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Evaluation of immunohistochemical staining with PMab-38, an anti-dog podoplanin monoclonal antibody, in various canine tumor tissues

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Abstract

Podoplanin (PDPN) is a type I transmembrane sialoglycoprotein with *O*-glycosylation and a high content of sialic acid. PDPN has been reported to be expressed in various human tumors and promote tumor progression, epithelial-mesenchymal transition, and distant metastasis. PDPN is also expressed in cancer-associated fibroblasts (CAFs), which promote tumor growth. We have developed novel tumor specific anti-PDPN antibodies. PMab-38, which is an anti-dog PDPN monoclonal antibody, recognized PDPN expression in canine squamous cell carcinoma (SCC) and malignant melanoma. However, there has been no research into PMab-38 recognition of other types of tumors and systemic normal tissue. The objective of this study was to evaluate the staining positivity of PMab-38 by immunohistochemical staining of various paraffin-embedded canine tumor and systemic normal tissues. Immunohistochemical analysis revealed that PMab-38 positively stained tumor cells in 9/11 (82%) SCC and 9/11 (82%) pulmonary adenocarcinoma tissues. In the tumor stroma, large spindle-shaped mesenchymal cells, which were suspected to be CAFs, were stained by PMab-38 in almost all tumor types: 9/10 (90%) for anal sac adenocarcinoma and 11/12 (92%) for transitional cell carcinoma tissues. Almost all normal tissues were negatively stained with PMab-38 except part of the kidney glomerulus. Taken together, our findings provide evidence that various types of tumor cells were strongly stained by PMab-38, but most normal cells were not stained. These results indicate that PDPN recognized by PMab-38 might be a target for tumor antigen targeting therapy. Further studies are required to investigate the anti-tumor effect in various canine tumors by antibody therapy using PMab-38.

Key Words: podoplanin, PMab-38, canine cancer

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Introduction

Podoplanin (PDPN) is a type I transmembrane sialoglycoprotein with *O*-glycosylation and a high content of sialic acid[27]. It has been reported that it is expressed in renal podocytes, lymphatic endothelial cells (LECs), and pulmonary type I alveolar cells[4,25,28]. It has been widely used as a specific marker for LECs because it is strongly expressed in them[25]. Since PDPN has essential functions in maintaining lymph node microarchitecture, PDPN deficient mice showed malformation of lymphatic nodules[3,33]. It has also been reported that PDPN interacts with C-type lectin-like receptor 2 (CLEC2) in platelets and induces PDPN-mediated platelet aggregation[4,33].

In human medicine, PDPN was reported to be expressed in various human tumors, such as squamous cell carcinoma (SCC), malignant melanoma (MM), malignant mesothelioma, hemangiosarcoma (HSA), osteosarcoma (OSA), germinoma, and malignant astrocytic[2,10,21]. Increasing evidence suggests that PDPN promoted tumor progression, epithelial-mesenchymal transition, and distant metastasis by facilitating tumor cell migration and inducing platelet aggregation[35]. Currently, it is understood that the tumor stroma contributes to tumor progression by interacting with tumor cells[16,18]. Cancer-associated fibroblasts (CAFs), one of the major components of the tumor stroma, were reported to over express PDPN[14,15,30,38]. As CAFs correlate with poor prognosis in human patients with tumors, it has been suggested that PDPN expressed in CAFs helps tumor progression and local invasion[22].

Recently, an increasing number of monoclonal antibodies (mAb) have been developed for therapeutic treatment of tumors[26]. Tumor antigen targeting cytotoxic antibodies, such as anti-CD20 and anti-human epidermal growth factor receptor 2 (HER2), have significantly improved the clinical outcome of patients with malignant tumors[19,34]. The best tumor antigens for cytotoxic antibody therapy are expressed only on the tumor cell's membrane but not on normal tissues' cell's membrane. However, only a few tumor antigens specifically expressed on tumor cells were developed. Although PDPN is strongly expressed in human tumor cells, it is also expressed in various normal tissue types. Therefore, PDPN has been thought to be an unsuitable target for cytotoxic antibody therapy.

