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Class II Major Histocompatibility Complex Expression and Cell Size Independently Predict Survival in Canine B-Cell Lymphoma

S. Rao, S. Lana, J. Eickhoff, E. Marcus, P.R. Avery, P.S. Morley, and A.C. Avery

Background: Class II major histocompatibility complex (MHC) is an independent predictor of outcome in human B-cell lymphoma. We assessed class II expression together with other markers for their impact on prognosis in canine B-cell lymphoma.

Hypothesis: Low class II MHC expression, large cell size, and expression of CD34 will predict a poorer outcome in canine B-cell lymphoma. Expression of CD5 and CD21 on tumor cells also may be associated with outcome.

Animals: One hundred and sixty dogs with cytologically confirmed lymphoma.

Methods: Patient signalment, treatment type, and flow cytometry characteristics were analyzed for their influence on outcome. A multivariable predictive model of survival was generated using 2/3 of the patients and validated on the remaining 1/3 of the dataset.

Results: Class II MHC expression had a negative association with mortality and relapse. Treatment type also influenced relapse and mortality, whereas cell size and patient age was only associated with mortality. CD34, CD21, and CD5 expression was not associated with disease outcome. The constructed model performed variably in predicting the validation group's outcome at the 6-month time point.

Conclusions and Clinical Importance: Low levels of class II MHC expression on B-cell lymphoma predict a poor outcome, as in human B-cell lymphoma. This finding has implications for the use of dogs to model human lymphomas. Class II expression, cell size, treatment, and age can be combined to predict mortality with a high level of specificity.

Key words: CD5; CD21; CD34; Immunophenotyping.

Canine lymphoma is a heterogeneous disease with a broad range of potential outcomes, from rapid clinical decline to a long, indolent disease course lasting more than a year. Features that reliably predict survival times (STs) are clinical stage, clinical substage, and immunophenotype,[1–5] where B-cell lymphoma has been shown to have a better prognosis than T-cell lymphoma. Other outcome predictors, less commonly described, include the presence of anemia,[6] histologic classification,[3,5] AgNor staining characteristics,[7] and survivin expression.[8]

Flow cytometry is now routinely used to determine the phenotype of lymphoma and leukemia,[9–11] and also to aid in the diagnosis of ambiguous cases. Flow cytometric studies provide objective data about cell size and level of antigen expression, which is used in human medicine to further subclassify lymphomas and leukemias into clinically relevant subcategories. In the present study, we asked if flow cytometric analysis of canine lymphoma, combined with signalment and treatment data, can provide information in addition to immunophenotype that would be prognostically useful.

One of the most consistent predictors of poor outcome in human B-cell lymphoma is low class II major histocompatibility complex (MHC) expression.[12–15] Decreased class II MHC expression is independent of other factors, including international prognostic index and histologic subtype of lymphoma.[12,14] Although the reason for poor outcome in these patients is not clear, several high quality gene expression profile studies have shown that the nature of the immune response to the tumor is prognostically more important than the tumor itself.[16,17] Thus, one explanation for decreased survival in patients with low class II expressing lymphomas is decreased presentation of tumor antigen to CD4 T cells, and a less effective host immune response. We tested the hypothesis that low class II expression also would be associated with a poor outcome in canine B-cell lymphoma.

A previous study from our laboratory showed that in dogs with peripheral B-cell lymphocytosis, representing either Stage V lymphoma or lymphocytic leukemia, cases with larger circulating B cells carried a worse prognosis than those with smaller B cells.[11] Therefore, in this study we hypothesized that B-cell lymphomas characterized by large cells would have a poor prognosis.

CD34 is a generally accepted marker of acute leukemia, and although CD34 expression on lymphomas has been reported,[9] its prognostic significance is not clear. In our previous study,[11] we found that lymphocytosis cases with expression of hematopoietic stem cell

Abbreviations:

CHOP	cyclophosphamide, doxorubricin, vincristine, prednisone
FBS	fetal bovine serum
FRT	first remission time
MFI	median fluorescence intensity
MHC	major histocompatibility complex
ST	survival time

From the Department of Clinical Sciences (Rao, Lana, Morley), the Flint Animal Cancer Center (Lana), the Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences (Marcus, P.R. Avery, A.C. Avery), and the Department of Statistics, College of Natural Sciences, Colorado State University, Fort Collins, CO (Eickhoff).

Corresponding author: A.C. Avery, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, 200 West Lake Street, Fort Collins, CO 80523; e-mail: Anne.avery@colostate.edu

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marker CD34 had the poorest outcome with a 16-day median ST. Here we asked if CD34 expression, which we detect on approximately 10% of B-cell lymphomas, would also be associated with a poor outcome.

As our understanding of the complex nature of canine lymphoma expands, we continue to identify factors that impact outcome, such as phenotype and type of treatment. Multivariable statistical models are useful for examining the interactions between these factors, but they also can be used to make specific predictions about outcome in individual patients by taking into account a specific constellation of variables in a particular patient. Here we create a statistical model to predict outcome in patients with different combinations of variables, and we test how well our model performs when used on a test group of patients with known clinical outcomes.

