

Anti-carbamylated Protein Antibodies Are Detectable in Various Connective Tissue Diseases

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ABSTRACT. Objective. Anti-carbamylated protein (anti-CarP) antibodies are possible diagnostic biomarkers of anticitrullinated protein antibody (ACPA)-negative rheumatoid arthritis (RA). We aimed to elucidate the prevalence of anti-CarP antibodies in non-RA connective tissue diseases (CTD) because CTD are important in the differential diagnosis of ACPA-negative RA.

Methods. The sera from 266 patients with RA and 616 patients with CTD and 80 healthy controls were examined using an in-house anti-CarP ELISA.

Results. The prevalence and the level of anti-CarP antibodies in several CTD were comparable to those in ACPA-negative RA.

Conclusion. Anti-CarP antibodies are not useful for differentiating ACPA-negative RA from CTD. (First Release May 15 2017; J Rheumatol 2017;44:1384–8; doi:10.3899/jrheum.161432)

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Anti-carbamylated protein (anti-CarP) antibodies were detected in various studies in 36%–45% of patients with rheumatoid arthritis (RA)^{1,2,3}. Remarkably, IgG anti-CarP antibodies were detected in 16% of anticitrullinated protein antibody (ACPA)-negative patients with RA. Because ACPA-negative RA is sometimes difficult to diagnose, a new diagnostic biomarker is needed, and it is possible that anti-CarP antibodies can be used in that way.

Because non-RA connective tissue diseases (CTD) are important in the differential diagnosis of ACPA-negative RA⁴, the prevalence of anti-CarP antibodies in non-RA CTD has to be evaluated to judge whether they are useful for diagnosing ACPA-negative RA. To this end, we tried to elucidate the prevalence of anti-CarP antibodies in non-RA CTD.

MATERIALS AND METHODS

Patients and clinical information. The sera of 882 patients and 80 healthy controls (HC) were collected, with written informed consent. Non-RA CTD patients overlapping RA were excluded from this study. Information about the disease name was obtained from each attending physician using questionnaires, and accordingly the diagnosis was based on each physician's judgment. The results of commercially available disease-specific autoantibody tests and information about arthritis in systemic lupus erythematosus (SLE) were obtained by retrospective chart review. The precise method of each disease-specific antibody test is shown in Supplementary Methods, available with the online version of this article. All data were analyzed anonymously. This study was designed in accordance with the Helsinki Declaration and was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee (approval number: E458).

Protein preparation. Fetal calf serum (FCS; Biowest) was carbamylated by potassium cyanate (KOCN; NACALAI TESQUE Inc.) following a reported method^{1,5}. Equal volumes of 2 mol/l of KOCN in distilled water and 4 mg/ml of FCS were mixed and incubated overnight at 37°C. Successful carbamylation was confirmed by liquid chromatography–tandem mass spectrometry.

Anti-CarP ELISA. We performed ELISA following the method described by Shi, *et al*¹. In brief, unmodified FCS (UmFCS) or carbamylated FCS (CarFCS) was used to coat Nunc Maxisorp plates (Thermo Fisher Scientific), followed by blocking with phosphate-buffered saline containing 2% bovine serum albumin (BSA-PBS). Patients' sera (150-fold dilution by BSA-PBS) were applied to the plates. After incubation and washing, these were added to the wells: rabbit anti-human IgG antibody (Thermo Fisher Scientific) diluted 10,000-fold with BSA-PBS, alkaline-phosphatase labeled anti-rabbit IgG (Promega) diluted 2000-fold with BSA-PBS, and 3,3'-diaminobenzidine (Sigma-Aldrich). The absorbency of each well was read at 405 nm. All samples were tested in duplicate. The standard curve was drawn using 1 patient's standard serum. Arbitrary units of anti-CarP level were calculated by subtracting the level against UmFCS from that against CarFCS. Negative values were regarded as zero. The cutoff value was set at the mean plus 2 SD of anti-CarP antibody levels in HC. The precision and repeatability of this in-house ELISA were evaluated by using coefficient of variation (CV%). Intraassay CV% was 8.4%, calculated by performing ELISA with 5 duplications of 8 samples on the same plate on the same day, and interassay CV% was 9.8%, calculated by 3 assays with 8 samples performed on different days.

Anticyclic citrullinated peptide antibodies. Anticyclic citrullinated peptide antibodies (anti-CCP) were measured by MESACUP-2 test CCP (Medical and Biological Laboratories) following the manufacturer's instruction.

