Prognostic value of CD44 variant isoform expression in dogs with multicentric high-grade B-cell lymphoma

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OBJECTIVE
To determine the prognostic value of CD44 variant isoform expression in dogs with multicentric high-grade B-cell lymphoma (BCL).

ANIMALS
45 dogs with multicentric BCL and 10 healthy control Beagles.

PROCEDURES
The medical record database of a veterinary teaching hospital was searched to identify dogs with BCL that were treated between November 2005 and April 2015. Information regarding overall response to chemotherapy, progression-free survival (PFS) time, and overall survival time was extracted from each record. Archived lymph node aspirate specimens from dogs with BCL and lymph node aspirate specimens from the 10 control dogs underwent real-time PCR analysis to determine mRNA expression of CD44 variant isoforms of exons 3, 6, and 7 and the CD44 whole isoform. For each isoform, mRNA expression was compared between dogs with BCL and control dogs. The mean relative expression of each isoform was used to classify dogs with BCL into either a high- or low-expression group, and overall response rate, PFS time, and overall survival time (ie, indices of prognosis) were compared between the 2 groups.

RESULTS
For all isoforms evaluated, mean relative mRNA expression for dogs with BCL was numerically lower than that for control dogs. Dogs with BCL and high CD44 isoform expression had a lower overall response rate, median PFS time, and median overall survival time, compared with dogs with BCL and low CD44 isoform expression.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that, for dogs with BCL, high expression of exons 3, 6, and 7 was associated with a poor prognosis. (Am J Vet Res 2018;79:961–969)

Lymphoma is one of the most common malignant tumors of dogs and accounts for 7% to 24% of all canine tumors and approximately 85% of all canine hematopoietic tumors. In dogs, lymphoma is considered a heterogeneous disease, and the response to treatment and outcome varies among affected individuals. Lymphoma is categorized on the basis of multiple factors including anatomic location, histologic classification, and immunophenotype. In dogs, multicentric BCL is the most common subtype and DLBCL is the most common histologic type of lymphoma. The CHOP protocol is one of the most commonly used treatments for dogs with high-grade BCL, resulting in a median disease-free period of 9 to 11 months and complete remission for 70% to 90% of treated dogs. However, a small number of dogs with high-grade BCL treated with the CHOP protocol do not respond to treatment and die soon after it is initiated. In human patients with lymphoma, many molecules are associated with the treatment response, including BCL2, BCL6, CD5, CD44 variant isoform, P53, and vascular endothelial growth factor. In dogs with lymphoma, a mutation in the P53 gene and overexpression of the P53 protein reportedly induce chemoresistance but occur with low frequency, and BCL2, BCL6, CD5, and vascular endothelial growth factor are not associated with disease outcome. Results of microarray analysis suggest that CD44 is one of the molecules associated with tumor pathogenesis and may have prognostic importance in dogs with BCL.

