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Anti-carbonic anhydrase III autoantibodies in vasculitis syndrome

(血管炎症候群における診断マーカーとしての抗 Carbonic Anhydrase III 抗体について)

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Abstract

Aim: To identify autoantibodies useful in the diagnosis of primary vasculitides.

Methods: The presence of antibodies against proteins in the lysate of mouse blood vessels was examined by two-dimensional electrophoresis followed by western blotting for the pooled serum sample from patients with various forms of vasculitis: polyarteritis nodosa (PAN), microscopic polyangitis (MPA), Wegener's granulomatosis (WG), and Takayasu's arteritis (TA). Autoantigenicity in patients with vasculitides was examined by western blotting and enzyme-linked immunosorbent assay (ELISA). Clinicopathological correlations between the positivity of the autoantibodies and clinical status of patients with the vasculitis were examined.

Results: The autoantigen detected in the lysate of pooled serum from patients with vasculitides was identified by mass spectrometry as carbonic anhydrase III (CAIII). ELISA showed significantly higher prevalence of anti-CAIII antibodies in MPA patients (MPA, 11/23 [47.8%]; healthy controls, 2/32 [6.3%]; $p < 0.001$). Further, anti-CAIII antibody-positive MPA patients had higher vasculitis activity scores compared to anti-CAIII antibody-negative patients, and a weak and not significant negative correlation was observed between anti-CAIII antibody levels and myeloperoxidase-anti-nuclear cytoplasmic antibody (MPO-ANCA) levels. No significant differences were found in

anti-CAIII autoantibody levels between MPA and the other primary vasculitides.

Conclusion: We found significantly high prevalence of anti-CAIII antibody levels in sera from MPA patients. Although the number of samples available in this study is small and anti-CAIII autoantibodies display weak specificity for MPA, anti-CAIII antibodies may be useful for diagnosing MPA in patients who have no ANCA, as well as for assessing disease activity.

Key words: autoantibodies, carbonic anhydrase III, vasculitis

Running title: Anti-CAIII antibodies in vasculitis.

Introduction

Vasculitides are clinical syndromes characterized by blood vessel wall inflammation that lead to tissue or end organ damage. One of the first descriptions of a specific systemic vasculitis was of polyarteritis nodosa (PAN) by Kussmaul and Maier in 1866.¹ Since its identification, PAN had long been taken in a generic sense to include all types of vasculitis, and the classification of vasculitis has been a challenging problem for decades. Currently, the Chapel Hill International Consensus Conference² has been accepted worldwide as the classification of 10 primary systemic vasculitides based predominantly on the vessels involved: giant-cell arteritis (GCA), Takayasu's arteritis (TA), PAN, Kawasaki disease, Wegener's granulomatosis (WG), Churg-Strauss syndrome (CSS), microscopic polyangiitis (MPA), Henoch-schönlein purpura (HSP), essential cryoglobulinemic vasculitis, and cutaneous leukocytoclastic angiitis (CLA).

The diagnosis of vasculitides is often difficult because the symptoms are similar to those of other autoimmune diseases. Uncertainties in clinical presentation, limited diagnostic laboratory tests, and difficulty obtaining appropriate tissues for histologic examination are limitations to proper diagnosis or classification. Although anti-nuclear cytoplasmic antibodies (ANCA) are useful diagnostic serological markers for ANCA-associated vasculitides (WG, MPA, and CSS), these markers are of limited

utility for detecting disease because a negative test results for ANCA does not exclude the presence of vasculitis.

In the present study, we assayed autoantibodies in a pooled serum sample (serum samples from one patient with each of PAN, MPA, WG, and TA) against lysates of mouse blood vessels using 2-dimensional electrophoresis (2-DE) followed by western blotting and subsequently identified the protein targeted by the autoantibodies using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. We then evaluated the autoantigenicity of the identified protein in patients with primary vasculitides (PAN, MPA, WG, allergic granulomatous angiitis [AGA], and TA).

Methods

Patients

Peripheral venous blood samples were obtained from 61 consecutive Japanese patients with primary vasculitides (PAN, 10; MPA, 23; WG, 15; AGA, 4; and TA, 9) and from 32 healthy controls (Table 1). We randomly selected serum samples from one patient with each of PAN, MPA, WG, and TA. These samples were used individually for 1-DE and western blotting using recombinant protein and used together (pooled serum sample) for 1-DE as well as 2-DE and western blotting using the lysate of mouse blood

vessels. Clinical data were systematically collated. Birmingham vasculitis activity scores (BVAS) were recorded at the start of the study. All patients provided informed consent, and the study was approved by the ethics committee of Fukushima Medical University, Japan.

