Table 1. Association analysis of *NFKBIE* and *RTKN2* with rheumatoid arthritis.

		Allele		Number of subjects		Frequency of allele 1			
Gene	dbSNP ID	(1/2)	Study set	Case	Control	Case	Control	Odds ratio (95% CI)	*P*-value[a]
NFKBIE	rs2233434	G/A	GWAS	2,303	3,380	0.254	0.216	1.24 (1.13–1.35)	2.2×10^{-6}
			Replication study-1	2,186	28,204	0.245	0.215	1.19 (1.10–1.27)	4.2×10^{-6}
			Replication study-2	3,396	3,756	0.239	0.209	1.19 (1.10–1.30)	1.1×10^{-5}
			Combined analysis	7,885	35,340	0.245	0.215	1.20 (1.15–1.26)	1.3×10^{-15}
RTKN2	rs3125734	T/C	GWAS	2,303	3,380	0.125	0.101	1.27 (1.13–1.43)	4.8×10^{-5}
			Replication study-1	2,185	28,218	0.129	0.110	1.20 (1.09–1.31)	1.4×10^{-4}
			Replication study-2	3,402	3,751	0.115	0.103	1.14 (1.02–1.26)	0.016
			Combined analysis	7,890	35,349	0.122	0.108	1.20 (1.13–1.27)	4.6×10^{-9}

[a]: Cochran-Armitage trend test was used for the GWAS and replication studies. Mantel-Haenszel method was used for the combined analysis.
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reporter assays with haplotype-specific expression vectors. In *NFKBIE*, the non-risk haplotype (A-C: rs2233434 (non-risk allele (NR))-rs2233433 (NR)) displayed an inhibitory effect on NF-κB activity compared with the mock construct, which reflected compulsorily binding of exogenous IκBε to the endogenous NF-κB, as shown in a previous study [16]. Of note, the risk haplotype (G-T: risk allele (R)-R) showed higher NF-κB activity than A-C (NR-NR) (Figure 3A), suggesting impaired inhibitory potential of G-T (R-R) products. No haplotypic difference was detected in the protein expression levels of these constructs (Figure 3C). We also examined two additional constructs of G-C (R-NR) and A-T (NR-R) haplotypes to evaluate the effect of each nsSNP (Figure S1A, S1B). Because NF-κB activity increased in the order of A-C<G-C<A-T<G-T (rs2233434-rs2233433: NR-NR<R-NR<NR-R<R-R) when cells were stimulated with TNF-α, the C>T substitution (Pro175Leu) in rs2233433 may have more impact on the protein function of IκBε compared with the A>G substitution (Val194Ala) in rs2233434. In contrast to the observations in *NFKBIE*, no clear difference was detected between the two common haplotype products of *RTKN2* in either their effect on NF-κB activity or protein expression levels, although both products enhanced NF-κB activity as reported previously (Figure 3B, 3D) [19]. These functional analyses of nsSNPs suggest that two nsSNPs (rs2233434 and rs2233433) in the *NFKBIE* region are candidates for causal SNPs.

ASTQ analysis suggested the existence of regulatory variants

As the majority of autoimmune disease loci have been implicated as eQTL [11], we speculated that variants in the *NFKBIE* and *RTKN2* loci would influence gene function by regulating gene expression, in addition to changing the amino acid sequences. To address this possibility, we performed allele-specific transcript quantification (ASTQ) analysis by using allele-specific probes targeting the nsSNPs in exons (rs2233434 for *NFKBIE* and rs3125734 for *RTKN2*, both of which were the GWAS landmarks). The genomic DNAs and cDNAs were extracted from peripheral blood mononuclear cells (PBMCs) in individuals with heterozygous genotype ($n=14$ for *NFKBIE* and $n=6$ for *RTKN2*) and from lymphoblastoid B-cell lines ($n=9$) for *NFKBIE*. As the expression levels of *RTKN2* were low in lymphoblastoid B cells, only PBMCs were used. When quantified by allele-specific probes, transcripts from the risk allele of *NFKBIE* showed 1.1-fold and 1.2-fold lower amounts (in PBMCs and lymphoblastoid B cells, respectively) than those from non-risk alleles ($P=0.012$ and 5.3×10^{-4}, respectively; Figure 3E and Figure S2). In contrast, 1.5-fold higher amounts of transcripts were observed in the risk allele of *RTKN2* ($P=0.016$; Figure 3F). These allelic imbalances suggested that both gene loci were eQTL and that there existed variants with *cis*-regulatory effects. Moreover, considering the inhibitory effects of *NFKBIE* and the activating potential of *RTKN2* on NF-κB activity, which might both be dose dependent (Figure 3G, 3H), these regulatory variants in the risk alleles should enhance NF-κB activity *in vivo*.

Integrated *in silico* and *in vitro* analysis to search for regulatory variants

To comprehensively search the two genomic regions for causal regulatory variants, we performed an integrated *in silico* and *in vitro* analysis with multiple steps (Figure 4 and Figures S3, S4). We first determined the target genomic region by selecting LD blocks containing disease-associated SNPs ($P_{GWAS}<1.0\times10^{-3}$) (Step 1). We then extracted SNPs with frequencies of >0.05 from HapMap and 1000 Genome Project databases in the region (Step 2). We excluded uncommon variants (MAF<0.05) from the analysis because of their low imputation accuracy in the GWAS (93% of uncommon variants in *NFKBIE* and 76% in *RTKN2* exhibited *Rsq* <0.6). There is neither structural variation (>1 kbps) nor indels (100 bps to 1 kbs) that are common in the population (frequency >0.01) in these loci. To evaluate the *cis*-regulatory potential of sequences around the SNPs *in silico*, we used the regulatory potential (RP) score [21,22]. This score was calculated based on the extent of sequence conservation among species or similarity with known regulatory motifs. We selected SNPs from the genomic elements with an RP score >0.1 (Step 3a). Subsequently, we selected SNPs from sites of transcriptional regulation as demonstrated by previous ChIP-seq studies (transcription factor binding sites [23,24] and histone modification sites [25,26]) or a DNase-seq study (DNase I hypersensitivity sites) [27] (Step 3b). Finally, these SNPs with regulatory potential were further screened out by the disease-association status ($P<0.05$) using an imputed GWAS dataset (Step 4). As a consequence, we selected 14 SNPs in *NFKBIE* and 10 SNPs in *RTKN2* that had regulatory potential predicted *in silico*.

To further investigate the regulatory potential of the SNPs, we evaluated 31-bp sequences around the SNPs by *in vitro* assays. First, we examined their ability to bind nuclear proteins by EMSAs (Step 5a) using nuclear extracts from lymphoblastoid B cells (PSC cells) and Jurkat cells. Of the 24 SNPs examined, nine

