Gene Profiling of Canine B-Cell Lymphoma Reveals Germinal Center and Postgerminal Center Subtypes with Different Survival Times, Modeling Human DLBCL

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Abstract
Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma subtype, and fewer than half of patients are cured with standard first-line therapy. To improve therapeutic options, better animal models that accurately mimic human DLBCL (hDLBCL) are needed. Canine DLBCL, one of the most common cancers in veterinary oncology, is morphologically similar to hDLBCL and is treated using similar chemotherapeutic protocols. With genomic technologies, it is now possible to molecularly evaluate dogs as a potential large-animal model for hDLBCL. We evaluated canine B-cell lymphomas (cBCL) using immunohistochemistry (IHC) and gene expression profiling. cBCL expression profiles were similar in many ways to hDLBCLs. For instance, a subset had increased expression of NF-κB pathway genes, mirroring human activated B-cell (ABC)-type DLBCL. Furthermore, immunoglobulin heavy chain ongoing mutation status, which is correlated with ABC/germinal center B-cell cell of origin in hDLBCL, separated cBCL into two groups with statistically different progression-free and overall survival times. In contrast with hDLBCL, cBCL rarely expressed BCL6 and MUM1/IRF4 by IHC. Collectively, these studies identify molecular similarities to hDLBCL that introduce pet dogs as a representational model of hDLBCL for future studies, including therapeutic clinical trials. Cancer Res; 73(16); 5029–39. ©2013 AACR.

Introduction
Non-Hodgkin lymphoma (NHL) is the seventh most common human systemic malignancy, estimated to affect approximately 70,000 people in the United States in 2013, and is a leading cause of cancer-related death (1). Despite improvements in treatments, mortality remains significant (1, 2), necessitating further progress in understanding disease biology and tailoring therapeutic interventions. Diffuse large B-cell lymphoma (DLBCL), the most common subtype of NHL, is recognized as a clinically heterogeneous disease (3). The international prognostic index (IPI), a score derived from clinical characteristics, groups patients into risk categories that range widely from 85% overall survival (OS) at 4 years in the lowest-risk category to 45% OS at 4 years in the highest-risk group (4, 5). This huge degree of variability within histologically identical DLBCLs prompted attempts to molecularly subcategorize the tumors (6). For example, gene expression profiling (GEP) identified two main subtypes, activated B-cell (ABC) and germinal center B-cell (GCB). These subcategories are thought to be reflective of the cell of origin, with GCBs arising from germinal center cells, and ABC-types arising from postgerminal center cells. In addition, ABC/GCB subtypes can be identified by examining somatic hypermutation (SHM), based on the finding that GCB lymphoma cells are continuously undergoing SHM, whereas ABC lymphomas arise from cells that have completed SHM and therefore contain static immunoglobulin heavy chain variable region (IGHV) sequences that are different than the germline sequences (7). These two categories of lymphomas have strikingly different survivals, independent of IPI, initially reported as ABC patient survival of 16% at 5 years and GCB patient survival of 76% at 5 years. This prognostic disparity was subsequently confirmed in multiple studies, even with the addition of rituximab to the standard cyclophosphamide, adriamycin, vincristine, and prednisone (CHOP) treatment regimen (6). Because it is currently impractical to conduct GEP on every patient with DLBCL, a variety of immunohistochemical algorithms have been developed to predict the cell of origin and/or survival of these patients.
(8–12). Antibodies recognizing antigens expressed by either germinal center or post-GCBs, such as CD10, MUM1/IRF4, BCL6, GCET1, and FoxP1 are typically included. Concordance with GEP results ranges from 80% to 93%, and most of these algorithms also predict OS independent of the IPI, although these results have not yet been consistently reproducible (4, 8–14).

