MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 39, Number 3, 2020

DOI: 10.1089/mab.2020.0010

Thr80 of Sheep Podoplanin Is a Critical Epitope of the Antisheep Podoplanin Monoclonal Antibody: PMab-256

Yukinari Kato,[1,2] Masato Sano,[1] Teizo Asano,[1] Yusuke Sayama,[1] and Mika K. Kaneko[1]

An antisheep podoplanin (sPDPN) monoclonal antibody (mAb), PMab-256, has recently been established. PMab-256 shows positive immunostaining for lymphatic endothelial cells, lung type I alveolar cells, and kidney podocytes. PDPN possesses three platelet-aggregation-stimulating (PLAG) domains, PLAG1, PLAG2, and PLAG3, and a PLAG-like domain (PLD). The binding epitope of many anti-PDPN mAbs is located in PLAG domains or PLD. The purpose of this study is to determine the binding epitope of PMab-256. Analysis of sPDPN deletion mutants revealed that the N-terminus of the PMab-256 epitope exists between amino acids 75 and 80 of sPDPN. Furthermore, analysis of sPDPN point mutations demonstrated that the critical epitope includes Thr80 of sPDPN, indicating that the PMab-256 epitope is in the PLD of sPDPN.

Keywords: sheep podoplanin, monoclonal antibody, epitope, PMab-256

Introduction

Sensitive and specific monoclonal antibodies (mAbs) against podoplanin (PDPN) of various species including human,[1] mouse,[2] and whale[3] are necessary for the analysis of expression and pathophysiological functions of PDPN. PDPN is used for discriminating lymphatic endothelial cells (LECs) from vascular endothelial cells[4] or type I alveolar cells from type II alveolar cells.[5] An antisheep podoplanin (sPDPN) mAb, PMab-256, was obtained using the Cell-Based Immunization and Screening (CBIS) method.[6–8] Like other typical anti-PDPN mAbs, PMab-256 demonstrated positive immunoreaction for LECs,[4] type I alveolar cells,[5] and kidney podocytes.[9]

The binding epitope of many anti-PDPN mAbs is located in three platelet aggregation-stimulating (PLAG) domains, PLAG1, PLAG2, and PLAG3,[10] at the N-terminus or a PLAG-like domain (PLDs)[3,11–14] in the middle of the PDPN protein. The purpose of this study was to determine the binding epitope of PMab-256.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). sPDPN mutation plasmids containing a RAP14 tag[15] were transfected into CHO-K1 cells using Lipofectamine LTX (Thermo Fisher Scientific, Inc., Waltham, MA). The RAP14 tag comprises 14 amino acids (aa), (DMVNPGLEDRIEDL), and is recognized by PMab-2 mAb[16] and LpMab-7.[1] Cells transiently transfected with deletion or point mutations were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Production of sPDPN mutants

Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding sPDPN (accession no.: XM_004013802.4) was subcloned into the pCAG vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and an RAP14 tag was added at the N-terminus. Deletion mutants of the sPDPN sequence were produced using a HotStar HiFidelity Polymerase Kit (Qiagen, Inc., Hilden, Germany) with oligonucleotides. Substitutions of aa with alanine in the sPDPN sequence were produced by QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies, Inc., Santa Clara, CA). Polymerase chain reaction fragments bearing

[1]Department of Antibody Drug Development, Graduate School of Medicine, Tohoku University, Sendai, Japan.
[2]New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

