Expression of podoplanin in various types of feline tumor tissues

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Running head: PODOPLANIN EXPRESSION IN FELINE TUMORS
ABSTRACT.

Podoplanin is expressed in various human tumors where it promotes tumor progression, epithelial-mesenchymal transition, and distant metastasis. Podoplanin is also expressed in cancer-associated fibroblasts and induces tumor malignancy. The objective of this study was to evaluate podoplanin expression in various types of feline tumor tissues. Immunohistochemical analysis revealed that podoplanin was expressed in cells of 13/15 (87%) squamous cell carcinomas and 5/19 (26%) fibrosarcomas. Moreover, cancer-associated fibroblasts expressed podoplanin in most tumor types, including 18/21 (86%) mammary adenocarcinoma tissues. Our findings demonstrate that various types of feline tumor tissues expressed podoplanin, indicating the importance of the comparative aspects of podoplanin expression, which may be used as a novel research model for podoplanin biology.

KEY WORDS: cat, feline tumor, immunohistochemistry, podoplanin
Podoplanin (PDPN) is a type I transmembrane sialoglycoprotein expressed in renal podocytes, lymphatic endothelial cells, and pulmonary type I alveolar cells [5, 6, 37]. It has been widely used as a prominent marker of lymphatic endothelial cells, where it is strongly expressed [24]. PDPN plays an essential role in the formation of a normal lymphatic system during embryonic development, as well as in platelet aggregation [2, 4, 12, 31].

In human medicine, PDPN was reported to be overexpressed in various types of tumors, including squamous cell carcinoma [18], malignant astrocytic tumors [19], malignant mesothelioma [25], hemangiosarcoma [6], osteosarcoma [1], and germinoma [17]. Many reports have demonstrated that PDPN expression is associated with tumor malignancy via promoting proliferation, epithelial-mesenchymal transition, and metastasis by inducing tumor cell migration and platelet aggregation; hence, PDPN has been considered as a therapeutic target [32]. In addition, cancer-associated fibroblasts (CAFs), a major component of the tumor stroma, have been reported to overexpress PDPN [11, 14, 28]. PDPN-positive CAFs have been reported to strongly contribute to tumor progression by promoting local invasion and lymph node metastasis [9, 21]. Importantly, a high ratio of PDPN-positive CAFs have been reported to be associated with poor prognosis in human patients with lung adenocarcinoma [21].

In veterinary medicine, PDPN was reported to be overexpressed in some naturally occurring tumors in dogs [17]. Cats living with humans have also been reported to develop various types of tumors that resemble those found in humans and dogs. Naturally occurring tumors in cats have increased in recent years [3], owing to increased life expectancy resulting from increased nutritional value of food, availability of vaccines for common infectious diseases, and advancements in veterinary medicine. Recently, analysis of the feline genome provided evidence of strong similarities with humans [22]. Because many features of tumors in cats are similar to those in humans, the study of
feline tumors would contribute to our understanding of tumor pathogenesis, progression, and therapy and could be considered as a novel research model [26].

Although the evaluation of PDPN expression in feline tumors has provided us very important findings, such as the comparative aspect of PDPN expression among species and a potential novel research model, there is no research on PDPN expression in feline tumor tissues because of the lack of feline PDPN-specific antibodies. To address this issue, we previously developed monoclonal antibodies against feline PDPN (fPDPN) that can be used to specifically recognize fPDPN expression in normal tissues using immunohistochemistry [7, 34]. Thus, the objective of this study was to evaluate the distribution of fPDPN expression in various types of feline tumor tissues by performing immunohistochemical analysis.

