Table 4. Standardized multiple linear regression analysis for air pressure 3 days before evaluations and synovitis among the 326 patients.

Synovitis	Mean±SD(median)	mean Beta±SD	P*
SJC	2.07±1.99 (1)	−0.126±0.0425	0.00023
TJC	2.08±2.19 (1)	−0.0893±0.0339	0.036
SJC+TJC	4.15±3.70 (2)	−0.215±0.0672	0.0015

*p-values for Student's t-test. SD:standard deviation.
doi:10.1371/journal.pone.0085376.t004

air pressure and RA joint synovitis instead of limited association between air pressure and a specific element of RA joint synovitis. At the same time, these results may suggest that composite measures composed of multiple elements showing weak association with air pressure are not sensitive enough to detect the influence of air pressure. In addition, because many medical doctors evaluate synovitis of many patients with RA, variations among doctors in each element would be amplified in the composite measure and result in difficulty to detect significant correlations. Considering the strong association between air pressure and sum of SJC and TJC, the fine direction of association between different elements and air pressure should partly overlap but partly differ. PVAS showed the lowest mean correlation coefficient among items of composite measures. This matches our observation that many of the RA patients feel correlation between their symptoms and air pressure. Although the number of data for pVAS is limited, 81 patients with more than 15 data for pVAS revealed a suggestive inverse correlation with air pressure (mean rho = −0.0469, data not shown). Accumulation of more data for each element would detect a significant association between each element and air pressure and a fine direction of association with air pressure in each element.

Because the number of joint assessments differed among patients and it was likely that analyses using all assessments would be influenced by the specific patients with large number of joint assessments, we performed the analyses focusing on the 326 patients with more than 20 assessments to avoid intra-patient correlations. We hypothesized that correlations between air pressure and joint synovitis should be largely different among patients but a large number of patients would result in a significant deviation. We calculated a correlation coefficient in each patient separately. The results supported our hypothesis and showed a significant deviation of the mean correlation coefficient from the null hypothesis. Figure 1 presenting patients with median correlations along with Table S2 indicated that overall distributions of correlations between air pressure and joint synovitis shifted to negative correlations. Considering the size of correlation coefficients, although RA patients show a negative correlation in average between air pressure and joint synovitis, the correlation greatly vary among patients and the correlation should not be generalized.

We found the strongest associations between air pressure and signs of synovitis three days before evaluations. This may indicate that slow mechanisms underlie the correlations or that joint synovitis is prone to unknown factors which reflect past air pressure.

When we classified patients into subgroups based on positivity of disease duration, smoking, Stage, Class and usage of biological agents during the observation period, we did not find significant difference among RA subsets (data not shown). The analysis of confounding factors revealed that the association of air pressure with joint synovitis was not derived from humidity and temperature, which were selected since air pressure, humidity and temperature are representatives of meteorological factors. Although both humidity and temperature showed correlations with air pressure (rho:0.19 and 0.58, between air pressure and humidity or temperature, respectively), their correlations could not explain the association between air pressure and RA synovitis. The analysis also revealed that humidity showed a negative association with joint synovitis that is independent from temperature and air pressure. A previous study reported that a combination of increase in humidity and decrease in air pressure were associated with worsening of joint pain [14]. Their findings matched our results for air pressure, but the association of humidity was opposite to ours. Thus, the association between humidity and joint synovitis is inconclusive and further studies are required. It is notable that mean temperature was not associated with joint synovitis in a multiple standardized linear regression analysis. As increase or decrease of blood flow due to temperature is supposed to influence signs of synovitis in RA, the lack of the association may be explained by rapid influence of finely conditioned temperature in hospitals.

It is difficult to assume the basic mechanisms underlying the correlation between joint synovitis and air pressure. One possible explanation is that air pressure directly presses joint structures in patients with RA. Low air pressure results in reduced outside pressure of joints which allow joints to be swollen more easily. Enlarged space of joints would allow more inflammatory cells to enter joint space and produce inflammatory cytokines. Another explanation is involvement of autonomic nerves to regulate threshold of pain. A Japanese group reported that both decreased air pressure and temperature led to worsening of joint pain in an animal model [22,23]. The group also showed that these correlations in the animal model were mediated by sympathetic nerve, whose excitement and increase of circulating noradrenaline were brought about by decrease of air pressure [24,25]. As the current study did not reveal a strong association between joint tenderness and air pressure, involvement of sympathetic nerve with pain cannot fully explain the current results. Variations of B-cell activity due to meteorological changes could be another possibility. Our previous study reported seasonal variation of IgG in rheumatic diseases [26]. Analysis incorporating altitude of residence for each patient, whose information is not available in the current study, would give a clue for the mechanism underlying the association.

We could not conclude whether air pressure directly influences RA synovitis or if it is just a confounding factor of yet-to-be-determined factors with direct effects on RA synovitis. However, our analysis supports the patients' subjective feelings of relationship between air pressure and joint synovitis. Another study addressing correlations between air pressure and joint synvoitis estimated by imaging including ultrasound seems necessary. Further experiments and analyses between air pressure and joint symptoms in humans would clarify the detailed mechanisms. It will be interesting to determine the characteristics of patients who are susceptible to change of air pressure.

Materials and Methods

Ethical statements

The analyses in the current study were performed under policy of data analysis of the KURAMA database approved by Kyoto University Hospital Ethical Committee [20]. Written informed consent to enroll in the database described below was obtained

from most of the patients, but for some patients the information regarding the construction of this database was disclosed instead of obtaining written informed consent. Participants who were informed regarding the construction of the database (instead of obtaining written informed consent) were allowed to withdraw from the study if desired. All data were de-identified and analyzed anonymously. This study was designed in accordance with the Helsinki Declaration.

Data of joint synovotis in patients with RA

A total of 23,064 evaluations of disease activity from 2,131 patients with RA were obtained with the corresponding dates of evaluations from the KURAMA database. The evaluation data contained some or all of the SJC, TJC in the 28 joints, ESR, pVAS and dVAS as well as DAS 28 as a composite measure for RA disease activity. The sum of SJC and TJC was also calculated for each evaluation. 326 patients with more than 20 evaluations of disease activity were extracted for further analysis.

Data of air pressure and other meteorological factors

Data of daily mean air pressure in Kyoto from 2005 to 2012 was obtained from the homepage of Japan Meteological Agency (http://www.jma.go.jp/jma/index.html). Data of daily mean temperature and humidity in Kyoto were also obtained in the same manner.

