Programmed Cell Death Ligand 1 Expression in Canine Cancer

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Abstract. Background: Antibody therapy targeting programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1) is a promising therapy in human cancer, but only limited information on PD-L1 expression in canine tumors is available. Materials and Methods: PD-L1 expression was examined in 31 canine tumor cell lines of various origins by flow cytometry and western blotting, and in canine tumor and normal tissue specimens by immunohistochemistry. Results: PD-L1 was only expressed on the cell surface of a small number of cell lines but was found expressed within the cells of almost all cell lines. Immunohistochemistry revealed that PD-L1 is frequently expressed in malignant melanoma, mammary gland tumor, mast cell tumor and lymphoma, but less frequently in soft-tissue sarcoma and hemangiosarcoma. PD-L1 was also expressed in some of the cells of normal canine tissue specimens. Conclusion: Canine tumors with PD-L1 expression that were identified in this study are potential candidates for antiPD-1 and antiPD-L1 therapy.

Incidence of canine cancer has been gradually increasing over the past two decades due to the prolonged lifespan of dogs (1). Compared to human patients, early detection of canine cancer is challenging and cancer is often diagnosed at an advanced stage. Various types of cancer, such as solid tumors, hematological tumors, adenocarcinoma and soft-tissue sarcoma, can be seen in dogs, and cancer therapy in dogs generally follows the same principles as in human medicine. The three main therapies for cancer, namely surgery, radiation therapy and chemotherapy, have since long been practiced in veterinary oncology. However, novel therapies are still required due to unfavorable outcomes with the currently available treatments.

To date, a number of emerging cancer therapies have been reported in human medicine. Among them, immunotherapy has been regarded as one of the most promising. In particular, antibody therapy targeting immune checkpoint molecules, such as cytotoxic T-lymphocyte antigen 4 (CTLA4), programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1), have shown favorable results in many clinical studies of human cancer (2, 3). CTLA4 and PD-1 are expressed in T-cells and work as immunosuppressive molecules to prevent excessive T-cell reaction. As a part of the normal immunosuppressive mechanism, PD-L1 on dendritic cells binds to PD-1 expressed on T-cells and this suppresses T-cell function (4). On the other hand, tumor cells also express PD-L1 on their cell surface and this has been reported to be involved in the evasion from host immunity through binding of PD-1 expressed on T-cells (5). Thus, antibodies against PD-1 and PD-L1 can be used as therapy to restore T-cell function, leading to enhancement of the host antitumor immunity. However, the role of immune checkpoint molecules in mammals other than humans and the mouse have not yet been elucidated. Even though canine cancers are good comparative models for spontaneously occurring cancer, only limited information on PD-1 and PD-L1 expression in canine cancer is available (6).
Committee Regulations. Tumor specimens previously used for experiments obtained from healthy beagle dogs kept for experimental purposes. The normal tissue specimens used in this study were provided by Dr. Munekazu Nakaichi (Yamaguchi University), Dr. Yasuhiko Okamura, Dr. Maxey Wellman (The Ohio State University), respectively. GL-1, Nody-1, CLGL-90, 17-71 and CLBL-1 were kindly provided by Dr. Hajime Tsujimoto (The University of Tokyo). LMeC, all canine mammary gland tumor cell lines and three malignant melanoma cell lines (CMeC1, CMeC2, KMeC, LMeC, CMGD2 and CMGD5), canine osteosarcoma (Abrams, D17, Gracie, Mackinley and Moresco), canine mammary gland tumor (CIPp, CIPm, CTBp, CTBm, CHMp, CHMm and CNMm), canine mast cell tumor (VIMC, CMMC, CoMS and HRMC) and canine lymphoma (GL-1, Ema, Nody-1, UL-1, CLGL-90, 17-71, CLBL-1, CLC and CLK) have been described in previous studies (7-9) and were cultured in R10 complete medium (RPMI-1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol). Cell lines from canine malignant melanoma (CMeC1, CMeC2, KMeC, LMeC, CMGD2 and CMGD5), canine osteosarcoma (Abrams, D17, Gracie, Mackinley and Moresco), canine mammary gland tumor cell lines (CIPp, CIPm, CTBp, CTBm, CHMp, CHMm and CNMm), canine mast cell tumor (VIMC, CMMC, CoMS and HRMC) and canine lymphoma (GL-1, Ema, Nody-1, UL-1, CLGL-90, 17-71, CLBL-1, CLC and CLK) have been described in previous studies (7-9) and were cultured in R10 complete medium (RPMI-1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol). All these cell lines were cultured at 37°C in a humidified incubator with 5% CO₂. HEK293T cells were provided by Dr. Kazuo Nishigaki (Yamaguchi University). Four canine malignant melanoma cell lines (CMeC1, CMeC2, KMeC and LMeC), all canine mammary gland tumor cell lines and three canine mast cell tumor cell lines (VIMC, CMMC, CoMS and CoMS) were kindly provided by Dr. Takayuki Nakagawa (The University of Tokyo). Two canine malignant melanoma (CMGD2 and CMGD5) and all canine osteosarcoma cell lines were kindly provided by Dr. Jaime Modiano (University of Minnesota) and Dr. Douglas Thamm (Colorado State University), respectively. Ema, CLC and CLK cell lines were established in our laboratory, HRMC, CL-1 and UL-1 were kindly provided by Dr. Hajime Tsujimoto (The University of Tokyo). GL-1, Nody-1, CLGL-90, 17-71 and CLBL-1 were kindly provided by Dr. Munekazu Nakaichi (Yamaguchi University), Dr. Yasuhiko Okamura, Dr. Maxey Wellman (The Ohio State University), Dr. Steven Suter (North Carolina University) and Dr. Barbara Rutgen (University of Veterinary Medicine, Vienna), respectively.

