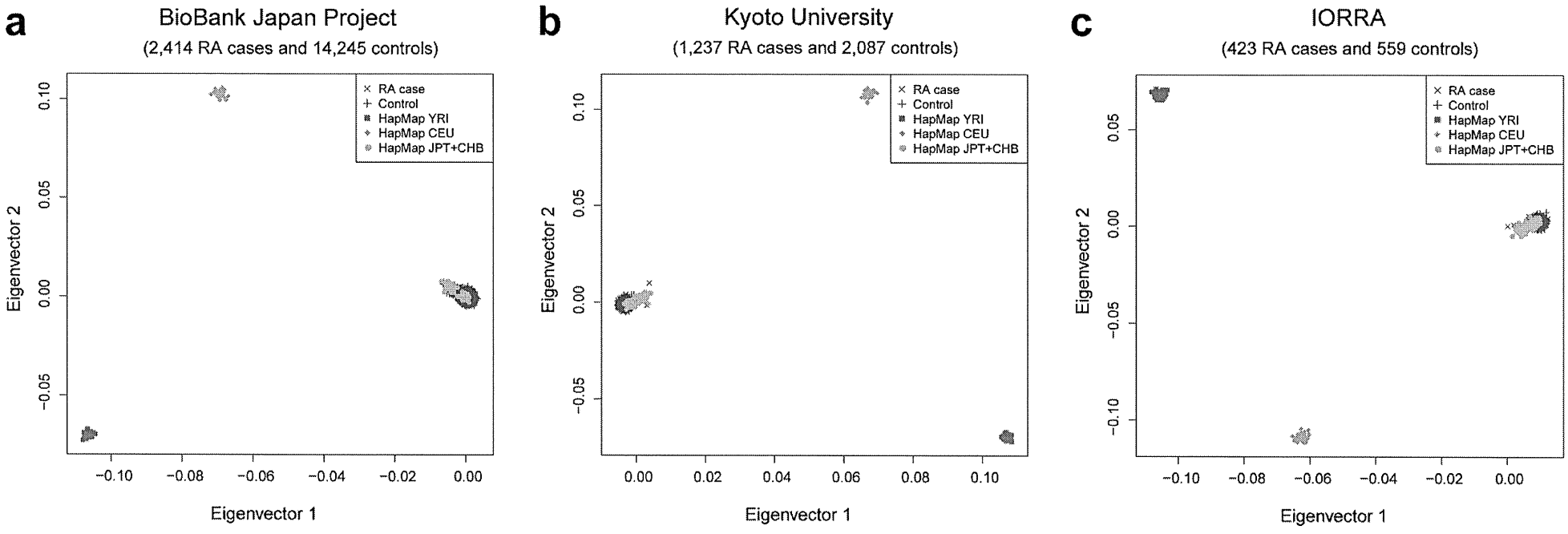
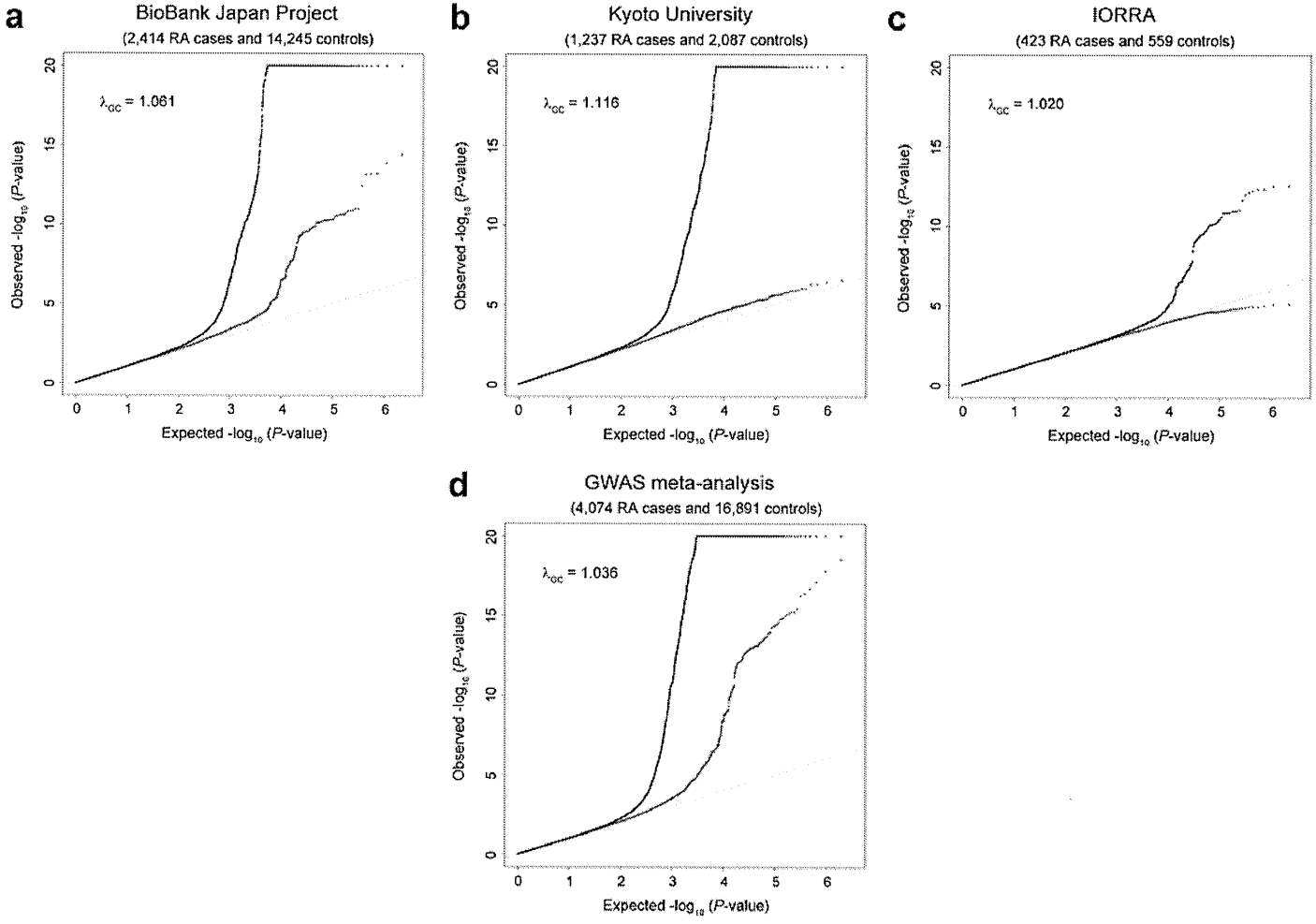
Supplementary Figure 1. Distribution of subjects in the results of principal component analysis (PCA).

Two dimensional display of subjects finally included in the GWAS conducted by **(a)** BioBank Japan Project, **(b)** Kyoto university, and **(c)** IORRA representing the results of PCA[21] performed along with HapMap European (CEU), African (YRI), Japanese (JPT), and Han Chinese (CHB) individuals (Phase II, release 24)[22]. The eigenvectors clearly separated the subjects into three clusters (YRI, CEU, and JPT + CHB clusters), and the distribution of the subjects in the GWAS was concordant with the JPT + CHB cluster as previously anticipated in the Japanese population[23].



