

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].

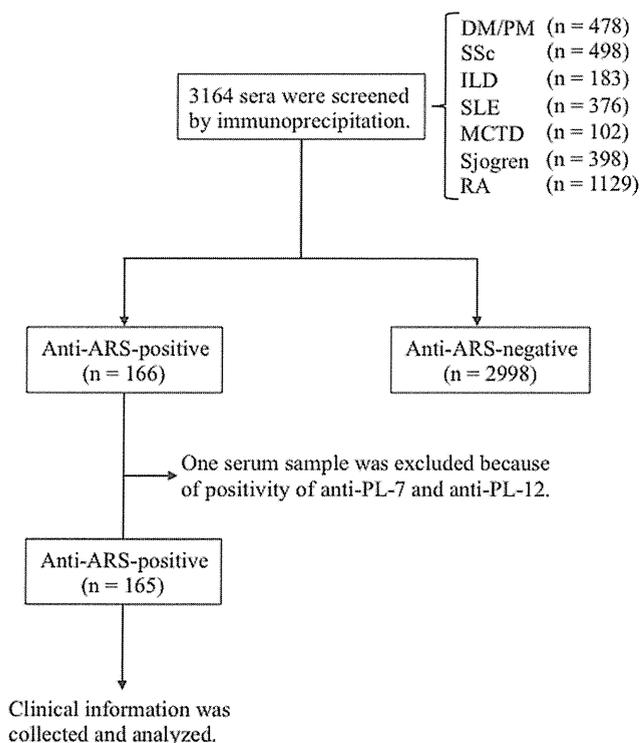


Figure 1. Enrollment and selection of patients. DM; dermatomyositis, PM; polymyositis, SSc; systemic sclerosis, ILD; interstitial lung disease, SLE; systemic lupus erythematosus, MCTD; mixed connective tissue disease, Sjogren; Sjogren's syndrome, RA; rheumatoid arthritis. doi:10.1371/journal.pone.0060442.g001

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

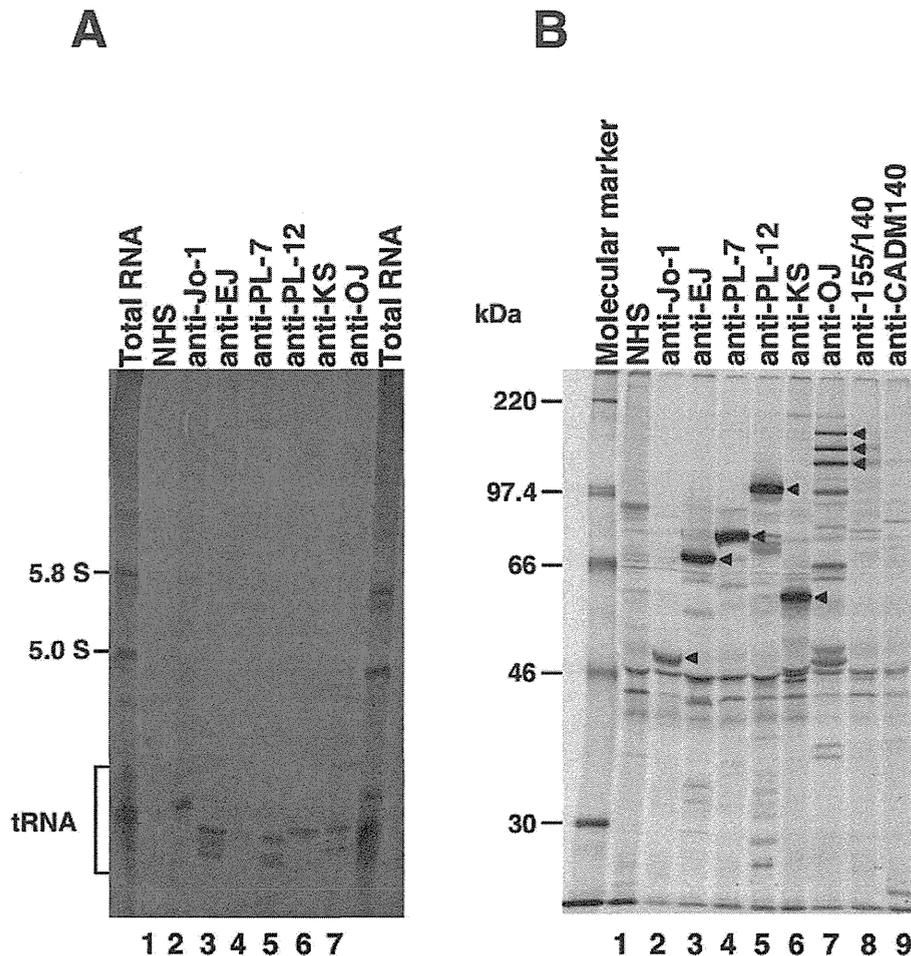


Figure 2. Representative immunoprecipitation assay for RNA with anti-aminoacyl-tRNA synthetase (anti-ARS) sera. **A**, Immunoprecipitation of histidyl-tRNA synthetase, glycyl-tRNA synthetase, threonyl-tRNA synthetase, alanyl-tRNA synthetase, asparaginyl-tRNA, and isoleucyl-tRNA synthetase by sera. K562 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.
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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-5m	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

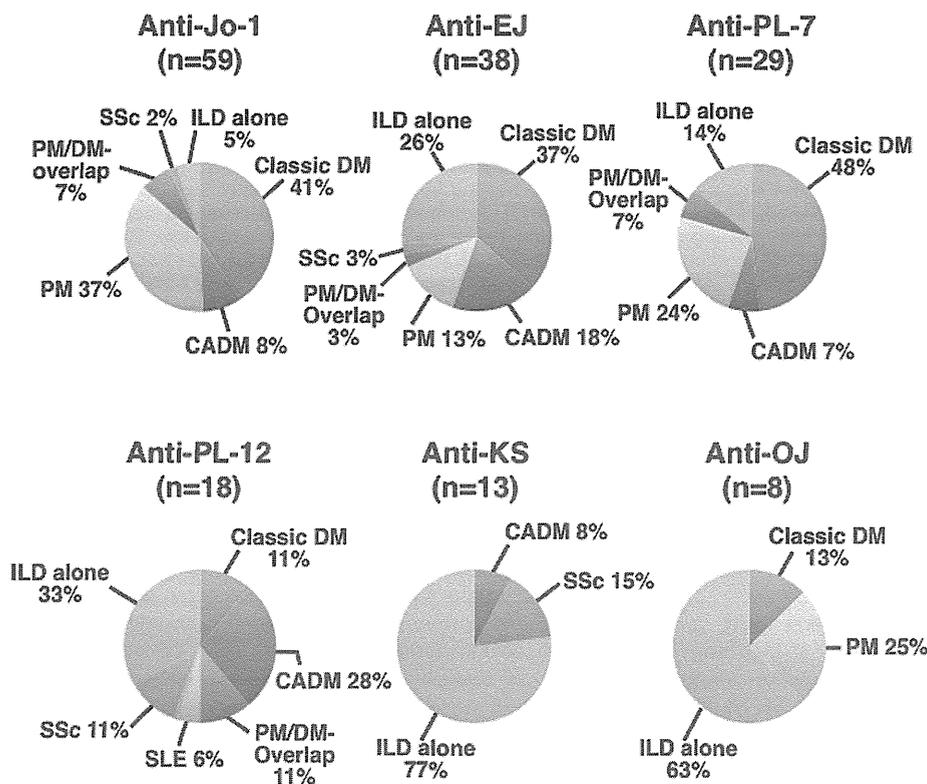


Figure 3. Prevalence of dermatomyositis (DM), clinically amyopathic DM (CADM), polymyositis (PM), PM/DM-overlap, systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and interstitial lung disease (ILD) alone, in each subgroup of anti-synthetase syndrome.

