การจำแนกรูปแบบปรากฏทางอิมมูน การวินิจฉัยทางอณูชีววิทยา การตรวจเซลล์มะเร็งที่หลงเหลือและการแสดงออกของโปรตีนดื้อยาในสุนัขป่วยด้วยโรคมะเร็งต่อมน้ำเหลืองที่ให้การรักษาด้วยเคมีบำบัดด้วยวิธี Modified COP, Modified CHOP และ Rescue Protocols

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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IMMUNOPHENOTYPE, MOLECULAR DIAGNOSIS, MINIMAL RESIDUAL DISEASE, AND MULTIDRUG RESISTANCE PROTEIN EXPRESSION IN MODIFIED COP, MODIFIED CHOP AND RESCUE PROTOCOLS OF TREATED CANINE LYMPHOMAS

Miss Sirintra Sirivisoot

A Dissertation Submitted in Partial Fulfillment of the Requirements
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สิรินทรา ศิริวิสูตร : การจำแนกรูปแบบปรากฏทางอิมมูน การวินิจฉัยทางอณูชีววิทยา การตรวจเซลล์มะเร็งที่หลงเหลือและการแสดงออกของโปรตีนดื้อยาในสุนัขป่วยด้วยโรคมะเร็งต่อมน้ำเหลืองที่ให้การรักษาด้วยเคมีบำบัดด้วยวิธี Modified COP, Modified CHOP และ Rescue Protocols (IMMUNOPHENOTYPE, MOLECULAR DIAGNOSIS, MINIMAL RESIDUAL DISEASE, AND MULTIDRUG RESISTANCE PROTEIN EXPRESSION IN MODIFIED COP, MODIFIED CHOP AND RESCUE PROTOCOLS OF TREATED CANINE LYMPHOMAS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. อนุเทพ รังสีพิพัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. สพ.ญ. ดร. สมพร เตชะงามสุวรรณ, ผศ. สพ.ญ. ดร. สิริขจร ตังควัฒนา, 149 หน้า.

โรคมะเร็งต่อมน้ำเหลืองพบได้บ่อยในสุนัข เซลล์เม็ดเลือดขาวลิมป์โฟไซท์ชนิดบีหรือชนิดที่เกิดความผิดปกติและแบ่งตัวเกิดเป็นมะเร็งต่อมน้ำเหลืองได้ วิธีการจำแนกชนิดเซลล์ขั้นพื้นฐานคือวิธีอิมมูนโนฮิสโตเคมี โดยใช้โปรตีนที่เฉพาะเจาะจงต่อลิมป์โฟไซท์ชนิดทีคือ CD3 และชนิดบีคือ CD79a อย่างไรก็ตามโปรตีนอีกชนิดที่จำเพาะต่อลิมป์โฟไซท์ชนิดบีคือ Pax5 หรือ B-cell specific activator ซึ่ง Pax5 มีความจำเพาะและความไวมากกว่า CD79a เพราะย้อมติดในมะเร็งต่อมน้ำเหลืองชนิดบีเท่านั้น วัตถุประสงค์แรกของงานวิจัยนี้เพื่อ 1) ตรวจวินิจฉัยมะเร็งต่อมน้ำเหลืองที่เกิดจากลิมป์โฟไซท์ชนิดบีด้วยวิธีอิมมูนโนฮิสโตเคมีจากตัวอย่างเนื้อเยื่อด้วยโปรตีน Pax5 เปรียบเทียบกับ CD79a เพื่อจำแนกลิมป์โฟไซท์ชนิดบีและ CD3 เพื่อจำแนกลิมป์โฟไท์ชนิดที ผลพบว่า 28 ตัวอย่างมะเร็งต่อมน้ำเหลืองชนิดบี พบการแสดงออกของโปรตีน Pax5 และ CD79a ขณะที่อีก 4 ตัวอย่างพบการแสดงออกของโปรตีน Pax5 เท่านั้น นอกจากนี้ 4 ใน 10 ตัวอย่างมะเร็งต่อมน้ำหลืองชนิดทีมีการแสดงออกของ CD79a ร่วมกับ CD3 อีกด้วย Pax5 มีความไวและความจำเพาะต่อมะเร็งต่อมน้ำเหลืองชนิดบีร้อยละ 100 ขณะที่ CD79a มีความไวร้อยละ 87.5 และความจำเพาะร้อยละ 71.4 ต่อมะเร็งต่อมน้ำเหลืองชนิดบี วิธีอิมมูนโนฮิสโตเคมีจากตัวอย่างเซลล์เป็นวิธีที่ง่ายและประหยัดมากกว่าตัวอย่างเนื้อเยื่อ เทคนิคปฏิกิริยาลูกโซ่โพลิเมอเรสพัฒนามาใช้ตรวจวินิจฉัยมะเร็งต่อมน้ำเหลืองชนิดบีหรือทีและเพื่อตรวจหาเซลล์มะเร็งหลงเหลือ วัตถุประสงค์ที่สองของงานวิจัยนี้เพื่อ 2) พัฒนาเทคนิคปฏิกิริยาลูกโซ่โพลิเมอเรสเพื่อวินิจฉัยเซลล์มะเร็งลิมป์โฟไซท์ชนิดบีหรือชนิดทีจากตัวอย่างเลือดและเซลล์ต่อมน้ำเหลืองและพัฒนาวิธีอิมมูนโนฮิสโตเคมีจากตัวอย่างเซลล์เปรียบเทียบผลกับตัวอย่างเนื้อเยื่อโดยใช้โปรตีน Pax5 และ CD3 ตัวอย่างเซลล์และตัวอย่างเนื่อเยื่อโดยใช้ Pax5 และ CD3 ให้ผลสอดคล้องกันใน 25 ตัวอย่างมะเร็งต่อมน้ำเหลืองชนิดบีและ 3 ตัวอย่างชนิดที เมื่อเปรียบเทียบผลทางวิธีอิมมูนโนฮิสโตเคมีกับเทคนิคปฏิกิริยาลูกโซ่โพลิเมอเรสเพื่อวินิจฉัยเซลล์มะเร็งลิมป์โฟไซท์ชนิดบีหรือชนิดทีจากตัวอย่างเลือดและเซลล์ต่อมน้ำเหลือง พบว่าให้ผลสอดคล้องร้อยละ 60 เท่านั้น ดังนั้นวิธีนี้จึงควรใช้ตรวจร่วมกับวิธีอิมมูนโนฮิสโตเคมีด้วยเสมอ มะเร็งต่อมน้ำเหลืองในสุนัขตอบสนองต่อการรักษาทางเคมีบำบัด แต่มักพบปัญหาการกลับมาเป็นใหม่หลังการรักษาเนื่องจากมีเซลล์มะเร็งที่หลงเหลืออยู่ในกระแสเลือดซึ่งสามารถตรวจพบด้วยวิธีทางอณูชีววิทยาเท่านั้น วัตถุประสงค์ที่สามของงานวิจัยนี้เพื่อ 3) ตรวจหาเซลล์มะเร็งหลงเหลือจากตัวอย่างเลือดโดยใช้เทคนิคปฏิกิริยาลูกโซ่โพลิเมอเรสในสุนัขที่ป่วยเป็นมะเร็งต่อมน้ำเหลืองชนิดบีและชนิดทีระหว่างการรักษาด้วยเคมีบำบัดโปรโตคอล modified L-COP (L-asparaginase, vincristine, cyclophosphamide, และ prednisolone) และ modified L-CHOP (L-asparaginase, vincristine, cyclophosphamide, doxorubicin และ prednisolone) เพื่อประเมินประสิทธิภาพและการตอบสนองต่อการรักษาระหว่างสองโปรโตคอล ผลพบว่าการตรวจพบเซลล์มะเร็งหลงเหลือจากตัวอย่างเลือดมีความสัมพันธ์กับขนาดของต่อมน้ำเหลืองระหว่างการรักษาและสามารถใช้ประเมินการตอบสนองและประสิทธิภาพโปรโตคอลการรักษาได้ สาเหตุหลักที่ทำให้การรักษาด้วยยาเคมีบำบัดล้มเหลวคือการดื้อยาระหว่างหรือหลังการตอบสนองต่อการรักษาทางเคมีโปรโตคอลเดิม และพบการกลับมาเป็นใหม่ เนื่องจากเซลล์มะเร็งมีการสร้างโปรตีนดื้อยามากขึ้นเพื่อขับยาออกได้มากขึ้น โปรตีนดื้อยาที่พบว่ามีบทบาทต่อภาวะดื้อยาในสุนัขคือ P-glycoprotein (Pgp) และ Breast cancer resistance protein (BCRP) ยากลุ่ม Tyrosine kinase inhibitor (TKI) สามารถยับยั้งการทำงานของ Pgp ได้ วัตถุประสงค์ที่สี่ของงานวิจัยนี้เพื่อ 4) พัฒนาโปรโตคอลกู้การรักษาในสุนัขที่ป่วยเป็นมะเร็งต่อมน้ำเหลืองที่มีการดื้อยาและพบการกลับมาเป็นใหม่ โดยการใช้ยา Lomustine (CCNU) หรือ L-asparaginase และ Vincristine ร่วมกับ Toceranib phosphate และตรวจการแสดงออกของโปรตีนดื้อยา Pgp และ BCRP จากตัวอย่างเลือดก่อนและหลังการรักษาด้วยปฏิกิริยาลูกโซ่โพลิเมอเรสแบบเรียลไทม์ ผลพบว่าการให้ Toceranib phosphate ร่วมกับยาเคมีบำบัด CCNU หรือ L-asparaginase และ Vincristine มีแนวโน้มที่จะลดระดับการแสดงออกของโปรตีนดื้อยา Pgp และ BCRP เมื่อเปรียบเทียบระดับการแสดงออกก่อนและหลังการได้รับยา ดังนั้นโปรโตคอลลกู้การรักษานี้อาจใช้เป็นหนึ่งในทางเลือกการรักษามะเร็งต่อมน้ำเหลืองที่มีการดื้อยาและกลับมาเป็นใหมในสุนัขได้

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ลายมือชื่อนิสิต .....
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KEYWORDS: CANINE LYMPHOMA / CHEMOTHERAPY / IMMUNOPHENOTYPE / MULTIDRUG RESISTANCE PROTEIN / MINIMAL RESIDUAL DISEASE / PARR

SIRINTRA SIRIVISOOT: IMMUNOPHENOTYPE, MOLECULAR DIAGNOSIS, MINIMAL RESIDUAL DISEASE, AND MULTIDRUG RESISTANCE PROTEIN EXPRESSION IN MODIFIED COP, MODIFIED CHOP AND RESCUE PROTOCOLS OF TREATED CANINE LYMPHOMAS. ADVISOR: ASSOC. PROF. DR. ANUDEP RUNGSIPIPAT, CO-ADVISOR: ASSOC. PROF. DR. SOMPORN TECHANGAMSUWAN, D.V.M., ASST. PROF. DR. SIRIKACHORN TANGKAWATTANA, D.V.M., 149 pp.

Multicentric lymphoma is the most common hematopoietic tumors in dogs. Both B- or T-lymphocyte clones can originate to lymphoma. A gold standard method to differentiate each lymphocyte lineage is immunohistochemistry (IHC) by detecting specific proteins of B and T cell. A protein marker for T and B cells is CD3 and CD79a, respectively. However, another pan pre B-cell marker is Pax5 or B-cell specific activator protein. It is more specific and sensitive than CD79a because it expresses only in B-cell lymphoma cases. The first purpose of this research was to 1) determine Pax5 expression in canine lymphomas by IHC and compare the results to CD79a and CD3 expression for lymphoid lineage. The result of this study showed that 28 B-cell lymphoma cases expressed both Pax5 and CD79a, nevertheless four cases of B cell illustrated only Pax5 expression. Furthermore, four from ten T-cell lymphoma samples presented dual CD3 and CD79 expression. Therefore, Pax5 has 100% sensitivity and specificity, whereas CD79a has 87.5% sensitivity and 71.4% specificity. Immunocytochemistry (ICC) is easier and cheaper than IHC for immunophenotyping. Clonality assay or PCR for antigen receptor rearrangement (PARR) could diagnose neoplastic lymphocyte clones and additionally detect minimal residual disease (MRD) in canine lymphomas. The second aim of this research was to 2) develop heteroduplex PARR for clonality test from peripheral blood and lymph node cytology and develop ICC for lineage determination compared to IHC using Pax5 and CD3. Immunophenotyping results by ICC and IHC with both Pax5 and CD3 showed strong correlation in 25 samples of B-cell lymphoma and three samples of T-cell lymphomas. When compared the clonality results between heteroduplex PARR and IHC, the percentage agreement was 60%. Thus, heteroduplex PARR could be used as an adjunctive method with IHC or ICC. Canine multicentric lymphoma generally responds well to chemotherapy, but tumor relapse after complete remission is prevalent because of MRD. MRD could assess by only molecular techniques. The third objective of this research was to 3) evaluate MRD from peripheral blood using heteroduplex PARR in canine multicentric B- and T-cell lymphomas during treatment with modified L-COP (L-asparaginase, vincristine, cyclophosphamide, and prednisolone)) and modified L-CHOP (L-asparaginase, vincristine, cyclophosphamide, doxorubicin and prednisolone) for determining treatment efficacy and treatment response between two protocols. MRD negative results showed the correlation to complete remission during treatment and could be useful for predicting treatment efficacy and clinical response. Drug resistance mechanisms is a major cause of treatment failure during treatment or after complete remission in refractory and relapsing canine lymphoma because tumor cells upregulate multidrug resistance protein for efflux toxic drugs out of cells. Two multidrug resistance proteins that have a role in drug resistance mechanism in canine multicentric B- and T-cell lymphoma are P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP). Furthermore, tyrosine kinase inhibitor (TKI) could inhibit Pgp function. The final aim of this research was to 4) develop rescue protocol for refractory/relapsed canine lymphoma with lomustine (CCNU) or L-asparaginase and vincristine concurrent with toceranib phosphate, and asses protein expression levels of Pgp and BCRP from peripheral blood before and after treatment using qRT-PCR. The results showed that toceranib phosphate or TKI with CCNU or L-asparaginase and vincristine tended to decrease the transcription expression levels of Pgp and BCRP when compared the levels prior to and after treatment. Hence, this rescue protocol could be another treatment option in refractory/relapsed canine lymphomas.

Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

ALP	alkaline phosphatase
ALT	alanine aminotransferase
APL	anaplastic plasmacytoid lymphoma
ABC	ATP-binding cassette
BCRP	breast cancer resistance protein
B-LBL	B-lymphoblastic lymphoma
BSA	bovine serum albumin
BSAP	b-cell specific activator protein
B-SLL	B-small lymphocytic lymphoma
BUN	blood urea nitrogen
CBC	complete blood count
CD	cluster of differentiation
CRD3	complementarity determining region 3
NHL	non-Hodgkin lymphoma
COP	cyclophosphamide, vincristine, and prednisolone
CHOP	cyclophosphamide, doxorubicin, vincristine, and prednisolone
CR	complete remission
DAB	diaminobenzidine
DLBCL	diffuse large B-cell lymphoma
DNA	deoxyribonucleic acid
FFPE	formalin-fixed paraffin-embedded
FL	follicular lymphoma
FNAB	fine needle aspiration biopsy
ICC	immunocytochemistry
IgH	immunoglobulin heavy chain

IHC	immunohistochemistry
H&E	hematoxyline and eosin
HL	Hodgkin lymphoma
hPARR	heteroduplex polymerase chain reaction for antigen receptor rearrangements
LLI	lymphocytic lymphoma of intermediate type
LN	lymph node
MMC	macronucleolated medium-sized cell
MI	mitotic index
MRD	minimal residual disease
MRP	multidrug resistance protein
NMZ	nodal marginal zone cell lymphoma
OST	overall survival time
PAX5	paired box gene 5
PCR	polymerase chain reaction
PD	progressive disease
PFS	progression-free survival
Pgp	p-glycoprotein
PR	partial response
PTCL	peripheral T-cell lymphoma
qRT-PCR	real-time reverse transcription polymerase chain reaction
RNA	ribonucleic acid
SD	stable disease
TCRγ	T-cell receptor gamma
TKI	tyrosine kinase inhibitor
WBC	white blood cell
WHO	world health organization

CHAPTER I

INTRODUCTION

1.1 The relation between all manuscripts in the thesis

This thesis consisted of four manuscripts that focused on assessment of a diagnostic marker and a diagnostic tool for immunophenotyping, evaluation of a clonality method for minimal residual disease detection and development of a rescue treatment in relapsed or resistant disease of canine multicentric lymphoma.

Firstly, the study aimed to use Pax5 protein to detect B-originated lymphoma by using immunohistochemistry (IHC) in multicentric, extranodal, and intestinal lymphoma and B lymphocytes in normal lymph node (LN) in dogs. The IHC applying Pax5 results showed the highly specific reaction to B-cell lineage; then the use of Pax5 was further developed for fine needle aspirated cytology from LN or immunocytochemistry. In addition, CD3 was also parallelly developed by using the same techniques as a T-cell marker. Secondly, heteroduplex polymerase chain reaction for antigen receptor rearrangements (hPARR) was generated to detect specific genes of B or T lymphocytes in canine lymphoma. This technique was beneficial to differentiate lymphoid lineages from various kinds of samples including peripheral blood or LN cytology, and was applicable as a diagnostic tool for minimal residual disease detection. Moreover, the hPARR results were compared with the IHC counterpart, a standard method, for comparison the sensitivity and specificity. Lastly, dogs with relapsed/refractory multicentric lymphoma showed resistant to a standard chemotherapy due to the up regulation of multidrug resistant protein (MRP) in tumor cells. Thus, the molecular targeted therapy to this protein could overcome the response to cytotoxic drugs. Tyrosine kinase inhibitor combining to rescue drugs were

applied to resistant or relapsed cases and they were determined the MRP transcription level before and after treatment.

All of manuscripts in partial fulfillment of the requirements for the degree of Doctor of Philosophy program in Veterinary Pathobiology.

1.2 Importance and rationale

Canine non-Hodgkin lymphoma (NHL) is the most common canine hematopoietic tumors with the incidence rate more than 83% (Vail and Young, 2007). Lymphoid malignancies can derive from B or T lymphocytes at various stages of their development. Canine NHL differentiates into B-cell lymphomas (60-80%), T-cell lymphomas (10-38%), mixed B- and T-cell lymphomas (22%), and null lymphomas (neither B cell nor T cell) (less than 5%) (Teske et al., 1994; Fournel-Fleury et al., 1997; Wilkerson et al., 2005). Each type of lymphoma has different prognosis. T-cell lymphomas are prone to be more aggressive than B-cell lymphomas which have shorter remission and overall survival times (Dobson et al., 2001; Ponce et al., 2004; Valli et al., 2013). In case of mixed B- and T-cell lymphomas, the remission rate and survival time are variable owing to the therapy response (Wilkerson et al., 2005). Thus, several techniques are developed for lineage determination, for example immunophenotyping method: immunocytochemistry (ICC), immunohistochemistry (IHC), or flow cytometry, and molecular method: PCR for antigen receptor gene arrangements (PARR).

While hematopoietic stem cells in the bone marrow differentiate to diverse maturational stages of B- or T-cell development, specific genes, transcription factors, and specific cellular antigens are useful for detection of discrete subsets. Cluster of differentiation (CD) antigens are the most common marker that recognized by antibody or immunophenotyping assay. Nowadays CD3 is used for indicating T-lineage lymphomas, whereas CD79a is expressed in B-cell lymphomas. However, CD79a has been found 10-40% in both canine and human T-cell lymphomas (Wilkerson et al., 2005; Guija de Arespacochaga et al., 2007; Willmann et al., 2009). Pax5 or B-cell specific activator protein (BSAP) is a transcription factor that is essential

for identity of B-lymphocyte development and differentiation. Its function is to control identity of B lymphocyte development from pro-B to mature B cells but not in plasma cells (Horcher et al., 2001; Dong et al., 2008). The expression of Pax5 remains detectable in all B-lineage neoplasms with 100% sensitivity and specificity and none of T-cell or myeloid malignancies are positive to Pax5 (Willmann et al., 2009; Desouki et al., 2010; Nasr et al., 2010). On the other hands, the expression of CD79a shows 100% specificity for B-cell malignancies, 79% sensitivity for B-acute lymphoblastic leukemia/lymphoma, and 97% sensitivity for Burkitt's lymphoma (Nasr et al., 2010).

Many studies report on using Pax5 antibody as a B-cell marker in humans lymphomas (Browne et al., 2003; Desouki et al., 2010; Nasr et al., 2010), nevertheless a small number of research has investigated in veterinary field. In addition, ICC has developed because of its quickness, simplicity and cheapness (Sapierzynski, 2010). When compared to IHC on tissue sections, ICC has the same accuracy and reliability for diagnosis and classification of lymphoma in dogs (Caniatti et al., 1996). ICC can apply on either cytological smears or cytospin preparations, but only two antibodies, CD3 for T-cell and CD79a for B-cell marker, were performed (Caniatti et al., 1996; Aulbach et al., 2010; Sapierzynski, 2010; Sapierzynski et al., 2012). Based on our knowledge, no study has reported on Pax5 expression from cytological samples in canine lymphomas.

PARR is a valuable method to detect DNA sequences of B- or T-cell clonality in lymphoid tumors. It determines antigen receptor gene rearrangements from complementarity determining region 3 (CDR3) of immunoglobulin and T-cell receptor genes that compose of each of multiple variable (V), diversity (D), and joining (J) segments. For B cells, genes that encode for immunoglobulin heavy (IgH) chain protein of CDR3 are V, D, and J regions, whereas T-cell receptor gamma (TCRγ) protein of CDR3 are only V and J regions that are joined. TCRγ gene is preferable for PARR analysis due to its simple complex for amplification without D segments (Burnett et al., 2003). PARR can detect IgH and TCRγ genes from various samples such as cavity fluid, peripheral blood, cytology, fresh tissue or paraffin-embedded

tissue (Burnett et al., 2003; Keller et al., 2004; Kaneko et al., 2009; Thilakaratne et al., 2010). Moreover, PARR also uses to differentiate LN hyperplasia, the early stages of lymphoma, mild or chronic lymphocytosis and minimal residual disease detection (Burnett et al., 2003). Nonetheless, due to the high sensitivity of the PARR, pseudoclonality or false positive can occur as a result of immunologic diversity of IgH and TCRγ genes and amplified multiple copies during the PCR amplification. Additionally, heteroduplex method could use to rule out pseudoclone in canine lymphoma. It helps to avoid misdiagnosis and resolve reliability of the results (Takanosu et al., 2010)

Minimal residual disease (MRD) is referred to the remnant neoplastic cells after treatment with cytotoxic drugs. In addition, it is a cause of tumor relapse in human and canine lymphomas during the remission period. According to a low level of MRD during the complete remission, it needs to be monitored by advanced molecular methods such as PARR or quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (Calzolari et al., 2006; Yamazaki et al., 2008; Manachai et al., 2014). If patients increase the MRD level through partial or complete remission, they need a rescue protocol. A standard chemotherapy for canine multicentric lymphoma is a CHOP-based protocol (cyclophosphamide, hydroxydaunorubicin/doxorubibin, vincristine, and prednisolone) (Garrett et al., 2002). Another treatment option is a high-dose COP protocol (cyclophosphamide, vincristine, and prednisolone) (Mellanby et al., 2002). A rescue therapy for refractory/relapsed canine multicentric lymphoma comprises of different antitumor drugs from standard protocol because of drug resistance mechanism. Many rescue protocols are developed such as lomustine (CCNU) and dacarbazine (DTIC), L-asparaginase, CCNU and prednisolone, and temozolomide or DTIC with doxorubicin. Nevertheless, dogs with refractory/relapsed disease usually die after treating with the rescue protocol one or two months because cancer cells overexpress multidrug resistance proteins and avoid cell death with anticancer drugs (Dervisis et al., 2007; Flory et al., 2008; Saba et al., 2009).

Even though multicentric lymphoma in dogs usually responds to the anti-neoplastic drugs and has a good remission at the first period, neoplastic lymphocytes then learn to survive by up-regulation of multidrug resistance protein (MRP) due to the induction of cytotoxic drugs. MRP is known as ATP-binding cassette (ABC) transporter proteins. The most common MRPs in human and animals are P-glycoprotein (Pgp; *ABCB1*), breast cancer resistance protein (BCRP; *ABCG2*), multidrug resistance-related protein 1 (*ABCC1*), and lung resistance-related protein (*LRP*). Pgp has not only shown the expression in normal various canine tissues: liver, colon, kidney, and adrenal cortex, but it also expressed in neoplastic tissues including malignant lymphomas (Ginn, 1996). Pgp showed a higher expression after the induction with vinca alkaloids and anthracyclines in canine lymphoma cells (Uozurmi et al., 2005). In addition, its expression was higher in dogs with lymphoma after chemotherapy (Moore et al., 1995; Bergman et al., 1996). IHC and western blot analysis with different antibody epitopes and scoring systems are unsuccessful to assess low levels of Pgp, difficult to evaluate due to primary cross-reactivity and insensitive for quantification (Dhaliwal et al., 2013). qRT-PCR is a sensitive and quantitative method to assess Pgp levels by determination the expression of *ABCB1/MDR1* (Culmsee et al., 2004). The *ABCB1* mRNA expression levels were relatively low in canine multicentric lymphomas compared to those in gastrointestinal lymphomas. Furthermore, four from ten chemotherapy-resistant dogs with multicentric high-grade lymphomas showed a high expression of *ABCB1* (Tomiyasu et al., 2010). Resistant multicentric B- and T-cell lymphomas increased the mRNA expression levels of *ABCB1* and *ABCG2*, respectively (Zandvliet et al., 2015). Thus, these two genes might play an important role in drug resistance mechanism in canine lymphomas.

