Journal of Pharmaceutical and Biomedical Analysis 115 (2015) 107–113

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Evaluation of a specific diagnostic marker for rheumatoid arthritis based on cyclic citrullinated peptide

Min Park[a,1], Jae-Chul Pyun[b,1], Hafeza Akter[a,c], Binh Thanh Nguyen[a,c], Min-Jung Kang[a,c,*]

[a] Molecular Recognition Research Center, Korea Institute of Science and Technology (KIST), Seoul 136-791, Republic of Korea
[b] Department of Material Science and Engineering, Yonsei University, Seoul 120-749, Republic of Korea
[c] Department of Biological Chemistry, Korea University of Science and Technology, Daejon 305-350, Republic of Korea

ARTICLE INFO

Article history:
Received 26 March 2015
Received in revised form 19 June 2015
Accepted 21 June 2015
Available online 25 June 2015

Keywords:
Rheumatoid arthritis
Osteoarthritis
CCP
ϵ-aminocaproic acid
Sequence modification
Diagnostic marker

ABSTRACT

A specific peptide marker for diagnosing rheumatoid arthritis (RA) was found based on cyclic citrullinated peptide (CCP) using the following three steps: (1) analysis of the binding epitope of autoimmune antibodies using ϵ-aminocaproic acid-modified peptides; (2) RA diagnosis using sequence-modified peptides; and (3) evaluation of the peptides' diagnostic performance for RA diagnosis. Ninety-five serum samples were analyzed by ELISA and compared using MedCalc (version 15.2.1). Microplate binding ϵ-aminocaproic acid was added to the N- or C-terminal of the CCP sequence. The N-terminal anchoring peptide assay showed 15% higher specificity compared with the C-terminal anchoring peptide assay. Based on this result, the hydrophilic C-terminal sequence of CCP was substituted with a hydrophobic amino acid. Among the sequence-modified peptides, CCP11A (in which alanine was substituted for the 11th amino acid of CCP) assay showed the highest sensitivity (87%) and specificity (100%) for RA diagnosis. Thus, CCP11A was selected as a possible specific marker peptide for RA diagnosis and further analyzed. The results of this analysis indicated that CCP11A showed better specificity than the CCP assay in both healthy individuals (11% better) and OA cohort (20% better). From these results, CCP11A was evaluated as a specific marker for diagnosing RA with higher diagnostic performance.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease of uncertain etiology. In RA patients, the synovium in the joint becomes inflamed due to an autoimmune reaction, and this chronic inflammation causes severe pain and work disability [1–6]. Growth of the inflamed synovium from chronic inflammation deforms the patient's joint or bone and leads to impaired activity [7,8]. Currently, there are no drugs or treatments to aid the complete recovery from RA. Late diagnosis of RA leads to deformed joints and even damage to other organs by invasion [9]. For these reasons, early diagnosis of RA is very important, both for symptom relief and for retarding disease progression. In addition, differentiating RA from other autoimmune diseases or types of arthritis is important. There are as many as 80 types of autoimmune diseases. Systemic lupus erythematosus (SLE), multiple sclerosis, and celiac sprue disease are the most representative autoimmune diseases with similar symptoms [10–12]. The most common types of arthritis are osteoarthritis (OA) and RA. In contrast with RA, OA is a mechanical disorder involving degradation of cartilage [13]. This degradation also causes pain and impaired activity [14]. The primary cause of OA is mechanical stress, whereas RA and SLE result from an autoimmune reaction [15]. An accurate differential diagnosis of RA from these autoimmune diseases or arthritis is essential for patient-specific treatment.

The rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) tests are the most widely used blood tests for the diagnosis of RA. The sensitivity of the RF test has been reported to be from 70% to 75% [16–18], and its specificity has been reported to be between 80% and 85% [16,17]. However, the RF antibody is reported not to be found in all RA patients [17–19]. Therefore, the specificity of the RF test has been limited. CCP is a 15-mer peptide including citrulline with a cyclic structure from disulfide bonding between cysteine sequences at both the N- and C-terminals. This peptide has been reported to bind with autoimmune antibodies in

* Corresponding author at: Molecular Recognition Research Center, Korea Institute of Science and Technology (KIST), Seoul 136-791, Republic of Korea. Fax: +82 2 958 5059.
E-mail address: mjkang1@kist.re.kr (M.-J. Kang).
[1] M.P. and J.C.P. contributed equally to this paper.

http://dx.doi.org/10.1016/j.jpba.2015.06.032

RA patients [16,20,21]. Citrulline is a post-translational modified peptide derived by peptidylarginine deiminases (PAD) [22]. This citrulline has been known to play an important role in the recognition of autoimmune antibodies in serum from RA patients [20]. The anti-CCP test shows high specificity (between 88% and 99%), and its sensitivity has been shown to range from 45% to 91% [18,20]. In case of Korean patients, the sensitivity of anti-CCP test has been reported between 72.0% and 82.3%, and its specificity has been reported to be from 92.0% to 96.0% [19,23,24]. The current disadvantage of the anti-CCP test is its lower specificity for RA diagnosis compared to other arthritis or autoimmune diseases [16]. Thus, evaluation of new biomarker with higher medical utility is important to improve the diagnosis of RA.