Materials and Methods

Study Subjects

Patients were enrolled in 2 phases. In the 1st phase, patients were selected from all dogs presented to the Veterinary Teaching Hospital at Colorado State University (CSU-VTH) between January 1, 2006 and April 30, 2009 on which flow cytometry of a lymph node aspirate was performed. Inclusion criteria were (1) the flow cytometry panel revealed that >60% of the large cells in the fine needle aspirate expressed B-cell marker CD21, (2) the patient had a definitive cytologic (133 cases) or histologic (28 cases) diagnosis of lymphoma as assessed by board-certified clinical or anatomic pathologists at one of several reference laboratories, and (3) there was no treatment for lymphoma before diagnosis but the dog subsequently underwent some form of chemotherapy. Dogs in our final group of patients were treated with either a multiagent, CHOP-based protocol (cyclophosphamide, doxorubricin, vincristine, prednisone), single agent doxorubricin (with or without prednisone),[18] or prednisone alone. Eighty-one patients met these criteria. Of these, 66 dogs had information about treatment and at least 1 month of follow-up, and they entered the study under enrollment phase 1.

In this 1st enrollment, only 1 dog had a CD34+ lymphoma. Therefore, a 2nd enrollment phase was conducted to increase the number of dogs with CD34+ lymphomas so that we could test the hypothesis that CD34 expression is associated with outcome. During the same time period (January 1, 2006–April 30, 2009) lymph node aspirates from 498 unique patients with B-cell lymphoma were submitted from clinics around the country. Of these, 70 expressed CD34. For each CD34+ dog in enrollment phase 2, a control CD34− B-cell lymphoma case was enrolled from the same submitting hospital as long as both dogs had been diagnosed within 4 months of each other. The 4-month time frame was chosen because it was the shortest time in which we could reasonably expect to obtain a 2nd B-cell lymphoma from the same clinic. We were able to identify 45 CD34+ cases with a CD34− B-cell lymphoma submitted from the same clinic. Three of these cases had an additional B-cell lymphoma submitted from the same clinic, and information was sought on all qualifying cases. Thus, the 2nd enrollment included 45 CD34+ cases and 48 CD34− controls with adequate information about treatment and at least 1 month of follow-up after initial diagnosis. In our final analysis, we found that the enrollment phase did not impact results of the study. Therefore, the 2 enrollment phases were combined and reanalyzed. The results presented here are for all patients considered together.

Flow Cytometry

Fine needle lymph node aspirates were performed on a peripheral lymph node by the attending clinicians. The aspirates were transferred into media consisting of RPMI 1,640 with 5% fetal bovine serum (FBS) if submitted at CSU-VTH. Referring hospitals transferred the aspirate into a sterile red-topped tube containing a mixture of 0.9 mL physiologic saline and 0.1 mL canine serum (either from the patient or from another dog). Samples were express shipped with cooling packs, and flow cytometry was conducted within 72 hours of collection.

Flow cytometry was carried out as described by Lana et al.[19] Briefly, samples were pelleted and washed twice with an erythrocyte lysis buffer, then resuspended in 300 μL phosphate-buffered saline (PBS) with 2% FBS. Twenty-five microliters of the final cell suspension was incubated with 25 μL of antibody mixture as listed in Table 1. All antibodies were purchased from AbD-Serotec, and were directly conjugated to fluorescein isothiocyanate or phycoerythrin. After a 20-minute incubation at room temperature, the samples were washed twice in PBS-2% FBS, resuspended, and 0.01% propidium iodide was added to exclude dead cells. Samples then were immediately analyzed on a Coulter XL flow cytometer. When possible, 5,000 cells in the lymphocyte gate were collected after dead cell exclusion. As a guideline to determine the percentage of cells stained with each antibody, gates were set based on the isotype controls (supplied by the same manufacturer) such that < 2% of cells were positive.

Data analysis were carried out with FCS Express.[a] Size classification was based on the forward light scatter of CD21 gated cells measured on a linear scale. Cells from cases of B-cell lymphoma in the study were larger than peripheral blood lymphocytes analyzed on the same day (generally from a different patient), and larger than CD5+ T cells in the same node. Peripheral blood lymphocytes were prepared using erythrocyte lysis (the same method used for isolating cells from lymph node aspirates) as described by Williams et al.[11] Therefore, we classified these cells as either medium or large lymphocytes. Cases in which CD21+ lymphocytes had a median forward scatter >720 U were assigned to cell size category "large,"

Table 1. Antibodies used for flow cytometry analysis.

Tube Number	FITC-Labeled Antibody	Clone	PE-Labeled Antibody	Clone
1	No antibody		No antibody	
2	MIgG1	Cat# MCA928A	CD45	YKIX16.13
3	CD18	YFC118.3	MIgG1	Cat# MCA928A
4	CD8	YCATE55.9	CD4	YKIX302.9
5	CD5	YKIX322.3	CD21	CA2.1D6
6	CD3	CA17.2A12	CD45	YKIX16.13
7	CD4	YKIX302.9	CD14	UCHM1
8	MHC class II	YKIX334.2	CD34	1H6

MHC, major histocompatibility complex; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

whereas the remaining cases were categorized as "medium." In order to translate this size value to other flow cytometers, cells classified as "large" had a median forward scatter value $>1.6\times$ the size of the CD5+ T cells detected in the same lymph node and the same staining tube.