Animal models are useful for better understanding cancer biology and therapeutic responses, with mice being the mainstay of lymphoma model organisms. However, mouse models present inherent properties that can make them less accurate model systems, namely impaired immunity and/or genetic homogeneity, neither of which accurately mimics spontaneously occurring tumors in a diverse population (15, 16). Their small size and metabolic differences can affect pharmacokinetic/pharmacodynamic parameters as well. These dissimilarities have resulted in therapies that seem promising in laboratory mice being ineffective when translated to humans. Therefore, more representative animal models that overcome these disadvantages would be a welcome advance.

Pet dogs develop a variety of naturally occurring cancers that are recognized as relevant models of their human counterparts (17–19), similar in clinical presentation, tumor biology, and response to therapy. Dogs provide additional advantages over traditional animal models in that they represent a spectrum of genetic diversity that is not present in most laboratory strains. A recent study showed that T- and B-cell lymphomas in dogs are separable by GEP (20), paving the way for this technology to be applied with larger sample sizes to further define canine B-cell lymphoma (cBCL). Through a series of studies that were sequentially progressive in resolution, we define aspects of cBCL that are similar to human DLBCL (hDLBCL), especially with respect to ABC/GCB subtype. To our knowledge, this is the first study to directly compare canine and human B-cell lymphomas using immunohistochemical algorithms, GEP analysis, and IGHV mutational status. Our data suggest that two subtypes of cBCL can be delineated using IGHV mutualional analysis and to a lesser extent, GEP. Interestingly, immunohistochemical algorithms that are used to separate human patients into GCB and ABC subtypes were not useful as prognostic markers in the dog population. This study represents a basis for using canine patients with lymphoma to understand both canine and human disease.

Materials and Methods

Subjects, sample acquisition

After owner’s consent, neoplastic lymph nodes were collected from 49 dogs presenting to the North Carolina State Veterinary Health Complex (NCSU VHC), or in some cases, referral veterinary clinics for evaluation and management between June 2008 and August 2010 (Table 1). Lymph nodes from referral clinics were shipped overnight in chilled physiological saline to NCSU VHC for processing. In cases where clients did not prefer lymph node resection, cells were collected via fine-needle aspiration (FNA) of enlarged peripheral lymph nodes. These were obtained from 19 additional dogs diagnosed with B-cell lymphoma via flow cytometry (68 total dogs in this study). FNAs were placed immediately into chilled RPMI/2% FBS, stored at 4°C and processed within 24 hours. These samples combined are referred to as cBCL throughout the text.

Lymph node morphology and immunohistochemistry

Lymph node morphology was evaluated using hematoxylin and eosin (H&E) staining of 35 available formalin-fixed and paraffin-embedded samples (NCSU VHC). Lymphoma was diagnosed and subtyped in alignment with the fourth edition of the World Health Organization (WHO) classification of hematopoietic and lymphoid tissues (21). B-cell phenotype was determined by IHC and/or flow cytometry using CD79a and CD3 antibodies (AbD Serotec). MUM1/IRF4 (Dako), PAX5, CD10, and BCL6 antibodies (Leica Microsystems) were used to both confirm B-cell phenotype (PAX5) and determine the cell of origin. External positive and negative control tissue was available for all antibodies. For all antibodies, a sample was called positive if more than 30% of the neoplastic cells were definitively staining (9).

RNA preparation and array processing

Excised lymph nodes were manually homogenized in chilled RPMI/2% FBS (Mediatech) and passed through a 70-μm filter. FNAs were washed twice in ice-cold PBS, pH 7.4 (Mediatech) followed by red blood cell (RBC) lysis (Roche Applied Science). After counting, 1 × 10⁷ cells were placed into TRIzol (Sigma-Aldrich) and then frozen at –80°C. RNA was prepared according to the manufacturer’s protocol and then purified over an RNeasy column (Qiagen). RNA quality was verified on a bioanalyzer (Agilent Technologies); all samples had an RNA integrity number (RIN) >9. Microarray gene expression data were obtained by the Lineberger Functional Genomics Core Facility using Affymetrix Canine Genome 2.0 Arrays. One microgram of total RNA was processed for microarray hybridization to Affymetrix GeneChips using the MessageAmp II-Biotin Enhanced Kit from Ambion (Life Technologies). Briefly, the RNA was reverse transcribed into cDNA using T7 OligodT primer. Following the first- and second-strand cDNA synthesis, in vitro transcription was carried out at 37°C for 14 hours to

