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

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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

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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.

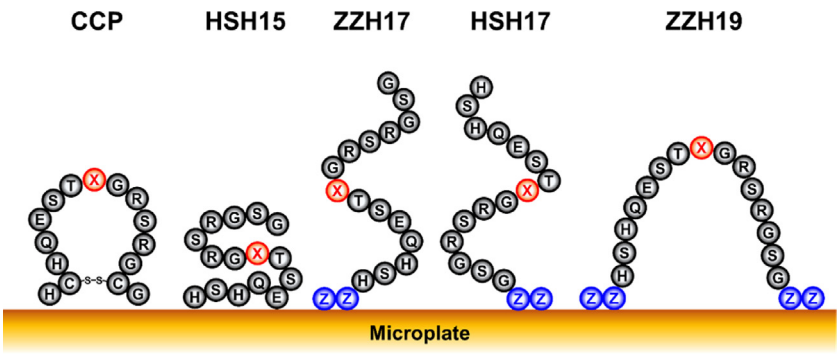

Fig. 1. Schematic diagram of peptide immobilization on the microplate. (X = citrulline, Z = ϵ-aminocaproic acid).

2.4. Statistics

To compare means among the four different cohorts of patients, Tukey's multiple comparison was used in this study. Significant differences between HI and the disease cohort were calculated by *t*-test. The test was regarded as statistically significant when the obtained *p*-value was less than 0.05. All analyses, including Tukey's multiple comparison, sensitivity, specificity, receiver operating curves (ROC), and areas under the curve (AUC) were calculated by MedCalc ver 15.2.1 (MedCalc Software, Mariakerke, Belgium). Significant differences among assays were calculated by the McNemar test.

3. Results and discussions

3.1. Analysis of binding epitopes of autoimmune antibodies

For the evaluation of the binding site between biomarker peptides and autoimmune antibodies in serum, RA patient serum was analyzed by ELISA using ϵ-aminocaproic acid modified peptides. ϵ-aminocaproic acid has a hydrophobic *n*-pentyl group as its side chain, so that was used as an anchor for peptide immobilization on the microplate [32]. The CCP peptide was linearized by the modification of cysteine to serine, which results in the deletion of disulfide bonding (HSH15). Then, the other peptides were changed by adding two ϵ-aminocaproic acids to the N- (ZZH17), C- (HSH17), or both the N- and C- (ZZH19) terminals of the linearized peptide, HSH15. The sequence-modified peptides are described in Table 1, and Fig. 1 shows the different binding structure of one linearized peptide and three modified peptides. Using these modified peptides, direct ELISA was performed for sera of RA, OA, and SLE patients.

Fig. 2 shows the results of Tukey's multiple comparison among the three disease cohorts (RA, OA, and SLE) and the HI. Autoimmune antibody levels from HI samples were significantly lower than those from disease samples in all assays ($p < 0.001$). The RF assay was less significant between RA cohort and HI ($p = 0.0002$) than that of CCP ($p < 0.0001$) and showed lower optical density (OD) values for RA cohort (0.2345) than for OA (0.2437) and SLE (0.2957) cohorts. In the CCP assay, the OD value of RA cohort 2.9-fold higher than that of HI (0.0874) and also higher than those for OA (0.2314) and SLE (0.2347) cohorts. For the diagnosis of RA, the citrullinated sequence in CCP plays a primary role [16]. The linearized peptide (HSH15) assay showed a lower OD value for RA cohort than for OA and SLE cohort, suggesting that the cyclic structure of CCP is essential for the exposure of citrulline toward autoimmune antibodies in serum. In the ϵ-aminocaproic acid-modified peptides assays, differences between the patient cohorts and HI were highly significantly different ($p < 0.0001$). ZZH19 has additional two ϵ-aminocaproic acid sequences at both the N- and C-terminals so that the central sequence, citrulline, is exposed by anchoring both the N- and C- terminals on a microplate (Fig. 1). For this reason, the ZZH19 assay showed higher OD values for RA cohort (0.4008) than for OA (0.3069) and SLE (0.3693) cohorts. The C-terminal anchored peptide, the HSH17 assay, also showed a lower OD value for RA cohort than for OA and SLE cohorts. In the case of the ZZH17 assay, the N-terminal was anchored on the microplate, and the C-terminal sequences were exposed toward autoimmune. The ZZH17 assay showed a higher OD value for RA cohort (0.2667) than for OA cohort (0.2344). These results suggest that the C-terminal sequences of CCP have a more specific affinity with autoimmune antibodies in RA patient's serum.

