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NOTE

Surgery

Detection of indoleamine 2,3-dioxygenase 1-expressing cells in canine normal and tumor tissues

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ABSTRACT. Cancer immunotherapy is a novel cancer treatment for canine tumors. Indoleamine 2,3-dioxygenase 1 (IDO1) is overexpressed in some human tumors and inhibits antitumor immunity. In this study, we comprehensively evaluated expression pattern of IDO1 and the nature of IDO1-expressing cells in canine normal and tumor tissues. In normal tissue samples, IDO1 expression was detected only in the lymph nodes, spleen, tonsil tissues, and colon tissues. In contrast, IDO1-positive tumor cells were observed in several tumor tissue types. This is the first study to evaluate IDO1 expression in canine normal and tumor tissues, and the results suggest that IDO1 is a promising target for novel cancer immunotherapy in dogs with tumors.

KEY WORDS: dog, immunohistochemistry, indoleamine 2,3-dioxygenase 1, tissue, tumor

Indoleamine-2,3-dioxygenase-1 (IDO1) is a cytosolic enzyme involved in the first rate-limiting step in metabolism of tryptophan (Trp) to kynurenine (Kyn) [21]. IDO1 also exerts immunosuppressive functions by preventing rejection of allogeneic fetuses by the maternal immune system [20]. Furthermore, studies have revealed the mechanism underlying acquired immune tolerance. For instance, overexpression of IDO1 causes depletion of Trp and accumulation of Kyn in tissues, arresting T cells in the G1 phase [21] and downregulating the T-cell receptor ζ-chain (TCRζ) in $CD8^+$ T cells [5]. Moreover, accumulation of Kyn activates the aryl hydrocarbon receptor (AHR) and induces the AHR-dependent $FoxP3^+$ regulatory T cells (Tregs) [17]. In cancer, tumor cells overexpress IDO1 in two types of expression mechanism, constitutive/intrinsic and induced/extrinsic expression, and IDO1 overexpressed by tumor cells promote the immunosuppressive tryptophan catabolism [15]. IDO1 is overexpressed in various human tumor types and is associated with reduced survival in patients with acute myeloid leukemia, small-cell lung carcinoma, melanoma, ovarian carcinoma, colorectal carcinoma, pancreatic carcinoma, and endometrial carcinoma due to strong suppression of antitumor immunity [11]. Therefore, several small molecule inhibitors targeting IDO1 are developed and currently being tested in clinical trials. In a clinical trial of IDO1 inhibitor monotherapy, approximately 34% of the patients with refractory and advanced neoplastic disease achieved stable disease [1]. In preclinical studies, combination therapy comprising IDO1 inhibitor and immune checkpoint inhibitors, such as antibodies targeting programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) synergistically suppressed tumor growth [25]. Therefore, combination therapy with IDO1 inhibitors and immune checkpoint inhibitors has high potential for novel cancer immunotherapy [18].

Cancer immunotherapy is a novel cancer treatment for canine tumors as well as human cancers. Recently, administration of antibodies targeting PD-1/PD-L1 has demonstrated robust clinical outcomes in dogs with tumors [8, 13, 14], suggesting the importance of antitumor immunity in canine tumors. As shown in human preclinical studies [1, 25], IDO1 inhibitor may provide anti-tumor effects by monotherapy or combination therapy with checkpoint inhibitors such as anti-PD1/L1 antibodies. Therefore, IDO1 may also be a promising target for cancer immunotherapy in dogs with tumors. However, the expression and potential of IDO1 as a therapeutic target in canine tumors remains unclear, as only two studies have investigated the expression of IDO1 in

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canine melanoma tissues [22, 23], and the potential antitumor response of IDO1 inhibitors in dogs with sarcomas is understudied [19]. In this study, we evaluated the comprehensive expression pattern of IDO1 and the nature of IDO1-expressing cells in normal and tumor tissues to elucidate the biological effect of IDO1.

