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The role of synthetic manufactured peptides containing common citrullinated epitopes in rheumatoid arthritis diagnosis

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ABSTRACT

Background: Anti-citrullinated peptide antibodies (ACPA) play an important role in rheumatoid arthritis (RA) diagnosis. In our study, we sought to assess the potential diagnostic value of synthetically manufactured peptides that contain epitopes believed to have a pathogenic role in RA.

Methods: Serum samples from RA patients and healthy controls were obtained. Two synthetic peptides were manufactured containing the common epitopes considered to play a pivotal role in the RA pathogenesis including the antigenic epitopes of filaggrin, beta-fibrinogen, collagen, vimentin and enolase. Three different ELISA kits for citrullinated peptides (namely: CCP3, Cit-ME-Vim and Cit-ME-Eno) were tested and compared. To *assess* the *diagnostic value* of the three *ELISA* tests, for *each* test the *optical densities* (*OD*) were recorded. The statistical power of each test was calculated measuring the area under the curve (AUC) corresponding with each peptide.

Results: Serum levels of ACPA recognized by the commercial CCP3 in RA and healthy controls were 1.31 ± 0.88 optic density units (ODU) and 0.21 ± 0.11 ODU, respectively. Cit-ME-Vim levels were 0.55 ± 0.46 ODU in RA subjects and 0.17 ± 0.182 ODU in healthy controls whereas Cit-ME-Eno was 0.61 ± 0.65 ODU in RA subjects and 0.22 ± 0.20 ODU in healthy controls. AUC results were as follows: CCP3, 0.89 [95%CI 0.75–0.87]; Cit-ME-Vim, 0.76 [95%CI 0.69–0.82]; Cit-ME-Eno, 0.73 [95%CI 0.65–0.79]. Statistical significance for all results was achieved ($p < .0001$). Sensitivity values for each kit are as follow: CCP3 70.42%; Cit-ME-Vim 63.38%; Cit-ME-Eno 40.85%, and specificity 91% for all tests.

Conclusion: Our study supports the presence of an added value for the Cit-ME-Vim peptides in the diagnosis of RA. Further studies are needed to replicate such findings.

1. Introduction

Rheumatoid Arthritis (RA) is a chronic autoimmune disease characterized by joint swelling and tenderness and the progressive tethering of the synovial tissue to the extent of loss of function accompanied with higher rates of mortality [1,2]. The disease prevalence is similar among all ethnic groups and the rate of incidence is estimated to be around 40 per 100,000 people as it affects 1% of adults in the developed world [3]. In terms of gender, women are found to be 2–3 times at higher risk of developing the disease compared to men [4].

RA is a multi-factorial disease and subjects with a set of alleles at the HLA-DRB1 gene locus are more prone to develop the disease [5]. Smoking was also found to be strongly correlated with diseases development [6]. Antibodies to citrullinated proteins/peptides (ACPA) are directed against proteins that have undergone a process of citrullination and become autoantigens such as collagen, filaggrin, vimentin, and fibrinogen [7,8]. The measurement of ACPA levels in the serum is correlated with the specificity for RA diagnosis, in other words, the higher the ACPA levels, the higher the likelihood of having RA. Therefore, this new serological marker has contributed greatly to the process of diagnosing the disease in terms of its specificity and accuracy, as the specificity rate of the ACPA test is found to be around 95–98% with a similar sensitivity rate to the rheumatoid factor (RF) [9–12].

As of today, the 2010 criteria for the diagnosis of RA replaced the 1987 criteria and now include the serological marker ACPA which are considered the most instrumental serological marker in diagnosing the disease [13]. However, these markers are found in only 70% of patients with RA [14]. Several reports noted that ACPA levels correlate with the