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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.
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 <i>P</i>
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

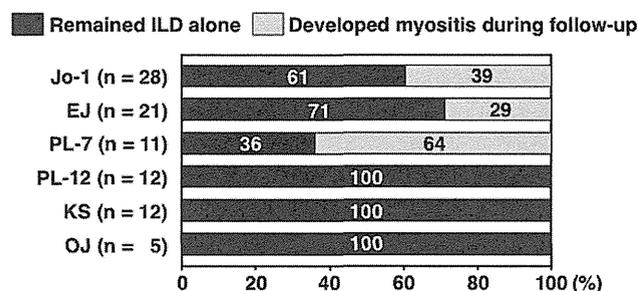
*Values are percentages of patients.

**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.

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A. ILD alone at initial presentation



B. Myositis alone at initial presentation

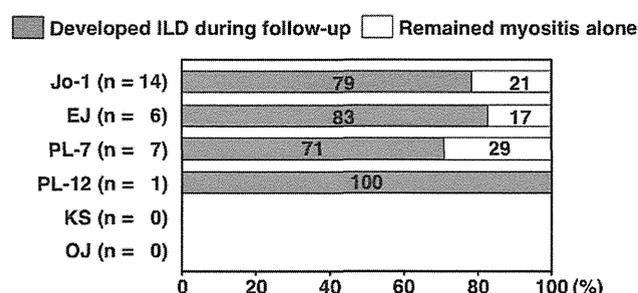


Figure 4. The clinical course of anti-synthetase syndrome patients who developed myositis or interstitial lung disease (ILD) with or without skin manifestations at disease onset.

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 anti-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-PL-12 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. Koderia EM KF MS HY SS KT M. Kuwana. Wrote the paper: YH MF M. Kuwana.

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Three Groups in the 28 Joints for Rheumatoid Arthritis Synovitis – Analysis Using More than 17,000 Assessments in the KURAMA Database

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Abstract

Rheumatoid arthritis (RA) is a joint-destructive autoimmune disease. Three composite indices evaluating the same 28 joints are commonly used for the evaluation of RA activity. However, the relationship between, and the frequency of, the joint involvements are still not fully understood. Here, we obtained and analyzed 17,311 assessments for 28 joints in 1,314 patients with RA from 2005 to 2011 from electronic clinical chart templates stored in the KURAMA (Kyoto University Rheumatoid Arthritis Management Alliance) database. Affected rates for swelling and tenderness were assessed for each of the 28 joints and compared between two different sets of RA patients. Correlations of joint symptoms were analyzed for swellings and tenderness using kappa coefficient and eigen vectors by principal component analysis. As a result, we found that joint affected rates greatly varied from joint to joint both for tenderness and swelling for the two sets. Right wrist joint is the most affected joint of the 28 joints. Tenderness and swellings are well correlated in the same joints except for the shoulder joints. Patients with RA tended to demonstrate right-dominant joint involvement and joint destruction. We also found that RA synovitis could be classified into three categories of joints in the correlation analyses: large joints with wrist joints, PIP joints, and MCP joints. Clustering analysis based on distribution of synovitis revealed that patients with RA could be classified into six subgroups. We confirmed the symmetric joint involvement in RA. Our results suggested that RA synovitis can be classified into subgroups and that several different mechanisms may underlie the pathophysiology in RA synovitis.

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Introduction

Rheumatoid arthritis (RA) is the most frequent inflammatory arthritis worldwide affecting 0.5 to 1% of the population [1]. As RA is a bone-destructive disease and functional impairment caused by joint damage is well correlated with swelling and tenderness of joints [2–3], the evaluation of joints in patients with RA is very important to assess disease activity and predict the risk of future joint deformity. ACR core set [4] and DAS (disease activity score) [5–6] were developed for evaluation of disease activity in RA. Recently, the three composite indices, namely, DAS28 [5], simplified disease activity index (SDAI) [7] and clinical

disease activity index (CDAI) [8] are frequently used for disease activity evaluation among rheumatologists. All of the three indices are shown to be well correlated with future joint destruction [7,9]. These three methods include the same 28 joints for evaluation of disease activity, namely, bilateral wrist, 1st to 5th metacarpal (MCP) joints and proximal interphalangeal (PIP) joints, elbow, shoulder, and knee joints. Though RA is known to show symmetric joint symptoms [10], the frequency of bilateral joint symptoms and the correlations between each joint symptom are not fully analyzed by using large numbers of joint assessments. There are several reports of successful prediction of joint damage using a reduced number of joints for evaluation by ultrasonogra-

phy [11–12]. These reports raise the possibility that some of the 28 joints are less frequently involved, and are less informative for disease activity. Analyses for characterization of joint symptoms would uncover correlations of unexpected joint symptoms and distribution of synovitis in RA.

Here, we analyzed the distribution of affected joints in the 28 joints in patients with RA using more than 17,000 joint assessments from 1,314 patients with RA and showed that synovitis in RA patients can be classified into three groups. We also showed that affected rates of the 28 joints greatly vary in RA patients, and that RA patients could be classified into subgroups based on the distribution of joint synovitis.

Results

Frequency order of joints involvement

We recruited 17,311 assessments for the 28 joints in 1,314 patients with RA from 2005 to 2011. A summary of the registered patients is listed in Table 1. The distribution of the number of patients with RA in each year and the number of joint assessments for each patient are shown in Figure S1. We analyzed how often each of the 28 joints was tender or swollen in patients with RA in 2011. From the analysis of 735 patients, we found that the frequency of joint swelling and tenderness in the 28 joints is widely different from joint to joint (Figure 1 and Table S1). The wrist joints were the most frequently affected joints for swelling and tenderness. The frequency of the right wrist joint being affected was more than four times as high as the least frequently affected joint. Many of the joints showed right-dominant tenderness (eleven of fourteen joints, $p = 0.057$, binomial test), indicating mostly right-handedness. We found strong correlations for the affected rates of each joint between swellings and tenderness except for shoulder joints (Spearman's rank-sum coefficient, $\rho = 0.70$ and $p = 3.8 \times 10^{-5}$, Figure 1, Table S1). Shoulder joints showed much higher frequencies of tenderness than those of swellings.

