MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 38, Number 3, 2019

DOI: 10.1089/mab.2019.0012

Epitope Mapping of Anti-Tiger Podoplanin Monoclonal Antibody PMab-231

Junko Takei,[1,2] Shunsuke Itai,[1,2] Yoshikazu Furusawa,[1,3,4] Shinji Yamada,[1] Takuro Nakamura,[1] Masato Sano,[1] Hiroyuki Harada,[2] Masato Fukui,[4] Mika K. Kaneko,[1] and Yukinari Kato[1,3]

Podoplanin (PDPN) is expressed on podocytes of the kidneys, type I alveolar cells of the lungs, and lymphatic endothelial cells. PDPN comprises three platelet aggregation-stimulating (PLAG) domains (PLAG1, PLAG2, and PLAG3) in the N-terminus and PLAG-like domains in the middle of the PDPN protein. We have previously reported on an anti-tiger PDPN (tigPDPN) monoclonal antibody (mAb), PMab-231, which was developed using the Cell-Based Immunization and Screening (CBIS) method. PMab-231 is very useful in flow cytometry, Western blotting, and immunohistochemical analyses; however, the binding epitope of PMab-231 remains to be elucidated. This study aimed to investigate the epitopes of PMab-231, which was developed by CBIS method, using enzyme-linked immunosorbent assay. The results revealed that the critical epitopes of PMab-231 are Glu29, Asp30, Asp31, Ile32, Met33, Thr34, Pro35, Gly36, and Glu38 of tigPDPN, which is corresponding to PLAG1/2. The findings of our study can be applied to the production of more functional anti-tigPDPN mAbs.

Keywords: podoplanin, PDPN, PMab-231, epitope mapping

Introduction

Podoplanin (PDPN) is a type I transmembrane sialoglycoprotein that is expressed in normal tissues, including renal corpuscles, type I lung alveolar cells, and lymphatic endothelial cells.[1,2] PDPN induces platelet aggregation by binding to C-type lectin-like receptor-2 (CLEC-2) on platelets.[1,3–9] The interaction between PDPN on lymphatic endothelial cells and CLEC-2 on platelets facilitates the separation of embryonic blood/lymphatic vessels.[10] Studies have reported the expression of human PDPN in several malignant tumors, such as oral squamous cell carcinomas,[11] malignant mesotheliomas,[12,13] lung cancers,[14] and malignant brain tumors.[15–18] PDPN expression has also been associated with malignant progression and cancer metastasis.[6,15,19] Although pathophysiological studies of alveolar cells of the lungs, podocytes of the kidneys, or lymphatic endothelial cells of the colon using anti-tigPDPN monoclonal antibodies (mAbs) are important in many disorders, mAbs against tigPDPN remain to be developed.

In our previous study, we used the Cell-Based Immunization and Screening (CBIS) method to develop specific and sensitive mAbs against the tigPDPN of 163 amino acids to facilitate the immunohistochemical analysis of paraffin-embedded tissue sections.[20] Screening identified strong signals against Chinese hamster ovary (CHO)/tigPDPN cells and weak or no signals against CHO-K1 cells in 18 of 958 wells (1.9%). One of the six clones, PMab-231 (IgG_{2a}, kappa), was finally selected using immunohistochemistry against paraffin-embedded feline normal tissue sections because tigPDPN and cat PDPN (cPDPN) shared 99% homology.[21] The dissociation constants (K_D) of PMab-231 for CHO/tigPDPN and CHO/cPDPN cells were determined to be 1.2×10^{-8} M and 1.9×10^{-8} M, respectively, indicating moderate affinity for CHO/tigPDPN and CHO/cPDPN cells. PMab-231 stained type I alveolar cells of the feline lungs and podocytes of the feline kidneys using immunohistochemistry. This study aimed to determine the binding epitope of PMab-231 to tigPDPN using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Enzyme-linked immunosorbent assay

Synthesized tigPDPN peptides using PEPScreen (Sigma-Aldrich Corp., St. Louis, MO) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA) at 10 μg/mL for 30 minutes at 37°C.

[1]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
[2]Department of Oral and Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan.
[3]New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.
[4]ZENOAQ RESOURCE CO., LTD., Koriyama, Japan.

