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DIAGNOSTIC VALUE OF ANTIBODIES TO CITRULLINE CONTAINING PEPTIDES IN PATIENTS WITH RHEUMATOID ARTHRITIS IN SOUTHERN IRAQ

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Abstract

A serological study has been done in Al-Nassiriyha, southern Iraq, from December 2006 to August 2007 designed to determine the frequency of anti-cyclic citrullinated peptide antibody (anti-CCP) in a group of patients with rheumatoid arthritis for specific period of time compared with other rheumatic diseases and healthy controls. Also to show the diagnostic value of anti-CCP antibody in relation to other markers [rheumatoid factor (RF), antinuclear factor (ANF), C-reactive protein (CRP) and cytokines such as interleukin–6 (IL-6), tumor necrosis factor–α (TNF-α)] to discriminate between those patients with and without RA.

Blood samples were taken from 121 patients with rheumatoid arthritis, 88 patients of other rheumatic diseases from AL-Hussein Teaching Hospital in AL-Nassiriyah and 120 healthy controls from medical personnel and school students who were healthy. All the information was taken from the patients and controls.

An Enzyme-linked immunosorbent assay was used for estimation of anti-CCP, IL-6, TNF-α and slide agglutination test for the estimation of RF, CRP and ANF. The seropositivity to anti-CCP in RA patients group was 61.2% among them 23% with low levels of anti-CCP, 25% with moderate levels and 51.3% with high levels (> 60 units /ml.).The high levels of anti-CCP persist for long durations up to 10 years (54.7%). However, anti-CCP was rarely detected among non-RA rheumatic diseases and healthy controls (2.3% and 2.5% respectively). Anti-CCP showed the greater specificity (97.6%) and sensitivity (61.2%) compared to the other marker used in this study. The overall agreement between the tested parameters in their ability to detect RA patient was higher for anti-CCP combined to IL-6 (91.7%) in comparison to the effect of other markers combination. Although anti-CCP was more common in RA patient with 2-10 years duration of illness, anti-CCP, IL-6 and TNF-α positivity as estimated by ELISA were persistently with high titers regardless the duration of illness. The majority of RA patients with high anti-CCP levels were among stage three of clinical RA. Smoking has a positive relation to the increased levels of all tested parameters compared to the non-smokers.

Introduction

Schellekens et al[1] showed that citrulline is an essential constituent of antigenic determinant for rheumatoid arthritis (RA) specific autoantibodies, that led to the development of anti-cyclic citrullinated peptides antibody (anti-CCP). The pathology of RA lesions reveals a prominent CD4 T-cells synovial infiltrate in close apposition to macrophages, resulting in high production of pro-inflammatory cytokines such as TNF-α, Interleukin-1 (IL-1) and proteolytic enzymes[2]. In addition, antibody producing B cells are abundantly present in the synovium producing autoantibodies such as rheumatoid factor (RF) and anti-CCP

that may be pathogenic by the formation and deposition of immune complexes in the lesion[3]. High levels of inflammatory mediators (IL-1β, IL-6, TNF-α) stimulate mesenchymal cells such as synovial fibroblast (Cynoviocytes), osteoclast and chondrocytes to release tissue-destroying Matrix Metaloproteinases (MMPs) causing further destruction[4-6]. RA activity reflects an ongoing imbalance between pro- and anti-inflammatory cytokines. TNF-α is a critical pro-inflammatory cytokine mediates many inflammatory processes in RA including immune-cell activation and proliferation, opoptosis and regulation of leukocytes movement[7]. IL-6 regulates the production of acute phase proteins by hepatocytes and activates osteoclasts to absorb bone[8]. High concentration 0f IL-6 have been detected in synovial fluid and serum of RA patients that highly correlate with disease activity[9]. RF is not very specific for RA because it can be found in other autoimmune diseases and in 3-5% of healthy populations, a percentage that increases up to 10-30% with aging[10]. Anti-CCP has been approved for the diagnosis of RA[11].The test is moderately sensitive (41-80% depending on the cohert studied) and approximately 90-98% specific for the diagnosis of RA[12,13]. Anti-CCP can appear in both early and advanced RA cases[14] and their presence predicts the development of RA in apparently healthy individuals before the occurrence of clinical symptoms and in patients with undifferentiated arthritis[15,16]. Investigators suggested the superiority of anti-CCP over IgM RF in predicting an erosive disease course[17]. In a study of early synovitis patients with a variety of diagnosis, the presence of anti-CCP had the highest predictive value of any laboratory test for the development of persistent synovitis and for development of erosive arthritis at two years after presentation[18]. The presence of anti-CCP in long term follow-up of patients is a risk factor for development of erosive arthritis[11,19].