To evaluate the expression of IDO1 in canine normal and tumor tissues, we performed immunohistochemistry (IHC) in normal tissues from 30 organs and 13 types of canine tumors. The normal tissues were collected from three experimental beagle dogs euthanized in another animal experiment certified by the laboratory animal committee of The University of Tokyo, and the tumor tissues were surgically removed from 110 tumor-bearing dogs at the Veterinary Medical Center of The University of Tokyo between 2011 and 2016. Permission for resection and collection of tissues and their usage was obtained from dog owners. The tissue samples were diagnosed by two veterinary pathologists, who are certified by the Japanese College of Veterinary Pathologists, at the Department of Veterinary Pathology, The University of Tokyo. IHC for IDO1 was performed on 4-µm thick paraffin-embedded sections from all normal and tumor tissues. After deparaffinization, heat-induced epitope retrieval was performed by autoclaving for 10 min at 121°C in citrate buffer (pH 6.0). After washing in Tris-buffered saline with 0.1% Tween 20 (TBS-T), endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 at room temperature for 10 min. The sections were blocked with 8% skim milk in TBS-T at room temperature for 1 hr, followed by incubation with primary antibody (goat polyclonal anti-human IDO1 antibody, ab134197, Abcam, Cambridge, UK) diluted 1:500, overnight at 4°C. Specificity of the anti-human IDO1 antibody against canine IDO1 was confirmed by western blotting (Supplementary Fig. 1). Negative control was incubated with purified goat IgG isotype antibody (Goat IgG Isotype Control, Invitrogen, Waltham, MA, USA) under identical conditions. After washing with TBS-T, all the sections were incubated with a horseradish peroxidase (HRP)-conjugated anti-goat antibody (Histofine Simple Stain MAX-PO (G), Nichirei Biosciences, Tokyo, Japan) at room temperature for 30 min. Further, the reaction products were visualized with 3,3′-diaminobenzidine (Liquid DAB+ Substrate Chromogen System, Dako Agilent Technologies, Santa Clara, CA, USA) solution for 10 min and counterstained with Mayer's hematoxylin. The expression of IDO1 was evaluated in 10 randomly selected high-power fields on each slide.

IDO1 expression in the normal tissues of three dogs was evaluated. IDO1 expression was detected only in some scattered middle dendritic cells (DCs) in the lymph nodes, spleen, tonsil tissues, and colon tissues (Fig. 1A and 1B, Table 1). In the lymph nodes and tonsil tissues, IDO1 staining was observed in the paracortical T-cell areas that contained T cells and antigen-presenting cells. In the spleen tissues, IDO1-positive cells were observed in the marginal zone, an area between the white pulp and red pulp, and contained macrophages and reticular cells. In the colon tissues, a few IDO1-positive cells were detected in the lamina propria, which contains T cells, B cells, and DCs. A majority of the IDO1-positive cells observed in these tissues were larger, with more abundant cytoplasm than that in lymphocytes, and the cytoplasm displayed dendritic appearance (Fig. 1B). Based on the localization and morphological features of IDO1-positive cells in the tissues, it is indicated that the IDO1-positive cells were mostly antigen-presenting cells.

