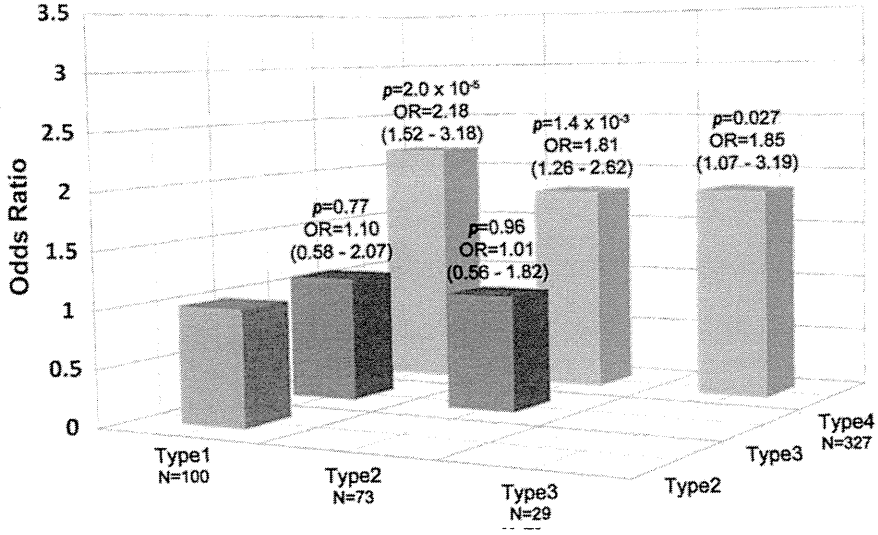

Figure 3. Histogram of odds ratios for genotype distribution of rs738409 between Matteoni types. Each box denotes the odds ratio (OR) comparing the corresponding Matteoni types on the horizontal axes. N represents the number of samples. Odds ratios and *p*-values are calculated for the higher Matteoni type per risk allele (G) on additive model by multivariable logistic regression adjusted for age, sex and BMI, and are shown with 95% CI above each box.
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identified on chromosome 1p (rs11206226) and chromosome 4p (rs1390096) neither of which has been reported as being associated with NAFLD in Caucasians (Table S1). Statistical calculation by taking their allele frequencies and effect sizes into account showed that approximately three times as many case and control samples are required to obtain sufficient statistical power (>0.8) for genome wide significance. Hence, further confirmation is required using a larger collection of patients and controls although they may be potential candidates of low-penetrance genes for susceptibility to NAFLD in Japanese.

Subsequent analyses through comparison of genotype distribution among four subgroups of NAFLD (type1 to type4) categorized by Matteoni's classification revealed that the seven NAFLD-associated SNPs in the *PNPLA3* gene were also significantly associated with the pathogenic status of NAFLD. There were also marked differences in genotype distribution of rs738409 between type4 subgroup and the other three groups ($p = 4.8 \times 10^{-6}$, OR = 1.96, 95%CI: 1.47–2.62 between type4 and pooled genotypes of type1 to type3). Moreover, a case/control analysis of rs738409 between Matteoni type4 and controls returned a surprisingly strong association ($p = 1.7 \times 10^{-16}$) which was much stronger than the initial analysis using all NAFLD cases ($p = 1.4 \times 10^{-10}$), whereas the analysis using Matteoni type1 to type3 as cases didn't show significance ($p = 0.41$). There were differences in the score of HOMA-IR and hs-CRP, indicators of insulin resistance and inflammation, respectively, between Matteoni type1 to type3 and type4 subgroups (Table 1). Our results provide compelling evidence that NASH corresponding to Matteoni type4 is both a clinically and genetically different disease subset from other spectrums of NAFLD. Previous studies showed association between *PNPLA3* and fatty liver, inflammation, fibrosis grade and NASH [13]. In our result, strong association between rs738409 and fatty liver was not observed by comparing control and Matteoni type1. In addition, strong association between rs738409 and lobular inflammation was not observed by comparing Matteoni Type1 and Type2. In contrast, a strong association between rs738409 and NASH was observed. Although we could not observe the strong association between rs738409 and fibrosis stage, strong association between rs738409 and Hyaluronic acid suggests that an association exists between *PNPLA3* and fibrosis.

We have also undertaken association analyses of rs738409 and clinical traits in the patients. The multivariable regression analysis adjusted for age, sex, BMI and Matteoni type followed by the correction for multiple testing revealed hyaluronic acid and HbA1c as being significantly associated with rs738409. Hyaluronic acid is one of the principle components of the extracellular matrix and its involvement in fibrosis has been previously suggested [20]. This may indicate another possible functional involvement of *PNPLA3* in the progression of liver fibrosis by influencing the circulating hyaluronic acid levels. A weak association of rs738409 and HbA1c levels was observed in our study population. However, there are no reports to date indicating such an association, and confirmation with different sample sets is needed for definitive conclusion. Also, the association between rs738409 and iron deposition was demonstrated by an ordinal logistic regression analysis. Since the association still remained after the results were adjusted with Matteoni type, rs738409 may play a functional role in the oxidative stress through iron absorption in the liver.

Recently, a genetic analysis of Japanese NAFLD patients was reported demonstrating a significant association in the increase of AST, ALT, ferritin levels and fibrosis stage (Brunt stage) and in the decrease of serum triglyceride with the risk allele (G) of rs738409 [12]. In our study, the association of rs738409 with AST ($p = 1.2 \times 10^{-4}$) and ALT ($p = 0.0016$) was reproduced and that of AST still remained after the results were adjusted for Matteoni type ($p = 0.038$). No association was observed for ferritin level. Brunt stage was available for Matteoni type4 patients only in our study. Although the odds ratio was slightly high (OR = 1.28, 95%CI: 0.95–1.72), it was not possible to examine the association. In addition, the inverse association of the risk allele of rs738409 with decrease of serum triglyceride was confirmed in our study ($p = 0.013$ after being adjusted for Matteoni type). For all of these

Table 3. Association of rs738409 with clinical traits.

Biochemical traits	Statistical calculation1		Statistical calculation 2	
Phenotype	**Coef. (S.E.)**	***p*-value**	**Coef. (S.E.)**	***p*-value**
Biological traits				
AST (IU/L)	0.22 (0.056)	**1.2×10^{-4}**	0.11 (0.052)	0.038
ALT (IU/L)	0.19 (0.058)	**0.0016**	0.093 (0.056)	0.098
GGT (IU/L)	−0.056 (0.061)	0.37	−0.088 (0.062)	0.16
Albumin (g/dL) *	0.015 (0.051)	0.77	−0.012 (0.052)	0.81
Total bilirubin (mg/dL)	−0.011 (0.063)	0.86	0.0059 (0.064)	0.93
Cholinesterase (unit) *	0.062 (0.040)	0.12	0.069 (0.041)	0.092
Type IV collagen 7S (ng/dL) *	−0.19 (0.064)	0.0025	−0.11 (0.062)	0.069
Hyaluronic acid (ng/dL)	0.30 (0.065)	**4.9×10^{-6}**	0.22 (0.063)	**4.6×10^{-4}**
Triglycerides (mg/dL)	−0.10 (0.058)	0.072	−0.15 (0.059)	0.013
Total cholesterol (mg/dL)	−0.066 (0.060)	0.27	−0.057 (0.061)	0.34
HbA1c (%)	−0.17 (0.053)	**0.0012**	−0.18 (0.054)	**0.0011**
IRI (μg/dL)	0.16 (0.063)	0.012	0.086 (0.061)	0.16
FPG (mg/dL)	−0.14 (0.049)	0.0047	−0.15 (0.05)	0.0035
HOMA-IR	0.084 (0.064)	0.19	0.0092 (0.062)	0.88
Hs-CRP (mg/dL)	−0.013 (0.048)	0.79	−0.031 (0.049)	0.52
Adiponectin (μg/mL)	0.048 (0.066)	0.47	0.12 (0.066)	0.072
Leptin (ng/mL)	0.11 (0.068)	0.11	0.10 (0.069)	0.15
Ferritin (ng/mL)	0.031 (0.047)	0.51	−0.0042 (0.048)	0.93
Uric acid (mg/dL)	−0.097 (0.061)	0.11	−0.11 (0.062)	0.067
PLT ($\times10^4$/μL)	−0.056 (0.020)	0.0052	−0.045 (0.020)	0.028
Immunological/histological traits				
ANA (0/1/2/3/4)	0.92 (0.70–1.21)	0.56	0.86 (0.65–1.15)	0.31
Brunt grade (1/2/3)	1.42 (1.06–1.92)	0.021	1.38 (1.02–1.87)	0.036
Brunt stage (1/2/3/4)	1.28 (0.95–1.72)	0.11		
Fat deposition (1/2/3/4)	1.44 (1.15–1.81)	0.0019	1.24 (0.98–1.56)	0.76
Iron deposition (0/1/2/3/4)	0.61 (0.47–0.80)	**3.0×10^{-4}**	0.62 (0.47–0.81)	**5.6×10^{-4}**

