

Epitope Mapping of Monoclonal Antibody PMab-52 Against Cat Podoplanin

Yao-Wen Chang,¹ Mika K. Kaneko,¹ Shinji Yamada,¹ and Yukinari Kato^{1,2}

The mucin-type membrane glycoprotein podoplanin (PDPN) is frequently overexpressed in numerous malignant cancers, including squamous cell carcinoma, germinal neoplasia, mesothelioma, lung cancer, oral cancer, and brain tumor. PDPN expression is strongly associated with cancer progression and poor prognosis. Furthermore, PDPN binds to C-type lectin-like receptor 2 (CLEC-2) on platelets, followed by PDPN-mediated platelet aggregation to facilitate tumor metastasis. We have previously reported a novel anti-cat PDPN (cPDPN) monoclonal antibody (mAb), PMab-52, which specifically detects cPDPN using flow cytometry analysis and successfully identifies cPDPN in feline squamous cell carcinomas. However, the specific binding epitope of cPDPN for PMab-52 remains unelucidated. In this study, a series of deletion or point mutants of cPDPN were utilized for investigating the binding epitopes of PMab-52 using flow cytometry and Western blotting. The findings of this study revealed that the critical epitopes of platelet aggregation-stimulating domain 4 (PLAG4) of cPDPN are responsible for the binding of PMab-52 to cPDPN.

Keywords: cat podoplanin, monoclonal antibody, epitope

Introduction

PODOPLANIN (PDPN/T1 α /AGGRUS), a highly *O*-glycosylated transmembrane glycoprotein,⁽¹⁻³⁾ is extensively distributed in normal tissues and cells, including kidney podocytes, lung alveolar cells, lymphatic endothelial cells, myofibroblasts, mesothelial cells, heart, and central nervous system.⁽⁴⁻⁸⁾ Versatile physiological functions of PDPN have been reported to play crucial roles in embryonic cardiac development,^(8,9) blood/lymphatic vessel separation,^(10,11) and high endothelial venule integrity.⁽¹²⁾ However, a high level of PDPN expression has been observed in several malignant tumors, including brain tumors,^(13,14) oral cancers,⁽¹⁵⁾ lung cancers,⁽¹⁶⁾ melanomas,⁽¹⁷⁾ mesotheliomas,⁽⁵⁾ breast cancers,^(18,19) central nervous system tumors,⁽²⁰⁾ and osteosarcomas.⁽²¹⁻²³⁾ Clinical data also indicated the expression of PDPN to be correlated with poor prognosis and tumor malignancy in lung carcinomas, oral squamous cell carcinomas, and breast cancers.^(8,24-27) Furthermore, PDPN facilitates hematogenous metastasis by eliciting tumor cell-induced platelet aggregation response through its interaction with platelet C-type lectin-like receptor 2 (CLEC-2).⁽²⁸⁻³¹⁾ These pieces of evidence imply the importance of developing anti-PDPN monoclonal antibodies (mAbs) for cancer therapeutic treatment.

We have recently established a novel PMab-52 mAb, which specifically detects cat PDPN (cPDPN) using flow

cytometry analysis and successfully recognizes cPDPN in feline squamous cell carcinomas.⁽³²⁾ However, the specific binding region of cPDPN for PMab-52 remains to be elucidated. In this study, we investigated the binding epitopes of PMab-52 by analyzing a series of deletion or point mutants of cPDPN using flow cytometry and Western blotting.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cPDPN mutation plasmids containing MAP tag were transfected into CHO-K1 cells using Lipofectamine LTX (Thermo Fisher Scientific, Inc., Waltham, MA). Transiently transfected cells with delete/point-mutants were cultured in 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 μ g/mL of streptomycin, and 25 μ g/mL of amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Production of cPDPN mutants

The cDNA of cPDPN was subcloned into a pCAG vector (Wako Pure Chemical Industries Ltd., Osaka, Japan), and an

¹Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.