Solubilization of blood vessels from Balb/c mice

Blood vessels of Balb/c mice were lysed using the Plasma Membrane Protein Extraction Kit (Bio Vision Research Products, Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, excised blood vessels (abdominal aorta, femoral and carotid arteries) from five Balb/c mice were washed and stripped of adhering tissue. Then, the blood vessels were cut into small pieces with a scissors, homogenized in 1 ml of homogenization buffer mix, sonicated on ice for 10 min, and centrifuged for 10 min at $700 \times g$ at 4°C . Following collection of supernatant containing solubilized membranes as well as cytosolic materials, the protein concentration was determined using the BioRad Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA), USA and samples were stored at -80°C until further assay.

SDS-PAGE (1-dimensional electrophoresis (1-DE)) and western blotting

The lysate of mouse blood vessels was resuspended in sample buffer so as to load

adequate amounts of protein (20 µg/lane) on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The separated proteins in the lysate were transferred onto polyvinylidene difluoride (PVDF) membranes. After incubation with 50 mmol/L Tris buffer/HCl (pH 7.5) (TBS) containing 0.05% Tween 20 and 1% bovine serum albumin (BSA) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), the membranes were incubated with pooled serum or serum from healthy controls diluted 1/100 in TBS containing 0.05% Tween20 (TBS-T) and 1% BSA. After incubation overnight at 4°C, the membranes were washed with TBS-T, then incubated for 60 min with alkaline phosphatase-conjugated Goat F(ab')₂ anti-human IgG (BIOSOURCE, Camarillo, CA) diluted 1/4,000 in TBS-T containing 1% BSA. After washing, the membranes were developed with the BCIP/NBT-Purple Liquid Substrate System for membranes (Sigma-Aldrich, St. Louis, MO, USA).

2-DE and 2-DE followed by western blotting

The proteins in lysate of mouse blood vessels were separated on 2-DE as described previously.^{3,4} Briefly, 20 µg of the protein was applied on 7-cm ReadyStrip IPG strips, pH 3-10 (Bio-Rad Laboratories, Inc.) and rehydrated at room temperature for 11 h. Isoelectric focusing (IEF) was performed for 12 h using a PROTEAN IEF Cell

(Bio-Rad Laboratories, Inc.). After IEF, the strips were equilibrated for 20 min in denaturation buffer I containing 6 mol/L urea, 2% SDS, 37.5 mM Tris-HCl, 20% glycerol, and 2% (w/v) dithiothreitol and then for 10 min in denaturation buffer II containing 6 M urea, 2% SDS, 37.5 mM Tris-HCl, 20% glycerol, and 2.5% (w/v) iodoacetamide. Strips were then embedded onto 10% SDS-polyacrylamide gels and gels were electrophoresed for 1 h. After electrophoresis, the proteins were visualized by staining the gel with a Silver Stain Plus Kit (Bio-Rad Laboratories, Inc.). For 2-DE followed by Western blotting, the proteins were transferred onto PVDF membrane. Then, the membrane was incubated in TBS-T containing 1% BSA for 1 h at room temperature for blocking. After the blocking step, the membrane was incubated with the pooled serum. After incubation at 4°C overnight, the membrane was washed with TBS-T and incubated with alkaline phosphatase-conjugated Goat F(ab')₂ anti-human IgG (BIOSOURCE) diluted 1/4000 in TBS-T containing 1% BSA. After washing with TBS-T, the membrane was developed with BCIP/NBT-Purple Liquid Substrate System for membranes (Sigma-Aldrich).

Identification of the target protein

The protein spot corresponding to the spot on 2-DE which reacted with IgG in the pooled serum on western blot was recovered from the 2-DE gel stained with

silver reagent and digested in trypsin. The digested peptides were desalted and eluted into α -cyano-4-hydroxycinnamic acid (α -CHCA) matrix solution and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Voyager-DE STR; Applied Biosystems, Foster City, CA, USA). The analysis was outsourced to APRO Science Corporation (Naruto, Japan). The NCBI nr (Taxonomy: Mammals) database was searched using MASCOT (Matrix Science, Tokyo, Japan) to identify the determined protein sequence. Only those proteins with a Mowse score above statistical significant identity ($p < 0.05$) were accepted as positive identifications.