Associations between distribution of rs738409 genotypes and clinical traits are calculated by multivariable regression. Statistical calculation1 is adjusted for age, sex and BMI, while the Matteoni types are additionally included as covariate in statistical calculation 2. Statistics are calculated by multivariable linear regression for biochemical traits and by multivariable ordinal logistic regression for immunological and histological traits.

Coefficients and odds ratios are calculated for the increase of each trait per risk allele (G). The *p*-values showing significance after Bonferroni's correction for multiple testing ($p = 0.0021$) was shown in bold.

*Reciprocal numbers are used for normalization and a negative coefficient implicates an increase in value according to the increase of the risk allele.

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biomarkers, however, the significance was lost after the correction for multiple testing.

A replication analysis of other genetic loci that had been reported for their association with NAFLD in East coast white Americans [14] was performed in our sample collection. We confirmed the association of rs780094 in *GCKR* with NAFLD in a case/control analysis but at a much weaker level ($p = 0.011$, OR = 0.82, 95%CI: 0.70–0.95) than that shown for the populations of European-descent. No associations were found for *LYPLAL1* and *NCAN* loci in our study. There are several reasons to explain such differences, such as the insufficient statistical power with a limited number of study subjects in our study due to the difficulty in the collection of a larger number of histologically diagnosed NAFLD patients. The difference in genetic background between the Japanese and Europeans is also conceivable. Indeed, the risk allele frequency of rs12137855 in *LYPLAL1* was 0.944 in our control subjects but approximately 0.79 in the European populations [14]. Similarly, there was a difference in the risk allele frequency of rs2228603 in *NCAN* (0.049 in Japanese and 0.08 in Europeans). Rs4240624 in *PPP1R3B* was not polymorphic in the Japanese while its risk allele frequency was 0.91 in Europeans.

Materials and Methods

Ethics Statement

In compliance with the Declaration of Helsinki, ethical approval for this study was given by the respective Institutional Review Board and subject written informed consent were obtained for all subjects (Ethical committee of Nara City Hospital; Ethical committee of Saiseikai Suita Hospital; Medical Ethics Committee of Kanazawa University; Ethics committee of Kyoto Prefectural University of Medicine; Ethical Committee of Aichi Cancer Center; Ethical Committee of Kochi Medical School, Kochi University; Ethics Committee of Tokyo Women's Medical University; Ethical Committee on Kawasaki Medical School and Kawasaki Medical School Hospital; Ethical Committee of

Juntendo University; Ethics Committee of Yamagata University School of Medicine; Ethical Committee of the Ikeda Municipal Hospital; Institutional Review Board and Ethics Committee of Kyoto University School of Medicine).

Study Population

A total of 543 patients histologically diagnosed for NAFLD in 2007–2009 were recruited through the Japan study of Nonalcoholic Fatty Liver Disease. Biopsy specimens were stained with H&E and Masson's trichrome for morphological review and assessment of fibrosis. Perl's Prussian blue was performed to evaluate iron load. Biopsy specimens were reviewed by a hepatopathologist (T.O). NAFLD patients were classified into four categories by liver histology according to the classification by Matteoni *et al* [2] as follows; type1: fatty liver alone, type2: fat accumulation and lobular inflammation, type3: fat accumulation and ballooning degeneration, type4: fat accumulation, ballooning degeneration, and either Mallory-Denk body or fibrosis. With these criteria, the 543 patients were classified as type1; 102, type2; 75, type3; 31 and type4; 335. The histological grade and fibrosis stage were also evaluated by the classification of Brunt *et al* [21] for advanced NAFLD cases (type3 and type4) as follows; grade 1: steatosis involving up to 66% of biopsy, occasional ballooned zone 3 hepatocytes and absence or mild portal chronic inflammation, grade2: steatosis, ballooning hepatocytes mild to moderate chronic inflammation, grade3: panacinar steatosis, ballooning and disarray obvious and mild or portal mild to moderate inflammation, stage1: perivenular and/or perisinusoidal fibrosis in zone3, stage2: combined pericellular portal fibrosis, stage3: septal/bridging fibrosis, stage4: cirrhosis. The degree of fat deposition was evaluated by amount of fat droplets as observed under the microscope as follows; 0: <5%, 1: 5–<10%, 2: 10–<34%, 3: 34–<67%, 4: >67%. The degree of iron deposition was categorized by the presence of granules of free iron observed under the microscope as follows; 0: absence by x400, 1: easily identifiable by x400 and rarely identifiable by x250, 2: identifiable by x100, 3: identifiable by x25, 4: identifiable at lower than x25.

Inclusion criteria for NAFLD patients were as follows; (i) no history of alcoholism, (ii) no history for HBV/HCV/HIV infection, (iii) diagnosed by liver biopsy, (iv) information regarding age and BMI available. The sex of two samples was unknown, and was imputed from the results of the genome scan. As general Japanese population controls, the genome scan results of 942 healthy Japanese volunteers from Aichi Cancer Center Hospital and Research Institute were used [22].

Anthropometric and Laboratory Evaluation

We employed conventional methods for the measurement of anthropometry (height, weight, amount of visceral fat and abdominal circumscript). BMI was calculated from the measurements. The following biochemical/hematological/immunological traits were also measured by conventional methods; aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), albumin, total bilirubin, cholinesterase, type IV collagen 7S, hyaluronic acid, triglyceride, total cholesterol, hemoglobin A1c (HbA1c), fasting immunoreactive insulin (IRI), fasting plasma glucose (FBS), high sensitive CRP (hs-CRP), adiponectin, leptin, ferritin, uric acid, and platelet (PLT) count. Anti nuclear antibody (ANA) was measured by ELISA and categorized by the detection limit in a serial dilution as follows; 0: <40x, 1: 40–80x, 2: 81–160x, 3: 160x, 4: >320x. Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated from the measurements. Patients were assigned a diagnosis of diabetes mellitus (DM) when they had documented use of oral hypoglycemic medication, a random glucose level >200 mg/dl, or FPG >126 mg/dl. Hyperlipidemia was diagnosed with the cholesterol level being >200 mg/dl and/or triglyceride level being >160 mg/dl. Hypertension was diagnosed when the patient was taking antihypertensive medication and/or had a resting recumbent blood pressure ≥140/90 mmHg on at least two occasions.

DNA Preparation

Genomic DNA was extracted from peripheral blood mononuclear cells by standard phenol-chloroform extraction and resuspended in TE buffer. DNA concentration and purity were measured with Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The samples were stored at −20°C until use.

