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Note

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

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Running head: IDO1 IN CANINE NORMAL AND TUMOR TISSUES

18   **Abstract**

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

27

28   **Keywords:** dog, immunohistochemistry, indoleamine 2,3-dioxygenase 1, tissue, tumor

29

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

51 Cancer immunotherapy is a novel cancer treatment for canine tumors as well as human cancers.
52 Recently, administration of antibodies targeting PD-1/PD-L1 has demonstrated robust clinical outcomes

53 in dogs with tumors [8, 13, 14], suggesting the importance of antitumor immunity in canine tumors. As
54 shown in human preclinical studies [1, 25], IDO1 inhibitor may provide anti-tumor effects by
55 monotherapy or combination therapy with checkpoint inhibitors such as anti-PD1/L1 antibodies.
56 Therefore, IDO1 may also be a promising target for cancer immunotherapy in dogs with tumors.
57 However, the expression and potential of IDO1 as a therapeutic target in canine tumors remains unclear,
58 as only two studies have investigated the expression of IDO1 in canine melanoma tissues [22, 23], and
59 the potential antitumor response of IDO1 inhibitors in dogs with sarcomas is understudied [19]. In this
60 study, we evaluated the comprehensive expression pattern of IDO1 and the nature of IDO1-expressing
61 cells in normal and tumor tissues to elucidate the biological effect of IDO1.

62 To evaluate the expression of IDO1 in canine normal and tumor tissues, we performed
63 immunohistochemistry (IHC) in normal tissues from 30 organs and 13 types of canine tumors. The
64 normal tissues were collected from three experimental beagle dogs euthanized in another animal
65 experiment certified by the laboratory animal committee of The University of Tokyo, and the tumor
66 tissues were surgically removed from 110 tumor-bearing dogs at the Veterinary Medical Center of The
67 University of Tokyo between 2011 and 2016. Permission for resection and collection of tissues and their
68 usage was obtained from dog owners. The tissue samples were diagnosed by two veterinary
69 pathologists, who are certified by the Japanese College of Veterinary Pathologists, at the Department of
70 Veterinary Pathology, The University of Tokyo. IHC for IDO1 was performed on 4- μ m thick paraffin-
71 embedded sections from all normal and tumor tissues. After deparaffinization, heat-induced epitope
72 retrieval was performed by autoclaving for 10 min at 121°C in citrate buffer (pH 6.0). After washing in
73 Tris-buffered saline with 0.1% Tween 20 (TBS-T), endogenous peroxidase activity was blocked by
74 incubation with 3% H₂O₂ at room temperature for 10 min. The sections were blocked with 8% skim milk
75 in TBS-T at room temperature for 1 h, followed by incubation with primary antibody (goat polyclonal

76 anti-human IDO1 antibody, ab134197, Abcam, Cambridge, UK) diluted 1:500, overnight at 4°C.
77 Specificity of the anti-human IDO1 antibody against canine IDO1 was confirmed by western blotting
78 (Supplementary Fig. 1). Negative control was incubated with purified goat IgG isotype antibody (Goat
79 IgG Isotype Control, Invitrogen, Waltham, MA, USA) under identical conditions. After washing with
80 TBS-T, all the sections were incubated with a horseradish peroxidase (HRP)-conjugated anti-goat
81 antibody (Histofine Simple Stain MAX-PO(G), Nichirei Biosciences, Tokyo, Japan) at room
82 temperature for 30 min. Further, the reaction products were visualized with 3,3'-diaminobenzidine
83 (Liquid DAB+ Substrate Chromogen System, Dako Agilent Technologies, Santa Clara, CA, USA)
84 solution for 10 min and counterstained with Mayer's hematoxylin. The expression of IDO1 was
85 evaluated in 10 randomly selected high-power fields on each slide.

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

97 Next, we performed IHC in 110 samples from 13 common canine solid tumor types, and the
98 overall areas of IDO1-positive and -negative cells were evaluated. Consistent with a previous report on

