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# A functional variant in *FcRH3*, encoding Fc Receptor Homolog 3, is associated with rheumatoid arthritis and several autoimmunities

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## Abstract

Rheumatoid arthritis (RA) is a common autoimmune disease with a complex genetic etiology. Herein we identify a single-nucleotide polymorphism (SNP) in the promoter region of *FcRH3*, a member of the Fc receptor homolog family, that is associated with RA susceptibility (OR=2.15,  $P=0.0000085$ ). This polymorphism alters the binding affinity of nuclear factor- $\kappa$ B and regulates

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COMPETING INTERESTS STATEMENT

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*FcRH3* expression. High *FcRH3* expression on B-cells and augmented autoantibody production were observed in individuals with the disease-susceptible genotype. Associations were also found between the SNP and susceptibility to autoimmune thyroid disease and systemic lupus erythematosus. *FcRH3* may thus play a pivotal role in autoimmunity.

Rheumatoid arthritis represents one of the most common autoimmune diseases, and is characterized by inflammation of synovial tissue and joint destruction. Although the disease is believed to result from a combination of genetic and environmental factors, the complete etiology of RA has not yet been clarified<sup>1</sup>. While specific haplotypes of human leukocyte antigen (HLA)-DRB1, usually referred to as shared-epitope (SE) sequences<sup>2</sup>, have been repeatedly reported as conferring RA-susceptibility<sup>3,4</sup>, other genetic components are also involved in the pathogenesis of RA<sup>5</sup>. This combination of HLA haplotypes and non-HLA genes accounting for disease susceptibility is also seen in other autoimmune diseases<sup>6–8</sup>. In autoimmune thyroid disease (AITD), for instance, studies have consistently shown that the *HLA-DR3* haplotype is associated with disease risk, in addition to a functional haplotype of a non-HLA gene, *CTLA4*, that has recently been associated with AITD susceptibility<sup>9</sup>.

Identification of non-HLA genes associated with RA susceptibility and other autoimmunities seems difficult, due to the low relative risk of disease resulting from these non-HLA genes compared with the strong relative risk from disease-associated HLA haplotypes. In a search for non-HLA determinants of disease susceptibility, whole genome studies have been conducted for both human autoimmune diseases and experimental animal models. These studies have revealed non-random clustering of susceptibility loci for clinically distinct diseases<sup>8,10</sup>. This overlapping of susceptibility loci for multiple autoimmunities suggests the existence of common susceptibility genes in those regions. Intense studies of loci-clustering regions has revealed genes commonly associated with multiple autoimmune diseases, such as *CTLA4* on 2q33 (ref. 9), *SLC22A4/A5* on 5q31 (ref.11) and *PTPN22* on 1p13 (ref. 12).

Cytoband 1q21-23 is one of the regions implicated in susceptibility to multiple autoimmune diseases. The Fcγ receptor (FcγR) II/III genes are located in 1q23 and a new family of genes, Fc receptor homologs (FcRHs)<sup>13,14</sup> (also known as IRTAs<sup>15,16</sup> or SPAPs<sup>17</sup>) clusters nearby in 1q21 (Fig. 1a). FcRHs have high structural homology with classical FcγRs, although ligands and function remain unclear. These receptors are strong candidates for involvement in autoimmunity, as they are believed to play important roles in the pathogenesis of RA and other autoimmune diseases<sup>18</sup>. Region 1q23 represents a candidate locus for susceptibility to systemic lupus erythematosus (SLE), and variants in the classical FcγR II/III genes would partially account for disease susceptibility<sup>6,19</sup>. The FcRH gene cluster in 1q21 is also reportedly associated with SLE (Gibson, AW. *et al.* American College of Rheumatology 66<sup>th</sup> Annual Scientific Meeting, 2003). Region 1q21 represents a candidate locus for psoriasis (*PSORS4* (ref. 7,<sup>20</sup>)) and multiple sclerosis<sup>21</sup>. The mouse syntenic region of human 1q21, on chromosome 3, also displays susceptibility loci for multiple autoimmune disease models<sup>8</sup>, including collagen-induced arthritis (*Mcia2* (ref. 22), *Eae3* (ref. 23), *Tmevd2* (ref. 24), *Idd10* and *Idd17* (ref. 25)). Although 1q21-23 is a strong candidate region for RA susceptible genes, as above mentioned, the association of classical FcγRs with disease susceptibility remains controversial<sup>26,27</sup>. The present study focused on the 1q21-23 region to identify RA-associated genes in Japanese subjects using linkage disequilibrium (LD) mapping.

## RESULTS

### Case-control study by SNP-based LD-mapping in 1q21-23

To evaluate the extent of association, we analyzed linkage disequilibrium (LD) with SNPs distributed in a 16-Mb region on 1q21-23, including the FcRH gene cluster and the classical

FcγRs (Fig. 1a). A total of 658 control subjects were genotyped for 742 SNPs from the JSNP database, and 491 SNPs were selected for evaluation of LD based on the following criteria: allele frequency >0.1; successful genotyping rate >0.95; and  $P > 0.01$  with Hardy-Weinberg Equilibrium (HWE) testing. The pairwise LD index  $\Delta^{28}$  was calculated for each pair of SNPs, identifying 110 LD blocks<sup>11</sup> at a threshold of  $\Delta > 0.5$  (Fig. 1a).

For association testing, the Japanese set of 830 cases and 658 controls used for LD block evaluation was examined. Initially, 94 RA cases were genotyped for 491 SNPs and their allele frequencies were preliminarily compared with 658 control subjects. A total of 9 SNPs were identified as displaying allele frequencies differing by >0.1 between 658 controls and 94 cases with  $P < 0.01$ . The remaining cases were genotyped for these 9 SNPs, and allele frequencies were tested for case-control association. The smallest  $P$  value was identified between an intronic SNP in the *FcRH3* gene and RA (fcrh3\_6,  $P = 1.8 \times 10^{-5}$ ; association was statistically significant in both RA subgroups (94 and 736)). This SNP was located in an LD block containing 4 of the 5 FcRH genes, with the fifth in the adjacent block. We thus used these 2 LD blocks to further evaluate the origin of this association (Fig. 1b), although our results do not exclude the presence of RA and/or other autoimmunity-associated variants in other LD blocks in 1q21-23.

