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Identification of citrullinated eukaryotic translation initiation factor 4G1 as novel autoantigen in rheumatoid arthritis

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Abstract

Antibodies against citrullinated proteins are highly specific for rheumatoid arthritis. We previously reported that functional variants of the gene encoding peptidylarginine deiminase type 4 were closely associated with RA. The purpose of this study was to investigate the citrullinated autoantigens recognized by serum samples from patients with RA. The human chondrocyte cDNA expression library was citrullinated by PADI4 and was immunoscreened with anti-modified citrulline antibodies and sera from patients with rheumatoid arthritis. One immunoreactive cDNA clone containing a 2480-base pair insert was isolated and sequence analysis revealed that the cDNA included a part of the eukaryotic translation initiation factor 4G1. Immunoreactivity against a recombinant citrullinated eIF4G1 fragment was observed with high specificity in 50.0% of RA patients. The levels of antibodies against citrullinated eIF4G1 were correlated with those of anti-CCP antibodies. Citrullinated eIF4G1 was identified as a candidate citrullinated autoantigen in RA patients. Citrullination of eIF4G1 may thus be involved in the pathogenesis of RA.

Keywords: Autoantigen; Citrulline; Eukaryotic translation initiation factor; Peptidylarginine deiminase type 4; Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common systemic autoimmune disease of unknown etiology characterized by synovial hyperplasia with inflammatory cell infiltration, which results in joint destruction. Numerous autoantibodies against a variety of autoantigens have been detected in sera from RA patients. However, many of these autoantibodies are not specific to RA. It was recently reported that antibodies directed against citrulline-containing proteins are highly specific to RA [1,2]. Citrulline is generated post-translationally from arginine by peptidylarginine deiminase (PADI). In a genomewide case-control study of single nucleotide polymorphisms, we reported that functional haplotypes of the gene encoding PADI4 were closely associated with RA and increased PADI4 mRNA stability probably resulting in increased protein citrullination and an increased chance of developing anti-citrullinated protein antibodies [3]. Several candidate citrullinated autoantigens, such as citrullinated fibrinogen [4] and citrullinated vimentin [5], were recently reported in RA. However, the role of these proteins in the pathogenesis of RA remains unknown.

To date, several candidate autoantigens have been identified in cartilage, including type II collagen [6,7], the cartilage proteoglycan component aggrecan [8], and human cartilage glycoprotein 39 [9]. Furthermore, cartilage has come under scrutiny because ubiquitous antigen glucose-6-phosphate isomerase, which is present on the cartilage surface, induces joint-specific autoimmune disease in the spontaneous mouse arthritis model K/BxN [10,11].

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In order to identify other citrullinated autoantigens involved in the pathogenesis of RA, we used RA sera and anti-modified citrulline antibodies to screen a human citrullinated chondrocyte cDNA expression library for targets of the autoimmune process in RA. Here, we report a novel citrullinated autoantigen, eukaryotic translation initiation factor 4G1 (eIF4G1) fragment, recognized specifically by sera from patients with RA. We further discuss the immunogenic features of the protein and its possible role as a substrate of PADI4.

Materials and methods

Human sera. Serum samples were obtained from a total of 100 patients with RA (84 females and 16 males; mean age: 61.1 years; range: 27–81 years). All patients satisfied the 1987 revised criteria of the American College of Rheumatology. A total of 34 serum samples were obtained from patients with other rheumatic diseases (29 females and 5 males; mean age: 43.5 years; range: 24–82 years), including systemic lupus erythematosus ($n = 18$), Sjögren's syndrome ($n = 4$), Churg–Strauss syndrome ($n = 2$), Behçet's disease ($n = 2$), polyarteritis nodosa ($n = 1$), systemic sclerosis ($n = 1$), mixed connective tissue disease ($n = 1$), polymyalgia rheumatica ($n = 1$), polymyositis/dermatomyositis ($n = 1$), anti-phospholipid syndrome ($n = 1$), pustulosis palmoplantaris ($n = 1$), and ulcerative colitis ($n = 1$). Patients were receiving treatment at the University of Tokyo Hospital. Written informed consent was obtained from all patients.

Control sera were obtained from 44 healthy donors (29 females and 15 males; mean age: 50.5 years; range: 23–78 years). All serum samples were stored at −20 °C until assay.

Immunological screening of cDNA libraries. Full-length human PADI4 cDNA was obtained by polymerase chain reaction using human bone marrow cDNA as a template [3] and was cloned into the prokaryotic expression vector pDEST17 (Invitrogen, San Diego, CA). His-tagged PADI4 was expressed in *Escherichia coli* BL21-SI (Invitrogen) by sodium chloride induction. Fusion protein was purified on a HiTrap column (Amersham Life Science, Cleveland, Ohio) according to the manufacturer's instructions. PADI activity was determined using *N*-benzoyl-L-arginine ethyl ester (BAEE, Sigma-Aldrich, St. Louis, MO) as a substrate, as described previously [12]. One unit of enzyme activity was defined as the activity required to produce 1 μmol of L-citrulline derivatives in 1 h at 37 °C.

XL1-Blue-MRF′ *E. coli* were infected with 1.5×10^4 plaque-forming units per plate (90×15 mm) of phage from the human chondrocyte lambda ZAP cDNA library following the manufacturer's instructions (Stratagene, La Jolla, CA). The resulting plaques were transferred onto nitrocellulose membranes treated with 20 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Membranes were incubated with 1 unit of recombinant PADI4 overnight at 37 °C and were modified with 2, 3-butanedione monoxime and antipyrine in a strong acid solution (Upstate Biotechnology, Lake Placid, NY). The membranes were then incubated with rabbit polyclonal anti-modified citrulline antibody (Upstate). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (Upstate) were used as secondary antibodies and were visualized with enhanced chemiluminescence (ECL) reagents on Hyperfilm (Amersham). cDNA expression libraries were also screened with pooled sera of 5 patients with RA. Pooled sera of patients with RA were preadsorbed with *E. coli* lysate in order to reduce background sera activity. Immunoscreening was repeated using the same assay procedure until the positive phage reached clonality.

DNA sequencing and database searches. Positive phage clones were converted into phagemids by in vivo excision with the helper phage ExAssist and *E. coli* SOLR (Stratagene). Recombinant plasmids were then purified on Qiagen Maxi Prep columns (Qiagen) and were subjected to DNA sequencing. The cDNA inserts were sequenced on ABI3700 capillary sequencers (Applied Biosystems) using standard T3 forward and T7 reverse primers. cDNA sequences were subjected to a BLAST search of the genetic databases of the National Center for Biotechnology Information.

Expression and purification of recombinant fusion proteins. cDNA encoding the eIF4G1 fragment (2480 bp) was cloned into the expression vector pDEST17. *E. coli* BL21-SI transformed with this recombinant pDEST17 plasmid was grown at 30 °C, and sodium chloride was added in order to induce expression of recombinant protein. Fusion protein was purified on a column charged with Ni–NTA agarose (Qiagen).

Enzyme-linked immunosorbent assay. Each well of the microplates (Nunc, Rocester, NY) was coated with 100 μl of 5 μg/ml recombinant eIF4G1 fragment in carbonate buffer overnight at 4 °C. Wells were washed with Tris-buffered saline (TBS) and incubated for 3 h at 37 °C with 0.01 U/well of recombinant PADI4 or PADI4 in 50 mM EDTA in order to detect reactivity against citrullinated or uncitrullinated eIF4G1 fragment. Wells without recombinant protein were simultaneously prepared for non-specific background examination. To confirm citrullination of eIF4G1 fragment, wells were washed with Tris-buffered saline with 0.05% Tween 20 (TBS-T) and then incubated with 0.1% ovalbumin. The modified citrullinated eIF4G1 was detected by anti-modified citrulline antibody (Upstate) according to manufacturer's instruction. To detect anti-eIF4G1 or anti-citrullinated eIF4G1 antibodies in human sera, wells were washed with TBS-T, followed by blocking with 5% skim milk. Patients and control sera were diluted at 1:100 with TBS-T containing 5% skim milk and were preincubated with bacterial lysate in order to adsorb the reactivity to bacterial proteins. After reacting with coated recombinant proteins for 2 h at room temperature, wells were washed 4 times with TBS-T. Bound antibodies were incubated with horseradish peroxidase-conjugated goat $F(ab')_2$ anti-human IgG antibody (Biosource, Camarillo, CA) diluted at 1:50,000 and reacted with 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate. Sample optical density (OD) values were calculated as OD values for antigen-coated wells minus those for uncoated wells. Anti-CCP titers were determined using DIASTAT Anti-CCP Test (Axis-Shield Diagnostics, Dundee, Scotland, UK) according to manufacturer's instruction.

Immunoblotting. Recombinant eIF4G1 fragment (200 ng/lane) was incubated with 0.3 U of recombinant PADI4 overnight at 37 °C and was separated by electrophoresis on 10% SDS–polyacrylamide gels. Separated proteins were transferred onto a nitrocellulose membrane (Amersham), blocked with TBS-T containing 10% skim milk, and were incubated with goat anti-human eIF4G1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or serum samples, diluted at 1:100 in TBS-T with 5% skim milk-containing bacterial lysate. Horseradish peroxidase-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology) or goat $F(ab')_2$ anti-human IgG antibodies (Biosource) were used as secondary antibodies and were detected with ECL reagents (Amersham).

DNA extraction and genotyping. DNA from RA patients ($n = 39$) was extracted and PADI4 genotype was determined as described previously [3].

Statistical analysis. Statistical analysis was performed using Mann–Whitney U test, Pearson's correlation test, and χ^2 test. p values of less than 0.05 were considered to be statistically significant.

