Flow cytometric evaluation of ki67 for the determination of malignancy grade in canine lymphoma

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Abstract

Ki67 is a nuclear antigen significantly correlated with degree of malignancy in human non-Hodgkin lymphomas. We wanted to assess the ability of flow cytometric evaluation of Ki67 index (Ki67I) in differentiating the grade of malignancy in canine lymphomas. Ki67I was determined on lymph node aspirates of 90 immunophenotyped lymphomas classified according to the updated Kiel classification: 80 high grade (HG, 62 B cell and 18 T cell) and 10 low grade (LG, 3 B cell and 7 T cell) lymphomas.

HG lymphomas showed significantly higher Ki67I compared with LG lymphomas ($P < 0.0001$). A significant difference in HG lymphomas was detected between B- and T-immunophenotypes.

Receiver operating characteristic (ROC) curve highlighted a high accuracy of Ki67I in recognizing HG lymphomas [area under the curve (AUC) = 99.4] and a cut-off value of 12.2% was established (sensitivity = 96.3% and specificity = 100%). Thus, we suggest the combination of Ki67I flow cytometric determination and immunophenotype as a reliable tool to classify canine lymphomas.

Keywords

dog, flow cytometry, Ki67, Kiel classification, lymphoma, malignancy grade

Introduction

Canine lymphoma represents a heterogeneous group of tumours characterized by clonal proliferation of neoplastic lymphoid cells with distinctive morphological and immunophenotypic features.

Lymphomas account for more than 20% of all canine neoplasms1 and approximately 83% of all haematopoietic tumours.2

The cytological evaluation of lymph node fine needle aspirates (FNAs) is generally accepted as a well-established tool for the diagnosis of canine lymphoma because it is simple, minimally invasive,

rapidly performed and inexpensive.3– 5

However, in the last years flow cytometric immunophenotyping emerged as a useful ancillary test to refine cytological diagnosis of canine lymphoma, contributing to the evaluation of tumour cell lineage, clonality, clinical staging and minimal residual disease evaluation.6– 9

In clinical practice canine lymphomas are mainly classified according to the updated Kiel classification that combines cytomorphological criteria with B- or T-immunophenotype allowing the recognition of HG and LG forms.4,10,11

The determination of the correct grading in canine lymphoma results fundamental to predict biological behaviour and response to therapy and it is useful to develop clinical treatment protocols.12,13 However, major limitations of the update Kiel classification are the difficulties in the correct identification of the different cytological subtypes and the detection of malignancy grade that requires high skills and experience in cytology.14 Moreover many criteria used for the morphological classification, such as percentage of neoplastic cells, cell size, pleomorphism or description of chromatin pattern depend on subjective interpretation, quality of the smears, quality of the staining and area of the slide examined. The use of a more objective and sensible parameter, in addiction to cytology, is needed to correctly define subtype and grading in canine lymphoma.

One of the most frequently used methods to determine the growth fraction of cell populations is the detection of proliferation markers such as the Ki67antigen.15 Ki67 is a non-histone nuclear protein expressed in all active phases of the cell cycle (G1, S, G2 and mitosis) and it is absent in resting cells (G0).16,17 Its use as a diagnostic and prognostic marker in various human malignancies has been well documented 18– 20 and a higher significant correlation has been found between the percentage of Ki67-positive cells [or Ki67 index (Ki67I)] and the histological grade of malignancy in non-Hodgkin lymphomas (NHL).21,22 Similarly, few studies high- lighted its correlation with tumour grade also in canine lymphomas.23– 25 All these studies evaluated Ki67I by immunohistochemistry. To the author's knowledge there are no reports on flow cytometric determination of Ki67I in canine lymphomas.

The aim of this study was to assess the ability of flow cytometric determination of Ki67I to discriminate between HG- and LG-lymphomas according to the updated Kiel classification. This could be a valid aid to refine cytological diagnosis, to increase reproducibility of grading procedures and to predict transfation from LG- to HG-lymphomas.

Materials and methods

Cases and specimens

Cases of lymphoma submitted to our laboratory for flow cytometric immunophenotyping between December 2011 and November 2012 were considered.

Inclusion criteria were presence of flow cytometric immunophenotyping, a conclusive cytological evaluation, flow cytometric determination of Ki67I, no administration of cortisone or chemotherapy before flow cytometric analyses.