It is well known that aberrant glycosylation of glycoproteins is a common feature of neoplastic transformation[32]. We focused on tumor specific modification of PDPN and developed novel tumor specific anti-PDPN antibodies, which targeted a specific epitope in various species[12]. Of these, the anti-dog PDPN (dPDPN) monoclonal antibody, PMab-38, recognized dPDPN expression in canine SCC and MM but not in pulmonary type I cells and LECs as in previous studies[7,9,23]. However, there has been no research screening for PMab-38 recognition in various types of tumors and systemic normal tissues. The objective of this study was to evaluate the staining positivity of PMab-38 by immunohistochemical analysis of various paraffin-embedded canine tumor and systemic normal tissues and to investigate the possibility of applying PMab-38 to canine patients with tumors.

Materials and methods

Specimens: A total of 134 paraffin-embedded tumor tissues were evaluated. These tissues were surgically removed from dogs at the Veterinary Medical Center of the University of Tokyo between 2012 and 2016. Permission for resected tissue collection and usage for this study was obtained from the owners. The tissue samples were diagnosed at the Department of Veterinary Pathology at the University of Tokyo. All samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and cut into 4-μm-thick serial sections. Tumor types included mammary adenocarcinoma (MAC, $n = 13$), SCC (n = 11), pulmonary adenocarcinoma (PAC, n =

11), MM (n = 10), fibrosarcoma (FSA, n = 10), OSA (n = 10), anal sac adenocarcinoma (ASAC, n = 11), peripheral nerve sheath tumor (PNST, n = 8), thyroid carcinoma/adenocarcinoma (TC/AC, n = 9), thymoma (THY, n = 9), hepatocellular carcinoma (HCC, n = 10), and HSA (n = 11). Healthy dog tissues were obtained from healthy beagles euthanized for other experiments. All procedures were approved by the Animal Care and Use Committee at the University of Tokyo.

Immunohistochemistry: Paraffin-embedded tumor sections were dewaxed and rehydrated in xylene and graded ethanol followed by antigen retrieval using Dako Target Retrieval Solution, pH 9.0 (Agilent Technologies, Santa Clara, CA, USA), at 100°C for 30 min in boiling water. After washing with tris buffered saline- 0.1% tween 20 (TBS-t), endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min. Specimens were then washed with TBS-t and incubated in 8% skimmed milk for 1 h at room temperature to reduce non-specific binding before overnight incubation with primary antibodies including mouse IgG_1 anti-dPDPN (PMab-38[9]) antibodies, anti-pan-dPDPN (PMab-48[37]) antibodies, or the anti-α-smooth muscle actin (α-SMA) antibody (clone: 1A4, Agilent Technologies) at 4°C in a humidified chamber. A negative control was incubated with the purified mouse IgG_1, κ isotype antibody (Clone: MG1-45, BioLegend, San Diego, CA, USA), under identical conditions. For PMab-38 immunostaining, a section of canine cutaneous SCC was used as the positive control[9,11]. After washing with TBS-t, sections were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Envision+ System-HRP Labelled polymer; K4001; Agilent Technologies) for 30 min at room temperature. Thereafter, the sections were washed with TBS-t, incubated with 3,3' diaminobenzidine (DAB; Dojindo Laboratories, Rockville, MD, USA) solution, and counterstained with Mayer's hematoxylin. For the negative control, sections were subjected to the same procedures.

The specimens were considered positive for PMab-38 if histological evidence of cell staining was present. Positive rates of each tumor types were investigated with PMab-38 on tumor cells and stroma cells separately. Staining specificity of PMab-38 was assured by the lack of staining in the negative control sections.

Results

1. Evaluation of PMab-38 staining in various types of canine tumor tissues

Immunohistochemical analysis using PMab-38 was performed in 134 specimens of various tumor types. The positive staining ratio for tumor cells varied among the tumor types. In evaluated tumor tissues, PMab-38 positively stained tumor cells in 9/11 (82%) SCC, 9/11 (82%) PAC, 4/10 (40%) FSA, 2/13 (15%) MAC, 1/10 (10%) OSA, and 1/10 (10%) ASAC. The ratio of positive tumor cells in the tumor tissues varied among samples. More than 50% of the tumor cells were stained in some samples of SCC and PAC but not in other samples, where less than 10% of tumor cells were stained. No staining was detected in the other six types of tumors, MM, TCC, PNST, TC/AC, THY, HCC, and HSA (Table 1).

