Consensus-based somatic variant-calling method correlates *FBXW7* mutations with poor prognosis in canine B-cell lymphoma

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17 *Translational Relevance*

18 Identifying tumor biomarkers associated with clinical outcomes has been a major driver in
19 improved success in treating many types of human cancers, including Non-Hodgkin lymphoma
20 (NHL). Since canine B-cell Lymphoma (cBCL) shares many clinically identifiable
21 characteristics with NHL, our detection of recurring mutations in certain genes in cBCL and their
22 association with clinical outcomes stands to benefit both humans and dogs. If common canine
23 lymphoma subtypes show mutational similarity to certain human subtypes, then therapies found
24 to be effective for a subtype in one species may be more likely to improve treatment response in
25 the analogous subtype in the other.

26

27 *Abstract*

28 INTRODUCTION:

29 Canine Lymphoma (CL) is the most commonly diagnosed malignancy in the domestic dog, with
30 estimates reaching 80,000 new cases per year in the United States. Understanding of genetic
31 factors involved in development and progression of canine B-Cell Lymphoma (cBCL), the most
32 common of the two major subtypes of CL, can help guide efforts to prevent, diagnose, and treat
33 disease in dogs. Such findings also have implications for human Non-Hodgkin Lymphoma
34 (NHL), as pet dogs have recently emerged as an important translational model due to the many
35 shared histopathological, biological, and clinical characteristics between cBCL and NHL.

36 OBJECTIVES:

37 We aimed to identify potential driver mutations in cBCL and detect associations between
38 affected genes and differential clinical outcomes.

39 METHODS:

40 Using exome sequencing of paired normal and tumor tissues from 71 dogs of various breeds with
41 cBCL, we identified somatic variants with a consensus approach: keeping variants called by both
42 MuTect2 and with high-confidence by VarScan 2. We predicted effects of these variants using
43 SnpEff then measured associations between mutated genes and survival times from clinical data
44 available for 62 cohort dogs using a multivariate Cox Proportional Hazards Model.

45 RESULTS:

46 Mutations in *FBXW7,* a gene commonly mutated in both human and canine cancers including
47 lymphoma, were associated with shorter overall survival (OS; p=0.01, HR 3.3 [1.4-7.6]). The
48 two most frequently mutated codons of *FBXW7* in our cohort correspond to the most frequently
49 mutated codons in human cancers.

50 CONCLUSIONS:

51 Our findings show that exome sequencing results can be combined with clinical data to identify
52 key mutations associated with prognosis in cBCL. These results may have implications for
53 precision medicine in dogs and also allow subsets of dogs to serve as models for specific
54 subtypes of human lymphoma.

55

56 *Introduction*

57 The opportunity for pet dogs to contribute to translational research in precision medicine
58 is unmatched by any other model species, as dogs receive the most medical assessment and
59 intervention of any non-human animal(1). Unsurprisingly, this interest in increased medical
60 surveillance for pet dogs comes with a demand from dog owners for continued advancements in
61 available treatments and improved outcomes. In the case of canine lymphoma, one of the most
62 common cancers in dogs, the parallels between canine and human disease create a unique
63 opportunity to improve both canine and human outcomes.

64 Published data continues to confirm that canine lymphoma shares many clinically
65 important characteristics with human lymphoma, including prevalence of B-cell over T-cell
66 subtypes, existence of multiple distinguishable histologic subtypes (e.g. Diffuse large B-cell
67 lymphoma is the most common subtype of B-cell lymphoma in both humans and dogs (2,3), varied
68 biological behavior between cases, wide-ranging responses to treatment between individuals, and
69 unexplained increase in incidence over the past several decades.

70 Biologically, pet dogs are more similar to humans than traditional mouse models in terms
71 of size and metabolism, and this can improve the relevance of pharmacokinetic and
72 pharmacodynamic data from drugs tested in the dog for application to human clinical research. Pet
73 dogs also share an environment with humans and are thus exposed to similar environmental risk
74 factors that are not present in the environments of laboratory species.

75 Additionally, dogs develop lymphoma spontaneously as humans do, in contrast to
76 genetically engineered mouse models (GEMMs) predisposed to lymphoma with specific known
77 genetic lesions or xenograft mouse models in which a human tumor is transplanted into an
78 immunocompromised mouse (2).

79 Lymphoma incidence rates vary widely among dog breeds, indicating that heritable factors
80 mediate lymphoma development. Significant progress is being made in identifying genes
81 associated with cancer risk in dogs, providing an opportunity to better understand oncogenic
82 mechanisms. Several genome-wide association studies (GWAS) with moderate to large cohorts of
83 case and control dogs (Golden Retrievers [4], Standard Poodles [5], Rottweilers, and Irish
84 Wolfhounds [6]) have revealed association signals near several known cancer genes (e.g. *TRPC6*,
85 *KITLG*, *CDKN2A* and *CDKN2B*).

