Bone Edema Determined by Magnetic Resonance Imaging Reflects Severe Disease Status in Patients with Early-Stage Rheumatoid Arthritis

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ABSTRACT. ***Objective***. To determine the significance of bone edema, detected by magnetic resonance imaging (MRI), in early-stage rheumatoid arthritis (RA).

Methods. We simultaneously examined serologic variables, MRI of wrist sites and finger joints of both hands, clinical disease activity score (DAS), and HLA-DR typing at entry in 80 patients with early-stage RA.

Results. The number of bones scored as positive for bone edema correlated with the number of sites scored as positive for MRI synovitis and MRI bone erosion, rate of enhancement (E-rate), and serum C-reactive protein (CRP), matrix metalloproteinase 3 (MMP-3), and interleukin 6 (IL-6). Findings for MRI synovitis and MRI bone erosion, E-rate, CRP, MMP-3, IL-6, seropositivity, and titer of anti-cyclic citrullinated peptide antibody (anti-CCP antibody), DAS28-CRP and HLA-DRB1*0405 allele carriership, were significantly higher in the positive versus the negative bone edema group.

Conclusion. Bone edema based on our scoring system may reflect severe disease status in patients with early-stage RA. However, its clinical value at entry in prognostication of RA should be examined through prospective clinical followup studies. (First Release Oct 1 2007; J Rheumatol 2007;34:2154–7)

Key Indexing Terms:
EARLY-STAGE RHEUMATOID ARTHRITIS MAGNETIC RESONANCE IMAGING
BONE EDEMA SEROLOGIC VARIABLES HLA-DRB1*0405 ALLELE

Evaluation of magnetic resonance imaging (MRI) in rheumatoid arthritis (RA) has been analyzed using the OMERACT 5 RA-MRI scoring system (RAMRIS)[1,2] and by others[3,4]; however, the scoring process is complicated. We evaluated MRI features by imaging only wrist sites and finger joints in early-stage RA using different qualification parameters[5,6].

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MATERIALS AND METHODS

Enrolled patients were from the Early Arthritis Clinic, First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University. The study consists of 80 patients with early-stage RA, who gave their informed consent to the protocol that was approved by the Institutional Review Board of Nagasaki University. Median disease duration from onset of symptoms to entry was 3 months. Disease duration in all 80 patients was < 2 years, similar to a recent report[7]. Diagnosis was made based on 1987 American College of Rheumatology (ACR) criteria for RA[8]. Thirty-six patients were already classifed as RA at entry, while the remaining 44, who were classified as undifferentiated arthritis at entry, developed to RA later. Median modified Genant-Sharp score at entry by plain radiography was 0.26.

The following variables were examined at entry. Serologic tests included matrix metalloproteinase-3 (MMP-3), anti-cyclic citrullinated peptide (anti-CCP), and interleukin 6 (IL-6). Clinical disease activity was qualified by DAS28 (Disease Activity Score 28-C-reactive protein (DAS28-CRP). High resolution analysis of HLA-DRB1 genotyping was performed, as described[7,9], by polymerase chain reaction. MR images of both wrists and finger joints (1.5 T system, Sigma, GE Medical Systems, Milwaukee, WI, USA) were evaluated for bone edema, bone erosion, and synovitis in 15 sites in each finger and wrist, i.e., distal radioulnar joint, radiocarpal joint, mid-carpal joint, first carpometacarpal joint, 2nd-5th carpometacarpal joint (together), 1st-5th metacarpophalangeal joints (proximal interphalangeal) separately (total 30 sites in both hands) as reported[5,6]. MR images were interpreted independently by 2 board-certified radiologists experienced in musculoskeletal imaging (MU and ST) who were blinded to patients' clinical status. Both radiologists read each image according to the definition as described[3-6], and disagreements were resolved by consensus. Degree of MRI features was evaluated per our recent report: synovitis; number of sites scored positive for MRI

synovitis and E-rate, bone edema; number of bones scored positive for bone edema, bone erosion; number of bones scored positive for MRI bone erosion, respectively[5].

Differences between groups were examined using the Mann-Whitney U test and chi-square test. A correlation between the 2 variables was calculated by Spearman's rank correlation. A p value < 0.05 denoted a statistically significant difference.

RESULTS

Representative MR images are shown in Figure 1. The number of bones scored as positive for bone edema clearly correlated with the number of sites scored as positive for MRI synovitis and MRI bone erosion, mean E-rate from 30 sites, bone erosion number, CRP, MMP-3, and IL-6 as shown in Figure 2.

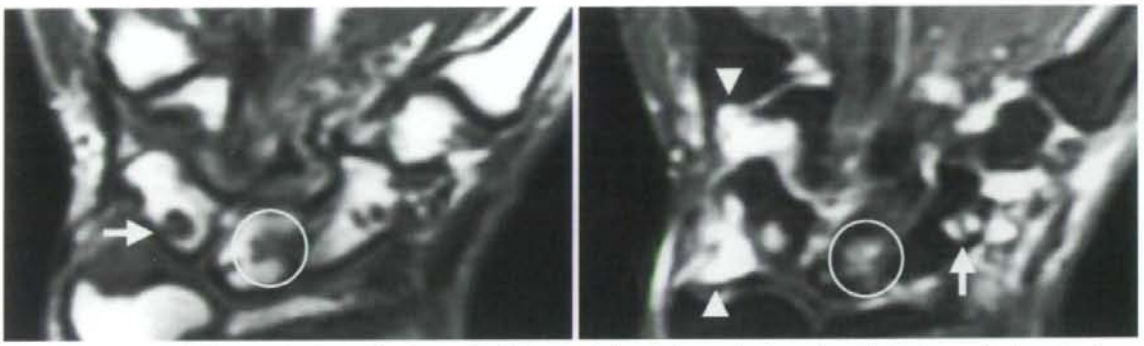

Figure 1. Representative MR images of patients with early-stage RA. T1-weighted spin-echo images show bone edema (circle), bone erosion (arrow), and synovitis (arrowheads). Right panel: gadolinium-diethylenetriamine-enhanced image.

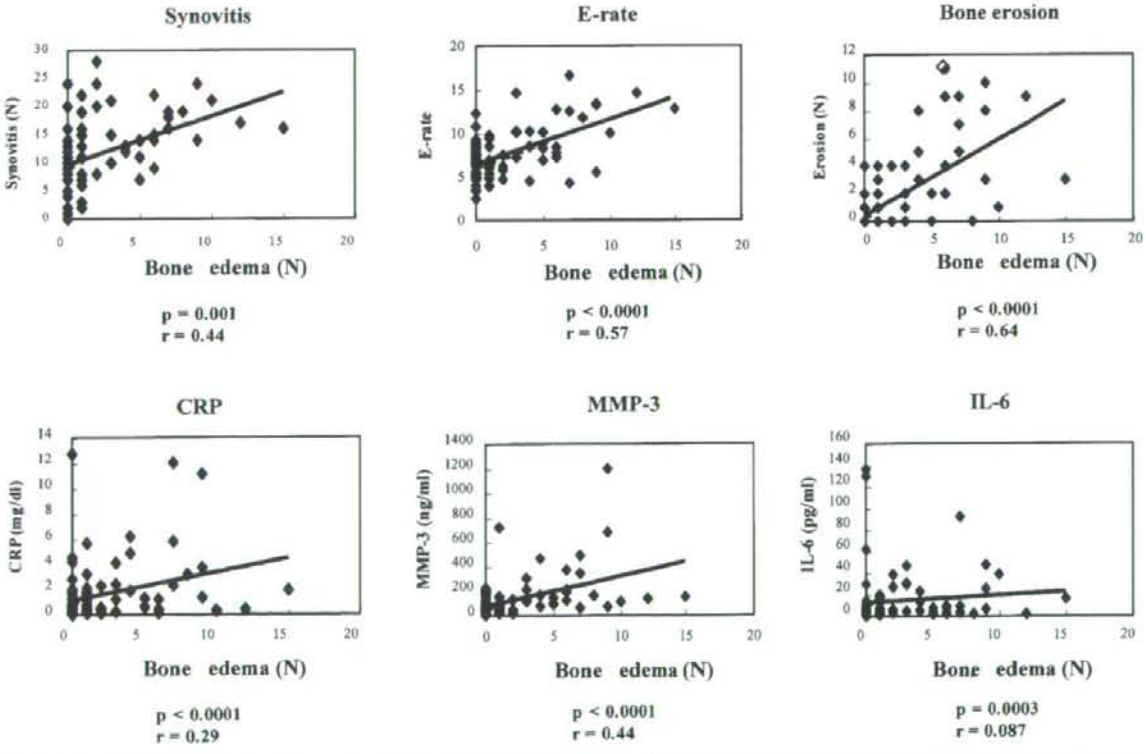

Figure 2. Positive correlation between the number of bones scored as positive for bone edema and the number of sites scored for MRI synovitis and MRI bone erosion, enhancement rate (E-rate), serum concentrations of CRP, MMP-3, and IL-6.

