Elevated levels of anti-carbamylated protein antibody in patients with rheumatoid arthritis: association with disease activity and bone destruction

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ABSTRACT

To measure the serum levels of anticarbamylated protein (CarP) antibodies in patients with rheumatoid arthritis (RA) in China and to evaluate the association of anti-CarP antibodies with clinical parameters and disease activity. 260 Chinese patients with RA, 40 patients with osteoarthritis (OA), 88 patients with spondyloarthritis (SpA) and 77 healthy controls were included. The serum levels of anti-CarP antibodies were detected by ELISA. Blood tests to detect the anticyclic citrullinated peptide (CCP) antibody level, rheumatoid factor (RF) level, erythrocyte sedimentation rate, C reactive protein level and Disease Activity Score in 28 joints using the erythrocyte sedimentation rate (DAS28-ESR) were performed by standard methods. Bone erosion was assessed by colour Doppler ultrasonography. A total of 18.8% of patients with RA and 9.4% of anti-CCP antibody and RF-double-negative patients were positive for anti-CarP antibody. The anti-CarP antibody level was significantly higher in patients with RA than in patients with OA or SpA and in healthy controls. Univariate and multivariate analyses showed that the level of anti-CarP antibody was positively correlated with DAS28-ESR; the higher a level of serum anti-CarP antibody, the higher the DAS28-ESR score. Anti-CarP-positive patients had higher disease activity scores than anti-CarP-negative patients. Moreover, anti-CarP-positive patients had a higher risk of developing bone erosion. The anti-CarP antibody was found to play an important role in the diagnosis of RA, especially in anti-CCP antibody and RF-double-negative patients. The anti-CarP antibody is a potential marker of disease activity and bone erosion in RA.

Significance of this study

What is already known about this subject?

- Autoantibodies, such as anticyclic citrullinated peptide (CCP) antibody and rheumatoid factor (RF), play a critical role in RA in the diagnosis of RA.
- Studies have shown that serological indexes (anti-CCP/RF-negative) were negative in some patients with RA.
- Previous cohorts of studies on the role of anticarbamylated protein (CarP) antibodies in patients with RA were mostly from Europe and North America.

What are the new findings?

- Anti-CarP antibodies were present in Chinese patients with RA and seronegative patients with RA .
- Anti-CarP antibody was positively correlated with disease activity of Chinese patients with RA.
- Anti-CarP-positive patients had a higher risk of developing bone erosion.

How might these results change the focus of research or clinical practice?

- This study demonstrated that anti-CarP antibodies can be also detected in the sera of Chinese patients with RA, and the study of anti-CarP antibody in disease activity and bone destruction in patients with RA provides new ideas for the diagnosis, treatment and prognosis of RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammatory infiltration of the synovium, leading to cartilage and bone destruction. Currently, early diagnosis and treatment are very important to improve the prognosis of patients with RA. Anticyclic citrullinated peptide (CCP) antibody and rheumatoid factor (RF) are two main autoantibodies closely associated with RA. They can be detected in the serum several years before the onset of symptoms; therefore, anti-CCP antibody and RF are used as important markers for the daily evaluation of RA.[1] Moreover, anti-CCP antibody and RF were included among the 2010 American College of Rheumatology/the European League Against Rheumatism criteria for RA.[2] Although great progress has been made in the study of anti-CCP antibody and RF in RA and their

important role in RA has been confirmed, researchers found that serological indexes were negative (anti-CCP/RF-negative) in some patients with RA.[3 4] Therefore, it is important to explore new markers for the diagnosis of RA.

Carbamylated proteins (CarPs) have been post-translationally modified; however, unlike citrullination, which requires enzyme catalysis, carbamylation is a chemical modification that converts lysine to homocitrulline in the presence of cyanate.[5] Cyanate comes from several sources in the body, including the spontaneous degradation of urea and catalysis of myeloperoxidase under inflammatory conditions, or is inhaled directly from the air.[6–8] Carbamylation not only can disrupt the structure and function of proteins and cellular processes but also can cause systemic reactions. Importantly, CarPs are targets of autoimmune reactions and immune tolerance, which can lead to the production of an antibody directed against CarPs, the anti-CarP antibody.[9]

Anti-CarP antibody, a new antibody family, has been detected in European, North American, and Japanese cohorts.[4 10–14] The presence of anti-CarP antibodies was associated with a higher disease activity score and increased radiological progression over time. However, so far, this has not been tested in a Chinese population. Furthermore, the role of anti-CarP antibody in the diagnosis, activity and prognosis of patients with RA has not been confirmed. In this study, we investigated the serum level of anti-CarP antibody in Chinese patients with RA and detected its association with disease activity and bone erosion.

