A novel anti-neutrophil extracellular trap antibody targeting myosin light chain 6 in microscopic polyangiitis

Short title: Anti-MYL6 antibody as ANETA

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This study was conducted with the permission of the Ethical Committee of the Faculty of Health Sciences, Hokkaido University (permission nos. 15-90 and 18-34) and in adherence with the Declaration of Helsinki. Written informed consent was obtained from all patients and healthy volunteers enrolled in this study.

To the Editor:

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is characterized by ANCA production in the serum. This disease entity includes microscopic polyangiitis (MPA). Previous studies have revealed that neutrophils excessively activated by ANCA and other serum factors are critically involved in AAV pathogenesis.[1] In particular, neutrophil extracellular traps (NETs) released from activated neutrophils play a pivotal role in tissue destruction in AAV.

NETs are extracellular web-like substances that consist of unraveled DNA coating with antimicrobial proteins.[2] Although NETs play roles in capturing and killing microbes invading the hosts, they are subject to degradation due to a risk of self-injury.[3] The principal physiological degrader of NETs is DNase I in the serum.

Some MPA patients possessed antibodies to NETs [anti-NETs antibody (ANETA)],[4] and a part of ANETA can affect NETs generation[5] and degradation.[6] Serum NETs degradation activity was lower in MPA patients than healthy controls.[4] The NETs degradation activity was significantly recovered in some ANETA^{+} MPA sera after IgG depletion using a protein G column but not in ANETA^{-} MPA sera. These findings suggested that some ANETA have NETs degradation inhibitory activity.[6] In the previous study, such ANETA was present in 2 of the 19 MPA patients enrolled, whereas ANETA was present in 10 of the 19 MPA patients (5). To date, the following facts have been clarified: (1) the antigen recognized by the ANETA with NETs degradation inhibitory activity is an ANCA antigen because it is also present in the cytoplasm of neutrophils before NETs induction, and (2) the antigen is different from known ANCA antigens.[6]

ANETA with NETs degradation inhibitory activity could deteriorate NETs-related diseases, including MPA. This study aimed to identify the antigen. At first, western blotting (WB) was carried out using neutrophil lysates as antigens and IgG eluted from

sera using a protein G column as antibodies. Results demonstrated that a 17 kDa band was detected by ANETA IgG with NETs degradation inhibitory activity eluted from the two MPA sera but not ANETA IgG without NETs degradation inhibitory activity and IgG without ANETA (**Figure 1a**). A piece of gel corresponding to the 17 kDa band was cut from the sodium dodecyl sulfate (SDS)-polyacrylamide gel and subjected to nanoscale liquid chromatography coupled to tandem mass spectrometry. The antigen candidates are listed in **Table S1**. Based on the order of priority and molecular weight, myosin light chain 6 (MYL6) was most likely the antigen recognized by the ANETA with NETs degradation inhibitory activity. WB using a commercially available anti-MYL6 polyclonal antibody as a primary antibody revealed that MYL6 was present in NETs and neutrophil lysates (**Figure 1b**). In addition, when recombinant MYL6 was electrophoresed through an SDS-polyacrylamide gel and subjected to WB, only ANETA IgG with NETs degradation inhibitory activity reacted with the corresponding 17 kDa band. Furthermore, when the commercially available anti-MYL6 polyclonal antibody was allowed to react with phorbol myristate acetate (PMA)-induced NETs 30 min before digestion by DNase I, NETs degradation was significantly inhibited compared to when control rabbit IgG was applied instead (**Figure 2**). These findings were consistent with the observation of recovered NETs degradation activity in ANETA^{+} MPA sera after IgG depletion.[6]

Myosin is a hexameric ATPase cellular motor protein composed of two heavy chains, two nonphosphorylatable alkali light chains, and two phosphorylatable regulatory light chains.[7] MYL6 is one of the nonphosphorylatable alkali light chains. Myosin mediates the morphological alteration and movement of cells by interacting with F-actin. F-actin is a cytoskeletal filamentous protein formed by the polymerization of spherical G-actin as a monomer. Because G-actin can bind to DNase I and absorb its enzymatic activity,[8] it is hypothesized that the anti-MYL6 antibody could interfere with the

molecular association between myosin and F-actin. This might inhibit G-actin polymerization or promote F-actin degradation into G-actin. Consequently, the increased G-actin would absorb the NETs degradation activity of DNase I.

