Diagnostic Value of Anti-Cyclic Citrullinated Peptide Antibody in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective***. To explore the diagnostic value of anti-cyclic citrullinated peptide antibody (anti-CCP) detected by ELISAin patients with rheumatoid arthritis (RA).

Methods. The synthesized cyclic citrullinated peptide was used as substrate for ELISA. Anti-CCP antibody was detected by ELISA in 191 patients with RA, 132 with rheumatic diseases other than RA, and 98 with nonrheumatic diseases. The antiperinuclear factor (APF), anti-keratin antibody (AKA), rheumatoid factor (RF), and HLA-DR4 gene complex were also tested in each RA patient. The results of these tests were compared with anti-CCPantibody to examine the correlation between them.

Results. Ninety (47.1%) patients with RA, 4 (3.0%) with other rheumatic diseases, and 2 (2.0%) with nonrheumatic diseases were found to be anti-CCPantibody positive by ELISA. The sensitivity of anti-CCP antibody was 47.1%, with a high specificity (97.4%) in RA. Anti-CCP antibody correlated with APF, AKA, RF, and HLA-DR4 gene complex.

Conclusion. A new modified anti-CCP antibody test had a moderate sensitivity (47.1%) but a high specificity (97.4%) in patients with RAand was found as a valuable supplement to diagnosis of RA. Anti-CCP correlated with APF, AKA, RF, and HLA-DR4 gene complex, but did not completely overlap with them. Anti-CCP antibody could be regarded as a new diagnostic marker for RA. (J Rheumatol 2003;30:1451–5)

Key Indexing Terms:
CYCLIC CITRULLINATED PEPTIDE ANTIBODY
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Rheumatoid arthritis (RA) is a systemic autoimmune disease that can lead to irreversible joint damage even in the first 2 years of disease. The most effective way to manage RA patients is early diagnosis and timely treatment with disease modifying antirheumatic drugs (DMARD), which prevent the exacerbation of disease, reduce the probability of joint destruction, and improve the outcome[1]. The only serologic indicator routinely used in RA diagnosis is the IgM rheumatoid factor (RF), which bears a low specificity because it can be found in a variety of patients with other autoimmune diseases as well as in healthy people, although at a lower frequency[2].

Two other antibodies, antiperinuclear factor (APF) and antikeratin antibodies (AKA), associated with antifilaggrin antibodies (AFA), have all been shown to be highly specific for RA, and can be detected in early stage RA[3-5]. All these antibodies target epitopes carried by profilaggrin or filaggrin molecules[6,7]. Protein maturation follows a common post-translational pathway, which involves peptidyl-arginine to citrulline deimination. Recently, a new serologic test based on the modified amino acid citrulline, anti-cyclic citrullinated peptide (anti-CCP), was developed. According to recent data, anti-CCP had an excellent specificity and a relatively high sensitivity for RA, especially for recent onset RA[8-10].

We describe a modified ELISA for detecting anti-CCP antibodies in patients with RA.

MATERIALS AND METHODS

Serum samples. We collected serum samples from 191 RAoutpatients from the clinic of the Peking Union Medical College Hospital. One hundred thirty-three were female and 58 were male, with a mean age of 47 years (range 15–77). All patients had a diagnosis of definite RAaccording to the revised criteria of the American College of Rheumatology (ACR)[11]. Sixty of these patients were classified as recent onset RA(symptom duration < 1 year at study entry).

To provide the data for specificity analysis, 230 control sera samples from 132 patients with non-RA rheumatic diseases, 98 patients with nonrheumatic diseases, and 90 healthy individuals were collected and tested (Tables 1 and 2).

Peptide synthesis and ELISA. The cyclic peptide (cfc1-cyc) was synthe-

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sized by the solid-phase strategy using fmoc chemistry as described[9]. The peptide was at least 95% pure as deduced from its elution pattern on reverse-phase high performance liquid chromatography. The identity of the peptide was confirmed by amino acid sequencing and mass spectrometry.