Genome-wide Genotyping and Quality Control

Genome scan was conducted for 543 patients with NAFLD and 942 healthy subjects using Illumina Human 610-Quad Bead Chip on a Bead Station 500G Genotyping System (Illumina, Inc., San Diego, CA, USA) and subjected to the following quality controls. Initially, ten patients and six control subjects were removed due to low call rates (<0.99). Regarding the SNP markers, 85,472 SNPs with minor allele frequency of smaller than 0.01 in either case or control group, 6,479 SNPs with lower success rates (<0.98) and 35 SNPs with distorted Hardy-Weinberg equilibrium ($p<10^{-7}$) were removed, resulting in 484,751 SNP markers being used for analysis. Principal component analysis by EIGENSOFT [17] including phase II HapMap (http://hapmap.ncbi.nlm.nih.gov/) samples identified no samples that were deviated from the Japanese population. Subsequently, the degree of kinship between individuals was examined by pi-hat in PLINK 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) [23]. Of the eight pairs of samples (four case pairs and four control pairs) showing high degrees of kinship (PI-HAT>0.4), the sample with the lower call rate in each pair was removed. After these steps, 529 case and 932 controls were used for the analysis.

Statistical Analysis

A case/control association analysis was performed by exact trend test between NAFLD patients and control subjects [24]. The correction of obtained *p*-values for population stratification was performed using EIGENSTRAT [17]. In addition, an association between Matteoni classification (type1 to type4) and additive model of genotype for each SNP was examined using Jonckheere-Terpstra test for NAFLD patients. Assessment of population stratification of inflation of *p*-value was carried out by the genomic control method for asymptotic trend test [25]. Association between each quantitative trait and the genotype of significant SNPs in NAFLD patients were calculated by multivariable linear regression or multivariable ordinal regression adjusted for age, sex and BMI. Each quantitative trait was transformed as follows; natural log for ALT, AST, HOMA-IR, HbA1c, IRI, triglyceride, total bilirubin, adiponectin, hs-CRP, hyaluronic acid, leptin, reciprocal number for albumin, cholinesterase, type IV collagen 7S and square root for uric acid and ferritin. The values of FPG, PLT, total cholesterol, amount of visceral fat, and abdominal circumscript were not transformed. For each trait, values that were within only 4 S.D. were included for analysis. LD indices were calculated by default setting of Haploview [26] and the LD block was defined manually.

Table 4. Replication study of previously reported SNPs.

			Genotyping Result and Allele Frequency of A2					Statistics		
			NAFLD					NAFLD vs. Control		Matteoni
dbSNPID	A1/A2	Gene	Control	Type 1	Type 2	Type 3	Type 4	*p*-value†	OR (95%CI)	*p*-value‡
rs12137855	C*/T	*LYPLAL1*	828/102/2	90/10/0	67/6/0	24/5/0	294/33/0	0.55	0.89	0.98
			(0.056)	(0.050)	(0.041)	(0.086)	(0.050)		(0.64–1.25)	
rs780094	T*/C	*GCKR*	321/433/178	34/54/12	28/34/11	17/11/1	133/139/55	0.011	0.82	0.92
			(0.423)	(0.390)	(0.383)	(0.224)	(0.381)		(0.70–0.95)	
rs4240624	G/A	*PPP1R3B*	–	–	–	–	–	–	–	–
rs2228603	C/T*	*NCAN*	842/88/2	93/7/0	65/8/0	28/1/0	292/31/4	0.80	1.05	0.58
			(0.049)	(0.035)	(0.054)	(0.017)	(0.059)		(0.75–1.48)	

Reference (A1) and non-reference (A2) alleles refer to NCBI Reference Sequence Build 36.3 with the effective allele marked by an asterisk. Genotyping results are shown by genotype count of A1A1/A1A2/A2A2 with allele frequency of A2 in parenthesis. †*P*-values are calculated by exact trend test with odds ratios (OR) calculated for A2 with 95% confidence interval (CI). ‡*P*-values are calculated by Jonckheere-Terpstra test in NAFLD patients for Matteoni type and additive model of genotype.
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Supporting Information

Figure S1 QQ plot of the GWA study comparing distribution of the observed and expected *p*-values. Upper box is expressed in antilog scale and the lower box is expressed in $-\log_{10}$ scale. The X- and Y-axis correspond to expected and observed *p*-values. Blue and red dots denote before and after correction by genomic control method ($\lambda = 1.04$), respectively.
(DOC)

Table S1 List of the SNPs showing $p<1.0\times10^{-5}$ in the GWA study. Reference (A1) and non-reference (A2) alleles refer to NCBI Reference Sequence Build 36.3 with the effective allele marked by an asterisk. Genotyping results are shown by genotype count of A1A1/A1A2/A2A2 with allele frequency of A2 in parenthesis. †*P*-values are calculated by exact trend test with odds ratios (OR) calculated for A2 with 95% confidence interval (CI). ‡*P*-values are calculated by Jonckheere-Terpstra test in NAFLD patients for Matteoni type and additive model of genotype. SNPs are ordered by chromosomal location.
(DOC)

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Author Contributions

Conceived and designed the experiments: FM TO. Performed the experiments: MT M. Kokubo. Analyzed the data: TK RY FM. Contributed reagents/materials/analysis tools: TK YS AU KM MT TT KY T. Saibara EH M. Kokubo SW SK YI M. Kawanaka T. Shima HP HT KT RY. Wrote the paper: TK MT RY FM TO.

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ACPA-Negative RA Consists of Two Genetically Distinct Subsets Based on RF Positivity in Japanese

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Abstract

HLA-DRB1, especially the shared epitope (SE), is strongly associated with rheumatoid arthritis (RA). However, recent studies have shown that SE is at most weakly associated with RA without anti-citrullinated peptide/protein antibody (ACPA). We have recently reported that ACPA-negative RA is associated with specific HLA-DRB1 alleles and diplotypes. Here, we attempted to detect genetically different subsets of ACPA-negative RA by classifying ACPA-negative RA patients into two groups based on their positivity for rheumatoid factor (RF). HLA-DRB1 genotyping data for totally 954 ACPA-negative RA patients and 2,008 healthy individuals in two independent sets were used. HLA-DRB1 allele and diplotype frequencies were compared among the ACPA-negative RF-positive RA patients, ACPA-negative RF-negative RA patients, and controls in each set. Combined results were also analyzed. A similar analysis was performed in 685 ACPA-positive RA patients classified according to their RF positivity. As a result, HLA-DRB1*04:05 and *09:01 showed strong associations with ACPA-negative RF-positive RA in the combined analysis ($p = 8.8 \times 10^{-6}$ and 0.0011, OR: 1.57 (1.28–1.91) and 1.37 (1.13–1.65), respectively). We also found that HLA-DR14 and the HLA-DR8 homozygote were associated with ACPA-negative RF-negative RA ($p = 0.00022$ and 0.00013, OR: 1.52 (1.21–1.89) and 3.08 (1.68–5.64), respectively). These association tendencies were found in each set. On the contrary, we could not detect any significant differences between ACPA-positive RA subsets. As a conclusion, ACPA-negative RA includes two genetically distinct subsets according to RF positivity in Japan, which display different associations with HLA-DRB1. ACPA-negative RF-positive RA is strongly associated with HLA-DRB1*04:05 and *09:01. ACPA-negative RF-negative RA is associated with DR14 and the HLA-DR8 homozygote.