²New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

MAP tag was added at the N-terminus. Deletion mutants of cPDPN sequence were performed using a HotStar HiFidelity PCR (Qiagen, Inc., Hilden, Germany) with oligonucleotides containing the desired mutations. Substitutions of amino acids to alanine in cPDPN sequence were conducted by QuikChange lightning site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA). PCR fragments bearing the desired mutations were inserted into pCAG vector using In-Fusion PCR cloning kit (Clontech, Palo Alto, CA).

Flow cytometry

Transiently transfected CHO-K1 cells were detached by 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) (Nacalai Tesque, Inc.) and collected in RPMI 1640 medium. After washing with 0.1% bovine serum albumin /phosphate-buffered saline, the cells were incubated with anti-cPDPN an-

tibody (PMab-52; 1 $\mu\text{g}/\text{mL}$) or control anti-MAP tag antibody (PMab-1; 1 $\mu\text{g}/\text{mL}$) for 30 minutes at 4°C followed by treatment with Alexa Fluor 488-conjugated antimouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA) and Oregon Green-conjugated antirat IgG (1:1000; Thermo Fisher Scientific, Inc.), respectively. Fluorescence data were collected using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Western blot analysis

Whole cell lysates of the deletion and point mutants were collected, lysed, and then boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.). The extracted proteins were electrophoresed using 5%–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Wako Pure Chemical Industries Ltd.) and transferred to polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt,