Patients with early-stage RA were divided into 2 groups: with or without bone edema. MRI evidence of synovial inflammation and bone erosion was more remarkable in bone edema-positive patients. Seropositivity and titer for anti-CCP antibody, CRP, MMP-3, IL-6, and DAS28-CRP were also higher in the bone edema-positive group (Table 1). Positive correlation between DAS28-CRP and the number of bones scored as positive for bone edema was also identified by Spearman's rank correlation test ($p = 0.00026$, $rs = 0.398$).

In 36 of the 80 (45%) patients with early-stage RA, we confirmed at least one HLA-DRB1*0405 allele, the most frequent shared epitope in Japanese RA patients[9]. This allele was more predominantly distributed in the bone edema-positive group versus the -negative group (Table 1). Our data also showed that significantly more patients who were positive for anti-CCP antibody and HLA-DRB1*0405 allele (N = 26) had a positive score for bone edema (77%) compared to patients who were negative for both these markers (N = 16), in whom bone edema was scored in only 25% ($p = 0.0013$).

DISCUSSION

Trying to solve the complexity of standard MRI scoring methods, we semiquantitatively evaluated MRI features. The present data suggest that our score, especially bone edema score, classified the disease status of early-stage RA.

Employing prospective analysis, van Gaalen, *et al* recently showed that patients with early-stage RA at entry who are positive for both anti-CCP antibody and HLA-DRB1 shared epitope developed severe erosive disease[7]. Considering bone edema is a forerunner of bone erosion[10], our findings that bone edema-positive early-stage RA patients are preferentially distributed in the subgroup positive for anti-CCP antibody and HLA-DRB1*0405 allele support the findings of van Gaalen, *et al* as well as the prognostic value of bone edema.

Our results suggest that bone edema in patients with early-stage RA develops through an inflammatory synovial microenviroment with specific HLA-antigen interaction, and our evaluation method for MRI may be an alternative for the standard methods. However, since a therapeutic regimen was already administered in some patients at entry to the study, its clinical value should be verified through prospective followup studies.

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Table 1. Clinical features in early-stage RA with or without bone edema. All the variables were highly significant in the bone edema-positive group.

Variable	Bone Edema, n = 45	No Bone Edema, n = 35	p
CRP, mg/ml*	2.08 ± 2.62	1.03 ± 2.33	< 0.0001
MMP-3, ng/ml*	178.2 ± 225.4	71.4 ± 61.9	0.002
No. of sites scored as positive for MRI synovitis*	13.9 ± 5.9	8.6 ± 5.8	0.0002
Mean E-rate from 30 sites*	8.34 ± 3.26	6.56 ± 2.10	0.03
Bone erosion			
%†	60.0	25.7	0.02
n*	2.71 ± 3.31	0.37 ± 0.81	0.02
IL-6, pg/ml	14.7 ± 17.2	13.1 ± 32.1	0.0003
Anti-CCP antibody			
%	77.8	52.3	0.02
U/ml*	218.2 ± 399.2	82.4 ± 182.2	0.01
DAS28-CRP	4.54 ± 1.02	3.88 ± 1.18	0.008
HLA-DRB1*0405, %	57.8	28.6	0.009

* Mann-Whitney U test. † Chi-square test, as described in text.

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Association of *STAT4* With Susceptibility to Rheumatoid Arthritis and Systemic Lupus Erythematosus in the Japanese Population

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Objective. ***STAT4*** **encodes a transcriptional factor that transmits signals induced by several key cytokines, and it might be a key molecule in the development of autoimmune diseases. Recently, a** ***STAT4*** **haplotype was reported to be associated with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) in Caucasian populations. This was replicated in a Korean RA population. Interestingly, the degree of risk of RA susceptibility with the** ***STAT4*** **haplotype was similar in the Caucasian and Korean populations. The present study was undertaken to investigate the effect of** ***STAT4*** **on susceptibility to RA and SLE in the Japanese.**

Methods. **We performed an association study using 3 independent Japanese RA case–control populations (total 3,567 cases and 2,199 controls) and 3 independent Japanese SLE populations (total 591 cases). All samples were genotyped using the TaqMan fluorogenic 5′ nuclease assay for single-nucleotide polymorphism (SNP) rs7574865, which tags the susceptibility haplotype. The association of the SNP with disease susceptibility in each case–control study was calculated using Fisher's exact test, and the results were combined, using the Mantel-Haenszel method, to obtain combined odds ratios (ORs).**

Results. **We observed a significant association of the** ***STAT4*** **polymorphism with susceptibility to both RA and SLE. The combined ORs for RA and SLE, respectively, were 1.27 ($P = 8.4 \times 10^{-9}$) and 1.61 ($P = 2.1 \times 10^{-11}$) for allele frequency distribution; these ORs were quite similar to those previously observed in the Caucasian population.**

Conclusion. **We conclude that** ***STAT4*** **is associated with RA and SLE in the Japanese. Our results indicate that** ***STAT4*** **is a common genetic risk factor for autoimmune diseases, with similar strength across major racial groups.**

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Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are chronic inflammatory autoimmune diseases characterized by pathologic infiltration of lymphocytes in target organs. Although the pathogenesis of these diseases remains unclear, dysregulated lymphocyte activation via the breakdown of self tolerance is believed to be implicated in their pathogenesis, and multiple genetic and environmental factors are important in the development of these diseases.

Recently, Amos et al conducted a genome-wide linkage scan using >5,700 single-nucleotide polymorphisms (SNPs) in 642 Caucasian families with affected sibling pairs; they found the best evidence of linkage at chromosomes 2q33 (1). Following the linkage analysis, Remmers et al performed a large case–control study of 13 selected candidate genes within the linkage region and found an association between a common haplotype located in the third intron of *STAT4* and susceptibility to RA and SLE (2). The association was replicated in several independent Caucasian RA and SLE populations, and also in a Korean RA population (2,3).

STAT4 encodes signal transducer and activator of transcription 4, the STAT protein family member that is uniquely activated by interleukin-12 (IL-12) through its receptor, which has an essential downstream role in Th1 cell differentiation and proliferation (4). In addition, it has been reported that STAT-4 is necessary for the development of Th17 cells (IL-17–producing CD4+ T cells) (5). Since Th1 cells and Th17 cells play an important role in chronic inflammatory disorders and since STAT-4 is considered to be a key molecule in both the Th1 and Th17 lineages, STAT-4 may play a crucial role in the development of autoimmune diseases such as RA and SLE.

Genetic association between HLA–DRB1 and RA susceptibility has been well established, and several other risk genes for RA outside the HLA region have been identified. However, while DRB1 has been repeatedly shown to be an RA risk locus in Caucasian and Asian populations, the other reported RA risk genes, such as *PTPN22*, *PADI4*, and *FCRL3*, have been difficult to replicate in other ethnic populations aside from the original populations first reported (6). These conflicting results suggest that the genetic background of the disease may vary among ethnic groups.

Interestingly, the degree of risk of RA susceptibility observed with the *STAT4* haplotype was found to be similar in the Caucasian and Korean populations (2,3). This finding indicates that the risk haplotype for RA susceptibility might be common across major racial groups. In the present study, we investigated the association of *STAT4* with RA susceptibility using large series of Japanese RA cohorts. We also tried to evaluate whether the gene is associated with RA outcome measures in a Japanese RA cohort. In addition, we tested the association between the gene and susceptibility to SLE in the Japanese population. This study is the first to investigate the association of *STAT4* with SLE in an Asian population.

PATIENTS AND METHODS

Subjects and disease criteria. All patients with RA fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for the disease (7). All patients with SLE met the ACR 1982 revised criteria for the disease (8).