Table 3
Sensitivity and specificity of CCP and ϵ-aminocaproic acid modified peptide assays.

Peptide name	Sensitivity (%)	Specificity (%)
RF	80	81
CCP	93	89
HSH15	100	69
ZZH17	87	85
HSH17	100	69
ZZH19	90	81

The sensitivity and specificity of the CCP-based assays for RA cohort were calculated (Table 3). The sensitivity and specificity of the RF assay were 80 and 81%, respectively. The CCP assay showed higher sensitivity (93%) and specificity (89%). The linearized peptide (HSH15) assay showed 100% sensitivity, whereas the specificity was much lower (20%) compared with that for the CCP assay. It also supports that the cyclic structure of CCP is essential for RA diagnosis. Among the three ϵ-aminocaproic acid-modified peptides assays, the ZZH17 assay showed the highest specificity (85%). The C-terminal anchoring peptide (HSH17) assay showed 16% lower specificity (69%) compared with the ZZH17 assay. These results indicate that C-terminal sequences are more significant than are N-terminal sequences for the diagnosis of RA.

3.2. RA diagnosis using sequence-modified peptides

We further modified the C-terminal sequences of CCP between citrulline and cysteine (GRSRG). Both arginine and serine are hydrophilic amino acids, and therefore the sequences were substituted with hydrophobic proline and/or alanine to examine differences in diagnostic performance. The sequences of the modified peptides are described in Table 1 (CCP10P, CCP11A, CCP12P, and CCPPAP), and these peptides were tested for their ability to diagnose RA. As shown in Fig. 3, the OD values from the patient cohorts were significantly higher than those from HI in all assays ($p < 0.001$). In the CCP assay, the OD value of RA cohort was higher than that for OA cohort (Fig. 2a). As shown in Fig. 3b, only the CCP11A test showed an 11% higher OD value for RA cohort (0.3091) compared with that for OA cohort (0.2689), whereas that of RA

