

Prevalence and Significance of Anti-Peptidylarginine Deiminase 4 Antibodies in Rheumatoid Arthritis

JINXIA ZHAO, YI ZHAO, JING HE, RULIN JIA, and ZHANGUO LI

ABSTRACT. *Objective.* To detect anti-peptidylarginine deiminase 4 (PAD4) antibody in patients with rheumatoid arthritis (RA) and to investigate its clinical significance in the pathogenesis of RA.

Methods. Serum samples were obtained from 109 patients with RA, 67 systemic lupus erythematosus (SLE), 48 primary Sjögren's syndrome (pSS), 41 systemic sclerosis (SSc), 34 osteoarthritis (OA), 23 dermatomyositis/polymyositis (DM/PM), and 19 ankylosing spondylitis (AS) and 106 healthy individuals. The presence of antibodies against recombinant human PAD4 (anti-PAD4) was examined by ELISA. Associations between anti-PAD4 and the clinical features of RA were evaluated.

Results. The prevalence of anti-PAD4 in RA patients (45.0%) was significantly higher than those of SLE (9.0%), pSS (4.2%), SSc (9.8%), OA (5.9%), DM/PM (13.0%), AS (0%), and controls (4.7%). The mean titer of anti-PAD4 in RA was also significantly higher than in SLE, other rheumatic diseases, and controls. Disease Activity Score-28 (DAS28), anti-cyclic citrullinated peptide (CCP) antibody, erythrocyte sedimentation rate, rheumatoid factor, IgM, and IgG in anti-PAD4-positive patients were all higher than in anti-PAD4-negative patients. There were positive correlations between anti-PAD4 and DAS28 score ($r = 0.333$, $p < 0.01$) and anti-CCP antibody ($r = 0.248$, $p < 0.05$).

Conclusion. The presence of anti-PAD4 in RA indicates that PAD4 may act as an autoantigen that may play a role in the pathogenesis of RA. (First Release April 1 2008; J Rheumatol 2008;35:969–74)

Key Indexing Terms:

PEPTIDYLARGININE DEIMINASE
ANTIBODIES

CITRULLINE
RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the joints that eventually leads to erosive changes and dysfunction. Antibodies against citrulline-containing epitopes such as antiperinuclear factor, antikeratin antibodies (AKA), anti-flaggrin antibodies, and anti-cyclic citrullinated peptide (CCP) antibodies are very specific in RA¹⁻⁴. Citrulline is formed by posttranslational modification of arginine residues by an enzyme named peptidylarginine deiminase (PAD). Five different isotypes of PAD have been identified in humans, including PAD1, PAD2, PAD3, PAD4, and PAD6⁵. In 2003, Suzuki, *et al* used a case-control linkage disequilibrium study to show that the gene of PADI4 is a susceptibility locus for RA and has correlation with production of anti-CCP antibody⁶, indicating that PADI4 may play a role in the

pathogenesis of RA. But little is known about how PADI4 is involved in the development of RA. Recently, Takizawa, *et al* found that sera from RA patients recognized recombinant human PAD4 using an enzyme-linked immunosorbent assay (ELISA), which implies that PAD4 acts as an autoantigen in RA⁷. However, in that study, only 42 patients with RA were tested, and no further analysis of association between anti-PAD4 and other disease features was assessed. We determined the prevalence of anti-PAD4 in a large cohort of Chinese patients with RA and other rheumatic diseases by ELISA, and investigated the associations between anti-PAD4 and the clinical and laboratory features of RA.

MATERIALS AND METHODS

Patients and serum samples. Serum samples were obtained from 341 patients admitted to the Department of Rheumatology and Immunology, People's Hospital, in the period January 2006 to March 2007. These included 109 patients with RA (83 women, 26 men; median age 58 yrs, range 31–84) who fulfilled the American College of Rheumatology criteria for RA⁸, 67 with systemic lupus erythematosus (SLE; 60 women, 7 men; median age 35 yrs, range 16–73), 48 with primary Sjögren's syndrome (pSS; 48 women; median age 60 yrs, range 30–79), 41 with systemic sclerosis (SSc, 37 women, 4 men; median age 48 yrs, range 16–76), 34 with osteoarthritis (OA; 24 women, 10 men; median age 62 yrs, range 42–85), 23 with dermatomyositis/polymyositis (DM/PM; 20 women, 3 men; median age 51 yrs, range 25–77), and 19 patients with ankylosing spondylitis (AS; 5 women, 14 men; median age 26 yrs, range 16–57). The median disease duration of RA was 7 years (range 0.3 to 40 yrs). Serum samples from 106 blood donors were used as healthy controls.

