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Research article

TRANSCRIPTOME ANALYSIS OF *ABCB1*, *ABCG2* AND THE *BCL2/BAX* RATIO IN REFRACTORY AND RELAPSED CANINE LYMPHOMAS UNDER TREATMENT AND RESCUE PROTOCOL

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The main problems that cause unresponsiveness to an anti-neoplastic drug are the overexpression of drug resistant and anti-apoptotic proteins in tumor cells. In a rescue protocol we evaluated the ability of toceranib phosphate concurrent with lomustine (CCNU) or L-asparaginase and vincristine to decrease drug resistant and apoptotic proteins in relapsed and refractory canine lymphomas. The peripheral blood samples were collected before and after the rescue treatment from fourteen dogs that were refractory to cyclophosphamide-vincristine-prednisolone (COP) or COP-doxorubicin (CHOP) treatment and had recurrent multicentric lymphoma. The mRNA expression level of *ABCB1*, *ABCG2*, *Bcl2* and *Bax* were determined by quantitative real-time PCR. The fold-change in *ABCB1*, *ABCG2*, *Bcl2* and *Bax* mRNA levels were analyzed in correlation with the progression-free survival (PFS). After the rescue treatment, the *ABCB1* and *ABCG2* mRNA expression levels were 1.57- and 1.85-fold lower ($p = 0.4$ and $p = 0.87$), respectively, compared to pre-treatment. Bcl2/Bax ratio was numerically but not significantly decreased 1.02-fold ($p = 0.74$). The overall response rate of this protocol was 50% with a median PFS of 79 days (range 14-207 days). The low medians of relative expression levels of *ABCB1*, *ABCG2* and Bcl2/Bax ratio group did not correlate with the clinical outcomes when compared to the high medians of relative expression levels, and likewise with the clinical stage, immunophenotype, histological grade and sub-stage. Therefore, the administration of a rescue drug with toceranib phosphate might be beneficial in refractory and relapsed canine lymphoma.

Key words: *ABCB1*; *ABCG2*; *Bax*; *Bcl2*; relapsed/refractory lymphoma

INTRODUCTION

One factor that causes failure in hematological cancer treatments, including canine lymphoma, is the multidrug resistant protein (MRP) mechanism. A member of the

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ATP-binding cassette (ABC) transporter MRPs, such as P-glycoprotein (Pgp; *ABCB1/MDR1*), breast cancer resistance protein (BCRP; *ABCG2*), multidrug resistance related protein 1 (MRP1; *ABCC1*), and lung resistance related protein (LRP; *LRP1*), are expressed as an intra- or extra-cellular membrane component of normal cells. However, they can become over-expressed in tumor cells due to induction by cytotoxic drugs, for example vincristine and doxorubicin [1], which are used as a standard treatment in canine lymphomas.

The Pgp plays an important role in the MRP mechanism in canine lymphomas. Their expression levels have been detected in previous studies by semi quantitative techniques, such as western blotting [2], and immunohistochemistry [3,4]. However, these methods have a low sensitivity and specificity. Conversely, quantitative real-time PCR (qRT-PCR) shows a greater accuracy in the detection of low ABCB1 transcript levels in normal canine tissues and canine lymphomas [5]. There have been a few studies on the resistant genes that are involved in resistant/relapsed canine multicentric B- and T-cell lymphomas, and these have reported the possible involvement of *ABCB1* and *ABCG2* in terms of their expression levels [6,7].

Receptor tyrosine kinases act as cell surface receptors that bind to growth factors and hormones. They are also involved in the control of cell growth and survival. The dysregulation of these receptors is involved in tumorigenesis [8]. In veterinary oncology, the tyrosine kinase inhibitor (TKI) was developed and shown to be an effective treatment drug in canine mast cell tumors and other solid tumors, including canine lymphomas [9-11]. Moreover, TKI had an inhibitory effect on Pgp function and reverted doxorubicin resistance in canine lymphoma cells [12].

Failure of cell apoptosis can result in cytotoxic drug resistance. In lymphoid malignancies, the common apoptotic signaling pathway is disturbed through the intrinsic pathway. This pathway is regulated by the related Bcl2 family protein, cell death promoters (BH3-only proteins) and cell death mediators (Bax and Bak). The impaired function of these apoptotic molecules can lead to oncogenesis [13]. For example, mutations in the *Bcl2* and tumor suppressor *p53* genes that cause loss of Bax activity are frequently involved in human or canine lymphomas [14-18]. Furthermore, in canine lymphomas, it was reported that T-cell lymphomas had a higher Bcl2/Bax ratio than B-cell lymphomas with poor clinical outcome in the high Bcl2/Bax level group [17]. Thus, Bcl2 and Bax expression might be a meaningful prognostic indicator in dogs with lymphoma.

The common chemotherapy for canine multicentric lymphoma is cyclophosphamide-vincristine-prednisolone (COP) or COP-doxorubicin (CHOP) regimes, while the general rescue drugs are lomustine (CCNU) or L-asparaginase. The aim of this study was to determine the favorable effect of the TKI (toceranib phosphate) concurrent with CCNU or L-asparaginase and vincristine as a rescue protocol in refractory and relapsing canine lymphomas that had previously been treated with COP or CHOP-based protocols. The transcript expression level of *ABCB1*, *ABCG2*, *Bax*, and *Bcl2*

were compared between pre- and post-treatment. The MRP (ABCB1 and ABCG2) transcript levels and Bcl2/Bax transcript ratio were compared with the clinical outcomes.

