

## CLINICAL REPORT

# Serum Anti-lysosomal-associated Membrane Protein-2 Antibody Levels in Cutaneous Polyarteritis Nodosa

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**Lysosomal-associated membrane protein-2 (LAMP-2) is a target antigen for anti-neutrophil cytoplasmic antibodies (ANCA), which are closely linked to a subset of primary systemic vasculitides. Cutaneous polyarteritis nodosa (CPN) is a necrotizing vasculitis of small to medium-sized arteries within the skin. We measured levels of serum anti-LAMP-2 antibody in 50 patients with CPN, 8 with microscopic polyangiitis (MPA), and 34 healthy persons. We also investigated the presence of ANCA in patients with CPN using indirect immunofluorescence (IIF), a direct ELISA and a capture ELISA specific for myeloperoxidase (MPO) and proteinase 3 (PR3). Serum anti-LAMP-2 antibody levels differed significantly between patients with CPN (0.263 U/ml) and those with MPA (0.180 U/ml) ( $p=0.0102$ ). Serum of all patients with CPN was negative for MPO-ANCA and PR3-ANCA by both direct ELISA and capture ELISA. In contrast, IIF assay revealed ANCA in 42 (84.0%) of the 50 CPN patients. Serum anti-LAMP-2 antibody levels in the perinuclear ANCA (P-ANCA) group were significantly elevated compared with the non-ANCA group ( $p=0.0147$ ). We suggest that anti-LAMP-2 antibody could play an important role in the pathogenesis of CPN in the presence of P-ANCA detected by IIF. Key words: cutaneous polyarteritis nodosa; lysosomal-associated membrane protein-2; anti-neutrophil cytoplasmic antibody; indirect immunofluorescence; microscopic polyangiitis.**

(Accepted May 9, 2012.)

Acta Derm Venereol 2013; 93: 70–73.

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Lysosomal-associated membrane protein-2 (LAMP-2) was first identified during a systematic search for anti-neutrophil cytoplasmic antibody (ANCA) antigens expressed on the surface of neutrophils and endothelial cells (1). LAMP-2 is a highly glycosylated protein and an abundant constituent of the lysosomal membrane, which is involved in lysosomal biogenesis and phagocytosis (2–6). LAMP-2 is also integrated into the membranes of neutrophil intracellular vesicles that contain myeloper-

oxidase (MPO) and proteinase 3 (PR3). Research suggests that autoantibodies against LAMP-2 are closely linked to a subset of primary vasculitides (7, 8). Willcocks et al. (9) proposed that anti-LAMP-2 antibodies bind to LAMP-2 on the surface of neutrophils and endothelial cells, and lead to ANCA-associated systemic vasculitis. In contrast, Roth et al. (10) suggested that antibodies against LAMP-2 might exist at very low titres in a minority of patients with ANCA-associated systemic vasculitis.

Cutaneous polyarteritis nodosa (CPN) is a necrotizing vasculitis of small to medium-sized arteries within the skin (11, 12). The aim of this study was to investigate the presence of ANCA in patients with CPN using indirect immunofluorescence (IIF), a direct enzyme-linked immunosorbent assay (ELISA) and a capture ELISA specific for MPO and PR3. We measured serum anti-LAMP-2 antibody levels in patients with CPN and examined correlations with clinical, serological and histopathological findings.

## MATERIALS AND METHODS

### Patients and sera

A total of 50 Japanese patients (17 men, 33 women; mean age  $\pm$  standard deviation (SD)  $47.0 \pm 16.8$  years) with CPN and 8 patients (3 men, 5 women; mean age  $\pm$  SD  $64.6 \pm 17.8$  years) with microscopic polyangiitis (MPA) seen at the Department of Dermatology, St Marianna University School of Medicine between October 2003 and December 2009 were examined. The patients were diagnosed according to the Japanese criteria (12) and the KAWAKAMI algorithm (13). The 50 patients included 16 who had been reported previously (14). All tissue specimens were obtained by skin biopsy, and were fixed in 10% formalin, step-sectioned and stained with haematoxylin and eosin (H&E). A diagnosis of CPN requires the presence of histological necrotizing vasculitis, such as fibrinoid degeneration, nuclear dust, neutrophilic infiltration and erythrocyte extravasation in the lower dermis and the subcutaneous fat. The following tests were negative or within the normal range: antinuclear antibodies, virus serology including hepatitis A, B and C. Two skin biopsies were obtained from most of the patients for routine and direct IF (DIF) staining according to standard procedures. Immunological assessments were performed on sera collected at the same time as the skin biopsies. Serum was obtained from each patient when the disease was active and the patient was not undergoing immunosuppressive therapy. All serum samples were collected and were immediately centrifuged at 1,500 g for 30 min at 4°C in our laboratory. Serum samples were stored at  $-80^{\circ}\text{C}$  until analysis.