In addition to the 25 SNPs among 491 used SNPs for LD block evaluation in the 2 LD blocks, 16 additional SNPs were identified in exons and 5'- and 3'-flanking regions of 5 FcRH genes and 1 pseudogene (*FcRH4* or *LOC343265*) by searching the public database and sequencing genomic DNA from Japanese RA-patients. These additional 16 SNPs were genotyped for the identical case and control samples (830 cases, 658 controls) to increase density of variants in the targeted region. A peak of association was observed in a short segment consisting of 4 SNPs in *FcRH3* ( $P < 1.0 \times 10^{-4}$ ) (Fig. 1c, **Supplementary Table 1** online). These comprised fcrh3\_3, fcrh3\_4, fcrh3\_5 and fcrh3\_6, located at nt -169, -110, +358 (5'-untranslated region of exon 2) and +1381 (intron 3; 204 and 859bp from the 3'- and 5'-end of the flanking exons) relative to the transcription initiation site, respectively.

The smallest  $P$  value without correction was observed in recessive-trait genotype comparison of fcrh3\_3 in *FcRH3* ( $P = 8.5 \times 10^{-7}$ ; odds ratio, 2.15; 95% confidence interval, 1.58–2.93) (Table 1). This  $P$  value was still significant when the most conservative Bonferroni correction was applied (comparisons for 507 SNPs;  $P_{\text{corr}} = 0.00043$ ). The 4 strongly associated SNPs were in LD with each other and 3 common haplotypes were inferred (Table 2; Fcrh3\_3, fcrh3\_5 and fcrh3\_6 showed strong LD with each other, with  $\Delta > 0.99$ , whereas fcrh3\_4 showed relatively weak LD with the other 3 SNPs (mean  $\Delta = 0.68$ )).

To identify causal variants in this segment based on genotype data, a forward stepwise-regression procedure was performed with cut-off  $P$  value to proceed next step being 0.01 (ref. 29). No SNP in FcRH genes other than *FcRH3* improved the model. None of the 4 SNPs in *FcRH3* were preferred over the others in these data (data not shown), which implicated that one of the SNPs in *FcRH3* may cause the disease, but there still remained the possibility that variants in other genes were truly associated with the disease.

To validate the case-control association test, samples (830 cases, 658 controls) were evaluated for the impact of population stratification on the case-control study. We selected 2069 SNPs, each of which was identified as a tagging SNP<sup>30</sup> in 2069 distinct LD segments that were previously identified by genotyping 74,842 SNPs distributed in autosomal chromosomes<sup>31</sup>. Analysis of population structure<sup>32</sup>, and the  $\chi^2$  sum<sup>33</sup>, were used for the evaluation of stratification. No significant evidence of population stratification was detected (**Supplementary Fig. 1** online), suggesting no or negligible stratification of our samples and

supporting the validity of the case-control association results by removing this confounder from further consideration.

### Regulatory effect of SNP -169 C/T on *FcRH3* expression

Since none of the 4 SNPs in *FcRH3* (*fcrh3\_3*, *fcrh3\_4*, *fcrh3\_5*, and *fcrh3\_6*) produces amino-acid substitutions, potential effects of the SNPs on transcription factor binding were assessed using TRANSFAC software. Nuclear factor- $\kappa$ B (NF- $\kappa$ B) was predicted to bind the sequence containing RA-susceptible allele *fcrh3\_3* (-169C) with a high score (core match 1.000, matrix match 0.957) and substitution to a non-susceptible allele T decreasing the score of NF- $\kappa$ B binding substantially (core match 0.760, matrix match 0.824). The other 3 SNPs were not predicted to bind to any transcriptional factor with high score and nucleotide substitution was not predicted to affect binding at any regulatory factor. We therefore focused on the 5'-flanking region of *fcrh3\_3* to explore the regulatory effects on expression of *FcRH3*.

Reporter gene analysis was performed using the genomic sequence of *FcRH3* from nt -523 to +203. Constructs were made corresponding to the 3 haplotypes using SNPs at nt -169 (C/T; *fcrh3\_3*) and -110 (G/A; *fcrh3\_4*) (Fig. 2a). These constructs were transfected into Raji cells, a Burkitt's lymphoma cell line that expresses *FcRH3* (ref. 13) and is derived from germinal center B-cells. Luciferase activity was significantly higher in cells transfected with -169C/-110G or -169C/-110A than in cells transfected with -169T/-110G. This suggests that -169C/T is critical for regulation of *FcRH3* expression. To clarify, we cloned single or 4 tandem copies of 30-bp oligonucleotides surrounding -169T/C and control oligonucleotides into a vector with the SV40 promoter. Cells transfected with a single copy of the C allele produced significantly greater luciferase activity than in cells transfected with a single copy of the T allele. More convincingly, transfection with 4 tandem copies of the C allele enhanced luciferase activity 20-fold over those cells transfected with 4 tandem copies of the T allele (Fig. 2a).

To elucidate specific nuclear factors that bind the disease-susceptible allele, we analyzed the sequence around -169C/T. These sequences were predicted by TRANSFAC software to display binding affinity for nuclear factor (NF)- $\kappa$ B, which regulates a wide variety of genes in the immune system. The disease-susceptible sequence GGGAAGTCCC (underlined nucleotide represents SNP at -169) displayed higher matrix similarity to the consensus NF- $\kappa$ B binding motif than the non-susceptible sequence GGGAAGTCCT. Electrophoretic mobility shift assay (EMSA) was then performed to examine whether differences between the susceptible -169C allele and the non-susceptible -169T allele affected binding of nuclear proteins from Raji cells. The 30-bp labeled oligonucleotides used in the luciferase assay were used again in this study. These sequences contain the predicted NF- $\kappa$ B binding site. Two major bands, I and II, were observed in the presence of nuclear extracts, and intensity of band I was higher for the susceptible -169C allele than for the non-susceptible -169T allele (Fig. 2b). Competition assays with unlabelled oligonucleotides revealed that these complexes were specific for the probes. In addition, competition assays with unlabelled probes of the C allele for T and the T allele for C demonstrated that the C allele was better able to compete for binding, a result consistent with the higher binding affinity exhibited by labeled C allele probes alone. A supershift experiment was also performed with antibodies specific for NF- $\kappa$ B components (p50, p52, p65, RelB, cRel). Supershifts were observed in some lanes with specific antibodies for p50, p65 and cRel (Fig. 2b). Among these, only anti-p50 antibody shifted band II, suggesting the presence of a p50-p50 homodimer. Band I had the highest intensity and a significant allelic difference, and was supershifted by anti-p50, anti-p65 and anti-cRel antibodies. Although these findings indicate that band I comprises a mixture of heterodimers, the greater shifts caused by anti-p50 and anti-cRel antibodies suggest that the main component is a p50-cRel heterodimer.

