การจำแนกชนิดของโปรตีนในซีรัมของสุนัขที่ป่วยเป็นโรคมะเร็งต่อมน้ำเหลืองด้วยเทคนิคทางโปรติโอมิกส์

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IDENTIFICATION OF SERUM PROTEOMIC PROFILE IN CANINE LYMPHOMAS

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ปิยนุช ฟองเหม : การจำแนกชนิดของโปรตีนในซีรัมของสุนัขที่ป่วยเป็นโรคมะเร็งต่อมน้ำเหลืองด้วยเทคนิคทางโปรติโอมิกส์ (IDENTIFICATION OF SERUM PROTEOMIC PROFILE IN CANINE LYMPHOMAS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร.อนุเทพ รังสีพิพัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. น.สพ. ดร. เกษม รัตนภิญโญพิทักษณ์, 48 หน้า.

มะเร็งต่อมน้ำเหลืองเป็นโรคที่พบมากที่สุดเป็นอันดับสามในสุนัข ซึ่งโรคมะเร็งต่อมน้ำเหลืองในสุนัขมีลักษณะคล้ายคลึงกับโรคมะเร็งต่อมน้ำเหลืองชนิดนอนฮอดจ์กินที่พบในคน (Human non-Hodgkin's lymphoma) การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษารูปแบบโปรตีโอมิกส์ในซีรัมของสุนัขที่เป็นมะเร็งต่อมน้ำเหลือง ซึ่งเป็นการศึกษาชนิดของโปรตีนต่าง ๆ จำนวนมากในคราวเดียวแบบองค์รวม และศึกษาการแสดงออกของโปรตีนที่เกิดการเปลี่ยนรหัสพันธุกรรมภายหลังการเกิดโรค ซึ่งบทวิจัยนี้เน้นการศึกษารูปแบบโปรตีโอมิกส์ในซีรัมของสุนัขที่เป็นมะเร็งต่อมน้ำเหลือง โดยใช้วิธี Tandem Mass Tag (TMT) เปรียบเทียบกับสุนัขที่ไม่เป็นโรค (กลุ่มควบคุม) โดยเก็บตัวอย่างเลือดจากสุนัขที่เป็นมะเร็งต่อมน้ำเหลืองจำนวน 20 ตัวและสุนัขกลุ่มควบคุม จำนวน 4 ตัว โดยแบ่งเกรดโรคมะเร็งต่อมน้ำเหลืองตามเกณฑ์ของ WHO clinical stage การยืนยันโรคใช้วิธีการตรวจทางเซลล์วิทยา (cytology) หรือการตรวจเนื้อเยื่อทางจุลพยาธิวิทยา (histopathology) หรือปฏิกิริยาลูกโซ่พอลิเมอเรส (hPAAR) และทำการแยกแยะชนิดมะเร็งต่อมน้ำเหลือง B-cell หรือ T-cell และใช้ขั้นตอนโปรตีโอมิกส์โดยเทคนิค Liquid chromatography mass spectrometry (LC-MS/MS) ผลการทดลองพบโปรตีนที่ตรงกับซีรัมสุนัข (*C. lupus familiaris*) ในฐานข้อมูลจำนวน 3 ชนิด พบโปรตีน 6 ชนิดที่มีความแตกต่างกันอย่างมีนัยสำคัญระหว่างกลุ่มควบคุมและมะเร็งต่อมน้ำเหลือง โดยพบโปรตีนที่มีการลดลงในกลุ่มที่เป็นมะเร็งต่อมน้ำเหลืองได้แก่ clusterin หรือ apolipoprotein J Ig heavy chain V region GOM beta-2 microglobulin apolipoprotein C-I และ Haptoglobin เทคนิคนี้สามารถตรวจหาโปรตีนที่มีปริมาณน้อยและมีความแม่นยำทางด้านสถิติสูง การตรวจพบโปรตีนดังกล่าวสามารถใช้ในพยากรณ์โรคในอนาคตซึ่งเป็นประโยชน์ในทางคลินิกปฏิบัติ

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต	.....
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PIYANOOT FONGHEM: IDENTIFICATION OF SERUM PROTEOMIC PROFILE IN CANINE LYMPHOMAS. ADVISOR: ASSOC. PROF. DR.ANUDEP RUNGSIPIPAT, D.V.M.,Ph.D., CO-ADVISOR: INSTRUCTOR DR. KASEM RATTANAPINYOPITUK, D.V.M.,Ph.D., 48 pp.

Lymphoma is the third most commonly diagnosed, malignant hematopoietic tumor in dogs, and it is similar to human non-Hodgkin's lymphoma. Proteomics refer to the large-scale study of proteins, including their structures as well as their complex protein mixtures. It also involves identifying the changes in the protein isoforms and posttranslational modifications expressed by genetic material under defined specific conditions. This study was aimed at identifying the serum proteomic profiles of canine lymphoma compared with healthy dogs by using Tandem Mass Tag (TMT). Blood samples from twenty lymphoma and four healthy dogs were collected. All cases were categorized according to the WHO clinical stage, confirmed either by cytological and heteroduplex polymerase chain reaction (hPAAR) or histopathological and immunophenotyping to determine B-cell or T-cell lymphoma, then serum proteomics were performed by liquid chromatography mass spectrometry (LC-MS/MS). Thirty-four individual proteins were identified from *C. lupus familiaris*. Six proteins were significantly different between control and lymphoma groups. Albumin was significantly decreasing, while five proteins were significantly increasing in lymphoma group such as clusterin or apolipoprotein J, Ig heavy chain V region GOM, beta-2-microglobulin, apolipoprotein C-I, and haptoglobin. This technique is able to detect low abundance proteins with a high degree of statistical confidence. These protein panels may be the possible candidate biomarkers for canine lymphoma in clinical oncology for the future.

Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
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LIST OF ABBREVIATIONS

°C	=	degree Celsius
µl	=	micrometer
2D	=	two-dimensional gel electrophoresis
2D-DIGE	=	two-dimensional fluorescence difference gel electrophoresis
2D-PAGE	=	two-dimensional polyacrylamide gel electrophoresis
Alb	=	albumin
ALP	=	alkaline phosphatase
ALT	=	alanine transaminase
Ba	=	basophil
Band	=	band neutrophil
bp	=	based pair
BUN	=	blood urea nitrogen
Cal	=	calcium
CD	=	cluster of differentiation
*c*NHL	=	canine non-Hodgkin lymphoma
Cr	=	creatinine
ELISA	=	enzyme-linked immunosorbent assay
Eo	=	eosinophil
FNAB	=	fine-needle aspiration biopsy
G	=	gram
GIST	=	global internal standard technology
H	=	hour
H&E	=	hematoxylin and eosin
Hb	=	hemoglobin
Hct	=	hematocrit
*h*NHL	=	human non-Hodgkin lymphoma
*h*PARR	=	heteroduplex polymerase chain reaction
ICAT	=	isotope coded affinity tag
IgH	=	immunoglobulin heavy chain
IHC	=	immunohistochemistry
iTRAQ	=	isobaric tag for relative and absolute quantitation
kDa	=	kilodalton
Kg	=	kilogram
LC-MS	=	liquid chromatography-mass spectrometry

Lym	=	lymphocyte
m/z	=	mass-to-charge
MALDI	=	matrix-assisted laser desorption ionization
MCH	=	mean corpuscular hemoglobin
MCHC	=	mean corpuscular hemoglobin concentration
MCV	=	mean corpuscular volume
mg	=	milligram
min	=	minute
mM	=	milimolar
Mo	=	monocyte
MS	=	mass spectromemtry
msec	=	millisecond
MW	=	molecular weight
Neu	=	neutrophil
n	=	Nano gram
nm	=	nanometer
PAX	=	paired-box
PCR	=	polymerase chain reaction
pI	=	Isoelectric point
Plt	=	platelet
PP	=	plasma protein
PPIA	=	Peptidyl-prolyl cis-trans isomerase A
PRDX1	=	Peroxiredoxin-1
RBC	=	red blood cell
RDW	=	red cell distribution width
SDC	=	sodium dodecyl sulfate
sec	=	second
SELDI	=	surface-enhanced laser desorption ionization
TCEP	=	Tris-(2-Carboxyethyl) phosphine
TCR	=	T-cell receptor
TEAB	=	Tris EDTA buffer
TMT	=	tandem mass tag
TOF	=	time-of- flight
TP	=	total protein
WBC	=	white blood cell
WHO	=	World Health Organization

CHAPTER I

INTRODUCTION

Proteomics has been developing rapidly in the last few decades. It refers to the large-scale study of proteins, including their structures, functions, and the differentiation of complex protein mixtures whereas proteome can be defined as the set of proteins expressed by the genetic material of an organism under defined environment conditions (Ceciliani et al., 2016; Morris, 2016a). Several protein studies have been performed to examine dynamic protein pathways involved in the pathogenesis of disease, such as, hereditary, infectious, and neoplastic disease. Canine lymphoma is a common malignant hematopoietic tumor in dog (Atherton et al., 2013a) and is similar to human non-Hodgkin's lymphoma (Ratcliffe et al., 2009). The most frequent form is multicentric form involving peripheral lymph nodes (McCaw et al., 2007) There are some studies focused on proteomic profiles of canine lymphoma (Gaines et al., 2007; McCaw et al., 2007; Wilson et al., 2008; Ratcliffe et al., 2009; Atherton et al., 2013a). In human, many neoplastic diseases which include breast, ovary, prostate, renal, lung cancers, non-Hodgkin's lymphoma, etc. have been reported in relation with their proteomic profiles (Chen et al., 2002; Rapkiewicz et al., 2004; Perroud et al., 2009; Poschmann et al., 2009; AL-RUWAILI et al., 2010). In the other hand, the application of proteomics in veterinary medicine is extremely limited.

The identification and validation of proteins as biomarkers associated with lymphoma will significantly be of advantage to dogs. Proteomic profiling may not only increase the understanding of canine lymphoma but also enhance the technique for early diagnosis and monitoring of the progression of the disease. However, the information about the proteomic profiles of canine lymphoma remains unclear and requires confirmation.

Therefore, the aim of this study was to identify proteomic profiles of canine lymphoma compared with healthy dogs by using isobaric mass tag-based protein quantification experiments (Tandem Mass Tag).

Objectives of Study

To identify the proteomic profiles of canine lymphoma compared between healthy dog, B-cell lymphoma and T-cell lymphoma dogs

Hypothesis

Proteomic profiles of canine lymphoma are potential candidate protein biomarker panel for disease diagnosis.

Advantages of study

The novel proteomic information of canine B-cell and T-cell lymphoma is available for clinically use as diagnostic and prognostic biomarkers.

CHAPTER II

LITERATURE REVIEW

Canine lymphoma

Dogs were used as a model of occurrence of tumor in human (Withrow et al., 1991; Vail and MacEwen, 2000; Rowell et al., 2011). The clinical presentation of canine non-Hodgkin lymphoma (cNHL) is varies, and depend on the type, stage, grading, and site of involvement as same as human non-Hodgkin lymphoma (hNHL). Conversely, Hodgkin-like lymphomas are rare (Marconato et al., 2013). For lymphoma, dogs were used as a model for human such as epidemiology, pathobiology, diagnosis, and therapy (Rowell et al., 2011; Marconato et al., 2013; Davis and Ostrander, 2014; Heather L. Gardner, 2016). Clinically, canine lymphoma often presents in advance stage (more than stage III) at diagnosis and commonly with severe clinical signs that cause of high mortality. Therefore, the goals of study in human and veterinary oncology are early diagnosis, prognosis, knowing pathogenesis, therapeutic response monitoring, and prognostic evaluation of NHL (Mobasheri, 2013).

The gold standard protocol for canine lymphoma diagnosis is biopsy. Fine-needle aspiration biopsy (FNAB) and surgical biopsy are an excellent, specific diagnosis procedure used to assess pathological processes in lymph node, including primary tumor localized and metastasis of lymph nodes. Histopathological interpretation of biopsy samples, using immunohistochemical (IHC) stains to confirm the precise cell of origin or specify subtype, to help with treatment decisions and prognosis (Morris, 2016b). However, biopsy technique is time-consuming, and had the limitation of tissue sampling. In some cases, biopsy cannot be perform due to the poor overall condition of the patients and the refusal from owner for surgical procedure (Sapierzyński et al., 2010) For the above reasons, tumor marker was invented and developed.

In the last few decades, proteomic studies have raised interest in prognostic and diagnostic biomarker proteins as regards to canine lymphoma (Gaines et al., 2007; McCaw et al., 2007; Wilson et al., 2008; Ratcliffe et al., 2009; Atherton et al., 2013a; Klopfleisch, 2015). Proteomics is the study of large-scale of proteins characterization, including their structures, functions, and differentiation of complex

protein mixtures with high thoughput (Ratcliffe et al., 2009). For Labeling with isobaric tags that by use metabolic labeling, ICAT, or enzymatic labeling and most other chemical labeling approaches for relative quantification are based on the mass difference between differentially labeled peptides. Nowadays, multiplexed sets of reagents for quantitative protein analysis have been developed. The isobaric tag for relative and absolute quantitation (iTRAQ) (Ross et al., 2004) and tandem mass tags (TMTs) (Thompson et al., 2003) technologies are commercially available. The multiplexing capabilities of isobaric chemical tag-based quantification, such as TMTs or iTRAQ support the analysis of up to 10 samples per mass spectromemtry (MS) injection. While peptide labeling with isobaric mass tags enables sensitive and precise multiplexed peptide and protein quantification, the accuracy of reporter ion-based quantification which detects coisolated peptide signals in the MS1 spectrum and subsequently estimated and adjusted it for cofragmentation in MS2 spectrum is reduced due to interferance from coelutiong and cofragmenting peptides as well as peptide fragments (Ahrné et al., 2016). In these procedures, both N-termini and lysine side chains of peptides in a digest mixture are labeled with different isobaric mass reagents in such a way that all derivative peptides are isobaric and chromatographically indistinguishable. Next, one peak in the MS1 scan will be isolated for fragmentation and quantitation and peptide fragmentation (MS2) spectra which is the survey scans and quantitative accuracy will depend on the isolation width of precursor ions for fragmentation. (Hsu and Chen, 2016). Protein fractionation and labeling techniques have improved protein identification to include the least abundant proteins (Chandramouli and Qian, 2009). Consequently, this information can be used to understand the consequences of a disease, its processes, and development and it can lead to early detection of disease. Finally, it enhances therapeutic administration to targets and development of precision medicine.

Immunophenotyping classification and molecular diagnosis in canine lymphoma

Phenotyping of canine lymphomas of Guija de Arespacochaga et al., 2007 (*n* = 82) and Ponce et al., 2010 (*n* = 608) studies have shown that 51.2 - 63.8% lymphomas in dogs are commonly linked to B-cell lymphoma. In the other hand, T-cell lymphomas accounted for 29.3 - 35.4% of lymphomas. The least of this tumor

are null cell (Guija de Arespacochaga et al., 2007b; Ponce et al., 2010). The prognosis of B-cell lymphoma was associated, with a better prognosis than T-cell lymphoma (Ponce et al., 2004). At the time of tumor development, specific cellular antigens and cluster of differentiation (CD) express can be used for identifying lineage stage. The specific protein markers for B-cell identification were CD79a or Pax5, while T-cell marker was CD3 (Ponce et al., 2004; Guija de Arespacochaga et al., 2007b; Willmann et al., 2009b; Ponce et al., 2010; Valli et al., 2011a; Sirivisoot et al., 2016b).

