Table 1. Characteristics of the study population*

	Patients	Controls
First set		
Institutions	Kyoto University, Tokyo Women's Medical University	Kyoto University, Tokyo Women's Medical University, BioBank Japan
Typing	TaqMan assay	Illumina HumanHap610 Quad BeadChip, Illumina HumanHap550 BeadChip, Affymetrix Genome-Wide Human SNP Array 6.0
Limited SSc/diffuse SSc, %	49.6/50.4	Not applicable
Anti–topo I/ACA, %	30.6/31.1	Not applicable
Interstitial lung disease, %	48.9	Not applicable
Age, mean ± SD years	50.9 ± 14.7	60.9 ± 12.5
Female, %	91.3	44.9
Replication set		
Institutions	Keio University, Sagamihara National Hospital, Kanazawa University	Kyoto University, BioBank Japan
Typing	TaqMan assay	Illumina HumanHap550 BeadChip, Illumina HumanHap610 Quad BeadChip
Limited SSc/diffuse SSc, %	63.8/34.6	Not applicable
Anti–topo I/ACA, %	29.5/35.2	Not applicable
Interstitial lung disease, %	43.2	Not applicable
Age, mean ± SD years	51.4 ± 14.1	59.3 ± 14.2
Female, %	87.3	48.4

* The first set included 415 patients with systemic sclerosis (SSc) and 16,891 control subjects. The replication set included 315 patients with SSc and 21,054 control subjects. Anti–topo I = anti–topoisomerase I; ACA = anticentromere antibody.

the control subjects with those of patients in the SSc subgroups based on the disease phenotypes. The subanalyses used the same control subjects as were used in the association studies. Intracase analyses based on phenotypes were also performed.

Odds ratios (ORs) and 95% confidence intervals were also calculated. The associations detected in the first and replication studies were then meta-analyzed using the inverse variance method. The resultant *P* values were corrected using the Benjamini-Hochberg false discovery rate (FDR) criterion, and corrected *P* values less than 0.05 were regarded as significant in both the combined study and the subanalyses. The efficiency of the current study was estimated by calculating the likelihood of detecting 3 significant markers (after correcting the *P* values using the FDR method) among 18 randomly selected markers. After the statistically significant markers were identified, the best-fit model for each association was analyzed using dominant, recessive, trend, and allelic chi-square tests or models. Statistical analyses were performed using R or SPSS (version 18) software.

RESULTS

Analyses of candidate genes for SSc in a Japanese population. The 415 patients with SSc and 16,891 control subjects in the first set were genotyped for the 18 markers that were shown to have associations or suspected associations with RA in our previous study. The HLA region was excluded from the genotyped markers, because this region has already been shown to be associated with SSc in Asians. The allele frequencies of the patients were compared with those of the control subjects, using a Cochran-Armitage trend test.

As a result, 3 markers that demonstrated associations with *P* values less than 0.01 in the first set (Table 2) were identified, namely, rs6932056 in the *TNFAIP3* region (P = 0.0000038, OR 1.69), rs10821944 in the *ARID5B* region (P = 0.0025, OR 1.25), and rs2841277 in the *PLD4* region (P = 0.0054, OR 1.25). Two loci that showed suggestive associations with *P* values less than 0.1 (Table 2) were also identified, namely, rs12529514 in the *CD83* region (P = 0.083, OR 1.18) and rs2280381 in the *IRF8* region (P = 0.095, OR 1.19). The *TNFAIP3* and *IRF8* regions were previously reported to display associations with SSc and lcSSc, respectively, in European populations (10,18). These 5 markers were selected as candidate susceptibility markers for SSc in Japanese and were subjected to validation.

Next, a replication study consisting of 315 patients with SSc and 21,054 control subjects was performed to validate the associations of the 5 markers with SSc. The patients were genotyped for the 5 markers. The genotypes of the control subjects for the 5 markers, except rs6932056, were extracted from the Illumina Infinium HumanHap610 Quad array, as reported previously (31). The genotypes for rs6932056 were imputed based on genome-scanning data using mach2dat soft-

Table 2. Association studies of Japanese patients with SSc*

				Allele 1 frequency								
				First set			Replication set			Combined study		
SNP	Chr	Gene	Allele 1/2	Controls	Patients	*P*	Controls†	Patients	*P*	*P*, patients vs. controls	OR (95% CI)	*P*, patients without overlapping RA vs. controls
rs766449	1	*PADI4*	T/C	0.40	0.37	0.12	–	–	–	–	–	–
rs11900673	2	*B3GNT2*	T/C	0.29	0.28	0.65	–	–	–	–	–	–
rs2867461	4	*ANXA3*	A/G	0.44	0.43	0.57	–	–	–	–	–	–
rs657075	5	*IL3-CSF2*	A/G	0.36	0.34	0.25	–	–	–	–	–	–
rs12529514	6	*CD83*	C/T	0.14	0.16	0.083	0.15	0.16	0.31	0.046	1.15 (1.00–1.33)	0.040
rs1571878	6	*CCR6*	C/T	0.49	0.47	0.28	–	–	–	–	–	–
rs6932056	6	*TNFAIP3*	C/T	0.069	0.11	3.8×10^{-6}	0.067	0.079	0.23	9.5×10^{-6}	1.50 (1.25–1.80)	5.4×10^{-6}
rs2233434	6	*NFKBIE*	G/A	0.21	0.21	0.93	–	–	–	–	–	–
rs10821944	10	*ARID5B*	G/T	0.36	0.41	0.0025	0.36	0.37	0.64	0.0073	1.16 (1.04–1.29)	0.010
rs3781913	11	*PDE2A-CENTD2*	T/G	0.69	0.69	0.91	–	–	–	–	–	–
rs4937362	11	*ETS1-FLI1*	T/C	0.68	0.68	0.88	–	–	–	–	–	–
rs2841277	14	*PLD4*	T/C	0.69	0.74	0.0054	0.69	0.73	0.012	0.00017	1.25 (1.11–1.41)	0.00052
rs3783637	14	*GCH1*	C/T	0.74	0.73	0.54	–	–	–	–	–	–
rs1957895	14	*PRKCH*	G/T	0.39	0.41	0.26	–	–	–	–	–	–
rs6496667	15	*ZNF774*	A/C	0.35	0.37	0.33	–	–	–	–	–	–
rs7404928	16	*PRKCB1*	T/C	0.62	0.63	0.51	–	–	–	–	–	–
rs2280381	16	*IRF8*	T/C	0.84	0.86	0.095	0.83	0.87	0.0099	0.0030	1.26 (1.08–1.47)	0.0021
rs2847297	18	*PTPN2*	G/A	0.34	0.34	0.85	–	–	–	–	–	–

* SSc = systemic sclerosis; SNP = single-nucleotide polymorphism; Chr = chromosome; OR = odds ratio; 95% CI = 95% confidence interval; RA = rheumatoid arthritis.
† The control rs6932056 genotypes used in the replication study were imputed using genome-scanning data obtained for 3,765 subjects.

ware, because rs6932056 was not included in the array. As a result, rs2841277 in the *PLD4* region and rs2280381 in the *IRF8* region showed relatively strong associations with SSc ($P = 0.012$, OR 1.25 and $P = 0.0099$, OR 1.37, respectively) (Table 2). Interestingly, we observed that all 5 of the markers that displayed associations in the first study also demonstrated the same association directions in the replication study.

The inverse variance method was used to combine the data for the first and replication studies. SNPs rs2841277 in the *PLD4* region, rs6932056 in the *TNFAIP3* region, and rs2280381 in the *IRF8* region showed significant associations with SSc even after correcting the associated *P* values using the FDR method for multiple testing (Table 2). Importantly, all 3 of these loci shared risk alleles with RA. Although rs6932056 in the *TNFAIP3* region did not show a strong association with SSc in the replication study, its association was significant in the combined study. The *PLD4* region was shown to be a novel susceptibility gene for SSc, and, for the first time, the *TNFAIP3* and *IRF8* regions were confirmed to be associated with SSc in Japanese.

The association between rs2841277 and SSc was then investigated in detail. When the 200-kbp region around rs2841277 was evaluated, 2 hypothetical genes and cell division cycle associated 4 gene (*CDCA4*) were located at the region, in addition to *PLD4*. *PLD4* was the only gene whose region showed moderate to strong linkage disequilibrium (LD) with rs2841277, indicating *PLD4* as a susceptibility gene (Figure 1A). We vigorously searched candidate markers in exons of *PLD4* that showed strong LD with rs2841277 and selected 2 markers registered in the 1000 Genomes Project (34) that displayed >5% frequency in genotyped subjects, namely, rs2841280 (Figure 1B) and rs894037 in exon 2. Genotyping of these polymorphisms revealed strong LD between rs2841280 (E27Q) and rs2841277 ($D' = 0.98$, $r^2 = 0.75$) and monomorphism of rs894037 in Japanese. An association study of rs2841280 using control genotypes obtained by imputation supported association of *PLD4* with SSc ($P = 6.3 \times 10^{-5}$) (see Supplementary Tables 1 and 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.002/art.37777/abstract).