1.3 Literature review

Canine lymphoma is one of the most common hematopoietic tumors in dogs. It accounts for 1.94% of all canine tumors and 76.6% of hematopoietic and lymphatic tumors in Bangkok (Rungsipipat et al., 2003). This neoplasm occurs mostly

in middle-aged to older dogs (median age 7.7 years). Sex is not related to risk factors. Breeds that have the high incidence of lymphomas include mongrels, Golden retrievers, German shepherds, Labrador retrievers, Rottweilers, Cocker Spaniels, Boxers and Doberman pinschers (Jagielski et al., 2002; Modiano et al., 2005). Nowadays the etiology induced canine lymphoma is remained unknown; however, there are a few factors that have been shown to encourage the risk of developing lymphoma in dogs such as genetic predisposition (Thomas et al., 2011), environmental factors (Ginn et al., 2014), and autoimmunity. Immune mediated diseases (e.g. immune-mediated thrombocytopenia, pemphigus foliaceus, and atopic dermatitis) are correlated to increase the risk of canine lymphoma (Keller, 1992; Foster et al., 2000; Santoro et al., 2007).

In general, lymphoma is originated from a clonal expansion of lymphoid cells, which is B or T cells. B-cell lymphoma is accounted for 51.2%, while the incidence rate of T-cell lymphoma is 29.3%. Nearly 20% represents as null cell lymphoma (Guija de Arespacochaga et al., 2007). According to the effect of lineage differentiation in a selected treatment protocol and a prognosis, it is important to determine immunophenotype. All B and T lymphocytes derive from pluripotent stem cells in the bone marrow. During their development, specific cellular antigens such as CD could be used for recognizing lineage stage. For B-cell lymphoma, pro-B to activated B lymphocytes express surface immunoglobulin, CD21 and CD79, whereas thymocytes and mature T cells in T-cell lymphoma reveal CD3, CD4 and CD8 (Wilkerson et al., 2005). The representative B-cell markers for canine NHL by IHC are CD79a and Pax5. The latter gains more specificity because it is undetectable in canine T-cell lymphoma (Willmann et al., 2009). Most studies have used CD79a and CD3 markers for indicating B- and T-cell origin, respectively (Ferrer et al., 1993; Guija de Arespacochaga et al., 2007; Ponce et al., 2010; Valli et al., 2013), but 10-40% of canine T-cell lymphomas were expressed both CD3 and CD79a antigens and some cases showed double negative staining for both markers. Thus, Pax5 is a useful marker in unclassified lymphoma cases (Wilkerson et al., 2005; Guija de Arespacochaga et al., 2007; Willmann et al., 2009).

Pax5 or BSAP is a nuclear protein in paired-box containing (PAX) family of transcription factors. It has several functions in B cell lymphopoiesis. During B cell commitment, Pax5 regulates progenitor cells in bone marrow to differentiate into B cell pathway by suppressing lineage-inappropriate genes and stimulating B cell-specific genes which have effects of transcriptional regulation, B cell signaling, cell adhesion, cell migration, cellular metabolism, antigen presentation, and B cell maturation (Nutt et al., 1999; Schebesta et al., 2007; Pridans et al., 2008). It also regulates chromatin-remodeling, histone-modifying, and basal transcription factor complexes in transcriptional process of B-cell commitment (McManus et al., 2011). Furthermore, it has an important role in pro-B cell stages by which provokes a contraction of the IgH locus to rule distal V_H-DJ_H recombination (Fuxa et al., 2004). Not only early B cell stage, all mature B cells need Pax5 for controlling the identity of B lymphocytes through their differentiation and development (Horcher et al., 2001). Many studies have used Pax5 protein as a pan pre-B cell marker in human Hodgkin lymphoma (HL), Burkitt lymphoma, B-acute lymphoblastic leukemia/lymphoma, and canine NHL (Browne et al., 2003; Dong et al., 2008; Willmann et al., 2009; Desouki et al., 2010; Nasr et al., 2010).

ICC is a simple, fast, reliable and inexpensive procedure to identify immunophenotype of tumor cells in canine lymphomas. Cytospin preparation or cytological smears from fine needle aspiration biopsy (FNAB) from LN were suitable for ICC (Caniatti et al., 1996; Aulbach et al., 2010; Sapierzynski, 2010). Routine cytopathology and ICC results could use as a final diagnosis without punch or surgery biopsy (Sapierzynski et al., 2010). However, samples from FNAB should have enough cells, good smear quality and minimal contamination.

PARR is a molecular method that uses for evaluation and manifestation of malignant lymphoid clonality. It assesses rearranged antigen receptor genes which encoded for IgH and TCRγ genes containing unique sequences: V, D, and J segments. Two primer sets, a single of V and two of J region primers, of IgH genes are used for B-cell detection, while T-cell neoplasms use one primer of V and two primers of J sequences of TCRγ genes. It could diagnose clonality in 91% of dogs with lymphoid

malignancy (Burnett et al., 2003). Tamura et al. (2006) designed specific primers to conserved sequences of IgH gene in canine B-cell lymphoma and they could differentiate malignant B lymphocytes from normal, reactive, and malignant T lymphocytes. Keller and Moore (2012) suggested multiplex PCR assay for evaluation of canine T-cell lymphoma to increase sensitivity and decrease false negative outcomes; however, it still has some drawbacks. Even though, PARR is a sensitive method but it can show false positive or pseudoclonality. Thus, duplicate, triplicate or heteroduplex analysis should be performed. Takanosu et al. (2010) reported that heteroduplex method after conventional PARR increased reliability and avoided misdiagnosis in canine lymphomas. Not only PARR has ability of lymphoma/leukemia diagnosis from various kinds of specimen including tissue biopsy, cavity fluid, fine needle aspirate, bone marrow and peripheral blood, but it could evaluate lineage determination, lymphoma staging, and MRD detection during treatment or after complete remission (Burnett et al., 2003; Keller et al., 2004; Calzolari et al., 2006; Lana et al., 2006; Thilakaratne et al., 2010; Manachai et al., 2014).

The important aspect about relapsing disease in canine multicentric lymphoma is MRD, which is referred to an escaping tumor cell after treated with cytotoxic drugs. During remission period, they could not be identified by a standard diagnostic method, thus PARR, qRT-PCR or flow cytometry are a valuable technique for MRD evaluation (Yamazaki et al., 2008; Aresu et al., 2014). If MRD could be detected in a patient, rescue chemotherapy should be considered.

Drug resistance is a major factor of an unsuccessful chemotherapeutic treatment in canine NHL. This mechanism is caused by ABC transporter proteins such as Pgp (*ABCB1*), multidrug resistance associated protein 1 (*ABCC1*), lung resistance-associated protein (*LRP*) and BCRP (*ABCG2*). MRP is overexpressed by intrinsic through mutation or transformation of malignant cells or acquired from induction with antitumor drugs. Cross resistance can develop between different drugs that use the same cellular pathway. Its functions are to reduce drug accumulation in the tumor cells, decrease apoptosis, increase detoxification and increase DNA repair. In canine multicentric lymphomas Pgp and BCRP are involved in refractory disease. From *in*

vitro study, Pgp was expressed in canine lymphoma cell lines after exposure to vincristine and doxorubicin (Uozurmi et al., 2005). Pgp expression was observed in normal canine tissues such as liver, colon, kidney, and adrenal cortex and also 27.3% (6/22) of malignant canine lymphomas (Ginn, 1996). Moore et al. (1995) reported that only one case from 30 dogs (3%) with lymphoma before chemotherapy expressed Pgp, and 3 of 8 dogs (38%) that became resistance to treatment showed Pgp expression. Western blot analysis was performed to detect Pgp levels but this technique was a semi-quantitative and could fail to detect low levels of Pgp (Lee et al., 1996). Pgp expression was increased after relapsing disease compared to the pretreatment samples with different percentage of C949 and C219 antibody using IHC method. Conversely, one study reported that 80% (24/31) of canine multicentric lymphoma prior treatment were positive to Pgp using C494 monoclonal antibody (Dhaliwal et al., 2013). Owing to variation in sensitivity of IHC, for example different primary antibody, methodology, and scoring system, qRT-PCR is developed to investigate MRP expression level (Culmsee et al., 2004). The low level of ABCB1 mRNA expression was detected in normal canine LN and untreated canine lymphomas. In addition, 4 from 10 chemotherapy-resistant dogs with multicentric high-grade lymphoma showed high expression levels of *ABCB1* after CHOP-based protocol (Tomiyasu et al., 2010). Moreover, resistant multicentric B- and T-cell lymphoma to chemotherapy showed high expressions of ABCB1 and ABCG2 mRNA, respectively (Zandvliet et al., 2015). Thus, these two proteins might be a major cause of relapsing or refractory canine multicentric lymphomas.

The treatment of choice in canine multicentric lymphomas is CHOP-based protocol. Another treatment option is COP-based protocol or without doxorubicin. A complete remission rate of COP in canine multicentric lymphoma is 60-70% and a median survival time is 6-7 months. Even if it has a low cost per each treatment but it has prolonged maintenance phase, shorter disease free interval and overall survival time. CHOP, or COP plus doxorubicin, shows a higher remission rate to 80-90% and overall survival times to 12 months (Garrett et al., 2002). Dogs usually have a complete remission after treatment, but relapsed/refractory disease could develop

due to MRP. Thus, rescue chemotherapy with different antitumor drugs is required. There are many rescue protocols for canine multicentric lymphoma, for example, CCNU and DTIC (Flory et al., 2008), L-asparaginase, CCNU and prednisolone (Saba et al., 2009), or temozolomide/DTIC combination with doxorubicin (Dervisis et al., 2007) with diverse response rates and survival times. However, there are only few studies have observed on therapeutic interventions to invert drug resistance mechanism in animals. A target therapy to tyrosine kinase receptor or masitinib, tyrosine kinase inhibitor (TKI), showed mild anti-proliferative effect on canine lymphoid cell lines, prevented Pgp function at a concentration up to or above 1μM and had a capable of switching doxorubicin resistance (Zandvliet et al., 2013). Hence, TKI could inhibit MRP expression and might be useful in recue protocol for relapsed/refractory canine lymphoma.

1.4 Research Hypothesis

1.4.1 Pax5 protein is a diagnostic immunophenotyping marker for canine B-cell lymphoma in CD79a negative case.

1.4.2 Heteroduplex PARR (hPARR) is a highly sensitive and specific test for lymphoid clonality when compared with ICC and IHC.

1.4.3 hPARR is a useful tool for MRD detection from peripheral blood and lymph node cytology in canine lymphomas during treatment with modified COP and modified CHOP protocols.

1.4.4 Pgp and BCRP is a negative indicator in relapsed/refractory lymphoma in dogs.

1.4.5 TKI concurrent with rescue drug (CCNU or L-asparaginase and vincristine) is succeeded to treat relapsed/refractory canine lymphoma.

1.5 Research Objectives

1.5.1 To classify immunophenotype of canine NHL with CD3, CD79a and Pax5 protein using ICC and IHC techniques.

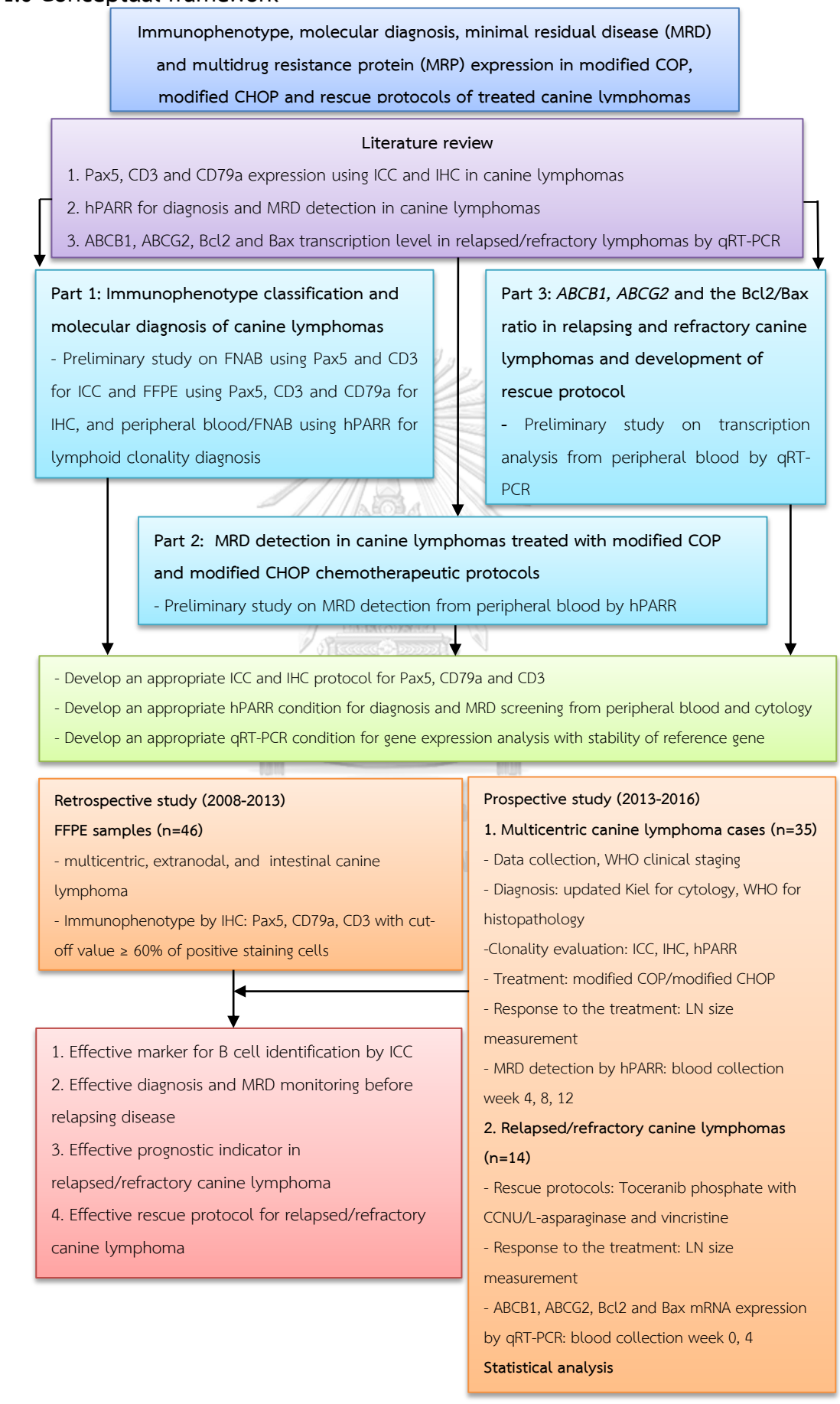
1.5.2 To develop clonality assay using hPARR compared to ICC and IHC techniques.

1.5.3 To evaluate MRD from peripheral blood in canine multicentric lymphoma during treatment with modified COP or modified CHOP chemotherapeutic protocols.

1.5.4 To analyze the transcription expression level of *ABCB1* and *ABCG2* in relapsing/refractory canine multicentric lymphomas after treated with modified COP or modified CHOP protocols.

1.5.5 To develop and evaluate a new rescue protocol using TKI concurrent with CCNU or L-asparaginase and vincristine in relapsing/refractory canine multicentric lymphomas.

1.6 Conceptual framework



1.7 Advantages of Study

ICC and hPARR can be applied as diagnostic tools for clonal classification in canine NHL without tissue biopsy for a cheaper cost and no need for generalized anesthesia in a weak dog. In addition, hPARR could be used for routinely MRD screening during the complete remission in canine multicentric lymphoma cases before tumor relapses. Not only MRD, MRP has a role on resistance to chemotherapy. A rescue protocol with TKI might be decreased the expression level of *ABCB1* and *ABCG2* in relapsed/refractory dogs and could be one option for treatment. Moreover, the Bcl2/Bax ratio might correlate with survival times and use as a prognostic factor in canine multicentric lymphoma.

1.8 Keywords

Canine lymphoma, chemotherapy, immunophenotype, minimal residual disease, multidrug resistance protein, PARR

CHAPTER II

2.1 Pax5 as a potential candidate marker for canine B-cell lymphoma

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(ISI, SCOPUS, Q2)

2.1.1 Abstract

Immunophenotyping is a valuable method for prognosis in canine malignant lymphoma. The general B-cell marker is CD79a; however, Pax5 or B-cell specific activator protein, a transcription factor that controls B-cell identity and cell maturation, could also be used as a B-cell indicator in canine lymphomas. This study aimed to use Pax5, CD79a and CD3 expression in immunohistochemistry of spontaneous canine lymphomas, in order to carry out diagnosis and histopathological classification according to the World Health Organization guidelines. Forty-six retrospective cases including 33 multicentric, eight extranodal, and five alimentary lymphomas in dogs were immunostained by anti-Pax5 and anti-CD79a antibodies for B-cell identification, and anti-CD3 antibody for T-cell identification. T-cell lymphomas (CD3+/Pax5–/CD79a–) accounted for 30.43% of cases (14/46), and four of the lymphomas (28.57%) presented with CD3+/Pax5–/CD79a+. Conversely, B-cell lymphomas (CD3–/Pax5+/CD79a+) accounted for 69.57% of cases (32/46) and 12.5% of these (4/32) showed only Pax5-positive cells (CD3–/Pax5+/CD79a–). Therefore, in dogs, Pax5 appears to be a more useful marker for staining all B-cell subtypes compared to CD79a. Immunophenotyping with both Pax5 and CD3 are necessary for lymphoid lineage identification in canine lymphomas.

2.1.2 Introduction

Malignant lymphoma is a common hematopoietic tumour in animals, especially in dogs. Canine lymphoma was found to represent approximately 1.94% of all canine tumours and 76.6% of all hematopoietic neoplasms in Bangkok, Thailand (Rungsipipat et al., 2012), similarly as in other countries (Weiss, 2006; Regan et al., 2013). Canine malignant lymphomas are normally derived from a clonal expansion of neoplastic B or T lymphocytes. B-cell lymphomas have a greater incidence rate (60–80%) than T-cell lymphomas (10–38%). However, mixed B- and T-cell lymphomas and null-cell type lymphomas have also been reported (Wilkerson et al., 2005). Many studies have reported that T-cell lymphomas are characterised by shorter survival times and disease-free intervals than lymphomas of B-cell origin (Ponce et al., 2004; Valli et al., 2013). Because of this dissimilar prognosis, histopathological classification with immunophenotyping is important for informing chemotherapeutic treatment in canine lymphomas (Rebhun et al., 2011). Histopathological classification, for example, the updated Kiel (Fournel-Fleury et al., 1997; Fournel-Fleury et al., 2002) or World Health Organization classifications (Valli et al., 2011), uses criteria based on the immunophenotype, cell morphology, and tissue architecture. Immunophenotyping data was also shown to be related to survival (Valli et al., 2013).

Gold standard methods, such as immunohistochemistry (IHC) or immunocytochemistry, have been developed for immunophenotyping classification in humans, dogs and cats. The typical protein markers for identification of B and T lymphocytes are CD79a and CD3, respectively (Ferrer et al., 1993; Ponce et al., 2004; Fernandez et al., 2005; Vezzali et al., 2010; Valli et al., 2011). *Pax5* is a member of the paired-box domain family of transcription factors that encodes the B-cell-specific activator protein. Its important roles are to control B-cell identity, development and differentiation. Pax5 protein is expressed in normal and neoplastic cells from the pro-B to mature B-cell stages (Horcher et al., 2001). It serves as a pan pre B-cell marker and was shown to be more specific than CD79a (Willmann et al., 2009). In human studies, Pax5 expression was restricted to B-cell malignancies including those that lacked CD20 and CD79a expression (Jensen et al., 2007). To the best of our

knowledge, only a few studies have investigated Pax5 expression in canine lymphoma using IHC. The aim of this study was to perform immunophenotyping on canine lymphoma cases using IHC techniques and staining for CD3, a T-cell marker and for the B-cell markers, CD79a and Pax5.

2.1.3 Materials and methods

2.1.3.1 Tissue samples

Thirty-four samples from biopsies and 12 samples from necropsies were submitted to the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University during the period 2008–2013. Patients presented with clinical signs of generalised lymphadenopathy, abdominal enlargement, or with chronic gastrointestinal signs. Thoracic radiography, abdominal radiography and abdominal ultrasonography were performed for anatomic classification. Thirty-three multicentric (at least clinical stage III), five alimentary, and eight extranodal (skin, liver, spleen, tongue, heart) lymphomas were included in this study. Tissue samples were fixed in 10% buffered formalin, routinely histologically processed, paraffin-embedded, stained with H&E and examined under a light microscope.

2.1.3.2 Histopathology

Histopathological changes of cell size and shape, nucleus size, mitotic index, number of nucleoli, chromatin density, cytoplasm characterisation of neoplastic lymphocytes and tissue architecture, were evaluated based on the World Health Organization classification by a veterinary pathologist (Vezzali et al., 2010; Valli et al., 2011). The number of mitoses per five high-power fields was noted as the mitotic index.

2.1.3.3 Immunohistochemistry

Four to six µm-thick sections were immunophenotypically classified using anti-CD3, anti-Pax5, and anti-CD79a, to identify T- and B-cell lineages, respectively. The immunohistochemical protocol was modified from Rungsipipat et al. (2012). Briefly, antigen retrieval for CD3 was achieved by heating slides with 10mM citrate

buffer (pH 6.0) in a microwave oven. To block endogenous enzymes, the slides were incubated in 3% H_2O_2 for 10 min and 1% bovine serum albumin at 37 ºC for 10 min, respectively. After washing the slides with 0.1M phosphate buffer solution (pH 7.4), they were incubated with ready-to-use monoclonal mouse anti-human CD3 antibody (LN 10, Leica, UK) at 4 ºC for 12–14 h. After washing, the slides were incubated with modified streptavidin-biotin-peroxidase complex or Envision polymer (Dako, Denmark) at room temperature for 45 min. Finally, they were immersed in 3, 3'-diaminobenzidine to develop the immunological reaction and counterstained with Mayer's haematoxylin before mounting.

Similarly, to determine B-cell lineages immunostaining with anti-Pax5 (clone 1EW, Leica, UK) was performed. Following deparaffinisation and dehydration with xylene and a graded series of alcohol, antigens were retrieved by heating in Tris/EDTA (pH 9.0) in an autoclave oven (121 ºC, 5 min), followed by incubation with monoclonal mouse anti-human Pax5 antibody (dilution 1: 50) at 4 ºC overnight. Slides were then rinsed with phosphate buffer solution; endogenous peroxidase was blocked with 3% H_2O_2 at room temperature for 10 min and non-specific background was blocked using 1% bovine serum albumin at 37 ºC for 10 min. The LSAB technique or Novolink detection system (Leica, UK) was used for conjugation to tissues at room temperature for 15 min. 3, 3'-diaminobenzidine was used as a chromogen and Mayer's haematoxylin was used for counterstaining.

CD79a was used as further B-lymphocyte marker. Citrate buffer pH 6.0 was used to retrieve antigens in an autoclave at 121 ºC for 5 min. After blocking steps (3% H_2O_2 at room temperature for 10 min and 1% bovine serum albumin at 37 ºC for 10 min), monoclonal mouse anti-human CD79a (clone HM57, Dako, Denmark) diluted to 1 : 100 was incubated with sections at 4 ºC for 12–14 h. Then, sections were incubated with Novolink polymer (Leica, UK) at room temperature for 15 min, lastly; colour was developed with 3, 3'-diaminobenzidine, and sections were counterstained with Mayer's haematoxylin, and mounted with Permount (Fisher Scientific, USA).

A normal lymph node from a dog necropsy was used as an antibody control. A positive identification of a B-cell lymphoma was made when at least 60% of neoplastic cells were positively stained with CD79a in their cytoplasm and with Pax5 in their nuclei. Identification of T-cell lymphomas was made when at least 60% of neoplastic cells were primarily stained with CD3 on the membrane of the T-cells (Willmann et al., 2009).

2.1.4 Results

In this study, out of a total of 46 dogs, 23 were males and 23 were females. Both pure (32/46) and mixed (14/46) breeds were included. The major purebreds were Golden retriever (9/32), Poodle (7/32), and Shih Tzu (4/32). The median age was eight years old (range3–15 years; Table 1). Based on anatomical classifications, multicentric lymphoma was diagnosed in 71.74% of cases (B-cell = 27/33 and T-cell = 6/33), intestinal lymphoma in 10.87% of cases (B-cell = 1/5 and T-cell = 4/5), and extranodal lymphoma in 17.39% of cases (B-cell = 4/8 and T-cell = 4/8). Additionally, 58.7% (27/46) of the canine lymphomas were categorized as high-grade. High-grade B-cell lymphomas represented 68.75% of cases (22/32; Table 2). The common histopathology of high-grade B- and T-cell lymphomas in this study were diffuse large B-cell lymphomas (Figure 1), which consisted of centroblastic and immumoblastic cells, and peripheral T-cell lymphomas, which composed of pleomorphic small to large-sized cells, respectively. The less common low-grade lymphomas were follicular lymphomas (Figure 2), T-cell small lymphocytic lymphomas (Figure 3) and cutaneous T-cell lymphomas (Figure 4). In addition, MI was on average 8/HPF in high-grade and 4/HPF in low-grade lymphomas.

Immunophenotyping using IHC revealed positive Pax5 staining in the nuclei of all B lineages (Figures 1B and 2B) as well as CD3 staining in the cytoplasm of T-lymphocytes in all cases (Figures 3C and 4C). However, CD79a staining gave a negative result in four cases of B-cell lymphoma (Dog No. 1, 2, 20, 42) and a positive result in four cases of T-cell lymphoma (Dog No. 33, 35, 36, 38) as shown in Table 1. Two from three follicular lymphomas were mainly reactive with Pax5 and CD79a in

the follicle area, and were stained moderately with CD3 in the paracortical area. Pearson's chi-squared test also revealed an association between immunophenotyping and histological grade (P = 0.036).

Table 1 Signalment, anatomical location (AL), histological classification (HC), and immunohistochemistry detection in 46 canine lymphomas between 2008 and 2013.