A total of 145 paraffin-embedded tumor tissues were evaluated. These tumor tissues were extracted surgically from the cats at the Veterinary Medical Center of the University of Tokyo between 2011 and 2017. Permission for collection and use of the resected tissues was obtained from the owners. The tissue samples were diagnosed by two veterinary pathologists (J.C. and K.U.) certified by the Japanese College of Veterinary Pathologists at the Department of Veterinary Pathology at The University of Tokyo. The tumor types included mammary adenocarcinoma (n=21), oral and skin fibrosarcoma (n=19), head and neck squamous cell carcinoma (n=15), mast cell tumor (n=11), ceruminous adenocarcinoma (n=10), colorectal adenocarcinoma (n=10), meningioma (n=10), nasal adenocarcinoma (n=10), osteosarcoma (n=10), peripheral nerve sheath tumor (n=10), thyroid adenoma (n=10), thymoma (n=6), and pulmonary adenocarcinoma (n=3). Normal skin, intestine, mammary gland, thyroid, and cerebral tissue from one healthy cat were used as normal controls. This study was approved by The University of Tokyo Animal Care and Use Committee (approval number: P15-099).
Immunohistochemical staining was performed using primary antibodies specific for fPDPN (mouse monoclonal antibody, clone: PMab-52, 2 µg/ml) [34], anti-α-smooth muscle actin (α-SMA) (mouse monoclonal antibody, clone: 1A4, 1:400, Agilent Technologies, Santa Clara, CA, USA), and anti-p63 (mouse monoclonal antibody, clone 4A4, 1:100, Biocare Medical, CA, USA). All tumor tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and cut into 4 µm thick serial sections. Each section was subsequently dewaxed and rehydrated in xylene and graded ethanol, followed by antigen retrieval using citrate buffer (pH 6.0) at 100°C for 30 min in boiling water. After washing with Tris-buffered saline and 0.1% Tween® 20 detergent (TBST), endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 10 min at 24-26 °C. The specimens were then washed with TBST and incubated in 8% skim milk for 1 hr at 24-26 °C to reduce non-specific binding before incubation with primary antibodies, including anti-fPDPN antibodies for 1 hr at 24-26 °C or anti-α-SMA antibodies overnight at 4 °C in a humidified chamber. After washing with TBST, the sections were incubated with a horseradish peroxidase-conjugated anti-mouse antibody (EnVision™+ System, HRP labeled polymer; K4001; Agilent Technologies) for 30 min at 24-26 °C. Thereafter, the sections were washed with TBST, incubated with 3,3′-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) solution for 3 min, and counterstained with Mayer's hematoxylin. Sections of feline lung tissues were used as positive controls for fPDPN [10], while sections of feline small intestine were used as positive controls for α-SMA [16, 27]. Negative controls did not include the primary antibodies. The specimens were considered to be positive for fPDPN if >10% of the tumor cells or stromal cells were stained in five randomly selected independent high-magnification (400×) fields [11, 15]. Five independent fields were selected randomly. Ratios of fPDPN-positive samples in each tumor type were investigated separately in tumor cells and stromal cells.
The fPDPN-positive sample ratios in the tumor cells varied among the tumor types. Feline PDPN expression was observed in the samples of squamous cell carcinoma (13/15; 87%), fibrosarcoma (5/19; 26%), and colorectal adenocarcinoma (2/10; 20%) (Table 1); in contrast, fPDPN-positive cells were not detected in peritumoral normal squamous epithelium (n=11) and normal intestinal glands (n=7), except in the lymph vessels (Supplementary Fig. 1). The ratio of positively stained tumor cells varied in all tumor samples. More than 50% of the tumor cells derived from some samples of squamous cell carcinoma and fibrosarcoma stained positive for fPDPN expression, but less than 10% of tumor cells tested positive for fPDPN expression in colorectal adenocarcinoma samples. The expression of fPDPN in positively stained samples was strong in the cell membranes of tumor cells and moderate in the cytoplasm of tumor cells. This result was consistent with the staining observed in the positive control feline lung tissues (Fig. 1 and Supplementary Fig. 1).

In positively stained samples, the cell membranes of tumor cells exhibited strong fPDPN expression, whereas their cytoplasm showed moderate fPDPN expression (Fig. 1). Staining patterns, including diffuse and focal staining patterns, were heterogeneous among positively stained samples. In addition, we could not identify any specific staining patterns based on the tumor type or clinical characteristics. The labeling intensity of the tumor cells was comparable in all positively stained tumor tissues.

In the tumor stroma, large spindle-shaped mesenchymal cells expressed fPDPN in almost all tumor types. The fPDPN-positive sample ratios in the tumor stromal were 9/15 (60%) for squamous cell carcinoma, 7/10 (70%) for colorectal adenocarcinoma, 18/21 (86%) for mammary adenocarcinoma, 3/10 (30%) for nasal adenocarcinoma, 2/10 (20%) for ceruminous adenocarcinoma and thyroid adenoma, respectively, and 1/10 (10%) for meningioma, peripheral nerve sheath tumor, and thymoma, respectively (Table 1). In fibrosarcoma and osteosarcoma samples, we did not evaluate...
the stromal cells separately, because we could not distinguish between tumor and stromal cells (Fig. 1).