Results

Identification and expression of eIF4G1

Screening of the human citrullinated chondrocyte cDNA library identified a total of 13 clones. One of the clones reacted strongly with both anti-modified citrulline antibodies and RA pooled sera. The nucleotide sequence of this clone, which contained a 2480-bp insert, matched the 3′-region of the eIF4G1 sequence (5317 bp, GenBank Accession No. AY082886, Fig. 1A). The eIF4G1 fragment was cloned into the expression vector pDEST17 and was expressed as a His-tagged fusion protein. The purified

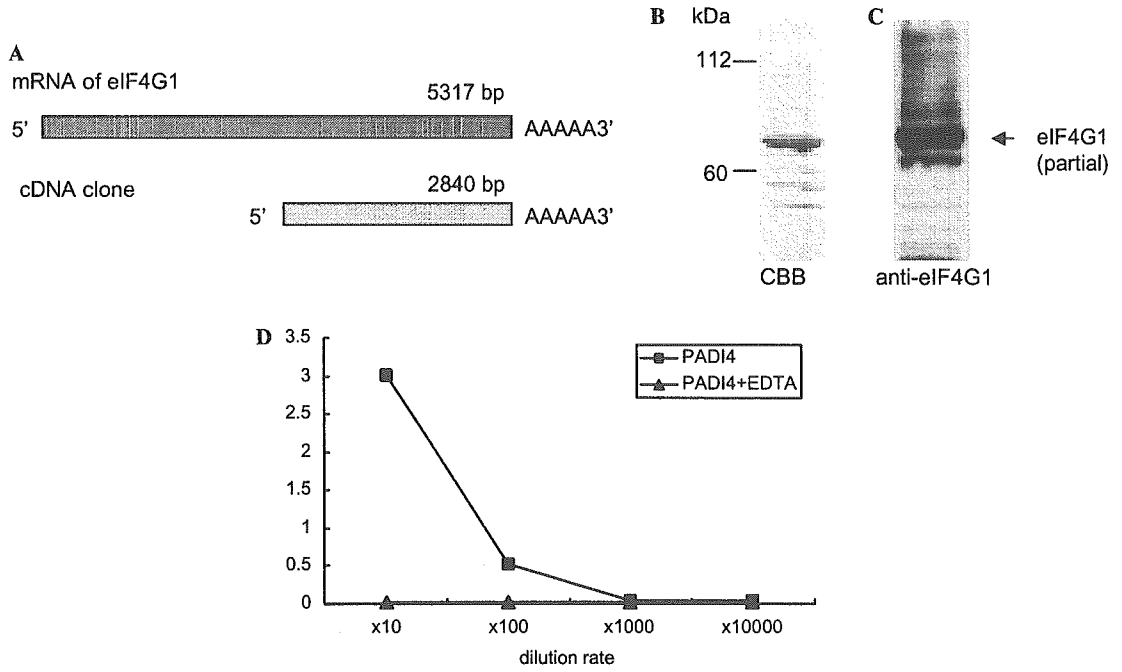

Fig. 1. Expression and immunoblotting of recombinant eIF4G1 fragment. (A) Insert of cDNA clone compared with the known eIF4G1 mRNA (bp, base pairs). (B) Purified recombinant eIF4G1 fragment was loaded onto 10% SDS–polyacrylamide gel and stained with Coomassie brilliant blue (CBB). (C) Separated proteins were transferred to nitrocellulose membrane, and immunoblotting using anti-eIF4G1 antibodies was performed. (D) Each dilution of recombinant eIF4G1 was incubated with PADI4 (■) or PADI4 in 50 mM EDTA (▲) and detected by anti-modified citrulline antibodies.

fusion protein had the expected molecular weight (Fig. 1B). Furthermore, immunoblotting confirmed that the recombinant eIF4G1 fragment reacted with anti-eIF4G1 antibodies (Fig. 1C). We also confirmed the citrullination of recombinant eIF4G1 fragment using ELISA (Fig. 1D).

Serum levels of antibodies against eIF4G1 and citrullinated eIF4G1

We used ELISA to investigate the prevalence of antibodies against uncitrullinated or citrullinated eIF4G1 fragment in patients with rheumatic diseases and in healthy individuals. As shown in Fig. 2, serum levels of IgG-type antibody against uncitrullinated eIF4G1 were higher in RA than in healthy controls ($p < 0.01$). Serum antibody levels against citrullinated eIF4G1 were also higher in RA than in controls ($p < 0.0001$) as well as in other rheumatic diseases ($p < 0.0001$). Anti-CCP antibody levels were also significantly higher in RA group than in healthy controls ($p < 0.0001$) or other rheumatic disease groups ($p < 0.0001$).

We defined the mean + 2SD value in control subjects as the cut-off value for anti-eIF4G1 or citrullinated eIF4G1 antibodies. The prevalence of anti-eIF4G1 antibodies in RA was 12.0%. After citrullination of eIF4G1, the prevalence of anti-citrullinated eIF4G1 increased to 50.0%. This percentage was significantly larger than those in controls and in other rheumatic diseases ($p < 0.001$, χ^2 test). All serum samples that were positive for anti-eIF4G1 were also positive for anti-citrullinated eIF4G1.

In RA, anti-citrullinated eIF4G1 antibody titers were significantly correlated with anti-cyclic citrullinated peptide (CCP) antibodies (Fig. 3). These results indicate that anti-citrullinated eIF4G1 antibodies are candidate citrullinated autoantigens in patients with RA.

Detection of antibodies against eIF4G1 and citrullinated eIF4G1 by immunoblotting

Positive serum samples were further examined by immunoblotting. We first confirmed that eIF4G1 was citrullinated by PADI4 using anti-modified citrulline antibodies. Of the 12 samples that were positive for anti-eIF4G fragment by ELISA, 9 were confirmed to recognize eIF4G1 fragment. Of the 50 samples that were positive for anti-citrullinated eIF4G1 by ELISA, 48 reacted to citrullinated eIF4G1. Representative results are shown in Fig. 4. Some RA sera also reacted with recombinant PADI4. These results agree with our previous findings that antibodies against PADI4 are present in RA patients [13].

Relationship between PADI4 haplotype and antibody against citrullinated eIF4G1

Table 1 shows the relationship between PADI4 haplotype and the presence of antibodies against citrullinated eIF4G1 or CCP in sera from patients with RA. Patients who were positive for antibody against citrullinated eIF4G1 were more likely to possess the susceptible allele (59.5%) than patients who were negative for antibody

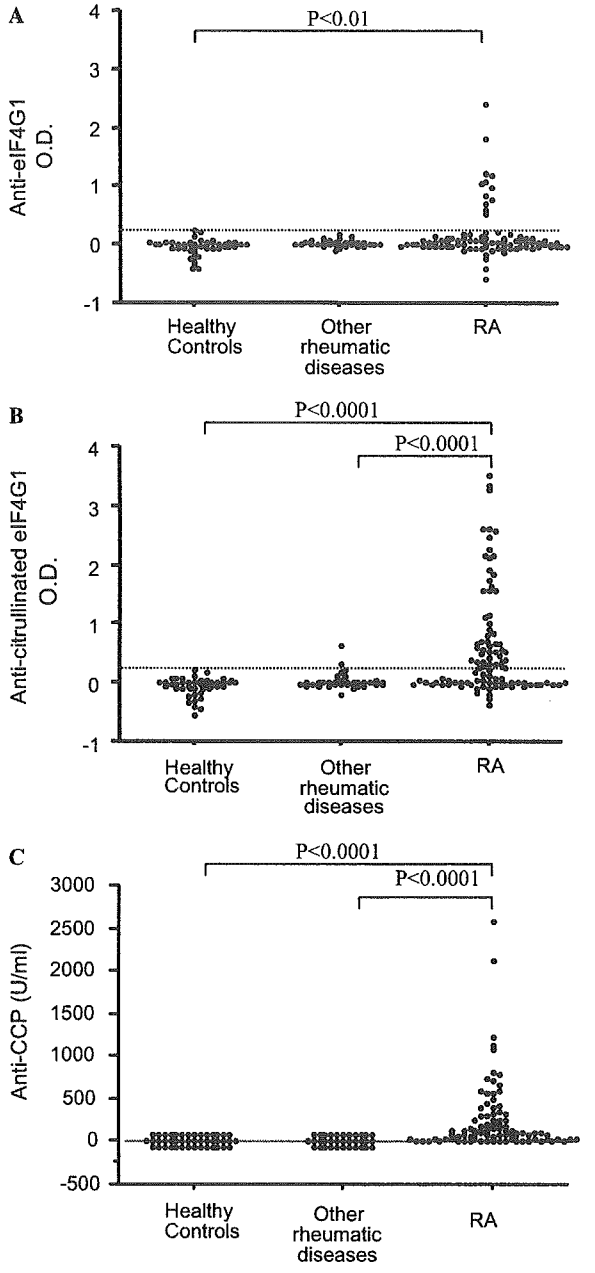

Fig. 2. Presence of autoantibodies against recombinant eIF4G1 fragment (A), citrullinated eIF4G1 fragment (B), and CCP (C) in sera from patients with RA, other rheumatic diseases, and healthy controls. Patient and control sera were diluted at 1:100. Dotted line represents cut-off value, which was calculated as the mean OD + 2SD in control subjects.

against citrullinated eIF4G1 (36.1%, $p = 0.03$, χ^2 test). On the other hand, the relationship between PADI4 haplotype and anti-CCP antibody was not significant ($p = 0.47$, χ^2 test).

Discussion

We identified citrullinated eIF4G1 fragment as a novel RA-specific autoantigen by immunoscreening a human chondrocyte cDNA expression library. eIF4G is a translational initiation factor and is recognized as the central organizing protein in recruitment of 43S preinitiation complex to mRNA [14]. There are two isoforms of eIF4G in mammals: eIF4G1 and eIF4G2. eIF4G1 is associated with two other factors, the cap-binding protein eIF4E and the RNA helicase eIF4A, in the large protein complex eIF4F [14]. Previous studies have shown that antibodies against eIF4G1 were present in a patient with squamous cell lung carcinoma [15]. However, the involvement of eIF4G1 or citrullinated eIF4G1 in the pathogenesis of RA is not certain.

RA is accompanied by the generation of numerous autoantibodies in the serum of patients. Although most of these autoantibodies are not specific to RA, autoantibodies against citrullinated proteins are reported to be highly specific for RA [1]. Candidate citrullinated autoantigens recognized in RA sera are filaggrin [16], keratin [17], vimentin [5], and fibrinogen or fibrin [4], but the true autoantigens in RA are unknown. This prompted research into other candidate citrullinated autoantigens.

Citrullination is mediated by PADI, a calcium-dependent enzyme that catalyzes the post-translational conversion of arginine residues to citrulline. There are five isoforms of PADI [18] and PADI4 is reported to be particularly important in RA [3]. Citrullination by PADI4 may play a critical role in breaking tolerance of RA by altering the antigenicity of native self-peptides. Therefore, we selected a cDNA expression library of proteins citrullinated by PADI4. In addition, this method enabled detection of trace or insoluble autoantigens.