Anti-CCP antibody was tested by ELISA essentially as described in detail by Schellekens, *et al*[9]. We modified the ELISAas follows. Synthetic peptide was diluted to a concentration of 1 µg/100 µl in 0.01 M phosphate buffered saline (PBS) (pH 7.4) and 96-well MaxiSorp microtitration plates (Nunc, Roskilde, Denmark) were coated by an overnight incubation at 4°C with the solutions. After blocking with 2% bovine serum albumin (BSA) in 0.01 M PBS (pH 7.4) at 37°C for 2 h, sera were diluted 100-fold in 2% BSA and incubated at 37°C for 1.5 h. After washing with 0.05% PBST [PBS/0.05% (v/v) Tween-20, 3 times], 100 µl anti-human IgG (Fab-specific, developed in goat) conjugated to peroxidase (Sigma Chemical, Rehovot, Israel) with a dilution of 1:1000 in 2% BSAwas added to the well and incubated at 37°C for 1 h. The wells were then washed 3 times, and reacted for 20 min with 3,3',5,5'-tetramethylbenzidine (Sigma Chemical, St. Louis, MO, USA). Color development was stopped with 2 M H_2SO_4. The result was read at 450 nm by a micro-ELISAreader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested twice and results were averaged.

To evaluate the repeatability and reproducibility of the test, 9 RA samples with 3 negative (< 99 units), 3 intermediate (about 500 units), and 3 high (≥ 1000 units) antibody concentrations and all 7 sera samples from non-RA subjects (6 non-RA, one healthy subject) with positive reactions were assessed 6 times by 3 independent groups of operators under identical experimental conditions on different days. The intra- and inter-test variations were less than 5% at all times.

Table 1. Characteristics of the study patients.

	RA	RD	Non-RD
No. of patients	191	132	98
Age, yrs, mean (range)	47 (15–77)	45 (16–72)	46 (16–75)
Female, %	69.6	77.3	68.4

RD: rheumatic disease other than RA; Non-RD: Nonrheumatic disease.

Detection of APF, AKA, RF, and HLA-DR4 gene complex. APF and AKA were detected by indirect immunofluorescence (IIF). Buccal mucosa cells were used for the APF test and cornified layer of rat esophagus cryostat sections were used for AKA detection. All the slides were read independently by 2 observers. IgM RF was measured by nephelometry, and a level > 20 IU/ml was considered positive. HLA-DR4 gene complex was detected by polymerase chain reaction[12].

Statistical analysis. Statistical analysis was performed using SPSS 10.0 software. The chi-square test was used for testing the significance of differences between 2 different groups of patients. Multiple regression analyses were used to assess the importance of the different variables relative to anti-CCPantibody status. Means, standard deviations, and confidence intervals (CI) were used where appropriate. Two-sided $p < 0.05$ was considered significant. Receiver operating characteristics (ROC) were used to calculate optimal cutoff values[13].

RESULTS

Sensitivity and specificity of anti-CCP antibody. Ninety of 191 (47.1%) RA patients were positive for anti-CCP antibody. Only 6 of 230 (2.6%) control sera (including 132 serum samples from other rheumatic diseases and 98 serum samples from nonrheumatic diseases) had a positive reaction (Table 2), including one patient with primary Sjögren's syndrome complicated with renal tubular acidosis, 2 with systemic sclerosis who had distal digital ulcer and bone erosion, one vasculitis, one tuberculosis, and one with viral infection. Only one of 90 healthy controls was positive for anti-CCP antibody. None of these 7 anti-CCP positive controls had symptoms or signs of RA. At a cutoff value of 99 units determined by ROC curve (Figure 1), the sensitivity of anti-CCP antibody was 47.1% (CI 40.0–54.2%) with a specificity of 97.4% (CI 95.3-99.5%). The positive predictive value and negative predictive values were 93.8% and 68.9%, respectively.

Of the 60 patients with early RA (symptom duration < 1 year at study entry), 26 were anti-CCPantibody positive and

Table 2. Results of anti-CCPantibody in RApatients and controls.

