Utility of Polymerase Chain Reaction for Analysis of Antigen Receptor Rearrangement in Staging and Predicting Prognosis in Dogs with Lymphoma

Susan E. Lana, Tracey L. Jackson, Robert C. Burnett, Paul S. Morley, and Anne C. Avery

In lymphoid neoplasia, molecular assays to confirm clonality rely on the fact that lymphoid cells generally contain DNA regions with unique sequences, resulting from recombination of the V, D, and J genes. The purpose of this study was to determine the utility of polymerase chain reaction (PCR) for antigen receptor rearrangements (PARR) for molecular staging and predicting prognosis in canine lymphoma. We hypothesized that the PARR assay would offer a sensitive method for detecting neoplastic cells in blood, and that the presence of such cells would be a negative prognostic finding compared with dogs with no detectable circulating tumor cells. Eighty-six patients with histologically or cytologically confirmed lymphoma were studied from initial diagnosis until death or euthanasia. All patients had PARR assays of a representative tumor-infiltrated lymph node and peripheral whole blood. Sixty-two patients had clonal rearrangements detected in the lymph node and were able to be staged by PARR. Seventeen patients (27%) had no detectable tumor in their blood and 45 (73%) were blood positive. Our findings showed that (1) PARR correlated with clinical stage in that the PARR assay was more likely to detect tumor cells in blood in stage 5 lymphomas, (2) PARR was more sensitive for detecting circulating tumor cells than visual assessment of blood or bone marrow because 80% of stage 3 lymphomas were blood-PARR-positive, and (3) PCR stage was not prognostic for disease-free interval (DFI) or survival.

Key words: B cell; Clonality; Hematologic malignancy; T cell.

In recent years, molecular characterization of hematologic malignancies has lead to the development of useful and sensitive diagnostic and prognostic tools. The amplification of novel genetic material created by translocation (eg, the bcr-abl gene in chronic myelogenous leukemia) allows for detection of as few as 1 neoplastic cell in 10^6 cells. Sensitive detection of circulating tumor cells before treatment to determine the extent of disease and after treatment to determine the efficacy of therapy has resulted in improved outcome and monitoring of many types of leukemias. Recurrent genetic translocations have not yet been described for canine lymphoma or for most human non-Hodgkin's lymphomas. Because lymphoma is the result of clonal lymphocyte expansion, neoplastic populations of lymphocytes can be identified by establishing the presence of clonal populations of lymphocytes. Detection of clonality relies on the fact that lymphocytes contain DNA regions that are unique in length and sequence. In B cells, this is a result of the recombination of the V, D, and J gene segments to produce the CDR3 (complementarity determining region 3) that encodes the antigen-binding portion of the immunoglobulin (Ig) heavy chain. In T cells, the unique CDR3 region encoding T cell receptor gamma (TCRγ) is produced by recombination of the V and J regions. Current methodology uses polymerase chain reaction (PCR) to detect antigen receptor rearrangements (PARR), with primers for conserved regions of the V and J genes used to amplify the desired CDR3 region, followed by size separation of the product. A dominant band indicates a population of cells bearing a single-sized receptor (ie, a clonal expansion). Detection of clonal lymphocyte populations by this method is well-established in human medicine and has been described in dogs. The limits of detection in dogs using currently available techniques is 1 neoplastic lymphocyte in a population of 100 heterogeneous, nonneoplastic lymphocytes.

In human patients, detection of clonality generally has been used to distinguish neoplasia from hyperplasia for detecting minimal residual disease after chemotherapy in leukemia, and as a sensitive test for detecting circulating tumor cells in peripheral blood. Sensitive detection of tumor cells has proven to have prognostic significance in some, but not all, situations.