MATERIALS AND METHODS

Blood sample collection

Peripheral blood samples (1 ml) were collected from 14 dogs between January 2013 and July 2016 at the Oncology Clinic, Small Animal Teaching Hospital, Chulalongkorn University (IACUC Number 13310074). Informed consent has been obtained for client-owned animals included in this study. Each dog was diagnosed as having multicentric lymphoma by histopathology. The immunophenotyping was performed with Pax5 and CD3 as described previously [19]. Five dogs had previously been treated with a modified COP regime, while the other nine dogs were previously treated with a modified CHOP regime. Five of 14 cases (1 COP-treated and 4 CHOP-treated dogs) had a complete remission after treatment and then the disease relapsed after 3-6 months, whereas the others (5 COP-treated and 4 CHOP-treated dogs) showed no response to the current chemotherapy with progressive disease (superficial lymph node size was increased in the sum longest diameter more than 20%). A rescue protocol with toceranib phosphate (Palladia, Zoetis, UK) was applied in these cases, as shown in Table 1, but before giving the rescue treatment, 1 ml of peripheral blood was collected. In addition, seven of these dogs had another 1 ml of blood collected four weeks after applying the rescue treatment. For calibration samples, 3 ml of blood was collected from three healthy dogs with no clinical history of disease. The blood samples from five dogs with naïve canine lymphomas were collected for comparison with animals with relapsed/refractory lymphomas. Peripheral blood samples were collected into EDTA tubes and then centrifuged at 5,000 rpm for 5 min for white blood cell (WBC) separation. The harvested WBC pellet was stored at -80 ºC until subsequent RNA isolation.

Table 1. Rescue protocol for relapsed/refractory canine lymphomas

Protocol*	Week						
	0	1	2	4	5	7	8
A (7 dogs)							
L-asparaginase 400 IU/kg SC	●						
Vincristine 0.5-0.7 mg/m^2 IV	●	●		●		●	
Toceranib phosphate 2.5 mg/kg/d PO EOD			●		●		●
B (7 dogs)							
CCNU 70 mg/m^2 PO	●						
Vincristine 0.5-0.7 mg/m^2 IV						●	
Toceranib phosphate 2.5 mg/kg/d PO EOD				●	●		●

*EOD = every other day, IV = intravenous, PO = per os, SC = subcutenous.

Follow up case

Every dog was evaluated for superficial lymph node size and checked for routine hematology (complete blood count) and serum chemistry (liver profiles; alanine aminotransferase and alkaline phosphatase levels, and kidney functions; blood urea nitrogen and creatinine levels) after each rescue phase. If dogs showed serious clinical signs, such as anorexia, vomiting, diarrhea or fever, and abnormal blood results, such as leukopenia, anemia, renal azotemia or increased serum levels of hepatic enzymes, then administration of the anti-neoplastic drug was stopped and supportive treatment was applied. The progression-free survival (PFS) was recorded from each dog during the rescue phase.

RNA isolation and cDNA synthesis

Total RNA was extracted from the WBC pellet using a Nucleospin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Contaminating genomic DNA in the RNA samples was removed by treatment with RQ1 RNase-Free DNase (Promega Corp., Madison, WI) at room temperature for 15 min to minimize the effect of pseudogenes. The RNA concentration was measured by a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA) before converting to cDNA. Thereafter, cDNA was synthesized by Omniscript Reverse Transcription (Qiagen, Hilden, Germany) using 50 ng of total RNA in a final volume 20 µl. All reactions were performed as recommended by the manufacturer's instructions. Samples were stored at -20 ºC until used.

Primer design and testing

The TATA box binding protein (TBP), beta actin (ACTB) and ribosomal protein S26 (RP S26) were chosen for normalization of the canine WBC pellet sample using the primer sequences (Table 2) as previously described [7,20,21]. To determine the best stability of these reference genes in the WBC pellets and individual variation in calibration samples, seven serial two-fold dilutions of the WBC pellet from each of three healthy dogs were performed. The evaluation of the candidate reference gene expression was calculated using five algorithms; the comparative delta Ct method [22], the geNorm [23], Normfinder [24], BestKeeper [25] and RefFinder [26]. Canine *ABCB1, ABCG2, Bax* and *Bcl2* primers (Table 2) were selected as previously described [5,7,21]. Conventional PCR was performed to confirm the PCR conditions for amplification efficiency and specificity, and subsequently used GoTaq Green Master Mix (Promega Corp.) with 0.4 µM of each primer and 2 µl of cDNA in a final reaction volume of 25 µl. The PCR thermal cycling was performed as 39 cycles of 95 ºC for 30 s, 50-60 ºC as a gradient for 1 min and 72 ºC for 1.3 min using a C-Master Pro thermal cycler (Dynamica Scientific Ltd., Newport Pagnell, United Kingdom). Afterwards the PCR products were separated by 2% (w/v) agarose gel electrophoresis, imaged by UV transillumination in the presence of a distinct band and the product bands were cut

out and purified by Nucleospin extract II kit (Macherey-Nagel). The products were then sent for commercial DNA sequencing to the SolGent analysis service (SolGent Co. Ltd., Daejeon, Korea).

Table 2. Primers for qRT-PCR

Gene	Sequence primer (5´-->3´)	Ta (ºC)	Size (bp)	GenBank no.
ABCB1	F 5´-CAG TGG TTC AGG TGG CCC T-3´ R 5´-CGA ACT GTA GAC AAA CGA TGA GCT-3´	81	79	NM001003215
ABCG2	F 5´-GGT ATC CAT AGC AAC TCT CCT CA-3´ R 5´-GCA AAG CCG CAT AAC CAT-3´	81.3	143	NM001048021
BAX	F 5´-TTC CGA GTG GCA GCT GAG ATG TTT-3´ R5´- TGC TGG CAA AGT AGA AGA GGG CAA-3´	82.3	79	KT693115
Bcl2	F 5´-CAT GCC AAG AGG GAA ACA CCA GAA-3´ R5´-GTG CTT TGC ATT CTT GGA TGA GGG-3´	80.2	76	NM001002949
S26	F 5´-CGT GCT TCC CAA GCT GTA CGT GA-3´ R 5´-CGA TTC CGG ACT ACC TTG CTG TG-3´	82	75	XM531628
TBP	F 5´-CTA TTT CTT GGT GTG CAT GAG G-3´ R 5´-CCT CGG CAT TCA GTC TTT TC-3´	80.3	96	XM849432
B-actin	F 5´-ATG GAA TCA TGC GGT ATC CAC-3´ R 5´-CTT CTG CAT CCT GTC AGC AA-3´	83	141	NM001195845