The expression of a given antigen in human lymphoma and leukemia is reported as "dim or low," "bright or high," or "partially expressed."[20] These adjectives are assigned based on comparison with the antigen's expression on the normal cellular counterpart and an isotype control. Accordingly, for this study we retrospectively assigned a patient's class II MHC expression as class II MHC low or class II MHC high if the neoplastic population had a median fluorescence intensity (MFI) below 226 U on a log scale. In order to translate this for use in other laboratories, this cutoff represented the bottom 15th percentile of all class II MFI for all patients in the study. For comparison, normal lymphocytes usually have an MFI between 400 and 600 U on our flow cytometer. We acknowledge that this cutoff value is likely to be more subjective when carried out in real time on clinical samples, and some patients will fall into a gray area. For similar statistical comparison, we also assigned cutoff values for MFI expression for CD5 and CD21 expression on neoplastic B cells, but neither of these variables showed statistical significance and were eliminated early in the analysis. CD34 expression, unlike class II MHC expression, was a dichotomous rather than a continuous variable. Therefore, cases in which $>5\%$ of B cells stained for CD34 above background isotype control were considered CD34+.

Data Analysis

For all data, descriptive statistics were calculated and variables summarized graphically. Two time-to-failure outcomes were investigated: ST and first remission time (FRT). ST was the time interval postonset of treatment to patient death, and FRT was the time interval from initiation of treatment to the date of first confirmed recurrence of cancer, as measured by recurrence of lymphadenopathy or extranodal mass assessed by cytological examination.

The independent variables evaluated in this study were age ($\leq$7 years, $>$7 years), sex (M, F), treatment type (prednisone only, single-agent doxorubricin [plus or minus prednisone], or multiagent [CHOP-based regimen]), cell size (large, medium), CD34 expression (positive, negative), MHC II expression (high, low), CD5 expression (high, low), and CD21 expression (high, low). Only 2 breeds (Golden Retrievers and Labrador Retrievers) had sufficient cases to be analyzed separately. The remaining breeds were grouped into the "other" category, and dogs designated as mixed or cross-breed comprised the 4th category.

Data from patients enrolled in the different phases were compared statistically (group versus ST, FRT, age, sex, and MHC II expression) by univariable Cox proportional hazards regression and chi-square tests. Significant differences were not identified among groups and we therefore elected to combine data from all enrollment phases for further analyses.

Cox proportional hazards regression analysis was used to investigate factors related to 2 outcomes (ST and FRT). Patients were censored if they had been lost to follow-up, died of conditions other than lymphoma, or were still alive at the end of the follow-up period. In order to assess the predictive ability of models, we used a split-sample approach to analysis so that reliability of the final multivariable models could be assessed using a separate subset of the data.[21] From all 160 observations, 2/3 of the observations (n = 106) were randomly selected for inclusion in the model building subset, and the remaining 1/3 (n = 54) were reserved for evaluation and validation of the final multivariable models. MS Excel 2007 was used for randomization.

Using the model-building subset, Kaplan-Meier survival curves were evaluated for ST and FRT relative to all independent variables. Independent variables also were evaluated in univariable Cox proportional hazards models using both outcome variables, and the proportionality assumption was evaluated. Variables with univariable P-values $<.25$ then were passed into the multivariable model building. Backward selection was used to determine final multivariable models using a critical α for retention of 0.05. Previously excluded variables were reintroduced to final models to ensure that exclusion was appropriate. If these variables had Type III P-values $<.10$ and had large HR suggesting there was a strong association with the outcome, they were retained in the multivariable models. Additionally, confounding was identified by $>20\%$ changes in parameter estimates when variables were individually removed from multivariable models; when identified, confounding variables were forced into multivariable models regardless of P-values. First-order interaction terms then were screened individually for all main effects that were included in the final models. Hazard ratios and 95% confidence interval (CI) were calculated from model results.

Adjusted ST Curves

Using the final multivariable model for ST, data from the modeling subgroup were used to develop ST curves that were adjusted for effects of other covariates included in the final model, stratified by the variables of interest (MHC class II expression and cell size).[22] Briefly, the statistical analysis software was used to calculate and output the predicted survival function estimates for each subject (PROC PHREG in by the BASELINE OUT statement; code available upon request). Subsequently, survival function estimates were calculated after adjusting for the covariates. These estimates were used to create adjusted curves for class II MHC and cell size.

Survival Model Validation

After multivariable models were finalized for ST and FRT, the validation subset was used to evaluate their predictive reliability. Using the model building subset, the baseline hazard (ie, the hazard probability when no covariates are present) of dying and first cancer recurrence were estimated by the statistical software for a 180-day time period after initiating treatment. The 180-day time point was chosen because most CHOP-based protocols last 6 months, and we wished to assess the likelihood of an event (death or relapse) within this time frame. This baseline hazard probability was adjusted for the covariate pattern of each observation in the validation subset using the regression parameter estimates, which provided an adjusted probability of events (mortality and cancer recurrence). For individuals with predictive probability estimates $\leq$0.50, it was interpreted that the model predicted that the event would not have occurred by 180 days, whereas it was interpreted that the model had predicted the event to have occurred if predictive probabilities were >0.50. These predicted outcomes then were compared with the actual patient outcomes and summarized by calculating the sensitivity and specificity and their associated 95% CI.