In dogs with lymphoma, clinical stage is considered a prognostic factor in some studies, with infiltration into the bone marrow or disease in the peripheral blood conveying a worse prognosis. Staging, in part, is based on subjective light-microscopic assessments of bone marrow and blood cytology. Visual detection of neoplastic cells in peripheral blood is relatively insensitive when compared with detection by PARR. Therefore, we hypothesized that detection of neoplastic cells in the peripheral blood by PARR would provide a more objective and discriminating method for determining prognosis in dogs with lymphoma compared with conventional clinical staging. Only a single study addressing the same question in human non-Hodgkin’s lymphoma (diffuse large B cell lymphoma) has been described. In that study, Mitterbauer-Hohendanner et al found that the presence of neoplastic lymphocytes in blood, bone marrow, or both by PARR was associated with a significantly poorer overall survival at 5 years (26%) compared with PARR-negative patients (66%).
Table 1. Clinical staging of lymphoma in dogs according to the World Health Organization (WHO).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tissue Involved</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Single lymph node</td>
</tr>
<tr>
<td>2</td>
<td>Regional lymph nodes, not crossing the diaphragm</td>
</tr>
<tr>
<td>3</td>
<td>Generalized lymph nodes, both sides of the diaphragm</td>
</tr>
<tr>
<td>4</td>
<td>Liver and/or spleen (±1–3)</td>
</tr>
<tr>
<td>5</td>
<td>Blood/bone marrow (±1–4)</td>
</tr>
</tbody>
</table>

Substage

a = without systemic signs
b = with systemic signs

Patients with PCR-positive but histologically negative bone marrow biopsies also had a decreased 5-year survival (37%), indicating that this molecular method may improve accuracy of staging in this population.

Here, we report the results of a similar study in a population of dogs with lymphoma.

Materials and Methods

Study Subjects

Between 2000 and 2003, blood and lymph node aspirates were collected on patients presented to the Oncology Service at Colorado State University for suspected or confirmed lymphoma. DNA was isolated and tested by the PARR assay on 111 patients. Additional diagnostic tests for staging purposes and treatment were performed without regard to results of the PARR assay. Samples from these patients were analyzed if they met the following inclusion criteria: (a) lymphoma was confirmed by cytology or histology, (b) treatment was carried out by the CSU Oncology Service, (c) complete clinical staging, including bone marrow aspirates was performed, according to World Health Organization (WHO) standards (Table 1), and (d) adequate follow-up was available. Eighty-six patients met these criteria. Disease-free interval (DFI) in days was calculated from the time remission was achieved to the time of disease progression. Survival time in days was calculated from the time of diagnosis to the time of death. Each patient was given a clinical stage designation and a PARR stage designation. Clinical stage (CS) was defined as WHO stage 1–5 (Table 1) and PARR stage (PS) was defined as 0 (no evidence of malignancy in the blood) or PARR stage 5 (presence of clonal malignancy in the blood). Patients were placed into 1 of 3 treatment categories: no treatment or prednisone alone, single-agent chemotherapy (typically doxorubicin), or multiple-drug therapy (typically a combination of 4 or 5 drugs or CHOP-based protocol).

PCR for PARR

DNA from lymph node and peripheral blood samples was isolated using a commercially available kit by previously described methods. For peripheral blood, DNA was extracted from 200 μL of whole blood. From this extraction, the quantity of DNA added to each reaction was equivalent to 100 and 500 ng of DNA. For lymph-node aspirates, DNA from the entire aspirated contents was isolated as described in Burnett et al using a QIAmp DNA minikit. The final volume used for DNA elution was 150 μL. Of this, 4.5 μL was used for each DNA amplification, representing 3% of the original aspirated material. This volume was routinely equivalent to between 100 and 500 ng of DNA per reaction. Amplification of immunoglobulin and T cell receptor gamma sequences was performed using previously described primers and conditions. Briefly, each sample was amplified by 2 sets of primers for immunoglobulin gene segments and 1 set of primers for TCRγ gene segments. A DNA positive control reaction also was run with each sample to amplify the constant region gene of IgM and ensure adequate DNA in the sample. A negative control also was run on each gel to ensure no contamination was present. A reaction was considered positive if 1 or more dominant and discrete clonal bands were present on the gel after electrophoresis. A reaction was considered negative (not clonal) if no bands, a diffuse smear, or a ladder of faint bands was observed. The presence of multiple products in the latter 2 cases represents the presence of a heterogeneous population of lymphocytes carrying rearranged antigen receptor genes of different sizes.