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The study protocol was approved by the Medical Ethics Committee of Peking University People's Hospital.

Expression and purification of human PAD4. The prokaryotic expression vector pDEST17-PAD14 (a kind gift from Prof. A. Suzuki, Laboratory for Rheumatic Diseases, SNP Research Centre, Institute of Physical and Chemical Research, Kanagawa, Japan) was introduced into *Escherichia coli* BL21-(DE3). 6×His-tagged PAD4 was expressed by 0.2 mM IPTG induction for 3 h at 25°C and purified by a Ni-sepharose FF (GE Healthcare).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of recombinant PAD4. Protein purity was assessed by SDS-PAGE. Samples were electrophoresed in 10% SDS polyacrylamide gels, and the gel was stained with Coomassie brilliant blue. In parallel experiments, gels were electrotransferred onto nitrocellulose membranes. The membranes were blocked in 5% nonfat dried milk in phosphate-buffered saline (PBS) at 4°C overnight and blotted with mouse anti-His tag monoclonal antibody at room temperature for 2 h. After repeated washing, the membranes were incubated with peroxidase-conjugated goat anti-mouse IgG for 1 h. Immunoblot was developed using DAB.

Detection of anti-PAD4 by ELISA. Recombinant PAD4 at a concentration of 5 µg/ml in PBS was incubated in 96-well microtiter plates (100 µl/well; Costar, Cambridge, MA, USA) overnight at 4°C. The wells were then washed with 0.05% Tween 20-PBS (PBS-T) 3 times and blocked with 5% (w/v) skim milk for 4 h at room temperature. Serum samples were diluted 1:200 with PBS-T containing 3% skim milk and 100 µl were added to each well. Wells without PAD4 were set up for each sample to examine the non-specific background. After incubation for 2 h at room temperature, the wells were washed with PBS-T 5 times. Then 100 µl of goat anti-human IgG conjugated to peroxidase diluted at 1:4000 was added to each well and incubated 1 h at room temperature. After washing with PBS-T 5 times, the bound antibodies were detected with O-phenylenediamine as substrate. The reaction was stopped by adding 100 ml of 2.5 M sulfuric acid to each well. Plates were read at wavelength absorbance of 492 nm (OD492nm). Each serum sample was assayed in duplicate. A positive serum sample was included on each plate as a positive control and reference to correct inter-assay variations. The titer of anti-PAD4 was expressed as arbitrary units (AU) and calculated as follows:

$$AU = \frac{[OD_{PAD4} - OD_{\text{nonspecific background}}]_{\text{test serum}}}{OD_{\text{nonspecific background}}_{\text{positive control serum}}} \times \frac{OD_{PAD4}}{100}$$

Clinical and laboratory data of RA patients. The following clinical and laboratory data were collected: disease duration, morning stiffness, rheumatoid nodules, pulmonary interstitial fibrosis, rheumatoid factor (RF), anti-CCP antibodies, AKA, immunoglobulins (IgG, IgM, IgA), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). Regarding the laboratory features, ESR was measured by the Westergren method; values ≤ 15 mm/h for men, ≤ 20 mm/h for women were considered normal. CRP, RF, and immunoglobulins were examined by immunonephelometry method. Values > 7.9 mg/l for CRP and > 20 IU/ml for RF were considered positive; normal ranges of IgG, IgM, and IgA were 6.94–16.18, 0.60–2.63, 0.68–3.78 g/l, respectively. Anti-CCP antibodies were tested using the second-generation ELISA kit (Euroimmun, Lubeck, Germany) and values > 1.0 were considered positive. AKA was tested by indirect immunofluorescence assay.

Radiographs of the hands of patients with RA were studied by an experienced radiologist blinded to patients' clinical and laboratory data. Radiographs were scored according to the Sharp–van der Heijde method⁹. The Disease Activity Score (DAS28) was calculated as described¹⁰.