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>BCL</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>CD44s</td>
<td>CD44 standard isoform</td>
</tr>
<tr>
<td>CD44v3</td>
<td>CD44 variant exon 3</td>
</tr>
<tr>
<td>CD44v6</td>
<td>CD44 variant exon 6</td>
</tr>
<tr>
<td>CD44v7</td>
<td>CD44 variant exon 7</td>
</tr>
<tr>
<td>CD44w</td>
<td>CD44 whole isoform</td>
</tr>
<tr>
<td>CHOP</td>
<td>Cyclophosphamide, hydroxydaunorubicin (doxorubicin), Oncovin (vincristine), and prednisolone</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-box-binding protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
CD44 is a type I transmembrane protein that is composed of extracellular, transmembrane, and cytoplasmic domains (Figure 1). The CD44 molecule has structural and functional diversities that arise from alternative splicing and variation in N- and O-glycosylation.26,29 The CD44S consists of 10 exons, is predominantly expressed in hematopoietic and epithelial cells,30 binds to hyaluronan at its extracellular domain, and performs physiological functions such as lymphocyte homing31 and migration.32 Various isoforms of CD44 generated by alternative mRNA splicing have been described in humans.30 Variant isoforms of CD44 consist of 11 to 20 exons with insertions of up to 10 exons at the membrane-proximal extracellular domain and, in humans, are expressed in many tissues such as the epidermis, thyroid gland, tonsils, lymph nodes, and thymus.33,34 In dogs, the CD44W is expressed in many tissues including macrophages, subsets of lymphocytes, epithelial cells, and the thymic medulla,35 and up to 48 types of mRNA from various variant isoform CD44 genes have been identified in the thymus and lymph nodes.36 Although the physiologic function of variant isoforms of CD44 has not been completely elucidated, the functions of some variants are known. The CD44V5 molecule contains specific posttranslational modifications that include a heparan sulfate site, which binds heparin-binding proteins such as fibroblast growth factor 2.37 Cells that express CD44v6 stimulate synthesis of hepatocyte growth factor, which activate the Akt pathway.38,39 The CD44V variant isoform containing exons 8 to 10 interacts with and stabilizes SLC7A11, thereby promoting cystine uptake for glutathione synthesis, and contributes to defense against reactive oxygen species through upregulation of the synthesis of reduced glutathione, the primary intracellular antioxidant.40

In human medicine, CD44 variant isoform expression is associated with resistance to anticancer agents used to treat many types of breast cancer,31 colorectal cancer,40 and ovarian cancer.42 CD44 variant isoform expression is used as a prognostic indicator for humans with NHL47 and DLBCL.43 In dogs, CD44w is expressed by mammary gland tumor cells14 and neoplastic cells associated with acute leukemia.45 Additionally, CD44 expression is associated with a poor prognosis for dogs with mammary gland tumors.46 The purpose of the study reported here was to determine the prognostic value of CD44 variant isoform expression in dogs with multicentric high-grade BCL.

Materials and Methods

Study design and animals

The study was largely retrospective in nature. The medical record database of the University of Tokyo Veterinary Medical Center was searched to identify dogs in which lymphoma was diagnosed between November 2005 and April 2015. To be included in the study, each dog had to have a diagnosis of multicentric high-grade BCL of the centroblastic or immunoblastic type as determined by the updated Kiel classification system,6 a complete medical record, and archived lymph node aspirate specimens available for histologic review and quantification of the expression of various CD44 variant isoforms. Dogs that were treated for any kind of cancer prior to diagnosis of high-grade BCL were excluded from the study.

Superficial popliteal and cervical lymph node aspirate specimens were obtained from 10 (4 females and 6 males) healthy adult Beagles to serve as controls for quantification of CD44 isoform expression. Those dogs were obtained from 2 laboratory animal suppliers5,6 and maintained as a research colony at the University of Tokyo in accordance with guidelines outlined in the Guide for the Care and Use of Laboratory Animals.87 The 10 dogs ranged in age from 3.8 to 8.7 years and in body weight from 7.8 to 14.2 kg. All dogs were considered healthy on the basis of results of a physical examination. Procedures used to obtain control specimens from the superficial popliteal and cervical lymph nodes were reviewed and approved by the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo (accession No. P15-65).

Data collection for dogs with high-grade BCL

For each of 45 dogs with high-grade BCL (ie, dogs with BCL) that met the study inclusion criteria, infor-
mation regarding radiographic and ultrasonographic findings and CBC (including peripheral blood cell morphology) and serum biochemical profile results were extracted from the medical record. Cytologic specimens obtained by fine-needle aspiration of enlarged superficial lymph nodes were reviewed to confirm that the neoplasm was lymphoid in origin. A PCR assay for detection of antigen receptor gene rearrangements was performed as described\(^4\) to confirm that the immunophenotype of the tumor was of B-cell lineage rather than T-cell lineage. The WHO clinical staging system\(^2\) was used to determine the clinical neoplastic stage (WHO clinical stage) and substage (WHO clinical substage) for each patient.

