RESEARCH ARTICLE

Preferential use of unmutated immunoglobulin heavy variable region genes in Boxer dogs with chronic lymphocytic leukemia

Emily D. Rout *, Robert C. Burnett, Julia D. Labadie, Janna A. Yoshimoto, Anne C. Avery

Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, United States of America

* Emily.Rout@colostate.edu

Abstract

Human chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease, and immunoglobulin heavy variable region (IGHV) gene mutational status is an important prognostic marker. IGHV mutational status has not been previously examined in canine CLL. We sequenced the IGHV-D-J rearrangements from 55 canine patients with CLL, including 36 non-Boxer and 19 Boxer dogs. The majority of non-Boxers (75%) had mutated IGHV genes, whereas the majority of Boxers (79%) had unmutated IGHV genes. IGHV3-41 and IGHV3-67 gene usage was significantly higher in Boxers with CLL compared to non-Boxers. Additionally, 11 Boxers with large B-cell lymphoma and the normal IGHV repertoire of six control dogs (three Boxers and three non-Boxers) were sequenced. IGHV3-41 was preferentially used in Boxers with other forms of lymphoma and without lymphoproliferative disease. However, preferential use of unmutated IGHV genes was unique to Boxers with CLL, suggesting Boxers may be a valuable model to investigate unmutated CLL.

Introduction

Human chronic lymphocytic leukemia (CLL) is the most common leukemia of adults in the Western world [1,2]. The disease has a variable clinical course, with wide ranges in time to progression and survival [3]. Analysis of the immunoglobulin genes has been crucial in understanding CLL pathogenesis and identifying subsets of patients with different clinical courses. Early studies identified restricted immunoglobulin heavy variable region (IGHV) gene usage in CLL compared to normal B-cells [4]. Later, studies demonstrated that the mutational status of the IGHV genes is highly prognostic and divides patients into subsets with different clinical outcomes [5,6]. Patients with mutated IGHV genes have a more favourable clinical course, while patients with unmutated IGHV genes have a poorer prognosis. Subsequently, subsets of unrelated CLL individuals were found to have highly similar to identical B-cell receptor immunoglobulins (stereotyped BCR) [7], which allowed for further stratification of patients and prognostication for certain subsets.
IGHV mutational status continues to be a major prognostic factor in human CLL [8] and more recently was shown to predict response to therapeutic agents [9]. The European Research Initiative on CLL has helped to establish standard methods for accurate analysis of mutational status [10,11]. Mutational status is determined by amplifying and sequencing the IGHV region, aligning the sequence to immunoglobulin databases, and calculating the percent identity between the case sequence and closest germline IGHV gene. Germline identity >98% is consistent with unmutated CLL, while cases with <98% identity constitute mutated CLL cases.

Canine B-cell chronic lymphocytic leukemia shares many features with human CLL. The disease is characterized by a clonal expansion of small B-cells in the peripheral blood. In people, the expanded B-cell population usually co-expresses CD5 and CD23 [12]. Canine CLL cells do not express the CD5 antigen and a CD23 antibody is not available in dogs. However, the clinical presentation and clinical course in dogs appear similar to that seen in human patients. The disease affects older dogs, with a median age at diagnosis ranging from 8–11 years [13–16]. Lymphadenopathy and splenomegaly are common, affecting approximately 50% of patients [13]. Cytologic review reveals the majority of lymphocytes are small with condensed chromatin and no apparent nucleoli, with fewer yet variable numbers of pro-lymphocytes. Anemia is relatively common, affecting 25–53% of patients across two studies, and thrombocytopenia and neutropenia are rare [13,14]. While it appears that many patients have indolent disease [14,17], one study [17] found a wide range in survival times (25 to >1000 days).

IGHV gene usage and mutational status have not previously been studied in canine CLL patients. Bao et al. [18] characterized the canine immunoglobulin heavy chain variable region, identifying 80 IGHV genes, 6 IGHD genes, and 3 IGJ genes. These gene names have been modified to adhere to the conventions of the international ImMunoGeneTics (IMGT) information system (http://imgt.cines.fr, [19–21]), and the new names are used in this study (personal correspondence from M-P Lefranc; unreferenced). IGHV genes were classified into three subgroups, with 76/80 genes belonging to subgroup IGHV3 (previously VH1). Recently, Martin et al. have expanded the canine immunoglobulin locus annotation, describing 83 IGHV genes and 6 IGJ genes [22]. Three studies have shown that canine IGHV-D-J rearrangements predominantly use IGHV3 subgroup genes [18,23,24], with IGHV3-38 (previously VH1-44) and IGHV3-19 (previously VH1-62) preferentially used in one study [18]. Heavy chain CDR3 length ranged from 7 to 17 amino acids (AA) in one study [18], and 5 to 27 AA in another [23]. IGHV gene usage and mutational status were investigated in canine diffuse large B-cell lymphoma [25,26], where IGHV3-38 was most frequently used.

We investigated IGHV gene usage and mutational status in a cohort of canine CLL patients, and compared the repertoire to patients with large B-cell lymphoma and to normal B-cells. We hypothesized that canine CLL patients would have a skewed IGHV gene repertoire and variable mutational status.

Materials and methods

Diagnostic criteria for CLL cases

CLL cases were selected from peripheral blood samples submitted to the Colorado State University Clinical Immunology (CSU-CI) laboratory for flow cytometric immunophenotyping. Flow cytometry was performed as previously described [27] and antibody combinations are listed in Table 1. CLL cases were defined as having >5,000 lymphocytes/μL, with a homogeneous expansion (>60%) of small lymphocytes expressing the B-cell marker CD21. Antibodies for CD19 and CD20 are not available in the dog but anti-CD21 reliably detects B-cells when combined with T-cell antibodies. Intracellular flow cytometry with CD79a and Pax5 antibodies is also available in the dog to detect B-cells, but these antibodies are not used in routine
Table 1. Antibody panels used for immunophenotyping.

| Tube | Antibody specificity and fluorochrome |
|------|--------------------------------------|
| Panel 1 (two color) |  |
| 1 | None |
| 2 | M’ IgG1-FITC/CD45-PE |
| 3 | CD18-FITC/M IgG1-PE |
| 4 | CD4-FITC/CD8-PE |
| 5 | CD5-FITC/CD21-PE |
| 6 | CD3-FITC/CD45-PE |
| 7 | CD4-FITC/CD14-PE |
| 8 | Class II MHC-FITC/CD34-PE |
| Panel 2 (multicolor) |  |
| 1 | M IgG1-FITC/M IgG1-PE/M IgG1-Alexa 647/M IgG1-Alexa 700/M IgG1-PE-Alexa-750/M IgG1-Pacific Blue |
| 2 | CD3-FITC/CD25-PE/CD5-APC/CD8-Alexa 700/CD4-Pacific Blue |
| 3 | Class II MHC-FITC/CD22-PE/CD21-Alexa 647 |
| 4 | Class II MHC-FITC/CD34-PE/CD5-APC/CD14-PE-Alexa 750 |
| 5 | Class II MHC-FITC/CD18-PE/CD5-APC/CD14-PE-Alexa 750/CD4-Pacific Blue |
| 6 | CD5-FITC/CD45-PE/CD21-Alexa 647 |

*Panel 1 samples were analyzed using a single laser Coulter XL (Beckman Coulter, Inc, Brea, CA).  
*Panel 2 samples were analyzed using a 3-laser Coulter Gallios (Beckman Coulter, Inc, Brea, CA).  
*M, mouse.

