Serum soluble programmed cell death protein 1 could predict the current activity and severity of antineutrophil cytoplasmic antibody-associated vasculitis: a monocentric prospective study

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ABSTRACT

Objective. We investigated whether serum soluble programmed cell death protein 1 (sPD-1) could predict the current activity and severity of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) based on Birmingham vasculitis activity score (BVAS) in patients with AAV.

Methods. Fifty-nine patients from a monocentric prospective cohort of AAV were included. On the same visit-day, blood samples were collected and isolated sera were stored. BVAS and other AAV-related parameters were assessed, and laboratory tests were performed. We defined the lower limit of the highest tertile of BVAS as the cut-off for severe AAV (BVAS ≥12). Serum sPD-1 was measured from stored serum samples.

Results. The mean age was 59.7 years (38 women). The mean BVAS was 8.9 and 18 patients had severe BVAS. Patients with severe AAV exhibited the higher mean serum sPD-1 than those without (380.7 pg/mL vs. 180.3 pg/mL). Serum sPD-1 (r=0.367), white blood cell count (r=0.288), haemoglobin (r=-0.590), serum albumin (r=0.670) erythrocyte sedimentation rate (ESR) (r=0.339) and C-reactive protein (CRP) (r=0.450) were significantly correlated with BVAS. Moreover, serum sPD-1 was meaningfully correlated with haemoglobin and serum albumin, but not ESR or CRP. In the multivariable linear regression analysis, only serum sPD-1 was significantly associated with BVAS (standardised β 0.274, p=0.024). We calculated the optimal cut-off of serum sPD-1 for severe AAV as 70.1 pg/mL. Severe AAV were more frequently identified in patients with serum sPD-1 ≥70.1 pg/mL than those without (RR 13.867).

Conclusion. Serum sPD-1 could predict the current activity and severity of AAV.

Introduction

Programmed cell death protein 1 (PD-1), which is one of the immune checkpoint proteins, regulates immune responses related to the stimulation and activation of T cells by binding to programmed cell death-ligand (PD-L) 1 (PD-L1) and PD-L2 producing inhibitory signals (1). In addition to a full-length isoform of PD-1, which has similar sequence to membranous PD-1, PD-1 has four different spliced mRNA transcripts, such as PD-1Δex2 (exon 2 deficiency), PD-1Δex3 (exon 3 deficiency), PD-1Δex2,3 (exon 2 and 3 deficiency), and PD-1Δex2,3,4 (exon 2, 3, and 4 deficiency). Among them, PD-1Δex3 is responsible for producing soluble PD-1(sPD-1) because it possesses the extracellular domain without the transmembrane domain (2). Secreted sPD-1 binds to PD-L1 or PD-L2 instead of PD-1 of T cells and could block the inhibitory effect of PD-1 on the stimulation and activation of T cells (3). PD-1 has been considered to play a critical role in viral hepatitis and autoimmunity: PD-1 expression in T cells is increased in patients with chronic viral hepatitis (A); PD-1 could alleviate autoimmunity by calming T cell activity, whereas PD-1 deficiency might result in accelerated autoimmunity (4, 5). A previous study demonstrated the correlation of serum sPD-1 and rheumatoid factor titre and 28-joint disease activity score in patients with rheumatoid arthritis. Furthermore, they suggested that pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-17, might enhance the expression of sPD-1 in CD4+ T cells (6).

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is characterised by necrotising vasculitis in small-sized vessels. AAV is categorised into 3 variants such as microscop-
ic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA) based on the 2012 Chapel Hill Consensus Conferences (CHCC) Nomenclature of Vasculitis (7). AAV can involve almost all the major organs, such as brain heart, lungs and kidneys, and furthermore, it was recently reported to influence on liver, particularly, subclinical but significant liver fibrosis (B). In the pathogenesis of AAV, pro-inflammatory cytokines prime neutrophils and drive them to release myeloperoxidase (MPO) or proteinase 3 (PR3). Secreted MPO and PR3 are subsequently presented to helper T cells by antigen presenting cells and contribute to the production of anti-MPO or anti-PR3 ANCs by autoreactive B cells. In the presence of ANCA and under the cytokine storm, activated neutrophils could initiate bulky inflammation on vessel walls, and then macrophages and T cells expand the inflammatory burden in adjacent tissues (8). Therefore, it is reasonably speculated that serum sPD-1 can predict the current activity and severity of AAV like 28-joint disease activity score (8). However, there is no report on the clinical implication of serum sPD-1 in AAV patients yet. Hence, in this study, we investigated whether serum sPD-1 could predict the current activity and severity of AAV based on Birmingham vasculitis activity score (BVAS) in patients with AAV (9).

