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Evaluation of circulating PD-1 and PD-L1 as diagnostic biomarkers in dogs with tumors

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Conflict of interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Song DW, Park HM; Data
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ABSTRACT

Background: Programmed cell death protein-1 (PD-1) and programmed cell death ligand-1 (PD-L1) have important roles in tumor evasion of the immune system.

Objectives: This study aimed to assess the diagnostic utility of circulating PD-1 and PD-L1 levels in healthy dogs and dogs with tumors.

Methods: Circulating PD-1 and PD-L1 levels in the serum of 71 dogs with tumors were compared with those of 52 healthy dogs by performing enzyme-linked immunosorbent assay (ELISA).

Results: The ELISA results revealed higher circulating PD-1 and PD-L1 levels in dogs with tumors (2.9 [2.2–3.7] ng/mL; median [IQR] and 2.4 [1.4–4.4] ng/mL, respectively) than in healthy dogs (2.4 [1.9–3.0] ng/mL; $p = 0.012$ and 1.4 [0.9–2.1] ng/mL; $p < 0.001$, respectively). Especially, there was a significant difference in circulating PD-1 levels between healthy dogs and dogs with malignant epithelial tumors (2.4 [1.9–3.0] ng/mL and 3.1 [2.6–4.4] ng/mL, respectively; $p < 0.01$). In addition, there was a significant difference in circulating PD-L1 levels between healthy dogs and dogs with lymphomas (1.4 [0.9–2.1] ng/mL and 2.7 [1.6–5.8] ng/mL, respectively; $p < 0.001$).

Conclusion: This study indicates that circulating PD-1 and PD-L1 have potential as tumor diagnostic biomarkers in dogs with tumors.

Keywords: Dog; tumor; biomarker; programmed cell death protein; ligand

INTRODUCTION

According to a previous report [1], approximately 5,300 dogs per 100,000 are diagnosed with tumors annually, a rate about 10 times higher than that in humans. In general, tumors develop spontaneously and are a common cause of death. Tumors can also reduce the quality of life in dogs [2,3]. However, until recently, few authors have reported on quantifiable biomarkers to diagnose tumors in veterinary medicine.

Programmed cell death protein-1 (PD-1) is an inhibitory receptor and, as previously described, is expressed on T and B cells [4]. It is also expressed by other cells such as natural killer cells, monocytes, and dendritic cells [5]. Among the CD28 superfamily members, PD-1 induces a negative signal via interaction with its ligand. Two PD-1 ligands have been

reported: PD-ligand 1 (PD-L1) and PD-L2. PD-L1 is expressed in various cell types, including non-hematopoietic cells, whereas PD-L2 is expressed in specific types of cells or tissues. One oncogenic theory involves evasion of the immune mechanism through the PD-1/PD-L1 pathway [4]. PD-L1 is not expressed in most normal tissues, but its expression is reported in various tumor cells [6,7]. In humans, many studies have detected PD-L1 in various tumor tissue types, including melanoma, leukemia, lymphoma, gastric, renal cell, and breast cancers, and non-small cell lung cancer (NSCLC) [8-13]. Furthermore, several studies observed serum soluble PD-L1 in the serum of patients with various tumors, including multiple myeloma, diffuse large B-cell lymphoma renal cell carcinoma, advanced gastric cancer, pancreatic adenocarcinoma, mesothelioma, and NSCLC [14-20]. However, there are few reports on PD-1 and PD-L1 in dogs, and the associations of diseases with PD-1 or PD-L1 remain to be revealed [3,21-23].

Few studies have attempted to assess PD-1/PD-L1 expressions in canine cancers, and there is no report on circulating PD-1 and PD-L1 levels in canine cancer. Therefore, this study aimed to compare the levels of circulating PD-1 and PD-L1 between healthy dogs and dogs with tumors and evaluate the diagnostic utility of PD-1 and PD-L1 in canine tumors.

