ORIGINAL ARTICLE

Association of New Generation Anti-CCP Antibodies with Disease Severity and Functional Status in Rheumatoid Arthritis Patients

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ABSTRACT

Introduction: Detection of anti-cyclic citrullinated peptide (anti-CCP) antibodies in patients with rheumatoid arthritis (RA) is associated with higher disease activity and lower functional ability. This study investigated the presence of the new generation of anti-CCP antibodies (anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA) and their association with disease severity and functional status of RA patients. **Methods:** A total of 46 RA patients and 40 healthy controls participated in this cross-sectional study that was conducted at the Rheumatology Clinic, Hospital Universiti Sains Malaysia. Blood samples were taken from all participants for anti-CCP2 IgG, anti-CCP2 IgA, and anti-CCP3.1 IgG/IgA analysis. Disease severity and functional status of RA patients were measured using the Disease Activity Score-28 (DAS28) and the modified Health Assessment Questionnaire (mHAQ) respectively. **Results:** Significantly higher proportion of RA patients were found with positive anti-CCP2 IgG (63.0%), anti-CCP2 IgA (37.0%), and anti-CCP3.1 IgG/IgA antibodies (63.0%) than the healthy controls. No significant association was found between anti-CCP antibodies status and mean DAS28 score of the RA patients. However, RA patients with negative anti-CCP2 IgG status had higher mean mHAQ score than patients with positive anti-CCP2 IgG status. **Conclusion:** Our study has demonstrated detection of the new generation anti-CCP antibodies in RA patients, supporting the use of autoantibodies in RA diagnosis. While no significant association was found between the presence of anti-CCP antibodies and disease severity of RA patients, the absence of anti-CCP2 IgG was associated with worse function and greater disability of the patients.
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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease. It is characterised by joint swelling, joint tenderness, and destruction of synovial joints (1). RA is one of the most common autoimmune diseases, affecting 0.24% of the world population, predominantly female (2). Rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies have been used to support RA diagnosis in addition to presence of inflammatory markers and clinical manifestation of symmetrical joints inflammation (3).

RF was the first autoantibody discovered in people with RA and has been used for decades to aid in RA diagnosis. However, RF is also commonly detected in other autoimmune diseases, hence limiting its specificity (4). Detection of anti-CCP antibodies using enzyme-linked immunosorbent assays (ELISA) was recently introduced to aid in diagnosis and prognosis of RA particularly in seronegative RF. Anti-CCP antibodies are produced from the citrullination process of extracellular proteins caused by inflammatory cell influx forming immune complexes which precipitate mostly in symmetrical joints leading to joints pain and damage (5).

Thus far, four generations of anti-CCP antibody test have been produced. The first generation of anti-CCP (anti-CCP1) antibody test was introduced in 2000 using cyclic citrullinated peptides derived from filaggrin protein as an antigen (6). In 2002, the second generation of anti-CCP (anti-CCP2) antibody test was developed using different cyclic peptides (7). The third generation of anti-CCP (anti-CCP3) antibody test containing unique peptide was introduced in 2012 (8), followed by the latest version in 2013 called anti-CCP3.1 that can detect the combination of IgA and IgG isotypes. This latest

generation, with higher sensitivity particularly in RF negative patients, has proven to be significantly better compared to previous anti-CCP antibody tests which can only detect single Ig (9).

The main goals of RA management and treatment are to achieve disease remission, arrest joints erosion, and prevent disability. Functional ability of RA patients is therefore routinely assessed to indicate response to treatment in addition to determination of disease activity by counting the number tender and swollen joints, and monitoring of serological and inflammatory markers (10). Increased anti-CCP antibodies titres have been shown to be associated with higher disease activity, poorer clinical outcomes, less sustained remission, and lower functional ability (11, 12, 13, 14). However, evidence on detection of anti-CCP antibodies in RA patients and its association with disease activity and functional ability is still lacking. This study aimed to demonstrate the presence of serum anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies in RA patients and their association with disease severity and functional status.

MATERIALS AND METHODS

Population and samples

A total of 46 RA patients and 46 healthy controls participated in this cross-sectional study. Data collection was done at the Rheumatology Clinic, Hospital Universiti Sains Malaysia, Kelantan from October 2019 to January 2020. Diagnosis of RA was established by the attending rheumatologist based on the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 classification criteria (1). Healthy controls were volunteered individuals who were free from any chronic medical illnesses. Participants in both groups must be at least 18 years old. Exclusion criteria for both RA and healthy controls were pregnant woman, lactating mother, smokers, patients with uncontrolled chronic medical illnesses such as diabetes mellitus, hypertension, cancer and cardiovascular diseases, presence of infectious diseases such as tuberculosis and AIDS, and autoimmune disease such as systemic lupus erythematosus (SLE).

