PROGNOSTIC SIGNIFICANCE OF KI67 EVALUATED BY FLOW CYTOMETRY IN DOGS WITH HIGH GRADE B-CELL LYMPHOMA


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Abstract

Ki67 can discriminate between high- and low-grade canine lymphomas, but its prognostic role in specific subtypes of the neoplasm is unknown. We assessed the prognostic significance of Ki67% (percentage of Ki67-positive cells), evaluated via flow cytometry, in 40 dogs with high-grade B-cell lymphoma, treated with a modified Wisconsin-Madison protocol (UW-25). The following variables were investigated for association with lymphoma specific survival (LSS) and relapse free interval (RFI): Ki67%, breed, sex, age, stage, substage, complete remission (CR). By multivariate analysis, Ki67% (P=0.009) and achievement of CR (P=0.001) were independent prognostic factors for LSS. Dogs with intermediate Ki67% (20.1-40%) presented longer LSS and RFI (median=866 and 428 days, respectively) than dogs with low (median=42 days, P<0.001; median=159 days, P=0.014) or high (median=173 days, P=0.038; median=100 days, P=0.126) values. Determination of Ki67 is a prognostic tool that improves the clinical usefulness of flow cytometric analysis in canine high-grade B-cell lymphoma.
**Introduction**

Canine lymphoma represents a heterogeneous group of neoplasms arising from the malignant transformation of lymphoid cells and is characterized by a broad range of clinical presentations and potential outcomes. Depending upon the grade of malignancy, lymphomas are cytologically grouped into two main categories. The most commonly encountered forms are high grade lymphomas, clinically aggressive and typically fatal within a short period of time when treatment is not instituted. Conversely low grade lymphomas are rare and characterized by an indolent disease course.\(^1,2\)

Defining the immunophenotype is also reported to be important in predicting prognosis.\(^3-7\) In fact multicentric T-cell forms, when compared with B-cell forms, seem to be associated with similar initial response rates, but have significantly lower response durability, even following an appropriate chemotherapy protocol.\(^2\)

Moreover, although several prognostic factors that independently influence the response rate and the survival time have been identified, the clinical outcome remains variable between identically treated lymphomas.\(^4\) In fact, dogs with similar signalment, stage and substage of disease, immunophenotype and tumor anatomic location may respond differently to the same treatment.\(^8\)

In recent years, many studies have stressed the prognostic significance of the evaluation of tumor biology and, in this context, the role of proliferative activity has received special attention. One of the most frequently used methods to evaluate the growth fraction of neoplastic populations is the detection of the Ki67 antigen.\(^9\) This proliferation-associated nuclear protein is expressed in all the active phases of the cell cycle (G1, S, G2 and mitosis), but it is absent in resting cells (G0).\(^10,11\) The exclusive expression in proliferating cells has made the antibodies raised against the Ki67 antigen an invaluable diagnostic tool for grading, assessing clinical behavior and outcome in various human malignancies.\(^12-15\)
In particular, in non-Hodgkin’s lymphoma, the human counterpart of canine lymphoma,\textsuperscript{16} Ki67 has been found to be an independent prognostic factor.\textsuperscript{17-19} However, contradictory results have been reported, mainly because of the heterogeneity within and among the different subtypes of the disease.\textsuperscript{13,20,21}

The proliferative activity has also been evaluated in few studies on canine lymphoma and, while Ki67 expression has shown a significant correlation with the grade of malignancy,\textsuperscript{22,23} its reliability as prognostic marker is still unclear.\textsuperscript{24,25} In all these studies the determination of Ki67 has been performed through immunohistochemistry in bioptic specimens. Moreover recently, our group has demonstrated that flow cytometric detection of Ki67 is a powerful and non-invasive alternative method able to discriminate between high and low grade canine lymphomas.\textsuperscript{26}

The aim of this study was to assess the prognostic significance of Ki67, evaluated by flow cytometry, in dogs with high grade B-cell lymphoma being treated with the same multidrug chemotherapy protocol.

Materials and methods

Case selection

Dogs with multicentric high grade B-cell lymphoma diagnosed at the Veterinary Teaching Hospital of the University of Turin between April 2011 and September 2014 were considered. The diagnosis was based on clinical presentation (lymph node enlargement), cytological examination of lymph nodes and flow cytometric analysis.

Inclusion criteria for the study were cytological diagnosis of high grade lymphoma according to the updated Kiel classification,\textsuperscript{27,28} presence of flow cytometric B-cell immunophenotype, flow cytometric Ki67 determination, treatment with a modified version of the University of Wisconsin-Madison chemotherapy protocol (UW-25)\textsuperscript{29} and the availability of follow up data. Dogs previously
treated with corticosteroid or chemotherapy agents were excluded. For each included dog, signalment data (breed, sex and age), when available, were retrieved and clinical stage (I-V) and substage (a or b) were assigned according to the World Health Organization (WHO) system. In particular, stage V was assigned when the neoplastic population, detected via flow cytometry, was ≥3% in peripheral blood and/or bone marrow.