Next, we performed IHC in 110 samples from 13 common canine solid tumor types, and the overall areas of IDO1-positive and -negative cells were evaluated. Consistent with a previous report on IDO1-positive cells in human tumor tissues [26], three different cell types were stained: tumor cells, immune-like cells in lymphocyte-rich stromal areas, and endothelial cells in stromal areas. IDO1-positive tumor cells were observed in several tumor types, including squamous cell carcinoma (SCC), anal sac adenocarcinoma (ASAC), and pulmonary adenocarcinoma (PAC). IDO1-positive immune-like cells were observed in various tumor types, and their appearance varied from lymphocytes to antigen-presenting cells. These cells were found in almost all transitional cell carcinoma (TCC) and thyroid carcinoma (TC) samples and half of SCC, PAC, and ASAC samples; whereas, some tumor types such as renal cell carcinoma, fibrosarcoma, and peripheral nerve sheath tumor tended to have few IDO1-positive immune-like cells than other tumor types. IDO1-positive endothelial cells were observed in only a few tumor types, such as TCC and SCC (Table 2). Representative images of positively stained canine tumor tissues are shown in Fig. 2. To describe the staining pattern of IDO1 in various tumor types, the following formula based on immunohistochemical scoring system of human IDO1 [24] was used: IDO1 expression based on the percentage of tumor cells with IDO1-positive staining (0=0%; 1=1–33%; 2=34–66%; 3 =≥66%) and the staining intensities (0 =no staining; 1 =low; 2 =moderate; 3 =strong) (Supplementary Fig. 2) were calculated as a composite IDO1 score by multiplying; and the scores were stratified as low=0–2, intermediate=3 and 4, and high=6 and 9. Intermediate and high scores were determined as IDO1-positive staining. In total, 34 of 110 tumors (30.9%) showed positive staining in the cytoplasm of tumor cells. The positive staining in the tumor cell cytoplasm was frequently broad and diffuse in SCC, ASAC, and TCC (Fig. 2B). All TCC samples displayed high IDO1 expression scores, and all SCC and ASAC samples were classified as having moderate or high IDO1 expression scores. In PAC, a few samples were scored with moderate expression scores, and IDO1-positive tumor cells were localized and observed at the tumor area in contact with the stroma (Fig. 2B). A majority of the other tumor types had fewer IDO1-positive tumor cells.

In this study, only immune-like cells expressed IDO1 in the systemic normal tissues, and IDO1-positive immune-like cells were detected in the lymph nodes, spleen, tonsil, and colon tissues. It has been reported that in the uterus of both humans and mice, the small intestine, cecum, and pancreas contain IDO1-positive cells [4, 26]; but, no IDO1-positive staining was found in these types of canine tissues in this study. It is likely that differences in species characteristics or in anti-IDO1 antibodies used in previous IHC studies lead to discrepancies in the staining pattern in normal tissues. The IDO1-positive immune-like cells detected in the lymph node, spleen, tonsil, and colon tissues resembled the appearance of antigen-presenting cells. IDO1-positive DCs have also been identified in the human glandular epithelium of the female reproductive tract and lymphoid organs, including the lymph nodes, spleen, tonsils, Peyer's patches, gut lamina propria, and thymus [26]. As IDO1-positive DCs have a strong tolerogenic capacity, such as against foreign antigens or food antigens [4], IDO1-expressing antigen-presenting cells in the canine normal tissues may