Quantification of transcript levels by qRT-PCR

The qRT-PCR was performed on triplicate cDNA samples in a 20 µl total reaction. Volume comprised of 10 µl of KAPA SYBR FAST Master Mix (Kapa Biosystems Inc., Wilmington, MA), 200 nM of forward and reverse primers and 5 ng of cDNA. The PCR amplification was performed in a Rotor-Gene Q (Qiagen) with a thermal profile of 95 ºC for 3 min followed by 40 cycles at 95 ºC for 3 s and 60 ºC for 30 s. The optimal temperature was determined by conventional PCR with temperature gradient from 50-60 ºC. The reaction was continued by a melting curve analysis, increasing the temperature stepwise each 5 s by 1 ºC, over the range of 65-95 ºC. A no template control and a positive control with known Cq value were included in each analysis to the assay specificity and Cq variation. Analysis of the qRT-PCR results was then performed using the Rotor-Gene Q Series software (Qiagen), where the ΔΔCq method was used to determine the fold differences in concentration between the target gene of interest and the normalization gene.

Two-fold serial dilutions of the calibrator sample were performed in triplicate for each cDNA dilution (six dilutions range from 2^{-1} to 2^{-6} ng). The slope of the log-linear portion of the calibration curve was used to calculate the PCR amplification efficiency.

Data analysis

The significance of variation in the transcript levels in canine lymphomas and relapsed/refractory lymphomas, substage, histopathological grade and immunophenotype were

analyzed by the Mann-Whitney U test. The influence of the clinical stage upon gene expressions levels was evaluated using the Kruskal-Wallis test. Wilcoxon-signed rank test was used to analyze the difference in gene expressions levels between pre- and post-rescue treatment dogs. The PFS was calculated from the initial rescue treatment until progressive disease or death from the treatment. The median PFS was noted and compared between low and high MRP transcript levels and the Bcl2/Bax ratio group, clinical stage, substage, histological grade and immunophenotype by Kaplan-Meier analysis with a log rank test. Alive dogs were censored when this study was analyzed. Statistics was performed using the SPSS statistics version 22 software (IBM Corporation, Armonk, NY), accepting significance at a p value of ≤ 0.05.

RESULTS

Clinical data

The signalments of 14 dogs were summarized in Table 3. They comprised six female (42.9%) and eight male (57.1%) dogs with a median of 7.5 years (range from 4-12 years). With respect to the lymphoma stage, nine (64.3%) were substage a (patients with no clinical signs) and five (35.7%) were substage b (patients with clinical signs), while five of each (35.7%) were stage III and stage IV, and four (28.6%) were stage V. For the phenotype, 10 (71.4%) were B-cell lymphomas and four (28.6%) were T-cell lymphomas.

Table 3. The signalment, WHO histopathology, and PFS of 14 relapsed/refractory canine lymphomas

Dog no.	Breed*	Age (y)	Gender†	WHO stage	Substage	Histopathology‡	PFS (d)
1	GR	11	Fs	IV	b	PTCL	70
2	Po	10	Mc	IV	b	T-ALCL	136
3	WHWT	6	Fs	V	b	DLBCL	196
4	Pu	7	Mc	III	a	DLBCL	127
5	MP	6	M	IV	b	B-SLL	79
6	BT	4	M	IV	b	PTCL	45
7	B	10	M	V	a	DLBCL	207
8	GR	7	M	III	a	DLBCL	21
9	Po	11	Fs	III	a	T-SLL	Alive
10	BT	8	M	III	a	DLBCL	Alive
11	Pu	5	Fs	III	a	DLBCL	25
12	ST	12	F	IV	a	DLBCL	29
13	LR	6	F	V	a	DLBCL	102
14	GR	12	M	V	a	DLBCL	14

*B = beagle, BT = bull terrier, GR = golden retriever, LR = Labrador retriever, MP = miniature pinscher, Pu = pug, Po = poodle, ST = shih tzu, WHWT = West Highland white terrier.
†M = male, Mc = castrated male, F = female, Fs = spayed female.
‡B- or T-SLL = B- or T-small lymphocytic lymphoma, DLBCL = diffuse large B-cell lymphoma, T-ALCL = anaplastic large T-cell lymphoma, PTCL = peripheral T-cell lymphoma.

The median PFS did not show any significant differences among the substages (a = 102 days, 95% CI 0-315.3 vs. b = 79 days, 95% CI 59.7-98.3; $p = 0.66$), WHO stage (III = 127 days, 95% CI 0-346 vs. IV = 70 days, 95% CI 16.3-123.7 vs. V = 102 days, 95% CI 0-280.4; $p = 0.39$), histological grade (low = 136 days , 95%CI 44.8-227.2 vs. high = 70 days, 95% CI 0-148.6; $p = 0.25$) and immunophenotype (B = 79 days, 95% CI 0-192.1 vs. T = 70 days, 95% CI 0-159.2; $p = 0.49$).

The overall response rate of the rescue protocol was 50%, where five (35.7%) dogs had a complete remission, two of each (14.3%) had a partial response and stable disease, and the other five (35.7%) dogs had a progressive disease and died after the initial treatment.