MATERIALS AND METHODS

Patients

Sera were obtained from 260 patients with RA (211 female and 49 male) who fulfilled the 2010 RA criteria[2]; we strictly ruled out other non-RA diseases. There was no prior treatment with hormones and biological agents. In addition, 40 patients with osteoarthritis (OA) and 88 patients with spondyloarthritis (SpA), respectively, satisfied the corresponding diagnostic criteria.[15–17] The aforementioned subjects were from the Department of Rheumatology, First Affiliated Hospital of China Medical University. Seventy-seven age-matched and sex-matched healthy persons from the physical examination center were included as controls. All serum samples were stored at −70°C until analysis.

Blood tests were used to assess the erythrocyte sedimentation rate (ESR), and levels of C reactive protein (CRP) were determined by the Westergren method and the immune transmission turbidimetry method, respectively; RF and anti-CCP antibody were detected by the immune transmission turbidimetry method (Roche, Switzerland) and electrochemiluminescence immunoassay (Roche, Switzerland), respectively. According to the manufacturers' guidelines, the positive level was set as >15 IU/mL for RF and >17 U/mL for the anti-CCP antibody. Clinical data on sex, age, smoking status, disease duration and disease-modifying antirheumatic drug (DMARD) therapy, the swollen joint count (SJC), and the tender joint count (TJC) were recorded on admission. Disease activity was scored by Disease Activity Score in 28 joints using the erythrocyte sedimentation rate (DAS28-ESR). Colour Doppler ultrasonography was performed with SUPERSONIC AIXP (Supersonic Imagine, France). Patients with RA were examined by the same professionally trained ultrasonographer and examination was repeated by another professionally trained ultrasonographer. The two ultrasound experts did not know the diagnosis and clinical data of all patients previously, and both were blinded to the ultrasonic examination results of the other ultrasonographer. Wrist joint, first–fifth metacarpophalangeal and proximal interphalangeal joint of both hands in patients with RA were assessed with colour Doppler ultrasonography for joint effusion, synovitis, bone erosions and power Doppler signal. The selected parameters of colour Doppler ultrasonography were defined as follows: joint effusion: a compressible anechoic intracapsular area (minimal to extensive amount of joint effusion); synovitis: anechoic or hypoechoic intracapsular area (synovial thickening); bone erosion: the presence of irregular bone surface and defects within a joint seen on two visible vertical planes; and power Doppler signal: flow signal could be seen in the synovium by power Doppler signal.[18 19]

Detection of anti-CarP antibody levels in serum samples

Serum anti-CarP antibody level was measured by ELISA according to the manufacturer's directions (Shanghai Guang Rui Biological Technology Co, China). Each sample was assessed in duplicate; the coefficient of variation between and within the assay were 6.5% and 4.5%, respectively. The optical density was measured spectrophotometrically at a wavelength of 450 nm. The levels of the anti-CarP were further detected by comparison to known standards. We used the mean±2 SD of the anti-CarP antibody levels from healthy controls as the anti-CarP antibody-positive cut-off value.

Statistical analysis

All data are presented as mean±SD (parametric) or median (non-parametric). The unpaired Student t-test and the non-parametric Mann-Whitney test were performed to compare variables between groups. The one-way analysis of variance (ANOVA) was used to compare the means of more independent groups. The χ^2 test was performed for qualitative variables. Pearson's or Spearman's correlation coefficient was used to test correlations between the serum levels of anti-CarP antibody and laboratory values. Multiple linear regression was used to analyze the covariates. All analyses were performed using SPSS V.20.0 and GraphPad V.7 software.