**Treatment efficacy and outcome**

All 45 dogs with BCL were initially treated with a modified University of Wisconsin-Madison chemotherapy protocol (ie, UW-25),\(^8\) which is a CHOP-based protocol. The UW-25 protocol was modified in that dogs were not administered L-asparaginase during week 1. That modification was made because results of multiple studies\(^5\) indicate L-asparaginase has no effect on the outcome for dogs with lymphoma that are treated with CHOP-based protocols.

Response to treatment was assessed on the basis of lymph node size in accordance with consensus criteria for peripheral nodal lymphoma in dogs established by the Veterinary Cooperative Oncology Group.\(^2\) For each dog, PFS was defined as the duration between initiation of chemotherapy and detection of progressive disease or death from any cause. Overall survival was defined as the duration between initiation of chemotherapy and death from any cause.

The modified UW-25 protocol was repeated for dogs that had tumor recurrence or relapse. Dogs that became refractory to the modified UW-25 protocol were treated with various rescue protocols such as L-asparaginase; a combination of lomustine, L-asparaginase, and prednisolone (LAP protocol); a combination of dexamethasone, melphalan, actinomycin D, and cytosine arabinoside (DMAC protocol); and nimustine\(^5\) as described.

**Quantification of CD44 mRNA**

Lymph node aspirate specimens collected from the dogs with BCL before initiation of the UW-25 protocol and those obtained from the 10 control dogs were placed in an RNA stabilization solution immediately after collection and stored frozen at \(-80^\circ\text{C}\) until analysis. From each specimen, total RNA was isolated by use of a commercial extraction kit\(^4\) and transcribed to cDNA.\(^6\) Genomic DNA was removed twice: the first time by use of DNase I in the total RNA extraction step and the second time by use of genomic DNA remover in the cDNA synthesis step. Lymph node RNA purity was evaluated by determining the ratio of spectrophotometric absorbance of the specimen at 260 nm to that at 280 nm (A260:A280 absorbance ratio). Primer pairs for amplification of CD44s and various CD44 variant isoform exons were designed from a canine-specific CD44 gene sequence by use of a PCR primer design web program.\(^5\) Specific primer pairs for CD44v4, CD44v5, CD44v6, and CD44v7 were designed to measure relative quantities by ΔΔCt methods\(^8\) (Appendix). Primers to quantify other variant isoforms of CD44 were difficult to design, and we chose not to pursue quantification of those isoforms. The primers for amplification of the internal control genes (ACTB [\(\beta\) actin], GAPDH [glyceraldehyde-3-phosphate dehydrogenase], TBP, and RPL32) were synthesized as described.\(^8\) The TBP gene was identified by a software program as the most suitable reference gene for the study.

For each control dog and dog with BCL, mRNA for CD44v3, CD44v6, CD44v7, and CD44w was quantified in triplicate by means of a real-time PCR assay, and the mean for each isoform was calculated and used for analysis. For each replicate, 25 ng of the cDNA being amplified was combined with 20 µL of PCR master mix containing SYBR green\(^1\) and 100 nM of both the forward and reverse primers for the gene being evaluated. A PCR thermal cycler\(^4\) was used for all assays with the following cycling conditions: 60 seconds at 95°C; 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 30 seconds; and then a dissociation step that consisted of 95°C for 60 seconds, 60°C for 30 seconds, and 95°C for 15 seconds to verify the presence of a single melting peak. The results for each replicate were reported as the Ct value or the cycle number at which the reported fluorescence crossed the fixed baseline threshold.

For each dog and isoform evaluated, the mean Ct value was calculated and compared with that for the referent gene (TBP). For both control dogs and dogs with BCL, the 2\(^{-\text{ΔΔCt}}\) method was used to calculate the fold change in mRNA expression for each CD44 isoform evaluated relative to that for TBP, and results were reported as the relative mRNA expression. Relative mRNA expression values < 1 indicated that the Ct value for the isoform in question was less than that for the referent gene, whereas relative mRNA expression values > 1 indicated that the Ct value for the isoform in question was greater than that for the referent gene.