Detection of anti-PAD4 by Western blotting. Recombinant PAD4 was electrophoresed in 10% SDS-PAGE. After transblotting onto a nitrocellulose membrane, the membrane was blocked with PBS containing 5% skim milk. The membrane was cut and incubated with serum samples diluted

1:100, which were positive for anti-PAD4 by ELISA. After repeated washing, membranes were incubated with peroxidase-conjugated goat anti-human IgG. Color development was performed using DAB.

Statistical analyses. Data analyses were performed using SPSS for Windows, version 13.0. For normally distributed data, the results were expressed as mean ± SD; differences between groups were analyzed with the t test. For data not distributed normally, expressed as median (range), differences were tested with the Mann–Whitney U test, and correlations were determined by computing Spearman rank correlation coefficients. The chi-square test was used to compare categorical data and percentages between groups. Bonferroni correction was applied to multiple comparisons. P values less than 0.05 were considered statistically significant.

RESULTS

Purification and identification of recombinant PAD4. The protein was identified by SDS-PAGE and Western blotting with anti-His tag monoclonal antibody. As shown in Figure 1, an exogenous protein roughly 70 kDa was observed on the SDS-PAGE, and Western blotting showed that the protein including 6×His-Tag was purified successfully by Ni-sepharose affinity chromatography.

Distribution of anti-PAD4. The distribution of anti-PAD4 in RA, other rheumatic diseases, and healthy controls is shown in Figure 2. The median AU values of anti-PAD4 were 85.6 (range 17.7–405.4) for RA, 44.6 (1.6–157.3) for SLE, 44.3 (1.3–165.2) for pSS, 49.0 (1.3–181.1) for SSc, 35.3 (8.0–111.9) for OA, 52.3 (3.4–137.8) for DM/PM, 28.3 (7.2–75.5) for AS, and 38.1 (4.8–122.9) for controls. The titer of anti-PAD4 in RA was significantly higher than those in other rheumatic diseases ($p < 0.001$) and in controls ($p < 0.001$). The cutoff value for positivity was defined as the 95th percentile of healthy sera (AU = 98.2). As shown in Table 1, the prevalence of anti-PAD4 was significantly higher in RA patients (45.0%) than in SLE (9.0%), pSS (4.2%), SSc (9.8%), OA (5.9%), DM/PM (13.0%), AS (0%), and controls (4.7%) ($p < 0.001$). There was no statistical difference among other combinations. The sensitivity and specificity of anti-PAD4 for RA were 45.0% and 93.5%, respectively. The positive and negative predictive values of anti-PAD4 were 69.0% and 84%, respectively.

Associations between anti-PAD4 and clinical features in RA. Forty-nine of the 109 patients with RA were found to be positive for anti-PAD4. Compared with RA patients without anti-PAD4, there were no significant differences in anti-PAD4-positive patients with respect to sex, age, and disease duration. The mean value of DAS28 in the anti-PAD4-positive patients was higher than that in anti-PAD4-negative patients. Patients with anti-PAD4 showed rheumatoid nodules more often than patients without anti-PAD4.

With regard to radiographic damage, we did a transversal radiographic assessment. We observed that RA patients with anti-PAD4 had significantly severe radiographic changes compared to those without anti-PAD4 (Table 2). No statistically significant differences were observed in duration of morning stiffness, cigarette smoking history, or experience