This study aimed on the determination of anti-CCP prevalence in RA patients compared to healthy control and other rheumatic diseases, the relation of anti-CCP to RF, CRP, and some cytokines as IL-6, TNF-α and to evaluate the combination of anti-CCP and RF and other markers in the prediction of RA.

Materials and methods

Study population: A case control study was carried out from January 2007 till August 2007, included 121 rheumatoid arthritis (RA) patients (95 females and 26 males); those fulfilled the revised American College of Rheumatology criteria[20] from the patients (RA group) consulted the orthopaedic/rheumatology clinics and the clinic of internal medicine at Al-Hussein Teaching Hospital in Thiqar governorate. The control group included 88 patients with rheumatic diseases other than RA (non-RA group): 73 females and 15 males, and 120 healthy individuals (31 males and 89 females) with no history or abnormal physical findings through their clinical examination by specialist and their routine laboratory investigations.

Blood was drawn from both patients and controls. Separated serum was divided into two parts. The first was used directly (fresh) for the detection of RF, CRP, and ANF by latex agglutination test kits provided by; RF: plasmatic, cat. No. 0109, UK, CRP: Biokit, Spain, ANF: STANDARD Co, UK. And the second part was kept frozen until use for the detection of anti-CCP by ELISA kits (IMMUN Co., DIAGNOSTICS/ Buffallo /NY/Cat No.8001) and TNF^{alpha} and IL-6 were detected by Enzyme amplified sensitivity immunoassay (EASIA) from BIOSOURCE EUROPE SA, Rue de I'Industrie, 8B-1 400 Nivelle, Belgium/ Cat No. KA175). All procedures were done according to the manufacturer's instructions. Anti-CCP titer was expressed in units determined by using the calibration sera for construction of the

calibration curve (provided by the manufacturers). Any sample with < 20 units considered negative for anti-CCP and the range of 20-39U as weak positive, 40-59 U as moderate positive and 60 U or more considered as strong positive. Any value of IL-6 greater than 6 pgm/ml considered positive and any value greater than 21 pgm/ml was considered positive for TNF-α by EASIA test. For the interpretation of RF; any agglutination in the sample equal or greater than 8 IU/ml considered positive and the lack of agglutination in the sample (< 8 IU/ml) considered negative. Clear agglutination for CRP in serum sample equal or greater than 6 mg/L (positive results) and the lack of agglutination (< 6 mg/L) considered negative for CRP and for ANF, the presence of agglutination within one minute considered positive for ANF (as recommended by the manufacturers).

Results

The prevalence of anti-CCP as estimated by ELISA was 61.2% (74/121) among RA group compared to 2.3% among rheumatic diseases other than RA and 2.5% among healthy control group (Table I). The seropositivity of the slide agglutination RF test was 57% (69/121) among RA patients and 21.6% and 9.2% among rheumatic disease and healthy control groups respectively. Also there were 81% of RA patients positive for CRP, 60.2% of rheumatic disease group positive to CRP while 8.3% of healthy controls showed CRP positive results. The test for ANF showed lower results among all the three groups which were 10%, 9.2%, and 3.3% among RA, rheumatic diseases and healthy control groups respectively.

The test for TNF-α and IL-6 by the use of EASIA showed that 47.1% and 54.5% of RA patients were positive for TNF-α and IL-6 respectively(Table I). However, rheumatoid diseases and healthy control groups showed lower percentages of seropositivity to these cytokines which was 17%, 5% for TNF- α in both groups, and 25% and 7.5% for IL-6 in both groups.