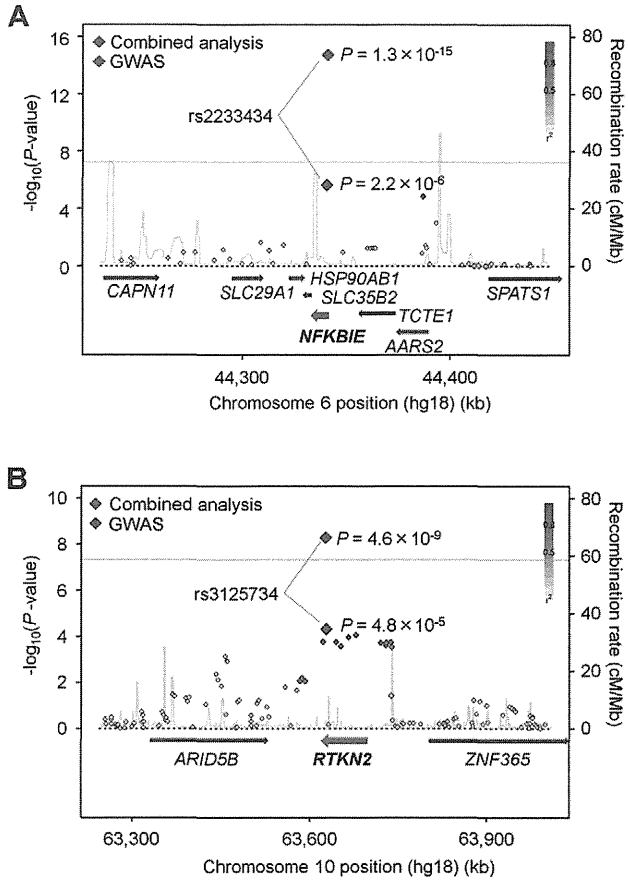

Figure 1. Association plots of *NFKBIE* and *RTKN2* regions. The diamonds represent the -$\log_{10}$ of the Cochran-Armitage trend *P*-values. Large diamonds show landmark SNPs in *NFKBIE* (rs2233434: A) and *RTKN2* (rs3125734: B). Red: GWAS, Blue: combined analysis. Red colors of each SNP indicate its r^2 with landmark SNP. Gray lines indicate the genome-wide significance threshold ($P<5\times10^{-8}$). For each plot, the -$\log_{10}$ *P*-values (y-axis) of the SNPs are presented according to their chromosomal positions (x-axis). Physical positions are based on NCBI build 36.3 of the human genome. Genetic recombination rates, estimated using the 1000 Genome Project (JPT and CHB), are represented by the blue line.
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SNPs displayed allelic differences, implying differential potential of transcriptional activity between these alleles (Figure 5A and Figure S5). We then evaluated the enhancing or repressing activity of the sequences by luciferase reporter assays (Step 5b). We cloned them into the pGL4.24 vector, which has minimal promoter activity, and transfected these constructs into HEK293A cells (for *NFKBIE* and *RTKN2*), lymphoblastoid B cells (for *NFKBIE*), and Jurkat cells (for *RTKN2*). Among the three SNPs examined in *NFKBIE*, the risk allele of rs2233424 (located −396 bps from the 5′ end) displayed stronger repression activity (Figure 2A and Figure 5B) than that of the non-risk allele. Among the six SNPs in *RTKN2*, the risk alleles of rs12248974 (approximately 10 kb from the 3′ end) and rs61852964 (−215 bps from the 5′ end) showed higher enhancing activity compared with the non-risk alleles (Figure 2A and Figure 5B). These results corresponded to the results of ASTQ analyses (Figure 3E, 3F). Other SNPs showed no allelic differences or had the opposite trend of transcriptional activity in the risk allele compared to the results of ASTQ analysis (Figure S6).

To confirm the regulatory potential of these SNPs, we investigated the correlation between genotypes and gene expression levels in lymphocytes utilizing the data from the previous eQTL studies. We evaluated the expression of *RTKN2* in primary T cells from Western European individuals by using Genevar software [28,29]. Though *NFKBIE* is also expressed in primary T cells, the genotypes of rs2233424 are not available. We thus evaluated gene expression data of lymphoblastoid B-cell lines obtained from HapMap individuals (Japanese (JPT) + Han Chinese in Beijing (CHB), European (CEU), and African (YRI)) [30,31] instead. The *NFKBIE* expression level decreased with the number of risk alleles of rs2233424 ($R=-0.18$, $P=0.020$), and the *RTKN2* expression levels increased with that of rs1432411 (a proxy for rs12248974, $r^2=0.97$) ($R=0.27$, $P=0.018$) (Figure 5C), corresponding to the results of the *in vitro* assays. The data for rs61852964 in *RTKN2* was not available. Among the SNPs that displayed opposite transcriptional activities in the reporter assays compared to the results of ASTQ, the data for rs2233434, rs77986492, and rs3852694 (a proxy for rs1864836, $r^2=1.0$) were available (Figure S7 and S8). These SNPs displayed the opposite direction of the correlation trend as compared to the results of reporter assays, but parallel to ASTQ, implying that the regulatory effects observed in the *in vitro* assays were cancelled out by the effects of other regulatory variants on the same haplotype *in vivo*.

Finally, we validated the associations of these regulatory (r)SNPs observed in the imputed GWAS dataset. We directly genotyped them by TaqMan assay and confirmed significant associations (Table S8). As the candidate causal variants (nsSNPs and rSNPs) and the landmark SNPs of GWAS were in strong LD at each locus (Figure 2A, 2B), we evaluated the independent effect of each SNP by haplotype analysis in both loci (Table S9 and S10) and the conditional logistic regression analysis in *RTKN2* (Table S11). The conditional analysis was not performed in *NFKBIE* because three candidate causal variants were in strong LD ($r^2>0.9$). However, the analyses for these two loci did not demonstrate any evidence of primary or independent effects across the candidate causal variants, and it remains a possibility that all of the functional variants were involved in the pathogenesis. In addition, although the landmark nsSNP (rs3125734) in *RTKN2* did not display any influence on NF-κB activity in our *in vitro* assays, rs3125734 might influence functions of *RTKN2* other than those in the NF-κB pathway; alternatively, it is still possible that rs3125734 tags the effects of other unknown variants, such as rare variants, in addition to the other two rSNPs (rs12248974 and rs61852964).

Discussion

In the present study, we performed a replication study of our previously reported GWAS and identified variants in *NFKBIE* and *RTKN2* loci that were associated with RA susceptibility. The associations of *NFKBIE* and *RTKN2* loci have not been reported in other populations with genome-wide significance. However, rs2233434 in *NFKBIE* showed a suggestive association (589 cases vs. 1,472 controls, $P=0.0099$, OR = 1.57, 95% CI = 1.11–2.21) in a previous meta-analysis in European populations [32]. The weak association signal in Europeans may be partially due to the lower frequency of the risk allele (0.04 in Europeans compared to 0.22 in Japanese). On the other hand, the association of rs3125734 in *RTKN2* was not observed in a GWAS meta-analysis of European populations (cases 5,539 vs. controls 20,169, $P=0.11$, OR = 1.04, 95% CI = 0.99–1.09). As the association of *RTKN2* locus was also implicated in Graves' disease in a Han Chinese population [33], the association in *RTKN2* locus may be unique to Asian populations.

To find the disease causal variants in disease-associated loci, target re-sequencing and variant genotyping with a large sample set followed by conditional association analysis examining the