Table 1. Determination of PMab-231 Epitope by Enzyme-Linked Immunosorbent Assay

Peptide	*Sequence*	*PMab-231*
24–43	STVRPEDDIMTPGVEDGMVT	++
34–53	TPGVEDGMVTLGAEDNVVTT	–
44–63	LGAEDNVVTTGAGADPEEST	–
54–73	GAGADPEESTGPTGLVPTNT	–
64–83	GPTGLVPTNTESITDFHIED	–
74–93	ESITDFHIEDGPTQESTGHA	–
84–103	GPTQESTGHANEESQSTTTL	–
94–113	NEESQSTTTLNVVTSHSIEK	–
104–123	NVVTSHSIEKIGEDTETTVE	–
114–129	IGEDTETTVEKDGLAT	–

++, 0.3 ≦ OD655 < 0.6; –, OD655 < 0.1.

Table 2. Determination of PMab-231 Epitope by Enzyme-Linked Immunosorbent Assay

Mutation	*Sequence*	*PMab-231*
S24A	ATVRPEDDIMTPGVEDGMVT	+
T25A	SAVRPEDDIMTPGVEDGMVT	+
V26A	STARPEDDIMTPGVEDGMVT	+
R27A	STVAPEDDIMTPGVEDGMVT	+
P28A	STVRAEDDIMTPGVEDGMVT	+
E29A	STVRPADDIMTPGVEDGMVT	–
D30A	STVRPEADIMTPGVEDGMVT	–
D31A	STVRPEDAIMTPGVEDGMVT	–
I32A	STVRPEDDAMTPGVEDGMVT	–
M33A	STVRPEDDIATPGVEDGMVT	–
T34A	STVRPEDDIMAPGVEDGMVT	–
P35A	STVRPEDDIMTAGVEDGMVT	–
G36A	STVRPEDDIMTPAVEDGMVT	–
V37A	STVRPEDDIMTPGAEDGMVT	+
E38A	STVRPEDDIMTPGVADGMVT	–
D39A	STVRPEDDIMTPGVEAGMVT	+
G40A	STVRPEDDIMTPGVEDAMVT	+
M41A	STVRPEDDIMTPGVEDGAVT	+
V42A	STVRPEDDIMTPGVEDGMAT	+
T43A	STVRPEDDIMTPGVEDGMVA	+

+, 0.1 ≦ OD655 < 0.3; –, OD655 < 0.1.

After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), the plates were incubated with purified PMab-231 (1 μg/mL), followed by a peroxidase-conjugated anti-mouse IgG (Agilent Technologies, Inc., Santa Clara, CA) dilution of 1:2000. Enzymatic reactions were performed using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA). These reactions were performed at 37°C using a total sample volume of 50–100 μL.

Results and Discussion

We have previously produced mAbs against HER2[22] or EGFR[23] using CasMab technology and mAbs against CD44, PD-L1, or CD133 using the CBIS method.[24–28] However, the binding epitopes of these mAbs (H_2Mab-77 against HER2, EMab-51 against EGFR, C_{44}Mab-5 against CD44, L_1Mab-13 against PD-L1, and CMab-43 against CD133) remain to be determined because these mAbs did not react with synthesized peptides. Conversely, the binding epitopes of mAbs produced by immunizing with synthesized peptides have been easily determined using deletion mutants and point mutants of synthesized peptides.[29,30]

We first synthesized a series of tigPDPN peptides, which are summarized in Table 1. Using ELISA, PMab-231 detected 24–43 peptides corresponding to the 24th–43rd amino acids (aas) of tigPDPN. We then synthesized point mutants of 24–43 peptides (Table 2). Using ELISA, PMab-231 detected S24A, T25A, V26A, R27A, P28A, V37A, D39A, G40A, M41A, V42A, and T43A. Conversely, it did not detect E29A, D30A, D31A, I32A, M33A, T34A, P35A, G36A, and E38A, indicating that Glu29, Asp30, Asp31, Ile32, Met33, Thr34, Pro35, Gly36, and Glu38 are included in the critical epitope of PMab-231. Although PMab-231 was produced using the CBIS method, the epitope of PMab-231 was fortunately determined by ELISA.

We previously developed mAbs against human,[31] mouse,[31] rat,[32] rabbit,[33] dog,[34] cat,[27] bovine,[35] horse,[36–38] and pig[39] PDPNs. PDPN comprises three tandem-repeat of the ''EDxxVTPG'' sequences, which were defined to be platelet aggregation-stimulating (PLAG) domains (PLAG1,