No.	Breed[a]	Age (years)	Sex[b]	Anatomical location	Histological classification	Immunophenotype		
						Pax5	CD79a	CD3
1	Mixed	na	F	Multicentric	B-SLL	+	–	–
2	CP	8	F	Multicentric	B-SLL	+	–	–
3	Mixed	11	M	Hepatic	LPL	+	+	–
4	LR	7	M	Multicentric	B-SLL	+	+	–
5	Sharpie	5	F	Hepatic	HSTL	–	–	+
6	Mixed	9	F	Alimentary	ITCL	–	–	+
7	GR	8	M	Multicentric	T-SLL	–	–	+
8	Poodle	na	F	Multicentric	B-SLL	+	+	–
9	Mixed	8	F	Splenic	FL	+	+	+[c]
10	Poodle	na	M	Multicentric	FL	+	+	+[c]
11	Mixed	13	F	Multicentric	FL	+	+	–
12	Schnauzer	7	M	Multicentric	NMZ	+	+	–
13	LR	10	F	Multicentric	NMZ	+	+	–
14	Poodle	9	M	Alimentary	ITCL	–	–	+
15	BT	10	M	Multicentric	PTCL	–	–	+
16	Shih Tzu	12	F	Alimentary	DLBCL	+	+	–
17	Dachshund	na	M	Splenic	DLBCL	+	+	–
18	GR	na	F	Multicentric	DLBCL	+	+	–
19	Mixed	8	M	Multicentric	DLBCL	+	+	–
20	GR	4	F	Multicentric	DLBCL	+	–	–
21	Mixed	12	M	Multicentric	DLBCL	+	+	–
22	Poodle	9	M	Multicentric	DLBCL	+	+	–
23	BT	7	F	Multicentric	DLBCL	+	+	–
24	GR	9	M	Multicentric	DLBCL	+	+	–
25	MP	10	F	Multicentric	DLBCL	+	+	–
26	Poodle	12	F	Multicentric	DLBCL	+	+	–
27	Shih Tzu	na	M	Multicentric	DLBCL	+	+	–
28	Shih Tzu	12	F	Multicentric	DLBCL	+	+	–
29	Shih Tzu	7	M	Multicentric	DLBCL	+	+	–
30	Mixed	7	M	Multicentric	DLBCL	+	+	–

No.	Breed[a]	Age (years)	Sex[b]	Anatomical location	Histological classification	Immunophenotype		
						Pax5	CD79a	CD3
31	CP	14	F	Multicentric	DLBCL	+	+	–
32	Mixed	10	M	Multicentric	DLBCL	+	+	–
33	Mixed	7	F	Splenic	HSTL	–	+	+
34	Boxer	3	M	Alimentary	ITCL	–	–	+
35	BT	3	M	Multicentric	PTCL	–	+	+
36	GR	8	M	Multicentric	PTCL	–	+	+
37	Mixed	6	M	Multicentric	PTCL	–	–	+
38	GR	na	F	Tongue	CTCL	–	+	+
39	Mixed	4	M	Cutaneous	CTCL	–	–	+
40	GR	15	F	Multicentric	PTCL	–	–	+
41	Poodle	11	M	Multicentric	B-LBL	+	+	–
42	Mixed	8	M	Multicentric	B-LBL	+	–	–
43	GR	na	F	Alimentary	ITCL	–	–	+
44	Poodle	7	F	Multicentric	B-LBL	+	+	–
45	GR	12	F	Multicentric	B-LBL	+	+	–
46	Mixed	6	F	Heart base	ALBL	+	+	–

A = alimentary; ALBL = anaplastic large B-cell lymphoma; B- or T-SLL = B-cell or T-cell small lymphocytic lymphoma; B-LBL = B-lymphoblastic lymphoma; BT = Bull terrier; C = cutaneous; CP = Cocker spaniel; DLBCL = diffuse large B-cell lymphoma; F = female; FL = follicular lymphoma; GR = Golden retriever; H = hepatic; HB = heart base; HSTL = hepatosplenic T-cell lymphoma; ITCL = intestinal T-cell lymphoma; LPL = lymphoplasmacytic lymphoma; LR = Labrador retriever; M = male; MC = multicentric; MP = Miniature pinscher; na = data not available; NMZ = nodal marginal zone lymphoma; PTCL = peripheral T-cell lymphoma; SP = splenic; T = tongue

[a]32 purebreeds, 14 mixed breeds

[b]23 males, 23 females

[c]Normal T lymphocytes showed positive staining in the pericortical area

Table 2 Histopathological classification and immunophenotyping based on the WHO classification.

B-cell	**No. of cases**	**T-cell**	**No. of cases**
Low grade: 31.25% (10/32)		**Low grade: 64.29% (9/14)**	
B-small lymphocytic lymphoma	4	T-small lymphocytic lymphoma	1
Lymphoplasmacytic lymphoma	1	Hepatosplenic T-cell lymphoma	2
Follicular lymphoma	3	Intestinal T-cell lymphoma	4
Nodal marginal zone lymphoma	2	Cutaneous T-cell lymphoma	2
High grade: 68.75% (22/32)		**High grade: 35.71% (5/14)**	
Diffuse large B-cell lymphoma	17	Peripheral T-cell lymphoma	5
B-cell lymphoblastic lymphoma	4		
Anaplastic large B-cell lymphoma	1		
Total	32		14

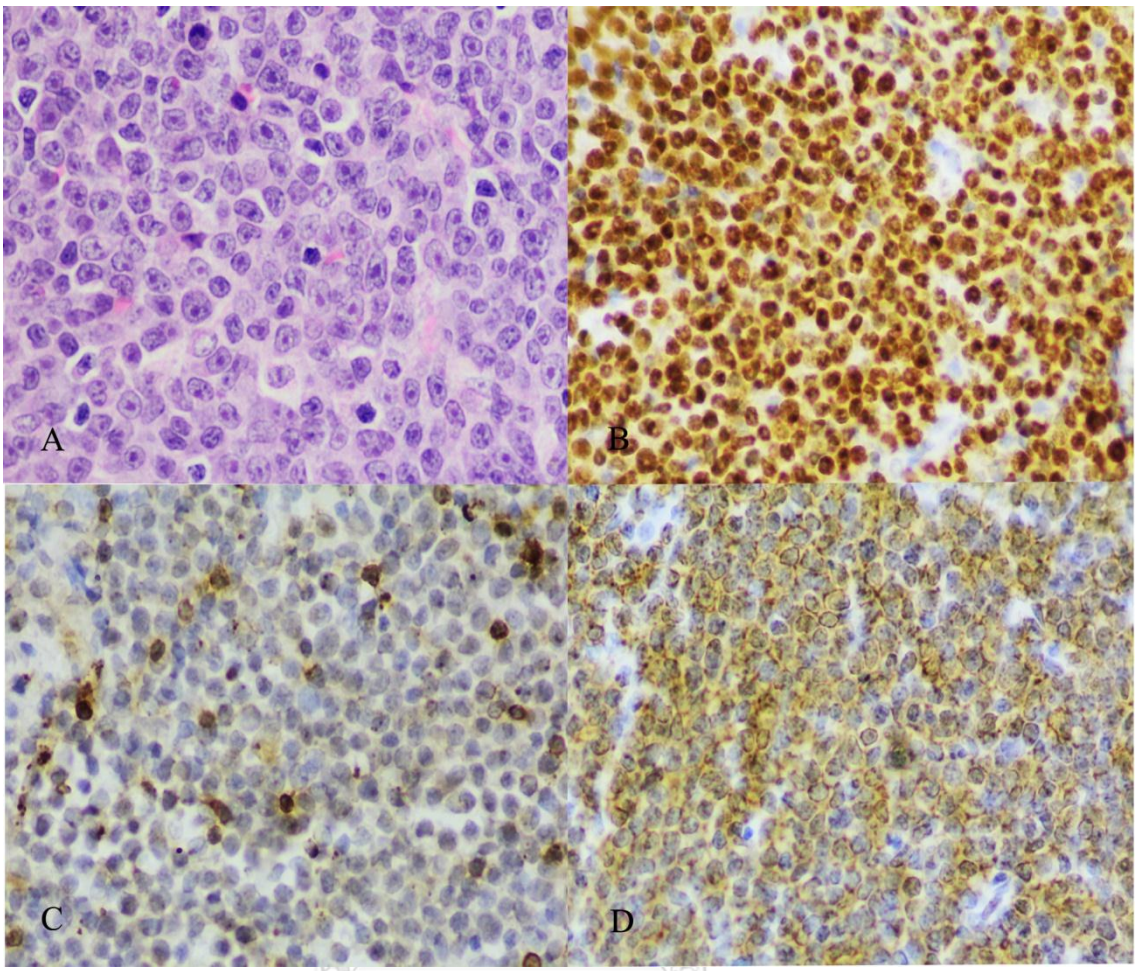

Figure 1 Diffuse large B-cell lymphoma. (A) Histopathology showed large neoplastic lymphocytes with round-to-cleaved nuclei, prominent nucleoli and abundant cytoplasm; H&E, x400. (B) Positive Pax5 staining in the nuclei of B cells; IHC, x400. (C) Neoplastic cells showed negative CD3 staining; IHC, x400. (D) CD79a antibodies revealed positive immunolabelling on the cytoplasmic border of B cells; IHC, x400.

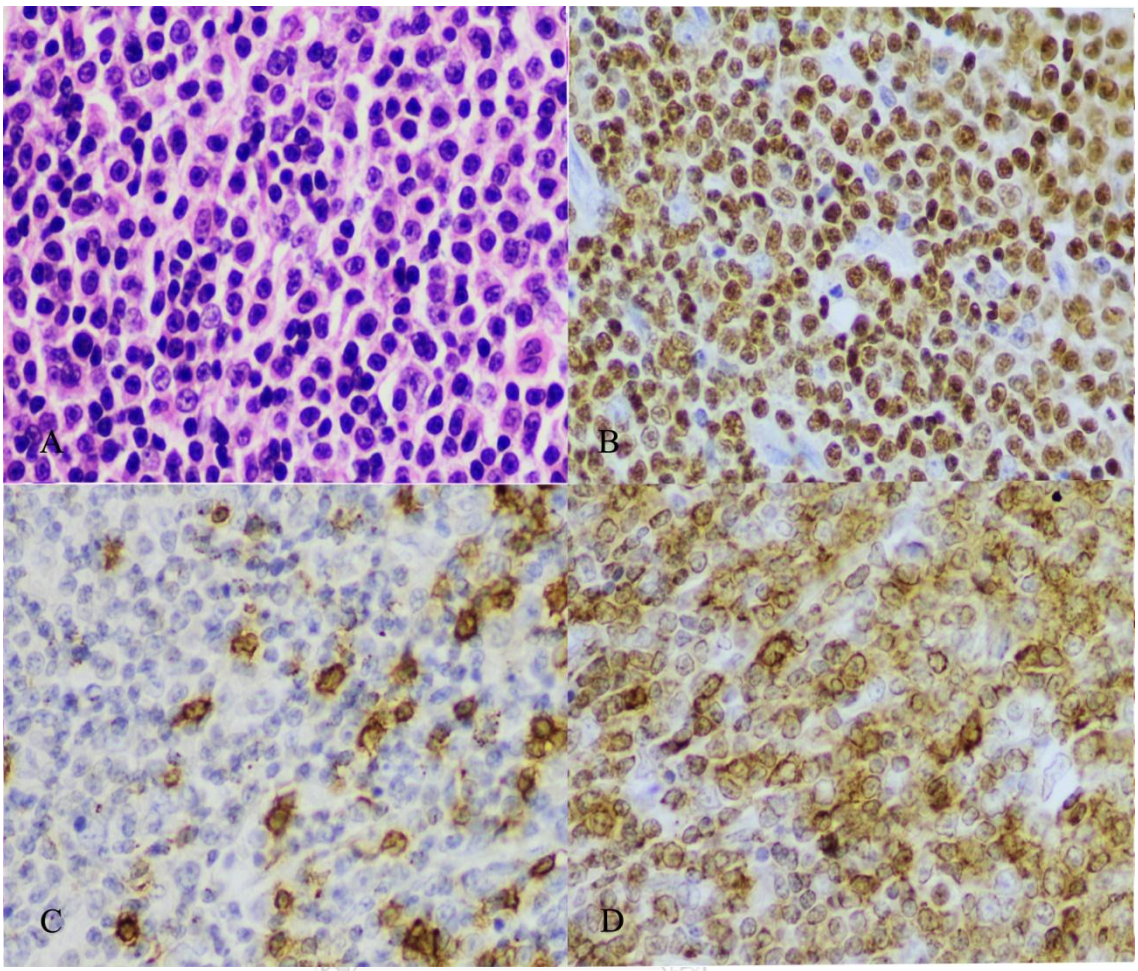

Figure 2 Follicular lymphoma. (A) Histopathology showed medium-sized and large-sized neoplastic lymphocytes; H&E, x400. (B) Positive Pax5 staining in the nuclei of B cells; IHC, x400. (C) Neoplastic cells showed some positive CD3 staining; IHC, x400. (D) CD79a staining revealed positivity cell in the cytoplasm of B cells; IHC, x400.

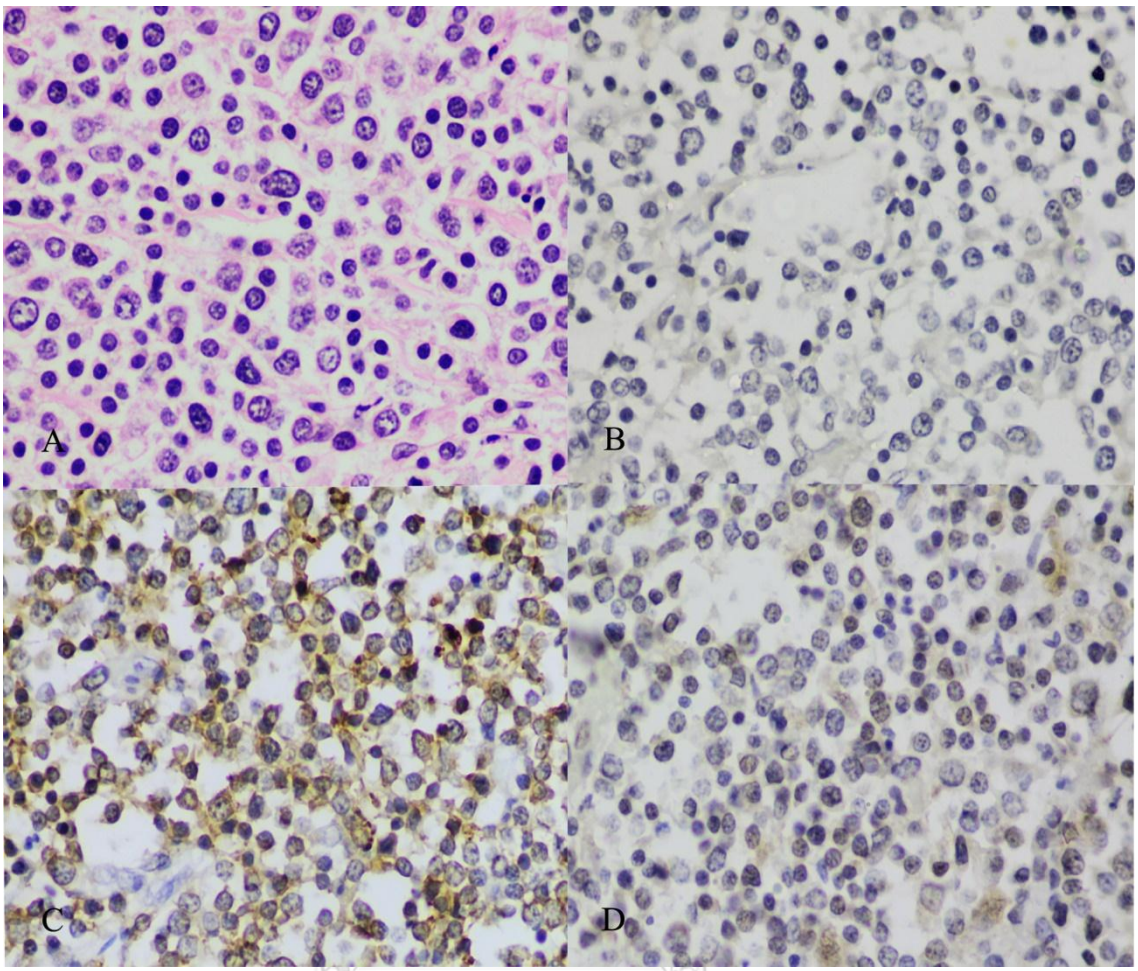

Figure 3 T-cell small lymphocytic lymphoma. (A) Histopathology showed small lymphoblasts with pleomorphic nuclear size and scant basophilic cytoplasm; H&E, x400. (B) Negative immunolabelling with Pax5 antibody; IHC, x400. (C) Neoplastic cells showed intense positive immunostaining with CD3 antibody; IHC, x400. (D) CD79a antibodies gave negative result; IHC, x400.

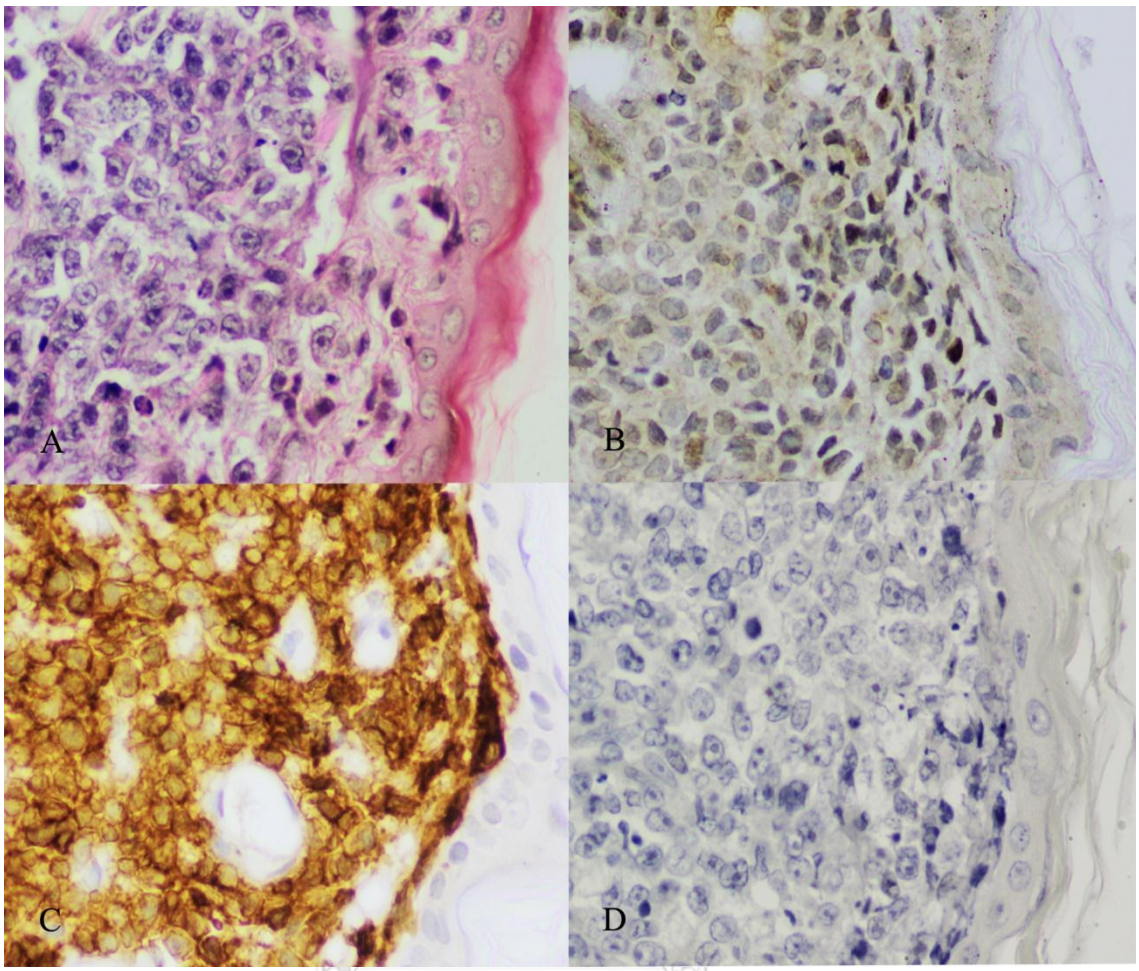

Figure 4 Cutaneous T-cell lymphoma. (A) Histopathology showed large lymphoblasts with pleomorphic nuclear size and moderate amounts of basophilic cytoplasm; H&E, x400. (B) Negative result with Pax5 antibody; IHC, x400. (C) Neoplastic cells showed positive staining with CD3 antibody; IHC, x400. (D) CD79a antibodies gave negative result; IHC, x400.

2.1.5 Discussion

Canine lymphoma in this study was classified into three anatomical locations: multicentric, alimentary, and extranodal, multicentric lymphomas have frequently been observed in dogs (Dobson et al., 2002; Rungsipipat et al., 2012). In this study, B-cell lymphoma was found in 69.57% of cases, whereas T-cell lymphoma was detected in the remaining 30.43%. This higher incidence rate of B-cell lymphoma is similar to that reported previously (Fournel-Fleury et al., 2002; Guija de Arespacochaga et al., 2007). Pax5 protein expression was observed in all B-derived lymphoma samples, while CD79a reactivity was not observed in four cases of B-cell lymphomas. Willmann et al. (2009) also described this problem. Moreover, four T-cell lymphomas expressed both CD3 and CD79a, which has been reported previously in both humans and dogs (Wilkerson et al., 2005; Guija de Arespacochaga et al., 2007; Willmann et al., 2009). Additionally, follicular

B-cell lymphomas showed positive staining with CD3, which might be a result of reactive T-cell population involvement. Although the Pax5 marker is superior to CD79a in B-cell identification, CD79a is still frequently selected for immunophenotyping in dogs with lymphoma (Vezzali et al., 2010; Valli et al., 2013). Both markers have high correlations through various B lymphocyte stages; however, Pax5 is expressed only during B-cell development and differentiation, and not in plasma cells (Horcher et al., 2001). Agostinelli et al. (2010) reported that DAK-Pax5 (clone 24) staining resulted in more intensely positive cells in most canine B-non-Hodgkin lymphomas apart from plasmacytomas. Nevertheless, the limitation of this antibody and IHC protocol might be a problem of its extensive application in animals.

In humans, Pax5 expression has been observed in B-lymphoblastic leukaemia/lymphoma, small lymphocytic lymphoma, diffuse large B-cell lymphoma, Burkitt lymphoma, Mantle cell lymphoma, Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (Browne et al., 2003; Desouki et al., 2010; Nasr et al., 2010). Furthermore, Pax5 expression was detected in B-cell lymphoma which occasionally lacks CD20 and CD79a expression (Jensen et al., 2007). Thus, Pax5 is accepted as a B-cell marker in

human medicine. In the veterinary field, Pax5 was chosen as a B-cell indicator in canine malignant lymphoma because of its specificity and sensitivity; CD79a-positive B-cells were reported to be expressed in 10–40% of canine and human T-cell lymphomas (Willmann et al., 2009). Pax5 staining was used for B-cell identification in a complicated case report on multicentric B-cell lymphoma with neurolymphocytosis (Schaffer et al., 2012). Pax5 was also employed in a study of inflammatory processes: spirocercosis-induced nodular formation in dogs (Dvir et al., 2011), and was also used in an immunocytochemistry by liquid-based cytology and tissue transfer technique for immunophenotyping in canine NHL (Stone and Gan, 2014; Fernandes et al., 2016). The sensitivity and specificity of Pax5 immunohistochemistry for B-cell lymphomas when compared to heteroduplex polymerase chain reaction for antigen receptor rearrangements of IgH primer sets for B-cells, were 48% and 100%, respectively (Sirivisoot et al., 2016). In our study, Cases No. 1, 2, 20 and 42 were diagnosed as B-cell lymphomas because significant numbers of Pax5-positive B-cells were present.

One complication to staining for Pax5 is the difficulty of choosing a suitable clone of Pax5 antibody for canine tissues. At first, we used DAK-Pax5 monoclonal anti-human antibody, but it failed to stain canine B lymphocytes. A previous study reported that a few cases of B-non-Hodgkin lymphomas and classical HLs from human and animal tissues showed negativity with this DAK-Pax5 clone (Agostinelli et al., 2010). However, when we used the anti-human Pax5 monoclonal antibody clone 1EW, this stained both normal and neoplastic B-cells. This discrepancy might occur due to species-specific differences, different epitopes, and the differing cross-reactivity of each antibody. The 1EW clone had 96–98% homologous identity with the canine epitope, similar to CD3 clone LN10 and CD79a clone HM57, which showed cross reactivity to canine antigens.

The immunophenotyping technique provides reliable information on the clonal origin of canine malignant lymphomas. Because of the pronounced dissimilarities in prognosis and treatment of B- and T-cell lymphomas, IHC is necessary for lineage determination. Besides its usefulness in precisely marking

lymphomas of B-cell origin, Pax5 staining might serve as a suitable diagnostic tool in unclassified lymphomas in dogs and also in other species.

2.1.6 Acknowledgements

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2.2 Application of immunophenotyping and heteroduplex PCR for antigen receptor rearrangements (hPARR) for diagnosis of canine lymphomas

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2.2.1 Abstract

Canine malignant lymphoma is classified into B- or T-cell origin, as in the human case. Due to differences in prognosis, a suitable method needs to be developed for lineage identification. To determine the accuracy of immunophenotypic and molecular information between three methods: immunocytochemistry (ICC), immunohistochemistry (IHC) and heteroduplex polymerase chain reaction for antigen receptor rearrangements (hPARR) in spontaneous canine lymphomas. Peripheral blood, fine needle aspiration and tissue biopsies from enlarged peripheral lymph nodes prior to treatment of 28 multicentric lymphoma patients were collected. Cytopathology and histopathology were examined and classified using the updated Kiel and WHO classifications, respectively. Anti-Pax5 and anti-CD3 antibodies as B- and T-cell markers were applied for immunophenotyping by ICC and IHC. Neoplastic lymphocytes from lymph node and white blood cell pellets from peripheral blood were evaluated by hPARR. In this study, low grade B-cell lymphoma accounted for 25% (7/28), high grade B-cell lymphoma for 64.3% (18/28) and high grade T-cell lymphoma for 10.7% (3/28). According to the WHO classification, 50% of all cases were classified as diffuse large B-cell lymphoma. In addition, ICC showed concordant results with IHC; all B-cell lymphomas showed Pax5+/CD3-, and all T-cell lymphomas exhibited Pax5-/CD3+. In contrast to hPARR, 12 B-cell lymphomas featured the IgH gene; seven presented the TCRγ gene; five cases showed both IgH and TCRγ genes, and one case were indeterminate. Three T-cell lymphomas showed the TCRγ gene. The percentage agreement between hPARR and ICC/IHC was 60%. Immunophenotyping should not rely on a single method. ICC or IHC with hPARR should be used concurrently for immunophenotypic diagnosis in canine lymphomas.