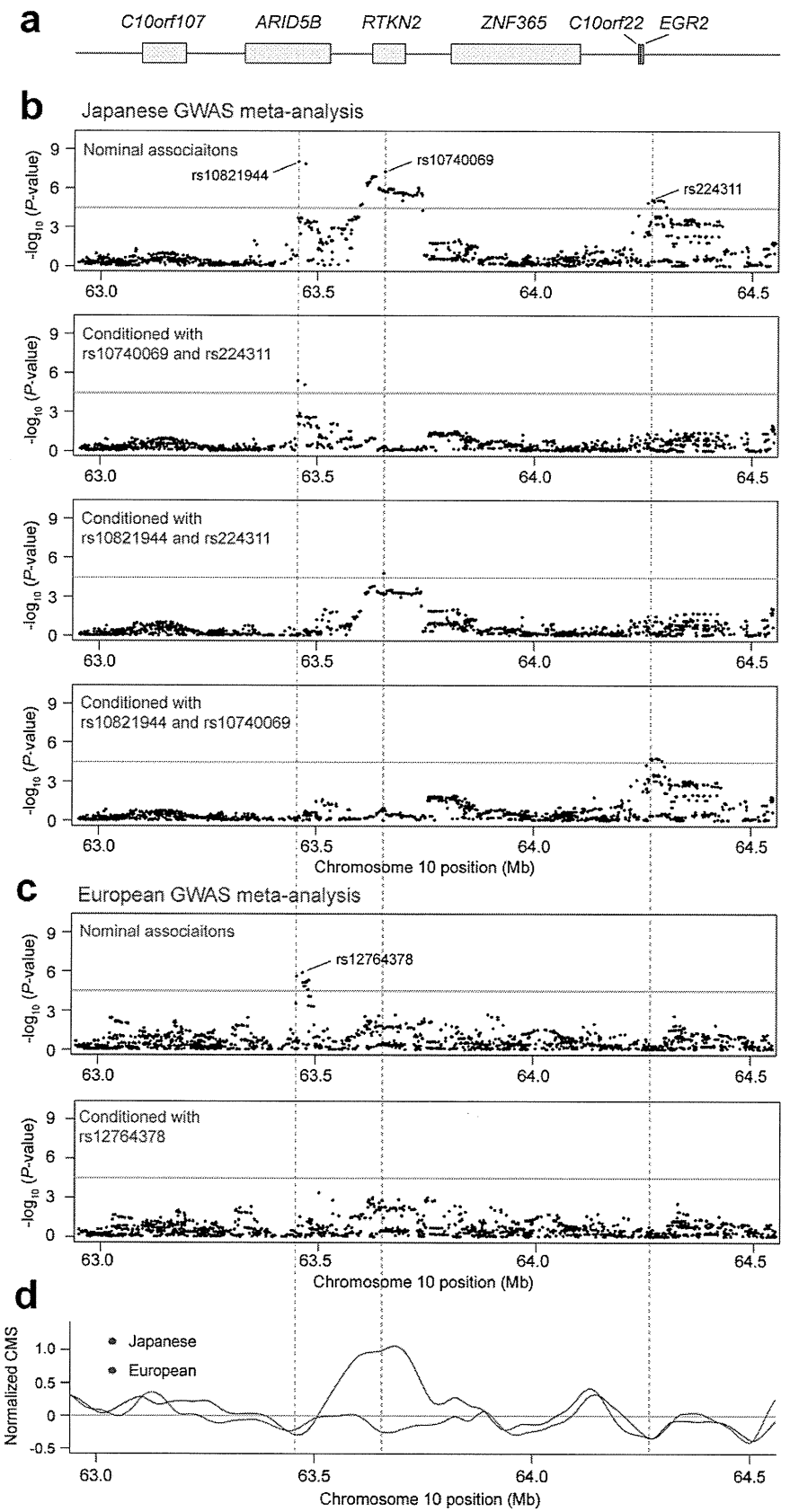
Supplementary Figure 2. Quantile-Quantile plots of *P*-values in the GWAS meta-analysis.

Quantile-Quantile (QQ) plots of the *P*-values in the GWAS conducted by **(a)** BioBank Japan Project, **(b)** Kyoto University, and **(c)** IORRA, and **(d)** the present GWAS meta-analysis. The QQ-plot of the *P*-values of all SNPs that passed the quality control criteria is indicated in black. The QQ-plot of the *P*-values after the removal of SNPs included in the MHC region is presented in blue. The horizontal axis represents the expected $-\log_{10}$ (*P*-values). The vertical axis indicates the observed $-\log_{10}$ (*P*-values) after the application of genomic control correction[24,25] with inflation factor, λ_{GC}, which is displayed in each of the plots. The gray line represents $y = x$, which corresponds to the null hypothesis. The SNPs for which the *P*-value were smaller than 1.0×10^{-20} are indicated at the upper limit of the plot.



Supplementary Figure 3. Regional plots of the SNPs in the *ARID5B* locus at 10q21.

(a) The RefSeq genes in the locus. Associations of the SNPs before and after the condition with the landmark SNP(s) **(b)** in the present Japanese GWAS meta-analysis and **(c)** the previous European GWAS meta-analysis[15]. Dots represent $-\log_{10}$ (*P*-values) of the SNPs in the GWAS meta-analysis. The gray horizontal line represents the region-wide threshold of $P = 0.05/1420 = 3.5 \times 10^{-5}$ (Bonferroni correction on the number of the assessed SNPs located between 63.0 Mbps and 64.5 Mbps). The red dotted lines represent the correspondence of the chromosomal positions of the landmark SNPs in **b** and **c**. In Japanese, three peaks of associations on, or near, *ARID5B*, *RTKN2*, and *EGR2* were observed ($P = 1.0 \times 10^{-8}$ at rs10821944, $P = 5.7 \times 10^{-8}$ at rs10740069, and $P = 8.5 \times 10^{-6}$ at rs224311, respectively; the first panel of **b**). These three variants indicated independent associations with each of the other SNPs (conditional $P = 4.3 \times 10^{-6}$, 1.7×10^{-5}, and 1.8×10^{-5}, respectively; the second to fourth panels of **b**). In contrast, there was only one peak at *ARID5B* observed in Europeans ($P = 1.2 \times 10^{-6}$ at rs12764378; the first panel of **c**) and no further association was observed in the conditional analysis with rs12764378 (the smallest conditional $P = 2.2 \times 10^{-4}$; the second panel of **c**), suggesting that the numbers of independent associations may be different in the locus between the two populations. **(d)** Spline curves of population-specific normalized composite of multiple signals (CMS), an index of positive selection[26]. Since the genetic locus with causal variants is likely to undergo natural selection, we evaluated population-specific selective signals of the locus using CMS calculated in HapMap Phase II East Asian and Caucasian individuals, respectively. Significance of natural selection pressure was assessed by comparing the mean value of population-specific CMS for the SNPs located ± 100 kbp of the landmark SNP with those from the SNPs located ± 100 kbp of randomly selected whole genome SNPs by a permutation procedure (× 20,000 iterations). Results suggested a significant positive selection pressure in Japanese near rs10740069 (permutation $P = 0.0092$), although no selective signal was implicated in Europeans ($\alpha = 0.05$), that was analogous to the regional associations on RA. This observation might suggest the existence of causal variant(s) in the locus, which may have specifically evolved in the Japanese population through a population-specific pressure of natural selection.


a
C10orf107
ARID5B
RTKN2
ZNF365
C10orf22
EGR2
b
Japanese GWAS meta-analysis
Nominal associaitons
rs10821944
rs10740069
rs224311
Conditioned with rs10740069 and rs224311
Conditioned with rs10821944 and rs224311
Conditioned with rs10821944 and rs10740069
-log10 (P-value)
Chromosome 10 position (Mb)
c
European GWAS meta-analysis
Nominal associaitons
rs12764378
Conditioned with rs12764378
d
Normalized CMS
Japanese
European

Supplementary Note

I. Descriptions of the participating cohorts

1. Rheumatoid arthritis (RA) case-control cohorts.

In the present study, we included a total of 9,351 RA cases and 38,575 controls not affected with autoimmune diseases in the Japanese population. The study consisted of three genome-wide association studies (GWAS; 4,074 RA cases and 16,891 controls) and two independent replication studies (5,277 RA cases and 21,684 controls). The GWAS were conducted by the BioBank Japan Project (2,414 RA cases and 14,245 controls)[16,27], Kyoto University (1,237 RA cases and 2,087 controls)[19], and the Institute of Rheumatology Tokyo Women's Medical University (IORRA: Institute of Rheumatology RA cohort; 423 RA cases and 559 controls), respectively. The replication study 1 consisted of 3,830 RA cases from the BioBank Japan Project[27] (*n* = 1,172), Center for Genomic Medicine at RIKEN (*n* = 1,014), and IORRA (*n* = 1,644)[28]. Control subjects (*n* = 17,920) were recruited from the BioBank Japan project[27]. The replication study 2 consisted of 1,447 RA cases and 3,764 controls from Kyoto University. All the RA cases satisfied the revised criteria of the American Rheumatism Association for rheumatoid arthritis[29]. All participants in each cohort provided written informed consent for participation in the study as approved by the ethical committees of each of the institutional review boards. Characteristics of the subjects and the genotyping platforms are provided in **Supplementary Table 1 and 2**. Detailed descriptions of the cohorts are described below.

