Int J Clin Exp Pathol 2016;9(10):10728-10733
www.ijcep.com /ISSN:1936-2625/IJCEP0033644

Original Article

Diagnostic value of third generation anti-cyclic citrullinated peptide assay in rheumatoid factor negative rheumatoid arthritis

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Received June 13, 2016; Accepted July 12, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Objective: This study aimed to evaluate the diagnostic usefulness of second and third generation anti-cyclic citrullinated peptide (anti-CCP2 and anti-CCP3) assays in Chinese patients with rheumatoid arthritis (RA), and to identify the potential advantages of anti-CCP3 diagnosis in rheumatoid factor (RF) negative RA patients. Methods: Serum samples were obtained from 148 RA patients and 120 controls (72 healthy subjects and 48 patients with rheumatic diseases). The routine screening of RF was performed using RFII Tina-quant®Turbidimetry reagents on a turbidimetry analyzer. Furthermore, the serum levels of anti-CCP2 and anti-CCP3 were detected using special kits. The individual proportions and comparisons for diagnostic capability of anti-CCP2 with anti-CCP3 were calculated for RA and RF-negative RA. Results: No significant differences were revealed in age, sex rate and disease duration between RA patients and controls ($P > 0.05$). The positive rates of RF, anti-CCP2 and anti-CCP3 were all significantly higher in RA patients than those in control group ($P < 0.01$). Meanwhile, the sensitivity of anti-CCP2 was lower than that of anti-CCP3 (77.7% vs 81.1%), whereas the specificity of anti-CCP2 was higher than that of anti-CCP3 (95.8% vs 92.5%). Besides, the sensitivity of anti-CCP3 was higher than anti-CCP2 (78.3% vs 71.6%) in RF-negative RA patients. Conclusion: The diagnostic performance of anti-CCP3 in RA patients was similar to anti-CCP2, while a superior specificity of anti-CCP3 was exhibited in RF negative RA patients compared with anti-CCP2.

Keywords: Rheumatoid arthritis, anti-cyclic citrullinated peptide, rheumatoid factor

Introduction

Rheumatoid arthritis (RA) is a chronic, autoimmune inflammatory disease characterized by joint inflammation, progressive erosion and cartilage destruction [1]. In clinic, early aggressive treatments always exhibit significant advantages for the overall outcomes of RA patients [2]. Effective serologic test is particularly important for early diagnosis and treatment of RA.As a laboratory criterion of RA in 1987 (American College of Rheumatology), rheumatoid factor (RF) test could measure the amount of the RF antibody in the blood by directly binding to the Fc-portion of immunoglobulin G [3]. However, the specificity of RF was only 50-70%, and positive RF could also be detected in rheumatic diseases, chronic inflammatory diseases, infections and even in healthy individuals (especially in the elderly) [4]. During the past decades, auto-antibodies directed at citrullinated epitopes are represented as a sensitive and specific marker for RA [5]. Anti-cyclic citrullinated peptide (CCP) antibody has been reported to greatly improve the diagnostic efficiency of RA [6]. RA patients with positive anti-CCP antibodies exhibited poor disease outcomes when compared to those without anti-CCP antibodies [7]. Therefore, determination of anti-CCP titers in patients with polyarthritis is obviously helpful for early diagnosis and evaluating the prognosis of RA.

In clinic, anti-CCP antibody has been widely used and RA patients with positive anti-CCP antibody titers are recommended to receive aggressive therapy in the early stages [8, 9]. For the development of anti-CCP, the first generation of anti-CCP antibody (anti-CCP1) was invented in 1998 by a mixture of CCP as a coat-

Table 1. Technical characteristics of the anti-cyclic citrullinated peptide (anti-CCP) assays

Assays	Second generation CCP	Third generation CCP
Assays kits	ImmunoscanCCPlus® test kit	QUANTA Lite CCP3 IgG ELISA
Antigen	anti-CCP2	anti-CCP3
Manufacturer	EURODIAGNOSTICA	INOVA
Specimen	Serum	Serum
Cutoff value	25 U/ml	20 Units
Measuring range	25-3200 U/ml	0-250 Unites