The two *in vitro* assays indicated the potent transcriptional activity of the disease-susceptible haplotype regulated by NF- $\kappa$ B, suggesting expression of *FcRH3* from a chromosome with the

disease-susceptible -169C allele be more than from a chromosome with non-susceptible -169T allele. To extend these findings, we quantified expression of *FcRH3* in peripheral blood B-cells from healthy donors using quantitative Taqman methods, and analyzed the effect of the number of susceptible copies on the transcript level by regression model. Regression analysis revealed a significant positive correlation between number of susceptible chromosomes and transcription level ( $R^2=0.49$ ,  $P=0.0076$ ) (Fig. 2c).

Allele-specific transcript quantification (ASTQ)<sup>9,34</sup> was also performed to confirm the effect of the SNP on transcription. Using an Eag I restriction fragment length polymorphism (RFLP) located at position +358 in exon 2 of *FcRH3* (*fcrh3\_5*, +358C/G), the relative contribution of each haplotype to transcript production in heterozygous individuals could be measured (Fig. 2d). The transcripts of 5 individuals with the -169C+358C/-169T+358G genotype were evaluated, and mean ratio (susceptible vs. non-susceptible haplotype) was 1.63, significantly higher than that of DNA amplicons (ratio=1.06,  $P<1 \times 10^{-5}$ ) from the same individuals. (The quantity of template DNA from the 2 haplotypes was equal.) These results show that the expression of *FcRH3* is higher in individuals with the disease-susceptible haplotype, and suggest that higher expression of *FcRH3* is a potential cause and component of the pathological mechanism(s) leading to RA.

### Expression of *FcRH3* mRNA

*FcRH3* expression in multiple tissues was then quantified using TaqMan methods. Expression of *FcRH3* transcripts was high in the spleen and tonsils (Fig. 3a), which are secondary lymphoid organs. Lower expression was observed in thymus and bone marrow. Analysis of human blood fractions revealed that CD19-positive cells, which represent the B-cell population, displayed the highest level of *FcRH3* expression among peripheral blood mononuclear cells. Lower expression was observed in CD4- and CD8-positive cells (Fig. 3b). Next, the effect of B-cell stimulation on *FcRH3* expression was examined. Peripheral blood B-cells from a healthy donor were cultured for 4 h using known B-cell stimulants, and mRNA of *FcRH3* was quantified (Fig. 3c). Expression of *FcRH3* was increased using stimuli comprising anti-CD40 antibody and lipopolysaccharide (LPS).

Expression of *FcRH3* transcripts in synovial tissue was then investigated using *in situ* hybridization methods. T and B cells are known as the key players with regard to inflammation in synovial tissue, producing pro-inflammatory cytokines and autoantibodies that might be pathogenic<sup>1</sup>. These cells are known to show three distinct histological patterns: diffuse infiltration; clustering in aggregates; and follicles with germinal center reaction<sup>35,36</sup>. Aggregations of T and B cells were observed in paraffinized synovial sections from patients with RA, using immunostaining with anti-CD3 and -CD20 antibodies, respectively (Fig. 3d, e). *In situ* hybridization assay with serial sections detected *FcRH3* mRNA in aggregated lymphocytes (Fig. 3f, g). Although strict differentiation between B and T cells was difficult, at least some aggregated B-cells were positive, with strong expression of *FcRH3* mRNA. RA synovium from two other patients displayed similar images of lymphocyte aggregates and *FcRH3* expression (Supplementary Fig. 2 online).

### SNP association with autoantibody and *HLA-DRB1* status

Higher *FcRH3* expression was suspected as leading to B-cell abnormalities in RA, so associations were examined in RA patients between genotype and two RA-related autoantibodies: rheumatoid factor (RF); and anti-cyclic citrullinated peptide (CCP) antibody. RF is a well-known autoantibody for the Fc region of IgG, and titers correlate with RA disease activity<sup>37</sup>. Anti-CCP antibody recognizes peptides containing citrulline, and is detected in RA with extremely high specificity<sup>38,39</sup>. RF titer in RA patients was positively correlated with the number of susceptible alleles with statistical significance ( $R^2=0.049$ ,  $P=0.0065$ ) (Table 3).



The positive ratio of anti-CCP antibody in RA patients also differed significantly among genotypes ( $P<0.05$ ), and correlated with numbers of susceptible alleles.

As genetic interactions between HLA and non-HLA loci have been described in susceptibility for RA and other autoimmune diseases<sup>26,40</sup>, genotype distributions for SNP -169C/T were compared among three RA-case subgroups stratified by number of *HLA-DRB1* shared-epitope (SE)-encoding alleles. We had previously genotyped *HLA-DRB1* in our population and observed significant associations between RA-susceptibility and SE-encoding alleles<sup>4</sup>. Allele frequency of RA-susceptible allele, SNP -169C, in the subgroup with two copies of SE alleles was significantly higher than in the subgroup with no SE alleles (SNP -169C allele frequency: SE +/+, 0.49 (n=113); SE +/-, 0.43 (n=376); SE -/-, 0.39 (n=215)) ( $P<0.05$ ).

### Replication study of association in RA, ATID and SLE

To confirm associations between the *FcRH3* variant and RA susceptibility, a replication study was conducted (540 RA patients, 636 control subjects). Allele-frequency comparison revealed a significant association between *fcrh3\_3* (-169C/T) and RA susceptibility ( $P=0.041$ , allele frequency: RA patients, 0.40; control subjects, 0.36) (**Supplementary Table 2** online). No significant differences were noted between two cohorts that consisted of the replication samples. These results further confirmed the association of the *fcrh3\_3* -169C SNP allele with RA susceptibility in Japanese.

