**Original Article**

# The prevalence and characterisation of *TRAF3* and *POT1* mutations in canine B-cell lymphoma

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**Abstract**

The genetic and mutational basis of canine lymphoma remains poorly understood. Several genes, including *TRAF3* and *POT1*, are mutated in canine B-cell lymphoma (cBCL), and are likely involved in the pathogenesis of this disease. The purpose of this study was to assess the prevalence of *TRAF3* and *POT1* mutations in a cohort of dogs with cBCL, compared to dogs with non-cBCL diseases (including 4 dogs with T-cell lymphoma [cTCL]). Forty-nine dogs were included (24 cBCL, 25 non-cBCL). Eleven dogs had matched non-tumour DNA assessed, to determine if mutations were germline or somatic. All dogs had *TRAF3* and *POT1* assessed by Sanger sequencing.

The prevalence of deleterious *TRAF3* and *POT1* mutations in cBCL was 36% and 17%, respectively. A deleterious *TRAF3* mutation was suspected to be germline in one of the five cases with matched non-tumour DNA available for comparison. No deleterious mutations were found in any non-cBCL cases. Several synonymous variants were identified in both genes in cBCL and non-cBCL samples, which likely represent polymorphisms. These results indicate *TRAF3* and *POT1* mutations are common in cBCL. Deleterious *TRAF3* and *POT1* mutations were only identified in dogs with cBCL, and not in dogs with non-cBCL diseases, suggesting they are important in the pathogenesis of cBCL. Future studies into the prognostic and therapeutic implications of these mutations are required.

*Key words:* Canine lymphoma; Genetics; *POT1; TRAF3*

## Introduction

Lymphoma is one of the most common malignancies in dogs (Merlo et al., 2008). The term lymphoma includes a diverse range of diseases with different presentations and prognoses and although their aetiology in dogs remains poorly understood, numerous factors including environmental, infectious, immunologic and genetic are likely to play a role.

Many genes contributing to lymphomagenesis have been identified in human oncology; however, relatively little is known about the genetic abnormalities associated with canine lymphoma. The high rate of lymphoma in specific breeds, and the propensity for specific breeds to develop certain types of lymphoma (e.g. T-cell lymphoma in Boxers) suggest an as yet poorly understood genetic basis for the disease (Modiano et al., 2005; Pastor et al., 2009). Recent advances in gene sequencing technologies, including next generation sequencing (NGS), have led to an increase in the identification of mutations in canine lymphoma (McDonald et al., 2018; Aresu et al., 2019; Giannuzzi et al., 2019). Previous studies have shown the ability of gene expression profiling to separate canine lymphoma patients into distinct subcategories (Frantz et al., 2013; Richards et al., 2013), and numerous genetic abnormalities have been identified in canine lymphoma including chromosomal aberrancies (Thomas et al., 2003), somatic and germline mutations (Veldhoen et al., 1998; Mudaliar et al., 2013; Elvers et al., 2015). In particular, mutations in *tumour necrosis factor associated factor 3* (*TRAF3*) and *protection of telomeres 1* (*POT1*) genes have been identified in human and canine B-cell lymphoma (Elvers et al., 2015).

*TRAF3* is a tumour suppressor gene (Shanique KE, 2015) and loss of *TRAF3* activity leads to upregulated nuclear factor kappa-B (NF- kB) activity and reduced pro-apoptotic

signalling, ultimately leading to increased B-cell survival. *TRAF3* mutations and upregulated NF-kB activity have been implicated in cBCL development (Rowell et al., 2011; Mudaliar et al., 2013; Richards et al., 2013; Bushell et al., 2015; Elvers et al., 2015). Mutations resulting in *TRAF3* inactivation have been identified in 30-44% of cBCL patients (Bushell et al., 2015; Elvers et al., 2015), and nine percent of human DLBCLs (Bushell et al., 2015).

Dysregulated telomerase activity is a common feature of neoplasia, occurring in ~90% of human tumours. Seventeen percent of cBCL have been reported to have a mutation of the *POT1* gene (Elvers et al., 2015) which has a prominent role telomerase activity (Loayza and De Lange, 2003). *POT1* is also frequently mutated in human chronic lymphocytic leukaemia (CLL) (Ramsay et al., 2013; Burns et al., 2018), and has prognostic significance in this disease (Hamblin et al., 1999).