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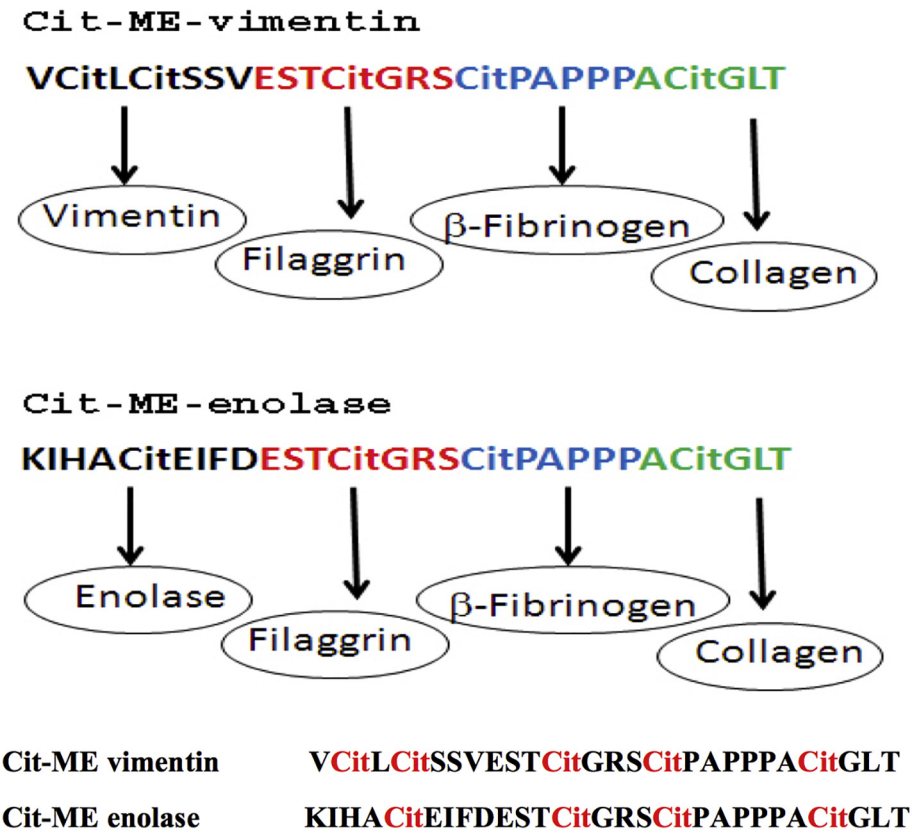

Fig. 1. Descriptive presentation of the amino-acids sequence of the Cit-ME-vimentin and Cit-ME–enolase, Cit represent the citrulline residues.

RA disease severity [15]. A synthetic protein kit was manufactured and termed cyclic citrullinated peptide (CCP) kit which includes one of the autoantigenic epitopes, filaggrin, which was used as the antigenic substrate for the anti-CCP1 antibody in ELISA. This new test yielded a low sensitivity of 41–68% and specificity of 98% for RA [16]. Later, second and third generation of diagnostic kits were developed (CCP-2 and CCP-3) and demonstrated a higher sensitivity compared to anti-CCP1 of 80% and a specificity of 98%. Tests have been conducted to compare diagnostic abilities of CCP2 compared to CCP3 showing CCP3 kit to have higher sensitivity values [17,18]. This study aimed to evaluate the diagnostic added value of using a synthetic peptide made of the common auto-antigens in the RA disease (vimentin, filaggrin, beta-fibrinogen, collagen, and enolase).

2. Materials and methods

2.1. Patients and controls selection

Serum samples from patients diagnosed with RA according to the American College of Rheumatology/European League against Rheumatism 2010 criteria at the Sheba Medical Center (located in Tel-Aviv, Israel) were recruited. Patients were considered potentially eligible and enrolled in the study if their diagnosis has been established by a specialist prior to the study. Patients were in different phases of their disease and their level of RA progression was not considered as a factor for this study. For comparison, we tested a group of control subjects not known to have RA nor any other inflammatory disease.

The current study received ethical approval from Tel-Hashomer's ethical committee. Patients as well as healthy controls signed their consent form in order to take part into this study.

2.2. Synthetic peptides

Two synthetic peptides were manufactured by GL Biochem group (Shanghai. China) in such a way to contain the common epitopes considered to play a pivotal role in RA pathogenesis including filaggrin, beta-fibrinogen, collagen, vimentin and enolase. Fig. 1 shows the sequence of the Cit-ME-Vim and Cit-ME-Enolase, Cit-ME-Vim peptide contained epitopic elements of filaggrin, beta-fibrinogen, collagen, and vimentin, while Cit-ME-Enolase contained filaggrin, beta-fibrinogen, collagen and enolase. Sera were analyzed for the presence of ACPA using the enzyme-linked immunosorbent assay (ELISA) Quanta Lite CCP3 IgG Kit (Inova Diagnostics. San Diego. CA).