Identification of canine T-cell lymphoma mainly used CD3 as a specific marker (Milner et al., 1996). There are some studies which identified T-cell and B-cell phenotyping by using CD3 and CD79a, respectively (Keller et al., 2007; Valli et al., 2013). Despite the fact that many cases showed expression of antigens in both CD3 and CD79a antigens, it also showed in canine B-cell lymphoma for 10-40%. Moreover, some cases show co-expressing or negative of B- and T-cell lineage markers (Thomas et al., 2003; Wilkerson et al., 2005; Guija de Arespacochaga et al., 2007a). Therefore, Pax5 is another useful marker in unclassified cases for prediction regarding prognosis and response of therapy (Willmann et al., 2009a).

Pax5 protein is a nuclear protein in paired-box (PAX) family of transcription factors. These proteins are important regulators in early development, and alterations in the expression of their gene are thought to contribute to neoplastic transformation, signaling of B-cell, adhesion of cell, migration of cell, transcriptional regulation, cellular metabolism, presentation of antigen, and B-cell maturation (Nutt et al., 1999; Delogu et al., 2006; Cobaleda et al., 2007; Pridans et al., 2008). Pax5 protein was used as a pan pre-B- and B-cell marker in human as Hodgkin lymphoma, B-cell non-Hodgkin lymphomas, and also precursor B-cell lymphoblastic neoplasms. In the other hand, it produced negative result in some tumors such as multiple myeloma, plasmablastic lymphoma, and plasma cell neoplasms. In animal studies, Pax 5 also has been used in cNHL subtyping (Feldman and Dogan, 2007; Willmann et al., 2009b; Sirivisoot et al., 2016b; Sirivisoot et al., 2016a).

Polymerase chain reaction (PCR) is a tool for diagnosis in case that has a limitation on sample collection by using blood for subtyping coordination for confirm lymphomas. PCR is used for evaluating and manifesting of malignant lymphoid clonality. Rearrangement of antigen receptor genes which code for immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) genes was contained in unique sequences; variable (V), diversity (D), and joining (J) segments. For B-cell identification, two primer sets, consensus VJ primers of IgH genes were used. Also, T-cell neoplasms use seven primers for V and J segments of TCR**γ** genes (Rezuke et al.,

1997). Gene rearrangements could be spotted in 91% of dog with lymphoma using PCR techniques even though less primer were used which is two J primers, and a single V primer for both TCRγ and IgH genes (Burnett et al., 2003). PCR for antigen receptor rearrangements (PARR) showed effective results for diagnosis in confusing cases and detection of minimal residual. For resolving false positive, heteroduplex might be useful to avoid misdiagnosis and raise reliability of the results. Peripheral blood sample was used to determine the consistency between samples and compared with immunophenotyping method (Takanosu et al., 2010; Sirivisoot et al., 2016a).

Proteomic study in canine lymphoma

Proteomic is mostly used in the study of protein biomarkers. In dog proteome, MS-based proteomic has the ability to analyze complex sample such as serum, tissue, urine, bronchoalveolar lavage fluid, cerebrospinal fluid, mammary cell line, and tear (Ceciliani et al., 2016; Morris, 2016a). Serum is expected to be an excellent source of protein biomarkers because it circulates through the body and encounters with all tissues, secreted, or shed by cells during different physiology or pathological processes. Using serum for biomarker identification is common because it's minimal invasion to the patient and obtains a high protein concentration. Matrix-assisted laser desorption ionization (MALDI) and surface-enhanced laser desorption ionization (SELDI) are the common MS techniques for detecting quantitative or qualitative changes in circulating serum or plasma proteins and analyzed by time-of-flight (TOF) MS. The first report of canine lymphoma biomarker using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and SELDI-TOF MS by compare protein profile from lymph node were performed in 13 healthy dogs to 11 B-cell lymphoma dogs. The study was found 93 differentially expressed spots and one upregulated protein (macrophage capping protein) and three down-regulated proteins (prolidase, triosephosphate isomerase and glutathione S transferase) (McCaw et al., 2007). Meanwhile, Gaines et al. (2007) compared serum samples from 87 healthy control dogs or non-lymphoma cancers and 29 B-cell lymphoma dogs by using ion exchange chromatography and SELDI-TOF MS. The results was shown 3 protein peaks with biomarker potential (Gaines et al., 2007). In another proteomic study, 3 lymphoma dogs and 2 dogs with transitional cell carcinoma of the bladder compare to 7 control dogs were studied by global internal standard technology (GIST) stable isotope labeling, LC and MALDI-TOF and found 11 fucosylated proteins. All three lymphoma dogs were found protein upregulated over 50% compared to

controls (Wilson et al., 2008). In another study, serum samples were collected from 92 non-lymphoma samples and 87 lymphoma dogs were found 19 serum peaks, two biomarkers in particular were able to differentiate between control and lymphomas (Ratcliffe et al., 2009). In 2013, Atherton et al. used a different method by using gel-based technique and MALDI-TOF to identify serum proteome of 3 lymphoma dogs compared with 2 normal healthy dogs. This study found 10 proteins in one lymphoma case but absent in control dogs. However, further studies are needed to evaluate in large set of samples, then, after identification and validation of candidate marker they need to be extent studies in large, well defined patient groups, confirm in the others techniques such as enzyme-linked immunosorbent assay (ELISA) and western blot techniques.

CHAPTER III

MATERIALS AND METHODS

1. Animals

Four healthy adult dogs (mixed breed) were used as the control group. The control group was small and large breed dogs that were healthy after physical examination with no known illness. The criteria for the selection are summarized as follows. First, the dogs were considerably healthy by physical examination. Second, the dogs were vaccinated and deworming. Third, they were not received any medication for 2 weeks prior the blood collection. Similarly, the lymphoma dogs used for the study also fulfilled the certain criteria to qualify. The lymphoma dogs were physical examined and categorized by gross anatomy as multicentric, mediastinal, and epitheliotropic lymphomas. The further examination included the hematological and biochemical profiles (complete blood count, liver function, kidney function, blood parasite, and leukemia). In addition, thoracic radiography, and abdominal ultrasonography were performed for clinical staging. Exclusion criteria are summarizing as follows. First, animal was excluded if it had been previously treated with any cytotoxic agents which might interfere with the result of the study. Second, animal had more than 7 days of corticosteroids prior to referral. Third, animal had a concurrent illness or if hyperproteinemia was detected. Cytological and histopathological from the enlarged peripheral lymph node were categorized according to the WHO clinical stage III or higher (Valli et al., 2011b). Selection and exclusion criteria were to eliminate potential subjectivity of the study.

Twenty dogs with lymphoma from the oncology clinic, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok were selected. All protocols of animal experiments were approved by the Ethics Committee of Chulalongkorn University Animal Care and Use Committee (CUACUC; APPROVAL NO. 1731053).

Four healthy adult dogs were used as the control group. The control group includes small and large breed dogs that were healthy after physical examination with no known illness. The criteria for the selection are summarized as follows. First, the dogs were considerably healthy by physical examination. Second, the dogs were vaccinated and dewormed. Third, they have not received any medication for 2 weeks prior the blood collection. Similarly, the lymphoma dogs used for the study also

fulfilled the certain criteria to qualify. The lymphoma dogs were physical examined and categorized by gross anatomy as multicentric, mediastinal, and epithelictropic lymphomas. The further examination included the hematological and biochemical profiles (complete blood count, liver function, kidney function, blood parasite, and leukemia). In addition, thoracic radiography, and abdominal ultrasonography were performed for clinical staging. Exclusion criteria are summarizing as follows. First, animal was excluded if it had been previously treated with any cytotoxic agents which might interfere with the result of the study. Second, animal had more than 7 days of corticosteroids prior to referral. Third, animal had a concurrent illness or if hyperproteinemia was detected. Cytological and histopathological from the enlarged peripheral lymph node were categorized according to the WHO clinical stage III or higher (Valli et al., 2011b). Selection and exclusion criteria were to eliminate potential subjectivity of the study.

2. Collection of sample

Blood samples (5 ml) were obtained from four healthy dogs and twenty lymphoma dogs. For routine health status check including complete blood cell; RBC, WBC, thrombocyte count, performed by automated machine (BC-5300 Vet Auto Hematology Analyzer, Mindray®, China), liver enzymes (alanine aminotransferase, alkaline phosphatase), and kidney profiles (blood urea nitrogen, creatinine) were performed by automated (CS-400 Auto-chemistry Analyzer, DiaSys®, Germany). Blood parasite was detected by blood smear and buffy coat under the microscope and SNAP® 4Dx® (IDEXX Laboratories) to make sure that they don't contain blood parasites.

The infiltrations of atypical lymphoid cells were determined by buffy coated smear for clinical staging. As the blood coagulates, serum sample was taken into plain tubes and made to clot, then separated by centrifugation and stored at -80°C for proteomic analysis.

3. Immunophenotyping classification and molecular diagnosis

Cytology diagnosis: Fine needle aspiration from superficial lymph node was collected. Cells from needle hub were flushed and smeared on the clean slides, fixed in absolute methanol, stained with Giemsa's stain, and classified as low or high grade lymphoma by pathologist according to update Kiel's classification under light microscope (Valli et al., 2011b).

Histopathological diagnosis: Lymph node incisional biopsy for histopathological diagnosis was carried out under sedation. Sedating the dogs were done with Zoletil® (Virbac, France) dosage 2 mg/kg intravenous route and locally anesthetized by using lidocaine (LBS, Thailand). For routine histopathological diagnosis, using 6 mm diameter punch biopsy was used to collect the sample and kept in 10% neutral buffered formalin and was sectioned at 4- to 6-µm thickness and stained with hematoxylin and eosin (H&E). Categorization of histopathological diagnoses was done according to the WHO classification of by pathologist (Valli et al., 2011b).

Immunophenotype: To phenotype the B- and T-cell lineage, lymphoma samples were classified by using anti-Pax5 and anti-CD3, respectively. Immunohistochemical protocol was modified from Sirivisoot et al., 2016b.

Polymerase chain reaction (PCR): Using mammalian genomic DNA miniprep kit, Genomic DNA of white blood cells (WBC) in EDTA-anticoagulated blood was extracted (Sigma-Aldrich, USA). DNA concentration was measured by NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). For PCR of antigen receptor rearrangement, white blood cell (WBC) pellet were done following with heteroduplex analysis. To control DNA amplification, Specific primers were chosen which were C_{μ} gene, IgH gene for B lymphocytes, TCR_{γ} gene for T lymphocytes follow as shown in table 1. The complete reaction volume was 25 µl contained 12.5 µl of GoTaq® Green Mater Mix (Promega, USA), 50-300 ng of DNA, and 200-300 nM of each primer. The protocol of PCR cycling was 94°C for 45 sec, 60°C for 30 sec, and 72°C for 30 sec and, the final extension was 5 min at 72°C., after PCR amplification 35 cycles. Lastly, Each PCR products were divided into 2 aliquots. One of them was used for heteroduplex analysis (*h*PAAR): incubation at 95°C for 5 min and re-annealing at 4°C for 30 min. Thereafter, visualizing of every sample was on 2% agarose gel electrophoresis (Sirivisoot et al., 2016a).

Table 1: Primers for heteroduplex PARR

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	
TCR$_{\gamma}$	V**γ**a	CGT GTA CTA CTG CGC TGC CTG G	55-90 bp
	V**γ**b	GGC TGT ATT ACT GTG CCT GCT GG	
	J**γ**b	TGT GCC AGG ACC AAG CAC TTT GTT	
IgH	V region	ACA CGG CC(A/C/G) TGT ATT ACT GT	80-150 bp
	J region	TGAGGAGACGGTGACC	

4. Proteomic techniques (TMT-Labeling Protocol)

4.1 Sample preparation

Determination of serum protein concentration was performed by modified Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). To prepare protein sample, preparing 500x of protein that dilute 1 μl of each samples: 499 μl of water and mixed reagent, the selection A: selection B proportion is 50: 1 then, making reaction for standard and protein samples in 96 well plates as follow in Table 2. The 200 μl of mixed reagents were added into each well. The reactions were incubated at 37°C for 30 min and measured at the OD 562 nm. Bovine Serum Albumin (BSA) was used as protein standard. Then, the protein concentration was calculated in Microsoft excel.

Table 2: Modified Pierce BCA Protein Assay making reaction for standard and protein samples in 94 well plate

Sample No.	STD (μl)	5% SDC in 25 mM TEAB (μl)	ddH_2O (μl)	Total (μl)
1	0	5	20	25
2	1	5	19	25
3	3	5	17	25
4	5	5	15	25
5	10	5	10	25
Protein Sample	5	0	20	25

SDC = sodium dodecyl sulfate TEAB = Tris EDTA buffer

4.2 Alkylation, reduction and digestion

Protein sample (500 µg) were transferred into a new 1.5 ml tube and adjust to a final volume of 100 µl with 100 mM Tris EDTA buffer (TEAB) then 5 µl of the 200 mM Tris-(2-Carboxyethyl) phosphine (TCEP) was added and incubated at 55°C for 1 h. Five µl of 375 mM of iodoacetamide were added and the solution was incubated for 30 min protected from the light at room temperature. Then six volumes (600 µl) of pre-chilled acetone were added, and the reaction was frozen at -20°C. The precipitation allows proceeding for overnight. Later, the sample was centrifuged at 8000 x g for 10 min at 4°C. The white pellets were suspended with 100 µl of 100 mM TEAB and measured the amount of protein by BCA assay. In the protein digestion process, the 500 µg of acetone-precipitated protein pellet samples were resuspended with 300 µl of 100 mM TEAB and ultrasonic sonicated using 30% amplitude, pluses for 10 sec and pulse off 5 sec for each 5-min cycle. Preparing digestion process was done by adding 25 µl of stock Trypsin (0.1 µg/µl) per 100 µg of protein into the reaction and incubating at 37°C overnight.

4.3 Peptide Quantitation Fluorometric Peptide Assay

Determination of the concentration of peptides was done by using Peptide Quantitation Fluorometric Peptide Assay (Thermo Scientific, Rockford, IL, USA). Standards and peptide samples assay were performed according to table 3. Ten µl of each standard or samples were pipette replicated into one well of the fluorescence-compatible microplate. Then, 70 µl of Fluorometric Peptide Assay Buffer and 20 µl of Fluorometric Peptide Assay Reagent were added into each well. The solution was incubated at room temperature for 5 min and measured the fluorescence signal using Ex/Em at 390nm/475 nm by spectrometer. Standard curve was plotted by using Microsoft excel program in order to determine the peptide concentration of each unknown samples.