Using the recombinant eIF4G1 fragment, we found that the mean sensitivity of the ELISA was 50.0%, with 97.4% specificity and 96.2% positive predictive value. The specificity and positive predictive value of this assay were comparable to those of the CCP-assay [19,20]. Of the anti-citrullinated eIF4G1-positive RA sera, most of them were positive for anti-CCP. Furthermore, anti-citrullinated eIF4G1 antibody titers were significantly correlated with anti-CCP antibody titers. This correlation suggests that anti-citrullinated eIF4G1 antibodies compose a subset of anti-CCP antibodies. It has been reported that antibodies against citrullinated proteins are heterogeneous [1]. Antibodies against citrullinated eIF4G1 may thus be one of several antibodies directed against citrullinated proteins.

Three samples that were positive for anti-eIF4G1 and two samples that were positive for anti-citrullinated eIF4G1 by ELISA did not recognize eIF4G1 or citrullinated eIF4G1 by immunoblotting. In these cases, antibodies may target conformational epitopes of eIF4G1.

It was recently reported that PADI4 regulated histone arginine methylation by converting methyl-arginine to citrulline, thus affecting chromatin structure [21,22]. In a similar way, PADI4 may catalyze other molecules, such as eIF4G1, under physiological conditions. Because PADI4 enzyme needs high calcium ion concentration for its enzyme activity [23], citrullination is reported to occur

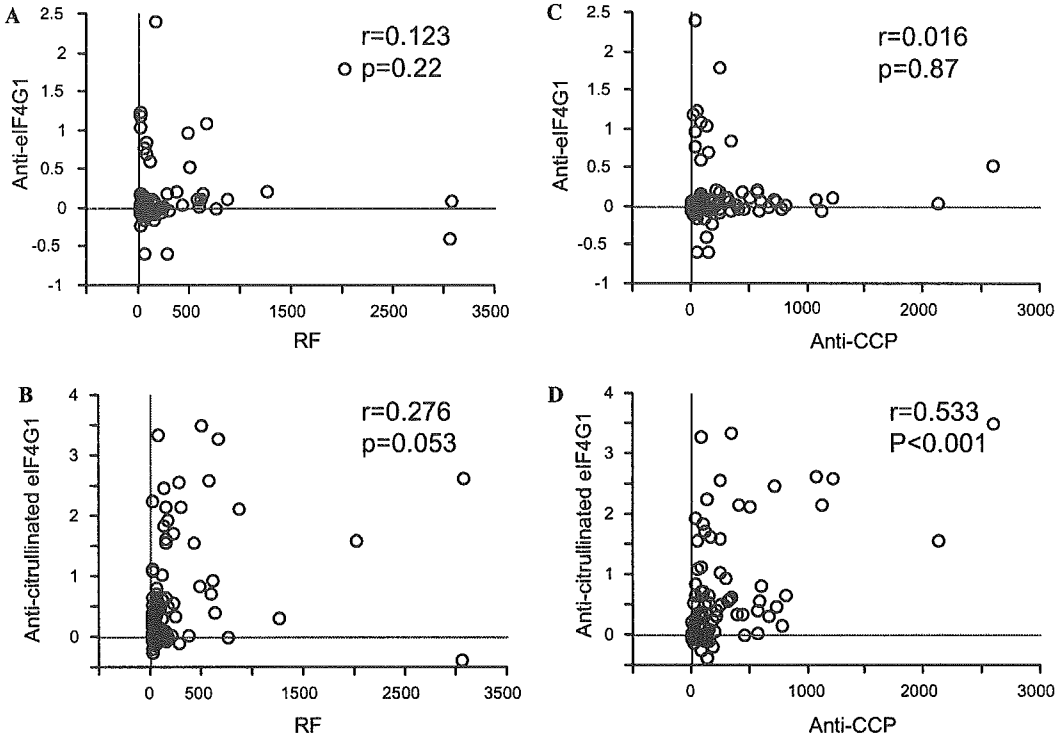

Fig. 3. Correlation between disease markers and anti-eIF4G1 or anti-citrullinated eIF4G1 antibody titer in patients with RA. Correlation between RF levels and anti-eIF4G1 (A) or anti-citrullinated eIF4G1 (B) levels. Correlation between anti-CCP levels and anti-eIF4G1 (C) or anti-citrullinated eIF4G1 (D) levels. Correlation coefficient (r) and p value are shown.

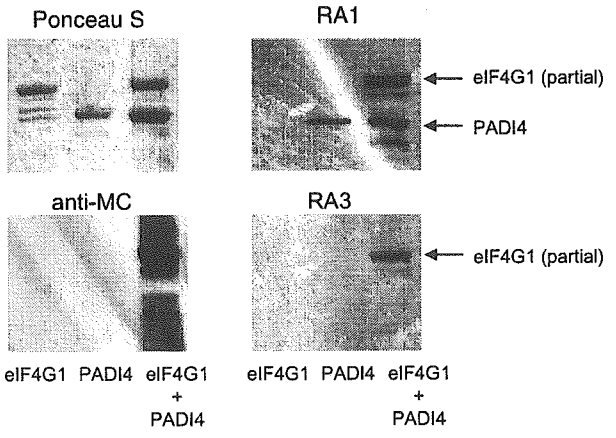

Fig. 4. Immunoreactivity of uncitrullinated or citrullinated eIF4G1. Recombinant eIF4G1 fragment in the presence or absence of PADI4 was separated by 10% SDS-PAGE and was transferred onto nitrocellulose membranes. Serum samples that were positive for anti-citrullinated eIF4G1 on ELISA were examined by immunoblotting. Control membranes were stained with Ponceau S. Citrullination of eIF4G1 fragment by PADI4 was confirmed using anti-modified citrulline antibodies. RA1, RA3: RA patient sera. Representative samples are shown.

Table 1
Association between PADI4 haplotype and antibody to citrullinated eIF4G1 or CCP

	PADI4 susceptible allele	PADI4 non-susceptible allele
Anti-citrullinated eIF4G1		
Positive	25	17
Negative	13	23
Anti-CCP		
Positive	33	37
Negative	5	3

during apoptosis [24,25]. Apoptosis induces degradation or modification of various proteins and the generation of new epitopes. If these proteins are cleared incorrectly, they could break tolerance by epitope spreading and this may trigger specific autoimmune responses [26]. eIF4G1 is reported to be cleaved by caspase 3 during the early stages of apoptosis [27]. Furthermore, our results showed that antibodies against uncitrullinated eIF4G1 were present in 12% of RA serum samples, which suggests that epitope spreading to uncitrullinated regions of eIF4G1 has occurred. In fact, all serum samples that recognized uncitrullinated eIF4G1 were positive for anti-citrullinated eIF4G1, indicating reactive epitopes spread from epitopes around the citrullinated regions to uncitrullinated parts of the molecule. Citrullinated eIF4G1 might play an important role in triggering autoimmunity in RA. To determine the independence of antigenicity of each antigen, we have done affinity purification and absorbing experiment

according to the method of Olmsted [28]. Serum absorbed with eIF4G1 showed higher titer of citrullinated eIF4G1 than that of eIF4G1, and affinity purified anti-eIF4G1 showed higher titer of eIF4G1 than that of citrullinated eIF4G1 (data not shown). However, as to the affinity purification and absorbing experiments using citrullinated eIF4G1, we could not show the independence of the antigenicity, probably because of the small amount of anti-citrullinated eIF4G1 antibodies in the patients' sera. Taken together, we can suggest that at least a part of patient's sera recognized different epitopes of eIF4G1 and citrullinated eIF4G1, according to our additional experiments. However, it is difficult to determine the overlapping of epitopes. Further investigation is needed whether citrullination of eIF4G1 occurs in vivo and citrullinated eIF4G1 actually contributes to breaking immunological tolerance.

We expressed only a portion of eIF4G1 and investigated its antigenicity in this experiment. The antigenicity of eIF4G1 or citrullinated eIF4G1 using full-length protein should be examined in the future. Recombinant eIF4G1 fragment contained 58 arginine residues, and it is uncertain which arginine residues are citrullinated in the recombinant protein. Further study is needed in order to identify which citrulline residues are important for antigenicity of epitopes in RA.

With regard to the association between PADI4 haplotype and the presence of antibodies against citrullinated eIF4G1, despite the small number, patients who were positive for antibodies against citrullinated eIF4G1 tended to possess a susceptible PADI4 allele. These findings indicate that PADI4 mRNA stability affects the generation of anti-citrullinated eIF4G1 antibodies and suggests the contribution of citrullinated eIF4G1 to the pathophysiology of RA.

In summary, we demonstrated that citrullinated eIF4G1 is a candidate autoantigen in RA patients. Our results show that citrullination of eIF4G1 may be involved in the pathogenesis of RA.

Acknowledgments

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A functional variant in *FCRL3*, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities

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Rheumatoid arthritis is a common autoimmune disease with a complex genetic etiology. Here we identify a SNP in the promoter region of *FCRL3*, a member of the Fc receptor-like family, that is associated with susceptibility to rheumatoid arthritis (odds ratio = 2.15, $P = 0.00000085$). This polymorphism alters the binding affinity of nuclear factor-κB and regulates *FCRL3* expression. We observed high *FCRL3* expression on B cells and augmented autoantibody production in individuals with the disease-susceptible genotype. We also found associations between the SNP and susceptibility to autoimmune thyroid disease and systemic lupus erythematosus. *FCRL3* may therefore have a pivotal role in autoimmunity.

Rheumatoid arthritis is 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, its complete etiology has not yet been clarified[1]. Specific haplotypes of human leukocyte antigen (HLA)-DRB1, usually referred to as shared-epitope sequences[2], have been repeatedly reported to confer susceptibility to rheumatoid arthritis[3,4]; other genetic components are also involved[5]. This combination of HLA haplotypes and non-HLA genes accounting for disease susceptibility is also observed for other autoimmune diseases[6–8]. In autoimmune thyroid disease (AITD), for instance, the *HLA-DR3* haplotype is associated with disease risk, as is a functional haplotype of a non-HLA gene, *CTLA4*, that has recently been associated with AITD susceptibility[9].