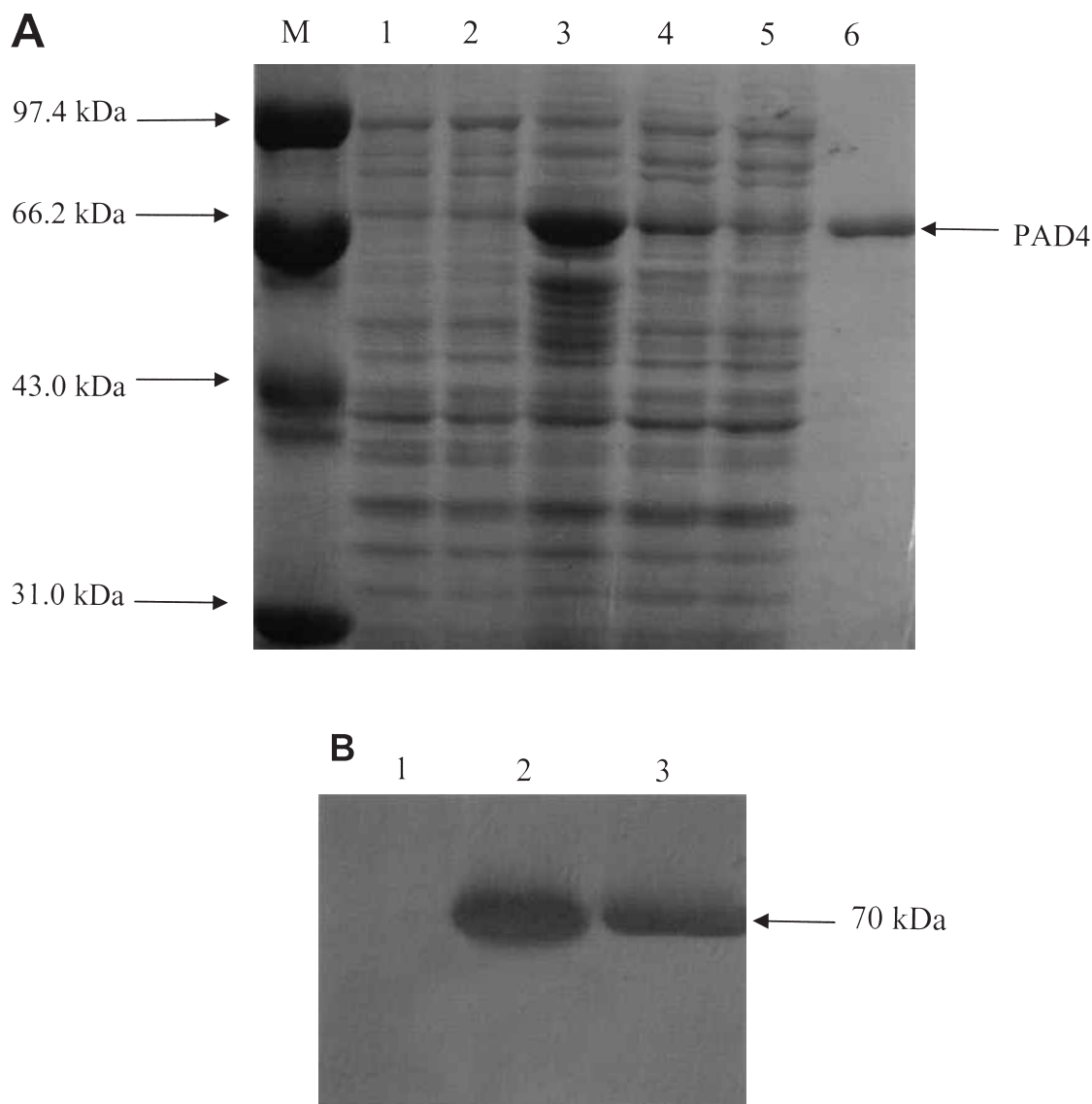


Figure 1. Detection of recombinant PAD4. **A.** Recombinant PAD4 revealed by SDS-PAGE with Coomassie blue staining. **M:** low molecular-mass marker proteins. Lane 1, 2: BL21-(DE3) cells without the plasmid. Lane 3: BL21-(DE3) cells carrying pDEST17-PAD4. Lane 4: flowthrough. Lane 5: wash. Lane 6: elution. **B.** Western blotting with anti-His tag monoclonal antibody in each lane. Lane 1: extracts of BL21-(DE3) cells without the plasmid. Lane 2: extracts of BL21-(DE3) carrying the plasmid induced by IPTG. Lane 3: the recombinant protein purified with Ni-sepharose affinity chromatography.

of pulmonary interstitial fibrosis between the 2 groups. Correlation assessment showed a positive correlation between anti-PAD4 and DAS28 scores ($r = 0.333$, $p < 0.01$; Figure 3).