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Introduction

Rheumatoid arthritis (RA) is the most common cause of chronic arthritis worldwide and results in severe joint destruction [1]. Genetic and environmental factors have been shown to be associated with its onset [2–3]. Among the susceptibility genes to RA, HLA-DRB1 has been shown to be the strongest genetic determinant of RA susceptibility, and its association with RA susceptibility has been repeatedly shown to be independent of ethnicity [4–5]. A common amino acid sequence extending from the 70th to 74th in the HLA-DRβ chain, which is known as the "shared epitope (SE)", is considered to be the reason for the association between HLA-DRB1 and RA, and the association between the SE and RA has been reported to be ethnicity-independent [6–8]. However, recent studies have shown that the SE is strongly associated with RA patients who have anti-citrullinated peptide/protein antibodies (ACPA), which is a highly specific marker of RA [9], but that it is not or only weakly associated with RA without ACPA [7,10–11]. Among the various HLA-DRB1 alleles, HLA-DR3 [12] and HLA-DR13 [13] were reported to be associated with ACPA-negative RA in populations of European descent, but these results were not confirmed in a

meta-analysis of a large Caucasian cohort [8]. In Asian populations, we recently reported that DRB1*12:01 is a HLA-DRB1 susceptibility allele for ACPA-negative RA in Japanese populations and that DRB1*04:05, the most common SE allele in Japanese, and *14:03 showed moderate associations with ACPA-negative RA susceptibility [14]. We also reported that DRB1*15:02 and *13:02 displayed protective associations with ACPA-negative RA and that being homozygous for HLA-DR8 was associated with ACPA-negative RA susceptibility. While a very small Japanese study suggested that HLA-DRB1*09:01 is associated with ACPA-negative RA [15], our study did not detect a significant association between them. These findings suggest that ACPA-negative RA is genetically different from ACPA-positive RA in terms of its associations with HLA-DRB1 alleles. While some specific alleles and diplotypes seem to be associated with ACPA-negative RA, the genetic characteristics of ACPA-negative RA have not been fully elucidated. Recently, UK group reported that SE is associated with ACPA-negative RF-positive RA in UK population [16]. However, whether this is true to other population is uncertain. Moreover, the associations of other alleles than SE with subgroups of ACPA-negative RA have never been reported. Here, we show that when we classified ACPA-negative RA into two subsets based on rheumatoid factor (RF) positivity, we were able to clearly distinguish them from each other according to their associations with HLA-DRB1 alleles, not only with SE, but with other alleles. We also compared ACPA-positive RA patients based on their RF positivity to examine whether we can apply this classification to ACPA-positive RA.

Results

HLA-DRB1 Alleles Associated with ACPA-negative RF-positive RA

We compared 179 ACPA-negative RF-positive RA with 1508 controls in collection 1 for their frequency of HLA-DRB1 alleles, followed by comparison of 267 ACPA-negative RF-positive RA with 500 controls in collection 2. Significant association was evaluated in the combined analysis. Regarding HLA-DRB1 alleles that were previously shown to be associated with ACPA-negative RA, we found that all of the alleles, namely, HLA-DRB1*12:01, *04:05, *13:02, *14:03, and *15:02 showed association tendency with ACPA-negative RF-positive RA in the combined study (Table 1). Interestingly, HLA-DRB1*04:05 ($p = 8.8 \times 10^{-6}$, odds ratio (OR): 1.57) showed the strongest association, while its association with entire ACPA-negative RA was moderate in the previous study. When we analyzed the associations of the SE, we found that it displayed a significant association ($p = 0.00013$, OR: 1.37). HLA-DRB1*04:05 was responsible for most of the association of SE because none of the other SE alleles showed significant associations with ACPA-negative RF-positive RA. We also found that HLA-DRB1*09:01, which was not associated with ACPA-negative RA as a single allele, was found to be significantly associated with ACPA-negative RF-positive RA ($p = 0.0011$, OR: 1.37). Importantly, these association tendencies written above were observed in both collections (Table 1). Logistic regression analysis was carried out to examine whether the susceptibility associations were dependent on a lack of protective alleles or vice versa. As a result, it was demonstrated that HLA-DRB1*04:05, *09:01, and *12:01 showed significant associations ($p<0.0005$), while the associations of HLA-DRB1*14:03, *13:02, and *15:02 were moderate to suggestive (Table S1). Next, we analyzed the dosage effects of the alleles and found that the association between HLA-DRB1*09:01 and ACPA-negative RF-positive RA showed a clear dosage effect (Figure S1). HLA-DRB1*12:01 also showed a dosage effect (data not shown due to small number). HLA-DRB1*04:05 did not show a dosage effect, suggesting that the effect of HLA-DRB1*04:05 on the predisposition to ACPA-negative RF-positive RA is a dominant effect.

HLA-DRB1 Alleles Associated with ACPA-negative RF-negative RA

Next we compared 274 ACPA-negative RF-negative RA with 1,508 controls, followed by comparison between 234 ACPA-negative RF-negative RA and 500 controls. Interestingly, we did not observe association of HLA-DRB1*04:05 and *09:01 with ACPA-negative RF-negative RA, while HLA-DRB1*12:01, *13:02, *14:03, and *15:02 were moderately associated with ACPA-negative RF-negative RA (Table 2). The SE was not associated with ACPA-negative RF-negative RA. DR14 was found to be significantly associated with ACPA-negative RF-negative RA and HLA-DRB1*14:03 and *14:06 comprised the association of HLA-DR14 (Table S2). These association tendencies in ACPA-negative RF-negative RA were observed in both sets (Table 2). Logistic regression analysis confirmed that none of the associations were mutually dependent and that the association of DR14 remained significant ($p = 0.00069$, Table S3). DR14 could not be evaluated the dosage effect because neither the cases nor controls included DRB1*14:03 or *14:06 homozygotes or the DRB1*14:03 and *14:06 diplotype.

HLA Diplotype Analysis: DR8 Homozygote and *12:01/*09:01 Diplotype

As we previously showed that the DR8 homozygote was significantly associated with susceptibility to ACPA-negative RA, we analyzed its associations with ACPA-negative RF-positive RA and RF-negative RA. As a result, we found that the HLA-DR8 homozygote is exclusively associated with ACPA-negative RF-negative RA in the combined study ($p = 0.00013$, OR: 3.08 for ACPA-negative RF-negative RA, Table 2; $p = 0.86$, OR: 1.08 for ACPA-negative RF-positive RA, Table 1). The effect of DR8 on the susceptibility to ACPA-negative RF-negative RA was not dose-dependent (OR: 1.04 for HLA-DR8 heterozygote).

We also found that the combination of HLA-DRB1*12:01 and *09:01, the diplotype that was most strongly associated with susceptibility to ACPA-negative RA in the previous study, was especially strongly associated with ACPA-negative RF-positive RA ($p = 5.0 \times 10^{-6}$, OR: 4.97 for ACPA-negative RF-positive RA; $p = 0.040$, OR: 2.46 for ACPA-negative RF-negative RA).

We found that the similar associations were seen between the alleles/diplotypes and ACPA-negative RF-positive erosive RA and ACPA-negative RF-negative erosive RA (except for that between HLA-DRB1*12:01 and the ACPA-negative RF-negative subset), even though the number of patients was limited (Table S4).

Comparison between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA

To compare the usage of HLA-DRB1 allele between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA, we directly compared the allele and diplotype frequencies between the two groups (Table 3). As expected, HLA-DRB1*09:01 and *04:05 showed significant differences in their frequencies between the two subsets ($p = 0.0018$ and 0.0034, respectively). The SE was more common in the ACPA-negative RF-positive RA patients ($p = 0.0047$), whereas DR14 was more prevalent in the ACPA-negative RF-negative RA patients ($p = 0.028$). The DR8 homozygote was more frequently seen in the ACPA-negative RF-negative RA patients than in the ACPA-negative RF-positive RA patients

Table 1. Association of HLA-DRB1 alleles with ACPA-negative RF-positive RA.