Identification of non-HLA genes associated with rheumatoid arthritis susceptibility and other autoimmunities seems difficult, because of 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 done for both human autoimmune diseases and experimental animal models. These studies have identified nonrandom clustering of susceptibility loci for clinically distinct diseases[8,10]. The overlap of susceptibility loci for multiple autoimmunities suggests that common susceptibility genes exist in those regions. Intense studies of loci-clustering regions identified genes commonly associated with multiple autoimmune diseases, such as *CTLA4* on 2q33 (ref. 9), *SLC22A4* and *SLC22A5* 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 at 1q23, and a new family of genes, Fc receptor-like genes (FCRLs, also known as FcRHs[13,14], IRTAs[15,16] or SPAPs[17]), clusters nearby at 1q21 (**Fig. 1a**). FCRLs have high structural homology with classical FcγRs, although their ligands and function are not yet known. These receptors are good candidates for involvement in autoimmunity, as they are believed to be involved in the pathogenesis of rheumatoid arthritis and other autoimmune diseases[18]. Region 1q23 is a candidate locus for susceptibility to systemic lupus erythematosus

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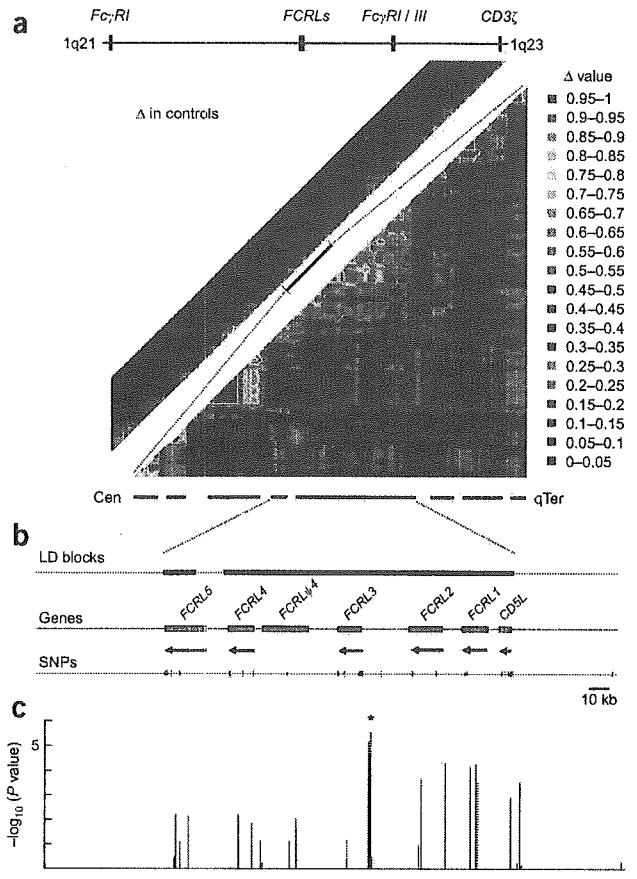


Figure 1 LD and association of the FCRL gene cluster. (**a**) Pairwise LD between SNPs, as measured by Δ in 658 controls. The 16-Mb region in 1q21–23 (upper left) and the 2-Mb region around the FCRL gene cluster (lower right) were evaluated. (**b**) Location of LD blocks, genes and 41 SNPs in the FCRL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

(SLE), and variants in the classical FcγR II/III genes partially account for disease susceptibility[6,19]. Region 1q21 is a candidate locus for susceptibility to psoriasis (*PSORS4*; refs. 7,20) and multiple sclerosis[21]. The mouse homologous region to human 1q21, on chromosome 3, also contains susceptibility loci for multiple autoimmune disease models[8], including collagen-induced arthritis (*Cia5*, also called *Mcia2* (ref. 22); *Eae3* (ref. 23); *Tmevd2* (ref. 24); *Idd10*; and *Idd17* (ref. 25)). Although 1q21–23 is a good candidate region for containing rheumatoid arthritis–susceptibility genes, the association of classical FcγRs with disease susceptibility remains controversial[26,27]. Here we focused on the 1q21–23 region to identify rheumatoid arthritis–associated genes in Japanese subjects using linkage disequilibrium (LD) mapping.

RESULTS

Case-control study by SNP-based LD mapping at 1q21–23

To evaluate the extent of association, we analyzed LD with SNPs distributed in a 16-Mb region on 1q21–23, including the FCRL gene cluster and the classical FcγRs (**Fig. 1a**). We genotyped 658 control subjects for 742 SNPs from the JSNP database and selected 491 SNPs with allele frequency >0.1, successful genotyping rate >0.95 and $P > 0.01$ with Hardy-Weinberg equilibrium testing for evaluation of LD. We calculated the pairwise LD index Δ (ref. 28) for each pair of SNPs, identifying 110 LD blocks[11] at a threshold of $\Delta > 0.5$ (**Fig. 1a**).

For association testing, we examined the Japanese set of 830 cases and 658 controls used for LD block evaluation. We initially genotyped 94 rheumatoid arthritis cases for 491 SNPs and compared their allele frequencies with those of 658 control subjects. We identified nine SNPs that had allele frequencies differing by more than 0.1 between 658 controls and 94 cases with $P < 0.01$. We genotyped the remaining cases for these nine SNPs and tested their allele frequencies for case-control association. We identified the smallest P value between an intronic SNP in the gene *FCRL3* and rheumatoid arthritis (fcrl3_6, $P = 1.8 \times 10^{-5}$; association was statistically significant in both rheumatoid arthritis subgroups (94 and 736 individuals)). This SNP was located in a LD block containing four of the five FCRL genes; the fifth was in the adjacent block. We therefore evaluated the origin of this association in these two LD blocks (**Fig. 1b**), although our results do not exclude the presence of variants associated with rheumatoid arthritis or other autoimmune diseases in other LD blocks at 1q21–23.

In addition to the 25 SNPs of the 491 that we used for LD block evaluation, we identified 16 additional SNPs in exons and 5′ and 3′ flanking regions of five FCRL genes and one pseudogene (*FCRLψ4*) by searching the public database and sequencing genomic DNA from Japanese individuals with rheumatoid arthritis. We genotyped these 16 SNPs in the identical case and control samples (830 cases, 658 controls) to increase the density of variants in the targeted region. We observed a peak of association in a short segment consisting of four SNPs in *FCRL3* ($P < 1.0 \times 10^{-4}$; **Fig. 1c** and **Supplementary Table 1** online): fcrl3_3, fcrl3_4, fcrl3_5 and fcrl3_6, located at nt −169, −110, +358 (5′ untranslated region of exon 2) and +1381 (intron 3; 204 bp and 859 bp from the 3′ and 5′ ends of the flanking exons) relative to the transcription initiation site, respectively.

We observed the smallest P value without correction in recessive-trait genotype comparison of fcrl3_3 in *FCRL3* ($P = 8.5 \times 10^{-7}$; odds ratio = 2.15; 95% confidence interval = 1.58–2.93; **Table 1**). This

Table 1 Case-control analysis of *FCRL3*

			Allele 1 frequency		Genotype 11 versus 12 + 22		
SNP	Location	Allele (1/2)	Affected individuals	Controls	OR (95% c.i.)	χ^2	P
fcrl3_3	−169	C/T	0.42	0.35	2.15 (1.58–2.93)	24.3	0.00000085
fcrl3_4	−110	A/G	0.25	0.18	3.01 (1.71–5.29)	16.1	0.000060
fcrl3_5	Exon 2	C/G	0.42	0.35	2.05 (1.51–2.78)	21.6	0.0000033
fcrl3_6	Intron 3	A/G	0.42	0.34	2.02 (1.49–2.75)	20.8	0.0000052

SNPs with $P < 0.0001$ in allele frequency comparison test are shown. c.i., confidence interval; OR, odds ratio.

Table 2 Haplotype structure and frequency in *FCRL3*

Haplotype	Sequence (fcrl3_3-4-5-6)	Frequency	
		Affected individuals	Controls
1	TGGG	0.58	0.65
2	CACA	0.25	0.19
3	CGCA	0.17	0.14

Haplotypes with frequency >0.01 are shown.

P value was still significant when the most conservative Bonferroni correction was applied (comparisons for 507 SNPs; corrected $P = 0.00043$). The four strongly associated SNPs were in LD with each other, and we inferred three common haplotypes (**Table 2**); fcrl3_3, fcrl3_5 and fcrl3_6 showed strong LD with each other ($\Delta > 0.99$), whereas fcrl3_4 showed relatively weak LD with the other three SNPs (mean $\Delta = 0.68$).

To identify causal variants in this segment on the basis of genotype data, we carried out a forward stepwise-regression procedure with a cut-off *P* value to proceed to the next step of 0.01 (ref. 29). No SNP in FCRL genes other than *FCRL3* improved the model. None of the four SNPs in *FCRL3* were preferred over the others in these data (data not shown). This result implied that one of the SNPs in *FCRL3* might cause the disease, but the possibility remained that variants in other genes were truly associated with the disease.

To validate the case-control association test, we evaluated the impact of population stratification on the case-control study (830 cases, 658 controls). We selected 2,069 SNPs, each of which was identified as a tagging SNP[30] in 2,069 distinct LD segments that were previously identified by genotyping 74,842 SNPs distributed in autosomal chromosomes[31]. We analyzed population structure[32] and the χ^2 sum[33] to evaluate stratification but detected no significant evidence of population stratification (**Supplementary Fig. 1** online). These results are suggestive of no or negligible stratification of our samples and support the validity of the case-control association results by removing this confounding factor from further consideration.

Regulatory effect of SNP −169C→T on *FCRL3* expression

Because none of the four SNPs in *FCRL3* (fcrl3_3, fcrl3_4, fcrl3_5 and fcrl3_6) produces amino-acid substitutions, we assessed potential effects of the SNPs on transcription factor binding using TRANSFAC software. Nuclear factor-κB (NF-κB) was predicted to bind the sequence containing the rheumatoid arthritis–susceptibility allele fcrl3_3 (−169C) with a high score (core match 1.000, matrix match 0.957); substitution with the nonsusceptible allele T decreased the score of NF-κB binding substantially (core match 0.760, matrix match 0.824). The other three 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 fcrl3_3 to explore the regulatory effects on expression of *FCRL3*.

