Serum peptides as candidate biomarkers for relapsing polychondritis

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Abstract

Background: For relapsing polychondritis (RP), no useful biomarkers have yet been identified. We analyzed serum peptide profiles to identify candidate biomarkers.

Methods: Patients with RP or rheumatoid arthritis (RA) and healthy control (HC) subjects were divided into training set (RP, n=19; RA, n=21; HC, n=17) and testing set (RP, n=18; RA, n=21; HC, n=18). Seven patients demonstrating granulomatosis with polyangiitis (GPA) were used for validation. The ion intensity of serum peptides was comprehensively measured by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry.

Results: One hundred sixty serum peptides were detected. In the RP group of the training set, 24, 8, and 7 peptides showed a ≥1.2-fold difference in ion intensity in comparison to the HC, RA, and HC+RA (non-RP) groups, respectively (p<0.05). Based on a supervised multivariate analysis of the ion intensity of 160 peptides, we generated 3 models that completely discriminated the RP group from the HC, RA, and non-RP groups (RP/HC-160P model, RP/RA-160P model, and RP/nonRP-160P model; AUROC, 1.000). By selecting 11, 9, and 14 peptides, the RP group was also completely discriminated from the 3 groups (RP/HC-11P model, RP/RA-9P model, and RP/nonRP-14P model; AUROC, 1.000). We attempted to identify the peptides with a ≥1.2-fold difference in ion intensity between the RP group and one of the 3 groups and the peptides comprising the RP/HC-11P, RP/RA-9P or RP/nonRP-14P models. Nineteen peptides were identified. Most were fragments of proteins associated with coagulation. To obtain biomarker models for RP which consists of a few peptides, we further generated 330 models, using all combinations of 3 or 4 peptides out of the 10 identified peptides of the RP/nonRP-14P model. Among them, 69 models provided ≥65.0% sensitivity and specificity in the training set (AUROC, 0.789-0.823). In the testing set and the testing set with GPA group, 4 models consisting of 4 peptides (RP/nonRP-4P-2, -10, -11, and -38 models) provided ≥70.0% sensitivity and specificity (AUROC, 0.779-0.815). Notably, the RP/nonRP-4P-2 model provided 83.3% sensitivity and 71.7% specificity in the testing set with GPA group (AUROC, 0.802).

Conclusion: Serum peptide profiles provided useful candidate biomarkers for RP and may be implicated in the pathophysiology of RP.

Trial registration: University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR), UMIN 000037212. Registered on 30 June 2019.

Background

Relapsing polychondritis (RP) is an autoimmune disease characterized by recurrent and progressive inflammation of cartilaginous tissue throughout the body [1-3]. Diagnostic criteria for RP have been proposed [4, 5] in which the diagnosis is based on the inflammatory symptoms of the following six tissue or organs of the pinna, joints, nose, eyes, respiratory tract, and inner ear, as well as histological findings. However, it is impractical to accurately diagnose RP in the early phase of the disease, before the typical symptoms and findings have developed. The differential diagnosis of RP from
rheumatoid arthritis (RA) and granulomatosis with polyangiitis (GPA) is also difficult especially in the cases with polyarthritis or saddle noses. A considerable percentage of RP patients are C-ANCA positive or PR3-ANCA positive [6-8], which makes the differential diagnosis more difficult.

At present, no specific diagnostic biomarkers for RP have been established. Thus far, autoantibodies to type II collagen [9] and matrilin 1 [10] have been detected as candidate biomarkers. Serum proteins of cartilage oligomeric matrix protein [11], macrophage migration inhibitory factor [12], and soluble triggering receptor expressed on myeloid cells-1 [13], and urinary type II collagen neoepitope [14] were reported to be putative markers for RP activity. However, the clinical use of these proteins as biomarkers for RP has not been achieved because they are frequently detected in other diseases (e.g., RA, GPA, and systemic lupus erythematosus), or because of the need for validation studies.