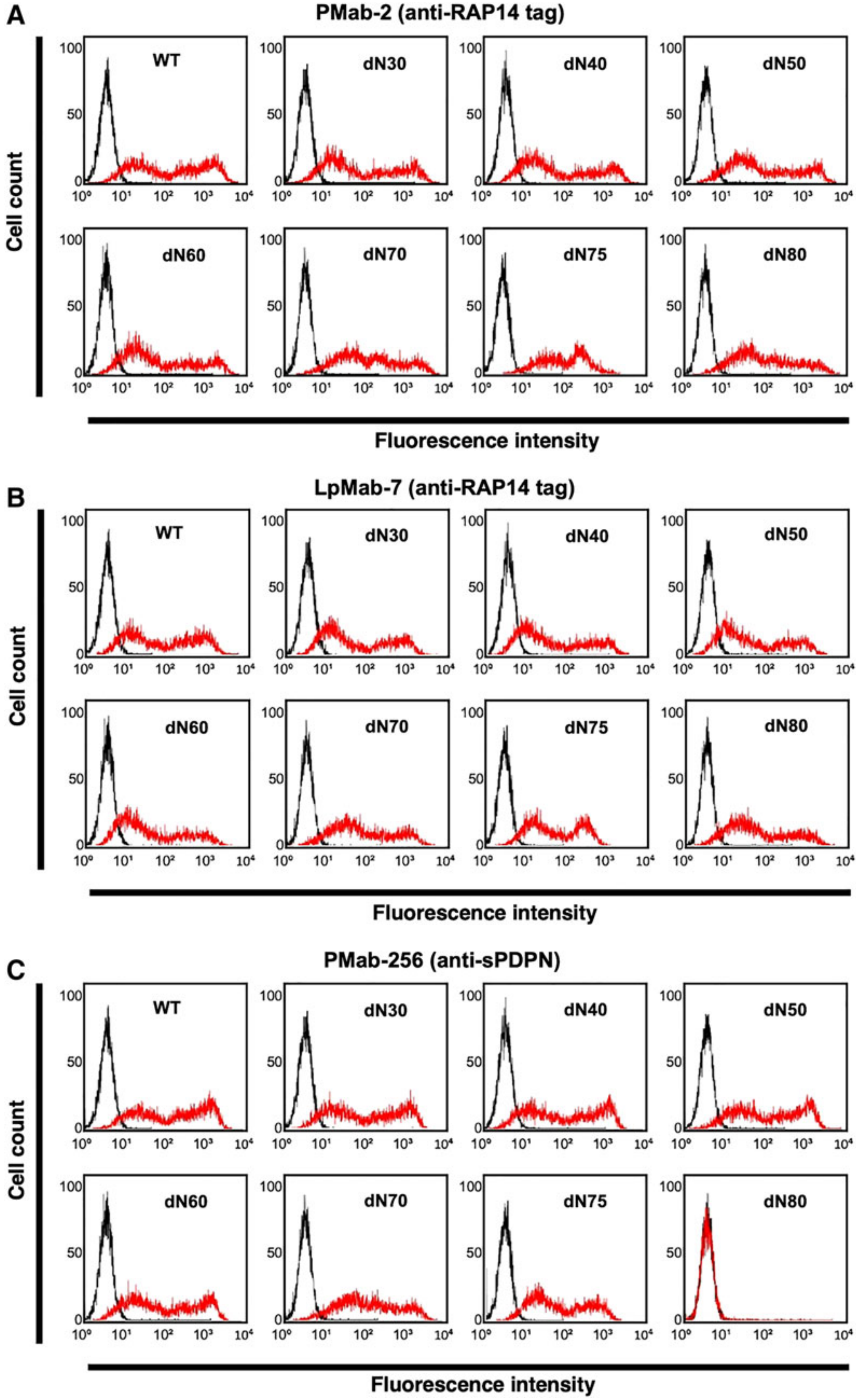

FIG. 1. Epitope mapping of PMab-256 using deletion mutations. **(A)** Deletion mutants of sPDPN were analyzed using flow cytometry. Mutants were incubated with PMab-2 (anti-RAP14 tag mAb; red line) or buffer control (black line) for 30 minutes at 4°C, followed by staining with secondary antibodies. **(B)** Deletion mutants of sPDPN were analyzed using flow cytometry. Mutants were incubated with LpMab-7 (anti-RAP14 tag mAb; red line) or buffer control (black line) for 30 minutes at 4°C, followed by staining with secondary antibodies. **(C)** Deletion mutants of sPDPN were analyzed using flow cytometry. Mutants were incubated with PMab-256 (anti-sPDPN mAb; red line) or buffer control (black line) for 30 minutes at 4°C, followed by staining with secondary antibodies. mAb, monoclonal antibody; sPDPN, sheep podoplanin.