Table 1. Characteristics of the patients and samples used in this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Number of dogs</td>
<td>68</td>
</tr>
<tr>
<td>Breeds</td>
<td>31 (11 LR, 7 GR, 6 GSD)</td>
</tr>
<tr>
<td>Spay/Neuter</td>
<td>(3 IF, 31 SF, 3 IM, 31 NM)</td>
</tr>
<tr>
<td>Age, y</td>
<td>Mean, 7.1 (range, 2–14)</td>
</tr>
<tr>
<td>B-cell lymphoma</td>
<td>Diagnosis 19 LN FNA, 49 LN biopsy</td>
</tr>
<tr>
<td></td>
<td>Stage 1 I II, 14 III, 18 IV, 28 V, 7 unknown</td>
</tr>
<tr>
<td></td>
<td>Substage 46 a, 17 b, 5 unknown</td>
</tr>
</tbody>
</table>

Abbreviations: GR, golden retriever; GSD, German shepherd dog; IF, intact female; IM, intact male; LN, lymph node; LR, labrador retriever; NM, neutered male; SF, spayed female
generate biotin-labeled amplified RNA (aRNA). The aRNA was fragmented at 94°C for 35 minutes and prepared for hybridization according to the Affymetrix Expression Analysis Technical Manual. The hybridization cocktail was hybridized to the arrays at 45°C for 16 hours. Following hybridization, the arrays were washed with the Affymetrix Fluidics Station 450 wash stations. The arrays were scanned with the Affymetrix GeneChip Scanner 3000 7G Plus with Autoloader. GeneChip Command Console Software was used to control all the instrumentation. Basic data analysis and quality of the arrays were conducted using Affymetrix Expression Console software. Data have been annotated and deposited according to Minimum Information About a Microarray Gene Experiment (MIAME) guidelines with Gene Expression Omnibus (GEO) accession number GSE43664.

Statistical analysis

Microarray data were normalized using robust multi-array average (RMA) software implemented by the Bioconductor package in R (22), version 2.12.1. Identification of differentially expressed genes (three or more samples varying by at least 4-fold from the median) and average linkage hierarchical clustering was done with Cluster 2.11 (23), and visualized with Java TreeView (24). The annotation file Canine_2.na31 was used to identify genes from probe set information.

To match probe sets between the dog and human arrays, we used the Homologene (build 64) and RefSeq (release 38, vertebrate mammalian sequences) databases from National Center for Biotechnology Information (NCBI).

OS was defined as the time from diagnosis until death from any cause. Progression-free survival was defined as the time from diagnosis until progression of disease or death from any cause. Outcomes were censored as of April 30, 2011. One dog was euthanized the day of diagnosis, and one was lost to follow up. These 2 dogs were not included in the survival analyses. Differences in survival by subgroup were tested with Cox proportional hazards regression, after testing that proportional hazards assumptions were met (25). Survival curves were estimated using the Kaplan–Meier estimator (26). Association between the hypermutation status and group categories was carried out using a χ² test of association. Association analysis was carried out in Stata v10 (www stata.com) and R (22).

Human DLBCL datasets

CEL files with GEO accession number GSE11318 contain expression data from 203 hDLBCL samples analyzed on Affymetrix Human Genome U133 Plus 2.0 expression arrays (27). These were downloaded and normalized with RMA. The systematic data biases between human gene expression data and dog gene expression data were detected and removed by Distance Weighted Discrimination (DWD) before the two datasets were combined (28). Twenty-five genes were then retrieved from the combined datasets and a two-way average linkage hierarchical cocluster was generated.