RESULTS

Clinical parameters of patients with RA

A total of 260 patients with RA were recruited. Their clinical parameters are listed in table 1. We assessed anti-CarP antibody levels in male and female patients, and we found no significant difference in anti-CarP antibody levels between the two groups (mean±SD, 30.7±38.7 vs 30.4±27.6, respectively; p=0.944 by unpaired t-test). In addition, anti-CarP antibody levels were not correlated with the other clinical features age, disease duration and smoking status.

Table 1 Clinical parameters of patients with rheumatoid arthritis and analysis of their correlation with serum anti-CarP antibody level

Characteristics	Value	Correlation with anti-CarP level	
		Correlation coefficient (r)	P value
Age (years)	55.0±13.6	−0.112	0.083
Disease duration (years)	6.4±7.6	−0.005	0.934
ESR (mm/hour)	43.9±19.5	0.012	0.864
CRP (mg/L)	34.8±33.3	0.004	0.957
RF (IU/mL)/positive rate (%)	159.9±223.8/79.7	0.003	0.957
Anti-CCP (U/mL)/positive rate (%)	358.7±170.9/79.4	0.028	0.672
SJC	4.4±0.9	0.111	0.120
TJC	9.2±4.0	0.148	0.038
DAS28-ESR	9.8±2.4	0.197	0.009

Values were presented as mean±SD. The relationship between the serum levels of anti-CarP antibody and parameters was analyzed by Pearson's correlation coefficient.
CarP, carbamylated protein; CCP, cyclic citrullinated peptide; CRP, C reactive protein; DAS28-ESR, Disease Activity Score in 28 joints using erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count.

Elevated levels of anti-CarP antibodies in patients with RA

Anti-CarP antibodies were detectable in the sera of 49 of 260 patients with RA (18.8%), 2 of 40 the patients with OA (5%), 4 of 88 patients with SpA (4.5%) and 5 of 77 healthy controls (6.5%). The serum anti-CarP antibody level was significantly higher in patients with RA than in patients with OA or SpA and healthy controls (p=0.036, p=0.002, and p=0.004, respectively, by one-way ANOVA) (figure 1A). However, there was no significant difference in anti-CarP antibody levels between the OA, SpA and healthy control groups (p=0.513 by one-way ANOVA) (figure 1A).

Association of anti-CarP antibodies with anti-CCP antibody and RF

Overall, 79.7% patients were anti-CCP antibody-positive and 79.4% patients were RF-positive. Anti-CarP antibodies were present in 9.4% of RF and anti-CCP antibody double-negative patients.

According to anti-CCP antibody and RF status, there were patients positive for anti-CarP antibody in each group. The percentage of anti-CarP antibody-positive patients in the anti-CCP antibody-positive group was higher than that in the anti-CCP antibody-negative group (16.4% vs 3.0%) (OR 6.5, 95% CI 3.0 to 15.3; p<0.05)