Amplification efficiency and specificity of qRT-PCR

For PCR specificity, the three internal reference genes and four genes of interest showed a single band of an appropriate size following agarose gel electrophoresis. The DNA sequences obtained from the PCR products were identical to the deposited gene sequences from the GenBank database (data not shown), while all the qRT-PCR reactions revealed a single melt peak in their respective melting curve analysis. For the PCR reaction efficiency, all the primer pairs gave 90%-110% amplification efficiency, determined from the standard curve analysis of the two-fold serial dilutions. The three reference genes (TBP, ACTB and RP S26) could be amplified from the WBC samples obtained from the five healthy dogs of different ages, breeds, sex and body weight. However, after analyzing the data with the five algorithms, TBP was found to be the most stable gene from the delta Ct, normFinder and geNorm (equal to RP S26) methods (data not shown), and therefore it was selected as the internal reference gene in this study. The subsequent expression of the mRNA levels of the five genes (*TBP, ABCB1, ABCG3, Bcl2* and *Bax*) was detected in all 21 samples with the highest mean Ct being 35.26 for *ABCB1* transcript expression.

Relative expression levels of MRP (ABCB1 and ABCG2) genes in relapsed/refractory lymphomas, immunophenotyping and prognosis

The transcript levels of *ABCB1* and *ABCG2* were compared between lymphomas prior to the treatment and relapsed/refractory lymphomas. The median relative transcript levels of *ABCB1* and *ABCG2* in lymphomas prior to treatment were 1.62- and 1.95-fold lower, respectively, than the relapsed/refractory lymphomas (Figure 1A), although this was only significantly different for *ABCG2* ($p = 0.01$) and not for *ABCB1* ($p = 0.56$).

Before rescue initiation, the T-cell lymphomas had 2.65- and 2.4-fold numerically higher *ABCB1* and *ABCG2* median relative transcript levels, respectively, than B-cell lymphomas, however; these were not significant ($p = 0.24$ and 0.14, respectively, Figure 1C). After the rescue treatment, relapsed/refractory lymphomas had median relative transcript levels of *ABCB1*1.57-fold lower than in the pre-treatment ($p = 0.4$).

In addition, the *ABCG2* transcript levels were decreased 1.85-fold after the rescue protocol ($p = 0.87$, Figure 2).

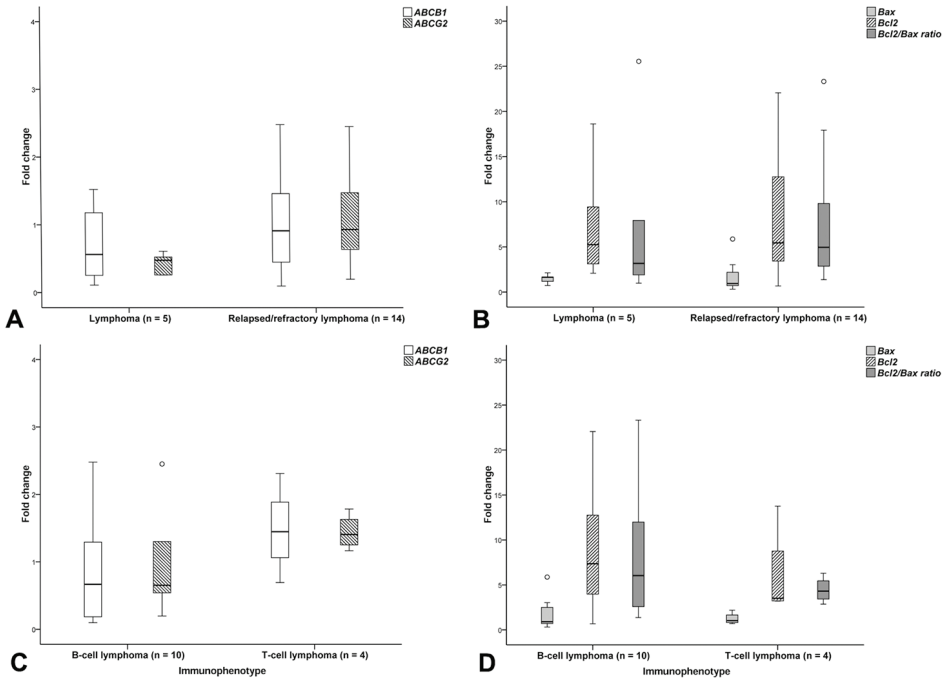

Figure 1. Box plots showing the relative quantities of mRNA for (A, C) *ABCB1* and *ABCG2* and (B, D) *Bcl2*, *Bax*, and Bcl2/Bax ratio in (A, B) five canine lymphomas at the first diagnosis and 14 relapsed/refractory lymphomas and (C, D) in 10 B-cell lymphomas and four T-cell lymphomas. The white circles indicate the outlier.

The transcripts levels of MRP (*ABCB1* and *ABCG2*) in the pre-treatment lymphomas were numerically, but not significantly, correlated with the PFS. The relapsed/refractory lymphomas with a low median transcript level of *ABCB1* had a median PFS of 102 days compared to the high levels group with a median PFS of 79 days ($p = 0.79$), while the median PFS of the low *ABCG2* transcript level group was 79 days compared to 25 days for the high expression group ($p = 0.76$).

Relative expression levels of Bax and Bcl2 and the Bcl2/Bax transcript ratio in relapsed/refractory lymphomas, immunophenotyping, and prognosis

Canine lymphomas had 1.74-fold higher median *Bax* transcript levels ($p = 0.5$), a 1.04-fold lower median *Bcl2* expression ($p = 0.75$), and a 1.56-fold lower *Bcl2*/*Bax* ratio ($p = 0.62$) than relapsed/refractory canine lymphomas with no statistically significant difference (Figure 1B).

When compared between B- and T-cell lymphoma, relapsing/refractory B-cell lymphomas had 1.23-fold lower median *Bax* transcript levels ($p= 0.95$) and 1.2-fold

higher median *Bcl2* transcript levels (p = 0.3) than T-cell lymphomas (Figure 1D). After the rescue treatment, relapsed/refractory lymphomas had 1.1-fold higher median relative transcript levels of *Bax* than in the pre-treatment (p = 0.4). *Bcl2* transcript levels had 1.4-fold lower than in pre-treatment (p= 0.61). Thus, relapsed/refractory lymphomas had a 1.02-fold lower *Bcl2*/*Bax* ratio than in the pre-treatment (p= 0.74, Figure 2).