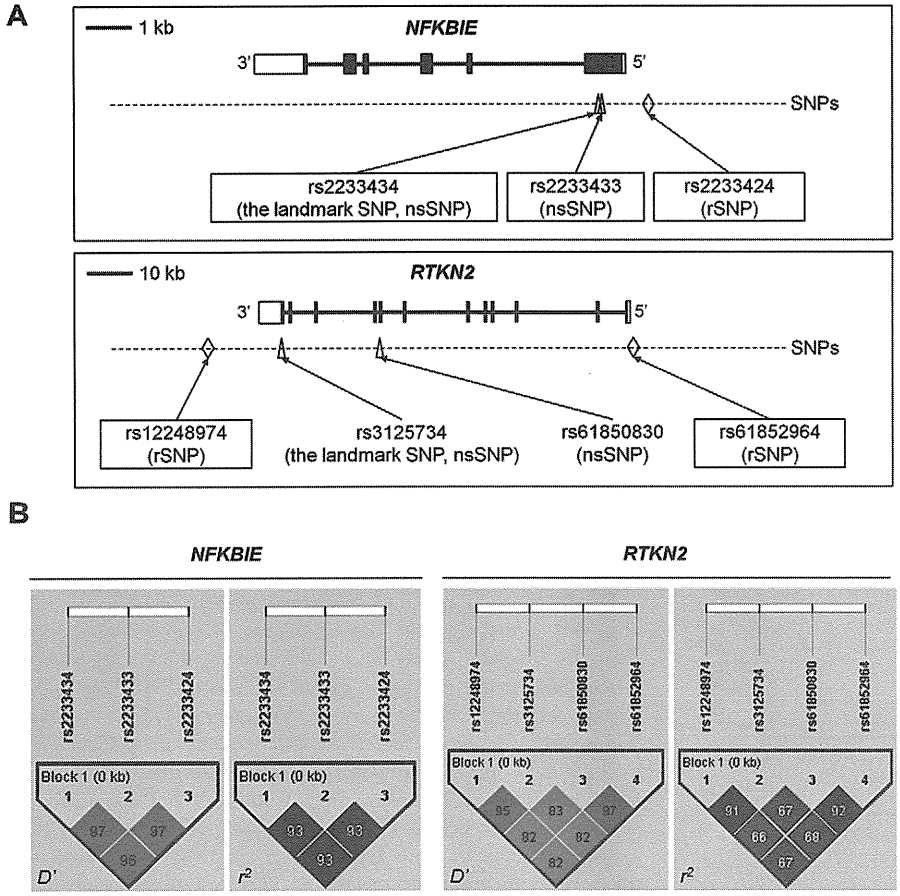

Figure 2. Genomic position and LD blocks. (A) Genomic position of non-synonymous (ns)SNPs and regulatory (r)SNPs in *NFKBIE* and *RTKN2*. *NFKBIE* (top) and *RTKN2* (bottom) correspond to transcripts NM_004556.2 and NM_145307.2, respectively. Exons are shown as boxes, where black boxes represent coding regions and open boxes represent untranslated regions. Intron sequences are drawn as lines. Open triangles represents nsSNPs and open diamond shapes indicate candidate rSNPs. dbSNP IDs of candidate causal variants were boxed in a solid line. (B) LD patterns for nsSNPs and candidate rSNPs in *NFKBIE* (left) and *RTKN2* (right) gene regions. LD blocks were constructed from genotype data of 3,290 control individuals of the GWAS. The diagrams show pairwise LD values as quantified using the D' and r^2 values.
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independent effects of each variant would be the first step. For this purpose, a recent attempt to fine-map the known autoimmunity risk loci in Celiac disease (MIM 212750) using an "Immunochip" brought us several insights [34]. First, no stronger signals compared to the GWAS signals were detected in most of the known loci, while additional independent signals were found in several loci. Second, none of the genome-wide significant common SNP signals could be explained by any rare highly penetrant variants. Third, although the fine-mapping strategy could localize the association signals into finer scale regions, it could not identify the actual causal variants due to strong LD among the variants, indicating that an additional approach, such as functional evaluation of candidate variants, is needed.

In the present study, we focused on common variants to find causal variants. Instead of re-sequencing additional samples, we utilized the 1000 Genome Project dataset, where the theoretically estimated cover rate for common variants (frequency of >0.05) in our population is >0.99 [12,35]. To fine-map the association signals, we performed imputation-based association analysis, where we could not find any association signals that statistically exceeded the effect of landmark SNPs (rs2233434 for *NFKBIE* and rs3125734 for *RTKN2*) in both gene regions (Figures S3 and S4). We also performed a conditional logistic regression analysis, and found no additional independent signals of association when conditioned on each landmark SNP (data not shown). Although the imputation-based association tests may yield some bias compared to direct genotyping of the variants, these results suggested that variants in strong LD with the landmark SNPs were strong candidates for causal variants.

Following the analysis of nsSNPs, we evaluated *cis*-regulatory effects of variants in the two regions by ASTQ analysis using both B-cell lines and primary cells (PBMC), the majority of which consisted of T and B lymphocytes. As the mechanism of gene-regulation is substantially different between cell types [26], ASTQ analysis in more specific cell types that are relevant to the disease etiology, such as Th1 and Th17 cells, would be ideal to evaluate the *cis*-regulatory effects of variants. In this context, a more comprehensive catalog of the eQTL database of multiple cell types should be established for genetic study of diseases. As our ASTQ analysis demonstrated *cis*-regulatory effects of variants in both regions, we then performed an integrated *in silico* and *in vitro* analysis to identify candidate regulatory variants. Accumulating evidence by recent ChIP-seq and DNase-seq studies suggested that *cis*-regulatory variants are

