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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

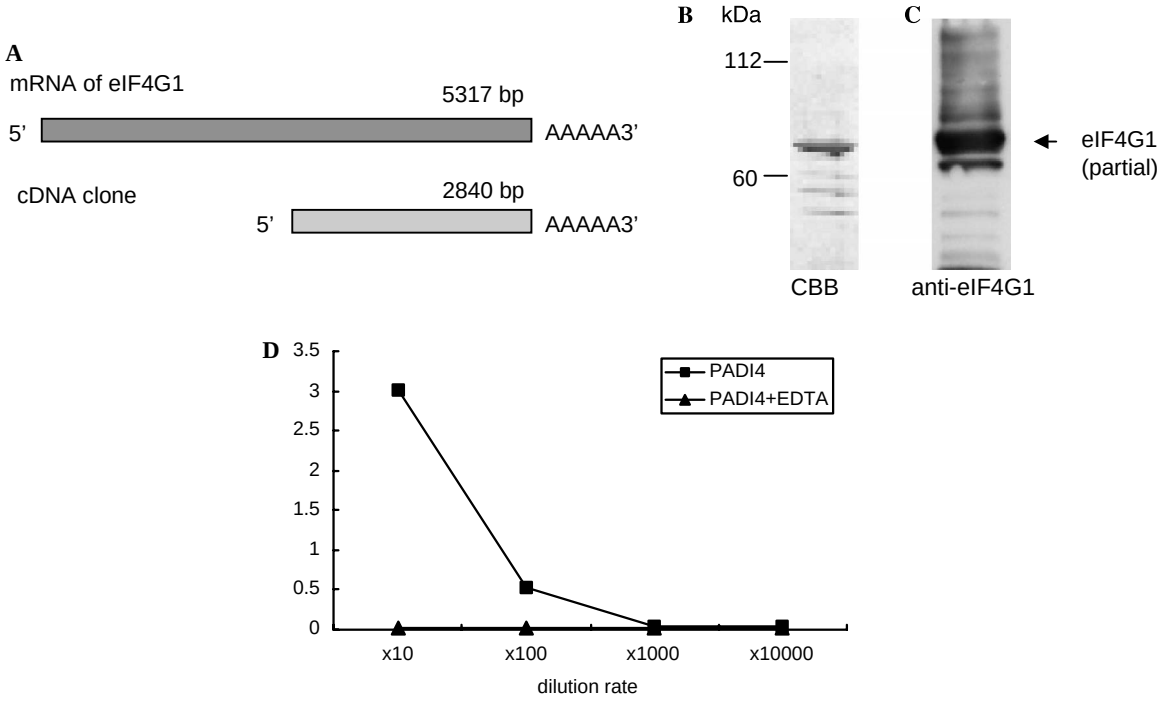

Fig. 1. Expression and immunoblotting of recombinant eIF4G1 fragment. (A) Insert of cDNA clone compared with the known eIF4G1 mRNA (bp, base pairs). (B) Purified recombinant eIF4G1 fragment was loaded onto 10% SDS–polyacrylamide gel and stained with Coomassie brilliant blue (CBB). (C) Separated proteins 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

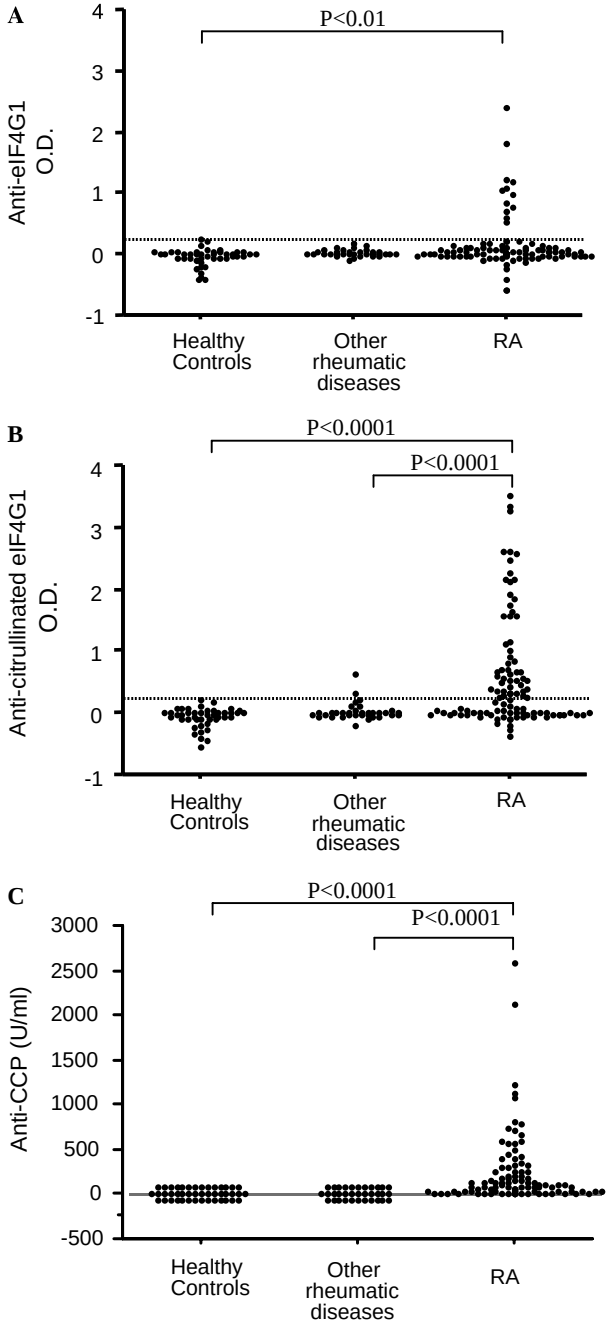

Fig. 2. Presence of autoantibodies against recombinant eIF4G1 fragment (A), citrullinated eIF4G1 fragment (B), and CCP (C) in sera from patients with RA, other rheumatic diseases, and healthy controls. Patient and control sera were diluted at 1:100. Dotted line represents cut-off value, which was calculated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