Western blotting

Ten randomly chosen canine DLBCLs (cDLBCL) were used for this experiment; 5 were “GCB-like,” that is, from group 1 in Fig. 5 (dogs 6, 8, 14, 28, and 44), and 5 were "ABC-like," that is, from group 2 in Fig. 5 (dogs 2, 3, 7, 21, and 49). Western blotting was conducted as previously described (29) except that cell pellets stored at −80°C were used as the starting material. Antibodies directed against phospho-NF-κB p65 (Ser536) and NF-κB p65 (Cell Signaling Technology) and β-actin as a load control (Sigma-Aldrich) were used. Bands were visualized with enhanced chemiluminescence (ECL) Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences Corp.). Densitometry was conducted using NIH ImageJ 1.46.

RNA isolation and qPCR

Total cellular RNA was extracted from TRIzol, and RNA (1 μg) was then reverse-transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s instructions. Quantitative PCR (qPCR) was conducted in a 25-μl reaction volume using SsoFast EvaGreen (Bio-Rad) Mastermix. Reactions used 400 nmol/L of each of forward and reverse primers prepared by the Nucleic Acids Core Facility [University of North Carolina at Chapel Hill (UNC-CH), Chapel Hill, NC]. The following primers were used: IRAK1BP1, 5′-GAGGCCAAAGAAGGTGTTG-3′ and 5′-GCT-TGGCCTGCGAGATTC-3′; STAT4, 5′-GCAGAGAGCTT-ACAGCCCA-3′ and 5′-TGCGGGACTCAGGTTTTCT-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CGGATCTAAGATGCGG-3′ and 5′-AGCCTTCTCTCAT-GGTGGTGAAGA-3′. Annealing temperature was 61.5°C for all reactions. The 10 cDLBCL samples used were the same as those used in the Western blotting experiment.

IGHV sequencing

The dog IGHV gene was sequenced according to the method previously described for the human IGHV gene (30). However, as 39 of 41 functional members of the canine IGHV repertoire are in a single family, only a single set of primers was used, rather than the seven family-specific sets used in humans. Each canine sample was amplified successfully with these primers: forward 5′-CCATGGAGTCTTGCTCCTGC-3′ containing the ATG at the start of the IGHV open reading frame, and reverse 5′-CTGAGGAGACGGTGACTGATAC-3′, located in the JH region, producing a 432-bp amplicon. RNA from each lymphoma sample was converted to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). The PCR conditions for cDNA amplification were 94°C for 3 minutes; followed by 35 cycles of (94°C for 1 minute; 55°C for 45 seconds; and 72°C for 1 minute) with a final elongation step of 72°C for 10 minutes. The PCR product was then cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) or the Topo II Cloning Kit. Colonies were screened by PCR or EcoRI restriction digest, and at least 12 clones from two separate PCR/cloning reactions were sequenced for each subject. S6 universal primer was used. Conventional deoxynucleotide sequencing was conducted by the DNA sequencing core at UNC-CH Genomic Analysis Facility, UNC-CH.

IGHV mutation status was then defined as “static,” that is, more than 50% of the subclones identical or nearly identical, or "ongoing," that is, less than or equal to 50% of the subclones identical or nearly identical.
Results

Lymph node phenotyping and morphology

Thirty-five canine lymph nodes that were CD79a-positive (CD79a⁺) and CD3-negative (CD3⁻), designated as large B-cell lymphomas, were selected for additional immunophenotyping. Of note, 28 of 35 (80%) of the dogs were morphologically characterized as DLBCL and 7 of 35 (20%) were morphologically characterized as late marginal zone lymphoma (MZL). Similar to hDLBCL, cDLBCL is characterized by a complete loss of lymph node architecture via the expansion of a population of large neoplastic B cells (Fig. 1A). Canine late MZL, in contrast to human MZL, is an aggressive lymphoma characterized by the expansion of large neoplastic B cells apparently arising from the marginal zone that, in the later stages of the disease, leads to the typical finding of “fading germinal centers” (Fig. 1B). Given that these two types of B-cell lymphomas have similar cellular morphology and clinical behavior, and that they account for the vast majority (>90%) of canine large B-cell lymphomas (31), we analyzed them together in this study.