Western blotting using recombinant CAIII as antigen

For detection of IgG antibodies to CAIII, 1 μ g/lane recombinant human CAIII (R&D Systems, Inc., Minneapolis, MN) was subjected to 15% SDS-PAGE under reducing conditions, and transferred onto PVDF membrane. After blocking with TBS-T containing 1% BSA, the membrane was probed individually with the serum from patients with each of PAN, MPA, WG, and TA (1/100 dilution in TBS-T containing 1% BSA). After incubation overnight at 4°C, the membrane was incubated with alkaline phosphatase-conjugated Goat F(ab')₂ anti-human IgG diluted 1/4,000 in TBS-T containing 1% BSA, followed by staining with BCIP/NBT-Purple Liquid Substrate

System for membranes. For the absorption test of recombinant CAIII, the patient serum sample which gave strong reactivity with recombinant CAIII on western blotting (50 µl) was added to 20 µg of recombinant CAIII and incubated at room temperature for 2 h, and then centrifuged at 10,000g for 10 min. The supernatant was diluted 1/100 with TBS-T containing 1% BSA and subjected to western blot.

Enzyme-linked immunosorbent assay (ELISA) using recombinant CAIII as antigen

Recombinant CAIII protein was immobilized on 96-well microtiter plates (CORSTAR EIA/RIA Plate, 96 well Half Area, Corning Incorporated, Corning, NY, USA) by coating each well with 0.25 µg of recombinant CAIII in 50 µl of 0.1 mol/L carbonate-bicarbonate buffer (pH 9.8) at 4°C overnight. To determine non-specific binding of each serum sample to the wells, a control well without CAIII was also examined. All procedures were performed at room temperature, except for the antigen coating. After each step, the wells were washed four times with TBS-T. The ELISA plates were incubated for 2 h with 100 µl of TBS-T containing 3% BSA. Serum samples (diluted 1/100 in TBS-T containing 3% BSA) were added to individual wells in duplicate. After incubating for 2 h, the bound IgG was detected with 50 µl of alkaline phosphatase-conjugated Goat F(ab')₂ anti-human IgG (diluted 1/4,000 in TBS-T containing 3% BSA), followed by the addition of Alkaline Phosphatase Yellow (pNPP)

Liquid Substrate (Sigma-Aldrich) and 3 mol/L NaOH to stop color development. The optical density (OD) at 405 nm was determined on a microplate reader (Benchmark Plus™ microplate spectrophotometer, Bio-Rad Laboratories, Inc.). In each serum sample, the specific binding of anti-CAIII antibodies was evaluated by subtracting the mean OD value of the control well from the mean OD value of the CAIII-coated well. The obtained antibody values were expressed in arbitrary units (AU) based on the positive and negative reference sera, which were set to 0.5 and 0.0 OD units, respectively. The reference sera were used to correct interplate/intraplate variability across this assay. The cut-off value was set as the mean + 2SD (standard deviation) of OD for sera of healthy controls.

Statistics

Fisher's exact probability test was used to compare differences in antibody prevalence rates among patients with various diseases and healthy controls. The Mann-Whitney U test was used to compare differences in disease activity or clinical manifestations between the antibody-positive and antibody-negative patients groups. Pearson correlation analysis was used to test differences in antibody and myeloperoxidase (MPO)-ANCA levels. $P < 0.05$ was considered to indicate statistical significance.

Results

Detection of autoantigens for vasculitis syndrome antibody using serum samples from patients with vasculitis syndrome

To identify autoantigens for vasculitis syndrome, we compared proteins reactive to serum antibody for a pooled serum sample (PAN, MPA, WG, and TA) and healthy controls. As shown in Figure 1A, a band of size 29 kDa was detected in the pooled serum.

2-DE separation and identification of autoantigen for vasculitis syndrome

To identify the protein corresponding to the 29-kDa band, the protein was separated by 2-DE over a pH range of 3.0 to 10.0 and detected by silver staining (Fig. 1B) and by western blotting using the pooled serum, which reacted to the 29-kDa band (Figure 1C). Protein from the spot was present in sufficient quantity to be identified by MALDI-TOF/TOF-MS and MS/MS analyses. The results of MS/MS analysis are summarized in Table 2. We identified the protein as carbonate anhydrase III (CAIII).