Statistical analysis. All statistical analyses were conducted using R version 3.1.1⁶. The Mann-Whitney U test was used for comparison of antibody levels. Pearson's chi-squared test or Fisher's exact test was used for categorical data. We drew the receiver-operator characteristic (ROC) curves and calculated their area under the curve (AUC) using the ROCR package⁷ in R.

RESULTS

Anti-CarP antibodies were positive in 53.6% (112/209) of ACPA-positive patients with RA and 21.4% (12/56) of ACPA-negative patients with RA, consistent with previous reports^{1,2,3}.

On the other hand, anti-CarP antibodies were also frequently seen in other CTD (Table 1). When non-RA patients with CTD were used as controls, the sensitivity and specificity of anti-CarP antibodies for diagnosing RA were 46.8% and 76.3%, respectively.

We then assessed whether the specificity could be improved by raising the cutoff value and permitting a lower sensitivity. However, a high anti-CarP level was mainly seen in ACPA-positive RA, and the level in ACPA-negative RA was not higher than that in non-RA CTD (Figure 1). AUC of ROC curves for non-RA CTD versus total RA and ACPA-negative RA were 0.663 and 0.461, respectively (Figure 2). Consequently, a high level of anti-CarP antibodies is specific to ACPA-positive RA, and anti-CarP antibodies are not informative for differentiating ACPA-negative RA from non-RA CTD.

Next, we tested anti-CCP antibody in 493 out of 617 non-RA patients with CTD and 12 out of 80 HC because the cross-reaction between anti-CCP and anti-CarP antibodies

has been a concern^{8,9}. Anti-CCP antibody was positive in 29 (5.9%) non-RA patients with CTD. The prevalence in each disease is shown in Table 1. In non-RA patients with CTD, the numbers of patients were as follows: anti-CarP-positive (CarP+)/anti-CCP positive (CCP+), 9; CarP+/anti-CCP-negative (CCP-), 105; anti-CarP-negative (CarP-)/CCP+, 20; and CarP-/CCP-, 359. There was no significant correlation between the positivity of anti-CarP and anti-CCP antibodies in non-RA patients with CTD ($p = 0.298$, Pearson's chi-squared test).

Then we obtained the results of commercially available disease-specific autoantibody tests in each patient by retrospective chart review, and assessed the correlation between each antibody and anti-CarP antibodies. The positivity of anti-SS-A/Ro antibodies was associated with that of anti-CarP antibodies (Supplementary Table 1, available with the online version of this article). The levels of anti-CarP antibodies were significantly higher in CTD that are usually autoantibody-positive than in those that are usually autoantibody-negative (Supplementary Figure 1, available with the online version of this article).

Finally, we reviewed the charts of patients with SLE and assessed the correlation between arthritis and anti-CarP positivity. Arthritis was present in 171 patients (71.0%) at least once during the course of the disease. Anti-CarP antibodies were positive in 36 patients with arthritis (21.1%) and 17 patients without arthritis (24.3%). There was no significant correlation between the positivity of anti-CarP and arthritis ($p = 0.582$).

DISCUSSION

It was recently reported that anti-CarP antibodies were seen in 27%-31.1% of primary Sjögren syndrome (pSS) cases^{10,11}, in 8.3%-16.8% of SLE cases^{11,12}, and in 5.8% of systemic sclerosis (SSc) cases¹¹. Our data expanded these findings and revealed that anti-CarP antibodies are detectable in various non-RA CTD.

Because our data lack information about the joint involvement of non-RA CTD except for SLE, the exact diagnostic value in differentiating ACPA-negative RA from other inflammatory arthritis in daily clinical practice is unknown. Shi, *et al* reported the good efficacy of anti-CarP antibodies in diagnosing ACPA-negative RA in their early arthritis cohort¹³. However, their cohort did not often contain CTD with autoantibodies such as SLE, SSc, mixed CTD, and pSS, which showed a higher prevalence of anti-CarP antibodies in our data (Figure 1). The difference in the efficacy of anti-CarP antibodies can be explained by the difference in the components of each cohort. Consequently, although the pre-test probability deeply affects the sensitivity and specificity of anti-CarP antibodies in daily clinical practice, our data suggest that anti-CarP antibodies cannot be used for differentiating ACPA-negative RA from non-RA CTD.

Table 1. Prevalence of anti-CarP antibodies and anti-CCP antibody in rheumatoid arthritis (RA) and other connective tissue diseases (CTD).