Because the 3 loci were associated with RA in a Japanese population, we analyzed whether the associations with SSc in the current study were contributed by patients with both RA and SSc. When 22 patients who had RA as well as SSc were excluded, significant associations for the 3 loci were still observed (Table 2). A

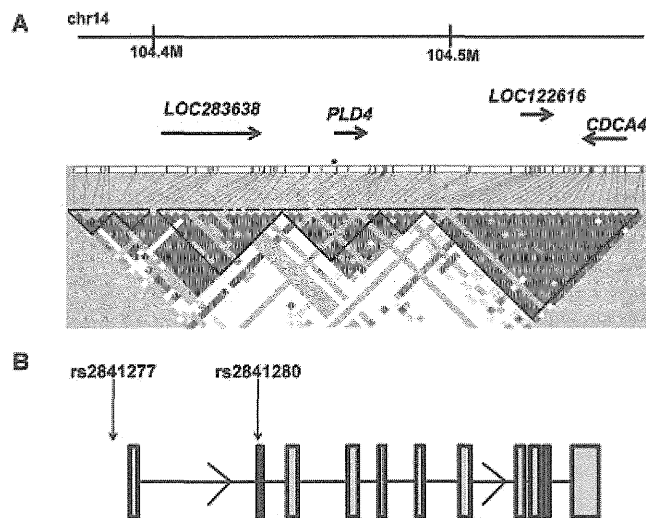


Figure 1. Linkage disequilibrium (LD) block around the *PLD4* region and the *PLD4* structure. **A,** LD block and genes around *PLD4*. The LD block is based on HapMap phase 3 data. **Asterisk** indicates rs2841277. **B,** Schematic view of *PLD4* structure. Rectangles represent exons of *PLD4*.

further stringent analysis excluding patients with other autoimmune diseases demonstrated significant associations of the 3 genes (see Supplementary Table 2). When we compared SSc patients with and those without other autoimmune diseases for the associated alleles, no differences were observed (data not shown).

Subanalysis of types of SSc. Previous studies have revealed that the genetic background of SSc varies between different types of SSc (11,18). Thus, subanalyses of the 5 regions examined in the combined study were performed, in which the allele frequencies of the control subjects were compared with those of the patients with lcSSc or dcSSc. The control subjects were the same as those used in the first study or the combined study. Although *PLD4* and *TNFAIP3* did not display a preference for either SSc phenotype, *IRF8* and *ARID5* showed suggestive preferences for lcSSc, and *CD83* showed a suggestive preference for dcSSc (Table 3).

We also investigated whether the susceptibility loci affect autoantibody status and severe complications. The association studies revealed an association of *TNFAIP3* with SSc patients who possess anticentromere antibodies (ACAs) (see Supplementary Table 3, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.37777/abstract), but intracase analyses did not demonstrate clear significance ($P = 0.043$). We did not observe other associations between the susceptibility loci and clinical phenotypes of SSc, in either case–control analyses or intracase analyses.

Efficacy of the current study. In the current study, a candidate gene analysis was performed based on a meta-analysis of RA GWAS, because many susceptibility genes for autoimmune disease have been reported

Table 3. Associations of the 2 SSc subtypes*

					Limited cutaneous SSc (n = 408)			Diffuse cutaneous SSc (n = 318)		
SNP	Chr	Gene	Allele 1/2	Controls, allele 1 frequency	Allele 1 frequency	*P*	OR (95% CI)	Allele 1 frequency	*P*	OR (95% CI)
rs766449	1	*PADI4*	T/C	0.40	0.39	0.52	0.94 (0.77–1.14)	0.36	0.11	0.85 (0.69–1.04)
rs11900673	2	*B3GNT2*	T/C	0.29	0.25	0.096	0.82 (0.66–1.03)	0.31	0.32	1.11 (0.9–1.38)
rs2867461	4	*ANXA3*	A/G	0.44	0.42	0.40	0.92 (0.75–1.12)	0.44	0.97	1.00 (0.82–1.22)
rs657075	5	*IL3-CSF2*	A/G	0.36	0.34	0.54	0.94 (0.76–1.15)	0.33	0.23	0.88 (0.72–1.08)
rs12529514	6	*CD83*	C/T	0.14	0.15	0.79	1.03 (0.85–1.25)	0.18	0.0075	1.32 (1.08–1.62)
rs1571878	6	*CCR6*	C/T	0.49	0.48	0.81	0.98 (0.80–1.19)	0.46	0.20	0.88 (0.72–1.07)
rs6932056	6	*TNFAIP3*	C/T	0.069	0.093	0.0062	1.40 (1.1–1.78)	0.10	0.00063	1.57 (1.21–2.04)
rs2233434	6	*NFKBIE*	G/A	0.21	0.20	0.60	0.94 (0.73–1.20)	0.22	0.70	1.05 (0.83–1.33)
rs10821944	10	*ARID5B*	G/T	0.36	0.40	0.0085	1.22 (1.05–1.41)	0.38	0.30	1.09 (0.93–1.29)
rs3781913	11	*PDE2A-CENTD2*	T/G	0.69	0.69	0.98	1.00 (0.81–1.24)	0.69	0.90	1.01 (0.82–1.25)
rs2841277	14	*PLD4*	T/C	0.69	0.73	0.0067	1.24 (1.06–1.45)	0.74	0.0049	1.29 (1.08–1.55)
rs2841280	14	*PLD4*	C/G	0.64	0.69	0.0011	1.30 (1.11–1.52)	0.69	0.0086	1.27 (1.06–1.51)
rs2847297	18	*PTPN2*	G/A	0.34	0.33	0.67	0.96 (0.78–1.18)	0.34	0.87	1.02 (0.83–1.25)
rs4937362	11	*ETS1-FLI1*	T/C	0.68	0.68	0.75	0.97 (0.78–1.19)	0.69	0.92	1.01 (0.82–1.25)
rs3783637	14	*GCH1*	C/T	0.74	0.73	0.69	0.96 (0.77–1.19)	0.73	0.65	0.95 (0.76–1.18)
rs1957895	14	*PRKCH*	G/T	0.39	0.40	0.84	1.02 (0.84–1.25)	0.42	0.16	1.15 (0.95–1.41)
rs6496667	15	*ZNF774*	A/C	0.35	0.39	0.088	1.19 (0.97–1.45)	0.34	0.75	0.97 (0.79–1.19)
rs7404928	16	*PRKCB1*	T/C	0.62	0.61	0.60	0.95 (0.78–1.16)	0.66	0.15	1.17 (0.95–1.44)
rs2280381	16	*IRF8*	T/C	0.84	0.88	0.0038	1.36 (1.11–1.68)	0.86	0.21	1.16 (0.92–1.45)

* SSc = systemic sclerosis; SNP = single-nucleotide polymorphism; Chr = chromosome; OR = odds ratio; 95% CI = 95% confidence interval.

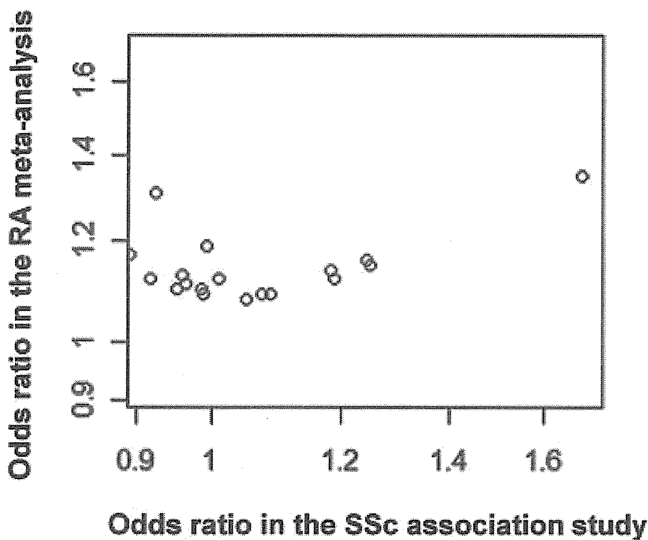


Figure 2. Comparison of associations for systemic sclerosis (SSc) and rheumatoid arthritis (RA). The odds ratios obtained for 18 genes in association studies of SSc and RA are plotted.

to be shared by a wide range of diseases. As a result, 3 susceptibility genes for SSc in Japanese were identified. Thus, we analyzed whether the candidate gene approach taken in the current study for detecting novel susceptibility genes for SSc was effective. When the likelihood of finding 3 susceptibility genes among 18 genes by chance was calculated, the likelihood was determined to be 2.5×10^{-8}. These results indicated that our approach to identifying novel susceptibility genes for systemic diseases is effective. It would be interesting to compare the risk direction of the genotyped markers between RA and SSc. Although the 3 susceptibility loci for SSc shared risk direction with RA, no correspondence of the risk directions of the markers between the 2 diseases was detected (Figure 2). This indicated that a large proportion of the 18 RA markers are not shared by SSc, and that the lack of association between the 13 markers and SSc was not attributable to the low power produced by the relatively small number of SSc patients included in this study.