**Statistical analysis**

Descriptive statistics were generated. The data distributions for the relative mRNA expression of CD44v3, CD44v6, CD44v7, and CD44w were assessed for normality by the Shapiro-Wilk W test. All variables were normally distributed, and results were reported as the mean ± SD. For each CD44 isoform evaluated, the mean relative mRNA expression for dogs with BCL was compared with that for control dogs by means of the Student \(t\) test. Dogs with BCL were then categorized as having either high or low expression of each CD44 isoform evaluated. The mean relative expression of each CD44 isoform for dogs with BCL
was used at the cutoff between the high and low expression groups.

The overall response rate was calculated as the proportion of dogs with BCL that had a favorable response to treatment at completion of the initial UW-25 protocol. Fisher exact tests were used to compare the overall response rate between dogs with high and low expression of each CD44 isoform evaluated. The Kaplan-Meier product limit method was used to calculate the median PFS time and overall survival time for dogs with BCL that had high and low expression of each CD44 isoform evaluated, and the resulting survival curves were compared by means of the log-rank test. Dogs for which chemotherapy was prematurely discontinued or that did not develop progressive disease during the observation period, were lost during follow-up, were still alive on the date of the last follow-up, or were euthanized at the owner’s request were censored from the PFS analyses. Dogs that were alive at completion of the observation period, lost during follow-up, or were euthanized at the owner’s request were censored from the overall survival analyses. Univariable and multivariable Cox regression analyses were used to identify factors associated with PFS and overall survival times; independent variables assessed in those analyses included age, sex, weight, WHO clinical stage and substage, presence of anemia (PCV < 35%), and relative expression of CD44v3, CD44v6, CD44v7, and CD44w. Post hoc power calculations were performed for the overall response rate, PFS, and overall survival as well as the Cox regression analyses. All analyses were performed by statistical software programs,1,2 and values of P < 0.05 were considered significant.

Results

Dogs with BCL

The 45 dogs with BCL included 4 castrated males, 18 sexually intact males, 14 spayed females, and 9 sexually intact females with a median age of 10.2 years (range, 2.6 to 14.1 years) and weight of 10.8 kg (range, 3.3 to 40.0 kg). The most commonly represented breeds were Pembroke Welsh Corgi (n = 7), Shih Tzu (6), Miniature Dachshund (4), and Golden Retriever (4). Individual dog data are provided elsewhere (Supplementary Table S1, available at avma journals.avma.org/doi/suppl/10.2460/ajvr.79.9.961).

Cytologic review of lymph node aspirate specimens revealed that 43 of the 45 dogs had centroblastic lymphoma, whereas the remaining 2 dogs had immunoblastic lymphoma. The owners of 5 dogs (3 with centroblastic lymphoma and 2 with immunoblastic lymphoma) consented to biopsy of peripheral lymph nodes, and histologic examination revealed that biopsy specimens from all 5 dogs had characteristics of DLBCL. The WHO clinical stage was classified as II, III, IV, and V for 2, 5, 15, and 23 dogs, respectively. The WHO clinical substage was classified as a for 25 dogs and b for 20 dogs.

Thirty of the 45 (67%) dogs died because of lymphoma-related complications, and 3 dogs were euthanized at the owners’ request because of lymphoma progression. Eleven (24%) dogs were censored during the PFS analysis, and 9 (20%) dogs were censored during the overall survival analysis. The WHO clinical stage and presence of anemia were significantly associated with PFS and overall survival times (Table 1). The median PFS (104 days) and overall survival (197 days) times for dogs with WHO clinical substage b were significantly less than the median PFS (271 days; \( P = 0.02 \)) and overall survival (337 days; \( P = 0.01 \)) times for dogs with WHO clinical substage a. Likewise, the median PFS (129 days) and overall survival (197 days) times for dogs with anemia were significantly less than the median PFS (228 days; \( P = 0.03 \)) and overall survival (271 days; \( P = 0.04 \)) times for dogs that did not have anemia.

