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Clinical Utility of Anti-Carbamylated Protein Antibody as a New Marker in Seronegative Rheumatoid Arthritis

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Abstract

Introduction: The development of rheumatoid arthritis (RA) is associated with the formation of a wide spectrum of autoantibodies, including rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs). A family of autoantibodies that recognize carbamylated proteins, Anti-CarP antibodies can be detected in sera of RA patients. The aim of the present study was to evaluate the role of anticarbamylated protein antibody (Anti-CarP antibodies) in diagnosis of seronegative (Negative RF and Negative ACCP) RA patients, in monitoring the severity of inflammation and degree of associated joint damage.

Methodology: Our study included 60 patients with seronegative RA (4 males and 56 females), their ages ranged between 29 and 70 years with a mean age of 48.5 ± 11.8 years, and 20 healthy controls of matched age and sex. Anti-CarP antibodies concentrations were measured by enzyme-linked immune-sorbent assay (ELISA).

Results: ACarPA was statistically significant increase in RA group compared to control group with no statistical significant differences between different RA groups. There was no statistical correlation between ACarPA and inflammatory markers (CRP and ESR). ACarPA had high diagnostic performance in differentiating RA from control and mild RA from control. There was no statistically significant difference in ACarPA between cases with or without osteolytic lesions in various RA studied groups.

Conclusion: Serum Anti-CarP Ab is a significant serological marker in sero-negative RA patients that has the potential to differentiate RA patients from control group.

Keywords: Rheumatoid arthritis; Anti-carbamylated protein antibody; Autoantibodies; Carbamylation; Rheumatoid factor; Anti-cyclic citrullinated peptides

Introduction

Rheumatoid arthritis (RA) is the most commonly occurring form of inflammatory polyarthritis. It is prevalent in approximately 0.8% of adults worldwide. If untreated, 20%-30% of RA patients become so severely debilitated within the first three years following initial diagnosis that they may become permanently disabled [1]. The initial presenting features of early RA do not substantially differ from other inflammatory arthritis. So prior to definite diagnosis patients with early RA are usually classified as undifferentiated arthritis which difficultly can be discriminated from other inflammatory arthritis [2]. Identification of RA at initial presentation and treatment at earlier stage can affect disease course, prevent the development of joint erosions or retard progression of erosive disease [3]. On the other hand, inappropriate treatment of patients who do not develop RA is harmful and should be avoided [2]. The development of RA is associated with the formation of a wide spectrum of autoantibodies, including rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs), the presence of which contribute substantially to the course and prognosis of the disease [4]. Despite the high diagnostic value of ACPAs and rheumatoid factors (RFs), there is still a need for novel biomarkers to further improve the diagnosis of RA Particularly in seronegative patients [5]. Approximately one-third of rheumatoid arthritis (RA) patients are seronegative for the 2 serological RA markers, rheumatoid factor (RF) and antibodies against cyclic citrullinated peptides (ACCP). Moreover, the sensitivities of both markers are lower in the diagnostically important early disease phase [6]. A family of autoantibodies that recognize carbamylated proteins, Anti-CarP antibodies can be detected in sera of RA patients. Anti-CarP antibodies and ACPA represent two different and independent autoantibody families, one recognizing carbamylated proteins and the other citrullinated proteins. Unlike citrullination which is catalyzed enzymatically, carbamylation (often referred to as homocitrullination) is a chemical modification. Although hCit and citrulline (Cit) are both posttranslationally modified amino acids and quite similar in structure, there are significant differences. hCit is one methylene group longer and is generated chemically from lysine by cyanate [7]. The carbamylation of amine groups leads to a change in the charge of the molecule. Carbamylated derivatives may therefore acquire biological and antigenic properties that are different from those of the

noncarbamylated molecules. On the other hand, carbamylation induce conformational changes in proteins leading to partial or complete loss of protein functions [8]. The objectives of this study were to evaluate the role of anticarbamylated protein antibody (Anti-CarP antibodies) in diagnosis of seronegative (Negative RF and Negative ACCP) RA patients, in monitoring the severity of inflammation and degree of associated joint damage.