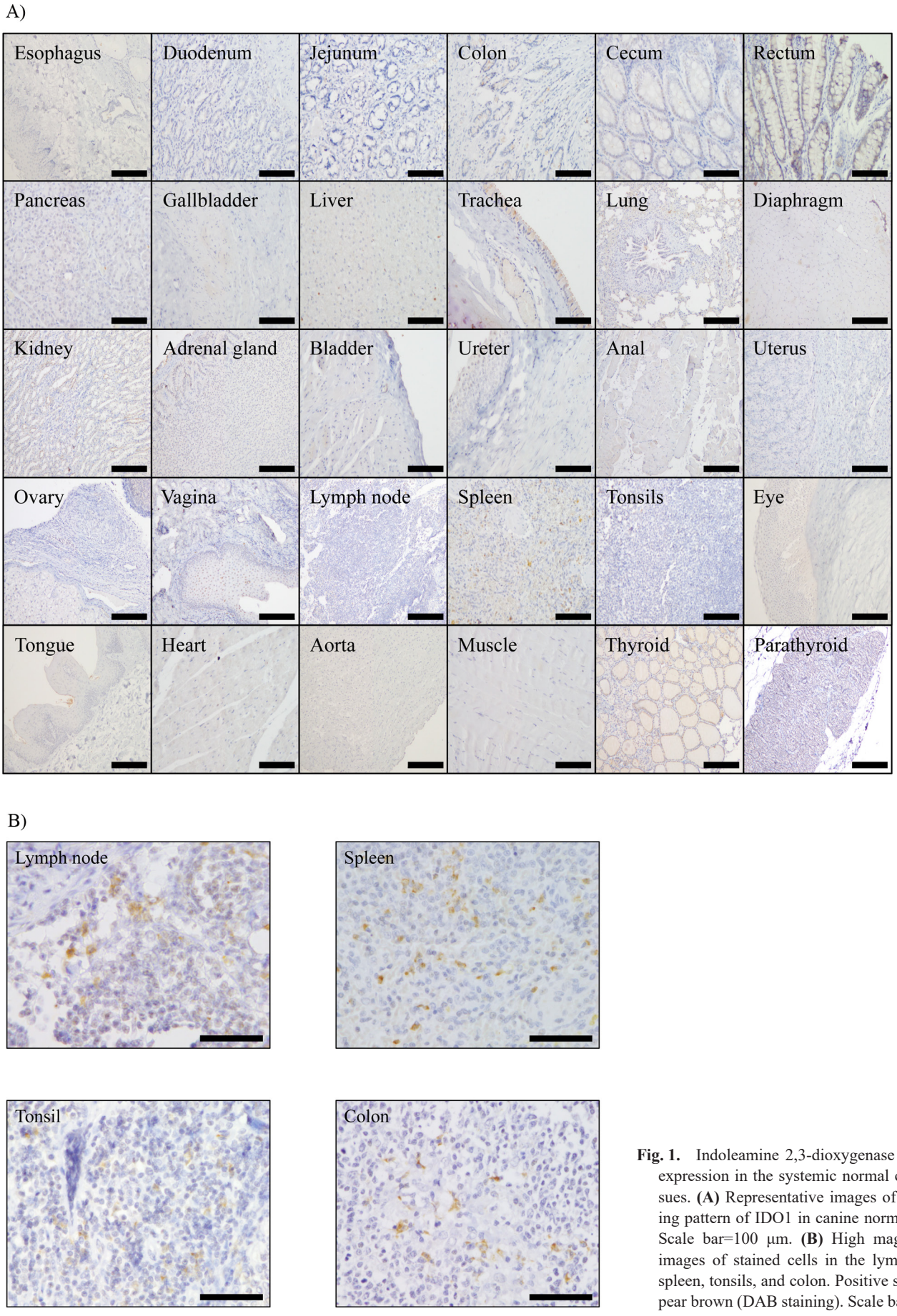

Fig. 1. Indoleamine 2,3-dioxygenase 1 (IDO1) expression in the systemic normal canine tissues. **(A)** Representative images of the staining pattern of IDO1 in canine normal tissues. Scale bar=100 µm. **(B)** High magnification images of stained cells in the lymph nodes, spleen, tonsils, and colon. Positive signals appear brown (DAB staining). Scale bar=50 µm.

Table 1. Indoleamine 2,3-dioxygenase 1 (IDO1) expression in the canine normal tissues

Organ, Tissues	IDO1-expression cells
Digestive tract	
Esophagus	None
Duodenum	None
Jejunum	None
Colon	Stromal cells in lamina propria
Cecum	None
Rectum	None
Pancreas	None
Gallbladder	None
Liver	None
Respiratory tract	
Trachea	None
Lung	None
Diaphragm	None
Urogenital system	
Kidney	None
Adrenal gland	None
Bladder	None
Ureter	None
Anal	None
Uterus	None
Ovary	None
Vagina	None
Lymphoid system	
Lymph node	Stromal cells in paracortical areas
Spleen	Stromal cells in marginal zone
Tonsils	Stromal cells in paracortical areas
Others	
Eye	None
Tongue	None
Heart	None
Aorta	None
Muscle	None
Thyroid	None
Parathyroid	None

be responsible for suppressing aberrant T-cell responses in the lymph nodes, spleen, tonsil, and colon tissues. Considering cancer immunotherapy, because no IDO1 is detected in the non-immune normal cells, therapy targeting IDO1 may not cause severe adverse effects, except in the immune system. Additionally, the expression patterns of IDO1 in the normal canine tissues were similar to those in normal human tissues. Clinical trials of IDO1 inhibitors in human cancer patients demonstrated no significant adverse events, including in the immune system. These results indicate the potential of IDO1 as a novel therapeutic target in dogs with tumors. Moreover, some types of canine tumors, such as TCC, SCC, and ASAC widely express IDO1. Several phase I/II studies suggest that combining IDO1 inhibitor with anti-PD1 antibody could boost response rates among patients with human bladder and non–small cell lung cancers [6, 12]. Although sample size is small, IDO1 inhibitor combined with anti-PD1 antibody also showed efficacy in patients with head and neck squamous cell carcinoma [18] and these findings suggest that IDO1 inhibitors may serve as a therapeutic tool in these tumor types.