To the authors' knowledge, serum anti-MYL6 antibody in autoimmune diseases has not yet been described in the literature. Anti-MYL6 antibody was detected in 2 of the 19 MPA patients enrolled in the previous study.[6] Although it is speculated that the presence of anti-MYL6 antibody with NETs degradation inhibitory activity could aggravate NETs-related diseases, including MPA, the sample size was too small to lead to statistically significant conclusions. Therefore, a mass study using a Japanese nationwide cohort of AAV patients ($n > 100$)[9] is now in planning. Currently, anti-MYL6 antibody can be detected only by WB. Because WB is not a high-throughput process, it is desired to establish an enzyme-linked immunosorbent assay (ELISA) in which the recombinant MYL6 is immobilized. The correlation between the presence of anti-MYL6 antibody detected by ELISA and the clinical parameters of AAV patients is an important subject to be faced in the next project.

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Author Contributions

A.I. designed the research. M.Y., F.H., Y.N., and S.M. performed the experiments. All authors discussed the results and contributed to the preparation of the manuscript.

Disclosures

The authors declare no conflicts of interest.

Availability of data and materials

The data sheets used and/or analyzed in this study are available from the corresponding author on reasonable request.

References

1. Nakazawa D, Masuda S, Tomaru U, Ishizu A. Pathogenesis and therapeutic interventions for ANCA-associated vasculitis. Nat Rev Rheumatol 2019;15:91-101.
2. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303:1532-5.
3. Hakkim A, Furnrohr BG, Amann K, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A 2010;107:9813-8.
4. Nakazawa D, Shida H, Tomaru U, et al. Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. J Am Soc Nephrol 2014;25:990-7.
5. Shida H, Hashimoto N, Kusunoki Y, et al. Anti-neutrophil extracellular trap antibody in a patient with relapse of anti-neutrophil cytoplasmic antibody-associated vasculitis: a case report. BMC Nephrol 2018;19:145.
6. Hattanda F, Nakazawa D, Watanabe-Kusunoki K, et al. The presence of anti-neutrophil extracellular trap antibody in patients with microscopic polyangiitis. Rheumatology 2019;58:1293-8.
7. Vicente-Manzanares M, Ma X, Adelstein RS, Horwitz AR. Non-muscle myosin II takes centre stage in cell adhesion and migration. Nat Rev Mol Cell Biol 2009;10:778-90.
8. Lazarides E, Lindberg U. Actin is the naturally occurring inhibitor of deoxyribonuclease I. Proc Natl Acad Sci U S A 1974;71:4742-6.
9. Ishizaki J, Takemori A, Horie K, et al. Usefulness of tissue inhibitor of metalloproteinase 1 as a predictor of sustained remission in patients with antineutrophil cytoplasmic antibody-associated vasculitis. Arthritis Res Ther

2021;23:91.