Methodology

Patient population

This study was performed on a cohort of 60 seronegative rheumatoid arthritis patients (4 males and 56 females), their ages ranged between 29 and 70 years with a mean age of 48.5 ± 11.8 years, and 20 healthy controls of matched age and sex from the rheumatology clinics of Al-Hussein and Sayed Galal hospitals, Faculty of Medicine, Al-Azhar University. All patients met the 2010 ACR/EULAR classification criteria for RA. Patients group was subdivided into three subgroups based on clinical evaluation for disease activity assessed using a 28 joint disease activity score (DAS-28). Subgroup Ia included 20 patients with severe RA (DAS-28 between 5.22-7.32). Subgroup Ib included 20 patients with moderate RA (DAS-28 between 4.00-4.96). Subgroup Ic included 20 patients with mild RA (DAS-28 between 2.35-3.20).

Laboratory tests

The following laboratory tests were done for all members of this study. CBC was assayed on the Medonic M20* hematology analyser of Sweden. Erythrocyte sedimentation rate (ESR) was performed by Westergren method. CRP was assayed spectrophotometrically on the Cobas Integra- 400 autoanalyser of Roche Anti-CarP antibodies concentrations were measured using a commercially available enzyme-linked immune-sorbent assay (ELISA) kit supplied by Shanghai Korain Biotech CO., LTD of China. The used method was designed for detection of human Anti-CarP antibodies in plasma, serum and other related tissue Liquids. This assay employed a double-antigen sandwich enzyme immunoassay technique which measured Anti-CarP antibodies. Using the microplate reader of 450 nm wavelength the absorbency (OD value) was measured. Cut off value was calculated (the average of negative control well+0.15)=(0.09+0.15=0.24). Samples with OD 0.24 were Anti-CarP antibodies positive. Samples with OD<0.24 were Anti-CarP antibodies negative.

Statistical analysis

The collected data were coded, tabulated and statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 18.0, IBM Corp., Chicago, USA, 2009. Descriptive statistics were done for quantitative data as minimum and maximum of the range as well as mean ± SD (standard deviation) for quantitative normally distributed data, while it was done for qualitative data as number and percentage. Inferential analyses were done for quantitative variables using independent t-test in cases of two independent groups with normally distributed data and ANOVA test with post hoc Tukey test for more than two independent groups with normally distributed data. In qualitative data, inferential analyses for independent variables were done using Chi square test for differences between proportions and Fisher's exact test for variables with small expected numbers. While correlations were done using Pearson correlation for numerical normally distributed data. ROC curve was used to evaluate the performance of different tests differentiate between certain groups. The level of significance was taken at P value<0.01 is highly significant, P value<0.05 is significant and P value>0.05 is non-significant difference.

Diagnostic characteristics were calculated as follows: Sensitivity=(True positive/True positive+False negative) × 100

Specificity=(True negative/True negative+False positive) × 100

Positive predictive value=(True positive/True positive+False positive) × 100

Negative predictive value=(True negative/True negative+False negative) × 100

Diagnostic accuracy=([True positive+True negative]/Total cases) × 100

Results

Samples were collected from 60 patients with seronegative RA (4 males and 56 females). Their ages ranged between 29 and 70 years with a mean age of 48.5 ± 11.8 years. They were subdivided into three subgroups based on clinical evaluation for disease activity assessed using a 28 joint disease activity score (DAS-28). Subgroup Ia included 20 patients with severe RA (DAS-28 between 5.22-7.32). They were 1 male and 19 female. Their ages ranged between 31 and 70 years with a mean age of 51.2 ± 12.4 years. Subgroup Ib included 20 patients with moderate RA (DAS-28 between 4.00-4.96). They were 2 males and 18 females. Their ages ranged between 31 and 70 years with a mean age of 48.6 ± 11 years. Subgroup Ic included 20 patients with mild RA (DAS-28 between 2.35-3.20). They were 1 male and 19 females. Their ages ranged between 29 and 70 years with a mean age of 45.7 ± 11.9 years. Samples were also collected from 20 age and sex matched apparently healthy subjects serving as control. They were 2 males and 18 females. Their ages ranged between 36 and 71 years with a mean age of 52.7 ± 11.7 years. There was no statistical difference between the studied groups as regards age and sex.