99 IDO1-positive cells in human tumor tissues [26], three different cell types were stained: tumor cells,
100 immune-like cells in lymphocyte-rich stromal areas, and endothelial cells in stromal areas. IDO1-
101 positive tumor cells were observed in several tumor types, including squamous cell carcinoma (SCC),
102 anal sac adenocarcinoma (ASAC), and pulmonary adenocarcinoma (PAC). IDO1-positive immune-like
103 cells were observed in various tumor types, and their appearance varied from lymphocytes to antigen-
104 presenting cells. These cells were found in almost all transitional cell carcinoma (TCC) and thyroid
105 carcinoma (TC) samples and half of SCC, PAC, and ASAC samples; whereas, some tumor types such as
106 renal cell carcinoma, fibrosarcoma, and peripheral nerve sheath tumor tended to have few IDO1-positive
107 immune-like cells than other tumor types. IDO1-positive endothelial cells were observed in only a few
108 tumor types, such as TCC and SCC (Table 2). Representative images of positively stained canine tumor
109 tissues are shown in Fig. 2. To describe the staining pattern of IDO1 in various tumor types, the
110 following formula based on immunohistochemical scoring system of human IDO1 [24] was used: IDO1
111 expression based on the percentage of tumor cells with IDO1-positive staining (0 = 0%; 1 = 1%–33%; 2
112 = 34%–66%; 3 = $\geq$ 66%) and the staining intensities (0 = no staining; 1 = low; 2 = moderate; 3 = strong)
113 (Supplementary Fig. 2) were calculated as a composite IDO1 score by multiplying; and the scores were
114 stratified as low = 0–2, intermediate = 3 and 4, and high = 6 and 9. Intermediate and high scores were
115 determined as IDO1-positive staining. In total, 34 of 110 tumors (30.9%) showed positive staining in the
116 cytoplasm of tumor cells. The positive staining in the tumor cell cytoplasm was frequently broad and
117 diffuse in SCC, ASAC, and TCC (Fig. 2B). All TCC samples displayed high IDO1 expression scores,
118 and all SCC and ASAC samples were classified as having moderate or high IDO1 expression scores. In
119 PAC, a few samples were scored with moderate expression scores, and IDO1-positive tumor cells were
120 localized and observed at the tumor area in contact with the stroma (Fig. 2B). A majority of the other
121 tumor types had fewer IDO1-positive tumor cells.

122 In this study, only immune-like cells expressed IDO1 in the systemic normal tissues, and IDO1-
123 positive immune-like cells were detected in the lymph nodes, spleen, tonsil, and colon tissues. It has
124 been reported that in the uterus of both humans and mice, the small intestine, cecum, and pancreas
125 contain IDO1-positive cells [4, 26]; but, no IDO1-positive staining was found in these types of canine
126 tissues in this study. It is likely that differences in species characteristics or in anti-IDO1 antibodies used
127 in previous IHC studies lead to discrepancies in the staining pattern in normal tissues. The IDO1-
128 positive immune-like cells detected in the lymph node, spleen, tonsil, and colon tissues resembled the
129 appearance of antigen-presenting cells. IDO1-positive DCs have also been identified in the human
130 glandular epithelium of the female reproductive tract and lymphoid organs, including the lymph nodes,
131 spleen, tonsils, Peyer's patches, gut lamina propria, and thymus [26]. As IDO1-positive DCs have a
132 strong tolerogenic capacity, such as against foreign antigens or food antigens [4], IDO1-expressing
133 antigen-presenting cells in the canine normal tissues may be responsible for suppressing aberrant T-cell
134 responses in the lymph nodes, spleen, tonsil, and colon tissues. Considering cancer immunotherapy,
135 because no IDO1 is detected in the non-immune normal cells, therapy targeting IDO1 may not cause
136 severe adverse effects, except in the immune system. Additionally, the expression patterns of IDO1 in
137 the normal canine tissues were similar to those in normal human tissues. Clinical trials of IDO1
138 inhibitors in human cancer patients demonstrated no significant adverse events, including in the immune
139 system. These results indicate the potential of IDO1 as a novel therapeutic target in dogs with tumors.
140 Moreover, some types of canine tumors, such as TCC, SCC, and ASAC widely express IDO1. Several
141 phase I/II studies suggest that combining IDO1 inhibitor with anti-PD1 antibody could boost response
142 rates among patients with human bladder and non-small cell lung cancers [6, 12]. Although sample size
143 is small, IDO1 inhibitor combined with anti-PD1 antibody also showed efficacy in patients with head

144 head and neck squamous cell carcinoma [18] and these findings suggest that IDO1 inhibitors may serve
145 as a therapeutic tool in these tumor types.