Table 3: Preparation of diluted peptide digests assay standards

Centrifuge tubes	Volume of Diluent (µl)	Volume of Digest (µl)	Final Standard Concentration for Peptide Mixture (µg/mL)	Final Standard Concentration for Individual Peptides (µM)
A	0	150 of Stock	1000	841.0
B	75	75 of Vial A	500	420.5
C	75	75 of Vial B dilution	250	210.3
D	75	75 of Vial C dilution	125	105.1
E	75	75 of Vial D dilution	62.5	52.6
F	75	75 of Vial E dilution	31.3	26.3
G	75	75 of Vial F dilution	15.6	13.2
H	75	75 of Vial G dilution	7.8	6.6
Blank	75	0	0	0

4.4 Labeling with TMT of the serum samples

Preparation of solution was done, by equilibrating the TMT Label Reagents to room temperature and adding 41 µl of anhydrous acetonitrile to each tube of labeling agents. The reagents were dissolved with occasional vortexing and spin down for 5 min. Thirteen µl of the TMT label reagents was added to each 30 µg peptide sample and the reaction was incubated for 1 h at room temperature. The 8 µl of 5% hydroxylamine was added to the sample and incubated for 15 min (in order to quench excessive TMT reagents). Both samples were combined at equal amounts and stored at -80°C.

Group I comprised of 6 lymphoma dogs, 2 healthy control samples by TMT with reporters at m/z=127N.1, 127C.1, 128N.1, 128C.1, 129N.1, 129C.1, 130N.1, 130C.1, respectively. In group II, 7 lymphoma dogs, 1 healthy control samples were labeled by another set of TMT with reporters at m/z=127N.2, 127C.2, 128N.2, 128C.2, 129N.2, 129C.2, 130N.2, 130C.2, respectively. In group III, 7 lymphoma dogs, 1 healthy control samples were labeled by another set of TMT with reporters at m/z=127N.3, 127C.3, 128N.3, 128C.3, 129N.3, 129C.3, 130N.3, 130C.3, respectively. 131.1, 131.2, and 131.3 were used as pool sample for group 1, 2, and 3, respectively (Figure 1 and 2).

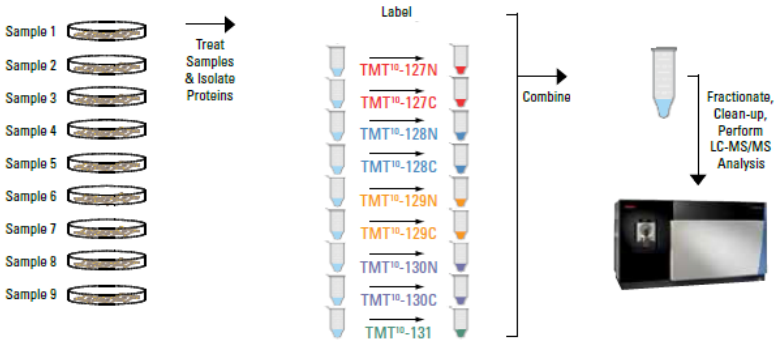

Figure 1. Labeling with TMT label reagents in the serum sample

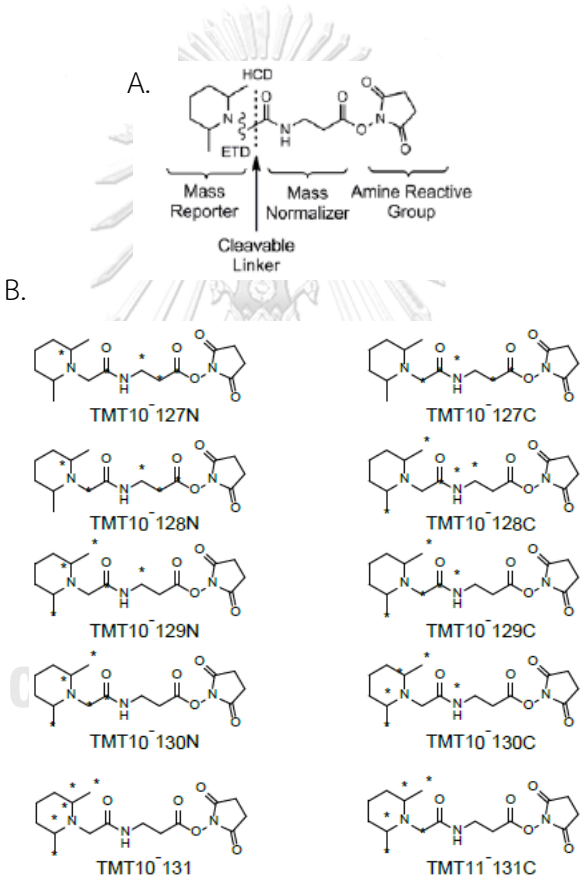

Figure 2. Chemical structures of the Thermo Scientific TMT Label Reagents.

A. Functional regions of the reagent structure including MS/MS fragmentation sites by higher-energy collision dissociation (HCD) and electron transfer dissociation (ETD).

B. TMT 10plex reagent and TMT11-131C reagent structures and isotope positions (*).

(From: TMT10plex™ Isobaric Mass Tag Labeling Kits and Reagents Thermo Fisher Scientific Inc. 2014)

4.5 Sample Fractionation Step

Elution solutions were prepared according to Table 4 and Figure 3. The protective white tip was removed from the bottom of the column and discard. The column was placed into a 2.0 ml sample tube and centrifuged at 5000 × g for 2 min to remove the solution and then the resin material was packed. The upper liquid part was discarded and the top screw cap was removed. Three hundred µl of acetazolamide (ACN) was loaded into the column. The spin column was replaced back into a 2.0 ml sample tube and centrifuged at 5000 × g for 2 min. ACN was discarded and the column was twice washing-step repeated with 300 µl 0.1% TFA solution. The column was conditioned and ready for use.

Meanwhile, it was noted that the Thermo Scientifics™ High pH Reversed-Phase Peptide Fractionation Kit was provided an optimized fractionation protocol and reagent to increase the number of proteins identified from complex samples by liquid chromatography-mass spectrometry (LC-MS) analysis.

In continuation, fractionalizations of the digested samples were done by dissolving 10-100 µg of digested sample in 300 µl of 0.1% TFA solution. The spin column was placed into a new 2.0 ml sample tube. Three hundred µl of the sample solution was loaded onto the column and centrifuged at 3000 × g for 2 min. Retain elute as “flow-through” fraction step was performed by place the column into a new 2.0 ml sample tube, load 300 µl of wash buffer (5% ACN, 0.1% TEA) onto the column and centrifuge again to collect the wash. Retain elute as “wash” fraction was performed by place the column into a new 2.0 ml sample tube, load 300 µl of the appropriate elution solution and centrifuge at 3000 × g for 2 min to collect the fraction. The remaining step of gradient fractions using the appropriate elution solutions in new 2.0 ml sample tubes were repeated twice. The liquid contents of each sample tube were evaporated to dryness using vacuum centrifugation. Dry samples were re-suspended in an appropriate volume of 0.1% formic acid (FA) before LC-MS analysis. The 12 fractions were dried under vacuum and stored at −80°C for further LC-MS/MS analysis.

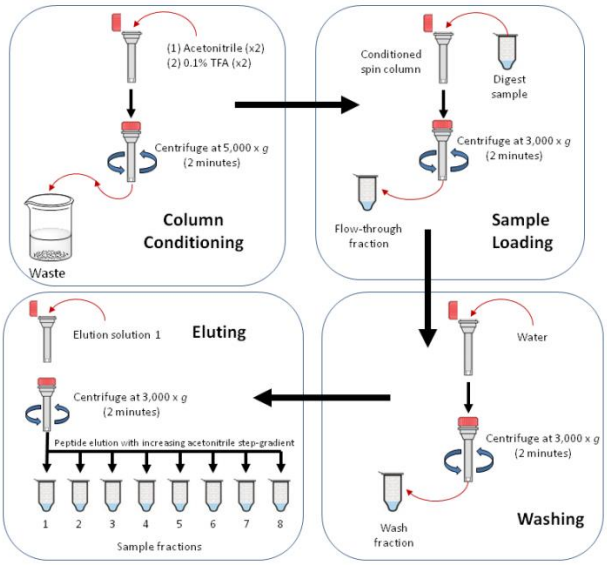

Figure 3. Spin column conditioning and sample fractionation workflow

(From: Pierce High pH Reversed-Phase Peptide Fractionation Kit.
Thermo Fisher Scientific Inc. 2016.)

Table 4: Preparation of elution solutions for Thermo™ Scientific TMT-labeled peptides

Fraction No.	Acetonitrile (%)	Acetonitrile (µl)	Triethylamine (0.1 %) (µl)
Wash	5.0	50	950
1	10.0	100	900
2	12.5	125	875
3	15.0	150	850
4	17.5	175	825
5	20.0	200	800
6	22.5	225	775
7	25.0	250	750
8	50.0	500	500

4.6 LC-MS/MS analysis

In each fraction, peptides were rehydrated in 0.1% (v/v) formic acid in water and injected with an autosampler (Thermo Scientific Easy-nLC1000). Peptides were first enriched on a reverse phase trap column (Acclaim PepMap™100, C18 3 µm, 100 Å, 75 µm × 2 cm, nanoViper) and then eluted to analytical column (PepMap™RSLC

C18, 2 μm, 100 Å, 50 μm × 15 cm). The mobile phase consisted of buffer (A) 0.1% formic acid in water, and buffer (B) 0.1% formic acid with 100% of acetonitrile. A flow rate of 0.3 μl/min was applied for the separation of peptides for 90 min. The gradient run was follow: 0–70 min flow rate 300 l/min of 5% B, 70-72 min flow rate 300 μl/min of 40% B, 72-90 min flow rate of 95% B. The mass spectrometer voltage was set to 2000 V and the heated capillary was kept at 250°C. All mass spectra were acquired in the positive ionization mode with m/z scan range of 350–1400. The LTQ-Orbitrap XL (Thermo Fisher Scientific, San Jose, USA) was operated in a top 10 configuration at 70,000 resolving power for a full scan, with enabled charge state screening, monoisotopic precursor selection enabled, and + 1, and unassigned charge states rejected. After master scan, three most intense ions were subjected for collision-induced dissociation (CID) fragmentation using an isolation window of 0.7, collision energy of 32, default charge state of 2 and activation time of 50 msec. Fragmentation of three most intense TMT-reporter-labeled ions were achieved with HCD fragmentation at 35,000 resolving power in the LTQ-Orbitrap using an isolation window of 0.7, collision energy of 100, default charge state of 2 and activation time of 100 msec.

4.7 Identification of protein and its quantification

Proteome Discoverer (v2.1) interfaced SEQUEST (Human IPI database v3.78, 302626 entries) was applied for data analysis having performed LC-MS/MS. Up to two missed cleavage sites were allowed during the database search. Peptides and proteins identification were filtered with charge state dependent cross correlation (Xcorr) ≥ 2.0 and peptide rank No. 1 with requiring at least two peptides per protein. The filters allow a 99% confidence level of protein identification with less than 1% false discovery rate. The Reporter Ions Quantitizer in the Proteome Discoverer were used to quantify the TMT reporter ion intensities at 126.13–131.14 m/z. Protein identification and quantification intensity ratios were exported to Microsoft Excel software. Reporter ion isotope correction factors were applied by subtracting the contribution of reporter ion isotopes to adjacent reporter ion intensities and adding these intensities back to the proper channel, after which data was normalized by median intensities for subsequent analyses.

4.8 Data analysis

In general, data were analyzed by using Thermo Scientific™ Proteome Discoverer™ software version 2.1. Similarly, analyses of the samples were done

specifically with Q Exactive™ Hybrid Quadrapole-Orbitrap™ Mass Spectrometer. Result from this part, absolute and relative protein values were explained and analysis between groups; canine B-cell, T-cell lymphoma and, healthy dog by One-way ANOVA then Post Hoc multiple comparisons Non-parametric Mann Whitney *U* tests were compared between two groups by using SPSS 23.0 Statistical Software (SPSS Inc.), The *p* value less than 0.05 is considered to have significance.

CHAPTER IV

RESULTS

Animals

Serum and tissue samples were collected from 24 dogs that were presented in the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. The dogs consisted of variety breeds, sexes, and ages included: American bully, American cocker spaniel, American Pit-bull, German shepherd, Golden retriever, Italian greyhound, Labrador retriever, Mixed, Pit-bull, Pomeranian, Saint Bernard, Shih Tzu, Spitz, and Thai Ridgeback. For the control group, the breeds were one American pit-bull, one Labrador retriever, one mixed breed, and one Shih Tzu. In table 5, four healthy adult dogs (small and large breed) were used as control group, which is 2 males and 2 females in an average age is 4.5 years (range, 3-7 years). The lymphoma group consisted of 13 males and 7 females in an average 10.5 years (range, 2-15 years) with the multicentric form (*n* = 16), mediastinal form (*n* = 3), and epithelictropic form (*n* = 1). Lymphoma groups were presented clinical stage III or higher, stage III (*n* = 9), stage IV (n = 8), and stage V (*n* = 3). There are 10 lymphoma dogs absence of systemic signs and 10 lymphoma dogs with presence of clinical sign (substages a and b, respectively).

In lymphoma group, the hematological profiles were shown dogs with mild anemia (*n* = 2), moderate anemia (*n* = 2), severe anemia (*n* = 1) leukocytosis (*n* = 2), and mild thrombocytopenia (*n* = 4), Detection of *antibodies* to *Ehrlichia canis* (*n* = 1) as shown in Table 7. Biochemical profiles of lymphoma dogs were shown in Table 8 that elevated BUN (*n* = 1), elevated liver enzyme (*n* = 9).

The histologic and immunophenotype from 14 multicentric lymphoma tissues were analyzed according to the WHO classification as data in table 6. There were 6 T-cell lymphomas and 14 B-cell lymphomas. Tissue biopsy were shown in Figure 4-7, the most common B-cell lymphoma is diffuse large B-cell lymphoma (High grade) and T-cell lymphoma is peripheral T-cell lymphoma (High grade). Three lymphoma dogs with mediastinal forms and 1 with epitheliotropic form were diagnosis by cytology and *h*PARR. The results of all samples were T-cell lymphoma (Figure 8 and 9).