The association between anti-carbamylated protein antibody level and seronegative rheumatoid arthritis patients

Our study showed that there were 13 positive cases out of 60 patients with RA (21.7%). As regards to values, there was a statistically significant increase regarding ACarPA in RA group compared to control group with no statistically significant difference between different RA groups. As regards to % of positive cases there was no statistical difference between different studied groups (Table 1).

Group	Quantitative			Qualitative		
	Mean ± SDOD	Range	HG	Positive	Negative	HG
Control	0.08 ± 0.04	0.02-0.15	a	0 (0.0%)	20 (100.0%)	a
Mild	0.24 ± 0.18	0.13-0.92	b	3 (15.0%)	17 (85.0%)	a
Moderate	0.25 ± 0.18	0.11-0.87	b	4 (20.0%)	16 (80.0%)	a
Severe	0.31 ± 0.20	0.14-0.85	b	6 (30.0%)	14 (70.0%)	a

RA	0.26 ± 0.19	0.10-0.92	--	13 (21.7%)	47 (78.3%)	--
P value All groups	<0.001*			and 0.053		
Control/RA	<0.001*			and 0.031*		

Table 1: Statistical comparison between the various studied groups as compared to one another regarding ACarPA. OD: Optical density, SD: Standard deviation, HG: Homogenous groups (homogenous groups had the same letter), P: Probability, RA: Rheumatoid arthritis, ACarPA: Anti-carbamylated protein antibody. P<0.01→ Highly significant, P>0.05→ Non-Significant.

Correlation study between ACarPA and laboratory markers used for follow up in control group and RA group

There was no statistically significant correlation between ACarPA and ESR or CRP in both control and RA groups. Meanwhile, there was a statistically significant positive correlation between CRP and ESR in RA group (Table 2).

Group	Lab		CRP	ACarPA
Control	**ESR mm/hr**	**r**	-0.017	0.226
		p	0.943	0.338
	CRP mg/dl	**r**		0.012
		p		0.96
RA	**ESR mm/hr**	**r**	0.713	0.112
		p	<0.001*	0.392
	CRP mg/dl	**r**		0.105
		p		0.425

Table 2: Correlation Study between ACarPA and laboratory markers used for follow up in control group and RA group. P: Probability, r: Correlation coefficient, ESR: Erythrocyte sedimentation rate, CRP: C reactive protein, ACarPA: Anti-carbamylated protein antibody, RA: Rheumatoid arthritis. P<0.01→ Highly significant. P>0.05→ Non-Significant.

Statistical comparison between conditions of osteolytic lesions regarding laboratory markers used for diagnosis and follow up between the various studied groups

There was no statistically significant correlation between ACarPA and CRP or ESR in various RA groups. Meanwhile, there was a statistically significant positive correlation between CRP and ESR in mild and moderate RA groups, but not in severe RA group (Table 3). Statistical comparison between conditions of osteolytic lesions regarding laboratory markers used for diagnosis and follow up between the various studied groups: There was no statistically significant difference in the levels of laboratory markers between cases with or without osteolytic lesions in various RA studied groups (Table 4).

