

**Original Full Paper****Immunohistochemical expression of Galectin-3 in canine tumors**Thiago H. M. Vargas^{1*}, Lidia H. Pulz², Ricardo F. Strefezzi¹¹Laboratório de Oncologia Comparada e Translacional, Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo.²Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

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Submitted October 12th 2017, Accepted January, 18th 2018**Abstract**

Galectin-3 (Gal-3) is a protein expressed by both normal and neoplastic cells. It participates in several biological processes such as cell proliferation, cell adhesion, apoptosis, tissue remodeling, and angiogenesis. Although it is known to serve as a valuable prognostic marker in several types of human cancer, there are few reports about its applicability as a marker in the veterinary oncology literature. The aim of the present study was to characterize Gal-3 expression in different types of canine tumors. Fifty-three tissue samples from 22 histologically different types of canine tumors were immunohistochemically evaluated for Gal-3 expression. Variations in the percentage of Gal-3-positive cells, localization of Gal-3 protein, and percentage of Gal-3-positive fibroblasts were observed. These preliminary results showed variable expression of Gal-3 among canine tumors. Further studies are needed in order to investigate the potential of this protein as a prognostic marker and a therapeutic target.

Key words: canine, Galectin-3, immunohistochemistry, cancer, prognosis.**Introduction**

Galectins are a family of proteins capable of binding to β -galactosidase and share primary structural homology in their carbohydrate-recognition domains (CRD). Galectin-3 (Gal-3) has a CRD that is connected to an extended non-lectin N-terminal domain. It is the most studied galectin type and participates in several biological processes such as cell proliferation, cell adhesion, apoptosis, and tissue remodeling (16).

Gal-3 is present in human epithelial cells, immune cells, sensory neurons, fibroblasts, chondrocytes, osteoclasts, osteoblasts, and keratinocytes (3). Although this protein is synthesized in the cytoplasm, it shuttles between the nucleus and/or other biological fluids. The action of Gal-3 on neoplasms depends on its subcellular location (2).

Gal-3 is involved in malignant transformation (8) and maintenance (5) of the transformed phenotype. It is

also selectively associated with activated K-Ras (6). In the cytoplasm, Gal-3 interacts with BCL2 and plays an anti-apoptotic role (29); however, in the nucleus, it plays a pro-apoptotic role, possibly related to Nucling activation (13). In the extracellular space, it mediates adhesion, migration, and invasion of tumor cells by interacting with laminin, fibronectin, elastin, and collagen (7,17). It also modulates the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), thereby regulating angiogenesis (14) and immune reactions by acting as a chemoattractant for monocytes and macrophages (21).

The expression of Gal-3 was demonstrated in several human neoplasms, such as osteosarcomas, melanomas, colorectal cancer, lymphomas, and carcinomas of the stomach, thyroid, lung, tongue, cervix, and breast (25).

In veterinary medicine, only few studies related to Gal-3 expression have been reported. Woo et al. (27)

described Gal-3 expression in canine gastric carcinoma samples and found that cancer cells showed stronger immunolabelling than normal epithelial cells. Johnson et al. (10) demonstrated Gal-3 expression in canine, human and murine hemangiosarcoma. De Oliveira et al. (18) showed an increase in Gal-3 expression in canine metastatic mammary carcinomas. Ribeiro et al. (20) found high serum levels of Gal-3 in dogs with mammary carcinoma, which may or may not be metastatic. In the same study, it was demonstrated that dogs with metastatic tumors maintained high levels of Gal-3 even after excision of the primary tumor. Due to scarce literature about Gal-3 expression in canine neoplasms, the aim of the present study was to characterize the immunohistochemical expression of Gal-3 in different canine tumors.

Material and Methods

Fifty-three tissue samples from 41 dogs were procured from the tumor archive of the Laboratory of Compared and Translational Oncology (LOCT) at the University of São Paulo. All samples were fixed in 10% formalin and routinely processed for histopathology. Four-micrometer sections were cut and stained with hematoxylin and eosin for confirmation of the diagnosis (Table 1).