86 The first report of tumor mutation profiling via exome sequencing in cBCL was done
87 using a variety of dog breeds (7). This was followed by another profiling study of exome
88 sequencing in three breeds, the Cocker Spaniel (B-cell Lymphoma); Golden Retriever (B-cell
89 and T-cell Lymphoma), and the Boxer (T-cell Lymphoma; 8). Recurrent mutations in B-cell
90 lymphoma were found in *TRAF3* in both studies, validating the importance of the NF-**κ**B
91 pathway in this disease, similar to its central role in human B-cell lymphomas.

92 Choosing a variant calling pipeline to identify somatic mutations in canine cancer exomes
93 is non-trivial. First, most tumor variant calling tools are designed for use with human or rodent
94 sequence data, so care must be taken to ensure that the algorithms can accommodate other
95 genomes. Most striking, however, is how poorly the variant calls of multiple established callers
96 agree with each other for the same sequence data. In a study of exome sequences from five
97 human breast cancer patients with somatic single nucleotide variants (SNVs) and indels called by
98 nine publicly available callers, 74% of total SNVs called (22,032 of 29,634) were called by only
99 one caller and not detected by the other eight. Another 14% were called by only two callers.
100 Indel calls overlapped even less with 88% (10,541 of 11,955) of indels called by only one caller
101 and another 9% called by only two callers (9).

102 Gene expression profiling has likewise been applied to canine lymphoma, using a
103 mixture of different breeds(10–13). Molecular subtypes based on dysregulated gene expression
104 again highlighted the importance of NF-κB genes, and established similarity to the human
105 disease in the aberrant upregulation of similar pathways, although not necessarily upregulation of
106 the same genes, as in human lymphoma(12,14). Similar to the case with mutation profiling, this
107 has implications for biomarker development and target identification in clinical studies, and
108 stresses the importance of developing canine-specific genomic and transcriptomic signatures.

109 *Methods*

110 **Diagnosis and Immunophenotyping**

111 Diagnosis of B-cell lymphoma was determined by board-certified veterinary oncologists
112 and pathologists using a combination of two or more of the following methods: morphological
113 characteristics of fixed, stained tumor biopsy; immunohistochemistry using standard anti-canine
114 B-cell, T-cell, and other leukocyte antigen antibodies including BLA36, CD3, CD18, CD21,
115 Pax-5, and Mac387; polymerase chain reaction (PCR) for antigen receptor rearrangement
116 (PARR); or flow cytometry (FCM).

117 **Sample collection**

118 Tissue samples from affected lymph nodes removed via excisional biopsy were provided
119 by coauthors. Whole blood was collected at the time of excisional biopsy to be used as matched
120 normal tissue.

121 **DNA Extraction**

122 Whole blood treated with the anticoagulant ethylenediaminetetraacetic acid (EDTA) was
123 used as the normal tissue for DNA extraction. Red blood cells were lysed, then samples were
124 centrifuged to form a pellet of white blood cells. These cells were then lysed and treated with a

125 salt solution to precipitate lipids, proteins, and cellular debris, leaving nucleic acids in the
126 supernatant. DNA was precipitated in alcohol, washed to remove salt, and hydrated in a
127 stabilizing buffer.

128 Formalin-fixed, paraffin-embedded (FFPE) tissue from affected lymph nodes removed
129 via excisional biopsy were used for extraction of tumor DNA. Extraction was performed using
130 the Qiagen QIAamp DNA FFPE Tissue Kit according to manufacturer instructions.

131 **Exome Library Preparation and Sequencing**

132 Pre-capture libraries were prepared with standard library preparation protocols using the
133 KAPA Hyper kit from Kapa Biosystems and then pooled at equal volume and sequenced on the
134 Illumina platform at low depth to determine exact relative abundances. Based on these
135 abundances, libraries were balanced optimally for whole exome sequencing using the SureSelect
136 Canine All Exon V2 bait set from Agilent. Library sequencing was performed on the Illumina
137 Hiseq 2500 platform.

138 **Exome Sequence Alignment**

139 Preprocessing and alignment of exome sequences (FASTQ to BAM) were performed
140 according to standard GATK best practices(15) using the canine reference genome file
141 canFam3.1 (available https://github.com/auton1/dog_recomb; 16).

142 **Variant Calling**

143 Several variant callers were initially tested for our dataset: VarScan 2 (17) Somatic
144 Sniper (18), Strelka (19) and MuTect (20). A consensus approach was tested in which variants
145 called by at least two of four callers were kept and annotated using SnpEff to predict effect. This
146 approach, herein referred to as the 4-caller method, was evaluated via visual inspection of
147 aligned sequencing reads at sites of variant calls using Integrated Genomics Viewer (IGV),

148 including identification of common causes for likely false positive and false negative calls by
149 each caller (e.g. varying minimum sequencing depth requirements, tolerance of three or more
150 alleles at one locus, tolerance of tumor/normal contamination).

