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Characterization of Monoclonal Antibody LpMab-7 Recognizing Non-PLAG Domain of Podoplanin

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Podoplanin (PDPN/Aggrus/T1α), a platelet aggregation-inducing type I transmembrane sialoglycoprotein, is involved in tumor invasion and metastasis. Furthermore, podoplanin expression was reported to be involved in poor prognosis of several cancers. Although many anti-podoplanin monoclonal antibodies (MAbs), such as NZ-1 and D2-40, have been established, those epitopes are limited to platelet aggregation-stimulating (PLAG) domain of podoplanin. In this study, we developed and characterized a novel anti-podoplanin MAb, LpMab-7, that is more sensitive than NZ-1 in immunohistochemistry. We identified the minimum epitope of LpMab-7 as Arg79-Leu83 of human podoplanin, which is different from PLAG domain, using ELISA, Western blot analysis, and flow cytometry. Because LpMab-7 has high sensitivity against podoplanin, LpMab-7 is expected to be useful for molecular targeting therapy for podoplanin-expressing cancers.

Introduction

Podoplanin is a platelet aggregation-inducing type I transmembrane sialoglycoprotein that is involved in tumor invasion and metastasis.[1,2] Expression of podoplanin has been reported in many tumors, including malignant brain tumors, mesotheliomas, lung cancer, esophageal cancer, testicular tumors, bladder cancer, and osteosarcoma.[1,3–14] Until now, several physiological functions of podoplanin have been reported. The podoplanin-CLEC-2 interaction is important for platelet aggregation and embryonic blood-lymphatic vascular separation.[1,2,15–19] Podoplanin is also very useful for detecting lymphatic endothelial cells.[20–22] The development of ectopic lymphoid follicles is dependent on Th17-expressing podoplanin.[23] Local sphingosine-1-phosphate release after podoplanin-CLEC-2-mediated platelet activation is critical for high endothelial venule integrity during immune responses.[24] Furthermore, the activation of CLEC-2 by podoplanin rearranges the actin cytoskeleton in dendritic cells to promote efficient motility along stromal surfaces.[25]

Many anti-podoplanin MAbs have been produced, and they are useful for detecting podoplanin, which functions physiologically in normal tissues. Almost all anti-podoplanin MAbs react with the platelet aggregation-inducing (PLAG) domain of human podoplanin.[7,26–30] Matsui and colleagues previously reported that rabbit polyclonal antibodies produced by immunizing recombinant rat podoplanin also recognize PLAG domains, which were shown to be immunodominant antigenic sites.[31] Recently, we established a platform on which to produce cancer-specific MAbs (CasMabs).[32] Using CasMab methods, we produced several anti-podoplanin MAbs against a non-PLAG domain. In this study, we characterized a novel anti-podoplanin MAb, LpMab-7, which possesses high sensitivity against podoplanin.

Materials and Methods

Cell lines and tissues

Chinese hamster ovary (CHO)-K1, SaOS2, and U-2 OS cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). OST cell line was obtained from the Riken BioResource Center (Ibaraki, Japan).[33] HuO9 cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).[33] NOS-1 cell line was donated by Dr. Teiichi Motoyama.[34] CHO-K1 cells were cultured in RPMI 1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. SaOS2, HuO9, U-2 OS, OST, and NOS-1 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Wako Pure Chemical Industries) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. This study examined osteosarcoma patients who underwent surgery at Yamagata University Hospital.[35] The ethical committee of

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the Yamagata University Faculty of Medicine approved our study. Informed consent for obtaining samples and for subsequent data analyses was obtained from each patient or the patient's guardian.

Flow cytometry

Cell lines were harvested by brief exposure to 0.25% Trypsin/1 mM EDTA (Wako Pure Chemical Industries).[16] After washing with phosphate-buffered saline (PBS), the cells were treated with primary antibodies (1 μg/mL) for 30 min at 4°C, followed by treatment with Alexa Fluor 488–conjugated anti-mouse IgG or anti-rat IgG (Cell Signaling Technology, Danvers, MA). Fluorescence data were collected using a Cell Analyzer EC800 (Sony, Tokyo, Japan).

