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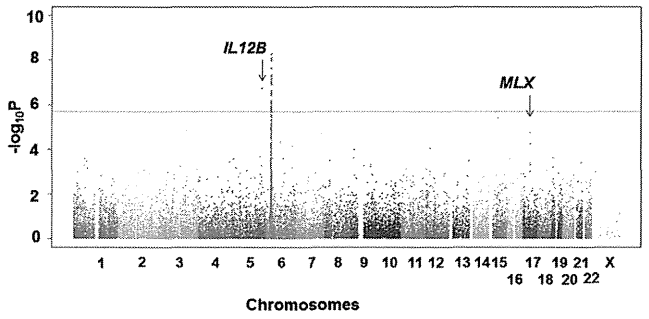

Figure 1. Manhattan Plot of Genome Scanning
The horizontal line indicates the significant level based on Bonferroni's correction. The HLA locus on chromosome 6 and the *IL12B* region on chromosome 5 reached the significant level.

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

Associations between Genotypes and Clinical Phenotypes of TAK

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

Statistical Analysis

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

Results

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

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

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

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Table 2. Results of Association Studies for TAK Susceptibility

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

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

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

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

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

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

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

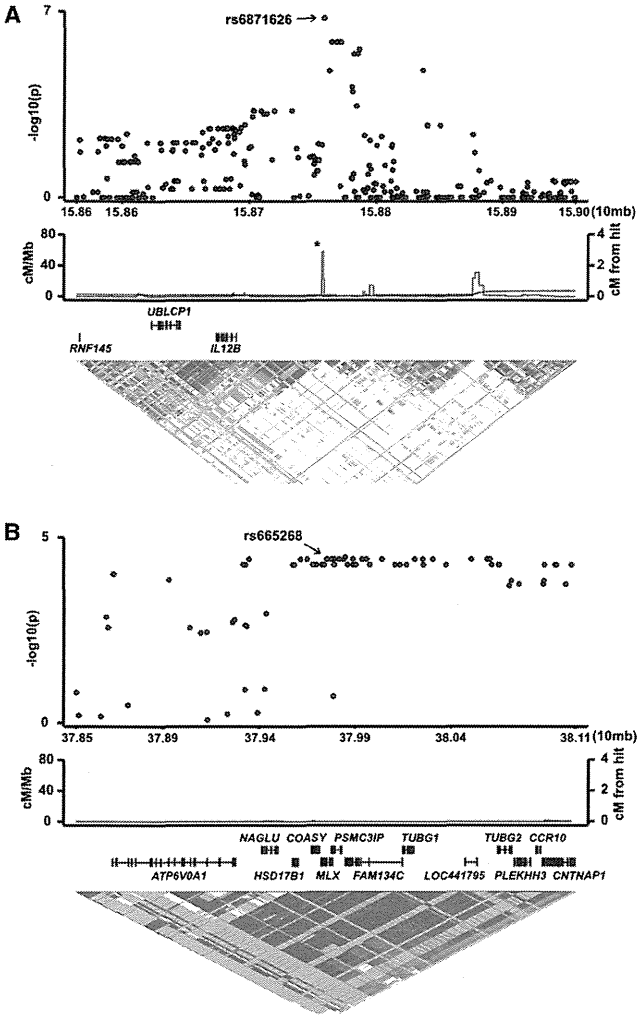

Figure 2. Associations of the *IL12B* and *MLX* Regions with the Susceptibility to TAK

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

Discussion

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

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

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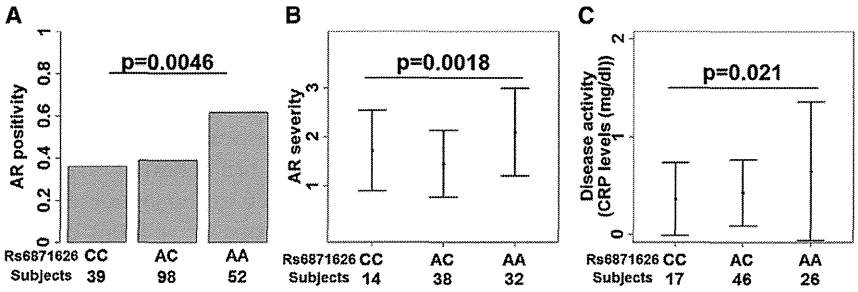