1.1 The BioBank Japan Project.

The BioBank Japan Project (http://biobankjp.org) started at the Institute of Medical Science, the University of Tokyo in 2003. To date, the BioBank Japan Project has collected up to 300,000 individuals with disease cases consisting of 47 various diseases[27]. These subjects were recruited from 12 medical institutes in Japan including, Osaka Medical Center for Cancer and Cardiovascular Diseases, the Cancer Institute Hospital of Japanese Foundation for Cancer Research, Juntendo University, Tokyo Metropolitan Geriatric Hospital, Nippon Medical School, Nihon University School of Medicine, Iwate Medical University, Tokushukai Hospitals, Shiga University of Medical Science, Fukujuji Hospital, National Hospital Organization Osaka National Hospital, and Iizuka Hospital. Subjects who were determined not to be of Japanese origin by self-report, by principal component analyses, or by our previous studies[16,23] were excluded from the current analyses.

1.2 Kyoto University.

Kyoto University collected DNA samples from 5,303 patients with connective tissue diseases including RA (*n* =

4,195)[29], systemic lupus erythematosus (n = 440), and systemic sclerosis (n = 191) as well as DNA samples of more than 10,000 healthy people and 4,000 patients with other diseases. RA patients were recruited at Kyoto University Hospital, Dohgo Spa Hospital, Sagamihara National Hospital, Niigata Rheumatic Center, and Saiseikai Takaoka Hospital. All subjects are Japanese by self-report. Possible overlapping samples with subjects in the BioBank Japan Project and IORRA cohorts were excluded from genome-scan and replication study.

1.3 Institute of Rheumatology Tokyo Women's Medical University (IORRA).

IORRA cohort is a long-term prospective observational cohort of RA patients at the Institute of Rheumatology, Tokyo Women's Medical University established in 2000[28]. For each patient, a survey composed of three domains (background variables, physician's evaluation, and laboratory test data) is conducted biannually to create a database. Of these, DNA samples were collected from 2,068 of these patients. Control samples for the IORRA case-control study are obtained from unrelated healthy Japanese adults who were recruited in Tokyo and Nagoya, Japan[30].

2. Systemic lupus erythematosus (SLE) case-control cohort.

We included 891 SLE cases and 3,384 matched controls, who were enrolled in the previously conducted GWAS in a Japanese populations[31]. SLE cases were collected from several medical institutes in Japan under the support of the Autoimmune Disease study group of Research in intractable Diseases (ADRD), Japanese Ministry of Health, Labor and Welfare. SLE cases met the revised American College of Rheumatology (ACR) criteria for SLE[32]. A portion of the control subjects overlapped with those used in the present GWAS on RA with 905 individuals coming from Kyoto University, and 2,479 individuals coming from the replication study 1. All subjects were of Japanese ancestry and provided written informed consent.

3. Greave's disease (GD) case-control cohort.

We included 1,783 GD cases and 3,384 controls (the same controls from the SLE case-control cohort). The GD cases have been included in our previous study[16]. Diagnoses of individuals with GD were established on the basis of clinical findings and results of routine examinations for circulating thyroid hormone and thyroid-stimulating hormone concentrations, thyroid-stimulating hormone receptors, ultrasonography, $^{[99m]}TCO4^-$ (or $[^{123}I]$) uptake, and thyroid scintigraphy. All subjects were of Japanese origin and provided written informed consent.

II. Genes in the newly identified rheumatoid arthritis (RA) susceptibility loci.

Genes which are located nearest to the landmark SNPs in each of the newly identified RA susceptibility loci are highlighted here. Nevertheless, additional genes neighboring the loci could also be candidates of causal origins. Further investigations into the functionality of these genes are warranted. It should be noted that cis-expression quantitative trait loci (cis-eQTL) analysis of landmark SNPs using lymphoblastoid B cell lines[1] suggested additional genes as possible candidates, such as *P4HA4* at 5q31, *AARS2* at 6p21.1, and *STARD10* at 11q13 (**Supplementary Table 4**), although relatively small sample size included in the eQTL study (n = 90) and lack of statistical power would provide limited conclusions. Among the landmark SNPs of the loci and the SNPs in linkage disequilibrium (LD) with them (r^2 > 0.8), rs2233434 at 6p21.1 and four SNPs in LD with rs2841277 at 14q32 had non-synonymous substitutions in *NFKBIE* (606A/G in exon 1) and *AHNAK2* (the strongest LD at rs9672139, r^2 = 0.97, 13726A/C in exon 7), respectively.

***B3GNT2* at 2p15.** *B3GNT2* (UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2) is a major synthase for polylactosamine, which has an important role for the regulation of lymphocyte activitiy[33]. The *B3GNT2* locus was reported to be associated with ankylosing spondylitis, an inflammatory arthritis in spine[34].

***ANXA3* at 4q21.** *ANXA3* (annexin A3) belongs to the annexin family, which encodes proteins related to calcium-dependent binding to phospholipid[35]. Annexin A3 is highly expressed in neutrophils and involved in the aggregation of cytosolic granules.

***CSF2* at 5q31.** *CSF2* (colony stimulating factor 2, also known as *GM-CSF*) regulates myeloid cell production and differentiation, and has been recognized as a promising therapeutic target of RA[36]. It should be noted that both the present study in Japanese and previous study in Europeans[15] observed suggestive associations ($P < 5.0 \times 10^{-4}$) at 17q12, where another well-known myeloid growth factor of *CSF3* (also known as *G-CSF*) is located.

***CD83* at 6p23.** *CD83* (cluster of differentiation, 83 molecule) is preferentially expressed on dendritic cells and is involved in antigen-presentation and lymphocyte activation[37]. To note, CD83-deficient mice lack T-cell development.

***NFKBIE* at 6p21.1.** *NFKBIE* (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon) encodes IκB epsilon (IκBε), which binds to components of NF-κB and inhibits the nuclear translocation of NF-κB[38].

***ARID5B* at 10q21.** *ARID5B* (AT rich interactive domain 5B, MRF1-like) is a transcription factor that mediates embryogenesis, and is known as a risk locus for acute lymphoblastic leukemia[39].

***PDE2A* and *ARAP1* at 11q13.** *PDE2A* (phosphodiesterase 2A, cGMP-stimulated) encodes an enzyme that

hydrolyzes cAMP and cGMP, and its activation mediated by tumor necrosis factor-α (TNF-α) would result in an increased endothelial permeability[40]. *ARAP1* (ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1), also known as *CENTD2*, has RHP-GAP and phosphatidylinositol (3,4,5) trisphosphate (PIP3)-dependent ARF-GAP activity *in vitro*.

***PLD4* at 14q32.** *PLD4* (phospholipase D family, member 4) is a transmembrane glycoprotein that lacks phospholipase activity, and is predominantly expressed in splenic marginal zone cells[41]. Unlike other PLD family genes (*PLD1-3*) implicated in numerous cellular activities, little is known about the physiological function of *PLD4*.