cBCLs are not separable by immunohistochemical stains that differentiate hDLBCLs

We studied immunohistochemical staining of PAX5 to corroborate our CD79a/CD3 data. This was supplemented with evaluation of three additional antibodies, CD10, BCL6, and MUM1/IRF4, which are used in hDLBCL as a surrogate for GEP to separate ABC and GCB subtypes. In our canine samples, 34 of 35 samples were PAX5-positive, confirming a B-cell phenotype, consistent with nearly universal PAX5-positivity in hDLBCL (Supplementary Fig. S1A; ref. 32). Eight of 35 (23%) samples were positive for CD10 staining, suggesting a GCB subtype using human algorithms, where CD10-positivity ranges from 26% to 40% (Supplementary Fig. S1B; refs. 9, 11). Notably, 2 of 7 (28%) MZLs were CD10-positive, reinforcing the distinction from human MZL, which is almost never CD10-positive (33, 34). Interestingly, only a single sample tested positive for BCL6 (Supplementary Fig. S1C) and a second single sample tested positive for MUM1/IRF4 (Supplementary Fig. S1D). This is in contrast to hDLBCL, in which 70% to 80% of cases are positive for BCL6 and 20% to 40% are positive for MUM1 (8, 12).

GEP does not distinguish MZL from DLBCL in cBCL

A total of 58 cBCLs, 39 with a histologic diagnosis and 19 obtained by FNA and diagnosed only as cBCL, were collected for transcriptional profiling. Genes that were differentially expressed were identified, and unsupervised hierarchical clustering using these 1,079 probe sets did not identify robust separation into subtypes, as has been reported with hDLBCL (Supplementary Fig. S2; ref. 6). Given that a subset of our samples were obtained by FNA and therefore did not have histologic diagnosis available, we first analyzed whether MZL could be distinguished from DLBCL by expression profiling. On the basis of population averages, more than 95% of our cBCL samples are expected to be either MZL or DLBCL, divided approximately as 25% MZL and 75% DLBCL (31). We analyzed the 39 samples with known histology, 7 MZL and 32 DLBCL, to see whether any genes were differentially expressed. Using two-class unpaired significance analysis of microarrays (SAM) analysis (35), no genes were statistically significantly differentially expressed. We also conducted principal component analysis with these 39 samples and there was no separation between MZL and DLBCL (Fig. 2), in agreement with previous results (20).

Expression of genes homologous to ABC/GCB signature genes distinguish two subtypes of cBCL

We hypothesized, on the basis of similar morphology and veterinary pathology classification standards, that the majority...
of cBCLs would be molecularly similar to hDLBCL (31). Therefore, we were interested in determining whether the two major subclasses of hDLBCL could be identified in cBCL. We analyzed all 58 cBCLs as a single group, given that there were no detectable differences in their gene expression profiles based on MZL/DLBCL distinction (possibly because late MZLs in dogs are similar to DLBCL). We used a 27-gene classifier that separates hDLBCL into ABC/GCB subtypes (36) and analyzed both human and canine lymphomas together. Two genes were omitted from this analysis: TBC1D27 did not have a known canine homolog and IGHM was so strongly expressed in some cBCL cases that it dominated the clustering when included. The hDLBCL samples clustered into two clear groups, corresponding to human ABC and GCB DLBCL, whereas the cBCL samples were interspersed among the human samples in both groups (Fig. 3). Next, we used this set of 25 human ABC/GCB classifier genes and conducted unsupervised hierarchical clustering of cBCL, which resulted in the separation into two distinct groups (Fig. 5A). There was a statistically significant progression-free survival difference between the groups, and a trend toward an OS difference (Fig. 5B). It should be noted that OS in pet dogs is not as robust an endpoint as it is in humans, because owners choose when to euthanize their pets, and factors other than extent of disease therefore affect this endpoint.

