Serum granzyme B is associated with otorhinolaryngological, pulmonary, and renal involvement of antineutrophil cytoplasmic antibody-associated vasculitis

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ABSTRACT

We investigated whether serum granzyme B (GrB) can reflect the inflammatory burden such as cross-sectional disease activity and organ-specific involvement in immunosuppressive drug-naïve patients with antineutrophil cytoplasmic antibody-associated vasculitis (AAV). Seventy-eight immunosuppressive drug-naïve patients with AAV were included in this study. At the time of the first classification, whole blood was obtained from each patient and sera was immediately isolated and stored at – 80°C. On the day of the blood sampling, we performed routine laboratory tests including antineutrophil cytoplasmic antibody tests and collected both clinical and laboratory data. AAV-specific indices included Birmingham Vasculitis Activity Score (BVAS) and Five-Factor Score (FFS). The median age of patients with AAV was 62 years and 26 patients were men. Serum GrB was not associated with the cross-sectional BVAS; however, patients with serum GrB positivity exhibited higher frequencies of otorhinolaryngological manifestation than those without (p=0.037). When serum GrB levels were compared after dividing the patients into two groups based on the presence of organ-specific involvement, patients with pulmonary involvement exhibited a significantly higher serum GrB than those without (p=0.042). On the other hand, patients with renal involvement showed a significantly lower serum GrB than those without (p=0.023). In addition, serum GrB was inversely correlated with the cross-sectional FFS (r=−0.249, p=0.028). Even though serum GrB could not reflect the inflammatory burden of AAV, serum GrB was associated with otorhinolaryngological, pulmonary, and renal involvement in immunosuppressive drug-naïve patients with AAV.

INTRODUCTION

Granzyme B (GrB) is one of the five human granzymes, which is a family of serine proteases. GrB is produced and stored in granules of cytotoxic T cells and natural killer cells and released on recognition of target cells. A released GrB primarily induces a perforin-mediated target cell apoptosis.[1] On the other hand, serum GrB has been considered as a biomarker for inflammatory diseases through two mechanisms: (1) GrB cleaves self-proteins to form new autoantigens, leading to a development of autoimmune diseases, and (2) GrB leaks from inflamed tissues, resulting in an increase in serum GrB.[1 2] The former is considered as a trigger that causes the autoimmune process, while the latter is considered as the consequence of the inflammatory burden. So far, there have been several reports on the clinical implications of extracellular GrB in autoimmune diseases. A previous study demonstrated that serum GrB in patients with systemic lupus erythematosus (SLE) was significantly higher than that in healthy controls and correlated with the SLE Disease Activity Index.[3] Another previous study elucidated that a high level of serum GrB in patients with rheumatoid arthritis (RA) was associated with rheumatoid factor and 1-year occurrence of bony erosions.[4]

Antineutrophil cytoplasmic antibody-associated vasculitis (AAV) is one of the systemic small vessels vasculitides and includes three variants, such as microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic granulomatosis with polyangiitis (EGPA).[5 6] Given that AAV pathogenesis is based on antigen–antibody reaction, complement pathways, effector cell activation, and endothelial cell dysfunction,[7] it is reasonably speculated that serum GrB can reflect the inflammatory burden similar to SLE and RA. However, there was no study reporting the clinical implication of serum GrB in patients with AAV to date. Hence, we investigated whether serum GrB can reflect the inflammatory burden, such as cross-sectional disease activity and organ-specific involvement, in immunosuppressive drug-naïve patients with AAV in this study.

METHODS

Patients

We included 78 patients with AAV in this study. All patients were enrolled in the prospective Severance Hospital ANCA-Associated

Vasculitides (SHAVE) cohort from November 2016 to March 2019. The SHAVE cohort is a prospective observational cohort of patients with MPA, GPA, and EGPA, begun in November 2016. All of the patients were classified as AAV at the Division of Rheumatology, Severance Hospital, for the first time, which enabled us to enroll immunosuppressive drug-naïve patients. They all satisfied the 2007 European Medicines Agency (EMA) algorithms for AAV and polyarteritis nodosa (the 2007 EMA algorithm) and the 2012 Chapel Hill Consensus Conferences (CHCC) Nomenclature of Vasculitis (the 2012 CHCC definitions).[5 6] Meanwhile, patients with clinically mimicking medical conditions of AAV, such as serious infection and malignancy, were excluded from this study. Also, we confirmed that all subjects had not received immunosuppressive drugs at enrollment using the Korean Drug Utilization Review system (online supplemental figure 1).