Table 5: The baseline characteristics of lymphoma dogs and control dogs

Variable	Lymphoma (*n* = 20)	Control (*n* = 4)
Breed		
Pure	17	3
Mixed	3	1
Ages (Median range)	10.5 (2-15 y)	4.5 (3-7 y)
Gender		
Male	13	2
Female	7	2
Immunophenotyping		
B cell lymphoma	14	
High grade	8	
Low grade	6	
T cell lymphoma	6	
High grade	2	
hPARR (Unclassified)	4	
Clinical staging		
III	10	
IV	8	
V	2	
Clinical substage		
a	10	
b	10	

Table 6: Signalment data, TNM stage, Histopathology and Immunophenotyping

ID	Breed	Sex	Age	Types	Clinical Stage		WHOs classification
Normal							
N1	Shih Tzu	Ms	9y	control			
N2	Mixed Siberian	Fs	3y	control			
N3	Labrador	Fs	3y	control			
N4	American Pit-bull	Ms	3y	control			
B cell lymphoma							
B1	Golden Retriever	F	10y4m	Multicentric	IIIa	Low B cell	B cell small lymphocytic lymphoma
B2	Shih Tzu	F	12y	Multicentric	Vb	Low B cell	T cell rich B cell lymphoma
B3	Golden Retriever	M	5y	Multicentric	IIIa	Low B cell	T cell rich B cell lymphoma
B4	Saint Bernard	M	11y7m	Multicentric	IIIa	Low B cell	Follicular lymphoma T cell rich B cell lymphoma
B5	Pit-bull	M	2y4m	Multicentric	IIIa	Low B cell	Lymphoplasmacytic lymphoma
B6	American cocker spaniel	M	13y	Multicentric	IIIa	Low B cell	Small lymphocytic lymphoma, follicular lymphoma
B7	Mixed	M	9y	Multicentric	IVb	High B cell	Diffuse large B cell
B8	Mixed	F	8y2m	Multicentric	IIIa	High B cell	Diffuse large B cell lymphoma HI CB
B9	Golden Retriever	M	10y	Multicentric	Vb	High B cell	Diffuse intermediate B cell lymphoma
B10	Thai Ridgeback	M	12y	Multicentric	IVb	High B cell	Diffuse large B cell
B11	Italian greyhound	F	14y	Multicentric	IVb	High B cell	Diffuse large B cell lymphoma HI CB
B12	Golden Retriever	M	8y	Multicentric	IIIa	High B cell	Diffuse large B cell lymphoma HI CB
B13	Shih Tzu	F	12y5m	Multicentric	IVa	High B cell	Diffuse large B cell
B14	Mixed	M	5y6m	Multicentric	IIIa	High B cell	Diffuse large B cell
T cell lymphoma							
T1	German shepherd	F	10y	Multicentric	IVb	High T cell	Peripheral T cell lymphoma (PTCL)
T2	American bully	M	6y7mt	Multicentric	Vb	High T cell	T cell lymphoblastic lymphoma
T3	Spitz	M	15y8m	Mediastinal	IVb		PCR = T cell
T4	Pomeranian	M	13y7m	Mediastinal	IVb		PCR = T cell
T5	Labrador retriever	M	10y	Epithelictrophic	IIIa		PCR = T cell
T6	Shih Tzu	F	7y9m	Mediastinal	IVb		PCR = T cell

N: normal, T: T cell lymphoma, B: B cell lymphoma, M: Male, Ms: Sprayed Male, F: Female, Fs: Spayed Female

Table 7: Summary of blood cell counts in lymphoma and control dogs

ID	Clinical Stage	WBC	N/L ratio	Lymphoblast	RBC	Hb	Hct	Platelets
N1		7900	3.27	-	7.37	14.7	45.1	338000
N2		12200	2.43	-	6.33	12.9	40	225000
N3		8400	2.16	-	8.23	16.7	50	267000
N4		10400	3.04	-	7.5	15.1	47	242000
B1	IIIa	19500	2.88	-	5	13	41	75000
B2	Vb	39400	31.00	-	3.46	9	25.4	161000
B3	IIIa	13500	4.41	-	5.56	13.4	38.2	406000
B4	IIIa	9930	6.31	-	4.86	11.5	33.7	328000
B5	IIIa	8440	4.28	-	3.23	7.9	24.8	219000
B6	IIIa	11700	3.45	-	4.91	11.4	32.7	72100
B7	IVb	15100	3.85	-	8.14	19.2	58.2	190000
B8	IIIa	16100	91.00	-	2.44	5.8	18.9	205000
B9	Vb	23400	0.55	-	6.08	14.2	43.6	531000
B10	IVb	38800	2.06	-	4.56	10.1	30.9	542000
B11	IVb	73800	22.75	-	4.73	11.2	35	158000
B12	IIIa	14600	6.69	-	5.03	13	37.6	211000
B13	IVa	5800	8.50	-	7.22	16.4	49.4	274000
B14	IIIa	9190	5.86	+	5.35	11.6	37.3	254000
T1	IVb	28600	4.71	-	5.2	11.7	36.4	210000
T2	Vb	33800	0.29	+	4.87	11.6	35.2	53000
T3	IVb	6500	5.71	-	5.43	11.9	36.6	103000
T4	IVb	14600	6.69	-	5.03	13	37.6	211000
T5	IIIa	17333	5.67	-	7.32	16.6	50.1	244000
T6	IVb	5000	1.69	-	5.58	13.8	39.3	495000

N: Normal, B: B cell lymphoma, T: T cell lymphoma,
WBC: White Blood Cell Count, N/L ratio: Neutrophil/Lymphocyte, RBC: Red blood cell count,
Hb: Hemoglobin, Hct: Hematocrit

Table 8: Summary of blood chemistry profiles in lymphoma and control dogs

ID	Clinical Stage	ALT	ALP	Cr	BUN	Cal	TP	Alb
N1		44	122	1.18	10	-	8.6	4.6
N2		44	-	1.13	-	-	-	-
N3		49	-	1.13	-	-	-	-
N4		39	-	39	-	-	-	-
B1	IIIa	360	257	0.6	13	-	-	2
B2	Vb	29	128	0.6	6	11.5	-	-
B3	IIIa	40	62	17	0.9	9.7	-	-
B4	IIIa	18	54	1.22	19	-	-	-
B5	IIIa	730	375	1.45	13	-	-	-
B6	IIIa	27	66	0.8	12.4	9.8	6.1	2.9
B7	IVb	30	52	1.1	18	-	-	-
B8	IIIa	54	-	1.6	-	9.9	6.4	2.2
B9	Vb	26	56	0.8	9	7.1	7.5	2.6
B10	IVb	14	48	0.9	11	-	-	-
B11	IVb	40	1661	0.5	34.9	6.8	4.6	2.2
B12	IIIa	241	798	0.7	19.6	-	-	-
B13	IVa	483	-	1.23	33	9	-	-
B14	IIIa	57	-	0.96	-	-	-	-
T1	IVb	390	990	1.8	24	-	-	-
T2	IIIb	273	790	0.9	38	8.2	5.6	2.8
T3	IVb	157	1039	1.7	49	-	3.6	2.3
T4	IVb	241	798	0.7	19.6	-	7	3.7
T5	IIIa	51	53	1.19	17	10.3	-	-
T6	IVb	180	1789	0.6	22	-	-	-

N: Normal, B: B cell lymphoma, T: T cell lymphoma,
ALT: Alanine transaminase, ALP: Alkaline phosphatase, Cr: Creatinine, BUN: blood urea nitrogen,
Cal: Calcium, TP: Total protein, Alb: Albumin

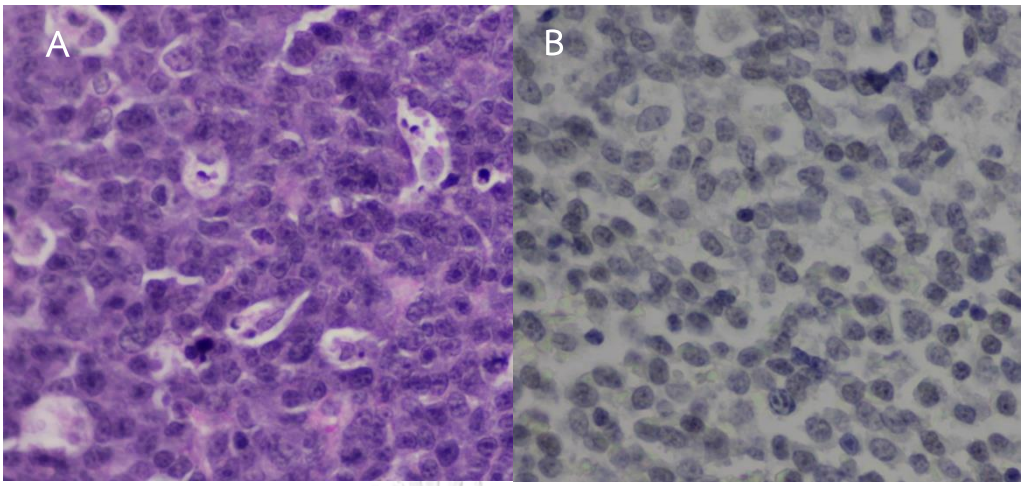

Figure 4. Diffuse large B-cell lymphoma (High grade)

A) Histopathology displayed round, oval or cleaved nuclei, with reticular chromatin and large central nucleolus (Giemsa, 400x)

B) Pax5 was positive staining in nucleus of malignant B lymphoblasts (IHC, 400x)

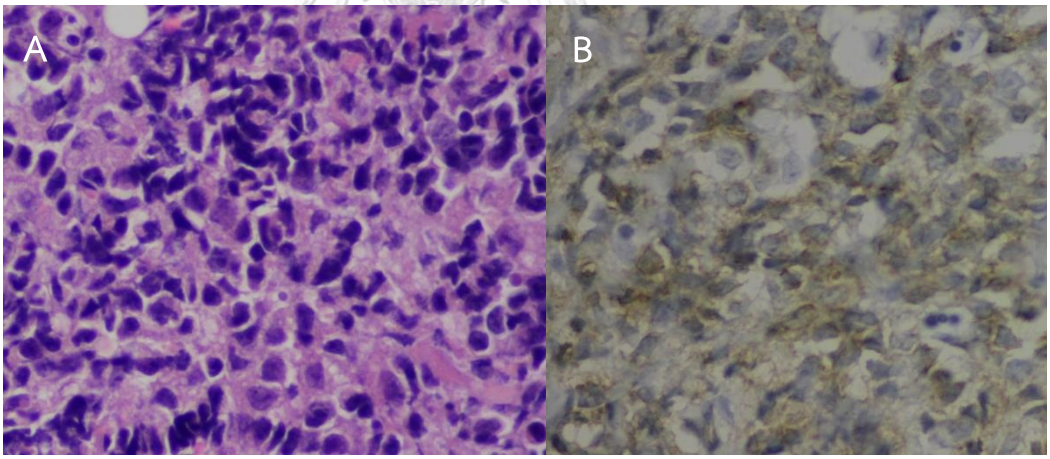

Figure 5. Peripheral T-cell lymphoma (High grade)

A) Histopathology showed various sizes of T cell lymphoma, small to large malignant T-cells, with irregularly cleaved nuclei and various amounts of cytoplasm (Giemsa, 400x)

B) CD3 was positive staining in cytoplasmic border of T lymphoblasts (IHC, 400x)

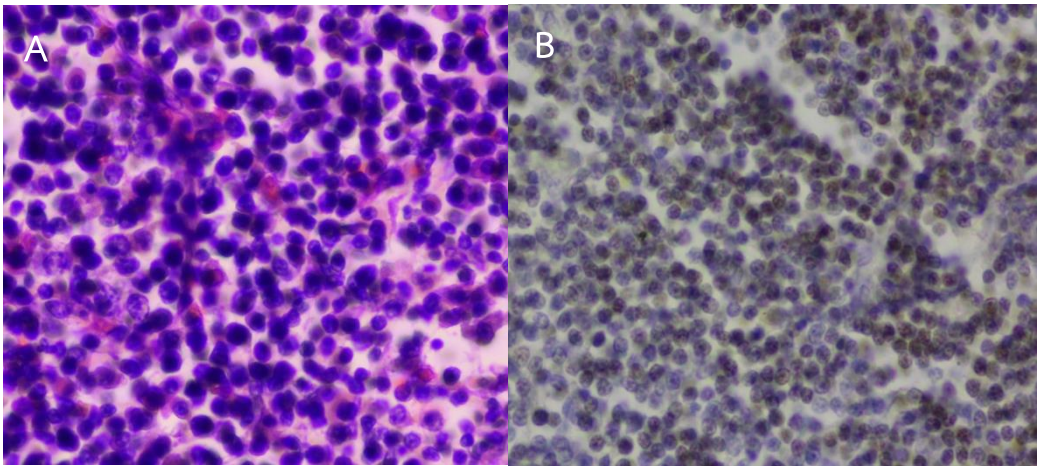

Figure 6. Lymphoplasmacytic B cell lymphoma (Low grade)

A) Histopathology displayed round, oval or cleaved nuclei, with reticular chromatin and large central nucleolus (Giemsa, 400x)

B) Pax5 was positive staining in nucleus of malignant B lymphoblasts (IHC, 400x)

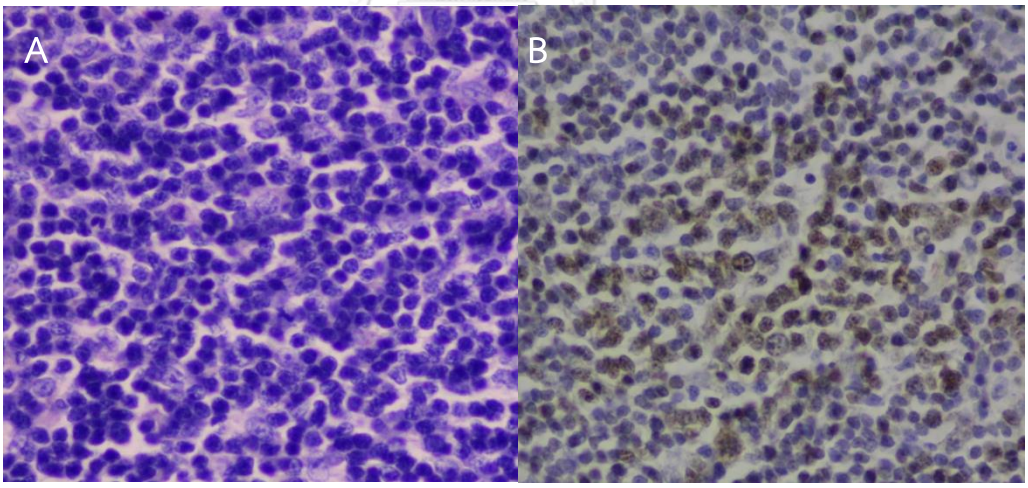

Figure 7. Follicular lymphoma T cell rich B cell (Low grade)

A) Histopathology displayed round, oval or cleaved nuclei, with reticular chromatin and large central nucleolus (Giemsa, 400x)

B) Pax5 was positive staining in nucleus of malignant B lymphoblasts (IHC, 400x)