Next, we tried to replicate the order of affected frequencies of the 28 joints and the correlation between tenderness and swellings in different RA patients. We obtained 579 patients whose joints data were not available for 2011, indicating we analyzed independent RA patients. We found that the order of the affected joint frequencies were well correlated for both swelling and tenderness among different sets of RA patients (Spearman's rank-

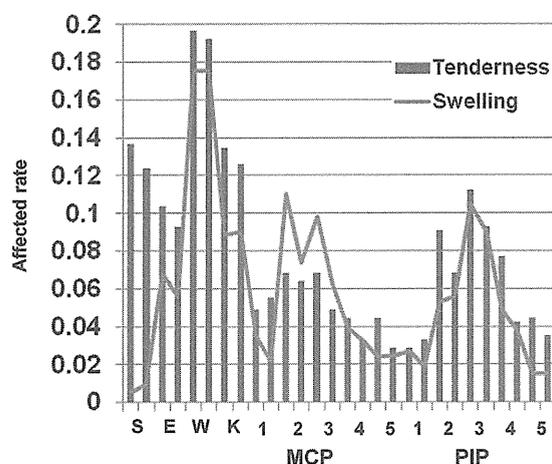


Figure 1. Affected rate of joint symptoms. Affected rate of joint symptoms. Each joint is arranged in the order of right and left. S:shoulder, E:elbow, W:wrist, K:knee. doi:10.1371/journal.pone.0059341.g001

Table 1. Summary of the KURAMA database.

The KURAMA database	
RA patients	1314
Age (mean \pm SD)	60.2 \pm 15.1
female ratio	81.70%
disease duration (years)	12.2 \pm 9.8
Stage*	2.75 \pm 1.17
Class*	1.87 \pm 0.69

*Stage and Class represent Steinbrocker's stage and class, respectively. SD: standard deviation.

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sum coefficient, $\rho = 0.815$ and 0.904 , $p = 1.3 \times 10^{-7}$ and $p = 4.6 \times 10^{-11}$ for swelling and tenderness, respectively, Figure S2). We also confirmed that rates of tenderness were well correlated with those of swellings in the 28 joints in the 579 patients ($\rho = 0.604$). These results indicate that some of the 28 joints are more likely to develop arthritis than the others in RA patients. The swelling and tenderness correlate with each other except for shoulder joints.

Whether the right-dominant involvement of joints in patients with RA is associated with joint destruction was analyzed. Joint destruction in the hand was evaluated for 246 patients with RA by modified Sharp score [13]. The six elements of the scores were separately analyzed, namely erosion of PIP, MCP, and wrist joints (we defined as joints other than MCP and PIP in hand) and narrowing of PIP, MCP, and wrist joints. We found that five out of six elements showed right-dominant destruction. In particular, narrowing and erosion of MCP joints showed a statistically significant right-dominance in binomial test ($p < 0.0050$, Table S2).

Three groups of 28 joints in RA synovitis

Next we analyzed correlations of joint symptoms between the 28 joints. We randomly picked up one assessment from each of the 1,314 patients to maximize the power. When the correlation of tenderness of the 28 joints was analyzed with kappa coefficient, we confirmed that each joint showed a symmetric involvement (Figure 2A). The results also showed that the tenderness of large joints and wrist joints are not correlated with the tenderness of PIP and MCP joints. We found that the tenderness of MCP joints was especially well correlated with each other and that PIP joints tenderness was well correlated with each other. The correlation of swelling in the 28 joints showed the same tendency as that of tenderness, namely, symmetric joint involvement, correlations between large joints and wrist joints, and no strong correlations between wrist joints and other small joints (Figure 2B).

Next we used eigen vectors of principal component analysis to assess the correlations of the 28 joints involvement. When we analyzed correlations of tenderness, eigen vectors revealed that PIP and MCP joints can be clearly distinguished from large joints and wrist joints (Figure 3A). PIP joints and MCP joints turned out to make independent groups after excluding large joints and wrist joints (Figure 3B). These three groups of affected joints were found both for tenderness and swelling (Figure 3C and 3D). We confirmed these three correlation groups in four independent resampling analyses by randomly picking up one assessment from each of the 1,314 patients four times (data not shown). The three groups were observed in the two independent sets of RA patients which were used in the analysis of joints involvement frequency

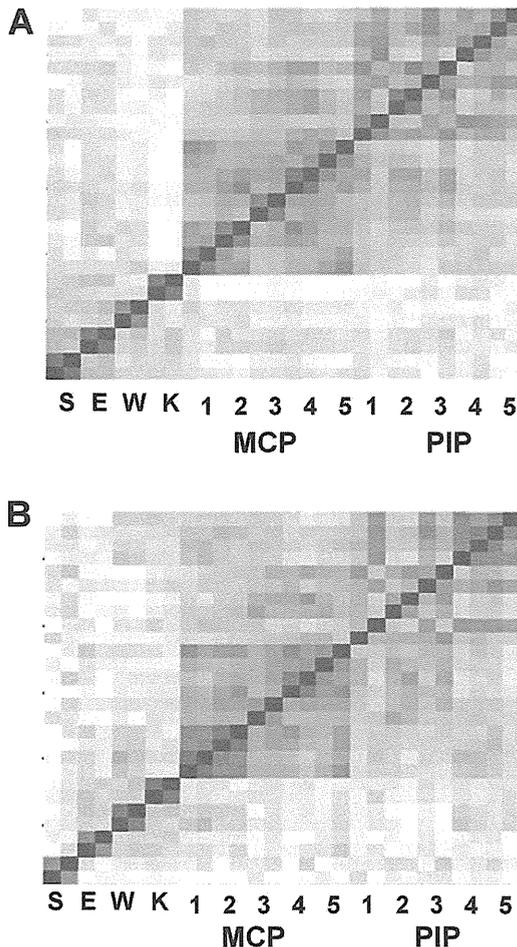


Figure 2. Correlations between the 28 joint symptoms. Brightness of the red color corresponds to the strength of correlations between joint tenderness (A) or swellings (B), using the Kappa coefficient. Each joint is arranged in the order of right and left. The joint order in the y axis is the same as the x axis. The result is a representative of five analyses based on resampled assessments. S:shoulder, E:elbow, W:wrist, K:knee.
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(Figure S3). In addition, no significant difference was observed in the relationship of the three groups of joint involvement when we divided the 1,314 patients into two groups according to the patients' caring physicians (Figure S4). We confirmed the three groups by resampling four times for each analysis (data not shown). These results indicate that these three groups were not due to specific patients, examiners, or time of evaluation.

Taken together, the correlation analyses using kappa coefficient and eigen vectors in principal component analysis indicated that there are three correlated groups of joints in RA synovitis, namely, large joints with wrist joints (which we express as "large and wrist joints"), PIP joints, and MCP joints.

Subgroups of patients with RA

We performed a clustering analysis of 5,383 evaluations of 28 joints from 1,314 patients with RA. Six subgroups of evaluations of 28 joints were observed (Figure 4). Each of the subgroups was characterized by 1) no synovitis (34.6%), 2) mild activity with dominant involvement of large and wrist joints (17.4%), 3) dominant involvement of MCP joints (18.3%), 4) dominant

involvement of PIP joints (9.3%), 5) active synovitis (4.1%), and 6) moderate activity with dominant involvement of large and wrist joints (16.4%) (Table S3). Whether patients with RA are classified into the same subgroups was analyzed. There were 998 patients with four or five evaluations, and of these, 734 were categorized into the regular groups across different evaluations, indicating that the patterns of synovitis in the same patients were stable. Analysis of joint destruction in each subgroup revealed that the sixth subgroup demonstrated dominant destruction of large and wrist joints compared with MCP and PIP joints ($p < 2.8 \times 10^{-5}$, Figure S5 and Figure S6).