151 As our aim for this study was to confidently identify an initial set of common mutations
152 that could be used to classify dogs into groups for precision treatment and ultimately match them
153 with analogous human subgroups, we first chose to keep the conservative caller MuTect2 (21);
154 an early, updated GATK 3-based version of the MuTect program used in the 4-caller method)
155 because of its low frequency of false positive calls in our dataset.

156 Initially, when MuTect2 was run using default parameters, its relatively high frequency
157 of false negative calls (i.e. removal of true somatic variants) was problematic. Two parameters in
158 particular were not optimized for our samples: "max_alt_alleles_in_normal_count" and
159 "max_alt_allele_in_normal_fraction." Both settings aim to keep germline variants from being
160 falsely identified as tumor variants by removing variants detected in the tumor sample that are
161 also detected in the paired normal sample. For lymphoma, a hematopoietic cancer, tumor DNA is
162 more likely to contaminate normal tissue (especially whole blood, the tissue used to extract
163 normal DNA in this study) than other cancer types. The default frequency and count of
164 sequencing reads with alternative alleles (detected in the tumor sample) tolerated in the normal
165 sample before variants were filtered from the called variant pool were 0.03 (3%) and 1,
166 respectively. Upon manual inspection of aligned reads in IGV, these default parameters were
167 indeed found to be too strict; in areas of low sequencing coverage, one contaminating tumor read
168 in the normal sample could easily represent more than 3% of total reads and in areas of high
169 sequencing coverage, the likelihood that two or more contaminating tumor reads were present
170 was significant. For our final variant calls, MuTect2 was run with default parameters except for

171 the two max_alt_allele settings: max_alt_alleles_in_normal_count was changed from the default
172 of 1 to 10,000,000 to essentially remove this filter from the variant calling process, and
173 max_alt_allele_in_normal_fraction was changed from the default of 0.03 (3%) to 0.10 (10%).
174 These changes decreased the frequency of false negative calls by MuTect2 in our dataset.
175 In making these adjustments to preserve true positives, however, we also increased false
176 positives.

177 In an effort to reduce false positives with minimal removal of true positives, we first used
178 two sets of germline mutation references to remove such variants from the pool of potential
179 driver mutations. One was a panel of normals (PON) file of germline variants generated using
180 the exome data from normal tissue from all dogs in the study, and the other was a dbsnp file
181 containing a catalog of germline single nucleotide polymorphisms (SNPs) called from 365 dog
182 and wolf genomes by a coauthor's laboratory (22). Variants that passed other thresholds to be
183 included as tumor SNVs or indels were removed if present in the PON or dbsnp files at allele
184 fractions greater than 10% as described above.

185 Next, we used a consensus approach by overlapping the remaining variants called by
186 MuTect2 with those called with high confidence by VarScan 2. These overlaps were identified
187 using the bcftools software package's -isec command (23). VarScan 2 was a less conservative
188 caller for our dataset especially in areas of high sequencing depth. Its sensitivity was an asset for
189 our purposes to increase likelihood of finding true positive calls, while the increased false
190 positives were likely to be filtered in the overlap with MuTect2 calls. If each were used alone,
191 MuTect2 (with our parameter changes) and VarScan 2 called an average of 92 and 1127 variants
192 per dog, respectively. Using the consensus method, we focused on an average of 35 mutations
193 per dog passed by both callers.

194 Functional annotation of variants was performed using SnpEff(24) to process a VCF file
195 of MuTect2 calls filtered for only those consensus mutations also called with high confidence by
196 VarScan 2.

197 **Cox Proportional Hazard Model**

198 Clinical data was gathered from medical records at the two participating referral hospital
199 (Cornell University Hospital for Animals and North Carolina State Veterinary Hospital) and
200 from referring veterinary records and communications where provided or available. Overall
201 survival time (OS) was censored to the last date the patient was known to be alive in cases lost to
202 follow up. For dogs that did not achieve remission and for dogs that were still in remission when
203 lost to follow-up, progression-free survival time (PFS) was set to missing and not included in
204 calculations.