Since this region was known to display associations with multiple autoimmune diseases, and because several variants were known to be involved in multiple autoimmunities, associations were investigated between SNP -169C/T and two other autoimmune diseases, AITD and SLE. A total of 509 Japanese patients with AITD (Graves' disease, n=351; Hashimoto's thyroiditis, n=158 patients) and 564 Japanese patients with SLE were recruited and compared with 2037 Japanese controls. In addition, AITD, SLE and RA cases were combined as subjects with an autoimmune phenotype and tested for associations with the SNP. Individual diseases as well as combination of two AITDs and combination of AITD, SLE and RA were significantly associated with the SNP (OR=1.52 and  $P=0.00000084$  in Japanese for a recessive model between all 4 autoimmunities considered in aggregate and controls; Table 4). As RA-specific autoantibodies were correlated with the number of susceptible alleles, anti-DNA antibody titer was higher in SLE patients with the -169C/C genotype than in subjects with other genotypes (294.1 IU/ml vs. 145.5 IU/ml; n=120;  $P=0.026$  by Student's t-test), a conclusion not further established by regression analysis ( $P=0.12$ ).

## DISCUSSION

LD mapping of 1q21-23 in Japanese subjects revealed multiple LD blocks in the region, and 1 block containing *FcRH3* displayed associations with RA. This association was replicated in a second Japanese case-control set. The RA-associated allele was also associated with increased risk of other autoimmune disorders, such as AITD (Graves' disease and Hashimoto's thyroiditis) and SLE. Recent reports on autoimmune disease-associated polymorphisms show that some disease-susceptible variants are often limited to specific ethnic groups<sup>12</sup> while other polymorphisms are widely dispersed among *Homo sapiens*, but significantly associated in only specific ethnic groups<sup>41,42</sup>. We evaluated haplotypes consisting of 4 SNPs in *FcRH3* in African American, European American, and Asian (Korean and Japanese) subjects. Weaker LD was identified in African Americans and substantial differences in allelic frequency were noted among three major ethnic groups (**Supplementary Table 3** online).

Though the evidence presented herein for *FcRH3* being an autoimmune susceptibility gene is very powerful, additional autoimmune disease-related genes may probably exist in this region in addition to *FcRH3*. For example, 1q23 represents a strong candidate locus for SLE-

susceptibility<sup>6</sup>, particularly involving the association of the classical FcγR genes with SLE-susceptibility in the Japanese population<sup>19</sup>, although those variants are not in LD with -169C/T in our Japanese population ( $\Delta < 0.05$ , Fig. 1a). Multiple SLE susceptibility genes are also syntenic to human 1q23 in murine models of SLE<sup>43</sup>.

Further evaluation of polymorphism associations showed that an SNP in the promoter region of *FcRH3* alters expression of *FcRH3* via NF-κB binding. Since higher expression of *FcRH3* was observed in individuals with susceptible alleles, and augmented autoantibody production was associated with the susceptible genotype, important steps in the sequence of events leading to autoimmunity must proceed through *FcRH3*. That the susceptible allele is associated with *HLA-DRB1* in RA is consistent with *FcRH3* functioning in the context of HLA class II restriction, which is usually seen in the interaction between T-cells and antigen-presenting cells, including B-cells. Moreover, together with the dominant expression of *FcRH3* on B-cells and the critical roles of B-cells suggested by a recent clinical trial of B-cell-depleting therapy<sup>44</sup>, the present findings might provide a genetic basis for B-cell abnormality in autoimmunity.

Although the precise function of *FcRH3* is unknown, the predicted molecular structure suggests that *FcRH3* is a membranous protein that conveys signals into cells through a cytoplasmic domain containing an immunoreceptor-tyrosine activation motif (ITAM) and an immunoreceptor-tyrosine inhibitory motif (ITIM)<sup>14</sup>. An *in vitro* study showing the binding of tyrosine kinases syk and ZAP70 to the ITAM region and tyrosine phosphatases SHP-1 and SHP-2 to the ITIM region<sup>17</sup> supports the proposed signaling function of *FcRH3*. In a previous study examining *in situ* hybridization in human tonsil, *FcRH3* was expressed in the germinal center, with particularly high expression in the light zone<sup>16</sup>, suggesting that *FcRH3* functions predominantly in centrocytes. The present finding that CD40 stimulation, which is important in germinal center formation<sup>45</sup>, upregulates *FcRH3* expression in B-cells could indicate that *FcRH3* is specifically expressed in germinal center centrocytes under the influence of CD40 signals. In the light zone, centrocytes undergo clonal selection and affinity maturation regulated by positive and negative signals from antigen receptors and co-receptors<sup>46</sup>. High expression of *FcRH3* and augmented autoantibody production in individuals with the disease-susceptible genotype is consistent with *FcRH3* influencing the fate of B-cells and augments the emergence of self-reactive cells in the germinal center.

Besides a role of *FcRH3* in lymphoid tissues, expression of *FcRH3* in synovial tissue might explain the pathological connection between *FcRH3* variants and RA. As we have shown, *FcRH3* is strongly expressed in aggregated lymphocytes. Although our synovial samples showed only T-cell/B-cell aggregates, lymphocytes in RA synovial tissue are known to form a germinal center-like structure, called an “ectopic germinal center”, where T-cell dependent antibody production and affinity maturation occur<sup>36</sup>. Ectopic germinal center formation is also seen in tissues from patients with AITD and SLE, and *FcRH3* might be involved in pathological autoimmune reaction in these disease-specific ectopic lymphocyte aggregates.

Considering that augmented expression of *FcRH3* is associated with susceptibility to autoimmune disorders, and that *FcRH3* expression is regulated in B-cells in the secondary lymphoid organ and is detected in lymphocytes of disease-specific tissues, *FcRH3* very probably is functional molecule in immunity and potentially pathogenic in autoimmune disorders.

## METHODS

### Subjects

Three independent cohorts of RA patients (n=830, 217, and 323), a cohort of SLE patients (n=564), and a cohort of AITD patients (n=509; comprising Graves' disease (n=351) and Hashimoto's thyroiditis (n=158)), were enrolled for the study through several medical institutes in Japan. Four independent cohorts of unaffected control subjects (n=658, n=262, n=374 and n=752) were recruited at various sites in Japan. All subjects were Japanese. RA patients (84.2% women; mean age,  $59.0 \pm 12.3$  years; 75.0% rheumatoid factor-positive) satisfied the revised criteria of the American Rheumatism Association for RA<sup>47</sup>. SLE patients followed the criteria of the American College of Rheumatology for SLE<sup>48</sup>. Diagnosis of AITD was established based on clinical findings and results of routine examinations for circulating thyroid hormone and thyroid stimulating hormone (TSH) concentrations, serum levels of antibodies against thyroglobulin, thyroid microsomes and TSH receptors, ultrasonography, [<sup>99m</sup>]TCO<sub>4</sub><sup>-</sup> (or [<sup>123</sup>I]) uptake and thyroid scintigraphy.