Lymphoma was diagnosed based on clinical pre- sentation (lymph node enlargement), cytological examination and flow cytometric analysis.6

Flow cytometric immunophenotype and Ki67 determination

Immunophenotype was performed on lymph node fine-needle aspirates within 24 h from collection as previously reported.9 The following monoclonal antibodies were used: CD45-PE (clone YKIX716.13, Serotec, Oxford, UK), CD3-FITC (clone CA17.2A12, Serotec, T cells), CD5-FITC (clone YKIX322.3, Serotec, T cells), CD4-FITC (clone YKIX302.9, Serotec, T-helper), CD8-PE (clone YCATE55.9, Serotec, T-cytotoxic/suppressor), CD21-PE (clone CA21D6 Serotec, B cells), CD79a (clone HM57 Serotec, B cells) and CD34-PE (clone 1H6 Pharmingen, precursor cells).

Proliferative activity was determined on lymph node aspirates using a fixation and permeabilization method with methanol. Briefly, 1×10^6 cells were harvested; after a lysing step (BD lysing solution, Becton Dickinson, San Jose`, CA, USA) cells were labelled with antiKi67-FITC monoclonal antibody (clone MIB-1, DAKO, Glostrup, DK) using Leucoperm reagents A and B (Serotec) following manufacturer's instructions except for an additional intermediate incubation step in 500 μL of frozen absolute methanol. Afterwards the samples were washed in phosphate buffered saline (PBS), resuspended and immediately acquired on a BD Accuri C6 flow cytometer (Becton Dickinson). A minimum of 10 000 events were acquired both for immunophenotype and Ki67 determination. Data were analysed using CFlow Plus software (Becton Dickinson); proliferative activity was expressed as percentage of Ki67 positive cells (KI67I). A gate of analysis was depicted on FSC versus SSC scattergram in order to exclude debris and background and the percentage of positive cells was calculated on SSC versus fluorescence intensity.

Cytological evaluation

Smears obtained by multiple FNAs of enlarged lymph nodes were air-dried fixed and stained with May-Grunwald-Giemsa. All cases were classified and allocated to the LG or HG group according to the updated Kiel scheme.10,11

Statistical analysis

The data obtained were processed using SPSS software ver. 19 (SPSS, Chicago, IL, USA).

Comparison of Ki67I values between B- and T- lymphomas, HG- and LG-lymphomas and between the different cytological types according to the Kiel classification was calculated using Student t test or Kruskal–Wallis, depending on the distribution of data. Values of $P < 0.05$ were considered statistically significant.

Receiver operating characteristic (ROC) curves were plotted and the area under the curve (AUC) was calculated to evaluate the accuracy of Ki67I in discriminating HG and LG forms and to identify the cut-off value associated to the best sensitivity and specificity in detecting HG lymphomas.

Results

In the considered period, 104 cases of lymphoma were immunophenotyped by flow cytometry. In four cases there was no smear available for cytological evaluation and in three cases the quality of the preparation was inadequate to classify the tumour; flow cytometric determination of Ki67 was not carried out in seven cases (five of which were pre-treated with cortisone). Therefore, 90 cases were included in the study. Cytological and flow cytometric analyses reported the percentage of atypical cells of suspected neoplastic origin to range between 70 and 98%.

B- and T-cell lymphomas were 65 and 25, respec- tively; 80 were HG (62 B cell and 18 T cell) and 10 were LG (3 B cell and 7 T cell) lymphomas. B-cell lymphomas were classified as follows: cen- troblastic monomorphic (n = 13), centroblastic polymorphic predominantly large cell (n = 32), centroblastic polymorphic predominantly small cell (n = 2), immunoblastic (n = 10), lymphoblas- tic (n = 3), plasmacytoid (n = 2) and macronu- cleolated medium-sized (putative marginal zone) (n = 3). T-cell lymphomas were classified as fol- low: lymphoblastic (n = 4), pleomorphic large cell (n = 5), pleomorphic mixed (n = 9), pleomorphic small cell (n = 2) and small clear cell (n = 5). Ki67I values summarized by Kiel categories, phenotype and grade of malignancy are reported in Table 1.

Considering all the samples together, Ki67I mean percentage was 33.2% (SD = 18.6%) and the median was 29.1% (range 1.3 – 85.0%).

No significant difference between B (median 28.7%; range 3.3 – 85.0%) and T-cell (median

35.5%; range 1.3 – 60.3%) lymphomas was detected, while an extremely significant higher Ki67I was found in HG (median 34.4%; range 7.8 – 85.0%) compared with LG lymphomas (median 5.3%; range 1.3 – 11.4%) ($P < 0.0001$, Fig. 1). Within HG lymphomas, T-cell phenotype (median 45.0%; range 17.6 – 60.3%) presented significant higher val- ues of Ki67I than B-cell forms (median 29.3%; range 7.8 – 85.0%) ($P < 0.05$, Fig. 2) Among the HG lymphomas the pleomorphic large T cell (median 54.5%), the lymphoblastic T cell (median 43.8%), the centroblastic polymorphic large B cell (median 39.5%) and the centroblastic monomor- phic B cell (median 37.2%) lymphomas showed significantly higher Ki67I than immunoblastic B- cell lymphomas (median 18.2%), resulting in the less proliferating HG lymphoma (Fig. 3).