The purpose of the current study was to assess the prevalence of *TRAF3* and *POT1* mutations in a cohort of dogs with cBCL, compared to dogs with T-cell lymphoma (cTCL) and non-lymphoma diseases.

## Materials and methods

### *Samples*

Samples were collected from canine patients presenting to the oncology service at the Small Animal Hospital, University of Glasgow, UK between 2010 and 2014. Samples included tissue biopsies for histopathology and tissue fine-needle aspirates. Aspirates had DNA extracted with 24 hours of collection and samples were stored frozen (-80 degrees Celsius) or, if submitted for histopathology, were formalin-fixed paraffin-embedded (FFPE). Matched non-tumour DNA samples were obtained from blood samples with no cytological

evidence of circulating neoplastic cells. Samples were submitted to Veterinary Diagnostic Services, University of Glasgow, UK for pathological assessment as part of routine diagnostic evaluation or collected post-mortem. Lymphoma diagnosis was based on cytology or histopathology results in combination with polymerase chain reaction for antigen receptor gene rearrangement (PARR) results, flow cytometry, and/or immunohistochemistry (see table 1). PARR was performed as previously described (Waugh et al., 2016). Immunophenotyping by flow cytometry was performed using a panel of antibodies comprising: CD5, CD21, CD45, CD3, CD4, CD8, CD34, CD79a, MHC II, MAC387, and CD14. Immunohistochemistry antibody panels were decided by the attending pathologist, with the majority including PAX5 and/or CD79a, and CD3. DNA samples from non-lymphoma cases (from peripheral blood, lymph node aspirates, or lymph node FFPE tissue) were collected from canine patients with a variety of diseases, in which there was no clinical suspicion of lymphoid neoplasia (see table 2).

Sample collection and subsequent research activity were approved by the Faculty of Veterinary Medicine Ethics and Welfare Committee (License number: 1a/09; 32a/15; Approval date: 26 June 2015) and written consent was obtained from owners at the time of initial presentation. The *TRAF3* and *POT1* polymerase chain reactions (PCR) performed in this study used DNA which had been previously extracted from blood and tissue samples. DNA was purified from blood and unfixed samples using DNeasy Blood and Tissue Kits (Qiagen, Manchester, UK), and from formalin-fixed paraffin-embedded (FFPE) samples using QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions.

*Polymerase chain reaction (PCR)*

Primer design

Primers were designed using Integrated DNA Technologies® (IDT, Leuven, Belgium) online PrimerQuest® Tool. Primers were designed to amplify exons 9, 10 and 11 of the canine *TRAF3* gene and exons 7, 8, 9, 10, 11, 15, 16, 17 of the canine *POT1* gene (Supplementary Table 1). Exons were selected based on the location of the majority of variants identified in previous publications (Bushell et al., 2015; Elvers et al., 2015). Primers were synthesised by IDT.

Reaction conditions

Amplification was performed in a 25µL reaction volume consisting of: 0.1 units of Invitrogen AccuPrime *TAQ* DNA Polymerase, High Fidelity (ThermoFisher Scientific, Paisley, UK); Invitrogen™ Accuprime™ PCR Buffer II (ThermoFisher Scientific); 0.5 µM forward primer; 0.5 µM reverse primer; and 20 ng DNA template. Template controls contained water instead of template DNA and were included after every seven samples. Samples previously shown to contain amplifiable DNA served as positive controls.

Thermal cycling was conducted using a BIO-RAD C1000 Touch™ Thermal Cycler (BIO-RAD, Kidlington, UK) under the following reaction conditions: initial activation at 94°C for 2 minutes; 40 cycles of 94°C for 15 s, 58°C for 30 s, 68°C for 30 s; and 68°C for 7 minutes.

Products were visualised using one percent agarose gel electrophoresis.

*DNA Purification*

PCR products were purified to remove residual primers and nucleotides using the MinElute® PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions. Samples were eluted in 20-40 μL of water.

*Sequence Analysis*

Direct Sanger Sequencing of purified amplicons was performed by Source Bioscience (Bellshill, UK). Sequence files were imported into CLC Genomics Workbench 6.5.1 (Qiagen) software. Chromatograms were visually inspected (see figure 1), and samples with poor quality traces were re-sequenced. Contiguous sequences were made by aligning forward and reverse reads. These were then compared against the canine *TRAF3* and *POT1* reference nucleotide sequence CanFam 3.1 (Hoeppner et al., 2014) and sequence variants identified. Mutated alleles were translated *in silico* to determine the effect of variants. Mutations were classed as deleterious if they were predicted to alter the amino acid sequence of the protein.