Unless otherwise noted, all antibodies were purchased from Bio-Rad, Hercules, CA. Clones are as follows: CD45 = YKIX716.13, CD18 = YFC118.3 (human CD18), CD4 = YKIX302.9, CD8 = YCA3E55.9, CD5 = YKIX322.3, CD21 = CA2.1D6, CD22 = RFB4 (human CD22, purchased from AbCam, Cambridge, MA), CD3 = CA17.2A12, CD14 = UCHM (human, used in panel 1) and CD14 = TUK4 (human, used in panel 2), class II MHC = YKIX334.2, CD34 = 1H6, CD25 = P2A10 (purchased from eBiosciences, San Diego, CA).

https://doi.org/10.1371/journal.pone.0191205.t001

immunophenotyping in our laboratory. B-cell size was classified as 'small' when the ratio of the geometric mean of B-cell to neutrophil forward scatter (FS) was <0.60, which correlates to a B-cell FS value <400 U on our flow cytometer. In previous studies, dogs meeting these diagnostic criteria predominantly had an indolent clinical course [17] and clinical characteristics [13] similar to mutated human CLL.

Clonality was confirmed in all cases using a PCR-based assay termed the PCR for antigen receptor rearrangements (PARR) assay [28,29], which detects clonal immunoglobulin gene rearrangements based on size and is similar to the method used in people [30].

CLL cases were selected at random from the CSU-CL database initially, with additional Boxers sequenced after discovering preferential IGHV gene usage in this breed.

Case selection for large B-cell lymphoma and control dog cohorts

Cases with large B-cell lymphoma were identified among lymph node aspirate samples submitted to the CSU-CL laboratory for immunophenotyping. To meet the criteria for large B-cell lymphoma, >60% of the large cells in the sample expressed CD21 [31], and the median FS of the CD21+ lymphocytes was >450 U. Histopathology was not performed, so further subtyping could not be determined.

Control dogs without evidence of lymphoproliferative disease were identified at necropsy or biopsy and lymph node sections were collected.
**Characterization of the canine IGHV locus**

In this study, we annotated the 80 germline genomic IGHV genes previously identified [18] using the guidelines established by the IMGT information system (http://imgt.cines.fr, [19–21]). A subset of these annotations are depicted in Fig 1. Productive rearrangements used for analysis had these conserved IMGT AA and motifs, and an open reading frame absent of stop codons or frameshift mutations.

Additionally, we identified new IGHV and IGHJ genes compared to previous annotations [18,22]. Contiguous germline DNA sequence from dog chromosome 8 (NCBI Reference Sequence: NW_003726071.1) encoding all previously identified IGHV genes through the immunoglobulin heavy constant mu (IGHM) gene was analyzed using the Geneious ‘Dotplot’ function with consensus IGHV and IGHJ gene probes generated via multiple sequence alignments (http://www.geneious.com, version 5.5.8 [32]). All identified genes were compared to those reported previously [18,22]. Newly identified genes were confirmed using BLAST search of dog genomic sequences (available at ncbi.nlm.nih.gov/projects/mapview, [33]) and/or dog BLAT search (available at genome.ucsc.edu, [34]), using the CanFam3.1 assembly (GenBank Assembly ID GCA_000002285.2).

**Sequencing of canine IGHV genes**

For CLL cases, genomic DNA was extracted from 200 μL peripheral whole blood using the QIAamp DNA mini kit (Qiagen, Germantown, MD). DNA was amplified by PCR with a consensus IGHV3 subgroup-specific forward primer binding the leader exon (L-PART1) and
Table 2. PCR amplification primer sequences and cycling conditions for IGHV sequencing.

| Primer sequence                          | Primer location | Cycling conditions                                                                 |
|------------------------------------------|-----------------|------------------------------------------------------------------------------------|
| **Protocol 1**                           |                 |                                                                                     |
| Forward: ATGGAGTGCTGTGCTCCGCT            | L-PART1         | Denaturation step of 15 minutes at 95°C, followed by 10 cycles of 94°C for 30 seconds, 64°C for 30 seconds with the temperature decreasing 0.5°C every cycle, and 72°C for 1.5 minutes each, followed by 30 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 1.5 minutes each, and final extension step of 7 minutes at 72°C. |
| Reverse primer pool*:                    |                 |                                                                                     |
| TGGATCTCTACGGTAAAGGGA                    | IGHJ1-INTRON    |                                                                                     |
| CCGAAAGAGACAGACAGGAGAGTAG               | IGHJ2-INTRON    |                                                                                     |
| CCCAGGGCTTCTGGAATAG                    | IGHJ3-INTRON    |                                                                                     |
| CCCAGGAGAAAGAGAGAGAGAGAGG               | IGHJ4-INTRON    |                                                                                     |
| ATCTCTCTTGCTACAGTTCTCT                  | IGHJ5-INTRON    |                                                                                     |
| CCCAGGAGAGAGAGAGAGAGAGAGAGG             | IGHJ6-INTRON    |                                                                                     |
| **Protocol 2**                           |                 |                                                                                     |
| Forward: ATGGAGTGCTGTGCTCCGCT           | L-PART1         | Denaturation step of 5 minutes at 95°C, followed by 40 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute each, and final extension step of 10 minutes at 72°C. |
| Reverse primer pool:                    |                 |                                                                                     |
| ACCCTGACCAGCACTGACCC                    | J-REGION IGHJ2, |                                                                                     |
| TGAGAGAGAGAGAGAGAGAGAGAGG               | J-REGION IGHJ4  |                                                                                     |
|                                                                 | J-REGION IGHJ6  |                                                                                     |

*Protocol 1 reverse primer pool: amplification was first attempted with a pool of IGHJ2, IGHJ4, and IGHJ6 primers. If the clone was not identified, amplification was attempted in a second wave with a pool of IGHJ1, IGHJ3, and IGHJ5 primers. These primer sequences are located in the introns downstream of the IGHJ genes (see Fig 1B).

