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Anti-podoplanin Monoclonal Antibody LpMab-7 Detects Metastatic Lesions of Osteosarcoma

Mika K. Kaneko,[1*] Hiroharu Oki,[1,2*] Satoshi Ogasawara,[1*] Michiaki Takagi,[2] and Yukinari Kato[1]

Osteosarcoma is the most common primary malignant bone tumor and is highly metastatic to the lungs. Therefore, the development of a novel molecular targeting therapy against metastasis of osteosarcoma is necessary. A platelet aggregation-inducing factor, podoplanin/aggrus, is involved in tumor metastasis. Furthermore, podoplanin expression was reported to be involved in the poor prognosis of osteosarcoma patients. However, the association between podoplanin expression and metastasis of osteosarcoma remains unclear because of the lack of highly sensitive anti-podoplanin monoclonal antibodies (MAbs). In this study, we used a novel anti-podoplanin MAb, LpMab-7, which is more sensitive than well-known anti-podoplanin MAbs in immunohistochemistry. Immunohistochemical analysis using LpMab-7 showed that podoplanin expression at primary lesions is observed in 15 out of 16 (93.8%) cases. Furthermore, podoplanin expression at metastatic lesions was higher compared with primary lesions in three out of four (75%) cases with lung metastasis. Because LpMab-7 has high sensitivity against podoplanin, it is expected to be useful for molecular targeting therapy for osteosarcomas.

Introduction

OSTEOSARCOMA IS THE MOST COMMON primary malignant bone tumor, and possesses a high rate of systemic spread, particularly to the lungs.[1] Survival rate was improved by the advance of aggressive systemic chemotherapy. Patients with no metastatic disease, approximately 70%, are considered to be long-time survivors.[2] In contrast, approximately 14% of the patients experience lung metastases at diagnosis.[3] Primary metastasis is one of the risk factors, and increases the mortality rate.[4] Moreover, multi-drug combination chemotherapy for osteosarcoma causes ototoxicity, cardiac toxicity, and secondary malignancies.[5] Therefore, a novel molecular targeting therapy against metastatic osteosarcoma should be established.

Podoplanin (PDPN/Aggrus/T1α) is a platelet aggregation-inducing type I transmembrane sialoglycoprotein, which is involved in tumor invasion and metastasis.[6,7] Expression of podoplanin has been reported in many tumors.[6,8–19] Several studies have reported that osteosarcoma tissues and cell lines such HOS, U-2 OS, and MG-63 express podoplanin.[17] Moreover, podoplanin expression was reported to be involved in the poor prognosis of osteosarcoma patients. However, the association between podoplanin expression and metastasis of osteosarcoma remains to be clarified because of the lack of high-sensitive anti-podoplanin monoclonal antibodies (MAbs). Although many anti-podoplanin MAbs have been developed, almost all anti-podoplanin MAbs react with the platelet aggregation-inducing (PLAG) domain of human podoplanin.[12,20–24] Rabbit polyclonal antibodies produced by immunizing recombinant rat podoplanin also recognize PLAG domains.[25] Recently, we developed several anti-podoplanin MAbs against a non-PLAG domain, including LpMab-7.[26] In this study, we investigated the usefulness of an anti-podoplanin MAb, LpMab-7, in immunohistochemistry.

Materials and Methods

Osteosarcoma tissues

This study examined 16 osteosarcoma patients who underwent surgery at Yamagata University Hospital (Yamagata, Japan).[27] The ethical committee of the Yamagata University Faculty of Medicine approved the study. Informed consent for obtaining samples and for subsequent data analyses was obtained from each patient or the patient's guardian. The pathological diagnosis of all specimens in this study was confirmed by a pathologist (Prof. Mitsunori Yamakawa, Yamagata University Faculty of Medicine).[27]

Immunohistochemical analyses

Podoplanin protein expression was detected immunohistochemically in paraffin-embedded tumor specimens. Briefly, 4-μm-thick histologic sections were deparaffinized in

[1]Department of Regional Innovation, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan.
[2]Department of Orthopaedic Surgery, Yamagata University Faculty of Medicine, Yamagata, Japan.
*These authors contributed equally to this work.