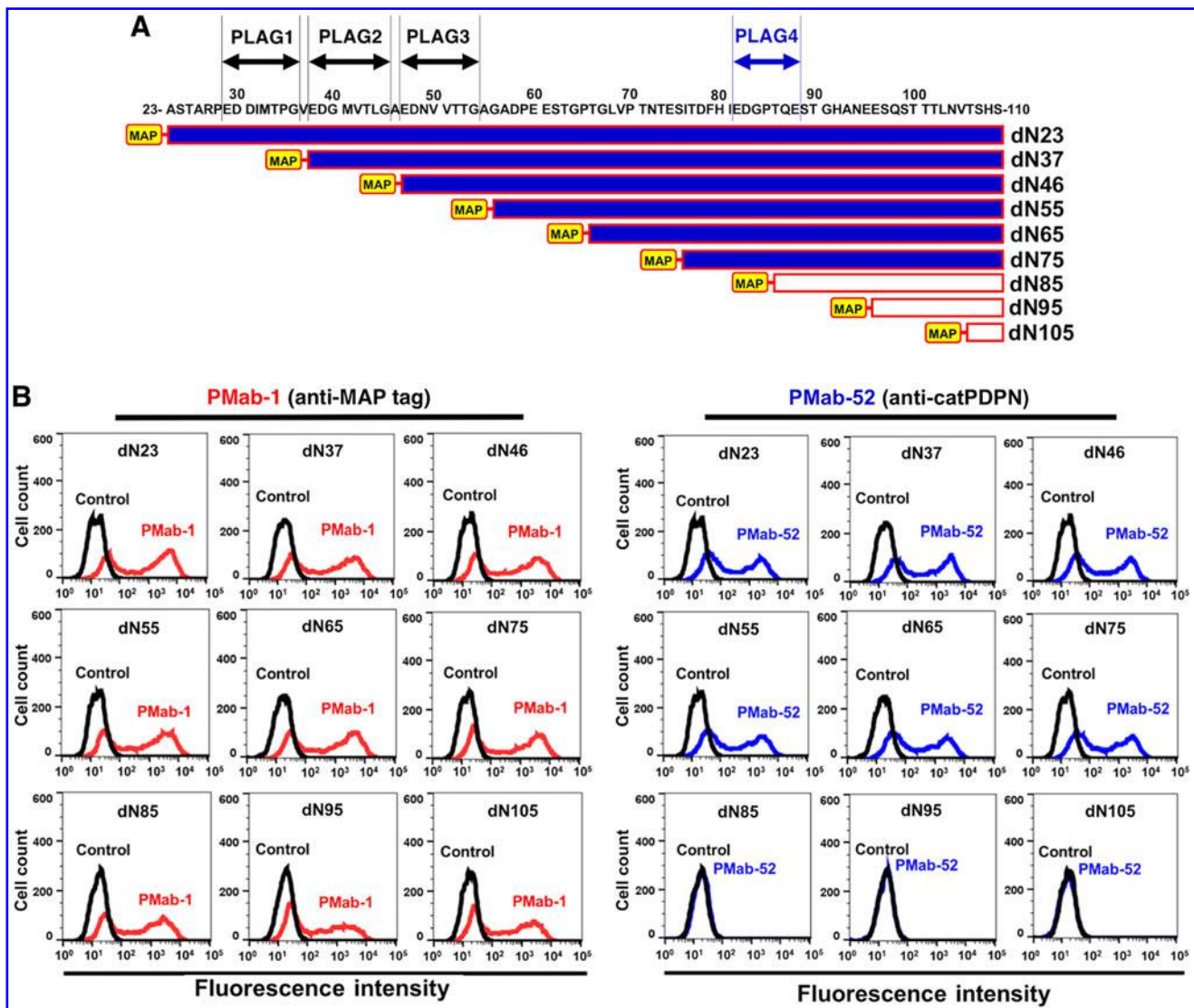


FIG. 1. Epitope mapping of PMab-52 using deletion mutants of cPDPN. **(A)** Illustration of nine cPDPN deletion mutants of dN23, dN37, dN46, dN55, dN65, dN75, dN85, dN95, and dN105. **(B)** Deletion mutants of cPDPN were analyzed using flow cytometry. Deletion mutants were expressed on CHO-K1 cells and were then incubated with anti-MAP tag PMab-1 (left panel, red line), PMab-52 (right panel, blue line), or buffer control (black line) for 30 minutes at 4°C, followed by treatment with corresponding secondary antibodies. CHO, Chinese hamster ovary; cPDPN, cat PDPN; PLAG4, platelet aggregation-stimulating domain 4.