Results

Population Characteristics

One hundred and sixty dogs were entered into the study. The characteristics of the population are described in Table 2. In order to both build a statistical model to predict prognosis and then test the accuracy of the model, we randomly chose 2/3 of the population (106 dogs) to build our model, and used the remaining 1/3 to validate it. There was no statistical difference in the proportion of animals in each predictive category between the model building and the validation groups.

Table 2. Characteristics of the patients in this study.

Variable[a]	Category	Total Dogs	# in Modeling Group	%	# in Validation Group	%
Age[b]	$\leq$ 7 years	57	36	35	21	39
	$>$ 7 years	100	67	65	33	61
Sex	Female	64	38	36	26	48
	Male	96	68	64	28	52
Treatment	Prednisone only	12	7	7	5	9
	Single agent	35	27	25	8	15
	Multiagent	113	72	68	41	76
Cell size	Medium	149	99	93	50	93
	Large	11	7	7	4	7
CD34	Negative	113	78	74	35	66
	Positive	46	28	26	18	34
MHC class II	High	138	91	86	47	89
	Low	21	15	14	6	11
Total		160	106		54	

MHC, major histocompatibility complex.

[a]Not all information was available for all patients. There were 3 patients for which age was not available. In 1 patient, information about CD34 and class II expression was not available.

[b]Median age of all dogs in the modeling group was 8.5 years (range 3.1–15.6) and in the validation group was 8 years (range 3.8–16.8).

Most dogs were $>$7 years old, male, and treated with multiagent therapy. The most common phenotype of B-cell lymphoma was medium sized, CD34 negative cells that expressed high levels of class II MHC.

CD34 Expression Is Dichotomous and Not Associated with Outcome

CD34 was expressed on a subset of cells in 46 cases of lymphoma. Seven cases had between 5 and 20% CD34 expression, and the remaining cases had between 20 and 95% CD34 positive cells. Expression of this antigen was dichotomous. That is, cells were easily divided into positive and negative (Fig 1), rather than exhibiting a range of expression as is seen with class II MHC in the majority of lymphomas (eg, Fig 2B). Univariable analysis demonstrated no difference in outcomes between lymphomas with CD34 expression and lymphomas without CD34 expression.

Class II MHC Expression and Cell Size Are Strong Predictors of Outcome in B-Cell Lymphoma

Univariable analysis was carried out on the model building group to determine which clinical variables impacted outcome (Table 3). Three variables had *P*-values $<$ .25 when analyzed in univariable models. For ST, these were treatment type, MHC class II category, and cell size category. For FRT, 4 variables passed univariable screening: treatment type, MHC class II category, cell size category, and sex (Table 2). As noted above, CD34 expression did not predict survival or remission, nor did the levels of CD5 and CD21. These variables were not included in the final analysis.

We then carried out multivariable analysis on the modeling group. Variables included in the final model for ST were treatment type, MHC class II expression, age, and cell size. Results of the analysis are shown in Table 4. Figure 2 shows examples of lymphomas that differ in cell size and class II MHC expression. Class II MHC expression proved to be a strong predictor of outcome. Controlling for differences in other factors in the multivariable model, patients with low MHC class II expression were 2.9 times more likely to die (95% CI = 1.4–5.9) in any time period compared with those with high expression. The adjusted median ST for dogs with low MHC class II expression was 120 days compared with 314 days for dogs with high MHC II expression (Fig 3). Our results are consistent with studies of human B-cell lymphoma patients, where low class II MHC is consistently found to be a poor prognostic indicator.

Lymphomas in which the tumor cells were very large (as assessed by forward light scatter) also had a significantly poorer outcome. Patients with large tumor cells were 2.8 times more likely to die (95% CI = 1.0–7.5) in any time period compared with those with small tumor cells. Dogs with large cells had an adjusted median survival of 155 days, whereas those with smaller cells had an adjusted median survival of 275 days (Fig 3).

Age and therapy also significant prognostic factors in the model. Younger dogs and dogs treated with prednisone only were more likely to die than older dogs and dogs treated with single agent or multidrug therapy.

Taken together, our data indicate that B-cell lymphomas can be subdivided by class II expression and size into clinically relevant prognostic groups. Our findings show that some subgroups of B-cell lymphoma have median ST similar to T-cell lymphomas reported in previously published studies.[4]

Multivariable Modeling Allows for Prediction of STs in Individual Patients

In order to determine the accuracy of our statistical model, we then asked how well the model predicted outcome in the remaining 54 cases (the validation group). Predictions of mortality probabilities at 6 months for each combination of variables are shown in Table 5.