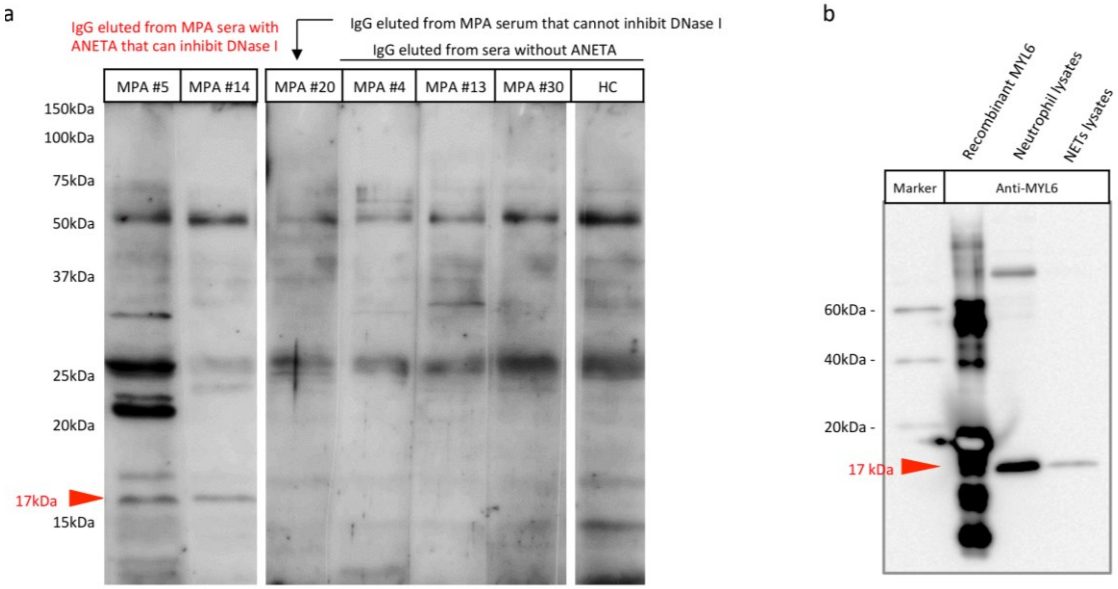

Figure 1. WB

(**a**) Neutrophil lysates from healthy volunteers (5 µl/lane) heated under reducing conditions were applied to 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). WB was carried out using IgG eluted from MPA sera containing ANETA with NETs degradation inhibitory activity (#5 and #14) as the primary antibody. For controls, IgG eluted from MPA sera containing ANETA without NETs degradation inhibitory activity (#20), MPA sera without ANETA (#4, #13, and #30), and healthy control (HC) sera were used. IgG was eluted from sera using Protein G HP SpinTrap (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions. (**b**) Recombinant MYL6 (Novus Biologicals, Centennial, CO, USA; 0.5 µg/lane), neutrophil lysates (5 µl/lane), and NETs lysates (5 µl/lane) heated under reducing conditions were applied to 15% SDS-PAGE. WB was carried out using a commercially available anti-MYL6 polyclonal antibody (Abcepta, San Diego, CA, USA) as the primary antibody.