Associations between anti-PAD4 and laboratory features in RA. The associations between anti-PAD4 and laboratory features in RA patients are analyzed in Table 2. The level of anti-CCP antibody in patients with anti-PAD4 was significantly higher than in those without anti-PAD4 ($p = 0.011$). There was a weak positive correlation between anti-PAD4 and anti-CCP antibody ($r = 0.248$, $p < 0.05$; Figure 4). The

values for ESR, RF, IgG, and IgM in patients with anti-PAD4 were significantly higher than in patients without anti-PAD4 ($p < 0.05$). Levels of CRP and IgA showed no statistical differences in anti-PAD4-positive and anti-PAD4-negative patients ($p > 0.05$). In addition, 20 of the 109 RA patients lacked RF. The prevalence of anti-PAD4 among these patients was 25.0% (5/20). Twenty-four patients (38.7%) with anti-PAD4 were found among 64 patients lacking AKA. In the patients without anti-CCP antibody, the positivity of anti-PAD4 was only 14.3% (2/14).

Detection of anti-PAD4 by Western blotting. It was previ-

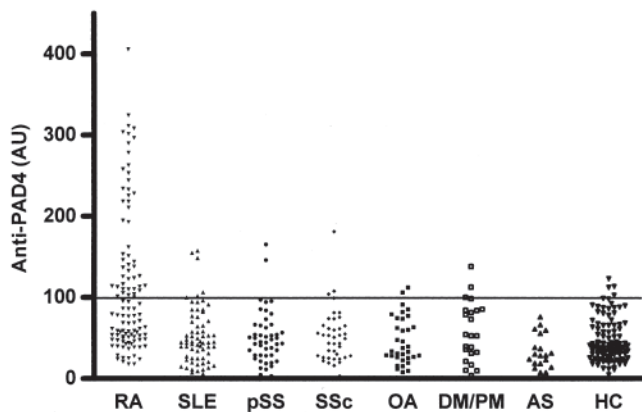


Figure 2. Distribution of anti-PAD4 antibodies in RA and other rheumatic diseases. Recombinant PAD4 was used as the antigen at a concentration of 5 µg/ml and sera were diluted 1:200. Sera were from 109 patients with rheumatoid arthritis (RA), 67 systemic lupus erythematosus (SLE), 48 primary Sjögren's syndrome (pSS), 41 systemic sclerosis (SSc), 34 osteoarthritis (OA), 23 dermatomyositis/polymyositis (DM/PM), 19 ankylosing spondylitis (AS), and 106 healthy controls (HC). Titer is expressed as arbitrary units. Cutoff level for positivity is defined as the 95th percentile of healthy sera (horizontal line).

Table 1. Prevalence of anti-PAD4 in patients with different rheumatic diseases and healthy controls.

| Group | No. Patients | No. Anti-PAD4-positive Patients (%) |
|----------|--------------|-------------------------------------|
| RA | 109 | 49 (45.0*) |
| SLE | 67 | 6 (9.0) |
| pSS | 48 | 2 (4.2) |
| SSc | 41 | 4 (9.8) |
| OA | 34 | 2 (5.9) |
| DM/PM | 23 | 3 (13.0) |
| AS | 19 | 0 (0) |
| Controls | 106 | 5 (4.7) |

* $p < 0.001$ ($\alpha = 0.007$) compared with other groups. RA: rheumatoid arthritis, SLE: systemic lupus erythematosus, pSS: primary Sjögren's syndrome, SSc: systemic sclerosis, OA: osteoarthritis, DM/PM: dermatomyositis/polymyositis, AS: ankylosing spondylitis.

Table 2. Clinical and laboratory features of RA patients with anti-PAD4.