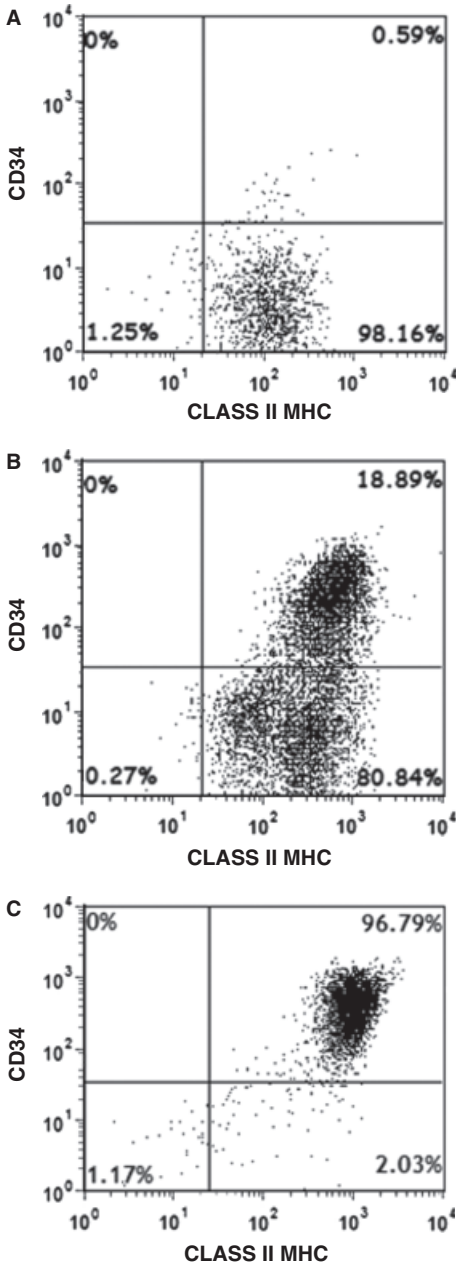

Fig 1. Examples of 3 different patterns of major histocompatibility complex (MHC) class II expression. (**A**) B-cell lymphoma that does not express CD34. (**B**) B-cell lymphoma in which approximately 20% of the cells express CD34. (**C**) B-cell lymphoma in which all tumor cells express CD34. Quadrants are set based on isotype controls such that there is 2% or less staining in an isotype-labeled quadrant. Tube 2 (Table 1) is used to set the vertical parameter (fluorescein isothiocyanate isotype control), and tube 3 to set the horizontal parameter (phycoerythrin isotype control).

These values then were compared with the observed outcomes in the validation subset. Considering all patients in the validation subset that survived for 180 day after initial treatment (n = 16), 88% (14/16; 95% CI = 60–98%) were correctly predicted to survive (mortality probability ≤ 0.50) by the multivariable model. For patients with uncensored ST that died before 180 days (n = 17), 42% (7/17; 95% CI = 19–67%) had predicted mortality probabilities >0.50 (not predicted to survive). The remaining dogs in the validation group were censored before 180 days.

Patients receiving multidrug therapy regimens then were evaluated separately. In multidrug treated animals, 93% of patients that survived for 180 days (14/15; 95% CI = 66–97%) were correctly predicted by the multivariable model. For patients with uncensored STs that received multidrug treatment regimens and died before 180 days (n = 9), the model correctly predicted this outcome for 22% of patients (2/9; 95% CI = 4–59%). Regarding FRT in patients that did not have a relapse before 180 days (n = 16), 94% (15/16; 95% CI = 68–99%) had predicted probabilities for first relapse of ≤ 0.50. In patients that relapsed before 180 days (n = 16), 25% (4/16; 95% CI = 8–53%) had predicted probabilities for relapse of >0.50.

Discussion

The most important findings of this study are (1) class II MHC expression on B-cell lymphomas influences outcome, a finding that closely parallels studies in humans; (2) large cell size as measured by light scatter also predicts outcome; (3) CD34 expression on B-cell lymphomas does not have any negative prognostic significance; and (4) by creating and validating a predictive model of death and survival using multiple variables, we can provide prognostic information to clinicians using data that are collected as part of routine immunophenotyping, as well as provide an estimate of the quality of that prognostic information.

The most common phenotype of B-cell lymphoma was class II high with medium-sized cells. The adjusted median STs, which incorporate all other variables, are 314 days for dogs with high class II MHC, and 275 days for dogs with medium-sized cells. This is in contrast to the minority subset of class II MHC low expressing lymphomas that were associated with an adjusted median ST of 120 days, and very large cells, which had an adjusted ST of 150 days. The largest previous study of overall ST in dogs treated with multiagent chemotherapy reported that ST for all B-cell lymphomas 330 days,[4] similar to our class II MHC high patients or medium-cell sized patients. Our study therefore highlights the fact that B-cell lymphoma is a heterogeneous disorder that can be placed into clinically relevant subcategories based on additional phenotypic information. Rebhun et al[8] made a similar observation when they divided B-cell lymphomas into high and low survivin expressers, in which dogs with high levels of survivin (detected by immunohistochemistry on biopsy sections) had an overall FRT time of 171 days, and low levels of survivin had an FRT of 321 days. Ponce et al[3] demonstrated that median ST of B-cell lymphoma could range from 15 days (for Burkitt-like lymphoma) to 510 days, depending upon the histologic classification of the tumor. Thus, although a B-cell phenotype frequently is considered a good prognostic indicator, together these studies show that some subsets of B-cell lymphoma are