Figure 3. Associations between rs6871626 Genotypes and Clinical Manifestations of TAK

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

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

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

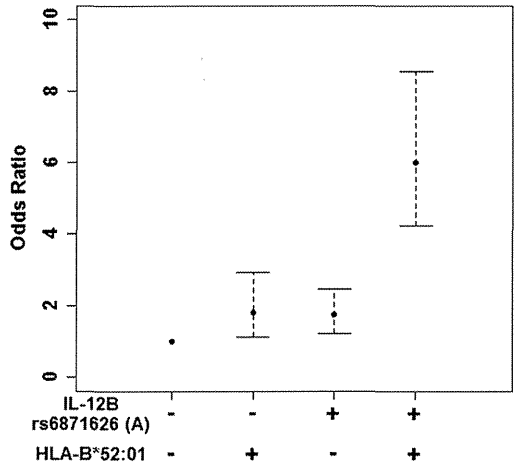

Figure 4. A Synergistic Effect between *IL12B* and HLA-B*52:01 on TAK Susceptibility

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

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

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

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Table 3. Synergistic Effects between *IL12B* and HLA-B*52:01 in Each Study

	RERI		AP		SI	
Study	**(95% CI)**	**p**	**(95% CI)**	**p**	**(95% CI)**	**p**
Genome scanning	2.90 (0.60–5.20)	0.014	0.50 (0.23–0.78)	0.00034	2.57 (1.08–6.09)	0.032
Replication study	3.87 (1.70–6.05)	0.00049	0.62 (0.42–0.81)	4.7×10^{-10}	3.76 (1.51–9.32)	0.0043
Combined study	3.46 (1.90–5.02)	1.4×10^{-5}	0.58 (0.42–0.73)	1.0×10^{-12}	3.24 (1.72–6.11)	0.00028

Abbreviations are as follows: RERI, relative excess risk; AP, attributable proportion; SI, synergy index; CI, confidence interval.

analyze whether these two loci are associated with TAK and whether the interactions are observed in other populations.

Taken together, the current study identified two susceptibility genes to TAK and provided evidence of a common immunological pathway exerted by the *IL12B* region that is involved in the etiology of TAK and other autoimmune disorders and of its synergistic role with HLA in the susceptibility to TAK.

Supplemental Data

Supplemental Data include six figures and one table and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/
Genevar (Gene Expression Variation), http://www.sanger.ac.uk/resources/software/genevar/
International HapMap Project, http://hapmap.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/
R statistical software, http://www.r-project.org/

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PLD4 as a Novel Susceptibility Gene for Systemic Sclerosis in a Japanese Population

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Objective. **Systemic sclerosis (SSc) is an autoimmune disease for which multiple susceptibility genes have been reported. Genome-wide association studies have shown that large numbers of susceptibility genes are shared among autoimmune diseases. Recently, our group identified 9 novel susceptibility genes associated with rheumatoid arthritis (RA) in a Japanese population. The aim of this study was to elucidate whether the 18 genes that displayed associations or suggestive associations for RA in our previous study are associated with SSc in Japanese.**

Methods. **We performed an association study that included 415 patients with SSc and 16,891 control subjects, followed by a replication study that included 315 patients and 21,054 control subjects. The 18 markers reported to display association with RA were analyzed for their associations with SSc in the first study, and 5 markers were further analyzed in the replication study. The inverse variance method was used to evaluate the associations of these markers with SSc in a combined study.**

Results. **In the phospholipase D4 gene (*PLD4*), rs2841277 displayed a significant association with SSc in Japanese patients ($P = 0.00017$). We observed that rs2841280 in exon 2 of *PLD4* was in strong linkage disequilibrium with rs2841277 and introduced an amino acid alteration. We also observed associations between SSc and rs6932056 in *TNFAIP3* and rs2280381 in *IRF8* ($P = 0.0000095$ and $P = 0.0030$, respectively), both of which displayed associations with SSc in a European population.**

Conclusion. **We determined that *PLD4* is a novel susceptibility gene for SSc in Japanese, thus confirming the involvement of *PLD4* in autoimmunity. Associations between SSc and *TNFAIP3* or *IRF8* were also detected in our Japanese population. SSc and RA appear to share relatively large proportions of their genetic backgrounds.**

Systemic sclerosis (SSc) is a connective tissue disease that affects 7–489 individuals per million worldwide and is characterized by the excess production of extracellular matrix molecules and fibrosis (1). Patients with SSc display skin sclerosis, obliterative microvasculopathy such as Raynaud's phenomenon, and multiorgan involvement. Severe complications of SSc sometimes develop, including interstitial lung disease, pulmonary hypertension, and renal crisis. These severe symptoms

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and complications of SSc result in a poor prognosis and a shortened lifespan (2,3). No effective method for preventing or curing SSc has been established (4).