Table 2. Clinical characteristics of rheumatoid arthritis (RA) patients and controls

Subjects	RA	Controls
Number	148	120
Female/Male (%)	72.9/27.1	71.6/28.4
Age (years)	50±15.3	52±12.6
Disease duration (years)	7.8±5.2	8.2±6.1 (48 rheumatic diseases)
Anti-CCP2 positive (%)	77.7	4.2*
Anti-CCP3 positive (%)	81.1#	7.5*
Rheumatoid factor positive (%)	52.0##	15.0*

*represents significant difference at $P < 0.01$ when compared with RA patients; # and ## represent significant difference at $P < 0.05$ and $P < 0.01$ when compared with anti-CCP2.

ing [10]. However, the sensitivity of anti-CCP1 for the diagnosis of RA were relatively low (40%) [11] and then it was replaced by second generation of anti-CCP antibody (anti-CCP2). By screening highly complex peptide libraries using highly reactive serum taken from RA patients, anti-CCP2 greatly improved the diagnosis efficiency of RA [12]. According to the statistics, the average specificity and sensitivity of anti-CCP2 were 95% and 68% for RA patients [13, 14]. Recently, an improved ELISA, third-generation of anti-CCP antibody (anti-CCP3) has been designed by combinatorial peptide engineering [6]. Anti-CCP3 consists of multiple citrullinated epitopes in a conformational structure, which could increase both epitope exposure and immunoreactivity [15]. Meanwhile, anti-CCP3 can enhance the clinical sensitivity of RA, and the high specificity can also be maintained in patients with rheumatic and infectious diseases [16]. Although both anti-CCP2 and anti-CCP3 are considered to be effective for RA diagnosis in some degrees, the diagnostic properties of these two methods in different populations are still needed to be studied. Meanwhile, since anti-CCP2 and anti-CCP3 assay has been increasingly used for RA diagnosis, it has become necessary to compare the clinical utility of these two methods among patients with rheumatic diseases.

In this study, anti-CCP2 and anti-CCP3 assays were performed in Chinese patients with RA, respectively. Our findings may identify the diagnostic usefulness of anti-CCP2 and anti-CCP3 on patients with RA and RF-negative RA, which could further improve the diagnostic accuracy in clinic.

Materials and methods

Subjects

A total of 148 RA patients were recruited from the local institute between Jan 1th and May 29th in 2014. All these RA patients met the diagnosis criterion of American College of Rheumatology [17]. Meanwhile, 72 healthy subjects and 48 patients with rheumatic diseases, including 12 Assystemic lupus erythematosus (SLE), 4 Sjogren's syndrome (SS), 7 Polymyositis/dermatomyositis (PM/DM), 6 Mixed connective tissue disease (MCTD), 5 Psoriatic arthritis (PsA), 4 Osteoarthritis (OA), 4 Ankylosing spondyl (AS), 3 Scleroderma (SSc) and 3 Allergic granulomatous angiitis (AGA), were selected as controls. The clinical features, including age, sex and disease duration, of all enrolled subjects were recorded. This study was approved by the local research ethics committee and informed consents were obtained from all individuals enrolled in this study.

Assays for rheumatoid factor

Routine screening of RF was performed on the serum samples obtained from all enrolled subjects using RFII Tina-quant®Turbidimetry reagents (Roche Diagnostics, Indianapolis, IN, USA) on a turbidimetry analyzer (Modular P800-1, Modular automatic biochemical analysis system, Roche, Switzerland). The positive RF has been identified as RF ≥ 30 U/ml [18].

Table 3. Comparison of diagnostic capabilities between CCP2 and CCP3 in 268 cases

Diagnostic methods	Diagnostic results	Gold standard		Total
		Positive	Negative	
Anti-CCP2	Positive	115[a]	5[b]	120
	Negative	33[c]	115[d]	148
	Total	148	120	268
Anti-CCP3	Positive	120[a]	9[b]	129
	Negative	28[c]	111[d]	139
	Total	148	120	268

[a]: true positive; [b]: false positive; [c]: false negative; [d]: ture negative.