L-PART1: first exon, encoding the first part of the leader sequence in an IGHV gene; J-REGION: coding region of the IGHJ genes (see Fig 1B).

https://doi.org/10.1371/journal.pone.0191205.t002

either a pool of reverse primers binding the IGHJ intron regions (protocol 1) or the IGHJ coding regions (protocol 2) (Table 2; Fig 1). Earlier cases were sequenced using IGHJ intron primers, but these primers did not amplify rearrangements as effectively as IGHJ coding region primers, so the majority of cases were sequenced with protocol 2. While either leader primers or FR1 region primers may be used to amplify the IGHV region, leader primers were selected because they allow analysis of the whole IGHV gene, while FR1 primers exclude the 5’ portion [10]. Amplified products were purified using the DNA Clean & Concentrator-5 kit (Zymo Research Corp, Irvine, CA), ligated into a pDrive T-vector (Qiagen, Germantown, MD) and transformed into competent TG1 bacteria (Zymo Research Corp, Irvine, CA). Inserts were directly sequenced by Sanger sequencing from multiple independent clones (8–16 total). Tumor-associated IGHV-D-J rearrangements were identified as predominant repeated sequences with identical CDR3 sequences and a nucleotide length equal to the fragment size detected in the PARR assay. The cloning and sequencing protocol was identical for large B-cell lymphoma cases, except that DNA was isolated from fresh lymph node aspirates.

For control dogs without lymphoproliferative disease, more IGHV-D-J rearrangements were sampled per dog, since there was not a clonal B-cell population present. For each case, the PCR and cloning conditions were the same, except that DNA was extracted from a section of fresh whole lymph node and 100 independent clones were selected for sequencing, rather than 8–16 clones.

Alignment and determination of mutational status and CDR3 length

Patient sequence from the first nucleotide of FR1 through the conserved 2nd-CYS in FR3 was queried against the CanFam3.1 assembly using NCBI BLAST (https://blast.ncbi.nlm.nih.gov). The most similar reference nucleotide sequence identified in this BLAST search was compared to our annotated library of germline reference IGHV genes (S1 Table) to determine IGHV
gene usage in the patient. When more than one reference germline IGHV gene was identified as a possible match, the intron sequence was used to confirm the patient IGHV gene identity. Mutational status was determined using guidelines adapted from human medicine [10]. The percentage of identity was calculated based on the number of nucleotide differences between the patient sequence and reference sequence in the V-REGION. Percent identity was calculated from the first nucleotide of FR1 to both the 2nd-CYS [35] and to codon 105 [10], but this boundary difference only changed the mutational status for one of the 389 sequences analyzed in this study. The sequence affected was a sequence from a control dog and did not have a statistical impact, so all sequences presented here were analyzed one way, to the 2nd-CYS. The following formula was used: IGHV identity (%) = 100 – (mutations/aligned IGHV region length × 100), with an insertion or deletion of multiple nucleotides counted as one mutation. Cases were classified as unmutated when the percent identity was >98% and mutated when percent identity was <98%, according to the convention used for human CLL [10,36]. Mutated CLL cases were further categorized into those with percent identity between 96%-97.9% and those with <96% sequence homology [37,38]. The CDR3 length was identified by determining the number of codons from the first codon following 2nd-CYS to the last codon preceding TRP-118 [21]. Additionally, a subset of IGHV-D-J rearrangements were analyzed using the NCBI IgBLAST web-based program (http://www.ncbi.nlm.nih.gov/igblast/, [39]) and IMGT/V-QUEST (http://www.imgt.org/IMGT_vquest/vquest, version 3.4.8 [35,40]), to confirm that the same results were obtained by other analysis methods. Custom reference databases were used in IgBLAST by uploading the canine germline IGHV, IGHD and IGHJ gene libraries.

Statistical analysis
A chi-square test and Fisher exact tests were performed to determine the statistical significance of differences in IGHV gene repertoire and mutational status.

Results
Study population
A total of 55 patients with CLL were included in the study, including 29 females and 26 males. The median age at the time of sample collection was 10.5 years (range, 4.9–15.6 years; one unknown). Initially, CLL cases were selected at random, with no bias for breed. However, as it became apparent that Boxer dogs had a unique IGHV gene usage, additional Boxer dogs were sequenced. Therefore, of the 55 CLL patients sequenced, 19 dogs (35%) were Boxers. Twenty-one breeds were represented in the non-Boxer group (Table 3). The peripheral B-cell count ranged from 7,300–816,600 cells/μL. Except for four more historic cases, all of the cases had a B-cell:neutrophil size ratio <0.55, which is the size cut-off used by Bromberek et al. [13] to define CLL cases. Four cases from 2011–2012 had a size ratio between 0.55–0.60, but were included because subjectively CD21+ lymphocytes appeared small by flow cytometry. Two of these four cases had a cytology report describing the lymphocytes as small and mature and suspicious for CLL, and two cases did not have cytology review.

Eleven Boxer dogs with large B-cell lymphoma were sequenced, including four females and seven males. The median age at the time of sample collection was 8.5 years (range, 7.1–13.1 years).