***PTPN2* at 18p11.** *PTPN2* (protein tyrosine phosphatase, non-receptor type 2) encodes the T cell protein tyrosine phosphatase (TC-PTP), a down-regulator for inflammatory responses, and has been implicated in other autoimmune diseases such as type 1 diabetes[4], Crohn's disease[4] and celiac disease[18].

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Quantitative effect of HLA-DRB1 alleles to ACPA levels in Japanese rheumatoid arthritis: no strong genetic impact of shared epitope to ACPA levels after stratification of HLA-DRB1*09:01

Anti-citrullinated peptide antibody (ACPA) is a highly specific serological marker for rheumatoid arthritis (RA).[1–3] Different HLA-DRB1 alleles have been shown to be associated with the susceptibility to ACPA-positive RA.[4 5] Former studies demonstrated that HLA-DRB alleles carrying a shared epitope (SE),[6] consisting of a conserved amino acid motif at positions 70–74 of the HLA-DRβ chain, were strongly associated with ACPA-positive RA and with higher ACPA levels in European and Japanese populations.[7–9] On the other hand, HLA-DRB1*09:01 was recently found to be negatively associated with ACPA levels in the Japanese.[9] These observations imply that combinations of HLA-DRB1 alleles differentially influence ACPA levels in ACPA-positive RA.

To address this question, we conducted a genetic association study employing 2457 ACPA-positive Japanese RA patients. ACPA was quantified by MESACUP CCP ELISA kit (MBL Co Ltd, Nagoya, Japan) with a cut-off level of 4.5 U/ml. The patients were then divided into three groups based on their ACPA titres: low (~4.5–13.5 U/ml), intermediate (~13.5–100 U/ml) and high (≥100 U/ml) groups. These groups were defined according to the 2010 ACR/EULAR classification criteria for RA and a measurement limit of the kit. HLA-DRB1 genotyping was carried out using either the Wakflow system (Wakunaga Pharmaceutical Co Ltd, Osaka, Japan) or the sequencing-based AlleleSEQR HLA-DRB1 typing kit (Abbott Japan, Nagoya, Japan). Frequencies of HLA-DRB1 alleles were compared among the three groups using the Cochran-Armitage Trend test. The relative predispositional effect (RPE) method was applied to identify the associations of more than one HLA-DBR1 allele sequentially according to their strength.[10] Briefly, associations of HLA-DRB1 alleles with ACPA categories were estimated for each allele using the Cochran-Armitage Trend test. When we detected the strongest association with a significant p value, the allele was excluded from the whole data and the same steps were repeated until no further significant alleles were found.

As expected from the previous studies,[9] HLA-DRB1*09:01 showed the strongest association with ACPA levels in a decreasing manner ($p=1.0\times10^{-21}$) and the SE alleles were significantly associated with an increasing effect ($p=3.2\times10^{-7}$) (table 1). In addition, HLA-DRB1*04:07 showed negative association with ACPA levels (p=0.0013), and HLA-DRB1*15:01 and HLA-DRB1*15:02 were positively associated with ACPA levels ($p=2.3\times10^{-5}$ and 0.0011, respectively) (table 1). Of note, the association between the SE and ACPA levels lost significance after stratification of HLA-DRB1*09:01 using RPE (p=0.16) whereas HLA-DRB1*04:07 and HLA-DRB1*15:01 remained significant after RPE (p=0.00034 and p=0.0011, respectively) (table 1). To confirm the dominant effect of HLA-DRB1*09:01

Table 1 Association of HLA-DRB1 alleles with ACPA levels

HLA-DRB1	Low n=594	Intermediate n=1510	High n=2810	p Value	RPE p Value	RPE (OR)	Effect on ACPA levels
SE							
SEall	216 (36.4%)	616 (40.8%)	1303 (46.4%)	3.2×10^{-7}	0.16†	1.08 (0.98–1.20)†	
DRB1*01:01	32 (5.4%)	96 (6.4%)	223 (7.9%)	0.0096			
DRB1*04:01	18 (3.0%)	47 (3.1%)	82 (2.9%)	0.78			
DRB1*04:04	2 (0.3%)	1 (0.1%)	14 (0.5%)	0.13			
DRB1*04:05	138 (23.2%)	409 (27.1%)	840 (29.9%)	0.00053			
DRB1*04:10	17 (2.9%)	33 (2.2%)	67 (2.4%)	0.71			
DRB1*10:01	6 (1.0%)	13 (0.9%)	28 (1.0%)	0.87			
DRB1*14:06	3 (0.5%)	14 (0.9%)	44 (1.6%)	0.013			
Non-SE							
DRB1*04:03	12 (2.0%)	30 (2.0%)	31 (1.1%)	0.019			
DRB1*04:06	17 (2.9%)	14 (0.9%)	57 (2.0%)	0.96			
DRB1*04:07	5 (0.8%)	11 (0.7%)	4 (0.1%)	0.0013	0.00034	0.30 (0.16–0.57)	(−)
DRB1*08:02	15 (2.5%)	30 (2.0%)	60 (2.1%)	0.74			
DRB1*08:03	36 (6.1%)	66 (4.4%)	119 (4.2%)	0.10			
DRB1*09:01	158 (26.6%)	334 (22.1%)	367 (13.1%)	1.0×10^{-21}	1.0×10^{-21}	0.56 (0.50–0.62)	(−)
DRB1*11:01	8 (1.3%)	27 (1.8%)	50 (1.8%)	0.57			
DRB1*12:01	14 (2.4%)	30 (2.0%)	68 (2.4%)	0.63			
DRB1*12:02	8 (1.3%)	26 (1.7%)	50 (1.8%)	0.52			
DRB1*13:02	22 (3.7%)	53 (3.5%)	102 (3.6%)	0.98			
DRB1*14:01	4 (0.7%)	32 (2.1%)	32 (1.1%)	0.64			
DRB1*14:03	6 (1.0%)	17 (1.1%)	37 (1.3%)	0.46			
DRB1*14:05	5 (0.8%)	19 (1.3%)	21 (0.7%)	0.36			
DRB1*15:01	20 (3.4%)	53 (3.5%)	180 (6.4%)	2.3×10^{-5}	0.0011	1.53 (1.21–1.92)	(+)
DRB1*15:02	36 (6.1%)	120 (7.9%)	276 (9.8%)	0.0011			
DRB1*16:02	4 (0.7%)	20 (1.3%)	29 (1.0%)	0.83			

HLA-DRB1 alleles with frequencies greater than 0.5% are shown. Significant levels were set as 0.0022 for HLA-DRB1 alleles after Bonferroni's correction for multiple testing.
†p Value and OR after removal of HLA-DRB1*09:01.
ACPA, anti-citrullinated peptide antibody; RPE, relative predispositional effect; SE, shared epitope.

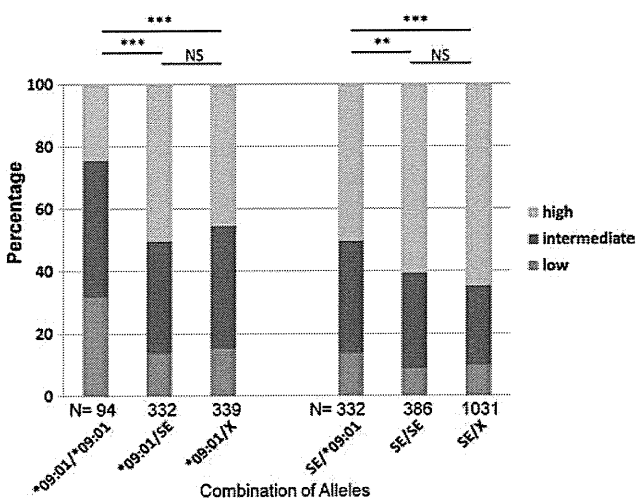

Figure 1 Comparisons of blood anti-citrullinated peptide antibody (ACPA) levels among HLA-DRB1*09:01, shared epitope (SE) and other alleles in combination. Frequencies of three rheumatoid arthritis subgroups based on ACPA levels were compared among different HLA-DRB1 combinations containing HLA-DRB1*09:01 and/or SE. X indicates HLA-DRB1 alleles other than HLA-DRB1*09:01 and SE. 'Low', 'intermediate' and 'high' categories correspond to patients with ACPA titres of ~4.5–13.5, ~13.5–100 and ≥100 U/ml, respectively. **p<0.005 and ***p<0.00005. NS, not significant.

on ACPA levels over SE, we compared ACPA levels in two sets: first between HLA-DRB1*09:01/*09:01 and HLA-DRB1*09:01/SE or HLA-DRB1*09:01/X, and second between SE/HLA-DRB1*09:01 and SE/SE or SE/X. We found that HLA-DRB1*09:01 showed a significant association with low ACPA category compared with the other two groups in both sets of analyses (p<0.005, figure 1). On the other hand, we could not observe any difference between SE and the other alleles.