Discussion

Since RA is a joint destructive autoimmune arthritis and joint damage occurs rapidly in the early stages of the disease course [14], the development of a quantitative scale which assesses disease activity and predicts joint damage is very important. After DAS and ACR core sets were introduced, DAS28, SDAI, and CDAI were developed to evaluate disease activity and easily calculate the disease activity score in patients with RA. All three indices were shown to be well correlated with future joint destruction and they share the same 28 joints for evaluation. Joint symptoms especially joint swelling is known to correlate with future joint damage [3]. While these indices were developed for use in clinical trials such as responsiveness to treatment, they are used by rheumatologists in daily clinical practice and they are reported to coincide very well among different examiners [9]. Characterizing the relative affected frequency of each joint and analysis of correlation between joint symptoms are important to analyze the basic mechanisms of synovitis and to efficiently select the joints to predict future joint destruction. However, there is no detailed analysis to address the correlations between the 28-joint symptoms.

In the current study, we characterized the 28-joint symptoms using large numbers of joint assessments. While we reported the affected rates of each joint in the 28 joints for tenderness and swelling of RA patients registered in the KURAMA database in 2011 as a representative (Table S1), these rates should not be generalized considering large effects of treatment especially biologics agents on joint symptoms. Thus, we focused on relative frequencies of joint involvement for the 28 joints. The affected frequency pattern was compared between the two sets of RA patients, and there were no apparent differences between the two sets for both tenderness and swelling. We also showed that joint symptoms in RA could be classified into three groups both for tenderness and swelling. Our analysis also demonstrated that patients with RA can be regularly classified into six subgroups based on patterns of joint symptoms. These results suggest that regular RA joint involvement pattern, including relative frequency and groups of joints, is largely maintained in RA patients. In addition, we confirmed that these patterns of joint involvement were not attributed to evaluators and fractions of RA patients.

It is interesting that the affected frequencies greatly varied from joint to joint, and the rate of the most highly affected joint was more than four times as high as the least-affected joint. The affected frequencies indicated that wrist joints were the most frequently affected. It should be noted that surface area may have influenced the sensitivity of detecting synovitis in physical exams when different joints were compared. The relatively high frequency of tenderness and swelling in large and wrist joints compared with MCP and PIP joints can be explained by this difference in surface area. However, surface area cannot fully explain the highest frequency of wrist involvement and different frequencies within the MCP or PIP joints. A dominant involve-

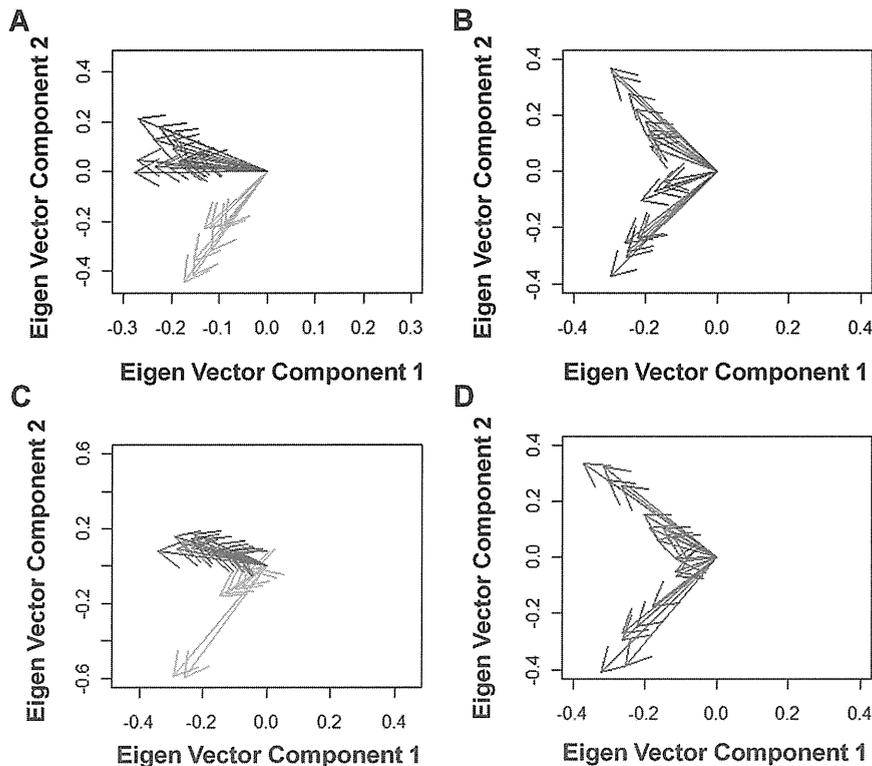


Figure 3. Relationship of the 28-joint involvement. The 1st and 2nd components of eigen vectors of the joint symptoms are plotted, using principal component analysis of the 28 joint involvement for tenderness (A) and swelling (C) or using that of the 20 joint involvement other than large and wrist joints for tenderness (B) and swelling (D). The results are representatives of five analyses based on resampled assessments. Green: large and wrist joints. Red: MCP joints. Blue: PIP joints.
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ment of right joints seemed to indicate a majority of the study population being right-handed in spite of the small difference of affected rates between bilateral joints. We also demonstrated that the right dominant involvement was also true for joint destruction. We could not compare the joint involvement and joint destruction between right-handed patients and left-handed patients due to a lack of information regarding handedness of patients.

Correlation analysis confirmed the well-known symmetric joint involvement in patients with RA. Strong correlations of tenderness and swelling in the same joints except for shoulder joints may indicate low sensitivity of shoulder swelling in the physical exams and common mechanisms of swelling and tenderness. It is striking that joint symptoms can be classified into three groups based on correlation analysis and principal component analysis. The

association observed between the symptoms in the wrist joints and the large joints is worth noting, since wrist joints are regarded as small joints according to ACR/EULAR criteria set in 2010. As wrist joints are much closer to other small joints than large joints, the relationship between wrist joints and large joints cannot be explained by the distance of joints. The distance of joints cannot explain the two different groups of MCP and PIP joints either. While symptoms of large and wrist joints are not related with those of MCP and PIP joints, they were not very strongly correlated with each other, compared with correlations among PIP joints or MCP joints. This may indicate that there are no common strong factors which predispose large and wrist joints to swelling and tenderness in patients with RA.

We also showed that patients with RA can be divided into six subgroups based on these three groups of joint involvement. More than 70% of patients are classified into regular subgroups, indicating that the pattern of synovitis in a patient with RA is stable. When patients who were regularly classified into the first subgroup of patients characterized by no synovitis were removed, more than 60% of patients were still classified into regular subgroups (data not shown), indicating that the stable patterns were observed regardless of activity of RA. As joint destruction was influenced by disease duration, disease activity, and treatment, we analyzed the relative distribution of joint destruction between the three joint groups in a patient with RA. We found that the sixth subgroup of patients, characterized by moderate activity with dominant involvement of large and wrist joints, demonstrated dominant destruction of wrist joints. This suggests that classifying patients with RA into appropriate subgroups would lead to prediction of patterns of joint destruction.