The first control cohort was used for evaluation of LD in 1q21-23 and was compared with the first RA cohort for initial identification of RA-associated LD block and SNPs. The second and third cohorts of RA and controls were used for replication test of the first cohort result. Graves' disease, Hashimoto's thyroiditis, SLE, combination of the two AITDs and combination of RA, SLE and AITDs were tested for associations using the total pool of controls. Control subjects in three other ethnic groups, Korean (n=100), African American (n=120) and European American (n=120), were enrolled for evaluation of *FcRH3* haplotypes. Synovial tissues were sampled from individuals with RA who underwent arthroplastic surgery. All subjects provided informed consent to participate in the study, as approved by the ethical committee of the SNP Research Center, RIKEN.

### SNPs and genotyping

SNPs were discovered in exons and 5'- and 3'-flanking regions of *FcRH1*, *FcRH2*, *FcRH3* and *FcRHψ4* (*LOC343265*) by direct sequencing of DNA from 24 patients. Other SNPs were selected from the JSNP database and Assay-On-Demand SNP database (Applied Biosystems). SNPs were genotyped using Invader and TaqMan assays<sup>41</sup> as indicated by manufacturers. Probe sets for the Invader assay were designed and synthesized by Third Wave Technologies, and those for the TaqMan assay were obtained from Applied Biosystems. When assessing the results of SNP genotyping, we generally exclude successful call rates <0.95 and values of  $P < 0.01$  obtained by HWE testing in control subjects. The error rate of Invader assay was 0.0023, which was estimated by 11,092 assays in 2 replicates using 118 randomly selected SNPs (internal control data).

### Luciferase assay

The promoter fragment of three haplotypes corresponding to nt -523 to +203 of *FcRH3* was cloned into the pGL3-Basic vector (Promega). Oligonucleotides were generated using the allelic sequences of nt -189 to -160 of *FcRH3* as follows: -169T (5' GGTGAGATTACGGGAAGTCCTTGATCTGTA3'); -169C (5' GGTGAGATTACGGGAAGTCCCTGATCTGTA3'); and Cont (GGAGTGTAATCGGGTTGACCAAGTACAGAT). A single copy or 4 tandem copies of these oligonucleotides were cloned into pGL3-Promoter vector (Promega). Raji cells (RCB1647; RIKEN Cell Bank) were grown in RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics. We electroporated (230 V and 975 μF)  $1 \times 10^7$  cells with 5 pmol of constructs and 1 pmol of pRL-TK vector (internal control for transfection efficiency) in a 0.4-cm gap cuvette. After 48 h, cells were collected and luciferase activity measured using the Dual-Luciferase Reporter Assay System (Promega).



## EMSA

EMSA and preparation of nuclear extract from Raji cells were performed as previously described<sup>49</sup>. Oligonucleotides -169T and -169C were labeled with digoxigenin -11-ddUTP using the DIG gel-shift kit (Roche). We incubated 5 µg of nuclear extract with 40 fmol of DIG-labeled nucleotide for 25 min at room temperature. For competition experiments, nuclear extract was pre-incubated with unlabeled oligonucleotide (100-fold excess) before adding digoxigenin-labeled oligonucleotide. For supershift assays, 4 µg of anti-p50, p52, p65, RelB and cRel antibodies and rabbit IgG (control antibody) (Santa Cruz Biotechnology) were incubated for 15 min at room temperature after incubation of the labeled probe. Protein-DNA complexes were separated on a non-denaturing 6% polyacrylamide gel in 0.5× Tris-Borate-EDTA buffer. The gel was transferred to a nitrocellulose membrane, and signals were detected using an LAS-3000 luminoimage analyzer (Fujifilm).

## RNA extraction and cDNA preparation

Peripheral blood was collected from consented healthy volunteers to obtain CD19-positive lymphocytes. Polymorphonuclear cells were separated by differential centrifugation using Lymphoprep resolving solution (AXIS-FIELD). CD19-positive lymphocytes were isolated using the MACS system with CD19 microbeads (Miltenyi Biotec), and cell purity >95% was confirmed using flow cytometry. Cells were stimulated with anti-CD40 Ab (Cymbus Biotechnology), anti-IgM Ab (Jackson Immunoresearch), IL-4 (eBioscience), APRIL (PeproTech), BAFF (PeproTech) and LPS (Sigma) for 4 h. Total RNA was isolated using RNeasy Mini Kit (Qiagen). RNAs of other normal tissues were quantified using Premium Total RNA (Clontech). Total RNA was reverse transcribed using TaqMan Gold RT-PCR reagents with random hexamers (Applied Biosystems), in accordance with the instructions of the manufacturer.

## Quantification of *FcRH3* expression using real-time RT-PCR

Real-time quantitative PCR was performed using an ABI PRISM 7900 (Applied Biosystems) and Assay-on-Demand TaqMan probe and primers (Hs00364720\_m1 for *FcRH3*), according to the manufacturer's instructions. A standard curve was generated from the amplification data for *FcRH3* primers using a dilution series of total RNA from Raji cells as templates, and data were normalized to GUS level.

## ASTQ

Allele-specific quantification was performed as previously described<sup>34</sup> with some modifications. Preparation of cDNA from B-cells was undertaken as described above. Both cDNA and genomic DNA were amplified by PCR for 37 cycles using primers specific for exon 2 of *FcRH3* (**Supplementary Table 4** online), and for an additional cycle using forward primer with Alexa Fluor 488 label at the 5'-end. Products were directly digested using Eag I by incubation at 37 °C for 12 h. Full digestion was monitored by the inclusion of PCR products from homozygote +358G/G. Digested products were then run on a 12.5% polyacrylamide gel, followed by quantification using an LAS-3000 analyzer.

## *In situ* hybridization and immunohistochemistry

*In situ* hybridization was performed as previously described<sup>50</sup>. Probes were obtained from PCR products using the sequence of *FcRH3* (nt 2052–2490, comprising the intracellular unique region that is poorly conserved among members of this family). An additional probe of the 5'-UTR sequence yielded similar results. Control probes were also examined, and yielded no specific hybridization (data not shown). Antibodies to CD3 (clone PS-1, Nichirei) and CD20 (clone L26, Zymed) were used for immunohistochemistry with an ABC Elite kit (Vector Labs)

according to the manufacturer's instructions. No specific staining was detected using mouse isotype IgG (data not shown).