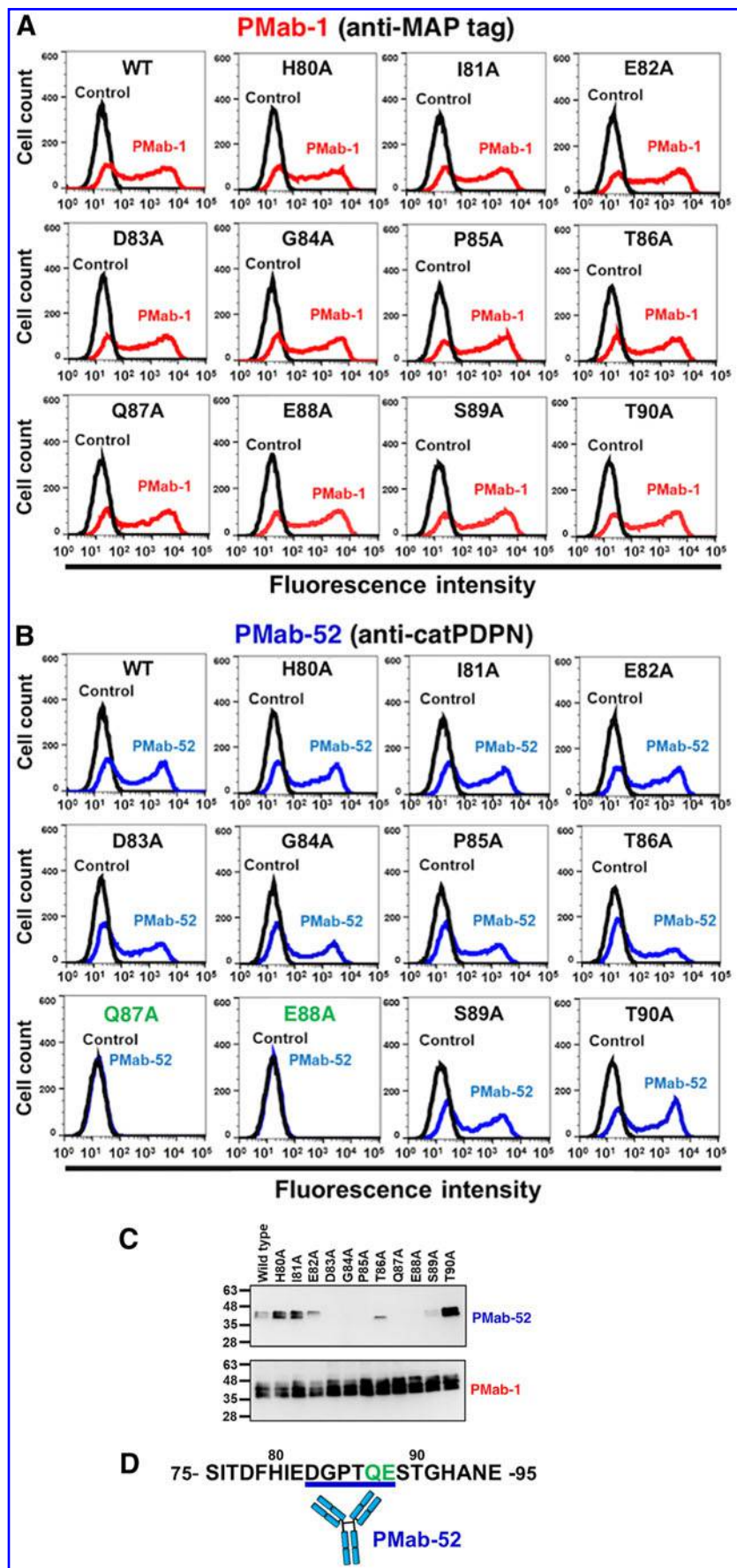


FIG. 2. Epitope mapping of PMab-52 using point mutants of cPDPN. **(A, B)** Flow cytometry. Transient point mutants expressing H80A, I81A, E82A, D83A, G84A, P85A, T86A, Q87A, E88A, S89A, and T90A of cPDPN reacted with PMab-1 **(A)** or PMab-52 **(B)** for 30 minutes at 4°C, followed by treatment with corresponding secondary antibodies. **(C)** The cell lysates from point mutants were collected for Western blotting with anti-cPDPN (PMab-52) antibody. The anti-MAP tag (PMab-1) antibody was used as a control. **(D)** Schematic illustration of the epitope recognized by PMab-52.

Germany). The membranes were blocked with 4% skim milk (Nacalai Tesque, Inc.) for 1 hour and then were incubated with 1 $\mu\text{g}/\text{mL}$ of PMab-52 and 1 $\mu\text{g}/\text{mL}$ of anti-MAP tag (PMab-1), then with peroxidase-conjugated antimouse and antirat IgG (1:1000; Agilent Technologies, Inc.), respectively. The proteins were finally detected using ImmunoStar LD (Wako Pure Chemical Industries Ltd.) with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Results and Discussion

We have previously established a novel PMab-52 mAb, which can be efficiently utilized for the immunohistochemical detection of cPDPN in normal feline tissues, including kidney, lung, and rectum.⁽³²⁾ Furthermore, PMab-52 specifically recognized cPDPN expression in feline squamous cell carcinomas, including carcinomas of the mouth floor, skin, ear, and tongue.⁽³³⁾ Flow cytometry and Western blotting analyses also revealed that PMab-52 specifically detects only cPDPN, not PDPNs of other species such as humans, dogs, cattle, rabbits, and mice.⁽³²⁾ On the basis of these results, the epitope mapping of PMab-52 could be beneficial in uncovering the pathophysiological function of cPDPN in feline squamous cell carcinomas and cPDPN-related antibody-based therapy.

First, we constructed nine deletion mutants of cPDPN (Fig. 1A). Transient transfections of cPDPN-mutant clones were produced using CHO-K1 cells, including dN23 (corresponding to 23–163 amino acids [aa]); dN37 (corresponding to 37–163 aa); dN46 (corresponding to 46–163 aa); dN55 (corresponding to 55–163 aa); dN65 (corresponding to 65–163 aa); dN75 (corresponding to 75–163 aa); dN85 (corresponding to 85–163 aa); dN95 (corresponding to 95–163 aa); and dN105 (corresponding to 105–163 aa). All deletion mutants of cPDPN contain N-terminal MAP tags and were analyzed using flow cytometry for epitope mapping of PMab-52. PMab-1 (anti-MAP tag mAb) detected all deletion mutants of cPDPN, including dN23, dN37, dN46, dN55, dN65, dN75, dN85, dN95, and dN105 (Fig. 1B, left). On the contrary, PMab-52 lost the reaction with dN85, dN95, and dN105 (Fig. 1B, right). Further experiments revealed that PMab-52 could detect deletion mutants of dN80 of cPDPN (data not shown). These results imply that the epitope-binding region of PMab-52 is located between the 80th and 90th amino acids, which contain new identified platelet aggregation-stimulating domain 4 (PLAG4), which plays a crucial role in PDPN-mediated cancer metastasis.⁽³⁴⁾