Materials and Methods

Cell lines. Human embryonic kidney HEK293T cell line was maintained in D10 complete medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol). Cell lines from canine malignant melanoma (CMeC1, CMeC2, KMeC, LMeC, CMGD2 and CMGD5), canine osteosarcoma (Abrams, D17, Gracie, Mackinley and Moresco), canine mammary gland tumor cell lines (CIPp, CIPm, CTBp, CTBm, CHMp, CHMm and CNMm), canine mast cell tumor (VIMC, CMMC, CoMS and HRMC) and canine lymphoma (GL-1, Ema, Nody-1, UL-1, CLGL-90, 17-71, CLBL-1, CLC and CLK) have been described in previous studies (7-9) and were cultured in R10 complete medium (RPMI-1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol). All these cell lines were cultured at 37°C in a humidified incubator with 5% CO₂. HEK293T cells were provided by Dr. Kazuo Nishigaki (Yamaguchi University). Four canine malignant melanoma cell lines (CMeC1, CMeC2, KMeC and LMeC), all canine mammary gland tumor cell lines and three canine mast cell tumor cell lines (VIMC, CMMC, CoMS and CoMS) were kindly provided by Dr. Takayuki Nakagawa (The University of Tokyo). Two canine malignant melanoma (CMGD2 and CMGD5) and all canine osteosarcoma cell lines were kindly provided by Dr. Jaime Modiano (University of Minnesota) and Dr. Douglas Thamm (Colorado State University), respectively. Ema, CLC and CLK cell lines were established in our laboratory, HRMC, CL-1 and UL-1 were kindly provided by Dr. Hajime Tsujimoto (The University of Tokyo). GL-1, Nody-1, CLGL-90, 17-71 and CLBL-1 were kindly provided by Dr. Munekazu Nakaichi (Yamaguchi University), Dr. Yasuhiko Okamura, Dr. Maxey Wellman (The Ohio State University), Dr. Steven Suter (North Carolina University) and Dr. Barbara Rutgen (University of Veterinary Medicine, Vienna), respectively.

Specimens. The normal tissue specimens used in this study were obtained from healthy beagle dogs kept for experimental purposes according to the Yamaguchi University Animal Care and Use Committee Regulations. Tumor specimens previously used for diagnostic purposes were obtained from several pathology laboratories in Japan (Laboratories of Veterinary Pathology of Yamaguchi University, Gifu University and University of Tokyo).