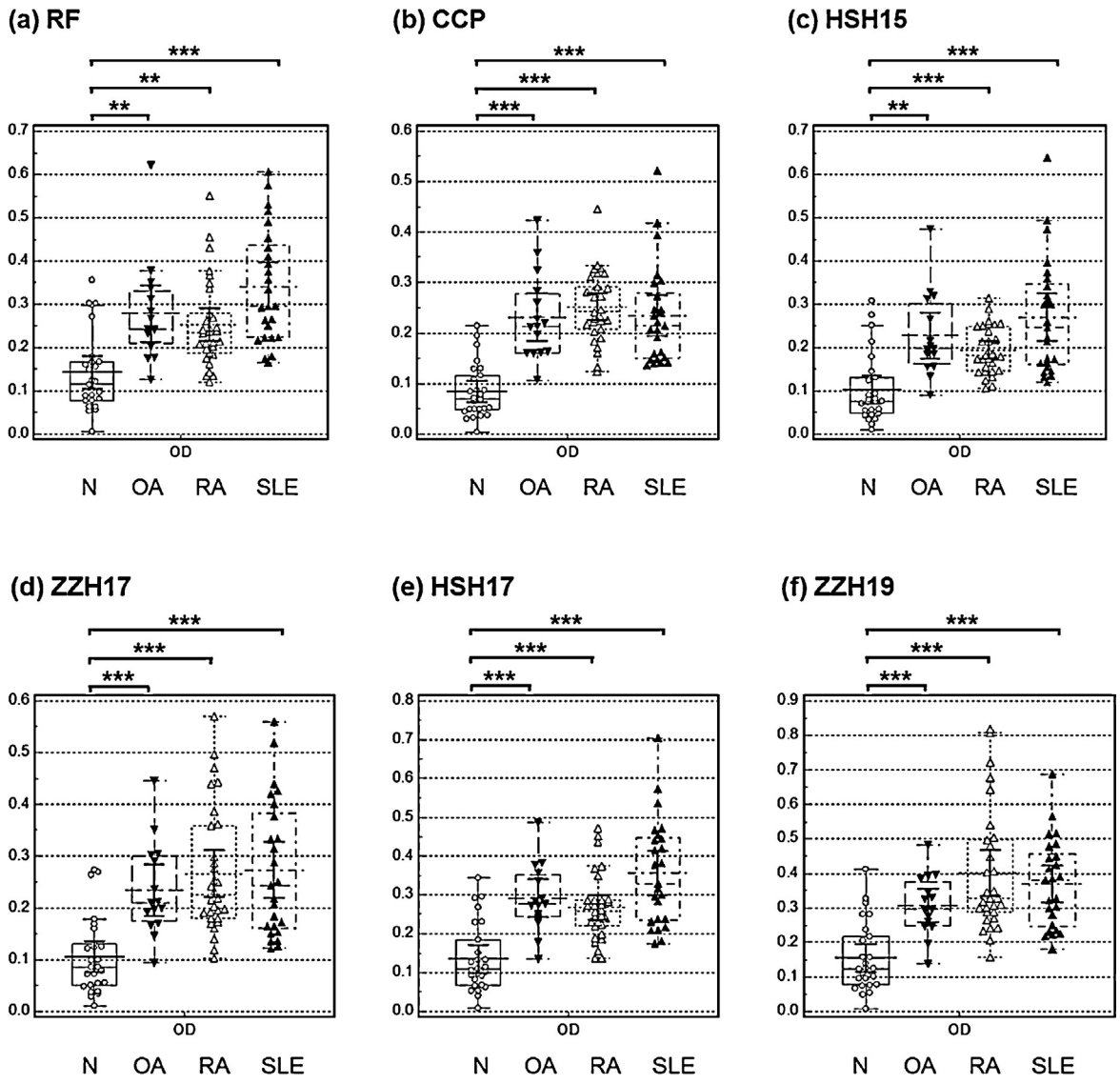

Fig. 2. ELISA assay of CCP and ϵ-aminocaproic acid-modified peptides. (*: $p < 0.005$, **: $p < 0.001$, ***: $p < 0.0001$). RA, OA, SLE, and healthy individuals (N) samples were tested using (a) RF, (b) CCP, (c) HSH15, (d) ZZH17, (e) HSH17, and (f) ZZH19 peptides.

cohort was lower than that of OA cohort for the CCP10P, CCP12P, and CCPPAP tests (Fig. 3). These results suggest that the diagnostic performance of test for RA can be improved by the substitution of a hydrophilic 11th amino acid to hydrophobic alanine.

The sensitivity and specificity of ELISA using sequence-modified peptides were calculated (Table 4). The results of these modified peptides showed increased specificities, especially the CCP11A, CCP12P, and CCPPAP assays. In the case of the CCP10P assay, its RA specificity was lowest (77%) among the peptides, so it was excluded as an RA diagnostic marker. The RA specificities of the CCP11A, CCP12P, and CCPPAP assays were 100%. The sensitivities of the CCP12P and CCPPAP assays were relatively lower (70% and 77% respectively) than that for the CCP11A assay. Among those assays, the CCP11A assay showed the highest sensitivity (87%) and specificity (100%) for RA diagnosis. The sensitivity was 6% lower than that of the CCP assay, whereas its specificity was 11% higher. The sensitivity of anti-CCP test has been reported to be between 45% and 91%, and its specificity has been reported to be from 88% to 99% [18–20]. Comparing with those results, CCP11A assay showed high level of both sensitivity and specificity. From these results, we selected CCP11A as a valuable specific marker peptide for RA diagnosis.

Table 4
Sensitivity and specificity of sequence modified peptide assays.