Western blot analysis

Cell lysates (10 μg) were boiled in SDS sample buffer (Nacalai Tesque, Kyoto, Japan).[36] The proteins were electrophoresed on 5–20% polyacrylamide gels (Wako Pure Chemical Industries) and were transferred onto a PVDF

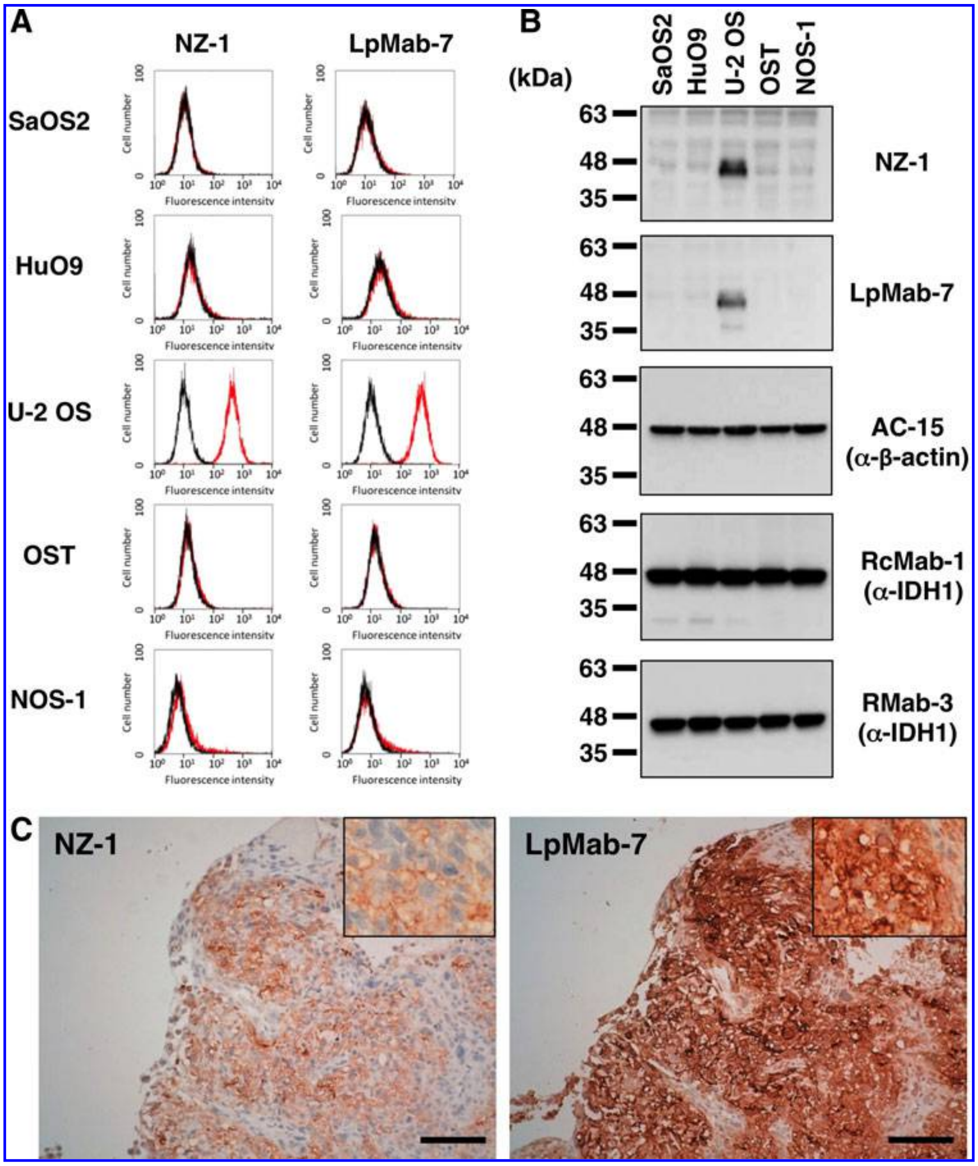

FIG. 1. Characterization of LpMab-7. (**A**) Flow cytometric analysis by NZ-1 and LpMab-7 against osteosarcoma cell lines. Five cell lines were treated with NZ-1 and LpMab-7 (1 μg/mL) for 30 min at 4°C, followed by treatment with Alexa Fluor 488 conjugated anti-rat IgG and anti-mouse IgG, respectively. Fluorescence data were collected using a Cell Analyzer EC800. Red line, NZ-1 or LpMab-7; black line, negative control. (**B**) Western blot analysis by NZ-1 and LpMab-7 against osteosarcoma cell lines. AC-15 (α-β-actin), RcMab-1 (α-IDH1), and RMab-3 (α-IDH1) were used as positive control. Total cell lysate of osteosarcoma cell lines (SaOS2, HuO9, U-2 OS, OST, NOS-1) were electrophoresed on 5–20% polyacrylamide gels and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μg/mL of primary antibodies and then with peroxidase-conjugated secondary antibodies; the membrane was detected using a Sayaca-Imager. (**C**) Immunohistochemical analysis by NZ-1 and LpMab-7 against osteosarcoma tissues. Serial sections were incubated with 5 μg/mL NZ-1 and LpMab-7, followed by biotin-labeled anti-rat IgG and anti-mouse IgG, respectively. Then, LSAB+ kit was used, and color was developed using DAB and counterstained with hematoxylin. Original magnification, ×200. Scale bar, 100 μm.

membrane (EMD Millipore, Billerica, MA). After blocking with 4% skim milk (Nacalai Tesque), the membrane was incubated with primary antibodies (1 μg/mL), then with peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark; 1:1000 diluted), and developed with the Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific, Waltham, MA) using a Sayaca-Imager (DRC, Tokyo, Japan).