Confirmation of immunoreactivity of CAIII in primary vasculitides syndrome by 1-DE western blotting for recombinant CAIII

To confirm the results obtained by 2-DE followed by western blotting, we evaluated antibody reactivity against recombinant CAIII using 1-DE western blotting. Recombinant CAIII reacted with sera from patients with MPA and TA but not PAN or WG (Fig. 2A). The specific antibodies in the serum of the patient with MPA (same sample of Lane 4 in Figure 2A) were absorbed with recombinant CAIII (Fig. 2B). These results indicate the presence of anti-CAIII antibodies in the serum of the patient with MPA.

ELISA using recombinant CAIII as an antigen

To determine the abundance of anti-CAIII antibodies in patients with primary vasculitides, ELISA assay using recombinant CAIII as the antigen was performed. Testing for IgG reactivity against recombinant CAIII immobilized onto an ELISA plate in serum samples from patients with various vasculitis syndromes (MPA, 23; WG, 15; PAN, 10; TA, 9, AGA, 4) and, as a control, healthy volunteers (n=32) showed a significantly higher level of CAIII-positive samples for patients with MPA (11/23, 47.8%) than for the control group (2/32, 6.3%) ($p < 0.001$) for a cutoff of 0.21 (Fig. 3). The mean intraplate and interplate coefficients of variation for the positive reference serum were 2.4% and 5.2%, respectively, and those for the controls were 3.2% and 7.5%. For other primary vasculitides, there were no differences in the prevalence of

anti-CAIII antibody compared to the healthy controls (TA, 1/9; PAN, 1/10; WG, 4/15; and AGA, 0/4). The summary of ELISA findings is shown in Table 3.

Assessment of clinicopathological correlation between anti-CAIII antibody ELISA and the clinical status in patients with MPA

As we observed a significantly higher prevalence of anti-CAIII antibody in MPA patients than in healthy controls, we looked for the presence of clinicopathological correlations between ELISA results and clinical status. First, we investigated the relationship between disease activity and anti-CAIII antibody ELISA level. Dividing the MPA patients into two groups, the anti-CAIII positive (n=11) and anti-CAIII negative (n=12) groups, we analyzed individual vasculitis activity score (BVAS). As shown in Figure 4, there was a significant correlation between anti-CAIII positivity and BVAS. Anti-CAIII positive patients had significantly higher BVAS scores compared to anti-CAIII negative patients (median BVAS [range]; 18 [12 to 31] vs. 9 [3 to 17], respectively; p=0.0013).

Further, anti-CAIII ELISA levels were stratified according to the cumulative presence of clinical manifestations of MPA. We could not detect any specific association between anti-CAIII ELISA levels and clinical manifestations in patients with MPA (data not shown).

Correlation between MPO-ANCA and anti-CAIII ELISA value in patients with MPA

Further, the correlation between the MPO-ANCA and anti-CAIII ELISA levels was also examined. A weak, and not significant, negative correlation was observed in MPA patients between anti-CAIII ELISA levels and MPO-ANCA levels. Pearson's correlation coefficient (r) and the corresponding p value for the correlation was $r=-0.304$ and $p=0.16$, respectively.

Discussion

In order to identify autoantibodies that are informative in the diagnosis of primary vasculitides, the reactivity of a pooled serum sample derived from patients with primary vasculitides (PAN, MPA, WG, and TA) toward the lysate of mouse blood vessels was tested. Antibodies reacting to a 29-kDa protein identified by MALDI-TOF MS as carbonic anhydrase III were detected.

Carbonic anhydrases (CaI EC 4.2.1.1) are a class of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide. Nine CA isozymes are reported in mammals^{5,6,7,8,9}: CAI-VII, IX, and XII. CAIII is distinguished from the other isozymes by several characteristics, particularly by its low specific activity as a carbon dioxide hydratase and resistance to acetazolamide.¹⁰ CAIII is abundant in red skeletal muscle,

where it comprises about 8% of the soluble protein,¹¹ and it is present in some other tissues and cell types. In addition to its carbon dioxide hydratase activity, CAIII shows tyrosine phosphatase activity, which is regulated by the cellular redox state, suggesting that CAIII may play a role in intracellular signaling, especially in response to oxidative stress.^{12,13}