Table 1. Clinicopathological Characteristics of Osteosarcoma Samples

										LpMab-7		*NZ-1*	
Alias	*Age*	*Gender*	*Site*	*Sample type*	*Sample class*	*Diagnosis*	*Grade*[a]	*Metastasis*[b]	*Status*	*Percentage*[c]	*Intensity*[d]	*Percentage*[c]	*Intensity*[d]
OS1	8	M	Tibia	Biopsy	Primaly	OB	Grade 1	+	DOD	−	−	+	+
OS2	16	M	Humerus	Biopsy	Primaly	OB	Grade 0	+	DOD	+ +	+	−	−
OS3	8	F	Clavicle	Biopsy	Primaly	OB	Grade 2	−	CDF	+ +	+ + +	+ +	+
OS4	29	M	Femur	Biopsy	Primaly	HGS	Grade 0	−	CDF	+ +	+ +	+	+
OS5	14	M	Tibia	Biopsy	Primaly	OB	Grade 1	+	DOD	+ +	+	−	−
OS6	74	F	Femur	Biopsy	Primaly	OB	Grade 1	+	DOD	+ + +	+ + +	+	+ +
OS7	12	M	Tibia	Biopsy	Primaly	OB	Grade 3	−	CDF	+ +	+ +	+	+
OS8	13	M	Tibia	Biopsy	Primaly	FB	Grade 3	−	CDF	+ +	+ +	+	+
OS9	6	M	Femur	Biopsy	Primaly	OB	Grade 3	−	CDF	+ +	+	+	+
OS10	10	M	Tibia	Biopsy	Primaly	OB	Grade 3	−	CDF	+ + +	+ + +	+ +	+ +
OS11	56	F	Femur	Biopsy	Primaly	OB	Grade 1	−	CDF	+ + +	+ + +	+ +	+ +
OS12	80	M	Mandible	Biopsy	Primaly	OB	No chemotherapy	−	AWD	+	+	−	−
OS13	59	F	Sacrum	Biopsy	Primaly	OB	No chemotherapy	−	DOD	+ + +	+ + +	+ +	+
OS14	14	F	Humerus	Biopsy	Primaly	OB	Grade 2	+	NED	+ + +	+ + +	+ + +	+ + +
OS15	16	M	Humerus	Biopsy	Primaly	CB	Grade 0	−	CDF	+	+	−	−
OS16	10	M	Tibia	Biopsy	Primaly	CB	Grade 1	−	CDF	+ + +	+ + +	+ +	+ +

OB, osteoblastic osteosarcoma; HGS, high-grade surface osteosarcoma; FB, fibroblastic osteosarcoma; CB, chondroblastic osteosarcoma; DOD, dead of disease; CDF, continuous disease-free; AWD, alive with disease; NED, no evidence of disease.

[a]Histological necrosis after preoperative chemotherapy: Grade 0, 0–50%; Grade 1, 51–90%; Grade 2, 91–99%; Grade 3, 100%.

[b]Lung metastasis existed before chemotherapy.

[c]−, no staining; +, <10%; ++ , 10–50%; and+ + +, >50%.

[d]−, no staining; +, weak; ++ , medium; ++ +, strong.