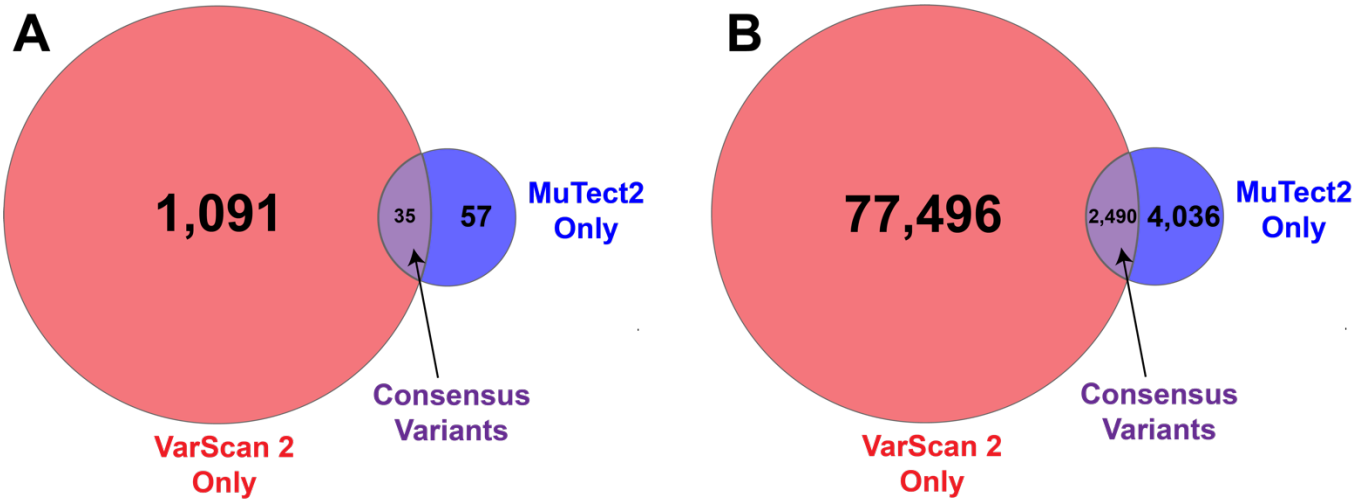
205 First, a univariate Cox regression was performed with the clinical data available from 62
206 of the dogs in the study using covariates (other than mutation status) known to affect survival
207 times in cancer patients (e.g. age, sex, initial treatment). The Cox proportional hazards model
208 used to test OS with mutation status by gene was fitted with non-colinear covariates that had
209 demonstrated effects in the univariate models, including age at diagnosis, sex, body weight at
210 time of diagnosis, and initial treatment factor (no treatment, induction chemotherapy only, full
211 course of single-agent chemotherapy, or full course of multi-agent chemotherapy). The Cox
212 proportional hazards model used to test PFS with mutation status by gene was fitted with the
213 same covariates plus another indicating treatment status at relapse (treated or untreated). A
214 mutation in a gene was considered to have a significant effect on time from remission to relapse
215 (PFS) or time from diagnosis to end of life (OS) if the p value of the mutation status's coefficient

216 in the model was less than 0.05. Data was input into the R packages "survival"(25) and

217 "survminer" (26).

218

219 *Results*



220

221 **Figure 1: Number of tumor variants kept by individual callers and using consensus method**

222 **shown as (A) average per dog and (B) combined for all dogs (n=71).** Consensus variants

223 shown in purple. Variants called by only VarScan 2 with high confidence in red and variants

224 called by only MuTect2 with adjusted parameters (see methods) in blue.

225

226

227 Compared to our initial 4-caller method (Supplemental Figure 1, Supplemental results),

228 our final method using consensus variants called by both MuTect2 and with high confidence by

229 VarScan 2 yielded many fewer variants, but kept variants were much more likely to be true

230 positive somatic mutations when sequencing read alignments were visually inspected.

231 In our dataset, *TRAF3* was the most commonly mutated gene in cBCL (n=20, 28% of

232 dogs). This is similar to a previous study sequencing cBCL in the Golden Retriever and Cocker

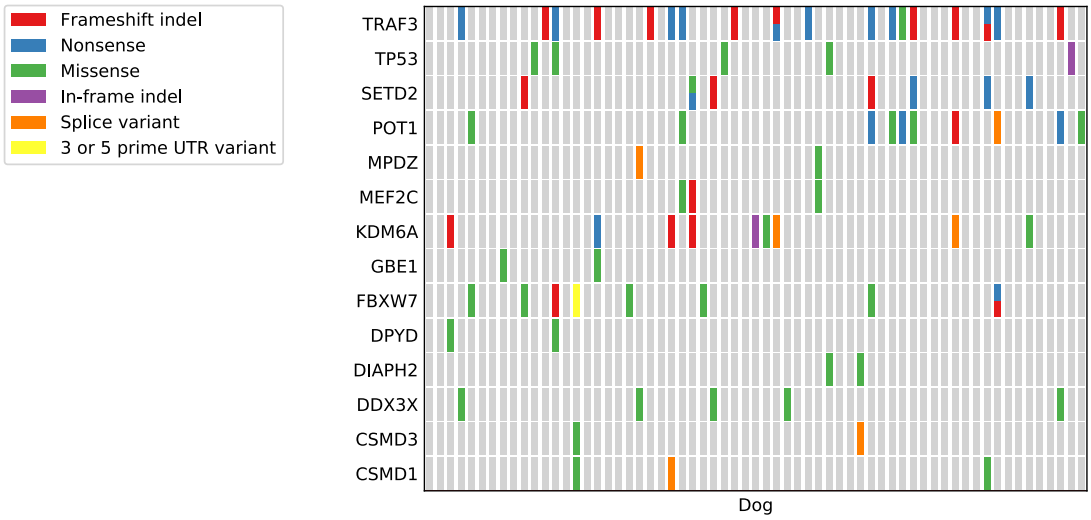
233 Spaniel in which 13 dogs of 64 (20.3%) had at least one somatic mutation in *TRAF3* (8). TRAF3

234 has a diverse role as a regulator of both the canonical and non-canonical NF-κB pathways known

235 to play a role in the pathogenesis of many cancers(7,27). In our dataset, *TRAF3* mutations were

236 not associated with significant differences in overall survival or progression-free survival.