Immunohistochemical analysis

Podoplanin protein expression was detected immunohistochemically in paraffin-embedded tumor specimens. Briefly, 4-μm-thick histologic sections were deparaffinized in xylene and rehydrated. Then, they were autoclaved in citrate buffer (pH 6.0; Dako) for 20 min. Sections were incubated with 5 μg/mL of primary antibodies overnight at 4°C followed by treatment with an LSAB+ kit or Envision+ kit (Dako). Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB; Dako) for 5 min, and then the sections were counterstained with hematoxylin (Wako Pure Chemical Industries).

Production of podoplanin mutants

The amplified human podoplanin cDNA was subcloned into a pcDNA3 vector (Life Technologies) and a FLAG epitope tag was added at the C-terminus. Substitution of amino acids to alanine in podoplanin was performed using a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA).[32,37] CHO-K1 cells were transfected with the plasmids using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Philadelphia, PA).

Enzyme-linked immunosorbent assay

Podoplanin peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific) at 1 μg/mL for 30 min.[16] After blocking with SuperBlock T20 (PBS) blocking buffer, the plates were incubated with culture supernatant or purified MAbs (1 μg/mL) followed by 1:1000 diluted peroxidase-conjugated anti-mouse IgG (Dako). The enzymatic reaction was conducted with a 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific) and was stopped using 1 M H_2SO_4. The optical density was measured at 450 nm using an iMark microplate reader (Bio-Rad Laboratories). These reactions were performed with a volume of 50 μL at 37°C.