MATERIALS AND METHODS

Serum samples

Stored serum samples of 71 dogs with tumors and 52 healthy dogs from 5 animal hospitals were retrospectively retrieved. Serum samples from dogs with tumors were obtained at the time of diagnosis. The dogs included in the study were presented between 2011 and 2020 for disease diagnosis, spay, castration, dental scaling, or routine health examination. All 123 dogs received a physical examination, a complete blood cell count (CBC), and serum chemistry analysis to determine their health condition. Sera remaining after laboratory testing were stored. Serum was collected from the jugular vein into 5 mL serum separating tubes (BD Vacutainers SST Tube, Becton Dickinson, USA). The tube was gently inverted about five times and then allowed to stand for 20–30 min at -4°C before centrifugation at 3,000 r/min for 15 min. The serum was aliquoted into cryovial and stored at -70°C until used.

Inclusion and exclusion criteria

Healthy dogs had no history of abnormalities and no evidence of tumors. Also, there were no remarkable physical examination and blood test results. The dogs with various tumors were included as the cancer group. Diagnosis of a solid tumor was based on histological examination and the diagnosis of lymphoma was based on cytologic examination of samples obtained via fine needle aspiration (**Table 1**). Dogs with cancer were further allocated into one of three general tumor types based on cytologic or histologic classification (**Table 2**). Specific terminology used depends on the tumor origin (epithelial, mesenchymal, and hematopoietic and lymphoreticular) and is also divided into benign or malignant. Dogs with diseases (cardiac disease, endocrinopathy, and systemic disorders) other than tumors were excluded from this study based on the possibility that a disease could affect the circulating PD-1 or PD-L1 level in serum.

Enzyme-linked immunosorbent assay

Levels of circulating PD-1 and PD-L1 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits for PD-1 (Canine PD1 ELISA Kit, MyBioSource Inc.,

Table 1. Characteristics of 52 healthy dogs and 71 dogs with epithelial (n = 18), mesenchymal (n = 14), and hematopoietic and lymphoreticular (n = 39) tumors in this study

Signalment	Healthy	Tumor types					
		Epithelial		Mesenchymal		Hematopoietic and lymphoreticular	
		Benign	Malignant	Benign	Malignant	Benign	Malignant
Number	52	1	17	4	10	2	37
Age (median [IQR])	4.0 [1.3–7.0]	15	11 [7.5–11.5]	11.5 [8.0–12.0]	13.0 [11.0–15.0]	4.5 [1.0–4.5]	11.0 [7.0–12.0]
Sex	F (16), SF (15), M (5), CM (16)	F (1), SF (0), M (0), CM (0)	F (4), SF (5), M (3), CM (5)	F (2), SF (1), M (0), CM (1)	F (5), SF (0), M (1), CM (4)	F (0), SF (0), M (0), CM (2)	F (5), SF (9), M (3), CM (20)
Breed	Maltese (18) Poodle (7) Mixed (4) Cavalier King Charles Spaniel (2) Chihuahua (2) Shih Tzu (2) Welsh Corgi (2) Golden Retriever (2) Others* (13)	Pomeranian (1)	Maltese (5) Shih Tzu (4) Yorkshire Terrier (2) Mixed (2) Others† (4)	Basenji (1) Golden Retriever (1) Jindo (1) Yorkshire Terrier (1)	Maltese (3) Shih Tzu (2) Yorkshire Terrier (2) Others‡ (3)	Shih Tzu (1) Spitz (1)	Maltese (11) Shih Tzu (9) Cocker Spaniel (6) Miniature pinscher (3) Yorkshire Terrier (2) Others§ (6)

IQR, interquartile range; F, female; SF, spayed female; M, male; CM, castrated male.

*Bichon Frise, Cocker Spaniel, Dachshund, Dalmatian, French Bulldog, Italian Greyhound, Jindo, Labrador Retriever, Pomeranian, Shetland Sheepdog, Soft-Coated Wheaten Terrier, Weimaraner, Yorkshire Terrier.

†Borzoï, Jindo, Schnauzer, Welsh Corgi.

‡Cocker Spaniel, Flat Coated Retriever, Labrador Retriever.

§Chihuahua, Coton de Tulear, Dachshund, Great Pyrenees, Poodle, Welsh Corgi.

