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PMab-38 Recognizes Canine Podoplanin of Squamous Cell Carcinomas

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Podoplanin, a type I transmembrane protein, is expressed in lymphatic endothelial cells. Although we previously developed an anticanine podoplanin monoclonal antibody (mAb), PMab-38, immunohistochemistry (IHC) showed that it did not react with canine lymphatic endothelial cells. Here, we determined whether PMab-38 recognizes canine podoplanin of squamous cell carcinomas (SCCs) and clarified its epitope. In IHC, PMab-38 reacted with 83% of SCCs (15/18 cases). Flow cytometry showed that the epitope of PMab-38 was different from that of the platelet aggregation-stimulating domain of the N-terminus, which was detected by almost all antipodoplanin mAbs such as D2–40 or NZ-1. PMab-38 is expected to be useful for investigating the function of podoplanin in canine tumors.

Keywords: canine podoplanin, monoclonal antibody, immunohistochemistry

Podoplanin is a type I transmembrane sialoglycoprotein, also known as Aggrus/T1α.[1–4] Canine podoplanin was first reported as gp40.[5] It is expressed in normal cells including renal podocytes, lymphatic endothelial cells, and pulmonary type I alveolar cells.[3] Podoplanin activates platelet aggregation by binding to C-type lectin-like receptor-2 (CLEC-2) on platelets,[6,7] and the interaction between podoplanin and CLEC-2 facilitates blood/lymphatic vessel separation.[8] The expression of human podoplanin has been reported in many malignant tumors, such as oral squamous cell carcinomas (SCCs),[9] malignant brain tumors,[10–12] lung cancers,[13] esophageal cancers,[14] malignant mesotheliomas,[15] testicular tumors,[16] and osteosarcomas.[17] Podoplanin expression is also associated with malignant progression and cancer metastasis.[6,10] However, canine podoplanin in tumors has not been investigated because of the absence of a specific and sensitive monoclonal antibody (mAb). We recently reported anticanine podoplanin mAb, PMab-38,[18] which is useful for immunohistochemistry (IHC). Furthermore, PMab-38 specifically reacts with canine podoplanin in flow cytometry and Western blotting.[18]

Here, we determined whether PMab-38 can recognize podoplanin of canine SCCs because human podoplanin is highly expressed in these cells and is involved in tumor malignancy.[9] We first checked the PMab-38 reactivity against SCCs (8 oral SCCs and 10 skin SCCs) in IHC. (Fig. 1 and Supplementary Fig. S1). Among them, only case 16 (1/18; 5.6%) was detected by PMab-38 in >50% of tumor cells. In total, tumor cells in 15 out of 18 cases (83%) were stained with PMab-38 in a membrane-staining pattern. Similarly, cancer-associated fibroblasts in 14 out of 18 cases (78%) were detected by PMab-38. Human podoplanin expression in several cancers contributes to poor prognosis[19]; therefore, PMab-38 might be useful for investigating the pathological function of canine podoplanin in the tumor microenvironment.

Next, we investigated the epitope of PMab-38 using flow cytometry. To this end, we produced several deletion mutants of canine podoplanin, which are expressed in CHO-K1 cells, including dN23 (corresponding to 23–169 amino acids [aa]), dN40 (corresponding to 40–169 aa), dN60 (corresponding to 60–169 aa), dN80 (corresponding to 80–169 aa), and dN100 (corresponding to 100–169 aa). Antipodoplanin mAbs usually detect the platelet aggregation-inducing (PLAG) domain, particularly PLAG1–PLAG3 (corresponding to 36–61 aa) near the N-terminus[20]; in contrast, PMab-38 reacted with dN23, dN40, dN60, and dN80. When antipodoplanin mAbs reacted with the PLAG domain, they could not react with dN60, dN80, and dN100, indicating that the PMab-38 epitope is far from PLAG1–PLAG3 (Fig. 2). Previously, we