2.2.2 Introduction

Immunophenotyping is essential for lymphocyte-cloned classification, prognosis and treatment option in human and canine lymphomas. Immunohistochemistry (IHC) is a classic method that is referred to a gold standard due to its accuracy (Valli et al., 2011; Thalheim et al., 2013). Regarding tissue specimens, it provides morphology of lymph nodes (LN) for histological assessment, but generalized anesthesia in dogs is required. Another immunophenotyping technique is immunocytochemistry (ICC). When compared to IHC, ICC is cheaper and easier. Fine needle aspiration biopsy (FNAB) can diagnose lymphoma by cytological classification; however, cell disruption, low cellularity or smear technique can lead to difficulty in interpretation (Aulbach et al., 2010; Sapierzynski, 2010). Nevertheless, liquid-based cytology, or keeping aspirated cells in a preservative fluid for multiple slide preparation, was developed to reduce these disadvantages (Wallace et al., 2015; Fernandes et al., 2016). The specific protein markers for B-cell identification were CD79a or Pax5, while T-cell marker was CD3 (Willmann et al., 2009; Valli et al., 2013). Flow cytometry is a good option for immunophenotyping data, but fresh sample from FNAB is needed, and specific antibody panels of B- and T- cells are required (Sozmen et al., 2005).

Polymerase chain reaction for antigen receptor rearrangements (PARR) showed effective results for diagnosis in confusing cases and detection of minimal residual disease (Burnett et al., 2003; Lana et al., 2006; Aresu et al., 2014). Not only samples from LN cytology, but also peripheral blood and formalin-fixed paraffin-embedded tissue were tested by this assay (Keller et al., 2007; Kaneko et al., 2009; Thilakaratne et al., 2010). However, false positive and false negative results by PARR are an obstruction when interpreting the results alone. In addition, pseudoclonality can occur as a result of immunologic diversity of immunoglobulin heavy chain (IgH) and T-cell receptor gamma (TCRγ) genes because a single copy is not amplified during PARR. Thus, duplicate, triplicate and heteroduplex analysis might be a helpful technique to rule out the pseudoclones, avoid misdiagnosis and resolve reliability of the results (Takanosu et al., 2010).

The purpose of this study was to evaluate immunophenotyping data between ICC and IHC, a gold standard method, using Pax5 and CD3. Heteroduplex PARR (hPARR) from peripheral blood and LN cytology was determined the consistency between samples and compared with immunophenotyping method. To achieve a goal, all results were analyzed and compared the agreement between ICC and hPARR with IHC for developing a diagnostic method for canine T- and B-cell lymphomas.

2.2.3 Materials and methods

2.2.3.1 Animals and sample collections

Twenty eight dogs presented to the Oncology Clinic, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, in 2013 to 2015 and clinically diagnosed as canine multicentric lymphoma were recruited for this study (IACUC Number 13310074). All cases were categorized according to the WHO clinical stage shown in Table 3, including peripheral LN measurement, thoracic and abdominal radiography and abdominal ultrasonography. Whole blood was collected for evaluation of complete blood cell count by automated, liver enzymes (ALT, ALP), kidney profiles (BUN, Creatinine) and blood parasite detection by blood morphology. The infiltration of atypical lymphoid cells was determined by buffy coated smear for clinical staging. If there were medium to large lymphoblasts and small lymphocytes more than 50%, it was classified as stage V. Microscopic diagnosis was based on cytological and histopathological results of the enlarged peripheral LN.

2.2.3.1.1 Cytology

FNAB from superficial LN was collected using 22 G needle with a 3 ml syringe. Cells from needle hub were flushed, smeared on silane-coated slides for cytopathological study and kept in sterile PBS-contained microcentrifuge tubes at -80 ℃ for molecular study. For routine cytopathological assessment, at least two smears were fixed in absolute methanol, stained with Giemsa and classified as low or high grade lymphoma by cell morphology according to updated Kiel criteria, assuming

that at least 80% of the cells presented on slides were blast cells excluding lymphocytic leukemia (Sapierzynski et al., 2010).

2.2.3.1.2 Histopathology

Biopsy specimen (popliteal, axillary or inguinal LN) was collected by a 6 mm diameter punch biopsy and preserved in 10% neutral buffered formalin for routine histopathological diagnosis. Histopathological changes in cell size and shape, nucleus size and mitotic figure, number of nucleoli, chromatin density, cytoplasm characterization of neoplastic lymphocytes and mitotic index were categorized based on the WHO classification (Vezzali et al., 2010; Valli et al., 2011) by a veterinary pathologist.

2.2.3.1.3 White blood cell pellet

Peripheral blood was also collected from patients. Buffy coat was separated from EDTA-anticoagulated blood by centrifugation at 5,000 rpm for 5 min and kept in sterile microcentrifuge at -80 °C until used for determining lymphoid lineage with hPARR.

2.2.3.2 Immunocytochemistry

At least four cytological smears on silane-coated slides from enlarged LN of individual case were dried and fixed in cold acetone at 4 °C for 10 min, and kept at 20 °C until used or stained directly. Antibody control for both markers was a cytological smear from a normal canine lymph node.

2.2.3.2.1 Immunocytochemical assays for B lymphocytes

Endogenous peroxidase in cells was blocked with 3% H_2O_2 and 1% bovine serum albumin (BSA, Merck Millipore, Germany) in 0.25% phosphate buffer saline-triton X-100 (PBST) used for non-specific blocking, followed by incubating with Pax5, monoclonal mouse anti-human antibody (1EW, Leica, UK) at a dilution of 1:50 at 4 °C overnight. The samples were conjugated and amplified by modified avidin-biotin-peroxidase complex (LSAB) using Novolink polymer (Leica, UK) at room temperature for 15 min. The slides were washed in PBS; color developed by 3, 3'-diaminobenzidine (DAB, Leica, UK) and then counterstained with Mayer's

hematoxylin for 1 min. Pax5 showed a nuclear staining in B cells. B-cell lymphoma was identified if at least 80% of the cells revealed expression of Pax5.

2.2.3.2.2 Immunocytochemical assays for T lymphocytes

Peroxidase and non-specific background were blocked by 3% H_2O_2 and 1% BSA, respectively. Then, a pan T-cell marker, CD3 monoclonal mouse anti-human antibody (LN10, Leica), was incubated at 4 ℃ overnight and Envision polymer (Dako, Denmark) was used to detect antigen with the LSAB method. The slides were developed in color with DAB (Dako, Denmark) and counterstained with Mayer's hematoxylin. Immunopositivity of CD3 was localized in cellular cytoplasm. T-cell lymphoma was diagnosed when at least 80% positive cells of a particular antibody were observed.

2.2.3.3 Immunohistochemistry

Four to six micrometer-thick sections were immunophenotypically classified using anti-CD3 and anti-Pax5 to identify T- and B-cell lineage, respectively. Briefly, antigen retrieval for CD3 was achieved by heating the slides with 10 mM citrated buffer (pH 6.0) in a microwave oven. To block endogenous enzymes, the slides were incubated in 3% H_2O_2 for 10 min and 1% BSA at 37 ℃ for 10 min, respectively. After washing, the slides were incubated with ready-to-use monoclonal mouse CD3 antibody (LN 10, Leica, UK) at 4 ℃ for 12-14 h. Then, the slides were incubated with modified avidin-biotin-peroxidase complex or Envision polymer at room temperature for 45 min. Finally, they were immersed in DAB to develop an immunological reaction and then counterstained with Mayer's hematoxylin before mounting. T-cell lymphoma was interpreted when at least 80% of the neoplastic cells were stained with CD3.

Similarly, for the immunostaining of anti-Pax5 (clone 1EW) to determine B-cell lineages, antigens were retrieved by heating the slides in Tris/EDTA (pH 9.0) with an autoclave oven (121 ℃, 5 min). Subsequently, they were incubated with anti-Pax5 antibody (dilution 1:50) at 4 ℃ overnight. The slides were rinsed with PBS, blocked peroxidase and non-specific background with 3% H_2O_2 at room temperature for 10 min and 1% BSA at 37 ℃ for 10 min. Novolink detection was conjugated on tissues at

room temperature for 15 min. DAB was used as chromogen and Mayer's hematoxylin was used for counterstaining. Interpretation of positive B-cell lymphoma was achieved when at least 80% of the neoplastic cells stained positively with Pax5.

2.2.3.4 hPARR

Genomic DNA of the cytological pellet and white blood cell (WBC) pellet was extracted with a mammalian genomic DNA miniprep kit (Sigma-Aldrich, USA). DNA concentration was measured by a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). Conventional PARR protocol was performed for both specimens, followed by heteroduplex analysis (Takanosu et al., 2010). Specific primers were chosen, the Cμ gene for controlling DNA amplification, IgH gene for B lymphocytes and TCRγ gene for T lymphocytes (Burnett et al., 2003). DNase/RNase-free distilled water was used as a negative control. ExcelBand 100 bp+ 3K DNA ladder (Smobio Technology, Taiwan) was used for sample size identification. Primer concentrations were 200-300 nM. The PCR cycling protocol was 94 ℃ for 45 s, 60 ℃ for 30 s and 72 ℃ for 30 s. After 35 cycles of PCR amplification, each PCR product was divided into two aliquots and each was run for heteroduplex analysis: incubation at 95 ℃ for 5 min and re-annealing at 4 ℃ for 30 min. Afterward, every PCR sample was visualized in 2% agarose gel electrophoresis with 10% ethidium bromide staining by AlphamanagerTM (Alpha Innotech, USA). A monoclonal band of each gene indicated a positive result. Biclonal or triclonal might have been observed and indicated two or three neoplastic clones. A negative result appeared as a polyclonal or no bands. B- and T-cell positive controls were histopathological diagnosis as B-and T-cell lymphoma.

2.2.3.5 Statistical analysis

Results of these three tests were classified as B-cell lymphoma if Pax5+/CD3-/ monoclonal band of IgH gene or T-cell lymphoma if Pax5-/CD3+/monoclonal band of TCRγ gene. Sensitivity, specificity and percent agreement of ICC and hPARR compared with IHC were calculated. Cohen's K test was run to determine the agreement between hPARR and ICC and IHC, respectively with SPSS version 22.0.

Table 3 WHO clinical staging criteria for canine lymphoma.

Stage	
I	A single lymph node is involved
II	Regional lymphadenopathy (restricted to one side of diaphragm)
III	Generalized lymphadenopathy
IV	Hepatosplenomegaly (with or without lymphadenopathy)
V	Involvement of bone marrow, central nervous system or extranodal sites
	Substage a: no clinical signs
	Substage b: clinical signs of illness

2.2.4 Results

2.2.4.1 Signalment

Twenty eight dogs consisted of 50% (14/28) male and 50% (14/28) female with the average age of 10 years (range 3-15 years). 78.6% (22/28) was pure and 21.4% (6/28) was mixed breeds as shown in Table 4. The majority of pure breeds were Golden Retriever and Shih Tzu. All cases were multicentric lymphoma. 28.6% (8/28) was classified as clinical stage III; 53.6% (15/28) had clinical stage IV and 17.9% (5/28) had clinical stage V. Only seven dogs with clinical stage III and IV showed normal blood profiles, whereas others had abnormal blood results such as neutrophilic leukocytosis, thrombocytopenia, and increased ALT or ALP. Three dogs with thrombocytopenia showed *Ehrlichia canis* infection. Five dogs with clinical stage V presented lymphocytosis and leukemic appearance.

2.2.4.2 Cytopathology and histopathology

After cytopathological and histopathological grading based on the updated Kiel and WHO classifications, 25% (7/28) was classified as low grade lymphoma and 75% (21/28) were categorized as high grade lymphoma (Table 4). For the cytopathology of low grade B-cell lymphoma, lymphocytic lymphoma presented small round cells (1 to 1.5 red blood cell in diameter) with small densely basophilic round nuclei, no nucleoli and minimal cytoplasm; prolymphocytic lymphoma had slightly larger and more vesicular nuclei; macronucleolated medium-sized cell (MMC) lymphoma showed medium-sized cells with fine chromatin, prominent nucleolus and a moderate amount of weakly basophilic cytoplasm; centroblastic-centrocytic lymphoma revealed a mixture of centrocytes (irregular notched or non-cleaved nuclei, dense chromatin and narrow cytoplasmic rim) and centroblasts (round nuclei, dispersed chromatin, prominent multiple nucleoli and narrow basophilic cytoplasm). For the high grade B-cell lymphoma, malignant lymphocytes were mainly large-sized cells. Polymorphic centroblastic lymphoma was composed of a few MMCs, a few immunoblasts and mostly centroblasts. Immunoblastic lymphoma had large size, large nucleolar volume with central or multiple nucleoli, extended basophilic

cytoplasm and moderate to high mitotic figures (Figure 5A). Lymphoblastic lymphoma had small to medium-sized cells with convoluted nucleus, fine chromatin, discrete nucleoli and moderate extension of basophilic cytoplasm. For T-cell lymphoma, pleomorphic small cell type illustrated small cells with irregular indented nuclei, clumped chromatin and scant cytoplasm; pleomorphic mixed type contained small-sized and large-sized cells with irregular nuclei and pale cytoplasm (Figure 6A). The pleomorphic large cell lymphoma had irregular nuclei, fine chromatin and pale to moderately basophilic cytoplasm.

The histopathology of lymph node punch biopsy was mainly classified into diffuse large B-cell lymphomas or DLBCL (Figure 5B) for 50% of cases. It was composed of centroblastic (multiple periphery nucleoli) and immunoblastic (single central nucleolus) lymphoma. A starry-sky pattern was frequently observed. Moreover, the mitotic index (MI) varied with the average number 8 MI/HPF. 10.71% of cases were follicular lymphoma (FC), which showed a mixture of centrocytes and centroblasts located throughout the follicles and CD3-positive T cells located between interfollicular or paracortical areas.

Three cases of B- lymphoblastic lymphoma (B-LBL) were immunostained with Pax5. The morphology presented a diffused pattern with homogenous medium-sized cells, round nuclei, fine chromatin and indistinct small nucleoli. Starry-sky and high MF were obviously seen. B small lymphocytic lymphoma (B-SLL) was presented less in this study (two cases). It illustrated small B lymphocytes with small round basophilic nuclei and scant cytoplasm, while B-cell lymphocytic lymphoma of intermediate type (LLI) showed slightly larger nuclei and more vesicular chromatin. One sample revealed nodal marginal zone cell lymphoma (NMZ). Macronucleolated medium-sized cells, small to medium nuclei, fine chromatin, prominent nucleolus and a moderate amount of cytoplasm were characteristic of this type. One anaplastic plasmacytoid B-cell lymphoma (APL) showed diffuse infiltration by medium to large cells with pleomorphic nuclei, prominent nucleoli and eccentric basophilic cytoplasm. Peripheral T-cell lymphoma (PTCL) was reported in three cases. It was

apparent that there was an intermixing of small to large cells with irregular indented nuclei and different amounts of cytoplasm (Figure 6B).

2.2.4.3 Immunocytochemistry and immunohistochemistry

In this study, B-cell lymphoma accounted for 89.3 % (25/28), whereas T-cell lymphoma accounted for 10.7% (3/28) of the cases. With immunophenotyping by ICC and IHC, all B-cell lymphomas showed immunostaining with anti-Pax5 antibody (Figure 5C and 5D). Though, CD3 showed positive staining in all cases of T-originated lymphoma (Figure 6C and 6D).

2.2.4.4 hPARR

In this study, DNA concentration of all WBCs and cytological pellets was between 4 ng to 260 ng with A260/280 equal to1.18 to 1.98. The conventional and hPARR classified clonal rearrangements into four types: IgH gene, TCRγ gene, dual genes and indeterminate. 12 dogs (42.9%) showed IgH gene rearrangements (Figure 7); 10 cases (35.7%) presented TCRγ gene rearrangements (Figure 8); five cases (17.9 %) showed dual genes rearrangement and one dog (3.6%) presented a polyclonal result of both genes (Table 5). Both peripheral WBC and LN cytology revealed similar results. However, two WBCs and one LN sample from three dogs did not show any distinct gene, so the result was restricted to the only apparent gene from the other samples.

2.2.4.5 Statistical data

The sensitivity and specificity of ICC and IHC for B- and T-cell lymphomas were similar. However, the sensitivity and specificity of IgH primer sets were 48% and 100%, respectively. The sensitivity and specificity of TCRγ primer sets were 100% and 72%, respectively (Table 5). The percentage agreement between ICC/IHC and hPARR was 60%. There was a slight agreement between ICC/IHC and hPARR, κ=0.2 (95%CI 0.002, 0.398), $P < 0.05$.

Table 4 Signalment, stage, cytological and histological classifications, immunophenotype and hPARR results of twenty-eight lymphoma cases.

Dog	Sex[a]	Age (y)	Breed[b]	WHO stage	Cytopathology (Updated Kiel)	Histopathology[c] (WHO)	Grade	Immunophenotype				hPARR
								ICC		IHC		
								Pax 5	CD 3	Pax 5	CD 3	
1	M	10	GR	III	Lymphocytic	B-SLL	Low	+	-	+		TCRγ
2	M	15	ST	V	Lymphocytic	B-SLL	Low	+	-	+	-	IgH
3	M	9	GR	V	Prolymphocytic	LLI	Low	+	-	+	-	Dual
4	F	13	Mixed	IV	Centroblastic-centrocytic	FC	Low	+	-	+	-[d]	TCRγ
5	M	8	CH	V	Centroblastic-centrocytic	FC	Low	+	-	+	-[d]	IgH
6	F	14	Mixed	IV	Centroblastic-centrocytic	FC	Low	+	-	+	-[d]	TCRγ
7	F	10	GR	III	MMC	NMZ	Low	+	-	+	-	IgH
8	F	11	LR	IV	Centroblastic monomorphic	DLBCL	High	+	-	+	-	IgH
9	M	14	ST	V	Centroblastic monomorphic	DLBCL	High	+	-	+	-	IgH
10	F	8	ST	III	Centroblastic polymorphic	DLBCL	High	+	-	+	-	Dual
11	F	10	Beagle	III	Centroblastic polymorphic	DLBCL	High	+	-	+	-	IgH
12	M	9	ST	IV	Centroblastic polymorphic	DLBCL	High	+	-	+	-	IgH
13	M	8	GR	IV	Centroblastic polymorphic	DLBCL	High	+	-	+	-	TCRγ
14	F	10	MP	IV	Centroblastic polymorphic	DLBCL	High	+	-	+	-	IgH
15	M	9	GR	V	Centroblastic polymorphic	DLBCL	High	+	-	+	-	IgH
16	F	14	GR	IV	Immunoblastic	DLBCL	High	+	-	+	-	TCRγ
17	F	12	ST	IV	Immunoblastic	DLBCL	High	+	-	+	-	IgH
18	M	7	ST	III	Immunoblastic	DLBCL	High	+	-	+	-	Dual
19	M	7	Mixed	III	Immunoblastic	DLBCL	High	+	-	+	-	TCRγ
20	F	15	CS	III	Immunoblastic	DLBCL	High	+	-	+	-	Dual
21	M	10	Mixed	III	Immunoblastic	DLBCL	High	+	-	+	-	Indeterminate
22	M	10	Mixed	IV	Lymphoblastic	B-LBL	High	+	-	+	-	IgH
23	F	7	Poodle	IV	Lymphoblastic	B-LBL	High	+	-	+	-	IgH
24	F	12	GR	IV	Lymphoblastic	B-LBL	High	+	-	+	-	Dual

Dog	Sex[a]	Age (y)	Breed[b]	WHO stage	Cytopathology (Updated Kiel)	Histopathology[c] (WHO)	Grade	Immunophenotype				hPARR
								ICC		IHC		
								Pax 5	CD 3	Pax 5	CD 3	
25	F	6	ST	IV	Anaplastic	APL	High	+	-	+	-	TCRγ
26	M	3	BT	IV	Pleomorphic mixed	PTCL	High	-	+	-	+	TCRγ
27	M	6	Mixed	IV	Pleomorphic mixed	PTCL	High	-	+	-	+	TCRγ
28	F	15	GR	IV	Pleomorphic large cell	PTCL	High	-	+	-	+	TCRγ

[a]M=male; F= female, [b]BT= Bull terrier; CH= Chihuahua; CS= Cocker spaniel; GR= Golden retriever; LR= Labrador retriever; MP= Miniature pinscher; ST= Shih Tzu, [c]APL = anaplastic plasmacytoid lymphoma; B-SLL= B-cell small lymphocytic lymphoma; LLI= B-cell lymphocytic lymphoma of intermediate type; FC= follicular lymphoma; NMZ = nodal marginal zone lymphoma; DLBCL = diffuse large B-cell lymphoma; B-LBL= B-cell lymphoblastic lymphoma; PTCL= peripheral T-cell lymphoma, [d]CD3 positive T-cells were located in the paracortical area

Table 5 Results and comparison between immunophenotype and clonal gene rearrangements in twenty-eight lymphoma dogs.

Clonal gene rearrangement	Pax5 (+), CD3 (-) (n = 25)	Pax5 (-), CD3 (+) (n = 3)
IgH (+), *TCRγ* (-)	12	0
IgH (-), *TCRγ* (+)	7	3
IgH (+), *TCRγ* (+)	5	0
IgH (-), *TCRγ* (-)	1	0

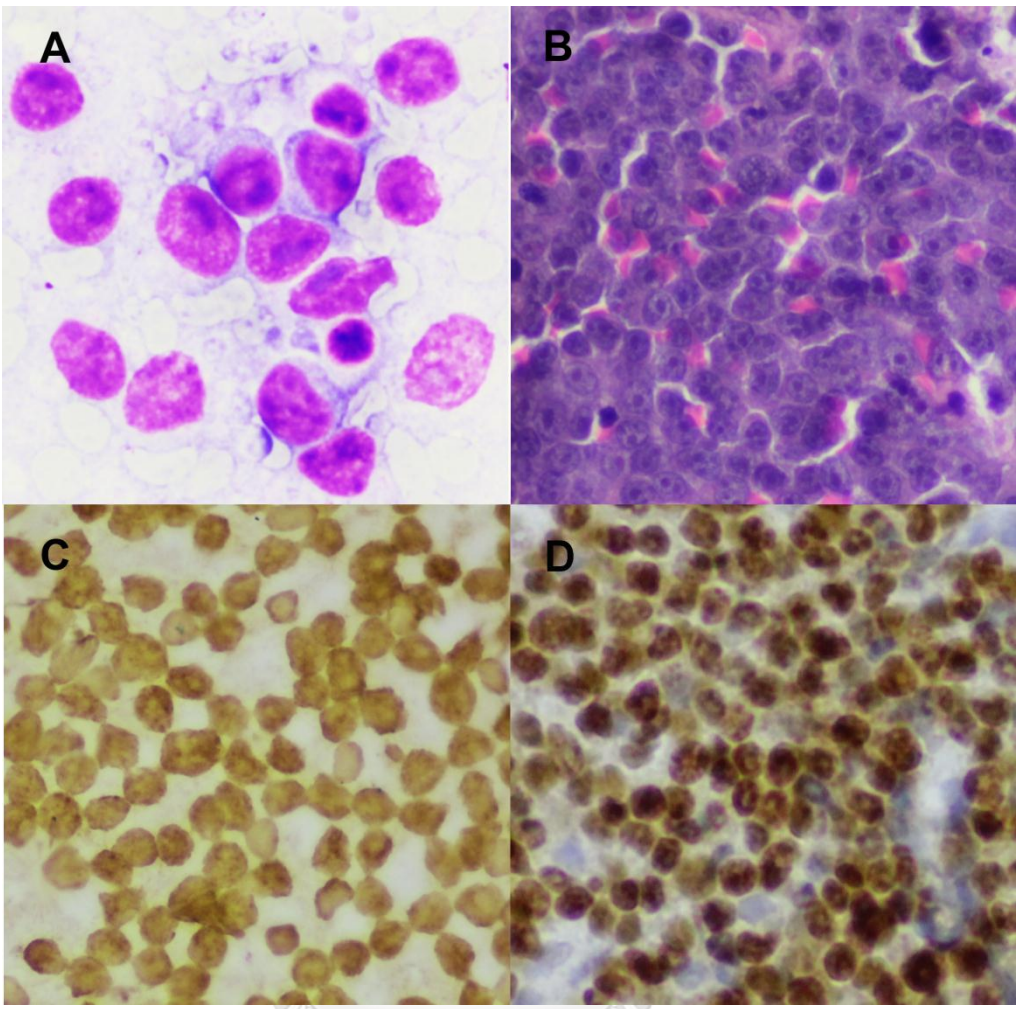

Figure 5 Diffuse large B-cell lymphoma (high grade). (A) Cytopathology revealed typical large B cells with a large nucleus and large centrally located nucleolus; Giemsa, x400. (B) Histopathology displayed round, oval or cleaved nuclei, with reticular chromatin and large central nucleolus; H&E, x400. (C) Pax5 was immunolabelled in nucleus of B cells; ICC, x400. (D) Pax5 showed positive staining in nucleus of malignant B lymphocytes; IHC, x400.