DISCUSSION

Because SSc can lead to severe complications, poor quality of life, and shortened survival, clarifying the characteristics of SSc is important. Clarification of the disease would aid the search for novel therapeutic targets and the development of new therapeutic strategies. Detecting susceptibility genes using GWAS or a candidate gene approach would also help to uncover the pathophysiology underlying SSc.

Previous studies have revealed that more than 15 markers and loci are associated with SSc. However, the markers detected so far cannot fully explain the genetics of SSc, indicating that many susceptibility genes are yet to be identified. Because a relatively large proportion of RA susceptibility genes are shared by other autoimmune diseases (24), a candidate gene approach using novel markers observed in GWAS of RA is a fascinating way of identifying new SSc markers. In fact, some of the novel susceptibility markers for RA identified in the meta-analysis were shown to be susceptibility markers for systemic lupus erythematosus (SLE) and Graves' disease (31).

In the current study, we successfully identified 3 susceptibility genes for SSc in Japanese. No studies have identified *PLD4* as an SSc-associated locus. The current study is also the first to detect *TNFAIP3* and *IRF8* as susceptibility genes for SSc in a Japanese population. The best-fit models for each association are shown in Supplementary Table 4, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.002/art.37777/abstract.

It is conceivable that these 3 associations might have been obtained due to the overlap of RA and SSc. Even after excluding the patients with both RA and SSc based on physicians' reports, the significant associations for the 3 loci were still observed (Table 3). Information regarding rheumatoid factor (RF) and anti–citrullinated protein antibody (ACPA) was available for 371 SSc patients without RA and 65 SSc patients without RA, respectively, of whom 21.6% and 10.8% were positive for RF and ACPA, respectively. These prevalences are compatible with those previously observed in SSc patients without RA (35,36). Moreover, we showed that the effect sizes and risk direction of the markers tested in this study were dissociated between SSc and RA. In addition, further stringent analysis comprising SSc patients without any autoimmune disease also showed the associations of the 3 loci. These results indicate that the associations of the 3 loci are not attributable to overlapping of RA or other diseases.

Although the associations of the *ARID5B* and *CD83* loci with SSc did not reach a stringently significant level in the combined study, the tendencies toward an association with SSc displayed by rs10821944 in the *ARID5B* locus and rs12529514 in the *CD83* region in the first study were maintained in the replication study. This indicates that these loci are potential susceptibility regions for SSc. Further replication studies are needed to

address the associations of these 2 loci with SSc in a Japanese population.

Because *TNFAIP3* was reported to be strongly associated with SSc in a European population (18), the significant associations detected in the combined study indicate that *TNFAIP3* displays general associations with SSc that go beyond ethnic boundaries. In addition, rs6932056, which displayed a strong association with SSc in a European population (18), is in strong LD with rs5029939 ($r^2 = 0.85$) in the Japanese population. SNP rs6932056 also displays strong LD with rs2230926, a missense mutation of *TNFAIP3* ($r^2 = 0.85$), in Japanese. The rs2230926 missense mutation leads to an amino acid alteration in the OTU (ovarian tumor) domain of the A20 protein, which is considered to result in decreased NF-κB signaling. Because we did not observe strong associations between rs6932056 and SSc in the replication study, it will be necessary to reexamine the association between *TNFAIP3* and SSc using independent sample sets of Japanese patients with SSc, in spite of the significant associations detected in this study.

PLD4 is a recently reported member of the phospholipase family without phospholipase D activity. *PLD4* is expressed in the spleen and early postnatal microglia in the white matter of mice (37). The phenotypes of *Pld4*-deficient mice have not been reported. In addition, little is known about the expression or distribution of *PLD4* in humans. Although the functions of *PLD4* are also poorly understood, it is known to be involved in the phagocytosis of microglia (38). The expression of *PLD4* around the marginal zone in the spleen might support the functional involvement of *PLD4* in immunologic systems. It is interesting that rs2841280, which alters an amino acid of PLD-4, is associated with SSc. Minor allele G of rs2841280 is associated in a protective manner. The impact of an amino acid alteration brought by rs2841280 on the effect of PLD-4 protein is not known.

When we analyzed the impact of the amino acid alteration using in silico analysis (SIFT software; http://sift.jcvi.org/), it was shown to result in a small effect. However, the association raises the possibility that this polymorphism leads functional modulation of PLD-4, and it is feasible to analyze the functional change of PLD-4 protein with rs2841280, using animal models of SSc. When we performed an in silico analysis of the effect of rs2841277 and rs2841280 on *PLD4* expression, we did not detect any clear associations between the 2 genotypes and *PLD4* transcription ($P > 0.05$) (39). Therefore, in spite of the association of these 2 mutations, it has not been confirmed whether one of these 2 polymorphisms is the causative mutation.

Although the detection of a P value less than 5×10^{-8} in a GWAS is stringent evidence of an association between a marker and a particular disease, the detection of suggestive associations between the *PLD4* region and SSc in European GWAS would indicate that associations exist between *PLD4* and SSc in other populations. However, when we examined the associations between the *PLD4* locus or nearby loci and SSc in GWAS involving a European population, we did not detect any strong associations ($P < 10^{-4}$) (8,9). According to the HapMap database, the European population displays a higher risk allele frequency for rs2841277 than the Japanese population. In addition, the HapMap database also indicates that the LD block spanning *PLD4*, which includes rs2841277, is similar in Europeans and Japanese. Nevertheless, a European population did not show a strong association between *PLD4* and SSc, suggesting that *PLD4* has a stronger effect on autoimmune diseases in Japanese than in Europeans. There is also a possibility that these 2 polymorphisms are only markers, and that a rare variant in LD with the 2 markers affects disease onset. A rare causative variant might explain a different association of *PLD4* with SSc between populations.

IRF8 was shown to be associated with SLE in a European population (40). Interferon regulatory factor 8 (IRF-8) protein is a transcription factor involved in the interferon pathway. The interferon pathway has been shown to be involved with a broad range of autoimmune diseases, including SSc (41). Thus, it is interesting that *IRF5* and *IRF8*, both of which belong to the IRF family, displayed associations with SSc. Although a European GWAS of SSc patients revealed suggestive associations between the *IRF4* locus and SSc, the results were not successfully replicated (8), indicating that the different functional roles of each IRF family molecule might influence the development of SSc. *IRF8* promotes B cell differentiation; however, the roles and importance of B cells in skin fibrosis in SSc patients have not been established (42–44). *IRF8* and its mutant variants are also known to be involved in the development of dendritic cells (45). Thus, the association between *IRF8* and SSc might indicate the involvement of B cells and dendritic cells in the development of SSc.

When the patients with SSc were classified as having either lcSSc or dcSSc and subanalyses were performed, *ARID5B*, *IRF8*, and *CD83* displayed stronger associations with one of the 2 phenotypes. However, the associations of these 3 markers with the phenotypes

were not strong enough to provide convincing evidence of a clear distinction between the genetic backgrounds of the 2 SSc phenotypes. When the associations of the SSc subtypes with the other 13 markers in the first set were analyzed, no strong association was detected ($P > 0.05$). Other subanalyses of the susceptibility loci in the combined set did not show significant results between disease phenotypes, due to lack of power. Because classification according to disease phenotypes resulted in limited numbers of subjects in each subset, we conducted this subanalysis only in the combined set. The association between *TNFAIP3* and ACAs should be confirmed in a large-scale association study.

Although GWAS are an extremely powerful way to detect novel susceptibility genes for diseases, GWAS of patients with SSc have been performed only in European populations. Our study detected strong evidence for the sharing of susceptibility genes between RA and SSc in a Japanese population. In addition, the current study indicated that a candidate gene approach based on the results of GWAS of other diseases that display pathologic signaling pathways or mechanisms similar to those associated with the disease being examined is an effective approach to identifying novel susceptibility genes.

It will be interesting to perform GWAS of Japanese patients with SSc and analyze the similarities and differences in the detected associations not only between Japanese and Europeans but also between Japanese patients with SSc and Japanese patients with RA.

ACKNOWLEDGMENT

We thank the staff of the BioBank Japan Project for collecting DNA samples from control subjects.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Terao had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Terao, Ohmura, Kawaguchi, Nishimoto, Kawasaki, Takehara, Furukawa, Kochi, Ota, Ikari, Sato, Tohma, Yamada, Yamamoto, Kubo, Yamanaka, Kuwana, Tsuchiya, Matsuda, Mimori.

Acquisition of data. Terao, Ohmura, Kawaguchi, Nishimoto, Kawasaki, Takehara, Furukawa, Kochi, Ota, Ikari, Sato, Tohma, Yamada, Yamamoto, Kubo, Yamanaka, Kuwana, Tsuchiya, Matsuda, Mimori.

Analysis and interpretation of data. Terao, Ohmura.