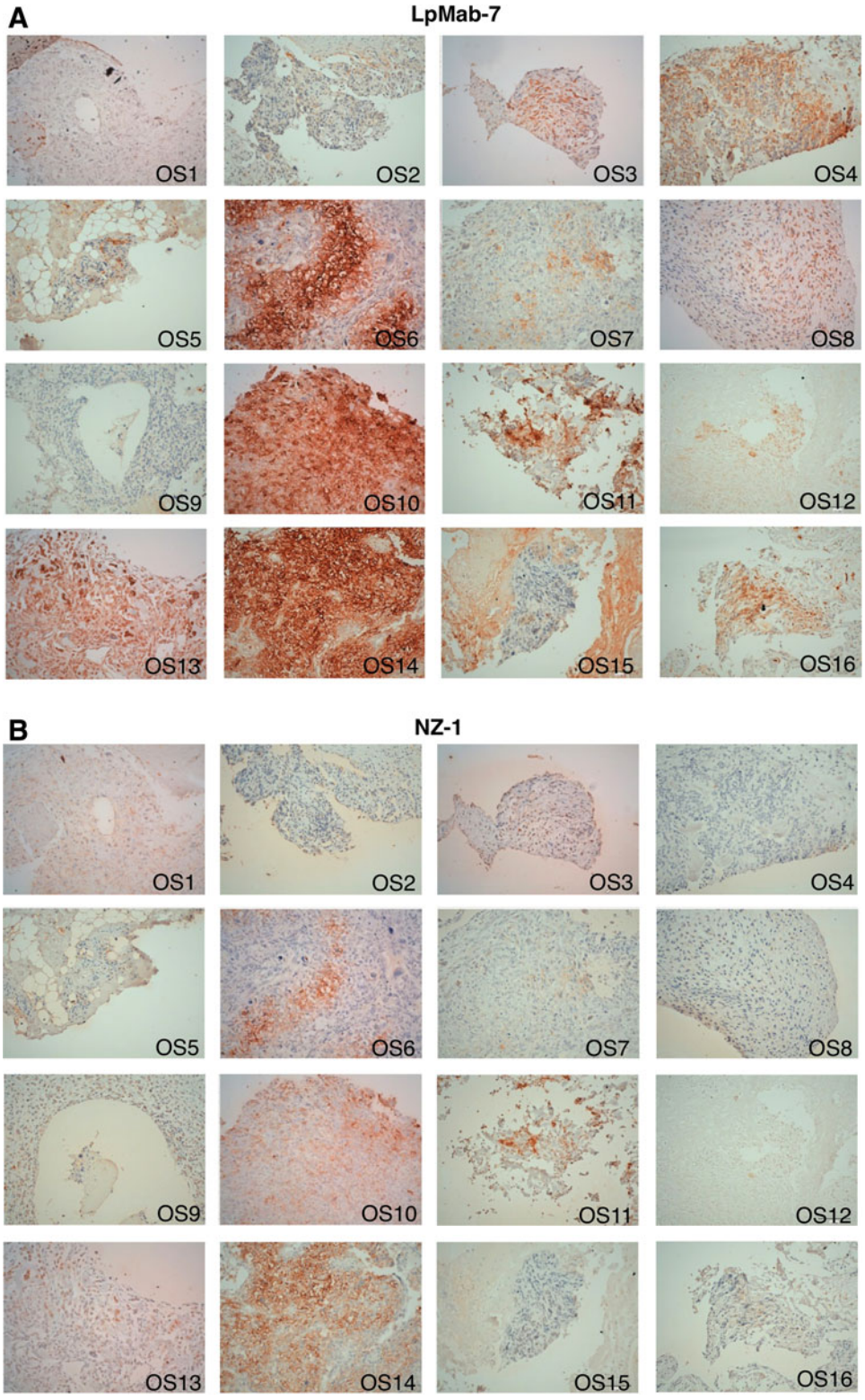

FIG. 1. Immunohistochemical analysis by LpMab-7 and NZ-1 against osteosarcoma tissues. Sections were incubated with 5 μg/mL LpMab-7 (**A**) and NZ-1 (**B**), followed by biotin-labeled anti-mouse IgG and anti-rat IgG, respectively. Then, the LSAB+ kit was used, and color was developed using DAB and counterstained with hematoxylin. Original magnification, ×200.