The sections were deparaffinized in xylene, rehydrated in graded alcohol, and rinsed in distilled water. Next, hydrogen peroxide solution (Cell Marque) was applied for 15 min to neutralize the endogenous peroxidase. Melanoma samples were subjected to melanin bleaching in 10% hydrogen peroxide solution in distilled water for 40 min at 65 °C. Antigen retrieval was achieved by heating the slides in citrate buffer (pH 6.0) in a steamer for 25 min. Non-specific antigen binding on the membrane was blocked by incubating it in skimmed milk solution (5%) for 15 min. The primary mouse monoclonal anti-Galectin-3 antibody (Abcam, clone A3A12, ab2785, 1:1000 dilution) was added and the slides were incubated overnight. Subsequently, the slides were incubated with the secondary antibody (Easylink ONE, EasyPath, EP-12-20502) for 15 min at room temperature. The reaction was visualized using diaminobenzidine (DAB) substrate and the slides were counterstained with Harris hematoxylin. For negative control, the primary antibody was replaced with normal mouse IgG and incubated in the same antibody dilution and under the same reaction conditions as the test samples. Samples of canine oral melanoma, which were previously shown to be positive for Gal-3 expression, were included in each run as the positive control.

Gal-3 expression in the immunostained cells was evaluated using light microscopy. The cells showing

nuclear and/or cytoplasmic staining were considered Gal-3-positive. The parameters evaluated for each sample included immunostaining intensity (weak or strong), percentage of positive tumor cells (C1, 0–25%; C2, 25–50%; C3, 50–75%; C4, 75–100%), percentage of positive nuclei (N1, 0–25%; N2, 25>50%; N3, 50–75%; N4, 75–100%), localization of stain (nuclear and/or cytoplasmic), and fibroblast staining (positive or negative, for typical fibroblasts, i.e., branched to fusiform stromal cell with elliptical nucleus).

Results

All tumor samples included in the study tested positive for Gal-3 expression. Immunostaining varied in terms of its intensity, percentage of Gal-3-positive tumor cells, percentage of positive nuclei, localization of the stain, and fibroblast staining (Table 1). The immunostaining patterns are shown in Figure 1-4. Staining intensity was strong in 79.2% (42/53) samples (Fig. 1). The percentage of Gal-3-positive tumor cells examined were scored, based on which 37.7% (20/53) of the samples were classified as C1, 34% (18/53) as C2, 18.9% (10/53) as C3, and 9.4% (5/53) as C4. When tumor tissue samples were classified according to the percentage of positive nuclei present, 37.7% (20/53) of the samples were classified as N1, 32.1% (17/53) as N2, 20.7% (11/53) as N3, and 9.4% (5/53) as N4. Cytoplasmic immunostaining was observed in all (53/53) samples, while nuclear staining was observed in 98.1% (52/53) samples (Fig. 2). Fibroblasts were positive for Gal-3 expression in 71.7% (38/53) of the samples (Fig. 3) and negative in 9.4% (5/53). The identification of positive fibroblasts was not done for 10 samples (18.9%), due to their similarity with neoplastic cells.

Discussion

All tissue samples included in this study tested positive for Gal-3 expression, with varying immunostaining intensities and variations in the location and percentage of Gal-3-positive tumor and stromal cells (both within and between the tumor types). Since fibroblast positivity was evaluated based on their typical morphology, the identification of this cell type was easier in epithelial and round cell tumors, but difficult for mesenchymal neoplasms, a limiting factor for the present study. For this reason, in some mesenchymal or spindle cell tumors, fibroblast positivity was not determined. Even so, we could detect fibroblast positivity in the majority of samples, indicating that, in addition to tumor cells, Gal-3 is expressed by other components of the tumor microenvironment.