### Measurement of autoantibodies

Rheumatoid factor in sera of RA patients was measured using latex-enhanced immunonephelometric assay. Anti-DNA antibody in sera of SLE patients was measured by radioimmunoassay. RA patients (n=147, 81.1% women; mean age, 63.9 ±10.6 years; 87.8% rheumatoid factor positive; mean Steinbrocker radiographic stage, 3.2) and SLE patients (n=120, 92.6% woman; mean age, 36.6 ±12.7 years) were part of the cohorts and from a single medical institute, respectively. For each patient, the maximum value of rheumatoid factor and anti-DNA antibody during the treatment period in the medical center or outpatient clinic was used. Anti-cyclic citrullinated peptide antibody was detected at a single time point using enzyme-linked immunosorbent assay, as previously described<sup>38</sup>.

### Statistical analysis

LD index  $\Delta^2$  was calculated and Figure 1a drawn using Excel software (Microsoft). Haplotype frequencies were estimated using HAPLOTYPER software. The  $\chi^2$  test was applied for contingency table tests for associations between allele/genotype distribution and phenotypes. *FcRH3* expression in B-cells and autoantibody production were regressed on the number of susceptible alleles (coded 0, 1, and 2). All other statistical analyses, unless otherwise stated, were performed using STATISTICA software (StatSoft).

### URLs

JSNP database (<http://snp.ims.u-tokyo.ac.jp/index.html>)

TRANSFAC (<http://www.gene-regulation.com>).

HAPLOTYPER (<http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm>)

### GenBank accession number

*FcRH3* mRNA, NM\_052939.