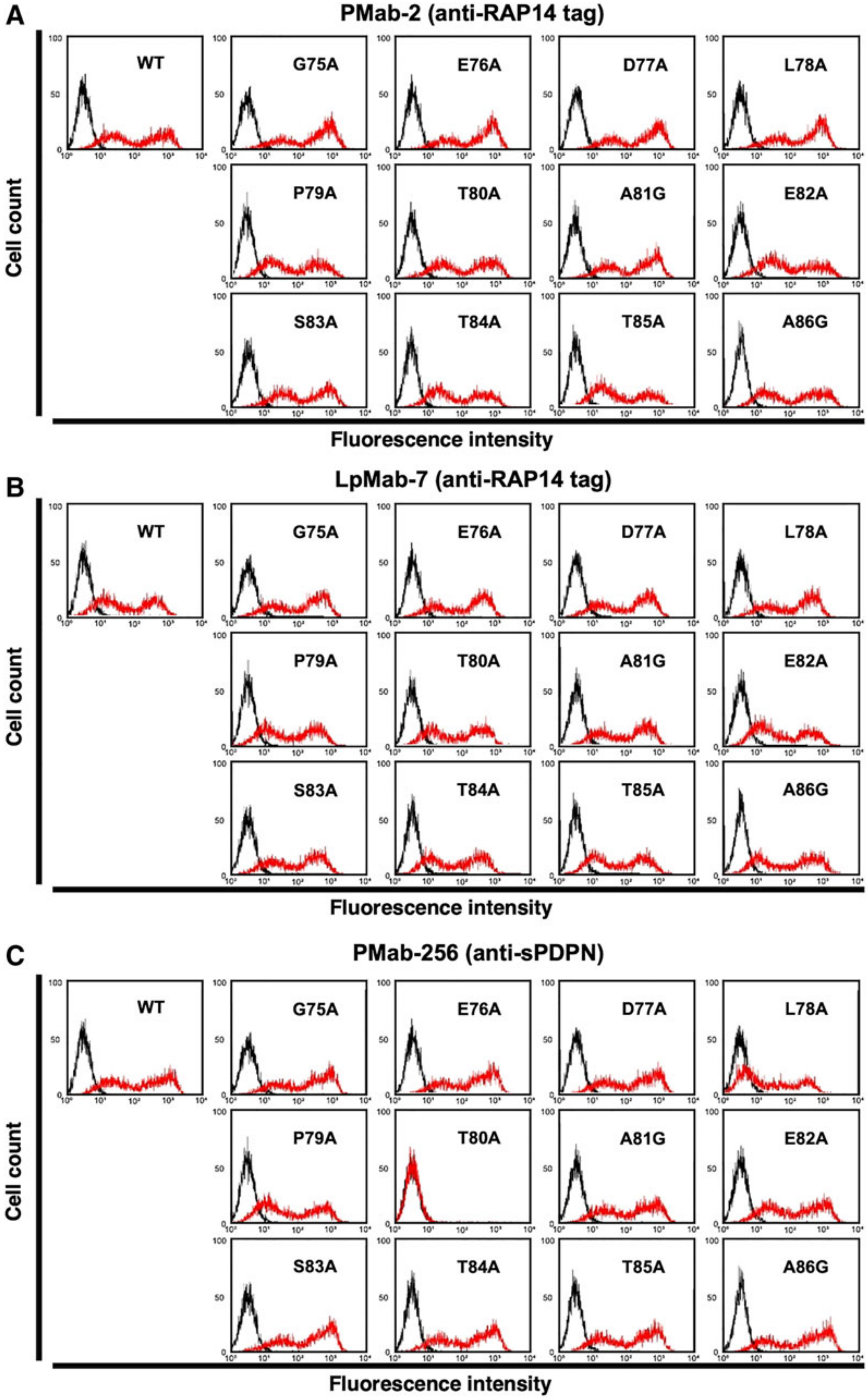

FIG. 2. Epitope mapping of PMab-256 using point mutations. **(A)** Transient point mutants expressing G75A, E76A, D77A, L78A, P79A, T80A, A81G, E82A, S83A, T84A, T85A, and A86G of sPDPN were incubated with PMab-2 (anti-RAP14 tag; red line), or buffer control (black line) for 30 minutes at 4°C, followed by staining with secondary antibodies. **(B)** Transient point mutants expressing G75A, E76A, D77A, L78A, P79A, T80A, A81G, E82A, S83A, T84A, T85A, and A86G of sPDPN were incubated with LpMab-7 (anti-RAP14 tag; red line) or buffer control (black line) for 30 minutes at 4°C, followed by staining with secondary antibodies. **(C)** Transient point mutants expressing G75A, E76A, D77A, L78A, P79A, T80A, A81G, E82A, S83A, T84A, T85A, and