Next, we investigated the epitope-binding region of PMab-52 by producing 11 point mutants of cPDPN, including H80A, I81A, E82A, D83A, G84A, P85A, T86A, Q87A, E88A, S89A, and T90A. All these mutants can be recognized by PMab-1 (Fig. 2A). Remarkably, we observed that PMab-52 reacted with all these mutants, except with Q87A and E88A using flow cytometry (Fig. 2B).

To identify the epitope of cPDPN for PMab-52 binding, we also performed Western blotting using these point mutants. The results revealed that PMab-52 could not recognize the point mutants of D83A-P85A and Q87A-E88A (Fig. 2C), which are located in the PLAG4 domain (₈₂-EDGPTQE₈₈). Both these results further confirmed that PMab-52 interacts with certain crucial amino acid residues (Asp83, Gly84, Pro85, Gln87, and Glu88) of the PLAG4 domain (Fig. 2D).

In conclusion, through this study, we characterized the crucial binding region of PLAG4 domain of cPDPN for PMab-52-specific binding. PMab-52 can be a useful tool in elucidating the pathophysiological function of cPDPN.

Acknowledgments

This research was supported in part by AMED under Grant Numbers: JP17am0301010 (Y.K.), JP17am0101078 (Y.K.), and JP17ae0101028 (Y.K.), and by JSPS KAKENHI Grant Number 17K07299 (M.K.K.) and Grant Number 16K10748 (Y.K.). This work was performed in part under the Cooperative Research Program of Institute for Protein Research, Osaka University, CR-17-05 and by the Grant for Joint Research Project of the Institute of Medical Science, the University of Tokyo.

Author Disclosure Statement

Y.K. received research funding from Ono Pharmaceutical Co., Ltd. All other authors have nothing to disclose.

References

1. Kato Y, Fujita N, Kunita A, Sato S, Kaneko M, Osawa M, and Tsuruo T: Molecular identification of Aggrus/T1alpha as a platelet aggregation-inducing factor expressed in colorectal tumors. *J Biol Chem* 2003;278:51599–51605.
2. Martin-Villar E, Scholl FG, Gamallo C, Yurrita MM, Munoz-Guerra M, Cruces J, and Quintanilla M: Characterization of human PA.26 antigen (T1alpha-2, podoplanin), a small membrane mucin induced in oral squamous cell carcinomas. *Int J Cancer* 2005;113:899–910.
3. 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.
4. Matsui K, Breitender-Geleff S, Soleiman A, Kowalski H, and Kerjaschki D: Podoplanin, a novel 43-kDa membrane protein, controls the shape of podocytes. *Nephrol Dial Transplant* 1999;14 Suppl 1:9–11.
5. Kimura N, and Kimura I: Podoplanin as a marker for mesothelioma. *Pathol Int* 2005;55:83–86.
6. 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.
7. Ugorski M, Dziegiel P, and Suchanski J: Podoplanin—A small glycoprotein with many faces. *Am J Cancer Res* 2016; 6:370–386.
8. Astarita JL, Acton SE, and Turley SJ: Podoplanin: Emerging functions in development, the immune system, and cancer. *Front Immunol* 2012;3:283.
9. Mahtab EA, Wijffels MC, Van Den Akker NM, Hahurij ND, Lie-Venema H, Wisse LJ, Deruiter MC, Uhrin P, Zaujec J, Binder BR, Schalijs MJ, Poelmann RE, and Gittenberger-De Groot AC: Cardiac malformations and myocardial abnormalities in podoplanin knockout mouse embryos: Correlation with abnormal epicardial development. *Dev Dyn* 2008;237:847–857.
10. Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, Chen CY, Xu B, Lu MM, Zhou D, Sebzda E, Santora MT, Merianos DJ, Stadtfeld M, Flake AW, Graf T, Skoda R, Maltzman JS, Koretzky GA, and Kahn ML: Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. *Blood* 2010;116:661–670.