Results

Characterization of LpMab-7

We recently immunized mice with human podoplanin (PDPN)-expressing LN229 glioma cells (LN229/PDPN), which possess cancer-type glycan patterns, including highly sulfated polylactosamine and aberrant sialylation, and developed a novel anti-podoplanin MAb, LpMab-7 (IgG_1, kappa).[32] First, podoplanin expression of five osteosarcoma cell lines (SaOS2, HuO9, U-2 OS, OST, and NOS-1) was investigated by flow cytometry and Western blot analysis. The anti-podoplanin MAb NZ-1 (rat IgG_{2a}, lambda) was used for positive control of detecting human podoplanin.[7] As shown in Figure 1A and B, both NZ-1 and LpMab-7 recognized podoplanin of U-2 OS. LpMab-7 detected 35 kDa band, which was not detected by NZ-1 (Fig. 1B). This 35 kDa band was also detected in CHO/PDPN and LN229/PDPN in our previous reports.[32] Immunohistochemical analysis showed that LpMab-7 detected podoplanin more sensitively than NZ-1 (Fig. 1C). The membranous/cytosolic staining pattern was observed. These results indicated that the novel anti-podoplanin LpMab-7 is much more useful in immunohistochemistry than previously established anti-podoplanin MAbs.

Table 1. Determination of NZ-1 and LpMab-7 Epitopes by ELISA

Peptides	*NZ-1*	*LpMab-7*
hpp23-36	–	–
hpp29-47	–	–
hpp38-51	+++	–
hpp49-68	–	–
hpp69-88	–	+++
hpp89-108	–	–
hpp109-128	–	–

+++, $2.0 \le OD450 < 3.0$; ++, $1.0 \le OD450 < 2.0$; +, $0.1 \le OD450 < 1.0$; –, negative; hpp, human podoplanin peptide.

Epitope mapping by ELISA, flow cytometry, and Western blot analysis

To determine the LpMab-7 epitope, we first performed ELISA using several synthetic peptides of human podoplanin (Table 1). NZ-1 reacted with 38-51 amino acids of human podoplanin (hpp38-51) that are the platelet aggregation-inducing (PLAG) domain. In contrast, LpMab-7 strongly recognized 69-88 amino acids of human podoplanin (hpp69-88), indicating that LpMab-7 epitope does not include *O*-glycan

Table 2. Determination of LpMab-7 Epitope by ELISA

Mutation	*Sequence*	*LpMab-7*
wild type	VTGIRIEDLPTSESTVHAQE	++++
V75A	ATGIRIEDLPTSESTVHAQE	++++
T76A	VAGIRIEDLPTSESTVHAQE	++++
G77A	VTAIRIEDLPTSESTVHAQE	++++
I78A	VTGARIEDLPTSESTVHAQE	++++
R79A	VTGIAIEDLPTSESTVHAQE	–
I80A	VTGIRAEDLPTSESTVHAQE	–
E81A	VTGIRIADLPTSESTVHAQE	–
D82A	VTGIRIEALPTSESTVHAQE	–
L83A	VTGIRIEDAPTSESTVHAQE	–
P84A	VTGIRIEDLATSESTVHAQE	+++
T85A	VTGIRIEDLPASESTVHAQE	++++
S86A	VTGIRIEDLPTAESTVHAQE	++++
E87A	VTGIRIEDLPTSASTVHAQE	++++
S88A	VTGIRIEDLPTSEATVHAQE	++++
T89A	VTGIRIEDLPTSESAVHAQE	+++
V90A	VTGIRIEDLPTSESTAHAQE	++++
H91A	VTGIRIEDLPTSESTVAAQE	++++
Q93A	VTGIRIEDLPTSESTVHAAE	++++
E94A	VTGIRIEDLPTSESTVHAQA	++++

++++, $OD450 \ge 3.0$; +++, $2.0 \le OD450 < 3.0$; ++, $1.0 \le OD450 < 2.0$; +, $0.1 \le OD450 < 1.0$; –, negative.

and is different from PLAG domain. We next performed an additional ELISA using 20 synthetic peptides, which included a point mutation among 75-94 amino acids of human podoplanin (Table 2). LpMab-7 did not react with R79A, I80A, E81A, D82A, and L83A, indicating that the critical amino acid sequence for LpMab-7 is RIEDL. To confirm this result, we performed flow cytometry and Western blot analysis. Flow cytometric analysis revealed that LpMab-7 did not react with I80A, E81A, and L83A, and very weakly recognized R79A and D82A, indicating that Ilu80, Glu81, and Leu83 are much more important for LpMab-7 (Fig. 2A). Western blot analysis also showed that LpMab-7 reaction was lost in point mutations of 79-83 amino acids (Fig. 2B). Taken together, the podoplanin epitope of LpMab-7 is Arg79-Leu83; and Ilu80, Glu81, and Leu83 are the most important amino acids.