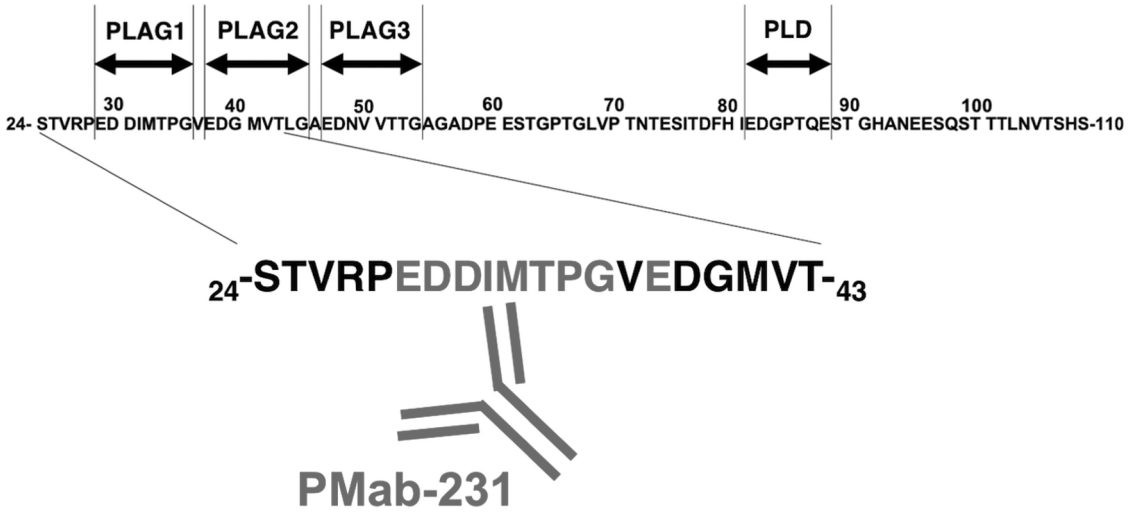

FIG. 1. Schematic illustration of the epitope recognized by PMab-231. The critical epitope of PMab-231 are shown in gray letters. PLAG, platelet aggregation-stimulating; PLD, PLAG-like domain.

PLAG2, and PLAG3) in the N-terminus of the PDPN protein.[4] There are several PLAG-like domains (PLDs) of the ''E(D/E)xx(T/S)xx'' sequence in the middle of the PDPN protein. PLDs are reportedly important for PDPN–CLEC-2 interactions.[40] Almost all mAbs against PDPNs reportedly react with PLAG domains or PLDs.[40,41] As summarized in Figure 1, PMab-231 also detected PLAG1 (Glu29, Asp30, Asp31, Ile32, Met33, Thr34, Pro35, and Gly36) and a part of PLAG2 (Glu38) of tigPDPN. Therefore, PLAG1 of tigPDPN was clarified to be the advantageous epitope for several applications, such as flow cytometry, Western blotting, and immunohistochemical analyses. These findings can be applied to the production of more functional anti-tigPDPN mAbs.

Acknowledgments

This research was supported in part by AMED under Grant Numbers: JP18am0101078 (Y.K.), JP18am0301010 (Y.K.), and JP18ae0101028 (Y.K.), and by JSPS KAKENHI Grant Number 17K07299 (M.K.K.) and Grant Number 16K10748 (Y.K.).

Author Disclosure Statement

Y.K. received research funding from ZENOAQ RESOURCE CO., LTD. The other authors have no conflict of interest.