| | Anti-PAD4-positive | Anti-PAD4-negative | p |
|--|--------------------|--------------------|-------|
| Morning stiffness, min (range) | 60 (5–240) | 60 (10–420) | 0.245 |
| Smoking, n (%) | 5/49 (10.2) | 8/60 (13.3) | 0.616 |
| Rheumatoid nodules, n (%) | 11/44 (25.0) | 3/55 (5.5) | 0.006 |
| Pulmonary interstitial fibrosis, n (%) | 8/40 (20.0) | 4/56 (7.1) | 0.060 |
| DAS28 scores, mean ± SD | 5.57 ± 0.83 | 5.06 ± 0.78 | 0.000 |
| Radiographic progression | | | |
| Erosion score | 7 (0–135) | 2 (0–30) | 0.001 |
| Joint-space narrowing score | 23 (2–70) | 7 (0–83) | 0.002 |
| Anti-CCP antibody | 4.7 (0–8.7) | 4.0 (0–6.6) | 0.034 |
| RF, U/ml | 247 (0–3430) | 187 (0–1820) | 0.036 |
| IgM, g/l | 1.5 (0.5–5.6) | 1.1 (0.4–5.3) | 0.039 |
| IgG, g/l | 16.1 (7.2–35.0) | 13.5 (4.3–29.3) | 0.000 |
| IgA, g/l | 2.9 (1.4–7.9) | 3.3 (1.2–7.9) | 0.605 |
| ESR, mm/h | 74 (4–140) | 58 (8–119) | 0.036 |
| CRP, mg/l | 23.4 (1–195) | 21.1 (1.44–141) | 0.974 |

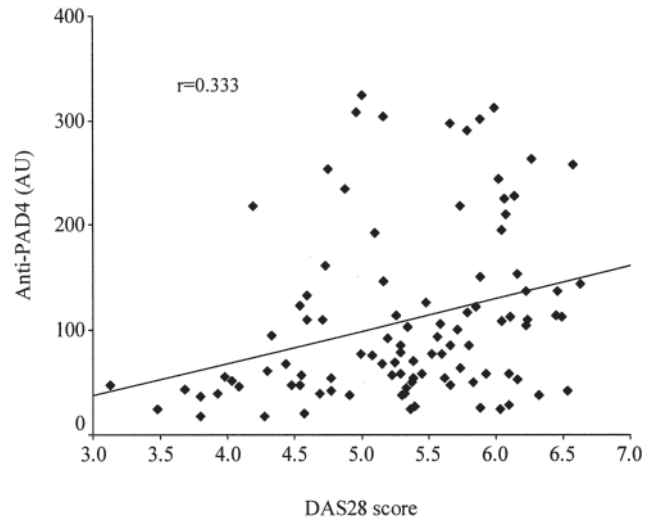


Figure 3. Correlation of anti-PAD4 and DAS28 scores in RA patients. The coefficient ($r = 0.333$) suggests a positive association between anti-PAD4 and DAS28 ($p < 0.01$).

ously reported that anti-PAD4 targeted mostly conformational determinants of PAD4⁷. Our Western blot analysis showing only one positive out of 8 ELISA-positive sera is consistent with that interpretation.

DISCUSSION

The role of PADI4 in the pathogenesis of RA has been extensively studied. Since the first positive association between PADI4 and RA was reported in a Japanese population⁶, several studies have been undertaken to replicate the association. Similar results were found in some European and North American populations^{11,12}. Other studies using European populations were inconsistent^{13–15}. Recently, Iwamoto, *et al*¹⁶ confirmed using a metaanalysis the positive association between PADI4 and RA not only in Japanese populations but also in populations of European descent.

Nissinen, *et al*¹⁷ showed that anti-rabbit-muscle PAD

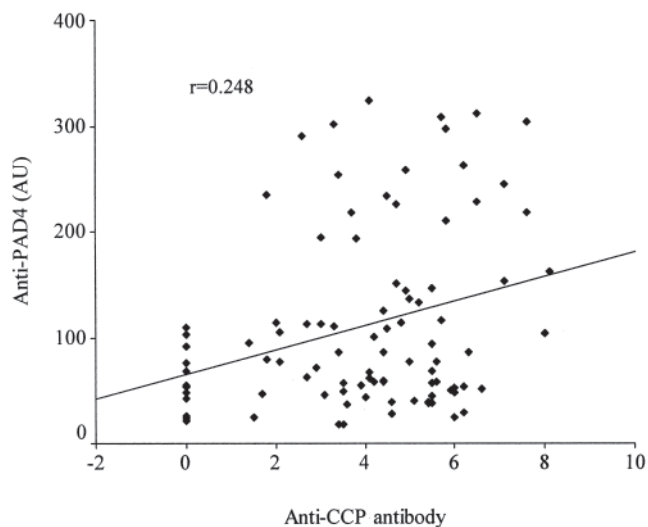


Figure 4. Correlation of anti-PAD4 and anti-CCP antibody in RA patients. The coefficient ($r = 0.248$) suggests a statistical connection between anti-PAD4 and anti-CCP antibody ($p < 0.05$).