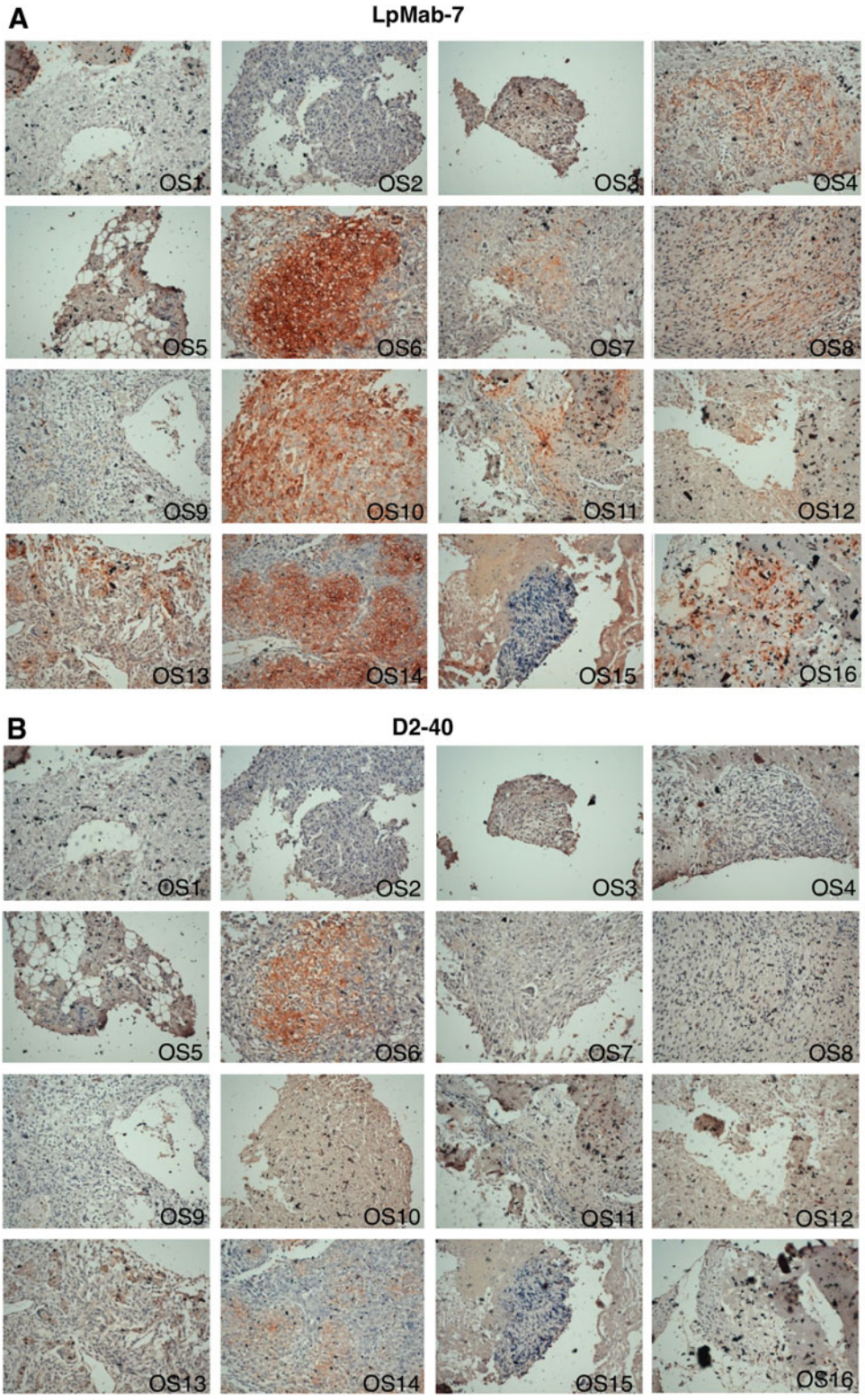

FIG. 2. Immunohistochemical analysis by LpMab-7 and D2-40 against osteosarcoma tissues. Sections were incubated with 5 μg/mL LpMab-7 (**A**) and 1:200 diluted D2-40 (**B**), followed by EnVision+. Color was developed using DAB and counterstained with hematoxylin. Original magnification, ×200.

Table 2. Immunohistochemical Analysis by LpMab-7 Against Primary and Metastatic Lesions of Osteosarcomas

							Primary lesion		*Metastatic lesion*	
Alias	*Age*	*Gender*	*Site*	*Diagnosis*	*Grade*[a]	*Status*	*Percentage*[b]	*Intensity*[c]	*Percentage*[b]	*Intensity*[c]
OS1	8	M	Tibia	OB	Grade 1	DOD	−	−	+ + +	+ + +
OS2	16	M	Humerus	OB	Grade 0	DOD	+ +	+	+ +	+ +
OS5	14	M	Tibia	OB	Grade 1	DOD	+ +	+	+ + +	+ + +
OS14	14	F	Humerus	OB	Grade 2	NED	+ + +	+ + +	+ + +	+ + +

OB, osteoblastic osteosarcoma; DOD, dead of disease; NED, no evidence of disease.
[a]Histological necrosis after preoperative chemotherapy: Grade 0, 0–50%; Grade 1, 51–90%; Grade 2, 91–99%; Grade 3, 100%.
[b]−, no staining; +, <10%; ++, 10–50%; + + +, >50%.
[c]−, no staining; +, weak; ++, medium; + + +, strong.

xylene and rehydrated. Then, they were autoclaved in citrate buffer (pH 6.0; Dako, Glostrup, Denmark) 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 the sections were counterstained with hematoxylin (Wako Pure Chemical Industries, Osaka, Japan). Staining was assessed semi-quantitatively from the percentage of tumor cells with membranous/cytoplasmic staining: 0, no staining; +, <10%; ++ , 10–50%; ++ +, >50%; and staining intensity: −, no staining; +, weak; ++ , medium; ++ +, strong.