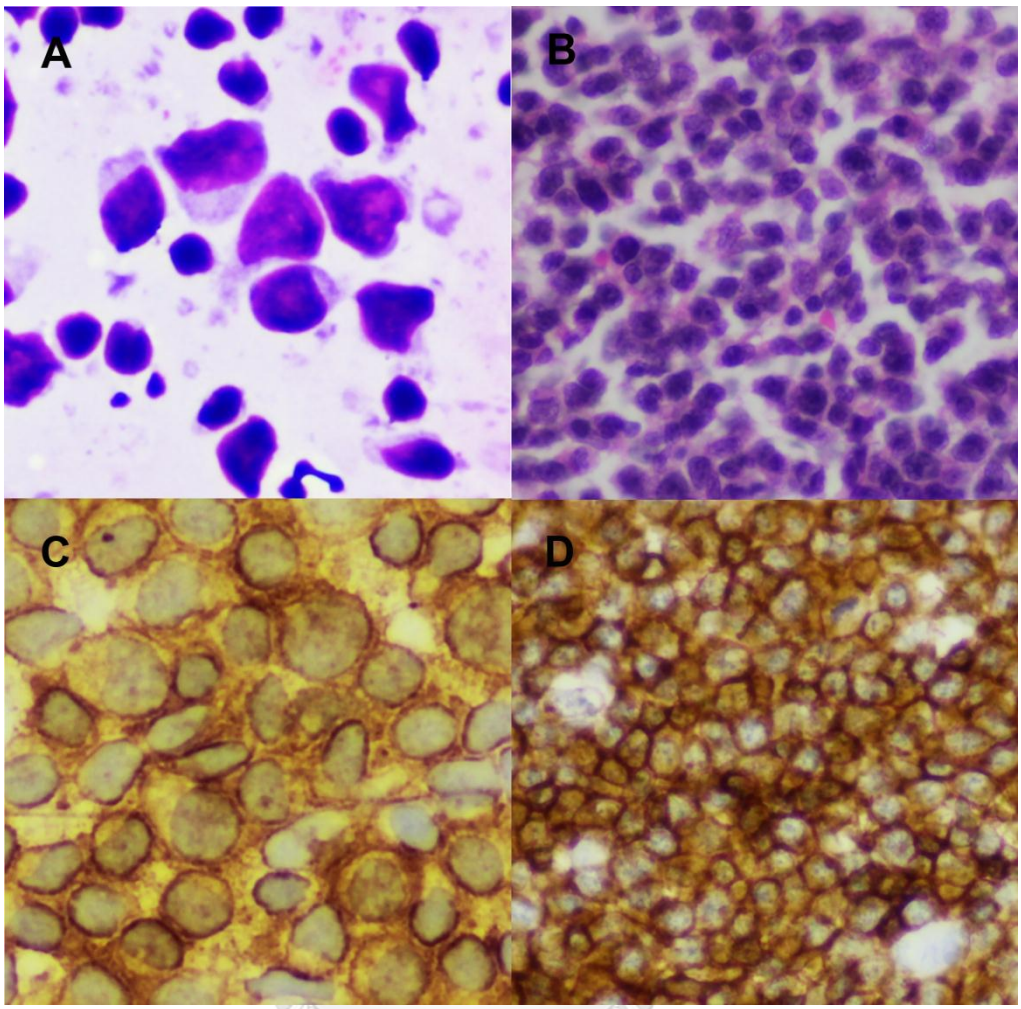

Figure 6 Peripheral T-cell lymphoma (high grade). (A) Cytopathology presented pleomorphic lymphoblastic cells with irregular nuclei, fine chromatin and pale basophilic cytoplasm; Giemsa, x400. (B) Histopathology showed various sizes of T-cell lymphoma, small to large malignant T cells, with irregularly cleaved nuclei and various amounts of cytoplasm; H&E, x400. (C) Positive immunostaining of CD3 revealed in cytoplasm of T lymphoblasts; ICC, x400. (D) CD3 immunostaining was observed in the cytoplasmic border of the T lymphoblasts; IHC, x400.

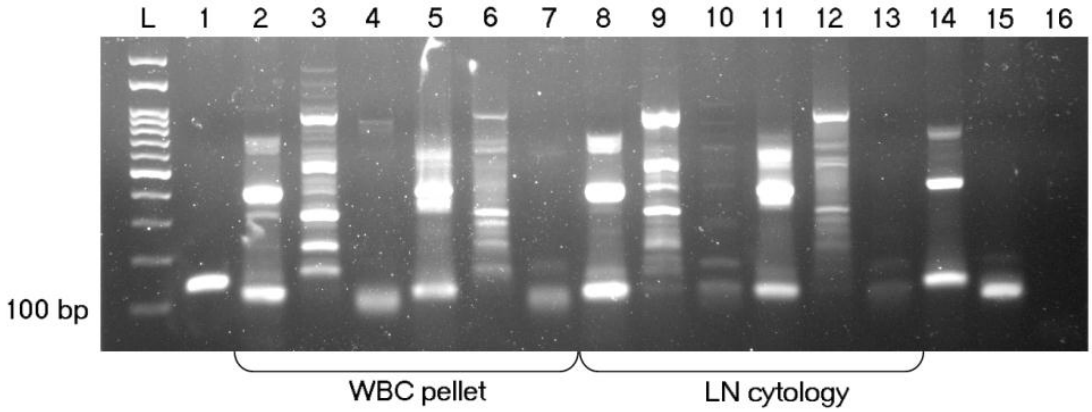

Figure 7 Conventional and hPARR results from WBC and LN specimens showed distinct bands of IgH genes. L = ExcelBand 100bp ladder; 1 = Cμ gene (130 bp); 2, 5, 8, 11 = IgH major gene (120 bp); 3, 6, 9, 12 = IgH minor gene (120 bp); 4, 7, 10, 13 = TCRγ gene (90 bp); 1-7 = sample from WBC; 8-13 = sample from LN cytology; 5-7 and 11-13 = heteroduplex analysis; 14 = B-cell lymphoma positive; 15 = T-cell lymphoma positive; 16= negative control.

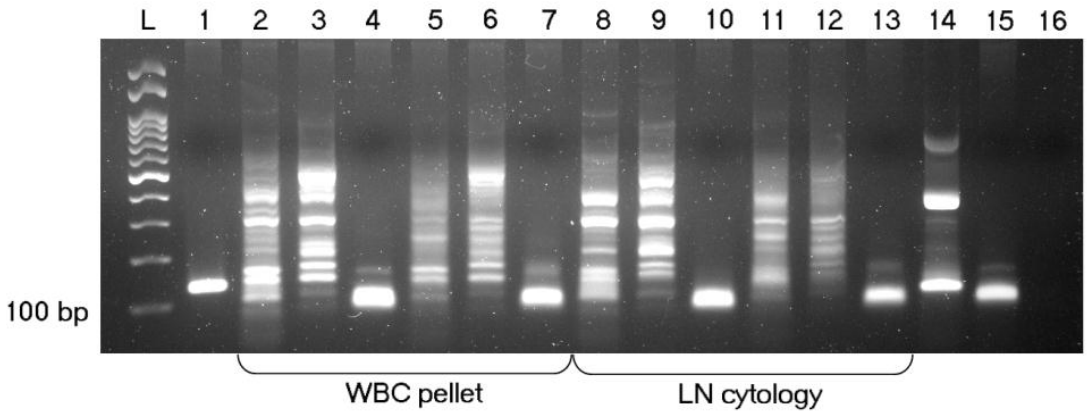

Figure 8 Conventional and hPARR results from WBC and LN specimens presented TCRγ genes. L = ExcelBand 100- bp ladder; 1 = Cμ gene (130 bp); 2, 5, 8, 11 = IgH major gene (120 bp); 3, 6, 9, 12 = IgH minor gene (120 bp); 4, 7, 10, 13 = TCRγ gene (90 bp); 1-7 = sample from WBC; 8-13 = sample from LN cytology; 5-7 and 11-13 = heteroduplex analysis; 14 = B-cell lymphoma positive; 15 = T-cell lymphoma positive; 16= negative control.

2.2.5 Discussion

In this study, high grade B-cell lymphoma was 64.3% and high grade T-cell lymphoma was 10.7%, while low grade B-cell lymphoma was 25% with no low grade T-cell lymphoma. High grade lymphomas, especially DLBCL and PTCL, were the most common type, which is similar to other studies. DLBCL is more regularly reported than PTCL in canine multicentric lymphoma (Wilkerson et al., 2005; Willmann et al., 2009; Ponce et al., 2010; Valli et al., 2013). In addition, immunophenotyping is necessary because B- and T-originated lymphoma have a different prognosis. High grade T-cell lymphoma has the shortest survival time; however, clinical stage or histopathological grading also has an impact on overall survival time (Kiupel et al., 1999; Valli et al., 2013). According to the WHO classification, the 28 dogs had multicentric lymphoma in at least clinical stage III. At the same time, all low grade lymphomas were in advanced stages, but the canine patients did not show any symptom at the presentation unless generalized lymphadenopathy. In contrast, most high grade lymphomas appeared as a systemic disease at the first visit.

Nowadays there are general criteria for canine lymphoma classification based on cellular morphology, lymph node architecture, and phenotype, for example, the updated Kiel (Fournel-Fleury et al., 1997; Fournel-Fleury et al., 2002), Working formulation (Guija de Arespacochaga et al., 2007), and WHO classification (Vezzali et al., 2010; Valli et al., 2011). As a result of emphasizing cytomorphology and immunophenotype data, this study used the updated Kiel scheme for categorizing LN cytology as was done in previous reports (Sozmen et al., 2005; Sapierzynski et al., 2010). On the other hand, the WHO system showed high accuracy and suitability for diagnostic classification of canine lymphoma tissues even if veterinary pathologists were not hematopathologists (Valli et al., 2011).

FL was the least common canine lymphoma. The incidence rate was 1% (Ponce et al., 2010; Vezzali et al., 2010). It can be divided into grade I to grade III, depending on the predominance of centrocytes or centroblasts. This study revealed a co-expression of B- and T-phenotype similar to previous studies (Wilkerson et al., 2005; Vezzali et al., 2010). Another rare subtype was anaplastic plasmacytoid, which

was reported in less than 1% (Ponce et al., 2010; Vezzali et al., 2010). It was diagnosed in one Shih Tzu dog and shown very aggressive disease with no response to standard chemotherapy.

To diagnose neoplastic lineage, IHC was used, as it is a standard and accurate method for histomorphology. In this study, all lymph node samples collected by punch biopsy were valuable for immunophenotyping by Pax5 and CD3. The Pax5 antibody was selected as a B-cell marker because it was expressed in pro B-cell throughout the activated B-cell stage, not in plasma cells and not accidently stained in T lymphocytes (Willmann et al., 2009). In addition, ICC was developed for lineage identification, as it is easier and cheaper than IHC. The ICC results in this study showed agreement with the IHC results with both the anti-Pax5 and anti-CD3 antibody. The evaluation of positivity for these two techniques was run blindly with the cut-off value 80% because of avoiding misdiagnosis with reactive lymph node. Few cytological smear glass slides produced a poor collection technique, such as a thick smear, low cellularity, and slow air dry or high watery sample, led to challenging for interpretation when compared with IHC. Other reports also found some complications using ICC with cytological smear samples, such as background staining, cell distortion and poor cellular specimen (Sapierzynski, 2010; Sapierzynski et al., 2012); however, the cytospin preparation technique showed advantageous immunocytochemical data with regard to little or no background staining (Valli et al., 2009; Aulbach et al., 2010).

PARR was a molecular assay used for the identification of a clonal proliferation, which could detect variable regions of B- and T-cell receptor genes (Burnett et al., 2003; Takanosu et al., 2010). It was also used to determine minimal residual disease in lymphoma cases during and after chemotherapy (Calzolari et al., 2006; Thilakaratne et al., 2010; Aresu et al., 2014; Manachai et al., 2014). The difficulty of this assay in this study was the presence of false positivity, false negativity and dual rearrangements. It was found that 13 B-cell lymphoma cases presented TCRγ (28%), dual (20%), and indeterminate (4%) genes by both conventional and hPARR. In this study, IgH primer sets had lower sensitivity than

TCRγ primer sets. This might be because primers cannot bind to V and J regions due to immunologic diversity of target genes, presence of mutations or aberrant gene rearrangements of tumor cells (Takanosu et al., 2010; Thilakaratne et al., 2010; Boone et al., 2013). Dual gene rearrangements might be caused by the presence of two clones of neoplastic cells, the transformation of single neoplastic clone to multiple clones or the presence of rearrangements on both chromosomes (Burnett et al., 2003). When comparing the sensitivity and specificity of ICC with IHC, a gold standard for immunophenotyping, there was no variation. Conversely, PARR showed lower sensitivity for IgH primers and lower specificity for TCRγ primers when compared to ICC/IHC. Thalheim et al. (2013) also reported the percent agreement between IHC and PARR which was 69%, while our result was 60%. It might be because the caseload in this study was limited; especially the number of T-cell lymphoma case, thus sensitivity, specificity and percent of agreement values might be controversial. Due to the unclear results in some cases, capillary electrophoresis or Gene Scan analysis in the detection of PARR products were developed for a superior solution (Jeon et al., 2007; Goto-Koshino et al., 2015).

In this study, both WBC-based and LN-based specimens were collected for PARR analysis. Both samples illustrated a parallel result of the clonal amplification. However, one sample of LN cytology and two samples of WBC pellet did not reveal clonal rearrangements; even though every patient had clinical stage III to V. Lana et al. (2006) also reported that 28% of cases with stage III to V had no detectable amplification gene from either peripheral blood or LN samples. This might be because samples did not contain specific DNA sequences, or current primers could not attach to receptor rearrangement genes in that sample.

Further investigation needs specific primers to individual genomic information of a particular clone of B- and T-cell receptor genes (Tamura et al., 2006; Yagihara et al., 2007; Keller and Moore, 2012). Thus, immunocytochemical and immunohistochemical techniques should be used for identifying lymphocyte lineage. However, concurrent methods such as heteroduplex PARR could be applied as an adjunct diagnostic tool either with ICC or IHC for sup-typing confirmation.

2.2.6 Acknowledgements

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2.3 Monitoring minimal residual disease in canine lymphomas treated with modified L-COP or L-CHOP protocols

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2.3.1 Abstract

Heteroduplex polymerase chain reaction for antigen receptor rearrangements (hPARR) was developed to monitor minimal residual disease (MRD) in canine B- and T-cell lymphomas treated with the modified L-COP or L-CHOP protocol. Thirty-five dogs were recruited in this study and determined their neoplastic lineage by immunophenotyping with Pax5 and CD3. Peripheral blood leucocytes were collected prior and during chemotherapy in week 4, 9 and 13 to detect MRD by hPARR. Twenty-eight dogs (80%) had B-cell lymphoma while seven dogs (20%) had T-cell lymphoma. A monoclonal band was detected in 11 cases that showed complete or partial remission before tumor relapse and no response to the current treatment without statistical difference in clinical outcomes, however; the treatment response had an association with MRD result ($P < 0.05$). Modified L-CHOP prolonged median progression-free survival than modified L-COP (215 days vs. 93 days; $P < 0.05$). Substage b had shorter progression-free survival than substage a (90 days vs. 215 days; $P < 0.05$). Clinical stage III affected median overall survival time when compared to clinical stage IV and V (432, 173, and 118 days, respectively; $P < 0.05$). hPARR could be used for screening refractory lymphoma unless lymph node measurement in routine clinical cases.

2.3.2 Introduction

Minimal residual disease (MRD) is a remnant of neoplastic cell that survives during or after chemotherapeutic treatment. It can be an indicator of tumor relapse and treatment outcome. Because of a low level of malignant cells during clinical remission, they should be monitored by advanced sensitive molecular methods, such as PCR, real-time PCR or flow cytometry (Calzolari et al., 2006; Yamazaki et al., 2008; Gentilini et al., 2010; Aresu et al., 2014). If the quantity of MRD in patients was increased through the current or after treatment, it indicated the poor prognosis and inadequate treatment efficacy in human and canine lymphomas (Yashima et al., 2003; Pott et al., 2006; Sato et al., 2013).

Polymerase chain reaction for antigen receptor rearrangements (PARR) is a molecular technique that detects alleles of immunoglobulin heavy chain (IgH) and T-cell receptor gamma (TCRγ) genes, the antigen-binding receptors of B and T lymphocytes, respectively. This technique showed a high sensitivity for detecting clonal B- or T-cell populations from various types of samples in dogs (Burnett et al., 2003). Because neoplastic lymphocytes could circulate in blood, peripheral blood is a good sample source for MRD screening (Keller et al., 2004; Lana et al., 2006; Thilakaratne et al., 2010). Aresu et al. (2014) compared the suitability of the flow cytometry and PARR methods to assess MRD from peripheral blood, lymph node (LN), and bone marrow samples in canine high-grade B-cell lymphoma. They found that PARR had a greater sensitivity than flow cytometry from peripheral blood. Moreover, the MRD positive cases screened by PARR showed a correlation with the time of relapse. Besides dogs that had been detected the MRD after complete remission had shorter survival time than the other dogs that were free of MRD (Manachai et al., 2014). Therefore, monitoring minimal residual disease might be a helpful method to detect refractory canine lymphoma.

Heteroduplex analysis with PARR (hPARR) was recommended for diagnosis of canine lymphoma because it decreased the chance of misdiagnosis due to pseudoclonality (Takanosu et al., 2010). After denaturation and slow re-annealing, it helped the formation of homoduplex molecules with identical size and sequence,

and defective the re-annealing of heteroduplex molecules with identical size and different sequence. Homogenous PCR products then migrated in expect size and showed the monoclonal band in high resolution gel, while heterogeneous products slowly migrated leading to diffuse smear pattern. Thus, it increased specificity of interpretation of PCR amplicons and validation of the clonal results.

The recommended chemotherapy for canine lymphomas is an L-CHOP protocol (L-asparaginase, vincristine, cyclophosphamide, doxorubicin, and prednisolone) (Garrett et al., 2002). However, a high-dose COP treatment (vincristine, cyclophosphamide, and prednisolone) might be one option because it has a cheaper cost (Dobson et al., 2001). Multicentric lymphoma treated with a COP combination with L-asparaginase (L-COP) or without L-asparaginase showed 70-80% of a complete remission rate and had median survival times at 6-7 months (Dobson et al., 2001; Jeffreys et al., 2005). Although, this treatment has longer maintenance phase, and shorter progression-free survival (PFS) and overall survival time (OST). On the other hand, the maintenance-free L-CHOP treatment has 90-100% of overall response rate and prolonged OST at 12 months (Garrett et al., 2002; Burton et al., 2013; Curran and Thamm, 2016).

Even though canine lymphoma is responsive to the chemotherapy but it could be resistant to anti-neoplastic drugs due to multidrug resistant genes (Zandvliet et al., 2015). The LN size evaluation concurrent with an adjunctive molecular tool might provide a tumor burden in non-target lesions unless than peripheral lymph nodes. The purposes of this study were to monitor MRD in canine B- and T-cell lymphomas treated with the modified L-COP (mL-COP) or modified L-CHOP (mL-CHOP) protocols by hPARR and evaluate the treatment efficacy. The clinical outcomes were compared the correlation to chemotherapeutic protocols, immunophenotype, clinical stage, substage and MRD results.

2.3.3 Materials and methods

2.3.3.1 Animals and sample collections

Thirty five dogs had been sent to the Oncology clinic, Small Animal Teaching Hospital, Chulalongkorn University in 2013 to 2015 (IACUC Number 13310074). They were clinically diagnosed as multicentric lymphoma by cytopathology and/or histopathology according to the WHO criteria. All cases were checked for complete blood count (CBC), liver enzymes: alanine aminotransferase (ALT) and alkaline phosphatase (ALP), and kidney function assessment: blood urea nitrogen (BUN), creatinine, total protein, albumin and globulin. Blood parasites were examined by thin blood smears and the presence of serum antigen of heartworm (*Dirofilaria immitis*), *Ehrlichia canis* and *Anaplasma platys* using the SNAP 4Dx Plus Test kit (IDEXX, USA). Clinical staging was done by CBC, chest and abdomen radiographs, abdominal ultrasound for determining liver and spleen metastasis and buffy-coated smear for leukemia assessment. After immunophenotyping, they received the mL-COP or mL-CHOP (Table 6) depending on owner decision and cardiac condition. Peripheral blood was collected into an EDTA tube in week 0 and during the treatment in week 4, 9 and 13. Dogs treated with mL-CHOP continued 25-week protocol, and dogs treated with mL-COP continued 1-year protocol. After achieving a complete remission, they were follow-up monthly or bimonthly. Physical examination, CBC, hepatic and renal chemistry profiles were checked before giving antineoplastic drugs. If patients had abnormal blood results, such as leukopenia, anemia, increased hepatic or renal values, the chemotherapy was postponed until the adverse events were normal and that time they obtained only supportive treatment.

2.3.3.2 Cytopathology/histopathology

Cytological specimens were collected by fine needle aspiration from enlarged superficial LNs in those cases that were inappropriate for generalized anesthesia and biopsy procedure. Four cytological smear slides were prepared for cytopathological classification based on Updated Kiel (Sapierzynski et al., 2010), stained by Giemsa, and for immunocytochemistry, immunostained by Pax5 and CD3. For the

histopathological study, a punch biopsy of 6 mm diameter was applied to collect enlarged superficial node and kept in 10% (w/v) neutral buffered formalin. Routine histological processing and paraffin wax embedding were performed. Four- to six-µm thickness sections were stained by Haematoxylin and Eosin (H&E) for histopathological classification based on the WHO criteria (Valli et al., 2011) , and immunostained by Pax5 and CD3 for immunophenotyping.

2.3.3.3 Immunocytochemistry (ICC)

After fixing the slides with cold acetone for 10 min, they were kept at -20 ºC until used. In brief, cells on the glass slide were blocked for endogenous peroxidase and protein background by 3% (v/v) H_2O_2 and 1% (w/v) bovine serum albumin (Merck Millipore, USA) in 0.25% phosphate buffer saline-triton X-100 (PBST), followed by incubating with the monoclonal mouse anti-human Pax5 antibody (1EW; Leica, USA) at a dilution of 1:50 at 4 ºC for 12-14 h. For T-cell detection, cells were treated as B cells above except for using the ready-to- use monoclonal mouse anti-human CD3 antibody (LN10; Leica, USA). In both cases the bound antibody was amplified the signal by Novolink polymer (Leica, USA) for 15 min and developed color by 3, 3'-diaminobenzidine (DAB; Leica). Slides were counterstained with Mayer's hematoxylin for 1 min. Negative controls used cytological smear from normal canine LN. Pax5 showed a nuclear staining in B cells while CD3 showed immunoreactivity in the cytoplasm of T cells. B-cell or T-cell lymphomas were identified if at least 60% of the cells revealed expression of Pax5 or CD3, respectively (Sirivisoot et al., 2017).

2.3.3.4 Immunohistochemistry (IHC)

In case of Pax5, antigens were revived by treatment of the slides in Tris/EDTA (pH 9.0) in autoclave oven (121 °C) for 5 min, while for CD3 the antigen was retrieved by heating the slides with 10 mM citrated buffer (pH 6.0) in a microwave for 6 min twice. Subsequently, the slides were blocked, incubated with the relevant monoclonal mouse anti-human antibody, and processed as described for ICC above. Normal canine lymph node obtained from necropsy was used as a staining control. Interpretations of positive B-cell or T-cell lymphomas were achieved when at least

60% of the neoplastic cells stained positively with Pax5 and CD3, respectively (Sirivisoot et al., 2017).

2.3.3.5 MRD by hPARR

The EDTA-anticoagulated blood was centrifuged 5,000 rpm for 5 min and the peripheral blood leucocytes were separated and kept at -20 ℃ for DNA extraction. Genomic DNA was extracted using a mammalian genomic DNA miniprep kit (Sigma-Aldrich, USA) and the obtained DNA concentration was measured by NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). Samples were then analyzed for IgH and TCRγ genes by hPARR analysis (Takanosu et al., 2010) using the four primer sets in Table 7 that were the Cμgene as a control for DNA amplification (Burnett et al., 2003), the IgH gene of B lymphocytes (Tamura et al., 2006) and two TCRγ gene primer pairs for T lymphocytes (Yagihara et al., 2007). The DNA and primer concentrations were 50-200 ng and 200-300 nM, respectively. The PCR was thermocycled with 35 cycles of 94 ℃ for 45 sec, 60 ℃ for 30 sec, and 72 ℃ for 30 sec. After conventional PARR, each PCR product was divided into two aliquots and one was run for heteroduplex analysis by incubation at 95 ℃ for 5 min and re-annealing at 4 ℃ for 30 min. Afterwards the PCR products were resolved by 2% (w/v) agarose gel electrophoresis and visualized with 10% (w/v) ethidium bromide staining by Alphamanager™ (Alpha Innotech, USA). DNase/RNase-free distilled water was used in place of the template as a negative control. ExcelBand 100 bp + 3K DNA ladder (Smobio Technology, Taiwan) was used for sample size identification. A monoclonal or biclonal band of each gene indicated hPARR positive, whilst the presence of polyclonal or no band was deemed to hPARR negative. Biopsy samples diagnosed as B- and T-cell lymphomas were used as B- and T-cell positive controls.

2.3.3.6 Follow up case

Peripheral LN measurement (target lesions: submandibular, prescapular, superficial inguinal and popliteal LNs) using caliper was recorded to assess treatment efficacy: complete response (CR; disappearance of all target lesions), partial response (PR; at least 30% decrease in the sum longest diameter of target lesions), stable disease (SD; target lesions neither sufficient decrease for PR nor sufficient increase for

PD), and finally progressive disease (PD; at least 20% increase in the sum longest diameter of target lesions) (Vail et al., 2010). If dogs showed PD and/or hPARR positive during the treatment in week 4, 9, and 13 (refractory lymphoma), they received a rescue drug, such as lomoustine or L-asparaginase with vincristine. Dogs with a complete treatment were follow-up every one or two months. Each case was noted for PFS and OST.

2.3.3.7 Statistical analysis

PFS and OST were compared between B- and T-cell originated lymphomas that were treated with the mL-COP and mL-CHOP protocols by Kaplan-Meier survival analysis. Dogs, which were still alive at the time of the analysis or lost to follow up, were censored for the survival analysis. Immunophenotyping, treatment, clinical stage, substage, hPARR result and L-asparaginase treatment were analyzed the relationship with clinical outcomes by Cox regression. Clinical response was observed the association with MRD result by Pearson's chi-square test. The SPSS 22 Statistics for Windows software (IBM, USA) was used for all analysis achieved and significance was accepted at the $P < 0.05$ level.