To improve the diagnosis of RA, lots of novel biomarkers were recently discovered such as autoantibodies including carbamylated protein (CarP), PAD type 4 (PAD4), v raf murine sarcoma viral oncogene homologue B1 (BRAF), and University Hasselt-RA. clone numbers (UH-RA. number) [25]. These biomarkers are non-CCP derived biomarker so they can diagnose RA in CCP-negative patients [25]. CarP contains homocitrulline which is post-translational modified amino acid derived by cyanate. The sensitivity and specificity of anti-CarP IgG test was reported to be 44.9% and 97.0%, respectively [26]. The sensitivity of the PAD4 test has been reported to be from 27.8% to 82.8%, and its specificity has been reported to be between 54.5% and 100.0% [27]. In case of BRAF p10 and p25 test, the sensitivity has been reported to be 35% and 19%, and the specificity was 93.0% and 100.0%, respectively [28]. The UH-RA clones resulted in poor sensitivity (Max. 29.4%) whereas the specificity was higher than 95% [29]. These biomarkers showed high specificity except PAD4-P28 (54.5%).

Currently, RA is primarily diagnosed by visual inspection, and blood testing is used as an auxiliary method for comprehensive analysis. In 1987, the American college of rheumatology (ACR) established standard classification criteria, and new classification criteria were jointly published by the ACR and the European league against rheumatism (EULAR) in 2010 [30,31]. The new classification criteria established a system where points are assigned between 0 and 10 for four areas: joint involvement, serologic parameters of the RF and anti-CCP tests, acute phase reactants, and the duration of arthritis. Among these four areas, half of the points are from joint involvement analyzed by visual inspection. The serologic parameters from specific biomarkers occupy only 30% of the total points. The high ratio of visual examination indicates that there is no specific and reproducible serum biomarker for RA diagnosis.

For developing a more specific marker of RA, we modified CCP by the addition of ϵ-aminocaproic acid for the analysis of binding sites between CCP-based peptides and autoimmune antibodies in serum from RA patients. The binding epitope of the autoimmune antibody was analyzed by ELISA using N-, C-, and both N- and C-terminal anchoring peptides. Then, sequence-substituted peptides were designed according to the epitope analysis, and RA samples were tested using these peptides.

2. Materials and methods

2.1. Materials

Cyclic citrullinated peptide (CCP) and modified peptides were synthesized with purities of 95% by Peptron Co. (Daejeon, Korea). The sequences of peptides are described in Table 1. Polyclonal anti-human immunoglobulin G (IgG) antibodies conjugated with horseradish peroxidase (HRP) were obtained from Abcam (Cambridge, UK). The 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for HRP detection and a 96 well-microplate were purchased from Thermo Scientific (Rockford, IL, USA). Premade phosphate buffered saline (PBS) was obtained from WelGene (Daejeon, Korea). Other chemical reagents, including Tween-20 and bovine serum albumin (BSA), were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Table 1
Sequences of CCP and modified peptide.

Name	Sequence of peptides	Modifications
CCP	HCHQESTXGRSRGCG	1X, C—C
RF	EGLHNHY	–
HSH15	HSHQESTXGRSRGSG	1X
ZZH17	ZZHSHQESTXGRSRGSG	1X, 2Z
HSH17	HSHQESTXGRSRGSGZZ	1X, 2Z
ZZH19	ZZHSHQESTXGRSRGSGZZ	1X, 4Z
CCP10P	HCHQESTXGPSRGCG	1X, C—C
CCP11A	HCHQESTXGRARGCG	1X, C—C
CCP12P	HCHQESTXGRSPGCG	1X, C—C
CCPPAP	HCHQESTXGPAPGCG	1X, C—C

X = citrulline, C–C = disulfide bonding (Cys–Cys), Z = ϵ-aminocaproic acid.

Table 2
Characteristics of patients.

Disease type	Number of patients	Age	Sex (F/M)
Rheumatoid arthritis (RA)	30	51.2 (25–74)	22/8
Osteoarthritis (OA)	15	47.3 (20–75)	13/2
Systemic lupus erythematosus (SLE)	25	38.3 (22–83)	24/1
Healthy individuals	25	52.6 (29–76)	15/10

2.2. Serum collection

Thirty serum samples from patient with RA, diagnosed according to the 2010 American rheumatism association (ARA) criteria, were collected at Kang-Nam St. Mary's Hospital [30]. Fifteen osteoarthritis (OA) patients' sera and 25 systemic lupus erythematous (SLE) patients' sera were obtained at Kang-Nam St. Mary's Hospital and Yonsei University College of Medicine, respectively. Serum from 25 healthy individuals (HI) was collected at Korea University Guro Hospital. There were 22 female and 8 male RA patients, and their mean age was 51.2 (range, 25–74) years. Patient information (age, gender) is described in Table 2. All serum samples from patients and HI were obtained according to the Declaration of the Helsinki. The study was approved by the institutional review board (IRB) of the Kang-Nam St. Mary's Hospital, Yonsei University College of Medicine, and Korea University Guro Hospital.

2.3. ELISA assay using CCP based peptides

The synthesized peptides were immobilized on a 96-well microplate by adding 100 μL of diluted solution in PBS with a concentration of 10 μg/mL. After overnight incubation at 4 °C, the solution was removed, and the non-binding site was blocked with 10 mg/mL BSA dissolved in PBS. After 1 h incubation at room temperature (RT), the blocked microplate was washed three times with 0.1% Tween-20 in PBS. For the autoimmune antibody detection, 100 μL of a serum sample diluted with PBS (1:200) was transferred into a well and incubated for 1 h at RT. After binding the autoimmune antibodies with the peptide, the microplate was again washed three times with same solution. Then, 100 μL of HRP-conjugated anti-human IgG antibody (100 ng/mL in PBS) was dispensed into each well for detection of autoimmune antibodies. After 1 h incubation at RT, the microplate was washed three times, and 100 μL of TMB substrate solution was treated at RT. After 5 min, the reaction was stopped by adding 100 μL of 2 M sulfuric acid. The optical density of the plate was measured at a 450-nm wavelength by microplate reader (Bio-rad, Hercules, US). All experimental steps were repeated three times.