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Prognostic significance of Ki67 and its correlation with mitotic index in dogs with diffuse large B-cell lymphoma treated with 19-week CHOP-based protocol

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Abstract. Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma in dogs. We evaluated Ki67 immunoexpression and mitotic index (MI) in dogs diagnosed with DLBCL and treated with a 19-wk CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) protocol. Twenty-nine lymph node samples from dogs diagnosed with DLBCL were analyzed for Ki67 immunostaining, and positive cells present in 1 cm^2 were counted in a grid reticle for comparison of survival times above and below the means. The Ki67 mean was 107, and the MI mean was 21. There was a significant ($p < 0.05$) difference in median survival time between Ki67 immunostaining above and below the mean, with no difference in MI groups. Ki67 values >107 positive cells per 5 HPF counted in a grid reticle were associated with shorter survival times in dogs with DLBCL treated with a 19-wk CHOP-based protocol.

Key words: Cell proliferation; dogs; immunohistochemistry; mitosis; prognosis.

Lymphoma is the most common hematopoietic tumor in dogs and the second most common tumor in this species, comprising 7–24% of all neoplasia cases.[12] In the United Kingdom, this tumor has an annual incidence of 107 cases per 100,000 dogs[4]; this rate in Brazil would suggest that over 55,000 dogs could be diagnosed with lymphoma each year based on a national canine population of 52 million (Instituto Brasileiro De Geografia E Estatística, Pesquisa Nacional de Saúde 2013. Portuguese. Available from: https://goo.gl/GnL-ZgS). Diffuse large B-cell lymphoma (DLBCL) is the most common histologic type of lymphoma in dogs and humans, and similarities in origin, molecular characterization, and treatment response have been observed in both species.[12] DLBCL has an aggressive and heterogeneous biological course, showing variable response to most commonly used chemotherapy protocols in patients with the same tumor stage and sub-stage.[7] Several prognostic factors have been identified in canine lymphoma[6,7,9]; however, only 2 studies have specifically analyzed prognostic factors in DLBCL, to our knowledge.[2,8]

Cell proliferation has been studied in different tumors, and several markers have been described in veterinary medicine.[6] Ki67 is a protein expressed at every phase of the cell cycle, except at G0, with greater expression during mitosis (M).[9] Ki67 is an important marker of cell proliferation for predicting the prognosis of DLBCL in humans.[9] MIB-1 is the monoclonal antibody most used to analyze Ki67 immunoexpression in some types of aggressive lymphomas. In veterinary medicine, the role of Ki67 as a prognostic factor in high-grade B-cell lymphomas has been demonstrated by flow cytometry[11]; results in paraffin-embedded tissues are still being debated.[6,18] As with Ki67, the mitotic index (MI) is an indicator of cell proliferation and used as a prognostic factor in human patients diagnosed with non-Hodgkin lymphoma.[1] Although some studies suggested that MI is not a good prognostic factor in canine lymphoma,[3,6] other studies have found a significant correlation between shorter survival time and MI.[16]

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We analyzed cell proliferation markers Ki67 and MI in dogs diagnosed with DLBCL to determine if a correlation existed between these markers and patient prognosis. Cases were selected from dogs with lymphoma admitted to veterinary centers in the state of Sao Paulo from January 2009 to September 2015. Cases were eligible for inclusion in this study if they met the following criteria: 1) histologic diagnosis of DLBCL examined by a single pathologist (FA Sueiro) and classified according to the WHO classification,[16] and 2) lack of previous chemotherapy treatment (including prednisone), and treatment using the 19-wk CHOP based protocol (cyclophosphamide, doxorubicin, vincristine and prednisone) recommended by the University of Wisconsin–Madison.[15] Clinical and epidemiologic data on the patients such as breed, weight, sex, clinical signs, and survival time were obtained from the patient's medical record. Clinical staging was established through physical examination, hemogram, basic serum biochemistry (creatinine and alanine aminotransferase), abdominal ultrasound, and thorax radiography in 3 projections. Bone marrow aspiration was only performed in patients with evidence of atypical lymphocytes in peripheral blood, lymphocytosis, or pancytopenia detected on initial hemogram. Sub-stages were classified as "a" when there were no clinical signs and "b" when the patient had clinical signs including gastrointestinal signs, respiratory signs, fever, depression, or lethargy. Survival time was considered from the date of diagnosis to the day of death or the date of last check-up at our hospital. All samples were analyzed by the same pathologist, and immunophenotyping was performed by immunohistochemistry, which used CD3 and CD79a antibodies to identify T- and B-lymphocytes, respectively. When CHOP chemotherapy was no longer effective at controlling remission, CCNU (lomustine) was implemented as first rescue protocol.