In this study, we comprehensively analyzed serum peptides to detect a novel and disease-specific biomarker for RP. Although 99.0% of serum proteome consists of 20 major proteins including albumin and immunoglobulin, the remaining 1% consists of a diverse spectrum of known and unknown proteins and peptides at low concentrations which would be an excellent source of biomarkers [15]. Thus far, we have detected serum peptide(s) that could work as disease-specific biomarkers for microscopic polyangiitis, dementia with Lewy bodies, and other conditions [16-19]. Herein, we similarly found novel candidate biomarkers for RP, each consisting of 4 serum peptides. Our analysis will provide a useful blood biomarker for the diagnosis of RP.

**Methods**

**Patients**

Eighty-seven patients with RP, RA, or GPA and 37 healthy control (HC) subjects were enrolled in this study (Supplementary Table 1). The diagnoses of RP [4, 5], RA [20, 21], and GPA [22, 23] were made according to the respective criteria. Peripheral blood was obtained from the above patients and HC subjects.

**The analysis of serum peptide profiles**

Serum peptides were purified by weak cation exchange (MB-WCX, Bruker Daltonics, Ettlingen, Germany). The ion intensity of peptides was measured using a matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer (MALDI-TOF/TOF MS, UltraflxXtreme, Bruker Daltonics). The ion intensity was compared among the RP, HC, and RA groups by ClinProTools (Bruker Daltonics). Peptides were designated as p + peptide mass/charge ratio (i.e., p1206 indicates a peptide with 1206 m/z). Amino acid sequences of peptides were analyzed using a nano-HPLC system (Eksigent nano-LC 400 system, AB sciex, Framingham, MA, USA) connected to a Triple TOF5600 system (AB sciex).

**Statistical analysis**

The significance of differences in peptide ion intensity between the RP group and the HC, RA, or HC+RA group was calculated using Student's t-test. Multivariate analyses of a principal component analysis
(PCA) and an orthogonal partial least square-discriminant analysis (OPLS-DA) were performed using the SIMCA-P⁺ software program (version 14; Umetrics, Umeå, Sweden). A receiver operating characteristic (ROC) analysis was conducted using the JMP software program (ver. 9.0.2, SAS Institute, Cary, NC, USA). The significance of the prediction values of the biomarker models for RP was calculated using a Wilcoxon signed-rank test.

**Results**

**Cohort setting for generation and validation of candidate biomarkers**

In this study, we aimed to discriminate RP from other inflammatory diseases and healthy condition using serum peptide profiles. As for other inflammatory diseases, RA was used when a biomarker model for RP was generated, and RA and GPA were used when the model was validated. The 117 cases and controls (RP, n=37; HC, n=37; RA, n=43) were divided into the 1st cohort (RP, n=19; HC, n=19; RA, n=22; total, n=60) and the 2nd cohort (RP, n=18; HC, n=18; RA, n=21; total, n=57) (Supplementary Table 1). The 1st cohort was used as a parent cohort from which a training set, used to generate the model, was derived. The 2nd cohort was used as a testing set for validation of the model together with the 7 GPA patients.

As a result, a total of 160 peptides were detected from the 60 serum samples of the 1st cohort (Supplementary Fig. 1). To exclude non-typical RP cases from the training set, serum peptide profiles of individual RP patients, HC subjects, and RA patients in the 1st cohort were analyzed by a PCA and compared in each group. All RP patients showed relatively similar serum peptide profiles (Fig. 1A). In contrast, 2 HC subjects (HC12, HC13) and 1 RA patient (RA22) were identified as outliers from the results of PCA (Fig. 1B, C). Excluding these 3 cases from the 1st cohort, 57 patients and subjects were used as the training set (Table 1, Fig. 2).