Statistical analysis

Correlations between mean air pressure on the day of joint evaluation and DAS28, SJC, TJC, ESR, pVAS, dVAS or sum of SJC and TJC were estimated by Spearman's correlation coefficients, using 23,064 evaluations of joint synovitis or evaluations in each of the 326 patients. The mean Spearman's correlation coefficients among the 326 patients with RA were estimated by Student's t-test under the null hypothesis that the mean was zero. Normality of distribution of correlation coefficients in the 326 patients was analyzed by Shapiro-Wilk test. A daily mean air pressure of the six days before the day of joint evaluation was also analyzed for correlations with signs of joint synovitis across the 326 patients with RA. To analyze independent effects of air pressure on joint synovitis from humidity and temperature, multiple standardized linear regression analysis was performed for 23,064 evaluations and each of the 326 patients with more than 20 evaluations. Mean beta values in the multiple standardized linear regression analysis among the 326 patients were assessed by Student's t-test under the null hypothsis that the mean beta values were zero. P-values less than 0.0071 were regarded as significant based on Bonferroni's correction. Data analysis was performed by R software (http://www.r-project.org/) or SPSS (ver 18).

Supporting Information

Figure S1 Fluctuations of air pressure and joint synovitis in the 326 patients. Fluctuations of each item are illustrated between 2005 and 2012. The three figures of SJC, TJC and combination of SJC and TJC are composed of 326 lines presenting fluctuations in the 326 patients.
(TIF)

Table S1 Correlation coefficients of RA joint synovitis in association with air pressure across different evaluations.
(DOC)

Table S2 Detailed information of the 326 RA patients.
(DOC)

Acknowledgments

We would like to thank Mr. Wataru Yamamoto at Kurashiki Sweet Hospital for his excellent support to establish and maintain the KURAMA database.

Author Contributions

Conceived and designed the experiments: CT MH FM HI TF TM. Analyzed the data: CT. Contributed reagents/materials/analysis tools: CT MH MF SN KO RN YI NY HY HI TF TM. Wrote the paper: CT MH.

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PLOS ONE

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."

Citation: Hamaguchi Y, Fujimoto M, Matsushita T, Kaji K, Komura K, et al. (2013) Common and Distinct Clinical Features in Adult Patients with Anti-Aminoacyl-tRNA Synthetase Antibodies: Heterogeneity within the Syndrome. PLoS ONE 8(4): e60442. doi:10.1371/journal.pone.0060442

Editor: Frederick Miller, National Institutes of Health, United States of America

Received November 13, 2012; **Accepted** February 27, 2013; **Published** April 3, 2013

Funding: This work was supported by a research grant on intractable diseases from the Ministry of Health, Labor and Welfare of Japan (to Manabu Fujimoto). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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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].

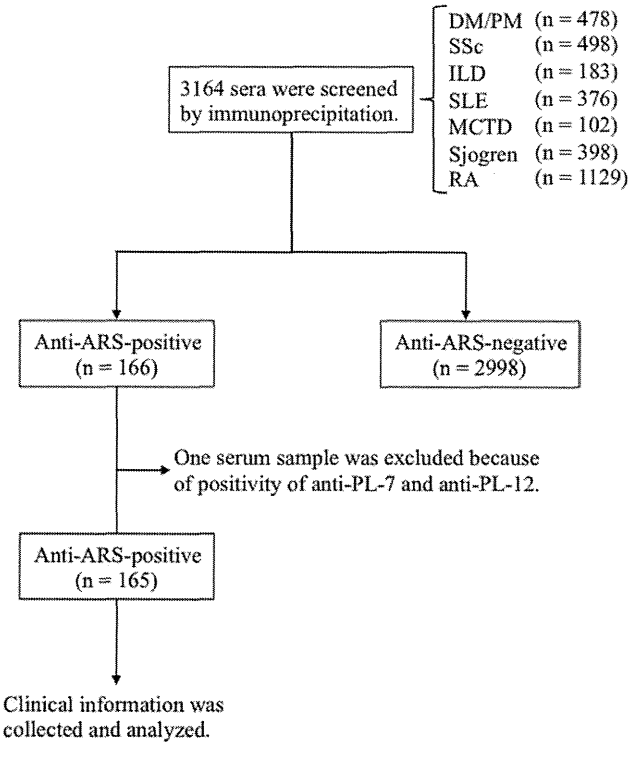

Figure 1. Enrollment and selection of patients. DM; dermatomyositis, PM; polymyositis, SSc; systemic sclerosis, ILD; interstitial lung disease, SLE; systemic lupus erythematosus, MCTD; mixed connective tissue disease, Sjogren; Sjogren's syndrome, RA; rheumatoid arthritis.
doi:10.1371/journal.pone.0060442.g001