REFERENCES

1. Chifflot H, Fautrel B, Sordet C, Chatelus E, Sibilia J. Incidence and prevalence of systemic sclerosis: a systematic literature review. Semin Arthritis Rheum 2008;37:223–35.
2. Kawut SM, Taichman DB, Archer-Chicko CL, Palevsky HI, Kimmel SE. Hemodynamics and survival in patients with pulmonary arterial hypertension related to systemic sclerosis. Chest 2003;123: 344–50.
3. Ioannidis JP, Vlachoyiannopoulos PG, Haidich AB, Medsger TA Jr, Lucas M, Michet CJ, et al. Mortality in systemic sclerosis: an international meta-analysis of individual patient data. Am J Med 2005;118:2–10.
4. Bhattacharyya S, Wei J, Varga J. Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. Nat Rev Rheumatol 2012;8:42–54.
5. Romano E, Manetti M, Guiducci S, Ceccarelli C, Allanore Y, Matucci-Cerinic M. The genetics of systemic sclerosis: an update. Clin Exp Rheumatol 2011;29:S75–86.
6. Arnett FC, Cho M, Chatterjee S, Aguilar MB, Reveille JD, Mayes MD. Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. Arthritis Rheum 2001;44:1359–62.
7. Terao C, Ohmura K, Katayama M, Takahashi M, Kokubo M, Diop G, et al. Myelin basic protein as a novel genetic risk factor in rheumatoid arthritis: a genome-wide study combined with immunological analyses. PLoS One 2011;6:e20457.
8. Radstake TR, Gorlova O, Rueda B, Martin JE, Alizadeh BZ, Palomino-Morales R, et al. Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. Nat Genet 2010;42:426–9.
9. Allanore Y, Saad M, Dieude P, Avouac J, Distler JH, Amouyel P, et al. Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. PLoS Genet 2011;7:e1002091.
10. Gorlova O, Martin JE, Rueda B, Koeleman BP, Ying J, Teruel M, et al. Identification of novel genetic markers associated with clinical phenotypes of systemic sclerosis through a genome-wide association strategy. PLoS Genet 2011;7:e1002178.
11. Rueda B, Broen J, Simeon C, Hesselstrand R, Diaz B, Suarez H, et al. The STAT4 gene influences the genetic predisposition to systemic sclerosis phenotype. Hum Mol Genet 2009;18:2071–7.
12. Dieude P, Guedj M, Wipff J, Avouac J, Fajardy I, Diot E, et al. Association between the IRF5 rs2004640 functional polymorphism and systemic sclerosis: a new perspective for pulmonary fibrosis. Arthritis Rheum 2009;60:225–33.
13. Gourh P, Agarwal SK, Divecha D, Assassi S, Paz G, Arora-Singh RK, et al. Polymorphisms in TBX21 and STAT4 increase the risk of systemic sclerosis: evidence of possible gene–gene interaction and alterations in Th1/Th2 cytokines. Arthritis Rheum 2009;60: 3794–806.
14. Dieude P, Guedj M, Wipff J, Ruiz B, Riemekasten G, Airo P, et al. NLRP1 influences the systemic sclerosis phenotype: a new clue for the contribution of innate immunity in systemic sclerosis-related fibrosing alveolitis pathogenesis. Ann Rheum Dis 2011;70: 668–74.
15. Bossini-Castillo L, Broen JC, Simeon CP, Beretta L, Vonk MC, Ortego-Centeno N, et al. A replication study confirms the association of TNFSF4 (OX40L) polymorphisms with systemic sclerosis in a large European cohort. Ann Rheum Dis 2011;70:638–41.
16. Dieude P, Guedj M, Truchetet ME, Wipff J, Revillod L, Riemekasten G, et al. Association of the CD226 Ser^{307} variant with systemic sclerosis: evidence of a contribution of costimulation pathways in systemic sclerosis pathogenesis. Arthritis Rheum 2011;63:1097–105.
17. Gourh P, Agarwal SK, Martin E, Divecha D, Rueda B, Bunting H, et al. Association of the C8orf13-BLK region with systemic sclerosis in North-American and European populations. J Autoimmun 2010;34:155–62.
18. Dieude P, Guedj M, Wipff J, Ruiz B, Riemekasten G, Matucci-Cerinic M, et al. Association of the TNFAIP3 rs5029939 variant

with systemic sclerosis in the European Caucasian population. Ann Rheum Dis 2010;69:1958–64.

19. Tsuchiya N, Kawasaki A, Hasegawa M, Fujimoto M, Takehara K, Kawaguchi Y, et al. Association of STAT4 polymorphism with systemic sclerosis in a Japanese population. Ann Rheum Dis 2009;68:1375–6.
20. Ito I, Kawaguchi Y, Kawasaki A, Hasegawa M, Ohashi J, Hikami K, et al. Association of a functional polymorphism in the IRF5 region with systemic sclerosis in a Japanese population. Arthritis Rheum 2009;60:1845–50.
21. Ito I, Kawaguchi Y, Kawasaki A, Hasegawa M, Ohashi J, Kawamoto M, et al. Association of the FAM167A–BLK region with systemic sclerosis. Arthritis Rheum 2010;62:890–5.
22. Hasebe N, Kawasaki A, Ito I, Kawamoto M, Hasegawa M, Fujimoto M, et al. Association of UBE2L3 polymorphisms with diffuse cutaneous systemic sclerosis in a Japanese population. Ann Rheum Dis 2012;71:1259–60.
23. Zhou X, Lee JE, Arnett FC, Xiong M, Park MY, Yoo YK, et al. HLA–DPB1 and DPB2 are genetic loci for systemic sclerosis: a genome-wide association study in Koreans with replication in North Americans. Arthritis Rheum 2009;60:3807–14.
24. Suzuki A, Kochi Y, Okada Y, Yamamoto K. Insight from genome-wide association studies in rheumatoid arthritis and multiple sclerosis. FEBS Lett 2011;585:3627–32.
25. Plenge RM, Seielstad M, Padyukov L, Lee AT, Remmers EF, Ding B, et al. TRAF1-C5 as a risk locus for rheumatoid arthritis: a genomewide study. N Engl J Med 2007;357:1199–209.
26. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, Behrens TW, et al. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 2007;357:977–86.
27. Plenge RM, Cotsapas C, Davies L, Price AL, de Bakker PI, Maller J, et al. Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. Nat Genet 2007;39:1477–82.
28. Thomson W, Barton A, Ke X, Eyre S, Hinks A, Bowes J, et al. Rheumatoid arthritis association at 6q23. Nat Genet 2007;39: 1431–3.
29. Begovich AB, Carlton VE, Honigberg LA, Schrodi SJ, Chokkalingam AP, Alexander HC, et al. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. Am J Hum Genet 2004;75:330–7.
30. Diaz-Gallo LM, Gourh P, Broen J, Simeon C, Fonollosa V, Ortego-Centeno N, et al. Analysis of the influence of PTPN22 gene polymorphisms in systemic sclerosis. Ann Rheum Dis 2011; 70:454–62.
31. Okada Y, Terao C, Ikari K, Kochi Y, Ohmura K, Suzuki A, et al. Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. Nat Genet 2012;44:511–6.
32. Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980;23:581–90.
33. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA Jr, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;15:202–5.
34. 1000 Genomes Project Consortium. A map of human genome variation from population-scale sequencing. Nature 2010;467: 1061–73.
35. Mimura Y, Ihn H, Jinnin M, Asano Y, Yamane K, Yazawa N, et al. Rheumatoid factor isotypes and anti-agalactosyl IgG antibodies in systemic sclerosis. Br J Dermatol 2004;151:803–8.
36. Santiago M, Baron M, Miyachi K, Fritzler MJ, Abu-Hakima M, Leclercq S, et al. A comparison of the frequency of antibodies to cyclic citrullinated peptides using a third generation anti-CCP assay (CCP3) in systemic sclerosis, primary biliary cirrhosis and rheumatoid arthritis. Clin Rheumatol 2008;27:77–83.
37. Yoshikawa F, Banno Y, Otani Y, Yamaguchi Y, Nagakura-Takagi Y, Morita N, et al. Phospholipase D family member 4, a transmembrane glycoprotein with no phospholipase D activity, expression in spleen and early postnatal microglia. PLoS One 2010;5: e13932.
38. Otani Y, Yamaguchi Y, Sato Y, Furuichi T, Ikenaka K, Kitani H, et al. PLD4 is involved in phagocytosis of microglia: expression and localization changes of PLD4 are correlated with activation state of microglia. PLoS One 2011;6:e27544.
39. Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N, et al. Relative impact of nucleotide and copy number variation on gene expression phenotypes. Science 2007;315: 848–53.
40. Cunninghame Graham DS, Morris DL, Bhangale TR, Criswell LA, Syvanen AC, Ronnblom L, et al. Association of NCF2, IKZF1, IRF8, IFIH1, and TYK2 with systemic lupus erythematosus. PLoS Genet 2011;7:e1002341.
41. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, et al. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. Ann Rheum Dis 2011;70:2029–36.
42. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci U S A 2003;100:12319–24.
43. Lafyatis R, Kissin E, York M, Farina G, Viger K, Fritzler MJ, et al. B cell depletion with rituximab in patients with diffuse cutaneous systemic sclerosis. Arthritis Rheum 2009;60:578–83.
44. Smith V, Van Praet JT, Vandooren B, Van der Cruyssen B, Naeyaert JM, Decuman S, et al. Rituximab in diffuse cutaneous systemic sclerosis: an open-label clinical and histopathological study. Ann Rheum Dis 2010;69:193–7.
45. Hambleton S, Salem S, Bustamante J, Bigley V, Boisson-Dupuis S, Azevedo J, et al. IRF8 mutations and human dendritic-cell immunodeficiency. N Engl J Med 2011;365:127–38.