SHAVE cohort

In the prospective SHAVE cohort, at the time of disease classification, we obtained whole blood from each patient with AAV after patient consent. Next, sera were immediately isolated from whole blood and stored at – 80°C. On the day of blood sampling, we performed routine laboratory tests including antineutrophil cytoplasmic antibody (ANCA) tests and collected both clinical and laboratory data according to the cohort protocol. ANCA was detected by antigen-specific assay for myeloperoxidase (MPO)-ANCA and proteinase 3 (PR3)-ANCA.[8 9] Investigated AAV-specific indices include Birmingham Vasculitis Activity Score (BVAS)[10 11] and Five-Factor Score (FFS).[12] We evenly applied BVAS to patients with MPA, GPA and EGPA to adjust the scoring system because BVAS for GPA has a different weight system compared with BVAS. 36-Item Short Form Survey scores, filled out by the patients, were also obtained.

Estimation of serum GrB level

Serum GrB level was measured from stored sera by ELISA kits (Abcam, Cambridge, UK) according to the manufacturer's instruction. Briefly, 50 µL of serum sample for each patients and 50 µL of antibody cocktail were added to each well. The plate was sealed and incubated at room temperature for 1 hour. Then, each well was washed three times using wash buffer. 3,3',5,5'-tetramethylbenzidine development solution (100 µL) was added to each well and the plate was incubated for 10 min at room temperature in the dark. Stop solution (100 µL) was added to each well and the respective optical density value was read at 450 nm using ELISA microplate reader. Since no data on serum GrB of healthy controls were available, we divided patients into the two groups based on the presence and absence of serum GrB (GrB positivity and negativity groups).

Statistical analyses

All statistical analyses were conducted using SPSS software V.23 for Windows. Continuous variables were expressed as median (IQR), and categorical variables were expressed as number (percentage). Significant differences in categorical variables between the two groups were analyzed using the χ^2 and Fisher's exact tests, and differences in continuous variables between the two groups were compared using the Mann-Whitney test. The correlation coefficient of continuous variables was obtained using the Pearson correlation analysis. P values less than 0.05 were considered statistically significant in all analyses.

RESULTS

Baseline characteristics

Forty-one patients were classified as MPA, 21 patients as GPA, and 16 patients as EGPA. The median age of patients with AAV was 62.0 years and 26 patients were men. MPO-ANCA (or P-ANCA) was detected in 45 patients and PR3-ANCA (or C-ANCA) was detected in seven patients. The median BVAS and FFS were 6 and 1, respectively. The most common clinical manifestation was pulmonary manifestation (64.1%) followed by renal (50.0%) and otorhinolaryngological manifestations (44.9%). The values of laboratory results are described in table 1.

Comparison of patient characteristics based on serum GrB positivity and organ involvement

First, we divided patients with AAV into two groups based on serum GrB positivity. The cross-sectional BVAS did not differ between patients with and those without serum GrB. In addition, there were no significant differences in demographic data, AAV variants, ANCA detection rates, and AAV-specific indices between the two groups. Among clinical manifestations at the classification, patients with serum GrB positivity exhibited a higher frequency of otorhinolaryngological manifestation than those without (63.6% vs 37.5%, p=0.037). However, no significant differences were found in frequencies of pulmonary and renal manifestations between the two groups. There were no laboratory results showing significant differences between patients with and those without GrB (table 1).

Moreover, we divided patients with AAV into two groups based on each organ-specific involvement and compared serum GrB. Unlike the result in table 1, there was no significant difference in serum GrB between patients with otorhinolaryngological involvement and those without. On the other hand, patients with pulmonary involvement exhibited a significantly higher serum GrB than those without (p=0.042), and patients with renal involvement showed a significantly lower serum GrB than those without (p=0.023) (figure 1).

Relationship between GrB and continuous variables

Serum GrB was inversely correlated with the cross-sectional FFS (r=−0.249, p=0.028). Meanwhile, serum GrB was significantly correlated with white blood cell and platelet counts (r=0.419, p<0.001 and r=0.470, p<0.001) (online supplemental table 1). However, serum GrB was not correlated with the cross-sectional BVAS.

DISCUSSION

In this study, we demonstrated that serum GrB positivity was associated with otorhinolaryngological involvement in immunosuppressive naïve patients with AAV. Furthermore, our data revealed that patients with pulmonary involvement had elevated serum GrB compared with those without, while serum GrB was lower in patients with renal involvement.