Relative mRNA expression of CD44 variant isoforms and CD44w

For the 10 healthy control dogs, the mean ± SD relative mRNA expression was 0.52 ± 0.29 (range, 0.18 to 1.09) for CD44v3, 0.21 ± 0.08 (range, 0.40 to 1.13) for CD44v6, 0.45 ± 0.26 (range, 0.17 to 1.06)...

Table 1—Results of univariable Cox regression analysis to assess the respective associations between various factors and median PFS time and overall survival time for 45 dogs with multicentric high-grade BCL that were examined at a veterinary teaching hospital between November 2005 and April 2015.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>No. of dogs</th>
<th>Median (95% confidence interval) PFS (d)</th>
<th>Median (95% confidence interval) overall survival (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&lt; 7 y</td>
<td>7</td>
<td>164 (104–310)</td>
<td>204 (129–477)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>22</td>
<td>139 (98–275)</td>
<td>204 (157–307)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>23</td>
<td>235 (198–310)</td>
<td>286 (203–499)</td>
</tr>
<tr>
<td>Weight</td>
<td>&lt; 10.8 kg</td>
<td>22</td>
<td>218 (164–333)</td>
<td>238 (198–499)</td>
</tr>
<tr>
<td></td>
<td>≥ 10.8 kg</td>
<td>23</td>
<td>168 (104–286)</td>
<td>204 (159–487)</td>
</tr>
<tr>
<td>WHO clinical stage</td>
<td>I, II, III, or IV</td>
<td>22</td>
<td>164 (139–333)</td>
<td>228 (184–499)</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>23</td>
<td>197 (100–446)</td>
<td>204 (157–421)</td>
</tr>
<tr>
<td>WHO clinical substage</td>
<td>a</td>
<td>25</td>
<td>271 (171–442)</td>
<td>337 (204–499)</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>20</td>
<td>104 (98–304)*</td>
<td>197 (107–297)*</td>
</tr>
<tr>
<td>Anemia</td>
<td>PCV &lt; 35%</td>
<td>12</td>
<td>129 (98–297)</td>
<td>197 (107–297)*</td>
</tr>
<tr>
<td></td>
<td>PCV ≥ 35%</td>
<td>33</td>
<td>228 (164–429)*</td>
<td>271 (198–337)*</td>
</tr>
</tbody>
</table>

*Within a variable and column, value differs significantly (\( P < 0.05 \)) from the corresponding value for the other category.
for CD44v7, and 7.66 ± 1.74 (range, 4.83 to 10.7) for CD44w. For the 45 dogs with BCL, the mean ± SD relative mRNA expression was 0.29 ± 0.43 (range, 0.03 to 2.15) for CD44v3, 0.13 ± 0.19 (range, 0.01 to 0.76) for CD44v6, 0.43 ± 0.56 (range, 0.02 to 3.13) for CD44v7, and 5.59 ± 9.09 (range, 0.47 to 53.45) for CD44w (Figure 2). Although the mean relative mRNA expression for each CD44 variant isoform in dogs with BCL was numerically lower than that for control dogs, that difference was not significant.

**Prognostic value of CD44 whole and variant isoform mRNA expression**

The overall response rate to chemotherapy for dogs with BCL and low mRNA expression was numerically greater than that for dogs with high mRNA expression for all isoforms evaluated, and that difference was significant for all isoforms evaluated except CD44v7 (Table 2). However, the $P$ value for comparison of the overall response rate for dogs with low expression of CD44v7 (19/31 [61%]) with that for dogs with high expression of CD44v7 (4/14 [29%]) was close to the cutoff for significance ($P = 0.055$). The median PFS and overall survival for dogs with high mRNA expression were significantly less than the median PFS and overall survival for dogs with low mRNA expression for all isoforms evaluated.