Canine lymphomas differentiated by human ABC/GCB–distinguishing genes can be better separated with a canine-specific set of genes

Hypothesizing that cBCL subtypes might be better distinguished by a canine-specific gene signature, we attempted to use the two groups of canine lymphomas defined by the human ABC/GCB signature genes (Fig. 4) to develop a more robust canine gene signature. Two-class unpaired SAM analysis was used to find genes that were differentially expressed between the “human ABC-like” and “human GCB-like” groups, and 1,180 probe sets were discovered using a cutoff false-discovery rate (FDR) of 5%. We used these to conduct hierarchical clustering of cBCL, which resulted in the separation into two distinct groups (Fig. 5A). There was a statistically significant progression-free survival difference between the groups, and a trend toward an OS difference (Fig. 5B). It should be noted that OS in pet dogs is not as robust an endpoint as it is in humans, because owners choose when to euthanize their pets, and factors other than extent of disease therefore affect this endpoint.

ABC/GCB genes are not conserved between dogs and humans, but pathways and biologic processes that distinguish germinal and postgerminal center groups are shared across species

We analyzed the 1,180 genes (Supplementary Table S1) that were statistically differentially expressed between cBCL groups when separated using two-class unpaired SAM analysis. Although individual genes were not uniformly conserved across species, pathways and processes were strikingly conserved. Ingenuity pathway analysis (Ingenuity Systems; www.ingenuity.com) was used to determine the top biologic processes and pathways enriched for members of the canine ABC/GCB gene list. Similar to the case in hDLBCL, B-cell activation, B-cell receptor signaling, and the NF-κB pathway were among the most statistically significantly associated processes. Two genes, IRAK1BP1 and STAT4, which were more highly expressed in group 1, the “GCB-like” group, were confirmed with qPCR in 10 cDLBCL samples (5 ABC-like and 5 GCB-like). Similar to the expression array data, the GCB-like group had...
higher expression of both genes, with an average 10.46-fold higher expression of IRAK1BP1 and 7.44-fold higher expression of STAT4. To further confirm biologic differences, we also conducted immunoblotting for p65 (RelA). This protein has previously been shown to be active in a majority of cDLBCLs, with activation of downstream targets in approximately half of cDLBCLs (37). In our samples, we observed higher total cellular expression of p65, concordant with the mRNA expression data. Phospho-p65 was also present in the majority of samples (Supplementary Fig. S3). One of the top two canonical pathways found in our pathway analysis was the B-cell receptor signaling pathway, with a $P$ value of 2.79 x $10^{-6}$ (Fig. 5A). To ensure that inclusion of MZLs was not obscuring our ability to define subgroups, we repeated the entire gene expression analysis using only known DLBCLs ($n = 32$), and found no substantial differences, other than loss of statistical significance in the survival analyses, presumably due to smaller sample size ($P = 0.10$ for PFS). Notably, MZLs were also found in both ongoing and static IGHV groups, but the sample size was too small for survival analysis ($n = 5$).

It has been reported in a small number of patients with hDLBCL that ABC/GCB phenotype is well correlated with IGHV status (ongoing vs. static; ref. 7). However, the two phenotypes were not well correlated in patients with cBCL, as shown in Fig. 6B. On the basis of hDLBCL, static IGHV mutation status should be correlated with the group of dogs that is more similar to ABC hDLBCL. However, the $\chi^2$ test showed no statistically significant difference, though it was trending in that direction ($P = 0.097$). In addition, a multivariate analysis using both IGHV status and GEP failed to show that either was independently predictive of survival.