Flow cytometry. Cell-surface expression of PD-1 and PD-L1 in the canine tumor cell lines was detected by incubation with antibodies to canine PD-1 (4F12-E6) and PD-L1 (G11-6) (prepared in our laboratory, article in preparation), followed by phycoerythrin (PE)-conjugated anti-rat IgG antibody (Southern Biotech, Birmingham, AL, USA). Cells were incubated with propidium iodide immediately before flow cytometric analysis in order to allow gating out of dead cells. The samples were analyzed using BD Accuri C6 (BD Biosciences, San Jose, CA, USA), and the obtained results were analyzed using FlowJo software (Treestar, San Carlos, CA, USA). The canine tumor cell lines were also incubated with 10 ng/ml interferon-gamma (IFN-γ) (R&D Systems, Minneapolis, MN, USA) for 24 h and collected for staining with antibody to canine PD-L1 before cell-surface expression of PD-L1 was detected using the same method described above.

Transfection. To confirm the crossreactivity of commercially available anti-human PD-L1 antibody to canine PD-L1 exogenously overexpressed in HEK293T cells, the plasmid vector coding canine PD-L1 (pMx-IP-cPDL1, constructed in our laboratory) or empty vector, pMx-IP (kindly provided by Dr. Toshio Kitamura (The University of Tokyo), was transiently transfected into HEK293T cells. Briefly, HEK293T cells were seeded at 7.5×10⁶ cells/well in 6-well plates one day prior to transfection. On the day of transfection, 1.25 μg of plasmid was mixed with PEI Max (Polysciences, Warrington, PA, USA) in the OPTI-MEM buffer, and was added onto culture with antibody to canine PD-L1 before cell-surface expression of PD-L1 was detected using the same method described above.

Western blot analysis. Plasmid-transfected HEK293T cells or canine tumor cell lines were collected and washed once with cold PBS. The cells were lysed for 15 mins at 4°C in lysis buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM phenylmethylsulfonyl fluoride and complete Mini EDTA-free protease inhibitor mixture (Roche Diagnostics, Tokyo, Japan), centrifuged at 20,000 x g for 15 min at 4°C, and the supernatant (cell lysate) was collected. The amount of protein in the cell lysate was measured with Micro BCA Protein Assay Reagent Kit (Thermo Scientific, Yokohama, Japan) according to the manufacturer’s recommendations. Equal amounts of samples (20-30 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 9% acrylamide gel before proteins were blotted onto Hybond ECL membranes (GE Healthcare, Tokyo, Japan). Immediately after blotting, the membranes were blocked with blocking buffer (Tribuffered saline with 0.05% Tween 20% and 5% skimmed milk; TBS-T) for 1 h at RT. The membranes were then incubated overnight at 4°C with primary antibody, rabbit monoclonal antibody to human PD-L1 (EPR1161; Abcam, Tokyo, Japan) or anti-FLAG M2 (Sigma-Aldrich, Tokyo, Japan) in TBS-T with 0.5% skimmed milk. The membranes were washed for three times with TBS-T for 10 min before incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:4,000 dilution; goat anti-mouse IgG polyclonal antibody; Merck Millipore, Tokyo, Japan) for 1 h at RT. The membranes were washed three times with TBS-T for 10 min, and visualized by immersion in Western Lightning Chemiluminescence Reagent (Perkin–Elmer, Yokohama, Japan).
Table I. Cell surface expression of canine programmed cell death-1 (PD-1) and programmed cell death-ligand (PD-L1) in canine tumor cell lines.

<table>
<thead>
<tr>
<th>PD-1</th>
<th>PD-L1</th>
<th>PD-L1</th>
</tr>
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<tbody>
<tr>
<td>(Name of cell lines with positive expression)</td>
<td>(Name of cell lines with positive expression)</td>
<td>(Cells treated with IFN-γ)</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>0/6</td>
<td>1/6 (KMcC)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>0/5</td>
<td>2/5 (D17 and Mackinley)</td>
</tr>
<tr>
<td>Mammary gland tumor</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>Mast cell tumor</td>
<td>2/4 (VIMC and CMMC)</td>
<td>3/4 (VIMC, CMMC and CoMS)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>2/9 (Ema and CLK)</td>
<td>2/9 (17-71 and CLBL-1)</td>
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IFN-γ: Interferon-γ.