**Patients and methods**

**Patients**

In this study, we included 59 patients with AAV from our prospective Severance Hospital ANCA associated VasculitidEs (SHAVE) cohort. All patients were first classified as AAV at the Department of Rheumatology, Yonsei University College of Medicine, Severance Hospital, from October 2000 to July 2018. They all fulfilled the 2007 European Medicines Agency algorithms and the 2012 CHCC Nomenclature of Vasculitis (1, 10). On the same visit-day, blood samples were collected and isolated sera were stored, clinical manifestations were reviewed and routine laboratory tests were performed. Particularly, on the visit-day of blood sampling, patients with serious medical conditions other than AAV were excluded in this study. This study was approved by the Institutional Review Board of Severance Hospital (4-2016-0901) and the patients’ written informed consent was obtained at the time of blood sampling.

**AAV-related parameters and definition of severe AAV**

On the visit-day of blood sampling, 3 categories of AAV-related parameters were assessed: BVAS (9) as an index for the activity and severity of AAV, vasculitis damage index (VDI) (11) as a damage index, and the Korean version of the short form-36 (12) as a functional index in AAV patients. Because BVAS for GPA has a different weight-system compared to BVAS, we evenly applied BVAS to MPA, GPA and EGPA patients to unify the scoring system. We evaluated items of both BVAS and VDI as clinical manifestations. We stratified AAV patients into three groups according to the tertile of BVAS and defined the lower limit of the highest tertile as the cut-off for the current severe AAV (BVAS ≥12). We also reviewed immuno-suppressive drugs which were administered on the same day of blood sampling.

**ANCA measurement**

Perinuclear (P)-ANCA and cytoplasmic (C)-ANCA were detected by immunofluorescent assay. MPO-ANCA and PR3-ANCA were measured by ELISA kit for anti-PR3 and anti-MPO (Inova Diagnostics, San Diego, USA) before 2013, and by the novel anchor coated highly sensitive (hs) Phadia ELiA (Thermo Fisher Scientific/Phadia, Freiburg, Germany) using human native antigens, performed on a Phadia250 analyser after 2013.

**Laboratory data**

We performed laboratory tests for variables which were previously known to be correlated with the activity of AAV. They include white blood cell and platelet counts (×10³), haemoglobin (g/dL), creatinine (mg/dL), serum albumin (g/dL), aspartate aminotransferase (AST) (IU/L), alanine aminotransferase (ALT) (IU/L), erythrocyte sedimentation rate (ESR) (mm/hr) and C-reactive protein (CRP) (mg/L).

**Serum sPD-1 measurement**

We obtained whole blood from each patient with AAV, isolated serum and stored it at -80°C on the same day of collecting clinical and laboratory data. Serum sPD-1 was measured from stored serum samples using human PD-1 DuoSet ELISA kits (R&D systems, Minneapolis, USA) according to the manufacturer’s instruction.

**Statistical analyses**

All statistical analyses were conducted using SPSS software (v. 23 for windows; IBM Corp., Armonk, NY, USA). The correlation coefficient among laboratory variables was obtained using the Pearson correlation analysis. The standardised correlation coefficient between laboratory variables and BVAS was assessed by the multivariable linear regression analysis using variables with significant differences in the univariable analysis. The optimal cut-off of serum sPD-1 for severe AAV was extrapolated by calculating the receiver operator characteristic (ROC) curve. The relative risk (RR) of the optimal cut-off of serum sPD-1 for severe AAV was analysed using contingency tables and the chi square test. p-values less than 0.05 were considered statistically significant.