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

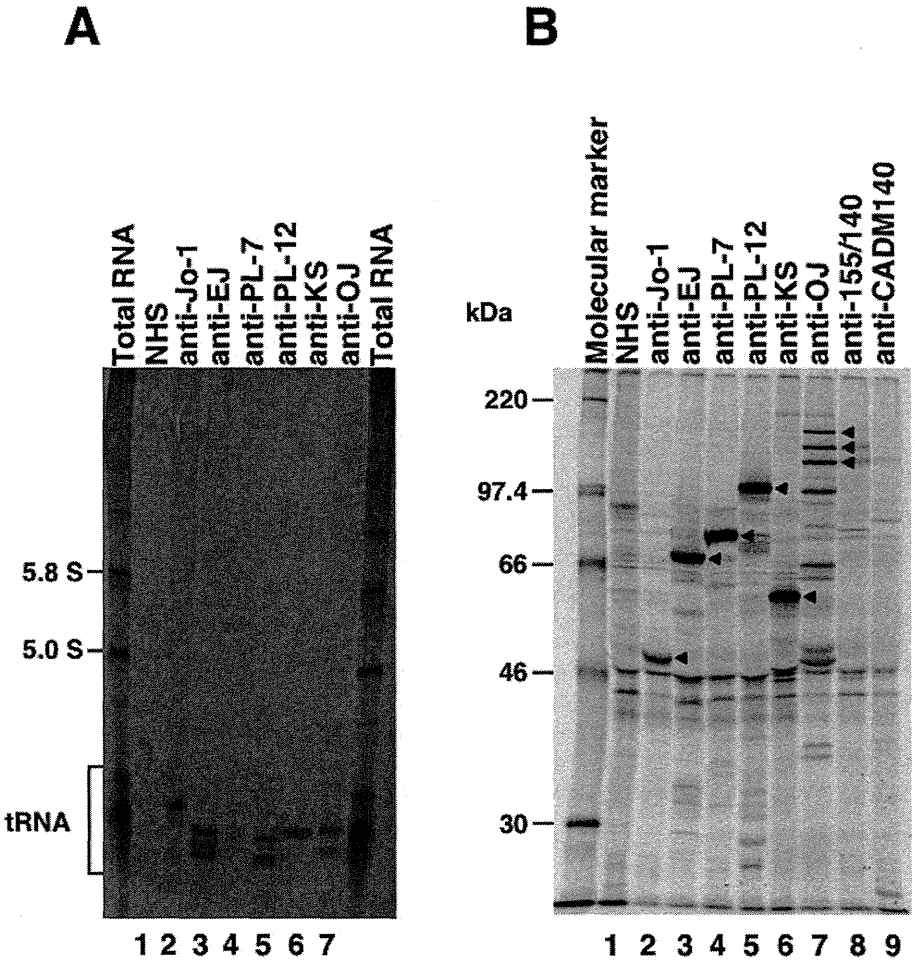

Figure 2. Representative immunoprecipitation assay for RNA with anti-aminoacyl-tRNA synthetase (anti-ARS) sera. A, Immunoprecipitation of histidyl-tRNA synthetase, glycyl-tRNA synthetase, threonyl-tRNA synthetase, alanyl-tRNA synthetase, asparaginyl-tRNA, and isoleucyl-tRNA synthetase by sera. K562 cell 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.
doi:10.1371/journal.pone.0060442.g002

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.
doi:10.1371/journal.pone.0060442.t001

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

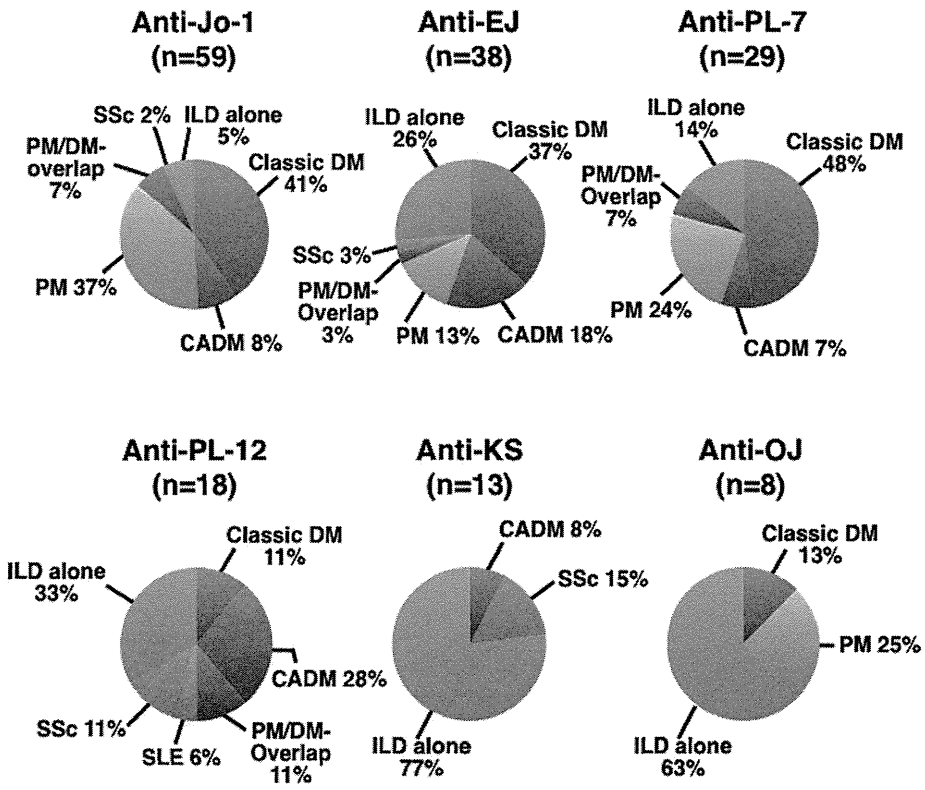

Figure 3. Prevalence of dermatomysitis (DM), clinically amyopathic DM (CADM), polymyositis (PM), PM/DM-overlap, systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and interstitial lung disease (ILD) alone, in each subgroup of anti-synthetase syndrome.
doi:10.1371/journal.pone.0060442.g003

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.

doi:10.1371/journal.pone.0060442.t002

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.
doi:10.1371/journal.pone.0060442.t003

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.
doi:10.1371/journal.pone.0060442.t004

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

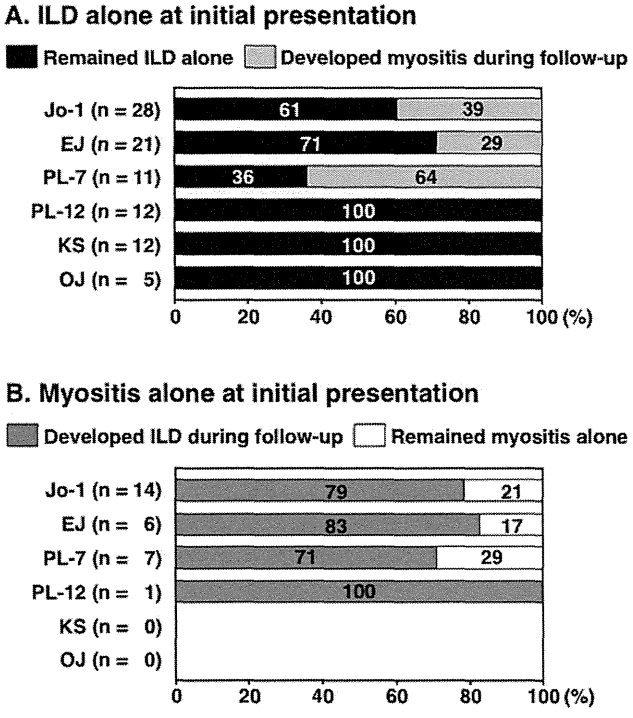

Figure 4. The clinical course of anti-synthetase syndrome patients who developed myositis or interstitial lung disease (ILD) with or without skin manifestations at disease onset. According to the clinical course, patients were classified into four types: remained with ILD alone, developed myositis during 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).
doi:10.1371/journal.pone.0060442.g004

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.
doi:10.1371/journal.pone.0060442.t006

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.