It is well known that SSc has genetic components (5); for example, a US study revealed that the incidence of SSc was much higher among the families of patients with SSc compared with the general population (6). Recent technologic developments enabled the use of genome-wide association studies (GWAS) to identify novel susceptibility loci for autoimmune diseases (7). GWAS of European patients with SSc revealed that *CD247* (8), HLA (8), *TNIP1*, *PSORS1C1*, and *RHOB* (9) are susceptibility loci for SSc. In addition, another GWAS identified associations between *IRF8*, *GRB10*, and *SOX5* and limited cutaneous SSc (lcSSc) in a European population (10). Furthermore, studies adopting a candidate gene approach based on subjecting genes to functional inference analysis led to the identification of *STAT4* (11), *IRF5* (12), *TBX21* (13), *NLRP1* (14), *TNFSF4* (15), *CD226* (16), *BLK* (17), and *TNFAIP3* (18) as novel susceptibility genes for SSc in Europeans. SSc association studies in Japanese populations confirmed that *STAT4* (19), *IRF5* (20), and *BLK* (21) are associated with SSc and identified *UBE2L3* as a susceptibility gene for diffuse cutaneous SSc (dcSSc) (22). An association between HLA and SSc was also detected in Asians (23). These findings suggest a clear overlap in the genetic background of SSc between different populations.

It is well known that susceptibility genes are shared by various autoimmune diseases (24). In fact, HLA (25), *STAT4* (26), and *TNFAIP3* (27,28), which are susceptibility genes for SSc, have also been reported to be associated with rheumatoid arthritis (RA). In addition, *PTPN22*, which was shown to be strongly associated with RA in a European population (29), showed a suggestive association with SSc in Europeans (30). The sharing of these susceptibility genes between RA and SSc raises the possibility that newly identified susceptibility genes for RA could also be susceptibility genes for SSc. Recently, a large Japanese consortium, the Genetic and Allied research in Rheumatic diseases Networking consortium, identified 9 novel susceptibility genes and 6 candidate susceptibility genes for RA using a meta-analysis of GWAS and replication studies (31). Four other genes, namely, HLA, *PADI4*, *CCR6*, and *TNFAIP3*, were also confirmed to display associations with RA. Here, we performed a 2-stage association study of Japanese patients with SSc, in which we genotyped these genes as candidate susceptibility loci.

PATIENTS AND METHODS

Study subjects. DNA samples were obtained from 415 patients with SSc at Kyoto University Hospital and Tokyo Women's Medical University; these samples comprised the first set. Independent DNA samples were obtained from 315 patients with SSc at Keio University Hospital, Sagamihara National Hospital, and Kanazawa University Hospital; these samples were used as the replication set. All patients were Japanese, all had a diagnosis of SSc as determined by a rheumatologist, and all fulfilled the 1980 American College of Rheumatology classification criteria for SSc (32). The patients with SSc for whom clinical information was available were classified as having lcSSc or dcSSc, according to the definitions developed by LeRoy et al (33). The control samples were described in detail in our previous study (31). The current study was approved by the local ethics committees at each institution, and written informed consent was obtained from all subjects. The basic characteristics of the study subjects are shown in Table 1.

Genotyping. The 9 novel susceptibility markers, 6 potentially associated markers, and 4 confirmed markers of RA that were identified in our previous study in a Japanese population (31) were chosen as candidate susceptibility markers for SSc in Japanese. Eighteen of the 19 markers (HLA was excluded; see Results), none of which had previously been reported to be associated with SSc in Japanese individuals, were genotyped in the current study. The 5 candidate markers in the first set that showed associations with P values less than 0.1 were further genotyped in the replication study. Single-nucleotide polymorphisms (SNPs) rs2841280 and rs894037 were chosen as candidate causative variants in the phospholipase D4 gene (*PLD4*) region. Because rs894037 was shown to be monomorphic in Japanese, rs2841280 was genotyped in 334 control subjects, in addition to all patients, for imputation reference. The patients in the first and replication studies were genotyped at Kyoto University or Tokyo Women's Medical University and at Keio University or University of Tsukuba, respectively, using TaqMan assays (Applied Biosystems). The genotyping methods in control subjects were described in detail in our previous study (31).