**Results**

*Dogs*

Forty-nine dogs were included in the study; these comprised 24 cases with cBCL and 25 cases with non-cBCL diseases (see table 1 and table 2). Four cBCL samples did not have sufficient sample available for *TRAF3* exon 9 sequencing (all other sequencing was performed), so an additional four cBCL cases were recruited for *TRAF3* exon 9 sequencing alone, giving a total of 53 cases for *TRAF3* exon 9 (28 cBCL).

Eleven lymphoma samples (10 cBCL, one cTCL) had matched non-tumour samples for comparison to determine whether mutations were somatic or germline. The age of dogs

ranged from 1.1 years to 14.5 years (median 7.5 years). 22 breeds and eight crossbreed dogs were included, and no breeds were overrepresented.

*Sequencing/Mutation analysis*

In total 11 separate exons were sequenced for each case (three from *TRAF3*, eight from *POT1*), based on the location of mutations in previous publications (Bushell et al., 2015; Elvers et al., 2015). In seven individual cases unsatisfactory DNA amplification and/or DNA sequencing data was obtained for one of the 11 exons sequenced (four *TRAF3* exon 9, one *TRAF3* exon 11, and one each for *POT1* exons 7 and 10). In one case unsatisfactory DNA amplification and/or DNA sequencing data was obtained for two of the 11 exons sequenced (*TRAF3* exon 9 and *POT1* exon 9). Since *TRAF3* exon 9 was predominantly affected by this, an additional four cBCL cases were recruited for *TRAF3* exon 9 sequencing alone, giving a total of 28 cBCL cases which had at least partial *TRAF3* sequencing.

*TRAF3*

Eleven deleterious mutations of *TRAF3* were found in 10 of 28 cBCL patients, giving a prevalence of 36%. No deleterious mutations were identified in the non-cBCL cases. The mutations are described in Table 3. One dog had two deleterious mutations identified.

Most (nine of 11) deleterious mutations were frameshift mutations, leading to premature termination of the translation and causing truncation of the protein. One further mutation was a nonsense mutation causing an identical effect. The final mutation was an in-frame deletion of 12 base pairs (four amino acids). Protein modelling was not performed however this mutation was assumed to be deleterious. Figure 2 shows the location of the mutations on the *TRAF3* protein, and the protein domains impacted.

Five of ten dogs with a deleterious *TRAF3* mutation had matched non-tumour DNA. In four dogs no mutation was identified in the matched tissue, indicating the mutations were somatic. In one dog with a frameshift mutation the wild-type allele was not detected (loss of heterozygosity), and an identical mutation was present in the matched tissue, suggestive of a germline mutation.

In addition to the above mutations, 39 instances of non-deleterious (synonymous) variants were identified in 31 dogs. These were spread across disease types, with a mutation identified in 15/28 cBCL and 16/25 non-cBCL cases. Ten dogs had two separate synonymous variants. Of the ten dogs with a deleterious mutation, five also had synonymous variants. Four variants were detected, of which three had been identified previously (Table 4). Since these variants were found in a variety of disease types including non-lymphoma cases, they likely represent polymorphisms. This is supported by the finding that the same synonymous variant was present in the non-tumour DNA (germline) in six of seven cases with matched DNA samples. In the final case, the synonymous variant was only identified in the lymphoma sample. In this case the wild-type allele was not present (loss of heterozygosity).

*POT1*

Four deleterious mutations of *POT1* were found in four of 24 separate cBCL patients, giving a prevalence of 17%. No deleterious mutations were identified in non-cBCL cases. The mutations are described in Table 3. In the one dog with matched non-tumour DNA, the deleterious mutation was not identified in the matched tissue, suggesting the mutation was somatic.

Two mutations caused premature truncation of the *POT1* protein (one frameshift, one nonsense). Two mutations were missense mutations in either the oligonucleotide/oligosaccharide-binding (OB) domain or C-terminal domain of the protein. Protein modelling was not performed however all mutations were assumed to be deleterious. Figure 3 shows the coding location of the mutations on the *POT1* protein, and which protein domains would be impacted.