Table 6 The mL-COP and mL-CHOP protocols for canine lymphoma treatment.

	Week											
Drugs	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**13**
Modified L-COP												
L-asparaginase 400 IU/kg SC	•											
Vincristine 0.5-0.75 mg/m^2 IV	•	•	•	•			•			•		•
Cyclophosphamide 250 mg/m^2 PO					•			•			•	
Prednisolone 2 mg/kg/d for 7 days	•	•	•	•	•	•	•	•	•	•	•	•
then 1 mg/kg EOD												
Modified L-CHOP												
L-asparaginase 400 IU/kg SC	•											
Vincristine 0.75 mg/m^2 IV	•		•			•		•			•	
Cyclophosphamide 250 mg/m^2 PO		•					•					•
Doxorubicin 30 mg/m^2 IV				•					•			
(if ≤ 15kg: 1 mg/kg)												
Prednisolone 2 mg/kg/d	•											
Prednisolone 1.5 mg/kg/d		•										
Prednisolone 1 mg/kg/d			•									
Prednisolone 0.5 mg/kg/d				•								

Table 7 Primer sets used in the hPARR method.

Product	Primer names	Primer sequence (5'-3')	Product size
Cμ	Sigmf1	TTC CCC CTC ATC ACC TGT GA	130 bp
	Srμ3	GGT TGT TGA TTG CAC TGA GG	
IgH	V region	ACA CGG CC(A/C/G) TGT ATT ACT GT	80-150 bp
	J region	TGA GGA GAC GGT GAC C	
TCRγ	Vγa	CGT GTA CTA CTG CGC TGC CTG G	55-90 bp
	Vγb	GGC TGT ATT ACT GTG CCT GCT GG	
	Jγb[a]	TGT GCC AGG ACC AAG CAC TTT GTT	

[a]Primer Jγb was used with either Vγa or Vγb

2.3.4 Results

2.3.4.1 Signalment

The signalment was shown in Table 8. Pure breeds were mainly Golden/Labrador retriever (eight dogs) and Shih Tzu (eight dogs). At the first presentation, thrombocytopenia (platelet count less than 160,000/µl) was reported in six dogs. One dog had anemia (RBC count less than $5.2x10^6$ cells/µl and a hematocrit volume less than 30%). Six dogs had elevated ALT/ALP (values more than 200 IU/L). One dog had elevated creatinine and BUN (values of more than 1.4 and 27 mg%, respectively). Three patients were infected with *Ehrlichia canis*: one dog by raised serum levels of antibodies against *E. canis* by the SNAP 4Dx Plus Test (dog no. 8), and two dogs by blood smear (dog no. 3 and 11).

2.3.4.2 Cytopathology, histopathology and immunophenotype

Four dogs were diagnosed as multicentric lymphoma based on their cytology due to their condition being unsuitable (too weak) to perform general anesthesia. Their cytological assessments by the updated Kiel criteria were one small lymphocytic lymphoma, one centroblastic monomorphic lymphoma and two centroblastic polymorphic lymphomas. The other 31 dogs were histopathologically classified from examination of the affected LN section using the WHO scheme based on architecture and immunophenotype, as shown in Table 9 In this study, B-cell lymphomas accounted for 80% (28/35) of the dogs and were categorized as 8.6% (3/35) low grade and 71.4% (25/35) high grade ,while T-cell lymphomas accounted for 20% (7/35) of the dogs and were 14.3% (5/35) low grade and 5.7% (2/35) high grade. The most common lymphoma subtypes in dogs were centroblastic diffuse large B-cell lymphoma (DLBCL CB; Figure 9A) and immunoblastic DLBCL (DLBCL IB). The ICC and IHC results of the B-cell lymphomas showed Pax5+/CD3- (Figure 9B, C), and T-cell lymphomas were Pax5-/CD3+ (Figure 10B, C). However, one case of a T-cell rich large B-cell lymphoma (T-cell rich LBCL) showed CD3+ T cells around large Pax5+ B cells.

2.3.4.3 MRD by hPARR

With the hPARR analysis of the 28 B-cell lymphomas, 16 cases presented a clonal band of the IgH gene, eight cases showed TCRγ genes (dog no. 1, 2, 10, 13, 16, 18, 26, and 27), and four cases (dog no. 3, 23, 25, and 28) showed dual gene rearrangements. In contrast, 6/7 cases of T-cell lymphomas showed a clonal band of the TCRγ gene and one (dog no. 31) showed both IgH and TCRγ genes (Table 10). Sensitivity and specificity of the IgH primer sets were 57.1% and 85.7%, respectively, while the TCRγ primer sets had 85.7% sensitivity and 57.1% specificity. The percentage agreement between hPARR and immunophenotyping method was 62.9%.

The mL-COP group consisted of 14 B-cell lymphomas and three T-cell lymphomas, while the mL-CHOP group was comprised of 14 B-cell lymphomas and four T-cell lymphomas. The first group, B-cell lymphomas treated with mL-COP protocol, dog no.2 showed no response to the treatment and presented hPARR positive in week 9 before PD. Dog no. 4 and 14 with CR showed hPARR positive during the treatment in week 9 or 13 prior to refractory disease. Dog no. 13 with PR had hPARR positive through the treatment before death duo to PD. Treatment efficacy of mL-COP was well responsive in other cases with CR and no molecular response.

The second group, B-cell lymphoma treated with mL-CHOP protocol, had only three dogs being found to be hPARR positive whilst receiving the anti-neoplastic drugs. Dog no. 15 with PR and 17 with CR had PD and had to change to a rescue protocol with L-asparaginase and vincristine. However, dog no. 19 with hPARR positive subsequently prolonged OST before death of an unknown cause. The other dogs responded well to the mL-CHOP treatment with no detectable molecular response (Figure 9D).

The third group, T-cell lymphomas treated with mL-COP protocol, dog no. 31 failed to achieve clinical response showed hPARR positive before PD and had to give the rescue drugs. Dog no. 29 and 30 with CR presented hPARR positive during the treatment, even though they had long PFS or OST (Figure 10D) similar to the last group, dog no. 32.

2.3.4.4 Treatment response and survival time

The overall response rate for mL-COP treatment was 88%: 13 dogs had CR and two dogs had PR. Two from 17 dogs had no response. Conversely, the overall response rate for the mL-CHOP treatment was 100%: 17 dogs had CR and one dog had PR. Two dogs that failed to achieve a CR (no. 2 and 31) had a PFS of 70 and 35 days, respectively, with MRD positive. Three dogs that had a PR (dog no. 7, 13 and 15) had a PFS of 24, 75 and 34 days, respectively, and presented hPARR positive before refractory disease. Six dogs were still alive during the time of analysis and two dogs lost for contact (Table 10). The treatment response had the association with MRD result; cases with CR to the treatment tended to have MRD negative (P = 0.03, Chi-square test). However, seven dogs with CR showed MRD positive before current treatment failure.

For the B-cell lymphoma dogs treated by the mL-COP protocol, they had a median PFS of 90 days (95% CI of 82.7 and 97.3 days) and a median OST of 114 days (95% CI of 0 and 312.7 days), whereas those treated with the mL-CHOP had a median PFS of 201 days (95% CI of 116.7 and 285.3 days) and a median OST 247 days (95% CI of 196.9 and 297.1 days). For the T-cell lymphoma dogs treated with the mL-COP, they had a median PFS of 118 days (95% CI of 0 and 250.8 days) and a median OST of 171 days (95% CI of 86.2 and 255.8 days), while those treated with mL-CHOP had a median PFS of 227 days and a median OST 272 days. Both B-cell and T-cell lymphomas treated with CHOP had longer median PFS (P = 0.04; Log-Rank test) and OST (P = 0.08; Log-Rank test) than COP-based protocol. By immunophenotyping, B-cell lymphomas had a median PFS of 155 days (95% CI of 121.3 and 188.7 days) and a median OST of 239 days (95% CI of 218.3 and 259.7 days), while T-cell lymphomas had a median PFS of 227 days (95% CI of 0 and 506.7 days) and a median OST of 272 days (95% CI of 17.9 and 526.1 days) with no statistical significance in the PFS (P = 0.213; Log-Rank test) or OST (P = 0.3; Log-Rank test).

When compared to other variables (Table 11), which affected the median PFS or OST, substage a had a significantly longer median PFS than substage b by both

Log-Rank test (Figure 11A) and Cox regression. The mL-CHOP also had an effect of prolonged median PFS than mL-COP by Log-Rank test (Figure 11B). Clinical stage III had a significantly higher OST than the other stages by both Log-Rank test (Figure 12) and Cox regression.

Table 8 The signalment of 35 canine lymphomas.

Gender	
Male	21
Female	14
Age (years)	3-13 (median 8.5)
Breed	
Pure	30
Mixed	5
Clinical stage	
III	19
IV	12
IV	4
Substage	
a	24
b	11

Table 9 Histopathological classification and immunophenotyping from 35 dogs.

B-cell lymphoma (Pax5+/CD3-)	**No.**	**T-cell lymphoma (Pax5-/CD3+)**	**No.**
Low grade		**Low grade**	
Small lymphocytic lymphoma (B-SLL)	2	Small lymphocytic lymphoma (T-SLL)	3
T-cell rich large B-cell lymphoma (T-cell rich LBCL)	1	Anaplastic large cell lymphoma (T-ALCL)	1
		Cutaneous T-cell lymphoma (CTCL)	1
High grade		**High grade**	
Diffuse large B-cell lymphoma Centroblastic (DLBCL CB)	15	Peripheral T-cell lymphoma (PTCL)	2
Diffuse large B-cell lymphoma Immunoblastic (DLBCL IB)	10		
Total	28		7

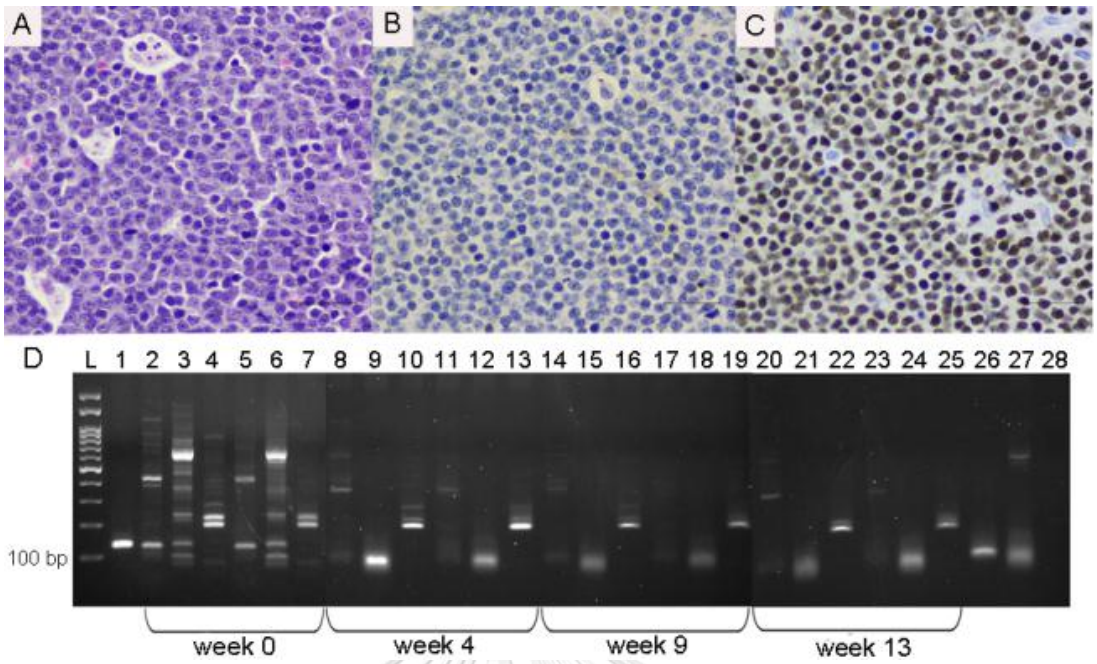

Figure 9 Diffuse large B-cell lymphoma, high-grade centroblastic type treated with mL-CHOP. (A) Histopathology showed starry-sky appearance with evident of tangible-body macrophages. (B) IHC revealed CD3-. (C) Pax5+ immunostained in the nuclei of B cells. Bar = 25 μm. (D) The hPARR analysis showed monoclonal IgH genes from WBC samples in week 0 and after week 4 to 13 a diffused pattern of both IgH and TCRγ genes was evident. L = ExcelBand 100bp ladder; 1 = Cμ gene (130 bp); 2, 5, 8, 11, 14, 17, 20, 23 = IgH genes (120 bp); 3, 6, 9, 12, 15, 18, 21, 24 = TCRγ-1 genes (90 bp); 4, 7, 10, 13, 16, 19, 22, 25 = TCRγ-2 gene (200 bp); 5-7, 11-13, 17-19, 23-25 = heteroduplex analysis; 26 = B-cell lymphoma; 27 = T-cell lymphoma; 28= negative control.

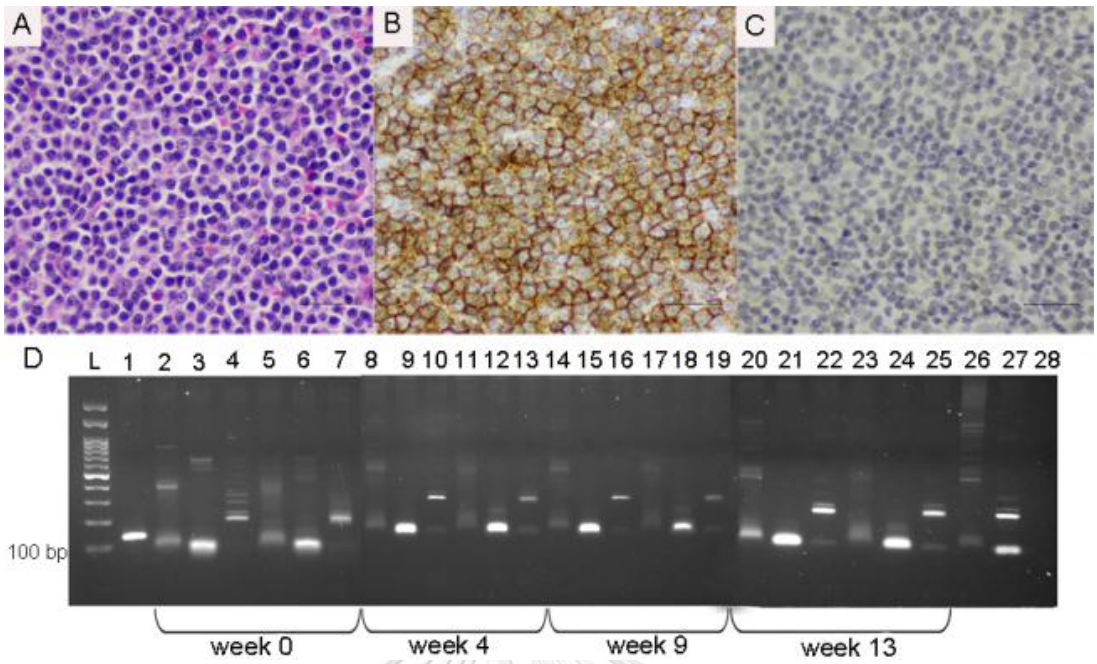

Figure 10 T-small lymphocytic lymphoma treated with mL-COP. (A) Histopathology presented small neoplastic T cells with cleaved, basophilic nuclei and scant cytoplasm. (B) CD3+ in the cytoplasmic membrane of T cells. (C) Pax5-. Bar = 25 µm. (D) The hPARR analysis showed a monoclonal TCRγ genes from WBC samples at week 0 to 13. L = ExcelBand 100bp ladder; 1 = Cμ gene (130 bp); 2, 5, 8, 11, 14, 17, 20, 23 = IgH genes (120 bp); 3, 6, 9, 12, 15, 18, 21, 24 = TCRγ-1 genes (90 bp); 4, 7, 10, 13, 16, 19, 22, 25 = TCRγ-2 gene (200 bp); 5-7, 11-13, 17-19, 23-25 = heteroduplex analysis; 26 = B-cell lymphoma; 27 = T-cell lymphoma; 28= negative control.

Table 10 The MRD results by hPARR from four treatment groups with PFS and OST.

Group	WHO Stage	Response	Pre-treatment	Post-treatment			PFS (days)	OST (days)
				Week 4	Week 9	Week 13		
B-cell lymphoma								
Modified COP								
B-SLL	III	CR	TCRγ	+	-	-	78	126
B-SLL	IV	No	TCRγ	-	+	R	70	227
T-cell rich LBCL	IV	CR	Dual	-	-	-	-	604[a]
DLBCL IB	IV	CR	IgH	-	-	+	103	239
DLBCL IB	III	CR	IgH	-	-	-	236	371
DLBCL CB	III	CR	IgH	-	-	-	-	144
DLBCL CB	III	PR	IgH	-	-	-	24	123
DLBCL CB	III	CR	IgH	-	-	-	-	84
DLBCL IB	III	CR	IgH	-	-	-	327	548[b]
DLBCL CB	IV	CR	TCRγ	-	-	-	89	91
DLBCL IB	III	CR	IgH	-	-	-	90	265
DLBCL CB	III	CR	IgH	-	-	-	-	218
DLBCL CB	V	PR	TCRγ	+	+	+	75	89
DLBCL CB	III	CR	IgH	-	+	+	69	93
Modified CHOP								
DLBCL IB	IV	PR	IgH	-	+	+	34	116
DLBCL IB	III	CR	TCRγ	-	-	-	-	826[a]
DLBCL CB	IV	CR	IgH	-	-	+	155	247
DLBCL CB	IV	CR	TCRγ	-	-	-	201	247
DLBCL CB	III	CR	IgH	+	+	+	-	338[b]
DLBCL CB	V	CR	IgH	-	-	-	224	286
DLBCL IB	V	CR	IgH	+	-	-	143	245
DLBCL CB	III	CR	IgH	-	-	-	215	238
DLBCL CB	III	CR	Dual	+	+	-	389	585
DLBCL CB	III	CR	IgH	-	-	-	169	432
DLBCL IB	IV	CR	Dual	-	-	-	-	161
DLBCL IB	IV	CR	TCRγ	-	-	-	130	159
DLBCL IB	III	CR	TCRγ	-	-	-	420	597[a]
DLBCL CB	III	CR	Dual	-	-	-	341	486

Group	WHO Stage	Response	Pre-treatment	Post-treatment			PFS (days)	OST (days)
				Week 4	Week 9	Week 13		
T-cell lymphoma								
Modified COP								
T-SLL	III	CR	TCRγ	+	+	+	450	862[a]
T-SLL	V	CR	TCRγ	+	+	+	-	118
T-ALCL	IV	No	Dual	+	R	n/a	35	171
Modified CHOP								
T-SLL	III	CR	TCRγ	+	+	+	-	527[a]
CTCL	III	CR	TCRγ	+	+	-	-	569[a]
PTCL	IV	CR	TCRγ	-	-	-	227	272
PTCL	IV	CR	TCRγ	-	-	-	103	173

CR= complete remission; PR = partial remission; PFS = progression-free survival; OST = overall survival time; R = relapse; B- or T-SLL = B- or T-small lymphocytic lymphoma; T-cell rich LBCL = T-cell rich large B-cell lymphoma; DLBCL IB = diffuse large B-cell lymphoma immunoblastic; DLBCL CB = diffuse large B-cell lymphoma centroblastic; T-ALCL = anaplastic large T-cell lymphoma; CTCL = cutaneous T-cell lymphoma; PTCL = peripheral T-cell lymphoma.

[a]still alive

[b]lost to follow-up

Table 11 Variables by univariable analysis as prognosis for PFS and OST in 35 dogs.

Parameter	Median PFS (days)	*P*-value	Median OST (days)	*P*-value
Immunophenotype				
B-cell (28)	155	0.213	239	0.303
T-cell (7)	227		272	
Substage				
A (24)	215	0.002	247	0.068
B (11)	90		173	
Clinical stage				
III (19)	236	0.060	432	0.040
IV (12)	103		173	
V (4)	118		118	
Minimal residual disease				
Present (11)	103	0.518	227	0.618
Absent (24)	201		247	
L-asparaginase				
With L-asparaginase (21)	155	0.574	245	0.955
Without L-asparaginase (14)	169		238	
Treatment				
mL-COP (17)	93	0.034	171	0.090
mL-CHOP (18)	215		272	

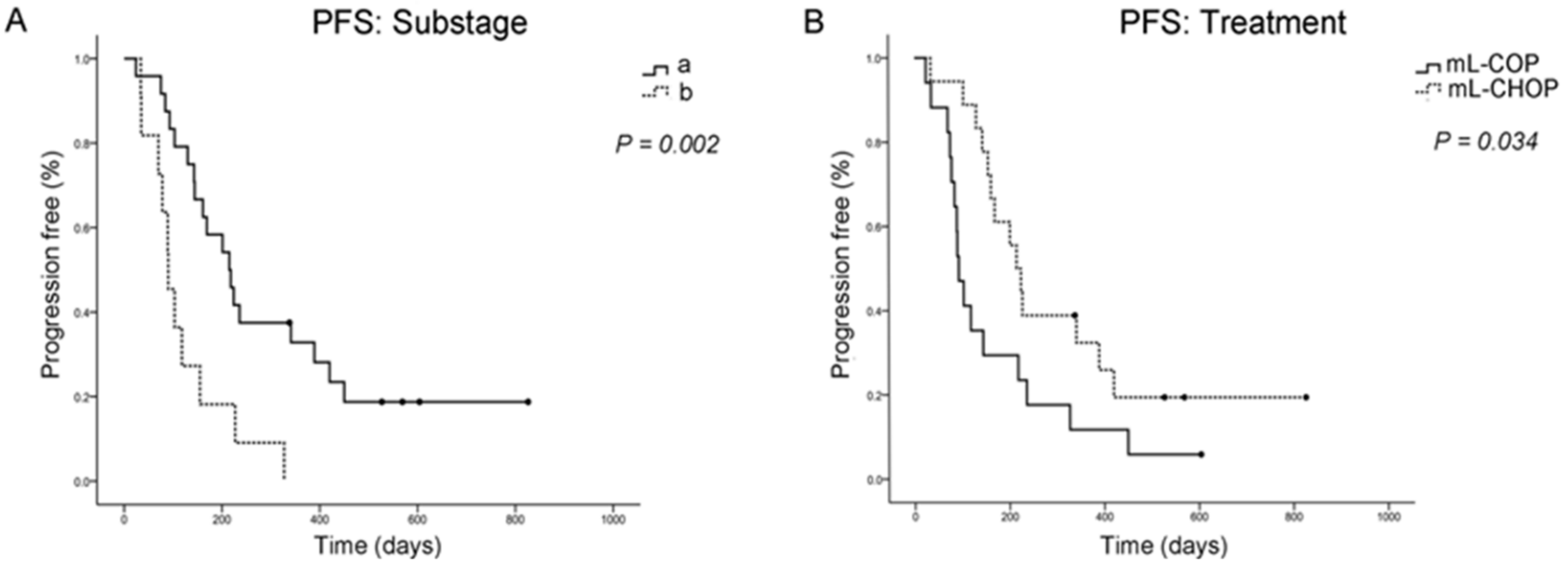

Figure 11 Kaplan-Meier analyses of (A) substage and (B) treatment affected to the PFS. Black circles indicate censored patients.

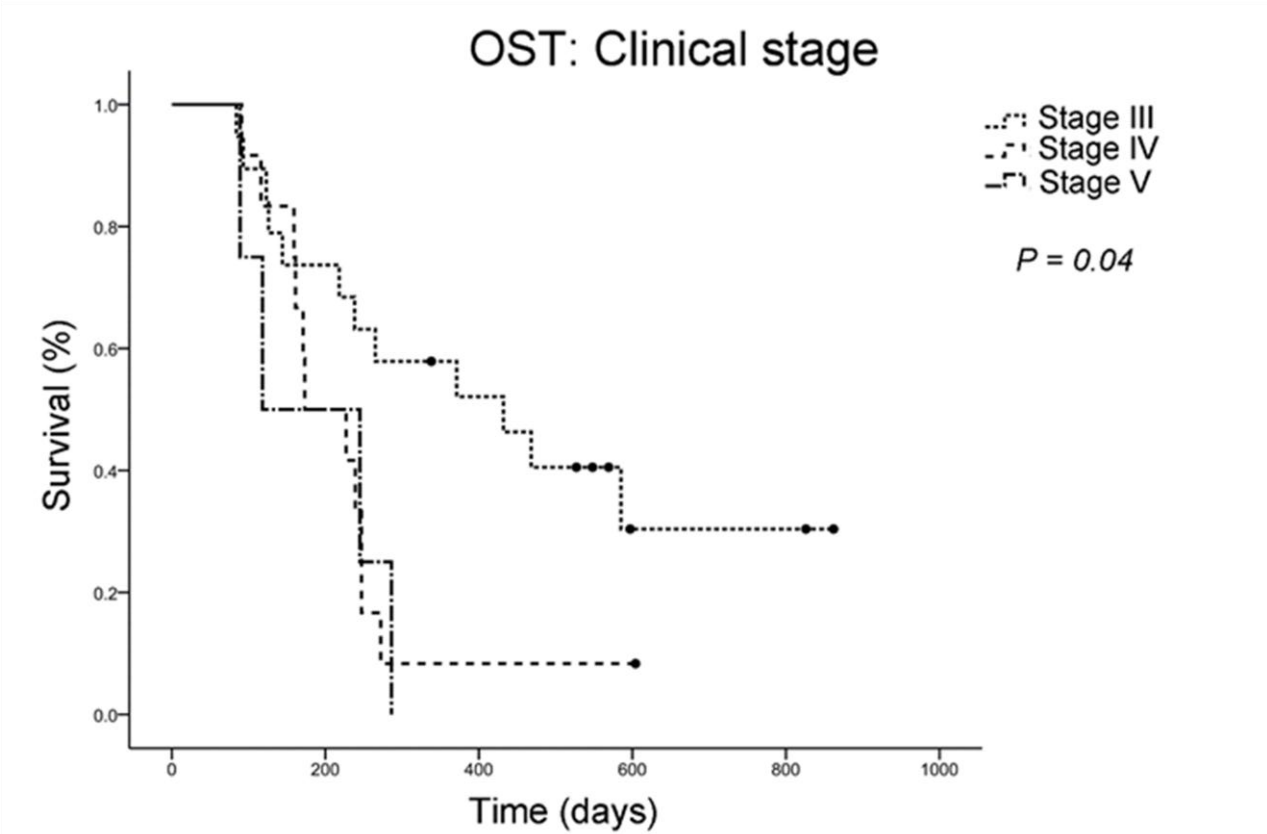

Figure 12 Kaplan-Meier analysis represented the clinical stage that affected to the prognosis of OST. Black circles indicate censored patients.