Peptide name	Sensitivity (%)	Specificity (%)
CCP10P	83	77
CCP11A	87	100
CCP12P	70	100
CCPPAP	77	100

3.3. Evaluation of CCP11A as a diagnostic marker for RA

As noted above, we selected CCP11A as a possible specific peptide marker for RA diagnosis. The ELISA results were further analyzed by MedCalc, and the obtained receiver operating characteristic ROC curves of CCP, CCP10P, and CCP11A are shown in Fig. 4. If the HI were regarded as negative controls, then the obtained areas under the curve (AUCs) from the ROC curves were 0.97, 0.84, and 0.98 for the CCP, CCP10P, and CCP11A assays, respectively. The CCP11A assay showed a higher AUC than did the CCP assay, indicating that CCP11A has better diagnostic performance compared

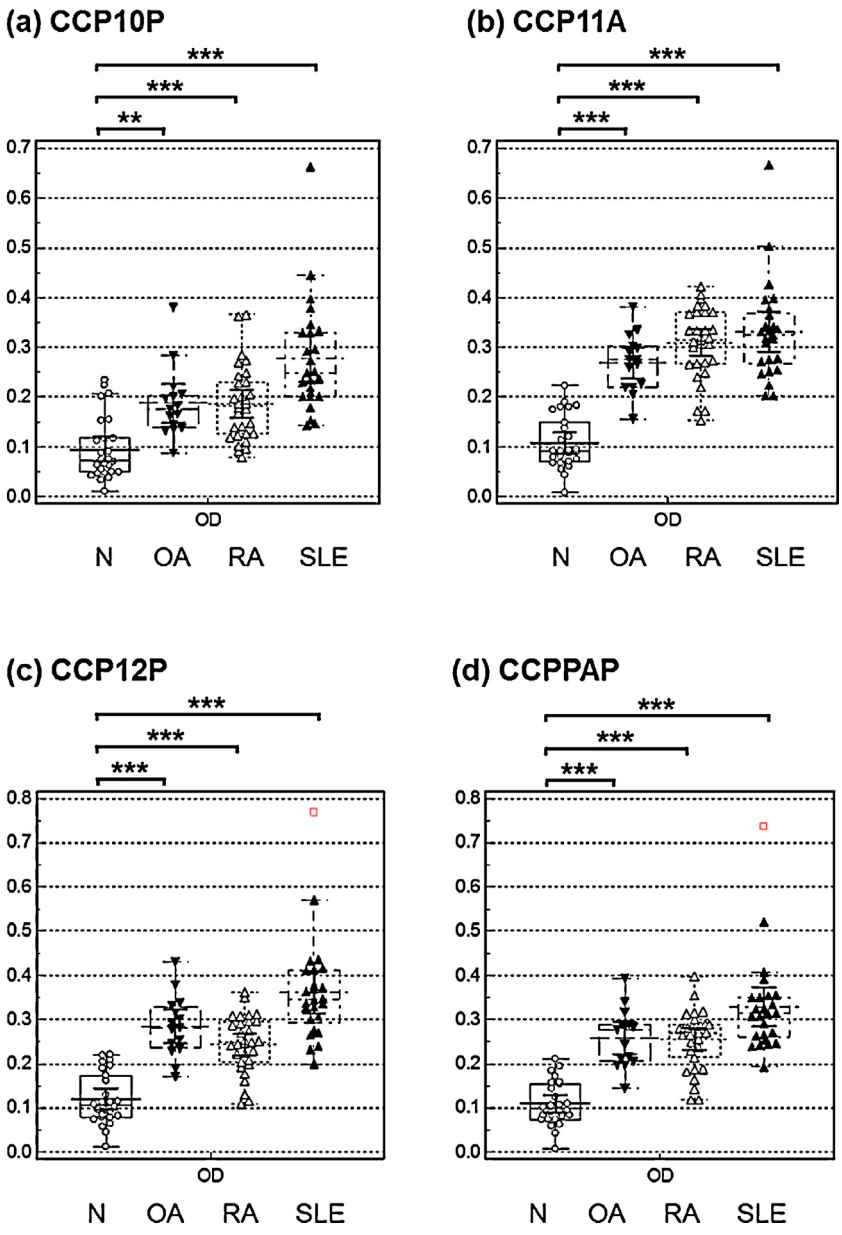

Fig. 3. ELISA assay of sequence-modified peptides from CCP (*: $p < 0.005$, **: $p < 0.001$, ***: $p = 0.0001$). RA, OA, SLE, and healthy individuals (N) samples were tested using (a) CCP10P, (b) CCP11A, (c) CCP12P, and (d) CCPPAP peptides.

to CCP for differentiating RA from HI. For higher diagnostic performance in RA, the differentiation of RA from other forms of arthritis is required. For this analysis, OA cohort was regarded as the negative controls, and ROC curves were obtained. From the ROC curves, the obtained AUCs were 0.61, 0.50, and 0.69 for CCP, CCP10P, and CCP11A, respectively. CCP11A also showed the highest AUC among them. Therefore, CCP11A had a higher diagnostic performance compared to CCP and CCP10P, and we evaluated CCP11A as a specific marker for differentiating RA from HI or OA cohort.