Statistical Analysis

Data were recorded in a computer spreadsheet, verified against written records, and summarized using descriptive statistics. The risk of death over time after diagnosis was evaluated using Cox proportional hazards regression modeling. The outcome variables for these analyses were DFI (days) and survival time (days). Subjects that were lost to follow-up before recurrence of cancer or death were censored in analyses as of the date of the last follow-up.

Fig 1. PARR assay on a dog with PCR stage 0 (A) and on 2 different dogs with PCR stage 5 (B, C) disease. Each panel shows amplification of blood (lanes 1–4) and lymph node (lanes 5–8). Each lane contains polymerase chain reaction (PCR) product from the following reactions: 1.5, positive control for DNA (primers for Cmu); 2, primers for detecting immunoglobulin gene rearrangements (Ig major as described in Burnett et al); 3, primers for detecting immunoglobulin gene rearrangements (Ig minor as described in Burnett et al); 4.8, primers for detecting TCRγ gene rearrangements. In A, no product is detectable in blood (lane 2) but 1 is evident in the lymph node (lane 6). In B and C, product is detectable in both blood and lymph node, and products are of the same size in each.
Table 2. Signalment and World Health Organization (WHO) stage of patients in the study.

<table>
<thead>
<tr>
<th>Signalment</th>
<th>N = 86</th>
</tr>
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<tbody>
<tr>
<td>Female</td>
<td>34 (1 intact)</td>
</tr>
<tr>
<td>Male</td>
<td>52 (9 intact)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.3</td>
<td>7.6</td>
<td>(1.7–13.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substage a</td>
<td>43</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Substage b</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Total n = 86 (%)</td>
<td>44 (51)</td>
<td>21 (24)</td>
<td>21 (24)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR stage</th>
<th>0</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total n = 62 (%)</td>
<td>17 (21)</td>
<td>45 (79)</td>
</tr>
</tbody>
</table>

Patient signalment and WHO staging data are presented in Table 2. Blood distribution was as follows: 21 mixed breed dogs (24%), 15 Golden Retrievers (17%), 8 Labrador Retrievers (9%), with all other breeds having 3 or fewer representatives in the study population. There were no detectable associations between analysis outcomes (DFI or survival) and breed, sex, or age in proportional hazards models ($P > .15$). Clinical stage and treatment were associated with statistically detectable differences in DFI and survival and therefore were included in multivariable models evaluating immunophenotype and PCR stage. Histologic classification was available on 42 dogs. Of those, 15 (36%) were considered to have small-cell type (small cell, small cell cleaved, or diffuse small cell) and 27 (64%) were considered to have large-cell type (lymphoblastic, immunoblastic, or diffuse large cell) lymphoma. Forty-four patients had no histopathologic classification and were simply diagnosed with lymphoma based on cytologic examination of lymph node aspirates.

**PARR Assay on Lymph Node and Blood**

Of the 86 patients assayed, 24 (28%) had no amplification product identified in either lymph node or peripheral blood and could not be given a PS designation. The remaining 62 dogs had DNA product amplified in lymph nodes and were assigned a PS designation of 0 (clonal lymphocytes not detected in peripheral blood) or 5 (clonal lymphocyte population detected in peripheral blood). Examples of each stage are shown in Figure 1. Of those 62, 17 were PS 0 (21%) and 45 were PS 5 (79%) (Table 2).

**Results**

**Population Characteristics**

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**Response to Treatment**

It was first determined if the patient population under study was similar to cohorts of lymphoma patients reported in the literature by analyzing their response to treatment by type of therapy, clinical stage, and immunophenotype. The results of univariate analysis for each variable are shown in Table 3. Of the 86 dogs, 12 (14%) had no response to initial treatment. For all dogs, mean and median DFI were 203 and 140 days, respectively (range, 0–1290) and mean and median survival were 273 and 216 days, respectively (range, 4–1,290).