Acknowledgments

We thank Ms. Masako Matsubara, Yuko Yamada, Tomoko Hayashi, and Natsuho Yoshifuji for technical assistance.

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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Two Susceptibility Loci to Takayasu Arteritis Reveal a Synergistic Role of the *IL12B* and *HLA-B* Regions in a Japanese Population

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Takayasu arteritis (TAK) is an autoimmune systemic vasculitis of unknown etiology. Although previous studies have revealed that HLA-B*52:01 has an effect on TAK susceptibility, no other genetic determinants have been established so far. Here, we performed genome scanning of 167 TAK cases and 663 healthy controls via Illumina Infinium Human Exome BeadChip arrays, followed by a replication study consisting of 212 TAK cases and 1,322 controls. As a result, we found that the *IL12B* region on chromosome 5 (rs6871626, overall $p = 1.7 \times 10^{-13}$, OR = 1.75, 95% CI 1.42–2.16) and the *MLX* region on chromosome 17 (rs665268, overall $p = 5.2 \times 10^{-7}$, OR = 1.50, 95% CI 1.28–1.76) as well as the *HLA-B* region (rs9263739, a proxy of HLA-B*52:01, overall $p = 2.8 \times 10^{-21}$, OR = 2.44, 95% CI 2.03–2.93) exhibited significant associations. A significant synergistic effect of rs6871626 and rs9263739 was found with a relative excess risk of 3.45, attributable proportion of 0.58, and synergy index of 3.24 ($p \leq 0.00028$) in addition to a suggestive synergistic effect between rs665268 and rs926379 ($p \leq 0.027$). We also found that rs6871626 showed a significant association with clinical manifestations of TAK, including increased risk and severity of aortic regurgitation, a representative severe complication of TAK. Detection of these susceptibility loci will provide new insights to the basic mechanisms of TAK pathogenesis. Our findings indicate that *IL12B* plays a fundamental role on the pathophysiology of TAK in combination with HLA-B*52:01 and that common autoimmune mechanisms underlie the pathology of TAK and other autoimmune disorders such as psoriasis and inflammatory bowel diseases in which *IL12B* is involved as a genetic predisposing factor.

Introduction

Takayasu arteritis (TAK [MIM 207600]) is an autoimmune systemic vasculitis that was first reported from Japan.[1] It is estimated that TAK affects around 0.004% of the population in Japan, especially young women aged between 15 and 35. Although TAK was originally thought to affect individuals of mainly Asian origin, individuals with TAK have been identified worldwide, though with lower prevalence compared to Asia.[2] TAK is characterized by the involvement of large arteries, especially the aorta and its large branches, and is grouped into "vasculitis affecting large vessels" according to the Chapel Hill classification.[3] Individuals with TAK develop a wide range of symptoms such as fatigue, syncope, and lowering of vision in addition to its characteristic complications including aortic regurgitation (AR), pulselessness, and difference of blood pressure between right and left upper limbs. Previous studies have revealed that genetic components are involved in the pathogenesis of TAK, and HLA-B*52:01 is so far the only established genetic factor across the world.[4–7] Other genetic components especially outside of the HLA locus have not been confirmed to date. Establishment of association with non-HLA regions would lead to a deeper understanding of the basics of TAK pathology and the development of a novel therapy for this vasculitis. Here, we performed a genome-scanning study of TAK to identify the genetic predisposing factors for TAK.

Subjects and Methods

Study Subjects

A total of 379 TAK cases and 1,985 controls were enrolled in this study. All the cases were diagnosed based on the criteria of

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http://dx.doi.org/10.1016/j.ajhg.2013.05.024.

Table 1. Summary of Study Subjects

	Case	Control
Genome Scanning		
Number	167	663
Age[a]	45.7 ± 15.2	53.5 ± 13.5
Female ratio	0.92	0.74
Age at onset[a]	30.5 ± 14.5	NA
Genotyping	Illumina Infinium Human-Exome BeadChip	Illumina Infinium Human-Exome BeadChip
Subjects with clinical information	AR:87; CRP:89	NA
Institutions	Kyoto University; Tokyo Women's Medical University	Kyoto University
Replication Study		
Number	212	1,322
Age[a]	46.6 ± 17.6	53.3 ± 13.4
Female ratio	0.94	0.62
Age at onset[a]	27.0 ± 11.8	NA
Genotyping	Taqman assay	Illumina Infinium Human Omni 2.5-4 BeadChip, Illumina Infinium Human Omni 2.5-8 BeadChip
Subjects with clinical information	AR:102; CRP:None	NA
Institutions	Tokyo Medical and Dental University; Kyoto University; Niigata University	Kyoto University

Abbreviations are as follows: NA, not applicable; AR, aortic regurgitation; CRP, C-reactive protein.
[a]Mean ± standard deviation (SD).

American College of Rheumatology[8] or guideline provided by Japanese Circulation Society.[9] The control subjects were collected as a part of the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (The Nagahama Study), a community-based prospective multiomics cohort study conducted by Kyoto University.[10] This study was approved by the local ethical committees at each institution, and written informed consent was obtained from each subject involved in the study.

Genome Scanning

Illumina Infinium Human Exome BeadChip arrays (Illumina) were used for genome scanning of the cases and the controls. The genome scanning was conducted in Center for Genomic Medicine, Kyoto University Graduate School of Medicine.

Quality Control of Genome Scanning

Polymorphisms showing success rates less than 0.95 in either cases or controls, departure from Hardy-Weinberg equilibrium (HWE) ($p < 1.0 \times 10^{-5}$), or minor allele frequencies less than 0.05 in both cases and controls were excluded from the analysis. Subjects who showed success rates less than 0.95 or evidence of relatedness with other subjects were also excluded. Kinship between study subjects were estimated by PLINK.[11] Quantile-quantile plot (QQ plot) was used to assess the population stratification of the study. Because 1,827 markers over 24,487 were located in the HLA locus in which polymorphisms are very closely linked with each other, the 22,660 markers in the non-HLA regions were used for QQ plot.

Replication Study

The SNPs with p values less than 1.0×10^{-5} in the genome scanning were selected for the replication study. Because the association found in the *HLA-B* region (MIM 142830) was largely attributable to HLA-B*52:01, rs9263739, a proxy of HLA-B*52:01, was selected as a representative of the HLA locus. In the replication study, case samples were genotyped by Taqman Assay (Applied Biosystems) and control genotypes were extracted from array data (Table 1).