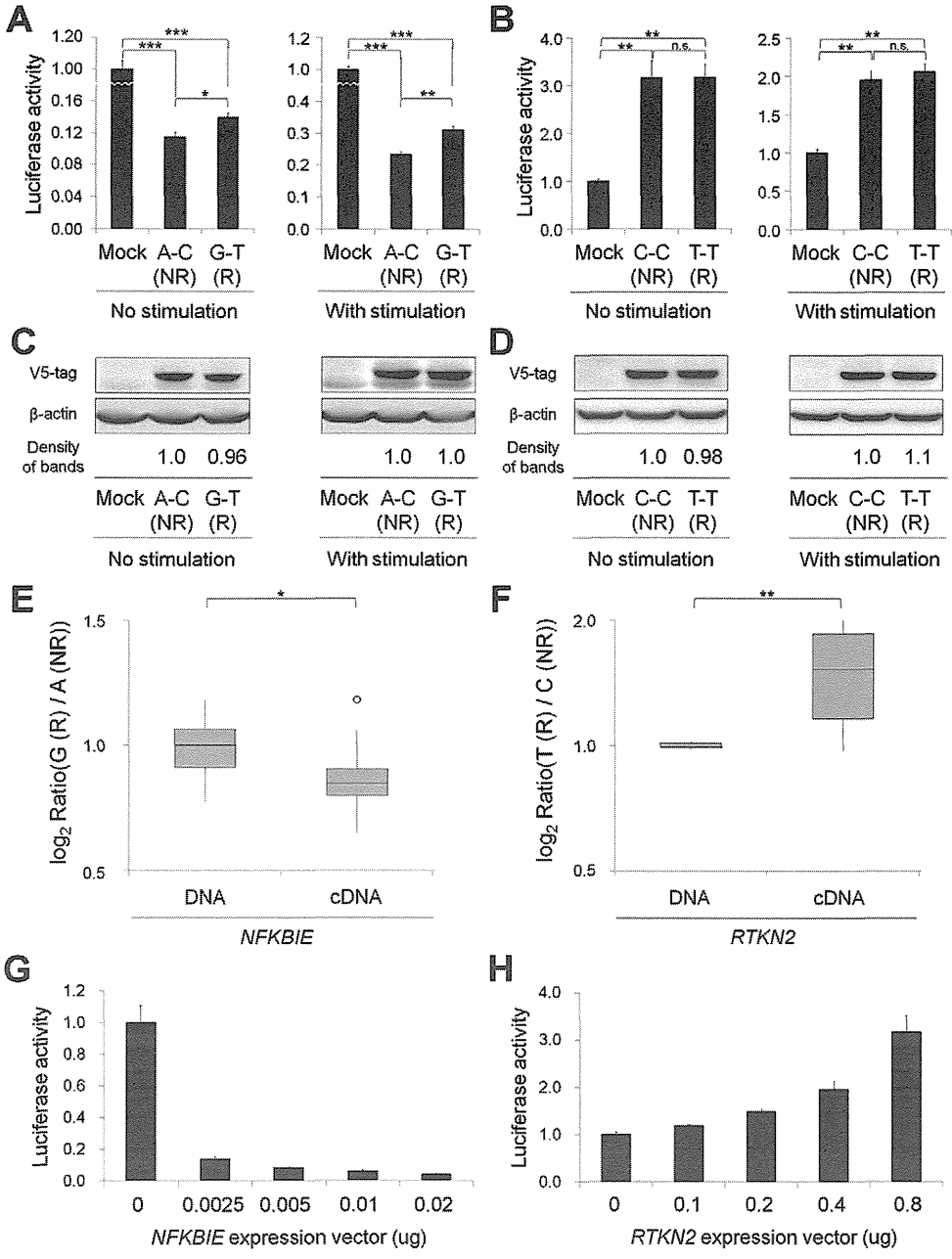

Figure 3. Functional evaluation of nsSNPs and allelic imbalance of expression in *NFKBIE* and *RTKN2*. (A, B) Effects of nsSNPs in *NFKBIE* (A) and *RTKN2* (B) on NF-κB activity by luciferase assays. Two haplotype constructs (A-C (rs2233434-rs2233433; non-risk (NR)) and G-T (risk (R)) for *NFKBIE* and C-C (rs3125734-rs61850830; NR) and T-T (R) for *RTKN2*) were used. The expression vector of each construct, pGL4.32[*luc2P*/NF-κB-RE] vector and pRL-TK vector were transfected into HEK293A cells. Data represent the mean ± s.d. Each experiment was performed in sextuplicate, and experiments were independently repeated three times. $^{*}P<0.05$, $^{**}P<1.0\times10^{-5}$, and $^{***}P<1.0\times10^{-10}$ by Student's *t*-test. n.s.: not significant. (C, D) Protein expression levels of each haplotype construct. Anti-V5 tag antibody was used in the Western blotting analysis to detect the expression of exogenous IκBε (C) and RTKN2 (D). Beta-actin expression was used as an internal control. The densities of the bands were quantified and normalized to that of the risk allele. (E, F) Allelic imbalance of expression in *NFKBIE* (E) and *RTKN2* (F). ASTQ was performed using samples from individuals heterozygous for rs2233434 (G/A) in *NFKBIE* and rs3125734 (T/C) in *RTKN2*. Genomic DNAs and cDNAs were extracted from PBMCs ($n=14$ for *NFKBIE* and $n=6$ for *RTKN2*). The y-axis shows the $\log_2$ ratio of the transcript amounts in target SNPs (risk allele/non-risk allele). The top bar of the box-plot represents the maximum value and the lower bar represents the minimum value. The top of box is the third quartile, the bottom of box is the first quartile, and the middle bar is the median value. The circle is an outlier. $^{*}P=0.012$, $^{**}P=0.016$, by Student's *t*-test. (G, H) Dose-dependent inhibition of *NFKBIE* (G) and activation of *RTKN2* (H) on NF-κB activity. Various doses of expression vectors carrying the non-risk allele of each gene were transfected into HEK293A cells with pGL4.32 and pRL-TK vectors.
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located in the key regions of transcriptional regulation [26,36], warranting the prioritization of variants before evaluation by *in vitro* assays. This could also minimize false-positive results of the *in vitro* assays. However, there may be additional causal variants, including rare variants, unsuccessfully selected at each step of our integrated screening. Therefore, the screening strategy

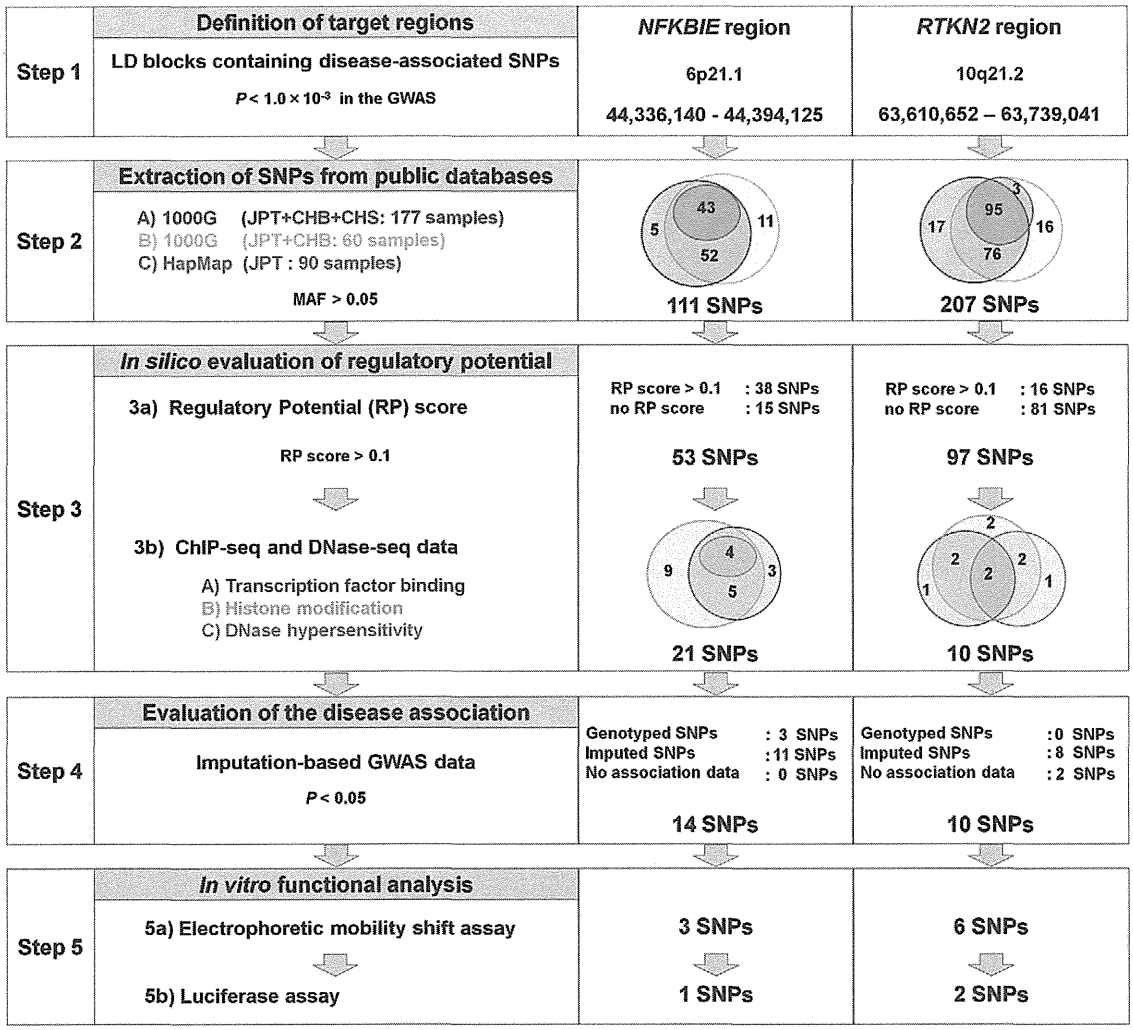

Figure 4. Overview of SNP selection using integrated *in silico* and *in vitro* approaches. The figure shows the SNP selection process (left) and the results of *NFKBIE* (middle) and *RTKN2* (right). (Step 1) LD blocks that contain disease-associated SNPs ($P_{GWAS}<1.0\times10^{-3}$) were selected. (Step 2) SNPs were extracted from three databases (A–C). 1000G, 1000 Genome Project; HapMap, International HapMap Project. A) JPT, CHB, and CHS samples ($n=177$) from the 1000G (the August 2010 release). B) JPT and CHB samples ($n=60$) from the pilot 1 low coverage study data of 1000G (the March 2010 release). C) JPT samples ($n=90$) from HapMap phase II+III (release #27). SNPs with minor allele frequency >0.05 were selected. (Step 3) Prediction of regulatory potential *in silico*. 3a) Regulatory potential (RP) scores were used for SNP selection, where an RP score >0.1 indicated the presence of regulatory elements. SNPs without RP scores were also selected. 3b) Prediction of regulatory elements by ChIP-seq data and DNase-seq data. (A) Transcription factor binding sites, (B) histone modification sites (CTCF binding, H3K4me1, H3K4me2, H3K4me3, H3K27ac, H3K9ac), and (C) DNase I hypersensitivity sites were evaluated. ChIP-seq and DNase-seq data derived from GM12878 EBV-transformed B cells were used for *NFKBIE* and *RTKN2*. DNase-seq data of Th1, Th2, and Jurkat cells were also used for *RTKN2*. (Step 4) Association data of the imputation-based GWAS using 1000G reference genotypes were used. SNPs with a significance level of $P<0.05$ were selected. SNPs without association data were also selected. (Step 5) EMSAs and luciferase assays were performed for evaluation of regulatory potentials *in vitro*.
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should be refined as the quality and quantity of genomic databases improves in the future.