From the ROC curves, the calculated sensitivities and specificities of diagnosing RA from OA are described in Fig. 5. The sensitivities of CCP, CCP10P, and CCP11A were 70%, 30%, and 63%, respectively, and the specificities were 60%, 93%, and 80%, respectively. The specificity of CCP10P was the highest among those three peptides. However the sensitivity was very poor (<30%), indicating that is not suitable as an RA diagnostic marker. The CCP11A assay showed a 20.0% higher specificity and a 6.7% lower sensitivity than the CCP assay, and it was further analyzed by the McNemar test. When the OA cohort was regarded as negative controls, the CCP and CCP10P assays showed a 38% difference ($p = 0.004$), and the CCP10A and CCP11A assays showed a 27% difference ($p = 0.04$). These data indicate that the CCP10P assay was significantly different from both the CCP and CCP11A assays, with poor diagnostic performance based on its low sensitivity (30%). Therefore, the hydrophilic 10th amino acid sequence of CCP plays an important role, and substitution of this sequence to a hydrophobic proline decreases its performance as a diagnostic marker for RA. It further suggests that this 10th amino acid sequence should not be substituted so that it will remain hydrophilic for an RA diagnostic marker. The difference between the CCP and CCP11A was 11% ($p = 0.18$), indicating that the positive and negative prediction rates for the CCP and CCP11A assays are statistically similar. However, CCP11A showed an 11% or 20% higher specificity than did the CCP assay when compared with HI or OA cohort, respectively. Therefore, CCP11A could be utilized as an improved specific marker for differentiating RA from HI or OA cohort without a significant difference from the CCP