Briefly, control genotypes in the first set were imputed based on the genome-scanning data, using mach2dat software with HapMap Phase II East Asian Populations as reference. The control genotypes for the replication study were extracted from genome-scanning data for the markers included on Illumina HumanHap610 Quad BeadChips. The genotypes for rs6932056 (which is not included in the array) were imputed based on the genome-scanning data, using mach2dat software with HapMap Phase II East Asian Populations as reference, and were used as control data for the replication set. The genotypes for rs2841280 (which is not included in the HapMap data or the array) were also imputed in control subjects, based on the genome-scanning data, using mach2dat software. Genotyping data for the 334 control subjects as determined by TaqMan assay in combination with genome-scanning data were used as reference.

Statistical analysis. The associations between the genotyped markers and SSc were analyzed using a Cochran-Armitage trend test in both the first and replication studies. Subanalyses were performed by comparing the genotypes of

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

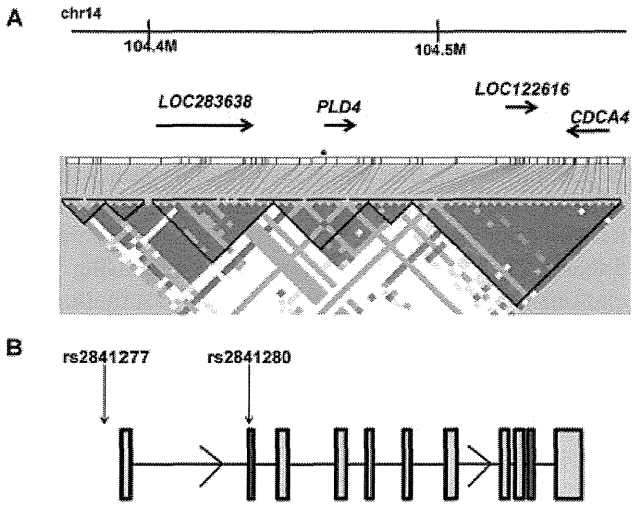

Figure 1. Linkage disequilibrium (LD) block around the *PLD4* region and the *PLD4* structure. **A,** LD block and genes around *PLD4*. The LD block is based on HapMap phase 3 data. **Asterisk** indicates rs2841277. **B,** Schematic view of *PLD4* structure. Rectangles represent exons of *PLD4*.

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*

SNP	Chr	Gene	Allele 1/2	Controls, allele 1 frequency	Limited cutaneous SSc (n = 408)			Diffuse cutaneous SSc (n = 318)		
					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.

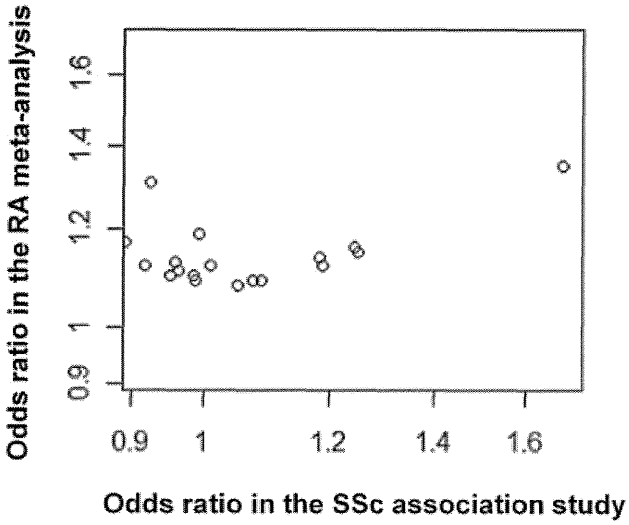

Figure 2. Comparison of associations for systemic sclerosis (SSc) and rheumatoid arthritis (RA). The odds ratios obtained for 18 genes in association studies of SSc and RA are plotted.