We identified multiple candidate causal variants in *NFKBIE* (two nsSNPs and one rSNP) and *RTKN2* (two rSNPs). We could not statistically distinguish the primary effect of each candidate causal variant, because these variants are in strong LD and on the same common haplotype. However, multiple causal variants could be involved in a single locus, which is also seen in another well-known autoimmune locus in 6q23 (*TNFAIP3* gene locus), where both an nsSNP and a regulatory variant have been shown to be functionally related to the disease [8,37]. The risk haplotype of nsSNPs in *NFKBIE* (rs2233433 and rs2233434) showed an enhancement of NF-κB activity, which might reflect an impaired inhibitory effect of IκB-ε on nuclear translocation of NF-κB. On the other hand, down-regulated *NFKBIE* expression and up-regulated *RTKN2* expression were observed at the risk haplotypes, which may be regulated in *cis* by the rSNPs (rs2233424 in *NFKBIE*, rs12248974 and rs61852964 in *RTKN2*). As overexpression studies have also demonstrated dose-dependent attenuation of NF-κB activity by *NFKBIE*, and dose-dependent enhancement by *RTKN2*, the *cis*-regulatory effects of these rSNPs should enhance the NF-κB activity in the risk allele. Taken together with the effect of nsSNPs in *NFKBIE*, the enhancement of NF-κB activity may play a role in the pathogenesis of the disease. This is further supported by evidence that previous GWAS for RA have also identified genes related to the NF-κB pathway, such as *TNFAIP3* [13], v-rel

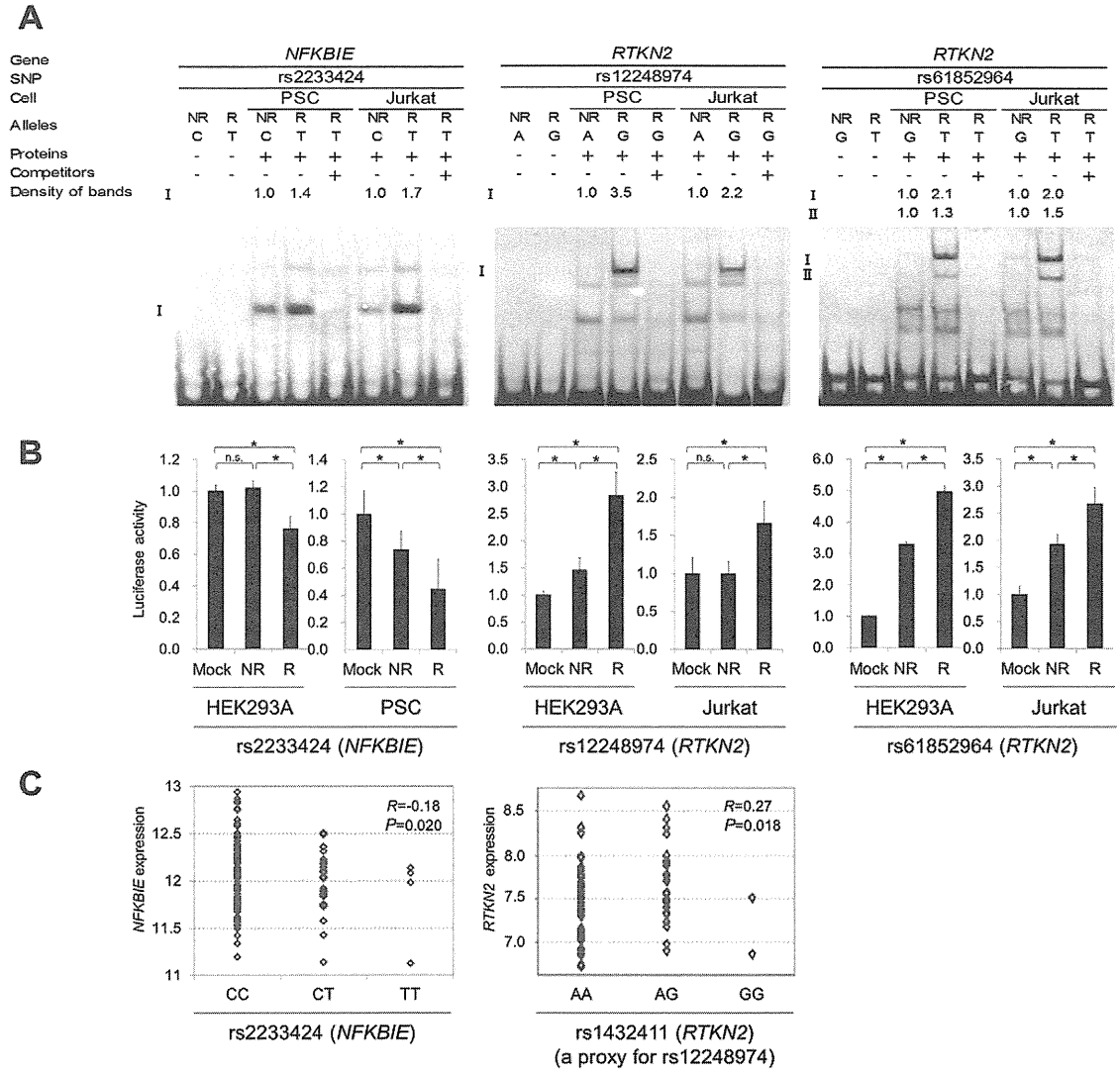

Figure 5. Evaluation of candidate regulatory SNPs *in vitro*. (A) Binding of nuclear factors from lymphoblastoid B-cells (PSC cells) and Jurkat cells to the 31-bp sequences around each SNP was evaluated by EMSA. Unlabeled probes in 200-fold excess as compared to the labeled probes were used for the competition experiment. The densities of the bands were quantified and normalized to that of the risk allele. rs2233424 in *NFKBIE* (C(NR)/T(R)) (left), rs12248974 (A(NR)/G(R)) (middle) and rs61852964 (G(NR)/T(R)) (right) in *RTKN2*. (B) Transcriptional activities were evaluated by luciferase assays. Each 31-bp oligonucleotide was inserted into the pGL4.24[*Luc2P*/minP] vector. Luc, luciferase; minP, minimal promoter. Transcfection was performed with HEK293A (for all the SNPs), PSC cells (for rs2233424), and Jurkat cells (for rs12248974 and rs61852964). rs2233424 (left), rs12248974 (middle), and rs61852964 (right). Data represent the mean ± s.d. Each experiment was performed in sextuplicate and independently repeated three times. *$P<0.05$ by Student's *t*-test. n.s.: not significant. (C) Liner regression analysis of the relationship between SNP genotype and gene expression level. *NFKBIE* expression data in lymphoblastoid B-cell lines of HapMap individuals (JPT+CHB, CEU and YRI; $n=151$), and *RTKN2* expression data in primary T cells from umbilical cords of Western European individuals ($n=85$) were used. The x-axis shows the SNP genotypes and the y-axis represents the $\log_2$-transformed gene expression level. R: correlation coefficient between SNP genotype and gene expression. Rs2233424 genotypes and *NFKBIE* expression level (left). The genotype classification by population: JPT+CHB, CC = 52, CT = 1; CEU, CC = 35, CT = 2; YRI, CC = 32, CT = 2, TT = 4. Rs1432411 genotypes and *RTKN2* expression level (right). Rs1432411 was used as a proxy SNP of rs12248974 ($r^2=0.97$).
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reticuloendotheliosis viral oncogene homolog (*REL* [MIM 164910]) [5], TNF receptor-associated factor 1 (*TRAF1* [MIM 601711]) [3], and CD40 molecule TNF receptor superfamily member 5 (*CD40* [MIM 109535]) [38].

In conclusion, we identified *NFKBIE* and *RTKN2* as genetic risk factors for RA. Considering the allelic effect of both genes, enhanced NF-κB activity may play a role in the pathogenesis of the disease. Because NF-κB regulates the expression of numerous genes, including inflammatory and immune response mediators, NF-κB and its regulators identified by GWAS are promising targets for the treatment of RA.

Materials and Methods

Ethics statement

All subjects were of Japanese origin and provided written informed consent for participation in the study, which was approved by the ethical committees of the institutional review boards.

Subjects

A total of 7,907 RA cases, 657 SLE cases, 1,783 GD cases, and 35,362 control subjects were enrolled in the study through medical

institutes in Japan under the support of the BioBank Japan Project, Center for Genomic Medicine at RIKEN, the University of Tokyo, Tokyo Women's Medical University, and Kyoto University. The same case and control samples were used in the previous meta-analysis of GWASs in the Japanese population (Table S1) [15] . RA and SLE subjects met the revised American College of Rheumatology (ACR) criteria for RA [39]. Diagnosis of individuals with GD was established on the basis of clinical findings and results of the routine examinations for circulating thyroid hormone and thyroid-stimulating hormone concentrations, thyroid-stimulating hormone receptors, ultrasonography, $^{[99m]}TCO_4^-$ (or $[^{123}I]$) uptake, and thyroid scintigraphy. DNAs were extracted from peripheral blood cells using a standard protocol. Total RNAs were also extracted from PBMCs of healthy individuals ($n=20$) using an RNeasy kit (QIAGEN, Valencia, CA, USA). Details of the samples are summarized in Table S1.