A86G of sPDPN were incubated with PMab-256 (anti-sPDPN mAb; red line) or buffer control (black line) for 30 minutes at 4°C, followed by staining with secondary antibodies.

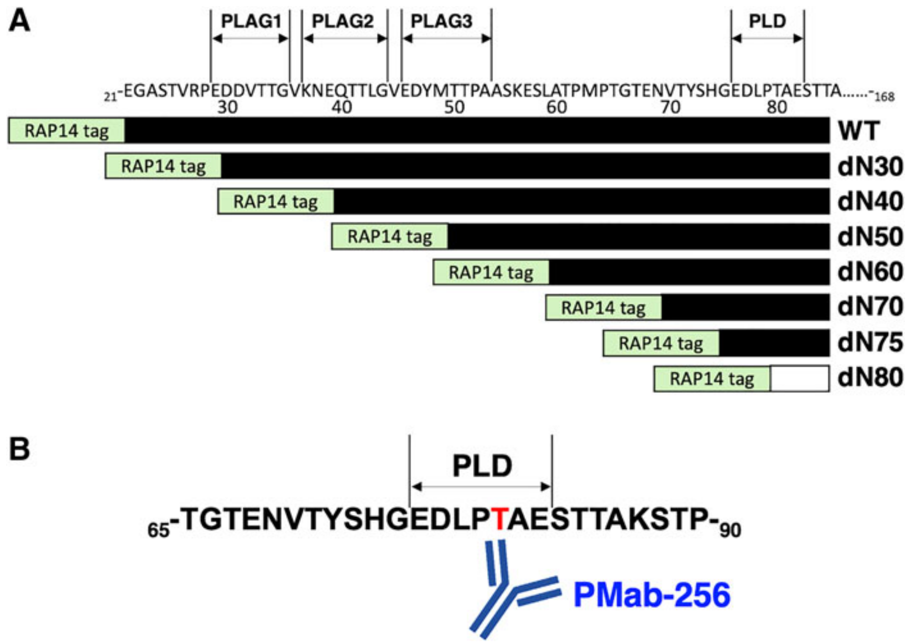

FIG. 3. Schematic illustration of the epitope recognized by PMab-256. **(A)** Illustration of WT sPDPN and its seven deletion mutants: dN30, dN40, dN50, dN60, dN70, dN75, and dN80. Black bars: positive reactions of PMab-256. White bar: negative reaction of PMab-256. **(B)** A red amino acid indicates a critical epitope of PMab-256. PLD, platelet-aggregation-stimulating-like domain; WT, wild type.

desired mutations were inserted into the pCAG vector using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan).