Combined Study and Association Study for Genotypes

Association studies of genotypes were performed by chi-square test based on 2 × 2 contingency tables. Combined study of the two studies was performed by inverse-variance method, assuming a fixed-effects model from the effect size (logarithm of odds ratio [OR]) in each study. A significant level for detecting susceptibility genes was set as 2.0×10^{-6}, which was obtained by Bonferroni's correction. A stringent cut-off level of 5.0×10^{-8} was also applied to assess overall significance.

Imputation of Genotypes

Mach dat2 software[12] was used for imputation of the whole genomes based on the results of genome scans with the use of the East Asian panel of HapMap phase II data as reference. SNPs with low imputation scores (Rsq < 0.3) were excluded from the analysis.

Calculation of Linkage Disequilibrium

LD between SNPs in the Illumina Infinium Human Exome BeadChip was assessed based on the genome-scanning data. HapMap project phase II data was used when SNPs were not contained in the array. LD between HLA-B*52:01 and SNPs was calculated by combining our previous HLA-genotyping data of the 173 TAK cases (C.T., unpublished data) by WAKFlow system (Wakunaga Pharmaceutical) with the genome-scanning data.

Estimation of Interaction

We used the method for evaluation of interaction proposed by Andersson et al.[13] Gene-gene interaction was defined as departure from additivity of two loci and measured by three indices based on calculation of relative risk (RR); relative excess risk due to interaction (RERI), attributable proportion (AP), and synergy index (SI). We considered an interaction as significant only when both RERI and AP were different from 0 and additionally SI was more than 1. The very low prevalence of TAK justifies to approximate OR by RR. For instance, when we assessed the interaction between rs9263739 and rs6871626 through these three indices, the subjects were classified into four groups: negative for both rs9263739 T allele and rs6871626 A allele, positive for rs9263739 T allele and negative for rs6871626 A allele, negative for rs9263739 T allele and positive for rs6871626 A allele, and positive for both rs9263739 T allele and rs6871626 A allele. Logistic models were used to calculate the indices.

In Silico Analysis of Association between the Gene Expression and rs6871626

We used two methods to assess the effect of rs6871626 on the *IL12B* (MIM 161561) expression. Gene expression data for *IL12B*

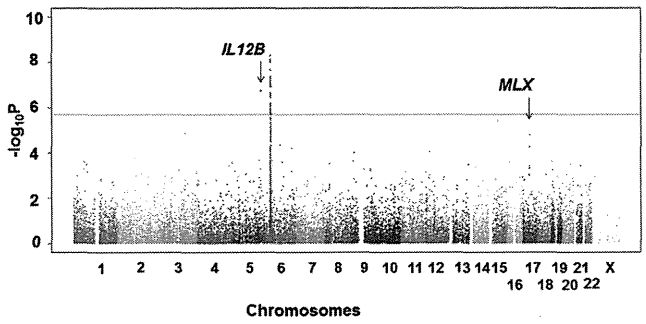

Figure 1. Manhattan Plot of Genome Scanning
The horizontal line indicates the significant level based on Bonferroni's correction. The HLA locus on chromosome 6 and the *IL12B* region on chromosome 5 reached the significant level.

in lymphoblastoid cells were obtained from GEO database (accession number GSE6536)[14] and analyzed for association with genotypes of rs6871626 obtained from HapMap project. Genevar software was used for analyzing the *IL12B* expression in adipose and skin in association with the rs6871626 genotypes.[15] Associations between genotypes and gene expression were evaluated by a linear regression analysis.

Associations between Genotypes and Clinical Phenotypes of TAK

Data of age at onset were analyzed for the association with the susceptibility alleles. AR, ischemic heart disease, and pulmonary infarction were selected for the association with genotypes as representative complications of TAK because cardiovascular event was the major cause of death in TAK individuals[16] and it was previously demonstrated that these phenotypes were associated with HLA-B*52:01,[17] suggesting that genetic backgrounds were at least partly responsible for these clinical manifestations. Data of the clinical manifestations were collected in Kyoto University Hospital or Tokyo Medical and Dental University by medical doctors who were blinded to genotype data reviewing clinical charts. Although AR evaluated by transthoracic echocardiography or angiography was positive for 44% of cases, other complications were found in less than 16%. Only AR was analyzed because of lack of power for other manifestations. Data for severity of AR assessed by the three categories[18] (mild, moderate, and severe) were also collected. C-reactive protein (CRP) was focused on as a biomarker reflecting disease activity. We calculated time-averaged CRP and dosage of prednisolone. Individuals who had visited hospitals for less than 500 days were excluded from the analysis of CRP. The associations between genotypes and clinical phenotypes were assessed by logistic regression analysis for existence of AR or linear regression analysis for severity of AR, time-averaged CRP, and age at onset. Time-averaged CRP was analyzed in condition with time-averaged dosage of prednisolone alone or in combination with rs3093059 genotypes in the *CRP* (MIM 123260) region. Associations between genotypes and clinical manifestations with p values less than 0.05 were regarded as significant.

Statistical Analysis

Statistical analyses were performed by PLINK v.1.07, R statistical software, or SPSS v.18.0.

Results

A summary of basic information of the subjects in our study is shown in Table 1. DNA samples from 167 cases and 663 healthy controls were genome scanned with the use of Illumina Human-Exome arrays containing 247,730 SNPs. One sample of the TAK cases and six samples in controls with success rates of less than 0.95 or with evidence of relatedness with other subjects (PI_HAT > 0.2 calculated by PLINK, see Subjects and Methods) were excluded from further analysis. The genotyping revealed that more than 80% of the markers in the array were monomorphic and 9% of the markers showed low minor allele frequency (<0.05) in the Japanese population, respectively. A total of 24,487 markers remained after filtering of SNPs that showed success rates of less than 0.95, deviation from HWE ($p < 1 \times 10^{-5}$) in either cases or controls, or minor allele frequencies of less than 0.05 in both cases and controls. The mean success rate of individuals was 0.999 after filtering.