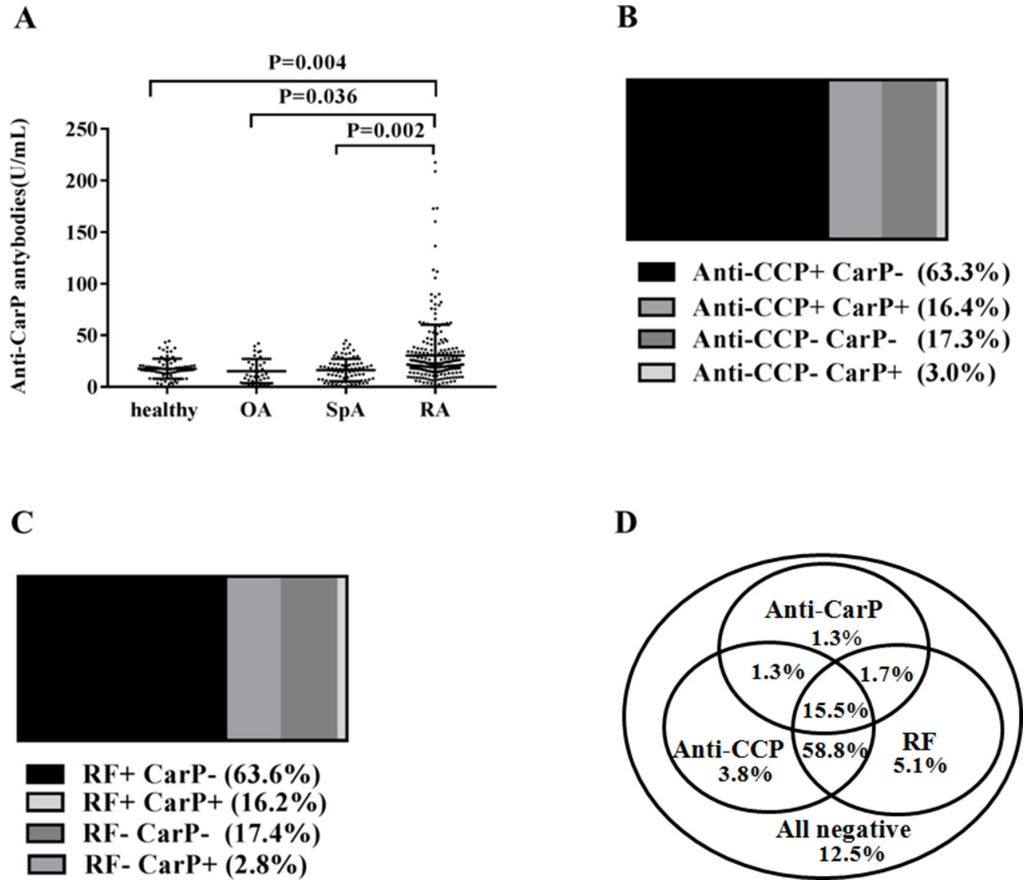

Figure 1 (A) Serum anti-CarP antibody levels in patients with RA, OA, SpA and healthy controls. (B,C) Distribution of anti-CarP antibodies based on anti-CCP antibody or RF. (D) Distribution of single-positive, double-positive and triple-positive autoantibodies. CarP, carbamylated protein; CCP, cyclic citrullinated peptide; OA, osteoarthritis; SpA, spondyloarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor.

(figure 1B), and the proportion of anti-CarP-positive patients in the RF-positive group was higher than that in the RF-negative group (16.2% vs 2.8%) (OR 6.8, 95% CI 3.0 to 15.9; p<0.05) (figure 1C). Patients positive for anti-CarP antibodies alone comprised 1.3% of the entire patients with RA population, while 1.3% of the patients with RA were positive for both anti-CarP antibody and anti-CCP antibody, 1.7% were positive for anti-CarP antibody and RF, and 15.5% were positive for all three autoantibodies (figure 1D).

Anti-CarP antibodies are associated with disease activity in patients with RA

Correlations between anti-CarP antibody levels and disease activity in patients with RA are presented in table 1 and figure 2A. A higher level of anti-CarP was associated with higher DAS28-ESR (r=0.197, p=0.009 by Pearson's correlation test), Statistical analysis of the relationship among the main covariates was made by multivariate linear regression, adjusted for other factors, including levels of anti-CCP antibody, RF, age, gender, disease duration and