Flow cytometry

Transiently transfected CHO-K1 cells were detached by 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.) and collected using 10% FBS in RPMI 1640 medium. After washing with 0.1% bovine serum albumin in phosphate-buffered saline, cells were incubated with anti-sPDPN antibody (PMab-256; 1 μg/mL) and an anti-RAP14 tag antibody (PMab-2; 1 μg/mL or LpMab-7; 1 μg/mL) for 30 minutes at 4°C. Alexa Fluor 488-conjugated antimouse IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA) was added to each cell suspension and incubation continued for 30 minutes at 4°C. Fluorescence data were collected and analyzed using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Results

We produced seven deletion mutants of sPDPN in CHO-K1 cells, namely, dN30, with aa 30–168 deletion; dN40, aa 40–168; dN50, aa 50–168; dN60, aa 60–168; dN70, aa 70–168; dN75, aa 75–168; dN80, aa 80–168; or wild type (WT) sPDPN, aa 27–168. All deletion mutants and WT containing the N-terminal RAP14 tag were recognized by PMab-2 or LpMab-7 (anti-RAP14 tag mAbs), indicating that the expression level of each construct is high (Fig. 1A). PMab-256 recognized dN30, dN40, dN50, dN60, dN70, and dN75, but not dN80 (Fig. 1B), suggesting that the N-terminus of the PMab-256 epitope exists between sPDPN aa 75 and 80.

Next, a series of point mutants of sPDPN, G75A, E76A, D77A, L78A, P79A, T80A, A81G, E82A, S83A, T84A, T85A, and A86G, were obtained. PMab-2 and LpMab-7 reacted with all point mutants (Fig. 2A). By contrast, PMab-256 did not react with T80A (Fig. 2B). Thr80 of sPDPN is essential for PMab-256 binding (Fig. 3).

Discussion

A variety of mAbs against pig,[17,18] horse,[19,20] Tasmanian devil,[21] alpaca,[22] tiger,[23] whale,[24] goat,[25,26] bear,[27,28] and sheep[29] PDPNs using the CBIS method are available.[6–8] An anti-sPDPN mAb PMab-256 is useful for immunohistochemical analyses using formalin-fixed paraffin-embedded tissues of sheep for detecting LECs of many organs, type I alveolar cells, and renal epithelial cells.[29] PMab-256 is also useful for Western blot analysis, indicating that PMab-256 recognizes sPDPN that is fixed in formalin and denatured by sodium dodecyl sulfate. Furthermore, PMab-256 detects sPDPN in flow cytometry.

PLAG and PLD domains bind C-type lectin-like receptor-2 and induce platelet aggregation and cancer metastasis.[30] Anti-PDPN mAbs, epitopes of which are located in PLAG domains or PLD, could neutralize platelet aggregation.[30] The epitope of PMab-256 is also located in PLD of sPDPN. PMab-256 is thus not only a useful mAb for research but also could be a functional mAb with the ability to neutralize induction of platelet aggregation.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported, in part, by AMED under Grant Nos. JP20am0401013 (Y.K.), JP20am0101078 (Y.K.), and JP20ae0101028 (Y.K.), and by JSPS KAKENHI Grant No. 17K07299 (M.K.K.) and Grant No. 19K07705 (Y.K.).