Genotyping and quality control

In the GWAS, RA cases and controls were genotyped using Illumina Human610-Quad and Illumina Human 550v3 Genotyping BeadsChips (Illumina, San Diego, CA, USA), respectively, and quality control of genotyping was performed as described previously [6]. For replication study of candidate loci, a landmark SNP was selected from each locus that satisfied $5\times10^{-8}<P_{GWAS}<5\times10^{-5}$ in the GWAS. If multiple candidate SNPs existed within ±100 kb, the SNP with the lowest *P*-value was selected. All case subjects in the replication study and both case and control subjects in the validation study of candidate causal variants were genotyped using TaqMan SNP genotyping assays (Table S12) (Applied Biosystems, Foster City, CA, USA) with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Because of the availability of DNA samples, only a part of the control subjects were genotyped for the validation study ($n=3{,}290$, 97.3%). To enlarge the number of subjects and enhance statistical power for replication studies, we used genotype data obtained from other GWAS projects genotyped using the Illumina platforms for the replication control panels (Table S1). All SNPs were successfully genotyped with call rates >0.98 and were in Hardy-Weinberg equilibrium (HWE) in control subjects ($P>0.05$ as examined by χ^2 test), except for rs2233434, which displayed a deviation from HWE ($P=0.00091$). To evaluate possible genotyping biases between the platforms, we also genotyped rs2233434 and rs3125734 by TaqMan assays for randomly selected subjects genotyped using other genotyping platforms ($n=376$), yielding high concordance rates of ≥0.99.

Association analysis

The associations of the SNPs were tested with the Cochran-Armitage trend test. Combined analysis was performed with the Mantel-Haenszel method. Haplotype association analysis and haplotype-based conditional association analysis were performed using Haploview v4.2 and the PLINK v1.07 program (see URLs) [40], respectively. The SNPs that were not genotyped in the GWAS were imputed using MACH 1.0.16 (see URLs), with genotype data from the 1000 Genome Project (JPT, CHB, and Han Chinese South (CHS): 177 individuals) as references (August 2010 release) [41]. All the imputed SNPs demonstrated *Rsq* values more than 0.60.

DNA re-sequencing

Unknown variants in the coding sequences of *NFKBIE* and *RTKN2* were revealed by directly sequencing the DNA of 48 individuals affected with RA. DNA fragments were amplified with the appropriate primers (Table S13). Purification of PCR products was performed with Exonuclease I (New England Biolabs, Ipswich, MA, USA) and shrimp alkaline phosphatase (Promega, Madison, WI, USA). The amplified DNAs were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), and signals were detected using an ABI 3700 DNA Analyzer (Applied Biosystems).

Construction of haplotype-specific expression vectors

The full coding regions were amplified using cDNAs prepared from an Epstein-Barr virus-transfected lymphoblastoid B-cell line (Pharma SNP Consortium (PSC), Osaka, Japan) for *NFKBIE* (NM_004556.2) and from Jurkat cells (American Type Culture Collection (ATCC), Rockville, MD, USA) for *RTKN2* (NM_145307.2) with appropriate primers (Table S14) and DNA polymerases. PCR products were inserted into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Camarillo, CA, USA) using the TaKaRa Ligation kit ver. 2.1 (Takara Bio Inc, Shiga, Japan), and mutagenized using the AMAP Multi Site-Directed Mutagenesis Kit (MBL, Nagoya, Japan). Each construct was then transformed into Jet Competent *Escherichia coli* cells (DH5α) (BioDynamics Laboratory Inc., Tokyo, Japan). These plasmids were purified using an Endofree Plasmid Maxi Kit (QIAGEN) after confirmation of the sequence.

NF-κB reporter assay

Human embryonic kidney (HEK) 293A cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France), 1% penicillin/streptomycin (Invitrogen), and 0.1 mM MEM Non-Essential Amino Acids (Invitrogen). Various doses of the haplotype-specific expression vector (0.0025–0.02 μg for *NFKBIE* and 0.1–0.8 μg for *RTKN2*), pGL4.32[*luc2P*/NF-κB-RE/Hygro] vector (Promega) (0.05 μg and 0.0125 μg, respectively), and pRL-TK vector (an internal control for transfection efficiency) (0.45 μg and 0.15 μg, respectively) were transfected into the HEK293A cells using the Lipofectamine LTX transfection reagent (Invitrogen) according to the manufacturer's protocol. The total amounts of DNAs were adjusted with empty pcDNA3.1 vector. After 22 h, cells were incubated with 1 ng/ml TNF-α (Sigma) for 2 h or with medium alone. Cells were collected, and luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega) and a GloMax-Multi+ Detection System (Promega). Each experiment was independently repeated three times, and sextuplicate samples were assayed each time.

Western blotting

After 24 h of transfection as described for the NF-κB reporter assay, cells were lysed in NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl at pH 8.0, and a protease inhibitor cocktail), and incubated on ice for 30 min. After centrifugation, the supernatant fraction was collected and 4×Sodium dodecyl sulfate (SDS) sample buffer was added. After denaturation at 95°C for 5 min, proteins were analyzed by SDS-polyachrylamide gel electrophoresis (PAGE) on a 5% to 20% gradient gel (Wako, Osaka, Japan) and were transferred to polyvinylidene difluouride (PVDF) membranes (Millipore, Billerica, MA, USA). Target proteins on the membrane were probed with antibodies (mouse anti-V5 tag (Invitrogen), anti-β-actin-HRP (an internal control), and goat anti-mouse IgG2a-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA)), visualized using enhanced chemiluminescence (ECL) detection reagent (GE Healthcare, Pollards Wood, UK), and detected using a LAS-3000 mini lumino-image analyzer

(Fujifilm, Tokyo, Japan). Band intensities were measured using MultiGauge software (Fujifilm).

Allele-specific transcript quantification (ASTQ) analysis

ASTQ analysis was performed as previously described [42]. Total RNAs and genomic DNAs were extracted from PBMCs and lymphoblastoid B-cell lines. cDNAs were synthesized using TaqMan reverse transcription reagents (Applied Biosystems). We selected SNPs (rs2233434 (A/G) for *NFKBIE* and rs3125734 (C/T) for *RTKN2*) as target SNPs. Allele-specific gene expression was measured by TaqMan SNP genotyping probes for these SNPs (Applied Biosystems). To make a standard curve, we selected two individuals that had homozygous genotypes of each target SNP. We mixed these DNAs at nine different ratios and detected the intensities. The $\log_2$ of (risk allele/non-risk allele intensity) for each SNP was plotted against the $\log_2$ of mixing homozygous DNAs. We generated a standard curve (linear regression line; $y = ax+b$), where y is the $\log_2$ of (risk allele/non-risk allele intensity) at a given mixing ratio, x is the $\log_2$ of the mixing ratio, a is the slope, and b is the intercept. We then measured the allelic ratio for each cDNA and genomic DNA from each individual by real-time TaqMan PCR. Based on a standard curve, we calculated the allelic ratio of cDNAs and genomic DNAs. Intensities were detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

Electrophoretic mobility shift assays (EMSA)

EMSA and preparation of nuclear extract from lymphoblastoid B-cell lines and Jurkat cells were performed as previously described [43]. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Following stimulation with 50 ng/ml phorbol myristate acetate (Sigma-Aldrich) for 2 h, cells were collected and suspended in buffer A (20 mM HEPES at pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA at pH 8.0, 1 mM DTT, 0.1% NP-40, and a protease inhibitor cocktail) for 10 min on ice. After centrifugation, the pellets were resuspended in buffer B (which contains buffer A with 500 mM NaCl). Following incubation on ice for 30 min and centrifugation to remove cellular debris, the supernatant fraction containing nuclear proteins was collected. Oligonucleotides (31-bp) were designed that corresponded to genomic sequences surrounding the SNPs (Table S15). Single-stranded oligonucleotide probes were labeled using a Biotin 3′ End DNA Labeling Kit (Pierce Biotechnology, Rockford, IL, USA), and sense and antisense oligonucleotides were then annealed. DNA-protein interactions were detected using a LightShift Chemiluminescent EMSA kit (Pierce Biotechnology). The DNA-protein complexes were separated on a non-denaturing 5% polyachrylamide gel in 1×TBE (Tris-borate-EDTA) running buffer for 60 min at 150 V. The DNA-protein complexes were then transferred from the gel onto a nitrocellulose membrane (Ambion, Carlsbad, CA, USA), and were cross-linked to the membrane by exposure to UV light. Signals were detected using a LAS-3000 mini lumino-image analyzer (Fujifilm). Allelic differences were analyzed using MultiGauge software (Fujifilm) by measuring the intensity of the bands.