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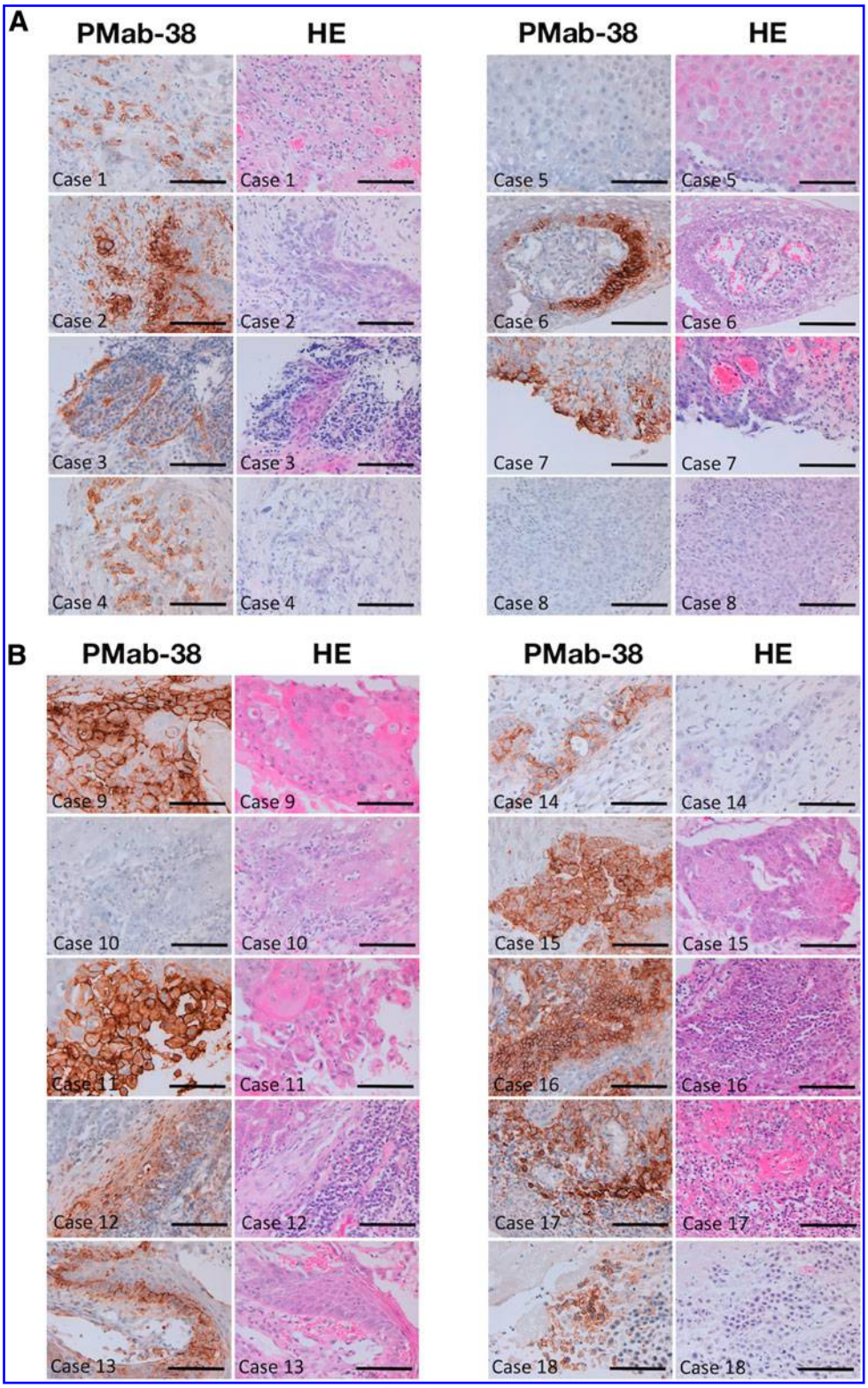

FIG. 1. Immunohistochemical analysis against canine SCCs by PMab-38. Canine tissues **(A,** oral SCCs; **B,** skin SCCs**)** were obtained from North Lab (Hokkaido, Japan). In total, 4 μm thick histologic sections were deparaffinized in xylene, rehydrated, and autoclaved in citrate buffer (pH 6.0; Dako, Glostrup, Denmark) for 20 minutes. Sections were incubated with 10 μg/mL of PMab-38 overnight at 4°C followed by treatment with Envision+ kit for 30 minutes (Dako). Color was developed using 3, 3-diaminobenzidine tetrahydrochloride (DAB; Dako) for 2 minutes, after which the sections were counterstained with hematoxylin (Wako Pure Chemical Industries Ltd., Osaka, Japan). Hematoxylin and eosin (HE) staining was also performed. Scale bar: 100 μm.