In this study, we aimed to identify HLA-DRB1 alleles showing quantitative effects on ACPA levels using a large collection of Japanese ACPA-positive RA patients. RPE was applied to avoid misleading frequency deviation by the allele with the strongest association to other associated alleles. We demonstrated that HLA-DRB1*09:01 was the strongest genetic determinant for lower ACPA levels, and the quantitative effects of HLA-DRB1 alleles carrying the SE were not a primary effect but merely an expected consequence of the decreased frequency of HLA-DRB1*09:01. We also identified two novel HLA-DRB1 alleles, HLA-DRB1*04:07 and HLA-DRB1*15:01, being associated with ACPA levels. It is interesting and feasible to perform similar studies in other populations and investigate whether or not the same set of HLA-DRB1 alleles are related to the quantitative effects beyond ethnicities and to examine if such alleles share conserved amino acid motifs.

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Extended report

A large-scale association study identified multiple HLA-DRB1 alleles associated with ACPA-negative rheumatoid arthritis in Japanese subjects

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ABSTRACT

Background HLA-DRB1 is associated with rheumatoid arthritis (RA). However, it has recently been suggested that HLA-DRB1 is only associated with patients with RA who have anticitrullinated peptide/protein antibodies (ACPA), which are specific to RA.

Objective To elucidate whether specific HLA-DR alleles are associated with ACPA-negative RA development.

Methods HLA-DRB1 typing was carried out in 368 Japanese ACPA-negative patients with RA and 1508 healthy volunteers as the first set, followed by HLA-DRB1 typing of 501 cases and 500 controls as the second set. The HLA-DRB1 allele frequency and diplotype frequency were compared in each group, and the results of the two studies were combined to detect HLA-DRB1 alleles or diplotypes associated with ACPA-negative RA.

Results HLA-DRB1*12:01 was identified as a novel susceptibility allele for ACPA-negative RA ($p=0.000088$, OR=1.72, 95% CI 1.31 to 2.26). HLA-DRB1*04:05 and *14:03 showed moderate associations with ACPA-negative RA ($p=0.0063$, OR=1.26, 95% CI 1.07 to 1.49 and $p=0.0043$, OR=1.81, 95% CI 1.20 to 2.73, respectively). The shared epitope was weakly associated with ACPA-negative RA, but no dosage effect was detected ($p=0.016$, OR=1.17, 95% CI 1.03 to 1.34). A combination of HLA-DRB1*12:01 and DRB1*09:01 showed a strong association with susceptibility to ACPA-negative RA ($p=0.00013$, OR=3.62, 95% CI 1.79 to 7.30). Homozygosity for HLA-DR8 was significantly associated with ACPA-negative RA ($p=0.0070$, OR=2.16, 95% CI 1.22 to 3.82). It was also found that HLA-DRB1*15:02 and *13:02 were protective against ACPA-negative RA ($p=0.00010$, OR=0.68, 95% CI 0.56 to 0.83 and $p=0.00059$, OR=0.66, 95% CI 0.52 to 0.84, respectively).

Conclusions In this large-scale association study multiple alleles and diplotypes were found to be associated with susceptibility to, or protection against, ACPA-negative RA.

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common causes of chronic arthritis and results in severe joint damage and a shorter life span.[1] Genetic factors have been shown to contribute to the onset of RA.[2] Among the genetic susceptibility loci detected to date, HLA-DRB1 has a strong impact on the predisposition to RA and has been repeatedly shown to be associated with RA in an ethnicity-independent manner.[3] It is widely accepted that the shared epitope (SE), a common amino acid sequence located from the 70th to the 74th amino acids of the HLA-DR β chain, explains the associations of specific HLA-DRB1 alleles with RA.[4] Anticitrullinated protein antibodies (ACPA) are a highly specific marker of RA.[5 6] Recent data have shown that the SE is associated with ACPA-positive RA but not associated or only weakly associated with ACPA-negative RA.[7–9] Many of the non-HLA susceptibility genes for RA detected to date, such as *PTPN22*[10] and *CTLA4*[11] have been shown to be associated with ACPA-positive RA alone, and no association between these genes and ACPA-negative RA has been detected. These findings suggest that ACPA-negative RA is genetically distinct from ACPA-positive RA.

Among HLA-DRB1 molecules, HLA-DR3[12] and HLA-DR13[13] were reported to be associated with ACPA-negative RA in populations of European descent, but the same results were not obtained in a meta-analysis of a large Caucasian cohort.[14] In Asian populations, there has only been a small study which showed that HLA-DRB1*09:01 might be associated with ACPA-negative RA,[15] while SEs, especially DRB1*04:05, *04:01 and *01:01, were associated with RA and ACPA-positive RA.[15 16] Thus, no specific alleles that convey susceptibility to, or are protective against, ACPA-negative RA have been identified in populations of European or Asian descent. In this large-scale Japanese case–control association study, we show that HLA-DRB1*12:01, *14:03 and *04:05 are susceptibility alleles for ACPA-negative RA and that HLA-DRB1*13:02 and *15:02 are protective against ACPA-negative RA. We also identified multiple diplotypes that convey susceptibility to, or are protective against, ACPA-negative RA.

MATERIALS AND METHODS

Study subjects

DNA samples were collected at Kyoto University Hospital from 184 patients with RA who were negative for ACPA, as reported previously,[7] and another 184 patients with RA without ACPA were recruited at Tokyo Women's Medical University. These two sample groups were used as the first

set. Independent DNA samples were collected from 501 ACPA-negative patients with RA at RIKEN under the support of BioBank Japan and were used as the second set. The 501 cases in the second set are a fraction of 2410 RA cases included in another manuscript (K Shimane *et al*, unpublished data). All patients were Japanese and diagnosed by rheumatologists to fulfil the 1987 American College of Rheumatology revised criteria for RA.[17] A first set of control DNA samples were collected from 1508 healthy control subjects at Aichi Cancer Center Hospital and from the DNA banks of the Pharma SNP Consortium, which contains DNA samples from healthy Japanese volunteers.[18] The second set of control DNA samples were collected from 500 healthy volunteers at the HLA laboratory. This study was approved by the local ethical committees at each institution, and written informed consent was obtained from all patients. Basic information about cases and controls is shown in table 1.

ACPA detection

ACPA were detected with the MESACUP CCP ELISA kit (Medical and Biological Laboratories Co, Ltd, Nagoya, Japan) according to the manufacturer's instructions at each institution. A cut-off value of 4.5 U/ml was used to assess ACPA positivity.

HLA-DRB1 genotyping

HLA-DRB1 typing was carried out with the WAKFlow system and described in detail elsewhere.[7] In the 184 cases collected at Kyoto University and all the controls in the two sets, genotyping was performed at the HLA laboratory (Kyoto, Japan), whereas it was carried out at RIKEN for all 501 cases in the second set. HLA-DRB1 genotyping of the 184 cases collected at Tokyo Women's Medical University was performed by a sequencing-based typing method using the AlleleSEQR HLA-DRB1 typing kit (Abbott, Tokyo, Japan), and allele assignment was performed using the Assign software.