**By Clinical Stage.** When all patients were considered, treated or not, clinical stage predicted survival (Table 3). When stratified by chemotherapy protocol, clinical stage predicted survival only in patients that received multi-
agent chemotherapy, and stage 3 patients that received multiagent therapy had significantly better median survival (stage 3 = 340 days) compared with stage 5 dogs that received multiagent therapy (stage 5 = 141 days, \( P < .05 \)).

**By Clinical Substage.** Regardless of clinical stage, substage was a significant indicator of DFI and survival. The median survival time when all patients were considered was 243 (substage a) versus 44 (substage b, \( P < .0001 \)). When untreated patients or patients treated with prednisone only were eliminated from analysis, median DFI and survival time of substage a patients was significantly greater than that observed for substage b patients (\( P = .0004 \), Table 3). Therefore, substage was strongly predictive of outcome, regardless of chemotherapy protocol or clinical stage.

**By Phenotype.** Phenotype was assigned based on the type of clonal gene rearrangement detected in the sample. The presence of a clonally rearranged immunoglobulin gene indicates a B cell phenotype, and a clonally rearranged T cell receptor gene indicates a T cell phenotype.\(^{15} \) In samples in which PARR and flow cytometry or PARR and immunohistology were performed concurrently, agreement was 97% for B cell tumors and 93% for T cell tumors. The discordant samples included cases in which a clonal rearrangement was undetectable and cases that harbored clonal rearrangements in both IgH and the TCR\( \gamma \). Therefore, when a single clonal receptor rearrangement can be identified, the nature of the rearrangement can be used to unambiguously assign a phenotype to the tumor. Patients in which no clonally rearranged receptor was detectable were designated N. Immunophenotype data are presented in Table 3. One patient had both a single immunoglobulin and a single T cell receptor rearrangement and could not be assigned a phenotype. This dog was included in the N category. There was no significant difference in the mean or median DFI or survival of patients based on phenotype. This outcome was true even when patients that had not been treated with chemotherapy were eliminated from the analysis.

**Relationship between Clinical Stage and PCR Stage.** Overall, 79% of all patients had detectable neoplastic cells in their peripheral blood (PCR stage 5), as evidenced by the presence of the same clonal population seen in the lymph node. By contrast, only 21% of the 62 patients that could be typed by PCR were characterized as clinical stage 5 (Table 4). Only 1 of the patients that was clinical stage 5 did not have tumor detected in peripheral blood, whereas 19 of the patients that were characterized as clinical stages 3 and 4 did not have detectable tumor in peripheral blood. Thus, patients with cytologically detectable tumor cells in their peripheral blood were more likely to be PCR positive.

**Utility of the PCR Assay for Predicting Outcome.** Treated patients with detectable tumor in their peripheral blood were more likely to be PCR positive. The presence of a clonally rearranged immunoglobulin gene indicates a B cell phenotype, and a clonally rearranged T cell receptor gene indicates a T cell phenotype. The presence of neoplastic cells in peripheral blood detected by PARR did not predict DFI or survival time. Blood positivity furthermore did not predict survival when examining the more homogeneous group of dogs with stage 3 B cell tumors. These findings evidenced by the presence of the same clonal population seen in the lymph node. By contrast, only 21% of the 62 patients that could be typed by PCR were characterized as clinical stage 5 (Table 4). Only 1 of the patients that was clinical stage 5 did not have tumor detected in peripheral blood, whereas 19 of the patients that were characterized as clinical stages 3 and 4 did not have detectable tumor in peripheral blood. Thus, patients with cytologically detectable tumor cells in their peripheral blood were more likely to be PCR positive.