References

1. 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.
2. 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.
3. 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.
4. 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.
5. Martin-Villar E, Scholl FG, Gamallo C, Yurrita MM, Munoz-Guerra M, Cruces J, and Quintanilla M: Characterization of human PA2.26 antigen (T1alpha-2, podoplanin), a small membrane mucin induced in oral squamous cell carcinomas. Int J Cancer 2005;113:899–910.
6. 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.
7. 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.
8. Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, Yamazaki Y, Narimatsu H, and Ozaki Y: Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. J Biol Chem 2007;282:25993–26001.
9. Nagae M, Morita-Matsumoto K, Kato M, Kaneko MK, Kato Y, and Yamaguchi Y: A Platform of C-Type lectin-like receptor CLEC-2 for binding O-glycosylated podoplanin and nonglycosylated rhodocytin. Structure 2014;22: 1711–1721.
10. Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, Chen CY, Xu B, Lu MM, Zhou D, Sebzda E, Santore 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. 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.
12. Kimura N, and Kimura I: Podoplanin as a marker for mesothelioma. Pathol Int 2005;55:83–86.
13. Abe S, Morita Y, Kaneko MK, Hanibuchi M, Tsujimoto Y, Goto H, Kakiuchi S, Aono Y, Huang J, Sato S, Kishuku M, Taniguchi Y, Azuma M, Kawazoe K, Sekido Y, Yano S, Akiyama S, Sone S, Minakuchi K, Kato Y, and Nishioka Y: A novel targeting therapy of malignant mesothelioma using anti-podoplanin antibody. J Immunol 2013;190:6239–6249.
14. 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. Tumor Biol 2005;26:195–200.
15. Mishima K, Kato Y, Kaneko MK, Nishikawa R, Hirose T, and Matsutani M: Increased expression of podoplanin in malignant astrocytic tumors as a novel molecular marker of malignant progression. Acta Neuropathol (Berl) 2006;111:483–488.
16. 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 (Berl) 2006; 111:563–568.
17. 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.
18. Kato Y, and Kaneko MK: A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. Sci Rep 2014;4:5924.
19. 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.
20. 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.
21. Yamada S, Itai S, Furusawa Y, Sano M, Nakamura T, Yanaka M, Handa S, Hisamatsu K, Nakamura Y, Fukui M, Harada H, Mizuno T, Sakai Y, Ogasawara S, Murata T, Uchida H, Tahara H, Kaneko MK, and Kato Y: Detection of tiger podoplanin using the anti-cat podoplanin monoclonal antibody PMab-52. Monoclon Antib Immunodiagn Immunother 2018;37:224–228.
22. Itai S, Fujii Y, Kaneko MK, Yamada S, Nakamura T, Yanaka M, Saidoh N, Chang YW, Handa S, Takahashi M, Suzuki H, Harada H, and Kato Y: H2Mab-77 is a sensitive and specific anti-HER2 monoclonal antibody against breast cancer. Monoclon Antib Immunodiagn Immunother 2017; 36:143–148.
23. Itai S, Kaneko MK, Fujii Y, Yamada S, Nakamura T, Yanaka M, Saidoh N, Handa S, Chang YW, Suzuki H, Harada H, and Kato Y: Development of EMab-51, a sensitive and specific anti-epidermal growth factor receptor monoclonal antibody in flow cytometry, Western blot, and immunohistochemistry. Monoclon Antib Immunodiagn Immunother 2017;36:214–219.
24. 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.
25. Yamada S, Itai S, Kaneko MK, and Kato Y: Detection of high PD-L1 expression in oral cancers by a novel monoclonal antibody L1Mab-4. Biochem Biophys Rep 2018;13: 123–128.
26. 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.
27. 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.
28. 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.
29. Furusawa Y, Yamada S, Itai S, Nakamura T, Fukui M, Harada H, Kaneko MK, and Kato Y: Elucidation of critical epitope of anti-rat podoplanin monoclonal antibody PMab-2. Monoclon Antib Immunodiagn Immunother 2018;37: 188–193.
30. 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.
31. Kaji C, Tsujimoto Y, Kato Kaneko M, Kato Y, and Sawa Y: Immunohistochemical examination of novel rat monoclonal antibodies against mouse and human podoplanin. Acta Histochem Cytochem 2012;45:227–237.
32. 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.
33. Honma R, Fujii Y, Ogasawara S, Oki H, Liu X, Nakamura T, Kaneko MK, Takagi M, and Kato Y: Establishment of a novel monoclonal antibody PMab-32 against rabbit podoplanin. Monoclon Antib Immunodiagn Immunother 2016;35:41–47.
34. Honma R, Kaneko MK, Ogasawara S, Fujii Y, Konnai S, Takagi M, and Kato Y: Specific detection of dog podoplanin expressed in renal glomerulus by a novel monoclonal antibody PMab-38 in immunohistochemistry. Monoclon Antib Immunodiagn Immunother 2016; 35:212–216.
35. Honma R, Ogasawara S, Kaneko M, Fujii Y, Oki H, Nakamura T, Takagi M, Konnai S, and Kato Y: PMab-44 detects bovine podoplanin in immunohistochemistry. Monoclon Antib Immunodiagn Immunother 2016;35: 186–190.
36. 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.
37. 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.
38. Furusawa Y, Yamada S, Itai S, Nakamura T, Yanaka M, Sano M, Harada H, Fukui M, Kaneko MK, and Kato Y: PMab-219: A monoclonal antibody for the immunohistochemical analysis of horse podoplanin. Biochem Biophys Rep 2019;18: DOI: 10.1016/j.bbrep.2019.1001.1009.
39. 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.
40. 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 2015;7:3934–3946.
41. Kaneko MK, Nakamura T, Honma R, Ogasawara S, Fujii Y, Abe S, Takagi M, Harada H, Suzuki H, Nishioka Y, and Kato Y: Development and characterization of anti-glycopeptide monoclonal antibodies against human podoplanin, using glycan-deficient cell lines generated by CRISPR/Cas9 and TALEN. Cancer Med 2017;6:382–396.

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: February 27, 2019
Accepted: April 24, 2019