**Comparison of serum peptide profiles between the RP group and the other groups**

For the generation of a biomarker model for RP, we attempted to discriminate the RP group from the other groups based on the ion intensity of serum peptides. To evaluate this possibility, we examined the number of peptides that showed a difference in ion intensity between the RP group and other groups. As a result, 29 peptides showed a significant difference in ion intensity between the RP and HC groups (p<0.05) (Table 2). Twenty-seven peptides showed a ≥1.2-fold difference in ion intensity in the RP group in comparison to the HC group (≥1.2-fold, 13; £-1.2-fold, 14). Among them, 6 peptides showed a ≥1.5-fold difference in ion intensity (≥1.5-fold, 2; £-1.5-fold, 4) in the RP group, and 1 peptide (p1466) showed approximately 2.0-fold higher ion intensity in comparison to the HC group (Table 2, 3). Similarly, 11 peptides showed significant difference in ion intensity between the RP and RA groups (p<0.05) (Table 2). Nine peptides in the RP group showed a ≥1.2-fold difference in ion intensity in the RP group in comparison to the RA group (≥1.2-fold, 4; £-1.2-fold, 5). We further compared the peptide ion intensity between the RP group and the HC+RA group (non-RP group). Thirteen peptides showed a significant difference in ion intensity (p<0.05). Among these 9 peptides showed a ≥1.2-fold difference in ion intensity.
(≥1.2-fold, 2; £-1.2-fold, 7). Taken together, the serum peptide profile of RP was differed from the serum peptide profiles of HC and RA. Based on these results, we subsequently generated biomarker models for RP based on the peptide ion intensity.

**Discrimination of the RP group using the ion intensity of single peptides**

First, we attempted to discriminate the RP group from the other groups using the ion intensity of single peptides. Using one of the above 29, 11 and 13 peptides, we attempted to discriminate the RP group from the HC, RA, and non-RP groups, respectively. As a result, no peptide completely discriminated the RP group from the other groups. However, 19, 7, and 9 peptides discriminated the RP group from the HC, RA, and non-RP groups with ≥65.0% sensitivity and specificity, respectively (Supplementary Fig. 2A).

We next validated the discriminative ability of these peptides using the testing set. Nine of the 19 peptides similarly discriminated between the RP and HC groups with ≥65.0% sensitivity and specificity. On the other hand, no peptide discriminated between the RP and RA groups and between the RP and non-RP groups with ≥65.0% sensitivity and specificity in the testing set (Supplementary Fig. 2B). It was difficult to discriminate the RP group from the non-RP group using ion intensity of single peptides.

**Generation of models to discriminate between the RP and HC groups by a multivariate analysis of peptide ion intensity**

Since the univariate analysis of peptide ion intensity could not discriminate between the RP group and the other groups, we subsequently attempted to discriminate between the RP group and the other groups using a multivariate analysis. First, an unsupervised multivariate analysis of PCA was applied for discrimination. The ion intensity of all 160 peptides were used for discrimination between the RP and HC groups, the RP and RA groups, and the RP and non-RP groups in the training set. However, the PCA did not discriminate the RP group from any of the other 3 groups (Fig. 1D).

Next, we performed an OPLS-DA as a supervised multivariate analysis to analyze the discriminative ability for RP. Specifically, the ion intensity of all the 160 peptides were used for discrimination between the RP and HC groups (RP/HC-160P model, Fig. 3A-D). As a result, the RP/HC-160P model completely discriminated between the 2 groups (Fig. 3A). The R²Y value of this model (0.829) indicated its high discriminative ability in the present cohort, whereas the Q² value of 0.173 indicated a relatively low predictive ability. To improve the predictive ability, we attempted to generate a new discriminant model by selecting the minimum number of peptides for complete discrimination. For this purpose, S-plot parameters (the x- and y-axes showed the magnitude and reliability of the peptides in the model, respectively) (Fig. 3C) and the variable importance in projection (VIP, x-axis indicated the contribution of each peptide to the generation of the model [average value is 1.0]) (Fig. 3D) were used for selection. As a result, we found that only 11 peptides were necessary for complete discrimination between the RP and HC groups (RP/HC-11P model, Fig. 3E-H). This RP/HC-11P model showed a higher Q² value of 0.431; thus, the predictive ability was improved by selection of the peptides (Fig. 3E).
Generation of models to discriminate between the RP and RA groups by a multivariate analysis of peptide ion intensity