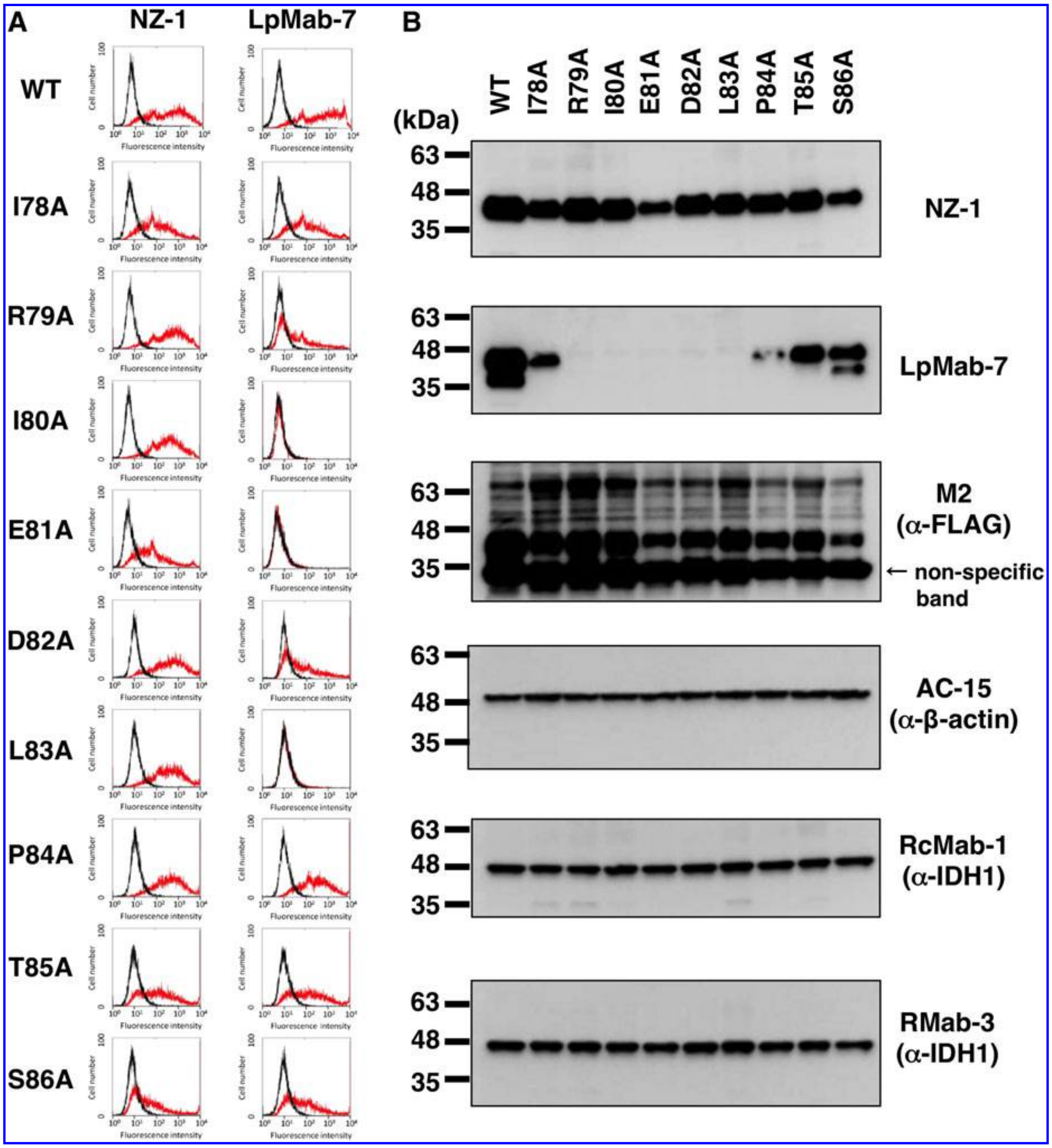

FIG. 2. Epitope mapping of LpMab-7 by ELISA, flow cytometry, and Western blot analysis. (**A**) Point mutants of human podoplanin were treated with NZ-1 and LpMab-7 (1 μg/mL) for 30 min at 4°C, followed by treatment with Alexa Fluor 488 conjugated anti-rat IgG and anti-mouse IgG, respectively. Fluorescence data were collected using a Cell Analyzer EC800. Red line, NZ-1 or LpMab-7; black line, negative control. (**B**) Total cell lysate of point mutants of human podoplanin were electrophoresed on 5–20% polyacrylamide gels and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μg/mL of primary antibodies and then with peroxidase-conjugated secondary antibodies; the membrane was detected using a Sayaca-Imager.

Discussion

Until now, investigative groups, including ours, have developed many anti-podoplanin MAbs. To neutralize platelet aggregation activity by blocking the association between podoplanin and CLEC-2, we produced NZ-1 MAb by immunizing rats with synthetic peptides of podoplanin PLAG domain.[7] NZ-1 possesses very high binding affinity, which was clarified by several methods including BIAcore, ELISA, flow cytometry, and Scatchard analysis.[14,38] NZ-1 inhibited tumor cell-induced platelet aggregation and tumor metastasis by its neutralizing activity, indicating that NZ-1 is a candidate of anti-metastatic neutralizing MAb.[7,16,36] Furthermore, NZ-1 is highly internalized into glioma cell lines and also accumulates efficiently into tumors *in vivo*.[14] NZ-1 and its rat-human chimeric anti-podoplanin antibody (NZ-8) possess antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against podoplanin-expressing glioblastoma or malignant mesothelioma cell lines.[10,36]