**Results**

**Baseline characteristics of patients with AAV**

The baseline characteristics of 59 patients with AAV (21 men and 38 women) are shown in Table I. The mean age was 59.7 year and the mean disease duration was 22.8 months. Of 59 patients, 30 patients were classified as MPA, 18 patients as GPA and 11 patients as EGPA. The mean BVAS was 8.9 and 18 patients had severe BVAS. The mean VDI and SF-36 physical and mental component summary were 3.1, 50.4 and 57.9, respectively. Thirty-five patients had MPO-ANCA (or P-ANCA) and 7 patients had PR3-ANCA (or C-ANCA). The mean white blood cell and platelet counts were 8,327.6/ mm³ and 282,900.9/mm³. The mean

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Glucocorticoid (72.9%) was the most frequently administered drug, followed by azathioprine (23.7%) and cyclophosphamide (15.3%).

Serum sPD-1 between patients with and without severe AAV and among 3 variants of AAV
We divided AAV patients based on severe AAV and compared the mean serum sPD-1 between patients with and without severe AAV. Patients with severe AAV exhibited the higher mean serum sPD-1 than those without (380.7 vs. 180.3 pg/mL) (Fig. 1A). We also compared the mean serum sPD-1 among patients with MPA (229.4 pg/mL), GPA (208.2 pg/mL) and EGPA (200.1 pg/mL), but we could find no significant difference (Fig. 1B).

Correlation between laboratory variables
In terms of the correlation between BVAS and laboratory variables, serum sPD-1 (r=0.367), white blood cell count (r=0.288), haemoglobin (r=0.590), serum albumin (r=0.670) ESR (r=0.339) and CRP (r=0.450) were significantly correlated with BVAS (Table II). Moreover, in terms of the correlation between serum sPD-1 and other laboratory variables, haemoglobin (r=-0.353) and serum albumin (r=-0.278) were significantly correlated with serum sPD-1, whereas, ESR and CRP were not correlated with serum sPD-1 (Table II).

Multivariable linear regression analysis
We conducted the multivariable linear regression analysis with laboratory variables with statistical significance.
Correlation of BVAS with laboratory variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Correlation Coefficient (β=β)</th>
<th>p-value</th>
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<tr>
<td>Serum sPD-1</td>
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<td>White blood cell count</td>
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<td>Haemoglobin</td>
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<td>Platelet count</td>
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<td>Creatinine</td>
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<td>Serum albumin</td>
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<td>AST</td>
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<tr>
<td>ALT</td>
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<tr>
<td>ESR</td>
<td>0.339</td>
<td>0.009</td>
</tr>
<tr>
<td>CRP</td>
<td>0.450</td>
<td>&lt;0.001</td>
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</tbody>
</table>

Correlation of serum sPD-1 with laboratory variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Correlation Coefficient (β=β)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>White blood cell count</td>
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<td>0.409</td>
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<tr>
<td>Haemoglobin</td>
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<td>Platelet count</td>
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<td>Creatinine</td>
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<tr>
<td>Serum albumin</td>
<td>-0.278</td>
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<tr>
<td>AST</td>
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<tr>
<td>ALT</td>
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<td>0.051</td>
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<tr>
<td>ESR</td>
<td>0.167</td>
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<tr>
<td>CRP</td>
<td>0.232</td>
<td>0.077</td>
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Discussion

In this study, we first investigated whether serum sPD-1 could predict the current activity and severity of AAV based on BVAS in a monocentric prospective cohort of AAV patients. We demonstrated that serum sPD-1 was significantly associated with BVAS in the multivariable linear regression analysis. We also provided an optimal cut-off of serum sPD-1 for severe AAV and found that severe AAV were more frequently identified in patients with serum sPD-1 ≥70.1 pg/mL than those without (51.6% vs. 7.1%, RR 13.867) (Fig. 2B).

How can serum sPD-1, which is rarely detectable in healthy individuals (13), predict the current activity and severity of AAV? PD-1 has a critical role in regulating T cell receptor (TCR) and its costimulatory signals. As a consequence of TCR ligation to antigen presenting cell, the expression of PD-1 on T cells is up-regulated and furthermore, it may be accelerated by TCR-mediated stimulation and activation by various pro-inflammatory cytokines (14). The persistent stimulation of TCR provokes the enhanced expression of PD-1 via phosphoinositide 3-kinase and protein kinase B (Akt), leading to T cell exhaustion (15). On the other hands, as aforementioned, since sPD-1 is one of the splice variants of PD-1 mRNA transcripts (2), the expression of sPD-1 might be increased along with the enhanced expression of PD-1 to counteract PD-1-mediated inhibitory effects on T cells (14). In patients with AAV, infiltrating T cells are often detected in the tissues of vasculitis, such as kidneys and lungs, which is supporting evidence that T cells also participate in the late phase of AAV along with B cell and macrophages (16). Thus, we assume that in patients with severe AAV, as more number of T cells are involved in the correlation analysis for BVAS.