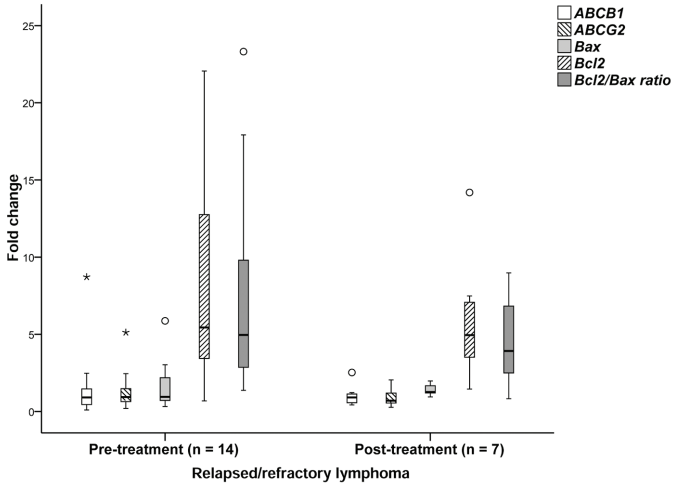

Figure 2. Box plot showing the fold-change in mRNA levels of *ABCB1*, *ABCG2*, *Bcl2*, *Bax*, and the Bcl2/Bax transcript ratio in 14 dogs with lymphoma before rescue treatment and seven dogs after the rescue treatment. The white circles indicate the outlier.

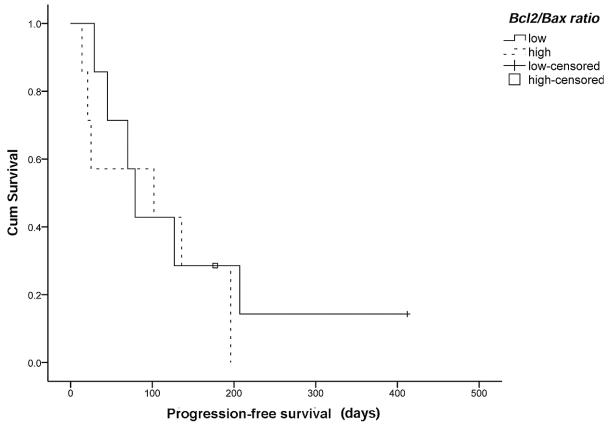

Figure 3. Kaplan-Meier survival graph illustrating the lymphoma survival time in 14 dogs with lymphomas grouped into those with a low and a high median Bcl2/Bax transcript ratio (Cum = cumulative).

Overall the *Bax* and *Bcl2* transcript levels and the *Bcl2*/*Bax* transcript ratio were not significantly associated with the clinical outcome. The median PFS of the high *Bcl2*/

Bax ratio group was 102 days, whereas the low *Bcl2*/*Bax* ratio group had a lower median PFS of 79 days ($p = 0.58$) (Figure 3). Regarding to the two rescue treatment groups, there were no differences in transcript levels of all genes and PFS between groups (data not shown).

DISCUSSION

The overall response rate for this rescue treatment with toceranib phosphate was 50% and patients had an overall median PFS of 79 days. Many rescue drugs have been developed for the treatment of relapsed/refractory multicentric lymphomas. However, because of drug resistant overexpression induced by previous COP- or CHOP-based protocols, the DNA alkylating agent CCNU and L-asparaginase (a hydrolytic enzyme from bacteria) are commonly used to treat dogs with relapsed/refractory lymphomas [27]. Toceranib phosphate, a receptor TKI, is known to retard tumor growth and angiogenesis via inhibition of the vascular endothelial growth factor receptor, platelet-derived growth factor receptor, Kit and Flt-3 [28]. It has been used to treat many tumors in dogs, including mast cell tumors, solid tumors, and lymphomas [9-11]. In human studies, TKIs inhibit the ATP binding site of receptor tyrosine kinases and prevent phosphorylation leading to cell death. They can block ABC drug transporter and possibly treat relapsed/resistant disease [29]. For example, Nilotinib has an inhibitory effect of the ABCB1 and ABCG2 function in chronic myeloid leukemia or Ponatinib shows the increased uptake of substrates of ABCB1 and ABCG2 [30,31]. Zandvliet et al. (2013) reported that masitinib inhibited Pgp activity and reverted doxorubicin resistance *in vitro*. From our study, dogs that were resistant to the COP and CHOP protocols tended to have a lower transcript level of the drug resistance proteins, Pgp and BCRP (*ABCB1* and *ABCG2*), after treatment with toceranib phosphate. However, further investigation with an increased number of cases is required.

Transcripts of *ABCB1* and *ABCG2* were detected in all WBC samples by qRT-PCR. From the three evaluated reference genes, TBP was found to be the most stable, followed by RP S26 and ACTB, as analyzed by RefFinder, and so TBP was chosen for normalization in this study, which is similar to previous studies [6,7,32]. When comparing the transcript levels of the two drug resistance proteins (*ABCB1* and *ABCG2*) between canine multicentric lymphomas at the time of their diagnosis and at the first time of relapse or becoming refractory to the current treatment, the *ABCB1* and *ABCG2* transcript levels were increased, consistent with that previously reported [6,7]. T-cell lymphomas were found to have higher transcript levels of both *ABCB1* and *ABCG2* than B-cell lymphomas in this study. However, it has previously been reported that T-cell lymphomas show only an increased expression of *ABCG2* [7]. In contrast, four out of ten dogs in the chemotherapy-resistant group increased expression of *ABCB1* [6]. Nevertheless, the sample size of our study was small and we evaluated the transcript levels of MRPs in WBC samples, while they analyzed them from lymph node cytology samples. Moreover, the up-regulation of ABCB1 and ABCG2 transcript levels did not significantly relate to a poor prognosis.