ROC curve analysis identified a high accuracy of Ki67I in discriminating between HG- and LG-lymphomas (AUC = 99.4%) and indicated a cut-off value of 12.2% to detect HG lymphomas with

a sensitivity of 96.3% and a specificity of 100% (Fig. 4).

Discussion

In this study flow cytometry was used for the first time to estimate the growth fraction of different types of canine lymphomas classified according to the updated Kiel classification adapted to the canine species.10

The evaluation of Ki67 is usually carried out through immunohistochemistry in bioptic specimens. However, lymphoma diagnosis and characterization in dogs is nowadays performed mostly through cytology and flow cytometric immunophenotyping using samples obtained by fine needle aspiration. Therefore, flow cytometric determination of Ki67 could be a useful tool to determine proliferative activity in the same sample used for immunophenotyping, thus avoiding a lymph node excision. The main advantages of flow cytometry over immunohistochemistry lie in its

ability to perform an objective and multiparamet- rical analysis in a relative short time and to use samples obtained by non-invasive technique .6– 8

In effect, in our study flow cytometric Ki67I was carried out using the same sample used for immunophenotype determination obtained by lymph node transcutaneous fine needle aspiration. Our results show an extremely significantly higher Ki67I in HG compared with LG lymphomas. Thus, flow cytometric Ki67I correlates with tumour grading, in accordance with previous immunohistochemical studies both in human26,27 and canine non-Hodgking lymphoma.23– 25

Our results indicate a cut-off of 12.2% to distinguish between LG- and HG-lymphomas. In a previous study performed on histologic sections Fournel-Fleury et al. reported a higher cut-off value (21%).23 This mismatch may be due to the different method used for the evaluation of Ki67 antigen expression (immunohistochemistry) and due to the to the difference in subtypes and number of lymphomas contributing to form the HG and LG groups. In particular, in the study by

Fournel-Fleury et al. four cases of mycosis fungoides were present showing the highest Ki67I values among the LG lymphomas and they are probably the reason for the higher cut-off reported in the study.

In accordance with other reports both in man21 and dog23,28 our study describes a wide range of Ki67 positivity in canine lymphomas with differences among categories and a certain heterogeneity within LG lymphomas, with T-cell lymphomas showing higher Ki67 levels. This heterogeneity opens the door to a possible role of Ki67 in subclassifying cases or stratifying prognostic groups within specific categories. Moreover the presence of HG lymphomas showing low Ki67I suggests a possible role of Ki67 determination in the early detection of lymphomas transforming from LG to HG forms. Further studies are needed to investigate these aspects. Further- more the lack of a group of non-neoplastic lymph nodes in our study does not allow to state if Ki67I is useful in the diagnosis of canine lymphomas in absence of an adequate sample to evaluate morphology. However, this is not within the aims of this article and for this purpose further study comparing Ki67I by flow cytometry in neoplastic versus non-neoplastic lymph nodes are needed.

In conclusion flow cytometric evaluation of Ki67I reflects the malignancy grade of canine lymphoma, regardless of phenotype and morphology. From our results flow cytometry appears as a powerful and non-invasive technique which is able to differentiate HG from LG lymphomas using a cut- off value of 12.2%. This could be useful to increase reproducibility of results, to solve diagnostic troubleshooting and to predict transformation of LG lymphoma in more aggressive forms.

Thus, we suggest combined flow cytometric determination of Ki67I and immunophenotype as a reliable tool to classify canine lymphomas in addition to cytology.

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Table 1. Ki67I values summarized by Kiel categories, phenotype and grade of malignancy

Grade of malignancy	Ki67I (%) median (range)	Phenotype	Ki67I (%) median (range)	Kiel classification	Ki67I (%) median (range)
High	34.4 (7.8–85.0)	B cell	29.3 (7.8–85.0)	Centroblastic monomorphic ($n = 13$)	37.2 (7.8–70.2)
				Centroblastic polymorphic large cell ($n = 32$)	39.5 (10.7–85.0)
				Centroblastic polymorphic small cell ($n = 2$)	22.5 (22.0–23.1)
				Immunoblastic ($n = 10$)	18.2 (10.0–26.7)
				Lymphoblastic ($n = 3$)	27.6 (27.0–39.6)
				Plasmacytoid ($n = 2$)	21.1 (13.0–29.2)
		T cell	45.0 (17.6–60.3)	Lymphoblastic ($n = 4$)	43.8 (28.9–58.1)
				Pleomorphic large cell ($n = 5$)	54.5 (43.2–60.3)
				Pleomorphic mixed cell ($n = 9$)	35.5 (17.6–54.0)
Low	5.3 (1.3–11.4)	B cell	6.9 (3.3–9.8)	Medium sized macronucleolated ($n = 3$)	6.9 (3.3–9.8)
		T cell	3.6 (1.3–11.4)	Pleomorphic small cell ($n = 2$)	9.4 (7.3–11.4)
				Small clear cell ($n = 5$)	3.3 (1.3–8.4)

Figure 1. Flow cytometric determination of Ki67I in HG and LG lymphomas (P < 0.0001).