237



238

239 **Figure 2: Map of selected functional categories of somatic mutations in most recurrently**

240 **mutated genes.**

241 Each column represents an individual dog, and colored bars indicate the dog had one or more

242 somatic mutations called via 2-caller consensus method in that gene. Only mutations with

243 greatest predicted effect are shown.

244

245

Overall Survival (n=62)	Univariate						Multi-variate				
Covariate	Beta	Hazard Ratio	95%CI for HR Low	95%CI for HR High	Wald Test	p Value	Beta	Hazard Ratio	95%CI for HR Low	95%CI for HR High	p Value
Age (yr)	0.1	1.1	1.0	1.2	3.5	0.06					
Sex	0.5	1.7	0.9	2.9	3.0	0.08					
Body Weight (kg)	0.0	1.0	1.0	1.0	17.0	0.20					
Treatment (4 Levels)					9021.0	<2E-16					
TRAF3	-0.1	0.9	0.5	1.6	0.1	0.70	0.3	1.4	0.7	2.7	0.37
DIAPH2	0.1	1.1	0.6	2.3	0.1	0.70	0.3	1.4	0.7	2.8	0.40
POT1	-0.6	0.5	0.3	1.1	2.8	0.09	-0.2	0.8	0.4	1.8	0.61
KDM6A	0.0	1.0	0.5	2.3	0.0	0.93	-0.2	0.8	0.3	2.0	0.67
FBXW7	0.8	2.1	1.0	4.6	3.7	0.06	1.2	3.3	1.4	7.6	0.01
SETD2	0.0	1.0	0.4	2.2	0.0	0.97	0.1	1.1	0.5	2.6	0.79
TP53	-0.1	1.0	0.4	2.4	0.0	0.91	0.1	1.1	0.4	3.2	0.82

Progression-Free Survival (n=35)	Univariate						Multi-variate				
Covariate	Beta	Hazard Ratio	95%CI for HR Low	95%CI for HR High	Wald Test	p Value	Beta	Hazard Ratio	95%CI for HR Low	95%CI for HR High	p Value
Age (yr)	0.1	1.1	1.0	1.2	1.4	0.23					
Sex	0.2	1.2	0.6	2.4	0.2	0.60					
Body Weight (kg)	0.0	1.0	1.0	1.0	2.4	0.12					
Treatment (4 Levels)					1.5	0.20					
TRAF3	0.3	1.3	0.6	2.7	0.5	0.50	0.4	1.5	0.6	3.5	0.39
DIAPH2	0.0	1.0	0.4	2.5	0.0	0.96	0.2	1.2	0.5	3.0	0.74
POT1	-0.6	0.5	0.2	1.3	2.1	0.10	-0.5	0.6	0.2	1.7	0.33
KDM6A	0.3	1.3	0.6	3.3	0.4	0.50	0.3	1.4	0.5	3.8	0.52
FBXW7	0.9	2.5	0.9	6.6	3.2	0.07	1.0	2.6	0.9	7.4	0.07
SETD2	0.1	1.1	0.4	2.8	0.0	0.90	-0.1	0.9	0.3	2.5	0.88
TP53	-1.3	0.3	0.0	2.1	1.6	0.20	-1.9	0.2	0.0	1.3	0.09

Table 1: Results of Univariate Cox Regression and Multivariate Cox Proportional Hazards Model

The next most frequently mutated gene was DIAPH2 in 17% of dogs (n=12), with only 2 dogs having missense variants with moderate predicted effect and the rest of dogs having one or

254 more mutations in introns of the gene. In dogs with clinical data unaffected by experimental
255 treatments or additional types of cancer (n=62), mutations in DIAPH2 were not associated with
256 significant changes in OS or PFS.

257 *ENSCAFG00000030258*, part of the immunoglobulin heavy chain, mu (IGHM) was the
258 next most frequently mutated gene with all mutations synonymous or outside of coding regions
259 in 15% of dogs (n=11). These mutations are not likely to be drivers in the transition to
260 malignancy and may instead be due to either hypermutation in the germinal center or allelic
261 variation(3). This is also likely true for another highly mutated gene, *ENSCAFG00000029236*,
262 part of the immunoglobulin light chain, lambda (IGL) with all mutations synonymous or outside
263 of coding regions, found in 14% of dogs (n=10).

264 *POT1* (protection of telomeres 1), a component of the telomerase ribonucleoprotein
265 (RNP) complex necessary for proper replication of chromosome ends, was mutated in 14% of
266 dogs (n=10). *POT1* has previously been implicated in cBCL, with 11/64 (17%) dogs with BCL
267 having non-silent, protein-coding mutations in one study of cBCL in two dog breeds (8). All 10
268 dogs with *POT1* mutations in our study had a single mutation in the gene with moderate to high
269 predicted effect. However, for dogs in our study, *POT1* mutations were not associated with
270 significant differences in overall survival or progression-free survival.