Convenience sampling method was used for recruiting the RA patients and healthy controls. Written informed consent was obtained from all subjects. Socio-demographic data including age, sex, ethnic group, marital status, education level, and employment status of RA patients and healthy controls were obtained using standardised data collection form, and clinical characteristics of RA patients including duration of disease, intake of RA medication, and presence of comorbidity were obtained from the medical records. The protocol of this study was approved by the Universiti Sains Malaysia Human Research and Ethics Committee (USM/JEPeM/16030138).

Blood samples collection

A standard venepuncture procedure was performed to obtain blood samples for anti-CCP2 IgG, anti-CCP2 IgA, and anti-CCP3.1 IgG/IgA antibodies tests. The blood samples were allowed to clot for one hour at room temperature. Centrifugation was performed for 5 minutes at 4,500 rpm using Universal 32R Centrifuge (Hettich, Germany) at 4°C. The sera were separated, placed in micro centrifuge tubes, and stored in the refrigerator at -80°C until assayed. Anti-CCP2 IgG and anti-CCP2 IgA antibodies levels were analysed using CCP2 IgG and anti-CCP2 IgA ELISA kit (Abnova, USA) respectively, whereas anti-CCP3.1 IgG/IgA antibody level was analysed using QUANTA Lite CCP3.1 ELISA kit (Inova Diagnostics, USA) according to the manufacturers' instructions. The levels of anti-CCP2 IgG and anti-CCP2 IgA of more than 18 IU/mL, and anti-CCP 3.1 IgG/IgA level of more than 20 IU/mL were considered positive. Additionally, the blood samples of RA patients were tested for inflammatory marker erythrocyte sedimentation rate (ESR) (mm/hour) using the Westergren method. The ESR was also used to determine disease activity.

RA disease activity

The Disease Activity Score-28 (DAS28) was used to measure disease activity of RA patients. The DAS28 is a scoring system that combines measurements of the following: 1) number of swollen joints out of 28 joints of the shoulder, elbow, wrist, hand, and knee, 2) number of tender joints out of the 28 joints of the shoulder, elbow, wrist, hand, and knee, 3) ESR, and 4) general health. The general health was assessed using the Visual Analogue Scale (VAS) to represent patients' perception of current pain intensity, indicated by marking on a 10cm line between "no pain" at the left terminus and "worst pain" at the right terminus. The distance (mm) from the left terminus indicates pain intensity ranging from 0 to 100. A higher score suggests greater pain intensity and poorer general health. In this study, an online DAS28 calculator (http://www.4sdawn.com/DAS28/) was used to compute and produce the overall DAS28 score. The disease activity was categorised according to the criteria of the European League against Rheumatism (EULAR) as follows: ≤3.2=low disease activity; >3.2 and ≤5.1=moderate disease activity and >5.1=high disease activity (15).

Functional status

A modified 8-item version of the Health Assessment Questionnaire (mHAQ) was used to assess the functional status of the RA patients. Patients' ability in performing the following activities of daily living over the past week was evaluated using the self-administered questionnaire: 1) dressing, 2) getting in and out of bed, 3) lifting cup or glass to mouth, 4) walking on flat ground, 5) body washing and drying, 6) bending and picking up clothing from floor, 7) turning faucets on and off, and 8) getting in and out of car. A 4-point rating scale (0=without any difficulty, 1=with some difficulty, 2=with much

difficulty, and 3=unable to do) was used to measure the patients' ability to perform the activities. The mHAQ score was obtained by adding the scores for all 8 items and dividing the number by 8. The mHAQ score may range from 0 to 3, with higher score indicating worse function and greater disability. The score can also be interpreted as follows: ≤1.3=mild disability, >1.3 and ≤1.8=moderate disability and >1.8=severe disability (16).

Statistical analysis

Data were analysed using IBM SPSS version 26.0. Numerical data were reported as mean and standard deviation (SD), and categorical data were expressed in frequency and percentage (%). Chi-square tests and independent t-tests were performed to compare differences in proportions and means respectively. The association of anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status with disease severity and functional status of the RA patients was determined at both univariable and multivariable levels using simple linear regression analysis (SLR) and multiple linear regression analysis (MLR), respectively. In MLR, the influence of age, sex, marital status, employment status, RA duration, RA medication intake, presence of comorbidity, pain severity (VAS score), and ESR were also tested.