USA) and PD-L1 (Canine PDL1 ELISA Kit, MyBioSource Inc.), following the manufacturer's protocols and a previous human study with some modification [24]. In brief, 96-well plates were incubated with standards, and serum samples were incubated with horseradish peroxidase (HRP)-conjugate reagent for 1 h at 37°C. After several aspiration/wash processes, chromogen solution was added to each well of the plates for 15 min at 37°C and protected from light. The substrate reaction was stopped by adding stop solution. Optical density was measured at 450 nm. Protein levels were calculated according to standard curves. The kit manufacturer indicates the intra- and inter-assay coefficients of variation were < 15% for PD-1 ELISA kit, while the intra- and inter-assay coefficients of variation of the PD-L1 ELISA kit were < 10% and < 12%, respectively.

Statistical analysis

The Mann-Whitney U test was used to compare two variables; the Kruskal-Wallis test compared three or more variables. Spearman correlation was used to assess the relationship between serum PD-1 and serum PD-L1 concentrations. Cut-off values for serum PD-1 and PD-L1 concentrations were determined by receiver operating characteristic (ROC) curve analysis. Logistic regression analysis was used to evaluate the relationships between various variables. A *p* value < 0.05 was considered statistically significant. Results are given as median and interquartile range (IQR) values. All analyses were performed using SPSS software (version 25.0, SPSS Inc., USA).

RESULTS

Serum concentrations of PD-1 and PD-L1

The concentrations (median [IQR]) of serum PD-1 and PD-L1 in healthy dogs were 2.4 [1.9–3.0] and 1.4 [0.9–2.1] ng/mL, respectively. No correlation was detected between PD-1 and PD-L1 serum concentrations (*p* = 0.452; *r* = -0.107). The median [IQR] circulating PD-1 and PD-L1 levels in dogs with tumors were 2.9 [2.2–3.7] and 2.4 [1.4–4.4] ng/mL, respectively.

Table 2. Number of 71 tumors according to cytologic and/or histologic diagnosis in this study

Epithelial (n = 18)	
Benign (n = 1)	
Mammary gland adenoma (n = 1)	
Malignant (n = 17)	
Mammary gland adenocarcinoma (n = 3)	
Transitional cell carcinoma (n = 2)	
Pulmonary adenosquamous cell carcinoma (n = 1)	
Thyroid adenocarcinoma (n = 1)	
Perianal gland adenocarcinoma (n = 3)	
Hepatocellular carcinoma (n = 1)	
Salivary gland adenocarcinoma (n = 1)	
Squamous cell carcinoma (n = 2)	
Nasal adenocarcinoma (n = 2)	
Rectal adenocarcinoma (n = 1)	
Mesenchymal (n = 14)	
Benign (n = 4)	
Lipoma (n = 4)	
Malignant (n = 10)	
Melanoma (n = 5)	
Osteosarcoma (n = 1)	
Liposarcoma (n = 1)	
Cutaneous hemangiosarcoma (n = 1)	
Fibrosarcoma (n = 1)	
Leiomyosarcoma (n = 1)	
Hematopoietic and Lymphoreticular (n = 39)	
Benign (n = 2)	
Histiocytoma (n = 2)	
Malignant (n = 37)	
Lymphoma (n = 31)	
Chronic lymphocytic leukemia (n = 1)	
Mast cell tumor (n = 4)	
Hemophagocytic histiocytic sarcoma (n = 1)	
Total number of tumors (n = 71)	

Specific terminology is used depending on the origin of the tumor (epithelial, mesenchymal, and hematopoietic and lymphoreticular). It is also divided into benign or malignant.

Circulating PD-1 and PD-L1 levels were significantly higher in dogs with tumors than in healthy dogs ($p = 0.012$ and $p < 0.001$, respectively) (**Fig. 1**).