Results

Detection of canine PD-1 and PD-L1 expression on the cell surface of canine tumor cell lines by flow cytometry. In order to elucidate canine PD-1 and PD-L1 expression in canine tumors, an extensive number of canine tumor cell lines were stained with antibodies against canine PD1 (4F12-E6) and PD-L1 (G11-6) for flow cytometric analysis (Table I). As expected, PD-1 was not detected in all of the cell lines, only in two mast cell tumor (VIMC and CMMC) and two lymphoma (Ema and CLK) cell lines. PD-L1 expression was observed in one out of six melanoma (KMeC), two out of five osteosarcoma (D17 and Mackinley), three out of four mast cell tumor (VIMC, CMMC and CoMS), two out of nine lymphoma (17-71 and CLBL-1) and none of the seven mammary gland tumor cell lines. Only the flow cytometric results of the canine tumor cell lines that express canine PD-1 and PD-L1 are shown in Figure 1. Interestingly, IFN-γ treatment of the cell lines for 24 h was able to induce cell-surface expression of PD-L1 in all except the lymphoma cell lines (Table I).

Confirmation of the cross-reactivity of antibody to human PD-L1 to canine PD-L1 protein by western blotting. As the results of the flow cytometric analysis showed that cell-surface expression of PD-L1 in canine tumor cell lines was far less than expected, we decided to analyze the expression of PD-L1 in various types of canine tumor using formalin-fixed paraffin-embedded specimens. Unfortunately, the anti-canine PD-1 and anti-PD-L1 used in the flow cytometric analysis were not able to detect canine PD-1 and PD-L1
molecules in these specimens. Therefore, in search of a suitable antibody for our purpose, we examined the cross-reactivity of the monoclonal antibody against human PD-L1 to the canine PD-L1 molecule. Western blotting using the anti-human PD-L1 antibody showed a strong band in the lane where lysate of HEK293T cells transfected with canine PD-L1 was loaded (Figure 2A). We also confirmed that the band correlated with transfected canine PD-L1 as the same band was detected by the anti-FLAG antibody on the same membrane. These results verified that the monoclonal antibody to human PD-L1 cross-reacts with canine PD-L1.

Detection of PD-L1 expression in canine tumor cell lines by western blotting. PD-L1 expression in the canine tumor cell lines was examined by western blotting using the same anti-human PD-L1 antibody. As shown in Figure 2B-F, one clear band at the expected molecular weight was detected in all cell lines, despite the varied expression in flow cytometric analysis (Table I). These results indicate that the antibody to human PD-L1 cross-reacts with canine PD-L1 molecules and all canine cell lines used in this study expressed PD-L1, even though most of the them did not exhibit any PD-L1 expression on the cell surface.

Screening of PD-L1 expression in canine normal tissue specimens by immunohistochemical analysis. Next, we determined the expression of PD-L1 in canine formalin-fixed parafin-embedded tissue specimens obtained from two healthy beagles (Figure 3 and Table II). PD-L1 expression was not found in the pituitary gland, thyroid gland, lung, heart, esophagus, pancreas, liver, adrenal gland and kidney. In the cerebrum and cerebellum, PD-L1 expression was detected in the glial cells, Purkinje cell axon and dendrites. The epithelial cells of the stomach and urinary bladder were positive in PD-L1 staining, but not the epithelial cells of the duodenum. In the thymus, spleen and lymph nodes, some of the lymphocytes were observed to be expressing PD-L1.

Detection of PD-L1 expression in canine tumor specimens by immunohistochemical analysis. The expression of PD-L1 was also examined in several types of canine tumor specimens. Specimens from malignant melanoma (n=15), osteosarcoma (n=3), mammary gland tumor (adenocarcinoma, n=6 and adenoma, n=9), mast cell tumor (n=13), malignant lymphoma (n=15), hemangiosarcoma (n=11), malignant fibrous histiocytoma (n=8), malignant nerve sheath tumor (n=11), hemangiopericytoma (n=10) and fibrosarcoma (n=6) were stained immunohistochemically. The results are shown in...
Figure 2. Demonstration of cross-reactivity of antibody to human PD-L1 and western blotting of programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1) in canine tumor cell lines. HEK293T cells were transfected with canine PD-L1-expressing plasmid. Cellular protein was then collected and western blotting was performed using antibody to human PD-L1 and anti-FLAG (M2) (A). Cellular protein of canine tumor cell lines, including melanoma (B), osteosarcoma (C), mammary gland tumor (D), lymphoma (E) and mast cell tumor (F) was collected before western blotting was performed using anti-human PD-L1 antibody. LMeC cell line was included in each membrane as a control. The results indicate that the antibody to human PD-L1 cross-reacts with canine PD-L1 molecules and all of the canine cell lines used in this study expressed PD-L1, although not necessarily on the cell surface (cf Figure 1).
Figure 4 and Table III. All lymphoma specimens were positively stained for PD-L1, while more than half of the tumor specimens from malignant melanoma, mammary gland tumor, mast cell tumor, malignant fibrous histiocytoma and fibrosarcoma exhibited positive PD-L1 staining. On the other hand, all three osteosarcoma specimens were negative for PD-L1, and less than half of the specimens from hemangiosarcoma, malignant nerve sheath tumor and hemangiopericytoma positively stained for PD-L1.