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

Yasuhito Hamaguchi[1], Manabu Fujimoto[1]*, Takashi Matsushita[1], Kenzo Kaji[1], Kazuhiro Komura[1], Minoru Hasegawa[1], Masanari Kodera[2], Eiji Muroi[3], Keita Fujikawa[4], Mariko Seishima[5], Hidehiro Yamada[6], Ryo Yamada[7], Shinichi Sato[8], Kazuhiko Takehara[1], Masataka Kuwana[9]

1 Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan, **2** Department of Dermatology, Social Insurance Chukyo Hospital, Nagoya, Japan, **3** Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, **4** Unit of Translational Medicine, Department of Immunology and Rheumatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, **5** Department of Dermatology, Ogaki Municipal Hospital, Ogaki, Japan, **6** Division of Rheumatology, Department of Internal Medicine, and Allergy, St. Marianna University, Kawasaki, Japan, **7** Center for Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **8** Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, Japan, **9** Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

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

Copyright: © 2013 Hamaguchi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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.

* E-mail: fujimoto-m@umin.ac.jp

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].

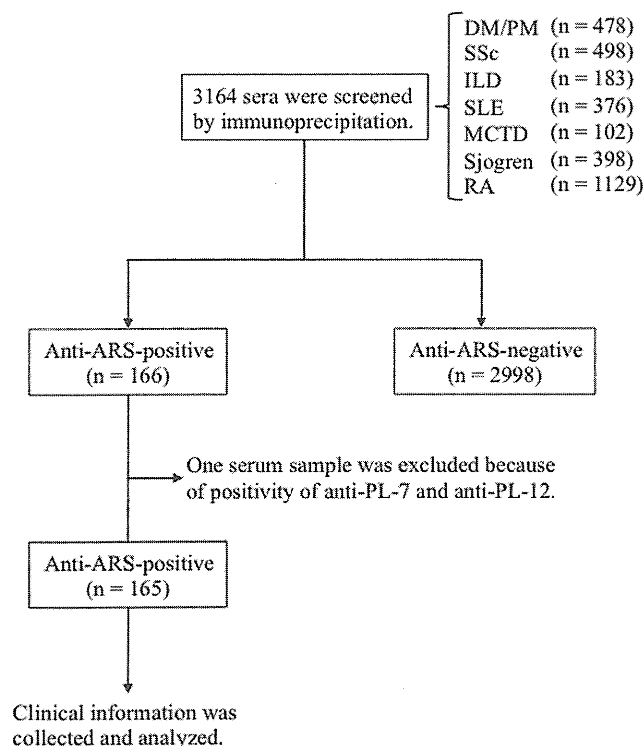


Figure 1. Enrollment and selection of patients. DM; dermatomyositis, PM; polymyositis, SSc; systemic sclerosis, ILD; interstitial lung disease, SLE; systemic lupus erythematosus, MCTD; mixed connective tissue disease, Sjogren; Sjogren's syndrome, RA; rheumatoid arthritis.
doi:10.1371/journal.pone.0060442.g001

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

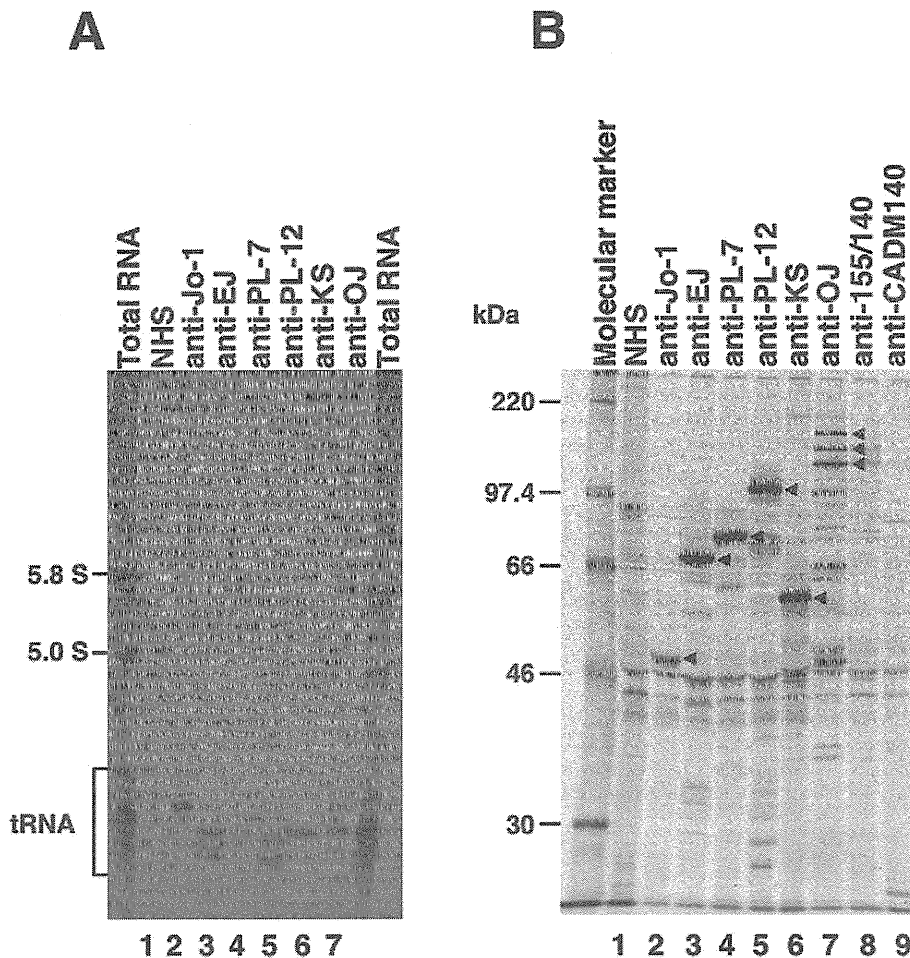


Figure 2. Representative immunoprecipitation assay for RNA with anti-aminoacyl-tRNA synthetase (anti-ARS) sera. A, Immunoprecipitation of histidyl-tRNA synthetase, glycyl-tRNA synthetase, threonyl-tRNA synthetase, alanyl-tRNA synthetase, asparaginyl-tRNA, and isoleucyl-tRNA synthetase by sera. K562 cell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

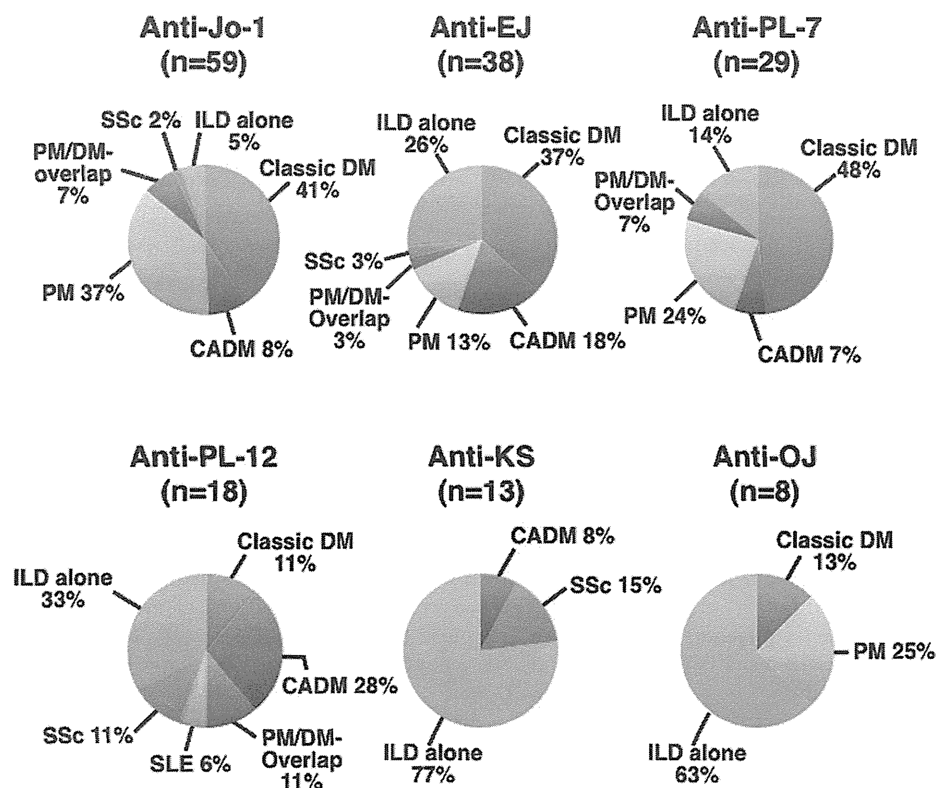


Figure 3. Prevalence of dermatomysitis (DM), clinically amyopathic DM (CADM), polymyositis (PM), PM/DM-overlap, systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and interstitial lung disease (ILD) alone, in each subgroup of anti-synthetase syndrome.
doi:10.1371/journal.pone.0060442.g003

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.