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.

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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.

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ORIGINAL ARTICLE

Validation of different sets of criteria for the diagnosis of Sjögren's syndrome in Japanese patients

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Abstract
Objective To validate the revised Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (SS) (JPN) (1999), The American-European Consensus Group classification criteria for SS (AECG) (2002), and American College of Rheumatology classification criteria for SS (ACR) (2012).
Methods The study subjects were 694 patients with SS or suspected SS who were followed-up in June 2012 at ten hospitals that form part of the Research Team for Autoimmune Diseases, The Research Program for Intractable Disease by the Ministry of Health, Labor and Welfare (MHLW). All patients had been checked for all four criteria of the JPN (pathology, oral, ocular, anti-SS-A/SS-B antibodies). We studied the clinical diagnosis made by the physician in charge and the satisfaction of the above criteria.
Results Of the 694 patients, 499 patients did not have other connective tissue diseases (CTDs). SS was diagnosed in 476 patients (primary SS in 302, secondary SS in 174), whereas non-SS was diagnosed in 218 patients (without other CTDs in 197, with other CTDs in 21) by the physician in charge. The sensitivities of JPN, AECG, and ACR in the diagnosis of all forms of SS (both primary and secondary SS) were 79.6, 78.6, and 77.5 %, respectively, with respective specificities of 90.4, 90.4, and 83.5 %. The sensitivities of the same systems in the diagnosis of primary SS were 82.1, 83.1, and 79.1 %, respectively, with specificities of 90.9, 90.9, and 84.8 %, respectively. The sensitivities of the same systems in the diagnosis of secondary SS were 75.3, 70.7, and 74.7 %, respectively, with specificities of 85.7, 85.7, and 71.4 %, respectively.
Conclusion The sensitivity of JPN to all forms of SS and secondary SS, the sensitivity of AECG to primary SS, and the specificities of JPN and AECG for all forms of SS, primary SS, and secondary SS were highest in the diagnosis of SS in Japanese patients. These results indicate that

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the JPN criteria for the diagnosis of SS in Japanese patients are superior to ACR and AECG.

Keywords Sjögren's syndrome · Criteria

Introduction

Sjögren's syndrome (SS) is an autoimmune disease that affects exocrine glands, including the salivary and lacrimal glands. It is characterized by lymphocytic infiltration into the exocrine glands, leading to dry mouth and eyes. A number of autoantibodies, such as anti-SS-A and SS-B antibodies, are detected in patients with SS. SS is subcategorized into primary SS, which is not associated with other well-defined connective tissue diseases (CTDs), and secondary SS, which is associated with other well-defined CTDs [1]. Primary SS is further subcategorized into the glandular form and the extraglandular form.

The revised criteria for the diagnosis of SS issued by the Japanese Ministry of Health (JPN) (1999) (Table 1) [2], as well as the American-European Consensus Group classification criteria for SS (AECG) (2002) (Tables 2, 3) [1], are usually used in both daily clinical practice and clinical studies in Japan. Thus, two sets of diagnostic systems are being applied for the same disease. This could result in a heterogeneous pool of SS patients. This heterogeneity of SS patients makes it difficult to analyze the diagnosis, efficacy of treatment, and prognosis of SS patients. A better alternative would be to use a unified set of criteria for the diagnosis of SS in Japan. Recently, The American College of Rheumatology (ACR) published the ACR classification criteria for SS (2012) (Table 4), which were proposed by the Sjögren's International Collaborative Clinical Alliance (SICCA) [3]. The new set of criteria is designed to be used worldwide, not only in advanced countries but also in developing countries. The SICCA established a uniform classification for SS based on a combination of objective tests that have known specificity to SS [3].

