17 years, with a range of 5–69 years at the diagnosis. There were 24 incident cases and 32 prevalent cases. Patients had been treated for 1.4 ± 2.3 years (0.0–8.1 years) at the beginning of the study. Time between the diagnosis and initiation of treatment was 0.4 ± 2.5 years (−0.1 to 18.9 years). Ninety-six percent of patients were initiated treatment <1 year after the diagnosis. At the time of diagnosis, 11 patients were in WHO functional class II, 39 in class III, and 6 in class IV. In 32 prevalent cases, one patient was in WHO functional class II, 20 in class III, and 11 in class IV at the time of diagnosis. By the time of referral, one patient improved from class IV to III, 16 patients remained in the same functional class, and 15 patients' conditions were deteriorated. Upon referral to our hospital, one patient was in WHO functional class II, 38 in class III, and 17 in class IV. Hemodynamic parameters measured at baseline were also evaluated: mPAP was 61 ± 15 mm Hg, cardiac index was 2.4 ± 0.9 L/min/m^2, and PVR was 1375 ± 611 dyn·s/cm^5.

HPAH and genetic testing

Eight families with 10 patients (18%) with a family history of pulmonary hypertension were included. Genetic analyses were conducted in 35 patients (including nine cases with HPAH). One patient with HPAH had not undergone genetic analyses. Four patients from two families (two patients from each pedigree included in this study) and two other patients with HPAH from two different families had a BMPR2 mutation. Of the remaining three patients with HPAH and 26 patients who seemed to be sporadic, no BMPR2 or ALK1 mutation was detected.

Treatment

All patients, except for one who responded to a calcium channel blocker, were receiving PAH-targeted drugs: prostacyclin analogs ($n = 52$, 93%), endothelin receptor antagonists ($n = 38$, 68%), and phosphodiesterase type 5 (PDE5) inhibitors ($n = 29$, 52%). Intravenous prostacyclin was highly prescribed ($n = 43$, 77%). Forty-two patients (75%) were treated with combination therapy. Thirteen patients (23%) were on warfarin and 53 patients (95%) were on oxygen therapy.

Overall survival

Seven patients died during the study period: one from alveolar hemorrhage and six from heart failure. Other than these patients, two patients were censored: one underwent lung transplantation and another died in a traffic accident, despite pulmonary hypertension being well controlled. Fig. 1A shows overall survival. Mean survival time from the diagnosis was 14.9 ± 0.8 years (95% CI, 13.4–16.4 years), with 1-, 2-, 3-, 5- and 10-year survival rates of 98, 96, 96, 96 and 78%, respectively.

Baseline data of survivors and non-survivors

Baseline characteristics of survivors and non-survivors are shown in Table 1. WHO functional class, 6MWD, BNP, and RAP were significantly worse in non-survivors than in survivors. There was no significant difference in remaining baseline hemodynamic parameters between survivors and non-survivors. Treatment was also evaluated. There was no significant difference in prescription rate, except for PDE5 inhibitors and triple PAH-targeted therapy. Non-survivors received PDE5 inhibitors less frequently than survivors (14% vs. 57%, $P < 0.05$) and none of the non-survivors received triple therapy.

Follow-up data

At follow-up, WHO functional class, 6MWD, and BNP were significantly improved (Table 2). Forty-three patients underwent follow-up right-heart catheterization >3 months after initial catheterization at our hospital. An average of the time by the follow-up catheterization evaluated in this study was 3.7 ± 2.8 years (0.1–11.7 years).

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