**Detection of Clonal Lymphocyte Populations in Bone Marrow.** In 34 dogs with positive results on lymph node, we also examined bone marrow for tumor by PARR. Eighty percent of clinical stage 5 dogs had tumor in their bone marrow by PARR (\( n = 10 \)). Sixty percent of dogs (\( n = 24 \)) with clinical stage 3 or 4 lymphoma had tumor in their bone marrow by PARR. As with the blood samples, PARR stage tended to correlate with clinical stage, and clinical stage 5 dogs had an increased tendency to have PARR-detectable tumor in their blood and bone marrow (data not shown).

We found no examples of patients that were PARR positive in bone marrow and not blood (\( n = 23 \)). Of the 11 dogs with negative bone marrow, 4 were positive in blood. These 4 blood-positive, marrow-negative cases consisted of clinical stage 3 (\( n = 1 \)), stage 4 (\( n = 2 \)), and stage 5 (\( n = 1 \)) cases. Having PARR-positive bone marrow did not correlate with disease outcome in any subgroup.

**Discussion**

In this study, we used a sensitive technique for detecting circulating neoplastic lymphocytes in the peripheral blood of dogs with lymphoma to determine if the assay could be used as a sensitive detector of circulating tumor cells and to determine if the presence of tumor in the blood was a negative prognostic factor. The PARR assay demonstrated that 76% of patients with stage 3 and 4 lymphoma, in which blood and bone marrow were visually judged to be free of tumor, nonetheless had circulating neoplastic lymphocytes. The presence of neoplastic cells in peripheral blood detected by PARR did not predict DFI or survival time. Blood positivity furthermore did not predict survival when examining the more homogeneous group of dogs with stage 3 B cell tumors. These findings
contrast with the one available similar study in human patients, in which the finding of circulating tumor cells predicted a worse outcome. Several differences between that study and ours, however, should be noted. Only diffuse large B cell lymphomas were included in the study of human patients. Our study population represented a much more diverse group with respect to histologic subtype, immunophenotype, and treatment. Effect of PCR stage may have been obscured by the potentially greater impact of these variables on prognosis.

We compared our population of lymphoma patients with those previously reported in the literature with regard to clinical stage, clinical substage and immunophenotype, and ability to predict outcome. Consistent with previous studies, clinical stage was predictive of survival in treated patients and clinical stage was strongly predictive. In our patients, immunophenotype did not have prognostic significance with respect to outcome. This finding is in contrast with several studies demonstrating that dogs with T cell lymphomas have shorter DFI and survival time. In human non-Hodgkin’s lymphoma, further classification into cytohistologic subtypes has shown that certain subgroups of T cell lymphoma exist that are divergent in response to therapy and prognosis. In veterinary medicine, a recent report by Ponce et al identified similar subgroups of canine T cell lymphoma. They demonstrated that T cell lymphomas classified as small cell had significantly better survival than all other tumor types, indicating that canine T cell lymphomas likely represent a diverse group of diseases with different biological behavior. In our patient population, only 8 of 13 T cell lymphoma cases had histologic samples available and 5 were classified as small cell or low grade. This finding could account for the improved outcome in these patients because low-grade lymphoma has been associated with longer survival. We also compared DFI and survival in patients with no detectable rearrangements. These patients were no different with respect to outcome than patients with detectable rearrangements. This finding suggests that tumors without detectable rearrangements do not represent a biologically unique subpopulation of neoplasms. More likely, these cases represent tumors that use V and J regions that are not compatible with the primers employed in the PARR assay.

In conclusion, our findings have several implications for testing patients with lymphoma using the PARR technique. First, because of the high rate of tumor detection in peripheral blood, it may be possible to use this assay as an objective monitor of response to therapy in a variety of different types of lymphomas. Studies addressing this question are underway. Second, we have shown that testing both blood and marrow in lymphoma patients offers no additional prognostic value. Clinical staging together with histologic phenotype continue to be the most important elements of prognosis in canine lymphoma. Additional studies using PARR may demonstrate that, within a single histologic subtype, the presence of circulating tumor cells offers prognostic value.

Footnotes

4. Qiagen, Valencia, CA
5. Excel 2002, Microsoft Corporation, Redmonds, WA

Acknowledgments

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References