2.3. ACPA detection laboratory techniques

Three different ELISA kits for citrullinated peptides (namely, CCP3, Cit-ME-Vim and Cit-ME-Eno) were tested and compared. First, peptides were diluted to 10 μg/ml in phosphate-buffered saline (PBS), then blocking was induced with bovine serum albumin (BSA) 2% diluted with PBS at 4 °C. Plates were washed 3 times with PBS solution Tween 0.05%. Afterwards, the plates underwent incubation for 2 h with the serum samples diluted 1:50 with BSA 2% and then washed again 3 times with PBS solution Tween 0.05%. After wash, the plates underwent incubation with HRP with anti-human IgG and underwent washing with specific horseradish peroxidase (HRP) wash (red) diluted to 1:40 with double distilled water (DDW) 5 times. Reactions were induced with 3,3′,5,5′-tetramethylbenzidine (TMB) for 30 min and stopped with sulfuric acid. Plates were read on an ELISA plate reader at a wavelength of 450 nm and measured in optic density units (ODU) for comparison.

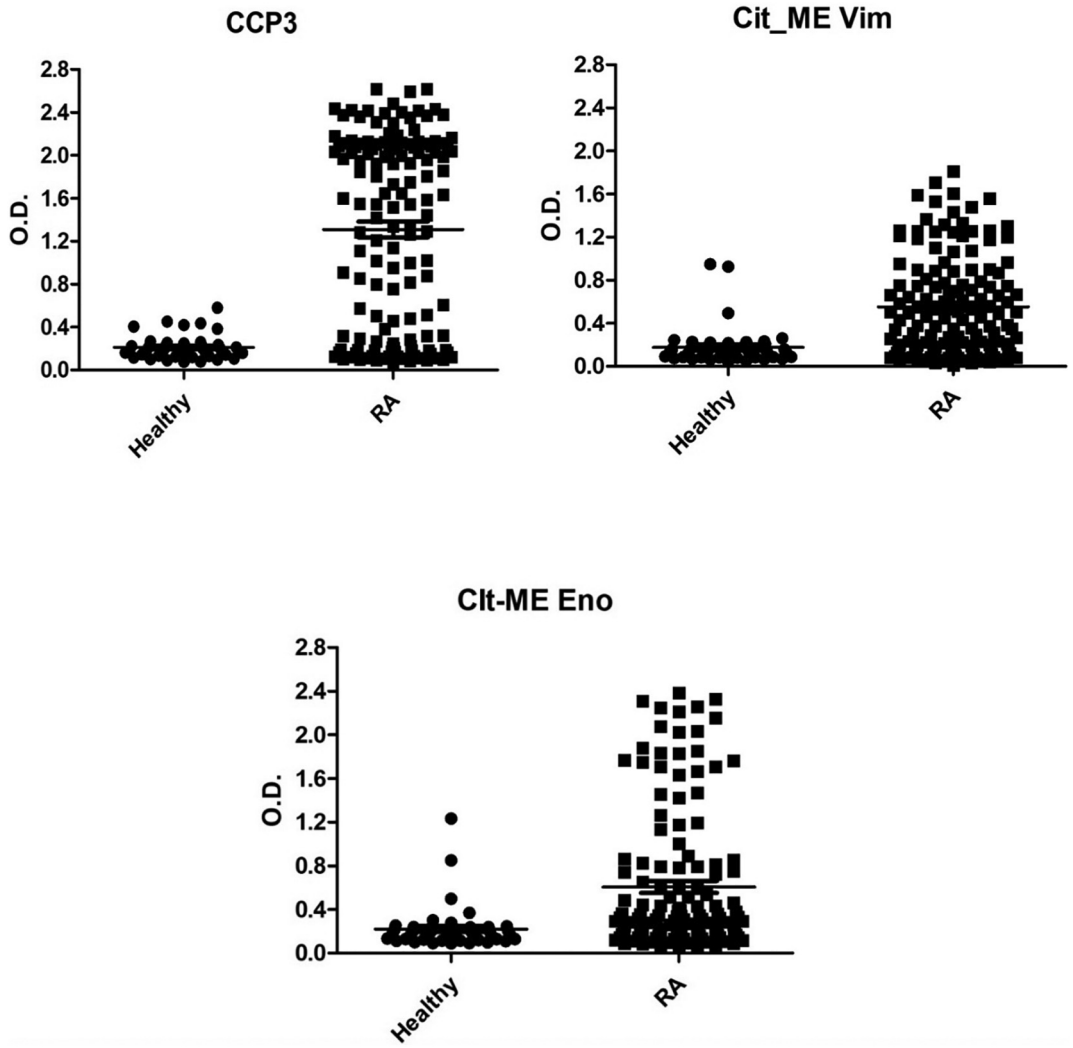

Fig. 2. Mean optic density (O.D.) values for CCP3, Cit-ME-Vim, and Cit-ME-Eno tests in RA and healthy controls.