146 Canine tumor cells in TCC, SCC, ASAC, and PAC expressed IDO1, and different expression
147 patterns were observed. Two types of IDO1 expression mechanisms have been reported in tumor cells. A
148 number of human tumors showed IDO1 expression associated with T-cell infiltration and signs of
149 inflammation. This IDO1 expression could be due to induction by interferon gamma (IFN- γ) produced
150 from infiltrating T cells and may induce IDO1 expression as a feedback mechanism for inflammation.
151 Consistent with other IDO1 expression mechanisms in tumor cells, constitutive/intrinsic IDO1
152 expression through autocrine cytokines, such as interleukin (IL)-6 [10] or prostaglandin E2 (PGE₂) [7]
153 have been reported in human tumors. In this study, IDO1-positive tumor cells were located at the
154 periphery of the tumor nodules in canine PAC cells. Environmental factors, such as inflammatory
155 cytokines produced in the stroma, may induce IDO1 expression. In contrast, in TCC, SCC, and ASAC,
156 the expression of IDO1 in tumor cells was broad and diffuse, suggesting that the activation of the IDO1
157 in these tumors is constitutive, rather than induced by environmental factors. In canine tumors,
158 constitutive expression of PGE₂ has been reported in TCC [9, 27], and these previous reports may
159 support our hypothesis that auto-paracrine PGE₂ signaling drives the overexpression of IDO1 in canine
160 TCC. These results suggest that canine tumors acquire the mechanism of immune escape via activation
161 of acquired or intrinsic IDO1 expression.

162 In this study, there were many IDO1-expressing immune-like cells in tumor tissues that contained
163 antigen-presenting cells. IDO1-positive DCs have also been identified in several types of human tumors.
164 It is well known that plasmacytoid DCs acquire a strong tolerogenic capacity under low tryptophan
165 conditions as they decrease antigen uptake [2], and IDO1-expressing DCs inhibit T cell priming and
166 differentiation of naïve T cells to Treg cells [3, 16]. Therefore, IDO1-positive immune-like cells in

167 tumor tissues may inhibit antitumor immunity in canine tumors, and their inactivation in canine tumor
168 tissues may serve as a promising strategy for enhancing antitumor immunity.

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

172

173

174 **CONFLICTS OF INTEREST**

175 The authors have nothing to disclose.

176

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181

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279

280 **FIGURE LEGENDS**

281 **Fig. 1.** Indoleamine 2,3-dioxygenase 1 (IDO1) expression in the systemic normal canine tissues.

282 (A) Representative images of the staining pattern of IDO1 in canine normal tissues. Scale bar = 100µm.

283 (B) High magnification images of stained cells in the lymph nodes, spleen, tonsils, and colon. Positive
284 signals appear brown (DAB staining). Scale bar = 50µm.

285 **Fig. 2.** Indoleamine 2,3-dioxygenase 1 (IDO1) expression in various canine tumors.

286 (A) Representative images of the staining pattern of IDO1 in canine tumor tissues. Scale bar = 100µm.

287 TCC: transitional cell carcinoma, SCC: squamous cell carcinoma, ASAC: anal sac adenocarcinoma,

288 PAC: pulmonary adenocarcinoma, MGT: mammary gland tumor, PNST: peripheral nerve sheath tumor,

289 FSA: fibrosarcoma, HSA: hemangiosarcoma, THY: thymoma, TC: Thyroid carcinoma, HCC:

290 hepatocellular carcinoma, OSA: osteosarcoma, RCC: renal cell carcinoma.

291 (B) High magnification images of stained cells in TCC and PAC. Tumor cell cytoplasm of TCC were

292 broadly and strongly stained. That of PAC were stained at the tumor area in contact with the stroma and

293 black arrow heads indicate these positively stained tumor cell. T: tumor, S: stromal area. Positive signals

294 appear brown (DAB staining). Scale bar = 50µm.

295 **Supplementary Fig. 1.** Specific detection of canine indoleamine 2,3-dioxygenase 1 (IDO1) expression

296 using the anti-IDO1 antibody. *IDO1* expression was evaluated by RT-qPCR and IDO1 protein level was

297 detected by Western blotting with actin (mouse monoclonal anti-actin antibody, MAB1501, Merck

298 Millipore, MA, USA) as loading control in TCC cells (TCCUB) and OSA cells (HOS) treated with

299 10ng/ml of recombinant canine IFN-γ (Recombinant Canine IFN-gamma Protein, R&D Systems,

300 Minneapolis, MN, USA). The data represents the mean±SD from triplicate measurements.

301 **Supplementary Fig. 2.** Indoleamine 2,3-dioxygenase 1 (IDO1) scoring of canine tumors.

302 Representative images of IDO1-positive staining. Scale bar, 100 µm.

Figure 1 A)

Figure 1 B)

Figure 2 A)

Figure 2 B)

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

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, SCC: squamous cell carcinoma, ASAC: anal sac adenocarcinoma carcinoma,
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.

Supplementary Figure 1

IDO1

Actin

Supplementary Figure 2

0

1

2

3