DIAGNOSTIC CLASSIFICATION AND DETECTION OF MINIMAL RESIDUAL DISEASE (MRD) IN CANINE LYMPHOMA

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Pathobiology

Department of Veterinary Pathology

Faculty of Veterinary Science Chulalongkorn University

Academic Year 2010

การวินิจฉัยจำแนกชนิดและตรวจหา Minimal Residual Disease (MRD) ของมะเร็งต่อมน้ำเหลืองในสุนัข

นาย นาวิน มานะชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา

คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2553

Thesis Title	DIAGNOSTIC CLASSIFICATION AND DETECTION OF MINIMAL RESIDUAL DISEASE (MRD) IN CANINE LYMPHOMA
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อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.น.สพ.ดร. อนุเทพ รังสีพิพัฒน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.สพ.ญ.ดร. สมพร เตชะงามสุวรรณ,จำนวน 57 หน้า

จุดประสงค์ของการศึกษาเพื่อวินิจฉัยและจำแนกชนิดของมะเร็งต่อมน้ำเหลืองในสุนัขตามลักษณะจุลพยาธิวิทยาและอิมมูนโนฟีโนไทป์ โดยใช้เกณฑ์ในการจำแนกแบบ NCI Working Formulation และ Updated Kiel classification จำนวน 50 ราย และตรวจหาจีนตัวรับแอนติเจน และ Minimal Residual Disease (MRD) ด้วยปฏิกิริยาลูกโซ่โพลิเมอเรส ของสุนัขที่เป็นมะเร็งต่อมน้ำเหลืองจำนวน 14 รายที่ทำการรักษาด้วยเคมีบำบัดและหายอย่างสมบูรณ์ ผลการศึกษาวิธีการจำแนกตาม NCI พบว่าชนิด Diffuse large cell สูงสุดถึงร้อยละ 32 ส่วนการจำแนกโดย Updated Kiel พบชนิด Centroblastic มีอุบัติการณ์สูงสุดร้อยละ 24 อิมมูนโนฟีโนไทป์พบ B cell (เซลล์มะเร็งแสดงออก IgM) ร้อยละ 60 และชนิด T cell (เซลล์มะเร็งแสดงออก CD3) ร้อยละ 40 ผลการตรวจหาจีนตัวรับแอนติเจนโดยปฏิกิริยาลูกโซ่โพลิเมอเรสจากตัวอย่าง เซลล์และเลือดจำนวน 14 ตัวอย่างทั้งก่อนและหลังให้เคมีบำบัดพบว่า ให้ผลบวกจำนวน 13 ตัวอย่าง และ ตรวจพบ MRD หลังการรักษาด้วยเคมีบำบัด ในช่วงการหายอย่างสมบูรณ์ จำนวน 7 ตัวอย่าง ซึ่งการตรวจหา MRD โดยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรสเป็นวิธีที่มีประสิทธิภาพในการตรวจสอบหลังการรักษาด้วยเคมีบำบัดและหายอย่างสมบูรณ์ ช่วยในการพยากรณ์โรคและเฝ้าระวังการเกิดโรคซ้ำ

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5175562231 : MAJOR VETERINARY PATHOBIOLOGY

KEYWORDS: CANINE LYMPHOMA/ DIAGNOSTIC CLASSIFICATION/ MINIMAL RESIDUAL DISEASE/ POLYMERASE CHAIN REACTION

NAWIN MANACHAI: DIAGNOSTIC CLASSIFICATION AND DETECTION OF MINIMAL RESIDUAL DISEASE (MRD) IN CANINE LYMPHOMA. THESIS ADVISOR: ASSOC.PROF.ANUDEP RUNSIPIPAT, D.V.M., Ph.D., THESIS CO-ADVISOR: SOMPORN TECHANGAMSUWAN, D.V.M., M.Sc., Ph.D., 57 pp

Objectives of this study were (i) to examine the histopathology and immunophenotype of canine lymphoma according to the NCI Working Formulation and updated Kiel classification adapted to the canine species, (ii) to detect clonal rearrangements of antigen receptor genes by PCR assay, (iii) to detect Minimal Residual Disease (MRD) in clinical complete remission of chemotherapeutic treated dogs. Fifty formalin-fixed paraffin-embedded tissue from dogs suffering from lymphoma were retrospectively studied. Histopathological results revealed the most common subtype classified by the NCI system was intermediate grade diffuse large cell type (32%) while by the updated Kiel was centroblastic lymphoma(24%).The immunophenotypic study displayed 40% T -subtype (CD3 expression) and 60% B cell (IgM expression) lymphoma. For the PCR assays determining clonality for antigen receptor rearrangement genes and MRD from cytologic and peripheral blood samples of 14 dogs with lymphoma either before chemotherapy and during remission, clonality was detected in 13 of the lymphomas before treatment. MRD was demonstrated in 7 dogs with lymphoma during remission. Detection of MRD during remission in canine lymphoma using PCR technique is considered as an useful tool for prognosis and monitoring relapsing disease.

Department: Veterinary Pathology Student's signature:…….

Field of study: Veterinary pathobiology Advisor's signature:……..

Academic year: 2010 Co-Advisor's signature…….