Results

Immunohistochemical analysis against primary osteosarcomas

Immunohistochemical analysis showed that LpMab-7 detected podoplanin more sensitively than NZ-1 does (Table 1, Fig. 1). LpMab-7 showed that podoplanin expression at primary osteosarcomas is observed in 15 out of 16 (93.8%) cases. In contrast, NZ-1 detected podoplanin in 12 out of 16 (75%) cases. In almost all cases, the intensity of LpMab-7 was also higher than NZ-1. Furthermore, the intensity of LpMab-7 is much higher than that of D2-40 (Fig. 2). The membranous/cytosolic staining pattern was observed. These results indicate that the novel anti-podoplanin LpMab-7 is much more useful in immunohistochemistry of osteosarcomas than the previously established anti-podoplanin MAbs.

Immunohistochemical analysis against metastatic osteosarcomas

LpMab-7 shows much higher sensitivity against podoplanin in immunohistochemistry; therefore, we compared the podoplanin expression in both primary osteosarcomas and their metastatic lesions. We obtained four sets of primary lesions and metastatic lesions from the same patients in this study (Table 2). In OS1, podoplanin expression was observed only in the normal osteocytes (Fig. 3A, right), not

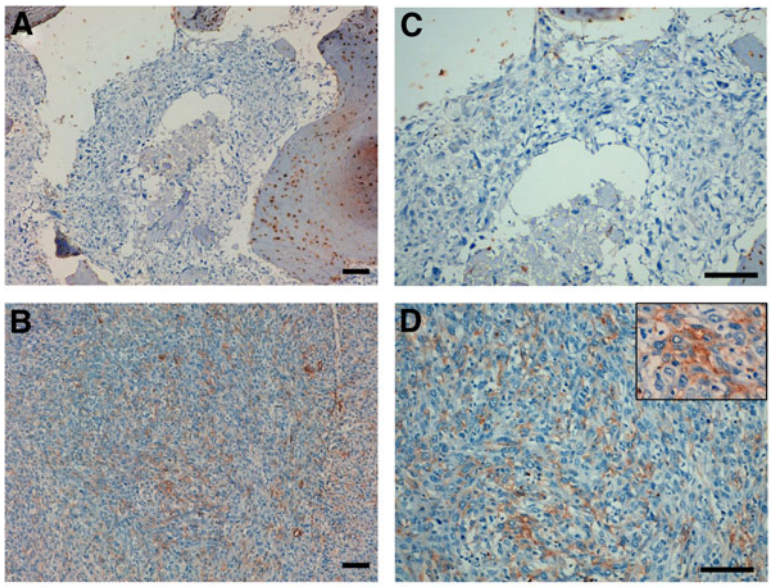

FIG. 3. Immunohistochemical analysis against primary and metastatic osteosarcomas using LpMab-7. Sections of primary (**A**,**C**) and metastatic (**B**,**D**) osteosarcomas (OS1) were incubated with 5 μg/mL LpMab-7, followed by Envision+ kit. Color was developed using DAB and counterstained with hematoxylin. Original magnification, ×100 (**A**,**B**); ×200 (**C**,**D**). Scale bar, 100 μm.

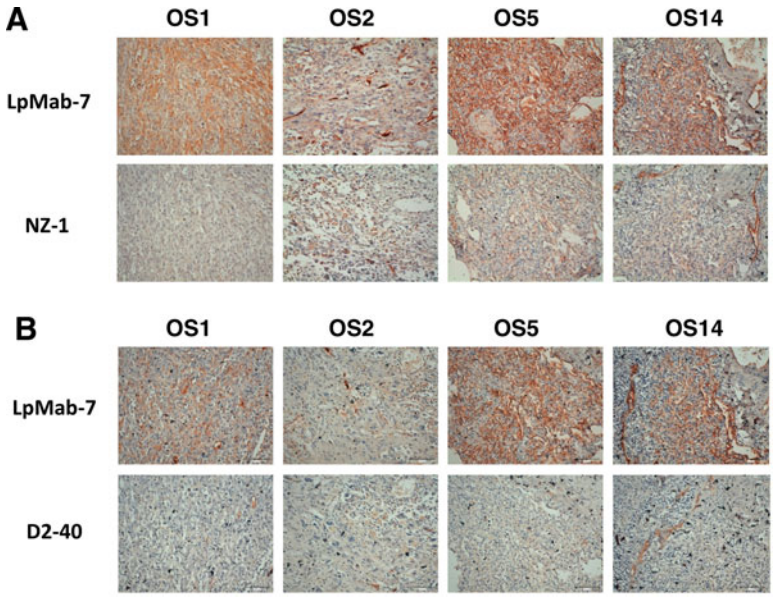

FIG. 4. Immunohistochemical analysis against metastatic osteosarcomas using three anti-podoplanin MAbs. (**A**) Sections of metastatic osteosarcomas (OS1, OS2, OS5, OS14) were incubated with 5 μg/mL LpMab-7 and NZ-1, followed by biotin-labeled anti-mouse IgG and anti-rat IgG, respectively. LSAB+ kit was used, and color was developed using DAB and counterstained with hematoxylin. (**B**) Sections were incubated with 5 μg/mL LpMab-7 and 1:200 diluted D2-40, followed by EnVision+. Color was developed using DAB and counterstained with hematoxylin. Original magnification, ×200.