Age, sex, WHO clinical stage and substage, and proportion of dogs with anemia did not differ significantly between dogs with high and low mRNA expression for any of the CD44 isoforms evaluated. Results of multivariable Cox regression analysis revealed that WHO clinical substage had the strongest association (and thus had the best prognostic value) with both PFS and overall survival time. Presence of anemia and mRNA expression of all CD44 variant isoforms evaluated were not significantly associated with PFS or overall survival. However, results of post hoc calculations indicated that the power to detect a significant association between those variables and PFS or overall survival time was low.

![Figure 2](https://example.com/figure2.png)
Table 2—Overall response rate to chemotherapy, median PFS time, and median overall survival time for the dogs of Table 1 on the basis of relative mRNA expression of various CD44 isoforms.

<table>
<thead>
<tr>
<th>CD44 isoform</th>
<th>Relative expression group</th>
<th>No. of dogs</th>
<th>Overall response rate (No. [%] that responded to chemotherapy)</th>
<th>Median PFS (95% confidence interval) (d)</th>
<th>Median overall survival (95% confidence interval) (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44v3</td>
<td>High</td>
<td>11</td>
<td>1 (9)</td>
<td>76 (60–171)</td>
<td>157 (124–499)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>34</td>
<td>22 (65)</td>
<td>265 (197–310)</td>
<td>297 (203–477)</td>
</tr>
<tr>
<td>CD44v6</td>
<td>High</td>
<td>10</td>
<td>1 (10)</td>
<td>76 (60–171)</td>
<td>157 (124–499)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>35</td>
<td>22 (63)</td>
<td>265 (197–310)</td>
<td>297 (203–477)</td>
</tr>
<tr>
<td>CD44v7</td>
<td>High</td>
<td>14</td>
<td>4 (29)</td>
<td>98 (60–197)</td>
<td>157 (124–499)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>31</td>
<td>19 (61)</td>
<td>235 (198–333)</td>
<td>271 (218–487)</td>
</tr>
<tr>
<td>CD44w</td>
<td>High</td>
<td>12</td>
<td>3 (25)</td>
<td>98 (60–171)</td>
<td>157 (124–499)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>33</td>
<td>20 (61)</td>
<td>265 (164–310)</td>
<td>297 (203–477)</td>
</tr>
</tbody>
</table>

For each CD44 isoform, the mean relative mRNA expression was used as the cutoff to define the high and low expression groups.

Within an isoform, value differs significantly (P < 0.05) from that for the low expression group.

**CD44 variant isoform mRNA expression patterns for dogs with BCL**

Among the 45 dogs with BCL, the most common CD44 variant isoform mRNA expression pattern was CD44v3low–CD44v6low–CD44v7low (n = 30), followed by CD44v3high–CD44v6high–CD44v7high (10). Four dogs had a CD44v3low–CD44v6low–CD44v7high mRNA expression pattern, and 1 dog had a CD44v3high–CD44v6low–CD44v7low mRNA expression pattern.

**Discussion**

Results of the present study indicated that relative mRNA expressions of CD44v3, CD44v6, CD44v7, and CD44w in lymph node aspirate specimens obtained from dogs with multicentric high-grade BCL (dogs with BCL) were numerically, but not significantly, lower than the relative mRNA expressions of those CD44 isoforms in lymph node aspirate specimens obtained from healthy control dogs. Results of another study also indicate that CD44w expression is lower in dogs with BCL than in healthy dogs. In human medicine, CD44w expression in lymph node cells of patients with acute B-cell lymphoblastic leukemia or lymphoma and Burkitt lymphoma is lower, whereas in lymph node cells of patients with mantle zone lymphoma is equal to or greater than that in healthy lymph nodes. CD44v3 expression varied greatly among the dogs with BCL evaluated in the present study, with the expression of CD44v3 whole and variant isoforms in the lymph nodes of some dogs with BCL being greater than or less than the expression of the corresponding isoforms in the lymph nodes of healthy dogs.