Inhibition of apoptosis is involved in tumor progression and leads to cytotoxic drug tolerance. In this study we detected changes in both Bax and Bcl2 mRNA transcript levels in relapsed and refractory canine lymphomas by qRT-PCR. Relapsed/refractory lymphomas had overexpressed Bcl2 and downregulated Bax transcript levels compared to those lymphomas before the cytotoxic treatment. However, the Bcl2/Bax ratio was previously found to be a useful prognosis tool in both humans and dogs [17,33]. Meichner et al. (2016) compared the ability to quantitatively detect the expressed protein and transcript levels of Bcl2 and Bax using Western blotting and flow cytometry for protein detection and qRT-PCR for transcript detection. The results were not different, and so transcript levels likely reflected protein expression levels, but flow cytometry was superior due to its relative speed and coherent result. In this study we used qRT-PCR to detect transcript levels, but the results showed a high degree of variation. The trend of Bcl2/Bax transcript ratio was higher in B-cell than T-cell lymphomas. Dogs with a higher Bcl2/Bax transcript ratio than the median value tended to have shorter survival times, similar to a previous study [17]. The limitation of this study was the small sample size, which might be why it failed to predict the clinical outcome and prognosis.

High grade T-cell lymphomas have a poorer prognosis than high grade B-cell lymphoma due to shorter progression-free and survival times [34,35]. The aggressive behavior of neoplastic T lymphocytes might be sensitive to overexpressed MRP and anti-apoptotic proteins. Programmed cell death occurs via intrinsic and extrinsic pathways. The extrinsic pathway is controlled by two phases which are death-stimuli dependent and degradation phases. After adaptor proteins are bound to the ligand of death receptors, the apoptotic signals are transduced and led to DNA fragmentation. The common cell death receptors are induced via Fas, Trail or TNF. Another apoptotic mechanism is intrinsic or mitochondrial pathway which is regulated by the Bcl2 protein family. When pro-apoptotic molecules, Bax and Bak, are activated, they trigger mitochondrial outer membrane permeabilization followed by release of cytochrome c and activation of caspases. The recent studies reported that Pgp could inhibit the activation of caspases in both T- lymphoblast cell lines and primary T-lymphocytes [36,37]. In addition, T-leukemic cell lines were sensitive to Fas-mediated apoptosis by activation of caspase cascade opposite to B-cell lines that were resistant to Fas- and Trail-induced apoptosis [38]. Thus, ABCB1 expression might affect T- cell survival during the anti-neoplastic drug treatment. ABCG2 is commonly expressed in lymphoid malignancy. In humans, ABCG2 transcription levels were relevantly up-regulated after exposing with daunorubicin and mitroxantrone in T-cell acute lymphoblastic leukemia [39]. However, the study on the resistant mechanism induced by *ABCG2* is limited in B- and T-cell lymphoma regardless to an efflux transporter. The doxorubicin-based protocol is a treatment of choice in canine lymphomas; ABCG2 may play an important role in progressive diseases in dogs.

Many details are reported that Bcl2 family proteins are involved in the pathogenesis of both B- and T-cell lymphoma by escaping apoptosis due to Bcl2 overexpression

and Bax/Bak downregulation. In a previous study, pro-apoptotic (Bax, Bak, Bid, and Bad) and anti-apoptotic (Bcl2 and Bcl-xl) clusters were investigated in human DLBCL. The pro-apoptotic profiles showed the higher expression in the germinal center B-cell lymphoma and could be used to identify subgroup of DLBCL [40]. The STAT-signaling pathway regulates Bcl2 expression and cell survival in PTCL. Thus, specific target therapy with Bcl2 inhibitors is developed as a single or combination treatment to overcome the resistant disease in human lymphoid malignancies [41].

In summary, the TKI toceranib phosphate concurrent with a rescue drug is likely to decrease the Pgp and BCRP (*ABCB1* and *ABCG2*) expression levels in canine relapsed/refractory lymphomas as well as anti-apoptosis molecules. However, increased amount of sample size is required to confirm its potentiality to treat relapsed/refractory B- and T-cell lymphomas.

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Author's contributions

SS collected samples, carried out the experiment, analyzed the data, and drafted the manuscript. PT collected samples. AR and ST (Techangamsuwan) conceived the study design and clinically revised the manuscript. ST (Tangkawattana) clinically revised the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Uozurmi K, Nakaichi M, Yamamoto Y, Une S, Taura Y: Development of multidrug resistance in a canine lymphoma cell line. Res Vet Sci 2005,78:217-224.
2. Moore AS, Leveille CR, Reimann KA, Shu H, Arias IM: The expression of P-glycoprotein in canine lymphoma and its association with multidrug resistance. Cancer Invest 1995,13:475-479.
3. Bergman PJ, Ogilvie GK, Powers BE: Monoclonal antibody C219 immunohistochemistry against P-glycoprotein: sequential analysis and predictive ability in dogs with lymphoma. J Vet Intern Med 1996,10:354-359.
4. Lee JJ, Hughes CS, Fine RL, Page RL: P-glycoprotein expression in canine lymphoma: a relevant, intermediate model of multidrug resistance. Cancer 1996,77:1892-1898.