**Flow cytometric immunophenotyping and Ki67 determination**

At time of initial staging, flow cytometric immunophenotyping was performed on lymph node fine-needle aspirate biopsies (FNABs), peripheral blood samples and/or bone marrow aspirates within 24h from collection as previously reported.

The following panel of monoclonal antibodies (mAbs) was used: CD45-Alexa647 (pan-leukocyte marker; clone YKIX716.1, AbD Serotec, Oxford, UK), CD3-FITC (T-cells marker; clone CA17.2A12, AbD Serotec), CD5-FITC (T-cells marker; clone YKIX322.3, AbD Serotec), CD4-Alexa647 (T-helper marker; clone YKIX302.9, AbD Serotec), CD8-PE (T-cytotoxic/suppressor maker; clone YCATE55.9, AbD Serotec), CD21-PE (B-cells marker; clone CA21D6, AbD Serotec), CD79b-FITC (B-cells marker; clone AT107-2, AbD Serotec) and CD34-PE (precursor cells marker; clone 1H6, Pharmingen Becton Dickinson, San Jose, CA, USA).

The proliferative activity was determined on the same lymph node FNABs used for immunophenotyping. Cells were labelled with antiKi67-FITC monoclonal antibody (clone MIB-1, DAKO, Glostrup, Copenhagen, Denmark) using a fixation and permeabilization method with methanol, as described previously.

A minimum of 10,000 events were acquired both for immunophenotype and Ki67 determination on BD Accuri C6 flow cytometer (Becton Dickinson). Data were analyzed using CFlow Plus software (Beckton Dickinson). A gate of analysis was depicted on forward (FSC) versus side scatter (SSC).
plot in order to exclude debris and background. The proliferative activity was expressed as the percentage of Ki67 positive cells (Ki67%) calculated on a SSC versus fluorescence intensity plot.

**Cytological evaluation**

Smears obtained by FNABs of enlarged lymph nodes were air-dried, fixed and stained with May-Grünwald-Giemsa. Each case was classified according to the updated Kiel classification\(^27,28\) and allocated to a specific grade of malignancy and cytological subtype.

**Follow up**

Information pertaining to the achievement of remission, occurrence of relapse, survival at the end of chemotherapy protocol, lymphoma specific survival (LSS), relapse-free interval (RFI), date and cause of death was collected.

Responses were classified as follows: complete remission (CR), which indicated reduction to normal size of all measurable lymph nodes; partial remission (PR), which indicated more than 50% but less than 100% reduction of all measurable lesions and stable disease (SD), which indicated less than 50% reduction or no change in the size of all measurable lesions. Relapse was defined as clinical reappearance and cytological evidence of lymphoma in any anatomical site in dogs having experienced CR. RFI was defined as the time in days from when a dog achieved CR until relapse. LSS was defined as the interval in days between the date on which chemotherapy was started and the date of death due to lymphoma related causes.

**Statistical analysis**

LSS and RFI for all dogs were estimated using the Kaplan-Meier product limit method. Contingency tables were prepared for each of following variables: Ki67% (low, intermediate, high),
breed (purebred or crossbred), sex (male or female), age (< or ≥ 10 years), stage (I-IV or V), substage (a or b), CR (yes or no). Pearson’s $\chi^2$ with z-test for column proportion comparisons and Bonferroni adjustment for multiple comparisons were calculated to test the association between each variable with the achievement of CR and survival at the end of first chemotherapy protocol (UW-25). Dogs that died for causes other than lymphoma and dogs that had not yet completed the protocol and did not meet the event (CR or death) were excluded from contingency tables. Ki67% cut-off values were defined rounding the thresholds of 25th and 75th percentiles to 20 and 405, respectively, and thus generating the following groups: low if Ki67≤20%, intermediate if Ki67 between 20.1% and 40%, high if Ki67>40%.

To evaluate the prognostic significance of each variable, univariate logistic regression for LSS and RFI was first used and variables with a P value <0.3 were then included in a multivariate Cox proportional hazards model progression analysis with a backward step selection. Kaplan-Meier curves were drawn for Ki67% groups and compared by log-rank test to assess the survival analysis. Dogs that were alive at the end of the study, lost to follow-up or dead due to causes other than lymphoma were censored for survival analysis. Differences were considered significant with P<0.05. Statistical analyses were performed using SPSS software (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA).