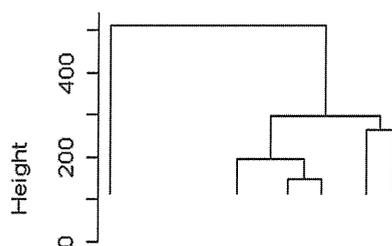


Figure 4. Six subgroups of evaluations of the 28 joints in RA. Results of clustering analysis with Ward method using randomly obtained 5,383 evaluations of the 28 joints in 1,314 patients were plotted.
doi:10.1371/journal.pone.0059341.g004

There are reports that evaluating fraction of joints by ultrasonography is a good way to predict future joint damage [11–12]. One study reported that 5 of the 28 joints with MTP2 and MTP5 joints, namely, wrist, MCP2, MCP3, PIP2, and PIP3 joints, are enough for ultrasonography evaluation [12]. Their data seems to be consistent with our results as they selected at least two joints from three different groups into which the 28-joint symptoms were classified. As ultrasonography usually surpasses physical examination in terms of the sensitivity to detect synovitis, it is interesting to analyze whether the assessments of synovitis using ultrasonography show the same pattern of synovitis over the 28 joints in RA.

Our results indicate that RA does not develop synovitis in the 28 joints with the same frequency and that the affected rate of each joint greatly varies from joint to joint. These different distributions of joint synovitis would lead to different distribution of joint destruction. Based on our results, the 28 joints can be categorized into three groups, and it is possible that some fractions of the 28 joints are less informative to assess disease activity than others. It would be interesting to develop a novel simplified joint core set, and analyze the correlation between joint damage and activity score based on this. It would be also interesting to characterize each of RA subsets in more detail.

Materials and Methods

Ethics Statement

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. This study including the consent procedure was approved by the ethics committee of Kyoto University Graduate School and Faculty of Medicine.

The KURAMA database

The KURAMA (Kyoto University Rheumatoid Arthritis Management Alliance) database was established in 2011 at Kyoto University to store detailed clinical information and specimens from patients with arthritis and arthropathy. The alliance is composed of rheumatic disease-associated departments in Kyoto University Hospital as well as its allied, integrating previous database and specimen collections in each department and allied. A template for electronic clinical charts developed at Kyoto University Hospital in 2004 to evaluate joint involvements in RA patients was used to obtain joint assessments. Rheumatologists evaluated swelling and tenderness of the 28 joints in patients with RA on each visit and filled in the template. The synovitis information of the 28 joints and data for C-reactive protein and erythrocyte sedimentation rate were extracted from electronic clinical charts [15] and stored in the KURAMA database.

Patients and data of joint assessment

A total of 17,311 joint assessments from 1,314 patients with RA from 2005 to 2011 were obtained in a retrospective manner from the KURAMA database. All of the patients fulfilled ACR revised criteria for RA in 1987 [10] or ACR and EULAR classification criteria for RA in 2010 [16–17].

Analysis of affected frequencies in the 28 joints

RA patients were subdivided depending on whether their data were available in 2011 or not, and the affected frequency in each of the 28 joints was calculated. We compared the order of the affected frequency in the 28 joints between the two patient sets with Spearman's rank-sum coefficient. We separately analyzed the affected rates of joints for swelling and tenderness. When multiple joint assessments in different visits were available in the same patient with RA, we randomly selected one of the assessments as representative in the patient. We compared frequencies between tenderness and swellings for the 28 joints with Spearman's rank-sum coefficient.

Clustering of patients with RA

Clustering analyses were performed by Ward method, using randomly-selected 5,383 evaluations of the 28 joints from 1,314 patients with RA. These evaluations did not contain more than six assessments from each patient to avoid excess influence of particular patients. Affected rates were calculated for the three groups of joints (namely PIP joints, MCP joints and large and wrist joints) in this clustering analysis. For example, when a patient showed tenderness and swelling for all PIP joints, the affected rate of PIP joints in the patient is 2. When a patient showed tenderness for four MCP joints, the affected rate of MCP joints is 0.4.

RA patients were regarded as belonging to a particular group when more than 60% of evaluations belonging to the same patients with four or five evaluations were classified into the same group.

Analysis between RA subgroups and joint destruction

Joint destruction of hand joints in 246 patients with RA was evaluated by modified Sharp score by a trained rheumatologist who was not informed of the patients' characteristics (KM). Joint destruction rates were defined for the three groups of joints as a sum of scores divided by the full score in the joints group. For example, when a patient shows 50 as a sum of scores in the large and wrist group, the patient's joint destruction rate for the group is 0.463 (50/108).

Correlation of the 28 joints and statistical analysis

Correlations of joint symptoms among the 28 joints were estimated separately for tenderness and swelling. We randomly obtained one assessment of the 28 joints in each patient as a representative of the patient's joint assessments for maximization of the power. Kappa coefficient was used to analyze coincidence of joint symptoms in each pair of the 28 joints. Eigen vectors obtained in principal component analysis were used to analyze the deviation of joint symptoms. We resampled joint assessments for each patient and created four other sets of joint assessments. The same correlation analyses were performed using the four resampled assessments to confirm the correlation shown in the first assessment set. Right dominance of the synovitis and joint destruction was analyzed by binomial test. Dominant destruction of joints was evaluated by paired-t test. Statistical analysis was performed by R software or SPSS (ver18).

Supporting Information

Figure S1 Distribution of joint evaluation counts and patients across different years. A) Distribution of number of RA patients according to numbers of 28-joint assessments. B) Distribution of number of patients with RA whose joint assessment data were available from 2005 to 2011 in the KURAMA database. (TIF)

Figure S2 Good correlations between joint involvement rates in different sets of RA patients. Rates of joint involvement for A) swelling and B) tenderness were compared between the two different sets of RA patients. X and Y axes represent rates in the first set of RA patients in 2011 and those in the second set in 2005 to 2010, respectively.
(TIF)

Figure S3 Three groups of joints regardless of different sets of RA patients. Analysis using one of four resampled assessments in one of the two sets of RA patients is shown as a representative. The 1st and 2nd components of eigen vectors of the joint symptoms are plotted, using principal component analysis of the 28 joint involvement for tenderness (A) and swelling (C) or using that of the 20 joint involvement other than large and wrist joints for tenderness (B) and swelling (D). Green: large and wrist joints. Red: MCP joints. Blue: PIP joints.
(TIF)

Figure S4 Three groups of joints regardless of different evaluators. Analysis using one of five resampled assessments by one of the two groups of medical doctors is shown as a representative. The 1st and 2nd components of eigen vectors of the joint symptoms are plotted, using principal component analysis of the 28 joint involvement for tenderness (A) and swelling (C) or using that of the 20 joint involvement other than large and wrist joints for tenderness (B) and swelling (D). Green: large and wrist joints. Red: MCP joints. Blue: PIP joints.
(TIF)

Figure S5 Dominant destruction of large and wrist joints in the sixth subgroup of patients with RA. Box plots indicating the joint destruction rates in the three joint groups in subjects belonging to the sixth subgroup.
(TIF)

Figure S6 Destruction of large and wrist joints among the six subgroups of RA. Differences in destruction rates were plotted for each subject in the six subgroups. The difference was defined as: A) destruction rate of group of large and wrist joints – destruction rate of MCP joints and B) destruction rate of group of large and wrist joints – destruction rate of PIP joints.
(TIF)

Table S1 Rate of joint involvement for 28 joints in RA.
(DOC)

Table S2 Right-dominant joint destruction in RA. Patients who showed unilateral higher or lower scores in each element were analyzed.
(DOC)

Table S3 Mean affected rates of the three joint groups in the six subgroups of patients with RA.
(DOC)

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Author Contributions

Evaluation of joint X-rays: KM. Conceived and designed the experiments: CT MH KO RY FM HI TF TM. Analyzed the data: CT. Contributed reagents/materials/analysis tools: CT MH KO RN KM N. Yamakawa H. Yoshifuji N. Yukawa DK TU H. Yoshitomi MF HI TF TM KY. Wrote the paper: CT.