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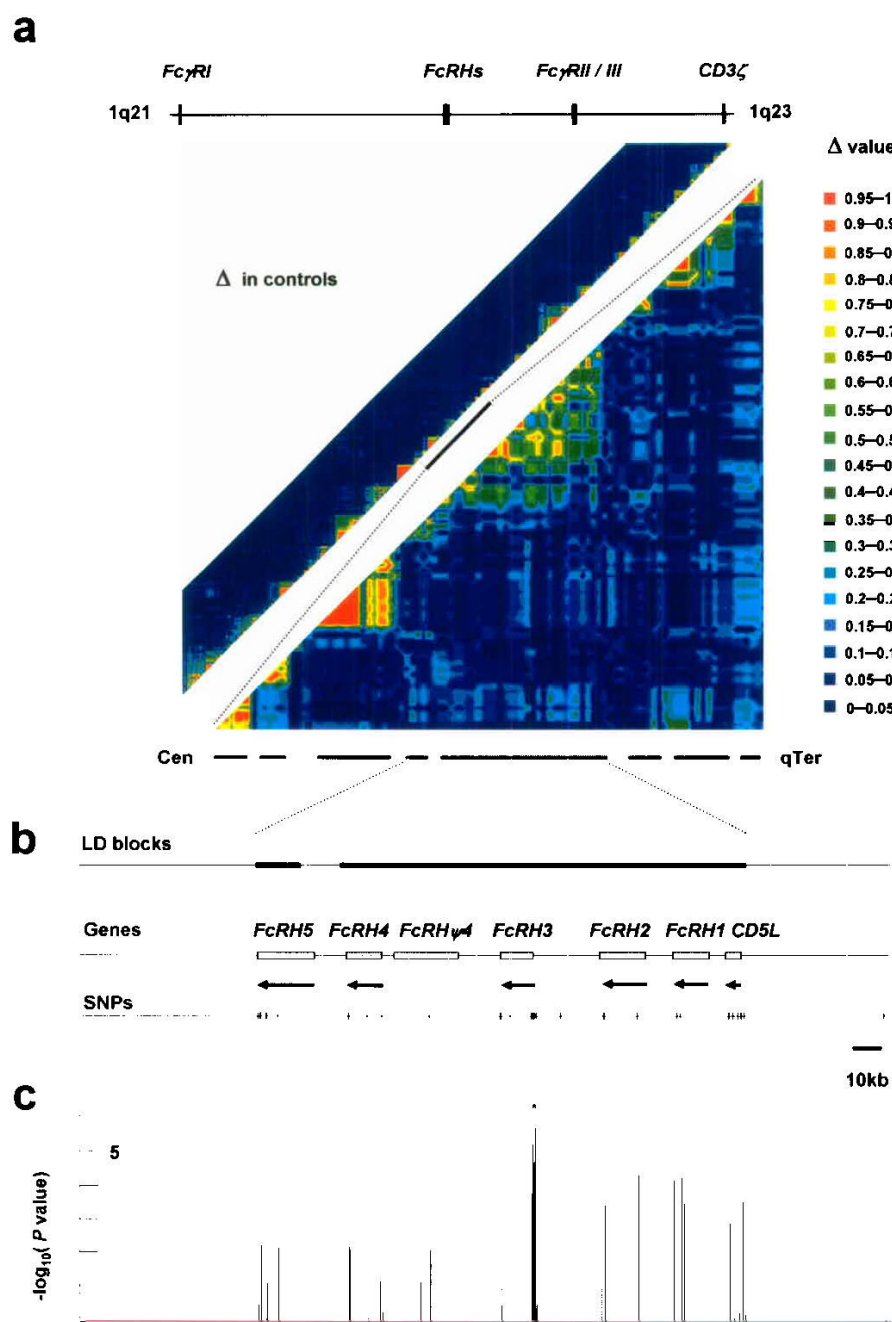
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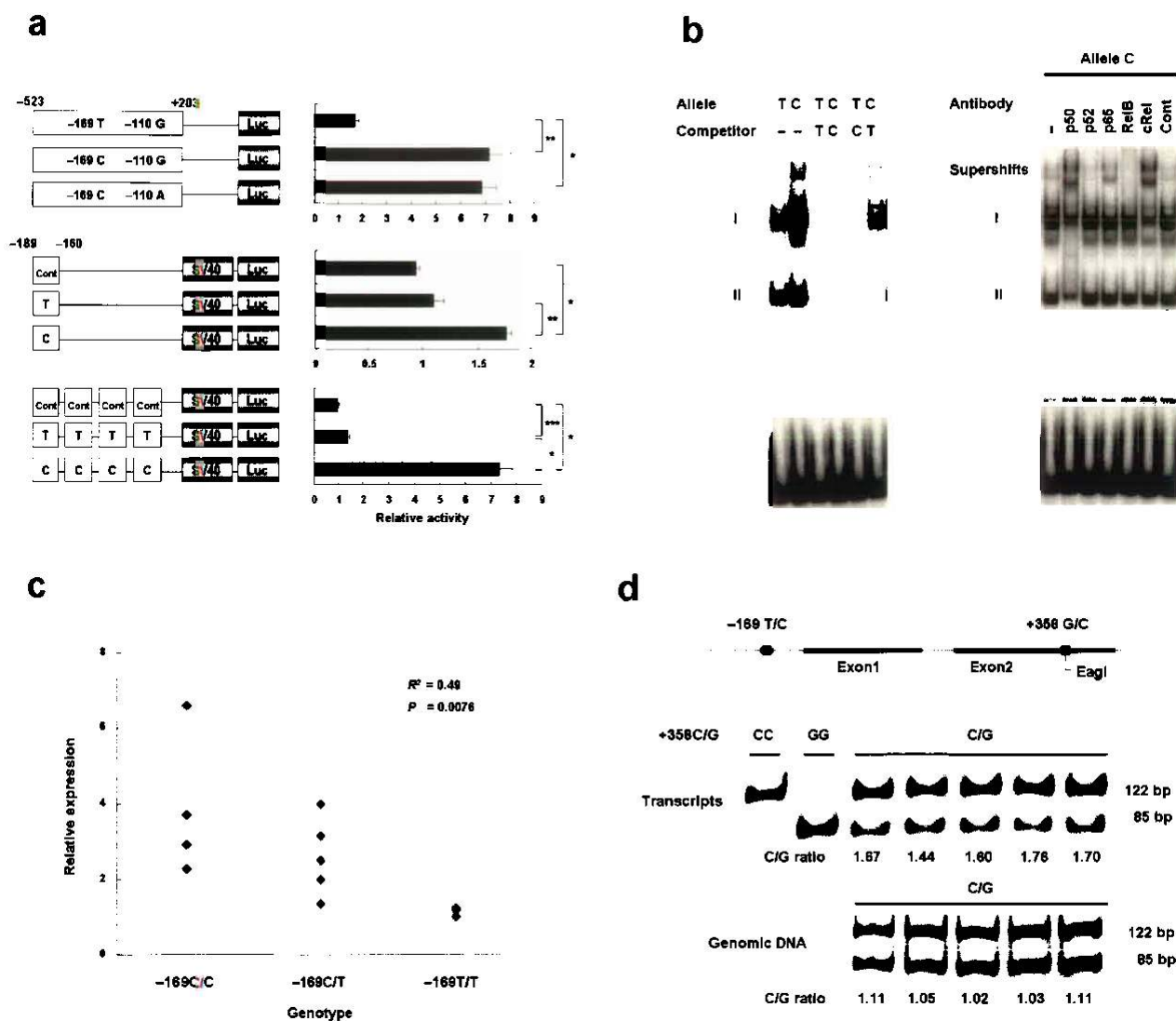
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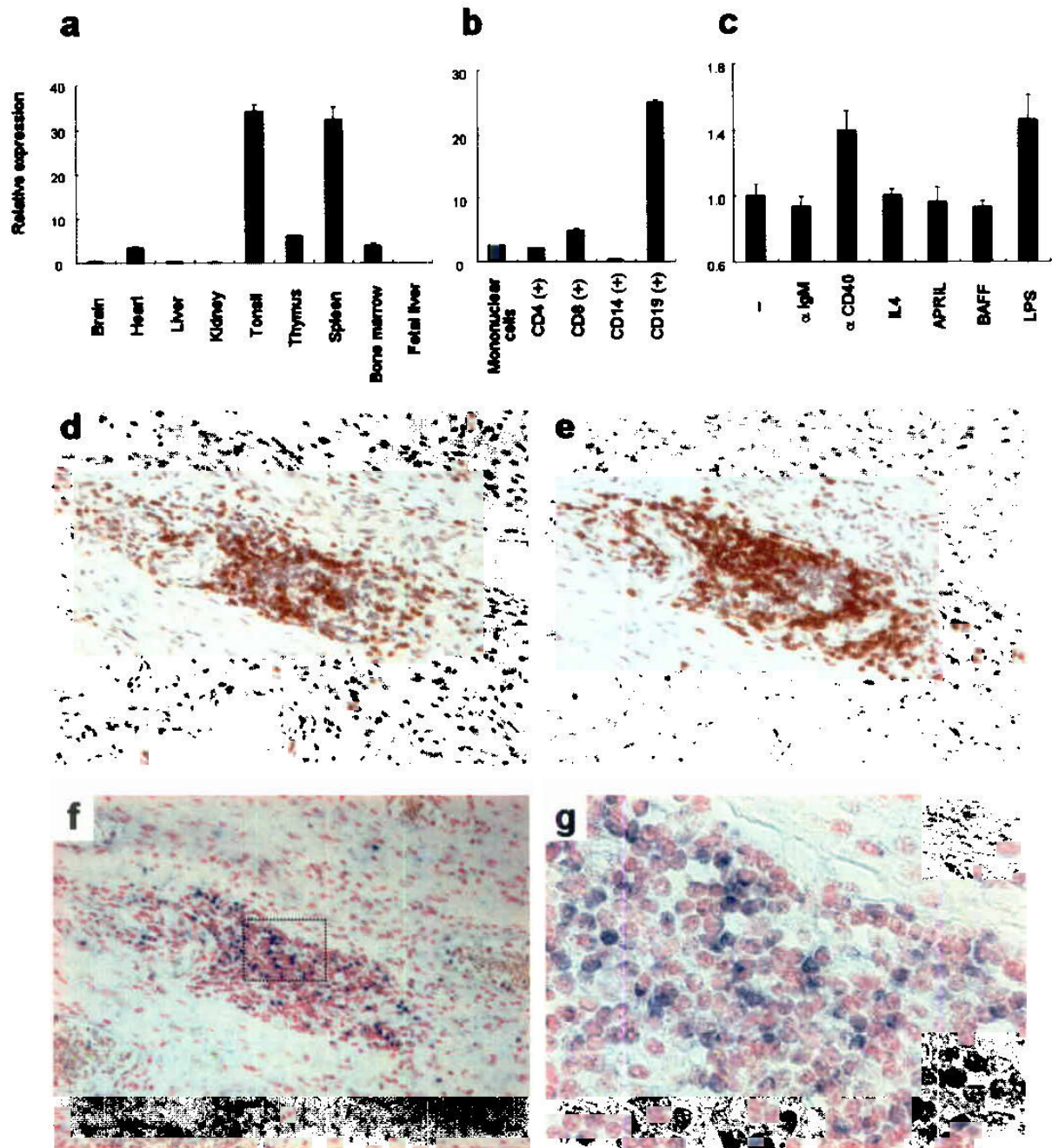
**Figure 1.**

LD and association of the *FcRH* gene cluster. **(a)** Pairwise LD between SNPs, as measured by  $\Delta$  in 658 controls. The 16-Mb region in 1q21-23 (upper left) and the 2-Mb region around the *FcRH* gene cluster (lower right) were evaluated. **(b)** Location of LD blocks, genes and 41 SNPs in the *FcRH* gene cluster. **(c)** Case-control association test with 41 SNPs in the *FcRH* gene cluster using 830 patients and 658 controls. \*Peak association.