#### ANCA detection by direct ELISA and capture ELISA

Serum MPO-ANCA and PR3-ANCA were measured by routine ELISA (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol. Each sample was also tested for the Wieslab capture MPO-ANCA (Euro-Diagnostica, Malmö, Sweden) and the Wieslab capture PR3-ANCA (Euro-Diagnostica) according to the manufacturer's protocol.

#### Indirect immunofluorescence assay for ANCA

A commercially available IIF kit (Medical & Biological Laboratories) was used for ANCA detection according to the manufacturer's instructions. Briefly, ANCA detection by IIF was performed on ethanol-fixed and on formalin-fixed neutrophils. Serum samples were diluted in phosphate-buffered saline (PBS) and screened at a dilution of 1:20. Interpretation of immunofluorescence results was based on the characteristics of the ethanol- and the formalin-fixed specimens and included the following patterns: (i) cytoplasmic-ANCA (C-ANCA) occurred as coarse, speckled cytoplasmic fluorescence with accentuation between the nuclear lobes in ethanol-fixed and in formalin-fixed neutrophils; (ii) perinuclear-ANCA (P-ANCA) presented as homogeneous fluorescent staining of the perinuclear cytoplasm with nuclear extensions in ethanol-fixed neutrophils and as cytoplasmic staining in formalin-fixed neutrophils; (iii) atypical ANCA occurred as a fine rim-like staining of the nuclear periphery or a combined pattern characterized by heterogeneous cytoplasmic staining in ethanol-fixed neutrophils and as weak cytoplasmic fluorescence in formalin-fixed neutrophils.

The IIF results were read in a blinded manner by two experienced dermatologists and one expert researcher.

#### Serum levels of anti-LAMP-2 antibody

Serum anti-LAMP-2 antibody levels were determined using an ELISA kit (Nipro, Kusatsu, Siga, Japan). Microtitre plates (Nunc Immunoplate, Roskilde, Denmark) were coated with 100 µl LAMP-2 antigen (1.0 µg/ml) (PEP-039, Thermo Scientific, Waltham, MA, USA) in coating buffer overnight at 4°C. The plates were then washed 3 times and kept at room temperature for 3 h. A total of 100 µl sera, diluted 1:50 in PBS, was added to each well. The plates were incubated at 25°C for 1 h; after washing, anti-human IgG-alkaline phosphatase antibody produced in goat (Sigma, St Louis, MO, USA) diluted 1:10,000 was added. Incubation was then resumed for 1 h at 25°C. 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) in substrate buffer was used as a substrate, and colour development was measured spectrophotometrically at 405 nm. We examined sera from 34 healthy persons as normal control samples.

#### Detection of other ANCA-associated antibody levels in serum of patients with cutaneous polyarteritis nodosa

The Wieslab ANCA panel kit (Euro-Diagnostica) was used in direct ELISA screening tests for qualitative analysis of anti-azurocidin antibodies, anti-bactericidal permeability increasing protein (BPI) antibodies, anti-cathepsin G antibodies, anti-elastase antibodies, anti-lactoferrin antibodies and anti-lysozyme antibodies, according to the manufacturer's protocol. The wells of the microtitre strips were coated with these purified ANCA antigens. During the first incubation, specific antibodies in diluted serum bound to the antigen coating. The wells were then washed to remove unbound antibodies and other components. A conjugate of alkaline phosphatase-labelled antibodies to human IgG bound to the antibodies in the wells in this second incubation. After a further washing step, detection of specific antibodies was obtained by incubation with substrate solution.

The amount of bound antibodies correlates with the colour intensity and is measured in terms of absorbance.

#### Statistical analyses

Differences among qualitative results were compared using the  $\chi^2$  test. Differences among quantitative parameters between groups were assessed using the Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant. All data are expressed as means  $\pm$  SD. All analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA).

The experimental protocol was approved by the St Marianna University, and informed consent was obtained from all patients.

## RESULTS

### ANCA detection

The serum of all 50 CPN patients was negative for MPO-ANCA and PR3-ANCA by both routine direct ELISA and capture ELISA. In contrast, the IIF assay revealed ANCA in 42 (84.0%) of the 50 patients. Among those positive patients, P-ANCA pattern was found in 13 (30.9%) and atypical ANCA pattern in 29 (69.0%), but there was no evidence of C-ANCA pattern in any of the patients. The clinical, serological and DIF features of the P-ANCA pattern, atypical ANCA pattern, and no ANCA- detected using the IIF assay are listed in Table I. The mean age of the no ANCA-detected group was significantly higher than the P-ANCA group ( $p=0.0347$ ) and atypical ANCA group ( $p=0.019$ ). There was also a significantly higher ratio of men to women in the no ANCA-detected group compared with the P-ANCA group ( $p=0.0015$ ) and atypical ANCA group ( $p=0.041$ ). Among the skin manifestations of CPN, skin ulcers were found in significantly greater numbers within the no-detected group compared with the P-ANCA group ( $p=0.023$ ) and the atypical ANCA group ( $p=0.0127$ ). Biopsies obtained from 33 of the patients underwent DIF staining and we found 22 (66.7%) with C3 deposits in the affected arteries. C3 deposits were found in all 8 patients in the no ANCA-detected group. The ratio of C3 deposit was significantly higher compared with the atypical ANCA group ( $p=0.0455$ ).