**Results**

**Lymphoma cases**

Forty cases met inclusion criteria and were enrolled in the study. Data about the identification of breed were reported for 39 cases. There were 2 (66.7%) purebred dogs (3 Labrador retrievers, 2 German shepherds, 2 Dobermans, 2 Bloodhounds, 2 Pitt bull terriers and 1 each of Italian Mastiff, Great Dane, Poodle, Dachshund, Beagle, Bernese mountain dog,
Cavalier King Charles Spaniel, Golden retriever, Jack Russell, Rottweiler, White Swiss shepherd, Cocker Spaniel, English bulldog, Lagotto romagnolo, American Staffordshire terrier) and 13 (33.3%) crossbred dogs. Sixteen dogs (42.1%) were males (1 castrated) and 22 (57.9%) were females (9 spayed), while in 2 cases the sex was unknown. The age was only reported for 37 dogs and the median age was 9 years (range, 4-15 years).

The included lymphomas were cytologically classified as follows: 8 (20%) centroblastic monomorphic, 24 (60%) centroblastic polymorphic predominantly large cell, 5 (12.5%) immunoblastic, 2 (5%) lymphoblastic and 1 (2.5%) plasmacytoid.

At time of diagnosis 27 dogs (67.5%) were in stage IV (10 substage a, 16 substage b and 1 unknown) and 13 (32.5%) in stage V (all substage b).

**Response to treatment**

CR was achieved in 25 (62.5%) dogs. Twelve out of these 25 (48%) relapsed (median RFI=180 days; range 28-530), 10 (40%) were still in CR at the end of the study (median follow up period=321 days; range 60-1005) and 3 (12%) died of causes unrelated to lymphoma after 34, 210 and 240 days from the beginning of chemotherapy, with lymphoma remaining in CR.

Relapses were treated with a second UW-25 or with other rescue protocols (DMAC; L-asparaginase+lomustine), depending on when the relapse occurred and owner compliance. At the end of the study, 8 out of 12 relapsed dogs (66.7%) were dead because of progressive disease (median LSS = 390 days; range 150-866), 3 (25%) were in PR (follow up period of 515, 800 and 1108 days) and 1 (8.3%) was in SD ( follow up period = 295 days).

Among 15 dogs that did not achieve CR, 11 (73.3%) died because of PD (median LSS=42 days; range 15-1100), 3 (20%) were in PR at the end of the study (follow up period of 28, 157 and 653 days) and 1 (6.7%) died of causes unrelated to lymphoma after 45 days. Estimated median RFI and
LSS for all dogs were 414 days (95% CI range 228-600 days) and 442 days (95% CI range 236-648 days), respectively.

**Proliferative activity**

The mean Ki67% was 33.8% (SD=14.2%) and the median was 30.7% (range 10-67%). Six cases presented low Ki67% (≤20%), 24 were in the intermediate group (20.1-40%) and 10 were in the high group (>40%).

**Survival at the end of chemotherapy protocol and achievement of CR**

Survival at the end of chemotherapy was significantly associated with the achievement of CR (P=0.001). In fact, 91.7% of the dogs that achieved CR were alived compared with 33.3% of dogs that did not reach CR (Table 1).

Ki67% showed near-significant association with both survival (P=0.063) and achievement of CR (P=0.075) at the end of chemotherapy protocol. In fact, percentages of both survival and CR were higher for dogs with intermediate Ki67% (85.7% and 81%, respectively) compared with dogs with low (50% and 33%) and high Ki67% (50% and 60%) (Table 1).

**Prognostic factors for LSS and RFI**

Ki67% (P=0.007) and achievement of CR (P=0.001) significantly influenced LSS on univariate analysis and were confirmed to be independent prognostic factors for LSS (P=0.009 and P=0.001 respectively) in the multivariate analysis (Table 2).

None of the variables significantly influenced RFI in the univariate analysis and none were of prognostic significance for RFI in the multivariate analysis (Table 2).
The Kaplan-Meier analysis showed that dogs with intermediate Ki67% have significantly longer LSS (median=866 days) than dogs with low (median=42 days; \( P<0.001 \)) and high Ki67% (median=173 days; \( P=0.038 \)) (Fig. 1).

Intermediate Ki67% was a significant predictor also for 1 year and 2 years survival (\( P=0.001 \) and \( P=0.004 \) versus low and high Ki67%, respectively, at both time points) (Fig. 1). Dogs with intermediate Ki67% reported also longer RFI (median =428 days) than dogs with low (median=159 days, \( P=0.014 \)) and high Ki67% (median=100 days, \( P=0.126 \)), although the difference with the high Ki67% group did not reach statistical significance (Fig. 2).

**DISCUSSION**

Ki67 is one of the most widely used markers of cell proliferation. Although it is considered an important factor for grading neoplasms and predicting their biological behavior,\(^\text{12,14}\) its clinical relevance is still being debated both in human and canine lymphomas. In a previous work,\(^\text{26}\) we assessed the feasibility of flow cytometric determination of Ki67 in canine lymphoma and we demonstrated its association with malignancy grade, regardless of phenotype and morphology.