5. Culmsee K, Gruber AD, von Samson-Himmelstjerna G, Nolte I: Quantification of MDR-1 gene expression in canine tissues by real-time reverse transcription quantitative polymerase chain reaction. Res Vet Sci 2004,77:223-229.
6. Tomiyasu H, Goto-Koshino Y, Takahashi M, Fujino Y, Ohno K, Tsujimoto H: Quantitative analysis of mRNA for 10 different drug resistance factors in dogs with lymphoma. J Vet Med Sci 2010,72:1165-1172.
7. Zandvliet M, Teske E, Schrickx JA, Mol JA: A longitudinal study of ABC transporter expression in canine multicentric lymphoma. Vet J 2015,205:263-271.
8. Lemmon MA, Schlessinger J: Cell signaling by receptor tyrosine kinases. Cell 2010,141:1117-1134.
9. London CA, Malpas PB, Wood-Follis SL, Boucher JF, Rusk AW, Rosenberg MP, Henry CJ, Mitchener KL, Klein MK, Hintermeister JG, Bergman PJ, Couto GC, Mauldin GN, Michels GM: Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. Clin Cancer Res 2009,15:3856-3865.
10. London C, Mathie T, Stingle N, Clifford C, Haney S, Klein MK, Beaver L, Vickery K, Vail DM, Hershey B, Ettinger S, Vaughan A, Alvarez F, Hillman L, Kiselow M, Thamm D, Higginbotham ML, Gauthier M, Krick E, Phillips B, Ladue T, Jones P, Bryan J, Gill V, Novasad A, Fulton L, Carreras J, McNeill C, Henry C, Gillings S: Preliminary evidence for biologic activity of toceranib phosphate (Palladia((R))) in solid tumours. Vet Comp Oncol 2012,10:194-205.
11. Pan X, Tsimbas K, Kurzman ID, Vail DM: Safety evaluation of combination CCNU and continuous toceranib phosphate (Palladia((R))) in tumour-bearing dogs: a phase I dose-finding study. Vet Comp Oncol 2016,14:202-209.
12. Zandvliet M, Teske E, Chapuis T, Fink-Gremmels J, Schrickx JA: Masitinib reverses doxorubicin resistance in canine lymphoid cells by inhibiting the function of P-glycoprotein. J Vet Pharmacol Ther 2013,36:583-587.
13. Llambi F, Moldoveanu T, Tait SW, Bouchier-Hayes L, Temirov J, McCormick LL, Dillon CP, Green DR: A unified model of mammalian BCL-2 protein family interactions at the mitochondria. Mol Cell 2011,44:517-531.
14. Dettwiler M, Croci M, Vaughan L, Guscetti F: Immunohistochemical expression study of proapoptotic BH3-only protein bad in canine nonneoplastic tissues and canine lymphomas. Vet Pathol 2013,50:789-796.
15. Jung JT, Kim DH, Kwak EK, Kim JG, Park TI, Sohn SK, Do YR, Kwon KY, Song HS, Park EH, Lee KB: Clinical role of Bcl-2, Bax, or p53 overexpression in peripheral T-cell lymphomas. Ann Hematol 2006,85:575-581.
16. Koshino A, Goto-Koshino Y, Setoguchi A, Ohno K, Tsujimoto H: Mutation of p53 Gene and Its Correlation with the Clinical Outcome in Dogs with Lymphoma. J Vet Intern Med 2016,30:223-229.
17. Meichner K, Fogle JE, English L, Suter SE: Expression of Apoptosis-regulating Proteins Bcl-2 and Bax in Lymph Node Aspirates from Dogs with Lymphoma. J Vet Intern Med 2016,30:819-826.
18. Sohn SK, Jung JT, Kim DH, Kim JG, Kwak EK, Park T, Shin DG, Sohn KR, Lee KB: Prognostic significance of bcl-2, bax, and p53 expression in diffuse large B-cell lymphoma. Am J Hematol 2003,73:101-107.

19. Sirivisoot S, Techangamsuwan S, Tangkawattana S, Rungsipipat A: Pax5 as a potential candidate marker for canine B-cell lymphoma. Vet Med (Praha) 2017,62:74-80.
20. Peters IR, Peeters D, Helps CR, Day MJ: Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of canine gene expression studies. Vet Immunol Immunopathol 2007,117:55-66.
21. Del Puerto HL, Martins AS, Moro L, Milsted A, Alves F, Braz GF, Vasconcelos AC: Caspase-3/-8/-9, Bax and Bcl-2 expression in the cerebellum, lymph nodes and leukocytes of dogs naturally infected with canine distemper virus. Genet Mol Res 2010,9:151-161.
22. Silver N, Best S, Jiang J, Thein SL: Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol 2006,7:33.
23. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002,3:RESEARCH0034.
24. Andersen CL, Jensen JL, Orntoft TF: Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 2004,64:5245-5250.
25. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP: Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett 2004,26:509-515.
26. Xie F, Xiao P, Chen D, Xu L, Zhang B: miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol 2012.
27. Saba CF, Thamm DH, Vail DM: Combination chemotherapy with L-asparaginase, lomustine, and prednisone for relapsed or refractory canine lymphoma. J Vet Intern Med 2007,21:127-132.
28. London CA, Hannah AL, Zadovoskaya R, Chien MB, Kollias-Baker C, Rosenberg M, Downing S, Post G, Boucher J, Shenoy N, Mendel DB, McMahon G, Cherrington JM: Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. Clin Cancer Res 2003,9:2755-2768.
29. Houghton PJ, Germain GS, Harwood FC, Schuetz JD, Stewart CF, Buchdunger E, Traxler P: Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 in vitro. Cancer Res 2004,64:2333-2337.
30. Tiwari AK, Sodani K, Wang SR, Kuang YH, Ashby CR, Jr., Chen X, Chen ZS: Nilotinib (AMN107, Tasigna) reverses multidrug resistance by inhibiting the activity of the ABCB1/Pgp and ABCG2/BCRP/MXR transporters. Biochem Pharmacol 2009,78:153-161.
31. Sen R, Natarajan K, Bhullar J, Shukla S, Fang HB, Cai L, Chen ZS, Ambudkar SV, Baer MR: The novel BCR-ABL and FLT3 inhibitor ponatinib is a potent inhibitor of the MDR-associated ATP-binding cassette transporter ABCG2. Mol Cancer Ther 2012,11:2033-2044.
32. Chimura N, Shibata S, Kimura T, Kondo N, Mori T, Hoshino Y, Kamishina H, Maeda S: Suitable reference genes for quantitative real-time rt-pcr in total RNA extracted from canine whole blood using the PAXgene system. J Vet Med Sci 2011,73:1101-1104.
33. Gulmann C, Espina V, Petricoin E, 3rd, Longo DL, Santi M, Knutsen T, Raffeld M, Jaffe ES, Liotta LA, Feldman AL: Proteomic analysis of apoptotic pathways reveals prognostic factors in follicular lymphoma. Clin Cancer Res 2005,11:5847-5855.
34. Frantz AM, Sarver AL, Ito D, Phang TL, Karimpour-Fard A, Scott MC, Valli VE, Lindblad-Toh K, Burgess KE, Husbands BD, Henson MS, Borgatti A, Kisseberth WC, Hunter LE,