11. Hess PR, Rawnsley DR, Jakus Z, Yang Y, Sweet DT, Fu J, Herzog B, Lu M, Nieswandt B, Oliver G, Makinen T, Xia L, and Kahn ML: Platelets mediate lymphovenous hemostasis to maintain blood-lymphatic separation throughout life. *J Clin Invest* 2014;124:273–284.
12. Herzog BH, Fu J, Wilson SJ, Hess PR, Sen A, McDaniel JM, Pan Y, Sheng M, Yago T, Silasi-Mansat R, McGee S, May F, Nieswandt B, Morris AJ, Lupu F, Coughlin SR, McEver RP, Chen H, Kahn ML, and Xia L: Podoplanin maintains high endothelial venule integrity by interacting with platelet CLEC-2. *Nature* 2013;502:105–109.
13. Kato Y, Vaidyanathan G, Kaneko MK, Mishima K, Srivastava N, Chandramohan V, Pegram C, Keir ST, Kuan CT, Bigner DD, and Zalutsky MR: Evaluation of anti-podoplanin rat monoclonal antibody NZ-1 for targeting malignant gliomas. *Nucl Med Biol* 2010;37:785–794.
14. Kato Y, and Kaneko MK: A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. *Sci Rep* 2014;4:5924.
15. Ochoa-Alvarez JA, Krishnan H, Pastorino JG, Nevel E, Kephart D, Lee JJ, Retzbach EP, Shen Y, Fatahzadeh M, Baredes S, Kalyoussef E, Honma M, Adelson ME, Kaneko MK, Kato Y, Young MA, Deluca-Rapone L, Shienbaum AJ, Yin K, Jensen LD, and Goldberg GS: Antibody and lectin target podoplanin to inhibit oral squamous carcinoma cell migration and viability by distinct mechanisms. *Oncotarget* 2015;6:9045–9060.
16. Kato Y, Kaneko M, Sata M, Fujita N, Tsuruo T, and Osawa M: Enhanced expression of Aggrus (T1alpha/podoplanin), a platelet-aggregation-inducing factor in lung squamous cell carcinoma. *Tumour Biol* 2005;26:195–200.
17. Abe S, Kaneko MK, Tsuchihashi Y, Izumi T, Ogasawara S, Okada N, Sato C, Tobiume M, Otsuka K, Miyamoto L, Tsuchiya K, Kawazoe K, Kato Y, and Nishioka Y: Antitumor effect of novel anti-podoplanin antibody NZ-12 against malignant pleural mesothelioma in an orthotopic xenograft model. *Cancer Sci* 2016;107:1198–1205.
18. Wahal SP, Goel MM, and Mehrotra R: Lymphatic vessel assessment by podoplanin (D2-40) immunohistochemistry in breast cancer. *J Cancer Res Ther* 2015;11:798–804.
19. Schoppmann SF, Berghoff A, Dinhof C, Jakesz R, Gnant M, Dubsy P, Jesch B, Heinzl H, and Birner P: Podoplanin-expressing cancer-associated fibroblasts are associated with poor prognosis in invasive breast cancer. *Breast Cancer Res Treat* 2012;134:237–244.
20. Mishima K, Kato Y, Kaneko MK, Nakazawa Y, Kunita A, Fujita N, Tsuruo T, Nishikawa R, Hirose T, and Matsutani M: Podoplanin expression in primary central nervous system germ cell tumors: A useful histological marker for the diagnosis of germinoma. *Acta Neuropathol* 2006;111:563–568.
21. Kunita A, Kashima TG, Ohazama A, Grigoriadis AE, and Fukayama M: Podoplanin is regulated by AP-1 and promotes platelet aggregation and cell migration in osteosarcoma. *Am J Pathol* 2011;179:1041–1049.
22. Ariizumi T, Ogose A, Kawashima H, Hotta T, Li G, Xu Y, Umezumi H, Sugai M, and Endo N: Expression of podoplanin in human bone and bone tumors: New marker of osteogenic and chondrogenic bone tumors. *Pathol Int* 2010;60:193–202.