For Ki67 immunostaining, 3-µm sections were cut from paraffin-embedded samples and mounted on silane-coated glass slides (Starfrost, Knittel Glass, Braunschweig, Germany). The slides were de-waxed and hydrated, and sections were subjected to antigen retrieval with citrate buffer (pH 6) in a Pascal pressure chamber (Dako North America, Carpinteria, CA). Endogenous peroxide blocking was carried out using blocking solution (Spring Biosciences, Pleasanton, CA) according to the manufacturer's recommendation. Blocking of nonspecific reactions was performed with protein block (Novocastra, Newcastle, UK), and sections were incubated with the primary antibody (monoclonal mouse MIB-1, Dako; 1:100) for 2 h at room temperature of 27°C. HistoFine (Nichirei Biosciences, Tokyo, Japan) was used as secondary antibody, and sections were stained with 3,3'diaminobenzidine (DAB Novolink, Leica Biosystems, Newcastle, UK). Samples were counterstained with Harris hematoxylin (Easypath, São Paulo, Brazil). A sample of canine lymphoma known to be positive to MIB-1 was used as the positive control. A negative control was obtained by replacing the primary antibody with the dilution solution.

For Ki67 evaluation, a single pathologist (FA Sueiro) selected areas with the highest proportion of immunopositive neoplastic lymphocytes at 100× magnification and manually counted the number of positively stained cells in 1 cm^2 using a 10 × 10 mm grid reticle in a light microscope (Eclipse E200, Nikon, Japan) at 400× magnification.[17] Five high power fields (HPF) were analyzed to calculate the mean number of positive cells for each case. For MI, the pathologist selected areas with the highest overall mitotic activity and counted the total mitotic figures present in 10 HPF (400×) as described previously.[13]

The cutoff points established for the mean numbers of Ki67-positive cells was 107, and the cutoff point for total MI was 21. Ki67 and MI values above or below the cutoff points were classified into "high" or "low" groups, respectively.

For statistical purposes, the Shapiro–Wilk test was used to assess normality of distribution of the continuous variables, and survival time curves were estimated and compared between the "high" and "low" groups of each marker by the Kaplan–Meier method and log-rank test. The degree of correlation between Ki67 and MI was estimated by Spearman correlation coefficient. The Fisher exact test was used to analyze potential associations between the markers and clinical stage (stages III or IV and sub-stages "a" or "b"). Significance was considered at $p < 0.05$.

The dog breeds most commonly diagnosed with DLBCL were mixed-breed dogs ($n = 6$), followed by Rottweilers ($n = 5$). Average age was 8.4 y (range: 4–14), with 18 females (12 intact and 6 spayed) and 11 males (8 intact and 3 castrated). Clinical staging classified 6 patients as stage III (generalized lymph node involvement), 22 as stage IV (liver or spleen involvement ± stage III), and 1 as stage V (blood or bone marrow involvement and/or other organ involvement ± any stage). Most dogs showed no clinical signs of the disease, with 18 patients being classified as sub-stage "a" and 11 as sub-stage "b" (Table 1).

Studies on dogs with DLBCL have reported that mixed-breed dogs are the most commonly affected,[5,8] as observed in our study in which they corresponded to 21% of patients. Rottweiler was the second most affected breed in our study with 17% of the total DLBCL cases. A 2015 study analyzed 51 dogs with DLBCL and observed that Rottweilers also corresponded to the second most affected breed (9.8%).[8] However, studies have not yet evaluated a significant number of animals that would confirm a breed predisposition.

The median survival time of the patients in our study was 245 d, similarly to previous studies on DLBCL.[16] Thirteen dogs died as a result of the disease before the end of the treatment protocol. Interestingly, 80% of Rottweiler (4 of 5) and 100% of mixed-breed patients (6 of 6) were in remission throughout the treatment.