References

1. Kato Y, and Kaneko MK: A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. Sci Rep 2014;4:5924.
2. Yamada S, Itai S, Kaneko MK, Konnai S, and Kato Y: Epitope mapping of anti-mouse podoplanin monoclonal antibody PMab-1. Biochem Biophys Rep 2018;15:52–56.
3. Sayama Y, Sano M, Kaneko MK, and Kato Y: Epitope analysis of an anti-whale podoplanin monoclonal antibody, PMab-237, using flow cytometry. Monoclon Antib Immunodiagn Immunother 2020;39:17–22.
4. Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, and Kerjaschki D: Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: Podoplanin as a specific marker for lymphatic endothelium. Am J Pathol 1999;154:385–394.
5. Rishi AK, Joyce-Brady M, Fisher J, Dobbs LG, Floros J, VanderSpek J, Brody JS, and Williams MC: Cloning, characterization, and development expression of a rat lung alveolar type I cell gene in embryonic endodermal and neural derivatives. Dev Biol 1995;167:294–306.
6. Itai S, Fujii Y, Nakamura T, Chang YW, Yanaka M, Saidoh N, Handa S, Suzuki H, Harada H, Yamada S, Kaneko MK, and Kato Y: Establishment of CMab-43, a sensitive and specific anti-CD133 monoclonal antibody, for immunohistochemistry. Monoclon Antib Immunodiagn Immunother 2017;36:231–235.
7. Yamada S, Itai S, Nakamura T, Yanaka M, Chang YW, Suzuki H, Kaneko MK, and Kato Y: Monoclonal antibody L1Mab-13 detected human PD-L1 in lung cancers. Monoclon Antib Immunodiagn Immunother 2018;37: 110–115.
8. Yamada S, Itai S, Nakamura T, Yanaka M, Kaneko MK, and Kato Y: Detection of high CD44 expression in oral cancers using the novel monoclonal antibody, C44Mab-5. Biochem Biophys Rep 2018;14:64–68.
9. Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, and Kerjaschki D: Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol 1997;151:1141–1152.
10. Kaneko MK, Kato Y, Kitano T, and Osawa M: Conservation of a platelet activating domain of Aggrus/podoplanin as a platelet aggregation-inducing factor. Gene 2006;378: 52–57.
11. Kato Y, Sayama Y, Sano M, and Kaneko MK: Epitope analysis of an antihorse podoplanin monoclonal antibody PMab-219. Monoclon Antib Immunodiagn Immunother 2019;38:266–270.
12. Kato Y, Takei J, Furusawa Y, Sayama Y, Sano M, Konnai S, Kobayashi A, Harada H, Takahashi M, Suzuki H, Yamada S, and Kaneko MK: Epitope mapping of anti-bear podoplanin monoclonal antibody PMab-247. Monoclon Antib Immunodiagn Immunother 2019;38:230–233.
13. Sano M, Kaneko MK, and Kato Y: Epitope mapping of monoclonal antibody PMab-233 against tasmanian devil podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:261–265.
14. Sayama Y, Sano M, Furusawa Y, Kaneko MK, and Kato Y: Epitope mapping of PMab-225 an anti-alpaca podoplanin monoclonal antibody using flow cytometry. Monoclon Antib Immunodiagn Immunother 2019;38:255–260.
15. Kato Y, Yamada S, Itai S, Kobayashi A, Konnai S, and Kaneko MK: Immunohistochemical detection of sheep podoplanin using an antibovine podoplanin monoclonal antibody PMab-44. Monoclon. Antib. Immunodiagn. Immunother 2018;37:265–268.
16. Oki H, Honma R, Ogasawara S, Fujii Y, Liu X, Takagi M, Kaneko MK, and Kato Y: Development of sensitive monoclonal antibody PMab-2 against rat podoplanin. Monoclon Antib Immunodiagn Immunother 2015;34:396–403.
17. Kato Y, Yamada S, Furusawa Y, Itai S, Nakamura T, Yanaka M, Sano M, Harada H, Fukui M, and Kaneko MK: PMab-213: A monoclonal antibody for immunohistochemical analysis against pig podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:18–24.
18. Furusawa Y, Yamada S, Itai S, Sano M, Nakamura T, Yanaka M, Fukui M, Harada H, Mizuno T, Sakai Y, Takasu M, Kaneko MK, and Kato Y: PMab-210: A monoclonal antibody against pig podoplanin monoclon. Antib Immunodiagn Immunother 2019;38:30–36.
19. Furusawa Y, Yamada S, Itai S, Sano M, Nakamura T, Yanaka M, Handa S, Mizuno T, Maeda K, Fukui M, Harada H, Kaneko MK, and Kato Y: Establishment of monoclonal antibody PMab-202 against horse podoplanin. Monoclon Antib Immunodiagn Immunother 2018;37:233–237.
20. Kato Y, Yamada S, Itai S, Kobayashi A, Konnai S, and Kaneko MK: Anti-horse podoplanin monoclonal antibody PMab-219 is useful for detecting lymphatic endothelial cells by immunohistochemical analysis. Monoclon Antib Immunodiagn Immunother 2018;37:272–274.
21. Furusawa Y, Yamada S, Itai S, Nakamura T, Takei J, Sano M, Harada H, Fukui M, Kaneko MK, and Kato Y: Establishment of a monoclonal antibody PMab-233 for immunohistochemical analysis against Tasmanian devil podoplanin. Biochem Biophys Rep 2019;18:100631.
22. Kato Y, Furusawa Y, Yamada S, Itai S, Takei J, Sano M, and Kaneko MK: Establishment of a monoclonal antibody PMab-225 against alpaca podoplanin for immunohistochemical analyses. Biochem Biophys Rep 2019;18:100633.
23. Furusawa Y, Kaneko MK, Nakamura T, Itai S, Fukui M, Harada H, Yamada S, and Kato Y: Establishment of a monoclonal antibody PMab-231 for tiger podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38: 89–95.
24. Kato Y, Furusawa Y, Itai S, Takei J, Nakamura T, Sano M, Harada H, Yamada S, and Kaneko MK: Establishment of an anticetacean podoplanin monoclonal antibody PMab-237 for immunohistochemical analysis. Monoclon Antib Immunodiagn Immunother 2019;38:108–113.
25. Furusawa Y, Yamada S, Nakamura T, Sano M, Sayama Y, Itai S, Takei J, Harada H, Fukui M, Kaneko MK, and Kato Y: PMab-235: A monoclonal antibody for immunohistochemical analysis against goat podoplanin. Heliyon 2019;5: e02063.
26. Takei J, Itai S, Harada H, Furusawa Y, Miwa T, Fukui M, Nakamura T, Sano M, Sayama Y, Yanaka M, Handa S, Hisamatsu K, Nakamura Y, Yamada S, Kaneko K, M., and Kato Y: Characterization of anti-goat podoplanin monoclonal antibody PMab-235 using immunohistochemistry against goat tissues. Monoclon Antib Immunodiagn Immunother 2019; DOI: 10.1089/mab.2019.0022.
27. Takei J, Furusawa Y, Yamada S, Nakamura T, Sayama Y, Sano M, Konnai S, Kobayashi A, Harada H, Kaneko MK,