We carried out reporter gene analysis using the genomic sequence of *FCRL3* from nt −523 to +203. We made constructs corresponding to the three haplotypes using SNPs at nt −169 (C→T, fcrl3_3) and −110 (G→A, fcrl3_4; **Fig. 2a**) and used them to transfect Raji cells, a Burkitt's lymphoma cell line that expresses *FCRL3* (ref. 13) and is derived from germinal center B cells. Luciferase activity was substantially greater in cells transfected with −169C −110G or −169C −110A constructs than in cells transfected with −169T −110G constructs. This suggests that SNP −169C→T is crucial for regulation of *FCRL3* expression. To clarify, we cloned single or four tandem copies of 30-bp

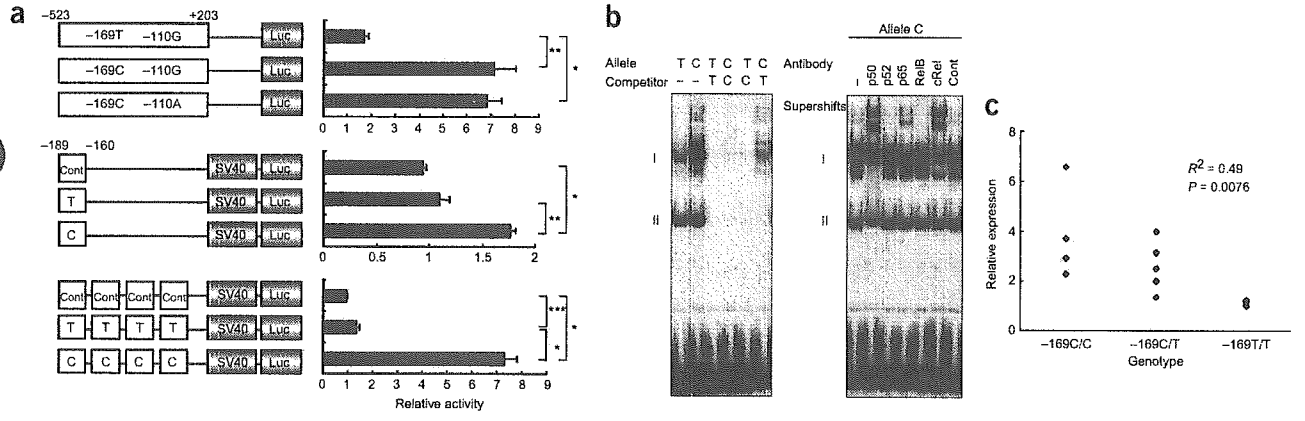
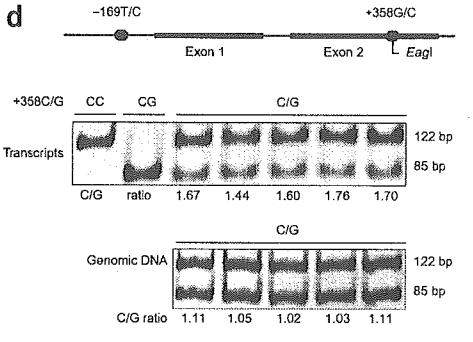


Figure 2 Correlation of *FCRL3* expression with allele and genotype. (**a**) Promoter activity of haplotypes in *FCRL3* (top) and enhancing activity of the 30-bp promoter region around −169C→T (middle and bottom), as evaluated by luciferase assay. Data represent mean ± s.e.m. Representative data from three experiments done in quadruplicate. $*P < 0.0001$; $**P < 0.001$; $***P < 0.01$ 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-κB components (right). (**c**) Expression of *FCRL3* measured by quantitative TaqMan PCR of RNA purified from $CD19^+$ B cells obtained from 13 healthy volunteers (C/C, $n = 4$; C/T, $n = 5$; T/T, $n = 4$). (**d**) ASTQ. *FCRL3* transcripts in B cells and genomic DNA from individuals ($n = 5$) with heterozygous genotypes (−169C/T +358C/G) 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.



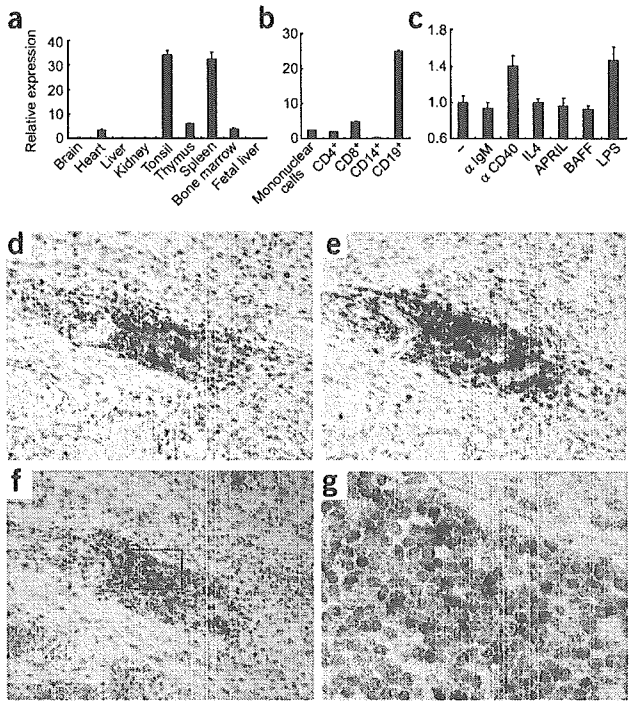


Figure 3 Expression patterns of *FCRL3* in human tissues and cells. (**a**) Relative expression of *FCRL3* in various tissues. (**b**) Relative expression of *FCRL3* in fractionated leukocytes using MTC panel (Clontech). (**c**) Relative expression of *FCRL3* in response to stimuli (antibody to CD40, 1 μg ml^{-1}; antibody to IgM, 1 μg ml^{-1}; IL-4, 10 ng ml^{-1}; APRIL, 10 ng ml^{-1}; BAFF, 10 ng ml^{-1}; LPS, 100 ng ml^{-1}) for 4 h. Representative data from three experiments done in triplicate. (**d**,**e**) Lymphocyte aggregates in rheumatoid arthritis synovium. T cells and B cells in serial sections were immunostained using antibodies to CD3 (**d**) and CD20 (**e**), respectively. (**f**,**g**) *FCRL3* mRNA expression (blue stain) in rheumatoid arthritis synovium as analyzed by *in situ* hybridization. Higher magnification views of synovium (**g**) are denoted by the box in **f** (magnifications: **d**–**f**, ×100; **g**, ×400). Counterstaining: **d**,**e**, hematoxylin; **f**,**g**, nuclear fast red.

oligonucleotides surrounding SNP −169C→T and control oligonucleotides into a vector with the SV40 promoter. Cells transfected with a single copy of the C allele produced substantially greater luciferase activity than cells transfected with a single copy of the T allele. More convincingly, transfection with four tandem copies of the C allele enhanced luciferase activity by a factor of 20 over transfection with four 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 have binding affinity for NF-κB, which regulates a wide variety of genes in the immune system. The disease-susceptible sequence (including −169C) had higher matrix similarity to the consensus NF-κB binding motif than the nonsusceptible sequence (including −169T). We then carried out electrophoretic mobility shift assays (EMSAs) to examine whether differences between the susceptible −169C allele and the nonsusceptible −169T allele affected binding of nuclear proteins from Raji cells. We used the same 30-bp labeled oligonucleotides used in the luciferase assay. These sequences contain the predicted NF-κB binding site. We observed two main bands, I and II, in the presence of nuclear extracts; the intensity of band I was higher for the susceptible −169C allele than for the nonsusceptible −169T allele (**Fig. 2b**). Competition assays with unlabeled oligonucleotides indicated that these complexes were specific for the probes. In addition, competition assays with unlabeled probes of the C allele for T and the T allele for C showed that the C allele was better able to compete for binding, a result consistent with the higher binding affinity of labeled C allele probes alone. We also carried out a supershift experiment with antibodies specific for NF-κB components (p50, p52, p65, RelB and cRel). We observed supershifts in some lanes with specific antibodies for p50, p65 and cRel (**Fig. 2b**). Among these, only antibody to p50 shifted band II, suggestive of the presence of a p50-p50 homodimer. Band I had the highest intensity and a substantial allelic difference and was supershifted by antibodies to p50, p65 and cRel. Although these findings indicate that band I comprises a mixture of heterodimers, the greater shifts caused by antibodies to p50 and cRel suggest that the main component is a p50-cRel heterodimer.

The two *in vitro* assays showed the potent transcriptional activity of the disease-susceptible haplotype regulated by NF-κB, suggesting that expression of *FCRL3* is greater from the disease-susceptible −169C allele than from the nonsusceptible −169T allele. To extend these findings, we quantified expression of *FCRL3* 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 identified a significant positive correlation between number of susceptible chromosomes and transcription level ($R^2 = 0.49$, $P = 0.0076$; **Fig. 2c**).

We also carried out allele-specific transcript quantification[9,34] to confirm the effect of the SNP on transcription. Using an *Eag*I restriction-fragment length polymorphism located at position +358 in exon 2 of *FCRL3* (fcrl3_5, +358C→G), we measured the relative contribution of each haplotype to transcript production in heterozygous individuals (**Fig. 2d**). We evaluated the transcripts of five doubly heterozygous individuals with genotype −169C/T +358C/G; the mean ratio (susceptible versus nonsusceptible 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 two haplotypes was equal.) These results show that the

Table 3 Genotype and autoantibodies in individuals with rheumatoid arthritis

	RF		Antibody to CCP	
Genotype	n[a]	Serum level[b] (IU/ml)	n[c]	Positivity (%)
−169C/C	29	479.9 ± 91.3[d]	17	100.0[e]
−169C/T	75	323.7 ± 47.3[d]	35	94.3[e]
−169T/T	44	216.4 ± 44.0[d]	19	73.7[e]

[a]$N = 148$. [b]Mean ± s.e.m. [c]$N = 71$. [d]$R^2 = 0.049$, $P = 0.0065$ by regression analysis. [e]$P = 0.029$ by Fisher's exact test.

Table 4 Association of SNP −169C→T with AITD and SLE

Disease	*n*	Genotype			Allele C frequency	Recessive-trait comparison		
		CC	CT	TT		OR (95% c.i.)	χ^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	2,437	438	1,167	832	0.42	1.52 (1.29–1.79)	24.2	0.00000084
Control	2,037	257	995	785	0.37			

*Rheumatoid arthritis represents sum of three sets (n = 1,364). c.i., confidence interval; GD, Graves' disease; HT, Hashimoto's thyroiditis; OR, odds ratio; RA, rheumatoid arthritis.

expression of *FCRL3* is higher in individuals with the disease-susceptible haplotype and suggest that higher expression of *FCRL3* is a potential cause and component of the pathological mechanism(s) leading to rheumatoid arthritis.