To discriminate the RP group from the RA group in the training set, the ion intensity of all 160 peptides of the patients was subjected to an OPLS-DA (RP/RA-160P model, Fig. 4A-D). As a result, the RP/RA-160P model completely discriminated between the 2 groups (Fig. 4A). This model provided a high $R^2_Y$ value of 0.843, but a low $Q^2$ value of 0.191, indicating low predictive ability. Selecting peptides with high magnitude, reliability (Fig. 4C), and VIP (Fig. 4D) in the RP/RA-160P model, we generated a new model in which the minimum number of peptides for complete discrimination was 9 (RP/RA-9P model, Fig. 4E-H). The $Q^2$ value of this model improved (0.471) with higher predictive ability again achieved by peptide selection (Fig. 4E).

Generation of models to discriminate between the RP and non-RP groups by a multivariate analysis of peptide ion intensity

Finally, we attempted to discriminate between the RP and non-RP groups in the training set by an OPLS-DA of the ion intensity of all 160 peptides (RP/nonRP-160P model, Fig. 5A-D). This RP/nonRP-160P model also completely discriminated the 2 groups and provided a high $R^2_Y$ of 0.781, but a low $Q^2$ of 0.151 (Fig. 5A). Therefore, we again selected peptides with high magnitude, reliability (Fig. 5C), and VIP (Fig. 5D) in the RP/nonRP-160P model to generate a better model with the minimum number of peptides. As a result, a combination of 14 peptides completely discriminated the 2 groups (RP/nonRP-14P model, Fig. 5E-H). The RP/nonRP-14P model provided a relatively high $R^2_Y$ value (0.639); however, the $Q^2$ value was still low (0.297), suggesting its low predictive ability (Fig. 5E).

Generation of models to discriminate between the RP and non-RP groups by a multivariate analysis of the ion intensity of a small number of peptides

To improve the predictive ability of the models to discriminate between the RP and non-RP groups, we selected peptides from the 14 peptides of the RP/nonRP-14P model. To achieve this, we subjected the 14 peptides to an MS/MS analysis, and identified 10 out of the 14 peptides (Table 3). We generated a discriminant model for RP by an OPLS-DA using the ion intensity of all 10 peptides (RP/nonRP-10P model). The RP/nonRP-10P model did not completely discriminate between the RP and non-RP groups; however, it provided the highest sensitivity of 100%, a high enough specificity of 65.8%, and a high AUROC of 0.892 in the training set (Table 4). Therefore, we further selected peptides from the 10 peptides, and attempted to generate models to discriminate between the RP and non-RP groups by an OPLS-DA which provided ≥65.0% sensitivity and specificity.

First, 10 models to discriminate between the RP and non-RP groups consisting of 9 peptides were generated in the training set by excluding one peptide from the 10 peptides. Among them, the model with the highest "sensitivity + specificity" value was selected and designated as the RP/nonRP-9P model. Similarly, the RP/nonRP-8P to 1P models were sequentially generated by excluding one peptide from the
former model. As a result of this generation, all the RP/nonRP-9P to -3P models provided ≥84.0% sensitivity, ≥81.0% specificity, and an AUROC of ≥0.895 in the training set. However, the RP/nonRP-2P model provided clearly lower discriminative ability with 79.0% sensitivity, 68.3% specificity, and AUROC of 0.769 (Table 4). From these results, we considered that a small number of 3 or 4 peptides out of the 10 peptides may provide high discriminative ability for RP.

Thus, we next generated models to discriminate between the RP and non-RP groups by using all combinations of 3 or 4 peptides out of the 10 peptides in the training set. Total of 330 models (RP/nonRP-4P-1 to -210 models and RP/nonRP-3P-1 to -120 models) were generated (Supplementary Table 2). We set the criteria for the selection of clinically useful models, that is, ≥65.0% sensitivity and specificity and an AUROC of ≥0.770 (the latter was better than the AUROC of the RP/nonRP-2P model [0.769]). We found that 57 models consisting of 4 peptides and 12 models consisting of 3 peptides met the criteria (Supplementary Table 3) and subjected the 69 models to a subsequent validation analysis.