and Kato Y: PMab-247 detects bear podoplanin in immunohistochemical analysis. Monoclon Antib Immunodiagn Immunother 2019;38:171–174.
28. Takei J, Yamada S, Konnai S, Ishinazaka T, Shimozuru M, Kaneko MK, and Kato Y: PMab-241 specifically detects bear podoplanin of lymphatic endothelial cells in the lung of brown bear. Monoclon Antib Immunodiagn Immunother 2019;38:282–284.
29. Kato Y, Furusawa Y, Sano M, Takei J, Nakamura T, Yanaka M, Okamoto S, Handa S, Komatsu Y, Asano T, Sayama Y, and Kaneko MK: Development of an anti-sheep podoplanin monoclonal antibody PMab-256 for immunohistochemical analysis of lymphatic endothelial cells. Monoclon Antib Immunodiagn Immunother 2020; 39:82–90.
30. Kato Y, Kaneko MK, Kunita A, Ito H, Kameyama A, Ogasawara S, Matsuura N, Hasegawa Y, Suzuki-Inoue K, Inoue O, Ozaki Y, and Narimatsu H: Molecular analysis of the pathophysiological binding of the platelet aggregation-inducing factor podoplanin to the C-type lectin-like receptor CLEC-2. Cancer Sci 2008;99:54–61.

Address correspondence to:
Yukinari Kato
New Industry Creation Hatchery Center
Tohoku University
2-1 Seiryo-machi
Aoba-ku
Sendai 980-8575
Japan

E-mail: yukinarikato@med.tohoku.ac.jp

Received: March 13, 2020
Accepted: April 19, 2020