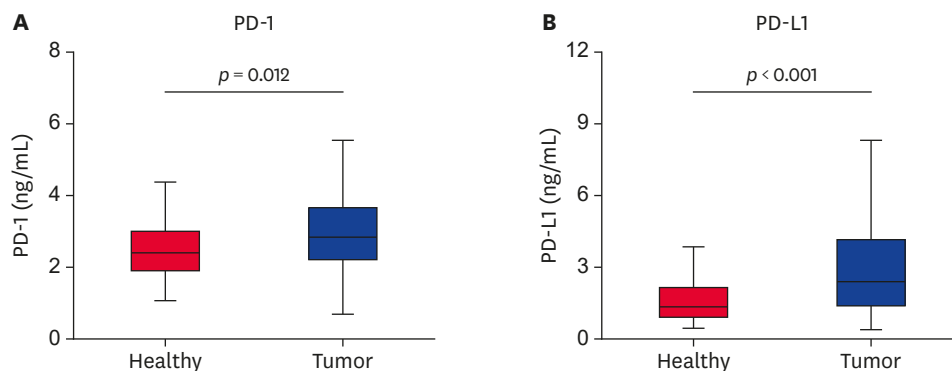


Fig. 1. Boxplots of PD-1 and PD-L1 concentrations in healthy dogs and dogs with tumors. (A) Dogs with tumors showed significantly higher PD-1 concentrations than healthy dogs. (B) Dogs with tumors showed significantly higher PD-L1 concentrations than healthy dogs. $p < 0.05$ was considered significant. PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1.

Comparison of PD-1 and PD-L1 concentrations between healthy dogs and dogs with tumors

The median [IQR] circulating PD-1 and PD-L1 levels were compared among healthy dogs, dogs with benign tumors, and dogs with malignant tumors. Serum concentrations of PD-1 for healthy dogs, dogs with benign tumors, and dog with malignant tumors were 2.4 [1.9–3.0], 2.8 [2.0–4.1], and 2.9 [2.2–3.6] ng/mL, respectively. Serum concentrations of PD-L1 in healthy dogs, dogs with benign tumors, and dogs with malignant tumors were 1.4 [0.9–2.1], 4.1 [1.5–4.5], and 2.3 [1.3–3.9] ng/mL, respectively. Circulating PD-1 and PD-L1 levels were significantly higher in dogs with malignant tumors than in healthy dogs ($p = 0.013$ and $p < 0.001$, respectively) (Fig. 2). Serum concentration of PD-1 for dogs with malignant epithelial, mesenchymal, and hematopoietic and lymphoreticular tumors were 3.1 [2.6–4.4], 2.7 [2.2–3.4], and 2.8 [1.9–3.6] ng/mL, respectively. The circulating PD-1 level was significantly higher in dogs with malignant epithelial tumors than in healthy dogs ($p < 0.01$) (Fig. 3A). The serum concentrations of PD-L1 for dogs with malignant epithelial, mesenchymal, and hematopoietic and lymphoreticular tumors were 1.8 [1.2–3.7], 1.8 [1.0–3.7], and 2.7 [1.6–5.8] ng/mL, respectively. The circulating PD-L1 level was significantly higher in dogs with malignant hematopoietic and lymphoreticular tumors than in healthy dogs ($p < 0.001$) (Fig. 3B).

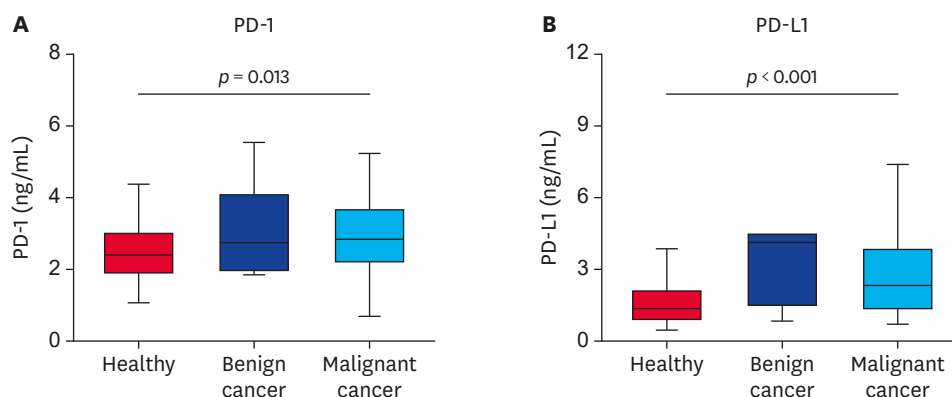


Fig. 2. Boxplots of PD-1 and PD-L1 concentrations in healthy dogs and dogs with tumors according to malignancy. (A) Dogs with malignant tumors showed significantly higher PD-1 concentrations than healthy dogs. (B) Dogs with malignant tumors showed significantly higher PD-L1 concentrations than healthy dogs. $p < 0.05$ was considered significant.

PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1.

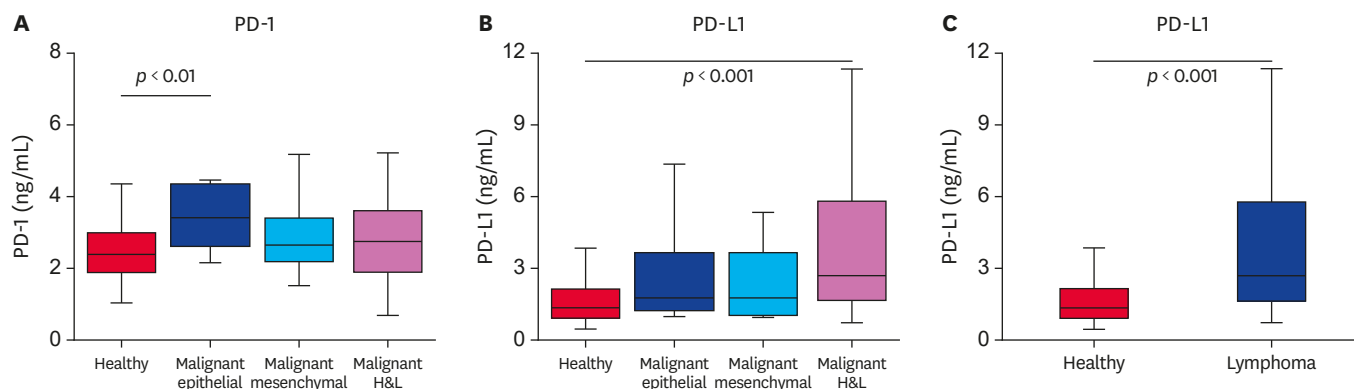


Fig. 3. Boxplots of PD-1 and PD-L1 concentrations for healthy dogs and dogs with tumors according to malignancy and type and lymphoma. (A) Dogs with malignant epithelial tumors showed significantly higher PD-1 concentrations than healthy dogs. (B) Dogs with malignant hematopoietic and lymphoreticular tumors showed significantly higher PD-L1 concentrations than healthy dogs. (C) Dogs with lymphomas showed significantly higher PD-L1 concentrations than healthy dogs. $p < 0.05$ was considered significant.

PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1.

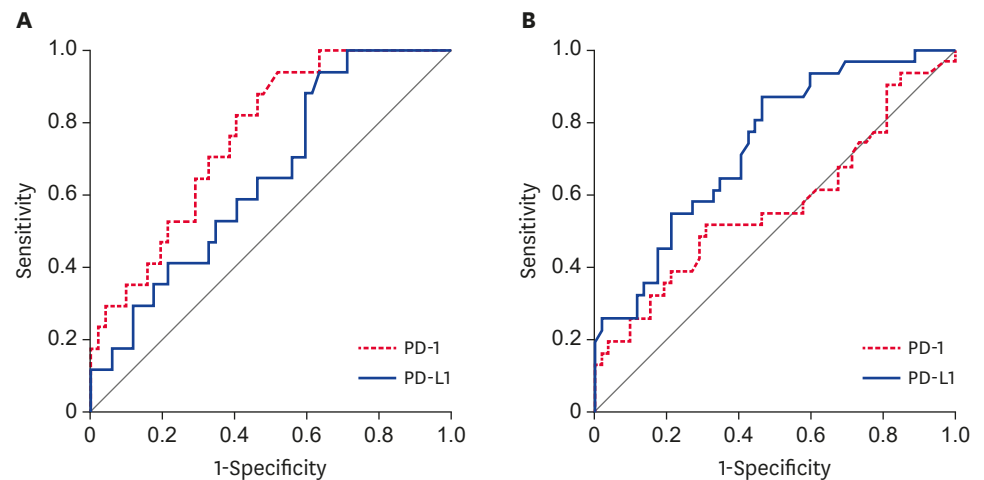


Fig. 4. ROC curve of PD-1 and PD-L1 concentrations for healthy dogs and dogs with malignant epithelial tumors and lymphomas. (A) ROC curve for predicting tumor in dogs with malignant epithelial tumors (AUC = 0.765; $p = 0.001$). (B) ROC curve for predicting tumor in dogs with lymphomas (AUC = 0.734; $p < 0.001$). PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1; ROC, receiver operating characteristic; AUC, area under the curve.