DNA samples were obtained from subjects in 3 RA case–control series (Table 1). DNA from the case subjects in the Tokyo Women's Medical University (TWMU) Institute of Rheumatology RA cohort (IORRA) case–control series was obtained from the IORRA DNA collection. The IORRA is an observational RA cohort with an enrollment of nearly 5,000 Japanese RA patients, and DNA samples were collected from 1,504 of these patients (mean age 59.3 years, 84% female, 88% rheumatoid factor [RF] positive) (9). This DNA collection was also used to analyze the effect of the single-nucleotide polymorphism (SNP) (see below) on RA outcome measures. Demographic, clinical, and treatment information on IORRA patients as of the spring of 2003 was obtained from the IORRA database, which includes the Disease Activity Score in 28 joints (DAS28) (10) and the Japanese version of the Health Assessment Questionnaire (J-HAQ) (11). Radiographs of the hands and feet of the IORRA patients, obtained when the duration of disease was 5 years, were reviewed retrospectively, and radiographic joint damage was assessed by a single skilled reader, using the modified Sharp/van der Heijde score (SHS) (12). The SHS includes a count of erosions and joint space narrowing in the hands and feet and has a range of 0 (no damage) to 448 (highest damage). DNA samples from popu-

Table 1. Case–control series for the studies of rheumatoid arthritis and systemic lupus erythematosus

Series	No. of patients	No. of controls
Rheumatoid arthritis		
IORRA	1,504	752
RIKEN	1,113	940
Tokushima	950	507
Systemic lupus erythematosus		
TWMU	238	752*
RIKEN	188	940†
Tokushima/Fukuoka	165	212

* Genotype information was obtained from the controls in the Tokyo Women's Medical University (TWMU) Institute of Rheumatology Rheumatoid Arthritis cohort (IORRA) rheumatoid arthritis series.
† Genotype information was obtained from the controls in the Institute of Physical and Chemical Research (RIKEN) series.

lation controls were obtained from the Pharma SNP consortium (http://www.jpma.or.jp/psc/index.html).

DNA from the case subjects in the Institute of Physical and Chemical Research (RIKEN) RA case–control series (mean age 60.4 years, 82% female, 70% RF positive) was obtained from the BioBank Japan Project DNA collection. As part of the BioBank Japan Project, DNA and serum samples along with clinical data have been collected from 300,000 patients with 47 diseases, including RA (13). Sixty-six hospitals affiliated with 12 institutions are participating in the project. Population-based control subjects were recruited through the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan.

Patients and controls in the Tokushima RA case–control series were recruited through the orthopedics clinic at University of Tokushima Hospital, its community affiliates, and the rheumatology clinic at Tokushima Kensei Hospital (Tokushima, Japan) (14). The mean age of the patients was 61.8 years, and 79% were female.

Cases with SLE were also obtained from 3 sources (Table 1). TWMU patients were recruited from Institute of Rheumatology and Aoyama Hospital, TWMU. RIKEN patients were recruited through the Specified Disease Treatment Research Program of the Japanese Ministry of Health, Labor, and Welfare. Several medical institutions nationwide are participating in the program. These 2 series included only cases; control genotype information was obtained from the RA case–control series in the IORRA and RIKEN, respectively. Patients and control subjects in the Tokushima/Fukuoka lupus case–control series were recruited from Kyushu University Hospital (Fukuoka, Japan) (15).

The ethics committee of each institution (TWMU, RIKEN, and University of Tokushima) granted approval for the study, and each individual subject signed an informed consent form after receiving a verbal explanation of the study.

SNP genotyping. A polymorphism located within intron 3 of *STAT4*, rs7574865, which tags the susceptibility haplotype, was selected for this study because it exhibited the best evidence for association in the primary study (2) and was one of the SNPs most significantly associated with RA susceptibility in the Korean replication study (3). This SNP has been considered to be in strong linkage equilibrium with a putative functional variant. Genotyping was performed using the TaqMan fluorogenic 5′ nuclease assay, according to the instructions of the manufacturer (Applied Biosystems, Tokyo, Japan). All polymerase chain reactions were performed using GeneAmp PCR System 9700 (Applied Biosystems), and end point fluorescence readings were performed with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems).

Statistical analysis. Allele frequencies of SNP rs7574865 in each case–control series were estimated by the allele counting method. Chi-square testing was used to identify significant departure from Hardy-Weinberg equilibrium.

Association of the SNP with susceptibility to RA or SLE in each study was estimated by Fisher's exact test; we compared the allelic effect of T (suspected risk allele) with G (common allele), and the genotypic effect of the homozygous genotypes with other genotypes. After assessing heterogeneity among the studies as determined based on Woolf's method, the Mantel-Haenszel test was used to evaluate combined odds ratios (ORs) and 95% confidence intervals (95% CIs), demonstrating the population-wide impact of the polymorphism on disease susceptibility.

Differences in patient characteristics among IORRA subjects with different rs7574865 genotypes were assessed by Kruskal-Wallis test or Fisher's exact test. The allelic effect of rs7574865 on the SHS was analyzed by linear regression analysis.

All statistical tests were implemented using the R software package, version 2.6.0 (http://www.r-project.org/).

RESULTS

On average, we achieved a genotyping success rate of 98.9%, with call rates of >98.2% for each case–control series. The genotype concordance rate was 100% as assessed by random retyping across different plates. Genotype distributions for SNP rs7574865 were in Hardy-Weinberg equilibrium in each case–control series.

Association of *STAT4* polymorphism with RA. The data summarized in Table 2 show the allele fre-

Table 2. Association of single-nucleotide polymorphism rs7574865 with RA in Japanese subjects*

Series, subjects	Genotype					Allele		GG vs. others		TT vs. others	
	GG	GT	TT	Total	MAF	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
IORRA											
RA	588	694	199	1,481	0.37	1.29 (1.13–1.48)	1.7×10^{-4}	1.38 (1.15–1.66)	3.9×10^{-4}	1.41 (1.05–1.89)	0.020
Controls	355	316	74	745	0.31						
RIKEN											
RA	447	502	160	1,109	0.37	1.31 (1.15–1.50)	4.8×10^{-5}	1.38 (1.16–1.66)	3.0×10^{-4}	1.48 (1.12–1.96)	0.0048
Controls	453	389	96	938	0.31						
Tokushima											
RA	365	448	128	941	0.37	1.17 (0.99–1.38)	0.056	1.20 (0.96–1.50)	0.11	1.30 (0.92–1.86)	0.13
Controls	216	230	54	500	0.34						
Combined						1.27 (1.17–1.37)	8.4×10^{-9}	1.34 (1.20–1.49)	1.9×10^{-7}	1.41 (1.19–1.67)	8.5×10^{-5}

* RA = rheumatoid arthritis; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).

Table 3. Association of single-nucleotide polymorphism rs7574865 with SLE in Japanese subjects*

Series, subjects	Genotype					Allele		GG vs. others		TT vs. others	
	GG	GT	TT	Total	MAF	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
TWMU											
SLE	76	103	48	227	0.44	1.73 (1.38–2.15)	8.3×10^{-7}	1.81 (1.31–2.50)	1.8×10^{-4}	2.43 (1.59–3.68)	2.9×10^{-5}
Controls	355	316	74	745	0.31						
RIKEN											
SLE	70	85	28	183	0.39	1.40 (1.10–1.77)	0.0059	1.51 (1.08–2.12)	0.015	1.58 (0.97–2.53)	0.053
Controls	453	389	96	938	0.31						
Tokushima/Fukuoka											
SLE	51	83	31	165	0.44	1.79 (1.31–2.45)	1.4×10^{-4}	2.07 (1.33–3.25)	0.0010	2.34 (1.22–4.59)	0.0059
Controls	102	91	19	212	0.30						
Combined						1.61 (1.40–1.85)	2.1×10^{-11}	1.74 (1.43–2.12)	4.9×10^{-8}	2.08 (1.59–2.72)	8.5×10^{-8}

* SLE = systemic lupus erythematosus; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).

quency and genotype distribution in RA patients and controls in each case–control series. We observed a significant difference in allele frequency and genotype distribution of the *STAT4* polymorphism between RA patients and controls in the IORRA and the RIKEN cohorts, while no significant difference was found in the Tokushima series. When study-specific ORs were combined using the Mantel-Haenszel method, the differences in allele frequency and genotype distributions of the SNP between patients and controls were significant (combined OR 1.27 [95% CI 1.17–1.37, $P = 8.4 \times 10^{-9}$]–1.41 [95% CI 1.19–1.67, $P = 8.5 \times 10^{-5}$]). There was no significant heterogeneity among the studies, as assessed by Woolf's method ($P > 0.05$).

Association of *STAT4* polymorphism with SLE. Table 3 shows the genotype distribution and minor allele frequency in the 3 SLE series. As with RA, we found differences in the allele frequency and genotype distributions of SNP rs7574865 between SLE patients and controls; these were significant in all 3 case–control series. No significant evidence of heterogeneity among the studies was identified by Woolf's method ($P > 0.05$), and the combined OR for the polymorphism as calculated by Mantel-Haenszel testing was 1.61 (95% CI 1.40–1.85, $P = 2.1 \times 10^{-11}$). Combined ORs for the recessive trait and the dominant trait were 2.08 (95% CI 1.59–2.72) and 1.74 (95% CI 1.43–2.12), respectively.