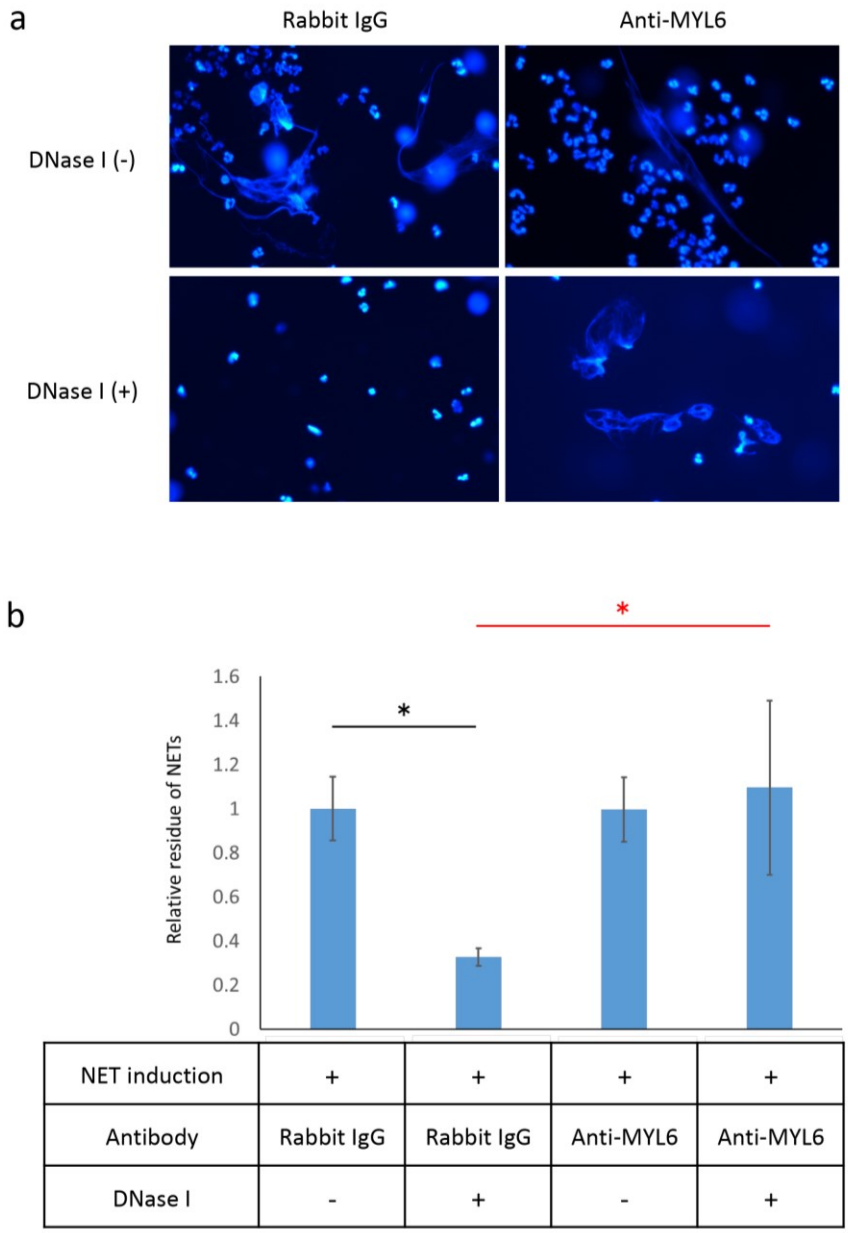

Figure 2. NETs degradation assay

(**a**) Peripheral blood neutrophils from healthy volunteers were seeded in the wells of four-well chamber slides (1×10^6/ml, 400 μl/well) and treated with 100 nM PMA for 3.5 h at 37°C. The anti-MYL6 polyclonal antibody (Abcepta, San Diego, CA, USA) or control rabbit IgG (Abcam, Cambridge, UK) was applied to the wells (1 μg/ml), and the chamber slides were allowed to sit at 37°C for 30 min. Thereafter, the medium was replaced by phosphate-buffered saline (PBS) with or without 1 U/ml DNase I, and the samples were incubated for 30 min at 37°C. After rinsing with PBS, the samples were fixed with 4% paraformaldehyde for 15 min at room temperature. After rinsing with PBS, the remaining samples were mounted with a 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting solution. Photomicrographs (magnification, ×200) were taken randomly (six fields per well of

chamber slides). Representative photomicrographs are shown. (**b**) The $DAPI^+$ area was measured using ImageJ version 1.50i (https://imagej.nih.gov/ij). The relative residue of NETs was calculated by setting the value of NETs without digestion under the presence of control rabbit IgG as 1. Experiments were repeated independently, and the reproducibility of results was confirmed. Data are the mean ± standard deviation. $*p < 0.05$ (one-way analysis of variance, followed by Tukey-Kramer's post hoc test).