Table 4. Comparison of diagnostic capabilities between CCP2 and CCP3

Parameters	CCP2		CCP3	
	Estimated value	95% CI	Estimated value	95% CI
Sensitivity	77.7%	0.70-0.84	81.08%	0.74-0.87
Specificity	95.83%	0.90-0.98	92.5%	0.86-0.96
Positive-predictive value	95.8%	0.90-0.98	93.02%	0.86-0.96
Negative-predictive value	77.70%	0.70-0.84	79.86%	0.72-0.86
Positive likelihood ratio	23	9.74-54.29	13.33	7.08-25.08
Negative likelihood ratio	0.29	0.21-0.39	0.25	0.18-0.35
Youden's index	0.74		0.74	
Odds ratio	80.15		52.85	

CI: confidence interval.

Anti-CCP assays

The serum levels of CCP in all enrolled subjects were detected using special kits of anti-CCP2 (EURO Diagnostic Immuoscanccplus®, SWEDEN) and anti-CCP3 (QUANTA Lite CCP3 IgG ELISA, Inova Diagnostics, San Diego, USA) following the procedures recommended by the manufacturers, respectively (**Table 1**).

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD) and analyzed using Student's t-test. A *p*-value less than 0.05 was considered to be significantly different. Meanwhile, the individual proportions (sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio) with 95% CIs were calculated on the website of http://vassarstats.net/clin1.html. In addition, relations among RF, anti-CCP2 and anti-CCP3 were analyzed using Spearman's rank correlation analysis.

Results

The clinical features of subjects enrolled in this study are shown in **Table 2**. No significant difference of age, sex and disease duration was revealed between RA patients and controls (P > 0.05). According to the manufacturers' cut-offs, the positive rate was 77.7% (115/148) for anti-CCP2 and 81.1% (120/148) for anti-CCP3 in RA patients. Moreover, 52.02% (77/148) RA patients were positive for RF. In the control group, 4.17% (5/120) subjects were positive for CCP2 (anti-CCP2: 2 SLE, 1 SS, 1 MCTD and 1 PsA) and 7.5% (9/120) subjects were positive for CCP3 (3 SLE, 2 SS, 1 MCTD, 1 PsA, 1 PM/DM and 1 SSc). However, these positive CCP subjects in control group were all patients with rheumatic diseases, but not healthy subjects. The positive rates of RF (52.0%), anti-CCP2 (77.7%) and anti-CCP3 (81.1%) were all significantly higher in RA patients than those in control group (P < 0.01). Diagnostic results for anti-CCP2 and anti-CCP3 are shown in **Table 3** in details.

Furthermore, the diagnostic capabilities of CCP2 and CCP3 were calculated and compared (**Table 4**). The sensitivity of anti-CCP2 was lower than anti-CCP3 (77.7% vs 81.1%), whereas the specificity of anti-CCP2 was higher than anti-CCP3 (95.8% vs 92.5%). Meanwhile, anti-CCP2 and anti-CCP3 shared with a same Youden's index of 0.74 and they both had high positive likelihood ratio (23 and 13.33, respectively) and low negative likelihood ratio (0.29 and 0.25, respectively).

Additionally, the sensitivity of anti-CCP2 and anti-CCP3 in 60 cases of RF-negative RA patients was further evaluated. As shown in **Table 5**, the positive rate of anti-CCP2 was found to be significantly lower than that of anti-

Table 5. Positive of second and third generation anti-cyclic citrullinated peptide (anti-CCP2, anti-CCP3) in rheumatoid factor (RF)-negative rheumatoid arthritis (RA) patients

		Anti-CCP2		
All patients (n=60)		Positive	Negative	Total
Anti-CCP3	Positive	40 (66.6%)	7 (11.6%)	47 (78.3%)*
	Negative	3 (5%)	10 (16.6%)	13 (21.6%)
	Total	43 (71.6%)	17 (28.3%)	60

*represents significant difference at $P < 0.01$ when compared with positive rate of anti-CCP2.