in osteosarcoma cells (Fig. 3A,C). In contrast, podoplanin in the metastatic lesion was detected by LpMab-7 (Fig. 3B,D). Of interest, the intensity of podoplanin expression at metastatic lesions was higher than primary lesions in three out of four cases (OS1, OS2, and OS5) with lung metastasis (Table 2). Because podoplanin expression at both primary and metastatic lesions of OS14 is high, the difference of intensity by LpMab-7 staining was not observed. In contrast, NZ-1 and D2-40 signals were very weak in metastatic lesions (Fig. 4). These results indicate that LpMab-7 is very useful for detecting podoplanin in metastatic lesions of osteosarcomas.

Discussion

We previously developed NZ-1 by immunizing rats with PLAG domain of podoplanin to inhibit platelet aggregation and cancer metastasis by blocking the association between podoplanin and CLEC-2.[12,28,29] NZ-1 possesses very high binding affinity, which was clarified by several methods, including Scatchard analysis ($K_D = 9.8 \times 10^{-10}$ M) and BIAcore ($K_D = 1.2 \times 10^{-10}$ M).[19,30] Furthermore, NZ-1 was internalized into glioma cell lines and also accumulated efficiently into tumors *in vivo*.[19] Rat-human chimeric anti-podoplanin antibody (NZ-8), which was produced from NZ-1, possesses antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against podoplanin-expressing glioblastoma or malignant mesothelioma cell lines.[15,29] Unexpectedly, the intensity of NZ-1 was very weak in metastatic lesions of osteosarcomas in this study, probably because (1) the NZ-1 epitope (42–47 amino acids)[20] was blocked by the attachment of glycan complex to podoplanin; (2) CLEC-2 or other counterparts inhibited the NZ-1 binding to PLAG domain; and (3) the conformation of podoplanin at metastatic lesions is different from that at primary lesions. In contrast, a novel anti-podoplanin MAb, LpMab-7, detects podoplanin not only at primary lesions but also at metastatic lesions of osteosarcomas, although the binding affinity of LpMab-7 ($K_D = 5.7 \times 10^{-10}$ M in ELISA and $K_D = 2.0 \times 10^{-8}$ M in flow cytometry) is lower than NZ-1.[26]

In this report, we mainly 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.[20] However, the intensity of LpMab-7 is much higher than that of D2-40 in immunohistochemistry of osteosarcomas, demonstrating that LpMab-7 sensitivity is not dependent on the species and the subclass. Because the concentration of D2-40 is unknown, we could not calculate the binding affinity of D2-40. The epitope of D2-40 in PLAG domain[20] is different from that of LpMab-7 in non-PLAG domain;[26,31] therefore, the epitope of LpMab-7 might be more critical for the high sensitivity in immunohistochemistry.

Indeed, there are many immunohistochemical methods suitable for each MAb; therefore, we should further consider the other methods for comparing those anti-podoplanin MAbs. Another research group previously reported that human podoplanin, detected by NZ-1, is highly expressed in osteosarcomas using 133 osteosarcoma tissues.[17] Although 33 metastatic lesions were investigated in that study, the signal intensity was not discussed. In contrast, we discuss both signal intensity and percentage in immunohistochemistry

using three anti-podoplanin MAbs. Furthermore, four sets of primary lesions and metastatic lesions from the same patients were used in this study, and podoplanin upregulation was observed in the metastatic lesions using LpMab-7. Further studies are necessary to clarify that podoplanin is significantly upregulated in the metastatic lesions compared with primary lesions of osteosarcomas. Because LpMab-7 detected metastatic lesions of osteosarcomas, it is expected to be useful for molecular targeting therapy for osteosarcomas.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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Address correspondence to:
Yukinari Kato
Department of Regional Innovation
Tohoku University Graduate School of Medicine
2-1 Seiryo-machi, Aoba-ku
Sendai, Miyagi 980-8575
Japan

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

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