271 *KDM6A* (lysine demethylase 6A) was mutated in 13% of dogs (n=9). Eight of these
272 mutations were in coding regions with moderate (n=3) or high (n=5) predicted effect and one
273 dog (ID 4101) had a splice region and intron variant with low predicted effect. The five
274 mutations with high predicted effect were annotated as loss-of-function (LOF) variants, which is
275 of translational significance given that gain-of-function mutations in *EZH2* (Enhancer of zeste
276 homolog 2), a gene encoding a histone-lysine N-methyltransferase enzyme, have been identified

277 in approximately 22% of diffuse large B-cell lymphoma (DLBCL) cases in humans.(28) EZH2
278 has an opposing effect to KDM6A, and therefore the presence of LOF mutations in *KDM6A* may
279 make these dogs an appropriate translational model for human cancers with GOF mutations in
280 *EZH2*. Pairing with clinical data did not show that KDM6A mutations associated with significant
281 differences in overall survival or progression-free survival.

282 *FBXW7*, F-box and WD repeat domain containing 7, was mutated in 13% of dogs (n=9
283 dogs), with 2 dogs each having 2 separate mutations (11 total mutations). The first of these two
284 latter dogs (ID 4117) had one frameshift mutation in codon 231 of 712 with high predicted effect
285 and one missense mutation at amino acid 484 of 712 with moderate predicted effect. Canine
286 codon R484 corresponds to one of the two most commonly mutated human codons of *FBXW7* in
287 cancer, codon R479 (29). The second dog with two mutations in *FBXW7* (ID 4127) had one
288 premature stop codon at amino acid 229 of 712 with high predicted effect and a frameshift
289 mutation at amino acid 329 of 712 with high predicted effect. Both dogs were heterozygous for
290 each of their two mutations with no fragments spanning both variant sites, so it is not known for
291 either dog whether the dog has two mutations in one copy of the *FBXW7* gene (and one wildtype
292 copy) or whether the dog has two mutated copies of *FBXW7*. For the 7 dogs with a single
293 mutation in *FBXW7*, 6 had mutations in coding regions with moderate or high predicted effect,
294 and one dog had a mutation 9 base pairs upstream of the transcription start site (i.e. in the
295 promoter region).

296 Dogs with mutations in *FBXW7* had significantly shorter overall survival using data from
297 62 dogs with OS data (p=0.01, Hazard Ratio 3.3; Figure 3) and reduction in progression-free
298 survival (PFS) that approached significance in the small subset of 35 dogs with known
299 achievement of remission and known relapse dates (p=0.07. Hazard Ratio 2.6; Figure 3).