RESULTS

All 46 RA patients completed the study, while six of 46 healthy controls were dropped from the study because they were later found to have uncontrolled chronic diseases including diabetes mellitus, hypertension, and hypercholesterolemia, and one of them also had gouty arthritis. Table I shows the socio-demographic characteristics of RA patients and healthy controls. The mean age of RA patients and healthy controls were 54.2 (SD 12.77) years old and 35.0 (SD 12.24) years old respectively. Most RA patients were 50 years old and above (63.0%), whereas most healthy controls were below 50 years old (85.0%). Most respondents in both RA patients and healthy control groups were females (80.4% and 87.5% respectively) and were from the Malay ethnic group (89.1% and 92.5% respectively). The highest education level for most RA patients was secondary school (52.2%) and most were unemployed (58.7%), whereas most healthy controls had post-secondary/tertiary education (77.5%) and were employed (65.0%). Except for sex and ethnicity, the distribution of other characteristics was significantly different between the groups.

The mean disease duration of RA patients was 4.7 (SD 4.86) years. Most patients were on medication for their condition (93.5%). The most common medication taken was methotrexate (34.8%), followed by a combination of methotrexate and prednisolone (17.4%), a combination of methotrexate and hydroxychloroquine (13.0%), and a combination of methotrexate, hydroxychloroquine, and sulfasalazine (8.7%). Some RA patients had comorbidities (43.5%), mainly hypertension and/or diabetes mellitus.

TABLE I: Socio-demographic characteristics of the respondents (n=86)

Variables	Frequency (%)		χ^2 statistics (df)*	P value
	RA patients (n=46)	Healthy controls (n=40)		
Age	54.2 (12.77)[a]	35.0 (12.24)[a]	7.07 (84)[b]	**<0.001**
Sex				
Male	9 (19.6)	5 (12.5)	0.78 (1)	0.376
Female	37 (80.4)	35 (87.5)		
Ethnicity				
Malay	41 (89.1)	37 (92.5)	-	0.719[c]
Others	5 (10.9)	3 (7.5)		
Marital status				
Single	6 (13.0)	20 (50.0)	13.85 (1)	**<0.001**
Married	40 (87.0)	20 (50.0)		
Educational Level				
Primary	8 (17.4)	0 (0.0)	20.92 (2)	**<0.001**
Secondary	24 (52.2)	9 (22.5)		
Post-secondary/ Tertiary	14 (30.4)	31 (77.5)		
Employment status				
Employed	19 (41.3)	26 (65.0)	4.82 (1)	**0.028**
Unemployed	27 (58.7)	14 (35.0)		

SD=standard deviation
*df=degree of freedom
[a] Mean (SD)
[b] t-statistics (df)
[c] Fisher's Exact test

The presence of anti-CCP antibodies in RA patients compared to healthy controls is shown in Table II. The anti-CCP2 IgG and CCP3.1 IgG/IgA were detected in most RA patients (63.0%) while anti-CCP2 IgA was found in less than half of the patients (37.0%). A significantly higher proportion of RA patients were found with positive anti-CCP2 IgG (P<0.001), anti-CCP2 IgA (*P*<0.001), and anti-CCP3.1 IgG/IgA antibodies (*P*<0.001) than healthy controls. The sensitivity and specificity of each anti-CCP antibody test are as follows: 63.0% and 97.5% respectively for anti-CCP2 IgG and anti-CCP3.1 IgG/IgA, and 37.0% and 100.0% respectively for anti-CCP2 IgA.

The mean DAS28 score of the RA patients was 3.52 (SD 1.13). Most patients had moderate disease activity (47.8%), followed by low (43.5%), and only few were severely affected (8.7%). The mean mHAQ score of the patients was 0.47 (SD 0.61), indicating mild functional disability. In addition, the mean ESR level was 43.6 (SD 31.72) mm/hour, and the mean VAS score of the patients was 3.63 (SD 2.07) mm.