We also analyzed the IGHV parental gene used in each lymphoma. Humans have 123 IGHV genes, including 79 pseudogenes, that fall into seven gene families (39). Dogs have a total of 80 IGHV genes, including 39 pseudogenes, which fall into three gene families, but the majority (76) are in VH1, the human VH3 family ortholog (38). The majority (57%) of cBCLs used VH1–44. This is the second most commonly used parental gene in normal canine B cells (23%; ref. 38). Parental gene usage in cBCL was significantly different from usage in normal B cells ($P < 0.0002$), indicating a bias in IGHV usage in cBCL. When
looking at MZL (2 of 5, or 40%, used VH1-44) and DLBCL (29 of 52, or 56% used VH1-44) separately, results were similar, although the number of MZLs was too small for statistical comparisons.

Discussion

In contrast to other available models, the population of pet dogs in the United States represents a diverse and abundant source of spontaneously occurring lymphomas (18, 19, 40). Recently, with improvements in genomic technologies and the advent of subspecialty veterinary care, including oncology, this readily available resource is now primed for use to augment clinic research. This study represents the first molecular analysis of cBCL combining modalities (GEP, IHC, and IGHV status) specifically aimed at defining molecular similarities to hDLBCL. We initially used antibodies against known human antigens that are expressed by either germinal center cells or post-GCBs in an effort to determine if cBCL can also be separated into these subtypes. Although the anti-human CD10, BCL6, and MUM1/IRF4 antibodies all cross-reacted with the canine antigens, the rare positivity for BCL6 and MUM1/IRF4 make human immunohistochemical algorithms less useful. This reinforces the notion that different proteins may be the hallmarks of cBCL subtypes, as compared with hDLBCL. Even if GCB and ABC correlates are not strictly conserved in dogs, discovering immunohistochemical stains to distinguish the germinal center and postgerminal center subtypes we characterized in this report will be important for prognostic purposes.

Our cBCLs were separable into two major histologic subtypes by lymph node morphology, which corroborates previous studies (21). The majority (32 of 39 or 82%) were cDLBCL and 7 of 39 (18%) were canine MZLs. Our analyses revealed no distinctions that reliably separated these two histologic subtypes, similar to a recently published analysis with a smaller number of cBCLs (10 DLBCL, five MZL; ref. 20). Gene
expression profiles were similar, with no genes differentially expressed between the two groups in our samples (albeit with only seven MZLs). Franz and colleagues also found similar gene expression profiles, but did find genes that were differentially expressed between cMZL and cDLBCL. However, it is possible that the smaller number of cBCLs in that study could account for this. In our study, MZLs and DLBCLs also were not distinguishable by IHC, IGHV mutation status, or by ABC/GCB subtyping. Furthermore, we repeated our entire analysis using only DLBCLs (n = 32), and found no substantial differences from our findings presented here, other than loss of statistical significance in the survival analyses, presumably due to smaller sample size (data not shown). Theoretically, canine MZL could begin as an indolent disease that is not detected early in its course and then progresses to a more aggressive form in the later stages when enlarged peripheral lymph nodes become apparent. In any case, it seems that by the time canine MZL is clinically detectable, the disease is as biologically aggressive as DLBCL and molecularly similar to it. Therefore, lymph node morphology in canine B-cell LSA may not be prognostic or clinically relevant at this time. More canine MZL cases will need to be studied before making any final conclusions.