**Validation of the models to discriminate between the RP and non-RP groups**

Finally, we validated the RP/nonRP-160P, -14P, -10P to -1P models, the 57 models derived from the RP/nonRP-4P model, and the 12 models derived from the RP/nonRP-3P model using the testing set and a cohort consisting of the testing set + the 7 GPA patients (the testing set + GPA group) (Table 4, Supplemental Table 3). As a result, 4 models generated with 4 peptides discriminated the RP and non-RP groups in the testing set and in the testing set + GPA group, providing ≥70.0% sensitivity and specificity and an AUROC of ≥0.770 (Table 4). These were the RP/nonRP-4P-2, -10, -11, and -38 models. No other model provided such high values in the validation analysis.

Among the above 4 models, the RP/nonRP-4P-2 model provided the highest sensitivity of 83.3%, with the same specificity of 71.7% as the other 3 models, and a high AUROC of 0.802 in the testing set + GPA group. Interestingly, all peptides constituting the RP/nonRP-2, -10, -11, and -38 models were the fragments of fibrinogen alpha chain (FIBA) (Table 3). The 4 models were made of different combinations of 4 of 6 peptides of p1466, p1617, p3264, p4091, p5335, and p5902.

In addition, the validation of the RP/HC-11P model (Fig.3EFGH) using the testing set showed relatively high sensitivity (66.7%) and specificity (72.2%; AUROC, 0.762), which suggested its usefulness in the discrimination of RP patients from healthy subjects (Supplementary Table 4). Validation of the RP/RA-9P model (Fig. 4E-H) revealed high sensitivity (88.9%) but low specificity (23.8%; AUROC, 0.585), indicating that it would be difficult to use this model for discrimination between RP and RA patients (Supplementary Table 4).

**Identification of the peptides**

To examine the difference in the pathophysiology of RP from that of RA and from the physiology of healthy condition, we attempted to identify the peptides that composed the RP/HC-11P model and the RP/RA-9P model in addition to the RP/nonRP-14P model. Peptides for which there was a ≥1.2-fold
difference in ion intensity between the RP group and the other groups were also subjected to identification (Table 2).

As a result, 19 peptides were identified (Table 3). Interestingly, 15 out of the 19 peptides were fragments FIBA, all of which were derived from fibrinopeptide A (FPA) or the C-terminal subdomain of the aC-domain (aCDC, Supplementary Fig. 3). As for the other 4 peptides, fragments of prothrombin (p4210) and apolipoprotein C-1 (p6628) were identified in the peptides that were included in the biomarker models for RP. A fragment of a2-antiplasmin (p3443) was identified in the peptides ion intensity of which were 1.2-fold or more different in the RP group. A fragment of a protein highly similar to coagulation factor XIII (p3952) was identified in both categories. Taken together, almost all of the identified peptides were derived from proteins related to coagulation and fibrinolysis.

Discussion

In this study, we attempted to generate serum peptide models that are useful for the diagnosis of RP. A total of 160 serum peptides were detected from the RP, HC, and RA groups in the training set (Supplementary Fig. 1, Fig. 2) which was a sufficient number for model generation. The comparison of peptide profiles between the RP and HC groups, the RP and RA groups, and the RP and HC + RA (non-RP) groups revealed significant differences (p < 0.05, Table 2), which suggested the high possibility of generating a discriminant model for RP. Interestingly, in the comparison of RP and HC the number of peptides that showed difference in ion intensity was greater than that observed in the comparison of RP and RA (Table 2). This suggested that the difference in peptide profiles between the inflammatory and healthy conditions was greater in comparison to that between the two inflammatory diseases.