Association studies were performed by chi-square test to compare allele frequencies between cases and controls. Population stratification was evaluated by QQ plot. The results indicated a lambda value of 1.05 in the QQ plot, indicating no excess population stratification in our study. Manhattan plot revealed that a region on chromosome 5 as well as the HLA locus showed significant associations that satisfied the genome-wide significant threshold obtained by Bonferroni's correction ($p = 2.0 \times 10^{-6}$; Figure 1). The associations were also confirmed by the imputed results (Figure S1 available online). rs4947248 in the *HLA-B* region, which is a known susceptibility gene to TAK, showed the strongest association ($p = 5.1 \times 10^{-9}$, OR = 2.17, 95% CI 1.67–2.82). rs9263739, a proxy of HLA-B*52:01 ($r^2 = 0.94$), similarly showed a significant association ($p = 8.0 \times 10^{-9}$, OR = 2.30, 95% CI 1.72–3.07; Table 2) and in moderate LD with rs4947248 ($D' = 0.95$, $r^2 = 0.58$). Because rs4947248 did not show evidence of an independent association from rs9263739 in logistic regression analysis ($p = 0.04$), we assumed that the top association in the HLA locus was attributable to HLA-B* 52:01. rs6871626 in the *IL12B* region on chromosome 5 also showed a significant association ($p = 1.8 \times 10^{-7}$, OR = 1.90, 95% CI 1.49–2.42; Table 2 and Figure 2A). Four other loci showed suggestive associations in our study ($p < 5.0 \times 10^{-5}$; Table 2). No departure from HWE was observed for these six SNPs ($p \geq 0.041$).

A replication study was performed with the use of DNA samples from 212 cases and 1,322 controls. The six SNPs with p values less than 5.0×10^{-5} in the genome scanning were genotyped in the replication study. rs9263739 was selected as a representative of the associations in the HLA locus. As a result, the significant associations of TAK with rs6871626 and rs665268 in the *MLX* (MAX dimerization protein [MIM 602976]) region on chromosome 17 as well as rs9263739 were replicated ($p = 1.1 \times 10^{-7}$, 0.0032, and 6.0×10^{-15}, respectively; Table 2, Figures 2A

Table 2. Results of Association Studies for TAK Susceptibility

						Genome Scan			Replication			Meta-analysis	
SNP	Chr	Position	Gene	Ref(A1)	Var(A2)[a]	Case A2freq	Cont A2freq	p	Case A2freq	Cont A2freq	p	p	OR (95% CI)
rs10934853	3	129521063	*EEFSEC*	A	C	0.59	0.45	1.3×10^{-5}	0.52	0.47	0.066	2.6×10^{-5}	1.40 (1.20–1.64)
rs6871626	5	158759370	*IL12B*	C	A	0.53	0.37	1.8×10^{-7}	0.53	0.39	1.1×10^{-7}	1.7×10^{-13}	1.75 (1.42–2.16)
rs9263739	6	31219335	*CCHCR1*	C	T	0.27	0.14	8.0×10^{-9}	0.30	0.14	6.0×10^{-15}	2.8×10^{-21}	2.44 (2.03–2.93)
rs1570843	6	84577239	*RIPPLY2*	C	T	0.62	0.50	4.6×10^{-5}	0.54	0.51	0.19	3.1×10^{-4}	1.34 (1.14–1.57)
rs12102203	15	49578851	*DMXL2*	G	A	0.64	0.49	3.8×10^{-6}	0.53	0.54	0.71	0.0081	1.24 (1.06–1.46)
rs665268	17	37975555	*MLX*	A	G	0.58	0.44	1.7×10^{-5}	0.49	0.42	0.0032	5.2×10^{-7}	1.50 (1.28–1.76)

Abbreviations are as follows: chr, chromosome; ref, reference allele; var, variant allele; CaseA2freq, variant allele frequency in cases; ContA2freq, variant allele frequency in controls; OR, odds ratio; CI, confidence interval. Positions are according to National Center for Biotechnology Information (NCBI) build 36.
[a]Risk alleles for TAK based on the results of the genome scanning are set as variant alleles.

and 2B). The suggestive association on chromosome 15 (Figures S1 and 1) was not replicated. Again, no departure from HWE was observed ($p \geq 0.11$).

A combined study in which the associations in the two studies were integrated by inverse-variance method demonstrated that rs6871626, rs665268, and rs9263739 showed significant associations ($p = 1.7 \times 10^{-13}$, 5.2×10^{-7}, and 2.8×10^{-21}; OR = 1.75, 1.50, and 2.44; 95% CI 1.42–2.16, 1.28–1.76, and 2.03–2.93, respectively; Table 2) satisfying the significance obtained by Bonferroni's correction. rs6871626 and rs9263739 satisfied the more stringent, widely accepted genome-wide significance ($p = 5.0 \times 10^{-8}$).

Because it was suggested that genetic components had influence on the manifestations of the disease,[17] we analyzed whether the variant of the *IL12B* region had clinical effects on the disease course or severity. Age at onset was not associated with rs6871626 ($p = 0.36$), whereas a significant association between rs6871626 and development of AR was observed in a recessive model ($p = 0.0046$; Figure 3A). Focusing on the cases with AR, a significant association between rs6871626 and severity of AR was observed in the recessive model ($p = 0.0018$; Figure 3B). Risk allele of rs6871626 (A allele) also demonstrated a significant association with increased level of time-averaged CRP, which was a representative marker of the disease activity ($p = 0.021$; Figure 3C). The association between rs6871626 and CRP levels was independent from rs3093059 in the *CRP* region ($p = 0.029$), which showed the strongest association with circulating CRP levels in Japanese.[19] These associations between rs6871626 and clinical manifestations were independent from rs9263739 (conditioned p value of rs6871626 ≤ 0.020). Although rs665268 also demonstrated a significant association with development of AR in a dominant model ($p = 0.0089$; Figure S2A), the association was not significant in condition with rs9263739 ($p = 0.080$). No significant associations were observed between rs665268 and other clinical phenotypes (Figures S2B and S2C).