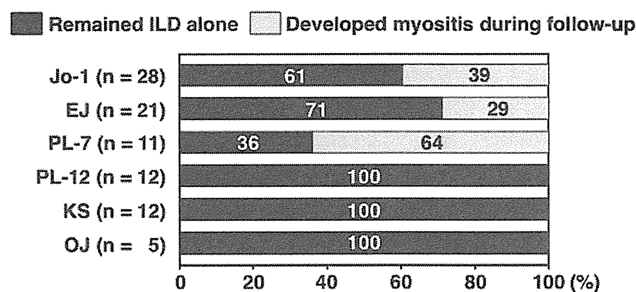
Anti-ARS	Age, y	Sex	Diagnosis	ILD	Cause of death	Time after diagnosis (y)
Anti-Jo-1	64	F	DM	+	ILD	0.3
Anti-Jo-1	38	F	DM	+	Infection	3
Anti-Jo-1	36	F	DM	+	ILD	5.5
Anti-Jo-1	62	M	PM	−	Gastric cancer	5
Anti-EJ	65	F	DM	+	ILD	2.5
Anti-EJ	55	F	ILD	+	ILD	0.6
Anti-EJ	55	F	DM	+	ILD	4.25
Anti-EJ	53	F	SSc	+	Infection	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



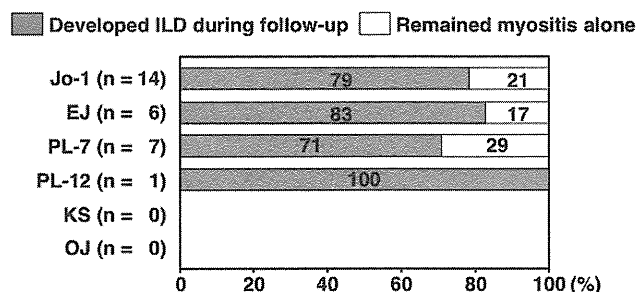


Figure 4. The clinical course of anti-synthetase syndrome patients who developed myositis or interstitial lung disease (ILD) with or without skin manifestations at disease onset. According to the clinical course, patients were classified into four types: remained with ILD alone, developed myositis during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

References

1. Targoff IN (2002) Laboratory testing in the diagnosis and management of idiopathic inflammatory myopathies. Rheum Dis Clin North Am. 28: 859–890, viii.
2. Mammen AL (2010) Dermatomyositis and polymyositis: Clinical presentation, autoantibodies, and pathogenesis. Ann N Y Acad Sci. 1184: 134–153.
3. Hamaguchi Y, Kuwana M, Hoshino K, Hasegawa M, Kaji K, et al. (2011) Clinical correlations with dermatomyositis-specific autoantibodies in adult Japanese patients with dermatomyositis: a multicenter cross-sectional study. Arch Dermatol. 147: 391–398.
4. Love LA, Leff RL, Fraser DD, Targoff IN, Dalakas M, et al. (1991) A new approach to the classification of idiopathic inflammatory myopathy: myositis-specific autoantibodies define useful homogeneous patient groups. Medicine (Baltimore). 70: 360–374.
5. Mierau R, Dick T, Bartz-Bazzanella P, Keller E, Albert ED, et al. (1996) Strong association of dermatomyositis-specific Mi-2 autoantibodies with a tryptophan at position 9 of the HLA-DR beta chain. Arthritis Rheum. 39: 868–876.
6. Komura K, Fujimoto M, Matsushita T, Kaji K, Kondo M, et al. (2005) Prevalence and clinical characteristics of anti-Mi-2 antibodies in Japanese patients with dermatomyositis. J Dermatol Sci. 40: 215–217.
7. Kaji K, Fujimoto M, Hasegawa M, Kondo M, Saito Y, et al. (2007) Identification of a novel autoantibody reactive with 155 and 140 kDa nuclear proteins in patients with dermatomyositis: an association with malignancy. Rheumatology (Oxford). 46: 25–28.
8. Targoff IN, Mamyrova G, Trieu EP, Perurena O, Koneru B, et al. (2006) A novel autoantibody to a 155-kd protein is associated with dermatomyositis. Arthritis Rheum. 54: 3682–3689.
9. Gunawardena H, Wedderburn LR, North J, Betteridge Z, Dunphy J, et al. (2008) Clinical associations of autoantibodies to a p155/140 kDa doublet protein in juvenile dermatomyositis. Rheumatology (Oxford). 47: 324–328.
10. Fujimoto M, Hamaguchi Y, Kaji K, Matsushita T, Ichimura Y, et al. (2012) Myositis-specific anti-155/140 autoantibodies target transcription intermediary factor 1 family proteins. Arthritis Rheum. 64: 513–522.
11. Sato S, Hirakata M, Kuwana M, Suwa A, Inada S, et al. (2005) Autoantibodies to a 140-kd polypeptide, CADM-140, in Japanese patients with clinically amyopathic dermatomyositis. Arthritis Rheum. 52: 1571–1576.
12. Sato S, Hoshino K, Satoh T, Fujita T, Kawakami Y, et al. (2009) RNA helicase encoded by melanoma differentiation-associated gene 5 is a major autoantigen in patients with clinically amyopathic dermatomyositis: Association with rapidly progressive interstitial lung disease. Arthritis Rheum. 60: 2193–2200.
13. Hirakata M, Mimori T, Akizuki M, Craft J, Hardin JA, et al. (1992) Autoantibodies to small nuclear and cytoplasmic ribonucleoproteins in Japanese patients with inflammatory muscle disease. Arthritis Rheum. 35: 449–456.
14. Yoshida S, Akizuki M, Mimori T, Yamagata H, Inada S, et al. (1983) The precipitating antibody to an acidic nuclear protein antigen, the Jo-1, in connective tissue diseases. A marker for a subset of polymyositis with interstitial pulmonary fibrosis. Arthritis Rheum. 26: 604–611.
15. Mathews MB, Reichlin M, Hughes GR, Bernstein RM (1984) Anti-threonyl-tRNA synthetase, a second myositis-related autoantibody. J Exp Med. 160: 420–434.
16. Bunn CC, Bernstein RM, Mathews MB (1986) Autoantibodies against alanyl-tRNA synthetase and tRNAAla coexist and are associated with myositis. J Exp Med. 163: 1281–1291.
17. Targoff IN (1990) Autoantibodies to aminoacyl-transfer RNA synthetases for isoleucine and glycine. Two additional synthetases are antigenic in myositis. J Immunol. 144: 1737–1743.
18. Hirakata M, Suwa A, Nagai S, Kron MA, Trieu EP, et al. (1999) Anti-KS: identification of autoantibodies to asparaginyl-transfer RNA synthetase associated with interstitial lung disease. J Immunol. 162: 2315–2320.
19. Betteridge Z, Gunawardena H, North J, Slinn J, McHugh N (2007) Anti-synthetase syndrome: a new autoantibody to phenylalanyl transfer RNA synthetase (anti-Zo) associated with polymyositis and interstitial pneumonia. Rheumatology (Oxford). 46: 1005–1008.
20. Hashish L, Trieu EP, Sadanandan P, Targoff IN (2005) Identification of autoantibodies to tyrosyl-tRNA synthetase in dermatomyositis with features consistent with antisynthetase syndrome. Arthritis Rheum 52 Suppl 9: S312.
21. Targoff IN (1992) Autoantibodies in polymyositis. Rheum Dis Clin North Am. 18: 455–482.
22. Hervier B, Devilliers H, Stanciu R, Meyer A, Uzunhan Y, et al. (2012) Hierarchical cluster and survival analyses of antisynthetase syndrome: Phenotype and outcome are correlated with anti-tRNA synthetase antibody specificity. Autoimmun Rev. 12: 210–217.
23. Kalluri M, Sahn SA, Oddis CV, Gharib SL, Christopher-Stine L, et al. (2009) Clinical profile of anti-PL-12 autoantibody. Cohort study and review of the literature. Chest. 135: 1550–1556.
24. Sato S, Hirakata M, Kuwana M, Nakamura K, Suwa A, et al. (2005) Clinical characteristics of Japanese patients with anti-PL-7 (anti-threonyl-tRNA synthetase) autoantibodies. Clin Exp Rheumatol. 23: 609–615.
25. Bohan A, Peter JB (1975) Polymyositis and dermatomyositis (second of two parts). N Engl J Med. 292: 403–407.
26. Sontheimer RD (2002) Would a new name hasten the acceptance of amyopathic dermatomyositis (dermatomyositis sine myositis) as a distinctive subset within the idiopathic inflammatory dermatomyopathies spectrum of clinical illness? J Am Acad Dermatol. 46: 626–636.
27. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, et al. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 25: 1271–1277.
28. Anonymous (1980) Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 23: 581–590.
29. Tomasova, Studynkova J, Charvat F, Jarosova K, Vencovsky J (2007) The role of MRI in the assessment of polymyositis and dermatomyositis. Rheumatology (Oxford). 46: 1174–1179.
30. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, et al. (2002) Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis. 61: 554–558.
31. Hamaguchi Y, Hasegawa M, Fujimoto M, Matsushita T, Komura K, et al. (2008) The clinical relevance of serum antinuclear antibodies in Japanese patients with systemic sclerosis. Br J Dermatol. 158: 487–495.
32. Hirakata M, Suwa A, Takada T, Sato S, Nagai S, et al. (2007) Clinical and immunogenetic features of patients with autoantibodies to asparaginyl-transfer RNA synthetase. Arthritis Rheum. 56: 1295–1303.
33. Kuwana M, Kaburaki J, Mimori T, Tojo T, Homma M (1993) Antoantibody reactive with three classes of RNA polymerases in sera from patients with systemic sclerosis. J Clin Invest. 91: 1399–1404.
34. Moroi Y, Peebles C, Fritzler MJ, Steigerwald J, Tan EM (1980) Autoantibody to centromere (kinetochore) in scleroderma sera. Proc Natl Acad Sci U S A. 77: 1627–1631.
35. Vancsa A, Gergely L, Ponyi A, Lakos G, Nemeth J, et al. (2010) Myositis-specific and myositis-associated antibodies in overlap myositis in comparison to primary dermatopolymyositis: Relevance for clinical classification: retrospective study of 169 patients. Joint Bone Spine. 77: 125–130.
36. Bandoh S, Fujita J, Ohtsuki Y, Ueda Y, Hojo S, et al. (2000) Sequential changes of KL-6 in sera of patients with interstitial pneumonia associated with polymyositis/dermatomyositis. Ann Rheum Dis. 59: 257–262.
37. Honda Y, Kuroki Y, Matsuura E, Nagae H, Takahashi H, et al. (1995) Pulmonary surfactant protein D in sera and bronchoalveolar lavage fluids. Am J Respir Crit Care Med. 152: 1860–1866.
38. Matsushita T, Hasegawa M, Fujimoto M, Hamaguchi Y, Komura K, et al. (2007) Clinical evaluation of anti-aminoacyl tRNA synthetase antibodies in Japanese patients with dermatomyositis. J Rheumatol. 34: 1012–1018.
39. Mammen AL (2011) Autoimmune myopathies: autoantibodies, phenotypes and pathogenesis. Nat Rev Neurol. 7: 343–354.
40. Connors GR, Christopher-Stine L, Oddis CV, Danoff SK (2010) Interstitial lung disease associated with the idiopathic inflammatory myopathies: what progress has been made in the past 35 years? Chest. 138: 1464–1474.
41. Gunawardena H, Betteridge ZE, McHugh NJ (2009) Myositis-specific autoantibodies: their clinical and pathogenic significance in disease expression. Rheumatology (Oxford). 48: 607–612.