Luciferase assay

Oligonucleotides (31-bp) were designed as described for the EMSAs (Table S15), and complementary sense and antisense oligonucleotides were annealed. To construct luciferase reporter plasmids, pGL4.24[*luc2P*/minP] vector (Promega) was digested with restriction enzymes (XhoI and BglII) (Takara Bio Inc), and annealed oligonucleotide was ligated into a pGL4.24 vector upstream of the minimal promoter. HEK293A ($n = 2.5\times10^5$), lymphoblastoid B-cell lines ($n = 2.0\times10^6$) and Jurkat ($n = 5.0\times10^5$) cells were transfected with the allele-specific constructs (0.4 μg, 1.8 μg and 2.5 μg, respectively) and the pRL-TK vector (0.1 μg, 0.2 μg and 0.25 μg, respectively) using the Lipofectamine LTX transfection reagent (for HEK293A and Jurkat cells) and Amaxa nucleofector kit (Lonza, Basel, Switzerland) (for lymphoblastoid B-cell lines). Cells were collected, and luciferase activity was measured as described for the NF-κB reporter assay. Each experiment was independently repeated three times and sextuplicate samples were assayed each time.

Correlation analysis between gene expression and genotypes

The expression data in lymphoblastoid B-cell lines derived from HapMap individuals ($n = 210$; JPT, CHB, CEU, and YRI) and in primary T cells from umbilical cords of Western European individuals ($n = 85$) from the database of the Gene Expression Variation (Genevar) project were used. SNP genotypes were obtained from HapMap and 1000 Genome Project databases. The expression levels were regressed with the genotype in a liner model. The statistical significance of regression coefficients was tested using Student's *t*-test.

Statistical analysis

We used χ^2 contingency table tests to evaluate the significance of differences in allele frequency in the case-control subjects. We defined haplotype blocks using the solid spine of LD definition of Haploview v4.2, and estimated haplotype frequency and calculated pairwise LD indices (r^2) between pairs of polymorphisms using the Haploview program. Luciferase assay data and ASTQ analysis data were analyzed by Student's *t*-test.

Web resources

The URLs for data presented herein are as follows:
PLINK, http://pngu.mgh.harvard.edu/~purcekk/plink
MACH, http://www.sph.umich.edu/csg/abecasis/mach/
UCSC Genome Browser, http://genome.ucsc.edu/;
Genevar, http://www.sanger.ac.uk/resources/software/genevar/
HapMap Project, http://www.HapMap.org/
1000 Genome Project, http://www.1000genomes.org
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/

Supporting Information

Figure S1 NF-kB activity was influenced by nsSNPs in *NFKBIE*. NF-κB activities were evaluated by luciferase assays. Allele specific construct, pGL4.32[*luc2P*/NF-κB-RE] luciferase vector, and pRL-TK vector were transfected into HEK293A cells. Four haplotypes (rs2233434-rs2233433; A-C, G-C, A-T, and G-T) were examined. (rs2233434: A = non-risk (NR), G = risk (R); rs2233433: C = NR, T = R). Twenty-two hours after transfection, cells were stimulated with medium alone (A) or TNF-α (B) for 2 h. Data represent the mean ± s.d. Each experiment was performed in sextuplicate, and experiments were independently repeated three times. $*P<0.05$ and $**P<1.0\times10^{-5}$ by Student's *t*-test. n.s.: not significant.
(TIF)

Figure S2 Allelic imbalance of expression in *NFKBIE*. ASTQ was performed using samples from individuals heterozygous for rs2233434 (G/A) in *NFKBIE*. Genomic DNAs and cDNAs were extracted from lymphoblastoid B cells ($n = 9$). The y-axis shows the $\log_2$ ratio of the transcript amounts in target SNPs (risk allele/non-risk

allele). The top bar of the box-plot represents the maximum value and the lower bar represents the minimum value. The top of box is the third quartile, the bottom of box is the first quartile, and the middle bar is the median value. The circle is an outlier. $*P = 5.3 \times 10^{-4}$ by Student's *t*-test.
(TIF)

Figure S3 SNP selection using *in silico* analysis in the *NFKBIE* region. Step 1: Definition of the target region. *P*-values of the SNPs in the GWAS (top) and genomic structure (middle), and the *D'*-based LD map (bottom). The green diamond shapes represent the -$\log_{10}$ of the Cochran-Armitage trend *P*-values. The dashed line indicates the significance threshold ($P < 1 \times 10^{-3}$). The LD map was drawn based on genotype data of the 1000 Genome Project (JPT, CHB and CHS: 177 samples) using Haploview software v4.2. LD blocks were defined by the solid spine method. The red box (top) represents the target region of the *in silico* analysis (Chr6: 44,336,140-44,394,125). Step 2: Target SNPs were extracted from public databases (HapMap and 1000 Genome Project). SNPs with MAF >0.05 were selected. Step 3: Evaluation of regulatory potential. Step 3a: The regulatory potential (RP) score was calculated for sequences surrounding the SNPs by ESPERR (evolutionary and sequence pattern extraction through reduced representations) method. SNPs with RP score >0.1 were selected. Step 3b: Subsequently, SNPs within the predicted, regulatory genomic elements were selected by using ChIP-seq data of transcription factor binding sites (Txn factor), histone modification sites (CTCF binding, H3K4me1, H3K4me2, H3K4me3, H3K27ac, H3K9ac) or DNase-seq data of DNase I hypersensitivity sites (DNase HS). ChIP-seq data and DNase-seq data used the signals derived from GM12878 EBV-transformed B cells. All these analyses of Steps 2 to 3 were performed by using the UCSC genome browser. Step 4: Evaluation of disease association. Association data of both genotyped (green diamonds) and imputed (black diamonds) SNPs in the GWAS samples were used. Red triangles represent 14 extracted SNPs *in silico*. The dashed line indicates the significance threshold ($P < 0.05$).
(TIF)

Figure S4 SNP selection using *in silico* analysis in the *RTKN2* region. SNP selection in the *RTKN2* region was performed the same as in the case of the *NFKBIE* region as described in Figure S3, except that we used DNase-seq data derived from Th1, Th2, and Jurkat cells in addition to GM12878 EBV-transformed B cells.
(TIF)

Figure S5 Results of EMSAs for candidate regulatory SNPs. Binding affinities of nuclear factors from lymphoblastoid B-cells (PSC cells) and Jurkat cells to the 31-bp sequences around each allele of the candidate regulatory SNPs were evaluated by EMSA. Nuclear factors from PSC cells were used for *NFKBIE*, and Jurkat cells were used for *RTKN2*. 14 SNPs in *NFKBIE* (A) and 10 SNPs in *RTKN2* (B) were tested. NR: non-risk allele; R: risk allele. Arrows indicate bands showing allelic differences in each SNP.
(TIF)

Figure S6 Luciferase assays for regulatory SNPs. Transcriptional activities of the 31-bp genomic sequences around the SNPs were evaluated by luciferase assays. Each oligonucleotide was inserted into the pGL4.24[*luc2P*/minP] vector upstream of the minimal promoter (minP), and allele-specific constructs were transfected into HEK293A cells. Relative luciferase activity is expressed as the ratio of luciferase activity of each allele-specific construct to the luciferase activity of the mock construct. Data represent the mean ± s.d. Each experiment was independently repeated three times, and each sample was measured in sextuplicate. $*P < 1 \times 10^{-3}$ by Student's *t*-test. n.s.: not significant. (A) rs2233434 and rs77986492 in the *NFKBIE* region. (B) rs3864793, rs1864836, rs4979765, and rs4979766 in the *RTKN2* region. NR: non-risk allele; R: risk allele.
(TIF)