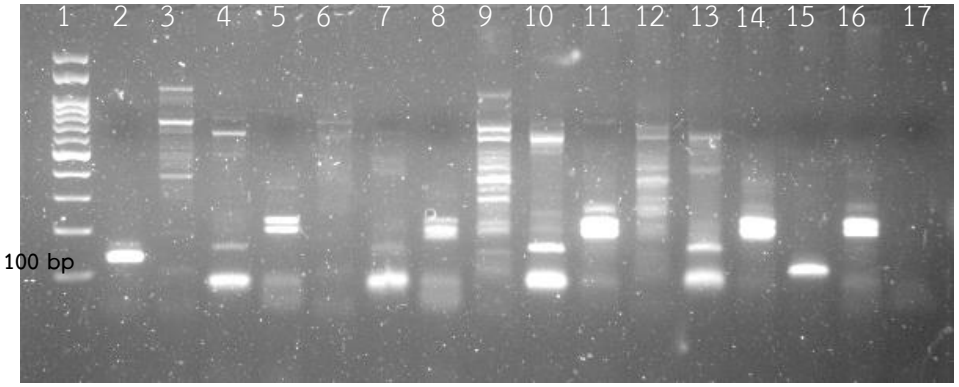

Figure 8. Conventional and Heteroduplex PARR Results from both peripheral white blood cells and pleural effusion specimens showed distinct bands of TCRγ genes of T-cell lymphoma number 3. 1 = ExcelBand 100bp ladder; 2-8 = sample from peripheral white blood cells; 9-14 = sample from pleural effusion; 2 = Cμ gene (130bp); 3, 6, 9, 12 = IgH gene (120bp); 4, 5, 7, 8, 10, 11, 13, 14 = TCRγ gene positive (90bp); 15 = B-cell lymphoma positive; 16 = T-cell lymphoma positive; 17 = Negative control

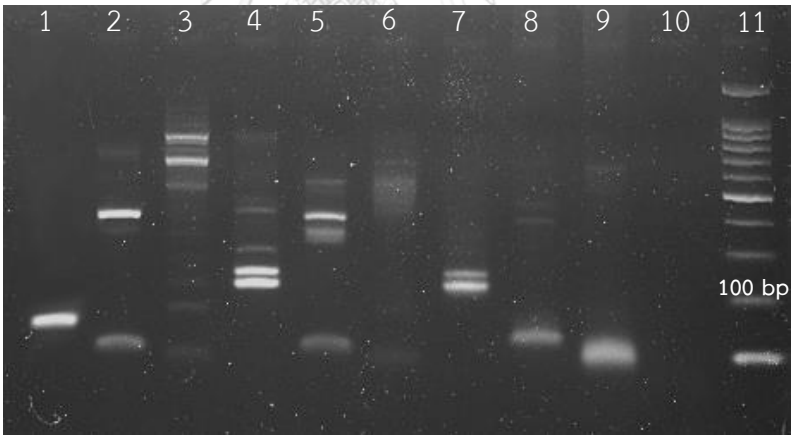

Figure 9. Conventional and Heteroduplex PARR Results from biopsy specimens showed distinct bands of IgH genes of B-cell lymphoma number 12. 1-10 = sample from peripheral white blood cells; 1 = Cμ gene (130bp); 2, 5 = IgH gene (120bp); 3, 4, 6, 7 = TCRγ gene positive (90bp); 8 = B-cell lymphoma positive; 9 = T-cell lymphoma positive; 10 = Negative control; 11= ExcelBand 100bp ladder

Proteome analysis

Proteins identification in serum from control dogs and lymphoma dogs (*C. lupus familiaris)* were shown in Table 9. There were 34 individual proteins such as actin (cytoplasmic 1), apolipoprotein A-I, apolipoprotein A-II, apolipoprotein A-IV, apolipoprotein C-I, apolipoprotein C-II, apolipoprotein C-III, apolipoprotein E, Beta-2-glycoprotein 1, Beta-2-microglobulin, CD 44 antigen, clusterin, collagen alpha-1(I) chain, fibrinogen alpha chain, fibrinogen beta chain, fibrinogen gamma chain, fibronectin, haptoglobin, hemoglobin subunit alpha, hemoglobin subunit beta, hepatocyte growth factor activator, Ig heavy chain V region GOM, Ig heavy chain V region MOO, Ig kappa chain V region GOM, Ig mu chain C region, Lysozyme C, spleen isozyme, metalloproteinase inhibitor-1, Myosin-13, nucleoside diphosphate kinase A, plasminogen, platelet glycoprotein ib alpha chain, serum albumin, serum amyloid A protein, transferrin receptor protein 1.

Heat map was shown the relation of protein intensities between control, T-cell, and B-cell lymphoma groups in Figure 10. There were three proteins that found in high absolute abundant consisted of albumin, haptoglobin, and apolipoprotein A-I, respectively. For control group, there is correlation in group. Meanwhile, some dogs of B-cell lymphoma had shown proteins expression complementally. In the other to, T-cell lymphoma didn't shown the significantly difference. There are 3 groups of protein by average sequence coverage (%) were used to categorize. Protein sequence that found in average sequence coverage less than 50% were apolipoprotein A-II, apolipoprotein C-II, apolipoprotein C-III, apolipoprotein E, clusterin, collagen alpha-1(I) chain, fibrinogen alpha chain, fibrinogen gamma chain, fibronectin, hemoglobin subunit alpha, hemoglobin subunit beta, Ig heavy chain V region MOO, Ig mu chain C region, lysozyme C, spleen isozyme, metalloproteinase inhibitor 1, myosin-13, nucleoside diphosphate kinase A, plasminogen, and platelet glycoprotein Ib alpha chain. The average sequence coverage of protein sequence between 51 to 75 percent were CD44 antigen, fibrinogen beta chain, haptoglobin, Ig heavy chain V region GOM, Ig kappa chain V region GOM, Serum albumin, serum amyloid A protein, and transferrin receptor protein 1 and more than 75 percent such as actin cytoplasmic 1, apolipoprotein A-I, apolipoprotein A-IV, apolipoprotein C-I, beta-2-glycoprotein 1, beta-2-microglobulin, hepatocyte growth factor activator as shown in Table 10.

In comparison between healthy dogs, B-cell and T-cell lymphoma dogs, six proteins were significantly different between control and lymphoma group such as albumin, clusterin or apolipoprotein J, Ig heavy chain V region GOM, beta-2-

microglobulin, apolipoprotein C-I, Haptoglobin. Albumin significant decreased (p = 7×10^{-7}), while five proteins significantly increased in lymphoma group such as Apo J (p = 0.014), Ig heavy chain V region GOM (p = 0.019), β2M (p = 0.023), Apo C-I (p = 0.024), and Hp (p = 0.030). Data was shown in Table 12, 13 and Figure 11.

Comparing serum proteins between 3 groups from healthy dogs and T-cell and B-cell lymphoma dogs was found that serum albumin of the normal dogs significantly increased than both groups with p = 0.011, and 0.003, respectively. The level of Apo J, Ig heavy chain V region GOM, and β2M significantly increased in B-cell lymphoma more than T-cell lymphoma with p = 0.005, 0.008, and 0.003, respectively. The level of Hp in T-cell lymphoma group was significant higher than healthy group (p = 0.03). Moreover, Apo C level in T-cell lymphoma group shown higher than B-cell lymphoma group (p = 0.032) as shown in Table 12.

Table 9: Summary of 34 canine proteins identified the serum of 20 lymphoma dogs and 4 healthy dogs.

Actin, cytoplasmic 1	Haptoglobin
Apolipoprotein A-I	Hepatocyte growth factor activator
Apolipoprotein A-II	Hemoglobin subunit alpha
Apolipoprotein A-IV	Hemoglobin subunit beta
Apolipoprotein C-I	Ig heavy chain V region GOM
Apolipoprotein C-II	Ig heavy chain V region MOO
Apolipoprotein C-III	Ig kappa chain V region GOM
Apolipoprotein E	Ig mu chain C region
Beta-2-glycoprotein 1	Lysozyme C, spleen isozyme
Beta-2-microglobulin	Metalloproteinase inhibitor-1
Clusterin	Myosin-13
CD 44 antigen	Nucleoside diphosphate kinase A
Collagen alpha-1(I) chain	Plasminogen
Fibrinogen alpha chain	Platelet glycoprotein ib alpha chain
Fibrinogen beta chain	Serum albumin
Fibrinogen gamma chain	Serum amyloid A protein
Fibronectin	Transferrin receptor protein 1

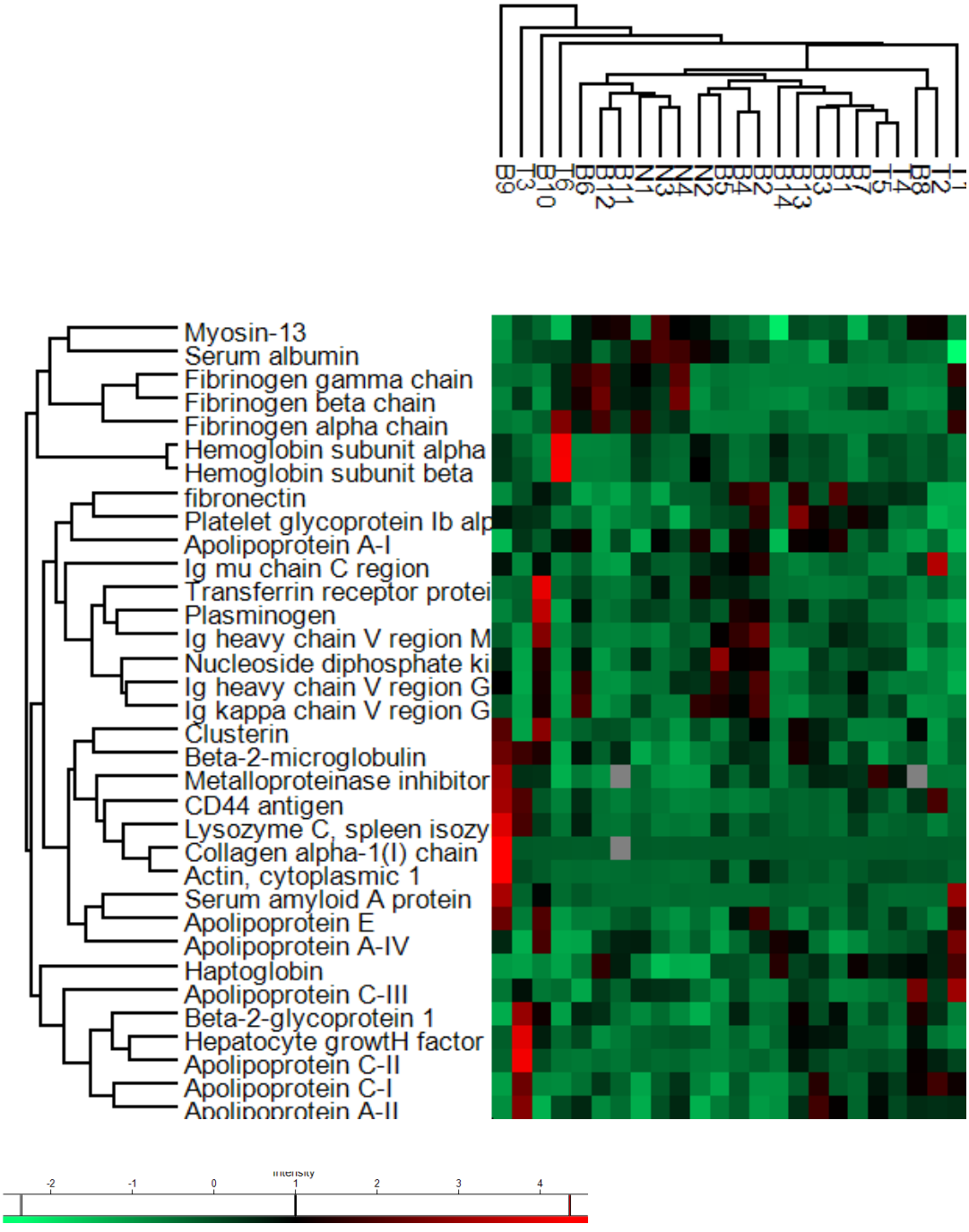

Figure 10. Heat map of protein intensities in 20 lymphoma dogs compared with 4 healthy dogs are shown. In total, 34 different proteins have been found in both groups. Green to red was showed the expression of protein from low to high intensities.

Table 10: Summary protein identified, Gene ID, Number of unique peptides, MW, Average sequence coverage (%)

No.	Accession No.	Protein name	Gene ID	Number of unique peptides	MW [kDa]	calc. pI	Average sequence coverage (%)
1	E2RAK7	Apolipoprotein A-II	APOA2	11	11.22	6.65	40.71
2	P12278	Apolipoprotein C-II	APOC2	8	11.22	4.88	28.57
3	P12279	Apolipoprotein C-III	APOC3	2	10.90	5.38	6.268
4	P18649	Apolipoprotein E	APOE	27	35.33	5.24	1.64
5	P25473	Clusterin	CLU	23	51.76	5.91	44
6	Q9XSJ7	Collagen alpha-1(I) chain	COL1A1	1	138.68	5.87	7.46
7	P68213	Fibrinogen alpha chain	FGA	4	2.96	6.48	21.37
8	P12800	Fibrinogen gamma chain	FGG	2	2.69	4.75	40.37
9	Q28275-1	Fibronectin	FN1	21	57.67	6.43	5.26
10	P60529	Hemoglobin subunit alpha	HBA	15	15.21	8.06	22.22
11	P60524	Hemoglobin subunit beta	HBB	27	15.99	8.05	14.73
12	P01785	Ig heavy chain V region MOO	N/A	3	12.70	4.72	48.51
13	P01874	Ig mu chain C region	N/A	2	48.87	6.13	24.33
14	P81709	Lysozyme C, spleen isozyme	N/A	2	14.57	8.81	11.54
15	P81546	Metalloproteinase inhibitor 1	TIMP1	2	22.84	8.44	4.22
16	Q076A3	Myosin-13	MYH13	1	223.20	5.68	9.66
17	Q50KA9	Nucleoside diphosphate kinase A	NME1	1	17.17	6.01	6.5
18	P80009	Plasminogen	PLG	19	36.65	8.1	18.52
19	Q28256	Platelet glycoprotein Ib alpha chain	GP1BA	4	74.09	5.91	0.31
20	Q28284	CD44 antigen	CD44	2	38.04	6.34	70.17
21	P02677	Fibrinogen beta chain	FGB	5	3.73	5	59.81
22	P19006	Haptoglobin	HP	56	36.43	6.09	63.1
23	P01784	Ig heavy chain V region GOM	N/A	1	12.42	5.4	59.09
24	P01618	Ig kappa chain V region GOM	N/A	2	12.00	6.61	54.06
25	P49822	Serum albumin	ALB	144	68.56	5.69	69.27
26	P19708	Serum amyloid A protein	SAA1	13	14.33	7.43	58.33
27	Q9GLD3	Transferrin receptor protein 1	TFRC	6	86.59	6.3	72.1

Table 10: Summary protein identified, Gene ID, Number of unique peptides, MW, Average sequence coverage (%), and One-way ANOVA of the serum proteomics (continue)