Previously, Robert-Pachot et al. reported that anti-CAIII autoantibodies have been detected in serum from patients with autoimmune diseases, such as rheumatoid arthritis (17%), systemic lupus erythematosus (33%), systemic sclerosis (13%), type I diabetes (20%), Addison's disease (28%), and autoimmune thyroiditis (5%).^{14,15} In this study, CAIII ELISA showed a high prevalence of anti-CAIII in MPA patients compared to the healthy controls. Our study also highlighted a clinicopathological correlation between the level of anti-CAIII antibody detected by ELISA and disease activity in patients with MPA. Interestingly, patients with MPA and higher BVAS tended to have higher titers of anti-CAIII antibodies compared to those with lower BVAS. We looked for associations between anti-CAIII antibody levels and clinical features, but none could be observed. The lack of statistical significance for differences of anti-CAIII autoantibodies between MPA and the other primary vasculitides means that anti-CAIII autoantibodies have only weak specificity for MPA. Considering that vasculitis also occur as a secondary feature

in other rheumatic diseases, elevated anti-CAIII antibody levels may reflect a common pathological condition among these diseases.

Microscopic polyangiitis is a systemic vasculitis that mainly affects small-caliber blood vessels and is associated with the presence of ANCA. The Chapel Hill International Consensus Conference criteria defined MPA as a necrotizing vasculitis with few or no deposits affecting small vessels, and it is frequently associated with necrotizing glomerulonephritis and pulmonary capillaritis. The diagnosis of MPA is made difficult by the great variability in the presenting manifestations and the intensity of the illness. There is no doubt that the ANCA test has utility in the diagnosis and monitoring of disease activity in patients with MPA. However, 15% to 40% of patients with MPA are negative for MPO-ANCA, and those patients are associated with difficulty in diagnosis and assessment of disease activity. Anti-CAIII antibodies could be useful for diagnosis of MPA in ANCA-negative patients because we observed a negative correlation in MPA patients between anti-CAIII ELISA levels and MPO-ANCA levels.

The pathogenic mechanism of anti-CAIII antibodies is not known. However, Räsänen et al.¹³ reported that CAIII functions as an oxyradical scavenger and thus protects cells from oxidative damage. Anti-CAIII may inhibit the function of CAIII to

protect cells from oxidative damage. Further study will be needed to clarify the mechanism of anti-CAIII antibodies.

This study has a limitation in that the number of patients is too small to draw definitive conclusions, and further investigation with larger sample sizes is needed. In consideration of the small sample size and the weak disease specificity of anti-CAIII antibody to MPA, the findings of (i) the high prevalence of anti-CAIII antibodies in MPA patients, (ii) a correlation between the anti-CAIII antibody titer and disease activity, and (iii) an association between the anti-CAIII antibody titer and serum MPO-ANCA level, all suggest the possibility that anti-CAIII antibodies may be useful markers in the diagnosis of MPA in ANCA-negative patients as well as in assessment of disease activity.

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Table 1. Demographic characteristics of patients with primary vasculitides.

	PAN	MPA	WG	AGA	TA
Number of patients	10	23	15	4	9
Sex (Male/Female)	5/5	12/11	5/10	2/2	2/7
Mean age (years)	64	70	62	50	41
Range (years)	42-76	20-82	25-81	40-68	23-62

AGA, allergic granulomatous angiitis ; MPA, microscopic polyangiitis; PAN, polyarteritis nodosa; TA, Takayasu's arteritis; WG, Wegener's granulomatosis.

Table 2. Identification of protein in lysate of mouse blood vessels by immunoglobulin G (IgG) in pooled sera samples of patients with vasculitides.

Protein name	Carbonic anhydrase III (Mus musculus)
NCBI accession number	gi 31982861
Calculated MW (Da/pI)	29348/6.8
Matched peptides	R. GGPLSGPYR. L R. VVFDDTYDR. S K. EPMTVSSDQMAK. L K. GDNQSPIELHTK. L K. HDPSLQPWSASYDPGSAK. T R. SLFSSAENEPPVPLVGNWRPPQPVK. G

Table 3. Prevalence of anti-CAIII positive serum samples by ELISA.

	n	Frequency of immunoreactivity		
		Number positive	Compared with MPA	Compared with healthy control
MPA	23	11 (47.8%)	-----	p<0.001
WG	15	4 (27%)	p=0.31	p=0.07
PAN	10	1 (10%)	p=0.06	p>0.999
TA	9	1 (11%)	p=0.10	p=0.53
AGA	4	0 (0%)	p=0.12	p>0.999
Healthy control	32	2 (6.3%)	p<0.001	-----

AGA, allergic granulomatous angüitis; MPA, microscopic polyangüitis; PAN, polyarteritis nodosa; TA, Takayasu's arteritis; WG, Wegener's granulomatosis.