The following HLA-DRB1 alleles were classified as belonging to the SE: DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, *04:16, *10:01, *13:03, *14:02 and *14:06.

Statistical analysis

The frequency of each genotype or diplotype among the ACPA-negative patients with RA was compared with that in the controls using a χ^2 test or Fisher's exact test. Ninety-five percent CIs, p values and ORs were also calculated. The relative risk (RR) of ACPA-negative susceptibility induced by homozygosity for each allele was calculated to estimate the dosage effect. We performed 1000 permutation tests to confirm the associations found for each allele. Logistic regression analysis was used to evaluate the effects of alleles by adjusting for the influence of other alleles. Statistical analysis was performed using the R statistic system (http://www.R-project.org) or SPSS (version 18). The power calculation was performed using an online power calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/).

RESULTS

Genotyping of the first set

We performed HLA-DRB1 genotyping in the 368 ACPA-negative patients with RA and 1508 healthy controls in the first set to compare the allele frequency of each genotype between the cases and controls (table 1). Tables 2 and 3 show the main results of our association study for single alleles and diplotypes, respectively. More detailed results are given in the online supplementary tables 1 and 2.

The SE showed a weak association with moderate effect (p=0.039), mainly due to HLA-DRB1*04:05. Among the other HLA-DRB1 alleles, HLA-DRB1*14:03, *12:01, and *09:01 resulted in moderate to potential susceptibility to ACPA-negative RA (p=0.022, 0.10, and 0.10, respectively). DRB1*13:02, *04:03, and *15:02 showed moderate to potentially protective effects (p=0.0072, 0.059, and 0.12, respectively).

Replication in the second set and combined analysis

We performed HLA-DRB1 genotyping of samples in the second set to replicate the results found in the first set, using the DNA samples from 501 ACPA-negative patients with RA and 500 sex-matched healthy controls and combined the results of the two association studies.

Among the susceptibility alleles found in the first set, HLA-DRB1*12:01 was confirmed to display a susceptible association (p=0.010 and 0.000088 for the second set and combined study, respectively; table 2). The susceptibility tendencies of *04:05 and *14:03 were replicated in the second set, and these alleles showed moderate associations with susceptibility to ACPA-negative RA in the combined analysis (p=0.0063 and 0.0043, respectively). DRB1*09:01 and *14:05 showed potential susceptibility to ACPA-negative RA in the pooled study (p= 0.062 and 0.080, respectively). The SE showed a weak association with susceptibility to ACPA-negative RA in the combined study (p=0.016), but we could not detect any dosage effect (table 3 and figure 1). Among the protective alleles detected in the first set, the protective effect of DRB1*15:02 was successfully replicated (p=0.002 and 0.00010 in the second set and combined study, respectively; table 2). Although the protective effect of DRB1*13:02 was not replicated in the second set, the combined analysis showed a significant protective effect (p=0.00059). The protective effect of DRB1*04:03 was confirmed in the second set, and the combined study demonstrated a weak protective association (p=0.038). To exclude the possibility that the associations of the susceptibility alleles were induced by the absence of protective alleles or vice versa, we applied logistic regression analysis. The logistic regression analysis suggested that none of the allelic associations—namely, those of HLA-DRB1*12:01, *14:03, *04:05, *13:02, and *15:02, depended on the effects of other alleles (online supplementary table 3). In addition, the permutation tests confirmed the associations of these five alleles (permutation p<0.0070, data not shown).

Next, we analysed the dosage effects of each protective or susceptibility allele. DRB1*12:01 showed a potential dosage effect, but only two patients were homozygous for DRB1*12:01 (figure 1). We could not detect any dosage effects of HLA-DRB1*04:05 or the SE. No patients were homozygous for *14:03

Table 1 Basic information for ACPA-negative patients with RA and controls

Classification	ACPA-negative RA	Control
Set 1		
Number	368	1508
Female (%)	79.7	52.9
Age (mean±SD)	54.7±16.1	46.5±15.3
Set 2		
Number	501	500
Female (%)	80.8	80.0
Age (mean±SD)	62.4±12.2	NA

ACPA, anticitrullinated peptide/protein antibody; NA, not available; RA, rheumatoid arthritis.

Table 2 Association of the HLA-DRB1 allele with ACPA-negative RA

	Set 1				Set 2				Pooled study			
	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)
Non-SE Susceptible												
*12:01	31 (4.2)	91 (3.0)	0.10	1.41 (0.93 to 2.14)	62 (6.2)	37 (3.7)	0.010	1.72 (1.13 to 2.60)	93 (5.4)	128 (3.2)	0.000088	1.72 (1.31 to 2.26)
*14:03	18 (2.4)	39 (1.3)	0.022	1.91 (1.09 to 3.36)	23 (2.3)	14 (1.4)	0.14	1.65 (0.85 to 3.23)	41 (2.4)	53 (1.3)	0.0043	1.81 (1.20 to 2.73)
*09:01	123 (16.7)	432 (14.3)	0.10	1.20 (0.96 to 1.49)	164 (16.4)	154 (15.4)	0.55	1.08 (0.85 to 1.37)	287 (16.5)	586 (14.6)	0.062	1.16 (0.99 to 1.35)
*14:05	19 (2.6)	63 (2.1)	0.41	1.24 (0.74 to 2.09)	29 (2.9)	18 (1.8)	0.11	1.63 (0.9 to 2.95)	48 (2.8)	81 (2.0)	0.080	1.38 (0.96 to 1.98)
Protective												
*15:02	75 (10.2)	369 (12.2)	0.12	0.81 (0.63 to 1.06)	73 (7.3)	113 (11.3)	0.0020	0.62 (0.45 to 0.84)	148 (8.5)	482 (12.0)	0.00010	0.68 (0.56 to 0.83)
*13:02	44 (6.0)	273 (9.1)	0.0072	0.64 (0.46 to 0.89)	52 (5.2)	52 (5.2)	0.99	1.00 (0.67 to 1.48)	96 (5.5)	325 (8.1)	0.00059	0.66 (0.52 to 0.84)
*04:03	14 (1.9)	97 (3.2)	0.059	0.58 (0.33 to 1.03)	23 (2.3)	28 (2.8)	0.47	0.82 (0.47 to 1.43)	37 (2.1)	125 (3.1)	0.038	0.68 (0.47 to 0.98)
SE												
*04:05	103 (14.0)	340 (11.3)	0.040	1.28 (1.01 to 1.62)	145 (14.5)	129 (12.9)	0.31	1.14 (0.89 to 1.47)	248 (14.3)	469 (11.7)	0.0063	1.26 (1.07 to 1.49)
*14:06	16 (2.2)	37 (1.2)	0.051	1.79 (0.99 to 3.23)	14 (1.4)	9 (0.9)	0.30	1.56 (0.67 to 3.62)	30 (1.7)	46 (1.1)	0.076	1.52(0.95 to 2.41)
*10:01	8 (1.9)	13 (0.4)	0.032	2.54 (1.05 to 6.15)	6 (0.6)	5 (0.5)	0.76	1.20 (0.36 to 3.94)	14 (0.8)	18 (0.4)	0.094	1.80 (0.90 to 3.63)
*04:04	4 (0.5)	6 (0.2)	0.10	2.74 (0.77 to 9.74)	3 (0.3)	2 (0.2)	0.66	1.50 (0.25 to 8.99)	7 (0.4)	8 (0.2)	0.16	2.03 (0.73 to 5.60)
*01:01	43 (5.8)	183 (6.1)	0.82	0.96 (0.68 to 1.35)	50 (5.0)	64 (6.4)	0.17	0.77 (0.52 to 1.12)	93 (5.4)	247 (6.2)	0.24	0.86 (0.67 to 1.10)
*04:01	12 (1.6)	35 (1.2)	0.30	1.41 (0.73 to 2.73)	10 (1.0)	10 (1.0)	1.0	1.00 (0.41 to 2.41)	22 (1.3)	45 (1.1)	0.64	1.13 (0.68 to 1.89)
*04:10	6 (0.8)	63 (2.1)	0.021	0.39 (0.17 to 0.89)	25 (2.5)	14 (1.4)	0.076	1.80 (0.93 to 3.49)	31 (1.8)	77 (1.9)	0.73	0.93 (0.61 to 1.41)
All SE	192 (26.1)	677 (22.4)	0.036	1.22 (1.01 to 1.47)	253 (25.3)	233 (23.3)	0.31	1.11 (0.91 to 1.36)	445 (25.6)	910 (22.7)	0.016	1.17 (1.03 to 1.34)