Recently, we have developed the novel platform, the CasMab method, to produce MAbs against membranous glycoproteins.[32] Using the CasMab method, we can obtain not only cancer-specific MAbs (CasMabs) but also non-CasMabs. Intriguingly, non-CasMabs also include the glycan within those epitopes or detects specific conformation of membranous glycoproteins expressed in cancers.[32,39] LpMab-7 recognized the usual 40 kDa band and the additional 35 kDa band, which has not been detected by previously reported anti-podoplanin MAbs. This 35 kDa band might be a low glycosylated form. Taken together, the ability to detect the 35 kDa band might lead to the high sensitivity of LpMab-7 in immunohistochemistry against podoplanin-expressing tumors.

In this report, we compared the reactivity of NZ-1 (rat IgG_{2a}, lambda) and LpMab-7 (mouse IgG_1, kappa). To remove the possibility that the difference of sensitivity in immunohistochemistry occurred by secondary antibodies, we also examined the difference of sensitivity between D2-40 (mouse IgG_1, kappa) and LpMab-7. D2-40 is commercially available and the most used MAb against human podoplanin in pathology or histology because D2-40 is also known as a lymphatic endothelial marker.[26] However, the intensity of LpMab-7 is much greater than that of D2-40 in immunohistochemistry of osteosarcomas (data not shown), demonstrating that LpMab-7 sensitivity is not dependent on the species and the subclass. Indeed, there are many immunohistochemical methods suitable for each MAb; therefore, we should further consider the other methods for comparing those anti-podoplanin MAbs. Although ADCC and CDC activities are very important for an antibody-based molecular targeting therapy, we could not investigate these activities because the subclass of LpMab-7 is IgG_1. The conversion of subclass into human IgG_1 or mouse IgG_{2a} is necessary to demonstrate ADCC/CDC activities. In conclusion, LpMab-7 is expected to be useful for molecular targeting therapy for podoplanin-expressing cancers.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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