As controls to the tumor tissues, normal tissues of the skin, intestine, mammary gland, thyroid, and cerebral tissue from one healthy cat were stained; fPDPN expression was not detected in any of these samples, except for endothelial cells in the thyroid tissue (Supplementary Fig. 2).

The fPDPN-positive large spindle-shaped mesenchymal cells were suspected to be CAFs, which have been reported to express PDPN in human tumor tissues. We evaluated α-SMA expression, which is generally utilized to detect CAFs [33], in fPDPN-positive spindle-shaped mesenchymal cells. Some parts of α-SMA positive cells in the stromal region co-expressed fPDPN (Fig. 2). Feline PDPN was strongly expressed in both the membranes and cytoplasm of α-SMA-positive cells in the stromal region. The staining intensity and the ratio of fPDPN positive cells in the stromal region were especially high in mammary adenocarcinoma samples compared to other tumor types (Table 1 and Fig. 2). In mammary adenocarcinoma samples, p63 expression which is a marker of myoepithelial cells, was evaluated, and no positive staining was observed in the tumor tissues (Supplementary Fig. 3).

Altogether, this study demonstrated the immunohistochemical expression of fPDPN in tumor cells and stromal cells in various types of feline tumor tissues. The tumor cell membranes showed strong fPDPN expression in squamous cell carcinoma, fibrosarcoma, and colorectal adenocarcinoma samples, similar to findings reported for canine and human tumors [13, 23]. However, feline meningioma and osteosarcoma, which were reported to cause the expression of PDPN in human homogenous tumor tissues, did not demonstrate strong staining (Table. 1). This discrepancy in PDPN expression in tumor tissues of different species indicates differences in the pathophysiology of PDPN expression. Additionally, although the feline normal lung tissues were positively stained, the feline lung tumor tissues were negatively stained. PDPN is expressed in type I alveolar epithelial cells in normal human lung tissues [37]. Therefore, differences in origin between the PDPN expressing cells in feline normal lung tissue and the PDPN negatively stained tumor cells in feline lung tumor tissues
might cause a difference in PDPN expression between normal and tumor tissues. Some feline tumor cells and α-SMA-positive cells in the stromal region expressed PDPN, which was reported to be related to malignancy of various tumors. Because of the limited sample volume and clinical information, we could not determine the clinical significance of fPDPN expression in feline tumors. Further studies are required to clarify the clinical significance of fPDPN.

Squamous cell carcinoma is the most common type of oral tumor in cats, accounting for approximately 70% of tumors in the feline oral cavity [29]. Feline oral squamous cell carcinoma is highly locally aggressive, often extending into the bone tissues, and can be associated with pain and inability to eat. In this study, most tumor cells in feline squamous cell carcinoma expressed fPDPN. High expression of PDPN in tumor cells in human oral squamous cell carcinoma tissues is strongly correlated with poor prognosis [8, 36]. PDPN expression in human squamous cell carcinoma is associated with various malignant mechanisms, including tumor cell invasion, metastasis, proliferation, and cancer stemness [20, 30]. These findings indicate that fPDPN expression in tumor cells may contribute to the aggressive behavior of feline squamous cell carcinoma, and fPDPN expressed on feline squamous cell carcinoma might be a candidate therapeutic target. Moreover, fPDPN-expressing feline squamous cell carcinoma might be a novel research model for PDPN-positive human oral squamous cell carcinoma.

We found that α-SMA positive cells in the stromal region expressed fPDPN in most types of tumors, as reported in humans and dogs [11, 13, 14, 28]. In mammary adenocarcinoma, colorectal adenocarcinoma, and squamous cell carcinoma samples, the ratios of positively stained α-SMA cells in the stromal region of tumor tissues were higher than those in other tumor types. Although it is difficult to completely distinguish between CAFs and myoepithelial cells from α-SMA expression in mammary adenocarcinoma [35], α-SMA positive cells in the stromal region of mammary adenocarcinoma were considered as CAFs because of their cell morphology, histological structures,
and negative expression of p63 (a myoepithelial cell marker). In human medicine, PDPN-expressing CAFs have been reported to promote local invasion and lymph node metastasis of tumor cells [14, 21]. Furthermore, it has been demonstrated that CAFs exist in feline tumors, and the presence of CAFs in feline oral squamous cell carcinoma was significantly associated with poor prognosis [15]. Therefore, fPDPN expressing α-SMA positive cells in the stromal region may be associated with malignancy of these tumors as well as human tumors.