No.	Accession No.	Protein name	Gene ID	Number of unique peptides	MW [kDa]	calc. pI	Average sequence coverage (%)
28	O18840	Actin, cytoplasmic 1	ACTB	12	41.71	5.48	77.33
29	P02648	Apolipoprotein A-I	APOA1	57	30.18	5.3	75.19
30	E2RE76	Apolipoprotein A-IV	APOA4	36	42.51	6.09	83.2
31	P56595	Apolipoprotein C-I	APOC1	18	9.74	9.41	100
32	P33703	Beta-2-glycoprotein 1	APOH	25	38.38	8.13	89.56
33	P19341	Beta-2-microglobulin	B2M	2	4.84	9.85	90.32
34	Q6QNF4	Hepatocyte growth factor activator	HGFAC	9	70.20	7.65	96.22

Table 11: Summary protein identified from the serum, biological processes, and molecular function

No.	Protein	Biological Process	Molecular Function
1	Apolipoprotein A-II	cell organization and biogenesis; metabolic process; regulation of biological response to stimulus; transport	enzyme regulator activity; protein binding; transporter activity
2	Apolipoprotein A-IV	cell organization and biogenesis; defense response; metabolic process; regulation of biological process; response to stimulus; transport	antioxidant activity; catalytic activity; enzyme regulator activity; metal ion binding; protein binding; transporter activity
3	Actin, cytoplasmic 1	ATP-dependent chromatin remodeling; platelet aggregation; retina homeostasis; substantia nigra development	ATP binding, identical protein binding, kinesin binding, nitric-oxide synthase binding, nucleosomal DNA binding, RNA polymerase II core promoter proximal region sequence-specific DNA, binding RNA polymerase II distal enhancer sequence-specific DNA binding, structural constituent of postsynaptic actin cytoskeleton, Tat protein binding
4	Ig kappa chain V region GOM	Miscellaneous	protein binding
5	Ig heavy chain V region GOM	antigen binding	protein binding
6	Ig heavy chain V region MOO	Miscellaneous	protein binding
7	Ig mu chain C region	adaptive immune response; antibacterial humoral response; B-cell receptor signaling pathway; complement activation; classical pathway; defense response to gram-negative bacterium; innate immune response; leukocyte migration; phagocytosis; engulfment; phagocytosis; recognition; positive regulation of B cell activation	protein binding
8	Apolipoprotein A-I	cell organization and biogenesis; metabolic process; regulation of biological process; response to stimulus; transport	enzyme regulator activity; protein binding; transporter activity
9	Fibrinogen beta chain	coagulation; defense response; response to stimulus	hemostasis; wound repair; thrombus formation; antibacterial immune response both innate and T-cell mediated pathways
10	Apolipoprotein C-II	metabolic process; regulation of biological process; transport	enzyme regulator activity
11	Apolipoprotein C-III	cell organization and biogenesis; metabolic process; regulation of biological process; transport	enzyme regulator activity; protein binding
12	Fibrinogen gamma chain	cell organization and biogenesis; coagulation; regulation of biological process; response to stimulus	protein binding

Table 11: Summary protein identified from the serum, biological processes, and molecular function (continue)

No.	Protein	Biological Process	Molecular Function
13	Apolipoprotein E	cell organization and biogenesis; metabolic process; regulation of biological process; response to stimulus; transport	enzyme regulator activity; protein binding; transporter activity
14	Haptoglobin	defense response; metabolic process; response to stimulus	antioxidant activity; catalytic activity; protein binding
15	Beta-2-microglobulin	response to stimulus	protein binding
16	Serum amyloid A protein	component movement; defense response; response to stimulus; acute-phase response	chemoattractant activity
17	Clusterin	cell death; metabolic process; regulation of biological process; response to stimulus	catalytic activity; protein binding
18	Beta-2-glycoprotein 1	metabolic process; regulation of biological process; response to stimulus	enzyme regulator activity; protein binding
19	Serum albumin	cell communication; regulation of biological process; response to stimulus; transport	DNA binding; metal ion binding
20	Apolipoprotein C-I	cell organization and biogenesis; metabolic process; regulation of biological process; transport	enzyme regulator activity
21	Hemoglobin subunit beta	transport	metal ion binding; transporter activity
22	Hemoglobin subunit alpha	transport	metal ion binding; transporter activity
23	Fibrinogen alpha chain	coagulation; defense response; response to stimulus	Hemostasis; wound repair; thrombus formation; antibacterial immune response both innate and T-cell mediated pathways
24	Plasminogen	coagulation; metabolic process; regulation of biological process	catalytic activity
25	Metalloproteinase inhibitor 1	regulation of biological process; response to stimulus	enzyme regulator activity; metal ion binding; protein binding
26	Lysozyme C, spleen isozyme	defense response; metabolic process; response to stimulus	catalytic activity
27	Myosin-13	cell organization and biogenesis; regulation of biological process	catalytic activity; motor activity; nucleotide binding; protein binding
28	Platelet glycoprotein Ib alpha chain	coagulation; regulation of biological process; response to stimulus	enzyme regulator activity; protein binding
29	fibronectin	defense response; regulation of biological process; response to stimulus	protein binding
30	CD44 antigen	cell adhesion; regulation of lamellipodium morphogenesis; wound healing; spreading of cells	hyaluronic acid binding

Table 11: Summary protein identified from the serum, biological processes, and molecular function (continue)

No.	Protein	Biological Process	Molecular Function
31	Nucleoside diphosphate kinase A	cell differentiation; metabolic process; transport	catalytic activity; metal ion binding; nucleotide binding
32	Hepatocyte growtH factor activator	metabolic process	catalytic activity; metal ion binding; protein binding
33	Transferrin receptor protein 1	cellular homeostasis; metabolic process; regulation of biological process; transport	receptor activity; transporter activity
34	Collagen alpha-1(I) chain	blood coagulation; blood vessel development; bone trabecula formation; cartilage development involved in endochondral bone morphogenesis; cellular response to amino acid stimulus, epidermal growth factor stimulus, fibroblast growth factor stimulus, fluoride, mechanical stimulus, retinoic acid, transforming growth factor beta stimulus, tumor necrosis factor; cellular response to vitamin E; collagen biosynthetic process; collagen catabolic process; collagen fibril organization; collagen-activated tyrosine kinase receptor signaling pathway, embryonic skeletal system development, endochondral ossification, extracellular matrix organization, face morphogenesis, intramembranous ossification, leukocyte migration, negative regulation of cell-substrate adhesion, osteoblast differentiation	metal ion binding; protein binding; structural molecule activity, extracellular matrix structural constituent, identical protein binding, metal ion binding, platelet-derived growth factor binding, protease binding, protein binding

Table 12: Summary protein identified, Cellular Component, Molecular function, and Multiple Comparisons by One-way ANOVA and Mann-Whitney *U* test.

Accession	Description	Cellular Component	Molecular Function	Multiple Comparisons		
				ANOVA Sig.	Man-Whitney test	Sig.
P01784	Ig heavy chain V region GOM	extracellular	protein binding	0.018	T > B	0.008
P19006	Haptoglobin (Hp)	extracellular	antioxidant activity catalytic activity protein binding	0.030	B > N	0.006
P19341	Beta-2-microglobulin (β2M)	extracellular	protein binding	0.023	B > N	0.003
P25473	Clusterin/ Apolipoprotein J (Apo J)	Cytoplasm Cytosol endoplasmic reticulum extracellular Golgi Membrane Mitochondrion Nucleus organelle lumen	catalytic activity protein binding	0.014	B > T	0.005
P49822	Serum albumin	Extracellular	DNA binding metal ion binding	7×10^{-7}	N > T N > B	0.011 0.003
P56595	Apolipoprotein C-I (Apo C)	endoplasmic reticulum extracellular	enzyme regulator activity	0.024	B > T	0.032

The mean difference is significant at the 0.05 level

T = T-cell lymphoma

B = B-cell lymphoma

N = normal

Table 13: Summary of protein identified, Mean, and SD of normal and lymphoma groups by One-way ANOVA

No.	Protein name	$\bar{x}$ + SD			*p* valve (ANOVA)
		Normal (*N* = 4)	T-cell lymphoma (*N* = 6)	B-cell lymphoma (*N* = 14)	
1	Apolipoprotein A-II	78.68 + 52.37	153.22 + 58.59	100.30 + 51.71	0.08
2	Apolipoprotein C-II	63.12 + 18.05	199.70 + 216.11	93.54 + 62.43	0.13
3	Apolipoprotein C-III	113.73 + 23.81	132.12 + 76.56	92.24 + 51.40	0.35
4	Apolipoprotein E	71.00 + 25.80	96.48 + 63.40	118.78 + 71.44	0.42
5	Clusterin	84.05 + 14.60	76.49 + 11.23	121.35 +38.71	0.01
6	Collagen alpha-1(I) chain	11.47 + 6.44	19.99 + 10.12	202.56 +725.08	0.74
7	Fibrinogen alpha chain	136.15 + 116.83	127.97 +174.76	66.59 +85.70	0.43
8	Fibrinogen gamma chain	165.18 + 123.37	90.28 + 92.79	76.13 + 93.93	0.30
9	Fibronectin	82.98 + 17.64	92.62 +30.56	116.43 + 47.16	0.26
10	Hemoglobin subunit alpha	91.35 +43.38	126.67 +152.85	52.85 + 32.53	0.22
11	Hemoglobin subunit beta	88.48 + 53.84	125.85 + 152.85	52.85 + 32.53	0.20
12	Ig heavy chain V region MOO	69.48 + 20.83	62.86 +32.34	132.51 +92.01	0.12
13	Ig mu chain C region	96.9 +24.35	53.03 +18.29	120.42 + 53.54	0.98
14	Lysozyme C, spleen isozyme	45.85 +24.53	104.78 + 126.78	131.93 + 146.63	0.52
15	Metalloproteinase inhibitor 1	66.02 + 17.81	116.6 +61.65	108.14 + 70.27	0.44
16	Myosin-13	125.5 + 21.96	107.53 +16.32	108.43 + 19.12	0.27
17	Nucleoside diphosphate kinase A	113.53 + 32.09	71.23 + 43.03	123.9 + 74.29	0.25
18	Plasminogen	106.65 + 9.21	74.52 + 16.04	115.91 + 44.60	0.09
19	Platelet glycoprotein Ib alpha chain	77.35 + 26.12	82.68 + 43.88	126.76 + 55.35	0.10
20	CD44 antigen	64.35 + 12.83	141.15 + 94.07	110.13 + 74.96	0.30
21	Fibrinogen beta chain	156.80 + 124.63	105.28 + 57.29	85.22 + 87.33	0.37
22	Haptoglobin	30.08 + 23.43	115.38 + 72.13	114.50 + 50.19	0.03
23	Ig heavy chain V region GOM	110.08 + 24.25	102.92 + 121.65	110.06 + 57.95	0.02
24	Ig kappa chain V region GOM	108.68 + 58.47	66.63 + 28.55	114.37 + 60.99	0.22
25	Serum albumin	122.32 + 4.82	90.72 + 13.97	96.62 + 7.64	7×10^{-7}
26	Serum amyloid A protein	11.55 + 6.83	155.85 + 329.50	116.84 + 237.12	0.66
27	Transferrin receptor protein 1	135.08 + 54.29	86.88 + 16.55	109.94 + 86.82	0.59
28	Actin, cytoplasmic 1	51.17 + 6.13	89.33 + 26.17	127.33 + 162.85	0.56
29	Apolipoprotein A-I	111.82 + 36.09	94.78 + 24.77	103.94 + 32.99	0.7
30	Apolipoprotein A-IV	83.90 + 27.43	103.88 + 66.07	104.92 + 44.50	0.92
31	Apolipoprotein C-I	82.00 + 39.96	166.17 + 71.62	92.88 + 48.58	0.02
32	Beta-2-glycoprotein 1	77.58 + 12.85	113.87 + 51.67	112.67 + 28.80	0.19
33	Beta-2-microglobulin	56.02 + 15.05	88.87 + 58.97	129.86 + 45.92	0.02
34	Hepatocyte growth factor activator	99.83 + 4.01	119.27 + 88.34	108.08 + 30.87	0.82

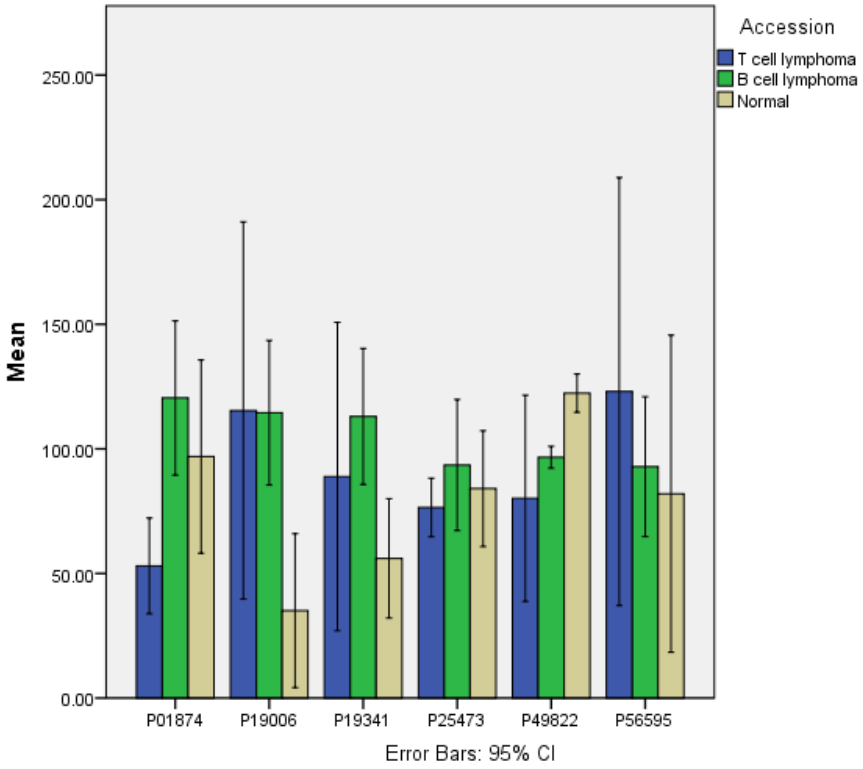

Figure 11. Summary of six proteins were significantly different between control and lymphoma group by One-way ANOVA

CHAPTER V

DISCUSSION

In general, disease causing agents elicit a response from the immune system and that is why the reaction and action of the proteins of the immune system can be used in diagnosing or treatment of diseases. Clinically, the rapid and accurate diagnosis is the key for the success in cancer treatment. Tissue biopsy and histopathology are time-consuming, and patients with poor condition may have to avoid invasive diagnostic processes such as sedation and bleeding complication. For the above reasons, tumor marker was invented and developed.

In human, follicular lymphoma and diffuse large B cell lymphomas is the main of adult B-cell malignancies as well as canine lymphomas. Several lymphoma entities have been studied using the proteomic approach. Their samples consisted of frozen tumor tissues, lymph nodes, and serum of the malignant lymphoma cases. All previous studies presented proteins/protein peaks with different clinical point. Most of studies had performed in the small patient cohorts. For example, the previous report suggested that there were the loss of expression of β2M, and gain of expression of PRDX1 and PPIA in the primary lymphoma tissues and cell lines in human by using SELDI-TOF MS (Wu et al., 2016).