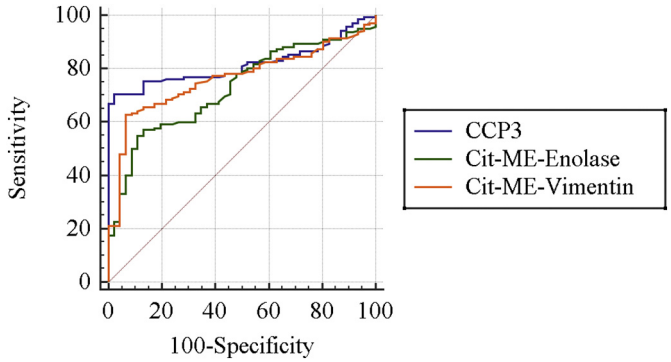

Fig. 3. Receiver operating characteristic (ROC) analysis for CCP3, Cit-ME-Vim and Cit-ME-Eno tests.

2.4. Statistical analysis

Quantitative data were presented as means ± standard deviation (SD). Cut-off values for home-made and commercial ELISA tests were computed by carrying out a receiver operating characteristic (ROC) analysis. In order to compare the different diagnostic values for each kit, sensitivity and specificity for each ELISA test were calculated. The intra-class correlation coefficient (ICC) was also computed and its magnitude was interpreted using the following rule of thumb by Cicchetti [19]; < 0.40 — poor; between 0.40 and 0.59 — fair. Between 0.60 and 0.74 — good; and between 0.75 and 1.00 — excellent.

The sample size was *a priori* estimated, with type 1 error set at 0.05 and type 2 error at 0.20. In order to distinguish between an expected AUC in the range 0.70–0.90 against the null hypothesis (AUC = 0.500) in a statistically significant fashion an overall sample size of 17–75 subjects was required.

Analysis and comparison of ROC curves were performed with the commercial software MedCalc v7.6 software, whereas all the other statistical analyses were performed using the commercial software "Statistical Package for Social Sciences" (SPSS version 16.0. IBM. USA). All *p*-values were two-sided and $p < .05$ was considered statistically significant.

3. Results

One hundred three serum samples from RA patients and 46 form healthy subjects were obtained during the study period (2012–2017), which largely exceeded our *a-priori* computed sample size.

Serum levels of antibodies recognized by CCP3 in RA and healthy subjects were 1.31 ± 0.88 (ODU) and 0.21 ± 0.11 (ODU), respectively (Fig. 2). Serum levels of antibodies recognized by Cit-ME-Vim levels were 0.55 ± 0.46 (ODU) in cases and 0.17 ± 0.182 (ODU) in controls whereas serum levels of antibodies recognized by Cit-ME-Eno were 0.61 ± 0.65 (ODU) in RA subjects and 0.22 ± 0.20 (ODU) in

Table 1
Pairwise comparison of ROC curves for each kit; CCP3, Cit-ME-Vim, and Cit-ME-Eno.

CCP3 *vs* Cit-ME-Eno	
Difference between areas	0.0892
Standard error[a]	0.0386
95% Confidence interval	0.0135 to 0.165
z statistic	2.311
Significance level	P = .0208
CCP3 *vs* Cit-ME-Vim	
Difference between areas	0.0530
Standard error[a]	0.0407
95% Confidence interval	−0.0267 to 0.133
z statistic	1.303
Significance level	*P* = .1927
Cit-ME-Eno *vs* Cit-ME-Vim	
Difference between areas	0.0362
Standard error[a]	0.0272
95% Confidence interval	−0.0170 to 0.0894
z statistic	1.333
Significance level	*P* = .1824

Table 2
Percentage of positive test for each kit in RA and healthy controls.

Population	N	CCP3	Cit-ME-Vim	Cit-ME-Eno
		N (%)	***N (%)***	***N (%)***
RA	143	101 (70.6%)	90 (63%)	59 (41%)
Healthy	46	4 (9%)	4 (9%)	4 (9%)

healthy individuals (Fig. 2).