Disease	n	Age, mean ± SD	Sex, F/M	Anti-CarP+, n (%)	Anti-CCP+, n (%)
RA (ACPA+)	209	57.1 ± 13.7	166/43	112 (53.6)	209/209 (100)
RA (ACPA-)	56	63.0 ± 12.8	46/10	12 (21.4)	0/56 (0)
SLE	241	41.3 ± 13.4	224/17	53 (22)	9/189 (4.8)
PM/DM	41	54.1 ± 12.6	34/7	3 (7.3)	5/41 (12.2)
SSc	100*	57.7 ± 13.2	94/6	23 (23)	3/61 (4.9)
MCTD and overlap syndrome	47	49.8 ± 13.2	43/4	18 (38.3)	4/35 (11.4)
Primary SS	73	57.9 ± 14.4	72/1	26 (35.6)	1/62 (1.6)
Vasculitis	37*	52.4 ± 17.5	29/8	8 (21.6)	2/37 (5.4)
BD	36*	47.2 ± 14.4	24/12	3 (8.3)	3/35 (8.6)
AOSD	13*	50.7 ± 17.4	9/4	2 (15.4)	1/11 (9.1)
PMR	13	70.1 ± 10.0	9/4	1 (7.7)	1/13 (7.7)
SpA	8*	46.5 ± 15.2	3/5	4 (50)	0/8 (0)
Others**	11*	52.8 ± 17.9	9/2	5 (45.5)	0/3 (0)
HC	80	ND	ND	3 (3.8)	0/12 (0)

*Including 3 overlapping patients (a patient with AOSD and SpA, a patient with BD and relapsing polychondritis in the group of Others, and a patient with SSc and vasculitis). They were counted in both disease groups. Anti-CCP was measured in 493 out of 617 non-RA CTD patients and 12 of HC. ** Includes relapsing polychondritis, sarcoidosis, primary antiphospholipid syndrome, and osteoarthritis. Anti-CarP: anti-carbamylated protein; ND: not determined; ACPA: anti-citrullinated protein antibody; anti-CCP: anticyclic citrullinated peptide antibodies; SLE: systemic lupus erythematosus; PM/DM: polymyositis/dermatomyositis; SSc: systemic sclerosis; MCTD: mixed CTD; SS: Sjögren syndrome; BD: Behçet disease; AOSD: adult-onset Still disease; PMR: polymyalgia rheumatica; SpA: spondyloarthritis; HC: healthy controls.