Patient Group	Total, n	Anti-CCPpositive sera, No. (%)
Rheumatoid arthritis	191	90 (47.1)
Early RA	60	26 (43.3)
Other rheumatic diseases	132	4 (3.0)
Systemic lupus erythematosus	15	0
Systemic sclerosis	20	2
Primary Sjögren's syndrome	15	1
Osteoarthritis	20	0
Ankylosing spondylitis	20	0
Reactive arthritis	15	0
Polymyositis/dermatomyositis	10	0
Vasculitis	17	1
Nonrheumatic diseases	98	2 (2.0)
Tuberculosis	20	1
Infectious endocarditis	10	0
Virus infections	30	1
Ulcerative colitis	18	0
Crohn's disease	10	0
Autoimmune hepatitis	10	0
Healthy individuals	90	1 (1.1)

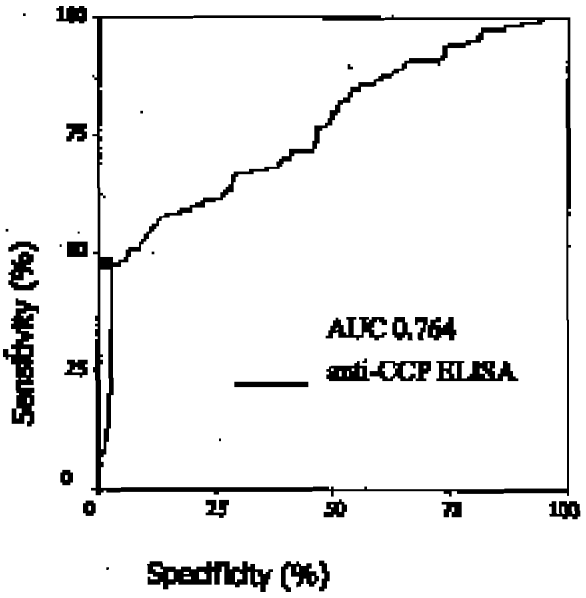

Figure 1. Receiver operating characteristics curve of anti-cyclic citrullinated peptide antibody (anti-CCP). ■ marks the optimal cutoff value for the anti-CCP ELISA (99 units). Area under the curve (AUC) was 0.764. The patients shown in Table 2 were included in this evaluation.

the sensitivity was 43.3%. There was no association between anti-CCP antibody and age or sex.

The repeatability and reproducibility of the assay we set up were reliable and the intra- and inter-test variations of the identical sample were less than 5% at all times.

Correlation among anti-CCP antibody, APF, and AKA. We also performed APF and AKAby IIF in 191 RApatients. Of the 191 sera samples, 61 were positive for anti-CCP antibody, APF, or AKA, and 83 were negative for all; 69 were positive for anti-CCPantibody or APF and 84 were negative for both; and 63 were positive for anti-CCP antibody or AKAand 89 were negative for both. The sensitivity of anti-CCP antibody was higher than for APF and AKA, and the difference between anti-CCP antibody and AKA was statistically significant ($p = 0.025$). The correlation of results of anti-CCP antibody, APF, and AKA was statistically significant ($p < 0.01$) (Table 3).

Table 3. Test results of anti-CCP antibody in relation to APF and AKA positive or negative in patients with RA.

Group	Anti-CCP(n) +	–	Total (n)
APF +/AKA +	61	11	72
APF +/AKA –	8	6	14
APF –/AKA +	2	1	3
APF –/AKA –	19	83	102
Total (n)	90	101	191

APF: antiperinuclear factor; AKA: anti-keratin antibody.

Correlation among anti-CCP, RF, and HLA-DR4 gene complex. We also tested RF in 191 sera samples from patients with recent onset RA. Of the 191 samples, 72 were positive for anti-CCP or RF and 60 were negative for both; 18 were positive for anti-CCP antibody or negative for RF and 41 were negative for anti-CCP antibody or positive for RF. The correlation of anti-CCP and RF was statistically significant ($p < 0.001$). We also investigated HLA-DR4 gene complex occurrence in the same series of samples of RA patients: HLA-DR4 gene complex was positive in 108 sera (56.5%) and negative in 83 sera. The correlation of results between anti-CCP antibody and HLA-DR4 gene complex was statistically significant ($p < 0.01$).