Six dogs without lymphoproliferative disease were sequenced, including three Boxer dogs and three non-Boxer dogs. The Boxers ranged in age from 6.0–8.4 years and included one female and two males. The non-Boxers included one Labrador Retriever, one mixed breed dog, and one Chihuahua. The non-Boxers ranged in age from 9.0–13.0 years and included two
| Case No. | Breed | Identity (%) | Mutational status | IGHV | IGHJ | CDR3 length |
|---------|-------|--------------|-------------------|------|------|-------------|
| 1       | SHI   | 91.7         | Mutated           | IGHV3-75 | IGHJ6 | 17          |
| 2       | CDT   | 88.9         | Mutated           | IGHV3-47 | IGHJ4 | 16          |
| 3       | MIX   | 92.4         | Mutated           | IGHV3-47 | IGHJ6 | 13          |
| 4       | COC   | 93.1         | Mutated           | IGHV3-47 | IGHJ4 | 22          |
| 5       | RAT   | 87.8         | Mutated           | IGHV3-41 | IGHJ4 | 11          |
| 6       | BDC   | 99.0         | Unmutated         | IGHV3-41 | IGHJ4 | 16          |
| 7       | BIC   | 99.0         | Unmutated         | IGHV3-41 | IGHJ4 | 18          |
| 8       | AIR   | 100.0        | Unmutated         | IGHV3-41 | IGHJ4 | 14          |
| 9       | MIX   | 89.2         | Mutated           | IGHV3-38 | IGHJ2 | 16          |
| 10      | CKP   | 89.9         | Mutated           | IGHV3-38 | IGHJ4 | 14          |
| 11      | SHI   | 91.3         | Mutated           | IGHV3-38 | IGHJ6 | 16          |
| 12      | MLT   | 93.1         | Mutated           | IGHV3-38 | IGHJ6 | 17          |
| 13      | JRT   | 93.8         | Mutated           | IGHV3-38 | IGHJ4 | 14          |
| 14      | BIC   | 95.8         | Mutated           | IGHV3-38 | IGHJ4 | 12          |
| 15      | BIC   | 95.8         | Mutated           | IGHV3-38 | IGHJ4 | 17          |
| 16      | CRN   | 96.5         | Borderline        | IGHV3-38 | IGHJ4 | 13          |
| 17      | MIX   | 97.6         | Borderline        | IGHV3-38 | IGHJ2 | 12          |
| 18      | CSH   | 97.6         | Borderline        | IGHV3-38 | IGHJ4 | 23          |
| 19      | LAB   | 100.0        | Unmutated         | IGHV3-38 | IGHJ4 | 15          |
| 20      | WET   | 100.0        | Unmutated         | IGHV3-38 | IGHJ4 | 16          |
| 21      | CCR   | 94.8         | Mutated           | IGHV3-35 | IGHJ4 | 14          |
| 22      | MIX   | 91.0         | Mutated           | IGHV3-19 | IGHJ4 | 13          |
| 23      | SHI   | 91.0         | Mutated           | IGHV3-19 | IGHJ2 | 14          |
| 24      | CRN   | 94.1         | Mutated           | IGHV3-19 | IGHJ4 | 13          |
| 25      | SHI   | 94.8         | Mutated           | IGHV3-19 | IGHJ4 | 13          |
| 26      | MIX   | 94.8         | Mutated           | IGHV3-19 | IGHJ4 | 14          |
| 27      | PIT   | 95.5         | Mutated           | IGHV3-19 | IGHJ4 | 10          |
| 28      | MIX   | 100.0        | Unmutated         | IGHV3-19 | IGHJ4 | 14          |
| 29      | MIX   | 97.9         | Borderline        | IGHV3-12 | IGHJ2 | 15          |
| 30      | POM   | 98.6         | Unmutated         | IGHV3-9  | IGHJ4 | 17          |
| 31      | MIX   | 99.0         | Unmutated         | IGHV3-9  | IGHJ6 | 12          |
| 32      | LAB   | 92.0         | Mutated           | IGHV3-5  | IGHJ4 | 16          |
| 33      | BOR   | 95.1         | Mutated           | IGHV3-5  | IGHJ4 | 13          |
| 34      | SHI   | 95.5         | Mutated           | IGHV3-5  | IGHJ2 | 14          |
| 35      | STS   | 98.6         | Unmutated         | IGHV3-5  | IGHJ2 | 27          |
| 36      | LAB   | 95.8         | Mutated           | IGHV3-2  | IGHJ4 | 15          |
| 37      | BOX   | 99.0         | Unmutated         | IGHV3-67 | IGHJ4 | 11          |
| 38      | BOX   | 99.7         | Unmutated         | IGHV3-67 | IGHJ4 | 10          |
| 39      | BOX   | 100.0        | Unmutated         | IGHV3-67 | IGHJ4 | 10          |
| 40      | BOX   | 96.9         | Borderline        | IGHV3-41 | IGHJ4 | 14          |
| 41      | BOX   | 97.9         | Borderline        | IGHV3-41 | IGHJ2 | 17          |
| 42      | BOX   | 98.3         | Unmutated         | IGHV3-41 | IGHJ4 | 17          |
| 43      | BOX   | 99.9         | Unmutated         | IGHV3-41 | IGHJ6 | 18          |
| 44      | BOX   | 99.7         | Unmutated         | IGHV3-41 | IGHJ4 | 11          |
| 45      | BOX   | 99.7         | Unmutated         | IGHV3-41 | IGHJ4 | 11          |
| 46      | BOX   | 99.7         | Unmutated         | IGHV3-41 | IGHJ6 | 22          |
| 47      | BOX   | 100.0        | Unmutated         | IGHV3-41 | IGHJ4 | 12          |

(Continued)
females and one male. Five of six animals were deceased at the time of lymph node collection. One animal was alive at sample collection, diagnosed with mast cell tumor disease, and had five months follow up since sample collection. Two dogs died of heart failure and the remaining four dogs had non-lymphoid neoplasms. All six dogs had polyclonal immunoglobulin and T-cell receptor rearrangements by PARR, providing additional support that the dogs did not have lymphoproliferative disease (results not shown).

**Newly identified IGHV and IGHJ genes**

A combination of BLAST/BLAT searching and Dotplot analyses were employed to identify seven new germline IGHV genes and three new germline IGHJ genes compared to those originally described by Bao et al. [18], bringing the totals to 87 IGHV and 6 IGHJ genes. All seven newly identified IGHV genes belong to the predominant IGHV3 subgroup, and were named: IGHV3-76, IGHV3-71, IGHV3-47-1, IGHV3-21-1, IGHV3-4, IGHV3-NL1, and IGHV3-NL2 (S1 Table). Three of these seven new IGHV genes were also annotated recently by Martin et al [22]. Five of the new IGHV genes mapped to chromosome 8 and two (designated NL) mapped to an unplaced genomic scaffold. Only IGHV3-76 and IGHV3-NL1 were considered functional. These two genes had open reading frames and conserved IMGT motifs, including the conserved amino acids: 23 (1st-CYS), 41 (CONSERVED-TRP), 89 (hydrophobic) and 104 (2nd-CYS). Two IGHV genes previously described [18] were reordered to reflect the genomic location on chromosome 8: IGHV3-32 (previously VH1-6) and IGHV3-78 (previously VH1-49P). The truncated sequence of VH1-42 that was previously published [18] was expanded and the more complete gene was renamed IGHV3-40. The three new IGHJ genes were clustered with the previously reported IGHJ genes on a 1.8 kb span 3’ to the IGHV region, and the IGHJ numbering system was revised from that which is already published [18], to IGHJ1-IGHJ6, 5’ to 3’ (Fig 2). This new annotation of the IGHJ genes is consistent with that described by Martin et al [22].