Among 6 variables with significance, only serum albumin, but not serum sPD-1, was significantly associated with BVAS. On the other hands, we also performed the multivariable linear regression analysis with 4 variables such as serum sPD-1, white blood cell count, ESR and CRP, as haemoglobin and serum albumin were remarkably correlated with serum sPD-1. Among 4 variables, only serum sPD-1 (standardised β 0.274, p=0.024) was significantly associated with BVAS (Table III).

Optimal cut-off of serum sPD-1 and relative risk for severe AAV

We calculated the optimal cut-off of serum sPD-1 for severe AAV as 70.1 pg/mL based on the ROC curve (AUC 0.794, 95% CI 0.672, 0.916, sensitivity 0.889 and specificity 0.634) (Fig. 2A). When we divided AAV patients into the two groups according to the optimal cut-off of serum sPD-1, we found that severe AAV were more frequently identified in patients with serum sPD-1 ≥70.1 pg/mL than those without (51.6% vs. 7.1%, RR 13.867) (Fig. 2B).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Standardised β</th>
<th>95% Confidence interval</th>
<th>p-value</th>
</tr>
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<tr>
<td>Serum sPD-1</td>
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<td>0.001, 0.010</td>
<td>0.024</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>0.135</td>
<td>0.000, 0.001</td>
<td>0.277</td>
</tr>
<tr>
<td>ESR</td>
<td>0.021</td>
<td>-0.065, 0.074</td>
<td>0.898</td>
</tr>
<tr>
<td>CRP</td>
<td>0.323</td>
<td>-0.004, 0.168</td>
<td>0.060</td>
</tr>
</tbody>
</table>

AAV: ANCA-associated vasculitis; ANCA: antineutrophil cytoplasmic antibody; BVAS: Birmingham vasculitis activity score; sPD-1: soluble programmed cell death protein 1; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; AST: aspartate aminotransferase; ALT: alanine aminotransferase.
Serum sPD-1 predicts BVAS of AAV / T. Yoon et al.

Fig. 2. Optimal cut-off of serum sPD-1 and relative risk for severe AAV.
A. We calculated the optimal cut-off of serum sPD-1 for severe AAV as 70.1 pg/mL based on the receiver operator characteristic curve. B. Severe AAV were more frequently identified in patients with serum sPD-1 ≥70.1 pg/mL than those without. sPD-1: soluble programmed cell death protein 1; AAV: antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis.

in the pathogenesis of AAV, the stimulation and activation of TCR could be amplified, and subsequently the transcription of PD-1 could be accelerated, leading to an increase in serum sPD-1. For these reasons, we suggest that serum sPD-1 could predict the current activity and severity of AAV.

Is serum sPD-1 correlated with circulating pro-inflammatory cytokines? Because the expression of PD-1 and sPD-1 is closely related to pro-inflammatory cytokines activating T cells, serum sPD-1 can be considered correlated with the circulating concentrations of these cytokines. However, it has still been controversial: a previous study reported no significant differences in serum IL-17 and IL-23 levels among patients with active AAV and healthy controls, whereas another previous study reported that different circulating cytokine levels might depend on ANCA specificity greater than variants of AAV (17, 18). We assume that there might be a time-sequence among the surge of pro-inflammatory cytokines, the stimulation and activation of T cells and the enhanced serum sPD-1: i) pro-inflammatory cytokines stimulate T cells; ii) the overstimulation of TCR increases the expression of PD-1 for regulating T cell functions; iii) as an epiphenomenon, serum sPD-1 may be elevated and at the same time, circulating T cell-related pro-inflammatory cytokines including IFN-γ and IL-17 can be offset by the inhibitory effect of PD-1; iv) thus, at the time point of measuring serum sPD-1 in AAV patients, the range of circulating concentration of pro-inflammatory cytokine may theoretically be wider. For these reasons, serum PD-1 can reflect the accumulated inflammatory burden and estimate BVAS independently from circulating pro-inflammatory cytokines in AAV patients.