In tumor stroma, large spindle-shaped mesenchymal cells were stained by PMab-38 in almost all of the tumor types and the positive ratios in the analyzed specimens were 9/11 (82%) for SCC, 6/11 (55%) for PAC, 3/13 (20%) for MAC, 9/10 (90%) for ASAC, 11/12 (92%) for TCC, 2/8 (25%) for PNST, 2/9 (22%) for TC/AC, 2/9 (22%) for THY, and 1/10 (10%) for HCC (Table 1). In FSA and OSA samples, we did not evaluate the cells separately because we could not distinguish tumor from stroma cells (Fig. 1). Surrounding normal tissues were not stained by PMab-38 in all cases.

Table 1. Evaluation of PMab-38 staining in various types of canine tumor tissues

Tumor types*[a]	Samples	Tumor cells (%)	Stroma (%)*[b]
SCC	11	9 (82)	9 (82)
PAC	11	9 (82)	6 (55)
MAC	13	2 (15)	3 (20)
FSA	10	4 (40)	ND
OSA	10	1 (10)	ND
ASAC	10	1 (10)	9 (90)
TCC	12	0 (0)	11 (92)
PNST	8	0 (0)	2 (25)
TC/AC	9	0 (0)	2 (22)
THY	9	0 (0)	2 (22)
HCC	10	0 (0)	1 (10)
HSA	11	0 (0)	0 (0)
MM	10	0 (0)	0 (0)

We analyzed the positive ratio of PMab-38 staining of tumor and stroma cell separately in each tumor type. The positive ratio of tumor cells was high in SCC and PAC, but tumor cells of some tumor types were not stained by PMab-38. However, stroma cells of almost all tumor types were stained by PMab-38, and stroma cells of SCC, PAC, ASAC, and TCC had a high positive ratio.
[a]SCC: Squamous cell carcinoma, PAC: Pulmonary adenocarcinoma, MAC: Mammary adenocarcinoma, FSA: Fibrosarcoma, OSA: Osteosarcoma, ASAC: Anal sac adenocarcinoma, TCC: Transitional cell carcinoma, PNST: Peripheral nerve sheath tumor, TC/AC: Thyroid carcinoma/adenocarcinoma, THY: Thymoma, HCC: Hepatocellular cell carcinoma, HSA: Hemangiosarcoma, MM: Malignant melanoma.
[b]ND: Not-determined

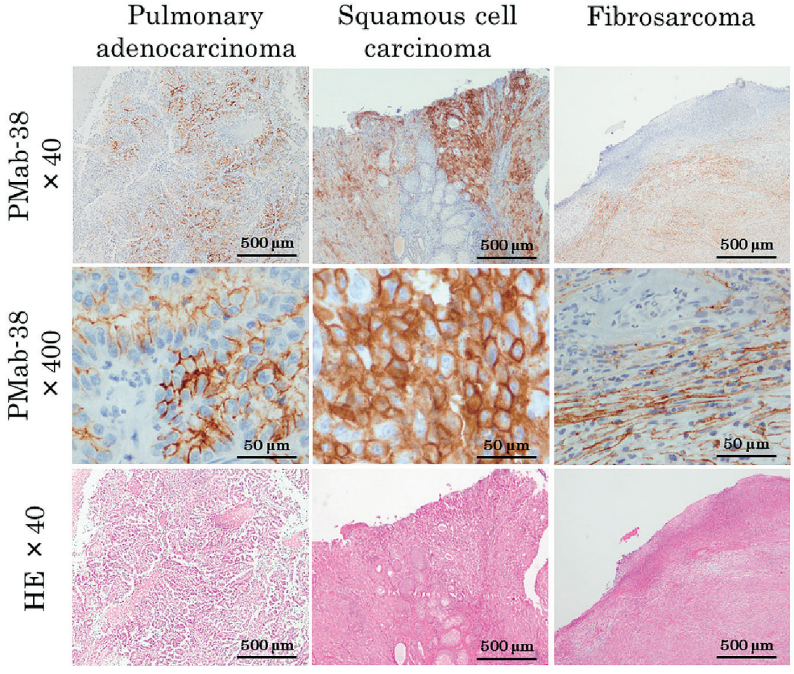

Fig. 1. Evaluation of the staining pattern of PMab-38. Representative staining patterns of PMab-38 in PAC, SCC, and FSA are shown. Tumor cell membranes of SCC and PAC were stained by PMab-38. In this SCC sample, the unstained area consisted of normal cells, and all tumor cells were stained. In PAC tissue, invasive tumor cells in the bronchial tube are shown, and most of them were stained by PMab-38. In FSA samples, some cells were stained, but we could not distinguish tumor cells from stroma cells.