Expression of *FCRL3* mRNA

We then quantified *FCRL3* expression in multiple tissues using TaqMan methods. Expression of *FCRL3* transcripts was high in the spleen and tonsils (**Fig. 3a**), which are secondary lymphoid organs. We observed lower expression in thymus and bone marrow. In human blood fractions, $CD19^+$ cells, which represent the B-cell population, had the greatest *FCRL3* expression among peripheral blood mononuclear cells. $CD4^+$ and $CD8^+$ cells had less expression (**Fig. 3b**). We next examined the effect of B-cell stimulation on *FCRL3* expression. We cultured peripheral blood B cells from a healthy donor for 4 h using known B-cell stimulants and then quantified *FCRL3* mRNA (**Fig. 3c**). Expression of *FCRL3* was increased by antibody to CD40 and lipopolysaccharide (LPS).

We then investigated expression of *FCRL3* transcripts in synovial tissue using *in situ* hybridization methods. T and B cells are the key players with regard to inflammation in synovial tissue, producing proinflammatory cytokines and autoantibodies that might be pathogenic[1]. These cells show three distinct histological patterns: diffuse infiltration, clustering in aggregates and follicles with germinal-center reaction[35,36]. We observed aggregations of T and B cells in paraffin-embedded synovial sections from individuals with rheumatoid arthritis, using immunostaining with antibodies to CD3 and CD20, respectively (**Fig. 3d,e**). *In situ* hybridization assay with serial sections detected *FCRL3* 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 *FCRL3* mRNA. Synovium from two other individuals with rheumatoid arthritis had similar lymphocyte aggregates and *FCRL3* expression (**Supplementary Fig. 2** online).

SNP association with autoantibody and *HLA-DRB1* status

Because we suspected that higher *FCRL3* expression led to B-cell abnormalities in rheumatoid arthritis, we examined associations in individuals with rheumatoid arthritis between genotype and two rheumatoid arthritis–related autoantibodies: rheumatoid factor (RF) and antibody to cyclic citrullinated peptide (CCP). RF is a well-known autoantibody for the Fc region of IgG, and titers correlate with rheumatoid arthritis disease activity[37]. Antibody to CCP recognizes peptides containing citrulline and is detected in rheumatoid arthritis with extremely high specificity[38,39]. RF titer in individuals with rheumatoid arthritis was significantly positively correlated with the number of susceptible alleles (R^2 = 0.049, P = 0.0065; **Table 3**). The positive ratio of antibody to CCP in individuals with rheumatoid arthritis also differed significantly among genotypes ($P < 0.05$) and correlated with number of susceptible alleles.

Because genetic interactions between HLA and non-HLA loci have been described in susceptibility for rheumatoid arthritis and other autoimmune diseases[26,40], we compared genotype distributions for SNP −169C→T among three rheumatoid arthritis subgroups stratified by number of *HLA-DRB1* shared-epitope alleles. We previously genotyped *HLA-DRB1* in our population and observed significant associations between rheumatoid arthritis susceptibility and shared-epitope alleles[4]. Allele frequency of the rheumatoid arthritis–susceptibility allele −169C was significantly higher in the subgroup with two copies of shared-epitope alleles (0.49, n = 113) than in the subgroup with no shared-epitope alleles (0.39, n = 215; $P < 0.05$).

Replication study of association in three autoimmunities

To confirm associations between the *FCRL3* variant and rheumatoid arthritis susceptibility, we carried out a replication study (540 individuals with rheumatoid arthritis, 636 controls). We compared allele frequency and found a significant association between fcrl3_3 (−169C→T) and rheumatoid arthritis susceptibility (allele frequency was 0.40 in individuals with rheumatoid arthritis versus 0.46 in controls; P = 0.041; **Supplementary Table 2** online). We noted no significant differences between two cohorts that consisted of the replication samples. These results further confirmed the association of the fcrl3_3 −169C allele with rheumatoid arthritis susceptibility in Japanese individuals.

Because this region is associated with multiple autoimmune diseases, and because several variants are involved in multiple autoimmunities, we investigated associations between SNP −169C→T and two other autoimmune diseases: AITD and SLE. We recruited 509 Japanese individuals with AITD (351 with Graves' disease and 158 with Hashimoto's thyroiditis) and 564 Japanese individuals with SLE and compared them with 2,037 Japanese controls. In addition, we combined AITD, SLE and rheumatoid arthritis cases 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 rheumatoid arthritis, were significantly associated with the SNP (odds ratio = 1.52, P = 0.00000084 in Japanese for a recessive model between all four autoimmunities considered in aggregate and controls; **Table 4**). As rheumatoid arthritis–specific autoantibodies were correlated with the number of susceptible alleles, antibody to DNA titer was higher in individuals with SLE with genotype −169C/C than in subjects with other genotypes (294.1 IU ml^{-1} versus 145.5 IU ml^{-1}; 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 identified multiple LD blocks in the region, and one block containing *FCRL3* was associated with rheumatoid arthritis. This association was replicated in a second Japanese case-control set. The rheumatoid arthritis–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 limited to specific ethnic groups[12] whereas others are widely dispersed but significantly associated with disease in only specific ethnic groups[41,42]. We evaluated four-SNP haplotypes in *FCRL3* in African American, European American and Asian (Korean and Japanese) subjects and found weaker LD in African Americans than in other groups and substantial differences in allelic frequency among the groups (**Supplementary Table 3** online).

Although the evidence presented here for *FCRL3* being an autoimmune disease–susceptibility gene is powerful, additional autoimmune disease–related genes probably exist in this region. For example, 1q23 is a good candidate locus for SLE susceptibility[6], particularly involving the association of the classical FcγR genes with SLE susceptibility in the Japanese population[19], although those variants are not in LD with SNP −169C→T in our Japanese subjects ($\Delta < 0.05$, **Fig. 1a**). Multiple SLE susceptibility genes are also homologous to human 1q23 in mouse models of SLE[43].

Further evaluation of polymorphism associations showed that a SNP in the promoter region of *FCRL3* alters expression of *FCRL3* through NF-κB binding. Because higher expression of *FCRL3* 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 *FCRL3*. That the susceptible allele is associated with *HLA-DRB1* in rheumatoid arthritis is consistent with *FCRL3* 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 *FCRL3* on B cells and the importance of B cells suggested by a recent clinical trial of B cell–depleting therapy[44], the present findings might provide a genetic basis for B-cell abnormality in autoimmunity.

Although the precise function of *FCRL3* is unknown, its predicted molecular structure suggests that it is a membranous protein that conveys signals into cells through a cytoplasmic domain containing an immunoreceptor-tyrosine activation motif and an immunoreceptor-tyrosine inhibitory motif[14]. An *in vitro* study showing the binding of tyrosine kinases syk and ZAP70 to the immunoreceptor-tyrosine activation motif region and of tyrosine phosphatases SHP-1 and SHP-2 to the immunoreceptor-tyrosine inhibitory motif region[17] supports the proposed signaling function of *FCRL3*. In a previous study examining *in situ* hybridization in human tonsil, *FCRL3* was expressed in the germinal center, with particularly high expression in the light zone[16], suggesting that *FCRL3* functions predominantly in centrocytes. The present finding that CD40 stimulation, which is important in germinal-center formation[45], upregulates *FCRL3* expression in B cells could indicate that *FCRL3* 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 coreceptors[46]. High expression of *FCRL3* and augmented autoantibody production in individuals with the disease-susceptible genotype is consistent with the idea that *FCRL3* influences the fate of B cells and augments the emergence of self-reactive cells in the germinal center.

In addition to its role in lymphoid tissues, expression of *FCRL3* in synovial tissue might explain the pathological connection between *FCRL3* variants and rheumatoid arthritis. *FCRL3* is strongly expressed in aggregated lymphocytes. Although our synovial samples showed only T-cell–B-cell aggregates, lymphocytes in rheumatoid arthritis 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[36]. Ectopic germinal-center formation also occurs in tissues from individuals with AITD and SLE, and *FCRL3* might be involved in pathological autoimmune reaction in these disease-specific ectopic lymphocyte aggregates.

Considering that augmented expression of *FCRL3* is associated with susceptibility to autoimmune disorders, and that *FCRL3* expression is regulated in B cells in the secondary lymphoid organ and is detected in lymphocytes of disease-specific tissues, *FCRL3* probably functions in immunity and potentially pathogenic in autoimmune disorders.

METHODS

Subjects. We enrolled three independent cohorts of individuals with rheumatoid arthritis ($n = 830$, 217 and 323), a cohort of individuals with SLE ($n = 564$) and a cohort of individuals with AITD ($n = 509$) comprising Graves' disease ($n = 351$) and Hashimoto's thyroiditis ($n = 158$) through several medical institutes in Japan. We recruited four independent cohorts of unaffected control subjects ($n = 658$, 262, 374 and 752) at various sites in Japan. All subjects were Japanese. Individuals with rheumatoid arthritis (84.2% women; age 59.0 ± 12.3 years (mean ± s.d.); 75.0% RF-positive) satisfied the revised criteria of the American Rheumatism Association for rheumatoid arthritis[47]. Individuals with SLE satisfied the criteria of the American College of Rheumatology for SLE[48]. Diagnosis of AITD was established on the basis of clinical findings and results of routine examinations for circulating thyroid hormone and thyroid-stimulating hormone concentrations, serum levels of antibodies against thyroglobulin, thyroid microsomes and thyroid-stimulating hormone receptors, ultrasonography, $^{[99m]}TCO_4^-$ (or $[^{123}I]$) uptake and thyroid scintigraphy.