42. Zong M, Lundberg IE (2011) Pathogenesis, classification and treatment of inflammatory myopathies. Nat Rev Rheumatol. 7: 297–306.
43. Hirakata M, Nagai S (2000) Interstitial lung disease in polymyositis and dermatomyositis. Curr Opin Rheumatol. 12: 501–508.
44. Yoshifuji H, Fujii T, Kobayashi S, Imura Y, Fujita Y, et al. (2006) Anti-aminoacyl-tRNA synthetase antibodies in clinical course prediction of interstitial lung disease complicated with idiopathic inflammatory myopathies. Autoimmunity. 39: 233–241.
45. Katzap E, Barilla-LaBarca ML, Marder G (2011) Antisynthetase syndrome. Curr Rheumatol Rep. 13: 175–181.
46. Mimori T, Imura Y, Nakashima R, Yoshifuji H (2007) Autoantibodies in idiopathic inflammatory myopathy: an update on clinical and pathophysiological significance. Curr Opin Rheumatol. 19: 523–529.
47. Targoff IN, Trieu EP, Plotz PH, Miller FW (1992) Antibodies to glycyl-transfer RNA synthetase in patients with myositis and interstitial lung disease. Arthritis Rheum. 35: 821–830.
48. Watanabe K, Handa T, Tanizawa K, Hosono Y, Taguchi Y, et al. (2011) Detection of antisynthetase syndrome in patients with idiopathic interstitial pneumonias. Respir Med. 105: 1238–1247.
49. Yamasaki Y, Yamada H, Nozaki T, Akaogi J, Nichols C, et al. (2006) Unusually high frequency of autoantibodies to PL-7 associated with milder muscle disease in Japanese patients with polymyositis/dermatomyositis. Arthritis Rheum. 54: 2004–2009.
50. Targoff IN, Arnett FC (1990) Clinical manifestations in patients with antibody to PL-12 antigen (alanyl-tRNA synthetase). Am J Med. 88: 241–251.
51. Sato S, Kuwana M, Hirakata M (2007) Clinical characteristics of Japanese patients with anti-OJ (anti-isoleucyl-tRNA synthetase) autoantibodies. Rheumatology (Oxford). 46: 842–845.

Functional Variants in *NFKBIE* and *RTKN2* Involved in Activation of the NF-κB Pathway Are Associated with Rheumatoid Arthritis in Japanese

Keiko Myouzen[1], Yuta Kochi[1,2]*, Yukinori Okada[1,2,3], Chikashi Terao[4,5], Akari Suzuki[1], Katsunori Ikari[6], Tatsuhiko Tsunoda[7], Atsushi Takahashi[3], Michiaki Kubo[8], Atsuo Taniguchi[6], Fumihiko Matsuda[4,9,10], Koichiro Ohmura[5], Shigeki Momohara[6], Tsuneyo Mimori[5], Hisashi Yamanaka[6], Naoyuki Kamatani[11], Ryo Yamada[12], Yusuke Nakamura[13], Kazuhiko Yamamoto[1,2]

1 Laboratory for Autoimmune Diseases, Center for Genomic Medicine (CGM), RIKEN, Yokohama, Japan, **2** Department of Allergy and Rheumatology, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan, **3** Laboratory for Statistical Analysis, CGM, RIKEN, Yokohama, Japan, **4** Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **5** Department of Rheumatology and Clinical Immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **6** Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan, **7** Laboratory for Medical Informatics, CGM, RIKEN, Yokohama, Japan, **8** Laboratory for Genotyping Development, CGM, RIKEN, Yokohama, Japan, **9** CREST Program, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan, **10** Institut National de la Santé et de la Recherche Médicale (INSERM), Unité U852, Kyoto University Graduate School of Medicine, Kyoto, Japan, **11** Laboratory for International Alliance, CGM, RIKEN, Yokohama, Japan, **12** Unit of Statistical Genetics, Center for Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **13** Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Abstract

Rheumatoid arthritis is an autoimmune disease with a complex etiology, leading to inflammation of synovial tissue and joint destruction. Through a genome-wide association study (GWAS) and two replication studies in the Japanese population (7,907 cases and 35,362 controls), we identified two gene loci associated with rheumatoid arthritis susceptibility (*NFKBIE* at 6p21.1, rs2233434, odds ratio (OR) = 1.20, $P = 1.3 \times 10^{-15}$; *RTKN2* at 10q21.2, rs3125734, OR = 1.20, $P = 4.6 \times 10^{-9}$). In addition to two functional non-synonymous SNPs in *NFKBIE*, we identified candidate causal SNPs with regulatory potential in *NFKBIE* and *RTKN2* gene regions by integrating *in silico* analysis using public genome databases and subsequent *in vitro* analysis. Both of these genes are known to regulate the NF-κB pathway, and the risk alleles of the genes were implicated in the enhancement of NF-κB activity in our analyses. These results suggest that the NF-κB pathway plays a role in pathogenesis and would be a rational target for treatment of rheumatoid arthritis.

Citation: Myouzen K, Kochi Y, Okada Y, Terao C, Suzuki A, et al. (2012) Functional Variants in *NFKBIE* and *RTKN2* Involved in Activation of the NF-κB Pathway Are Associated with Rheumatoid Arthritis in Japanese. PLoS Genet 8(9): e1002949. doi:10.1371/journal.pgen.1002949

Editor: Panos Deloukas, The Wellcome Trust Sanger Institute, United Kingdom

Received March 31, 2012; **Accepted** July 12, 2012; **Published** September 13, 2012

Copyright: © 2012 Myouzen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was conducted as a part of the BioBank Japan Project that was supported by the Ministry of Education, Culture, Sports, Sciences, and Technology of the Japanese government. 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.