2.3.5 Discussion

This study had applied hPARR to monitor MRD during the treatment for detecting refractory lymphoma. The treatment efficacy was well responsive in dogs with hPARR negative and untraceable LNs, while hPARR positive was associated with PR or PD. The rescue drugs or secondary protocol, such as lomustine, doxorubicin or the second shot of L-asparaginase were given in refractory cases. Three T-cell lymphomas had hPARR positive during the treatment even though they had a CR, thus in the case of neoplastic T-cell clones, molecular result and LN measurement should be interpreted concurrently. In our study WBC samples at diagnosis showed clonal bands dissimilar to previous reports (Lana et al., 2006; Aresu et al., 2014; Hiyoshi-Kanemoto et al., 2016). However, we used the different primer sets which might affect sensitivity and specificity of the assay. In addition, our study did not perform bone marrow biopsy in every case. We used the blood smear technique to detect lymphoblasts. If the sample had lymphoblasts more than 50%, the dog had been classified into clinical stage V. Thus, it might be a stage migration from clinical stage III/IV to stage V duo to hPARR positive from all WBC samples (Flory et al., 2007).

PARR analysis can be used for either diagnosis or MRD detection by the use of designed suitable primers against the IgH and TCRγ genes (Tamura et al., 2006; Yagihara et al., 2007). A few difficulties in the result interpretation were observed. Firstly, the disagreement of immunophenotyping data and monoclonality from leucocytes samples in week 0. Even though the primer sets of the IgH genes designed by Tamura et al. (2006) showed 100% sensitivity and specificity in their study, the sample size was small. Our study found that this primer pair had a sensitivity of 57.1%. However, we detected genes from peripheral blood leucocytes while they used fine needle aspiration from LN. The TCRγ primer sets from Yagihara et al. (2007) represented a T-cell clonal assay in this study. According to their result, we only chose the primer sets of Vγa-Jγb and Vγb-Jγb. Our study reported a dissimilar sensitivity and specificity to theirs, but this may reflect experimental differences since they analyzed clonal TCRγ gene rearrangements from LNs and resolved the amplicons on 7.5% polyacrylamide gels. One study compared the

visualizing PCR products methods of TCRγ gene rearrangement. They found that agarose gel analysis had lower sensitivity than polyacrylamide gel and Genescan analysis (Costa et al., 2004). Different tumor cellularity between the peripheral blood leucocytes and LN samples could have decreased the sensitivity of the primers. During the treatment, anti-neoplastic drugs killed the tumor cells, and so the enlarged LNs became smaller and contained necrotic tumor cells that were not appropriate for extraction of genomic DNA. Therefore, peripheral blood was more suitable to assess the MRD at the end of treatment by PARR (Aresu et al., 2014). Secondly, the product size of the monoclonal band of Vγb-Jγb should be in 55-90 bp size range, but in our study, it was observed at 200 bp. After DNA sequencing, the amplicons were found to have 100% nucleotide identity to canine TCRγ chain (FR828700.1), ruling out false and non-specific amplicons. Moreover, another study reported that the product size of Vγb or the V5-2 region was 140-190 bp (Keller and Moore, 2012). Lastly, some leucocytes samples during the remission phase (week 9 and 13) presented a monoclonal pattern of TCRγ genes. This could be due to the up-regulation of normal T-cell population or to chemo-resistant T-cell lymphoma clones (Calzolari et al., 2006).

Even though PARR is a sensitive method for clonal recognition, pseudoclonality is an impediment due to the immunological diversity of IgH and TCRγ genes (Takanosu et al., 2010). Therefore, heteroduplex analysis was helpful to denature and renature polyclonal populations from the real monoclonal population. Our result observed that pseudoclones after heteroduplex analysis appeared as faint bands as reported before (Takanosu et al., 2010). However, duplicate or triplicate bands could increase the reliability of the result if it was performed concurrently with the heteroduplex analysis, but it is more laborious and costly. Rather, capillary electrophoresis and gene scanning have been shown more advantageous in recent studies (Gentilini et al., 2009; Keller and Moore, 2012; Goto-Koshino et al., 2015).

Dogs with lymphoma showed lesser relapsing rate after doxorubicin administration than vincristine and cyclophosphamide (Wang et al., 2016). Our study investigated treatment efficacy between mL-COP and mL-CHOP treatment by MRD

detection with hPARR. However, with the high variations and low sample sizes of B- and T-cell lymphoma groups for subset, the lack of meaningful differences by univariate and multivariate analysis between COP and CHOP protocol is inclusive.

Fourteen dogs did not receive L-asparaginase for the first treatment in this study (8 mL-COP B-cell lymphoma, 5 mL-CHOP B-cell lymphoma, and 1 mL-COP T-cell lymphoma). In most cases (nine dogs), the owners denied this drug due to its high cost, and in a few cases (five dogs) the dog had abnormal blood results during first diagnosis, so vincristine without L-asparaginase was applied as the first therapy. Four out of the 21 (19%) dogs treated with L-asparaginase and vincristine showed neutropenia, which resulted in delayed treatment. There was no difference in the median PFS and OST between treatment with and without L-asparaginase in both the COP and CHOP protocols, in accord to the previous studies (Jeffreys et al., 2005; MacDonald et al., 2005). One study observed the efficacy of L-asparaginase combined with CHOP and its toxicity. The minimal toxicities were leukopenia and particularly neutropenia (neutrophils less than 1,500 cells/μl). L-asparaginase concurrent with vincristine might cause myelosuppression because L-asparaginase decreased hepatic clearance of vincristine and increased its toxicity (Northrup et al., 2002). Another adverse event was GI signs. Moreover, there was no advantageous response of L-CHOP treatment over CHOP (MacDonald et al., 2005). Also, comparison between COP and L-COP for the treatment of lymphoma in 76 dogs revealed that L-COP treatment did not increase the clinical remission or prolong the progression-free interval compared to the COP treatment (Jeffreys et al., 2005). From our observation, both B- and T-cell lymphomas responded well with the first dose of L-asparginase concurrent with vincristine, as LN size was decreased more than 75%.

All points of view, MRD detection from peripheral blood leucocytes by hPARR might be beneficial in clinical practice for determining refractory lymphomas during the treatment. Because of the painless sampling method, it should be used concurrent with LN measurement as an indicator when the current treatment efficacy has failed. In addition, the L-CHOP protocol tended to be a suitable

treatment in both multicentric B- and T-cell lymphomas as it could prolong PFS and OST in dogs.

2.3.6 Acknowledgements

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2.4 Transcriptome analysis of ABCB1, ABCG2 and the Bcl2/Bax ratio in refractory and relapsed canine lymphomas under treatment and a rescue protocol

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2.4.1 Abstract

The main problems that cause unresponsive 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 substage. Therefore, the administration of a rescue drug with toceranib phosphate might be beneficial in refractory and relapsed canine lymphoma.

2.4.2 Introduction

One factor that causes failure in hematological cancer treatments, including canine lymphoma, is the multidrug resistant protein (MRP) mechanism. A member of the ATP-binding cassette (ABC) transporter MRPs, such as P-glycoprotein (Pgp; *ABCB1/MDR1)*, breast cancer resistant protein (BCRP; *ABCG2)*, multidrug resistant related protein 1 (MRP1; *ABCC1)*, and lung resistant related protein (LRP; *LRP),* are expressed as an intra- or extra-cellular membrane component of normal cells. However, they can become over-expressed in the tumor cells due to induction by cytotoxic drugs, for example vincristine and doxorubicin (Uozurmi et al., 2005), 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 (Moore et al., 1995), and immunohistochemistry (Bergman et al., 1996; Lee et al., 1996). 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 (Culmsee et al., 2004). 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 (Tomiyasu et al., 2010; Zandvliet et al., 2015).

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 (Lemmon and Schlessinger, 2010). In the veterinary oncology, 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 (London et al., 2009; London et al., 2012; Pan et al., 2016). Moreover, TKI had inhibitory effect to Pgp function and revert doxorubicin resistance in canine lymphoma cells (Zandvliet et al., 2013).

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 proteins of pro-survival proteins (Bcl2), cell death promoters (BH3-only proteins) and cell death mediators (Bax and Bak). The impaired function of these apoptotic molecules can lead to oncogenesis (Llambi et al., 2011). 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 (Sohn et al., 2003; Jung et al., 2006; Dettwiler et al., 2013; Koshino et al., 2016; Meichner et al., 2016). 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 (Meichner et al., 2016). 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 the correlation with the clinical outcomes.

2.4.3 Materials and methods

2.4.3.1 Blood sample collection

Peripheral blood samples (1 ml) were collected from 14 dogs between January 2013 and July 2016 at Oncology clinic, Small Animal Teaching Hospital, Chulalongkorn University (IACUC Number 13310074). Each dog had been diagnosed as having multicentric lymphoma by histopathology. The immunophenotyping was

performed with Pax5 and CD3 as described previously (Sirivisoot et al., 2017). 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, Pfizer Inc., Ascoli, Italy) was applied in these cases, as shown in Table 12, 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 tube 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.

2.4.3.2 Follow up case

Every dog was evaluated for the 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.

2.4.3.3 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.

2.4.3.4 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 13) as previously described (Peters et al., 2007; Del Puerto et al., 2010; Zandvliet et al., 2015). 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 (Silver et al., 2006), the geNorm (Vandesompele et al., 2002), Normfinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004) and RefFinder (Xie et al., 2012). Canine *ABCB1, ABCG2, Bax* and *Bcl2* primers (Table 13) were selected as previously described (Culmsee et al., 2004; Del Puerto et al., 2010; Zandvliet et al., 2015). 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).

2.4.3.5 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.

2.4.3.6 Data analysis

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

Table 12 Rescue protocol for relapsed/refractory canine lymphomas.

Protocol[a]	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				•	•		•

[a]EOD = every other day; IV = intravenous; PO = per os; SC = subcutaneous.

Table 13 Primers for qRT-PCR.

Gene	Sequence primer (5′→3’)	Ta (°C)	Size (bp)	GenBank no.
ABCB1	F 5′-CAG TGG TTC AGG TGG CCC T-3′	81	79	NM001003215
	R 5′-CGA ACT GTA GAC AAA CGA TGA GCT-3′			
ABCG2	F 5′-GGT ATC CAT AGC AAC TCT CCT CA-3′	81.3	143	NM001048021
	R 5′-GCA AAG CCG CAT AAC CAT-3′			
BAX	F 5′-TTC CGA GTG GCA GCT GAG ATG TTT-3′	82.3	79	KT693115
	R5′- TGC TGG CAA AGT AGA AGA GGG CAA-3′			
Bcl2	F 5′- CAT GCC AAG AGG GAA ACA CCA GAA-3′	80.2	76	NM001002949
	R5′- GTG CTT TGC ATT CTT GGA TGA GGG-3′			
S26	F 5′-CGT GCT TCC CAA GCT GTA CGT GA-3′	82	75	XM531628
	R 5′- CGATTCCGGACTACCTTGCTGTG-3′			
TBP	F 5′-CTA TTT CTT GGT GTG CAT GAG G-3′	80.3	96	XM849432
	R 5′- CCT CGG CAT TCA GTC TTT TC-3′			
ß-actin	F 5′-ATG GAA TCA TGC GGT ATC CAC-3′	83	141	NM001195845
	R 5′- CTT CTG CAT CCT GTC AGC AA-3′			

2.4.4 Results

2.4.4.1 Clinical data

The signalments of 14 dogs were summarized in Table 14. They were comprised of 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 sign) 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%) was stage V. For the phenotype, 10 (71.4%) were B-cell lymphomas and four (28.6%) were T-cell lymphomas.

The median PFS did not show any significant difference among the substage (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 death after the initial treatment.

2.4.4.2 Amplification efficiency and specificity of the qRT-PCR

For the 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, and 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.

2.4.4.3 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 13A), 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 13C). After the rescue treatment, relapsed/refractory lymphomas had a 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 14)

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).

2.4.4.4 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 13B).

When compared between B- and T-cell lymphoma, relapsing/refractory B-cell lymphomas had 1.13-fold lower median *Bax* transcript levels (*P*= 0.95) and a 2.1-fold higher median *Bcl2* transcript levels (*P* = 0.3) than T-cell lymphomas (Figure 13D). After the rescue treatment, relapsed/refractory lymphomas had 1.1- fold lower 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 14).

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 numerically lower median PFS of 79 days (*P* = 0.58) (Figure 15). Regarding to the two rescue treatment groups, there were no differences in transcript levels of all genes and PFS between groups (data not shown).

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

Dog no.	Breed[a]	Age (years)	Gender[b]	WHO stage	Substage	Histopathology[c]	PFS (days)
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

[a]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.

[b]M = male; Mc = castrated male; F = female; Fs = sprayed female.

[c]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.

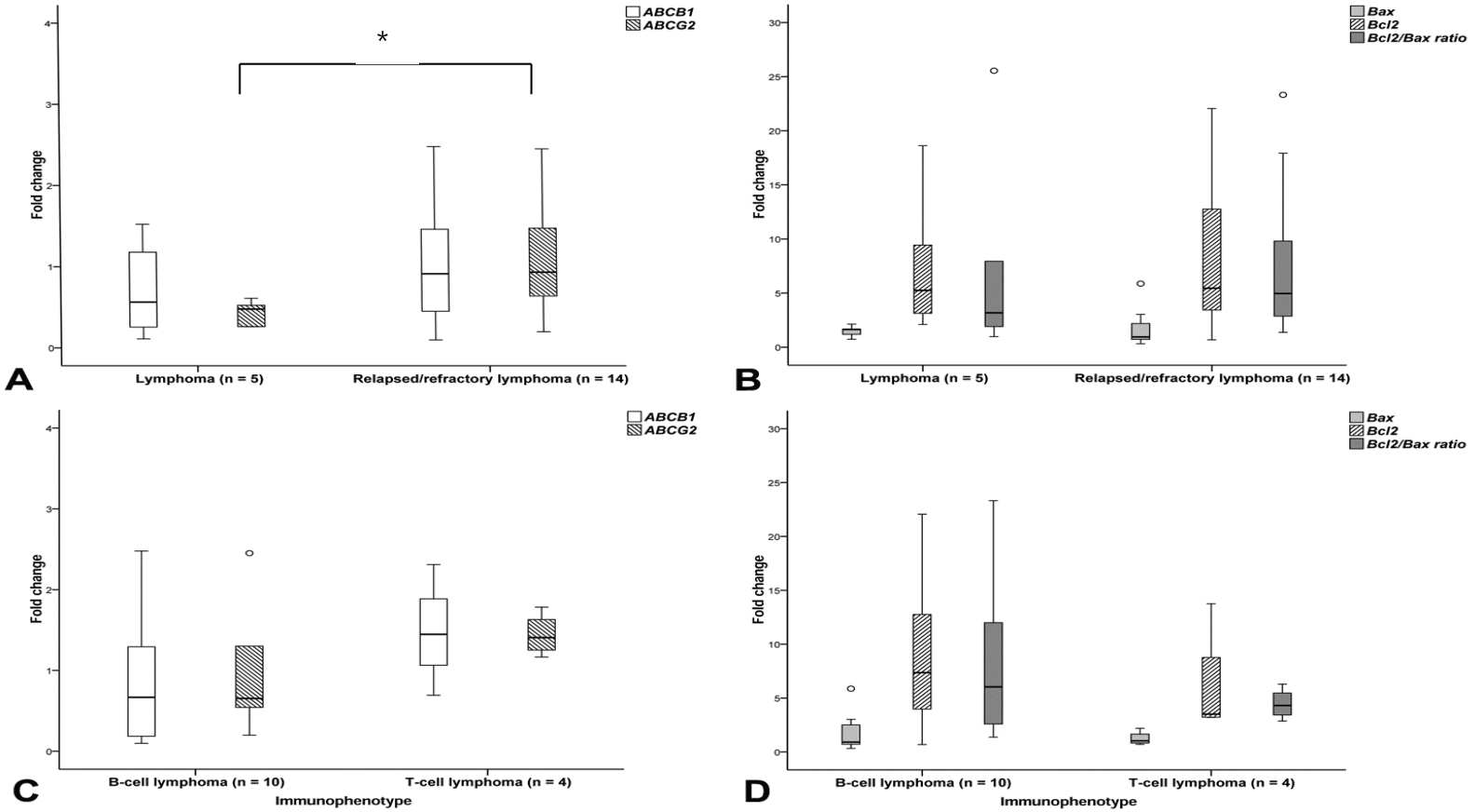

Figure 13 Box plots illustrated the relative quantities of mRNA for (A, C) *ABCB1* and *ABCG2* and (B, D) *Bcl2*, *Bax*, and the Bcl2/Bax ratio in (A, B) five canine lymphomas at the first diagnosis and 14 relapsed/refractory lymphomas and (C, D) in 10 B-cell lymphomas and four T-cell lymphomas. The white circles indicated the outlier. The star indicated the statistical significance.

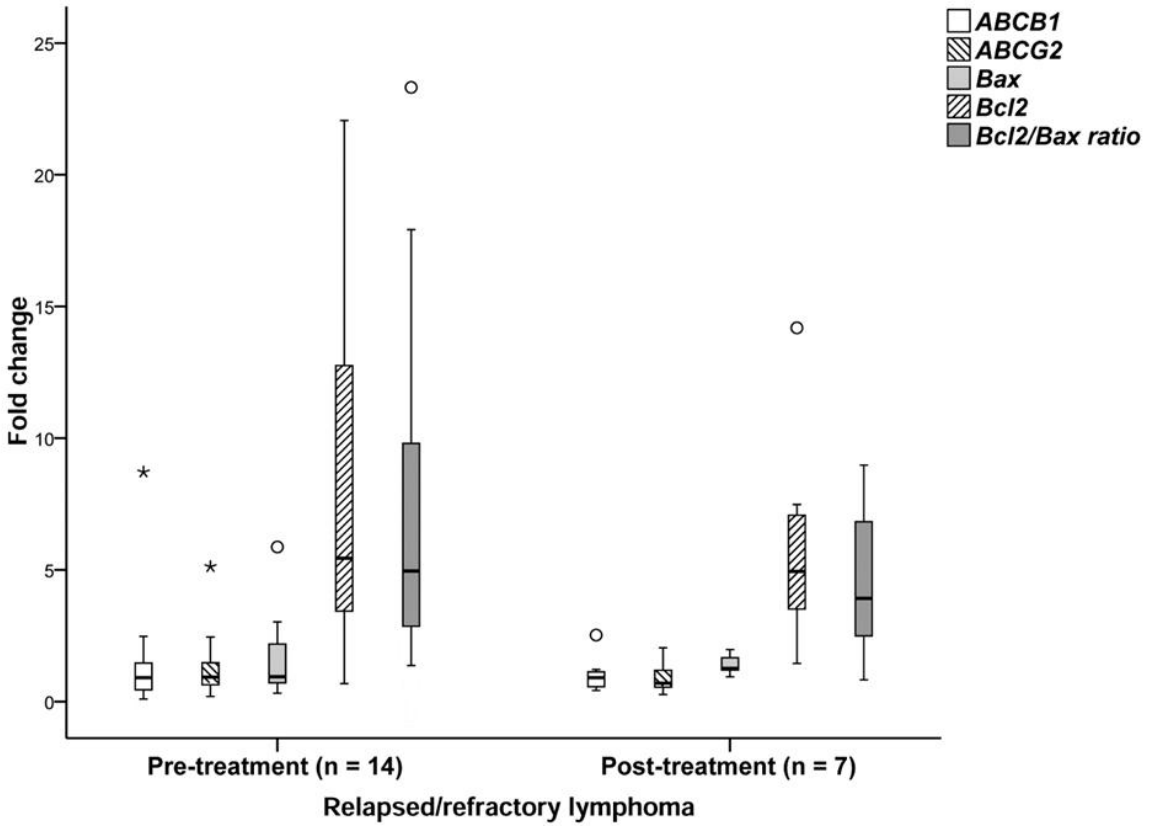

Figure 14 Box plot presented the fold-change in mRNA levels of *ABCB1*, *ABCG2*, *Bcl2*, *Bax*, and the Bcl2/Bax transcript ratio in seven dogs with lymphoma before and after rescue treatment. The white circles and stars indicated the outlier.

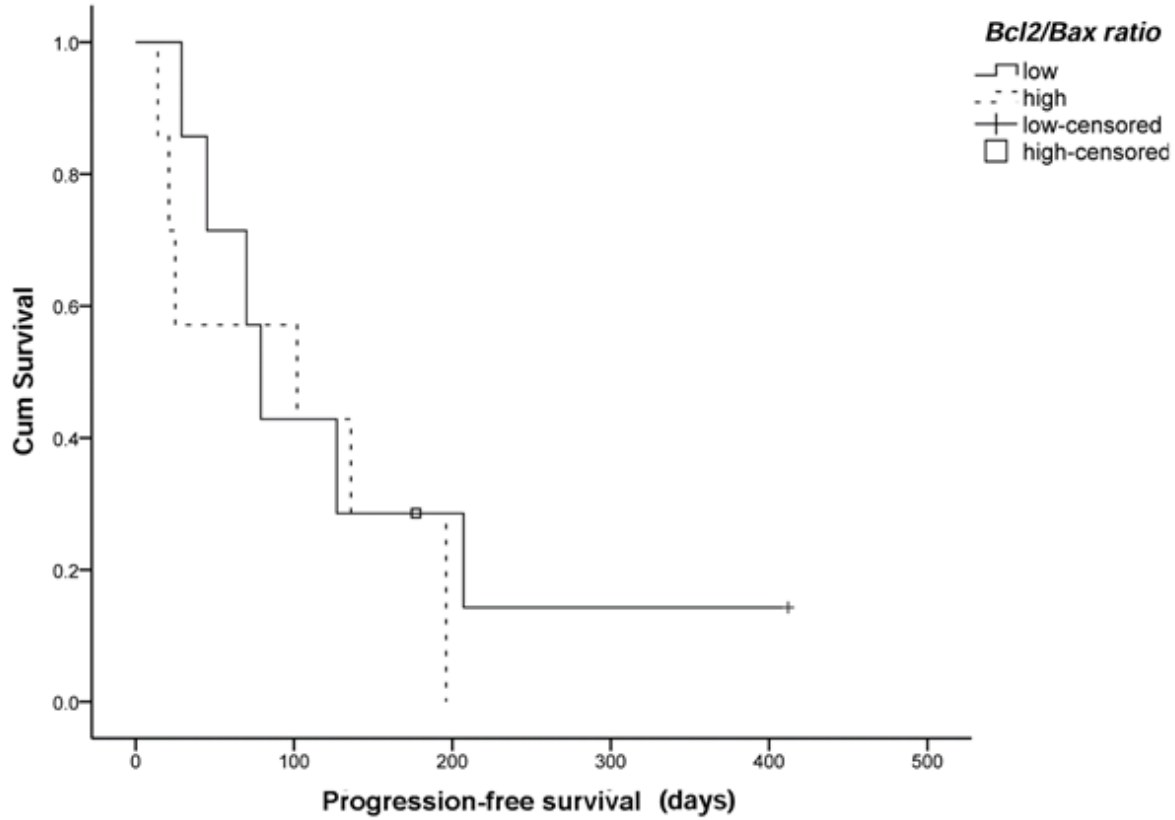

Figure 15 Kaplan-Meier survival graph illustrated the progression-free survival in 14 dogs with lymphoma grouped into those with a low and a high median Bcl2/Bax transcript ratio (Cum = cumulative).

2.4.5 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 (Saba et al., 2007). 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 (London et al., 2003). It has been used to treat many tumors in dogs, including mast cell tumors, solid tumors, and lymphomas (London et al., 2009; London et al., 2012; Pan et al., 2016). In human studies, TKIs inhibit the ATP binding site of receptor tyrosine kinases and prevent the phosphorylation leading to cell death. They can block ABC drug transporter and possibly treat relapsed/resistant disease (Houghton et al., 2004). For example, Nilotinib has inhibitory effect of ABCB1 and ABCG2 function in chronic myeloid leukemia or Ponatinib shows the increased uptake of substrates of ABCB1 and ABCG2 (Tiwari et al., 2009; Sen et al., 2012). Zandvliet et al. (2013) reported that masitinib inhibited Pgp activity and reverted doxorubicin resistance *in vitro* study. From our study, dogs that were resistant to the COP and CHOP protocols tended to have a lower transcript level of the drug resistant proteins, Pgp and BCRP (*ABCB1* and *ABCG2*), after treatment with toceranib phosphate. However, further investigation with an increased number of cases is required.