A univariate analysis using the ion intensity of a single peptide and an unsupervised multivariate PCA using the ion intensity of all the 160 peptides did not completely discriminate the RP group from the HC, RA, and non-RP groups (Supplementary Fig. 2, Fig. 1D). In contrast, an OPLS-DA, a supervised multivariate analysis, using the ion intensity of 160 peptides completely discriminated the RP group from the HC group, the RA group, and the non-RP group (Fig. 3–5). Furthermore, the OPLS-DA completely discriminated the RP group from the 3 above-mentioned groups using the ion intensity of 11, 9, and 14 peptides, respectively (Fig. 3–5). The OPLS-DA was useful for generation of the biomarker models for RP. Focusing on the discrimination of RP and non-RP, however, the RP/non-RP-160P model and the RP/nonRP-14P model showed low sensitivity and/or specificity (30–60%) in the testing set and the testing set + GPA group (Table 4). It was considered that those models were not useful for the discrimination of RP.

To establish a clinical laboratory examination using serum peptides, the number of peptides should be reduced to one or at most a few, and the amino acid sequences of the peptides should be identified. Accordingly, we next selected 10 identified peptides from the 14 peptides of the RP/nonRP-14P model and attempted to generate models with high discriminative ability for RP by further selecting peptides from the 10 peptides (Supplementary Table 2). The models generated with 2 or 3 peptides selected from
the 10 identified peptides did not provide $\geq 65.0\%$ sensitivity and specificity in the testing set (Table 4, Supplementary Table 2, 3). However, we succeeded in creating 4 biomarker models for RP with $\geq 70.0\%$ sensitivity and specificity and an AUROC of $\geq 0.770$ in the testing set, using 4 differently selected peptides (RP/nonRP-4P-2, -10, -11, -38 models) (Supplementary Table 2, 3, Table 4). At least 4 peptides may be required to generate a principal component that sufficiently discriminates RP from non-RP in the OPLS-DA.

These 4 models also provided $\geq 70.0\%$ sensitivity and specificity of 70.0% in the testing set + GPA group, suggesting their usefulness for the discrimination for RP not only from RA but also from GPA (Table 4, Supplementary Table 3). In particular, the RP/nonRP-4P-2 model provided 83.3% sensitivity, 71.7% specificity, and an AUROC of 0.802 in the testing set + GPA group, suggesting its high usefulness for clinical application. Since no diagnostic biomarkers for RP are currently available, these 4 RP/nonRP-4P models may become the first clinically useful RP biomarkers. We can simultaneously measure the serum concentration of 4 peptides by establishing an ELISA for each peptide. Each of the RP/nonRP-4P models showed its own potential usefulness. For examples, the RP/nonRP-4P-2 model showed a high discriminative ability for the RP group (83.3%) and the RA group (61.9%), and the RP/nonRP-4P-11 model showed a high discriminative ability for the GPA group (71.4%) and the HC group (94.4%) (Supplementary Fig. 4). Specifically, the high discriminative ability for HC subjects (94.4%) would be a great advantage of the model because healthy individuals constitute the majority of an actual cohort. Since the 4 RP/nonRP-4P models consist of only 6 peptides (p1466, p1617, p3264, p4091, p5335, and p5902), quantification of the 6 peptides and the use of all of the 4 RP/nonRP-4P models may increase the accuracy of the discrimination of RP.