	1st set				2nd set				combined analysis			
HLA-DRB1 allele	[a]ACPA (-)RF(+)RA	[a]control	p	OR	[a]ACPA (-)RF(+)RA	[a]control	p	OR	[a]ACPA (-)RF(+)RA	[a]control	p	OR
*04:05	65 (18.2%)	340 (11.3%)	0.00015	1.75 (1.30–2.34)	88 (16.5%)	129 (12.9%)	0.055	1.33 (0.99–1.79)	153 (17.2%)	469 (11.7%)	8.8×10^{-6}	1.57 (1.28–1.91)
*09:01	70 (19.6%)	432 (14.3%)	0.0086	1.45 (1.10–1.92)	99 (18.5%)	154 (15.4%)	0.11	1.25 (0.95–1.65)	169 (18.9%)	586 (14.6%)	0.0011	1.37 (1.13–1.65)
*12:01	13 (3.6%)	91 (3%)	0.53	1.21 (0.67–2.19)	35 (6.6%)	37 (3.7%)	0.012	1.83 (1.14–2.93)	48 (5.4%)	128 (3.2%)	0.0014	1.73 (1.23–2.43)
*13:02	21 (5.9%)	273 (9.1%)	0.043	0.63 (0.40–0.99)	18 (3.4%)	52 (5.2%)	0.10	0.64 (0.37–1.1)	39 (4.4%)	325 (8.1%)	0.00013	0.52 (0.37–0.73)
*14:03	7 (2.0%)	39 (1.3%)	0.31	1.52 (0.68–3.43)	13 (2.4%)	14 (1.4%)	0.14	1.76 (0.82–3.77)	20 (2.2%)	53 (1.3%)	0.040	1.71 (1.02–2.88)
*15:02	43 (12.0%)	369 (12.2%)	0.90	0.98 (0.70–1.37)	37 (6.9%)	113 (11.3%)	0.0060	0.58 (0.4–0.86)	80 (9.0%)	482 (12.0%)	0.010	0.72 (0.56–0.93)
SE	106 (29.6%)	677 (22.4%)	0.0024	1.45 (1.14–1.85)	150 (28.1%)	233 (23.3%)	0.039	1.29 (1.01–1.63)	256 (28.7%)	910 (22.7%)	0.00013	1.37 (1.17–1.62)
DR14	29 (8.1%)	253 (8.4%)	0.85	0.96 (0.64–1.44)	48 (9.0%)	73 (7.3%)	0.24	1.25 (0.86–1.83)	78 (8.7%)	326 (8.1%)	0.55	1.08 (0.83–1.40)
Diplotype												
DR8/DR8	3 (1.7%)	17 (1.1%)	0.46	1.49 (0.28–5.24)	5 (1.1%)	8 (1.6%)	0.76	0.70 (0.12–2.94)	6 (1.3%)	25 (1.2%)	0.86	1.08 (0.44–2.65)
*12:01/*09:01	5 (2.8%)	10 (0.66%)	0.0041	4.30 (1.45–12.74)	9 (3.3%)	3 (0.60%)	0.0051	5.76 (1.42–33.42)	14 (3.1%)	13 (0.6%)	5.0×10^{-6}	4.97 (2.32–10.66)

OR: odds ratio.
SE: shared epitope: HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, *04:16, *10:01, *14:02, and *14:06.
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($p = 0.021$). When we applied logistic regression analysis to the HLA-DRB1*09:01, *04:05, and HLA-DR14, their associations were revealed to be significant and do not depend on each other ($p = 0.00067$ and 0.00072, respectively, Table S5), except for that of DR14 ($p = 0.30$).

Comparison between ACPA-positive RF-positive RA and ACPA-positive RF-negative RA

Next, we analyzed whether these allele usage differences are also seen in ACPA-positive RA. We collected data about the HLA-DRB1 genotypes of 154 ACPA-positive RF-negative RA patients and 531 ACPA-positive RF-positive RA patients. As the SE and HLA-DRB1*09:01 were found to be associated with ACPA-positive RA, we analyzed the differences in the frequencies of these alleles [17]. In comparison with the healthy controls, SE and HLA-DRB1*09:01 were associated with a predisposition to ACPA-positive RF-positive RA as well as ACPA-positive RF-negative RA and displayed comparable odds ratios in logistic regression analysis (Table 4). No HLA-DRB1 alleles showed a strong specific association with a particular subset. When we directly compared the two subsets of ACPA-positive RA, no alleles displayed significant associations (Figure 1, Table S6). However, whether the two subsets of ACPA-positive RA share most of HLA-DRB1 susceptibility associations is inconclusive due to the small number of RF-negative subset.

Discussion

In this study, we demonstrated that classifying Japanese ACPA-negative RA patients based on their RF positivity successfully divided them into two genetically different subsets, which displayed different associations with HLA-DRB1. We showed that HLA-DRB1*09:01 and *04:05, strong susceptibility alleles to ACPA-positive RA, were also associated with ACPA-negative RF-positive subset, and that DR14 and the DR8 homozygote were associated only with the ACPA-negative RF-negative subset (Figure 1). Since the titer of RF fluctuates along with disease activity much more than that of ACPA, we were very careful to take the maximum RF titer when multiple titers were available for a particular patient, in order to prevent the RF positive subset from being contaminated with RF negative RA patients. The Recent UK population study reported the association of SE with ACPA-negative RF-positive RA [16]. Our study not only confirmed this association in Japanese RA, but also showed that the association of SE with ACPA-negative RF-positive RA is mainly due to the effect of HLA-DRB1*04:05 and that HLA-DRB1*09:01, HLA-DR14, and homozygote of HLA-DR8 are specifically associated with subsets of ACPA-negative RA.

These above-mentioned association tendencies were observed in the first set and successfully replicated in the second set, indicating that we can avoid population stratification or sampling bias. The effect sizes (odds ratio) of the alleles were comparable in each cohort (Tables 1 and 2) and the associations in the combined analysis reached significant level, although the p-values in each set did not reach the significance level due to the limited number of samples they contained. These data indicate that our results are reliable, at least in Japanese populations, although further replication studies including other populations are favorable. In the current study, we used logistic regression analysis to confirm independency of associated alleles in each comparison. When we used relative predispositional effects (RPE) method [18] to stratify associated alleles, we obtained the similar results to those we obtained by logistic regression analysis (data not shown).

Table 2. Association of HLA-DRB1 alleles with ACPA-negative RF-negative RA.

	1st set				2nd set				combined analysis			
HLA-DRB1 allele	ACPA(-)RF(-)RA	control	*p*	OR	ACPA(-)RF(-)RA	control	*p*	OR	ACPA(-)RF(-)RA	control	*p*	OR
*04:05	69 (12.6%)	340 (11.3%)	0.37	1.13 (0.86–1.49)	57 (12.2%)	129 (12.9%)	0.70	0.94 (0.67–1.31)	126 (12.4%)	469 (11.7%)	0.52	1.07 (0.87–1.32)
*09:01	74 (13.5%)	432 (14.3%)	0.61	0.93 (0.72–1.22)	65 (13.9%)	154 (15.4%)	0.45	0.89 (0.65–1.21)	139 (13.7%)	586 (14.6%)	0.46	0.93 (0.76–1.13)
*12:01	28 (5.1%)	91 (3.0%)	0.012	1.73 (1.12–2.67)	27 (5.8%)	37 (3.7%)	0.070	1.59 (0.96–2.65)	55 (5.4%)	128 (3.2%)	0.00071	1.74 (1.26–2.40)
*13:02	28 (5.1%)	273 (9.1%)	0.0023	0.54 (0.36–0.81)	34 (7.3%)	52 (5.2%)	0.070	1.59 (0.96–2.65)	62 (6.1%)	325 (8.1%)	0.033	0.74 (0.56–0.98)
*14:03	12 (2.2%)	39 (1.3%)	0.10	1.71 (0.89–3.29)	10 (2.1%)	14 (1.4%)	0.30	1.54 (0.68–3.49)	22 (2.2%)	53 (1.3%)	0.047	1.65 (1.00–2.73)
*15:02	51 (9.3%)	369 (12.2%)	0.051	0.74 (0.54–1.00)	36 (7.7%)	113 (11.3%)	0.033	0.65 (0.44–0.97)	87 (8.6%)	482 (12.0%)	0.0020	0.69 (0.54–0.87)
SE	131 (23.9%)	677 (22.4%)	0.45	1.09 (0.88–1.34)	103 (22%)	233 (23.3%)	0.58	0.93 (0.71–1.21)	234 (23.0%)	910 (22.7%)	0.80	1.02 (0.87–1.2)
DR14	69 (12.6%)	253 (8.4%)	0.0016	1.57 (1.19–2.09)	51 (10.9%)	73 (7.3%)	0.021	1.55 (1.07–2.26)	120 (11.8%)	326 (8.1%)	0.00022	1.52 (1.21–1.89)
Diplotype												
DR8/DR8	12 (4.4%)	17 (1.1%)	9.1×10^{-5}	4.02 (1.90–8.51)	7 (3.0%)	8 (1.6%)	0.21	1.90 (0.68–5.29)	19 (3.7%)	25 (1.2%)	0.00013	3.08 (1.68–5.64)
*12:01/*09:01	4 (1.5%)	10 (0.66%)	0.25	2.22 (0.50–7.76)	4 (1.7%)	3 (0.60%)	0.22	2.88 (0.48–19.80)	8 (1.6%)	13 (0.6%)	0.040	2.46 (1.01–5.96)