This study showed the distribution of fPDPN expression in feline normal and tumor tissues. However, our data is limited. Firstly, the co-expression of fPDPN and α-SMA in stromal cells could not be confirmed by double staining. Secondly, number of normal control samples was small; for example, we obtained only one normal tissue sample from mammary gland, thyroid, and cerebrum and no tissue from normal nasal cavity, ear canal, and thymus.

In conclusion, fPDPN expression was observed in various types of feline tumor tissues for the first time. The cell membranes of squamous cell carcinoma tumor cells strongly express fPDPN. Remarkably, α-SMA positive cells in the stromal region of several feline tumors extensively expressed fPDPN. These novel findings suggest the importance of comparative aspects of PDPN expression in naturally occurring tumors among humans, cats, and dogs, and the possibility of feline tumors as a new research model for PDPN biology.

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**FIGURE LEGENDS**

**Figure 1.** Evaluation of the expression pattern of feline podoplanin (fPDPN) in tumor cells. (A) A representative image of hematoxylin and eosin (HE) staining in feline squamous cell carcinoma. (B, C) Representative immunostaining images of podoplanin in squamous cell carcinoma. Tumor cell membranes were broadly and strongly stained, while the cytoplasm of tumor cells were moderately stained in squamous cell carcinoma. Black arrow heads indicate tumor cells which fPDPN strongly express on tumor cells membranes. (D) A representative image of HE staining in feline fibrosarcoma. (E, F) Representative immunostaining images of fPDPN in fibrosarcoma are shown. In fibrosarcoma samples, part of the cells was stained, but we could not distinguish tumor cells from stroma cells.

**Figure 2.** Evaluation of the expression pattern of feline podoplanin (fPDPN) in stroma cells. A representative image of hematoxylin and eosin staining (A) and immunostaining images of fPDPN (B) and α-smooth muscle actin (α-SMA) (C) in feline mammary adenocarcinoma are shown. Large spindle-shaped mesenchymal cells in the tumor stroma expressed α-SMA and part of the α-SMA positive cells expressed fPDPN.
| Tumor types                        | Samples | Tumor cells (%) | Stroma cells (%) |
|-----------------------------------|---------|-----------------|------------------|
| Mammary adenocarcinoma           | 21      | 0 (0)           | 18 (86)          |
| Fibrosarcoma                     | 19      |                 | 5 (26)*          |
| Squamous cell carcinoma          | 15      | 13 (87)         | 9 (60)           |
| Mast cell tumor                  | 11      | 0 (0)           | 0 (0)            |
| Colorectal adenocarcinoma        | 10      | 2 (20)          | 7 (70)           |
| Nasal adenocarcinoma             | 10      | 0 (0)           | 3 (30)           |
| Ceruminous adenocarcinoma        | 10      | 0 (0)           | 2 (20)           |
| Thyroid adenoma                  | 10      | 0 (0)           | 2 (20)           |
| Meningioma                       | 10      | 0 (0)           | 1 (10)           |
| Peripheral nerve sheath tumor    | 10      | 0 (0)           | 1 (10)           |
| Osteosarcoma                     | 10      | 0 (0)*          |                  |
| Thymoma                           | 6       | 0 (0)           | 1 (10)           |
| Pulmonary adenocarcinoma         | 3       | 0 (0)           | 0 (0)            |

* We could not distinguish tumor cells from stromal cells.
Squamous cell carcinoma

Fibrosarcoma

Fig. 1
Mammary adenocarcinoma

Fig. 2
Supplementary figure. 1. Evaluation of the expression pattern of fPDPN in normal lung tissues (A, B), normal squamous epitheliums (C), and normal intestinal glands (D).
Supplementary figure 2. Evaluation of the expression pattern of fPDPN in the normal lung (A), skin (B), intestine (C), mammary gland (D), thyroid (E) and cerebrum (F).
Supplementary figure 3. Evaluation of the expression pattern of p63 in the normal mammary gland (A, D) and mammary adenocarcinoma (B, E) and the expression pattern of α-SMA in mammary adenocarcinoma (C, F).