Next, we investigated the interaction between the *IL12B* and *HLA-B* loci to TAK susceptibility. The risk of TAK in the population positive for both rs6871626 A allele and rs9263739 T allele surpassed the product and sum of the risk in those who were positive for either rs6871626 A allele or rs9263739 T allele alone (Figure 4). The analysis revealed that those who were positive for both had OR of 6.00 (95% CI 4.22–8.55), whereas those who were positive for either rs9263739 T allele or rs6871626 A allele showed OR of 1.80 (95% CI 1.11–2.93) or 1.74 (95% CI 1.23–2.47), respectively. Interaction measures revealed RER of 3.46 ($p = 1.4 \times 10^{-5}$, 95% CI 1.90–5.01), AP of 0.58 ($p = 1.0 \times 10^{-12}$, 95% CI 0.42–0.73), and SI of 3.24 ($p = 0.00028$, 95% CI 1.72–6.11). This significant interaction between *IL12B* and *HLA-B* on TAK susceptibility could be observed in both studies (Table 3). The synergistic interaction effects between rs6871626 and rs9263739 were not evident in the clinical manifestations associated with rs6871626 (Figure S3). When we analyzed the interaction between the *MLX* and *HLA-B* regions, we observed suggestive interaction with RER of 1.73, AP of 0.43, and SI of 2.29 ($p \leq 0.027$; Figure S4 and Table S1). The associations between the interaction and clinical manifestations were not significant (Figure S5).

IL12B encodes a common subunit of the IL12 and IL23 protein, known as p40. Because previous studies showed that the *IL23R/IL12RB2* (MIM 607562/601642) region was associated with Behçet disease[20] (MIM 109650), another connective tissue disease where vasculitis is involved in its pathology, we investigated this region for the possible associations in the current study. As a result, no suggestive association was found, either in our study or in the imputed results (Figure S6).

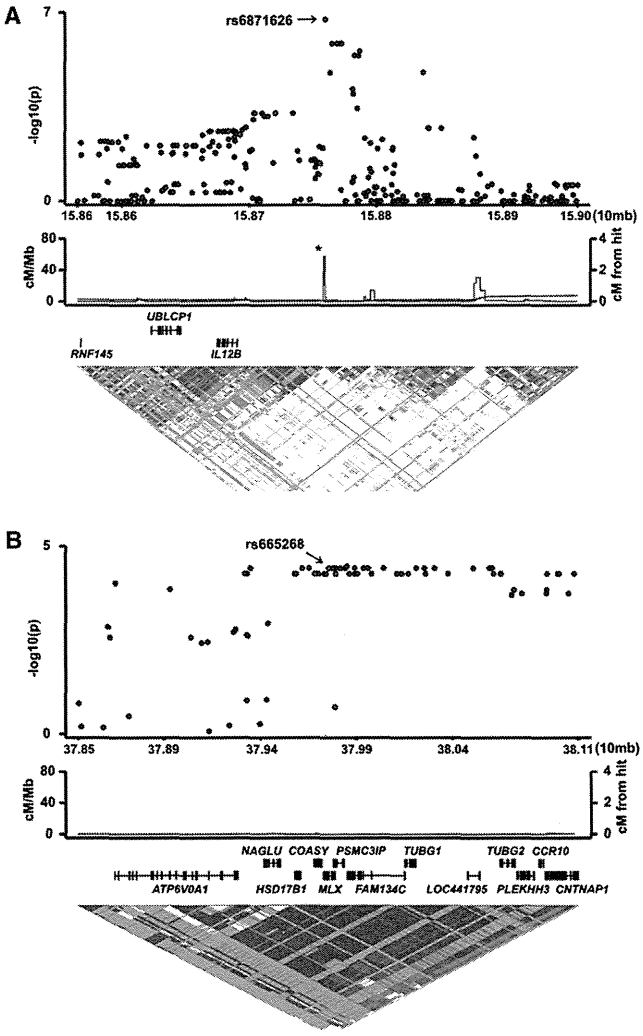

Figure 2. Associations of the *IL12B* and *MLX* Regions with the Susceptibility to TAK

Associations of SNPs in the (A) *IL12B* and (B) *MLX* regions in the genome scanning are plotted according to the position of the markers. Red circles indicate results of the current genome scanning. Blue circles indicate results of the imputation analysis based on the current results. The middle panel indicates recombination rates. The lower panel indicates LD of markers. Asterisk indicates a recombination hot spot in the *IL12B* region.

Discussion

This study provides a convincing evidence of associations between non-HLA genes and TAK susceptibility along with a synergistic role of susceptibility genes to TAK. The lack of evidence for associations of non-HLA genes with TAK so far is attributable to the lack of GWASs of TAK performed to date. Low prevalence of this disease had made it difficult to collect DNA samples to obtain sufficient power to detect susceptibility genes and perform a GWAS. Previous studies have revealed that the *IL12B* region was associated with a wide variety of autoimmune disorders and infectious diseases, including psoriasis[21–23] (MIM 177900), ankylosing spondylitis[24] (MIM 106300), Crohn disease[25] (CD [MIM 266600]), ulcerative colitis[26] (UC [MIM 191390]), and leprosy[27] (MIM 609888). rs6871626 showed a significant association with UC and leprosy over the genome-wide significance. Notably, rs6871626 A allele is susceptible to UC but protective against leprosy. A previous study from Turkey reported a suggestive association of TAK with rs3212227 in the 3′ UTR of the *IL12B* region.[28] rs3212227 is not in strong LD with rs6871626 in the Japanese population ($r^2 = 0.11$) and in Europeans ($r^2 = 0.06$) because of a recombination hot spot adjacent to rs6871626 (Figure 2A). In fact, an imputed association of rs3212227 with TAK in the current study resulted in only a suggestive association ($p = 0.0027$). There is a possibility that rs6871626 was responsible for the suggestive association between rs3212227 and TAK reported in the Turkish population. The association between gene expression and SNPs in the *IL12B* region appears to be complicated and inconsistent across different studies. rs3212227 in the 3′ UTR and rs17860508, an ins/del polymorphism in the promoter region of *IL12B*, were shown to have potential effects on the gene expression.[29,30] However, the previous studies showed that the association patterns varied according to the cell type and the protocol used for stimulation.[31–33] No previous report analyzed the effects of rs6871626 on the gene expression of *IL12B*. Although our in silico analysis failed to show the effects of rs6871626 on *IL12B* expression (data not shown, see Subjects and Methods), specific cell types or stimulus could lead to a significant association. Because a recent study showed that a haplotype of SNPs in the *IL12B* region could influence the gene and protein expression of *IL12B*,[22] a combination of rs6871626 and other SNPs in the *IL12B* region might lead to consistent results.