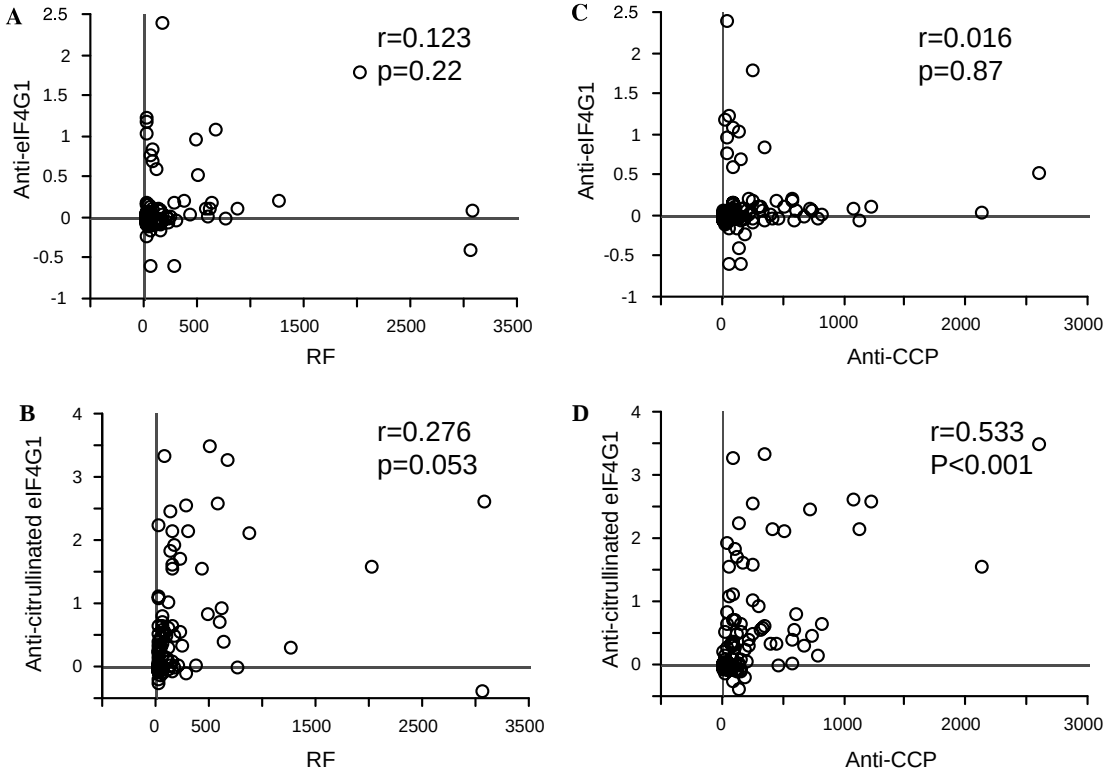

Fig. 3. Correlation between disease markers and anti-eIF4G1 or anti-citrullinated eIF4G1 antibody titer in patients with RA. Correlation between RF levels and anti-eIF4G1 (A) or anti-citrullinated eIF4G1 (B) levels. Correlation between anti-CCP levels and anti-eIF4G1 (C) or anti-citrullinated eIF4G1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

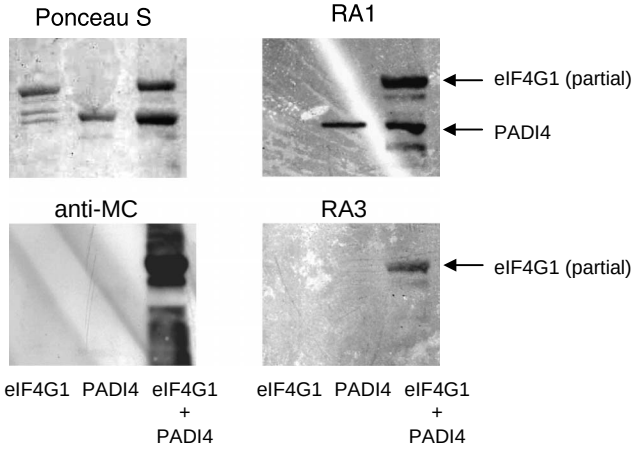

Fig. 4. Immunoreactivity of uncitrullinated or citrullinated eIF4G1. Recombinant eIF4G1 fragment in the presence or absence of PADI4 was separated by 10% SDS-PAGE and was transferred onto nitrocellulose membranes. Serum samples that were positive for anti-citrullinated eIF4G1 on ELISA were examined by 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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