Table 1 Comparison of variables at diagnosis between patients with GrB positivity and negativity

Variables	All patients (N=78)	Serum GrB negativity (N=56)	Serum GrB positivity (N=22)	P value
Variants of AAV, n (%)				0.147
MPA	41 (52.6)	33 (58.9)	8 (36.4)	
GPA	21 (26.9)	12 (21.4)	9 (40.9)	
EGPA	16 (20.5)	11 (19.6)	5 (22.7)	
Demographic data				
Age (years)	62.0 (21.3)	62.0 (22.0)	64.5 (16.3)	0.762
Male gender, n (%)	26 (33.3)	19 (33.9)	7 (31.8)	0.859
ANCA, n (%)				
MPO-ANCA (or P-ANCA)	45 (57.7)	32 (57.1)	13 (59.1)	0.875
PR3-ANCA (or C-ANCA)	7 (9.0)	3 (5.4)	4 (18.2)	0.075
ANCA negativity	26 (33.3)	21 (37.5)	5 (22.7)	0.213
AAV-specific indices				
BVAS	6.0 (10.3)	6.0 (11.5)	6.5 (8.5)	0.857
FFS	1.0 (1.0)	1.0 (1.0)	1.0 (1.3)	0.380
Clinical manifestations, n (%)				
General	26 (33.3)	19 (33.9)	7 (31.8)	0.859
Cutaneous	9 (11.5)	7 (12.5)	2 (9.1)	0.672
Mucous membranes/eyes	5 (6.4)	4 (7.1)	1 (4.5)	0.673
Otorhinolaryngological	35 (44.9)	21 (37.5)	14 (63.6)	0.037
Pulmonary	50 (64.1)	34 (60.7)	16 (72.7)	0.320
Cardiovascular	4 (5.1)	2 (3.6)	2 (9.1)	0.320
Gastrointestinal	0 (0)	0 (0)	0 (0)	N/A
Renal	39 (50.0)	30 (53.6)	9 (40.9)	0.314
Nervous	17 (21.8)	11 (19.6)	6 (27.3)	0.463
Laboratory results				
White blood cell count (/mm^3)11.8 (3.7)	7685.0 (4,240.0)	7155.0 (3,980.0)	8580.0 (7,100.0)	0.035
Hemoglobin (g/L)	118 (37)	117 (37)	120 (45)	0.863
Platelet count (×1000/mm^3)	252.5 (136.0)	252.0 (112.8)	263.5 (234.3)	0.105
Fasting glucose (mg/dL)	101.0 (35.3)	102.0 (38.8)	94.0 (28.3)	0.222
Blood urea nitrogen (mg/dL)	18.9 (17.1)	18.7 (22.2)	19.7 (11.4)	0.240
Creatinine (mg/dL)	0.9 (1.6)	0.9 (1.9)	0.9 (1.1)	0.235
Total protein (g/dL)	6.4 (0.9)	6.6 (1.0)	6.4 (0.7)	0.623
Serum albumin (g/dL)	3.7 (0.9)	3.7 (0.9)	3.7 (1.0)	0.356
Aspartate aminotransferase (IU/L)	18.0 (9.0)	18.0 (8.8)	17.0 (9.0)	0.399
Alanine aminotransferase (IU/L)	18.0 (12.3)	18.5 (14.0)	17.0 (11.3)	0.281
Total bilirubin (mg/dL)	0.5 (0.3)	0.5 (0.4)	0.5 (0.3)	0.564
ESR (mm/hr)	37.5 (43.3)	35.5 (36.0)	39.4 (61.5)	0.216
CRP (mg/L)	2.4 (14.6)	1.9 (9.6)	8.0 (30.6)	0.237

Values are expressed as a median (IQR) or n (%).
AAV, antineutrophil cytoplasmic antibody-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; BVAS, Birmingham Vasculitis Activity Score; C, cytoplasmic; CRP, C reactive protein; EGPA, eosinophilic granulomatosis with polyangiitis; ESR, erythrocyte sedimentation rate; FFS, Five-Factor Score; GPA, granulomatosis with polyangiitis; GrB, granzyme B; MPA, microscopic polyangiitis; MPO, myeloperoxidase; N/A, not applicable; P, perinuclear; PR3, proteinase 3.