Stratified analyses of clinical and laboratory variables in RA patients. Among 1,504 patients with available DNA samples, 1,335 participated in the IORRA clinical data collection in the spring of 2003, and information on their demographic, clinical, and treatment details as of that time could be obtained from the IORRA database (Table 4). Consistent with previous findings by Lee et al in a Korean population (3), there was no significant genotypic association with age at disease onset or sex. We also found no significant differences among the genotypes in age, disease duration, family history of RA, RF status, DAS28 score, or J-HAQ score. There was a trend toward an association of risk allele with elevated levels of inflammation markers and patient's assessment of global health, but these were not significant. Only glucocorticoid usage and glucocorticoid dosage were found to differ significantly among the genotypes, with the difference increasing in a stepwise manner according to the number of risk alleles (median dosage 0, 1, and 2.5 mg equivalent prednisolone, respectively, among patients with the GG, GT, and TT genotypes).

The SHS after 5 years of disease could be measured in 163 patients, of whom 160 were genotyped. Although a trend toward an effect of the risk allele on the SHS was observed, it was not significant ($P = 0.22$) (median score 40, 45, and 46, respectively, among patients with the GG, GT, and TT genotypes [n = 67, 79, and 14, respectively]).

We did not perform a stratified analysis on anti–cyclic citrullinated peptide antibody (anti-CCP) positivity, since anti-CCP data were not available on most of the patients from the IORRA DNA collection. However, Lee and colleagues suggested that, at least among Asians, the risk of RA susceptibility associated with the *STAT4* variant may not be restricted to the anti-CCP positive disease subset (3).

DISCUSSION

This study is the first to investigate the association of a *STAT4* polymorphism with genetic susceptibility to lupus in any Asian population, and susceptibility to

Table 4. Genotypic differences in clinical or laboratory variables in RA patients*

	Total	Genotype			P†
		GG	GT	TT	
No. (%) of patients	1,335	521 (40)	610 (46)	183 (14)	
Age, years	60 (53–68)	61 (53–68)	60 (53–68)	60 (52–66)	0.14
Female, no. (%)	1,125 (84)	441 (85)	507 (83)	157 (86)	0.63
Disease duration, years	10 (5–16)	10 (5–17)	10 (5–16)	10 (5–17)	0.71
Age at RA onset, years	48 (39–57)	49 (40–57)	49 (40–57)	48 (38–55)	0.18
Family history of RA, no. (%)	415 (32)	157 (31)	187 (31)	63 (35)	0.54
RF positive, no. (%)‡	1,195 (90)	468 (90)	543 (89)	164 (90)	0.92
RF titer, IU/ml‡	116 (48–282)	115.5 (49–296)	116 (49–278)	122 (46–283)	0.97
Treatment§					
NSAID, no. (%)	980 (73)	378 (73)	446 (73)	143 (78)	0.32
DMARD, no. (%)	1,228 (92)	478 (92)	560 (92)	171 (93)	0.78
Glucocorticoid, no. (%)	719 (54)	266 (51)	325 (53)	117 (64)	0.01
Prednisolone, mg	1 (0–4.9)	0 (0–4)	1 (0–5)	2.5 (0–5)	0.01
DAS28	3.6 (2.7–4.5)	3.5 (2.7–4.4)	3.6 (2.8–4.5)	3.8 (2.8–4.6)	0.39
TJC	1 (0–3)	1 (0–3)	1 (0–3)	1 (0–3)	1.00
SJC	1 (0–3)	1 (0–3)	1 (0–3.75)	1 (0–4)	0.25
Patient's global assessment by VAS, mm	27 (10–54)	24 (9–54)	27 (11–55)	33 (14–53)	0.12
ESR, mm/hour	28 (16–48)	27 (16–46)	29 (16–48)	31 (16–54)	0.51
CRP, mg/dl	0.7 (0.2–1.6)	0.6 (0.2–1.6)	0.7 (0.2–1.6)	0.95 (0.3–1.9)	0.08
J-HAQ	0.625 (0.125–1.375)	0.625 (0.125–1.375)	0.625 (0.125–1.25)	0.625 (0.125–1.375)	0.96

* Data on some variables were missing for a small number of patients (maximum 3.1%). Data on genotype were missing for 21 patients (1.6%); therefore, values in the individual columns under Genotype are for 1,314 patients (521 for GG, 610 for GT, 183 for TT). Except where indicated otherwise, values are the median (interquartile range). RA = rheumatoid arthritis; NSAID = nonsteroidal antiinflammatory drug; DMARD = disease-modifying antirheumatic drug; DAS28 = Disease Activity Score in 28 joints; TJC = tender joint count (28 joints); SJC = swollen joint count (28 joints); VAS = visual analog scale; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; J-HAQ = Japanese version of the Health Assessment Questionnaire.

† By Kruskal-Wallis test or Fisher's exact test.

‡ The highest rheumatoid factor (RF) value measured in the cohort project during 2000–2006 for each individual was used. Cutoff for positivity = 15.0 IU/ml.

§ Biologic agents were not available in Japan at this time (spring 2003). Glucocorticoid dosage was calculated as the prednisolone equivalent dosage in milligrams.

RA in a Japanese population. Although replication studies using other ethnic populations are essential for establishing any genetic association, results are often reported as negative in the other populations. One of the reasons for this is that the degree of genetic risk differs among ethnic groups.

Concerning RA genetics, many study groups worldwide have made great efforts to newly identify susceptibility genes and to replicate findings of other groups, particularly using Caucasian or Asian populations. However, findings for most susceptibility genes identified outside the HLA region have not been replicated in the populations different from the population used in the primary study. A typical example of this is an association between *PTPN22* and susceptibility to RA. A missense SNP in *PTPN22* known as R620W was discovered as a common genetic risk factor for several autoimmune diseases including RA in a Caucasian population, and the finding has been replicated in many Caucasian RA cohorts (16). However, the risk allele is extremely rare in Asians, and attempts to validate the association in Asian populations have been unsuccessful (17). In contrast, the association between *PADI4* and RA susceptibility is thought to be strong among Asian populations, and indeed, most replication studies in Asian populations have succeeded in validating this association. However, a meta-analysis of studies using Caucasian populations revealed the combined OR for the association to be as low as 1.1 (18), and as a result, replication studies in Caucasian populations have seldom validated the association.

Failure to replicate a genetic association in a different ethnic population from the population used in the primary study is often due to low statistical power. To avoid this problem, it is important to make the sample size as large as possible, as we did in the present multicenter Japanese case–control study. We collected 3,567 RA cases and 591 SLE cases to validate the association between *STAT4* and susceptibility to RA and lupus in the Japanese. Consistent with previous reports (2,3), we observed a significant association of the *STAT4* polymorphism with both RA and SLE susceptibility in

the Japanese. Although the risk allele frequency in control populations is slightly different between Caucasians (22%) and Japanese (30–34%), the OR shown by investigation of the allele frequency distribution of rs7574865, 1.27, is exactly the same as in the Caucasian populations. Also, the impact of the risk allele on susceptibility to SLE in the Japanese population was found to be similar to that obtained in the previous meta-analysis of studies of Caucasian populations (1.61) (2). These results suggest that the responsible functional variant, which remains unknown, is ancient in origin. Further independent studies using populations of other ethnicities would help to prove the hypothesis.

Autoimmune diseases are initiated by breakdown of self tolerance, and thus, they may share a common pathogenesis. Indeed, some RA susceptibility genes have been identified as common risk factors for clinically different autoimmune phenotypes. One of them is *PTPN22*, which has been reported as a disease susceptibility gene for type 1 diabetes, autoimmune thyroid disease, lupus, Addison's disease, and juvenile idiopathic arthritis, in addition to RA (16). *CTLA4*, one of the genes associated with lupus and RA, especially in Asians, has also been suggested to be a disease-associated gene in a variety of other autoimmune diseases (19). Both *PTPN22* and *CTLA4* negatively regulate T cell activation and maintain peripheral tolerance, and T cells play a central role in the immunopathogenesis of autoimmune diseases. STAT-4 is suggested to be a key molecule in both the Th1 and Th17 lineages, and therefore may be involved in a common pathway of pathogenesis in autoimmune diseases.

It is reasonable to speculate that a variant on *STAT4* could also affect disease activity in autoimmune diseases through dysregulation of the Th1 and Th17 pathways. Although we did not find evidence of association between *STAT4* and disease activity in RA, we did observe a trend toward an effect of the risk allele on elevated levels of inflammation markers and patient's global assessment. Both the fact that glucocorticoid usage and dosage increased significantly in a stepwise manner in parallel with the number of risk alleles and the knowledge that glucocorticoid treatment significantly reduces levels of inflammation markers suggest that the polymorphism on *STAT4* might be associated with disease activity in RA. Although a trend toward an effect of risk allele on radiographic damage in the first 5 years was observed, it was not significant, similar to findings in the Korean study (3). However, while differences in other clinical variables among the genotypes were tested using DNA from 1,335 patients, the effect on radiographic severity was tested only in 163 patients, due to the unavailability of suitable radiographs in the others (20). As a result, the statistical power of the study of association with radiographic severity was rather limited. There were also other potential sources of artifacts that should be considered in interpretation of these preliminary data. A large prospective study, accounting for the genotypes of *STAT4*, is needed to definitively answer the question of its associations with clinical and laboratory features.