Transcript 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 (Tomiyasu et al., 2010; Chimura et al., 2011; Zandvliet et al., 2015). When comparing the transcript levels of the two drug resistant 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 level were increased, consistent with that previously reported (Tomiyasu et al., 2010; Zandvliet et al., 2015). 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* (Zandvliet et al., 2015). In contrast, four out of ten dogs in a chemotherapy-resistant group increased expression of *ABCB1* (Tomiyasu et al., 2010). Nevertheless, the sample size of our study was small and we evaluated the transcript levels of theses 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 a 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 in both humans and dogs (Gulmann et al., 2005; Meichner et al., 2016). 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 reflect protein expression levels, but flow cytometry was superior due to its relative speed and coherent result. 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 T-cell than B-cell lymphomas. Dogs with a higher Bcl2/Bax transcript ratio than the median value tend to have shorter survival times, similar to a previous study (Meichner et al., 2016). 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 poor prognosis than high grade B-cell lymphoma due to shorter progression-free and survival times (Frantz et al., 2013; Valli et al., 2013). 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 Bcl2 family proteins. When pro-apoptotic molecules, Bax and Bak, are activated, they trigger mitochondrial outer membrane permeabilization followed by releasing cytochrome c and activating caspases. The recent studies reported that Pgp could inhibit the activation of caspases in both T- lymphoblast cell lines and primary T-lymphocytes (Gollapud and Gupta, 2001; Tainton et al., 2004). 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 (Luciano et al., 2002). Thus, ABCB1 expression might affect T- cell survival during the anti-neoplastic drug treatment. ABCG2 is commonly expressed in lymphoid malignancy. In human, ABCG2 transcription levels were relevantly up-regulated after exposing with daunorubicin and mitroxantrone in T-cell acute lymphoblastic leukemia (Bram et al., 2009). However, the study on the resistant mechanism induced by *ABCG2* is limited in B- and T-cell lymphoma regardless to an efflux transporter. Doxorubicin-based protocol is a treatment of choice in canine lymphomas; ABCG2 may play an important role in progressive disease in dogs.

Many evidences 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 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 (Bai et al., 2004). 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 (Cayrol et al., 2017).

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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CHAPTER III

GENERAL DISCUSSION AND CONCLUSION

3.1 General discussion and conclusion

In human studies, Pax5 protein is a pan-pre and pan-B-cell marker. It was expressed in all cases of precursor and mature B-cell NHL. It was also showed an association with CD20, but it showed higher sensitivity in case of pre-B acute lymphoblastic leukemia and classical HL (Torlakovic et al., 2002). When compared Pax5 expression to other pan-B-cell markers (CD20, CD22, CD79a), it demonstrated in 100% of classical HL, B-acute lymphoblastic leukemia/lymphoma, and Burkitt lymphoma (Browne et al., 2003; Nasr et al., 2010). Furthermore, Pax5 could not detect in anaplastic large cell lymphoma, T-cell lymphoblastic leukemia/lymphoma, acute myeloid leukemia, melanoma, plasmacytoma, and multiple myeloma (Tiacci et al., 2004; Jensen et al., 2007; Agostinelli et al., 2010; Desouki et al., 2010). According to specificity and sensitivity of Pax5 marker to B-lineage lymphoma, Willmann et al. (2009) studied the expression of Pax5 with CD79a and CD3 in canine NHL. They found that all cases of B-cell lineage expressed Pax5; however, five cases of T-cell lymphoma were detected both CD3 and CD79a without Pax5 staining. Similar to our study, 28.57% (4/14) of T-cell NHL also displayed both markers indicated the valuable of Pax5 to B-cell NHL in dogs. Not only in human and dogs, a relevance of Pax5 as a B-cell marker was studied in feline NHL. Eighty-three percentage of all B-cell lymphomas stained with Pax5 and indicated the usefulness of Pax5 in cats (Felisberto et al., 2016).

When compared the method of immunophenotype between cytological and histological samples, the results revealed a potent association (Fisher et al., 1995). In

the previous studies, CD21 and CD79a were used as a B-cell marker, and CD3 was used as a T-cell marker on FNAB from LN (Caniatti et al., 1996; Aulbach et al., 2010; Sapierzynski, 2010). Our study used Pax5 and CD3 on cytological smear compared with formalin-fixed paraffin-embedded (FFPE) tissue. ICC showed resembles results to IHC with both markers. However, to preserve cellular morphology, increases cellularity and decrease background on slides, liquid-based cytology with fixed sediment method was developed to decrease the disadvantages of smear method (Fernandes et al., 2016).

Molecular clonality assay is a useful technique for differential diagnosis of B or T lineages in canine lymphomas. Specific primer sets were firstly created based on lymphocytes antigen receptor genes. Complementarity determining region 3 (CRD3) contains variable (V), diversity (D), and joining (J) genes after rearrangement. Due to its characteristic for each lymphocyte clone; primer design is based on coverage to this region. The main target of T cells is TCRγ locus because it is remained in $\alpha\beta$ and $\gamma\delta$ T lymphocytes, whereas the primary target of B cells is IgH chain locus because it rearranged without light chain usage. A clonal rearrangement assay or polymerase chain reaction for antigen receptor rearrangements (PARR) is developed to differentiate hyperplastic disease from lymphoma in dogs (Vernau and Moore, 1999; Burnett et al., 2003). According to high sensitivity of PARR, many previous studies reported the advantageousness of this method. It could be determined lineage differentiation in ambiguous result of immunophenotype, clinical staging by assessment circulating neoplastic lymphocytes in peripheral blood, and minimal residual disease detection before or after chemotherapy and during remission (Keller et al., 2004; Wilkerson et al., 2005; Calzolari et al., 2006; Lana et al., 2006; Thilakaratne et al., 2010; Aresu et al., 2014). Though, the difficulty of PARR is false positive and false negative, the improvement of primer gene coverage was designed to increase sensitivity for IgH and TCRγ genes (Tamura et al., 2006; Yagihara et al., 2007; Gentilini et al., 2009; Chaubert et al., 2010). Our study used the primer sets from Burnett et al, Tamura et al and Yagihara et al. Sensitivity and specificity of primer sets from Burnett et al were 48% and 100% for IgH, and 100% and 72% for

TCRγ gene, respectively. Conversely, Sensitivity and specificity of the IgH primer sets from Tamura et al were 57.1% and 85.7%, and the TCRγ primer sets from Yagihara et al had 85.7% sensitivity and 57.1% specificity. Nowadays genome sequence of IgH and TCRγ locus in *Canis familiaris* is published (Massari et al., 2009; Bao et al., 2010). Therefore, the modification of this primer set through sequencing data with V/J coverage is necessary for further investigation.

Resistant to anti-neoplastic drugs is a common reason of a treatment failure in canine lymphomas due to MRP. Tumor cells learn to avoid cell death by chemotherapy via overexpression of MRP and pump the drugs out of the cells. MRPs that associate drug resistance mechanism in canine multicentric lymphoma after CHOP treatment are Pgp (*ABCB1*) and BCRP (*ABCG2*) (Tomiyasu et al., 2010; Zandvliet et al., 2015). Tyrosine kinase inhibitor (TKI) is one of target therapy for cancer treatment in human and animals. Masitinib showed mild antiproliferative effect on canine lymphoid cell lines, inhibited Pgp function, and reverted doxorubicin resistance (Zandvliet et al., 2013). Furthermore, both canine B- and T-cell lymphoma expressed c-kit and platelet-derived growth factor receptors which are crucial targets of TKI (Giantin et al., 2013; Arico et al., 2014). There is a report on acarabrutinib that used to treat canine B-cell lymphoma with overall response rate of 25% and median PFS of 22.5 days (Harrington et al., 2016). Another study used masitinib to treat canine epitheliotropic lymphoma with overall response rate of 70% and median time to progression of 85 days (Holtermann et al., 2016). Our study used toceranib phosphate concurrent with CCNU or L-asparaginase and vincristine in clinical cases as a rescue treatment for relapsed/refractory canine multicentric lymphomas. Overall response rate was 50% and median PFS was 79 days. In addition, this protocol decreased both *ABC*B1 and *ABCG2* transcription levels; therefore it might be an alternative rescue protocol for canine multicentric lymphoma.

In summary, ICC could use for diagnosis of immunophenotype in canine multicentric lymphoma regardless to FFPE samples using Pax5 and CD3 as a B- or T-lineage identification. ICC is faster and cheaper than IHC with the concordant results. Additionally, clonality test with hPARR is useful as an adjunctive diagnosis of

lymphoma lineage and MRD screening for determining treatment efficacy in canine multicentric lymphoma during chemotherapy. Peripheral blood is a suitable sample for clonality assay when compared to FNAB or FFPE samples during the complete remission; however, clinical stage migration should be concerned. Furthermore, a rescue protocol, CCNU or L-asparaginase and vincristine with toceranib phosphate, should be considered as a treatment option in relapsed/refractory canine multicentric lymphoma because they tended to decrease Pgp, BCRP and Bcl2 protein expression.

3.2 Limitations of the research

The major limitations of this study are the variation of canine multicentric lymphoma cases and a small number of sample groups. Dogs in our study had different WHO clinical stage, substage, histological grade, and immunophenotype, thus all factors failed to correlate with clinical outcomes. Moreover, inadequate sample size between B- and T-cell lymphoma treated with COP and CHOP protocol showed a weak relation to MRD, and failed to predict prognosis as well as suitable treatment for each group. In case of relapsed/refractory canine lymphoma, the difficulty to collect blood sample after the rescue treatment was a main issue because most patients did not well tolerated to a rescue drug or too weak and death a few days after treatment. According to a small sample size MRP, apoptotic and anti-apoptotic protein expressions did not show significant difference when compared to immunophenotype, after treatment, and PFS.

3.3 Suggestions for further investigation

A study on molecular clonality assay using hPARR should use more specific primers for IgH and TCR genes. Primer sets of B cell that used in this study had low sensitivity because IgH genes frequently have a somatic hypermutation during the immune response. In addition, primer coverage to V and J regions was unavailable during the time of this study. However, today a public database of whole genome sequences in dogs are more accessible, thus they provide more information on

specific primer design. Primer sets of T cell in this study showed quite high sensitivity and specificity, nevertheless the improvement of primer coverage and separating PCR product method could decrease false positive and false negative results and increase sensitivity and specificity of hPARR for diagnosis and MRD evaluation in canine lymphoma.

A study on treatment efficacy of COP or CHOP or rescue treatment efficacy with MRP and Bcl2-family proteins determination should be focused on one population group, such as B- or T-cell lymphoma, DLBCL or PTCL, low or high grade, to decrease a variation in the study, and increase a strong result and interpretation.

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APPENDIX

Antigen retrieval solution preparation

0.1 M Phosphate buffer saline (PBS) 10X

Sodium chloride	40	g
Potassium chloride	1	g
Disodium hydrogen phosphate	5.75	g
Potassium dihydrogen phosphate	1	g
Distilled water	500	ml

Tris/EDTA buffer pH 9.0 (10 mM Tris Base, 1 mM EDTA)

Tris Base	1.21	g
EDTA	0.37	g
Distilled water	1000	ml

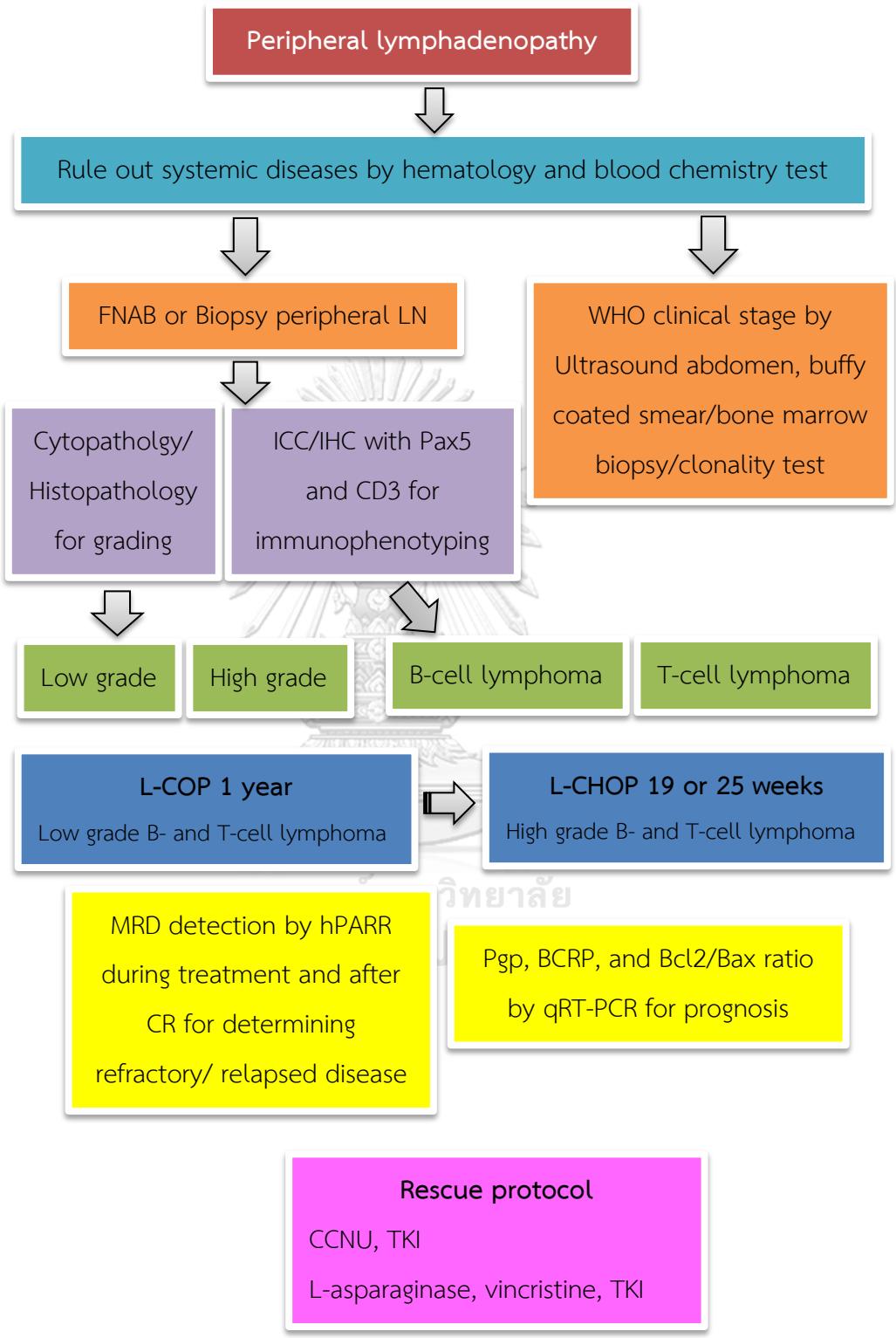
10 mM citrate buffer pH 6.0

Citric acid	2.1	g
Distilled water	900	ml

PBS with Triton X-100

PBS pH 7.2	100	ml
Triton X-100	250	µl

Guideline when approaching canine multicentric lymphomas



Canine multicentric lymphoma cases conclusion from 2013-2015

No	Breed	Gender	Age (years)	Clinical stage	Cytopathology (updated Kiel)	Histopathology (WHO)	Immunophenotype	Clonality result	Treatment	MRD result	PFS (days)	OST (days)
1	GR	M	10	III	Lymphocytic	B-SLL	B cell	TGRγ	No treatment	n/a	n/a	n/a
2	ST	Mc	15	V	Lymphocytic	B-SLL	B cell	IgH	CHOP	n/a	-	76
3	GR	M	9	V	Prolymphocytic	LLI	B cell	Dual	L-CHOP	n/a	65	67
4	Mixed	F	13	IV	Centroblastic/centrocytic	FC	B cell	TGRγ	No treatment	n/a	-	7
5	Chihuahua	M	8	V	Centroblastic/centrocytic	FC	B cell	IgH	VP	n/a	n/a	n/a
6	Mixed	Fs	14	IV	Centroblastic/centrocytic	FC	B cell	TGRγ	m-COP	n/a	-	64[c]
7	LR	F	10	III	Immunoblastic	DLBCL	B cell	IgH	L-COP	-	236	371
8	LR	Fs	11	IV	Centroblastic monomorphic	DLBCL	B cell	IgH	COP	n/a	-	36
9	ST	Mc	14	V	Centroblastic monomorphic	DLBCL	B cell	IgH	Vincristine	n/a	n/a	n/a
10	ST	F	8	III	Centroblastic polymorphic	DLBCL	B cell	Dual	No treatment	n/a	n/a	n/a
11	Beagle	Fs	10	III	Centroblastic polymorphic	DLBCL	B cell	IgH	LVP	n/a	n/a	n/a
12	ST	M	9	IV	Centroblastic polymorphic	DLBCL	B cell	IgH	COP	n/a	n/a	n/a
13	GR	Mc	8	IV	Centroblastic polymorphic	DLBCL	B cell	TGRγ	COP	n/a	-	25[c]

Canine multicentric lymphoma cases conclusion from 2013-2015 (Cont')

No	Breed	Gender	Age (years)	Clinical stage	Cytopathology (updated Kiel)	Histopathology (WHO)	Immunophenotype	Clonality result	Treatment	MRD result	PFS (days)	OST (days)
14	MP	F	10	III	Centroblastic polymorphic	DLBCL	B cell	IgH	COP	-	327	548[b]
15	GR	M	9	V	Centroblastic polymorphic	DLBCL	B cell	IgH	VP	n/a	-	24
16	GR	Fs	14	IV	Immunoblastic	DLBCL	B cell	TGRγ	VP	n/a	-	7
17	ST	F	12	IV	Immunoblastic	DLBCL	B cell	IgH	L-COP	Week 13	103	239
18	ST	M	7	IV	Immunoblastic	DLBCL	B cell	IgH	L-COP	Week 9, 13	34	116
19	Mixed	M	7	III	Immunoblastic	DLBCL	B cell	TGRγ	CHOP	-	-	826[a]
20	CP	Fs	15	III	Immunoblastic	DLBCL	B cell	IgH	COP	-	-	84
21	Mixed	Mc	10	III	Immunoblastic	DLBCL	B cell	Indeterminate	Vincristine	n/a	-	10
22	Mixed	M	10	IV	Lymphoblastic	B-LBL	B cell	IgH	L-CHOP	n/a	-	37
23	Poodle	Fs	7	IV	Lymphoblastic	B-LBL	B cell	IgH	L-COP	n/a	-	45
24	GR	Fs	12	IV	Lymphoblastic	B-LBL	B cell	Dual	COP	n/a	-	28
25	ST	Fs	6	IV	Anaplastic	APL	B cell	TGRγ	L-COP	n/a	-	99[b]
26	BT	M	3	IV	Pleomorphic mixed	PTCL	T cell	TGRγ	L-CHOP	-	227	272
27	Mixed	Mc	6	IV	Pleomorphic mixed	PTCL	T cell	TGRγ	Vincristine	n/a	-	9
28	GR	Fs	15	IV	Pleomorphic large cell	PTCL	T cell	TGRγ	VP	n/a	-	7

Canine multicentric lymphoma cases conclusion from 2013-2015 (Cont')

No	Breed	Gender	Age (years)	Clinical stage	Cytopathology (updated Kiel)	Histopathology (WHO)	Immunophenotype	Clonality result	Treatment	MRD result	PFS (days)	OST (days)
29	Mixed	M	6	III	Centroblastic monomorphic	DLBCL	B cell	IgH	L-COP	-	-	144
30	Pomeranian	M	5	III	Centroblastic polymorphic	DLBCL	B cell	IgH	L-COP	-	24	123
31	ST	Fs	11	IV	Centroblastic polymorphic	DLBCL	B cell	TGRγ	COP	-	89	91
32	ST	Mc	8	III	Lymphocytic	B-SLL	B cell	TGRγ	COP	Week 4	78	126
33	GR	M	n/a	III	Immunoblastic	DLBCL	B cell	IgH	COP	-	90	265
34	LR	M	9	IV	Centroblastic/centrocytic	T-cell rich LBCL	B cell	Dual	L-COP	-	-	604[a]
35	MP	M	5	IV	Lymphocytic	B-SLL	B cell	TGRγ	COP	Week 9, 13	70	227
36	Pug	Fs	4	III	Centroblastic monomorphic	DLBCL	B cell	IgH	COP	-	-	218
37	GP	M	7	III	Centroblastic monomorphic	DLBCL	B cell	IgH	COP	Week 9, 13	69	93
38	GR	M	12	V	Centroblastic polymorphic	DLBCL	B cell	TGRγ	L-COP	Week 4, 9,13	75	89
39	ST	M	12	IV	Centroblastic polymorphic	DLBCL	B cell	IgH	L-CHOP	Week 13	155	247

Canine multicentric lymphoma cases conclusion from 2013-2015 (Cont')

No	Breed	Gender	Age (years)	Clinical stage	Cytopathology (updated Kiel)	Histopathology (WHO)	Immunophenotype	Clonality result	Treatment	MRD result	PFS (days)	OST (days)
40	Westy	M	13	IV	Immunoblastic	DLBCL	B cell	TGRγ	CHOP	-	201	247
41	ST	F	3	III	Centroblastic polymorphic	DLBCL	B cell	IgH	L-CHOP	Week 4, 9,13	-	338[b]
42	FB	F	5	V	Centroblastic polymorphic	DLBCL	B cell	IgH	L-CHOP	-	224	286
43	LR	F	5	V	Immunoblastic	DLBCL	B cell	IgH	L-CHOP	Week 4	143	245
44	Mixed	M	10	III	Centroblastic polymorphic	DLBCL	B cell	IgH	CHOP	-	215	238
45	Westy	Fs	5	III	Centroblastic polymorphic	DLBCL	B cell	Dual	L-CHOP	Week 4, 9	389	585
46	GR	M	7	III	Centroblastic polymorphic	DLBCL	B cell	IgH	CHOP	-	169	432
47	GR	M	11	IV	Immunoblastic	DLBCL	B cell	IgH	L-CHOP	-	-	161
48	ST	F	12	IV	Immunoblastic	DLBCL	B cell	TGRγ	L-CHOP	-	130	159
49	BT	M	7	III	Immunoblastic	DLBCL	B cell	TGRγ	L-CHOP	-	420	597[a]
50	Pug	Mc	6	III	Centroblastic polymorphic	DLBCL	B cell	Dual	CHOP	-	341	468

Canine multicentric lymphoma cases conclusion from 2013-2015 (Cont')

No	Breed	Gender	Age (years)	Clinical stage	Cytopathology (updated Kiel)	Histopathology (WHO)	Immunophenotype	Clonality result	Treatment	MRD result	PFS (days)	OST (days)
51	Poodle	Fs	10	III	Prolymphocytic	T-SLL	T cell	TGRγ	COP	Week 4, 9,13	450	862[a]
52	Mixed	Fs	9	V	Prolymphocytic	T-SLL	T cell	TGRγ	L-COP	Week 4, 9,13	-	118
53	Poodle	Mc	10	IV	Aggressive large granular	T-ALCL	T cell	Dual	L-COP	Week 9	35	171
54	Mixed	M	13	III	Mycosis fungoides	CTCL	T cell	TGRγ	L-CHOP	Week 4, 9	-	569[a]
55	ST	M	11	III	Prolymphocytic	T-SLL	T cell	TGRγ	L-CHOP	Week 4, 9,13	-	527[a]
56	GR	Fs	11	IV	Pleomorphic mixed	PTCL	T cell	TGRγ	L-CHOP	-	103	173

Mc = castrated male; M=male; F= female; Fs = sprayed female; BT= Bull terrier; CS= Cocker spaniel; FB = French bulldog; GP = German Shepherd; GR= Golden retriever; LR= Labrador retriever; MP= Miniature pinscher; ST= Shih Tzu; APL = anaplastic plasmacytoid lymphoma; B- or T-SLL = B- or T-small lymphocytic lymphoma; CTCL = cutaneous T-cell lymphoma; LLI= B-cell lymphocytic lymphoma of intermediate type; FC= follicular lymphoma; NMZ = nodal marginal zone lymphoma; DLBCL = diffuse large B-cell lymphoma; B-LBL= B-cell lymphoblastic lymphoma; PTCL= peripheral T-cell lymphoma; T-cell rich LBCL = T-cell rich large B-cell lymphoma; T-ALCL = anaplastic large T-cell lymphoma; LVP = L-asparaginase, vincristine and prednisolone ; VP = vincristine and prednisolone; PFS = progression-free survival; OST = overall survival time.

[a]still alive

List of publications and conferences proceedings

1. **Sirivisoot S**, Teewasutrakul P, Techangamsuwan S, Tangkawattana S and Rungsipipat A 2018. Monitoring minimal residual disease in canine lymphomas treated with either the mL-COP or the mL-CHOP chemotherapeutic protocols. Acta Vet Hung. 66 (1). Accepted November 7, 2017.
2. **Sirivisoot S**, Teewasutrakul P, Tangkawattana S, Rungsipipat A and Techangamsuwan S 2017. Transcriptome analysis of ABCB1, ABCG2 and the Bcl2/Bax ratio in refractory and relapsed canine lymphomas under treatment and a rescue protocol. Acta Vet (Beogr). Accepted October 31, 2017.
3. Thaiwong T, **Sirivisoot S**, Takada M, Yuzbasiyan-Gurkan V and Kiupel M 2017. Gain-of-function mutation in *PTPN11* in histiocytic sarcomas of Bernese Mountain Dogs. Vet Comp Oncol. 1–9: https://doi.org/10.1111/vco.12357.
4. **Sirivisoot S**, Techangamsuwan S, Tangkawattana S and Rungsipipat A 2017. Pax5 as a potential candidate marker for canine B-cell lymphoma. Vet Med (Praha). 62: 74-80.
5. **Sirivisoot S**, Techangamsuwan S, Tangkawattana S and Rungsipipat A 2016. Application of immunophenotyping and heteroduplex polymerase chain reaction (hPARR) for diagnosis of canine lymphomas. Asian Pac J Cancer Prev. 17: 2909-2916.
6. Piewbang C, **Sirivisoot S**, Lacharoje S, Rungsipipat A and Techangamsuwan S 2016. Immunophenotyping of cutaneous extramedullary plasmacytoma in two dogs. Thai J Vet Med. 46: 723-727.
7. Siripoonsub J, **Sirivisoot S**, Lacharoje S, Rungsipipat A and Techangamsuwan S 2016. Equine multicentric B-cell lymphoma associated with disseminated arteriosclerosis in a Gelding. Res J Vet Pract. 4:11-16.
8. Imerbsin R, Chumpolkulwong K, Hanrujirakomjohn A, Saithasao M, **Sirivisoot S**, Tayamun S, Inamnuay L and Lombardini E 2014. Seminal vesicle carcinoma-in-situ in an adult rhesus macaque: a case report and review of the literature. Thai J Vet Med. 44: 385-390.
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