According to the previous study, ANCA type was associated with serum levels of some cytokines, which are related to serum PD-1, in AAV patients (18). Therefore, we investigated the association of ANCA type and high level of serum sPD-1 using the chi-square analysis. In terms of MPO-ANCA (or P-ANCA), 22 of 35 AAV patients with MPO-ANCA (or P-ANCA) (62.5%) exhibited serum sPD-1 ≥70.1 pg/mL, whereas, 9 of 24 AAV patients without MPO-ANCA (or P-ANCA) (37.5%) exhibited serum sPD-1 ≥70.1 pg/mL. In other words, MPO-ANCA (or P-ANCA) positivity showed a tendency of contributing the elevated level of serum sPD-1, however, it was not statistically significant (Suppl. Fig. 1). Meanwhile, in terms of PR3-ANCA (or C-ANCA), there was no significant association between PR3-ANCA (or C-ANCA) and the proportion of serum sPD-1 ≥70.1 pg/mL.

Despite the significant correlation between serum sPD-1 and BVAS, serum sPD-1 was not correlated with ESR and CRP which may meaningfully reflect the current activity and severity of AAV. Whereas, serum sPD-1 was negatively and remarkably correlated with haemoglobin and serum albumin which were also inversely correlated with BVAS. For this reason, in the multivariable linear regression analysis, we excluded haemoglobin and serum albumin among 6 variables with statistical significance in the correlation analysis and found an independent association of serum sPD-1 with the current activity and severity of AAV. Although we could not clarify this discrepancy, we believe a direction that is not parallel to traditional acute phase reactant, ESR and CRP, can be rather advantageous to predict the current activity and severity of AAV from diverse angles.

Serum sPD-1 was not affected ANCA positivity or clinical manifestations, but it was associated with concurrently administered azathioprine. Patients receiving azathioprine had a lower serum sPD-1 level than those not. Although the number of patients receiving azathioprine was not large enough to further evaluate the mechanism, we assumed that most patients receiving azathioprine might be in the status of inactive AAV, as azathioprine is mainly used as a maintenance therapeutic regimen. A finding that patients receiving cyclophosphamide and rituximab, which are the most widely used induction therapeutic drugs and generally administered to patients with severe AAV, exhibited higher levels of serum sPD-1 may support our assumption.

In this study, the mean serum sPD-1 in all AAV patients was 217.5 pg/mL, but the median serum sPD-1 was only 74.7 pg/mL. Because there was no report on serum sPD-1 in AAV patients to date, we compared it to that in patients with rheumatoid arthritis and acute respiratory distress syndrome. A previous study reported the median serum sPD-1 in RA patients, similar to our results (around 50 pg/mL vs. 74.7 pg/mL) (6), whereas another study reported the much higher mean serum sPD-1 in patients with ARDS than our results (11,429 pg/mL vs. 217.5 pg/mL) (19). With these results together, we assume that serum sPD-1 may differ in diverse autoimmune diseases.
Our study has several advantages. We provided valuable information on serum sPD-1 in AAV and we first reported the clinical implication in AAV patients. Since we selected patients based on the inclusion criteria, obtained blood samples on the same day of assessing clinical manifestation and performing laboratory tests, and measured laboratory variables in their stored serum samples in a prospective cohort of AAV patients, we could overcome the limitations of a retrospective study by blocking a time-gap between clinical and laboratory data. Furthermore, this study was conducted in a monocentric cohort, we could minimise the inter-observer variation. Also, because we included only Korean patients with AAV in our cohort, the concern on ethnic or geographical differences may be negligible.

Since the mean follow-up period of our prospective cohort was not longer than 2 years, 59 patients were included in this study and only 15 of 59 patients (25.4%) had the disease duration over 2 years. For this reason, the number of patients was not large enough to represent Korean patients with AAV and furthermore we could not measure serially serum sPD-1 with proper interval, particularly before and after induction or maintenance treatment, and investigate a dynamic change in serum sPD-1 as BVAS altered. Also we could not compare serum sPD-1 in AAV patients with those in healthy individuals, as serum sPD-1 was not detected in controls.

However, we believe that this study is valuable as a pilot study to elucidate the clinical role of serum sPD-1 in AAV patients. Furthermore, future prospective studies with larger AAV patients and serial measurement of serum sPD-1 along with BVAS will help physicians apply serum sPD-1 to the real clinical settings. In conclusion, serum sPD-1 could predict the current activity and severity of AAV.

References