23. Kaneko MK, Oki H, Ogasawara S, Takagi M, and Kato Y: Anti-podoplanin monoclonal antibody LpMab-7 detects metastatic lesions of osteosarcoma. *Monoclon Antib Immunodiagn Immunother* 2015;34:154–161.
24. Xu Y, Ogose A, Kawashima H, Hotta T, Ariizumi T, Li G, Umezumi H, and Endo N: High-level expression of podoplanin in benign and malignant soft tissue tumors: Immunohistochemical and quantitative real-time RT-PCR analysis. *Oncol Rep* 2011;25:599–607.
25. Yuan P, Temam S, El-Naggar A, Zhou X, Liu DD, Lee JJ, and Mao L: Overexpression of podoplanin in oral cancer and its association with poor clinical outcome. *Cancer* 2006;107:563–569.
26. Yurugi Y, Wakahara M, Matsuoka Y, Sakabe T, Kubouchi Y, Haruki T, Nosaka K, Miwa K, Araki K, Taniguchi Y, Shiomi T, Nakamura H, and Umekita Y: Podoplanin expression in cancer-associated fibroblasts predicts poor prognosis in patients with squamous cell carcinoma of the lung. *Anticancer Res* 2017;37:207–213.
27. Pula B, Jethon A, Piotrowska A, Gomulkiewicz A, Owczarek T, Calik J, Wojnar A, Witkiewicz W, Rys J, Ugorski M, Dziegiel P, and Podhorska-Okolow M: Podoplanin expression by cancer-associated fibroblasts predicts poor outcome in invasive ductal breast carcinoma. *Histopathology* 2011;59:1249–1260.
28. 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.
29. Chang YW, Hsieh PW, Chang YT, Lu MH, Huang TF, Chong KY, Liao HR, Cheng JC, and Tseng CP: Identification of a novel platelet antagonist that binds to CLEC-2 and suppresses podoplanin-induced platelet aggregation and cancer metastasis. *Oncotarget* 2015;6:42733–42748.
30. Kunita A, Kashima TG, Morishita Y, Fukayama M, Kato Y, Tsuruo T, and Fujita N: The platelet aggregation-inducing factor aggrus/podoplanin promotes pulmonary metastasis. *Am J Pathol* 2007;170:1337–1347.
31. Kaneko MK, Kunita A, Abe S, Tsujimoto Y, Fukayama M, Goto K, Sawa Y, Nishioka Y, and Kato Y: Chimeric anti-podoplanin antibody suppresses tumor metastasis through neutralization and antibody-dependent cellular cytotoxicity. *Cancer Sci* 2012;103:1913–1919.
32. Yamada S, Itai S, Nakamura T, Yanaka M, Saidoh N, Chang YW, Handa S, Harada H, Kagawa Y, Ichii O, Konnai S, Kaneko MK, and Kato Y: PMab-52: Specific and sensitive monoclonal antibody against cat podoplanin for immunohistochemistry. *Monoclon Antib Immunodiagn Immunother* 2017;36:224–230.
33. Itai S, Yamada S, Kaneko MK, Harada H, Kagawa Y, Konnai S, and Kato Y: Expression of cat podoplanin in feline squamous cell carcinomas. *Monoclon Antib Immunodiagn Immunother* 2017;36:243–250.
34. Sekiguchi T, Takemoto A, Takagi S, Takatori K, Sato S, Takami M, and Fujita N: Targeting a novel domain in podoplanin for inhibiting platelet-mediated tumor metastasis. *Oncotarget* 2016;7:3934–3946.

Address correspondence to:

Yukinari Kato
 New Industry Creation Hatchery Center
 Tohoku University
 2-1 Seiryomachi
 Aoba-ku
 Sendai 980-8575
 Japan

E-mail: yukinari-k@bea.hi-ho.ne.jp

Received: December 7, 2017

Accepted: January 4, 2018