The functional variant in *STAT4* that is responsible for increased disease susceptibility remains unknown. Since the susceptibility haplotype is located within intron 3 of *STAT4*, it is considered to be responsible for splice variation or regulatory effects of STAT-4. However, it might be also possible that the putative functional variant could be responsible for a biologic effect on intragenic RNA or other factors. Studies to investigate the functional variant on the susceptibility haplotype remain to be performed.

In conclusion, using Japanese RA and SLE case–control series with large samples, we confirmed *STAT4* polymorphism as a common genetic risk factor for these autoimmune diseases. The strength of the association was found to be similar across major racial groups.

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AUTHOR CONTRIBUTIONS

Dr. Ikari 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 design. Ikari, Kochi, Inoue.

Acquisition of data. Ikari, Kochi, Yamamoto, Shimane, Nakamura, Toyama, Kawaguchi, Terai, Hara, Tomatsu, Yamanaka, Horiuchi, Tao, Yasumoto, Hamada, Yasui, Inoue, Itakura, Okamoto, Kamatani, Momohara.

Analysis and interpretation of data. Kobayashi, Ikari, Kaneko, Kochi, Mochizuki, Tsukahara, Inoue.
Manuscript preparation. Kobayashi, Ikari, Kochi, Inoue.
Statistical analysis. Kobayashi, Ikari.

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Clinical and Immunogenetic Features of Patients With Autoantibodies to Asparaginyl–Transfer RNA Synthetase

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Objective. **We have previously described anti-KS autoantibodies and provided evidence that they are directed against asparaginyl–transfer RNA (tRNA) synthetase (AsnRS). The aim of the present study was to identify patients with anti-AsnRS autoantibodies and elucidate the clinical significance of this sixth antisynthetase antibody. In particular, we studied whether it was associated with the syndrome of myositis (polymyositis or dermatomyositis [DM]), interstitial lung disease (ILD), arthritis, and other features that had been previously associated with the 5 other anti–aminoacyl–tRNA synthetase autoantibodies.**

Methods. **More than 2,500 sera from patients with connective tissue disease (including myositis and ILD) and controls were examined for anti-AsnRS autoantibodies by immunoprecipitation (IP). Positive and control sera were tested for the ability to inhibit AsnRS by preincubation of the enzyme source with the serum. The HLA class II (DRB1, DQA1, DQB1, DPB1) alleles were identified from restriction fragment length polymorphism of polymerase chain reaction–amplified genomic DNA.**

Results. **Anti-AsnRS antibodies were identified in the sera of 8 patients (5 Japanese, 1 American, 1 German, and 1 Korean) by IP of the same distinctive set of tRNA and protein that differed from those precipitated by the other 5 antisynthetases, and these antibodies showed specific inhibition of AsnRS activity. Two of these patients had DM, but 7 of 8 (88%) had ILD. Four patients (50%) had arthritis, and 1 had Raynaud's phenomenon. This antisynthetase was very rare among myositis patients (present in 0% of Japanese myositis patients), but it was found in 3% of Japanese ILD patients. Thus, most patients with anti-AsnRS had chronic ILD with or without features of connective tissue disease. Interestingly, all 4 Japanese patients tested had DR2 (DRB1*1501/1502), compared with 33% of healthy controls.**

Conclusion. **These results indicate that anti-AsnRS autoantibodies, like anti–alanyl–tRNA synthetase autoantibodies, have a stronger association with ILD than with myositis and may be associated with the DR2 phenotype.**

The aminoacyl–transfer RNA (aminoacyl-tRNA) synthetases are a family of cytoplasmic enzymes that catalyze the formation of aminoacyl-tRNA from a specific amino acid and its cognate tRNA and play a crucial role in protein synthesis. Autoantibodies to certain of these synthetases (histidyl–, threonyl–, alanyl–, isoleucyl–, and glycyl–tRNA synthetases) have been identified in patients with inflammatory myopathies (1–6). Among these "antisynthetase autoantibodies," the most common is anti–Jo-1 (anti–histidyl–tRNA synthetase [anti-HisRS]), found in 20% of patients with polymyositis/dermatomyositis (PM/DM) (7–11). Anti–PL-7 (anti–threonyl–tRNA synthetase [anti-ThrRS])

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and anti–PL-12 (anti–alanyl–tRNA synthetase [anti-AlaRS]) autoantibodies are less common, found in 3–4% of all patients with PM/DM (4,5,11–13), while anti-OJ (anti–isoleucyl–tRNA synthetase [anti-IleRS]) and anti-EJ (anti–glycyl–tRNA synthetase [anti-GlyRS]) autoantibodies are the least common, occurring in <2% (6,14,15), although the frequency may vary in different populations (16).

Characteristic clinical features have been found in patients with anti-HisRS and other antisynthetase autoantibodies (1,9,10). These features include myositis, interstitial lung disease (ILD), arthritis, Raynaud's phenomenon, fever with exacerbations, and the skin lesion of the fingers referred to as mechanic's hands, and they appear to form a distinct syndrome referred to as the "antisynthetase syndrome" (8–11). Although the similarity of the clinical features associated with different antisynthetases is impressive (17,18), certain differences have been noted, which must be considered preliminary due to the small reported number of patients with non-HisRS antisynthetases (1,9,19). Patients with anti-AlaRS appear to be more likely than those with anti-HisRS to have ILD and/or arthritis either without myositis or with little evidence of muscle disease. Absence of significant myositis over the full disease course in patients with anti-HisRS is rare (<5%), although it may occur. Clinically significant myositis was seen in 60% of US patients with anti-AlaRS (13), whereas none of 6 Japanese patients with anti-AlaRS autoantibodies fulfilled criteria for myositis (20). Among patients with anti-IleRS, 2 of 10 had ILD without evidence of myositis, and 1 had ILD with subclinical myositis (14). In addition, antisynthetases may occur in either PM or DM, but PM is usually more common with anti-HisRS (10,16,21), and DM is usually more common with other antisynthetases, especially anti-GlyRS (15,22).

We recently described anti-KS autoantibodies and provided evidence that the KS antigen is asparaginyl–tRNA synthetase (AsnRS) (23). This sixth antisynthetase was found in sera from 3 patients with ILD and/or inflammatory arthritis without evidence of myositis. It immunoprecipitated a 65-kd protein and a unique tRNA that was distinct from that precipitated by any previously described antisynthetase or other reported tRNA-related antibody. Each of the 3 sera and their IgG fractions showed significant inhibition of AsnRS activity, but did not inhibit any of the other 19 aminoacyl–tRNA synthetase activities.

In this report, we describe the clinical and immunogenetic features of 5 additional patients with anti-AsnRS autoantibodies, most of whom had the syndrome of ILD with arthritis and/or myositis. Immunoprecipitation (IP) and aminoacylation inhibition studies with sera from these patients provide additional evidence that anti-KS (anti-AsnRS) reacts with asparaginyl–tRNA synthetase.

PATIENTS AND METHODS

Sera. Serum samples from a collection of sera from ~800 patients seen at the current or previous collaborating centers of the authors (Keio University, Tokyo, Japan; Kyoto University, Kyoto, Japan; Seoul National University, Seoul, Korea; Clinic and Research Institute for Rheumatic Diseases Aachen, Aachen, Germany; University of Oklahoma Health Sciences Center, Oklahoma City; National Institutes of Health, Bethesda, MD) or sera referred there for testing were stored at −20°C and were tested for the presence of anti-AsnRS autoantibodies. Sera from the following patients were included: 1) patients with PM or DM according to the criteria described by Bohan and Peter (24,25); 2) patients with a condition suggesting the clinical diagnosis of myositis; 3) patients with ILD who had no evidence of myositis and did not meet criteria for other connective tissue diseases; and 4) patients with serum anticytoplasmic antibodies, regardless of diagnosis. Approximately 1,700 other sera have also been tested, including sera from patients with other conditions including systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis, as well as sera from normal subjects. Many of the sera were tested in studies of other autoantibodies. All samples were obtained after the patients gave their informed consent, as approved by the corresponding institutional review boards. Stored sera known to contain autoantibodies against synthetases for histidine, threonine, alanine, glycine, and isoleucine were used as controls.

ILD was considered to be present if an interstitial infiltrate was observed on chest radiography. DM was considered to be present if a heliotrope rash and/or Gottron's papules were observed.