Allele number and the frequency of each HLA-DRB1 allele in ACPA-negative patients with RA (n=368 and allele number=736 in the 1st set and n=501 and allele number=1002 in the 2nd set) and healthy controls (n=1508 and allele number=3016 in the 1st set and n=500 and allele number=1000 in the 2nd set) as well as the p value and OR of each allele for the development of ACPA-negative RA are shown. p Values were calculated using Fisher's exact test or the χ^2 test.
†Number of alleles (allele frequency).
ACPA, anticitrullinated peptide/protein antibody; SE, shared epitope; RA, rheumatoid arthritis.

in the cases or controls. Both DRB1*13:02 and *15:02 showed potential dosage effects.

Diplotype analysis

When we analysed the effects of HLA-DRB1 allele diplotypes on the predisposition to ACPA-negative RA, we found that a combination of DRB1*09:01 and *12:01 demonstrated susceptible effects in both sets (p=0.025, 0.020 and 0.00013 in the first, second and combined study, respectively; table 3). DRB1*08:03 homozygosity showed a weak susceptible association without any dosage effects (table 3,supplementary table 1). Although we found no susceptibility effect of DRB1*08:02 homozygosity, the combination of DRB1*08:02 and *08:03 also resulted in weak susceptibility (supplementary table 2). When we analysed DR8 allele homozygosity, we found that it displayed a moderate susceptibility association in the combined analysis (p=0.0070, table 3). Any combination of two of the three susceptibility alleles—namely, HLA-DRB1*12:01, *14:03, and *04:05, showed a potentially susceptible effect (supplementary table 2).

The HLA-DRB1*08:03 and *15:02 diplotype showed the strongest protective effect (p=0.00011, table 3). We found that the diplotypes with protective effects (*08:03/*15:02, *15:02/*15:02 and *13:02/*15:02) all included HLA-DRB1*15:02 (table 3).

DISCUSSION

Recent studies have suggested that ACPA-negative RA is a genetically different subset of RA.[7 8] While SE is very strongly associated with ACPA-positive RA, it is reported as not associated or only weakly associated with ACPA-negative RA. In populations of European descent, HLA-DR3 and DR13 were reported to be susceptibility alleles,[12 13] but a recent meta-analysis of a large Caucasian cohort did not find any such association.[14] In Japanese subjects, only DRB1*09:01 was reported to be associated with ACPA-negative RA, using small numbers of patients and controls (28 and 265, respectively).[15 16] HLA-DR3 is rare in the Japanese population, and we found only one HLA-DR3 allele in our cohorts.

Although genetic factors contribute to the development of ACPA-negative RA as much as ACPA-positive RA,[19] little is known about the ACPA-negative RA susceptibility alleles of HLA and non-HLA genes.

Here, we performed a case–control association study using a large number of ACPA-negative patients with RA and controls and showed that multiple alleles and diplotypes are associated

Table 3 Associations between HLA-DRB1 allele diplotypes and ACPA-negative RA

			Set 1				Set 2				Pooled study			
Effect	Allele 1	Allele 2	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)
Non-SE														
Susceptible	*09:01	*12:01	7 (1.9)	10 (0.7)	0.025	2.90 (1.1 to 7.68)	13 (2.6)	3 (0.6)	0.020	4.41 (1.25 to 15.58)	20 (2.3)	13 (0.7)	0.00013	3.62 (1.79 to 7.30)
	*08:03	*08:03	5 (1.4)	7 (0.5)	0.054	2.95 (0.93 to 9.36)	7 (1.4)	4 (0.8)	0.36	1.76 (0.51 to 6.04)	12 (1.4)	11 (0.6)	0.021	2.54 (1.12 to 5.78)
	*04:05	*14:05	5 (1.4)	7 (0.5)	0.054	2.95 (0.93 to 9.36)	5 (1.0)	2 (0.4)	0.26	2.51 (0.48 to 13.00)	10 (1.2)	9 (0.5)	0.033	2.59 (1.05 to 6.39)
Protective	*08:03	*15:02	3 (0.8)	35 (2.3)	0.095	0.35 (0.11 to 1.13)	1 (0.2)	14 (2.8)	0.00047	0.070 (0.010 to 0.53)	4 (0.5)	49 (2.4)	0.00011	0.18 (0.07 to 0.51)
	*15:02	*15:02	2 (0.5)	16 (1.1)	0.36	0.51 (0.12 to 2.23)	1 (0.2)	9 (1.8)	0.011	0.11 (0.010 to 0.86)	3 (0.4)	25 (1.3)	0.024	0.27 (0.08 to 0.91)
	*13:02	*15:02	3 (0.8)	28 (1.9)	0.16	0.43 (0.13 to 1.44)	3 (0.6)	7 (1.4)	0.20	0.42 (0.11 to 1.65)	6 (0.7)	35 (1.7)	0.029	0.39 (0.16 to 0.94)
SE	SE	SE	26 (7.1)	87 (5.8)	0.35	1.24 (0.79 to 1.95)	27 (5.4)	30 (6.0)	0.68	0.89 (0.52 to 1.52)	53 (6.1)	117 (5.8)	0.78	1.05 (0.75 to 1.47)
Serotype	DR8	DR15	8 (2.2)	72 (4.8)	0.027	0.44 (0.21 to 0.93)	10 (2.0)	23 (4.6)	0.021	0.42 (0.20 to 0.90)	18 (2.1)	95 (4.7)	0.00075	0.43 (0.26 to 0.71)
	DR13	DR15	6 (1.6)	55 (3.7)	0.051	0.44 (0.19 to 1.02)	6 (1.2)	11 (2.2)	0.22	0.54 (0.20 to 1.47)	12 (1.4)	66 (3.3)	0.0039	0.41 (0.22 to 0.77)
	DR8	DR8	13 (3.5)	17 (1.1)	0.00097	3.21 (1.55 to 6.67)	10 (2.0)	8 (1.6)	0.64	1.25 (0.49 to 3.20)	23 (2.7)	25 (1.3)	0.0070	2.16 (1.22 to 3.82)

Diplotype number and the frequency of each HLA-DRB1 diplotype in ACPA-negative patients with RA (n=368 and 501 in the 1st and 2nd set, respectively) and healthy controls (n=1508 and 500 in the 1st set and 2nd set, respectively) as well as the p value and OR of each diplotype for the development of ACPA-negative RA are shown. p Values were calculated using Fisher's exact test or the χ^2 test.
†Number of alleles (allele frequency).
ACPA, anticitrullinated peptide/protein antibody; SE, shared epitope; RA, rheumatoid arthritis.

Figure 1 Suggestive dosage effect of associated alleles on anticitrullinated peptide/protein antibody (ACPA)-negative rheumatoid arthritis susceptibility. The OR for each genotype is shown. Different colours indicate the number of copies of each allele. The numbers of homozygotes of *12:01, *14:05, *15:02, and *13:02 in cases are limited (2, 2, 3 and 3, respectively). Since no patients in this study were homozygous for DRB1*14:03, only the result for *14:05 is shown in this figure. SE in the figure includes DRB1*04:05, which is shown separately.

with ACPA-negative RA in Japanese people. Although the controls in the first set had different age and sex ratio values from those of the patients and we could not obtain age data for the 500 controls in the second set, the effects of the above-mentioned difference and lack of data on our results were considered to be limited. The HLA locus is located on chromosome 6 and is not affected by sex or age. Indeed, regression analysis did not significantly alter our association results (data not shown).