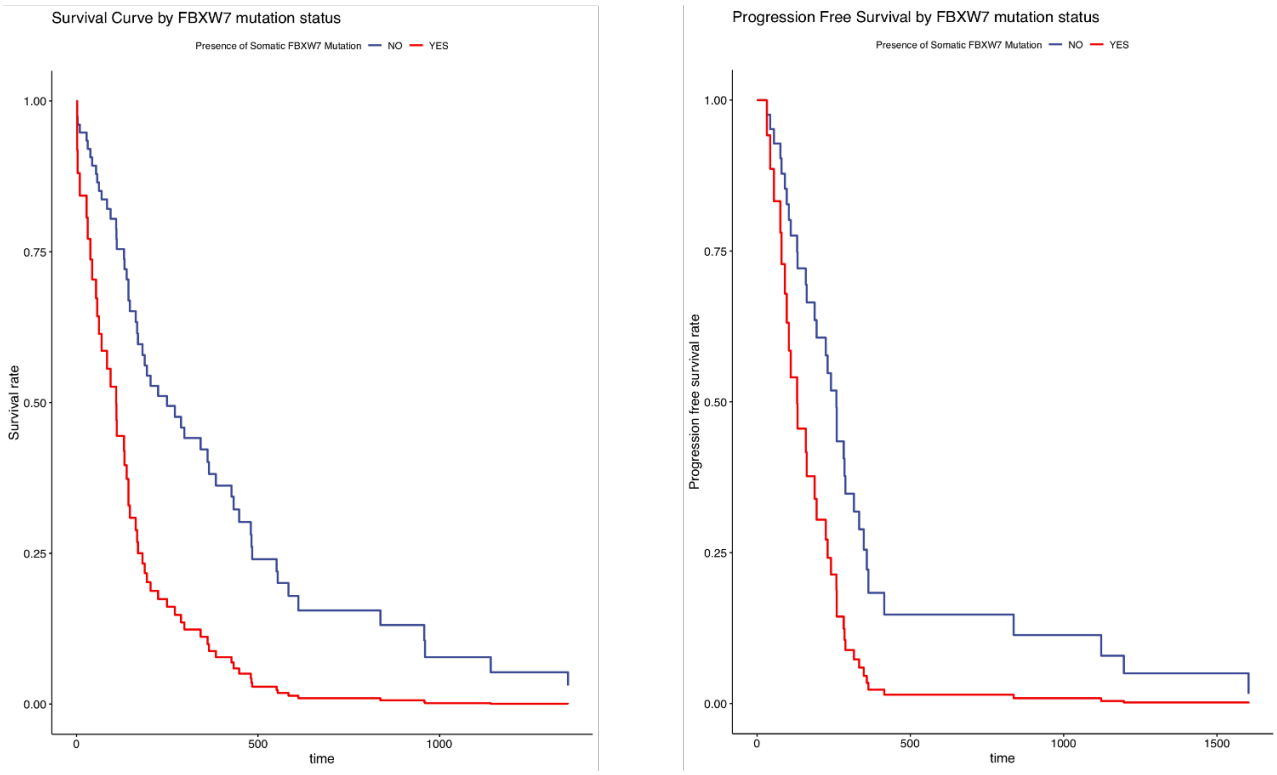


Figure 3: Survival curve (left, n=62) and Progression-free survival curve (right, n=35) of dogs with (red) or without (blue) a somatic mutation called in *FBXW7*.

Dogs with a called somatic mutation in *FBXW7* had a significantly shorter overall survival time (p=0.006, HR 3.26) and a shorter progression-free interval that approached significance (p=0.07).

In a previous study of canine lymphoma from three dog breeds (8) *FBXW7* was the second most commonly mutated gene in canine B-cell tumors of the Golden Retriever and Cocker Spaniel combined and most commonly mutated gene in cBCL tumors of the Golden Retriever alone. Forty-one percent of these mutations were in canine codon R470. This codon in the dog corresponds to the second of the aforementioned two most commonly mutated human codons, R465 (29). In our study, 2 dogs had tumors with missense mutations in canine codon R470, and 2 (as mentioned above) had mutations in the other corresponding commonly mutated

314 codon, but the remaining 7 mutations were elsewhere. Four other mutations were also in the
315 sequence encoding the WD40 repeating domain of the FBXW7 protein (both of the two
316 mutations in dog 4127 and one mutation in dog 4117 described above plus one dog with a
317 frameshift at canine codon 401), and two dogs both had a missense mutation in canine codon
318 524.

319 *FBXW7* is commonly mutated in many human cancers (mutation frequency across all
320 types has been reported at approximately 6%, with variation by cancer type ; 30). Mutations are
321 frequently found in a heterozygous state and, because of FBXW7's action as a dimer, these
322 mutations can still easily affect protein function in terms of stability and substrate affinity (31).
323 Mutations in *FBXW7* reducing protein function have been implicated in resistance to
324 chemotherapy and poor prognosis in human cancers including non-small cell lung cancer and
325 pancreatic cancer due to stabilization of antiapoptotic protein MCL-1(32,33).

326 *SETD2* was mutated in 11% of dogs (n=8). One dog (ID 4167) had 2 mutations in
327 *SETD2*, one with a premature stop codon at amino acid 1541 of 2562 with high predicted effect
328 and the other a missense variant with moderate predicted effect at amino acid 1578. The dog is
329 heterozygous for both mutations, and it is unknown whether the dog has two mutations in one
330 allele of *SETD2* (and one wildtype copy) or whether the dog has two mutant *SETD2* alleles. For
331 the 7 dogs with a single mutation in *SETD2*, all had mutations in coding regions with high
332 predicted effect. SETD2 has many diverse roles in the body and thus mutations can have a
333 variety of phenotypes including loss of genomic stability, disruption of the p53 apoptosis
334 response, changes to recruitment of splice machinery and splice sites used, and altered
335 recruitment of proteins in DNA damage response (34). Mutations in *SETD2* were not
336 significantly correlated with overall survival or progression-free survival in our study.

337 *TP53* was mutated in 8% of dogs (n=6). This tumor suppressor is the most frequently
338 mutated gene detected in human cancers with over half containing a mutation in the gene
339 (35,36), and it has also been shown to be frequently mutated in canine cancers including
340 lymphoma (1), for which the mutations have been associated with decreased response to
341 treatment and shorter survival times(37). In our cohort, *TP53* mutation was not associated with
342 significant changes to overall survival time, but a decrease in the progression-free survival for
343 dogs with a mutation in the gene approached significance (p=0.09). Due to its common presence
344 in many diverse human cancers, TP53 function is repeatedly targeted for cancer therapies. The
345 common presence of *TP53* mutations in dogs could make them more useful for development of
346 such targeted therapies.