The prevalence of anti-CCP, IL-6 and TNF-α in relation to the duration of illness of RA patients is presented in table II.

The percentages of sero-positivity to anti-CCP was increased as the duration of illness increased. On the other hand, the percentages of patients with IL-6 (53%) and TNF- α (52.6%) were peaked by 2-10 years of illness duration, then dropped to lower levels beyond the 10^{th} years of duration.

Table III shows the levels of anti-CCP, IL-6 and TNF- α where the majority presented with high levels of the three markers; anti-CCP (51.3 %), IL-6 (50%) and TNF-α (51%) at the time of presentation. Although RA patients presented with variable rates of illness duration, anti-CCP persist for longer periods with high levels of antibody titers (Tables II & III).

The ability of each test to predict a positive or negative case is presented in table IV. Although there were a variation in the sensitivity and specificity of various tests, anti-CCP antibody detection has a powerful prediction ability to detect RA positive cases in 93.7 % and to point the negative RA in 81.2 % of the tested population. TNF- α rises has almost an equal prediction values for a positive (73%) or negative (74.5%) RA cases. However IL-6 and RF seropositivity showed closed ability in prediction of positive RA (68% and 69% respectively) and negative RA (76.2% and 77.4% respectively) cases.

Table V shows the agreement of anti-CCP seropositivity with the presence of some RA related laboratory parameters together with combined anti-CCP sensitivity and specificity with one of the variables. The presence of anti-CCP together with IL-6 agreed in 91.7% of tested sera with a combined sensitivity and specificity of 98.3% and 83.6%

respectively. The presence of anti-CCP combined to TNF-α show a degree of agreement in 84.3 % with sensitivity of 98.2 % and specificity of 71.8 %. The degree of agreement of anti-CCP with RF was 79.3 % with a sensitivity of 85.6 % and specificity of 71.2%. However, the presence of anti-CCP together with CRP and ANF agreed in lower percentages which were 66.9 % and 42.1 % respectively. Also the sensitivity and specificity of this combination was lower for both; 67.4%, 66.6 % and 65.2 %, 39.4 % respectively.

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Table I: Serological parameters in patients with rheumatoid arthritis, rheumatic diseases and healthy controls.

Study groups / Test	Rheumatoid arthritis N+ve / Tested (%) N = 121 N %	Rheumatic diseases N+ve/Tested (%) N = 88 N %	Healthy control N+ve/Tested (%) N = 120 N %
Anti- CCP	74 61.2	2 2.3	3 2.5
	χ^2 = 109.091	df = 2	P = 0.000
RF	69 57.0	19 21.6	11 9.2
	χ^2 = 42.023	df = 2	P = 0.000
CRP	98 81.0	53 60.2	10 8.3
	χ^2 = 56.920	df = 2	P = 0.000
ANF	12 10.0	8 9.1	4 3.3
	χ^2 = 4.909	df = 2	P = 0.142
IL-6	66 54.5	22 25.0	9 7.5
	χ^2 = 38.614	df = 2	P = 0.000
TNF-α	57 47.1	15 17.0	6 5.0
	χ^2 = 40.696	df = 2	P = 0.000

Table II: Prevalence of anti-CCP, IL-6 & TNF-α in relation to the duration of illness (RA group).

Duration of illness (Y)	Anti-CCP N+ve (%)	IL-6 N+ve (%)	TNF-α N+ve (%)
< 2 years	23 (54.8%)	23 (34.5%)	18 (33.5 %)
2 – 10 years	42 (63.6 %)	35 (53.0%)	30 (52.6 %)
> 10 years	9 (69.2 %)	8 (12.1%)	9 (15.8 %)
Total	74 (61.2 %)	66 (54.5%)	57 (47.1 %)

Table III: The levels of anti-CCP, IL-6 and TNF-α in positive cases:

Type of marker (No. +ve)	Levels of serologic parameter		
	Low N (%)	Moderate N (%)	High N (%)
Anti-CCP (n=74)	17 (23.0 %)	19 (25.6 %)	38 (51.3%)
IL-6 (n= 66)	14 (21.2 %)	19 (28.8 %)	33 (50.0%)
TNF-α (n= 57)	12 (21.0 %)	16 (28.0 %)	29 (51.0 %)

Low titer ELISA of anti-CCP : 20 – 39 units, Mod.:40 – 59 units, High ≥ 60 units
Low IL- 6 EASIA 7 – 15.1 pg/ml, Mod.:15.2 – 40 pg/ml, High:> 40 pg /ml.
Low TNF-α EASIA 21- 40 pg/ml., Mod.: 41- 60 pg/ml, High: > 60 pg /ml.

Table IV: Comparison of the sensitivity and specificity between serological parameters. (control = 208, RA = 121)

Test	sensitivity	specificity	PPV	NPV
Anti-CCP	61.2 %	97.6 %	93.7 %	81.2 %
ANF	9.9 %	94.2 %	50.0 %	64.2 %
TNF- α	47.1 %	90.0 %	73.0 %	74.5 %
RF	57.0 %	85.6 %	69.7 %	77.4 %
IL – 6	54.5 %	85.1 %	68.0 %	76.2 %
CRP	81.0 %	69.7 %	60.8 %	86.3 %

PPV: positive predictive value. NPV: Negative predictive value.

Table V: Comparison of the overall agreement of the locally available laboratory tests to diagnose rheumatoid arthritis.

Test	Overall agreement %	Sensitivity %	Specificity %
Anti-CCP X IL- 6	91.7	98.3	83.6
Anti-CCP X TNF-α	84.3	98. 2	71.8
Anti-CCP X RF	79.3	85.6	71.2
Anti-CCP X CRP	66.9	67.4	65.2
Anti-CCP X ANF	42.1	66.6	39.4

Discussion

This is the first attempt to our knowledge to present the results of RA serological study in AL-Nassiriyah governorate, southern Iraq. The present study showed that the seropositivity of anti-CCP antibodies generally higher in RA patients (61.2 %) than in rheumatic diseases and normal individuals. This is because these antibodies raised against different epitopes in citrullinated peptides present in RA patients sera[21,22]. The figure obtained in this study almost comparable to 62.5 % reported in Iran on anti-CCP antibody[23]. and 70% an overall prevalence in Netherlands[24].

The current study showed that the seropositivity of anti-CCP was increased with increased duration of illness. The exact explanation of this is not so clear, although there are some possible reasons might be due to

several micro-organism produces arginine to citrulline deiminase activities[25] or due to the hypothesis of humoral responses to bacteria that may lead to stimulation of the inflammatory process for RA development[26] by triggering the immune system to generate a T cell and, next, B cell responses to citrullinated antigens. However, the over time stability of anti-CCP titers has been reproduced by many studies[27-29], whereas the changes in levels have varied across study populations[30]. On the other hand, decreases in anti-CCP titers has been recently correlated with clinical improvement after infliximab therapy in patients with RA[31,32]. This treatment is unavailable in our country and the other DMARDs do not reduce anti-CCP titers.

After thorough search in the locally available conventional literature and then Internet we obtained only few studies investigating serum levels of TNF-α and IL-6 in RA patients. A study carried out by Lene S.et al[33] who observed the circulating levels of IL-6 were significant elevated in RA patients compared with healthy subjects ($p<0.001$) and showed that in the early RA patients, serum IL-6 was lower compared to patients with erosive RA where serum IL-6 was significantly higher. Also, there are no strong correlation between age and the IL-6 concentration[33] and no difference in IL-6 concentrations between men and women[34,35]. Another study carried out by Norbert et al (2008) showed that IL-6 in combination with anti-CCP has the highest classification power for the diagnosis of RA[36]. These two studies have a good agreement with our results obtained from this study. However, another studies found that only 10—21% of RA patients had normal serum IL-6[37,38]. In contrast it has been reported that approximately 66% of RA patients had normal serum IL-6[39]. These differences are probably due to different assay used and different RA populations with different disease activity.