Serum concentrations of PD-1 and PD-L1 in healthy dogs and dogs with lymphoma

The median [IQR] serum PD-1 level in dogs with lymphoma was 2.8 [1.9-3.6] ng/mL, and in healthy dogs, it was 2.4 [1.9-3.0] ng/mL. There was no significant difference in circulating PD-1 levels between healthy dogs and dogs with lymphoma ($p = 0.290$). The serum PD-L1 level in dogs with lymphoma was 2.4 [1.6-4.8] ng/mL, while in healthy dogs, it was 1.4 [0.9-2.1] ng/mL. The circulating PD-L1 level was significantly higher in dogs with lymphoma than in healthy dogs ($p < 0.001$) (Fig. 3C).

Correlations of serum concentrations of PD-1 and PD-L1 with tumor occurrence

Logistic regression results showed that the serum PD-1 level was significantly associated with malignant epithelial tumor presence (odds ratio = 2.402; B = 0.876; $p = 0.024$), whereas the PD-L1 level (odds ratio = 1.703; B = 0.532; $p = 0.003$) was significantly associated with lymphoma. To evaluate the potential of PD-1 and PD-L1 as biomarkers for tumors, receiver operating characteristic (ROC) curves were analyzed in healthy dogs and dogs with tumors. The analysis indicated that PD-1 level could be used to differentiate dogs with malignant epithelial tumors from healthy dogs, with an ROC area under curve (AUC) of 0.765 (95% confidence interval [95% CI], 0.647-0.883; $p = 0.001$) (Fig. 4A). Moreover, the PD-L1 level could be used to differentiate dogs with lymphoma from healthy dogs, with an ROC AUC of 0.734 (95% CI, 0.626-0.842; $p = 0.001$) (Fig. 4B).

DISCUSSION

Tumors can evade the host's immune system via the PD-1/PD-L1 pathway, and while growing, they undergo three phases — elimination, equilibrium, and escape [25]. In the elimination phase, most abnormal cells are eliminated by immune cells, but cancer cells proliferate. During the equilibrium phase, tumor size is stable because a few abnormal cells may escape the immune system. In the escape phase, abnormal cells escaping the immune system continue to grow and become tumorous. Overexpressions of PD-1 and PD-L1 in tumor

tissue are often reported in various cancers, suggesting the PD-1/PD-L1 pathway is a possible mechanism for cancer to evade the immune system [6,7]. Also, the PD-1/PD-L1 pathway has been associated with the suppression of T-cell-based immune functions and could mediate tumor immunosuppression [4]. In contrast to membrane-bound forms, circulating forms of PD-1 and PD-L1 have significant roles in tumor pathogenesis [26]. Circulating PD-1 and PD-L1 have been reported in cancer, suggesting they have a crucial role in tumors [17-20,26,27].

In veterinary medicine, recent studies have demonstrated that PD-1 and PD-L1 are involved in immune evasion by canine cancer. However, there are no reports assess circulating PD-1 and PD-L1 concentrations in various canine cancers. This study demonstrated that the circulating PD-1 level in dogs with malignant epithelial tumors was significantly higher than that in healthy dogs. Also, the circulating PD-L1 level in dogs with lymphoma was significantly higher than that in healthy dogs.

Soluble forms of molecules are known to be generated via cleavage of the membrane-bound form or by translation of alternative splice variants [26], actions that are thought to have occurred in the generation of circulating PD-1 and PD-L1 molecules in the dogs in this study. Both soluble and membrane forms of PD-1 and PD-L1 affect the immune system and contribute to immune system escape. Therefore, circulating PD-1 and PD-L1 in dogs with tumors in this study would have induced escape from the T-cell-based immune system.