Additionally, a single identical non-deleterious (synonymous) variant was identified in 18 dogs, including 9/24 cBCL and 9/25 non-cBCL cases. This variant (Table 4) had been described previously, and likely represents a polymorphism. Consistent with this, the variant was detected in both tumour and non-tumour DNA from the single dog with matched samples.

Two dogs had a deleterious mutation identified in both *TRAF3* and *POT1* genes.

**Discussion**

The results of this study support previous findings that *TRAF3* and *POT1* mutations are frequent in cases of cBCL. The majority of the mutations identified were novel, however a single *TRAF3* mutation had previously been reported in cBCL (Sherry et al., 2001), and a single *POT1* mutation had previously been identified at the homologous location in a case of human melanoma (Shi et al., 2014). In our study *TRAF3* and *POT1* mutations were present in 36% and 17% of cBCL patients respectively. This is similar to previous studies which found mutations in 30-44% and 17% of cBCL patients respectively (Bushell et al., 2015; Elvers et al., 2015). Deleterious mutations of *TRAF3* and *POT1* were only identified in dogs with cBCL, supporting the idea that these genes are important in the pathogenesis of cBCLs.

The cBCL cases included in this study were not further subtyped, and therefore it is likely that different cBCL subtypes were included. Similar to human BCL, cBCL comprises a diverse group of distinct diseases. The most common BCL subtype in humans and dogs is diffuse large B-cell lymphoma (DLBCL) (Valli et al., 2011), and it is likely that the majority of cBCL included in this study were DLBCL. Histology and immunohistochemistry on all cases would be required to confirm this. As cBCL comprise a heterogeneous group of diseases, it is likely that different subtypes have different mutational characteristics, and future studies to assess how *TRAF3* and *POT1* mutations relate to different cBCL subtypes are required.

*TRAF3* is a member of the tumour necrosis factor receptor-associated factor (TRAF) family of cytoplasmic adaptor proteins (Shanique KE, 2015). It is considered a tumour suppressor gene, and loss of TRAF3 function has been implicated in human tumours including several types of lymphoma, and multiple myeloma as well as other malignancies (Bushell et al., 2015). Pro-survival signalling results in trimerization of TRAF3 with other proteins, inhibiting its function and ultimately inducing the transcription of anti-apoptotic Bcl2 family proteins, leading to B-cell survival (Shanique KE, 2015). TRAF3 also negatively regulates NF-kB (Sun, 2011; Bushell et al., 2015), and reduced TRAF3 activity leads to an upregulation of NF-kB activity.

The TRAF3 protein has several domains with specific functions (Figure 2). The coiled coil domains are crucial for interactions between the TRAF proteins and their substrates (Hacker et al., 2011). The meprin and TRAF homology (MATH) domain is necessary for receptor interaction and interactions with adaptor proteins (Hacker et al., 2011). All mutations identified in the current study would have resulted in premature truncation or

change of the TRAF3 protein before or within these domains, and therefore would be expected to disrupt normal *TRAF3* function.

Approximately 90% of human cancers upregulate telomerase and increased telomerase activity can predispose to neoplasia (Kim et al., 1994). POT1 has a prominent role in telomerase activity as part of the shelterin complex (Loayza and De Lange, 2003). POT1 is made up of three main domains: two N-terminal OB domains which have an affinity for single stranded DNA (specifically TTAGGG), and the *POT1* C-terminal domain which is critical for heterodimerisation and function as part of the shelterin complex (Ramsay et al., 2013; Pike et al., 2019).

The deleterious mutations in *POT1* identified in this study occurred within the OB or C-terminal domains (figure 3) and are predicted to impact normal POT1 protein function. Two of the four mutations were missense, with one occurring in the OB domain, and one occurring in the C-terminal domain. Missense mutations in the OB domains are predicted to affect POT1 function by decreasing interaction between aromatic residues and disrupting electrostatic interactions between the ssDNA and POT1 protein, thus reducing DNA affinity (Ramsay et al., 2013). A missense mutation affecting the identical amino acid residue in the OB domain of the highly conserved human POT1 protein has been detected in malignant melanoma (Shi et al., 2014); using several computational models, the authors predicted a deleterious effect on POT1 function.