Table III shows the difference in mean DAS28 scores and mean mHAQ score of RA patients according to their anti-CCP antibodies status. No significant difference was found in the mean DAS28 scores between patients with positive anti-CCP antibodies status and patients

TABLE II: Comparison of anti-CCP antibodies status in RA patients and healthy controls

Variable	Frequency (%)		χ^2 statistics (df)*	P-value
	RA patients (n=46)	Healthy controls (n=40)		
Anti-CCP2 IgG				
Positive	29 (63.0)	1 (2.5)	34.53 (1)	**<0.001**
Negative	17 (37.0)	39 (97.5)		
Anti-CCP2 IgA				
Positive	17 (37.0)	0 (0.0)	18.43 (1)	**<0.001**
Negative	29 (63.0)	40 (100.0)		
Anti-CCP3.1 IgG/IgA				
Positive	29 (63.0)	1 (2.5)	34.53 (1)	**<0.001**
Negative	17 (37.0)	39 (97.5)		

*df=degree of freedom

TABLE III: Differences in mean DAS28 scores and mean mHAQ scores of RA patients (n=46)

Variable	Mean (SD)		t statistics (df)*	P-value
	Positive	Negative		
DAS28 score				
Anti-CCP2 IgG	3.40 (1.03)	3.73 (1.28)	-0.95 (44)	0.349
Anti-CCP2 IgA	3.25 (0.94)	3.68 (1.21)	-1.27 (44)	0.209
Anti-CCP3.1 IgG/IgA	3.50 (1.11)	3.57 (1.18)	-0.20 (44)	0.842
mHAQ score				
Anti-CCP2 IgG	0.31 (0.45)	0.74 (0.75)	-2.41 (44)	**0.020**
Anti-CCP2 IgA	0.41 (0.55)	0.50 (0.65)	-0.47 (44)	0.640
Anti-CCP3.1 IgG/IgA	0.35 (0.48)	0.67 (0.75)	-1.76 (44)	0.085

SD=standard deviation
*df=degree of freedom

with negative anti-CCP antibodies status. Patients with negative anti-CCP2 IgG status had significantly higher mean mHAQ score compared to patients with positive anti-CCP2 IgG status. However, no significant difference was found in mean mHAQ score between patients having different anti-CCP2 IgA and anti-CCP 3.1 IgG/IgA status.

Results of simple linear regression analysis of factors associated with disease severity of RA patients showed that ESR was the only factor found to be significantly associated with the mean DAS28 score. There was a significant positive linear relationship between ESR and disease severity (P<0.001). An increase in ESR level by 1 mm/hour had increased the mean DAS28 score by 0.02 unit (95% CI=0.013, 0.030). The influence of anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status, as well as other tested variables was not significant. Multivariable analysis indicated that ESR remained the only factor significantly associated with disease severity (Adjusted b=0.02, 95% CI=0.013, 0.030) (Table IV), indicating that RA patients with higher ESR level had higher disease severity. With an R2 value of 0.370, the model predicted that ESR explained 37.0% of the variance in mean DAS28 score.

Results of simple linear regression analysis of factors associated with functional status of RA patients showed that VAS pain score and anti-CCP2 IgG status were significantly associated with the mean mHAQ score. There was a significant positive linear relationship between VAS pain score and functional status (P<0.001) as well as between anti-CCP2 IgG status and functional status (P=0.020). An increase in VAS pain score by 1 mm had increased the mean mHAQ score score by 0.18 unit (95% CI=0.109, 0.250). In addition, RA patients with negative anti-CCP2 IgG status had higher mean mHAQ score than patients with positive anti-CCP2 IgG status by 0.43 unit (95% CI=0.069, 0.781). The influence of anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status, as well as other tested variables was not significant. Multivariable analysis showed that both VAS pain score (Adjusted b=0.17, 95% CI=0.097, 0.236) and anti-CCP2 IgG status (Adjusted b=0.30, 95% CI=0.001, 0.591) remained significantly associated with functional status (Table IV). These findings indicate that RA patients with higher pain score and RA patients with negative anti-CCP2 IgG status had worse function and greater disability than their respective counterparts. With an R2 value of 0.427, the model predicted that VAS pain score and anti-CCP2 IgG status explained 42.7% of the variance in mean mHAQ score.