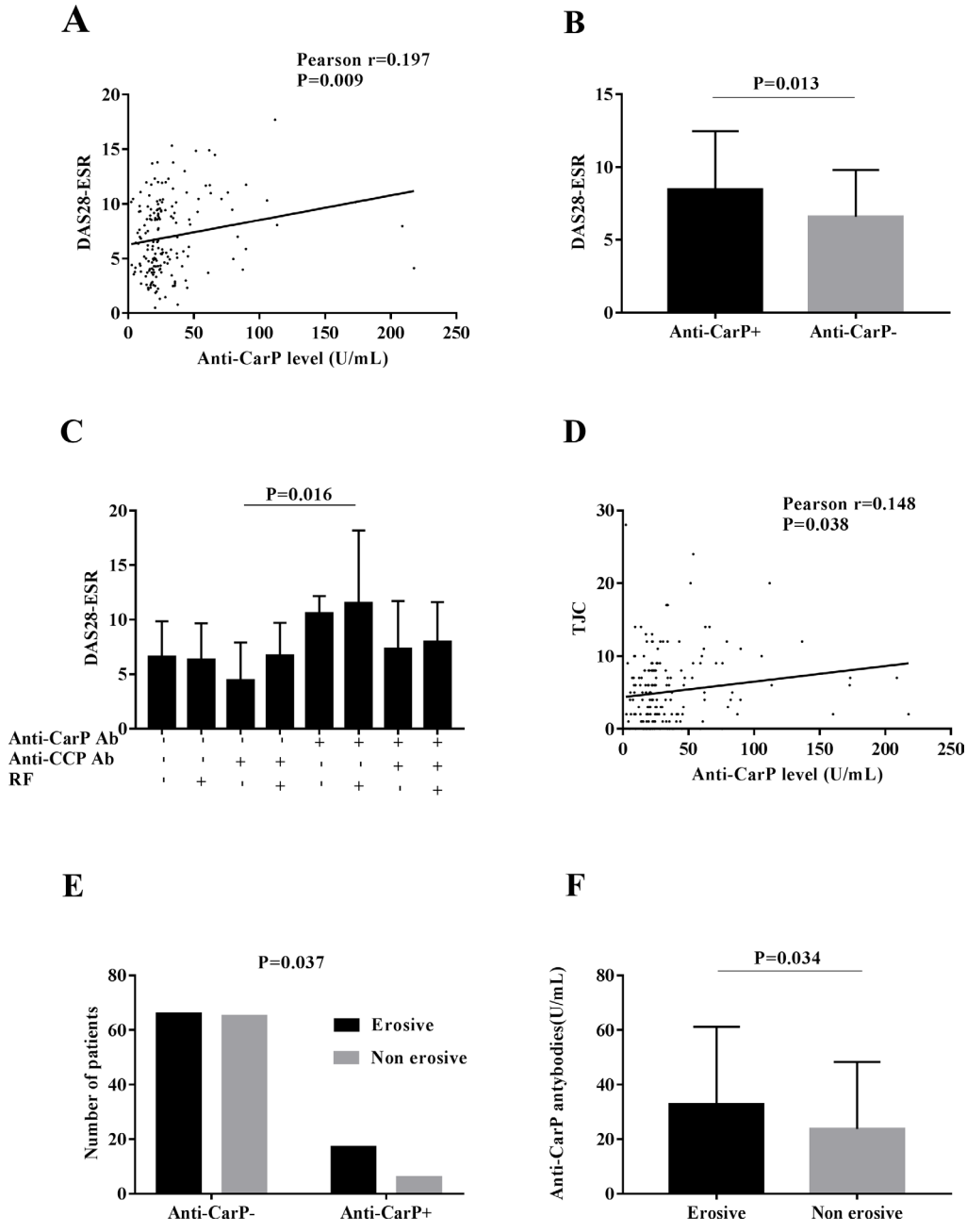

Figure 2 (A) Correlation between anti-CarP Ab levels and DAS28-ESR. (B) Comparison of DAS28-ESR between anti-CarP Ab-positive group and anti-CarP antibody-negative group. (C) DAS28-ESR according to Ab status (presence or absence anti-CarP, anti-CCP and RF). (D) Correlation between anti-CarP Ab levels and TJC. (E) The number of patients with erosion versus non-erosion in the anti-CarP-positive group and in the anti-CarP-negative group. (F) Anti-CarP Ab levels in patients with erosive disease and non-erosive disease. Ab, antibody; CarP, carbamylated protein; CCP, cyclic citrullinated peptide; DAS28-ESR, Disease Activity Score in 28 joints using the erythrocyte sedimentation rate; RF, rheumatoid factor; TJC, tender joint count.

Table 2 Multiple linear regression analysis of serum anticarbamylated protein antibody level

	B	β′	95% CI	P value
DAS28-ESR	1.37	0.21	0.1 to 2.7	0.045
Medications	11.30	0.20	0.2 to 22.4	0.047

Adjusted for anticyclic citrullinated peptide antibody, rheumatoid factor, age, gender, disease duration and medication use.
DAS28-ESR, Disease Activity Score in 28 joints using erythrocyte sedimentation rate.

medication use; this maintained significance in the multivariate analysis (p=0.045) (table 2), suggesting that serum anti-CarP antibody was significantly associated with disease activity. Anti-CarP-positive patients had higher disease activity, as indicated by higher DAS28-ESR scores than anti-CarP antibody-negative patients (mean±SD, 8.5±4.0 vs 6.6±3.1, respectively; p=0.013 by unpaired t-test) (figure 2B). Patients who were positive for both RF and anti-CarP antibodies were found to have higher DAS28-ESR values than those who were positive for anti-CCP antibodies alone (mean±SD, 11.5±6.6 vs 4.5±3.5, respectively; p=0.016 by one-way ANOVA) (figure 2C).