Group	Lab		CRP	ACarPA
Mild	**ESR mm/hr**	**r**	**0.546**	-0.236
		p	**0.013***	0.316
	CRP mg/dl	**r**		0.176
		p		0.458
Moderate	**ESR mm/hr**	**r**	**0.606**	-0.117
		p	**0.005***	0.624
	CRP mg/dl	**r**		-0.153
		p		0.520
Severe	**ESR mm/hr**	**r**	**0.369**	0.214
		p	**0.109**	0.364
	CRP mg/dl	**r**		0.005
		p		0.982

Table 3: Correlation Study between ACarPA and laboratory markers used for follow up of RA in various studied group. P: Probability, r: Correlation coefficient, ESR: Erythrocyte sedimentation rate, CRP: C reactive protein, ACarPA: Anti-carbamylated protein antibody, RA: Rheumatoid arthritis. P<0.01→ Highly significant. P<0.05→ Significant. P>0.05→ Non-Significant.

Group	Marker	Lesion	No lesion	P	Osteolytic lesion
Mild	**ESR mm/hr**	22.3 ± 9.8	24.4 ± 9.4	0.648	12 (60.0%)
	CRP mg/dl	0.3 ± 0.2	0.4 ± 0.3	0.539	
	ACarPA OD value	0.26 ± 0.23	0.20 ± 0.07	0.459	
Moderate	**ESR mm/hr**	38.9 ± 15.4	33.3 ± 15.5	0.466	14 (70.0%)
	CRP mg/dl	1.3 ± 1.0	0.6 ± 0.3	0.075	
	ACarPA	0.27 ± 0.23	0.19 ± 0.13	0.395	
	OD vaue				
Severe	**ESR mm/hr**	58.1 ± 14.7	54.6 ± 12.0	0.64	15 (75.0%)
	CRP mg/dl	2.7 ± 1.5	3.5 ± 2.2	0.331	
	ACarPA	0.32 ± 0.21	0.29 ± 0.21	0.822	
	OD value				
	ESR	41.1 ± 19.8	35.2 ± 17.0	0.267	41 (68.3%)

	mm/hr				
RA	CRP mg/dl	1.5 ± 1.4	1.3 ± 1.8	0.561	
	ACarPA	0.28 ± 0.21	0.22 ± 0.13	0.228	
	OD value				

Table 4: Statistical comparison between laboratory markers associated with osteolytic lesions in RA patients OD: Optical density, P: Probability, ESR: Erythrocyte sedimentation rate, CRP: C reactive protein, ACarPA: Anti-carbamylated protein antibody, RA: Rheumatoid arthritis. P>0.05→ Non-Significant.

Diagnostic performance of ACarPA

Receiver operating characteristic curve (ROC) analysis was applied to assess the diagnostic performance of ACarPA. It showed that: ACarPA had high diagnostic performance in differentiating RA from control and mild RA from control (Figures 1 and 2; Tables 5 and 6).

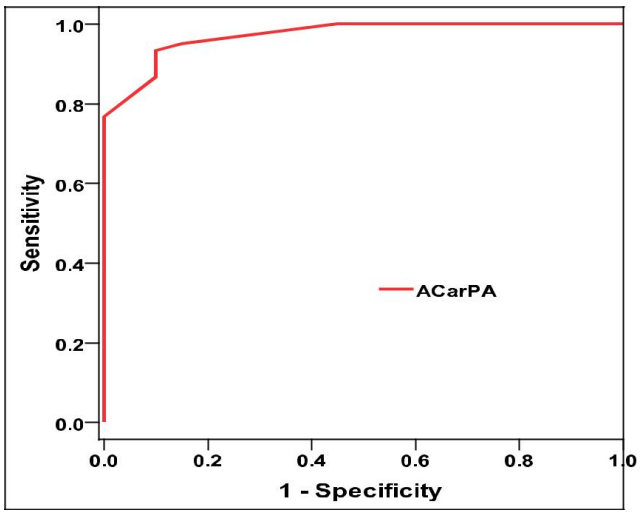

Figure 1: ROC curve for ACarPA to discriminate RA from control. Meanwhile the best diagnostic cutoff for ACarPA to differentiate mild RA from control group was 0.13. This had a diagnostic sensitivity of 100.0%, specificity 85.0%, negative predictive value 100.0%, positive predictive value 87.0% and the area under curve (AUC) was 0.979 as shown in Table 6 and Figure 2.