Table 1. Tumor types included in the study and features of Gal-3 immunostaining (percentage of immunolabeled cells, intensity of staining, percentage of immunolabeled nuclei, staining location and fibroblast immunolabeling).

| Tumor | Gal-3 positivity (%) | Gal-3 intensity | Nuclear positivity (%) | Location | Fibroblasts |
|--------------------------------|----------------------|-----------------|------------------------|----------|-------------|
| Mammary tumors | | | | | |
| Papillary carcinoma | 25-50 | Strong | 25-50 | N/C | - |
| Solid Carcinoma | 0-25 | Strong | 0-25 | N/C | + |
| Tubulopapillary carcinoma | 25-50 | Strong | 25-50 | N/C | + |
| Comedocarcinoma | 25-50 | Strong | 0-25 | N/C | + |
| Myoepithelioma | 50-75 | Strong | 0-25 | N/C | + |
| Adenosquamous carcinoma | 0-25 | Strong | 75-100 | N/C | + |
| Carcinoma in a mixed tumor | 25-50 | Strong | 25-50 | N/C | + |
| Lung adenocarcinoma | 75-100 | Strong | 50-75 | N/C | - |
| | 25-50 | Strong | 25-50 | N/C | - |
| Sebaceous adenoma | 25-50 | Strong | 0-25 | N/C | + |
| Squamous cell carcinoma | 25-50 | Strong | 0-25 | N/C | + |
| | 50-75 | Strong | 0-25 | N/C | + |
| | 25-50 | Strong | 25-50 | N/C | + |
| | 25-50 | Strong | 25-50 | N/C | + |
| | 50-75 | Strong | 25-50 | N/C | + |
| Fibroma | 75-100 | Strong | 75-100 | N/C | n.d. |
| Hemangioma | 0-25 | Weak | 0-25 | N/C | n.d. |
| | 25-50 | Strong | 25-50 | N/C | + |
| | 0-25 | Strong | 50-75 | N/C | n.d. |
| Hemangiopericytoma | 50-75 | Strong | 0-25 | N/C | n.d. |
| | 50-75 | Strong | 25-50 | N/C | n.d. |
| Hemangiosarcoma | 0-25 | Weak | 0-25 | N/C | + |
| | 50-75 | Strong | 0-25 | N/C | n.d. |
| | 0-25 | Strong | 50-75 | N/C | + |
| Lipoma | 25-50 | Strong | 0-25 | N/C | + |
| Mast cell tumor | 0-25 | Strong | 0-25 | N/C | + |
| Melanocytoma | 0-25 | Weak | 50-75 | N/C | + |
| | 75-100 | Strong | 50-75 | N/C | + |
| Melanoma | 75-100 | Strong | 50-75 | N/C | - |
| | 0-25 | Weak | 0-25 | C | - |
| | 0-25 | Weak | 0-25 | N/C | n.d. |
| | 0-25 | Weak | 0-25 | N/C | + |
| | 0-25 | Strong | 0-25 | N/C | + |
| | 0-25 | Weak | 0-25 | N/C | + |
| | 50-75 | Strong | 0-25 | N/C | + |
| | 25-50 | Strong | 25-50 | N/C | n.d. |
| | 0-25 | Strong | 25-50 | N/C | + |
| | 0-25 | Strong | 25-50 | N/C | + |
| | 25-50 | Strong | 25-50 | N/C | + |
| | 25-50 | Strong | 25-50 | N/C | + |
| | 25-50 | Strong | 25-50 | N/C | + |
| | 75-100 | Strong | 25-50 | N/C | + |
| | 0-25 | Weak | 50-75 | N/C | + |
| | 0-25 | Weak | 50-75 | N/C | + |
| | 25-50 | Strong | 50-75 | N/C | + |
| | 25-50 | Strong | 50-75 | N/C | + |
| | 0-25 | Strong | 75-100 | N/C | + |
| | 0-25 | Weak | 75-100 | N/C | n.d. |
| Osteosarcoma | 50-75 | Strong | 25-50 | N/C | + |
| | 0-25 | Weak | 0-25 | N/C | + |
| | 25-50 | Strong | 75-100 | N/C | n.d. |
| Trichoblastoma | 50-75 | Strong | 50-75 | N/C | + |
| Tricholemmoma | 50-75 | Strong | 0-25 | N/C | + |