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Concise report

Association of Takayasu arteritis with HLA-B*67:01 and two amino acids in HLA-B protein

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Abstract

Objective. Takayasu arteritis (TAK) is a rare autoimmune arteritis that affects large arteries. Although the association between TAK and HLA-B*52:01 is established, the other susceptibility *HLA-B* alleles are not fully known. We performed genetic association studies to determine independent *HLA-B* susceptibility alleles other than HLA-B*52:01 and to identify important amino acids of HLA-B protein in TAK susceptibility.

Methods. One hundred patients with TAK and 1000 unrelated healthy controls were genotyped for *HLA-B* alleles in the first set, followed by a replication set containing 73 patients with TAK and 1000 controls to compare the frequencies of *HLA-B* alleles. Step-up logistic regression analysis was performed to identify susceptibility amino acids of HLA-B protein.

Results. Strong associations of susceptibility to TAK with HLA-B*52:01 and HLA-B*67:01 were observed ($P = 1.0 \times 10^{-16}$ and 9.5×10^{-6} , respectively). An independent susceptibility effect of HLA-B*67:01 from HLA-B*52:01 was also detected ($P = 1.8 \times 10^{-7}$). Amino acid residues of histidine at position 171 and phenylalanine at position 67, both of which are located in antigen binding grooves of the HLA-B protein, were associated with TAK susceptibility ($P \leq 3.8 \times 10^{-5}$) with a significant difference from other amino acid variations ($\Delta AIC \geq 9.65$).

Conclusion. HLA-B*67:01 is associated with TAK independently from HLA-B*52:01. Two amino acids in HLA-B protein are strongly associated with TAK susceptibility.

Key words: Takayasu arteritis, genetic association study, HLA-B, aortitis, vasculitis.

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Introduction

Takayasu arteritis (TAK) is a relatively rare systemic arteritis that affects mainly large branches of the arteries, including the aorta, carotid artery, subclavian artery and coronary artery. TAK affects mainly young females. Both environmental and genetic components have been shown to be involved with the onset of TAK [1]. Among genetic components, HLA-B52, mainly B*52:01, has shown a strong association with TAK with odds ratios (ORs) of ~3 [2, 3]. The association with HLA-B52 has been repeatedly shown in various studies and the association is established beyond ethnicity [4, 5]. Other associations between TAK and HLA alleles have not been confirmed due to the low prevalence of the disease and the lack of

large-scale comprehensive genetic analysis using TAK patients. While a previous study showed that HLA-B39 is also associated with TAK [6], another recent study failed to replicate the association [7]. The recent study also suggested that a relatively rare *HLA-B* allele, HLA-B*67:01, was associated with TAK susceptibility (corrected $P=0.023$) [7], but there are no replication studies. No studies have addressed independent associations of *HLA-B* alleles from HLA-B*52:01. Moreover, no large studies have ever been performed to analyse which amino acid residues are important for TAK susceptibility. As some TAK patients develop autoantibodies against aortic endocardium [8], the detection of susceptibility amino acids to TAK would lead to identification of a possible antigen that provokes an autoimmune response in TAK patients.

Materials and methods

Study subjects

DNA samples of 100 Japanese patients with TAK were collected at Kyoto University Hospital and Tokyo Women's Medical University. DNA samples of 73 patients with TAK were also collected at Kyoto University Hospital. Two patients in the first set are a parent and a child. Each of 1000 control DNA samples in the first and replication sets were collected at the HLA laboratory from unrelated healthy individuals. All the patients were diagnosed with TAK based on ACR criteria [9] or guidelines of the Japanese Circulation Society [10] or were registered to the Japanese national registry for rare and intractable diseases (<http://www.nanbyou.or.jp/english/index.htm>). Information on classifications of TAK based on criteria by Hata *et al.* [11] and of complications of aortic regurgitation (AR) were obtained from 75 and 85 patients, respectively, by reviewing clinical charts from the Kyoto University Hospital. A summary of the subjects is shown in supplementary Table S1, available at *Rheumatology* Online. This study was approved by the local ethics committees at each institution (Kyoto University Graduate School and Faculty of Medicine Ethics Committee, Tokyo Women's Medical University Genome Ethics Committee and Tokyo Women's Medical University Ethics Committee) and written informed consent was obtained from all subjects.

HLA-B genotyping

The cases and controls were genotyped for *HLA-B* alleles in four-digit resolution using the WAKFlow system at the Kyoto University Hospital and HLA laboratory, respectively.

Amino acid sequences of *HLA-B* alleles

Amino acid sequences were obtained for each *HLA-B* allele for four-digit resolution from the IMGT database (<http://www.ebi.ac.uk/ipd/imgt/hla/>). Amino acid variations were searched across all the *HLA-B* alleles contained in the study population. In total, 99 variants of amino acids over 53 positions were determined in the

HLA-B alleles. A three-dimensional structure analysis of the *HLA-B* protein was performed using UCSF Chimera software (University of California, San Francisco, San Francisco, CA, USA) [12].

Statistical analysis

The association between TAK and *HLA-B* alleles was investigated using 2×2 contingency tables and tested for statistical significance using the χ^2 test or Fisher's exact test for alleles with a frequency $>1\%$ in either the cases or the controls. Heterogeneity between the two studies was analysed using the Breslow–Day test. The ORs and 95% CIs were also calculated. The relative predispositional effect (RPE) method [13] was applied to identify the associations of more than one *HLA-B* allele sequentially according to their strength. The associations between clinical phenotypes and HLA-B*67:01 were assessed by the Wilcoxon rank sum test for age at onset or Fisher's exact test for other clinical phenotypes. Crude P -values were indicated unless we mentioned the use of corrected P -values.

The 99 amino acids over 53 positions were used as independent variables in logistic regression analysis (supplementary Table S2, available at *Rheumatology* Online). When we found only two amino acids in the same position, we set one independent variable in this position. When we found more than three amino acids in the same position, we distinguished all amino acids as independent variables. Step-up multiple logistic regression analyses for TAK susceptibility were performed to identify susceptibility amino acids in *HLA-B* in the combined study until an amino acid whose P -value did not reach significance was chosen. The Akaike information criterion (AIC) was also calculated.