The age of onset is similar to those reported in the literature, where DLBCL is more frequent in animals aged 7.5–8 y.[5,8] The majority of patients in our study were classified as stage IV (76%) and did not show clinical signs of

Table 1. Clinical characteristics of 29 dogs with diffuse large B-cell lymphoma. Ki67-low and mitotic index (MI)-low are groups below cutoff points (mean) of 107 and 21, respectively; Ki67-high and MI-high are groups above the mean.

	n	Ki67-low (*n* = 21)	Ki67-high (*n* = 8)	MI-low (*n* = 17)	MI-high (*n* = 12)
Age (range)	8.4 (4–14)	8.8 (5–14)	7.2 (7–10)	8.5 (4–14)	8.1 (5–13)
Sex*					
Male	11 (38)	9 (43)	2 (25)	8 (47)	3 (25)
Female	18 (62)	12 (57)	6 (75)	9 (53)	9 (75)
Breed*					
Mixed-breed dog	6 (21)	6	0	6	0
Rottweiler	5 (17)	4	1	2	3
Schnauzer	3 (10)	1	2	2	1
Other purebred dogs	15 (52)	10	5	7	8
Stage*					
III	6 (21)	5	1	3	3
IV	22 (76)	15	7	14	8
V	1 (3)	1	0	0	1
Sub-stage*					
a	18 (62)	14	4	12	6
b	11 (38)	7	4	5	6

* Numbers in parentheses are percentages.

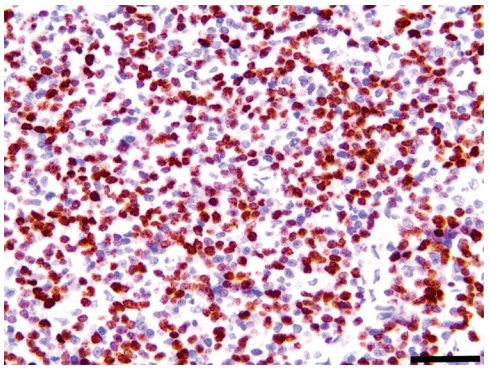

Figure 1. Photomicrograph of Ki67 nuclear staining in the lymph node of a dog diagnosed with diffuse large B-cell lymphoma. Positive cells were counted in 1 cm^2 using a 10 × 10 mm grid reticle. MIB-1, Histofine, DAB, 40×. Bar = 50 μm.

the disease (sub-stage "a", 64%), in agreement with previous findings.[12]

Ki67 staining was restricted to the nucleus (Fig. 1) and was positive in all samples. The mean value of Ki67 was 107 (range: 1–446) and 21 for MI (range: 0–73). Based on the mean value for Ki67, we evaluated 2 groups: Ki67-low (<107, n = 21) and Ki67-high (≥107, n = 8). The median survival time for Ki67-low (294 d) was significantly longer ($p < 0.05$) than for Ki67-high dogs (63 d; Table 2). Based on the mean MI, we also identified 2 groups: MI-low (<21, n = 17) and MI-high (≥21, n = 12). No statistically significant difference was detected in the median survival time between groups MI-low (294 d) and MI-high (129 d; Fig. 2). A statistically significant relationship was not observed between Ki67 and MI values, or between either factor and stages or sub-stages ($p > 0.05$).

Ki67 and MI have been considered prognostic factors in dogs with mast cell tumor and melanoma.[13,14,17] In the 2 most recent reports discussing prognosis of dogs with DLBCL,[2,8] no relationship was found between Bcl2 and MYC immunoexpression and prognosis,[2] whereas the other study reported that a lymphocyte-to-monocyte ratio <1.2 (flow cytometry) is a prognostic factor in dogs with high-grade B-cell lymphomas.[8] Similarly, other authors quantified Ki67 by flow cytometry and established it to be a prognostic factor in dogs with B-cell lymphomas.[11] This result is consistent with our study, which demonstrated that a correlation exists between Ki67 immunostaining and shorter survival times in dogs with DLBCL. Interestingly, the former study,[11] did not allow for a cutoff point to be determined given the lack of significant difference when the survival time between groups was compared with values above and below the median. A significant association or correlation was reported when Ki67 values were calculated by percentile, which enabled 3 groups to be established (low, intermediate, and high) with correlation between the intermediate group and shorter survival times.[11] We also compared survival times from cases with central values of Ki67 immunoexpression with those of extreme values and found no association (data not shown). Although the statistical analysis in the previous study[11] differs from our study, the results suggest that Ki67-low is a good prognostic marker in dogs with B-cell lymphoma, especially DLBCL.