We evaluated LD at 1q21–23 in the first control cohort compared with the first rheumatoid arthritis cohort to identify the rheumatoid arthritis–associated LD block and SNPs. The second and third rheumatoid arthritis and control cohorts were used for replication testing of results from the first cohorts. We tested Graves' disease; Hashimoto's thyroiditis; SLE; the combination of the two AITDs; and the combination of rheumatoid arthritis, SLE and the two AITDs for associations using the total pool of controls. We enrolled control subjects from three other ethnic groups, Korean ($n = 100$), African American ($n = 120$) and European American ($n = 120$), for evaluation of *FCRL3* haplotypes. We sampled synovial tissues from individuals with rheumatoid arthritis 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. We identified SNPs in exons and 5′ and 3′ flanking regions of *FCRL1*, *FCRL2*, *FCRL3* and *FCRLψ4* by direct sequencing of DNA from 24 individuals. We selected other SNPs from the JSNP and Assay-On-Demand SNP databases (Applied Biosystems). We genotyped SNPs using Invader and TaqMan assays[41] as indicated by the 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 excluded successful call rates <0.95 and values of $P < 0.01$ obtained by Hardy-Weinberg equilibrium testing in control subjects. The error rate of Invader assay was 0.0023, which was estimated by 11,092 assays in two replicates using 118 randomly selected SNPs (internal control data).

Luciferase assay. We cloned the promoter fragment of three haplotypes corresponding to nt −523 to +203 of *FCRL3* into the pGL3-Basic vector (Promega). We generated oligonucleotides using the allelic sequences of nt −189 to −160 of *FCRL3*. We cloned a single copy or four tandem copies of

these oligonucleotides into pGL3-Promoter vector (Promega). We grew Raji cells (RCB1647; RIKEN Cell Bank) in RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics. We electroporated (230 V and 975 μF) 1×10^7 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, we collected cells and measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

EMSA. We carried out EMSA and preparation of nuclear extract from Raji cells as previously described[49]. We labeled oligonucleotides −169T and −169C with digoxigenin -11-ddUTP using the DIG gel-shift kit (Roche). We incubated 5 μg of nuclear extract with 40 fmol of digoxigenin-labeled nucleotide for 25 min at room temperature. For competition experiments, we preincubated nuclear extract with unlabeled oligonucleotide (100-fold excess) before adding digoxigenin-labeled oligonucleotide. For supershift assays, we incubated 4 μg of antibodies to p50, p52, p65, RelB or cRel and rabbit IgG (control antibody; Santa Cruz Biotechnology) for 15 min at room temperature after incubation of the labeled probe. We separated protein-DNA complexes on a nondenaturing 6% polyacrylamide gel in 0.5× Tris-Borate-EDTA buffer. We transferred the gel to a nitrocellulose membrane and detected signals using an LAS-3000 luminoimage analyzer (Fujifilm).

RNA extraction and cDNA preparation. We collected peripheral blood from healthy volunteers to obtain $CD19^+$ lymphocytes. We separated polymorphonuclear cells by differential centrifugation using Lymphoprep resolving solution (AXIS-FIELD). We isolated $CD19^+$ lymphocytes using the MACS system with CD19 microbeads (Miltenyi Biotec) and confirmed that cell purity was >95% using flow cytometry. We stimulated cells with antibodies to CD40 (Cymbus Biotechnology) or IgM (Jackson Immunoresearch), with Il-4 (eBioscience), with APRIL (PeproTech), with BAFF (PeproTech) or with LPS (Sigma) for 4 h. We isolated total RNA using RNeasy Mini Kit (Qiagen). We quantified RNA in other normal tissues using Premium Total RNA (Clontech). We reverse-transcribed total RNA using TaqMan Gold RT-PCR reagents with random hexamers (Applied Biosystems) in accordance with the instructions of the manufacturer.

Quantification of *FCRL3* expression using real-time RT-PCR. We carried out real-time quantitative PCR using an ABI PRISM 7900 (Applied Biosystems) and Assay-on-Demand TaqMan probe and primers (Hs00364720_m1 for *FCRL3*) in accordance with the manufacturer's instructions. We generated a standard curve from the amplification data for *FCRL3* primers using a dilution series of total RNA from Raji cells as templates and normalized data to GUS level.

ASTQ. We carried out ASTQ as previously described[34] with some modifications. We prepared cDNA from B cells as described above. We amplified both cDNA and genomic DNA by PCR for 37 cycles using primers specific for exon 2 of *FCRL3* (**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. We monitored full digestion by the inclusion of PCR products from +358G/G homozygotes. We then separated digested products on a 12.5% polyacrylamide gel and quantified them using an LAS-3000 analyzer.

***In situ* hybridization and immunohistochemistry.** We carried out *in situ* hybridization as previously described[50]. We obtained probes from PCR products using the sequence of *FCRL3* (nt 2052–2490, comprising the intracellular unique region that is poorly conserved among members of this family). An additional probe of the 5′ untranslated sequence yielded similar results. We also examined control probes, which yielded no specific hybridization (data not shown). We used antibodies to CD3 (clone PS-1, Nichirei) and CD20 (clone L26, Zymed) for immunohistochemistry with an ABC Elite kit (Vector Labs) in accordance with the manufacturer's instructions. No specific staining was detected using mouse isotype IgG (data not shown).

Measurement of autoantibodies. We measured RF in sera of individuals with rheumatoid arthritis using latex-enhanced immunonephelometric assay. We measured antibody to DNA in sera of individuals with SLE by radioimmunoassay. Individuals with rheumatoid arthritis (n = 147, 81.1% women; age 63.9 ± 10.6 years (mean ± s.d.); 87.8% RF-positive; mean Steinbrocker radiographic stage 3.2) or SLE (n = 120, 92.6% women; age 36.6 ± 12.7 years (mean ± s.d.)) were part of the cohorts or from a single medical institute, respectively. For each individual, we used the maximum value of RF and antibody to DNA measured during the treatment period in the medical center or outpatient clinic. We detected antibody to CCP at a single time point using enzyme-linked immunosorbent assay, as previously described[38].

Statistical analysis. We calculated LD index Δ (ref. 28) and drew **Figure 1a** using Excel software (Microsoft). We estimated haplotype frequencies using HAPLOTYPER software. We applied the χ^2 test for contingency table tests for associations between allele-genotype distribution and phenotypes. *FCRL3* 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 done using STATISTICA software (StatSoft).

URLs. The JSNP database is available at http://snp.ims.u-tokyo.ac.jp/index.html. TRANSFAC is available at http://www.gene-regulation.com/. HAPLOTYPER is available at http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm.

GenBank accession number. *FCRL3* mRNA, NM_052939.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Anti-citrullinated collagen type I antibody is a target of autoimmunity in rheumatoid arthritis ☆

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Abstract

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, but its autoimmune mechanisms are not clearly understood. Recently, anti-citrullinated peptide antibodies have been specifically observed in sera of RA patients. Furthermore, we identified RA-susceptible variant in a gene encoding citrullinating enzyme, peptidylarginine deiminase type 4 (PADI4). Therefore, we hypothesized that proteins which are modified in RA synovium by PADI4 act as autoantigens. Subsequently, we obtained human collagen type I (huCI) as one of the autoantigens using a RA synoviocyte cDNA library by immunoscreening. We also investigated that the levels of anti-citrullinated huCI were significantly higher in RA patient sera than in normal control sera with high specificity (99%) and positively correlated with the levels of anti-cyclic citrullinated peptide (anti-CCP) antibodies. We concluded that huCI is a novel substrate protein of PADIs and that citrullinated huCI is a candidate autoantigen of RA.

Keywords: Autoantibodies; Peptidylarginine deiminases; Rheumatoid arthritis; Human type I collagen; Human type II collagen

Rheumatoid arthritis (RA) is a major autoimmune disease and affects ~1% of the world population. Many autoantibodies have been found in sera of RA patients, including antibodies against rheumatoid factor (RF). However, the antibodies that are most specific to RA are autoantibodies against citrulline-containing proteins or peptides. These are the so-called anti-citrullinated protein antibodies, including APF (anti-perinuclear factor), AKA (anti-keratin antibodies), AFA (anti-filaggrin antibodies), and anti-Sa. Anti-CCP antibody is an anti-citrullinated protein antibody that is reasonably sensitive (41–88%) and extremely specific (~98%) for RA, and has been used in clinical applications with high reliability [4,23,24]. The anti-CCP antibody system is also suited to the diagnosis of early RA [33]. Although the highly reliable anti-CCP antibody assay system (second generation) is designed to detect antibodies that recognize a mixture of synthetic peptides containing citrulline, the precise sequences of those peptides are not known. To clarify the pathologic mechanism of anti-citrullinated peptide antibodies in RA, it is important to identify native citrullinated peptides that are recognized by RA sera. Native citrulline-containing peptides are only produced by enzymatic conversion of peptidylarginine to citrulline, because citrulline is a non-coded amino acid in vivo. The enzymes involved in this conversion are

☆ *Abbreviations:* RA, rheumatoid arthritis; PADI, peptidylarginine deiminase; huCI, human collagen type I; huCII, human collagen type II; anti-MC, anti-modified citrulline; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; pNPP, *p*-nitrophenyl phosphate.

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peptidylarginine deiminases (PADIs). Five PADI isozymes have been detected in humans, and two of them (PADI2 and PADI4) have been detected in RA synovial tissue [35]. Previously, we found that the PADI4 gene is associated with RA [28]. These facts strongly suggest that citrullination of self proteins (particularly by PADI4) and production of autoantibodies against those citrullinated proteins play pathologic roles in RA. Consequently, identification of the substrates of PADIs is important for investigation of autoimmunity in RA.

PADIs are enzymes that participate in post-translational modification of proteins by catalyzing citrullination of arginine residues. PADIs have five mammalian isoforms, which are expressed in different tissues and different stages of development [37]. All PADIs are dependent on calcium ions for activity. Arginine residues in a variety of proteins are modified by PADIs, but free L-arginine is not a substrate of PADIs [30]. Recently, citrullinated histone was reported to have an ability to antagonize transcriptional induction by regulating histone Arg methylation levels [8,38,41]. Although the physiologic role of peptidylcitrulline is unknown, it has been reported that citrullination is related to several diseases including autoimmune diseases [35,37,40].

Many substrates that are citrullinated by PADIs have been identified; e.g., histones, nucleophosmin/B23 [12], keratins [25], filaggrin [32], vimentin [2,34], myelin basic protein (MBP) [42], and fibrin(ogen) [18]. Not all of those proteins are expressed in RA-specific tissues, such as synovial tissue or joints, but all are recognized by RA autoantibodies. Also, there appears to be cross-reaction with anti-citrullinated antibodies. On the other hand, citrullinated fibrin(ogen) has been detected in synovial membranes of RA patients, and is recognized by RA autoantibodies [18]. However, it is unclear whether citrullinated fibrin(ogen) or other citrullinated peptides are pathogenic autoantigens of anti-citrullinated antibodies in RA. Therefore, we designed a survey to identify further candidate citrullinated proteins that are important in the pathology of RA autoimmunity.