2. Evaluation of the staining pattern of PMab-38 in tumor cells

Cell membranes of tumor cells were strongly stained by PMab-38 in positively stained samples (Fig. 1). Positively stained tumor cells showed various staining patterns, including diffuse and focal staining, but we did not find any specific pattern in accordance with tumor types. The intensity of PMab-38 staining of tumor cells was equivalent in all positively stained samples. In some PAC samples, invasive tumor cells in the bronchial tube were also positively stained with PMab-38.

3. Evaluation of PMab-38 staining in tumor stroma cells

Since large spindle-shaped mesenchymal cells were stained by PMab-38 in tumor stroma, these cells were suspected to be CAFs, which express PDPN in some types of human tumors. In this study, we analyzed whether canine CAFs were recognized by PMab-38 according their morphology and α-SMA staining, which is generally utilized to detect CAFs[36,39].

Positively stained cells by α-SMA staining and PMab-38 staining were compared in this study[36] (Fig. 2). Parts of α-SMA positive cells were stained by PMab-38. PMab-38-positive CAFs tended to be located adjacent to tumor nests. CAF membranes were strongly stained, and their cytoplasm was weakly stained by PMab-38. Staining intensity and the ratio of PMab-38 positive CAFs were especially high in TCC samples compared to other tumor types.

4. Evaluation of PMab-38 staining in systemic normal tissues

To explore the PMab-38 reaction against normal tissues, we examined PMab-38 staining of systemic normal tissues including skin, adipose, mammary gland, muscle, patella, brain, tonsil, thyroid, lymph node, esophagus, lung, heart muscle, liver, pancreas, spleen, kidney, large intestine, urinary bladder, and uterus tissues. Although normal tissues were positively

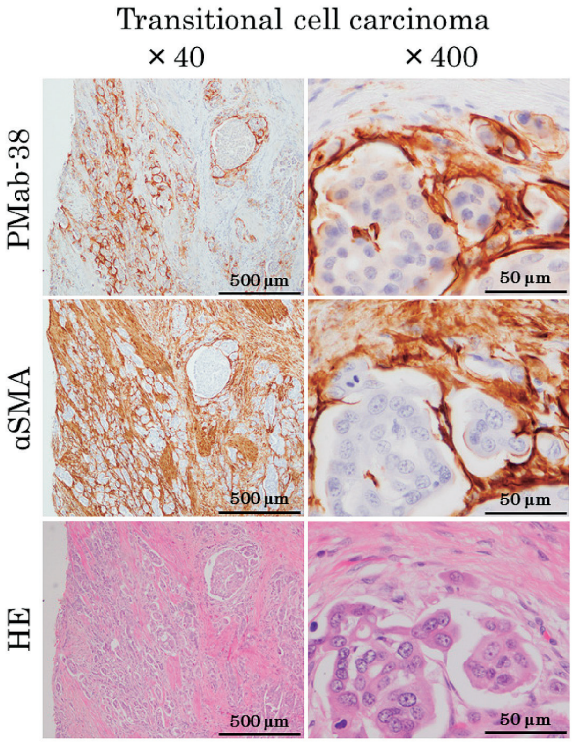

Fig. 2. Evaluation of PMab-38 staining in tumor stroma cells. Representative staining pattern for PMab-38 and α-SMA in TCC are shown. Large spindle-shaped mesenchymal cells in the tumor stroma were clearly stained by α-SMA and part of the α-SMA positive cells were stained by PMab-38. CAFs stained by PMab-38 tended to be located adjacent to the tumor nests.

stained with anti-pan-dPDPN antibody, most normal tissues were not positively stained with PMab-38 (Fig. 3, Fig. S1). Lung and lymphatic nodules, which were reported to express PDPN[5,25], were also negatively stained by PMab-38. Although the intensity was weaker than SCC, part of the normal glomerulus in the kidney, which might be podocytes, was positively stained by PMab-38 as previously reported[9] (Fig. S2).