Table S1. Candidates for the ANETA antigen

Protein	gene	MW	Score	Peptide	Coverage	Accession	*Note**
Peptidyl-prolyl cis-trans isomerase A	PPIA CYPA	18,012	444	13	70	P62937	*NP_066953.1*
Myosin light polypeptide 6	MYL6	16,961	362	14	76	P60660	*AAH06781.2*
Actin, cytoplasmic 1	ACTB	41,737	307	15	46	P60709	*NP_001092.1*
Triosephosphate isomerase	TPI1 TPI	30,791	243	8	43	P60174	*NP_000356.1* *NP_001152759.1*
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH GAPD	36,053	236	6	25	P04406	*CAA25833.1*
Cytidine deaminase	CDA CDD	16,185	229	4	40	P32320	*NP_001776.1*
hCG2016877, isoform CRA_c		43,255	217	6	17		*EAW74247.1*
Alpha-enolase	ENO1 ENO1L1	47,169	210	9	17	P06733	*NP_001419.1*
Adenylyl cyclase-associated protein 1	CAP1 CAP	51,901	157	5	15	Q01518	*NP_006358.1*
Annexin A1	ANXA1 ANX1	38,714	152	2	7	P04083	*NP_000691.1*
Lactotransferrin	LTF GIG12	78,182	152	3	5	P02788	*NP_002334.2*
Ubiquitin-conjugating enzyme E2 N	UBE2N BLU	17,138	102	4	36	P61088	*NP_003339.1*

Myosin-9	MYH9	226,532	101	2	1	P35579	*NP_002464.1*
Glucose-6-phosphate 1-dehydrogenase	G6PD	59,257	94	5	11	P11413	*NP_000393.4*
Matrix metalloproteinase-9	MMP9 CLG4B	78,458	83	2	3	P14780	*BAH13736.1* *NP_004985.2*
Arachidonate 5-lipoxygenase-activating protein	ALOX5AP FLAP	18,157	77	4	25	P20292	*NP_001620.2*
Glucose-6-phosphate isomerase	GPI	63,147	74	4	9	P06744	*BAG62702.1* *NP_000166.2*
Rho-related GTP-binding protein RhoG	RHOG ARHG	21,309	72	3	15	P84095	*AAM21121.1*
Rho GDP-dissociation inhibitor 1	ARHGDIA GDIA1	23,207	68	1	7	P52565	*NP_004300.1*
Transaldolase	TALDO1 TAL	37,540	65	1	3	P37837	*NP_006746.1*
Ras-related C3 botulinum toxin substrate 2	RAC2	21,429	64	3	20	P15153	*NP_002863.1*
Gelsolin	GSN	85,698	61	3	4	P06396	*NP_000168.1*
Endoplasmic reticulum resident protein 29	ERP29 C12orf8	28,993	58	1	4	P30040	*NP_006808.1*
Golgi-associated plant pathogenesis-related protein 1	GLIPR2 C9orf19	17,218	56	1	8	Q9H4G4	*BAC11019.1* *NP_071738.1*
Cell division control protein 42 homolog	CDC42	21,259	56	5	22	P60953	*NP_001782.1*
6-phosphogluconate dehydrogenase, decarboxylating	PGD PGDH	53,140	54	1	2	P52209	*NP_002622.2*

Ras-related protein Rab-3D	RAB3D GOV	24,267	54	1	6	O95716	*NP_004274.1*
Proteolipid protein 2	PLP2 A4	16,691	51	1	9	Q04941	*NP_002659.1*
Actin-related protein 2	ACTR2 ARP2	44,761	51	1	4	P61160	*EAW99911.1* *NP_005713.1*
Serpin B10	SERPINB10 PI10	45,403	48	2	6	P48595	*NP_005015.1*
Rab GDP dissociation inhibitor beta	GDI2 RABGDIB	50,663	44	2	5	P50395	*NP_001485.2*
Ubiquitin-conjugating enzyme E2 D2	UBE2D2 PUBC1	16,735	42	1	7	P62837	*NP_003330.1*
Proteasome subunit alpha type-7	PSMA7 HSPC	27,887	40	1	4	O14818	*NP_002783.1*
Protein S100-A9	S100A9 CAGB	13,242	39	3	20	P06702	*NP_002956.1*

*Accession in Mascot Search Results

MW, molecular weight