The associations between rs6871626 and clinical manifestations of TAK suggest the fundamental effects of IL-12p40 protein on TAK progression as well as TAK onset. We found that HLA-B*52:01 was associated with AR as reported previously ($p = 0.00014$). This finding supported the accuracy of our data. Although the risk allele of rs6871626 was associated with a significant dose-dependent increase in risk and severity of AR and in circulating CRP levels ($p = 0.013$, 0.030, and 0.023, respectively), these associations were more evident in a recessive manner. This raises a possibility that those who are homozygous for rs6871626 have strong disease activity that exceeds the additive disease activity of cases with single risk alleles, leading to severe destruction of aortic valve. Genetic variations in *IL12B* are known to influence the risk of psoriasis[21–23] and CD.[25] Because ustekinumab, a monoclonal antibody against IL-12p40, is an effective treatment for both diseases,[34,35] our findings would raise a possibility of its therapeutic use for TAK by targeting the IL-12/23 pathway. A previous study reported that the level of IL-12 protein was increased in TAK cases compared to healthy populations,[36] whereas there have been no reports addressing the circulating levels of IL-23 in TAK cases. IL-12 directly leads to type 1 helper T cell proliferation[37] and IL-23 upregulates IL-17 production and

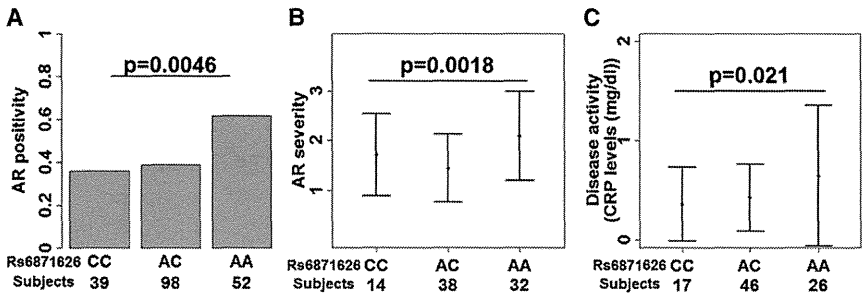

Figure 3. Associations between rs6871626 Genotypes and Clinical Manifestations of TAK

An association between rs6871626 genotypes and (A) development of AR, (B) severity of AR, and (C) time-averaged CRP levels in TAK cases. The p value was calculated by (A) logistic regression analysis, (B) linear regression analysis, and (C) linear regression analysis with time-averaged dosage of prednisolone as covariate. The recessive model is applied to all calculations. Severity of 1 to 3 in AR corresponds to mild, moderate, and severe, respectively. Mean ± SD are indicated for (B) and (C).

supports survival of activated Th17 cells.[38] Further analyses addressing circulating T cells in individuals with TAK or cell types infiltrating the artery specimen obtained from cases would provide clues to specify a critical pathway in TAK pathology.

The synergistic effect between rs6871626 and HLA-B*52:01 was notable. Those carrying both risk alleles had OR of 6.00 in comparison with those not carrying any risk alleles. Combination of rs6871626 and HLA-B*52:01 showed tendency of severe clinical phenotypes. Thus, we assume that increase of subjects and extraction of subjects who are homozygous for rs6871626 and positive for HLA-B*52:01 would provide evidence for significant effects of the combination on the disease phenotypes. The synergistic effect of these two loci raises a possibility that immune-related cells that recognize a yet-to-be-determined antigen through HLA-B*52:01 can be overactivated by IL-12/23 whose expression or function is modulated by rs6871626. In vitro analysis of immune-related cells from cases with TAK or healthy individuals would provide functional evidence of this synergistic role in the TAK pathogenesis.

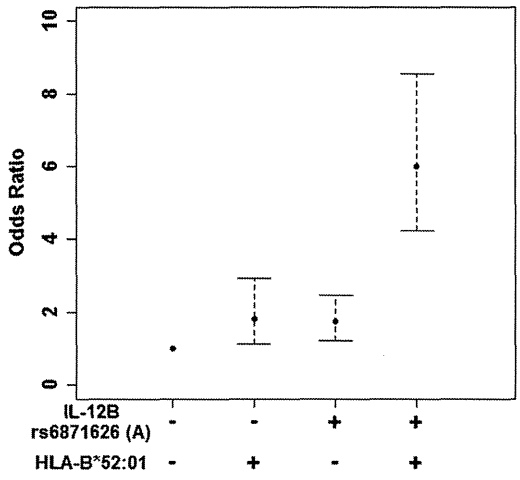

Figure 4. A Synergistic Effect between *IL12B* and HLA-B*52:01 on TAK Susceptibility

ORs are shown for the four strata of subjects according to combination of rs6871626 and rs9263739 genotypes. Those who are negative for rs9263739 T allele, a proxy of HLA-B*52:01, and rs6871626 A allele are used as reference. ORs and 95% CI are indicated.

rs665268 is a missense mutation of *MLX* that alters the 139th glutamine to arginine (Gln139Arg). MLX is a member of the basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor family and regulates gene expression by forming heterodimers with Mad protein.[39] The 17q21 region, whose associations with other autoimmune diseases including psoriasis[40] and CD[41] were shown, contains a number of genes including immune-related genes and polymorphisms that are in strong LD with each other (Figure 2B), so the corresponding gene to TAK susceptibility was inconclusive. Because risk allele frequency of rs665268 is comparable to that of rs6871626, the lack of associations between rs665268 and clinical manifestations and the weaker interaction between rs665268 and HLA-B*52:01 compared to rs6871626 might be a reflection of the milder effect of rs665268 on TAK progression. No interaction was observed between rs665268 and rs6871626 (data not shown).

We set the relatively low cut-off value of imputation score (Rsq $\geq$ 0.3) in the imputation analysis to increase sensitivity at the expense of specificity, but we failed to find other candidates of susceptibility loci. Another imputation analysis based on the data from the 1000 Genomes Project[42] revealed the same signals as the current study (data not shown). However, because the array used in the current study focused on SNPs in exons or nearby genes, it did not fully cover the whole genome with dense markers even in imputation analysis. There is a possibility that other SNPs not tagged by the markers on the array are associated with TAK. When the associations in the HLA locus were conditioned by rs9263739 or rs4947248, the most significantly associated SNPs, suggestive association signals in this locus could still be observed (the smallest p value = 5.5×10^{-5}, data not shown). The use of arrays with denser markers especially in intergene regions and using an increased number of cases could lead to the discovery of other susceptibility regions or independent associations in the HLA locus. Considering that both of the non-HLA susceptibility loci to TAK found in the current study are also associated with psoriasis and inflammatory bowel diseases, further analysis of TAK susceptibility genes would reveal other overlapping loci and common autoimmune mechanisms between TAK and other autoimmune diseases. It is feasible to