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In our previous study [14], HLA-DRB1*09:01 was not significantly associated with ACPA-negative RA, in spite of the association it displayed in combination with HLA-DRB1*12:01. In the current study, we showed that HLA-DRB1*09:01 displayed a strong dose-dependent association with ACPA-negative RF-positive RA, but not with ACPA-negative RF-negative RA. These findings were confirmed by a direct comparison between the two subsets. A small study in Japan suggested that HLA-DRB1*09:01 is associated with ACPA-negative RA [15]. Our results suggest that their study mainly included ACPA-negative RF-positive RA patients. HLA-DRB1*09:01 was shown to reduce the ACPA titer in Japanese ACPA-positive RA patients [19–20]. Therefore, HLA-DRB1*09:01 might increase the titer of RF and decrease that of ACPA, although our study also showed that HLA-DRB1*09:01 is associated with ACPA-positive RF-negative RA.

HLA-DRB1*04:05, which is a major component of the SE in Asians [17], was shown to be significantly associated with ACPA-negative RA in our previous study. The current study showed that it is only associated with ACPA-negative RF-positive RA. This predisposition was also confirmed by direct comparison of the two subsets. As we could not detect a dosage effect of HLA-DRB1*04:05, its susceptibility effect might occur in a dominant manner. It is interesting that of the many SE alleles only HLA-DRB1*04:05 is associated with ACPA-negative RF-positive RA. This does not seem to be due to the relatively low frequencies of the other SE alleles (Table 1). Therefore, the common amino acid sequence that extends from the 70^{th} to the 74^{th} amino acid of the HLA-DRβ chain might not be important for the development of ACPA-negative RF-positive RA. As immunization of citrullinated peptide induced arthritis in HLA-DR4 transgenic mice [21] and citrullinated peptides were shown to have higher affinity to HLA-DR4 [22], high affinity of SE to citrullinated antigen is hypothesized to be the link between SE and RA development. Our findings may raise possibility of another mechanism of SE in developing arthritis.

It is quite interesting that HLA-DRB1*04:05 and *09:01, strongly associated alleles with ACPA-positive RA, are associated with ACPA-negative RF-positive RA. Although there are genetic similarities between ACPA-negative RF-positive RA and ACPA-positive RA, they should be considered to be different subsets as SE alleles other than HLA-DRB1*04:05 are not associated with ACPA-negative RF-positive RA and the HLA-DRB1*09:01 and *12:01 diplotype is strongly associated with ACPA-negative RF-positive RA.

When we analyzed the HLA-DR14 serotype, it showed a strong association with ACPA-negative RF-negative RA, largely due to HLA-DRB1*14:03 and *14:06. When we compared the frequency of DR14 in each ACPA-negative subset after stratifying the data according to the presence of HLA-DRB1*09:01 and *04:05, DR14 did not display a significant effect. In this sense, the specific association of DR14 with ACPA-negative RF-negative RA needs to be confirmed.

The HLA-DR8 homozygote displayed an association with ACPA-negative RA in our previous study [14]. The current study demonstrated that its association is specific to ACPA-negative RF-negative RA. As the number of HLA-DR8 homozygote is limited, further replication is necessary for this association. No association between the HLA-DR8 and 14 diplotype and susceptibility to ACPA-negative RF-negative RA was found (data not shown).

It is interesting that HLA-DR14 and HLA-DR8, associated serotype with ACPA-negative RF-negative RA, were reported association with psoriatic arthritis [23]. HLA-DR14 is often linked with HLA-Cw*06, susceptibility serotype to psoriasis arthritis in European [24]. HLA-Cw*06 is rare in Japanese (<1%) and the

Table 3. Direct comparison of HLA-DRB1 allele frequency between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA.

HLA-DRB1	ACPA(−)RF(+)RA Number of allele (%)	ACPA(−)RF(−)RA Number of allele (%)	*p*	OR (95%CI)
*09:01	169 (18.9%)	139 (13.7%)	0.0018	1.47 (1.15–1.88)
*04:05	153 (17.2%)	126 (12.4%)	0.0034	1.46 (1.13–1.89)
*08:02	24 (2.7%)	52 (5.1%)	0.0068	0.51 (0.31–0.84)
*14:06	8 (0.9%)	21 (2.1%)	0.037	0.43 (0.19–0.97)
SE	256 (28.7%)	234 (23.0%)	0.0047	1.35 (1.09–1.65)
DR14	78 (8.7%)	120 (11.8%)	0.028	0.72 (0.53–0.97)
DR8/DR8	6 (1.3%)	19 (3.7%)	0.021	0.35 (0.14–0.89)

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strong association between HLA-Cw*06 and HLA-DR14 is not observed in Japan (<10%). While psoriatic arthritis is not reported to be associated with these serotypes in Japan, association between these serotypes and arthritis is interesting.

It could be argued that ACPA-negative RA includes some non-RA arthritic diseases such as psoriasis, seronegative spondyloarthropathy and other collagen vascular diseases. Thus, we analyzed the associations between the above-mentioned alleles and diplotypes with ACPA-negative RA displaying bone erosion to examine whether the same association patterns were present in this strictly defined cohort. The typical bone erosions of RA are rarely seen in other arthritic disorders. As a result, we found the same associations. Therefore, we are convinced that our findings were not caused by the contamination of our study population by patients with other diseases. Since RF sometimes normalizes after treatment, the RF-negative RA patients whose RF titers were not measured at multiple points might not have been RF-negative. So, we re-analyzed our data by excluding the RA patients for whom consecutive RF titers were not available. As a result, we found the same tendency of associations for each allele and diplotype in each subset (data not shown), indicating that these subsets are stable.

Analysis using ACPA-positive RF-positive RA and ACPA-positive RF-negative RA patients compared with healthy controls did not result in distinct differences in HLA-DRB1 association. The SE is associated with both ACPA-positive RF-positive and RF-negative RA. HLA-DRB1*09:01 was found to be associated with both subsets after stratifying the patients according to their SE alleles. We also did not detect an association between HLA-DR14 or the HLA-DR8 homozygote and either subset. While 154 ACPA-positive RF-negative RA patients in our study are too small in number to detect the difference in HLA-DRB1 alleles with weak effect size between the two ACPA-positive subsets, these results suggest that there are no big differences in the HLA usage of the two subsets in ACPA-positive RA. To confirm our results and to detect possible different frequency of other HLA-DRB1 alleles in the two ACPA-positive subsets, replication study is necessary.

Table 4. Logistic regression analysis of HLA-DRB1 alleles with ACPA-positive RF-positive RA and ACPA-positive RF-negative RA.

	ACPA(+)RF(+)RA		ACPA(+)RF(−)RA	
HLA-DRB1	*p**	OR (95%CI)*	*p**	OR (95%CI)*
SE	$<2\times10^{-16}$	3.21 (2.72–3.78)	$<2\times10^{-16}$	3.03 (2.33–3.94)
*09:01	2.4×10^{-9}	1.83 (1.5–2.25)	0.0035	1.67 (1.17–2.37)

*p-values and odds ratios in logistic regression analysis using SE and HLA-DRB1*09:01.

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In the current study, we performed multiple comparisons in each subset and between subsets. The associations should be evaluated in the combined analysis with significant level corrected by Bonferronii's method and independency of each association should be evaluated by logistic regression analysis or RPE method. In this sense, p-values around cut-off level in the combined analysis should be taken with caution and the associations should be confirmed by independent study.