Significant levels were set according to Bonferroni's correction, namely, 0.0028 for *HLA-B* alleles, 0.05 for clinical phenotypes and 0.00051 for amino acids. Amino acid variations were considered significant over other variations when the logistic regression model containing the variation showed the smallest AIC with the difference in AIC >7 in comparison with those containing other alleles ($\Delta\text{AIC} > 7$) [14]. Permutation tests were performed 10 000 times to assess the probability that the observed improvements of deviance in multiple logistic regression analysis were obtained by chance. Deviances of the best-fitting model using one, two and three amino acids were calculated in each permutation test and sequential improvements of deviance by the best three amino acids were compared with the observed improvements. Deviance of logistic regression analysis is defined as $-2 \times \log$ likelihood of logistic models with degrees of freedom of k , where $k-1$ is the number of alleles in the models.

Results

The strong association of TAK with HLA-B*52:01 was confirmed in the first set ($P=2.6 \times 10^{-13}$). A significant association with HLA-B*67:01 was also observed ($P=0.00081$; Table 1), and no other *HLA-B* alleles showed significant associations with TAK. The replication

TABLE 1 Associations of HLA-B alleles with TAK susceptibility

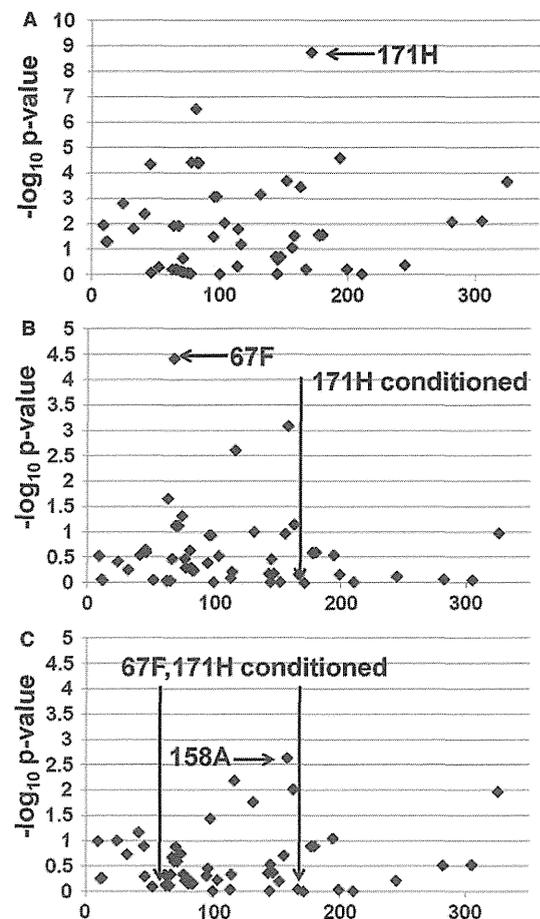
HLA-B allele ^a	First set				Replication set				Combined study		
	Case	Control	P	OR (95% CI)	Case	Control	P	OR (95% CI)	P	OR (95% CI)	RPE P
B*07:02	7	122	0.14	0.56 (0.26, 1.21)	6	119	0.36	0.68 (0.29, 1.57)	0.085	0.61 (0.34, 1.08)	
B*13:01	1	28	0.51	0.35 (0.0086, 2.17)	1	27	1.00	0.50 (0.012, 3.10)	0.32	0.42 (0.049, 1.59)	
B*15:01	16	153	0.86	1.05 (0.61, 1.80)	11	152	0.98	0.99 (0.52, 1.87)	0.90	1.03 (0.68, 1.54)	
B*15:18	1	27	0.31	0.51 (0.0089, 2.25)	0	35	0.17	0 (0, 1.52)	0.059	0.18 (0.0046, 1.08)	
B*35:01	10	160	0.13	0.61 (0.31, 1.17)	9	164	0.38	0.74 (0.37, 1.47)	0.084	0.66 (0.41, 1.06)	
B*39:01	3	68	0.20	0.43 (0.086, 1.34)	8	61	0.11	1.84 (0.86, 3.93)	0.96	0.99 (0.53, 1.84)	
B*40:01	6	108	0.14	0.54 (0.24, 1.25)	9	115	0.84	1.08 (0.53, 2.17)	0.33	0.77 (0.45, 1.31)	
B*40:02	14	177	0.38	0.78 (0.44, 1.36)	6	153	0.11	0.52 (0.22, 1.19)	0.11	0.68 (0.43, 1.09)	
B*40:06	8	92	0.70	0.86 (0.41, 1.81)	1	102	0.0085	0.13 (0.0032, 0.74)	0.057	0.52 (0.27, 1.03)	
B*44:03	13	125	0.89	1.04 (0.58, 1.88)	6	115	0.41	0.7 (0.30, 1.62)	0.70	0.91 (0.56, 1.47)	
B*46:01	11	87	0.45	1.28 (0.67, 2.44)	6	102	0.6	0.8 (0.34, 1.85)	0.87	1.04 (0.63, 1.73)	
B*48:01	5	60	0.69	0.83 (0.33, 2.09)	3	61	0.80	0.67 (0.13, 2.08)	0.45	0.76 (0.37, 1.57)	
B*51:01	10	161	0.12	0.60 (0.31, 1.16)	14	169	0.63	1.15 (0.65, 2.04)	0.39	0.83 (0.54, 1.27)	
B*52:01	60	232	2.6x10 ⁻¹³	3.27 (2.34, 4.55)	34	235	4.8 x 10 ⁻⁵	2.28 (1.52, 3.43)	1.6 x 10 ⁻¹⁶	2.82 (2.19, 3.64)	1.6 x 10 ⁻¹⁶
B*54:01	12	133	0.72	0.90 (0.49, 1.65)	9	149	0.57	0.82 (0.41, 1.63)	0.57	0.85 (0.54, 1.35)	
B*55:02	2	47	0.31	0.42 (0.049, 1.62)	5	58	0.72	1.19 (0.47, 3.01)	0.72	0.77 (0.35, 1.66)	
B*59:01	2	41	0.43	0.48 (0.056, 1.88)	1	39	0.52	0.35 (0.0085, 2.08)	0.21	0.43 (0.086, 1.31)	
B*67:01	9	27	0.00081	3.44 (1.60, 7.43)	5	19	0.0061	3.70 (1.36, 10.05)	9.5 x 10 ⁻⁶	3.62 (1.97, 6.66)	1.8 x 10 ⁻⁷

^aAlleles with a frequency of >1% in either the case or control are indicated.

study was performed to confirm the findings in the first set. As a result, the association of HLA-B*67:01 with TAK was observed ($P=0.0061$; Table 1), as well as that of HLA-B*52:01 ($P=4.8 \times 10^{-5}$). HLA-B*40:06 showed a protective association against TAK ($P=0.0085$). In the combined analysis, solid evidence of associations of HLA-B*67:01 and HLA-B*52:01 with TAK was obtained [$P=9.5 \times 10^{-6}$ and 1.0×10^{-16} , OR=3.62 (95% CI 1.97, 6.66) and 2.82 (95% CI 2.19, 3.64), respectively; Table 1]. Any signs of heterogeneity between the two studies were observed for these two alleles ($P \geq 0.18$). No other alleles, including HLA-B*40:06, showed significant associations. To assess the independence of HLA-B*67:01's association from HLA-B*52:01 and to detect further candidates of independent susceptibility HLA-B alleles, RPE analysis was performed in the combined study. As a result, HLA-B*67:01 showed a significant association with TAK susceptibility that was independent of HLA-B*52:01 ($P=1.8 \times 10^{-7}$; Table 1). The analysis did not show further candidates with independent associations with TAK ($P \geq 0.17$). Since previous studies suggested associations between HLA-B alleles and disease phenotypes [15], we analysed the associations between HLA-B*67:01 and age at onset, female ratio, classifications of TAK or complications of AR. As a result, we did not observe significant associations ($P \geq 0.61$; supplementary Tables S3 and S4, available at *Rheumatology* Online).