Results of ROC analysis for each kit (CCP3, Cit-ME-Vim and Cit-ME-Eno) are shown in Fig. 3. AUC results were as follows: CCP, 0.89 [95%CI 0.75–0.87]; Cit-ME-Vim, 0.76 [95%CI 0.69–0.82]; Cit-ME-Eno, 0.73 [95%CI 0.65–0.79]. Statistical significance for all results was achieved ($p < .0001$).

CCP3 resulted to have the highest AUC whilst Cit-ME-Eno had the lowest. The difference between CCP3 and Cit-ME-Eno were statistically significant ($p = .0208$) (Table 1). Concordance among the three kits was good, namely 0.638 [95%CI 0.521–0.730].

The cut-off values for optic density for each test are as follows: CCP3, 0.385 ODU; Cit-ME-Vim, 0.243 ODU; Cit-ME-Eno, 0.385 ODU. The cut off values were calculated according to ROC analysis that sets the same value of specificity for each kit of 91%. Sensitivity values for each kit were found as follow: CCP3, 70.42%; Cit-ME-Vim, 63.38%; Cit-ME-Eno, 40.85%, and specificity of 91% for all tests. The percentage of positive tests for each kit in RA and healthy subjects are reported in Table 2. Thirty-four percent of the serum samples in the RA group were positive for all three tests, whereas 15% of the serum samples in the same RA group were negative for all three tests.

4. Discussion

In our current study, we examined the diagnostic value of synthetic peptides made up from epitopes that contain common autoantigens believed to play a key role in RA pathogenesis reporting an added value of Cit-ME (mainly for Cit-ME-Vim) peptides in RA diagnosis in comparison to CCP3 peptide.

The presence of ACPA among RA patients is considered one of the serological hallmarks of RA and is one of the criteria for its diagnosis [13].

ACPA are considered to be specific for RA with a specificity of 98%, notwithstanding the high specificity rate, the sensitivity rate of 70% [14,20]. This percentage affects the way we diagnose and we treat the disease as an early presence of ACPA implies a more erosive disease with more apparent radiological findings [21]. Another study suggests that the presence of ACPA in early stages of the disease, along with a monotherapeutic treatment approach, produce a more progressive disease [22].

In previous studies where anti-mutated citrullinated vimentin (anti-MCV) was used, it was found to be a potent diagnostic tool comparable with that of CCP3 maintaining a sensitivity value of 82% (compared to 72% for CCP3) and a specificity value of 98% (compared to 96% for CCP3) [23]. Another study, utilizing filaggrin/collagen/vimentin chimeric peptide was found effective in diagnosing different subgroups of RA patients who were negative according to the CCP3 test [14]. Our study found a sensitivity value of 70% for CCP3, 63% for Cit-ME-Vim, and 41% for Cit-ME-Eno. A possible explanation for the Cit-ME-Vim's sensitivity rate is that vimentin in our study did not undergo mutation, as opposed to anti-MCV, which might explain the further potency of vimentin in this regard [23]. In regards to Cit-ME-Eno, results show the lowest sensitivity rate out of the three tests. Although enolase plays a vital role in the citrullination process, it is not present among all of RA's patients [24,25].

However, it is noteworthy that 11% of the cases were found to be positive for Cit-ME-Vim whereas resulted negative to CCP3 and Cit-ME-Eno. Furthermore, one case was found positive for Cit-ME-Eno and negative for CCP3 and Cit-ME-Vim, which makes up for < 1% of the cases.

The accumulative percentage of all the cases that were found positive for Cit-ME peptides (positive in either one of them, or both simultaneously) and negative for CCP3 is 13%. This indicates that Cit-ME peptides had an added value for diagnosing RA in comparison to the use of only CCP3 kit. It is worthwhile to consider utilization of ELISA with Cit-ME-Vim for patients that are suspected to have RA and were diagnosed as seronegative with CCP3.

Our study has several limitations such as not including an examination to evaluate the potential synergistic diagnostic role of both Cit-ME-Vim and Cit-ME-Eno together. Another limitation, our study did not take into consideration the phases of the disease with each patient, as it would have given us more information regarding the timing of the diagnosis and each test's characteristic according to the disease's timeline.

In conclusion, our study supports that synthetic peptides containing common citrullinated epitopes may have an added value over the commercially available kits for the diagnosis of RA. However, this study necessitates further examination and search for other different subgroups of RA patients who are, as of today, considered ACPA negative.

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