Figure Legends

Figure 1

(A): Western blot of lysate from mice blood vessels with serum from healthy control (Lane 1) and pooled serum from patients with each of polyarteritis nodosa (PAN), microscopic polyangiitis (MPA), Wegener's granulomatosis (WG), and Takayasu's arteritis (TA) (Lane 2). A band of 29 kDa was detected with pooled vasculitis syndrome serum in Lane 2.

(B): Two-dimensional electrophoresis (2-DE) separation and western blotting of lysate from mice blood vessels. Lysates from mice blood vessels were separated by 2-DE and stained with silver reagent. The circle indicates the protein spot corresponding to the immunoreactive spot in (C).

(C): The separated proteins were transferred onto PVDF membrane and then were reacted with pooled serum from patients with vasculitis. Circle indicates immunoreactive spot corresponding to the protein spot in (B).

Figure 2

(A) Western blot showing reactivity to recombinant carbonic anhydrase III (CAIII) in serum from patients with vasculitis. Lane 1, serum from patient with polyarteritis nodosa (PAN), Lane 2, serum from patient with Takayasu's arteritis (TA), Lane 3, serum

from patient with Wegener's granulomatosis (WG), Lane 4, serum from patient with microscopic polyangiitis (MPA). Blot reactivity was observed in serum samples from patients with TA and MPA (Lanes 2, 4). (B) Western blotting with non-treated serum and absorbed serum. Lane 1, Western blotting with serum incubated with recombinant CAIII to absorb anti-CAIII antibodies before blotting. Lane 2, Western blotting with serum from patient with untreated MPA.

Figure 3

Quantitative analysis of immunoglobulin G (IgG) reactivity against the recombinant protein carbonic anhydrase III (CAIII) in individual serum sample of patients with vasculitis. Serum samples from 23 patients with microscopic polyangiitis (MPA), 15 with Wegener's granulomatosis (WG), 10 with polyarteritis nodosa (PAN), nine with Takayasu's arteritis (TA), four with allergic granulomatous angiitis (AGA), and 32 healthy controls were incubated with recombinant CAIII immobilized onto an enzyme-linked immunosorbent assay plate. The mean optical density (OD) values for the recombinant protein were normalized by mean OD values of the background immunoreactivity and expressed as arbitrary units (AU) based on the positive and negative reference sera. Horizontal lines indicate the mean for each group and the broken line indicate the cutoff level at the mean + 2 standard deviation (SD) of the

healthy control. In patients with MPA, 11 of 23 serum samples were positive for CAIII. In contrast, of the 32 healthy controls, 2 were positive for CAIII reactivity. The difference between patients with MPA and healthy controls was statistically significant ($p < 0.001$).

Figure 4

Comparison between carbonic anhydrase III (CAIII) enzyme-linked immunosorbent assay values and disease activity of vasculitis (Birmingham vasculitis activity scores, BVAS). Patients with microscopic polyangiitis (MPA) were divided into two groups, the anti-CAIII positive group and anti-CAIII negative group. BVAS were significantly higher in the anti-CAIII positive group compared to the anti-CAIII negative group. Horizontal bars indicate the median BVAS in each group.