CCP3 (71.6% vs 78.3%, $P < 0.01$). Among these patients, anti-CCP2 and anti-CCP3 were both positive in 40 cases (66.6%) and both negative in 10 cases (16.6%). Differently, anti-CCP2 and anti-CCP3 was revealed to be positive in 3 and 7 cases in the remaining 10 patients, respectively (**Table 5**).

As to further reveal the relations among RF, anti-CCP2 and anti-CCP3, Spearman's rank correlation analysis was performed. As a result, the Spearman's rank correlation coefficients of RF and anti-CCP2, RF and anti-CCP3, and anti-CCP2 and anti-CCP3 were 0.5397, 0.5415 and 0.8864, respectively.

Discussion

RA is a common and serious systemic inflammatory disorder that primarily affects joints [19]. In the diagnosis of RA, the sensitivity and specificity of serologic markers were particularly important [20]. Until now, RF and anti-CCP are the most commonly used diagnostic methods on RA in clinic [21]. Although the specificity of RF was relatively low, anti-CCP exhibits superior diagnostic and prognostic value on RA [22]. In this study, significantly higher positive rates of RF, anti-CCP2 and anti-CCP3 were found in RA patients compared to controls, illustrating that both RF and anti-CCP are capable of RA diagnosis in some degrees. However, the low positive rate of RF (52%) indicated RF was a moderately insensitive test for RA, which was consistent with the previous study [23]. It has been reported that anti-CCP was present in patients with autoimmune disorders such as SLE, SS, MCTD, SSc, PM/DM, PsA, primary biliary cirrhosis and miscellaneous joint symptoms [6, 9]. In our study, positive anti-CCP in non-RA patients was all found in patients with other rheumatic diseases (SLE, SS, MCTD, PsA, PM/DM and SSc). Therefore, these findings may be not confirmed false positives, but indicated an increased risk for inflammatory joint disease including RA in these patients.

Recently, several studies have been conducted to assess and compare the diagnostic performances of various anti-CCP assays, including anti-CCP2 and anti-CCP3 on different populations, while the related results were controversial. As reported, higher diagnostic sensitivity (61.3-75%) was found in anti-CCP3 as compared with that in anti-CCP2 (60.2-72%) [24, 25]. Besides, some investigations also suggested that anti-CCP3 exhibitednot noly more sensitivity than anti-CCP2 test but also sustaining high specificity [26]. However, the diagnostic performance of anti-CCP2 and anti-CCP3 was revealed to be similar in RA patients [27], and greater sensitivity of anti-CCP3 than anti-CCP2 may only apply to early RA patients [7]. These discrepant results may be explained by the cohort size, cohort composition and the manual performance of the CCP ELISA. In this study, we specifically assessed the performance of CCP2 and CCP3 in RA. In agreement with Jaskowski et al. and Swart et al. [28, 29], higher positive rate was found in anti-CCP3 than anti-CCP2 (81.1% vs 77.7%) in RA patients. However, the specificity of anti-CCP3 was a bit lower than that of anti-CCP2 (92.5% vs 95.8%). Meanwhile, the Spearman's rank correlation coefficients of anti-CCP2 and anti-CCP3 was 0.8864, indicating that the diagnostic performance of anti-CCP3 in RA patients was similar to that of anti-CCP2. Furthermore, a higher specificity was exhibited in anti-CCP3 than anti-CCP2 (78.3% vs 71.6%) for RF-negative RA patients. It has been reported that anti-CCP3 was more prevalent than anti-CCP2 in RF-negative RA patients [29] and anti-CCP3 was effective in discrimination of RF-negative RA with a disease duration of ≤ 5 years [28]. Our result confirmed previous studies and underlined that anti-CCP3 was pronounced effective in the diagnosis of RF-negative RA [28].

In conclusion, anti-CCP is identified to be more effective than RF for the diagnosis of RA, and

anti-CCP3 had a higher diagnose sensitivity of RF-negative RA than anti-CCP2 in Chinese people. However, this study was still limited with insufficient subjects, and differences revealed on anti-CCP assays though intense efforts have gone into standardizing CCP detection [30].

Disclosure of conflict of interest

None.

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