We have shown that ACPA-negative RA includes two genetically distinct subsets in Japanese population: RF-positive and RF-negative RA. This is the first report in Asians to show that these subsets are genetically distinct. We have to clarify the clinical difference between these two subsets. We also have to clarify whether non-HLA genes display different associations with each subset. So far, many genome wide association studies (GWAS) of RA and ACPA-positive RA have been performed, and more than twenty genes or loci have been shown to be susceptibility loci [25–38]. However, no GWAS studies have detected susceptibility genes for ACPA-negative RA with genome-wide significance [39]. This is probably due to the relatively small number of patients studied, but it might be overcome by stratifying ACPA-negative RA patients into RF-positive and RF-negative subsets. Since RA susceptibility genes usually cross ethnic boundaries [40], global collaboration might result in a fruitful dissection of these minor subsets.

Materials and Methods

Ethics Statement

This study was approved by the local ethical committees at each institution, namely, Kyoto University Graduate School and Faculty of Medicine, Ethics Committee, Tokyo Women's Medical University Genome Ethics Committee, and the ethics committee of RIKEN, and written informed consent was obtained from all patients.

Study Subjects

DNA samples were collected from ACPA-negative RA patients at Kyoto University Hospital, Tokyo Women's Medical University [41], and RIKEN with the support of BioBank Japan. All patients were Japanese and had been diagnosed by rheumatologists

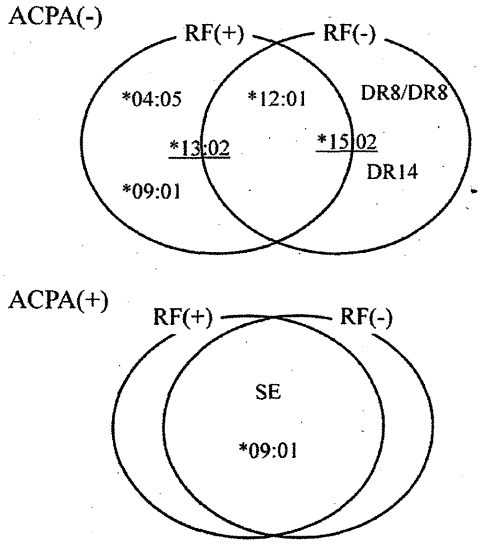

Figure 1. Summary of the HLA-DRB1 alleles associated with ACPA-negative RA and ACPA-positive RA. The relationships between the RF-positive and RF-negative subsets of ACPA-negative and ACPA-positive RA in terms of their associations with HLA-DRB1 alleles are illustrated. While the two subsets of ACPA-positive RA seem to share most associations with HLA-DRB1, the two ACPA-negative RA subsets possess specific alleles and HLA-DRB1 diplotypes. The underlined alleles are protective alleles.
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according to the 1987 American College of Rheumatology revised criteria for RA [42]. The control DNA samples were collected at Aichi Cancer Center Hospital, the DNA banks of the Pharma SNP Consortium [43], and HLA laboratory. A more detailed description of the collection procedure was given in a previous study [14]. We performed association studies using similar study design of the two collections to our previous study; namely, collection 1 for 456 ACPA-negative RA and 1508 healthy subjects, and collection 2 for 501 ACPA-negative RA and 500 healthy people. RF data were available for 453 out of 456 cases in collection 1 and all of 501 cases in collection 2. 179 patients were RF-positive in collection 1 and 267 patients were RF-positive in collection 2. We also collected DNA samples from 531 ACPA-positive RF-positive RA patients at Kyoto University Hospital and 154 ACPA-positive RF-negative RA patients at Kyoto University and Tokyo Women's Medical University.

ACPA Detection

The MESACUP CCP ELISA kit (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan) was used to detect 2nd generation ACPA in each RA patient, according to the manufacturer's instructions. A cut-off value of 4.5 U/ml was used to define ACPA positivity.

RF Detection

The serum RF concentrations of samples in collection 1 were quantified using a latex agglutination turbidimetric immunoassay. An ELISA assay was used to determine the RF levels of samples in collection 2. When multiple values for RF had been obtained at different visits, we used the maximum RF value for each patient. The cut off values of each detection kit in each hospital were employed.

HLA-DRB1 Genotyping

The HLA-DRB1 typing methods were previously described [14]. Briefly, the WAKFlow system or the AlleleSEQR HLA-DRB1 typing kit (Abbott, Tokyo, Japan) was used for the HLA-DRB1 typing. 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, *14:02, and *14:06.

Statistical Analysis

The frequency of each allele or diplotype was compared among the ACPA-negative RF-positive RA, ACPA-negative RF-negative RA patients, and the healthy controls in each set and combined set using the chi-square test or Fisher's exact test. The same analyses were performed in ACPA-positive RA patients classified according to their RF possession. Ninety-five percent confidence intervals (CI) for the OR were also calculated. Logistic regression analysis was used to evaluate the effects of each allele by adjusting for the influence of strongly-associated alleles. Single alleles were regarded as significant when they showed p-values of less than 0.0026 in a combined study, which is obtained by Bonferroni's correction. For diplotype analyses, we regarded 0.025 as the cut off level for significance because we performed just two tests. All statistical analyses were performed using the R statistic system (http://www.R-project.org) or SPSS (version 18).

Supporting Information

Figure S1 Dosage effects of HLA-DRB1*04:05 and *09:01 alleles on ACPA-negative RF-positive RA susceptibility. Each column represents the odds ratio for developing ACPA-negative RF-positive RA associated with possessing one (red column) or two (green column) alleles of HLA-DRB1*04:05 or *09:01.
(TIF)

Table S1 Logistic regression analysis of associated alleles with ACPA-negative RF-positive RA. *p-values and odds ratios in logistic regression analysis using the six alleles listed above.
(DOC)

Table S2 Association between HLA-DR14 and ACPA-negative RF-negative RA.
(DOC)

Table S3 Logistic regression analysis of associated alleles with ACPA-negative RF-negative RA. *p-values and odds ratios in logistic regression analysis using HLA-DR14 and three HLA-DRB1 alleles listed above.
(DOC)

Table S4 Association of HLA-DRB1 with ACPA-negative RA erosive subsets. [a]Total allele number is 268. [b]Total allele number is 212.
(DOC)

Table S5 Logistic regression analysis of assoicated alleles with ACPA-negative RF-positive RA, compared with ACPA-negative RF-negative RA. *p-values and odds ratios in logistic regression analysis using HLA-DRB1*09:01, *04:05, and HLA-DR14. [a]HLA-DRB1 alleles which showed $p<0.05$ in Table 3 were used for analysis.
(DOC)

Table S6 Comparison between ACPA-positive RF-positive RA and ACPA-positive RF-negative RA. [a] Alleles with frequency more than 1% in any groups are shown.
(DOC)

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Author Contributions

Conceived and designed the experiments: CT KO KI YK RY FM TM. Performed the experiments: CT KI YK EM K. Yurugi MK AS HS. Analyzed the data: CT. Contributed reagents/materials/analysis tools: KI EM KS AM SH K. Takasugi KM K. Tajima SM HY K. Yamamoto HS TM. Wrote the paper: CT KO.

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Full Length

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PLD4 as a novel susceptibility gene for systemic sclerosis in a Japanese population

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PLD4 association with Japanese systemic sclerosis

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PLD4 association with Japanese systemic sclerosis

ABSTRACT

Background

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, we reported nine novel susceptibility genes for Japanese rheumatoid arthritis (RA).

Objective

To elucidate whether the 18 genes that displayed associations or suggestive associations for Japanese RA in our previous study are associated with SSc in Japanese.

Method

We performed an association study involving 415 SSc patients and 16,891 controls, followed by a replication study, which involved 315 cases and 21,054 controls. The 18 markers were analyzed for their associations with SSc in the first study, and five 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

Rs2841277 in the *PLD4* gene displayed a significant association with SSc in Japanese patients ($p=0.00017$). We detected that rs2841280 in exon2 of *PLD4* is in strong linkage disequilibrium with rs2841277 and introduces amino acid alteration. We also found associations between SSc and rs6932056 in *TNFAIP3* and rs2280381 in *IRF8* ($p=0.0000095$ and 0.0030), both of which displayed associations with SSc in a European population.