N = nucleus; C = cytoplasm; n.d. = not determined; "+" = positive; "-" = negative

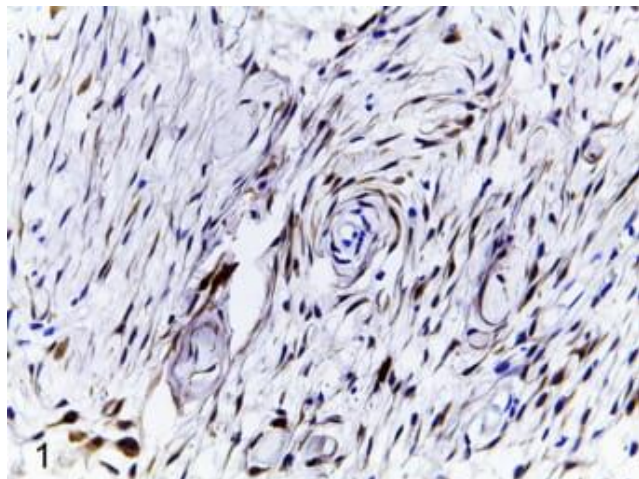


Figure 1. Photomicrograph of a hemangiopericytoma showing strong staining of neoplastic cells for Gal-3. Immunohistochemistry, counterstained with Harris hematoxylin, Obj. 40x.

Large variations in Gal-3 staining patterns have been reported in several human neoplasms. Upregulation of Gal-3 expression was associated with the progression of gastric, lung, colorectal, and pancreatic cancers; hepatocellular, nasopharyngeal, and esophageal carcinomas; large B-cell lymphoma, squamous cell carcinoma, melanoma, astrocytoma, and glioblastoma (25). Downregulation or suppression of Gal-3 expression was found to be associated with neoplastic progression in non-melanoma skin cancer, but not in squamous cell carcinoma, breast and endometrial cancers, prostate cancer, and chronic lymphocytic leukemia (25).

In the present study, Gal-3 expression in the neoplastic melanocytes from canine oral melanomas was low in the majority of the samples. Additionally, low nuclear expression of Gal-3 was observed in 65% (13/20) of the tumors. Brown et al. (1) reported higher nuclear expression of Gal-3 in human benign nevi and thin melanoma, compared to its expression in metastatic and thicker melanomas. Prieto et al. (19) described strong differences in the cytoplasmic and nuclear expression patterns of Gal-3 in benign nevi, dysplastic nevi, and melanoma, and showed that high nuclear:cytoplasmic ratio of Gal-3 expression was associated with metastatic disease, shorter disease-free intervals, and overall survival. Metastatic melanoma cell lines also expressed more Gal-3 protein and mRNA in comparison to the non-metastatic ones (26).

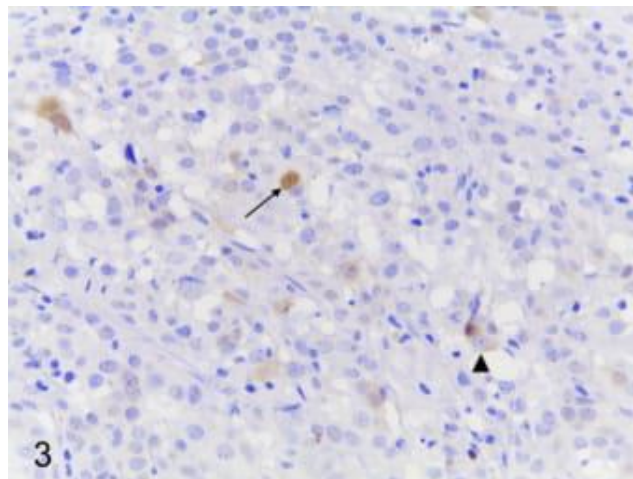


Figure 2. Photomicrograph of an oral melanoma showing weak staining for Gal-3. Gal-3 staining is confined to the nucleus (arrow) or cytoplasm (arrowhead). Immunohistochemistry, counterstained with Harris hematoxylin, Obj. 40x.