Discussion

This study demonstrated that cell membranes of tumor cells were stained by PMab-38 in more than half of the SCC and PAC samples and a portion of the FSA, MA, OSA, and

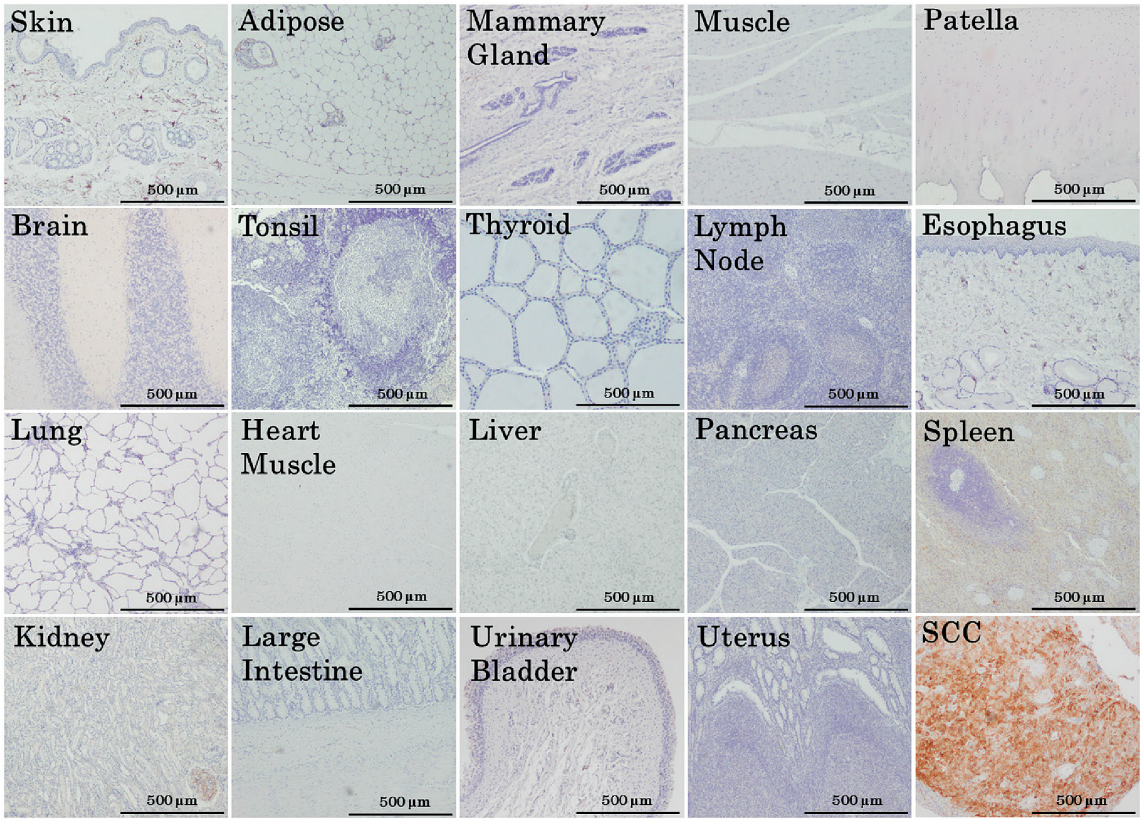

Fig. 3. Evaluation of PMab-38 staining in systemic normal tissues. Systemic normal tissues were stained by PMab38. SCC tissue was used as a positive control for PMab-38 staining. Most of the normal tissues were not positively stained with PMab-38. Only part of the kidney glomerulus was stained, but the lung and lymph nodes were not stained by PMab-38.

ASAC samples. In human medicine, it was reported that PDPN was expressed in many tumors, such as SCC, MM, HSA, OSA, malignant mesothelioma, Kaposi's sarcoma, testicular seminoma, dysgerminoma, and glioblastoma. However, it was reported that human PAC did not express PDPN[2,10,21]. Interestingly, in this study, most canine PAC was positively stained with PMab-38. In contrast to the human report, all canine HSA samples were not stained. Although there are discrepancies between dog and human tumors, there is a correlation between tumor types that express PDPN in humans and dogs. Our findings indicated that PMab-38 might be used for evaluating the prognosis or in antibody therapy for various types of tumors positively stained by PMab-38.