Conclusions

We determined *PLD4*, phospholipase D4, as a novel susceptibility gene for SSc in Japanese, confirming the involvement of *PLD4* in autoimmunity. Associations between SSc and *TNFAIP3* or *IRF8* were also detected in our Japanese population. SSc and RA seem to share relatively large proportions of their genetic backgrounds.

INTRODUCTION

Systemic sclerosis (SSc) is a connective tissue disease, which affects 7 to 489 people per one million individuals worldwide and is characterized by the excess production of extracellular matrix molecules and fibrosis (1). SSc patients display skin sclerosis, obliterative microvasculopathy such as Raynaud's phenomenon, and multi-organ involvement. SSc patients sometimes develop severe complications including interstitial lung diseases, pulmonary hypertension and renal crisis. These severe complications and symptoms result in SSc patients displaying a poor prognosis and shorter lifespan (2-3). There is no established effective therapeutic method for preventing or curing sclerosis (4). It is well known that SSc has genetic components (5), for example, a US study revealed that SSc displayed a much higher incidence among the families of patients compared with the general population (6). Recent technological developments have enabled us to use genome-wide association studies (GWAS) to identify novel susceptibility loci for autoimmune diseases (7). GWAS of European SSc patients have revealed that *CD247* (8), HLA (8), *TNIP1*, *PSORS1C1*, and *RHOB* (9) are susceptibility loci for SSc. In addition, another GWAS study identified associations between *IRF8*, *GRB10*, and *SOX5* and limited cutaneous SSc in a European population (10). In addition, studies adopting a candidate gene approach based on subjecting genes to functional inference analysis have 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 European populations. SSc association studies of Japanese populations have confirmed that *STAT4* (19), *IRF5* (20), and *BLK* (21) are associated with SSc and identified the *UBE2L3* gene as a susceptibility gene for diffuse cutaneous SSc(22). An association between HLA and SSc has also been detected in Asians (23). These results indicate that there is 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, are also reported to be associated with rheumatoid arthritis (RA). In addition, *PTPN22*, which was found 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 nine novel susceptibility genes and six 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 two-stage association study of Japanese SSc patients, in which we genotyped these genes as candidate susceptibility loci.

METHODS

Study subjects

DNA samples were collected from 415 SSc patients at Kyoto University Hospital and Tokyo Women's Medical University. These two sample groups were used as the first set. Independent DNA samples were collected from 315 SSc patients at Keio University Hospital, Sagamihara National Hospital, and Kanazawa University Hospital. These sample groups were used as the replication set. All patients were Japanese and had been diagnosed by rheumatologists to fulfill the American College of Rheumatology classification criteria for SSc developed in 1980 (32). The SSc patients for whom clinical information was available were classified as having the limited cutaneous (lcSSc) or diffuse cutaneous type of SSc (dcSSc) according to the definitions developed by Leroy et al. (33). The control samples were described in detail in our previous study (31). This study was approved by the local ethical committees at each institution, and written informed consent was obtained from all patients. The basic information about the cases and controls are shown in Table 1.

Genotyping

The nine novel susceptibility markers, six potentially associated markers, and four confirmed markers of Japanese RA reported in our previous study (31) were chosen as candidate susceptibility markers for Japanese SSc. Eighteen of the nineteen markers (HLA was excluded, see the Results section for details), none of which had previously been reported to be associated with SSc in Japanese, were genotyped in the current study. The candidate markers that displayed p-values of less than 0.1 in the first set were further genotyped in the replication study. Rs2841280 and rs894037 were chosen as candidates of causative variant in *PLD4* region. Since rs894037 turned out to be monomorphic in a Japanese population, rs2841280 was genotyped for 334 controls for imputation reference in addition to all cases. The cases in the first and replication studies were genotyped using Taqman assays (Applied Biosystems) at Kyoto University or Tokyo Women's Medical University for the first set and Keio University or University of Tsukuba for the replication study. 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 HapMap Phase II East-Asian Populations as reference by mach2dat software. The control genotypes for the replication study were extracted from genome-scanning data for the markers included on the Illumina HumanHap610-Quad BeadChip. The genotypes for rs6932056, which is not included in the array, were imputed based on the genome-scanning data using HapMap Phase II East-Asian Populations as reference by mach2dat software and used as the control data for the replication set. The genotypes for rs2841280, which is not included in Hapmap data nor in the array, were also imputed in controls based on the genome-scanning data using genotyping data of the 334 controls by Taqman assay in combination with genome-scanning data as reference by mach2dat software.

Statistical analysis

The associations between the genotyped markers and SSc were analyzed using the Cochran-Armitage Trend test (trend test) in the first and replication studies. Subanalyses were performed by comparing the genotypes of the controls with those of SSc subgroups based on the disease phenotypes. The subanalyses used the same control subjects as the association studies. Intra-case analyses based on phenotypes were also performed. Odds ratios 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), and corrected p-values of less than 0.05 were regarded as significant in the combined study and subanalyses. The efficiency of the current study was estimated by calculating the likelihood of detecting three significant markers (after correcting the p-values using the FDR method) among eighteen randomly selected markers. After detecting statistically significant markers, we analyzed the best fitting model for each association, using dominant, recessive, trend, and allele chi test or model. Statistical analyses were performed using the statistical software R or SPSS (ver18).

RESULTS

Candidate gene analyses of SSc in Japanese

We genotyped the 415 SSc patients and 16,891 controls in the first set for the eighteen markers that were reported to have associations or suspected associations with RA in our previous study. We excluded the *HLA* region from the genotyped markers as this has already been shown to be associated with SSc in Asians. We compared the allele frequencies of the cases with those of the controls using the Cochran-Armitage

Trend test.

As a result, we found three markers that demonstrated associations with p-values of less than 0.01 in the first set (Table 2); namely, rs6932056 in the *TNFAIP3* region (p=0.0000038 and OR=1.69), rs10821944 in the *ARID5B* region (p=0.0025 and OR=1.25), and rs2841277 in the *PLD4* region (p=0.0055 and OR=1.25). We also found that two loci showed suggestive associations with p-values of less than 0.1 (Table 2); namely, rs12529514 in the *CD83* region (p=0.083 and OR=1.18) and rs2280381 in the *IRF8* region (p=0.095 and OR=1.19). The *TNFAIP3* and *IRF8* regions have been reported to display associations with SSc and limited cutaneous SSc, respectively, in European populations (10, 18). We selected these five markers as candidate susceptibility markers for SSc in Japanese populations and subjected them to validation.

Next, we performed a replication study consisting of 315 SSc patients and 21,054 controls to validate the associations of the five markers with SSc. The case subjects were genotyped for the five markers. The genotypes of the control subjects for the five markers, except for rs6932056, were extracted from the Illumina Infinium HumanHAP610-Quad array (for details, see method), as reported previously (31). Those for rs6932056 were imputed based on genome-scanning data using the mach2dat software (for details, see method), as 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 and 0.0099, OR=1.25 and 1.37, respectively, Table 2). Interestingly, we found that all five of the markers that displayed associations in the first study also demonstrated the same association directions in the replication study. We used the inverse-variance method to combine the data for the first and replication studies. 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 false-discovery rate method for multiple testing (Table 2, see method). Importantly, all of these three loci shared risk alleles with RA. While rs6932056 in the *TNFAIP3* region did not show a strong association with SSc in the replication study, its combined association was significant. The *PLD4* region was shown to be a novel susceptibility gene for SSc, and the *TNFAIP3* and *IRF8* regions were confirmed to be associated with Japanese SSc for the first time.

It is interesting to investigate in detail the association between rs2841277 and SSc. When we evaluated the 200kbp region around rs2841277, two hypothetical genes and the other gene, *CDCA4*, cell division cycle associated 4, are located at the region in addition to *PLD4*. *PLD4* was the only gene whose region showed moderate to strong linkage disequilibrium with rs2841277, indicating *PLD4* as a susceptibility gene (Figure