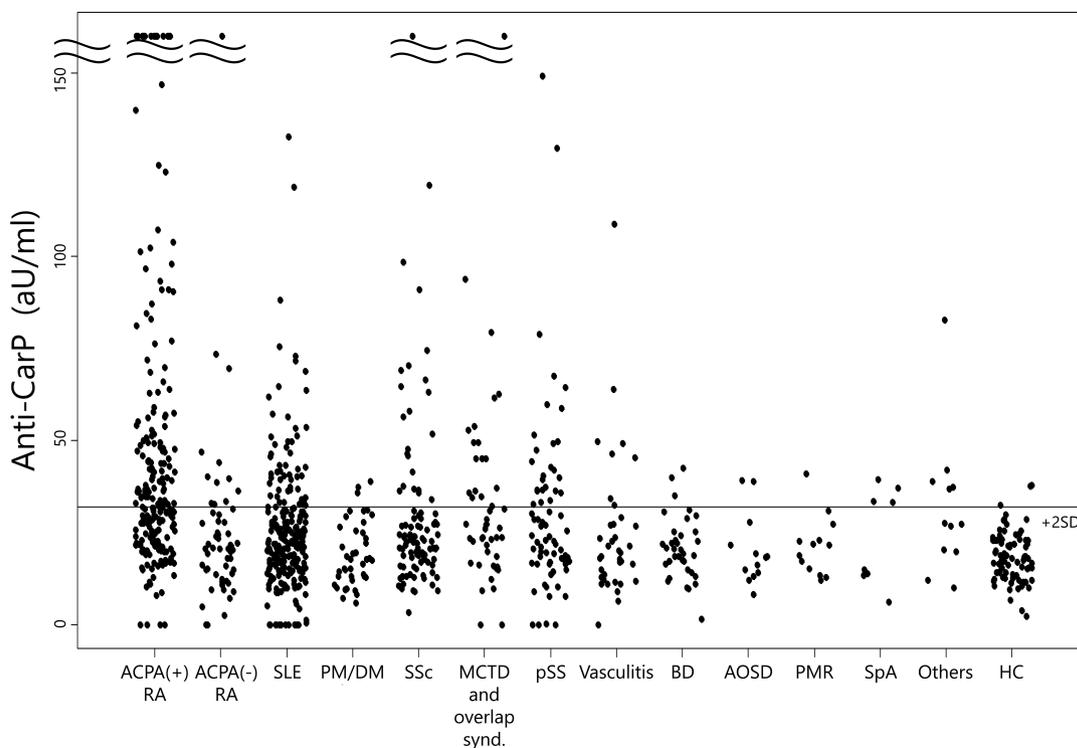


Figure 1. Anti-CarP levels in RA and other CTD. The horizontal line represents the cutoff value (mean + 2 SD of anti-CarP antibody levels in healthy controls). Anti-CarP: anti-carbamylated protein; RA: rheumatoid arthritis; CTD: connective tissue disease; ACPA: anticitrullinated protein antibodies; SLE: systemic lupus erythematosus; PM/DM: polymyositis/dermatomyositis; SSc: systemic sclerosis; MCTD: mixed CTD; pSS: Sjögren syndrome; BD: Behçet disease; AOSD: adult-onset Still disease; PMR: polymyalgia rheumatica; SpA: spondyloarthritis; HC: healthy controls.

Our data showed that the prevalence of anti-CarP antibodies tended to be higher in CTD that are usually autoantibody-positive than in those that are usually auto-

antibody-negative (Supplementary Figure 1, available with the online version of this article). And the positivity of anti-CarP antibodies were significantly correlated with that

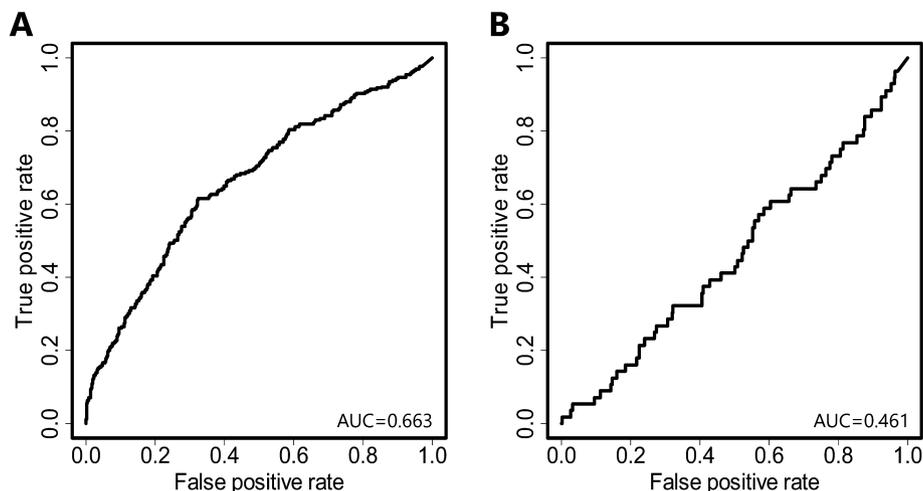


Figure 2. Receiver-operator characteristic curves of anti-CarP antibodies for RA versus non-RA CTD (A), and anticitrullinated protein antibody-negative RA versus non-RA CTD (B). AUC: area under the curve; anti-CarP: anti-carbamylated protein; RA: rheumatoid arthritis; CTD: connective tissue disease.

of anti-SS-A/Ro antibodies. This suggests the existence of a common pathway to produce anti-CarP antibodies and several autoantibodies.

On the other hand, although protein carbamylation occurs in various situations, such as uremia¹⁴ and inflammation in atherosclerotic lesions¹⁵, it was reported that anti-CarP antibodies are seen only in CTD¹⁶. Although we could not show the association between the presence of anti-CarP antibodies and joint symptoms in patients with SLE, Bergum, *et al* reported that anti-CarP antibodies in pSS are associated with more severe symptoms and stronger inflammation in minor salivary glands¹⁰. These findings suggest that induction of anti-CarP antibodies requires not only protein carbamylation, but also some other immune response.

Finally, our data showed that there is no significant association between the presence of anti-CarP and anti-CCP antibodies in non-RA CTD groups. Although the cross-reaction between anti-CCP and anti-CarP antibodies has been a concern^{8,9}, our data indicate that the detection of anti-CarP antibodies is not the result of cross-reaction of anti-CCP antibody.

The mechanism to develop anti-CarP antibodies and its pathological roles are of interest, but our study revealed that anti-CarP antibodies are not useful for differentiating ACPA-negative RA from non-RA CTD.

ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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