Figure 1

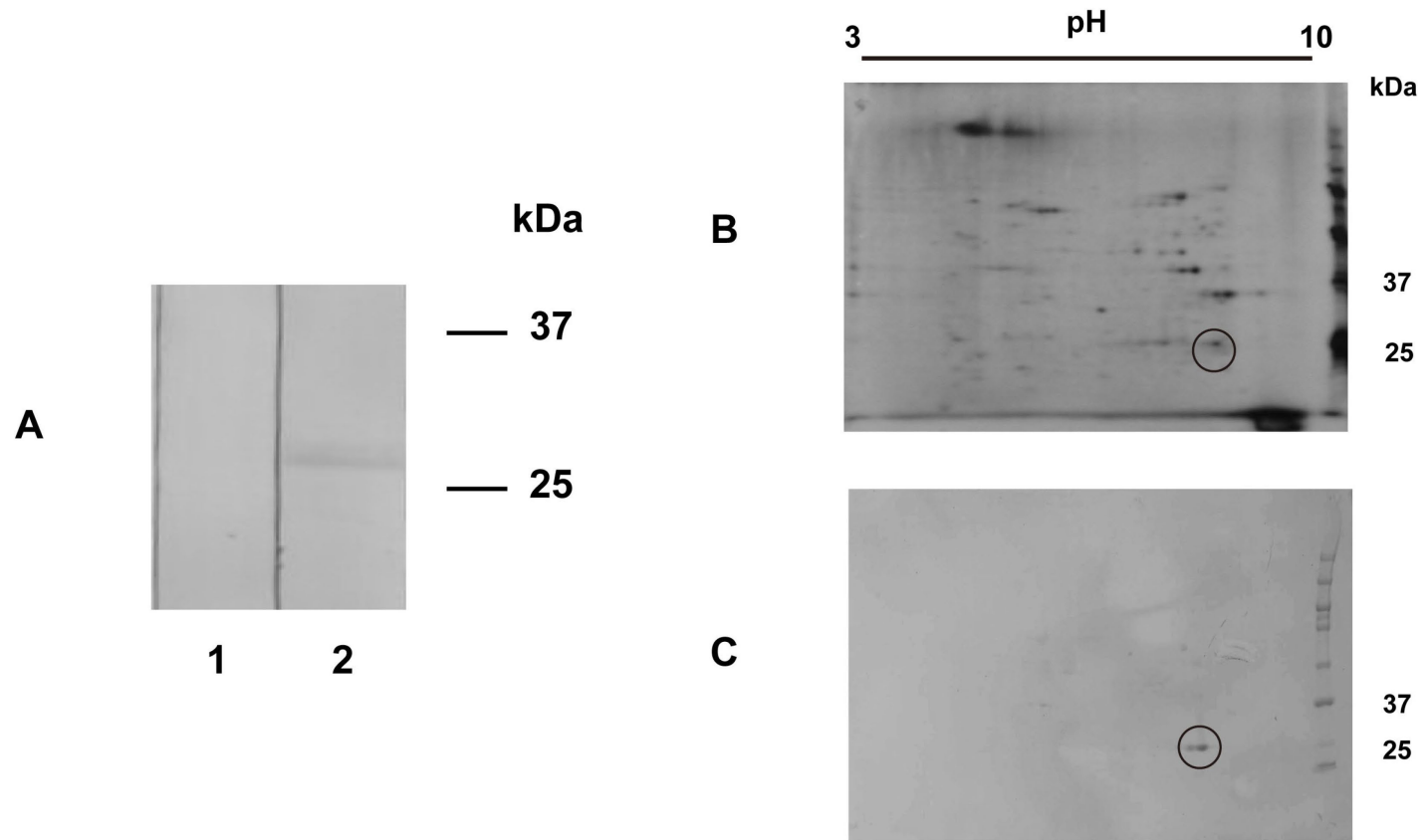


Figure 2

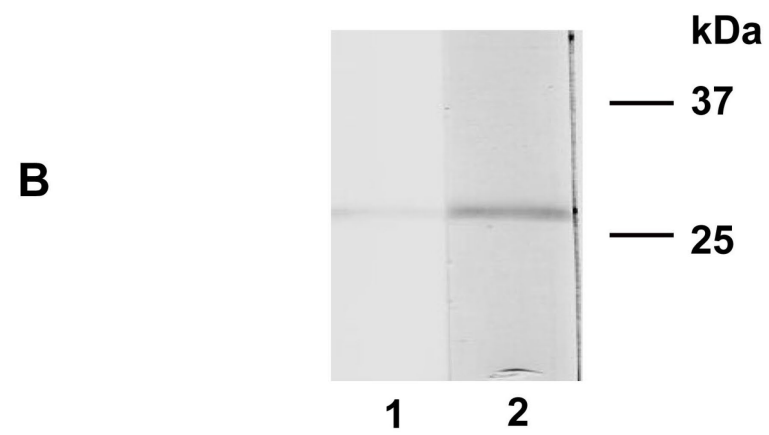
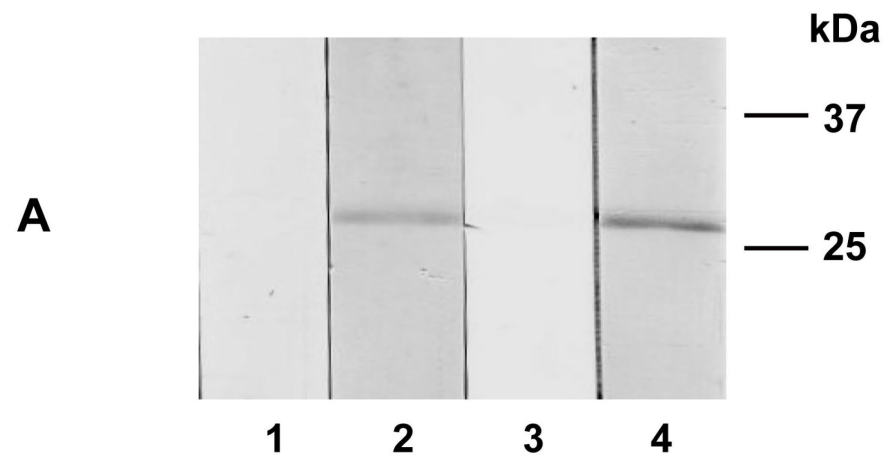