There are two main techniques applying in the proteomic researches, including 1) gel base; one-dimensional gel electrophoresis (1DE), two-dimensional gel electrophoresis (2DE), two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and 2D gel-free; LC-MS/MS, Label free. Labeling isobaric tags is a gel-free technique that based on the mass difference between differentially labeled peptides. In the current experiment, TMT is a new useful and suitable technique because it can analyse the samples up to 10 samples per single mass spectromemtry (MS) injection. Due to TMT reagents, or isobaric mass tags which is reporter region, but use different elemental isotope with a neutron difference of 0.0063 Da. in various position. The tags contain four regions such as a mass reporter region (M), a cleavable linker region (F), a mass normalization region (N) and a protein reactive group (R). The combined M-F-N-R regions of the tags have the same total molecular weights and when amine-reactive chemical groups in biomolecular probes for labeling and crosslinking primary amines include NHS esters (N-hydroxysuccinimide esters), imidoesters, and lysine. During chromatographic or electrophoretic separation and in single MS mode, molecules labeled with different tags are indistinguishable. Upon

fragmentation in MS/MS mode, sequence information is obtained from fragmentation of the peptide back bone and quantification data are simultaneously obtained from fragmentation of the tags, giving rise to mass reporter ions.

The advantages of TMT are enables sensitive, high resolution, precise multiplexed peptide and protein quantification, easy to perform due to automation, unlimited number of experimental and higher accuracy than other labeling-based methods comparing with gel base techniques which has low thoughput and dynamic range. Therefore, the benefits of using LC-MS may be helpfully used for clinical application and preclinical diagnosis by serum biomarker. However, there are some disadvantages, including the incomplete peptide labeling and high cost.

One of the major components of the serum proteomic is Ig heavy chain V region GOM which is the large polypeptide subunit of an antibody (immunoglobulin). These heavy chain types which play important role in the maturation of B cells vary between different animals. All heavy chains contain a series of immunoglobulin domains, usually with one variable domain (V_H) that is important for binding antigen and several constant domains (C_H1, C_H2, etc.). Production of the viable heavy chain is a key step in B cell maturation. If the heavy chain is able to bind to a surrogate light chain and move to the plasma membrane, then the developing B cell can begin producing its light chain. This light chain which is isolated from a myeloma protein cannot fight cancer on its own until it fuses with the heavy chain to make up a complete immunoglobulin. Increased expression of Ig heavy chain V region GOM in human was found in multiple myeloma, chronic lymphatic leukemia, and lymphoma. Furthermore, it was found that monoclonal and polyclonal were associated with reactive is used as a biomarker in dog such as X-linked hereditary nephropathy (Nabity et al., 2012), snake envenomation (Hrovat et al., 2013), pyometra (Maddens et al., 2011), leishmaniasis (Solano-Gallego et al., 2003), leptospirosis (Zaragoza et al., 2003), hypercortisolism (Smets et al., 2012), inflammatory conditions, chronic inflammatory processes, severe infections, and immune-mediated disorders (Tothova et al., 2016).

The current results also revealed an increasing of haptagloblin (Hp) in the lymphoma dogs. Hp is a colorless protein produced by the liver for binding hemoglobin released from red blood cell destruction. Hp is one of the acute phase protein (APPs) in the nonspecific inflammatory reaction. In dogs, the glycosylation pattern of Hp can vary in dogs with various inflammatory, autoimmune, and neoplastic diseases. The biological functions of Hp involve in host's defense response to infection and inflammation. Hp has a bactericidal effect by binding

hemoglobin and limiting the availability of hemoglobin iron for bacterial growth. It also inhibits granulocyte chemotaxic and phagocytosis (Ceron et al., 2005). Increased expression of Hp in the animals was found in inflammatory diseases and trauma nephrotic syndrome (Tothova et al., 2016). Moreover, Mischke et al. (2007) reported that C-reactive protein and Hp can be used as a tumor marker in canine lymphoma and the commercial canine CRP ELISA kit had been developed in 2016. Jeffrey et al. also found the increasing of C-reactive protein, haptoglobin, and thymidine kinase in canine lymphoma by using SELDI-TOF and ELISA techniques. However, haptoglobin production may be induced by the administration of exogenous corticosteroids, but the lymphoma dogs in this study had not been treated with any corticosteroids.

Beta-2-microglobulin (β2M) is a small polypeptide, which is the light chain subunit of the major histocompatibility complex class I antigen. β2M presents in nearly all nucleated cells and most biological fluids, including serum, urine, and synovial fluid. β2M acts as an apoptosis-inducing factor and it's able to stimulate various growth and progression (Ling Li et al., 2016). β2M elevation was found in several lymphoproliferative disorders including chronic lymphocytic leukemia, lymphoma, Hodgkin's disease, and multiple myeloma (Hagop et al., 1992). There are many studies in human that used serum β2M as an indicator in a new prognostic model and be used as a prognostic factor with malignant lymphoma patients (Changhoon et al., 2014). In human, several studies showed the increased levels of β2M in cases such as inflammatory diseases, infections, active hepatitis, chronic liver diseases, liver cirrhosis, hypercholesterolemia, iron deficiency anemia, nephrotic syndrome (Tothova et al., 2016), and early stages of X-linked hereditary nephropathy (Nabity et al. 2012). Our result showed that β2M increased in the lymphoma groups. Although, the mechanism underlying the prognosis value of serum β2M in canine malignant lymphomas remain unclear but previous findings showed that elevated serum β2M was associated with advanced disease stage, indicating a greater tumor burden. In human follicular lymphoma, β2M is a potential target for cancer therapy and being investigated in the pre-clinical setting. The biologic agents against β2M have shown the promising anti-cancer activity and become a useful biomarker for treatment with anti-β2M therapeutic drugs. Therefore, β2M may be one of the potential biomarker for canine lymphoma.

Clusterin or apolipoprotein J (Apo J) is a heterodimeric highly conserved secreted glycoprotein. It has being expressed in a wide variety of tissues and found in all human fluids. Its function has been reported in several physiological processes such as sperm maturation, lipid transportation, regulation of complement, function

complement inhibitions, tissue remodeling, membrane recycling, cell-cell and cell-substratum interactions, stabilization of stressed proteins in a folding-competent state, promotion or inhibition of apoptosis, and drug-induced acute kidney injury (Ioannis et al., 2002; Zhou et al., 2014). In animal studies, increasing of clusterin was found in inflammatory diseases, trauma nephrotic syndrome (Tothova et al., 2016). Decreasing of serum clusterin was found in DCM dogs due to increased vascular permeability and the venous return loss and response to heart failure (Kocaturk et al.,2015). The current study demonstrated that clusterin increased in lymphoma dogs. Previous report indicated that the hypothetical indirect tumorgenesis role of clusterin may involve in the introduction or up-regulation of a cellular background of genomic instability. Moreover, it could also be speculated due to its implication in cell-cell and cell-substratum interactions, apoptotic process, and in a cytoprotective role of plasma membrane (Trougakos and Gonos, 2002).

In this study also was found the increased of apolipoprotein C-I (Apo C-I) in lymphoma dogs compared to healthy dogs. Apo C is a small polypeptide of 27 amino acids, associated with transport of chylomicrons, triglyceride, cholesterol, and fatty acids etc. It is normally found in plasma and responsible for the activation of the esterified lecithin cholesterol with an important role in the exchange of esterified cholesterol between lipoproteins and in removal of cholesterol from tissues. The main function of Apo C-I is inhibition of cholesteryl ester transfer protein (CETP), probably by altering the electrical charge of HDL molecules. In human, it was found that the increasing of Apo C-I associated with Alzheimer's disease (AD) (Caroline Petit-Turcotte et al., 2001). In veterinary research, previous report indicated that Apo C mRNA is only detectable in liver tissue of dogs by using slot blot hybridization of total RNA isolated from various tissues (Chi-Cheng Luo et al., 1989). So, it may suggest that increased of Apo C-I may be affected by the damage of liver induced by lymphoma subsequently.

Serum protein has 35-50% of albumin in total (Keneko et al., 1997). Albumin is important in maintaining homeostasis, transporting substances, and acting as a free-radical scavenger (Hankins et al., 2006). Serum albumin is the major phase negative acute phase protein and its synthesis may be markedly reduced during the acute inflammation. During the acute phase response, the positive acute phase proteins is markedly increased, in which the hepatic protein synthesis due to albumin synthesis is down-regulated and amino acids are shunted toward the synthesis of the positive acute phase protein (Aldred et al., 1993). The decreased levels of serum albumin were shown in liver disease, chronic hepatitis, cirrhosis, liver failure, renal diseases,

nephritic syndrome, chronic malnutrition, gastrointestinal diseases, protein-losing enteropathy, internal parasitism, and acute inflammation (Tothova et al., 2016). Similarly, there are significantly increased levels of urinary markers in dogs with lymphoma and osteosarcoma (Kovarikova, 2015). Although, our current study also found the decrease of albumin in lymphoma dogs, but it might be considered that there are many factors affect the albumin level. Albumin may be not suitable for the specific biomarker for lymphoma.

Some protein expression in this current study was differed from the previous study was found that kininogen was absent for the sera of dogs with lymphoma, but present in normal dogs (Atherton et al., 2013b; Atherton et al., 2013a). Kininogen is a precursor for the inflammatory protein bradykinin, cofactor for coagulation factor XII in the intrinsic coagulation pathway, and part of a pro-inflammatory response in lymphoid neoplasm. Our study did not demonstrate kininogen in both groups. It may cause by the interference of high abundant protein in serum and the incomplete peptide labeling.

For this report, B-cell and T-cell lymphoma proteomic profiling was also analysed compared with healthy dogs. The result were found significantly increasing in expression of several proteins such as apolipoprotein A-II and Plasminogen but it was not clearly different.

The aim of the study is to clarify the serum proteomic changes as biomarkers to ascertain the stage and severity of canine lymphomas for rapid and proper treatment. Serum biomarker which is a secretory protein will be the new choice. By implication, effective biomarkers for cancer screening facilitate disease identification in sub-clinically affected patients and lead to subsequent improvements in clinical outcome. There are also many variable of result depend many the condition study. For this reason, biopsy still is the gold standard for diagnosis but application of serum proteomic will help for early detection of the subclinical symptom. Both of techniques should be done together accuracy and precision diagnosis. Potential applications of biomarkers to veterinary oncology may be useful for diagnosis, staging, prognosis, and monitoring the response to therapy. By the way, this study had some limitations. First, the number of the dogs available for the studies is not enough to represent variables of the whole population such as breeds, sex variation, and clinical stage. Secondly, the depletion column should be applied for elimination the interference of high abundant protein in the proteomic process. Further study with appropriate number of animals, various breeds should be investigated.

CONCLUSION

Proteomic profiles of canine lymphoma demonstrated the different protein pattern between healthy dog, B-cell lymphoma and T-cell lymphoma dogs. The current studies demonstrated six proteins were significantly different between control and lymphoma group such as albumin, clusterin or apolipoprotein J, Ig heavy chain V region GOM, beta-2-microglobulin, apolipoprotein C-I, and haptoglobin. These protein panels may be the possibly candidate biomarkers for canine lymphoma in the future. However, there are several limitations such as the low number of the samples and the variation of the pateints. Therefore, the further study should be investigated in the proper number of population and should use the advance or helpful techniques such as depletion column for more precise proteomic profiling.

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APPENDIX

Appendix A: Summary of 34 canine proteins identified and abundances scaled of the serum of lymphoma dogs and healthy dogs

Accession	Description	Abundances (Scaled)							
		T1	T2	T3	T4	T5	T6	B1	B2
E2RAK7	Apolipoprotein A-II	134.1	131.9	267.2	112.3	160.7	113.1	170.7	50
E2RE76	Apolipoprotein A-IV	222.1	127.7	43.5	94.4	89.5	46.1	50.1	123.2
O18840	Actin, cytoplasmic 1	65.3	97.3	107.3	109	110.9	49.2	56.8	183.3
P01618	Ig kappa chain V region GOM	65.2	53.3	40.1	112.6	87.4	41.2	88.9	210.8
P01784	Ig heavy chain V region GOM	33.9	61	38	65.7	79.4	40.3	99	202.4
P01785	Ig heavy chain V region MOO	31.5	88.1	28.1	90.3	97.5	41.7	147.7	286.4
P01874	Ig mu chain C region	46.2	351.1	52	55.1	55.4	57.7	92.7	213.2
P02648	Apolipoprotein A-I	58.3	89.6	112.5	77.4	104.5	126.4	146.7	125.1
P02677	Fibrinogen beta chain	160	28.9	127.3	36.6	134.6	144.3	63.1	36.5
P12278	Apolipoprotein C-II	127	184	631.5	94.1	113.3	48.3	84	77.1
P12279	Apolipoprotein C-III	277.6	120.5	149.8	85.3	77	82.5	63.7	67.4
P12800	Fibrinogen gamma chain	257.2	29.5	53.6	28.4	30.4	142.6	28.6	23.6
P18649	Apolipoprotein E	211.9	56.5	64	122.4	85.3	38.8	70.3	222.7
P19006	Haptoglobin	219.2	132.5	33.5	144.8	131.3	31	76.8	78.6
P19341	Beta-2-microglobulin	91.6	58.8	202.8	85.3	51.7	43	75.1	124
P19708	Serum amyloid A protein	828.1	19	34.4	10.8	33.3	9.5	11.1	8
P25473	Clusterin	94.5	66.6	62.5	79.2	78	78.1	107	139.6
P33703	Beta-2-glycoprotein 1	82.8	120.4	212.4	90.3	108.7	68.6	136.5	89.8
P49822	Serum albumin	63.5	92.8	98.3	96.1	91.5	102.1	102	99.4
P56595	Apolipoprotein C-I	181.7	222.4	258.9	122.4	152.9	58.7	97.4	51.3
P60524	Hemoglobin subunit beta	36.1	74	69.2	77.3	62.1	436.4	55.3	51.7
P60529	Hemoglobin subunit alpha	38.2	79.8	58.2	79.3	88.9	415.6	61.4	59.5
P68213	Fibrinogen alpha chain	287.6	15.1	14	25.3	17.3	408.5	14	11.9
P80009	Plasminogen	54.2	67.9	94.5	85	85.3	60.2	110.5	149.6
P81546	Metalloproteinase inhibitor 1	81.5	82.1	133.4	154.1	210.2	38.3	139.5	133.2
P81709	Lysozyme C, spleen isozyme	67.8	23.5	360.8	74.8	59.4	42.4	50.3	113.4
Q076A3	Myosin-13	99.7	133.3	112.1	105.3	111.1	83.7	109.9	95.5
Q28256	Platelet glycoprotein Ib alpha chain	45	33.9	128.3	73.8	142.4	72.7	134.2	191.3
Q28275-1	Fibronectin	53.4	54.6	102.2	121.4	114.2	109.9	190.9	184
Q28284	CD44 antigen	82.7	256.5	265	77.4	107.9	57.4	76.9	68.6
Q50KA9	Nucleoside diphosphate kinase A	37.7	59.6	34.3	123.2	128	44.6	94.8	181.7
Q6QNF4	Hepatocyte growtH factor activator	67.8	84.8	298.6	79.3	93.5	91.6	140.3	72.7
Q9GLD3	Transferrin receptor protein 1	57.5	94.4	88.5	93.4	106.2	81.3	67.4	111.9
Q9XSJ7	Collagen alpha-1(I) chain	11.6	26.8	37.2	16.8	11.4	16.1	4.8	4