### Serum levels of anti-LAMP-2 antibody in patients with cutaneous polyarteritis nodosa and microscopic polyangiitis

Serum anti-LAMP-2 antibody levels differed significantly between 50 patients with CPN (0.263 U/ml, median 0.210, quartile 0.133) and 8 patients with MPA (0.180 U/ml, median 0.16, quartile 0.085) ( $p=0.0102$ ; Fig. 1). Mean serum anti-LAMP-2 antibody level in the 34 healthy persons was 0.223 U/ml (median 0.206, quartile 0.109). A significantly elevated serum anti-LAMP-2 antibody level was measured in the P-ANCA

Table I. Comparison of clinical, serological and direct immunofluorescence (IF) features of the perinuclear anti-neutrophil cytoplasmic antibody (P-ANCA) pattern (13 patients), atypical ANCA pattern (29 patients), and no ANCA detected (8 patients) using indirect IF assay in 50 patients with cutaneous polyarteritis nodosa

Parameters	P-ANCA (n=13)	Atypical ANCA (n=29)	No ANCA detected (n=8)	Total (n=50)
Age, years, mean ± SD	44.31 ± 15.81	44.55 ± 16.26	60.50 ± 15.89*	47.04 ± 16.84
Gender, n (%)				
Female	12 (92.3)	19 (65.5)	2 (25.0)	33 (66.0)
Male	1 (7.7)	10 (34.5)	6 (75.0)*	17 (34.0)
Arthralgia, n (%)	8 (61.5)	21 (72.4)	7 (87.5)	36 (72.0)
Myalgia, n (%)	7 (53.8)	14 (48.3)	6 (75.0)	27 (54.0)
Skin ulcer, n (%)	7 (53.8)	15 (51.7)	8 (100.0)*	30 (60.0)
Purpura, n (%)	10 (76.9)	20 (69.0)	8 (100.0)	38 (76.0)
Livedo, n (%)	13 (100.0)	23 (79.3)	8 (100.0)	44 (88.0)
C3 deposit, n (%)	6 (66.7) n=9	10 (55.6) n=18	6 (100.0) n=6*	22 (66.7) n=33

\*p<0.05.

P-ANCA vs. No ANCA detected: Age p=0.0347; Men p=0.0015; Skin ulcer p=0.023; C3 deposit p=0.114.

Atypical ANCA vs. No ANCA detected: Age p=0.019; Men p=0.041; skin ulcer p=0.0127; C3 deposit p=0.0455.

SD: standard deviation.

group by IIF assay (0.288 U/ml, median 0.250, quartile 0.200) compared with the no ANCA-detected group (0.171 U/ml, median 0.169, quartile 0.020) (p=0.0147; Fig. 2). Similar trends were seen in serum anti-LAMP-2 antibody levels between the P-ANCA group and the 34 healthy persons (p=0.0287; Fig. 2). All 50 patients with CPN and 8 patients with MPA were negative for anti-azurocidin antibodies, anti-BPI antibodies, anti-cathepsin G antibodies, anti-elastase antibodies, anti-lactoferrin antibodies and anti-lysozyme.

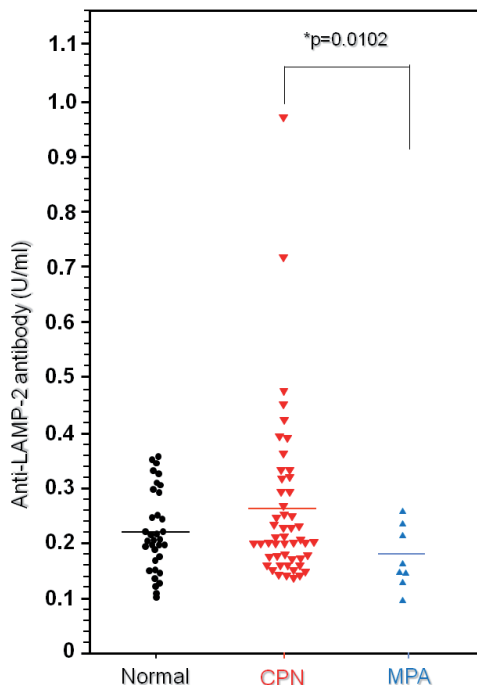


Fig. 1. Serum anti-lysosomal-associated membrane protein-2 (anti-LAMP-2) antibody levels in patients with cutaneous polyarteritis nodosa (CPN) and microscopic polyangiitis (MPA) by enzyme-linked immunoassay (ELISA). Normal: healthy persons. Serum anti-LAMP-2 antibody levels in 50 patients with CPN were significantly higher than in 8 patients with MPA (p=0.0102).