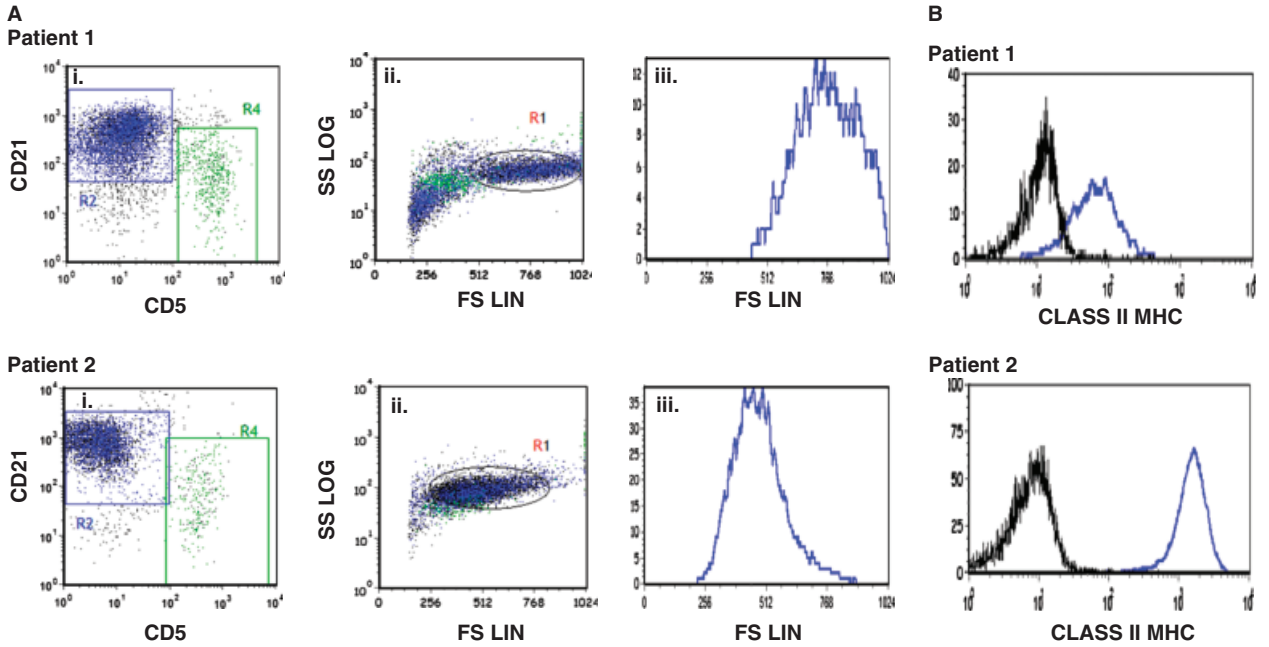

Fig 2. Determination of size and class II expression levels on tumor cells from 2 different patients. Patient 1 was a 9-year-old male neutered Rottweiler with large cells and low class II major histocompatibility complex (MHC) expression. Patient 2 was a 9-year-old male neutered mixed breed dog with medium-sized cells and high class II MHC expression. (**A**) Determination of median cell size. To determine cell size, a blue color gate was drawn around B cells (histogram i, gate R2; the green gate is drawn around T cells to show the resident lymphocyte population). This allowed us to identify the lymphoma population in the size and scatter gate (histogram ii, gate R1). Histogram iii shows only those cells which were found in gates R1 and R2. Cell size was determined as the median forward scatter value of the cells in histogram iii. Cells from patient 1 had a median forward scatter of 770, whereas cells from patient 2 had a median forward scatter of 481. (**B**) Determination of class II MHC expression. The level of class II MHC expressed by lymphomas cells (cells within gate R1) is shown (blue histograms) together with the isotype control (black histogram). Patient 1 had low levels of class II MHC, with a median fluorescence of 62, whereas patient 2 had a high level of class II MHC with a median fluorescence of 1,567.

associated with median survivals that are as poor as those reported for T-cell lymphoma.

We consider the most important finding of this study to be the association between low class II MHC levels and poor survival because this finding has implications for the growing interest in the study of tumor vaccines in people and dogs. Class II MHC expression was the strongest predictor of outcome, and thus it parallels findings in people with diffuse large B-cell lymphoma.[12–15] Similar to our findings, human B-cell lymphoma with low class II MHC expression comprised only a small subset of B-cell lymphomas, and that group of patients had significantly poorer outcomes. Class II expression did not correlate with any histologic subtype of diffuse large B-cell lymphoma in people, but rather was an independent predictor of outcome.