Because the expression of standard and variant isoforms of CD44 is associated with prognosis in human patients with various forms of cancer,5,6 we wanted to investigate whether the same was true in dogs with BCL. In another study, CD44w expression was negatively associated with disease-free survival for dogs with BCL. In the present study, when the mean relative mRNA expression for a given CD44 isoform was used as the cutoff to classify dogs as having high or low expression of that isoform, dogs with high expression had a worse overall response rate, median PFS time, and median overall survival time (proxies for prognosis) than did dogs with low expression across all isoforms evaluated. Moreover, when the BCL-affected dogs of the present study were classified as having high expression of CD44v6, they typically had high expression of all 3 CD44 variant isoforms and vice versa. Dogs with high expression of CD44v6 had the next to lowest overall response rate to chemotherapy (1/10 [10%]) and the lowest median PFS (76 days) and median overall survival (157 days) times, which suggested it was a negative prognostic indicator. In human patients with NHL, CD44v6 expression is inversely related to overall survival and is used as an independent prognostic indicator. In the present study, results of the multivariable analysis did not identify CD44v6 expression as an independent prognostic indicator; instead, those results suggested that CD44v6 expression might be associated with WHO clinical substage in dogs with BCL. Results of post hoc power analysis indicated that it would have been necessary to evaluate approximately 100 dogs with BCL to detect a moderate effect size (d = 0.5) between CD44v6 expression and WHO clinical substage. Additional research is necessary to elucidate whether CD44 variant isoform expression can be used as a prognostic indicator in dogs with BCL. Dogs of the present study with high expression of CD44v3 had the lowest overall response rate (1/11 [9%]) and a median PFS time and median overall survival time that were equal to those for dogs with high expression of CD44v6. CD44 variant exon 3 expression is not associated with prognosis in human patients with high-grade NHL.

In human patients with DLBCL, CD44v6 expression is positively associated with the development of resistance to CHOP-based chemotherapy protocols and a poor prognosis. Results of the present study suggested that high expression of CD44 variant isoforms, particularly CD44 variant exon 3 and CD44v6, was associated with a poor prognosis (low overall response rate, median PFS time, and median overall survival time), which might have been caused by the development of resistance to the CHOP-based chemotherapy protocol (UW-25) used to treat the dogs. The role of CD44v3 and CD44v6 in the development of chemoresistance in dogs with BCL requires further research.
The mechanism by which CD44 variant isoforms induce chemoresistance has not been elucidated for either human or veterinary patients. Some studies indicate that CD44v6 activates the Akt pathway and inhibits apoptosis9,90 and that CD44v6 interacts with the Oct4, Sox2, and Nanog proteins, resulting in cisplatin resistance.62 Development of a canine lymphoma cell model with high CD44 variant isoform expression would be useful for investigating the relationship between CD44 variant isoform expression and chemoresistance.

In the present study, the most common CD44 variant isoform mRNA expression pattern identified for dogs with BCL was CD44v3low–CD44v6low–CD44v7low (n = 30), followed by CD44v3high–CD44v6high–CD44v7high (10). CD44 variant isoform mRNA containing variant exons 3, 6, and 7 has been identified in canine lymphoid tissues.36 CD44 variant isoform mRNA containing variant exons 3 to 10 (CD44v3-10) is common in rats53,64 and humans.65,66 The fairly high prevalence (10/45 [22%]) of BCL-affected dogs with the CD44v3high–CD44v6high–CD44v7high expression pattern and the fact that high expression of those isoforms was negatively associated with overall response rate, PFS time, and overall survival time may indicate that CD44v3-10 expression is upregulated in tumor cells of dogs with BCL. Only 4 dogs of the present study had a CD44v3low–CD44v6low–CD44v7high expression pattern, and 1 dog had a CD44v3high–CD44v6low–CD44v7low expression pattern. Various CD44 variant isoforms containing a variant exon 7 have been identified in the lymph nodes of healthy dogs.55 Further research is necessary to investigate the expression patterns of CD44 variant isoforms and the associations of those patterns with the clinical characteristics of dogs with lymphoma.