To identify novel substrates of PADIs, which are candidate autoantigens of anti-CCP and anti-citrullinated peptide antibodies, we targeted proteins expressed in RA synovium. Because the anti-CCP antibody assay is designed to detect peptide epitopes, we also focused on citrulline-containing peptides rather than structures or various modifications of proteins. In order to survey proteins expressed by RA synovium regardless of their expression level, we adopted a λ-ZAP library of cDNA from RA synoviocytes as the origin of our proteins, and expressed proteins were citrullinated in vitro.

As a result of immunoscreening, we identified human collagen type I (huCI) peptides as a substrate of PADIs. Collagens, including CI, are structural proteins found in connective tissues of animals, and comprise the main extracellular support system. Type II collagen (CII)-induced arthritis (CIA) is a widely used model of arthritis [7,16,27,39]. Anti-CII antibodies were also commonly found in both RA (IgG, 41–72.4%) and non-RA (e.g., osteoarthritis and infective arthritis) (IgG, 36–88%) sera [3,31]. However, there have been no reports of a pathogenic role of CI in RA, although CI is distributed in bone, tendon, vascular tissues, synovial tissues, and skin (where it has functions related to its unique mechanical properties). In the present study, we identified human collagen type I (huCI) peptides as a candidate native substrate of PADIs. We also demonstrated that anti-citrullinated huCI peptide antibody is specific to RA, and that anti-citrullinated huCI peptide antibody appears to share some diagnostic features with anti-CCP antibody, although there are some distinguishing features.

Materials and methods

Human sera and synovial tissues. Human serum was obtained from three groups of subjects: 117 patients diagnosed with RA according to the criteria of the American Rheumatism Association [1]; 47 healthy blood donors (control); 37 patients with non-RA diseases (systemic lupus erythematosus, 19 patients; Sjogren's syndrome, 4 patients; Bechet's disease, 2 patients; other rheumatic disease, 12 patients). All non-RA patient serum and healthy control serum were anti-CCP negative. Mean age of the RA patients was 61.1 years (range, 33–81 years), and 66% of RA cases were positive for rheumatoid factor. No relationship was observed between age and anti-CCP antibody ($R^2 = 0.00001$). Mean age of non-RA patients and the healthy controls was 45.7 years (range, 20–82) and 40.5 years (range, 23–84), respectively. Informed consent was obtained from all subjects.

Preparation of PADIs. His-tagged human PADI4 (hPADI4) was expressed in *Escherichia coli*, BL21-SI, and was purified using a HiTrap protein purification system (Amersham Biosciences, Piscataway, NJ), as previously described [21]. Rabbit PADI2 (rPADI2) was obtained from Sigma. We used hPADI4 or rPADI2 in assays for in vitro citrullination of substrates according to assay condition requirements, after adjusting their enzymatic activity unit for artificial substrates, as described elsewhere [21].

Immunoscreening. A human RA synoviocyte cDNA library in λ-ZAP (Stratagene, La Jolla, CA) was used for the immunoscreening of citrullinated proteins. Induction of protein expression was performed according to the manufacturer's instructions. Briefly, the phage library was plated at 5×10^3 pfu/plate on a series of 100-mm NZY agar plates and was incubated at 37 °C for 5 h. After transfer to nitrocellulose membrane, protein expression was induced on the membranes by incubation with 20 mM IPTG at 37 °C for 4 h. After the membranes were washed, citrullination by hPADI4 was performed on a membrane with 0.075 U/ml hPADI4, 100 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, and 5 mM DTT at 37 °C for 1 h. Detection of citrullinated proteins was performed using an anti-MC detection kit (Upstate, Waltham, MA). The positive clones were converted to a pBluescript II SK (+). The sequence of the inserted DNA was determined using an ABI PRISM 3700 Sequencer (Applied Biosystems, Foster City, CA).

Identification of citrullinated sites of human CI by LC/MS/MS. Human CI was citrullinated by rPADI2, which is generally used for analysis of anti-citrullinated antibodies, in a reaction buffer containing 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, and 5 mM DTT for 12 h at 50 °C. Then, 100 μg huCI, with or without enzyme treatment, was separate by SDS–PAGE, and bands on Coomassie brilliant blue-stained gel were excised and digested with trypsin. The mixture of the digested peptides was analyzed by LC/MS/MS (APROscience, Toku-

shima, Japan). A Q-TOF2 mass spectrometer (Micromass, Manchester, UK) equipped with MAGIC 2002 (Michrom BioResources, Auburn, CA) was also used. Elution of peptides was performed using an acetonitrile gradient in 0.1% formic acid. The MS/MS data were searched against the SWISS-PROT database with protease specificities under consideration of fixed modification (propionamide-Cys) and variable modifications (citrullination of Arg, deamination of Asn and Gln, hydroxylation of Pro, Pyro-glu of N-terminus of Gln, and oxidation of Met) using the Mascot program (Matrix Science, Boston, MA).

Western blotting using human antisera. Citrullinated proteins were separated by SDS–PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes (Bio-Rad). Membrane strips were probed with human sera (1:50) in blocking buffer (5% skimmed milk in T-TBS). Goat anti-human IgG peroxidase-conjugated antibodies were used for the detection of primary antibody, and peroxidase activity was visualized using ECL Western blotting reagents (Amersham).

Detection of citrullination of collagen type I and type II by ELISA. We coated 96-well ELISA plates (Corning, Corning, NY) with 100 μl of acid-extracted huCI (Becton, Dickinson and Company) and huCII (Collagen Research Center, Tokyo, Japan, or Becton, Dickinson and Company) per well in 0.01 M acetic acid at 5 μg/ml, and performed citrullination by rPADI2 at 50 °C. After washing with T-PBS and adding 0.1% ovalbumin in TBS, the plate was treated with 1% glutaraldehyde in PBS. Then, the plate was washed with 0.2 M Tris–HCl (pH 7.8) and distilled water. The modified citrullinated collagen was detected by anti-MC antibody (Upstate) using the standard method.

Detection of anti-citrullinated antibodies in RA patients and normal sera by ELISA. After coating and deimination as described above, anti-citrullinated collagen antibodies were detected by a modification of a previously reported method [29]. Briefly, citrullinated collagen coated plates were blocked with 5% skimmed milk in T-TBS. Then, 100 μl of each diluted serum was added, followed by incubation for 2 h at room temperature. After washing, 100 μl of 1:20000 dilution of goat $F(ab')^2$ anti-human IgG AP (Biosource, Camarillo, CA) was added, followed by incubation at room temperature for 1 h. Plates were developed at room temperature with 100 μl of pNPP substrate (Sigma) per well, and the absorbance at 405 nm was measured using a Fusion plate reader (PerkinElmer, Boston, MA). Standard control serum was analyzed, to assure standardized conditions for ELISA of anti-citrullinated collagen. All tests were run in triplicate. Testing for significant differences between means was performed using Student's *t* test (Microsoft Excel).

Results

Identification and confirmation of (in vitro) citrullination of proteins from synoviocyte cDNA library

To identify novel self molecules that are citrullinated as targets of RA-specific autoantibody, we immunoscreened $\sim 10^6$ pfu lambda of a ZAP cDNA expression library that was constructed using synoviocytes from RA patients. The expressed proteins were citrullinated and expressed proteins that were not treated with the citrullinating enzyme were used as a negative control (Fig. 1A). Three of the positive clones were obtained from a second screening plate and were identical with the huCI α1 gene. To confirm that the huCI was citrullinated, we performed Western blotting (Fig. 1B). We detected citrullination of huCI using ELISA (Figs. 1C and D). Our results indicate that PADIs citrullinated huCI.

After in vitro citrullination of huCI by rPADI2, we performed LC/MS/MS to identify the citrullination site of huCI. The coverage of LC/MS/MS analysis was 45% for huCI α1 precursor and 55% for huCI α2 precursor. We identified 20 citrullination sites of 35 arginine residues in analyzed fragments of huCI α1 and identified 27 citrullination sites of 45 arginine residues in analyzed fragments of huCI α2 (Figs. 1E and F). We did not observe sequence specificity of recognition of targeted arginine residues by the enzyme for conversion from peptidylarginine to peptidylcitrulline. These findings are consistent with those of a previous study [21]. We also confirmed that huCII was citrullinated by PADIs in vitro (data not shown), in addition to their citrullination of huCI.

IgG from RA patients' sera recognized citrullinated human collagen type I in vitro

Reactivity of sera to citrullinated and uncitrullinated huCI was analyzed by Western blotting using 10 RA sera and five normal control sera (Table 1). There was a slight difference in reactivity toward uncitrullinated huCI between sera of RA patients and sera of normal controls. Sera of RA patients were reactive toward the citrullinated huCI.

Additionally, we detected anti-citrullinated huCI by ELISA using serum from a RA patient with a high anti-CCP titer and anti-CCP-negative serum from a healthy control (Fig. 2A). Serum from the RA patient was highly reactive with citrullinated huCI, but serum from the healthy control was not reactive. We also performed time-course analysis (Fig. 2B). The level of anti-citrullinated huCI antibodies detected was increased by citrullination, and was dependent on the enzyme reaction time.

High titers of anti-citrullinated collagen antibody are present in RA sera, but not in normal sera

To investigate whether RA patients, non-RA patients, and normal healthy controls had serum antibodies against citrullinated collagens, ELISA was used to analyze panels of sera from RA patients, non-RA patients, and normal healthy controls.

Next, we measured levels of anti-collagen antibodies in the present subjects. The relative levels of anti-huCII were significantly higher for RA patients than for normal controls ($p = 0.0026$, Student's *t* test; Fig. 3B), and 22 of 56 sera (39%) from RA patients and 1 of 9 sera (11%) from healthy controls were positive for anti-huCII antibodies. However, the relative levels of anti-huCII were significantly higher for non-RA patients than for normal controls ($p = 0.00014$, Student's *t* test; Fig. 3B), and 8 of 13 sera (62%) from non-RA patients were positive for anti-huCII antibodies. In contrast, there