Figure S7 The correlation between *NFKBIE* expression and rs2233434 and rs77986492 genotypes. Linear regression analysis of the relationship between SNP genotypes and *NFKBIE* expression. Gene expression data from EBV-transformed lymphoblastoid B cell lines of HapMap individuals (JPT+CHB, CEU, and YRI). (A) rs2233434 ($n = 204$) and (B) rs77986492 ($n = 152$). The genotype classification by population: rs2233434 (JPT+CHB, AA = 61, AG = 28, GG = 1; CEU, AA = 52, AG = 2; YRI, AA = 53, AG = 72) and rs77986492 (JPT+CHB, CC = 52, CT = 24; CEU, CC = 35, CT = 2; YRI, CC = 38, CT = 1). The x-axis shows SNP genotypes and the y-axis represents the $\log_2$-transformed *NFKBIE* expression level. *R*: the correlation coefficient between *NFKBIE* expression and SNP genotype.
(TIF)

Figure S8 The correlation between *RTKN2* expression and rs3852694 genotypes. Linear regression analysis of the relationship between the rs3852694 genotype and *RTKN2* expression. Rs3852694 was used as a proxy SNP of rs1864836 ($r^2 = 1.0$). Gene expression data in primary T cells from umbilical cords of Western European individuals ($n = 85$) were presented by using Genevar software. The x-axis shows the rs3852694 genotypes (AA, AG, GG) and the y-axis represents the $\log_2$-transformed *RTKN2* expression level. *R*: the correlation coefficient between *RTKN2* expression and rs3852694 genotype.
(TIF)

Table S1 Summary of samples.
(DOC)

Table S2 Association results of the GWAS and 1st replication study.
(DOC)

Table S3 Association analysis of *NFKBIE* and *RTKN2* with autoimmune diseases.
(DOC)

Table S4 Association analysis of nsSNPs with RA.
(DOC)

Table S5 Haplotype association study of nsSNPs in *NFKBIE*.
(DOC)

Table S6 Haplotype association study of nsSNPs in *RTKN2*.
(DOC)

Table S7 Predicting the effects of nsSNPs on protein function.
(DOC)

Table S8 Association analysis of candidate rSNPs with RA.
(DOC)

Table S9 Haplotype association study of candidate causal SNPs in *NFKBIE*.
(DOC)

Table S10 Haplotype association study of candidate causal SNPs in *RTKN2*.
(DOC)

Table S11 The conditional haplotype-based association analysis of candidate causal SNPs in *RTKN2*.
(DOC)

Table S12 Probes and Primers used for TaqMan assays.
(DOC)

Table S13 Primers used for DNA re-sequencing.
(DOC)

Table S14 Primers used for construction of expression vectors.
(DOC)

Table S15 Oligonucleotides used for EMSAs and Luciferase assays.
(DOC)

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Author Contributions

Conceived and designed the experiments: K Myouzen, Y Kochi, Y Okada, C Terao, K Ikari, K Ohmura, R Yamada, K Yamamoto. Performed the experiments: K Myouzen, Y Kochi, C Terao, A Suzuki, K Ikari, K Ohmura. Analyzed the data: K Myouzen, Y Kochi, Y Okada, C Terao, T Tsunoda, A Takahashi, R Yamada. Contributed reagents/materials/analysis tools: M Kubo, A Taniguchi, F Matsuda, K Ohmura, S Momohara, T Mimori, H Yamanaka, N Kamatani, Y Nakamura. Wrote the paper: K Myouzen, Y Kochi, Y Okada, C Terao, K Yamamoto.

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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	§ACPA (-)RF(+)RA	§control	*p*	OR	§ACPA (-)RF(+)RA	§control	*p*	OR	§ACPA (-)RF(+)RA	§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)	3 (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 70th to the 74th 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

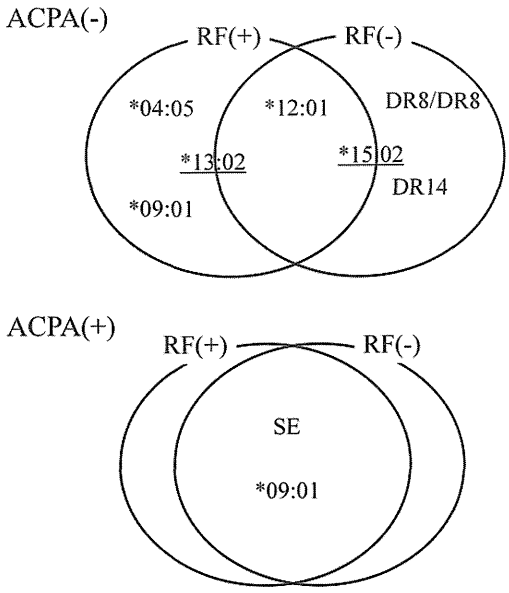

Figure 1. Summary of the HLA-DRB1 alleles associated with ACPA-negative RA and ACPA-positive RA. The relationships between the RF-positive and RF-negative subsets of ACPA-negative and ACPA-positive RA in terms of their associations with HLA-DRB1 alleles are illustrated. While the two subsets of ACPA-positive 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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Genetic Polymorphisms of the Human PNPLA3 Gene Are Strongly Associated with Severity of Non-Alcoholic Fatty Liver Disease in Japanese

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) includes a broad range of liver pathologies from simple steatosis to cirrhosis and fibrosis, in which a subtype accompanying hepatocyte degeneration and fibrosis is classified as nonalcoholic steatohepatitis (NASH). NASH accounts for approximately 10–30% of NAFLD and causes a higher frequency of liver-related death, and its progression of NASH has been considered to be complex involving multiple genetic factors interacting with the environment and lifestyle.

Principal Findings: To identify genetic factors related to NAFLD in the Japanese, we performed a genome-wide association study recruiting 529 histologically diagnosed NAFLD patients and 932 population controls. A significant association was observed for a cluster of SNPs in *PNPLA3* on chromosome 22q13 with the strongest *p*-value of 1.4×10^{-10} (OR = 1.66, 95%CI: 1.43–1.94) for rs738409. Rs738409 also showed the strongest association ($p=3.6\times10^{-6}$) with the histological classifications proposed by Matteoni and colleagues based on the degree of inflammation, ballooning degeneration, fibrosis and Mallory-Denk body. In addition, there were marked differences in rs738409 genotype distributions between type4 subgroup corresponding to NASH and the other three subgroups ($p=4.8\times10^{-6}$, OR = 1.96, 95%CI: 1.47–2.62). Moreover, a subgroup analysis of NAFLD patients against controls showed a significant association of rs738409 with type4 ($p=1.7\times10^{-16}$, OR = 2.18, 95%CI: 1.81–2.63) whereas no association was obtained for type1 to type3 ($p=0.41$). Rs738409 also showed strong associations with three clinical traits related to the prognosis of NAFLD, namely, levels of hyaluronic acid ($p=4.6\times10^{-4}$), HbA1c ($p=0.0011$) and iron deposition in the liver ($p=5.6\times10^{-4}$).

Conclusions: With these results we clearly demonstrated that Matteoni type4 NAFLD is both a genetically and clinically different subset from the other spectrums of the disease and that the *PNPLA3* gene is strongly associated with the progression of NASH in Japanese population.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a broad range of pathologies from fatty liver (simple steatosis), steatonecrosis, and steatohepatitis to cirrhosis [1–3]. NAFLD often accompanies other lifestyle-related pathologies of metabolic syndrome such as diabetes mellitus, hypertension and dyslipidemia, and the number of NAFLD patients is increasing worldwide along with the escalation in the incidence of metabolic syndrome [4]. Prevalence of NAFLD is considered as approximately 8% in Japanese and 6–35% in Europeans [4,5]. The majority of NAFLD