antibody can be detected in sera from patients with RA, SLE, and pSS, suggesting that PAD may be a novel autoantigen in inflammatory rheumatic diseases. Roth, *et al*¹⁸ also observed a significantly increased frequency of anti-rabbit-muscle PAD among RA patients (31%) compared to controls (3.4%), and a correlation was found between anti-PAD and anti-CCP antibody¹⁴. Takizawa, *et al*⁷ developed an ELISA system using recombinant human PAD4, and found for the first time that 21 out of the 42 patients with RA (50%), 2 of 19 with SLE (10.5%), 1 of 23 with other collagen diseases (4.3%), and 1 of 40 healthy controls (2.5%) were positive for anti-PAD4. The prevalence and the titers of anti-PAD4 were significantly higher in RA patients than in other rheumatic disease patients and controls, indicating that PAD4 may act as an autoantigen in some patients with RA.

However, it remains unclear if anti-PAD4 has a relation with disease severity and other laboratory features. To further investigate the clinical significance of anti-PAD4 in RA, we investigated antibody against recombinant human PAD4 in a large cohort of Chinese RA patients by ELISA, and found that the prevalence and titers of anti-PAD4 were higher in RA than in other rheumatic diseases and healthy individuals, similar to a previous report⁷. Although the sensitivity of anti-PAD4 was only 45.0%, the specificity did reach 93.5%, which was higher than for anti-rabbit-muscle PAD antibody. We also investigated the relationship between anti-PAD4 and disease activity and laboratory measures in patients with RA. Anti-PAD4 and DAS28 showed a positive correlation ($r = 0.333$, $p < 0.001$). With regard to radiographic damage, the RA patients with anti-PAD4 showed more severe radiographic changes than patients without anti-PAD4 in a transversal radiographic assessment.

Further, a positive correlation between anti-PAD4 and anti-CCP antibody was found in our study. This raised a

question that the recombinant PAD4 might contain citrulline residues generated by self-deimination, which would partly explain the observed correlation with anti-CCP antibodies. Since PAD enzymes rely strongly on the presence of calcium for activity and no reagent containing calcium was used during the experiment, it is unlikely that the recombinant PAD4 could deiminate itself. The correlation between anti-PAD4 and anti-CCP antibody indicates that the 2 antibodies both play roles in the pathogenesis of RA.

How the immunological tolerance to PAD4 is broken in RA remains unknown. One possibility is that anti-PAD4 is formed after the production of anti-CCP antibody by epitope spreading, for the target antigens of anti-CCP antibodies are citrullinated proteins modified by PAD¹⁸. After the generation of immune response to citrullinated proteins, immune tolerance to the enzyme PAD4 can also be broken by epitope spreading, since they act as an enzyme and substrates. Another theory is that PAD4 acts as an autoantigen separately, since anti-PAD4 can be detected in patients without anti-CCP antibody. PAD4 may be overexpressed in RA patients with a susceptible haplotype, which leads to the breakdown of immunological tolerance to PAD4 and production of anti-PAD4. At the same time, as more PAD4 enzymes are being produced, this leads to increased citrullination of proteins and an increased chance of developing anti-CCP antibodies. The mechanism of how the anti-PAD4 and anti-CCP antibodies contribute to perpetuation of the inflammation and the chronicity of RA remains to be elucidated.

In addition, we observed that 5 of 20 (25%) patients with RA lacking RF were anti-PAD4-positive. In the patients without AKA, the prevalence of anti-PAD4 was 38.7% (24/64). These results indicated that anti-PAD4 may be helpful for the diagnosis of RA patients lacking RF or AKA.

In summary, as an enzyme responsible for protein modification, PAD4 not only participates in the generation of multiple specific autoantigens for RA, i.e., citrullinated proteins, but also acts as an autoantigen in RA by itself, and has a positive association with disease severity. Further studies of the molecular mechanism of anti-PAD4 production will be of great significance in understanding the pathogenesis of RA.

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