Table 1 The revised Japanese Ministry of Health criteria for the diagnosis of SS (1999)

1. Histopathology
Definition: Positive for at least one of (A) or (B)
(A) Focus score ≥1 (periductal lymphoid cell infiltration ≥50) in a 4 mm^2 minor salivary gland biopsy
(B) Focus score ≥1 (periductal lymphoid cell infiltration ≥50) in a 4 mm^2 lacrimal gland biopsy
2. Oral examination
Definition: Positive for at least one of (A) or (B)
(A) Abnormal findings in sialography ≥stage 1 (diffuse punctate shadows of <1 mm)
(B) Decreased salivary secretion (flow rate ≤10 ml/10 min according to the chewing gum test or ≤2 g/2 min according to the Saxon test) and decreased salivary function according to salivary gland scintigraphy
3. Ocular examination
Definition: Positive for at least one of (A) or (B)
(A) Schirmer's test ≤5 mm/5 min and rose bengal test ≥3 according to the van Bijsterveld score
(B) Schirmer's test ≤5 mm/5 min and positive fluorescein staining test
4. Serological examination
Definition: Positive for at least one of (A) or (B)
(A) Anti-Ro/SS-A antibody
(B) Anti-La/SS-B antibody
Diagnostic criteria: diagnosis of SS can be made when the patient meets at least two of the above four criteria

Upon comparing these three classification sets, there are some differences among them in their purpose and the items adopted in the set (Table 5). The JPN criteria (1999) are intended as an aid for diagnosis, whereas the AECG criteria (2002) and the ACR criteria (2012) are intended for classification purposes in clinical studies and trials. Although the ACR criteria include only three objective items (Tables 4, 5) and are the simplest among the three sets, the ACR criteria may not identify SS patients with negative findings in labial salivary gland biopsy, because the ACR criteria do not include salivary secretion analysis and imaging studies. On the other hand, the JPN criteria combined oral examinations such as salivary secretion, sialography, and salivary gland scintigraphy with three objective items adopted in the ACR criteria (Table 5). Only the AECG criteria include ocular and oral symptoms, which may cause false positives in patients with non-SS conditions such as aging or visual display terminals (VDT) syndrome (Table 5).

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Table 2 The American-European Consensus Group classification criteria for SS (2002)

I. Ocular symptoms: a positive response to at least one of the following questions
1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?
II. Oral symptoms: a positive response to at least one of the following questions
1. Have you had a daily feeling of dry mouth for than 3 months?
2. Have you had recurrently or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?
III. Ocular signs—that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests
1. Schirmer's test, performed without anaesthesia ($\leq$5 mm in 5 min)
2. Rose bengal score or other ocular dry eye score ($\geq$4 according to van Bijsterveld's scoring system)
IV. Histopathology: in minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score $\geq$1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acuni and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue
V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests
1. Unstimulated whole salivary flow ($\leq$1.5 ml in 15 min)
2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts
3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer
VI. Autoantibodies: presence in the serum of the following autoantibodies
1. Antibodies to Ro (SS-A) or La (SS-B) antigens, or both

Table 3 The American-European Consensus Group classification criteria for SS (2002) rules for classification

For primary SS
In patients without any potentially associated disease, primary SS may be defined as follows:
(A) The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (histopathology) or VI (serology) is positive
(B) The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)
For secondary SS
In patients with a potentially associated disease (for instance, another well-defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS
Exclusion criteria:
Past head and neck radiation treatment
Hepatitis C infection
Acquired immunodeficiency disease (AIDS)
Pre-existing lymphoma
Sarcoidosis
Graft vs. host disease
Use of anticholinergic drugs (for a time shorter than 4-fold the half life of the drug)

The purpose of the present study was to validate the JPN criteria, AECG criteria, and ACR criteria for the diagnosis of SS in Japanese patients. The study identified the differences among these three classification sets.

Patients and methods

Study population

The study subjects were 694 patients (51 males and 643 females) with a diagnosis of SS or suspected SS who had been checked for all four criteria of the JPN (pathology, oral, ocular, anti-SS-A/SS-B antibody), and were followed up in June 2012 at ten hospitals across Japan (Kanazawa Medical University Hospital, Nagasaki University Hospital, Hyogo Medical University Hospital, Keio University Hospital, Tokyo Women's Medical University Hospital, Tsurumi University Hospital, Kyushu University Hospital, University of Occupational and Environmental Health Hospital, Kyoto University Hospital, and University of Tsukuba Hospital) that form part of the Research Team for Autoimmune Diseases, The Research Program for Intractable Disease of the Ministry of Health, Labor and Welfare (MHLW).