Diagnostic characteristics for the best cut-off points of ACarPA to diagnose and follow up of different RA groups

The best diagnostic cutoff for ACarPA to differentiate RA from control group was 0.13. This had a diagnostic sensitivity of 96.7%, specificity 85.0%, negative predictive value 89.5%, positive predictive value 95.1% and the area under curve (AUC) was 0.971 (Table 6; Figure 1).

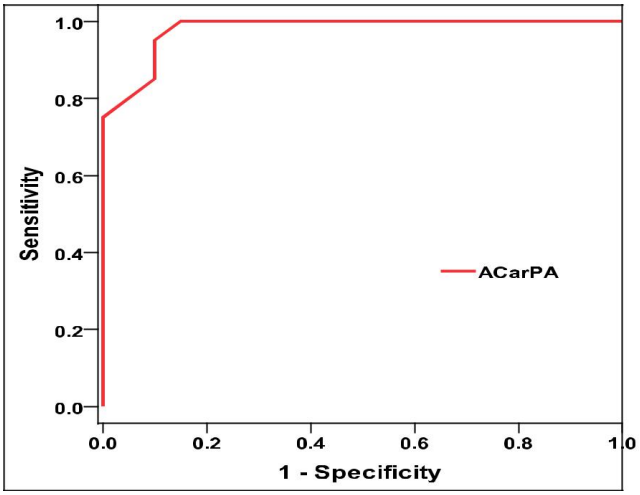

Figure 2: ROC curve for ACarPA to discriminate mild RA from control.

Factors	AUC	SE	P	95% CI
RA from control				
ACarPA	0.971	0.016	<0.001*	0.936-1.000
Mild from control				
ACarPA	0.979	0.017	<0.001*	0.500-1.000
Moderate from mild				
ACarPA	0.529	0.094	0.756	0.500-0.714
Severe from moderate				
ACarPA	0.649	0.088	0.108	0.500-0.821

Table 5: Diagnostic performance of ACarPA. AUC: Area under curve, SE: Standard error, CI: Confidence interval, P: Probability, ACarPA: Anti-carbamylated protein antibody, RA: Rheumatoid arthritis. P<0.01 → Highly significant. P>0.05→ Non-Significant.

Characters	Value	95% CI	Value	95% CI
RA from control		**Mild RA from control**		
ACarPA ≥ 0.13		**ACarPA ≥ 0.13**		
Sensitivity	96.70%	88.5%-99.6%	100.00%	83.2%-100.0%
Specificity	85.00%	62.1%-96.8%	85.00%	62.1%-96.8%
DA	93.80%	86.0%-97.9%	92.50%	79.6%-98.4%
PPV	95.10%	86.3%-99.0%	87.00%	66.4%-97.2%
NPV	89.50%	66.9%-98.7%	100.00%	80.5%-100.0

Table 6: Diagnostic characteristics for the best laboratory markers cut-off points to diagnose and follow up of different RA groups. CI: Confidence interval, DA: Diagnostic accuracy, PPV: Positive Predictive

value, NPV: Negative Predictive value, ACarPA: Anti-carbamylated protein antibody, RA: Rheumatoid arthritis.