In this study we investigated the prognostic significance of Ki67, as evaluated by flow cytometry in dogs with high grade B cell lymphoma treated with the UW-25 chemotherapy protocol. We focused on the most common type of canine lymphoma to limit heterogeneity with regards to some clinical prognostic features, such as malignancy grade and immunophenotype.\(^\text{4}\) Likewise in our case series, all dogs were treated with the same chemotherapeutic protocol to avoid treatment bias in the response, although the LSS of relapsed dogs may have been influenced by receiving multiple reinduction or rescue protocols.
The achievement of CR and the intermediate Ki67% values were associated with the survival at the end of chemotherapy protocol, suggesting their prognostic role, even though the association with the Ki67 did not reach a statistical significance.

Based on multivariate analysis, Ki67% and CR were found to be independent prognostic factors for LSS, while none of the investigated variables had prognostic significance for RFI. Moreover, the Kaplan-Meier analysis confirmed that an intermediate Ki67% was associated with a better prognosis with longer LSS and RFI compared with dogs with low or high Ki67%.

These findings are discordant with the results of few previous studies that evaluated the prognostic significance of Ki67 in dogs with lymphoma. In the work by Kiupel et al. Ki67 expression showed no prognostic value, while Phillips et al. reported that Ki67 was predictive for duration of first RFI but not overall survival. Differences in inclusion criteria and method of Ki67 determination could account for these discrepancies. In fact, in the previous studies, both high and low grade lymphomas and both B and T-cell immunophenotype were included. Moreover, Ki67 detection was carried out through immunohistochemistry on bioptic specimens, while we used flow cytometric analysis of FNABs. Furthermore, the different selection of the cutoff values to define groups may have influenced the results. In our work, we used an approach similar to that of Phillips et al., using median and the 75th percentile to differentiate two prognostic groups and we get near significant results with the latter (data not shown). Observing that the longest survival times were associated with intermediate Ki67% values, we assessed the prognostic significance of Ki67% dividing cases in three different groups using quartiles. Furthermore, we rounded the quartile cutoff values to 20% and 40% in order to simplify the use in clinical practice. Unfortunately, a direct comparison of our cut-off values with those assessed by Phillips et al. is not possible because they did not reported the actual percentages that define quartiles in their caseload. However, this comparison, although interesting, would presumably be limited by the different method use to
measure Ki67 expression. In this regard, Kiupel et al.\textsuperscript{25} did not get significant results despite the application of thresholds similar to ours (Ki67<20%; 21-40%; 41-60%; >60%).

Contradictory results on prognostic significance of Ki67 have also been reported in human non-Hodgkin’s lymphoma, because of the heterogeneity of the different subtypes of this disease.\textsuperscript{19} Many studies have determined Ki67 in aggressive diffuse large B-cell lymphomas, with a wide range of expression.\textsuperscript{21} In accordance with our results, in the largest one Jerkeman et al.\textsuperscript{32} found that patients with either low (<60%) or high Ki67 (>90%) expression demonstrated a trend toward overall lower survival than patients with moderate expression (60-90%). Moreover, a low proliferation index was associated with a low failure-free survival compared with moderate or high indexes. This behavior is likely because lymphomas with a low proliferation rate exhibit resistance to cycle specific cytotoxic chemotherapy, given that the majority of cells are resting in the G0 phase of the cell cycle. Conversely, in cases with high proliferation rates, treatment failure may be caused through regrowth or by the increasing likelihood of further mutations.

In addition to the proliferative activity, we also found that achievement of CR was an independent prognostic factor for LSS, as reported in previous studies, where obtaining CR led to prolonged survival for dogs with aggressive lymphoma.\textsuperscript{7,33,34} Stage and substage did not shown prognostic significance for LSS or RFI, in contrast with some authors\textsuperscript{8,35,36} but, in accordance with others.\textsuperscript{37,38} These differences may be because of the inclusion of different types of lymphoma, different therapeutic strategies and to the different methods and cut-offs used to stage the disease.

Major limits of this work are its retrospective nature and the limited number of cases. Prospective studies considering a large number of lymphomas are required to confirm the clinical usefulness of a Ki67-based stratification of patients.

In conclusion, flow cytometric determination of Ki67 was found to be an independent predictor for LSS in treated high grade B cell lymphomas; intermediate values were associated with the best
We previously demonstrated that this determination is useful in discriminating between low and high grade lymphomas. Thus, we suggest the introduction of Ki67 in the routine panel of labeling in order to add diagnostic and prognostic value to the flow cytometric analysis.

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