Appendix A: Summary of 34 canine proteins identified and abundances scaled of the serum of lymphoma dogs and healthy dogs (continue)

Accession	Description	Abundances (Scaled)							
		B3	B4	B5	B6	B7	B8	B9	B10
E2RAK7	Apolipoprotein A-II	215.9	96.9	73.8	138.2	91	135.2	60	33.3
E2RE76	Apolipoprotein A-IV	78.9	83.6	51	48.8	65.2	103.3	125.7	192.8
O18840	Actin, cytoplasmic 1	82.2	40.2	149.1	53.4	84.5	93.2	675.6	80.2
P01618	Ig kappa chain V region GOM	90.1	153.9	180.7	211.1	128.7	49.4	96.2	172.4
P01784	Ig heavy chain V region GOM	106.8	137.3	184.7	183.9	146.6	68.5	150.8	166.8
P01785	Ig heavy chain V region MOO	85.8	234.5	187.9	101.5	130.8	36.9	88.6	321.2
P01874	Ig mu chain C region	73	183.2	157.4	89.9	88.1	118.4	162.9	168.9
P02648	Apolipoprotein A-I	133.5	141.5	78.8	140	92.1	70.9	59.9	96.5
P02677	Fibrinogen beta chain	48.7	46.9	63.3	217	113	22.7	37	21.6
P12278	Apolipoprotein C-II	128	58.9	31.6	41.5	96.4	249.6	75.6	111.4
P12279	Apolipoprotein C-III	103.7	106.7	51.2	38.9	67	249.7	75.4	73.8
P12800	Fibrinogen gamma chain	27.1	33	41.9	262.3	34.8	21.6	49.5	32.6
P18649	Apolipoprotein E	104.6	153.4	43	64.1	112.8	88.9	259.7	237.1
P19006	Haptoglobin	126	98.4	100.9	68.2	169.9	154.5	39.4	55.7
P19341	Beta-2-microglobulin	142.5	131.9	86.6	147.6	120.7	99.8	236	181.1
P19708	Serum amyloid A protein	16.4	12	12.6	70.3	158.3	13.6	881.3	331.5
P25473	Clusterin	125.3	115.8	77.9	93.6	77.9	137.2	182.5	207.3
P33703	Beta-2-glycoprotein 1	107.2	126.9	82.1	125.7	89.1	155.5	62.3	149.8
P49822	Serum albumin	84.3	96.5	109.7	108.3	92.7	93.7	88.1	101.5
P56595	Apolipoprotein C-I	201.7	99.4	36.2	80.1	87.3	178	42.5	61.3
P60524	Hemoglobin subunit beta	78.1	32.5	84.4	20	18.4	53.9	106.3	21.3
P60529	Hemoglobin subunit alpha	87.2	42.2	90	22.6	22	58.4	110.2	22.2
P68213	Fibrinogen alpha chain	11.8	13.1	12.1	168.6	14	43.2	29.6	63.3
P80009	Plasminogen	89.2	157	97.2	134.5	113.3	74.5	75.6	240.4
P81546	Metalloproteinase inhibitor 1	143.6	94.5	131	80	131.1		287.2	131.7
P81709	Lysozyme C, spleen isozyme	72.9	66.5	150.8	187.8	134.7	81.5	617.7	137.9
Q076A3	Myosin-13	107.5	104.3	108.3	124.2	85.5	133.9	92.5	104
Q28256	Platelet glycoprotein Ib alpha chain	171.7	117.2	109.8	89.4	173.7	79.4	144.9	123.5
Q28275-1	Fibronectin	99.4	170	113.5	56.9	119	115.9	70.4	134.5
Q28284	CD44 antigen	88.4	58.4	69.6	141.4	146.1	137.8	349.4	99.5
Q50KA9	Nucleoside diphosphate kinase A	125.5	177.6	293.5	158.3	113.1	34.8	139.4	202.1
Q6QNF4	Hepatocyte growtH factor activator	144.9	92.5	76.3	112.9	93.7	147.4	98.3	138.8
Q9GLD3	Transferrin receptor protein 1	65.3	149.2	143.8	116.1	72.7	58.1	79.5	393.1
Q9XSJ7	Collagen alpha-1(I) chain	5.6	5	11.6	5	6	6.1	2721.7	19.9

Appendix A: Summary of blood cell counts in lymphoma and control dogs

ID	WBC	Mo	Neu	Lym	Eo	Ba	Blast	Band	RBC	Hb	Hct	MCV	MCH	MCHC
Reference ranges	6000 -17000	3-10 %	60-75 %	12-30 %	2-10 %	0-05 %	0 %	0-4 %	5.5-8.5 $\times 10^{6}$ cell/mm^{3}	12-18 g/dl	37-55 %	60-77 Fl	20-25 Pg	32-35 g/dl
N1	7900	1	72	22	5	0	0	0	7.37	14.7	45.1	61.2	19.9	326
N2	12200	3	68	28	1	0	0	0	6.33	12.9	40	63.2	20.4	32.2
N3	8400	1	67	31	1	0	0	0	8.23	16.7	50	60.8	20.3	33.4
N4	10400	2	73	24	1	0	0	0	7.5	15.1	47	62.7	20.1	32.1
B1	19500	3	72	25	0	0	0	0	5	13	41	68.2	22.4	31.8
B2	39400	1	93	3	2	0	0	1	3.46	9	25.4	73	26	35.5
B3	13500	3	75	17	5	0	0	0	5.56	13.4	38.2	69	24	34.9
B4	9930	3	82	13	2	0	0	0	4.86	11.5	33.7	69.3	23.7	34.2
B5	8440	1	77	18	4	0	0	0	3.23	7.9	24.8	76.9	24.5	31.9
B6	11700	1	76	22	0	1	0	0	4.91	11.4	32.7	-	-	-
B7	15100	2	77	20	1	0	0	0	8.14	19.2	58.2	71	23.6	33
B8	16100	5	91	1	3	0	0	0	2.44	5.8	18.9	77	23.8	30.8
B9	23400	1	34	62	3	0	0	0	6.08	14.2	43.6	72	23.3	32.5
B10	38800	1	66	32	1	0	0	0	4.56	10.1	30.9	68	22.2	32.7
B11	73800	4	91	4	0	0	0	1	4.73	11.2	35	-	-	-
B12	14600	0	87	13	0	0	0	0	5.03	13	37.6	0	0	0
B13	5800	5	85	10	0	0	0	0	7.22	16.4	49.4	68	22.7	33.2
B14	9190	1	82	14	1	0	2	0	5.35	11.6	37.3	69.7	21.7	31.1
T1	28600	3	80	17	0	0	0	0	5.2	11.7	36.4	71	22.3	31.3
T2	33800	3	19	66	1	0	11	0	4.87	11.6	35.2	72	23.7	32.9
T3	6500	0	80	14	4	0	0	2	5.43	11.9	36.6	67	22	32.6
T4	14600	0	87	13	0	0	0	0	5.03	13	37.6	-	-	-
T5	17333	0	85	15	0	0	0	0	7.32	16.6	50.1	-	-	-
T6	5000	2	61	36	1	0	0	0	5.58	13.8	39.3	71	24.7	35

N: Normal, B: B cell lymphoma, T: T cell lymphoma,

Mo: Monocyte, Neu: Neutrophil, Lym: Lymphocyte, Eo: Eosinophil, Ba: Basophil, Blast: Lymphoblast,

Band: Band neutrophil, RBC: Red blood cell count, Hb: Hemoglobin, MCV: mean corpuscular volume,

MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration

Appendix B: Summary of blood chemical profiles in lymphoma and control dogs

ID	Platelets	RDW	PP	Platelets smear	ALT	ALP	Cr	BUN	Cal	TP	Alb
Reference ranges	200000-500000	12-15 %			10-100 U/L	20-120 U/L	0.5-1.5 mg/dl	7-27 mg/dl	8.9-11.4 mg/dl	5.2-7.9 g/dl	2.7-4.2 g/dl
N1	338000	-	-	Adequate	44	122	1.18	10	-	8.6	4.6
N2	225000	14.4	-	Adequate	44	-	1.13	-	-	-	-
N3	267000	14.4	-	Adequate	49	-	1.13	-	-	-	-
N4	242000	14.4	-	Adequate	39	-	39	-	-	-	-
B1	75000	-	-	Decrease	360	257	0.6	13	-	-	2
B2	161000	15.2	8	Adequate	29	128	0.6	6	11.5	-	-
B3	406000	15	24	Adequate	40	62	17	0.9	9.7	-	-
B4	328000	12.3	8.8	Adequate	18	54	1.22	19	-	-	-
B5	219000	16.2	7.2	Adequate	730	375	1.45	13	-	-	-
B6	72100	-	-	Decrease	27	66	0.8	12.4	9.8	6.1	2.9
B7	190000	15.8	7.8	Adequate	30	52	1.1	18	-	-	-
B8	205000	19.2	8.8	Adequate	54	-	1.6	-	9.9	6.4	2.2
B9	531000	16	9.8	Increase	26	56	0.8	9	7.1	7.5	2.6
B10	542000	14.4	10.8	Adequate	14	48	0.9	11	-	-	-
B11	158000	-	-	Decrease	40	1661	0.5	34.9	6.8	4.6	2.2
B12	211000	0	0	Adequate	241	798	0.7	19.6	-	-	-
B13	274000	13.5	7.6	Adequate	483	-	1.23	33	9	-	-
B14	254000	12.8	9.2	Adequate	57	-	0.96	-	-	-	-
T1	210000	-	-	Adequate	390	990	1.8	24	-	-	-
T2	53000	16.2	8.6	Decrease	273	790	0.9	38	8.2	5.6	2.8
T3	103000	15.5	6	Decrease	157	1039	1.7	49	-	3.6	2.3
T4	211000	-	-	Adequate	241	798	0.7	19.6	-	7	3.7
T5	244000	-	-	Adequate	51	53	1.19	17	10.3	-	-
T6	495000	14.7	9.8	Adequate	180	1789	0.6	22	-	-	-

N: Normal, B: B cell lymphoma, T: T cell lymphoma,

RDW: Red blood cell distribution width, PP: Plasma Protein, ALT: Alanine transaminase,

ALP: Alkaline phosphatase, Cr: Creatinine, BUN: blood urea nitrogen, Cal: Calcium, TP: Total protein,

Alb: Albumin

Appendix C: Summary of 34 canine proteins identified and abundances scaled of the serum of lymphoma dogs and healthy dogs

Accession	Description	Abundances (Scaled)							
		B11	B12	B13	B14	N1	N2	N3	N4
E2RAK7	Apolipoprotein A-II	85	63.8	132.2	58.3	35.9	33.5	107	138.3
E2RE76	Apolipoprotein A-IV	129	110.8	143.5	163	131.6	91.4	86	66.2
O18840	Actin, cytoplasmic 1	50.5	47.9	74.6	111.1	59	48.4	52.6	44.7
P01618	Ig kappa chain V region GOM	61.9	45.9	52.1	59.1	110.3	190.8	67.4	66.2
P01784	Ig heavy chain V region GOM	51.2	53	72.3	62.6	76	116.1	75.7	119.8
P01785	Ig heavy chain V region MOO	46	48.2	84.1	55.5	59.9	78.8	45.9	93.3
P01874	Ig mu chain C region	27.9	35.7	56.1	73.4	128.9	132.9	92.5	86
P02648	Apolipoprotein A-I	104.2	69.7	138	58.2	63.8	145.5	132.5	105.5
P02677	Fibrinogen beta chain	153.5	315.7	23.7	30.4	165	22.1	119.1	321
P12278	Apolipoprotein C-II	48.2	62.8	198.8	45.7	77.5	37.1	65.4	72.5
P12279	Apolipoprotein C-III	87.8	116.5	121	68.6	130.2	78.5	125.6	120.6
P12800	Fibrinogen gamma chain	149	305.1	27.8	28.9	199.5	16.9	132.7	311.6
P18649	Apolipoprotein E	91.9	63	56.6	94.8	104.7	75.3	59.8	44.2
P19006	Haptoglobin	137	199.1	107.7	190.9	63.8	22.2	9.6	24.7
P19341	Beta-2-microglobulin	132.4	91.6	177.4	71.4	42.5	69.3	68.8	43.5
P19708	Serum amyloid A protein	12.3	19.5	41.3	47.5	14.4	9.3	3.3	19.2
P25473	Clusterin	100.5	102.8	148.4	83.1	79.4	102.5	86.7	67.6
P33703	Beta-2-glycoprotein 1	91.1	120.7	146.8	93.9	78.6	61.1	92.5	78.1
P49822	Serum albumin	103.1	93.7	90.2	89.5	120.3	116.6	127.4	125
P56595	Apolipoprotein C-I	90.5	126.8	96	51.8	36.6	65.3	96.9	129.2
P60524	Hemoglobin subunit beta	25	18.8	61.6	112.6	97.2	160.9	54.3	41.5
P60529	Hemoglobin subunit alpha	32.2	23.1	71.3	133.2	107.8	144.9	61.7	51
P68213	Fibrinogen alpha chain	113.7	317.8	45.9	73.2	289.1	6.1	111	138.4
P80009	Plasminogen	86.9	80.9	122.6	90.5	112	114.1	106.9	93.6
P81546	Metalloproteinase inhibitor 1		60.6	105.2	76.3	92.3	55.5	61.8	54.5
P81709	Lysozyme C, spleen isozyme	40.7	47.1	62.6	83.1	82	29.7	31.5	40.2
Q076A3	Myosin-13	136.5	133.9	111.3	70.7	95.9	127.2	148.9	130
Q28256	Platelet glycoprotein Ib alpha chain	66.6	49.4	248.3	75.3	79.3	95	95.3	39.8
Q28275-1	Fibronectin	58	68.6	168.1	80.8	83.1	96	58	94.8
Q28284	CD44 antigen	59.5	74.7	82.3	89.2	80.8	56.3	68	52.3
Q50KA9	Nucleoside diphosphate kinase A	72	42	50.2	49.9	139.6	141.5	77.4	95.6
Q6QNF4	Hepatocyte growtH factor activator	80.3	67.9	153.6	93.5	94.7	100.2	99.9	104.5
Q9GLD3	Transferrin receptor protein 1	64.7	71.3	70.9	75.1	96.7	200.4	158.6	84.6
Q9XSJ7	Collagen alpha-1(I) chain		12.9	14.2	19.1	17.4	8.4	3.9	16.2