Next, which amino acid residues contribute to TAK susceptibility was analysed by a step-up multiple logistic regression analysis in the combined study, using 99 amino acid variations over the HLA-B protein (see the Materials and methods section). Logistic regression analysis revealed the strongest association of histidine residue at the position of the 171st amino acid residue ($P=1.8 \times 10^{-9}$; Fig. 1 and supplementary Table S5, available at *Rheumatology* Online), with a Δ AIC of 11.17 in comparisons with other amino acids. In the case of conditioning with the 171st histidine, phenylalanine at the 67th amino acid residue showed the strongest association ($P=3.8 \times 10^{-5}$; Fig. 1), with an Δ AIC of 9.65. An alanine residue at the 158th position showed a suggestive association in conditioning with these two amino acids ($P=0.0024$; Fig. 1) without significant Δ AIC (0.13), indicating uncertainty of the selection of this amino acid as the third susceptibility amino acid. We did not find any further amino acid variations with significant associations in the condition with these three amino acids ($P \geq 0.065$). A total of 10 000 permutation tests revealed that improvements in deviance from these three amino acids were less likely to be obtained by chance (permutation P -value 0.0001, 0.0001 and 0.0024, respectively). The 171st and 67th amino acid residues are located in peptide binding grooves, implying that an antigen binding capacity conferred by these amino acids might influence TAK susceptibility (supplementary Fig. S1, available at *Rheumatology* Online). When HLA-B*52:01 was excluded from the association studies, the two amino acid variations did not show significant associations (data not shown), indicating that the associations of the two amino acids were brought by HLA-B*52:01.

Fig. 1 Associations of amino acids of the HLA-B protein with TAK susceptibility.



P -values are plotted according to the amino acid positions in (A) single logistic regression analysis, (B) in the condition with histidine at position 171 and (C) with 171 histidine and 67 phenylalanine.

Discussion

This is the first study to provide solid evidence of an HLA-B TAK susceptibility allele independent of HLA-B*52:01 and to report on TAK susceptibility amino acids in HLA-B protein. As the top two residues of 171 and 67 were located at peptide binding grooves and have shown their significance in peptide binding [16, 17], the susceptibility effects on TAK appear to be reasonable.

HLA-B39 did not show an association in the current study, or in another recent study [7]. Thus association between HLA-B39 and TAK is not likely. Although the previous study suggested the association of TAK with HLA-B*67:01, the association was not conclusive [7]. The current study has clearly revealed that HLA-B*67:01 is associated with TAK independently of HLA-B*52:01. The association and independence of

HLA-B*67:01 were observed in the two independent sets. The two relatives in our study did not have HLA-B*67:01, thus having no effect on the conclusion. When we analysed the previous data in which only serological *HLA-B* type was available [6], HLA-B67 showed a suggestive association with TAK after excluding HLA-B52 (95 cases and 232 controls) [6]. As HLA-B*67:01 is present specifically in East Asians, this association should be specific to East Asians. The RPE analysis did not reveal an association of HLA-B*40:06. This indicates that the protective association of HLA-B*40:06 in the replication set was conferred by positive associations of HLA-B*52:01 and HLA-B*67:01. While we detected the association of HLA-B*67:01 with TAK, HLA-B*67:01 did not show significant associations with age at onset, female ratio, TAK classifications and AR. Based on the fact that the *P*-values are far from significant, it is not very likely that HLA-B*67:01 has a strong effect on clinical phenotype. Further detailed clinical information, including affected branches of the aorta, and disease activity were available for only a limited number of patients in our study, so we could not analyse the association between the susceptibility allele and detailed clinical phenotypes.

The 171st histidine was found to be a susceptibility amino acid and the 67th phenylalanine was found to be a protective amino acid. While the permutation *P*-value supported the existence of the third susceptibility amino acid, alanine at position 158 did not show a significant Δ AIC in comparison with other amino acids. Thus we cannot specify the third susceptibility amino acid to TAK. The significant associations of the top two amino acids were mainly attributed to HLA-B*52:01. No other alleles share the two amino acids with HLA-B*52:01. Although HLA-B*51:01 has similar amino acid sequences to HLA-B*52:01 with two different amino acid residues, it is not associated with TAK [4, 7]. This lack of association can be explained by our findings because HLA-B*51:01 includes the 67th phenylalanine, the protective allele against TAK. HLA-B*67:01 did not have histidine in position 171. The effects of amino acids in HLA-B*67:01 were not very apparent due to its low frequency. These two amino acids did not explain TAK susceptibility more efficiently than HLA-B*52:01 (Δ AIC over the null model = 53.94 and 66.01, respectively), indicating that these amino acids cannot explain all the susceptibility effects of HLA-B*52:01. This suggests the existence of a haplotypic effect of amino acids in HLA-B*52:01 or further susceptibility amino acids.

The 171st tyrosine forming an A pocket in the α 2 domain is one of the highly conserved amino acid residues among *HLA-B* alleles. The conversion into histidine has been shown to modulate the binding groove, coordinating the N-terminus of the binding peptides in HLA-B14 and HLA-B*51:01 [17, 18]. The 67th serine in the B pocket of the peptide binding groove in HLA-B27 has been shown to influence peptide presentation [16]. These reports suggest important roles for the two amino acids in TAK susceptibility by modulating peptide binding. A previous Mexican study, which included 19 patients

with TAK, suggested that serine at 67 and glutamic acid at 63 was associated with TAK [19]. The previous report seemed to support our results, as the current study showed non-phenylalanine amino acid residues at position 67, including serine, were positively associated with TAK. As the current study did not specify the third susceptibility amino acid, the specific association of alanine at position 158 with TAK is inconclusive. Thus these combinations of amino acids should be regarded as incomplete and further investigation is required.

As previous studies surveying susceptibility HLA alleles beyond *HLA-B* did not find significant alleles beyond the *HLA-B* alleles [6, 7], it is not very likely that amino acids in other HLA alleles linked with HLA-B*52:01 and HLA-B*67:01 can explain the TAK susceptibility of the HLA locus. Nevertheless, it is undeniable that other genes in the HLA locus, without belonging to HLA alleles, play a critical role in TAK susceptibility. Amino acid residues in the HLA locus should be analysed to grasp the whole picture of the relationship between the HLA locus and TAK susceptibility. It would be interesting to determine what kinds of protein bind to the alleles containing the susceptibility amino acid residues.

Rheumatology key messages

- HLA-B*67:01 is an independent susceptibility allele to TAK from HLA-B*52:01.
- Amino acids at positions 171 and 67 of the HLA-B protein show TAK susceptibility effects.

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Supplementary data

Supplementary data are available at *Rheumatology Online*.