Our results partially contradict others that concluded that a higher number of Ki67 cells was associated with a better response to chemotherapy in multicentric lymphoma and therefore a longer disease-free interval with no difference found in overall survival time.[10] However, one of those studies evaluated random areas instead of areas of highest Ki67

Table 2. Survival times of 29 dogs with diffuse large B-cell lymphoma based on Ki67 and mitotic index (MI) scores, with Ki67 cutoff of 107 and MI cutoff of 21.

Animal	Ki67	Ki67 group	MI	Survival time (d)
1	6.2	Low	3	539
2	12.2	Low	32	147
3	16.4	Low	18	336
4	22.2	Low	49	343
5	30.8	Low	4	553
6	33.4	Low	34	357
7	39.0	Low	29	392
8	40.8	Low	15	245
9	41.4	Low	73	133
10	48.0	Low	13	490
11	49.6	Low	8	140
12	56.0	Low	10	28
13	57.4	Low	0	252
14	76.8	Low	3	336
15	82.0	Low	48	14
16	90.4	Low	23	105
17	91.4	Low	7	294
18	96.4	Low	14	420
19	98.0	Low	51	126
20	100.4	Low	4	378
21	106.2	Low	11	287
22	112.8	High	44	133
23	155.2	High	6	14
24	159.2	High	38	56
25	161.2	High	19	7
26	176.2	High	37	7
27	319.2	High	41	84
28	386.8	High	0	70
29	446.0	High	10	420

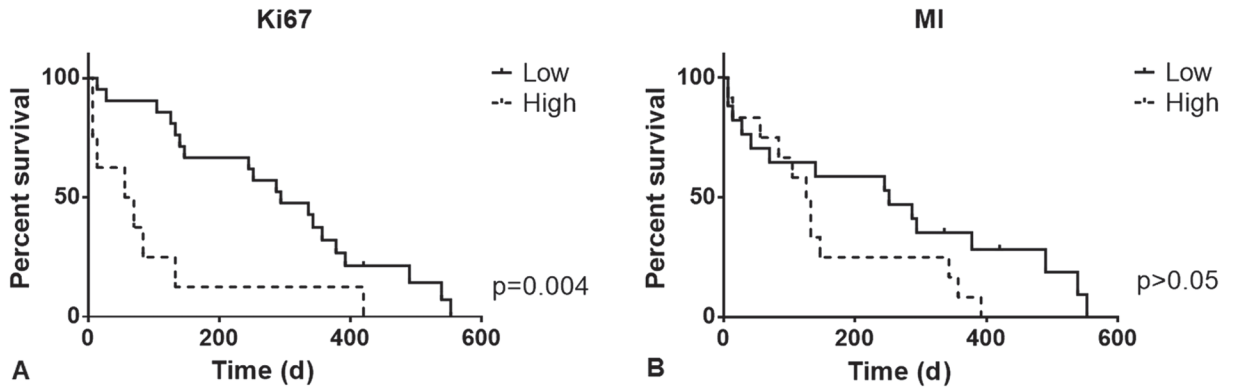

Figure 2. Survival curve in dogs diagnosed with diffuse large B-cell lymphoma. **A.** Survival time was statistically significant ($p = 0.004$) between groups Ki67-low ($n = 21$) and Ki67-high ($n = 8$). **B.** No significant difference was identified ($p > 0.05$) between group mitotic index (MI)-low ($n = 17$) and MI-high ($n = 12$).

immunoexpression for counting.[10] Another study observed no significant correlation between survival time and the number of Ki67-positive cells.[6] It is important to note that the counting technique used in our study was different than the one reported in these other studies.

The cutoff point established for MI was not a prognostic factor in dogs with DLBCL in our study. This result is in disagreement with others who determined the same MI value (21) and observed shorter survival time in 26 dogs with MI >21, comparing them to 353 dogs with MI <20 with

multicentric lymphoma.[16] However, that publication did not differentiate DLBCL from other histologic types such as indolent lymphomas and other low-grade lymphomas, which resulted in a heterogeneous group that may have affected findings.

Our study has limitations such as its retrospective nature and the relatively small number of cases. A lack of homogeneity of clinical stages, especially in stage V, may contribute to the lack of association found between clinical staging and cell proliferation.

Declaration of conflicting interests

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