Canine tumor cells in TCC, SCC, ASAC, and PAC expressed IDO1, and different expression patterns were observed. Two types of IDO1 expression mechanisms have been reported in tumor cells. A number of human tumors showed IDO1 expression associated with T-cell infiltration and signs of inflammation. This IDO1 expression could be due to induction by interferon gamma (IFN-γ) produced from infiltrating T cells and may induce IDO1 expression as a feedback mechanism for inflammation. Consistent with other IDO1 expression mechanisms in tumor cells, constitutive/intrinsic IDO1 expression through autocrine cytokines, such as interleukin (IL)-6 [10] or prostaglandin E2 (PGE_2) [7] have been reported in human tumors. In this study, IDO1-positive tumor cells were located at the periphery of the tumor nodules in canine PAC cells. Environmental factors, such as inflammatory cytokines produced in the stroma, may induce IDO1 expression. In contrast, in TCC, SCC, and ASAC, the expression of IDO1 in tumor cells was broad and diffuse, suggesting that the activation of the IDO1 in these tumors is constitutive, rather than induced by environmental factors. In canine tumors, constitutive expression of PGE_2 has been reported in TCC [9, 27], and these previous reports may support our hypothesis that auto-paracrine PGE_2 signaling drives the overexpression of IDO1 in canine TCC. These results suggest that canine tumors acquire the mechanism of immune escape via activation of acquired or intrinsic IDO1 expression.

In this study, there were many IDO1-expressing immune-like cells in

Table 2. Frequency and expression pattern of indoleamine 2,3-dioxygenase 1 (IDO1) in common canine solid tumors. Moderate and high scores were determined as IDO1-positive staining

Tumor types	Positive cases	Expression score in tumor cells			Positive in immune-like cells	Positive in endothelial cells
		Low	Moderate	High		
TCC	19/19	0	0	19	18	3
SCC	5/8	3	3	2	4	1
ASAC	3/8	5	3	0	4	0
PAC	2/8	6	2	0	5	0
MGT	0/14	14	0	0	3	0
PNST	0/9	9	0	0	1	0
FSA	0/9	9	0	0	1	0
HSA	0/8	8	0	0	3	0
THY	0/8	8	0	0	2	0
TC	0/5	5	0	0	5	0
HCC	0/5	5	0	0	0	0
OSA	0/5	5	0	0	2	0
RCC	0/4	4	0	0	0	0

TCC: transitional cell carcinoma, SCC: squamous cell carcinoma, ASAC: anal sac adenocarcinoma, PAC: pulmonary adenocarcinoma, MGT: mammary gland tumor, PNST: peripheral nerve sheath tumor, FSA: fibrosarcoma, HSA: hemangiosarcoma, THY: thymoma, TC: Thyroid carcinoma, HCC: hepatocellular carcinoma, OSA: osteosarcoma, RCC: renal cell carcinoma.