**CLL IGHV repertoire and mutational status**

IGHV-D-J rearrangements from 55 CLL patients were examined (Table 3). Eleven IGHV genes were represented in the CLL patient cohort. In non-Boxers, IGHV3-38 (33.3%) and IGHV3-19 (19.4%) were the most commonly used IGHV genes, followed by IGHV3-41

---

**Table 3. (Continued)**

| Case No. | Breed* | Identity (%) | Mutational status b | IGHV | IGHJ | CDR3 length c |
|----------|--------|--------------|---------------------|------|------|---------------|
| 48       | BOX    | 100.0        | Unmutated           | IGHV3-41 | IGHJ4 | 13            |
| 49       | BOX    | 100.0        | Unmutated           | IGHV3-41 | IGHJ4 | 16            |
| 50       | BOX    | 100.0        | Unmutated           | IGHV3-41 | IGHJ6 | 17            |
| 51       | BOX    | 97.9         | Borderline          | IGHV3-38 | IGHJ4 | 13            |
| 52       | BOX    | 100.0        | Unmutated           | IGHV3-38 | IGHJ4 | 10            |
| 53       | BOX    | 100.0        | Unmutated           | IGHV3-38 | IGHJ4 | 14            |
| 54       | BOX    | 96.5         | Borderline          | IGHV3-19 | IGHJ4 | 12            |
| 55       | BOX    | 99.3         | Unmutated           | IGHV3-5 | IGHJ4 | 10            |

*Breed abbreviations: SHI, Shih-Tzu; CDT, Coton de Tulear; MIX, Mixed Breed; COC, Cocker Spaniel; RAT, Rat Terrier; BDC, Bearded Collie; BIC, Bichon Frise; AIR, Airedale Terrier; CKP, Cockapoo; MLT, Maltese; JRT, Jack Russell Terrier; CRN, Cairn Terrier; CSH, Chihuahua, Shorthair; LAB, Labrador Retriever; WET, Soft Coated Wheaten Terrier; CCR, Chinese Crested; PIT, Pit Bull Terrier; POM, Pomeranian; LBD, Labradoodle; BOR, Border Collie; STS, Schnauzer; BOX, Boxer.

bMutational status: mutated (<96% similarity); borderline (96–98% similarity); unmutated (>98% similarity).

*Heavy chain complementary-determining region 3 amino acid length.

https://doi.org/10.1371/journal.pone.0191205.t003
Fig 2. Dog IGHJ locus. The organization of the six canine IGHJ genes is shown, with the genomic locations on chromosome 8 identified (CanFam3.1, NCBI Accession NC_006590.3). Gene segments are shown as light grey bars, recombination signal sequences as dark grey bars, and splice signal sequences as black triangles. The three new IGHJ genes are IGHJ1, IGHJ2 and IGHJ5. The three IGHJ genes previously described [18] have been renamed from JH1, JH2 and JH3 to IGHJ3, IGHJ4 and IGHJ6, respectively, based on their genomic position. IGHC, immunoglobulin heavy constant genes.

https://doi.org/10.1371/journal.pone.0191205.g002

(11.1%) and IGHV3-5 (11.1%) (Fig 3A). In Boxers with CLL, IGHV3-41 (57.9%) was most commonly used, followed by IGHV3-38 (15.8%) and IGHV3-67 (15.8%) (Fig 3B). IGHV gene usage was significantly different between non-Boxers and Boxers for IGHV3-67 (p = 0.037) and IGHV3-41 (p < 0.001).

Among non-Boxers with CLL, 9/36 (25%) cases were classified as unmutated and 27/36 (75%) cases were mutated. Among Boxers with CLL, 15/19 (79%) cases were unmutated, while 4/19 (21%) cases were mutated. The majority of mutated non-Boxer CLL cases had a percent identity < 96% (24/36 (66.7%) cases), and few cases with a 96%–97.9% identity (3/36 (8.3%) cases). All four mutated Boxer CLL cases had a percent identity between 96%–97.9%, with none of the cases having a percent identity < 96%. The frequency of unmutated cases was significantly higher in Boxers with CLL compared to other breeds (p < 0.001).

The IGHJ gene repertoire was not significantly different between non-Boxer and Boxer CLL patients. Across the cohort of 55 patients, 3 IGHJ genes rearranged. IGHJ4 (72.7%) was the most frequently used IGHJ gene, followed by IGHJ6 (14.5%) and IGHJ2 (12.7%). The heavy chain CDR3 mean length was 14.6 AA (range, 10–27 AA) across all CLL cases. There was not a significant difference in CDR3 length between breed groups or between mutated and unmutated cases.

These results indicate that Boxers with CLL have preferential rearrangement of IGHV3-41 and that the majority of cases are unmutated, regardless of IGHV gene usage.

Large B-cell lymphoma IGHV repertoire and mutational status

To determine whether Boxers with other forms of lymphoproliferative disease have preferential rearrangement of IGHV3-41 or unmutated IGHV genes, IGHV-D-J rearrangements from 11 Boxers with large B-cell lymphoma were examined. IGHV3-41 (63.6%) was most commonly used, at a frequency similar to that seen in the Boxer CLL cohort (Fig 3C). 4/11 (36.4%) cases were classified as unmutated, and 7/11 (63.6%) cases were mutated. Among mutated cases, one case had a percent identity between 96%–97.9%, and remaining mutated cases had < 96% homology to germline. The IGHJ gene repertoire was similar to CLL, with IGHJ4 (72.7%) most frequently used, followed by IGHJ6 (18.2%) and IGHJ2 (9.1%). The CDR3 mean length was 13.8 AA (range, 10–20 AA). These results indicate that Boxers with large B-cell lymphoma preferentially use IGHV3-41, as seen in Boxers with CLL, but in the majority of cases the IGHV genes are mutated rather than unmutated.
Fig 3. Distribution of IGHV gene usage and mutational status in (A) non-Boxer dogs with CLL (n = 36), (B) Boxer dogs with CLL (n = 19), and (C) Boxer dogs with large B-cell lymphoma (n = 11). IGHV gene usage is reported as the percentage of patients using an IGHV gene within that cohort. There were significant differences in the IGHV gene usage between non-Boxers and Boxers with CLL for IGHV3-67 (p = 0.037) and IGHV3-41 (p < 0.001). There were significantly more unmutated cases in the Boxer CLL cohort, compared to non-Boxers with CLL (p < 0.001) and Boxers with large B-cell lymphoma (p = 0.026).