Data collection and analysis

We collected clinical data from the above ten hospitals using a questionnaire. We retrospectively examined the clinical diagnosis made by the physician in charge, as well as the satisfaction of the JPN, AECG, and ACR criteria. Because lissamine green ocular staining had not been adopted in Japan at the time of clinical examination, we regarded patients who had a positive rose bengal test or fluorescein staining test as having satisfied the ocular staining score in the ACR classification system.

We regarded the clinical diagnosis made by the physician in charge as the gold standard for the diagnosis of SS in this study. We compared the sensitivities and specificities of the JPN, AECG, and ACR diagnostic systems in the diagnosis of SS (both primary and secondary SS), primary

Table 4 The American College of Rheumatology classification criteria for SS (2012)

The classification of SS, which applies to individuals with signs/symptoms that may be suggestive of SS, will be met in patients who have at least 2 of the following 3 objective features:
1. Positive serum anti-SS-A/Ro and/or anti-SS-B/La or (positive rheumatoid factor and ANA titer $\geq$1:320)
2. Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score $\geq$1 focus/4 mm^2
3. Keratoconjunctivitis sicca with ocular staining score $\geq$3 (assuming that individual is not currently using daily eye drops for glaucoma and has not had corneal surgery or cosmetic eyelid surgery in the last 5 years)
Prior diagnosis of any of the following conditions would exclude participation in SS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests:
History of head and neck radiation treatment
Hepatitis C infection
Acquired immunodeficiency syndrome
Sarcoidosis
Amyloidosis
Graft vs. host disease
IgG4-related disease

SS, and secondary SS. Agreement between the three was assessed via the kappa coefficient.

Results

Diagnosis of SS (primary and secondary SS) and non-SS

Of the 694 patients, 499 patients did not have other well-defined CTDs, whereas 195 patients did. SS was diagnosed in 476 patients (302 primary SS, 174 secondary SS), whereas non-SS was diagnosed in 218 patients (197 without other CTDs, 21 with other CTDs) by the physician in charge (Table 6).

Sensitivities and specificities of the three diagnostic systems for SS

The sensitivities of JPN, AECG, and ACR in the diagnosis of all SS (302 primary SS and 174 secondary SS) were 79.6, 78.6, and 77.5 %, respectively, whereas the respective specificities in the diagnosis of all SS were 90.4, 90.4, and 83.5 %. The sensitivities of JPN, AECG, and ACR in the diagnosis of 302 primary SS were 82.1, 83.1, and 79.1 %, respectively, with specificities of 90.9, 90.9, and 84.8 %, respectively. The sensitivities of JPN, AECG, and ACR in the diagnosis of 174 secondary SS were 75.3, 70.7, and 74.7 %, respectively, with specificities of 85.7, 85.7, and 71.4 % (Table 7).

Table 5 Comparison of the items adopted in the JPN and AECG and ACR criteria

	JPN	AECG	ACR
Ocular symptoms	×	○	×
Oral symptoms	×	○	×
Ocular signs			
Schirmer's test	○	○	×
Ocular staining	○	○	○
Labial salivary gland biopsy	○	○	○
Salivary gland involvements			
Salivary secretion	○	○	×
Sialography	○	○	×
Scintigraphy	○	○	×
Autoantibodies			
SS-A	○	○	○
SS-B	○	○	○
ANA	×	×	○
RF	×	×	○

SS-A anti-SS-A antibody, *SS-B* anti-SS-B antibody, *ANA* anti-nuclear antibody, *RF* rheumatoid factor, ○ adopted, × not adopted, *JPN* the revised Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

Table 6 Diagnosis of SS and non-SS

	Associated with other CTDs		Total
	No	Yes	
Clinical diagnosis			
SS	302 (primary SS)	174 (secondary SS)	476
Non-SS	197	21	218
Total	499	195	694

Clinical diagnosis diagnosis of SS by the physician in charge

CTDs connective tissue diseases

Comparisons of the satisfaction of the three diagnostic systems

Figure 1 displays Venn diagrams showing comparisons of the satisfaction of the three diagnostic systems. Among all SS patients ($n = 476$), more patients satisfied only the AECG criteria ($n = 42$) rather than only the JPN criteria ($n = 8$) or the ACR criteria ($n = 6$). The same tendency was also observed in patients with primary SS only and in those with secondary SS only. The diagrams indicate that the JPN and ACR diagnostic systems are similar, whereas the AECG diagnostic system is different from the other two. Table 8 shows the agreement among the three