* E-mail: ykochi@src.riken.jp

Introduction

Rheumatoid arthritis (RA [MIM 180300]) is an autoimmune disease [1] with a complex etiology involving several genetic factors as well as environmental factors. Previous genome-wide association studies (GWAS) for RA have discovered many genetic loci [2–6], although the causal mechanisms linking the variants in these loci and disease etiology are largely unknown, except for in a few cases [6–8]. In contrast to mutations in Mendelian, monogenic diseases, most disease-associated variants in complex diseases, including autoimmune diseases, have moderate effects on disease susceptibility. This is because the disease causal variants in complex diseases are thought to have moderate effects on gene function, while amino acid changes introduced by the mutations of monogenic diseases have critical impacts on protein function [9]. Moreover, it has been demonstrated that the majority of autoimmune disease loci are expression quantitative trait loci (eQTLs) [10,11], indicating that accumulation of quantitative, but not qualitative, changes in gene function likely predisposes individuals to the disease. This renders it difficult to pinpoint the causal variants in the GWAS loci, especially in eQTLs, because all the variations in strong linkage disequilibrium (LD) with the marker SNP in a GWAS, the majority of which are not covered by the SNP array, are possible candidates for causal variants.

In recent years, with the emergence of next-generation sequencing technologies, the way we approach disease-causing variants has dramatically changed. First, a comprehensive map of human genetic variations is now available owing to the 1000 Genome Project [12], which allows us to grasp most of the potential common variants. This also enables us to perform genotype imputation of SNPs that are not directly genotyped in the GWAS, and consequently, to test them for association. Second, genomic studies using new technologies, such as chromatin immunoprecipitation-sequencing (ChIP-seq) and DNase I hypersensitive sites sequencing (DNase-seq), have advanced our understanding of how each genomic cluster regulates gene

Author Summary

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting approximately 1% of the general adult population. More than 30 susceptibility loci for RA have been identified through genome-wide association studies (GWAS), but the disease-causal variants at most loci remain unknown. Here, we performed replication studies of the candidate loci of our previous GWAS using Japanese cohorts and identified variants in *NFKBIE* and *RTKN2* gene loci that were associated with RA. To search for causal variants in both gene regions, we first examined non-synonymous (ns)SNPs that alter amino-acid sequences. As *NFKBIE* and *RTKN2* are known to be involved in the NF-κB pathway, we evaluated the effects of nsSNPs on NF-κB activity. Next, we screened *in silico* variants that may regulate gene transcription using publicly available epigenetic databases and subsequently evaluated their regulatory potential using *in vitro* assays. As a result, we identified multiple candidate causal variants in *NFKBIE* (2 nsSNPs and 1 regulatory SNP) and *RTKN2* (2 regulatory SNPs), indicating that our integrated *in silico* and *in vitro* approach is useful for the identification of causal variants in the post–GWAS era.

transcription. If disease-associated variants are present in a critical site for gene regulation suggested by the ChIP-seq and DNase-seq studies, the disease-associated variants might possibly influence gene transcription levels such as through altered transcription factor-DNA binding avidity.

In the present study, we first performed replication studies of candidate loci in our previous GWAS and identified two association signals with genome-wide significance ($P<5\times10^{-8}$) in nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon (*NFKBIE* [MIM 604548]) and rhotekin 2 (*RTKN2*) loci. By utilizing publicly available datasets yielded by the above-mentioned genomic studies, we then performed integrated *in silico* and *in vitro* analysis to identify plausible causal variants in *NFKBIE* and *RTKN2* loci.

Results

Identification of rheumatoid arthritis susceptibility genes

We previously performed a GWAS of RA using a Japanese case-control cohort (2,303 cases and 3,380 controls) and identified significant associations in major histocompatibility complex, class II, DR beta 1 (*HLA-DRB1* [MIM 142857]), and chemokine (C-C motif) receptor 6 (*CCR6* [MIM 601835]) loci ($P_{GWAS}<5\times10^{-8}$) [6]. To reveal additional risk loci from those showing moderate associations in the GWAS (31 loci, $5\times10^{-8}<P_{GWAS}<5\times10^{-5}$), we selected a landmark SNP from each locus and genotyped it for an additional cohort (replication-1: 2,187 cases and 28,219 controls) (Table S1, S2). Among the 31 SNPs genotyped, seven SNPs were nominally associated with RA ($P<0.05$), which included SNPs in the tumor necrosis factor, alpha-induced protein 3 (*TNFAIP3* [MIM 191163]), and signal transducer and the activator of transcription 4 (*STAT4* [MIM 600558]) gene loci that were previously reported to be associated with RA [13,14] (Table S2). In a combined analysis of the GWAS and the 1st replication study, we identified two associations with genome-wide significance ($P<5\times10^{-8}$) in *NFKBIE* (6p21.1, rs2233434, $P=4.1\times10^{-11}$, odds ratio (OR) = 1.21, 95% confidence interval (CI) = 1.14–1.28) and in *RTKN2* (10q21.2, rs3125734, $P=3.7\times10^{-8}$, OR = 1.23, 95% CI = 1.14–1.32) (Table 1 and Figure 1). *NFKBIE* was previously reported as a novel RA susceptibility gene locus in a meta-analysis of three GWASs for RA in the Japanese population, which included the GWAS set that the present study used [15]. *RTKN2* is located in the same region (10q21) as *ARID5B*, in which a significant association signal was also reported in the meta-analysis [15]. In our GWAS set, however, two significant signals were observed at rs3125734 (*RTKN2*: $P=4.8\times10^{-5}$) and rs10821944 (*ARID5B*: $P=7.4\times10^{-4}$), the former of which was tested as a landmark in the replication study. These two SNPs were in weak LD ($r^2=0.11$) and the independent effect of each SNP was observed after conditioning on each SNP (*RTKN2*: $P=1.5\times10^{-3}$, *ARID5B*: $P=0.024$, respectively). This indicated that two independent associations existed in this region, and the association of *RTKN2* is novel. We also confirmed the association in the *STAT4* locus [14] with genome-wide significance (2q32.2, rs10168266, $P=3.2\times10^{-8}$, OR = 1.16, 95% CI = 1.10–1.22) (Table S2). The associations in *NFKBIE* and *RTKN2* were further replicated in the 2nd replication cohort (3,417 cases and 3,763 controls; rs2233434, $P=1.1\times10^{-5}$, OR = 1.19, 95% CI = 1.10–1.30 and rs3125734, $P=0.016$, OR = 1.14, 95% CI = 1.02–1.26, respectively), confirming the associations in these loci (a combined analysis of three sets; rs2233434, $P=1.3\times10^{-15}$, OR = 1.20, 95% CI = 1.15–1.26 and rs3125734, $P=4.6\times10^{-9}$, OR = 1.20, 95% CI = 1.13–1.27, respectively) (Table 1 and Figure 1). We also genotyped these SNPs for individuals with systemic lupus erythematosus (SLE [MIM 152700]) ($n=657$) and Graves' disease (GD [MIM 275000]) ($n=1,783$). We identified a significant association of *RTKN2* (rs3125734) with GD ($P=3.4\times10^{-5}$, OR = 1.24, 95% CI = 1.12–1.37), whereas no significant associations were detected in *NFKBIE* (rs2233434) with either disease or in *RTKN2* (rs3125734) with SLE (Table S3).

Functional analysis of non-synonymous SNPs

NFKBIE and *RTKN2* genes are both involved in the NF-κB pathway: *NFKBIE* encodes IκB epsilon (IκBε), a member of the IκB family [16], and its binding to NF-κB inhibits the nuclear translocation of NF-κB [17]; *RTKN2* encodes a member of Rho-GTPase effector proteins highly expressed in $CD4^+$ T cells [18] and is implicated in the activation of the NF-κB pathway [19]. Considering that the NF-κB pathway is critical for the pathogenesis of RA [20], these two genes could be strong candidates in these regions. To identify disease-causing variants, we first sequenced the coding regions of the genes using DNA from patients ($n=48$) to find variants that alter amino acid sequences. We identified four non-synonymous (ns)SNPs, which were all registered in the dbSNP database: two nsSNPs in *NFKBIE* (rs2233434 (Val194Ala) and rs2233433 (Pro175Leu)) and two in *RTKN2* (rs3125734 (Arg462His) and rs61850830 (Ala288Thr)), where rs2233434 and rs3125734 were the same as the landmark SNPs in the GWAS (Figure 1 and Figure 2A). The two nsSNPs of each locus were in strong LD (Figure 2B) and were both associated with disease (Table S4). In the haplotype analysis, a single common risk haplotype with a frequency >0.05 was observed in each locus, and significant associations with disease risk were detected (*NFKBIE*, $P=5.3\times10^{-8}$, Table S5; *RTKN2*, $P=5.7\times10^{-5}$, Table S6).

To investigate the effect of these nsSNPs on protein function, we evaluated them by *in silico* analysis using PolyPhen and SIFT software, which predicts possible impacts of amino acid substitutions on the structure and function of proteins, but all four nsSNPs were predicted to have little effect (Table S7), contrasting with the effect of Mendelian disease mutations [9]. We next examined their influence on the NF-κB activity in cells by performing NF-κB