Current evidence indicates that down-regulation of class II MHC is likely because of decreased expression of its principal transcription factor, class II transactivator.[23] It is not clear if low class II expression is indicative of other transcriptional changes that directly impact the ability of tumor cells to proliferate and survive, or if the decrease in class II expression diminishes the ability of these cells to present antigen to CD4 T cells, thereby inhibiting immunosurveillance. These mechanisms are not mutually exclusive, but several gene expression profiling studies of human B-cell lymphomas have shown that the response to the tumor is a more important prognostic factor than the nature of the tumor itself.[16,17] Thus, our findings suggest that additional studies of the T-cell immune response in canine B-cell lymphoma are warranted, and helps strengthen the dog as a model of human B-cell lymphoma.

During the course of routine immunophenotyping, we noted that approximately 10% of B-cell lymphomas had a CD34+ component, a finding also described by Gelain et al.[9] The consensus view is that the presence of CD34+ cells in the peripheral blood is diagnostic for acute leukemia[24,25] and carries a poor prognosis,[11] and we therefore hypothesized that the expression of CD34 on B-cell lymphomas would predict a poor clinical outcome as well. We were surprised to find that CD34 expression had no influence on patient outcome in this study, and we conclude that CD34+ B-cell lymphomas are a different category of lymphoproliferative disorder than CD34+ leukemias. Although our study raises the possibility that CD34+ B-cell lymphomas with a leukemic component might be misdiagnosed as acute leukemia based on immunophenotyping, we feel this is unlikely. CD34+ acute leukemias are uniformly class II MHC negative (unpublished data), and do not express CD21, whereas the CD34+ lymphomas in this study were all class II MHC positive (an example is depicted in Fig 1), and all expressed CD21. In our experience, CD34+ class II MHC+ cells are rarely found in peripheral blood. Furthermore, the cytologic appearance and clinical

Table 3. Univariable analysis of the modeling group (106 dogs).

			Survival Time (ST) (Probability of Mortality)			Remission Time (FRT) (Probability of Recurrence)		
Variable	Category	N	Hazard Ratio	95% CI	Type 3 *P*-Value	Hazard Ratio	95% CI	Type 3 *P*-Value
Age (years)[a]	≤ 7	36	Reference		.32	Reference		.57
	> 7	67	0.78	0.48–1.27		0.86	0.51–1.44	
Sex	Female	38	Reference		.77	Reference		.22
	Male	68	0.93	0.56–1.53		0.724	0.43–1.22	
Breed[b]	Other	60	Reference		.84	Reference		.97
	GLDR	16	0.83	0.41–1.68		1.01	0.5–2.05	
	LAB	12	0.76	0.35–1.66		1.11	0.49–2.53	
	MIX	17	1.09	0.56–2.12		1.17	0.6–2.28	
Treatment	P	7	Reference		.055	Reference		.0063
	S	27	0.34	0.13–0.9			0.228	.08–0.65
	M	72	0.35	0.15–0.84		0.204	0.08–0.54	
MHC class II	High	91	Reference		.011	Reference		.0064
	Low	15	2.30	1.22–4.34		3.13	1.38–7.1	
CD34	Negative	78	Reference		.91	Reference		.63
	Positive	28	0.97	0.57–1.65		0.86	0.47–1.59	
Cell size	Medium	99	Reference		.097	Reference		.23
	Large	7	2.19	0.87–5.55		1.75	0.7–4.39	
CD21	Low	18	Reference		.69	Reference		.97
	High	88	1.15	0.59–2.25		0.99	0.52–1.87	
CD5	Low	92	Reference		.67	Reference		.35
	High	14	0.86	0.42–1.73		0.67	0.29–1.55	

MHC, major histocompatibility complex.

[a]Ages were not available for 3 animals.

[b]"Other" refers to all dogs for which a breed was indicated. "Mix" refers to all dogs where the breed was indicated as mixed or cross (eg, Lab-cross). No breed was indicated for 1 dog.

presentation of acute leukemias generally make their diagnosis straightforward.

Cell size was found to be a significant predictor of ST but not FRT. After controlling for other variables in the analysis, the dogs in the large cell size category showed a 2.8 times higher probability of mortality at any given time point compared with dogs in the other size category. We speculate that these very large B lymphocytes might correspond to a more immature cell type that has a more aggressive neoplastic behavior. We currently are working to correlate the flow cytometric, cytologic, and histologic classification of cells in this group, with the hypothesis being that these cases represent a distinct morphologic subtype of B-cell lymphoma.

In addition to class II MHC expression and cell size, the multivariable analysis found age to be prognostic, with younger dogs having a poorer outcome. To our knowledge, this is the 1st report in which age has been identified as a prognostic factor in lymphoma. The most likely explanation is that as lymphoma is categorized into

Table 4. Multivariable analysis of patients in the modeling group (106 patients).

Variable	Category	Hazard Ratio	95% CI	Type 3 *P*-Value
Survival time model				
MHC class II	High	Reference		.005
	Low	2.87	1.39–5.93	
Treatment	Prednisone only	Reference		.009
	Single agent	0.3	0.11–0.79	
	Multiagent	0.24	0.09–0.59	
Age	≤ 7 years	Reference		.029
	> 7 years	0.55	0.32–0.94	
Cell size	Medium	Reference		.046
	Large	2.77	1.02–7.54	
First remission time model				
MHC class II	High	Reference		.004
	Low	3.49	1.51–8.08	
Treatment	Prednisone only	Reference		.004
	Single agent	0.23	0.08–0.64	
	Multiagent	0.18	0.07–0.5	

MHC, major histocompatibility complex.