Our study showed that HLA-DRB1*12:01 is strongly associated with ACPA-negative RA and that HLA-DRB1*14:03 and HLA-DRB1*04:05 in SE are moderately associated with ACPA-negative RA in Japanese people. All three susceptibility alleles showed susceptibility associations with ACPA-negative RA when found in combination with one of the other two alleles. Our data also suggested a dosage effect of HLA-DRB1*12:01, while no dosage effect of HLA-DRB1*04:05 was detected, with decreased OR of DRB1*04:05 in homozygotes compared with heterozygous patients. In addition, we showed that the HLA-DRB1*09:01 and HLADRB1*12:01 diplotype and HLA-DR8 homozygosity are strong susceptibility combinations for ACPA-negative RA. We also determined HLA-DRB1*13:02 and *15:02 as protective alleles against ACPA-negative RA with a potential dosage effect. The combination of DRB1*08:03 and *15:02 had a strong protective effect in our study. Using logistic regression analysis, we confirmed that the effects of these susceptibility and protective alleles do not depend on each other (supplementary table 3). Although we searched for common amino acid sequences among the susceptibility alleles, we could not detect any meaningful sequences common to HLA-DRB1*12:01, *14:03, and/or *04:05. We also failed to detect a common amino acid sequence among the protective alleles HLA-DRB1*15:02 and *13:02.

Although the association of SE with ACPA-negative RA cannot be concluded, our large-scale study showed that it is weakly associated with ACPA-negative RA. As we observed a lower OR of the SE in homozygotes than in heterozygous patients, confirmation of this association in other studies are needed. We consider that the SE is associated with ACPA-negative RA but has a much weaker effect than in ACPA-positive RA. Both the

relatively small effect of SE on ACPA-negative RA and the small number of cases in previous reports might have resulted in non-significant p values for such tendencies.

HLA-DRB1*12:01, which was found to be associated with ACPA-negative RA susceptibility in our study, was reported to be associated with type 1 diabetes mellitus (T1D) in Latin America, but no similar association has been reported in Japan.[20 21] While a Japanese study showed RA with the anti-glucose-6-phosphate isomerase antibody is associated with HLA-DRB1*12:01,[22] no large-scale studies have reported an association between HLA-DRB1*12:01 and RA. As RA shares susceptibility genes with T1D such as *PTPN22*,[23] the determination of HLA-DRB1*12:01 as a potential common risk allele for both T1D and ACPA-negative RA is interesting. Although HLA-DRB1*12:01 showed a possible dosage effect, further confirmation is necessary as only two homozygous patients were among the cases. The allele frequency of HLA-DRB1*12:01 in a European population is 1–4%,[24] and so far there are no reports showing an association with ACPA-negative RA.[14] HLA-DRB1*12:02, the other allele of HLA-DR12, showed no association with ACPA-negative RA.

HLA-DRB1*14:03 was reported to be associated with Grave's disease in Japanese patients,[25] but its role in RA is unknown. Although our samples did not contain any patients who were homozygous for the allele owing to its low allele frequency, it showed a moderate association with ACPA-negative RA susceptibility. Among the other non-SE DR14 alleles, DRB1*14:05 displayed a tendency towards ACPA-negative RA susceptibility, while *14:01 and *14:07 did not. In total, DR14 alleles, including *14:06 in SE, showed moderate susceptibility effects on ACPA-negative RA (supplementary table 1).

Although one European study suggested that HLA-DR15 has a protective effect against ACPA-negative RA, its effect on ACPA-negative RA has not been fully examined.[13] We showed that HLA-DR15 has strong protective effect against ACPA-negative RA and a possible dosage effect. HLA-DRB1*15:02 is reported to be associated with Japanese T1D in a protective manner.[21]

Among HLA-DR13 alleles, HLADRB1*13:02 was reported to be protective against ACPA-positive RA.[26 27] Its protective effect was also reported in Japanese patients with RA.[16] Its effect on ACPA-negative RA has not been established.[13 14] Our study suggested that HLA-DRB1*13:02 has a protective effect against ACPA-negative RA. As the second set in our study did not show any differences in allele frequency between the patients and controls, further validation of our findings is necessary. HLA-DRB1*13:01, a major component of DR13 in populations of European descent, had no effect in our study, where we included DRB1*13:01 in eight alleles in cases and 23 alleles in controls (p=0.59).

HLA-DR8 has also been reported to be associated with some arthropathic autoimmune diseases, such as juvenile idiopathic arthritis[28] and psoriatic arthritis[29] in European subjects. The associations indicate that these arthropathies share common pathological mechanisms. Interestingly, the combination of DR8 and DR15 had a strong protective effect against ACPA-negative RA. Considering that DR8 did not show susceptibility association as a single allele, it seems to induce ACPA-negative RA susceptibility in a recessive manner. Among the DR8 alleles, DRB1*08:03 appeared to have a strong effect on ACPA-negative RA susceptibility.

Although we did not detect a dosage effect of HLA-DRB1*04:03, it showed a potentially protective effect against ACPA-negative RA in the combined study. Further studies are necessary to confirm the association.

As DRB1*09:01 has been shown to be associated with a decreased ACPA titre in ACPA-positive RA,[30] it is likely to be associated with ACPA-negative RA. While DRB1*09:01 showed a potential susceptibility association (p=0.062), the combination of DRB1*09:01 and *12:01 showed strong susceptibility association (p=0.00013). DRB1*09:01 also showed a possible dosage effect. From this viewpoint, we consider that DRB1*09:01 has a potential susceptibility effect on ACPA-negative RA. Owing to the relatively high allele frequency of DRB1*09:01, another independent association study or appropriate classification of ACPA-negative RA could produce significant results.

In addition to the different associations of the SE with ACPA-negative RA and ACPA-positive RA, we found multiple alleles associated with ACPA-negative RA that are not shared by ACPA-positive RA. These showed that ACPA-negative RA is a distinct subset of RA. Moreover, when we focused on ACPA-negative erosive RA to exclude the possibility of our results being affected by non-RA arthritic diseases, the effects of all the following alleles were maintained: *12:01, *14:03, *04:05, *13:02 and *15:02 (data not shown).

This is the first large-scale association study involving Japanese ACPA-negative patients with RA and the detection of multiple alleles and diplotypes associated with susceptibility to, or protection against, ACPA-negative RA. To evaluate whether our cohort had sufficient power to detect HLA-DRB1 genotype associations, we applied a risk allele with 5% frequency in the general population (see 'Materials and methods'). Our power calculation showed that this study had power values of 81% for finding genotype associations with an OR of 1.4 at the 0.05 significance level. When we set the OR to 1.2, our study had power values of 31%. These results suggest that our study has sufficient power to detect associated alleles that are present in relatively high frequencies (such as 5%) and a moderate OR of 1.4. On the contrary, our study has insufficient power to detect associations involving a weak OR such as 1.2. There is a possibility that ACPA-negative RA is associated with more HLA-DRB1 alleles or diplotypes that display a low allele frequency and/or a low OR. Further studies using ACPA-negative RA samples in Japan are necessary to find such associations.

While association studies using ACPA-negative patients with RA of European descent only found a few weak associations and none of them were subsequently replicated, our study successfully determined multiple alleles with relatively strong effects on ACPA-negative RA. From this viewpoint, we suppose that Japanese ACPA-negative patients with RA have a relatively similar genetic background compared to European patients. Population stratification within European population may also be assumed. Nevertheless, the validation of our results in Asian countries is necessary, and large-scale genome-wide association studies of ACPA-negative RA are also required to elucidate the pathogenesis of ACPA-negative RA.

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