DISCUSSION

The critical strategy to prevent joint damage in RA treatment is to initiate DMARD therapy early in the disease course. RA is diagnosed primarily according to clinical manifestations and serologic test that is limited to the presence of IgM RF. RF is highly sensitive for RA patients, but has a low specificity, as it can be found in a variety of conditions such as other nonrheumatic autoimmune diseases and infectious diseases, and even in healthy elderly individuals.

APF and AKA are not popular because of problems with antigen preparation and test methods. Although the AFA could be detected by Western blot and ELISAand showed a high specificity, the sensitivity of the AFA varied from 12% to 54%, depending on the method for purification of filaggrin[14,15]. APF, AKA, and AFA are all present in the early stage of the disease[4-7,14,15]. Recently, several studies have shown that antibodies specific for RA bound to antigenic determinants that contained citrulline residues, including filaggrin and Sa system[16-18], but CCP was the most specific one for RA, and could be detected early in the disease course. Schellekens, *et al* synthesized a modified peptide variant, a single cyclic peptide with a mimic ß-turn conformation, named cyclic citrullinated peptide (CCP)[8,9]. CCP was recognized by RA autoantibodies with relatively high sensitivity in a reliable and convenient test format.

We evaluated the diagnostic accuracy of a modified ELISAfor anti-CCPantibody in Chinese patients. In the 191 RA subjects studied, the diagnostic sensitivity of the test was 47.1% and the specificity was as high as 97.4%. Our result confirmed those obtained initially by other authors[9,10,16,19,20]. Table 4 shows the prevalence differences of anti-CCP antibody in RApatients in the literature, which ranged from 68% to 41%. Our result of 47% was within the range of prevalence. In our assay, we compared the Costar assay plates, used in the work of Schellekens and Kroot[8,10], to Nunc assay plates, and radioimmunoassay buffer [1% BSA, 0.35 M NaCl, 0.01 M Tris HCl buffer, pH 7.6, 1% (v/v) Triton X-100, 0.5% (w/v) Na-deoxycholate, 0.1% sodium dodecyl sulfate] supplemented with 10% normal rabbit serum to our working diluting buffer (2% BSA). The

Table 4. Percentage of serum samples positive for anti-CCP antibody in patients with RA and controls — summary of published reports.

First Author, year[ref]	RA* Samples		Control Samples	
	No.	Anti-CCP+, %	No.	Anti-CCP+, %
Schellekens, 2000[9]	134	68	815	2
Kroot, 2000[10]	273	66	NA	NA
Goldbach-Mansky, 2000[16]	106	41	132	9
Bizzaro, 2001[19]	98	41	232	3
Our results, this article	191	47	320	2

NA: Not available. CCP: Cyclic citrullinated peptide antibody.

difference between the results was not statistically significant (results not shown). So, the various geographical areas were considered as the primary factor affecting prevalence of anti-CCP antibody. In our cohort, the sensitivity of RF in RApatients was higher than that of anti-CCPantibody (59% vs 47%), but was lower than the average sensitivity (about 60%–80%) outside China. Further research is required to illustrate the differences including geographical factors between our results and others'.

Our data clearly indicate that reactivity to CCP did not capture the complete spectrum of APF and AKA reactivity. A potential explanation for this might relate to the demonstration that citrulline has been shown to be an essential constituent of the antigenic determinants recognized by APF and AKA, but individual RA patients responded to different antigenic determinants.

Anti-CCP positivity correlated with RF positivity. Combining RF with anti-CCP antibody could provide a much higher sensitivity without compromising specificity for the diagnosis of RA. There was a direct correlation between anti-CCP and HLA-DR4 gene complex, in agreement with the fact that both assays predicted more progressive disease course[9,10,21,22].

In conclusion, anti-CCP antibody detection by an easy-to-use ELISAavoided many of the problems of the APF and AKA tests regarding quantification of results and standardization of the assay. The anti-CCP antibody response has been shown to be very specific for RA, to be able to diagnose RAat a very early stage of the disease, and to predict a more destructive disease course[10,22,23]. Moreover, when combined with the APF or AKA and RF, it provided high specificity and thus could be very helpful in the differential diagnosis of RA. Anti-CCP has the potential to become another serologic marker for RA.

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