**Figure 2.**

Correlation of *FcRH3* expression with allele and genotype. (a) Promoter activity of haplotypes in *FcRH3* (top) and enhancing activity of the 30-bp promoter region around -169 C/T (middle and bottom), as evaluated by luciferase assay. Data represent mean  $\pm$ SEM. Representative data from three experiments performed in quadruplicate. \* $P < 0.0001$ , \*\* $P < 0.001$ , and \*\*\* $P < 0.01$  by Student's *t*-test. (b) Binding affinity of nuclear factors to the 30-bp promoter region around -169C/T, evaluated by EMSA. Allelic difference and competition experiment (left) and supershift experiment using antibodies for NF- $\kappa$ B components (right). (c) Expression of *FcRH3* measured by quantitative Taqman PCR of RNA purified from CD19-positive B-cells obtained from 13 healthy volunteers (C/C,  $n=4$ ; C/T,  $n=5$ ; T/T,  $n=4$ ). (d) Allele-specific transcript quantification (ASTQ). *FcRH3* transcripts in B cells and genomic DNA from individuals ( $n=5$ ) with heterozygous genotype (-169C/+358C/-169T/+358G) were amplified and quantified using an Eag I restriction fragment length polymorphism located at position +358. The 122-bp and 85-bp bands represent transcripts of the +358C allele and +358G allele, respectively. Transcripts from homozygous individuals (+358C/C and +358G/G) are shown as controls for digestion.



**Figure 3.**

Expression patterns of *FcRH3* in human tissues and cells. **(a)** Relative expression of *FcRH3* in various tissues. **(b)** Relative expression of *FcRH3* in fractionated leukocytes using MTC panel (Clontech). **(c)** Relative expression of *FcRH3* in response to stimuli (anti-CD40 Ab, 1  $\mu$ g/ml; anti-IgM Ab, 1  $\mu$ g/ml; IL-4, 10 ng/ml; APRIL, 10 ng/ml; BAFF, 10 ng/ml; LPS 100ng/ml for 4 h). Representative data from 3 experiments performed in triplicate. **(d, e)** Lymphocyte aggregates in RA synovium. T-cells and B-cells in serial sections were immunostained using anti-CD3 (d) and anti-CD20 (e) antibodies respectively. **(f, g)** *FcRH3* mRNA expression (blue stain) in RA synovium as analyzed by *in situ* hybridization. Higher magnification views of

synovium (g) are denoted by the *box* in f (magnifications: d, e, f,  $\times 100$ ; g,  $\times 400$ ). Counterstaining: d, e, hematoxylin; f, g, nuclear fast red.

**Table 1**Case-control analysis of *FcRH3*

ID	SNPs <sup>a</sup> Location	Allele 1/2	Allele frequency		Genotype 11 versus 12+22		<i>P</i>
			Patients	Controls	OR (95% CI)	$\chi^2$	
fcrh3_3	-169	C/T	0.42	0.35	2.15 (1.58–2.93)	24.3	0.00000085
fcrh3_4	-110	A/G	0.25	0.18	3.01 (1.71–5.29)	16.1	0.000060
fcrh3_5	Exon2	C/G	0.42	0.35	2.05 (1.51–2.78)	21.6	0.0000033
fcrh3_6	Intron3	A/G	0.42	0.34	2.02 (1.49–2.75)	20.8	0.0000052

<sup>a</sup> SNPs with *P*<0.0001 in allele frequency comparison test



**Table 2**Haplotype structure and frequency in *FcRH3*

Haplotype <sup>a</sup>	fcrh3_3/4/5/6	Haplotype frequency	
		Patients	Controls
Haplotype1	TGGG	0.58	0.65
Haplotype2	CACA	0.25	0.19
Haplotype3	CGCA	0.17	0.14

<sup>a</sup>Haplotypes with frequency >0.01

**Table 3**

Genotype and autoantibodies in patients

Genotype	Rheumatoid factor		Anti-CCP antibody	
	n (N=148)	Serum level $\pm$ SEM (IU/ml)	n (N=71)	Positivity (%)
-169 C/C	29	479.9 $\pm$ 91.3 <sup>a</sup>	17	100.0 <sup>b</sup>
-169 C/T	75	323.7 $\pm$ 47.3 <sup>a</sup>	35	94.3 <sup>b</sup>
-169 T/T	44	216.4 $\pm$ 44.0 <sup>a</sup>	19	73.7 <sup>b</sup>

<sup>a</sup>  $R^2=0.049$ ,  $P=0.0065$  by regression analysis.

<sup>b</sup>  $P=0.029$  by Fisher's exact test.

**Table 4**

Association of SNP -169C/T with AITD and SLE

Disease	Number of subjects	Genotype			Allele C frequency	Recessive-trait comparison		
		CC	CT	TT		OR (95% CI)	$\chi^2$	P
GD	351	72	179	100	0.46	1.79 (1.34–2.39)	15.7	0.000074
HT	158	30	74	54	0.42	1.62 (1.07–2.47)	5.2	0.022
AITD total	509	102	253	154	0.45	1.74 (1.35–2.24)	18.5	0.000017
SLE	564	100	259	205	0.41	1.49 (1.16–1.92)	9.8	0.0017
RA+AITD +SLE <sup>a</sup>	2437	438	1167	832	0.42	1.52 (1.29–1.79)	24.2	0.00000084
Controls	2037	257	995	785	0.37			

<sup>a</sup>RA represents sum of three sets (n=1364).

GD = Graves' disease; HT = Hashimoto's thyroiditis; AITD = Autoimmune thyroid disease; SLE = Systemic lupus erythematosus.