Similar to hDLBCL, we found heterogeneity in gene expression using microarray analysis. Genes that distinguish ABC and GCB subtypes in hDLBCL separate cBCL into two groups. Using this distinction to select a canine-specific set of differentially expressed genes, yields two distinct groups with statistically different survival times. Furthermore, these canine-specific "ABC/GCB" discriminating genes, while different from the human ABC/GCB gene list, are involved in the same pathways and processes (e.g., NF-κB signaling and B-cell receptor signaling). The importance of B-cell receptor signaling in canine lymphoma has been shown previously by responses to ibrutinib, an inhibitor of B-cell receptor signaling, in a subset of patients with cBCL (41). NF-κB signaling in cBCL is complex, showing both similarities and differences to hDLBCL in both our results and in other reports (37). Our protein studies of NF-κB support differential expression of pathway members found by GEP, although future studies using nuclear extracts/staining will be needed to definitively show activation of the pathway in canine ABC-like DLBCL. These and other studies, including generation of canine lymphoma cell lines and more detailed study of molecular aberrations in these lymphomas, will be necessary to definitively determine whether both ABC and GCB subtypes exist in cBCL.

Figure 5. Clustering of cBCLs using "dog specific" ABC/GCB-classifier genes. A, hierarchical clustering with 1,180 "dog specific" ABC/GCB genes. Differentially expressed genes categorized as being in the "B-cell receptor pathway," are listed on the left. B, Kaplan–Meier survival curves and Cox regression were conducted on the two groups.
Like ABC/GCB gene expression pattern, IGH status (ongoing SHM vs. completed SHM) also identified two cBCL groups with statistically different survival times. In hDLBCL, these two phenotypes (ABC/GCB gene expression pattern and completed/ongoing SHM) are reported to be overlapping in most cases. However, this observation is based on a limited number of samples ($n = 14$) and may not be as perfectly correlated as previously reported (7). If that is the case, ongoing SHM may be a better predictor of survival in hDLBCL, as it is in cBCL. Further study of SHM in hDLBCL is therefore warranted.

Several limitations of our study should be highlighted. The enrolled dogs were not part of a clinical trial, so while they were generally treated with a standard first-line CHOP-based protocol, variability in treatment did occur. Uniform treatment should increase the statistical power of future results. Another source of heterogeneity is the type of biopsy ($39$ excisional biopsies and $19$ FNAs for the gene expression studies). Both types of biopsies were equally represented in both classes of canine lymphomas in both Figs. 5 and 6 ($\chi^2$ test; $P > 0.25$ in both cases), making bias caused by type of biopsy unlikely in this study. Additional excisional biopsies will be needed to adequately power studies of whether stromal expression signatures are relevant in cBCL. Finally, as many different breeds were included, our study does not address the existence of breed-specific lymphoma subtypes, which could be of interest. Our data create a reference group against which future breed-specific cohorts can be compared.

In conclusion, our work represents a first combination approach using detailed immunohistochemical and molecular characterization of cBCL, thus providing a pathway for this widely available resource to be further developed as a large-animal model for the study of hDLBCL. Our data are consistent with a germinal center and postgerminal center phenotype in cBCL, although how closely these mimic human GCB and ABC subtypes clinically remains to be determined. As molecular similarities are better defined, pet dogs will be useful in clinical trials with new agents that target particular molecular subtypes of lymphoma with aberrations that are shared between canines and humans. Given the lack of shared specific genes that are aberrantly expressed, comparative translational oncology will likely focus more on conserved pathways that are deranged rather than specific gene products. Targeting these shared pathways in patients with canine lymphoma will allow the rapid development of new therapies by gathering pharmacokinetic/pharmacodynamic and efficacy data from the same model organism. This study provides an important step toward the development of a more faithful and representative animal model for the development of hDLBCL therapeutics.

Figure 6. IGH mutation status predicts survival in cBCL. The IGHV gene was subcloned and sequenced in $53$ cBCL, which were then categorized as "ongoing" (many different subclones, indicating ongoing SHM) or "static" (subclones identical or nearly identical, indicating completed SHM). A, Kaplan–Meier survival curves and Cox regression were conducted on the two groups. B, overlap between groups defined by canine "ABC/GCB" genes (Fig. 5) and IGHV status is shown. $P = 0.097$ for the $\chi^2$ test.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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