Interestingly, 15 of the 19 identified peptides that included the 6 peptides of the 4 RP/nonRP-4P models were fragments of FIBA (Table 3). All of the 15 FIBA peptides were derived from either of FPA or $\alpha$CDC (Supplementary Fig. 3), which is similar to the results of our previous studies [17–19, 24]. FPA, located at the N-terminus of FIBA, is cleaved by thrombin for release prior to fibrin formation [25]. In this study, since the ion intensity of the full length FPA (p1519, Ser-dehydrated; p1617, Ser-phosphorylated) tended to be increased in the RP group in comparison to the HC group (Supplementary Fig. 3), the production of FIBA and/or release of FPA may be increased in RP. The ion intensity of FPA-derived fragments of p1206 and p1466 was also increased in the RP group in comparison to the HC group ($p < 0.05$) (Supplementary Fig. 3, Table 3), which may suggest the increased catalysis of FPA in RP. In contrast, the ion intensity of the full length $\alpha$CDC (p5902) and the relatively long fragments of $\alpha$CDC (p5335, p3264, and p3193) were decreased in the RP group in comparison to the HC group ($p < 0.05$) (Supplementary Fig. 3, Table 3). The $\alpha$C-domain is known to augment the lateral aggregation of protofibrils for the formation of thick fibrin fibers [25]. Therefore, the decrease of $\alpha$CDC and its major fragments may reflect the decreased production of FIBA, the high usage of FIBA for fibrin formation, or the increased catalysis of FIBA or $\alpha$CDC in RP. At least, the trend toward an increase in relatively short fragments of $\alpha$CDC-derived p2555, p2661, and p2770 may indicate the increased catalysis of $\alpha$CDC (Supplementary Fig. 3, Table 3). The $\alpha$CDC-derived p3264 and p4091 were used in all the 4 RP/nonRP-4P models, while the FPA-derived p1466 were used in 3 out of the 4 RP/nonRP-4P models, which may suggest that the advantage of RP/nonRP-4P models was
partially due to the above putative disease characteristics of RP. Since not only FIBA-derived but also prothrombin- and α2-antiplasmin-derived peptides were also identified (Table 3), it was suggested that dysregulation of blood coagulation and the fibrinolytic system may be involved in the pathophysiology of RP. In addition, thrombin, plasmin, and neutrophil elastase have been reported to cleave FPA and αCDC at the indicated sites in Supplementary Fig. 3 [25, 26]. The change in the activity of these enzymes may be involved in a complicated manner in the formation of serum peptide profiles of RP.

One of the limitations of this study is the limited control disease types and the small overall sample size. To demonstrate the usefulness of the RP/nonRP-4P-2, -10, -11, -38 models, it is necessary to validate the discriminant models for RP using a large number of cases and controls that includes diseases other than RA and GPA. Another is that the comprehensive analysis system of ClinProTools used in this study is a method used to screen for useful peptides, and is not fully optimized for reproducible quantification. Thus, an ELISA for the respective peptides and/or a method for mass spectrometry quantification with internal standard control peptides should be established for the accurate quantification of each peptide in the near future.

**Conclusion**

As diagnostic candidate biomarkers for RP, we generated the RP/nonRP-4P-2, -10, -11, -38 models, each of which consisted of 4 serum peptides. Notably, the RP/nonRP-4P-2 model consisting of p1466, p1617, p3264, and p4091 provided 83.3% sensitivity and 71.7% specificity in the validation using the testing set + GPA group. Most of the identified peptides that were important for the discrimination of RP were fragments of proteins associated with coagulation, with the majority derived from FIBA. Dysregulation of the coagulation system may be involved in the pathophysiology of RP. Further validation of the 4 RP/nonRP-4P models by quantification of the peptides using ELISA or mass spectrometry may lead to the establishment of the first useful biomarker for RP.

**Abbreviations**

αCDC: C-terminal subdomain of the αC-domain  
ANCA: anti-neutrophil cytoplasmic antibody  
AUROC: area under the receiver operating characteristic curve  
ELISA: enzyme-linked immunosorbent assay  
FIBA: fibrinogen α  
FPA: fibrinopeptide A  
GPA: granulomatosis with polyangiitis
HC: healthy control subjects

OPLS-DA: orthogonal partial least square-discriminant analysis

PCA: principal component analysis

RA: rheumatoid arthritis

RP: relapsing polychondritis

VIP: variable importance of projection

**Declarations**

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

_Ethics approval and consent to participate_

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki revised in 2013 and was approved by the ethics committee of St. Marianna University School of Medicine. All of the participants gave their informed consents.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

TS designed study protocol supervised by MSK. MF, YN, YT, SO, KK, and YY collected blood samples and collected patients’ information. TS, MS, and KN collected experimental raw data. TS, MS, TU, KO, AT, MA, and NS analyzed the collected data under supervised by MSK. TS wrote the manuscript. TK and MSK supervised the manuscript. All the authors read and approved the final manuscript.

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References


Tables

Tables 1 to 4 are available in the Supplementary Files section

Figures
Figure 1

Score scatter plots of the PCA using the serum peptide ion intensity.