In the present study the majority of detected mutations were suspected to be somatic. In the five dogs with *TRAF3* mutations and matched tissue, one had the mutation identified in the matched tissue, suggesting a germline variant. However, the matched DNA samples were

289 collected from peripheral blood, so detection of the mutation in circulating neoplastic cells, or
290 free tumour DNA in the blood, cannot be completely excluded. While it is possible that this
291 variant is a germline polymorphism, we did not find this variant in any of the non-cBCL
292 cases, as was seen with the synonymous variants. This suggests that the change may be a
293 genuine germline mutation. A previous study found 17.5% of cBCL *TRAF3* mutations were
294 germline (Bushell et al., 2015), which is similar to the current study (one of five dogs with
295 matched tissue). A *POT1* mutation was not detected in the matched tissue from the single dog
296 with available matched tissue, indicating a somatic mutation. However, germline *POT1*
297 mutations in humans have been associated with a predisposition to developing malignancies,
298 including some types of lymphoma (McMaster et al., 2018). Germline mutations in *TRAF3*
299 and *POT1* could result in a genetic predisposition to developing cBCL. Genome wide
300 association studies (GWAS) are needed to confirm this.
301
302 The prevalence of *TRAF3* mutations in our study was similar to previous studies (36%
303 vs 30-44%) (Bushell et al., 2015; Elvers et al., 2015). This is despite sequencing only exons
304 9, 10 and 11 of the *TRAF3* gene. These three exons constitute 51% of the entire *TRAF3*
305 sequence and a previous study of *TRAF3* mutations in cBCL (Bushell et al., 2015) found that
306 the majority of mutations occurred within exons 9, 10 and 11; however, some mutations were
307 identified in the other exons. As we did not sequence the entire gene, it is possible that our
308 results may underestimate the true prevalence of mutations in our population.
309
310 The prevalence of *POT1* mutations in our study was identical to previously reported
311 data (17%) (Elvers et al., 2015). In our study we sequenced only eight of 20 exons of the
312 *POT1* gene, based on the location of mutations identified in a previous study (Elvers et al.,
313 2015). In human medicine, *POT1* mutations associated with neoplasia have been reported to

cluster in the OB domains (Pinzaru et al., 2016). The eight exons sequenced in the present study represent 40% of the total gene, including 87% of the OB domains. As not all of the gene was sequenced, some significant mutations may have been missed, and the true prevalence of *POT1* mutations could have been higher than reported in this study. However, as we included the majority of the OB-domains, it is likely that our results captured the majority of mutations present, and our data were consistent with that of a previous study (Elvers et al., 2015).

Two dogs in this study had deleterious mutations identified in both the *TRAF3* and *POT1* genes. This could suggest that mutation of one gene could predispose to mutation of the other or could reflect that some tumours have significant genetic instability, resulting in increased instances of mutations. Future studies assessing the prognostic and therapeutic implications of dogs with mutations of both genes are required.

Most of the identified mutations (nine of 11 *TRAF3* and four of four *POT1*) were heterozygous, meaning these dogs also had a wild-type allele detected. However, both *TRAF3* and *POT1* mutations can have a dominant-negative effect, allowing heterozygous mutations to result in phenotypic changes. Mutations to the C-terminal TRAF3 domains (identified in this study) can prevent normal TRAF3 recruitment (Force et al., 1997), and *POT1* mutations can also function in a dominant-negative manner (Loayza and De Lange, 2003; Ramsay et al., 2013).

Our study has several limitations. Similar to many veterinary studies, our sample size is small. Given the prevalence of the mutations, this leads to identification of only a small overall number of mutations. We used bulk Sanger sequencing, which is insensitive to

mutations occurring at ~<20% allele frequency (MacConaill, 2013), so mutations may have been missed if they occurred in only a small proportion of the tumour cells, or if there was a significant number of non-tumour cells within the samples tested. This means the true prevalence of *TRAF3* and *POT1* mutations may have been underestimated. Also, the majority of samples did not have matched non-tumour tissue to assess whether the variants were germline or somatic. Despite the small numbers of matched samples, the proportion of germline and somatic mutations found in our study was similar to previous studies. Only a portion of the genes were sequenced in this study, which may have led to some mutations being missed, and an underestimation of the total number of mutations. Ideally, sequencing of the full genes would have been performed. Despite this it is likely that we were able to identify the majority of mutations present.