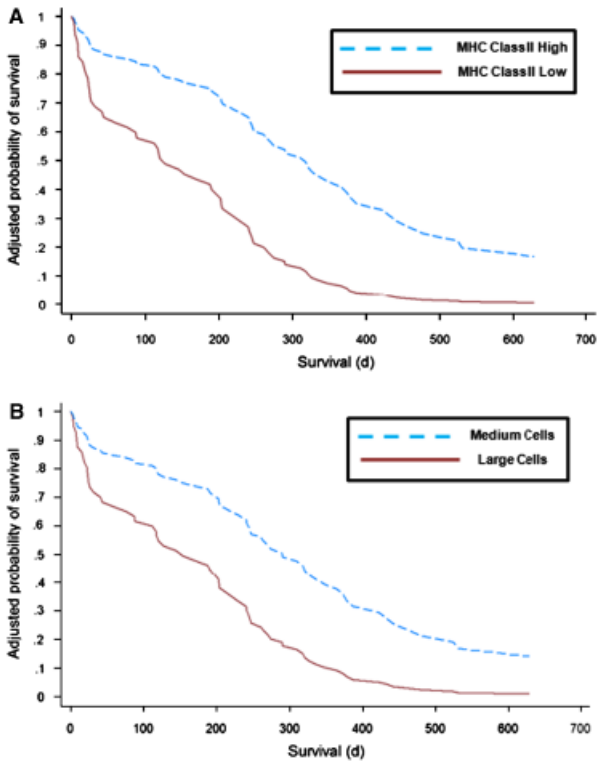

Fig 3. Adjusted survival curves for patients with class II major histocompatibility complex (MHC) low lymphomas (solid line) and class II MHC high lymphomas (broken line). The data are adjusted survival curves calculated using the modeling group (104 patients). (**A**) Adjusted survival curves showing decreased survival in cases with low levels of class II MHC. (**B**) Adjusted survival curves showing decreased survival in cases with large cells.

increasingly homogeneous subgroups, other factors such as age emerge as clinically relevant.

One goal of this study was to develop clinically relevant prognostic information using data obtained by routine flow cytometry. We therefore created a statistical model that used the significant prognostic factors (age, class II MHC, size of cells, and treatment) to predict survival and remission times. The model predicts, for example, that older dogs with high levels of class II MHC and medium-sized cells have a 0.17 (95% CI 0.08–0.26) probability of dying before 6 months when treated with a multidrug protocol —the category of patients with the best prognosis. Comparing model predictions to the actual outcome in a limited number of observations from the data validation subset, we found that this model was very specific for predicting death (93% specific) but not sensitive (22%). Therefore among all prediction errors, model estimates regarding the probability of death was underestimated for some animals. On the other hand, patients with low class II expressing cells, which are large, have between 0.78 and 0.94 probability of dying by 6 months, depending on age. These predictions have broad CIs (Table 5) because they are based on fewer patients than the good prognosis categories. However, because the model is specific for predicting death but not sensitive, it is likely that the error will be an underestimate of mortality, and those dogs with low class II MHC, large cells, or both will have a generally poor prognosis. With larger datasets, it will be useful to create models that incorporate additional clinical variables that can be measured simply and objectively. The use of quality of life outcomes rather than euthanasia or death would help eliminate variability in outcome because of non-disease-related issues (such as owner income). This in turn will allow clinicians to identify patients with more uniform and accurate prognosis that can be targeted for clinical trials.

Table 5. Probability of mortality at 6 months based on the model generated with the prediction group (106 dogs).

Class II Expression	Size	Age	Probability of Mortality 180 Days	95% CI
High	Medium	≤ 7	0.3	0.16–0.41
		> 7	0.17	0.08–0.26
	Large	≤ 7	0.62	0–0.87
		> 7	0.41	0.01–0.65
Low	Medium	≤ 7	0.63	0.16–0.84
		> 7	0.42	0.16–0.61
	Large	≤ 7	0.94	0–1
		> 7	0.78	0.07–0.95

Although histologic classification was not assessed in these patients, recent studies have shown that the majority of B-cell lymphomas can be classified as diffuse large cell.[26,27] Therefore it is likely that the majority of cases described here also fall into this category, and we ultimately hope that flow cytometric categories will correlate with histologic subtype. Only 1 study so far has correlated ST with a contemporary histologic classification scheme that includes immunophenotype.[3] With additional histologic studies and consensus among pathologists to use a single classification scheme, we anticipate that categorization of canine lymphomas will become standard practice for diagnosis and clinical trials. Finally, as canine lymphoma becomes a better defined disease, we anticipate that gains made in the treatment of human lymphoma will be more easily applied to dogs, and that dogs will be a stronger model for the human disease.

Footnotes

[a] De Novo Software, Ontario, Canada

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