347 *Discussion*

348 In our initial attempts to optimize a variant calling pipeline for exome sequencing of
349 canine lymphoma, we used a consensus approach of accepting variants called by 2 or more of 4
350 variant callers. Overlap between callers was very small and even kept calls had a high likelihood
351 of being false positives when manually curated. We adjusted our approach using MuTect2, an
352 updated version of the caller that had performed best in our previous attempt in terms of calling
353 mostly true positive calls as determined by visual inspection. However, we found that this caller
354 often rejected true positive mutations due to tumor contamination in the normal sample reads,
355 which the caller interpreted as the mutation being germline in origin. As tumor contamination
356 into normal tissue is a common problem in hematopoietic cancers(38), we first relaxed standards
357 for rejecting mutations found in normal tissue as germline mutations to allow for a small amount
358 of contamination of tumor tissue into normal tissue. We reduced the introduction of false
359 positives created by this change by only accepting mutations scored as high confidence by

360 VarScan 2, a less conservative caller that performed well in our initial approach with a greater
361 likelihood of keeping true positives compared to MuTect but simultaneous increase in the calling
362 of false positives. By using the extremely conservative consensus approach of these two callers,
363 we were still able to identify commonly mutated genes in canine B-cell lymphoma in our dataset
364 and found a significant relationship to clinical outcomes based on mutations in one gene. This
365 would be expected if the set of mutations identified in our study was enriched for actual "driver"
366 mutations important to the establishment and progression of lymphoma (rather than "passenger"
367 mutations arising due to genome instability or other malignancy factors that have little to no
368 effect on pathogenesis (39) that confer a selective advantage on that cell and its offspring. These
369 cells would become the predominant population and thus their mutations will be sequenced more
370 frequently and called with increased frequency and confidence by variant callers.

371 Dogs with mutations in *FBXW7* show promise for translational research because of their
372 similarity to humans in terms of codons affected and the shared, negative effect of coding
373 mutations on prognosis. Prevalence of *FBXW7* mutations differed by breed in a study comparing
374 exome sequencing data with Golden Retrievers much more likely to have a mutated allele than
375 Cocker Spaniels with B-cell lymphoma (8). No two dogs of the nine with *FBXW7* mutations in
376 our study were from the same breed (though one was a purebred Labrador Retriever and one was
377 a Labrador Retriever cross) but our sample size was small. Continued profiling of canine
378 lymphoma is necessary to reveal if breed may be useful as a proxy for tumor profile in making
379 clinical decisions in canine lymphoma.

380 Dogs with mutations in *TP53* are also poised to contribute to the development and testing
381 of drugs. Because of its overwhelming frequency of mutations in human cancers, intense focus
382 has been placed on developing new drugs targeting *TP53*-mutated cancers. With its diverse

383 potential contributions to malignancy, many options exist for potential mechanistic targets for
384 changes to TP53 activity. With so many resources aimed at such drugs and so many affected dog
385 and human patients, it is crucial to test each potential therapy for safety and efficacy as soon and
386 as quickly as possible. Here, the condensed lifespan of the dog compared to human could be of
387 great benefit, as tumors occur and dogs relapse over a shorter span of time such that studies
388 could be completed in less time (2).

389 Future directions for this work should include the study of differential gene expression by
390 somatic mutation to increase understanding of the downstream effects of these variants and the
391 biological pathways involved in tumor progression. Additionally, gene expression work in cell
392 lines with single gene knockouts or GOF mutations in human and dog cell lines will provide a
393 more controlled experiment regarding the effects of each somatic mutation.

394 Information from this study increases our potential to create canine lymphoma subtypes
395 based on genes mutated, and this will aid in assessing their similarity to human lymphoma
396 subtypes. Additional clinical genomic data is needed with dogs phenotyped by breed,
397 histopathological lymphoma subtype, and clinical outcomes. Such data will also help establish
398 whether differences between our study and others in terms of codons affected, differences in the
399 frequency that specific genes are mutated, and effect of mutations on clinical outcomes is due to
400 differences in breeds sampled or the heterogeneity of the canine lymphoma mutation landscape.

401 The outlook for dogs becoming increasingly valuable as translational models for
402 lymphoma continues to improve as dogs receive increased medical surveillance and treatment,
403 costs of sequencing decrease, and additional molecular similarities between human and canine
404 lymphoma are discovered. Studies like the Golden Retriever Lifetime Study(40) may help with
405 the contribution of serial biological samples (metabolites, cfDNA, circulating tumor cells) that

406 can be used to develop and improve more sensitive detection methods for the development
407 and/or return of cancer. Additionally, such studies could bank healthy tissue earlier before cancer
408 develops in the bloodstream, thus preventing many of the issues with tumor contamination in
409 normal tissue leading to rejection of true somatic mutations as germline variants.

410 As clinical data from more dogs is paired with genomic data, we will be able to begin
411 making precision recommendations for dogs with certain mutation profiles. As these mutations
412 are further researched and studied, we will gain a better understanding of how these mutations
413 affect gene expression and tumor behavior and gain insight into the mechanisms of cancer
414 pathogenesis and differential response to treatments between types. Ultimately, this work can
415 improve both canine and human outcomes in patients with lymphoma.

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