Breen M, O'Brien TD, Modiano JF: Molecular profiling reveals prognostically significant subtypes of canine lymphoma. Vet Pathol 2013,50:693-703.

35. Valli VE, Kass PH, San Myint M, Scott F: Canine lymphomas: association of classification type, disease stage, tumor subtype, mitotic rate, and treatment with survival. Vet Pathol 2013,50:738-748.
36. Tainton KM, Smyth MJ, Jackson JT, Tanner JE, Cerruti L, Jane SM, Darcy PK, Johnstone RW: Mutational analysis of P-glycoprotein: suppression of caspase activation in the absence of ATP-dependent drug efflux. Cell Death Differ 2004,11:1028-1037.
37. Gollapud S, Gupta S: Anti-P-glycoprotein antibody-induced apoptosis of activated peripheral blood lymphocytes: a possible role of P-glycoprotein in lymphocyte survival. J Clin Immunol 2001,21:420-430.
38. Luciano F, Ricci JE, Herrant M, Bertolotto C, Mari B, Cousin JL, Auberger P: T and B leukemic cell lines exhibit different requirements for cell death: correlation between caspase activation, DFF40/DFF45 expression, DNA fragmentation and apoptosis in T cell lines but not in Burkitt's lymphoma. Leukemia 2002,16:700-707.
39. Bram EE, Stark M, Raz S, Assaraf YG: Chemotherapeutic drug-induced ABCG2 promoter demethylation as a novel mechanism of acquired multidrug resistance. Neoplasia 2009,11:1359-1370.
40. Bai M, Skyrlas A, Agnantis NJ, Kamina S, Kitsoulis P, Kanavaros P: Cluster analysis of apoptosis-associated bcl2 family proteins in diffuse large B-cell lymphomas. Relations with the apoptotic index, the proliferation profile and the B-cell differentiation immunophenotypes. Anticancer Res 2004,24:3081-3088.
41. Cayrol F, Praditsuktavorn P, Fernando TM, Kwiatkowski N, Marullo R, Calvo-Vidal MN, Phillip J, Pera B, Yang SN, Takpradit K, Roman L, Gaudiano M, Crescenzo R, Ruan J, Inghirami G, Zhang T, Cremaschi G, Gray NS, Cerchietti L: THZ1 targeting CDK7 suppresses STAT transcriptional activity and sensitizes T-cell lymphomas to BCL2 inhibitors. Nat Commun 2017,8:14290.

ANALIZA TRANSKRIPTOMA *ABCB1*, *ABCG2* I *BCL2/BAX* KOD LIMFMA PASA REFRAKTARNIH NA TERAPIJU I PASA U RELAPSU TOKOM TERAPIJE

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Osnovni problemi vezani za nepostojanje odgorova na antitumorske lekova je rezistencija i povećana ekspresija antiapoptoičnih proteina u ćelijama tumora. U okviru protokola lečenja obavljena je evaluacije sposobnosti toceranib-fosfata konkurentnog sa lomustinom (CCNU) ili L-asparaginazom i vinkristinom, a u cilju smanjivanja rezistencije na lekove i apoptoične proteine kod limfoma pasa koji su bili ili u relapsu ili su bili potpuno refraktarni na tretman. Uzorci periferne krvi su uzimani pre i posle terapije od 14 pasa koji su bili refraktarni na tretman sa ciklofosfamid-vinkristin-

prednisolonom (COP) ili COP-doksorubicinom (CHOP) i kod kojih su bili prisutni rekurentni multicentrični limfomi. Nivo ekspresije *ABCB1*, *ABCG2*, *Bcl2* i *Bax* bio je određivan kvantitativnom real time - PCR metodom. Stepen promena nivoa *ABCB1*, *ABCG2*, *Bcl2* i *Bax* analiziran je u korelaciji sa preživljavanjem bez progresije simptoma (PRS). U poređenju sa polaznim vrednostima, a posle tretmana, nivo ekspresije *ABCB1* i *ABCG2* informacone RNK bio je za 1,57 odnosno1,85 puta niži (p=0,4 i p=0,87). Odnos *Bcl2/Bax* je bio snižen ali ne značajno smanjen, 1.02 puta (p=0,74). Celokupan odgovor na ovaj protokol bio je 50% sa srednjom vrednošću PFS od 79 dana (u rasponu od 14 do 207 dana). Niske srednje vrednosti nivoa relativne ekspresije *ABCB1, ABCG2* kao i proporcija *Bcl/Bax* grupe nije bio u korelaciji sa kliničkim ishodom u poređenju sa visokim srednjim vrednostima nivoa relativne ekspresije, a samim tim i sa kliničkim stanjem pacijenata, imunofenotipom, histološkim promenama i stadijumom bolesti. Otuda, primena lekova za tretman zajedno sa toceranib- fosfatom može da dâ pozitivne efekte u slučaju tretmana kod refraktarnih i pasa i pasa u relapsu.