IP. IP from HeLa cell extracts was performed as previously described (6,10). Ten microliters of patient sera was mixed with 2 mg of protein A–Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) in 500 μl of IP buffer (10 m*M* Tris HCl at pH 7.5, 500 m*M* NaCl, 0.1% Nonidet P40 [NP40]) and incubated with end-over-end rotation (Labquake shaker; Lab Industries, Berkeley, CA) for 2 hours at 4°C. The IgG-coated Sepharose was washed 4 times in 500 μl of IP buffer using 10-second spins in a microfuge tube, and resuspended in 400 μl of NET-2 buffer (50 m*M* Tris HCl at pH 7.5, 150 m*M* NaCl, 0.05% NP40).

For analysis of RNAs, this suspension was incubated with 100 μl of extracts, derived from 6×10^6 cells, on the rotator for 2 hours at 4°C. The antigen-bound Sepharose was then collected with a 10-second centrifugation in the microfuge, washed 4 times with NET-2 buffer, and resuspended in 300 μl of NET-2 buffer. To extract bound RNAs, 30 μl of 3.0*M* sodium acetate, 30 μl of 10% sodium dodecyl sulfate (SDS), and 300 μl of phenol/chloroform/isoamyl alcohol (50:50:1; containing 0.1% 8-hydroxyquinoline) were added to the Sepharose beads. After agitation in a Vortex mixer and

spinning for 1 minute, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 20 μl of electrophoresis sample buffer, composed of 10*M* urea, 0.025% bromphenol blue, and 0.025% xylene cyanol FF (Bio-Rad, Hercules, CA) in Tris–borate–EDTA buffer (90 m*M* Tris HCl at pH 8.6, 90 m*M* boric acid, and 1 m*M* EDTA). The RNA samples were denatured at 65°C for 5 minutes and then resolved by 7*M* urea–10% polyacrylamide gel electrophoresis (PAGE), with silver staining (Bio-Rad).

For protein studies, antibody-coated Sepharose was mixed with 400 μl of ^{35}S-methionine–labeled HeLa extract derived from 2×10^5 cells and rotated at 4°C for 2 hours. After 4 washes with IP buffer, the Sepharose was resuspended in SDS sample buffer (2% SDS, 10% glycerol, 62.5 m*M* Tris HCl at pH 6.8, 0.005% bromphenol blue). After heating at 90°C for 5 minutes, the proteins were fractionated by 10% SDS-PAGE, enhanced with 0.5*M* sodium salicylate, and dried. Labeled proteins were analyzed by autoradiography.

Aminoacylation. Aminoacylation inhibition reactions were performed as described previously, with minor modification (6,26). Six microliters of HeLa cell extract diluted 1:10 in Tris buffered saline was incubated with 3 μl of a 1:10 dilution of serum for 2 hours at 4°C. This was combined with 17 μl of reaction solution (50 m*M* Tris HCl at pH 7.5, 0.02*M* NaCl, 0.01*M* $MgSO_4$, 1 m*M* dithiothreitol) containing 8 units of yeast tRNA, 3 μl of ^{14}C-asparagine or other ^{3}H-labeled amino acid, and 1 μl of 200 m*M* cold amino acid. Ten-microliter aliquots were tested at 10 minutes and 20 minutes, spotted onto filter paper treated with 5% trichloroacetic acid (TCA), washed 5 times with 5% TCA, then with ethanol, then dried for counting. Results of inhibition testing were expressed as the percent inhibition of the average activity seen with the normal serum included in that experiment, as follows: % inhibition = [(average counts per minute with normal serum) − (cpm with test serum)] × 100/(average cpm with normal serum). Inhibition of >50% compared with the activity with normal serum was considered significant. In previous studies, although nonspecific effects on aminoacylation reactions by serum were common, nonspecific inhibition was usually <25%, and inhibition >50% reliably reflected specific antibody effects (6,7,12,13,26).

DNA typing of the HLA class II (DRB1, DQA1, DQB1, DPB1) alleles by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). Genomic DNA was isolated by phenol extraction of SDS-lysed and proteinase K–treated peripheral blood leukocytes, and then amplified by the PCR procedure using an automated PCR thermal cycler (PerkinElmer Cetus, Norwalk, CT). The primers used for specific amplification of the polymorphic exon 2 domains of the DRB1, DQA1, DQB1, and DPB1 genes were previously described (27). Amplified DNA was digested by all-specific restriction endonucleases and subjected to electrophoresis using a 12% polyacrylamide gel. Digested fragments were detected by staining with ethidium bromide, and HLA genotypes were determined on the basis of the RFLP patterns generated as previously described (27).

Other. Ouchterlony double immunodiffusion was performed as described previously, using HeLa cell extract as antigen (10).

Cases. *Patient 1.* The patient, a 61-year-old Japanese woman, noticed chest pain, followed 3 months later by dyspnea on mild exertion. Chest radiography and computed tomography (CT) scanning showed bilateral basilar infiltrates. The patient had hypoxemia, with a restrictive pattern on pulmonary function tests. No muscle weakness was observed, and the creatine kinase (CK) level was normal (67 IU/liter). A lung biopsy specimen obtained by video-assisted thoracic surgery showed mild interstitial chronic inflammation and interstitial fibrosis lacking a temporal heterogeneity pattern, and a diagnosis of fibrotic nonspecific interstitial pneumonia was made.

Patient 2. The patient, a 51-year-old German woman, developed a nonproductive cough and dyspnea on exertion. Chest radiography showed bibasilar interstitial fibrosis, and pulmonary function tests showed a restrictive pattern with decreased diffusing capacity for carbon monoxide (DLco). A diagnosis of ILD was made, and the patient's pulmonary function remained stable throughout her disease course. She had polyarthralgia and developed erythema and keratosis of the palms and fingers consistent with mechanic's hands, but no cutaneous scleroderma, Raynaud's phenomenon, or DM rash (Gottron's papules or heliotrope rash) was observed. No muscle weakness was found, and the CK level was normal (56 IU/liter at the first visit) each time it was measured. When the patient was age 58 years, ovarian carcinoma was found, and surgery with subsequent irradiation was performed. She died of metastatic ovarian carcinoma at age 63 years.

Patient 3. The patient, a 72-year-old American woman, developed an itchy red eczematous rash that was thought to be due to a medication for hypertension. The rash was soon accompanied by progressive weakness, myalgias, mild dyspnea, and difficulty swallowing. She was started on prednisone and methotrexate, and 6 months after the rash had first appeared, she was referred to the Arthritis and Rheumatism Branch of the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health. There was a widespread maculopapular rash of the trunk, extremities, and head, and Gottron's papules were observed. Proximal muscle weakness was present, and her CK level was 358 IU/liter. Magnetic resonance imaging of the thighs showed both atrophy and probable inflammation on the STIR images. A biopsy of the deltoid muscle showed changes of an active inflammatory myopathy. No malignancy was identified. She was treated with pulse methylprednisolone. However, her muscle weakness and rash were not significantly improved, and infectious complications limited the therapeutic options. Her disease course was subsequently complicated by herpes zoster and the Ramsay-Hunt syndrome as well as by skin infections and cellulitis, mastoiditis, heart failure, and a cerebrovascular accident.

Patient 4. The patient, a 53-year-old Korean woman with intermittent episodes of productive cough due to bronchiectasis, noticed easy fatigability and myalgia in 1994 and later developed muscle weakness and was admitted to Seoul National University Hospital in February 1995. Proximal muscle weakness in her extremities and a dark pigmentation over the extensor surface of both knees were observed. The CK level was elevated at 3,808 IU/liter. The findings on electromyogram and muscle biopsy were consistent with inflammatory myopathy. A diagnosis of DM associated with ILD was made, and she was treated with prednisolone (60 mg/day). Her muscle enzyme levels gradually normalized, and her muscle weakness improved. Her chest radiograph and high-resolution