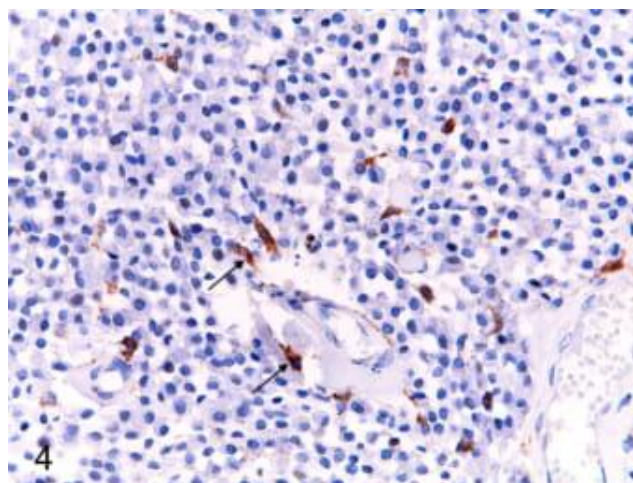


Figure 3. Photomicrograph of a cutaneous mast cell tumor showing fibroblast positivity for Gal-3 (arrows). Immunohistochemistry, counterstained with Harris hematoxylin, Obj. 40x.

We also tested Gal-3 expression in seven different histologic subtypes of mammary tumors. A considerable variation in the percentage of immunolabeled cells, ranging from C1 to C3, was observed in these tumors. However, the majority of the mammary tumor samples showed strong immunostaining in the nucleus and cytoplasm. Regarding fibroblast positivity, papillary-type was the only negative carcinoma. Imer et al. (9) revealed that low expression of Gal-3 was associated with decreased survival rate and an increase in the locoregional recurrence of human breast cancer. Another study showed that high expression of Gal-3 was associated with increased chemoresistance (31).

Concerning canine mammary tumors, De Oliveira et al. (18) verified that benign tumors exhibited stronger Gal-3 immunostaining compared to malignant counterparts, in which the immunostaining intensity was low and confined to the cytoplasm. However, higher expression of Gal-3 was detected in the tumors undergoing metastases, particularly in intravascular tumor cells (27). Recently, the same research group showed that serum levels of Gal-3 are higher in bitches with malignant mammary tumors, but these levels remain persistently elevated only in the metastatic cases (18, 20).

Two other studies in dogs described Gal-3 expression in canine gastric carcinomas (27) and hemangiosarcoma (10). The first examined 9 canine gastric carcinomas and detected stronger immunolabelling in neoplastic cells, while normal epithelial cells showed faint staining for Gal-3 (27). Johnson et al. (10) found 100% positivity in 17 canine hemangiosarcomas and 10 human angiosarcomas. Additionally, they tested the effects of two Gal-3 inhibitors on murine angiosarcoma cells in vitro and demonstrated significant reduction in cell survival, as well as higher sensitivity to doxorubicin (10).

Several studies have reported the involvement of Gal-3 in almost every hallmark of cancer, such as angiogenesis (14, 15), apoptosis (31, 15, 13), invasion and metastasis (3, 17, 22, 25, 30), cell proliferation (4, 11, 12, 24, 32) and evasion from immune destruction (5, 6, 16, 21). These findings suggest that, at least for human oncology, the detection of Gal-3 in cancer tissues may serve as a valuable prognostic marker and a therapeutic target.

As in the case of humans, we showed that many canine tumors also expressed Gal-3. Moreover, canine tumor cells showed considerable variations in intensity of immunostaining, percentage of Gal-3-positive cells, and localization of the Gal-3 protein. Owing to the extensive role of Gal-3 under pathological conditions and considering the findings of human and canine oncology literature, we hypothesize that this protein may prove to be an important diagnostic and/or prognostic biomarker for veterinary oncology in the future. Further studies are required to investigate the prognostic value of Gal-3 expression in a greater number of cases of each canine neoplasm.

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