Additionally, the anti-CarP antibody level was significantly positively correlated with disease activity index and the TJC; high serum anti-CarP levels were associated with high TJC (r=0.148, p=0.038 by Pearson's correlation test) (figure 2D) .

However, the anti-CarP antibody level was not significantly correlated with the inflammatory parameters ESR and the CRP level. In addition, the anti-CarP level was not correlated with the other medical parameters, such as the SJC (p=0.12).

Anti-CarP antibody levels and bone erosion in patients with RA

Data on bone erosion detected by colour Doppler ultrasonography were available for 154 patients. The presence of anti-CarP antibodies was associated with erosion in patients with RA (73.9% of anti-CarP antibody-positive patients vs 50.4% of anti-CarP antibody-negative patients) (OR 2.8, 95% CI 1.0 to 7.4; p=0.037 by χ^2 test) (figure 2E). We evaluated the association of anti-CarP antibodies with bone erosion in different subgroups of patients according to anti-CCP antibody and RF status. Within the anti-CCP antibody-negative/RF-negative/anti-CarP-positive patients did not significantly associate with a higher percentage of bone erosion than the anti-CCP antibody-negative/RF-negative/anti-CarP-negative patients (p=0.137). However, triple-positive patients had a higher percentage of bone erosion (68.8% vs 31.2%) (OR 4.8, 95% CI 1.1 to 18.4; p<0.05 by χ^2 test), and there was a significantly greater percentage of bone erosion in anti-CarP antibody-positive/RF-positive patients compared with anti-CarP antibody-negative/RF-negative patients (77.8% vs 41.2%, respectively) (OR 3.7, 95% CI 1.0 to 11.5; p<0.05 by χ^2 test).

Moreover, patients with bone erosion had higher levels of anti-CarP antibodies than patients without bone erosion (mean±SD, 32.9±28.3 vs 23.8±24.5, respectively; p=0.034 by unpaired t-test) (figure 2F). Therefore, these results suggest that anti-CarP antibody has important prognostic value for disease progression in patients with RA. However, anti-CCP antibody levels and RF positivity were not associated with more severe joint destruction.

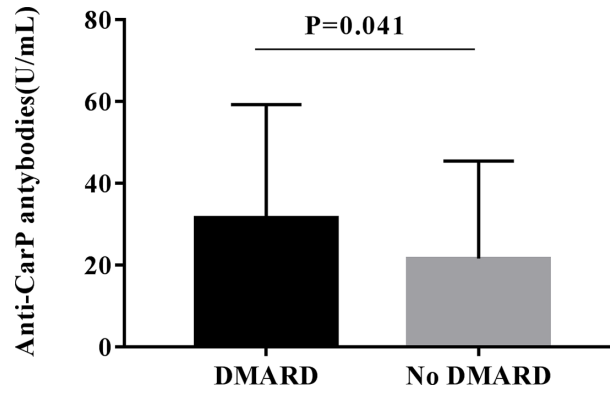

Figure 3 Anti-CarP levels in patients who received DMARDs and patients who did not receive DMARDs. CarP, carbamylated protein; DMARD, disease-modifying antirheumatic drug.

Treatment and anti-CarP antibodies

Patients who were treated with DMARDs had higher levels of anti-CarP antibodies than patients who did not receive DMARDs (mean±SD, 31.7±27.6 vs 21.6±23.9, respectively; p=0.041 by unpaired t-test) (figure 3).

DISCUSSION

This study is the first to show that anti-CarP antibody levels are elevated in Chinese patients with RA and correlated with disease activity, bone erosion and the use of prescription DMARDs. In this study, serum anti-CarP antibody levels were significantly higher in patients with RA compared with healthy controls and disease controls, and patients with OA or SpA. We observed the presence of anti-CarP antibody in 18.8% of patients with RA. Importantly, anti-CarP antibodies were detected in 9.4% of CCP-negative and RF-negative patients. However, according to previous studies, the prevalence of the anti-CarP antibody is 30%–45%, and anti-CarP antibody was detected in approximately 8%–30% of seronegative patients with RA.[4 12 20–23] Our results are slightly different from those of previous reports. We believe that this discrepancy may be related to differences in the different genetic backgrounds of the patients. Based on current and previous studies, we infer that anti-CarP antibodies undeniably play a pivotal role in RA, especially in patients seronegative for anti-CCP antibody and RF.