https://doi.org/10.1371/journal.pone.0191205.g003
Normal canine IGHV repertoire and mutational status

IGHV-D-J rearrangements from six control dogs without lymphoproliferative disease were examined, including three non-Boxers and three Boxers. The number of unique productive clones obtained for each animal ranged from 27 to 65 (Fig 4). Across the six dogs, which included 323 unique IGHV-D-J rearrangements, the most commonly used IGHV genes were
IGHV3-19 (28.2%), IGHV3-47 (24.5%), and IGHV3-41 (24.1%), followed by IGHV3-38 (6.5%), IGHV3-5 (6.2%) and IGHV3-2 (5.0%). There were significant differences in IGHV gene usage between individual animals, and between breeds. Boxers (Fig 4A–4C) used IGHV3-41 (p<0.001) and IGHV3-47 (p<0.001) significantly more than non-Boxers, and non-Boxers (Fig 4D–4F) used IGHV3-19 (p<0.001) and IGHV3-38 (p<0.001) significantly more than Boxers. There was no significant difference in mutational status between Boxers and non-Boxers. These results indicate that Boxers with normal B-cells preferentially use IGHV3-41 and IGHV3-47 compared to other breeds, and the majority of rearrangements are mutated.

**Discussion**

In this study, we investigated IGHV gene usage and mutational status in canine patients with CLL. Among non-Boxer patients with CLL, we found that the majority of cases (75%) were mutated, using the homology cut-off value of 98% as established in human CLL. The ratio of mutated to unmutated cases was slightly higher than seen in the human population [6,41]. Boxers were analyzed separately as they were preferentially sequenced over other breeds due to their skewed use of unmutated IGHV genes. Among Boxers with CLL, 79% of cases were unmutated and all of the mutated cases had a percent identity between 96%-97.9%, demonstrating that none of the Boxer CLL cases sequenced were highly mutated. These data suggested that the Boxer breed may be a useful model for unmutated CLL. However, the reference dog genome sequence was obtained by sequencing a Boxer [42]; therefore, we were concerned that the high homology between the Boxer CLL cases and reference genome may be due to breed bias. To address this question, we sequenced IGHV genes from additional Boxer dogs with large B-cell lymphoma and without lymphoproliferative disease and found that these dogs used predominantly mutated IGHV genes. The majority of IGHV genes from Boxers with large B-cell lymphoma (64%) were mutated. There was no significant difference in the number of unmutated IGHV gene rearrangements in normal Boxers compared to other breeds, suggesting use of unmutated IGHV genes in Boxers is specific to CLL.

We examined gene usage in CLL patients as well as the normal IGHV gene repertoire in six control dogs. IGHV3-38 and IGHV3-19 were most commonly used in non-Boxers with CLL and in the normal gene repertoire of two of the three non-Boxer control dogs. One of the three control non-Boxers had a gene repertoire more similar to the Boxers. Bao et al. [18] demonstrated a bias for IGHV3-38 and IGHV3-19 amongst three healthy dogs, and IGHV3-38 was preferentially rearranged in canine cases with diffuse large B-cell lymphoma [25,26]. Boxers with CLL, large B-cell lymphoma, and without lymphoproliferative disease preferentially used IGHV3-41, indicating IGHV3-41 gene usage is high in Boxers, regardless of disease status. IGHV3-47 was commonly used in all three control Boxers without lymphoproliferative disease, but was not used in Boxers with CLL or large B-cell lymphoma. IGHV3-67 was one of the more commonly used IGHV genes in Boxers with CLL, but was not used in any of the non-Boxers with CLL, Boxers with large B-cell lymphoma, or normal B-cell repertoires examined. This suggests IGHV3-67 may be preferentially used in Boxers with CLL, but the number of cases is small and additional cases are needed to verify this finding. A limitation of this study is that the leader primer used for sequencing is specific to the IGHV3 subgroup, which likely had a small effect on the normal repertoire of control dogs. The IGHV3 subgroup is the dominant subgroup in multiple studies [18,23,24], accounting for 90% or more of rearranged genes in one study [23], and we were able to identify the clone in 92% of CLL cases we attempted to sequence with an IGHV3-specific primer.
This study contributes to our understanding of the canine immunoglobulin heavy chain variable region. Seven new IGHV genes and three new IGHJ genes were identified compared to the original annotation from Bao et al [18]. Some of the additions may be attributed to different reference genome builds. Martin et al. recently described three of these new IGHV genes and the new IGHJ genes [22]. Slight differences in our annotations may be due to different consensuses used to search for new IGHV genes. Additionally, all of the IGHV genes were assessed for functionality and annotated using the IMGT guidelines. All productive rearrangements used for analysis had the four previously described conserved AA and the conserved Gly-X-Gly motif following codon 118. Eight of 389 sequences examined in this study had a residue other than the highly conserved PHE or TRP at codon 118, but were considered adequate for interpretation because the Gly-X-Gly was conserved [11].

A challenge of mutational analysis in dogs is the lack of knowledge about polymorphisms in IGHV genes across dog breeds and individuals. Ideally, canine CLL sequences would be compared to reference genome sequences from the same breed, but those are not available at this time. Additionally, the homology cut-off of 98% which distinguishes clinically distinct subsets of human CLL patients [5,6,43] may need to be adjusted to distinguish subsets of canine patients with different prognoses. The next phase of this study is to perform a large-scale outcome study in dogs with CLL to correlate clinical outcome with mutational status. An additional challenge is the lack of antibodies available to differentiate CLL from other canine B-cell neoplasms. The case criteria used in this study define an entity in dogs that most closely resembles CLL in people in its clinical presentation [13]. However, this entity may represent a different small B-cell neoplasm with lymphocytosis, such as leukemic mantle cell lymphoma [44], or group of small B-cell neoplasms. We consider leukemic mantle cell lymphoma a less likely differential because this histologic subtype appears quite rare in dogs [45–47]. Future steps include obtaining histopathology and gene expression profiling to correlate canine findings with that seen in human CLL.

These results contribute to our understanding of the canine immunoglobulin genes and are the first to examine mutational status in a canine population with CLL. This study identified Boxers with CLL as having predominantly unmutated IGHV gene rearrangements and no highly mutated rearrangements. This suggests that this breed may be a valuable model to study CLL associated with unmutated IGHV genes.

Supporting information
S1 Table. Canine IGHV genes, including the IMGT name, previous name, genomic location, and functionality information. (DOCX)

Acknowledgments
The authors thank Dr. Marie-Paule Lefranc and the international ImMunoGeneTics information system, for their invaluable expertise and assistance.