## Conclusions

This study adds further support to the idea that *TRAF3* and *POT1* mutations are important in the pathogenesis of cBCL. Future studies looking at potential prognostic and therapeutic implications of these mutations is needed. Studies assessing *POT1* and *TRAF3* germline mutations and predisposition to neoplasia would also add to our understanding of lymphomagenesis in the dog.

## Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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## Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at https:// doi.org/...

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509 **Table 1**

510 Canine B-cell lymphoma (cBCL) and canine T-cell lymphoma (cTCL) cases included in the
511 present study.

| Case group | Diagnosis | Tests for lymphoma diagnosis and immunophenotyping | Samples for DNA extraction |
|---|---|---|---|
| cBCL (*n*=28) | cBCL (*n*= 28) | Cytology, flow cytometry and PARR (*n*= 20)<br>Histology, IHC, PARR and flow cytometry (*n*= 4)<br>Cytology and PARR (*n*= 2)<br>Histology, IHC and PARR (*n*= 1)<br>Cytology and flow cytometry (*n*= 1) | Lymph node aspirates (*n*= 28) |
| cTCL (*n*=4) | High-grade (*n*=3)<br>TZL (*n*=1) | Cytology, flow cytometry and PARR (*n*=2)<br>Histology, IHC and PARR (*n*=1)<br>Histology, IHC, flow cytometry and PARR (*n*=1) | Lymph node aspirates (*n*=4) |

512 IHC, immunohistochemistry; PARR, PCR for antigen rearrangement; TZL, T-zone
513 lymphoma.

514
515 **Table 2**

516 Non-lymphoma cases included in the present study.

| Diagnosis | Samples for DNA extraction |
|---|---|
| Lysosomal storage disease (*n*=1) | FFPE lymph node |
| Soft tissue sarcoma (*n*=2) | FFPE lymph node (*n*=2) |
| Idiopathic immune-mediated thrombocytopenia (*n*=1) | Peripheral blood |
| Idiopathic immune-mediated neutropenia (*n*=1) | Bone marrow aspirate |
| Glaucoma (*n*=2) | Peripheral blood (*n*=2) |
| Idiopathic epilepsy (*n*=1) | Lymph node aspirate |
| Idiopathic immune-mediated polyarthritis (*n*=2) | Lymph node aspirate (*n*=2) |
| Allergic skin disease (*n*=1) | Peripheral blood |
| Portosystemic shunt (*n*=1) | Peripheral blood |
| Idiopathic/infectious lymphadenopathy which resolved with antibiotics (*n*=1) | Lymph node aspirate |
| Arrhythmogenic right ventricular cardiomyopathy (*n*=1) | Lymph node aspirate |
| Inflammatory bowel disease (*n*=1) | FFPE intestinal biopsy |
| Polyneuropathy (*n*=1) | Lymph node aspirate |
| Lymph node lipomatosis (*n*=1) | FFPE lymph node |
| Non-neoplastic reactive lymphadenopathy (diagnosis based on: histopathology and PARR [*n*=1], histopathology and IHC [*n*=1], histopathology, IHC, and PARR [*n*=1], cytology and PARR [*n*=1]) | FFPE lymph node (*n*=3)<br>Lymph node aspirate (*n*=1) |