Figure 3

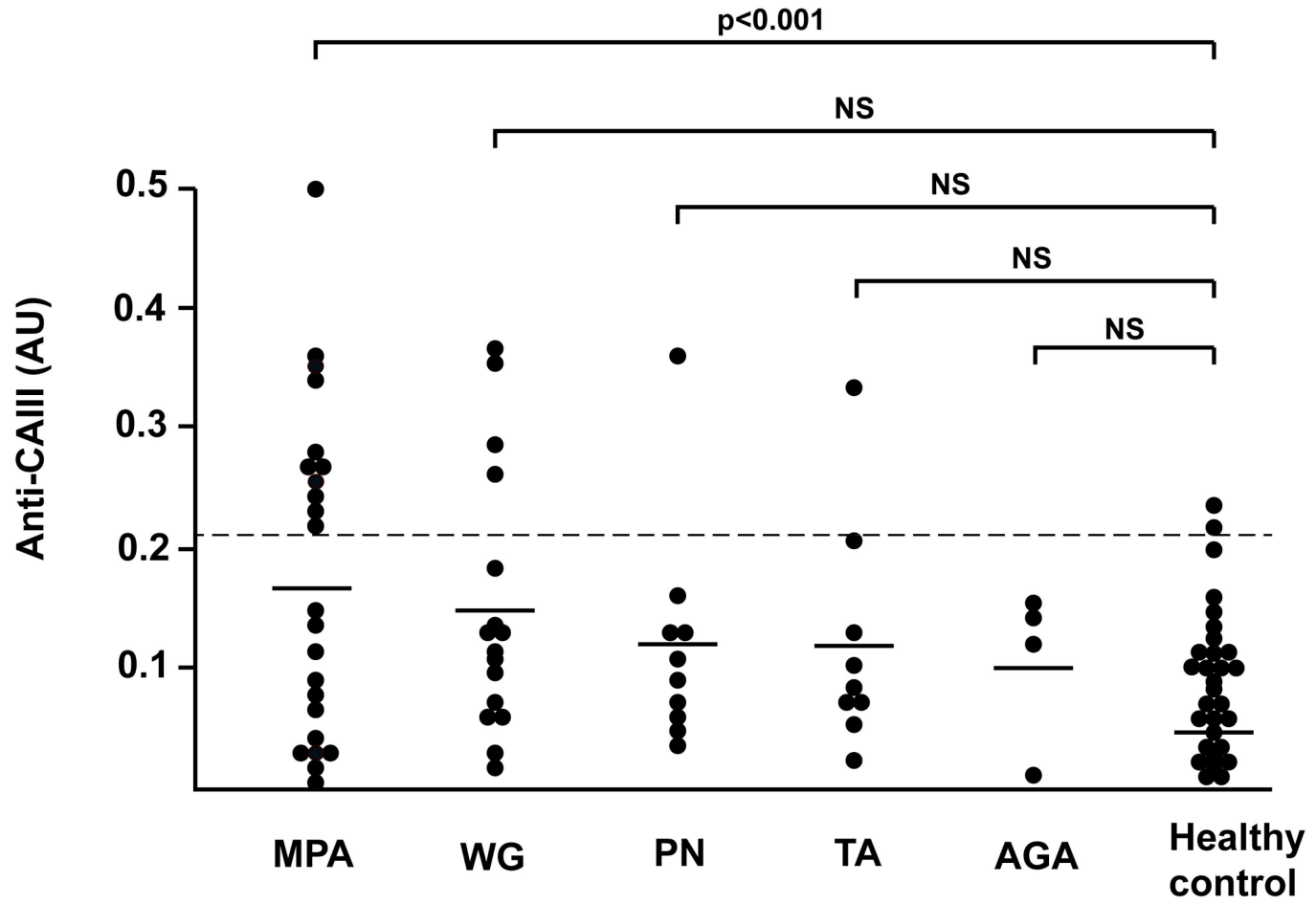


Figure 4