Author Contributions
Conceptualization: Emily D. Rout, Robert C. Burnett, Anne C. Avery.
Formal analysis: Julia D. Labadie.
Investigation: Emily D. Rout, Robert C. Burnett, Janna A. Yoshimoto.
Writing – original draft: Emily D. Rout.
References

1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. World Health Organization Classification of Tumours of Hematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2008.

2. Nabhan C, Rosen S. Chronic Lymphocytic Leukemia A Clinical Review. JAMA. 2014; 312: 2265–2276. https://doi.org/10.1001/jama.2014.14553 PMID: 25461996

3. Rai KR, Jain P. Chronic lymphocytic leukemia (CLL)-Then and now. Am J Hematol. 2016; 91: 330–340. https://doi.org/10.1002/ajh.24282 PMID: 26690614

4. Fais F, Ghiotto F, Hashimoto S, Sellars B, Valetto A, Allen SL, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest. 1998; 102: 1515–25. https://doi.org/10.1172/JCI3009 PMID: 978864

5. Damle BRN, Wasi T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood. 1999; 94: 1840–7. PMID: 10477712

6. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999; 94: 1848–54. PMID: 10477713

7. Agathangelidis A, Darzentas N, Hadzidimitriou A, Brochet X, Murray F, Yan X-J, et al. Stereotyped B-cell receptors in one third of chronic lymphocytic leukemia: towards a molecular classification with implications for targeted therapeutic interventions. Blood. 2012; 119: 4467–4476. https://doi.org/10.1182/blood-2011-11-393694 PMID: 22415752

8. The International CLL-IPI working group. An international prognostic index for patients with chronic lymphocytic leukemia (CLL-IPI): a meta-analysis of individual patient data. Lancet Oncol. 2016; 17: 779–790. https://doi.org/10.1016/S1470-2045(16)30029-8 PMID: 27185642

9. Sutton L-A, Hadzidimitriou A, Baliakas P, Agathangelidis A, Langerak AW, Stilgenbauer S, et al. Immunoglobulin genes in chronic lymphocytic leukemia: key to understanding the disease and improving risk stratification. Haematologica. 2017; 102: 968–971. https://doi.org/10.3324/haematol.2017.165605 PMID: 28566340

10. Ghia P, Stamatopoulos K, Belessi C, Moreno C, Stilgenbauer S, Stevenson FK, et al. ERIC recommendations on IGHV gene mutational status analysis in chronic lymphocytic leukemia. Leukemia. 2007; 21: 1–3. https://doi.org/10.1038/sj.leu.2404457 PMID: 17167528

11. Langerak AW, Davi F, Ghia P, Hadzidimitriou A, Murray F, Potter KN, et al. Immunoglobulin sequence analysis and prognosis in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. Leukemia. 2011; 25: 979–84. https://doi.org/10.1038/leu.2011.49 PMID: 21455216

12. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Do H. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute–Working Group 1996 guidelines. 2008; 111: 5446–5457.

13. Bromberek JL, Rout ED, Agnew MR, Yoshimoto J, Morley PS, Avery AC. Breed Distribution and Clinical Characteristics of B Cell Chronic Lymphocytic Leukemia in Dogs. J Vet Intern Med. 2016; 30: 215–222. https://doi.org/10.1111/jvim.13814 PMID: 26740174

14. Comazzi S, Gelain ME, Martini V, Riondato F, Miniscalco B, Marconato L, et al. Immunophenotype predicts survival time in dogs with chronic lymphocytic leukemia. J Vet Intern Med. 2011; 25: 100–6. https://doi.org/10.1111/j.1939-1676.2010.0640.x PMID: 21092008

15. Tasca S, Carli E, Caldin M, Menegazzo L, Furlanello T, Gallego LS. Hematologic abnormalities and flow cytometric immunophenotyping results in dogs with hematopoietic neoplasia: 210 cases (2002–2006). Vet Clin Pathol. 2009; 38: 2–12. https://doi.org/10.1111/j.1939-165X.2008.00099.x PMID: 19171020

16. Adam F, Villiers E, Watson S, Coyne K, Blackwood L. Clinical pathological and epidemiological assessment of morphologically and immunologically confirmed canine leukemia. Vet Comp Oncol. 2009; 7: 181–95. https://doi.org/10.1111/j.1746-5829.2009.00189.x PMID: 19691647

17. Williams MJ, Avery AC, Lana SE, Hillers KR, Bachand AM, Avery PR. Canine lymphoproliferative disease characterized by lymphocytosis: Immunophenotypic markers of progression. J Vet Intern Med. 2008; 22: 596–601. https://doi.org/10.1111/j.1939-1676.2008.0041.x PMID: 18346150
18. Bao Y, Guo Y, Xiao S, Zhao Z. Molecular characterization of the VH repertoire in Canis familiaris. Vet Immunol Immunopathol. 2010; 137: 64–75. https://doi.org/10.1016/j.vetimm.2010.04.011 PMID: 20483487

19. Lefranc MP, Giudicelli V, Duroux P, Jabado-Michaloud J, Folch G, Aouniti S, et al. IMGT, the international ImMunoGeneTics information system 25 years on. Nucleic Acids Res. 2015; 43: D413–D422. https://doi.org/10.1093/nar/gku1056 PMID: 25378316

20. Lefranc MP. Immunoglobulin and T cell receptor genes: IMGT® and the birth and rise of immunoinformatics. Front Immunol. 2014; 5: 1–22.

21. Lefranc MP, Pommie´ M-P, Kaas Q, Duprat E, Bosc N, Guiraudou D, et al. IMGT unique numbering for immunoglobulin and T cell receptor constant domains and Ig superfamily C-like domains. Dev Comp Immunol. 2005; 29: 185–203. https://doi.org/10.1016/j.dci.2004.07.003 PMID: 15572068

22. Martin J, Ponstingl H, Lefranc M-P, Archer J, Sargan D, Bradley A. Comprehensive annotation and evolutionary insights into the canine (Canis lupus familiaris) antigen receptor loci. Immunogenetics. 2017; https://doi.org/10.1007/s00251-017-1028-0 PMID: 28924718

23. Steiniger SCJ, Dunkle WE, Bammert MF, Wilson TL, Krishnan A, Dunham SA, et al. Fundamental characteristics of the expressed immunoglobulin VH and VL repertoire in different canine breeds in comparison with those of humans and mice. Mol Immunol. 2014; 59: 71–78. https://doi.org/10.1016/j.molimm.2014.01.010 PMID: 24509215