517 FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry; PARR, PCR for
518 antigen receptor rearrangement.

519

519 **Table 3**

520 Deleterious *TRAF3* and *POT1* mutations in canine B-cell lymphoma (cBCL).

| Gene | Exon | Position | Mutation | Type | Allele frequency | Amino acid change | Protein domains affected | Present in matched sample (if tested) | Rs# (if previously reported) |
|---|---|---|---|---|---|---|---|---|---|
| *TRAF3* | 9 | 8:70782999 | T-deletion | Frameshift | Heterozygous | Ile302MetfsTer20 | TRAF | | |
| | 9 | 8:70782945 | A-T point | Nonsense | Heterozygous | Lys286Ter | TRAF | N | rs851689319 |
| | 9[a] | 8:70783037 | CCAAAATA insertion (duplication) | Frameshift | Heterozygous | Leu317ProfsTer9 | TRAF | N | |
| | 9 | 8:70783003 | A-insertion | Frameshift | Wild-type allele not detected | Glu303GlufsTer9 | CC | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | 8:70788018 | ACAG-deletion | Frameshift | Heterozygous | Asp324AlafsTer6 | CC | |
| 10 | 8:70788012-70788026 | AGTAATAGACAGCC-deletion | Frameshift | Wild-type allele not detected | Arg321ArgfsTer102 | CC | Y |
| 11 | 8:70789277 | A-deletion | Frameshift | Heterozygous | Tyr446SerfsTer7 | TRAF | |
| 11 | 8:70789371-70789383 | ATGCGTGGAGAG deletion | In-frame deletion | Heterozygous | Val477_His480del | MATH | |
| 11 | 8:70789530 | TC-insertion | Frameshift | Heterozygous | Ala510AlafsTer14 | MATH | N |
| 11 | 8:70789589 | A-deletion | Frameshift | Wild-type allele not detected | Lys549LysfsTer9 | MATH | |
| 11[a] | 8:70789131 | C-deletion | Frameshift | Heterozyg | Arg397AlafsTer21 | MATH | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | ous | | | | |
| *POT1* | 9 | 14:11033763 | T-deletion | Frameshift | Heterozyg ous | Phe309LeufsTer3 | OB | | |
| | 9 | 14:11033690 | C-T | Missense | Heterozyg ous | Arg284Cys | OB | N | [b] |
| | 15 | 14:11053090 | C-T | Nonsense | Heterozyg ous | Arg583Ter | C-domain | | |
| | 16 | 14:11056601 | T-C | Missense | Heterozyg ous | Phe643Ser | C-domain | | |

Y, yes; N, no; NT, not tested; CC, coiled-coil domain; MATH, meprin and TRAF homology domain; OB, oligonucleotide/oligosaccharide binding domain.

[a] Same dog.

[b] Mutation detected in a homologous location in human melanoma.

**Table 4**

*TRAF3* and *POT1* synonymous variants identified in the present study.

| Gene | Exon | Position | Mutation | Type | Total (n) | Allele Frequency (n) | Disease (cBCL, non-cBCL) | Present in matched sample | Rs# (if previously reported) |
|---|---|---|---|---|---|---|---|---|---|
| *TRAF3* | 9 | 8:70783013 | A-G | Synonymous | 24 | Heterozygous 13<br>Homozygous 11 | cBCL 5<br>Non-cBCL 8<br>cBCL 6<br>Non-cBCL 5 | Present in 4/5 matched samples | rs24528193 |
| | 10 | 8:70788076 | C-T | Synonymous | 1 | Heterozygous 1 | Non-cBCL 1 | No matched samples. | |
| | 11 | 8:70789480 | G-A | Synonymous | 11 | Heterozygous 7<br>Homozygous | cBCL 4<br>Non-cBCL 3<br>cBCL 1 | Present in 1/1 matched | rs853019186 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 4 | Non-cBCL 3 | samples | |
| | 11 | 8:70789213 | C-T | Synonymous | 3 | Heterozygous | cBCL 1 | Present | rs851492892 |
| | | | | | | 2 | Non-cBCL 1 | in 1/1 | |
| | | | | | | Homozygous | cBCL 1 | matched | |
| | | | | | | 1 | | samples | |
| *POT1* | 8 | 14:11030215 | A-G | | 18 | Heterozygous | cBCL 5 | Present | rs24528193 |
| | | | | Synonymous | | 10 | Non-cBCL 5 | in 1/1 | |
| | | | | | | Homozygous | cBCL 5 | matched | |
| | | | | | | 8 | Non-cBCL 3 | samples | |

527 cBCL, canine B-cell lymphoma; cTCL, canine T-cell lymphoma.

528

**Figure legends**

Fig. 1. Example mutations as depicted on sequencing chromatograms (CLC Workbench). A) Heterozygous C deletion (asterisk) resulting in frameshift, B) heterozygous C-T nonsense mutation (arrow), C) homozygous G-A synonymous variant (arrowhead). The reference sequence is depicted at the top of each example.

Fig. 2. Position of *TRAF3* mutations in relation to protein domains. An overview of the variants identified, showing the amino acid position and predicted protein domain of each variant. TRAF, tumour necrosis factor activating factor; RING, really interesting new gene; MATH, meprin and TRAF homology.

Fig. 3. Position of *POT1* mutations in relation to protein domains. An overview of the variants identified, showing the amino acid position and predicted protein domain of each variant. POT1, protection of telomeres 1; OB, oligonucleotide/oligosaccharide-binding.