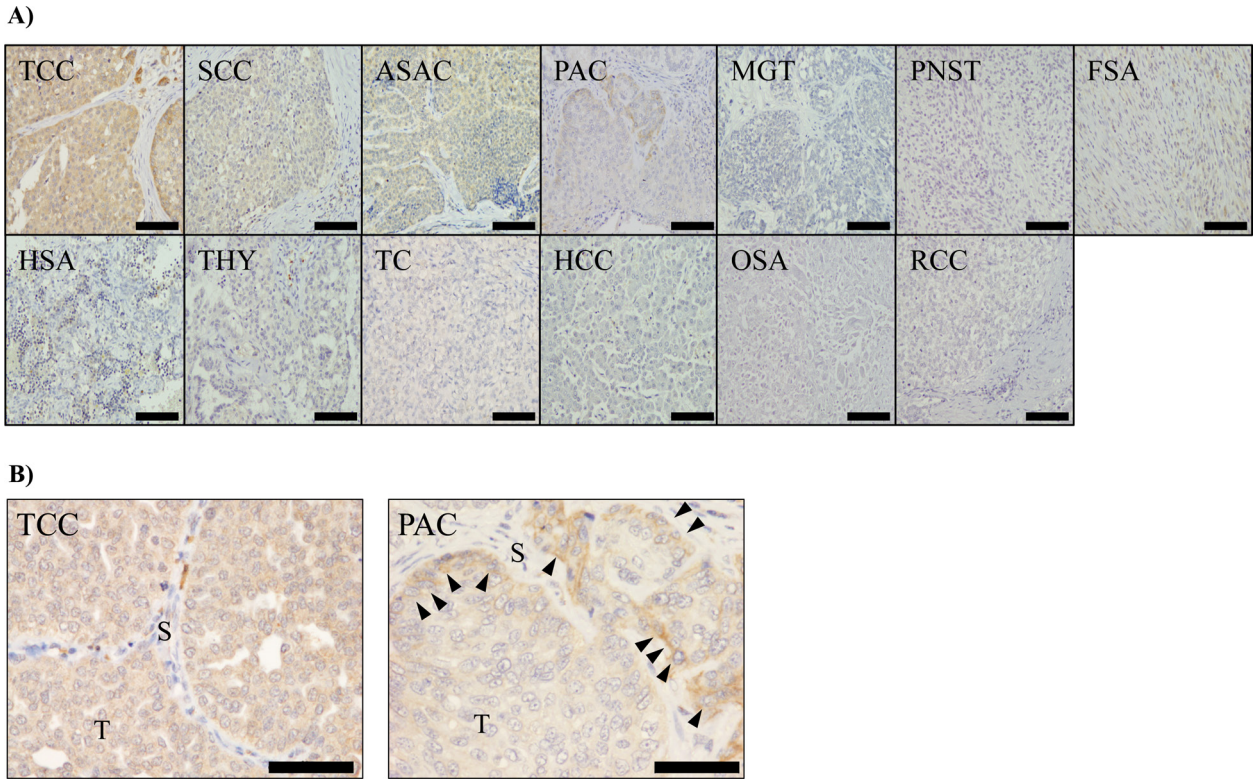

Fig. 2. Indoleamine 2,3-dioxygenase 1 (IDO1) expression in various canine tumors. **(A)** Representative images of the staining pattern of IDO1 in canine tumor tissues. Scale bar=100 µm. TCC: transitional cell carcinoma, SCC: squamous cell carcinoma, ASAC: anal sac adenocarcinoma, PAC: pulmonary adenocarcinoma, MGT: mammary gland tumor, PNST: peripheral nerve sheath tumor, FSA: fibrosarcoma, HSA: hemangiosarcoma, THY: thymoma, TC: Thyroid carcinoma, HCC: hepatocellular carcinoma, OSA: osteosarcoma, RCC: renal cell carcinoma. **(B)** High magnification images of stained cells in TCC and PAC. Tumor cell cytoplasm of TCC were broadly and strongly stained. That of PAC were stained at the tumor area in contact with the stroma and black arrow heads indicate these positively stained tumor cell. T: tumor, S: stromal area. Positive signals appear brown (DAB staining). Scale bar=50 µm.

tumor tissues that contained antigen-presenting cells. IDO1-positive DCs have also been identified in several types of human tumors. It is well known that plasmacytoid DCs acquire a strong tolerogenic capacity under low tryptophan conditions as they decrease antigen uptake [2], and IDO1-expressing DCs inhibit T cell priming and differentiation of naïve T cells to Treg cells [3, 16]. Therefore, IDO1-positive immune-like cells in tumor tissues may inhibit antitumor immunity in canine tumors, and their inactivation in canine tumor tissues may serve as a promising strategy for enhancing antitumor immunity.

This is the first study to demonstrate extensive profiling of IDO1-expressing cells in canine normal and tumor tissues. The results suggest that IDO1 is a promising target for novel cancer immunotherapy in dogs with tumors.

CONFLICTS OF INTEREST. The authors have nothing to disclose.

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