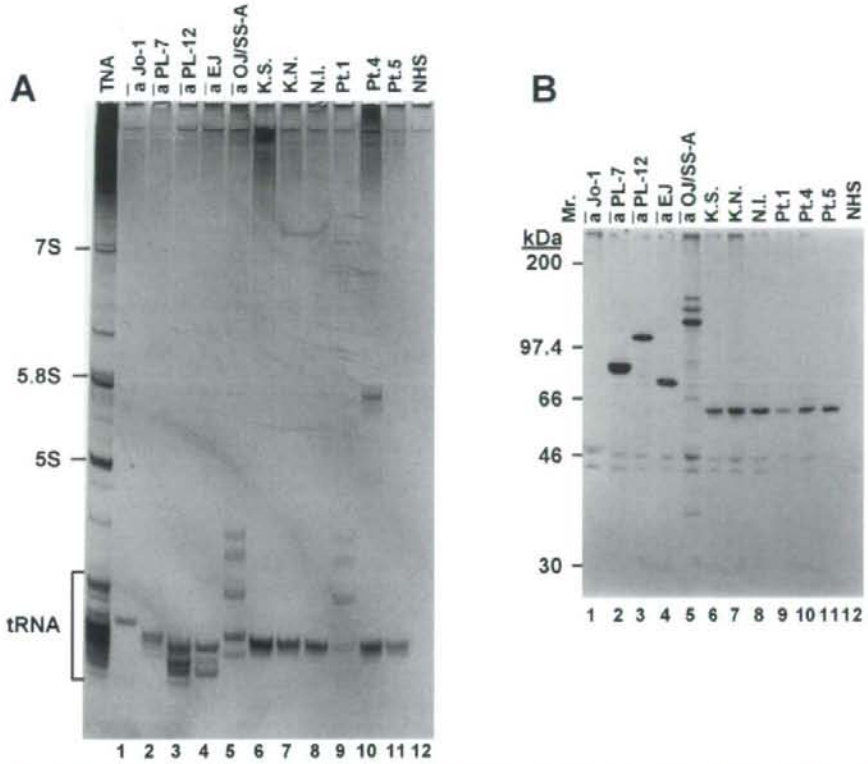

Figure 1. A, Immunoprecipitation (IP) for nucleic acids with anti-KS and control sera. Shown are patterns of transfer RNA (tRNA) resulting from 7*M* urea–10% polyacrylamide gel electrophoresis (PAGE) of phenol-extracted immunoprecipitates from HeLa cell extract, developed with silver stain. TNA = total nucleic acids, with the 5.8S and 5S small ribosomal RNAs and the tRNA region indicated. Antisynthetase sera used for IP are indicated. Lane 1, Anti–histidyl–tRNA synthetase (a Jo-1); lane 2, anti–threonyl–tRNA synthetase (a PL-7); lane 3, anti–alanyl–tRNA synthetase (a PL-12); lane 4, anti–glycyl–tRNA synthetase (a EJ); lane 5, anti–isoleucyl–tRNA synthetase (a OJ/SS-A); lanes 6–11, anti-KS sera from patients KS, KN, and NI in the previous study (23) and from patients 1, 4, and 5 in the present study; lane 12, normal human serum (NHS) control. The tRNA pattern with anti-KS sera is easily distinguishable from that of other antisynthetases. **B,** IP for proteins with anti-KS and control sera. Autoradiogram of 10% sodium dodecyl sulfate–PAGE of immunoprecipitates from ^{35}S-methionine–labeled HeLa cell extract. Mr. = molecular weight markers. Antisynthetase sera used for IP are indicated as in **A**. Anti-KS sera immunoprecipitated a very strong protein band from ^{35}S-methionine–labeled HeLa cell extracts (lanes 6–11), migrating at 65 kd, that was clearly different from the bands immunoprecipitated by sera with the described antisynthetases.

CT scan showed bilateral basilar interstitial fibrosis, and pulmonary function tests showed a restrictive pattern with decreased DLco. Her muscle weakness gradually improved, and the CK level normalized in January 1996. Prednisolone was tapered and discontinued in March 1996.

Patient 5. The patient, a 64-year-old Japanese man with a previous history of prostatic carcinoma, was admitted to the hospital due to bilateral infiltrates on chest radiography. He did not notice cough or dyspnea at that time, but a chest CT scan revealed bibasilar interstitial fibrosis. A transbronchial lung biopsy was performed, with histology showing usual interstitial pneumonia. He was started on prednisolone (40 mg/day), resulting in slight improvement seen on his chest radiograph. Prednisolone was tapered and discontinued in April 1998. He then developed polyarthritis and was treated with a nonsteroidal antiinflammatory drug. No muscle weakness was found, and the CK level was normal (50 IU/liter at the first visit) throughout his disease course.

RESULTS

Identification of anti-KS (anti-AsnRS) antibodies. Sera from all 8 patients (the 3 patients with ILD and/or inflammatory arthritis without evidence of myositis in our previous study [patients KS, KN, and NI; see

Table 1. Clinical features of 8 patients with anti-KS antibodies*

	Patient							
	KS	KN	NI	1	2	3	4	5
Age at onset, years/sex	36/F	44/F	61/F	60/F	51/F	72/F	53/F	65/M
Ethnic background	Japanese	Japanese	Japanese	Japanese	German	US	Korean	Japanese
ILD	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Myositis	No	No	No	No	No	Yes	Yes	No
DM rash	No	No	No	No	No	Yes	Yes	No
Arthritis	Yes	No	No	No	Yes	Yes	No	Yes
Malignancy	No	No	No	No	Ovarian cancer	No	No	Prostate cancer
Raynaud's phenomenon	No	Yes	No	No	No	No	No	No
Other autoantibodies	No	No	No	Anti-SSA/Ro	No	No	No	No
Diagnosis	ILD with arthritis	Idiopathic ILD	Idiopathic ILD	Idiopathic ILD	Idiopathic ILD	DM	DM	ILD with arthritis

* ILD = interstitial lung disease; DM = dermatomyositis.

ref. 23] and the 5 additional patients described above) were shown to immunoprecipitate a characteristic, identical pattern of tRNA, with a strong predominant nucleic acid band of tRNA size, accompanied by a faster faint band (Figure 1A). This gel pattern of tRNA was clearly distinguishable from the pattern of tRNA precipitated by the 5 other described antisynthetases (Figure 1A) and was identical in mobility and appearance to that of serum KS, the originally reported anti-KS serum (23) (Figure 1A).

A very strong band from ^{35}S-methionine–labeled HeLa cell extracts (Figure 1B), migrating at 65 kd, that was also identical in mobility to that of serum KS, was found by IP for all 8 sera, with 5 representative sera shown in Figure 1B. This was clearly different from the characteristic bands immunoprecipitated by sera with the other described antisynthetases (Figure 1B).

Five of the newly recognized anti-KS antibody–positive sera were tested for their ability to inhibit the in vitro enzymatic function of AsnRS (aminoacylation of $tRNA^{Asn}$). Four of the 5 new anti-KS sera significantly inhibited (by >50% at 10 minutes) AsnRS activity compared with normal serum or other controls (serum from patient KS by 87%, serum from patient KN by 99%, serum from patient NI by 91%, serum from patient 1 by 82%, serum from patient 2 by 100%, serum from patient 3 by 18%, serum from patient 4 by 87%, and serum from patient 5 by 91%). This inhibition was strong and comparable with that seen with serum KS, for 4 of the 5 new anti-KS sera. Purified IgG from the third new serum (from patient 3) showed significant, but not strong, inhibition (52%) that increased at 20 minutes (to 84%).

There was no significant inhibition of other synthetases. Normal control serum and anti-KS–negative myositis serum did not show significant inhibition of AsnRS, although sera with other antisynthetases inhibited the expected enzymes. These results indicated that sera with anti-KS by IP showed specific inhibition of AsnRS, further supporting previous data indicating that anti-KS reacted with AsnRS.

Clinical findings. The clinical features of the 5 newly identified patients (patients 1–5) and the 3 patients with anti-AsnRS reported previously (patients KS, KN, and NI) (23) are summarized in Table 1. All patients with anti-AsnRS antibodies were middle-aged or elderly, and 7 of them were women. Five patients were Japanese, 1 was from the US, 1 was German, and 1 was Korean. Seven of these 8 patients (88%) had ILD, documented in each case by both chest radiography and pulmonary function tests. In addition, 2 patients had myositis and a diagnosis of DM. Their clinical courses of ILD were classified as the chronic type. Four patients (50%) had nonerosive arthritis or arthralgia. Raynaud's phenomenon was seen in only 1 patient. None of the patients had sclerodactyly or overlap syndromes with other connective tissue diseases. Malignant diseases (ovarian carcinoma and prostatic carcinoma) were observed in 2 patients. Regarding other autoantibodies, anti-SSA/Ro antibodies were detected in only 1 patient.

Anti-AsnRS was found in 0% of Japanese patients with myositis, but was found in 3% of Japanese patients with "idiopathic" ILD. Thus, most patients with anti-AsnRS antibodies had chronic ILD with or without features of PM/DM or other connective tissue disease.

Immunogenetic features. The HLA class II gene was determined in 4 Japanese patients (Table 2). All 4 patients had DR2 (DRB1*1501 or DRB1*1502) compared with 33% of healthy local controls. It should be noted that all patients with anti-AsnRS antibodies had DR2, but the frequency of DR2 did not reach statistical significance ($P > 0.05$).