The main source of circulating PD-1 is tumor-specific T cells, and the circulating PD-1 level decreases as the tumor reduces [28,29]. In human medicine, the magnitude of the association between circulating PD-1 and tumor prognosis was inconsistent in previous studies of different tumors (i.e., NSCLC and hepatocellular carcinoma) [30,31]. Circulating PD-1 may be both the cause and result of the tumor. PD-L1 present on cell surfaces of immune and tumor cells can be a source of circulating PD-L1 [19,32-35]. However, in other studies, there is little association between PD-L1 expression in tumor tissue and the level of circulating PD-L1; these studies indicate that circulating PD-L1 may be produced in the tumor microenvironment [20,26,33,36]. Circulating PD-L1 from tumors or mature dendritic cells enhances T lymphocyte apoptosis, leading to immune evasion by the tumor [34]. Similar to the result of our study, the circulating PD-L1 level is elevated in humans with diffuse large B-cell lymphoma (DLBCL) and is associated with the prognosis [20]. In this study, dogs with benign tumors showed a higher median circulating PD-L1 level than that in healthy dogs. It is possible that the PD-L1 level can also increase in benign tumors, which can subsequently develop into malignant tumors. However, since there was no statistical significance of the differences associated with benign tumors, the result in this study should be verified in future studies with larger sample numbers. Further studies are also needed on the role of circulating PD-1 and PD-L1 in the diagnosis, prognosis, and mechanism of tumors.

In humans, the circulating PD-L1 level increased with age, but the difference between the 1-10-years-old group and 51-70-years-old group was only about 0.3 ng/mL, a relatively small amount [33]. In contrast, the circulating PD-L1 level in healthy dogs was negatively correlated with age in this study (data not shown, Spearman correlation between age and PD-L1 in healthy dogs, $r = -0.275$, $p = 0.049$). Despite this negative correlation between PD-L1 and age, the circulating PD-L1 level was higher in dogs with tumors than that in healthy dogs. Based these results, PD-L1 was mainly associated with tumors, but a weak correlation with age was observed in this study. Further large-scale studies should be undertaken to clarify the association between PD-L1 and these factors.

Recently, immunotherapy targeting PD-1 and PD-L1 has been used for the treatment of some human malignant tumors. Blockade of the PD-1 or PD-L1 pathway enhances T-cell responses to cancer antigens. An anti-PD-1 monoclonal antibody, Nivolumab, has been shown to be safe and effective in patients with some malignant tumor types, and a treatment response of 36% was noted in patients with PD-L1-positive cancers [37]. In another study, pembrolizumab treatment produced a response rate of 45.2% in patients with NSCLC [38]. However, human immunotherapies targeting PD-1 and PD-L1 have shown different results depending on the expression levels of PD-1 and PD-L1 in tumor tissues. Nivolumab show no response in PD-L1-negative cancer patients, and only 16.5% of patients with NSCLC responded to pembrolizumab when 1 to 49% of the tumor cells were PD-L1 positive [37,38]. However, PD-1 and PD-L1 have not been considered precision biomarkers because of differing immunohistochemistry (IHC) cutoffs, tissue preparation methods, processing procedures, and biopsy type (primary versus metastatic); moreover, PD-L1 expression differs between oncogenic and induced types [39]. Circulating PD-1 and PD-L1 levels have shown potential as a less invasive and more efficient biomarker [14-16,40]. In veterinary medicine, some canine cancers, including oral malignant melanoma, are reported to express PD-L1, and specific anti-PD-1 or PD-L1 monoclonal antibodies have induced immune-cell activation *in vitro* [3,21-23]. Thus, circulating PD-1 and PD-L1 have the potential to serve as diagnostic biomarkers in dogs with tumors.

To the best of our knowledge, this is the first report describing circulating PD-1 and PD-L1 levels in various canine tumors. This study demonstrated that the circulating levels of PD-1 and PD-L1 in dogs with tumors are higher than those in healthy dogs. The findings indicate PD-1 and PD-L1 offer an easy and effective method to predict tumor presences and may be helpful in selecting an appropriate immunotherapy. Further studies are needed to assess the alteration of circulating PD-1 and PD-L1 levels during treatment and long-term follow-up periods. As the number of benign tumors in this study was small, further studies on circulating PD-1 and PD-L1 levels in larger groups of dogs with benign tumors are required.

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