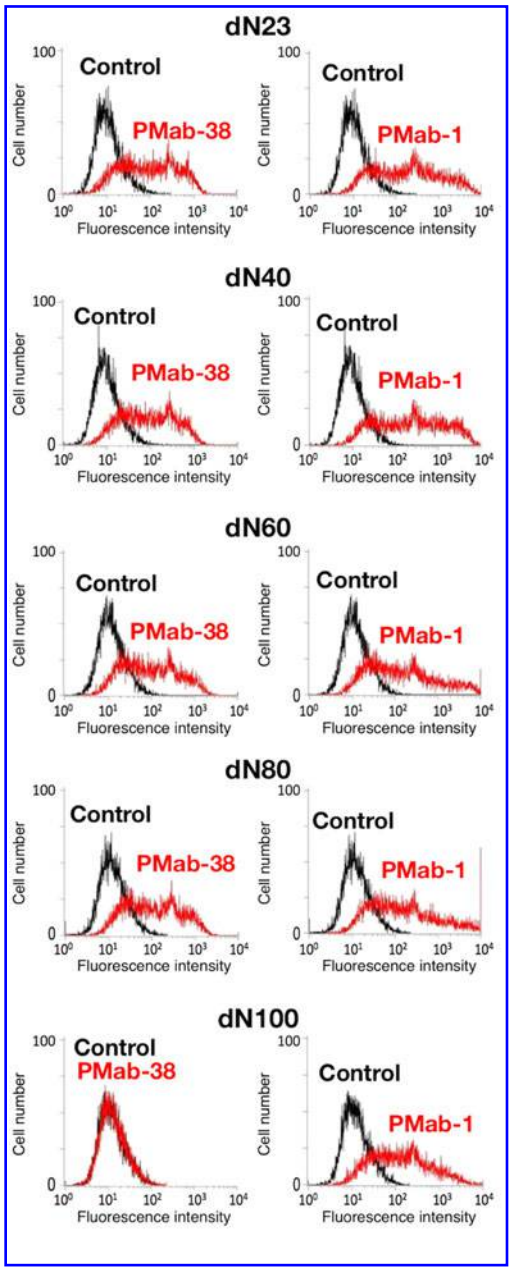

FIG. 2. Epitope mapping of PMab-38. Amplified canine podoplanin cDNA was subcloned into a pCAG vector (Wako) with an MAP tag, which was added to the N-terminus. MAP tag is a peptide: GDGMVPPGIEDK, which is derived from mouse podoplanin. The signal sequence of IL-2 was inserted into the N-terminus. Deletion mutants of canine podoplanin were generated using sense primers and an antisense primer for dN23, dN40, dN60, dN80, and dN100, as summarized in Supplementary Table S1. CHO-K1 cells were transfected with the plasmids using electroporation. Each deletion mutant of CHO/canine podoplanin was cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Each cell was treated with PMab-38 or PMab-1 (anti-MAP tag) at a concentration of 1 μg/mL for 30 minutes at 4°C followed by treatment with Oregon green-conjugated antimouse IgG or antirat IgG (1:1000 diluted; Thermo). Fluorescence data were collected using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

produced an antihuman podoplanin mAb, LpMab-7, the epitope of which is Arg79-Leu83 of human podoplanin,[17] which corresponds to His88-Gly90 of canine podoplanin. LpMab-7 demonstrated high sensitivity compared with other antihuman podoplanin mAbs,[21] suggesting that mAbs against these epitopes have high sensitivity and specificity against not only human podoplanin but also canine podoplanin.

Taken together, these data show that PMab-38 could be useful for uncovering the pathophysiological function of podoplanin in canine tumors. PMab-38 did not react with the lymphatic endothelium in our previous study[18]; therefore, the PMab-38 epitope might be involved in cancer specificity of canine podoplanin.

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Author Disclosure Statement

No competing financial interests exist.

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