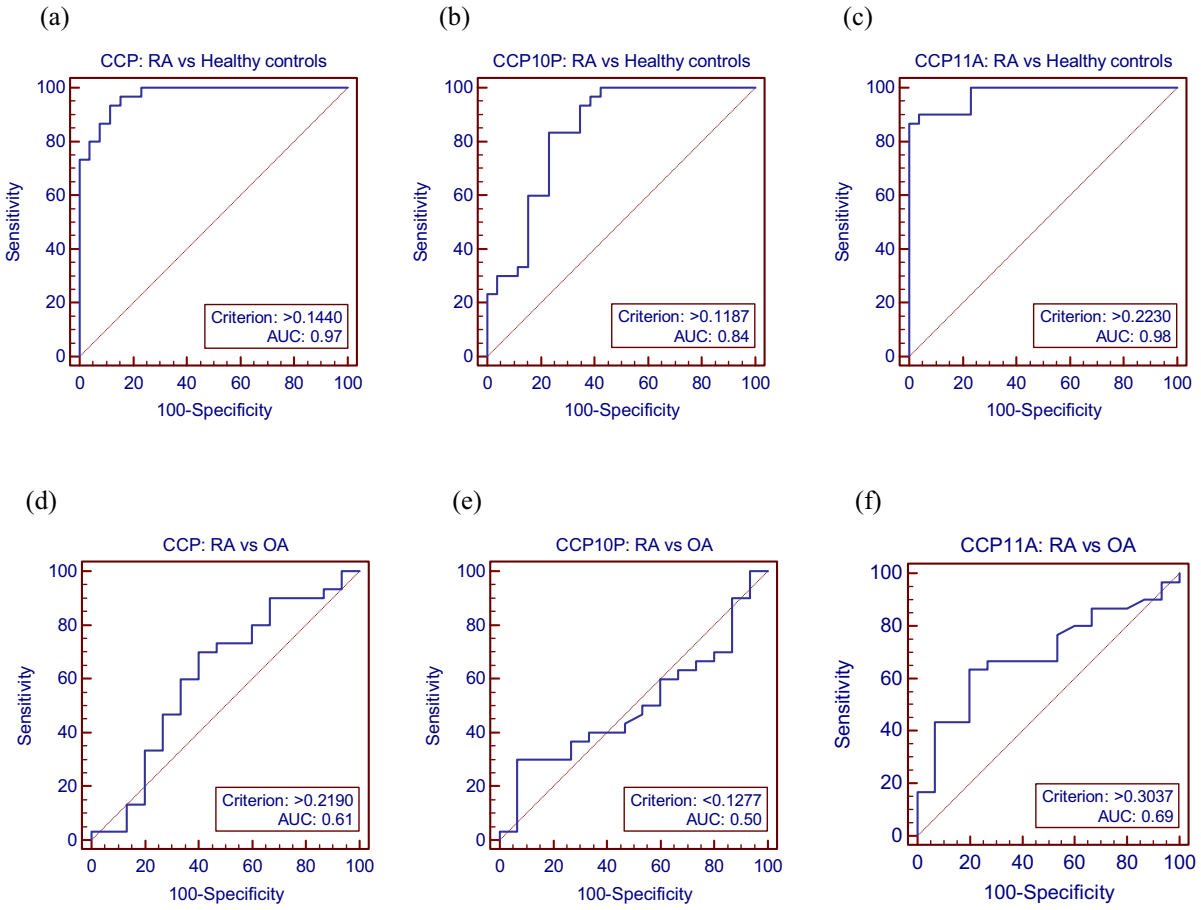

Fig. 4. Receiver operating curves of CCP, CCP10P, and CCP11A test results. RA cohort was compared with healthy individuals by (a) CCP, (b) CCP10P, and (c) CCP11A tests and with OA cohort by (d) CCP, (e) CCP10P, and (f) CCP11A tests.

result. When the SLE cohort was regarded as negative control, both CCP and CCP11A assays showed poor specificity less than 40% (data not shown). This poor specificity means that CCP-based assay has limitation to diagnosis among the autoimmune disease. The positive percentage of RA patients who were predicted as negative by CCP has been reported to be from 16% to 50% using BRAF and CarP [25,26,28,33]. To improve the specificity from other autoimmune diseases, combination of CCP11A with those biomarkers should be useful for RA diagnosis.

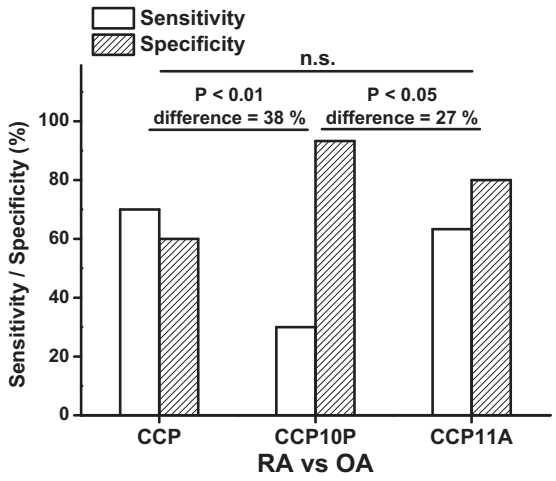

Fig. 5. Sensitivity and specificity of the CCP, CCP10P, and CCP11A tests. RA cohort was compared with OA cohort. n.s. = not significant.

4. Conclusion

The binding site of the autoimmune antibody in RA patient serum was evaluated using an ϵ-aminocaproic acid-modification of CCP. From our results, the C-terminal sequences of CCP were confirmed to have more specific affinity with autoimmune antibodies, and the cyclic structure of CCP was shown to be essential for RA diagnosis. When the sequence of CCP was substituted with a hydrophobic amino acid, the substitution of the 10th amino acid decreased RA diagnostic performance and substitution of the 11th amino acid improved specificity. Thus, CCP11A was evaluated as a specific marker for diagnosing RA with higher diagnostic performance than CCP. Despite its improved specificity, the CCP-based marker is limited in its specificity for differentiating RA from other autoimmune diseases. Until now, the immunogenicity of the anti-CCP antibody has not been reported. The discovery of its exact immunogenicity would greatly improve the evaluation of more specific biomarkers for early diagnosis of RA.

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