## DISCUSSION

Serum anti-LAMP-2 antibody levels differed significantly between 50 patients with CPN and 8 patients with MPA (p=0.0102). MPA is one of the primary systemic vasculitis that is MPO-ANCA positive. The data from the present study did not support a positive relationship between anti-LAMP-2 antibodies and MPA. Conventional direct ELISA for detection of ANCA often lacks sensitivity because epitopes of the target antigen are hidden by binding to the ELISA plate. A recently developed capture assay for detection of ANCA using monoclonal antibodies to capture the antigen has been found to be more sensitive than conventional ELISA (15). Neither MPO-ANCA nor PR3-ANCA was detected in any of the serum samples in CPN patients by direct ELISA or by capture ELISA. However, ANCA was detected in 42 (84.0%) of the 50 patients with CPN by IIF assay (13 were positive for the P-ANCA pattern and 29

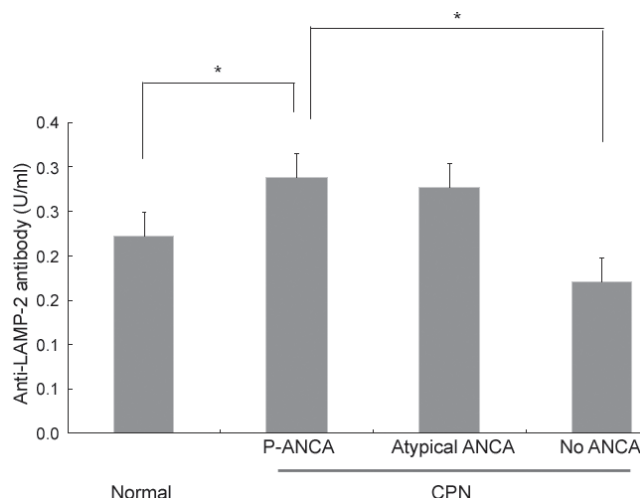


Fig. 2. Differences in serum anti-lysosomal-associated membrane protein-2 (anti-LAMP-2) antibody levels among perinuclear anti-neutrophil cytoplasmic antibody (P-ANCA), atypical ANCA, and no ANCA in human cutaneous polyarteritis nodosa. Normal: healthy persons. \*p<0.05.



for the atypical ANCA pattern). These findings suggest that antibodies against ANCA-specific antigens in neutrophils other than MPO and PR3 could be involved in the pathogenesis of CPN patients. Previous studies have indicated that ANCA target antigens include azurocidin, BPI, cathepsin G, elastase, lactoferrin, and lysozyme in addition to LAMP-2 (16–20). In the present study, we did not detect autoantibodies against azurocidin, BPI, cathepsin G, elastase, lactoferrin, and lysozyme antibodies in any of the sera from the CPN patients. In contrast, we found that CPN patients with a P-ANCA pattern detected by IIF had a significantly elevated serum anti-LAMP-2 antibody level compared with the no ANCA detected group and control subjects. Based on these findings, we propose that the anti-LAMP-2 antibody plays an important role in the pathogenesis of human CPN in the presence of P-ANCA.

Deposition of complement in the affected arteries could be involved in some cases of vasculitis including CPN. The ratio of C3 deposit within the necrotizing vasculitis, based on DIF staining of skin biopsy specimens in the no ANCA-detected group, was significantly higher than the P-ANCA group and atypical ANCA group among our CPN patients. The prevalence of skin ulcers in the no ANCA-detected group was significantly higher than the P-ANCA group and atypical ANCA group among our CPN patients. CPN patients in whom ANCA was not detected by IIF assay had a more frequent occurrence of complement deposit and skin ulcers than did CPN patients with ANCA. The underlying pathogenic mechanisms involving immune complexes appear to be different between no ANCA-detected patients and ANCA-detected patients. There was also a significantly higher trend in the age and men:women ratio. The present study, in combination with clinical and immunological observations, suggests that CPN may be a heterogeneous disease. Using the IIF assay to identify ANCA, patients with CPN can be categorized into ANCA positive and negative. This should be validated in larger studies involving a more detailed clinical investigation.

#### ACKNOWLEDGEMENTS

This work was supported by grants from the Scientific Research Fund of the Ministry of Health, Labour and Welfare, Japan (Grant-in-Aid for Scientific Research).

*The authors declare no conflicts of interest.*

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