The current study showed that the seropositivity and titers of cytokines IL-6 and TNF-α were significantly higher in RA patients serum compared to the control groups. However, cytokines other than IL-6 and TNF-α might be involved in immunological mechanisms which constitute another limitation of this study, as we were unable to conduct assays for additional cytokines. We found that patients in the 2- 10 years duration of RA had higher levels of TNF-α than patients in other groups. This may be because the study mainly depends on already diagnosed RA cases with regular attendance to the rheumatology clinic and TNF-α levels may reflect RA reactivity[40].

This study establishes the value of anti-CCP antibody in the diagnosis of RA in comparison to IL-6, TNF-α, ANF, C-reactive protein and RF tests. The specificity obtained for the anti-CCP test (97.6%) was similar to that found by other studies: 91–98 %[41,11,42]. However, there is a considerable variation in the diagnostic sensitivity of anti-CCP, ranging from 41–68%[43,11,42]. This variation can be attributed to the different serum dilutions tested or more probably, to the different cut-off values used in the interpretation of the results. In the present study, the sensitivity of anti-CCP antibody was 61.2%, did not significantly differ from that obtained for RF (57%), IL-6 (54.5%) and TNF–α (47.1%) but was significantly higher than that obtained for ANF (9.9%) and lower than CRP (81%), hence, anti-CCP antibody test has moderate sensitivity and excellent specificity. These results are consistent with some studies in Switzerland[44].

It is clear that combining anti-CCP with IL-6 tests carries the best outcome in diagnosing RA and this combination

is superior to the anti-CCP with RF tests. This finding is in consistent with another study carried out by Norbert et al (2008)[36] showed that the best marker is the combination of anti-CCP and interleukin (IL)-6 resulted in a sensitivity gain of (85.9% vs.78.3%) at a minor loss in the specificity of 90.3% vs. 91.9% compared with anti-CCP as the best single marker and found the anti-CCP alone or anti-CCP in combination with IL-6 has the highest classification power for the diagnosis of RA[36]. It is possible that the combinations of CRP level and anti-CCP antibody close to the time of diagnosis may strongly predict incident disease[41]. However, we do not have data on combination of CRP level and anti-CCP antibody to examine this question, but the current study showed that the combination between anti-CCP and CRP has a sensitivity of 67.4% and specificity of 65.2%, which is not highly encouraging.

The results of combining anti-CCP with RF in this study are different to those calculated in Iran by Sakineh-Khatoun et al, 2007 (sensitivity 62.9 %; specificity 92.3 %)[23]. It also different from Schellekens et al.(2000) findings (sensitivity 39 %; specificity 98 %)[11].

The overall agreement measured for anti-CCP-RF combination in our study (79.3 %) differs from one study measured by S. Bas et al.,2002 (36 %)[43] and another study conducted in Greek showed the presence of either antibody (anti-CCP antibody and/ or RF) with increased sensitivity (75.5%), whereas the presence of both antibodies (anti-CCP antibody and RF) with increased specificity 98.2%[45].

Anti-CCP antibodies (CCP1 test) have been used successfully to diagnose persisted arthritis as compared to self-limited arthritis[18], or to differentiate RA (CCP2 test) from early undifferentiated arthritis[16]. Our findings of sensitivity (61.2%) and specificity of (97.6%) for anti-CCP in RA are in agreement with these studies. However, measurement of anti-CCP antibodies may have clinical utility in helping to establish a diagnosis of early RA or in RF-negative patients.

Anti-CCP test has the most powerful predictivity and validity compared to other concerned tests, a probability need to be put in mind that the anti-CCP specificity may be improved if ELISA kit we used of the second generation[23] .An important fact should be mentioned regarding false positive (those not showing typical clinical RA), cases allocated by the anti-CCP, may have value since the more recent studies showed anti-CCP could be detected l.5 – 9 years before the onset of arthritis and progression from undifferentiated polyarthritis to RA in 93 % of anti-CCP positive patients[17]. This simply means that many of the early positive anti-CCP tests will develop RA in the future and the test can be used as predictor to the disease advances.

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