The anti-CarP antibody is a member of a novel family of antibodies. Steinbrecher *et al* first proposed in 1984 that CarP can be used as an immunogen to stimulate the body's immune response to produce autoantibodies.[24] Turunen *et al* and Kunnu *et al* demonstrated the presence of anti-CarP antibody in rabbit and mouse disease models.[25 26] By 2010, the discovery of anti-CarP antibodies in human serum and autoimmune arthritis animal models had deepened understanding of the correlation between autoantibodies and RA.[27] Shi *et al* reported the presence of anti-CarP antibodies in the sera of patients with RA and that the prevalence of IgG and IgA anti-CarP antibodies was 45% and 43%, respectively. Moreover, 16% and 30% of anti-CCP

antibody-negative patients with RA were positive for IgG and IgA anti-CarP antibodies, respectively. Researchers have also shown that anti-CarP antibodies and anti-CCP antibodies represent two different and independent autoantibody families.[4]

Currently, most research content on anti-CarP antibodies focuses on their role in bone destruction in patients with RA, and little research is focused on the relationship between anti-CarP antibodies and disease activity. The principles of RA treatment call for early, standardized treatment and regular testing and follow-up, and the goal is to achieve disease remission or low disease activity, so disease activity should not be ignored in long-term RA detection. In our study, we evaluated the relationship between anti-CarP antibodies and disease activity. We found that anti-CarP antibodies were positively correlated with disease activity and that patients in the anti-CarP antibody-positive group had more disease activity than patients in the anti-CarP antibody-negative group. We also evaluated the prognosis of patients in the anti-CarP antibody-positive group. The incidence of bone destruction in anti-CarP antibody-positive patients was higher than that in anti-CarP-negative patients. The association between anti-CarP antibody levels and disease activity, as measured by DAS28-ESR score and bone destruction, was especially strong when associated with positive anti-CCP antibody or RF status.[9 11 28] Therefore, we believe that the combination of anti-CarP antibody level, anti-CCP antibody level and RF level is of considerable value for the disease progression and prognosis of RA.

However, based on anti-CarP antibody status, we did not observe a significantly higher proportion of patients with high disease activity in the anti-CarP antibody-positive group than in the anti-CarP antibody-negative group.

Additionally, because smoking is a source of carbamylation, we analyzed the association between smoking and anti-CarP antibody level. In our study, there were 170 people with complete information on smoking status, but smoking status was not associated with anti-CarP antibody levels, consistent with the results of Jiang *et al*.[12]

The anti-CarP antibody has not been used in clinical testing, and its cut-off value is not as clear as those for anti-CCP antibody and RF. In the European case–control cohort, Jiang *et al* established the cut-off value as the mean plus two times SD[12]; the cut-off for positivity was defined by receiver operating characteristic curves within the study from Northern Sweden cohort,[22] and in the study of Yee *et al*, the cut-off was defined after testing 100 apparently healthy donors as the 95% percentile.[23] So, the verification of the significance of anti-CarP in the study of RA and other rheumatic diseases still requires a large amount of valid data.

In conclusion, patients with different genetic backgrounds may have different anti-CarP antibody levels. Anti-CarP antibodies were present in Chinese patients with RA, and serum anti-CarP antibody levels were detected in patients with RA and were found to be correlated with disease activity and bone erosion. Anti-CarP antibodies could be a disease activity marker. The results of this study support the hypothesis that anti-CarP antibodies can contribute to the diagnosis, progression, and prognosis of RA.

Contributors HS, LX and JL designed the study and revised the manuscript. BZ and YL performed the experiments. BZ drafted the manuscript. BZ, XL and ZG analyzed the data.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study was approved by the ethics committee of the First Affiliated Hospital of China Medical University (2019-125-2) and complies with the Declaration of Helsinki. Written informed consent was given by all patients.

Provenance and peer review Not commissioned; internally peer reviewed.

Data availability statement Data are available upon reasonable request. no additional data.

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