A limitation of the present study was that fine-needle aspirate specimens obtained from the lymph nodes of the healthy dogs did not undergo cytologic evaluation to confirm that those nodes were cytologically normal. However, we believe it was unlikely that any of those lymph nodes were neoplastic because they were not enlarged and all control dogs were considered clinically normal on the basis of results of a physical examination. Other limitations of this study included the fact that the location of the lymph nodes aspirated was not recorded or was unknown for the dogs with BCL, and the expression and function of all CD44 variant isoform proteins could not be examined.

In the present study, the mean relative mRNA expressions of CD44v3, CD44v6, CD44v7, and CD44v10 in lymph node aspirate specimens obtained from dogs with BCL were numerically, but not significantly, lower than the mean relative mRNA expressions of those CD44 isoforms in lymph node aspirate specimens obtained from healthy control dogs. However, dogs with BCL that had high expression of CD44v3, CD44v6, and CD44v7 had a lower overall response rate to chemotherapy, median PFS time, and median overall survival time (ie, a poorer prognosis) than dogs with BCL that had low expression of those CD44 isoforms, likely because those isoforms are associated with the development of chemoresistance.

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Footnotes
a. Kitayama Labe Co Ltd, Nagano, Japan.

b. Zenoaq, Fukushima, Japan.


e. ReverTra Ace qPCR RT Master Mix with gDNA Remover, Toyobo Co Ltd, Osaka, Japan.


g. geNorm for Windows, version 3.0, Ghent University, Ghent, Belgium.

h. Thunderbird SYBR qPCR Mix, Toyobo Co Ltd, Osaka, Japan.

i. Thermal Cycler Dice Real Time System TP800, Takara Bio, Shiga, Japan.

j. JMP, version 11.2.0, SAS Institute, Cary, NC.


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53. Saba CF, Thamm DH, Vail DM. Combination chemother-
apy with t-asparaginase, lomustine, and prednisone for re-


**Appendix**

Primer pairs used for real-time RT-PCR assays to determine mRNA expression of various CD44 isoforms in lymph node aspirate specimens obtained from 45 dogs with multicentric high-grade BCL and 10 healthy adult Beagles (controls).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Forward primer (5’-3’)</th>
<th>Position</th>
<th>Reverse primer (5’-3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44w</td>
<td>NM_001197022</td>
<td>CGCTCCGTGGCCTTGGCTTTGATT</td>
<td>1,020–1,042</td>
<td>CCCACCTGCTCCATTGCAATTGTT</td>
<td>1,106–1,129</td>
</tr>
<tr>
<td>CD44v3</td>
<td>L28932</td>
<td>CAAGTATCATCTCAGACGACG</td>
<td>260–279</td>
<td>GCTGGAGATAAAAATTGCATAC</td>
<td>349–372</td>
</tr>
<tr>
<td>CD44v6</td>
<td>L28932</td>
<td>GCAGTGGGTGGAGAATTTGAT</td>
<td>657–676</td>
<td>AGCGTGCCTGCTGATAATTCATGC</td>
<td>16–735</td>
</tr>
<tr>
<td>CD44v7</td>
<td>L28932</td>
<td>CAAGACAGCCTCCAGATCA</td>
<td>745–764</td>
<td>TTGGATGTTGAGATTGCTG</td>
<td>813–832</td>
</tr>
<tr>
<td>TBP*</td>
<td>XM849432</td>
<td>CTATTTCCTGGTCATCATAG</td>
<td>1,331–1,352</td>
<td>CCTGGCATCAGTCTTTTC</td>
<td>1,407–1,426</td>
</tr>
</tbody>
</table>

*Housekeeping gene; primer sequences used were reported previously.*

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