Discussion

In this study, we elucidated the expression of canine PD-L1 in canine tumor cell lines and tumor specimens. All tumor cell lines, irrespective of tumor type, expressed PD-L1 protein as shown by western blotting. On the contrary, PD-L1 expression of most cell lines was not detected by flow cytometric analysis, indicating that PD-L1 was not expressed on the cell surface but was found in the cells of most cell lines. However, PD-L1 expression was detected by flow cytometry after induction by IFNγ, a potent PD-L1 inducer (11), in all except the lymphoma cell lines. These results are consistent with those of Maekawa et al. (6) who showed that out of eight cell lines (four melanoma, two mast cell tumor, and two osteosarcoma), only two mast cell tumor cell lines expressed PD-L1; PD-L1 expression was successfully induced by IFNγ in six of the cell lines, while the two remaining osteosarcoma cell lines did not respond to IFNγ treatment. The results in our study showed that PD-L1 expression on all of the five osteosarcoma cell lines was successfully induced by IFNγ. The discrepancy between these two results is most likely a reflection of the characteristics of the different cell lines. Maekawa et al. also showed that primary tumor cells obtained from a cancer-bearing dog exhibited PD-L1 expression on the cell surface (6). This suggests that tumor cell lines may have lost cell surface expression of PD-L1 during the cell culture process due to the absence of microenvironmental factors.

To the best of our knowledge, this study is the first to show PD-L1 expression in normal canine tissue specimens. Transcriptional expression of PD-L1 has been detected in the placenta, heart, lung, liver, spleen, lymph nodes and thymus of the mouse (12, 13). In a different study, mouse PD-L1 protein expression was detected in the spleen, thymus, heart, pancreas, small intestines and placenta (14). The results of previous reports differed regarding the expression pattern of PD-L1 from that of the normal canine non-hematopoietic tissue specimens found in this study. Perhaps this reflects the species-specific function of PD-L1 in dogs.

PD-L1 expression in canine tumor specimens has only been reported by Maekawa et al. (6). Their article presented very preliminary data on canine tumors as analysis was performed in only a limited number of tumor types. The results of their immunohistochemical analysis showed that all oral malignant melanoma (8/8), 4/6 mast cell tumors and 7/10 renal cell carcinomas expressed PD-L1. One of the objectives of our study was to expand the current pool of data on PD-L1 expression in various tumor types so that potential canine
Figure 3. Immunohistochemical analysis of programmed cell death-ligand 1 (PD-L1) expression in normal canine tissue specimens. Tissue specimens from two healthy beagles were stained immunohistochemically with anti-human PD-L1. Representative immunostaining of cerebrum (A), cerebellum (B), pituitary gland (C), stomach (D), duodenum (E), urinary bladder (F), ovary (G), thymus (H), spleen (I) and lymph node (J) are shown. The upper and lower panels represent hematoxylin and eosin staining and immunohistochemical staining of each tissue specimen, respectively. The scale bars of A-F and G-I are 40 μm and 160 μm, respectively.
Figure 4. Immunohistochemical analysis of PD-L1 expression in canine tumor specimens. Canine tumor specimens from cancer-bearing dogs were stained with antibody against human PD-L1. All specimens were immunohistochemically stained, except for malignant melanoma (A), that was stained using immunofluorescence. Representative immunostaining of malignant melanoma (A), mammary gland tumor (adenocarcinoma) (B), mammary gland tumor (adenocarcinoma) (C), mast cell tumor (D), lymphoma (E), hemangiosarcoma (F), malignant fibrous histiocytoma (G), malignant nerve sheath tumor (H), hemangiopericytoma (I) and fibrosarcoma (J) are shown. The upper and lower panels represent hematoxylin and eosin staining and immunofluorescent/immunohistochemical staining of each tumor specimen, respectively. The scale bar is 40 μm.
tumor candidates can be narrowed down for therapy using antibodies PD-1 and PD-L1. Consistent with Maekawa et al., our study showed that PD-L1 expression can be detected in high proportions in oral malignant melanoma (11/15) and mast cell tumor (11/13) (6). Although treatment options are available for canine mast cell tumor (7, 15), there is still a lack of effective treatment for oral malignant melanoma (16). That we found PD-L1 expression in a high number of specimens of canine malignant melanoma indicates that this type of cancer is a good candidate for antibody therapy against PD-1 and PD-L1, and the benefitted effects of such antibody therapy in actual clinical cases are highly anticipated.