In this study, we did not detect positive staining of PMab-38 in any of the MM samples (n = 10). Previous research reported that 9 of 10 amelanotic MM samples were positively stained by PMab-38[23]. This difference may be related to the histlogical differences of canine MM because all of our samples were melanotic MM. As evaluated, sample numbers were small in both studies, and therefore, further investigation for this heterogeneity might be needed.

In this study, both oral and tonsillar SCC were stained by PMab-38. Oral SCC, which accounts for 20–30% of canine oral tumors, is locally invasive and systemic therapy is needed when the lesion is unresectable[29]. Tonsillar SCC has characteristics of rapid growth with infiltration of underlying tissues, early invasion of regional lymph nodes, and frequent metastasis to the lungs and other distant organs[6]. Since,

responses to chemotherapy are poor for these tumors, new therapies are desired to improve clinical outcomes for dogs with SCC. A cytotoxic antibody therapy using PMab-38 might be a good candidate therapeutic option for dogs with SCC.

It has been reported that PDPN expression is related to tumor malignancy, such as tumor invasion and metastasis. In human keratinocytes and MCF7 breast tumor cells, the forced expression of PDPN led to a drastic change in morphology and promoted cell spreading, migration, and invasion[35]. PDPN expression was also reported to be related to lymph node and lung metastasis by introducing lymphangiogenesis and platelet aggregation[1,8,13,17]. In PAC tissues in this study, tumor cells which invaded bronchial tubes were stained by PMab-38. This finding suggests the possibility that PDPN function in canine tumor cell invasion is similar to human reports. Furthermore, inhibition of dPDPN expressing tumor cells using PMab-38 may show anti-tumor effects via suppression of invasive and metastatic tumor cells.

We found that canine CAFs were stained by PMab-38 in almost all types of tumors, similar to reports in humans[2,10,21]. Both staining intensity and the ratio of positive CAFs in tumor tissues were higher than other tumor types, especially in TCC samples. In the other tumor types, the staining pattern of PMab-38 was heterogenic within tumor types. Although the underlying mechanism of PDPN expression in CAFs remains to be investigated, it was reported that fibroblasts had clonal heterogeneity and the protein expression pattern differed among clones[31], which could explain the heterogenetic staining pattern of PMab-38.

Several reports have shown that human and canine CAFs played an important role in tumor progression and dissemination by secreting cytokines and growth factors[16,39]. Moreover, it was reported that human melanoma with PDPN-positive CAFs showed significantly increased lymphatic invasion and sentinel lymph node metastasis[10]. The evidence regarding the negative function of CAFs has propelled the development of CAF-targeting therapies. Ohshio *et al.* reported that CAFs targeting therapy with antifibrotic agent enhanced anti-tumor immune responses with a dendric cell-based vaccine therapy in syngeneic mouse models[24]. Mertensers *et al.* demonstrated a cytotoxic drug for CAFs suppressed tumor growth and improved host survival through the mechanism of CAF apoptosis in a synergistic rat model of cholangiocarcinoma[20]. These preclinical studies may bring about a novel anti-tumor strategy in dogs, such as cytotoxic antibody therapy of chimeric PMab-38 that recognizes dPDPN expression in CAFs.

Although various normal tissues expressed PDPN, all normal tissues were negatively stained with PMab-38 except part of the kidney glomerulus. This staining difference between tumor tissues and normal tissues confirmed that PMab-38 recognized the dPDPN epitope, which was expressed in tumor tissues but not normal tissues. The mechanism of tumor specific epitope expression is unknown and further study to uncover the mechanism is needed. Positive staining in part of the glomerulus had the same pattern as the previous report. However, the staining pattern of the normal glomerulus was weaker compared to that of tumor tissues. This finding suggests that cytotoxic antibody therapy using PMab-38 will be less damaging to normal organs.

To the best of our knowledge, this is the first report of PMab-38 reactivity against various canine tumors and systemic normal tissues. Cell membranes of tumor cells were strongly stained by PMab-38 in SCC and PAC, but most normal cells were not stained. Remarkably, CAFs of most TCC and ASAC samples were extensively stained by PMab-38. These results of immunohistochemical staining with PMab-38 indicate that dPDPN recognized by PMab-38 might be a target for tumor antigen targeting therapy.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.67.1.25

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