TABLE IV: Factors associated with disease severity and functional status of RA patients by multiple linear regression analysis (n=46)

Outcome	Variable	Adjusted b#	(95% CI)	t-statistic	P-value
Disease severity	ESR (mm/hour)	0.02	(0.013, 0.030)	5.09	**<0.001**
Functional status	Pain score (mm)	0.17	(0.097, 0.236)	4.83	**<0.001**
	Anti-CCP2 IgG Positive*				
	Negative	0.30	(0.001, 0.591)	2.03	0.049

CI=confidence interval
# Regression coefficient
*Reference category

DISCUSSION

In this study, significantly higher proportion of RA patients were found with positive anti-CCP2 IgG (63.0%) and anti-CCP3.1 IgG/IgA (63.0%) antibodies than the healthy controls but only 37.0% were positive for anti-CCP2 IgA antibody. In agreement, studies among other population groups also demonstrated higher positive status of serum anti-CCP antibodies in RA patients compared to the control patients (9, 17, 18). This study therefore provided evidence supporting the use of anti-CCP antibodies tests to aid in RA diagnosis, in addition to the conventionally used RF. The specificity of anti-CCP2 IgG, anti-CCP2 IgA, and anti-CCP3.1 IgG/IgA test in this study was very high, indicating that the anti-CCP antibodies tests correctly returned negative results for almost all people who did not have RA, with reasonable sensitivity to correctly detect disease in RA patients.

Serum concentration of RF and anti-CCP antibodies were found to be associated with RA disease activity due to their high specificity and sensitivity as well as the ability to predict progressive damage to the joints (11, 18). The DAS28 score is commonly used by rheumatologist to monitor disease progression in RA patients. The association of disease severity and the status of serum autoantibodies had been investigated since the discovery of RF to evaluate and improve the management of RA. In this study, most RA patients had either moderate or low disease activity. This finding corresponds with the fact that our participants were mostly being treated with disease modifying anti-rheumatic drugs (DMARDs), and our findings indicated their favourable responses to the treatment.

ESR is an inflammatory marker which is commonly used in diagnosis and management of RA. In this study, higher ESR was associated with higher disease activity. However, we found no significant association between RA disease activity with anti-CCP2 IgG, anti-CCP2 IgA, and anti-CCP3.1 IgG/IgA antibodies. In agreement, studies done among RA patients in Tunisia and Tьrkiye also reported no significant association between RA disease activity and anti-CCP status (12, 19). While the French ESPOIR cohort study among patients at the early-stage RA found significantly higher DAS28 score in patients with positive anti-CCP2 antibody compared to anti-CCP2 negative patients (20), contradicting findings were reported by Reed et al. in the Swedish population-based EIRA cohort study whereby negative anti-CCP2 antibody status was associated with higher disease activity (21).

The association of RA autoantibodies with disease severity and functional status are still poorly understood with limited findings. In our study, higher mHAQ score or poorer functional ability of RA patients was associated with negative anti-CCP2 IgG antibody status. This finding contradicts earlier reports that lower functional status was associated with positive anti-CCP status (13, 14). In this study, we used the mHAQ and DAS28 as tools to determine functional status and disease severity respectively, and the measurement was done only at one point of time without radiographical investigation to determine the presence and extent of joint destructions. Nevertheless, we demonstrated pain as another predictor of poor functional status in RA patients. RA is a chronic disease, and presence of autoantibodies has been shown to be a strong predictor of joint erosion (22). Therefore, the absence of anti-CCP2 IgG antibody in presence of functional disability may indicate that joint destructions may have already occurred, leading to pain and poor functional status despite the negative anti-CCP2 IgG status.

In this study, participants in the RA group and healthy control group were not matched for age because it was not feasible to find adequate age-matched healthy controls who were free from any chronic medical illnesses or with controlled chronic medical illnesses within the study period. Our literature review also showed that the mean age of RA patients in Malaysia was 50 years old (23), in agreement with our findings. The difference in mean age between RA patients and healthy controls in our study was significant, and this may pose a limitation to this study because increasing age has been shown to be associated with increased production of several autoantibodies including anti-CCP antibodies in people without RA (24). Nevertheless, we are confident that the confounding effect of age on the study outcomes has been adequately controlled in the multivariable analysis.

CONCLUSION

Anti-CCP2 IgG, anti-CCP2 IgA, and anti-CCP3.1 IgG/IgA antibodies were detected in a substantial proportion of RA patients than the healthy controls. The tests were highly specific and therefore support the use of anti-CCP antibodies detection in RA diagnosis because the tests rarely misclassify healthy individuals as having RA. Most of RA patients had moderate or low disease severity and mild functional difficulty, suggesting favourable response to treatment. The significant inverse association found between anti-CCP2 IgG antibody and functional status of RA patients warrants further studies. Incorporation of other investigations such as radiographic scoring to quantify joint destruction that is largely irreversible and may certainly influence functional status of patients is recommended.

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