Results of the RP group (A), the HC group (B), the RA group (C), and all 3 groups (D) in the training set. The two individuals in the HC group and 1 patient in the RA group who were identified as outliers are specifically indicated by their ID numbers.
Figure 2

Serum peptide profiles of the RP, HC, and RA groups in the training set.

One hundred sixty serum peptide ion peaks were detected from 19 RP patients, 17 HC subjects, and 21 RA patients in the training set. The average ion intensity of the peptides in the RP, HC, and RA group is shown.
Models to discriminate between the RP and HC groups.

Two models were generated using the peptide ion intensity of the 19 RP patients and the 17 HC subjects in the training set. First, the ion intensity values of the 160 peptides were subjected to an OPLS-DA (RP/HC-160P model, ABCD). A. The score scatter plot of the RP/HC-160P model. The x axis indicates the
first principal component for the discrimination. B. The loading scatter plot of the RP/HC-160P model. Parameters localized further away from the center of the x-axis contribute more to the discrimination. C. The S-plot of the RP/HC-160P model. The magnitude (x axis) and reliability (y axis) of the peptides are visualized. D. The VIP of the RP/HC-160P model. Twenty-eight of the 160 peptides relatively highly contributed to the generation of this model (VIP >1.0). The second model between the RP and HC groups was generated using a minimum of 11 peptides (RP/HC-11P model, EFGH). The score scatter plot (E), loading scatter plot (F), S-plot (G), and VIP (H) of the RP/HC-11P model are shown. The numbers in brackets in H indicate the order of VIP scores of the peptides in the RP/HC-160P model (D).
Figure 4

Models to discriminate between the RP and RA groups.

Two models were generated using the peptide ion intensity of the 19 RP patients and the 21 RA patients in the training set. First, ion intensity values of the 160 peptides were subjected to an OPLS-DA (RP/RA-160P model, A-D). A. Score scatter plot of the RP/RA-160P model. X axis indicates the first principal
component for the discrimination. B. Loading scatter plot of the RP/RA-160P model. Parameters localized further away from the center of the x-axis contribute more to the discrimination. C. The S-plot of the RP/RA-160P model. The magnitude (x axis) and reliability (y axis) of the peptides are visualized. D. The VIP of the RP/RA-160P model. Thirty-five of the 160 peptides relatively highly contributed to the generation of this model (VIP >1.0). The second model between the RP and RA groups was generated using a minimum of 9 peptides (RP/RA-9P model, EFGH). The score scatter plot (E), loading scatter plot (F), S-plot (G), and VIP (H) of the RP/RA-9P model are shown. The numbers in brackets in H indicate the order of the VIP scores of the peptides in the RP/RA-160P model (D).
Figure 5

Models to discriminate between the RP and non-RP groups.

Two models were generated using the peptide ion intensity of the 19 RP patients and the non-RP subjects (HC subjects, n=17; and RA patients, n=21) in the training set. First, the ion intensity values of the 160 peptides were subjected to an OPLS-DA (RP/nonRP-160P model, A-D). A. The score scatter plot of the
The x axis indicates the first principal component for the discrimination. B. The loading scatter plot of the RP/nonRP-160P model. Parameters localized further away from the center of the x-axis contribute more to the discrimination. C. The S-plot of the RP/nonRP-160P model. The magnitude (x axis) and reliability (y axis) of the peptides are visualized. D. VIP of the RP nonRP-160P model. Thirty-six of the 160 peptides relatively highly contributed to the generation of this model (VIP >1.0). The second model between the RP and nonRP groups was generated using a minimum of 14 peptides (RP/nonRP-14P model, EFGH). The score scatter plot (E), loading scatter plot (F), S-plot (G), and VIP (H) of the RP/nonRP-14P model are shown. The numbers in brackets in H indicate the order of the VIP scores of the peptides in the RP/nonRP-160P model (D).

**Supplementary Files**

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- SupplementaryFigure1.tif
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- SupplementaryFigure3.tif
- SupplementaryFigure4.tif
- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
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- SupplementaryTable4.xlsx
- Table1.xlsx
- Table2.xlsx
- Table3.docx
- Table4.xlsx