Table 2. HLA class II genes in Japanese patients with anti-KS autoantibodies

	Patient			
	KS	KN	NI	1
DR	2/5	2/1	2/2	2/4
DRB1*	1502/1101	1501/0101	1502/1502	1501/0405
DQA1*	0103/0501	0102/0101	0103/0103	0102/0303
DQB1*	0601/0301	0602/0501	0601/0601	0602/0401
DPB1*	0901/1401	0201/0501	0901/0901	0201/0402

DISCUSSION

We have identified anti-KS (anti-AsnRS) autoantibodies in 8 patients with ILD and DM, by IP of the same distinctive set of tRNA and protein that differed from those precipitated by the other 5 antisynthetases. Most of the anti-KS sera showed specific inhibition of the enzyme target, AsnRS, without inhibiting other synthetases.

Several interesting characteristics of the previously studied antisynthetases have been described: 1) they are associated with a distinctive clinical syndrome referred to as the antisynthetase syndrome, 2) they are directed at functionally related enzymes (performing the same function for different amino acids), 3) they do not cross-react with other synthetases, and 4) they tend to be mutually exclusive. Anti-AsnRS antibodies seem to have the same features. No serum with any other antisynthetase has had antibodies to AsnRS, and none of the 8 anti-AsnRS sera reported here showed signs of reaction with other synthetases. The mechanism of this phenomenon remains unknown.

Multiple tRNA bands immunoprecipitated by anti-AsnRS were found on urea-PAGE. The patterns of tRNA for each of the 8 patients were very similar, highly restricted compared with total tRNA, and distinctive compared with the pattern of other anti–aminoacyl tRNA synthetase autoantibodies. These bands are likely to represent different forms of tRNA for asparagine, which can include tRNA with different asparagine anticodons (uracil-uracil-adenine, uracil-uracil-guanine) or tRNA with the same anticodon but differences in other parts of the sequence. Most sera with anti-HisRS, anti-ThrRS, anti-GlyRS, and anti-IleRS had not been described to react directly with tRNA, suggesting indirect precipitation of tRNA. However, approximately one-third of anti-HisRS–positive sera were reported to contain autoantibodies recognizing $tRNA^{His}$ (28). Most anti-AlaRS sera react directly with the sets of $tRNA^{Ala}$ with the inosine-guanine-cytosine anticodon (29). We previously found that the 3 original anti-KS (anti-AsnRS) sera did not immunoprecipitate any RNA from deproteinized HeLa extracts (23). This suggests that anti-AsnRS antibodies can precipitate $tRNA^{Asn}$ indirectly, through its affinity for AsnRS, although the possibility of conformational epitopes on the tRNA has not been excluded (28). Further analysis will be necessary to determine the sequence and specificity of tRNA immunoprecipitated by anti-AsnRS.

The specific inhibition of AsnRS function by most of the sera found to have anti-KS is consistent with findings observed for other antisynthetases. It should be noted that our anti-KS sera also demonstrated inhibition of enzymatically active recombinant AsnRS (30). Most sera with any of the 5 reported antisynthetases specifically inhibit the aminoacylation of the respective tRNA, indicating inhibition of the enzymatic function of the synthetase (3,5–7,12). This functional inhibition may indicate that the autoantibodies are recognizing the active sites of the synthetases. In contrast, it has been reported that animal antisera raised against synthetases do not consistently show such inhibition, suggesting that active sites tend not to be immunogenic for animals (31). Hypothetically, this could relate to relative conservation of the active site. However, there might be an alternative mechanism for inhibition. For example, binding of antibodies outside the active site may alter the structure of the enzyme or interfere with enzyme activity sterically. Further studies of the precise epitope on the aminoacyl–tRNA synthetase might help to explain the development of these autoantibodies.

Each of the 5 previous antisynthetases was first identified in patients with myositis and then found to be associated with ILD. In previous studies, these autoantibodies were associated with myositis with a high frequency of ILD (50–80%) and arthritis (50–90%) (1,2,17,18), as well as an increase in Raynaud's phenomenon (60%), fever with exacerbations (80%), and the skin lesion of the fingers referred to as mechanic's hands (70%) when compared with the overall population of patients with myositis (9–11). The similarities between patients with different antisynthetases have been noted, whereas certain differences have been found, which must be considered preliminary due to the small reported number of patients with non-HisRS antisynthetases. Absence of significant myositis over the full disease course in patients with anti-HisRS is rare (<5%) (32), whereas patients with anti-AlaRS are more likely than patients with anti-HisRS to have ILD and/or arthritis without clinical evidence of myositis (19). Anti-ThrRS

resembles anti-HisRS more than anti-AlaRS in Japanese patients (33).

In the present study, 7 of 8 patients (88%) with anti-AsnRS autoantibodies had ILD, some with other associated features of connective tissue disease including arthritis and Raynaud's phenomenon. In this respect, anti-AsnRS appears to resemble anti-AlaRS more than anti-HisRS. It is noteworthy that the 2 patients with both anti-AsnRS and myositis were among the 3 patients from outside Japan, while none of 5 patients from Japan had myositis. Thus, as with patients with anti-AlaRS, for patients with anti-AsnRS, the frequency of ILD without myositis may be higher in Japanese patients. However, most of the group of patients with ILD without myositis who were tested in this study were from Japan.

The features of these 8 patients with anti-KS appeared to reside within the spectrum of the antisynthetase syndrome that has been associated with other antisynthetases. ILD is one of the most important features of the antisynthetase syndrome, and Raynaud's phenomenon and arthritis, as seen in some patients with anti-AsnRS, are also likely to be part of the syndrome. The syndrome associated with anti-AsnRS may be one end of the spectrum of patients with antisynthetase. This highlights the clinical importance of looking for such antibodies in patients with ILD even if there are no signs of myositis or connective tissue diseases.

The typical cutaneous features of DM were observed in 2 patients with anti-AsnRS antibodies. PM has been reported to be much more common (60–80% or more) than DM in patients with anti-HisRS in most studies, whereas DM was most frequent with anti-GlyRS (15) and was also found to be common among patients with anti-AlaRS (13). Like anti-GlyRS and anti-AlaRS antibodies, anti-AsnRS antibodies were more associated with DM in the small number of patients available.

Malignancy has been reported to be unusual in patients with antisynthetases. In our studies, 2 patients were found to have malignancy during their disease course. However, malignancy in these patients may not be related to the DM or ILD, since these malignancies occurred separated in time from each other.

Immunogenetic studies of connective tissue disease have been performed, but HLA associations produced conflicting results. However, a strong correlation of HLA class II antigens with some autoantibodies has been reported (34). With regard to antisynthetase antibodies, HLA–DR3 (DRB1*0301), DQA1*0501, or DQA1*0401 was found to be significantly increased in myositis patients with antisynthetases (9,21). In Japanese patients, we have reported that 7 of 9 patients (78%) with anti-HisRS tested had the HLA class II DRB1*0405;DQA1*0302;DQB1*0401 haplotype, compared with 22% of healthy controls (odds ratio [OR] 13, $P = 0.002$), while 4 of 7 patients (57%) with anti-AlaRS had the DRB1*1501;DQA1*0102;DQB1*0602 haplotype, compared with 9% of healthy controls (OR 14, $P = 0.006$) (35). Interestingly, all 4 Japanese patients tested had DR2 (DRB1*1501/1502), compared with 33% of healthy controls, although a definite statistical association could not be established. These results suggest that the stronger association of anti-AlaRS and anti-KS with ILD may be related to the DR2 phenotype. However, it has been noted that different ethnic groups exhibit different immunogenetic profiles that link with specific autoantibodies (36). Therefore, further studies including analysis of more patients with anti-KS antibodies in different ethnic groups and major histocompatibility complex–restricted T cell responses could provide important clues for understanding the possible mechanisms for the development of antisynthetase antibodies.

The mechanism for the association of antisynthetases with ILD is unknown, but it seems to be related to etiologic factors (37). Recently, a new association of anti-HisRS–positive PM and ILD was reported in a patient with hepatitis C virus infection (38). It was hypothesized that viruses might interact with the synthetases and induce autoantibodies by molecular mimicry or antiidiotype mechanisms in the anti-HisRS–positive patient with myositis associated with ILD (3,39). Another mechanism for generating autoantigenic epitopes of synthetase by granzyme B cleavage in apoptosis was also described recently (40,41). However, these proposed mechanisms remain speculative, and further studies could provide important clues for understanding the possible mechanisms for the development of these antibodies. Studies of these antibodies may provide insight into the etiologic and pathogenetic mechanisms of ILD and myositis.

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AUTHOR CONTRIBUTIONS

Dr. Hirakata 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 design. Hirakata.
Acquisition of data. Hirakata, Nagai, Genth, Song, Targoff.
Analysis and interpretation of data. Hirakata, Suwa, Takada, Sato, Mimori.
Manuscript preparation. Hirakata, Takada, Targoff.
Statistical analysis. Hirakata, Suwa, Targoff.

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