24. Braganza A, Wallace K, Pell L, Parrish CR, Siegel DL, Mason NJ. Generation and validation of canine single chain variable fragment phage display libraries. Vet Immunol Immunopathol. 2011; 139: 27–40. https://doi.org/10.1016/j.vetimm.2010.07.026 PMID: 20817275

25. Richards KL, Motsinger-Reif AA, Chen H-W, Fedoriv Y, Fan C, Nielsen DM, et al. Gene profiling of canine B-cell lymphoma reveals germinal center and postgerminat center subtypes with different survival times, modeling human DLBCL. Cancer Res. 2013; 73: 5029–39. https://doi.org/10.1158/0008-5472.CAN-12-3546 PMID: 23783577

26. Chen H-W, Small GW, Motsinger-Reif A, Suter SE, Richards KL. VH1-44 gene usage defines a subset of canine B-cell lymphomas associated with better patient survival. Vet Immunol Immunopathol. 2014; 157: 125–30. https://doi.org/10.1016/j.vetimm.2013.09.020 PMID: 24323268

27. Seelig DM, Avery P, Webb T, Yoshimoto J, Bromberek J, Ehrhart EJ, et al. Canine t-zone lymphoma: Unique immunophenotypic features, outcome, and population characteristics. J Vet Intern Med. 2014; 28: 878–886. https://doi.org/10.1111/jvim.12343 PMID: 24655022

28. Burnett RC, Vernau W, Modiano JF, Olver CS, Moore PF, Avery AC. Diagnosis of canine lymphoid neoplasia using clonal rearrangements of antigen receptor genes. Vet Pathol. 2003; 40: 32–41. https://doi.org/10.1354/vp.40-1-32 PMID: 12827711

29. Rout ED, Shank AMM, Waite AHK, Siegel A, Avery AC, Avery PR. Progression of cutaneous plasmacytoma to plasma cell leukemia in a dog. Vet Clin Pathol. 2017; 46: 77–84. https://doi.org/10.1111/vcp.12463 PMID: 28186653

30. van Dongen JJM, Langerak AW, Bruggeman M, Evans PAS, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003; 17: 2257–2317. https://doi.org/10.1038/sj.leu.2403202 PMID: 14671650

31. Rao S, Lana S, Eickhoff J, Marcus E, Avery PR, Morley PS, et al. Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. J Vet Intern Med. 2011; 25: 1097–105. https://doi.org/10.1111/j.1939-1676.2011.0767.x PMID: 21781170

32. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012; 28: 1647–1649. https://doi.org/10.1093/bioinformatics/bts199 PMID: 22543367

33. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215: 403–10. https://doi.org/10.1016/S0022-2836(05)80360-2 PMID: 2231712

34. Kent WJ. BLAT—The BLAST-Like Alignment Tool. Genome Res. 2002; 12: 656–664. https://doi.org/10.1101/gr.229202 PMID: 11932250

35. Giudicelli V, Brochet X, Lefranc M-P. IMGT/V-QUEST: IMGT standardized analysis of the immunoglobulin (IG) and T cell receptor (TR) nucleotide sequences. Cold Spring Harb Protoc. Cold Spring Harbor Laboratory Press; 2011; 2011: 695–715. https://doi.org/10.1101/pdb.prot5633 PMID: 21632778

36. Rosenquist R, Ghia P, Hadzidimitriou A, Sutton L-A, Agathangelidis A, Bialiak P, et al. Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: updated ERIC recommendations. Leukemia. 2017; 31: 1477–1481. https://doi.org/10.1038/leu.2017.125 PMID: 28439111
37. Stanganeli C, Travella A, Bezares R, Slavutsky I. Immunoglobulin gene rearrangements and mutational status in argentannian patients with chronic lymphocytic leukemia. Clin Lymphoma, Myeloma Leuk. 2013; 13: 447–457.e2. https://doi.org/10.1016/j.clml.2013.02.019 PMID: 23665144

38. Cahill N, Sutton L-A, Jansson M, Murray F, Mansouri L, Gunnarsson R, et al. IGHV3-21 gene frequency in a Swedish cohort of patients with newly diagnosed chronic lymphocytic leukemia. Clin Lymphoma Myeloma Leuk. 2012; 12: 201–6. https://doi.org/10.1016/j.clml.2012.01.009 PMID: 22464020

39. Ye J, Ma N, Maddon TL, Ostell JM. IgBLAST: an immunoglobulin variable domain sequence analysis tool. Nucleic Acids Res. 2013; 41: 34–40. https://doi.org/10.1093/nar/gkt382 PMID: 23671333

40. Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for Ig and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res. 2008; 36: 503–508. https://doi.org/10.1093/nar/gkn316 PMID: 18503082

41. Ghia P, Stamatopoulos K, Belessi C, Moreno C, Stella S, Guida G, et al. Geographic patterns and pathogenetic implications of IGHV gene usage in chronic lymphocytic leukemia: The lesson of the IGHV3-21 gene. Blood. 2005; 105: 1678–1685. https://doi.org/10.1182/blood-2004-07-2606 PMID: 15466924

42. Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature. 2005; 438: 803–819. https://doi.org/10.1038/nature0338 PMID: 16341006

43. Tobin G, Thunberg U, Laurell A, Karlsson K, Áleskog A, Willander K, et al. Patients with chronic lymphocytic leukemia with mutated VH genes presenting with Binet stage B or C form a subgroup with a poor outcome. Haematologica. 2005; 90: 465–469. PMID: 15820941

44. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood. 2016; 127: 2375–2391. https://doi.org/10.1182/blood-2016-01-643569 PMID: 26980727

45. Vezzali E, Parodi AL, Marcato PS, Bettini G. Histopathologic classification of 171 cases of canine and feline non-Hodgkin lymphoma according to the WHO. Vet Comp Oncol. 2010; 8: 38–49. https://doi.org/10.1111/j.1476-5829.2009.00201.x PMID: 20230580

46. Valli VE, San Myint M, Barthel A, Bienzle D, Caswell J, Colbatzky F, et al. Classification of canine malignant lymphomas according to the World Health Organization criteria. Vet Pathol. 2011; 48: 198–211. https://doi.org/10.1177/0300985810379428 PMID: 20861499

47. Valli VE, Vernau W, de Lorimier L-P, Graham PS, Moore PF. Canine indolent nodular lymphoma. Vet Pathol. 2006; 43: 241–56. https://doi.org/10.1354/vp.43-3-241 PMID: 16672571