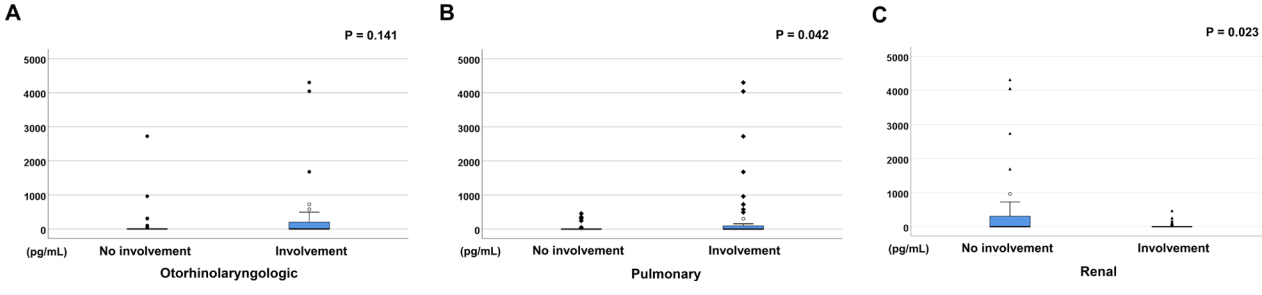

Figure 1 Organ-specific involvement and serum GRB. Patients with pulmonary involvement had increased serum GRB compared with those without, whereas those with renal involvement had lower serum GRB than those without. Serum GRB level tended to be higher in those with otorhinolaryngological involvement but was not significant. GRB, granzyme B.

However, serum GrB was not associated with the cross-sectional BVAS or other acute-phase reactants. In terms of otorhinolaryngological and pulmonary involvement of AAV, the following results were in line with the previous studies that reported serum GrB was associated with disease activity or progression in several systemic inflammatory diseases such as SLE, RA, Crohn's disease, and low-grade systemic inflammation in type 2 diabetes mellitus.[3 4 13 14] However, the results of lower serum GrB in patients with AAV with renal involvement showed a different pattern from that of otorhinolaryngological or pulmonary involvement.

Since this study is the first study on the clinical relationship between serum GrB and renal involvement of AAV, two previous studies that evaluated GrB in patients with lupus nephritis (LN) could be a reference.[3 15] Nonetheless, it is noticeable that a discordant result was found between the two studies. Kok *et al* provided evidence that both serum and tissue GrB levels were associated with kidney damage in SLE.[3] In contrast, Rabani *et al* demonstrated an impaired function of B cells to produce GrB in patients with LN and insisted that the reduced regulatory function of B cell via GrB might initiate LN as well as cause disease exacerbation.[15]

Although it is difficult to generalize, B-cell differentiation by interleukin-21 may have two directions: one is the differentiation into plasma cells producing autoantibodies and another is to stimulate B cells to secrete GrB.[16] We assumed that serum GrB could play a role of perpetuating autoimmunity by exposing new autoantigens or could act as indicator of inflammation reflecting B-cell activation. Therefore, it may be hypothesized that GrB could provoke inflammation or reflect increased inflammation. Contrastingly, in the present study, it was shown that patients with renal manifestation exhibited a significantly lower serum GrB than those without. These results show that serum GrB level itself may not be clinically useful in estimating the inflammatory burden of AAV, and also, a precise pathophysiological mechanism of GrB leading to renal involvement in AAV should be identified.

In the correlation analysis, serum GrB was inversely correlated with the cross-sectional FFS. FFS consists of five items such as age ≥65 years, no otorhinolaryngological involvement, renal insufficiency, gastrointestinal signs, and cardiac insufficiency.[12] Pulmonary involvement, however, was not included in FFS. In this study, patients with serum GrB positivity more frequently had otorhinolaryngological involvement, and there was no difference in renal involvement. Therefore, serum GrB could be inversely correlated with FFS because all of the factors that increase serum GrB contributed to the reduction of FFS. Notably, serum GrB was not correlated with the cross-sectional BVAS. The non-significant correlation between serum GrB and BVAS might be complicated by the inverse correlation of renal involvement and a positive association with otorhinolaryngological involvement and pulmonary involvement.

For the first time, we demonstrated that serum GrB could not reflect the cross-sectional activity of AAV, although it may be associated with organ-specific involvement of otorhinolaryngological, pulmonary, and renal involvement in immunosuppressive drug-naïve patients with AAV. However, our study has several limitations. First, the number of patients might not have been large enough to have a statistical power because we included only immunosuppressive-naïve patients who were initially classified as AAV. Second, the level of GrB was only assessed in the sera, and the expressions of GrB in tissues or peripheral blood mononuclear cells could not be evaluated. Third, the dynamic change of serum GrB through serial measurements were not measured. Therefore, we believe that future studies with a larger number of patients with AAV and serial measurements of GrB will provide more reliable and validated data.

In conclusion, serum GrB could not reflect the inflammatory burden of AAV. However, serum GrB was associated with otorhinolaryngological, pulmonary, and renal involvement in immunosuppressive drug-naïve patients with AAV.

Contributors TY, JY and S-WL researched the literature and conceived the study. TY, SSA and S-WL collected and analyzed the data. JY and S-WL wrote the first draft of the manuscript. JJS and Y-BP contributed in data collection and revised the draft of the manuscript. All authors reviewed and approved the final version of the manuscript.

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