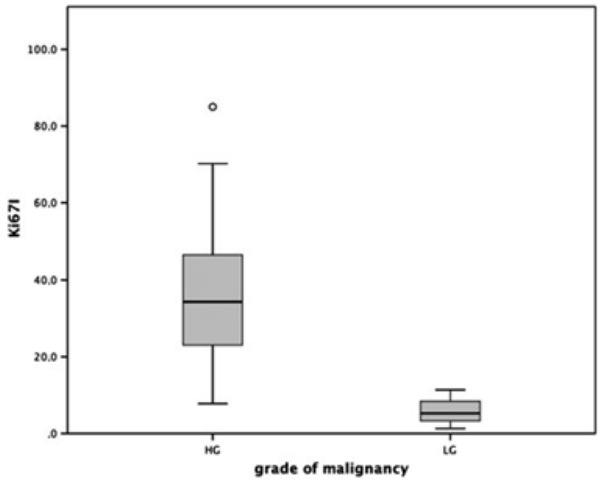

Figure 2. Flow cytometric determination of Ki67I in B- and T-cell high grade lymphomas ($P < 0.05$).

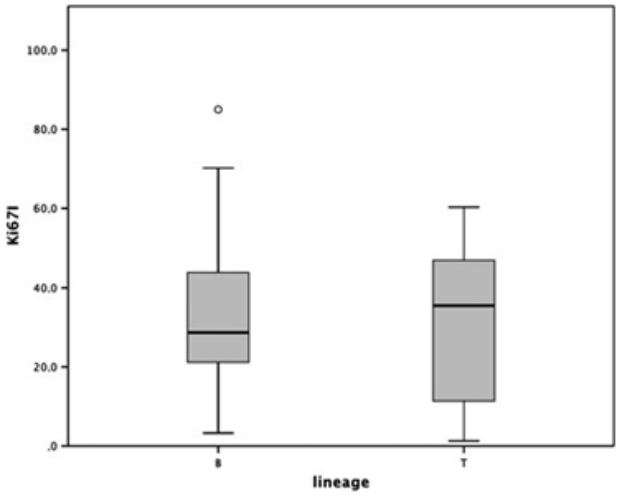

Figure 3. Flow cytometric determination of Ki67I in different types of lymphoma classified according the updated Kiel classification. Median, minimum and maximum values are represented. CM, centroblastic monomorphic lymphoma; CPPL, centroblastic polymorphic predominantly large cell lymphoma; CPPS, centroblastic polymorphic predominantly small cell lymphoma; IB, immunoblastic lymphoma; LB, lymphoblastic lymphoma; MMC, macronucleolated medium-sized; PC, plasmocytoid lymphoma; PL, pleomorphic large cell lymphoma; PM, pleomorphic mixed cell lymphoma; PSC, pleomorphic small cell lymphoma; SC, small clear cell lymphoma.

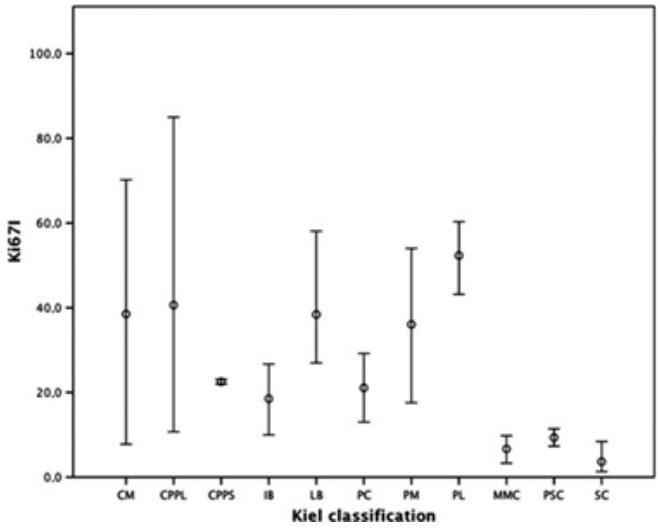

Figure 4. ROC curve analysis for the identification of a cut-off value for Ki67I to discriminate between low and high grade lymphomas.