Discussion

In our study, serum ACarPA levels showed statistically significant increase in RA group compared to control group with no statistically significant difference between different RA groups. These results are in agreement with Shi et al. in 2014 who found that anti-CarP antibodies were present in 27% of the serum samples that were drawn from RA patients compared to 4% of the matched control samples. Anti-CarP antibodies were present in both ACPA positive and ACPA negative patients [9]. Shi et al. in 2015 also demonestrated that anti-CarP antibodies were present in 44% of RA patients. Anti-CarP antibodies were also found in 11% of inflammatory osteoarthritis patients, 9% in psoriatic arthritis 16% in reactive arthritis (bacterial and viral), 4% in remitting seronegative symmetrical synovitis with pitting edema, 9% in sarcoidosis, 15% in spondylarthropathy with peripheral arthritis, 10% in undifferentiated arthritis and 8% in gout [10]. Brink et al. in 2015 also reported that the concentration of anti-CarP antibodies was significantly increased in the pre-symptomatic RA individuals compared with controls and also increased significantly as RA disease progress [11]. Similarly, Verheul et al. in 2016 reported that levels of anti-CarP antibodies were significantly elevated in RA patients, in comparison with those of the control group [12]. In a similar study, Othman et al. in 2017 demonstrated that the level of anti-CarP antibodies were significantly increased in the RA patients (both RF positive and RF negative) compared with healthy controls, with no significant differences between different RA groups [13]. Moreover, Shi et al. in 2011 found that IgG antibodies recognizing carbamylated (homocitrulline-containing) antigens were present in sera of over 45% of RA-patients. Likewise, anticarbamylated protein (anti-CarP) IgA antibodies were observed in 43% of RA-sera. In line with this observation, 16% of ACPA-negative RA-patients harbored IgG anti-CarP antibodies, whereas 30% of these patients tested positive for IgA anti-CarP antibodies [7]. Pecani et al. in 2016 also reported that the prevalence of anti-CarP antibodies was significantly higher in patients with RA compared to healthy controls and non-RA disease. Isolated anti-CarP positivity was detected in about one third of patients with RA who were seronegative for ACPA and RF. Beside patients with RA, anti-CarP antibodies were present in a small number of patients with SLE and SS (16.8% of patients with SLE and 31.1% of patients with SS). That increase in SLE and SS could be attributed to inflammation and kidney involvement due to high concentration of cyanate in these conditions. So anti-CarP antibodies is not considered as a specific marker in any of connective tissue diseases [14]. In contrast to our findings, Hoyos et al. in 2016 found that anti-CarP antibodies did not add any significant advantage to the diagnosis of RA as compared with RF or ACPA [15]. Moreover, Challener et al. in 2016 also reported that ACarP antibodies were present in only a small percentage (6.1-8.9%) of seronegative RA patients [16]. Differences in preparation of the antigen could lead to conflicting results between investigators. Our study also reported that there was no statistically significant difference in the levels of anti-CarP antibodies between cases with or without osteolytic lesions in various RA studied groups. In contrast to our findings, Brink et al. in 2015 reported that the presence of anti-CarP antibodies was related to radiological destruction at diagnosis and to the radiological progression observed once the disease had developed [11], Yee et al. in 2015 also found that anti-CarP antibodies were correlated with joint erosion score. No correlation between ACPA and joint erosion score was observed [17]. Humphreys et al. in 2016 also demonstrated that patients with anti-CarP antibodies were more disabled and had higher disease activity early in the disease and continued to have more functional disability and disease activity compared with anti-CarP antibody negative patients [18]. In the present study, there was no statistically significant correlation between ACarPA and ESR or CRP in both control and RA groups. Meanwhile, there was a statistically significant positive correlation between CRP and ESR in mild and moderate RA groups but not in marked RA group. In contrast to our findings Othman et al. in 2017 correlation analysis indicated that there was a statistically significant positive correlation between anti-CarP antibodies and CRP. Certainly, a possible limitation of our study was the small number of cases involved [13]. Receiver operating characteristic curve (ROC) analysis was applied to assess the diagnostic performance of anti-CarP antibodies in diagnosis and follow up of sero-negative RA in various studied groups. It showed that: anti-CarP antibodies had high diagnostic performance in discriminating RA from control and mild RA from control. These findings are going with studies done by Shi et al. in 2015, Pecani et al. in 2016 and Othman et al. in 2017 with variable degrees [10,13,14].

Conclusion

In view of our study, we observed that serum Anti-CarP Ab is a significant serological marker in sero-negative RA patients that has the potential to differentiate RA patients from control group.

Recommendations

Serum anti-carbamylated protein antibody is recommended to be measured in suspected cases of rheumatoid arthritis together with usual markers (RF and ACCP) in order to increase the diagnostic accuracy of rheumatoid arthritis.

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