In our study, PD-L1 expression was observed in all of the canine lymphoma specimens, including low-grade lymphoma and intermediate- to high-grade lymphoma, as well as both T-cell and B-cell lymphoma. We also confirmed that PD-L1 is expressed in some lymphocytes in the spleen and lymph nodes of healthy beagles. The results for normal canine specimens indicate that PD-L1 expression does not seem to be specific to tumor cells. However, in preliminary studies conducted in our laboratory, PD-L1 expression was not detected in canine peripheral blood mononuclear cell by flow cytometry (article in preparation). Since we did not examine the specific types of cells in the spleen and lymph nodes that expressed PD-L1 in this study, further studies are warranted to clarify this matter. Interestingly, PD-L1 expression was observed only in certain types of human lymphoma (17-20), and the expression of PD-L1 in human diffuse large B-cell lymphoma cells has been correlated to a worse prognosis (20). Therefore, future studies should include in-depth analysis of PD-L1 expression in a larger number of canine lymphoma specimens in order to gain a comprehensive understanding of the pathogenesis involving PD-L1 in dogs.

This study is also the first to report on the results of the analysis of PD-L1 expression in tumor specimens that included osteosarcoma, mammary gland tumor, hemangiosarcoma and soft-tissue sarcomas (malignant fibrous histiocytoma, malignant nerve sheath tumor, hemangiopericytoma and fibrosarcoma). These results contribute to a very important part of canine oncology because the tumors that were examined are common in dogs and are highly malignant. Osteosarcoma and hemangiosarcoma are common in larger-breed dogs and are known to have a grave prognosis (21). None of the osteosarcoma specimen (0/3) and only a few of the hemangiosarcoma specimens (2/11) were found to have PD-L1 expression. Since only three canine osteosarcoma specimens from this study and a small number of human osteosarcoma specimens (22) have been examined for the expression of PD-L1, more clinical data on osteosarcoma are needed in order to come to an acceptable conclusion on the PD-L1 expression of this tumor type. On the other hand, more than half of the canine mammary gland tumor specimens, including both adenoma and adenocarcinoma, exhibited PD-L1 expression, which was consistent with the results for human breast cancer (23). Soft-tissue sarcoma is also common in dogs, and 18 out of 35 specimens exhibited PD-L1 expression. Among the four types of soft-tissue sarcoma, malignant fibrous histiocytoma and fibrosarcoma had a higher percentage of specimens that expressed PD-L1 as compared to malignant nerve sheath tumor and hemangiopericytoma. As antibodies to immune checkpoint proteins for human soft-tissue sarcoma are currently being tested in various clinical trials (24), similarly, canine soft-tissue sarcoma might be a good candidate for such therapy.

In this study, we were unable to analyze the correlation of PD-L1 expression and the prognosis of the canine cancer patients because there was a lack of patient data due to the retrospective nature of the study. In human medicine, many studies have reported on the positive correlation of the expression of PD-L1 in tumor cells and worse prognosis of patients with cancer (10, 23, 25-29). We also did not examine the rate of CD8+ T-cell infiltration in the tumor specimens, the functional analysis of PD-1 on CD8+ T-cells and the functional analysis of PD-L1 on tumor cells. These studies are essential to providing basic data for the evaluation of the potential of immune therapy using antibodies to PD-1 and PD-L1 in canine oncology.

In conclusion, canine mast cell tumor, malignant melanoma, mammary gland tumor, and soft-tissue sarcoma appear to be good tumor candidates for therapy using antibodies to PD-1 and PD-L1. Similarly to human medicine, there are high expectations for immune therapy in veterinary oncology and we hope to narrow the gap between the two as part of our efforts to provide better veterinary care.

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