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my advisor, Assoc. Prof. Dr. Anudep Rungsipipat and my co-advisor, Dr. Somporn Techangamsuwan for their merciful helps and guidance through the whole course of this work. My appreciation is also expressed to the thesis committee members, Assoc. Prof. Dr. Achariya Sailasuta and Assoc. Prof. Dr. Chaleow Sarakij, for their critical comments. My gratitude also goes to Dr. Pattarakit Theewasutakul, senior staff of Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University for his support and advice. I would like to express my special thanks to Mr. Supradit Wangnaitham and Mr. Sittichoke Lacharoje, staffs of the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University for their technical assistances. I am also grateful to my colleagues , Miss Suphatthra Jittimanee, Mr. Supalerk Nuntawan na Ayudhya and Miss Komkiew Pinpimai for their friendship in many ways that support me to complete this work.

Furthermore, I am grateful to Asst. Prof. Dr. Nopadon Pirarat, Asst. Prof. Dr. Theerayuth Kaewamatawong and all instructors & staffs of Veterinary Pathology Division for always creating a pleasant, enjoyable social and academic atmosphere for me. Without all mentioned above this work would have been impossible.

Finally, my indebtedness is also due to my family especially my parent (Mr. Opas and Mrs. Supranee Manachai) and my brother & his wife (Mr. Montree Manachai, Mrs. Panida Manachai). Their endless love and support encourage me preserving through the course of study. I am a lucky person to born in a very kind and careful family.

CONTENTS

Page

CHAPTER Page

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

bp	Base pair
BSA	Bovine serum albumin
CBC	Complete blood count
CD	Cluster of differentiation
CDR3	Third complementary determining region
CLL	Chronic lymphocytic leukemia
CR	Complete response
°C	Degree Celsius
D gene	Diversity gene
DAB	3,3'-diaminobenzidine-HCL
DNA	Deoxyribonucleic acid
EDTA	Ethylene daimine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FNA	Fine needle aspiration
FISH	Fluorescent in situ hybridization
GALT	Gut associated lymphoid tissue
hrs	Hours
H&E	Hematoxylin & eosin
HTLV-1	Human T-cell lymphotropic virus 1

IgH	Immunoglobulin heavy chain
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IU	International Unit
J gene	Joining gene
mg	Milligram (s)
m^2	Square meter
MRD	Minimal Residual Disease
µl	Microlitre
µm	Micrometer
N	Number of samples
NCI	National Cancer Institute
NHLs	Non-hodgkin's lymphomas
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rpm	Round per minute
s	Seconds
SD	Stable disease
TCRγ gene	T cell receptor gamma gene
UV	Ultraviolet

V gene	Variable gene
VMDP	Veterinary Medicine Data Base Program
WHO	World Health Organization
2,4-D	2, 4 dichlorphenoxy acetic

CHAPTER I

INTRODUCTION

Lymphoma is the most common hematopoietic neoplasm and one of the five common malignant neoplasm in human patients (Groves et al., 2000) and in dogs (Dobson et al., 2002) In recent years, the incidence of canine lymphoma has increased significantly. The reasons for this remain unknown, but in man both improvements in diagnostic methods and spread of as yet undefined risk factors may have contributed (Fisher and Fisher, 2004). Many studies in human have shown that the prevalence of different lymphoma subtypes depends strongly on the geographic region, and a causal relationship with specific risk factors has been demonstrated (Fisher and Fisher, 2004). In Thailand, no previous report about classification of canine lymphomas was been performed. Canine lymphoma gained considerable veterinary interest because it is one of the most treatable 'cancers' in small animal medicine, so these are rationale to classify canine lymphomas in Thailand and examine correlation between subtypes of canine lymphoma and malignancies for further treatment and prognosis.

An accurate histological diagnosis is an essential prerequisite not only for an accurate prognosis, but also a selection of a suitable therapeutic protocol (Cater et al., 1986; Greenlee at al., 1990; Teske et al., 1994). Therapeutic approach for canine lymphoma is challenged widely as new discoveries in the fields of chemotherapy, immunotherapy and molecular target therapeutics hold significant promise for improvement in duration of survival and remission rates. The technique of immunophenotyping to distinguish between T cell and B cell subtypes of canine lymphoma performing by immunohistochemistry is now more readily available to the practicing veterinarian. In addition, the detection of molecular markers for is currently investigated. Although there is certain uniformity in clinical presentation among dogs with lymphomas, the biologic behavior, histological subtype, immunophenotype, therapeutic response and outcome show a great deal of heterogeneity. Most clients are very satisfied with

results of chemotherapy because side effects are mild and the treatment provides prolonged and good-quality survival time (Bay et al., 2003).

In recent years, molecular characterization of lymphoproliferative malignancies has led to the development of useful and sensitive diagnostic and prognostic tools. This assay has become increasingly popular in human medicine. Since microscopic technique is often unable to recognize a residual neoplastic population (Calzolari et al., 2006). The molecular detection of residual malignant cells so called namely minimal residual disease (MRD), which is sensitive detection of circulating tumor cells before treatment to determine the extend of disease and after treatment to determine an efficacy of therapy, has resulted in improved outcome and monitoring of lymphomas (Lana et al., 2006). One of the most practical technique for MRD assessment is polymerase chain reaction (PCR) assay (Gentilini et al., 2009). Many different molecular markers have been evaluated in order to increase the sensitivity of the diagnostic tools; among these, the clonal rearrangements of immunoglobulin heavy chain (IgH) and T-cell receptor γ chain (TCR γ) genes, have been investigated.

The objectives of this study were to classify subtypes of canine lymphomas based on their morphology, immunophenotypes and clonal rearrangements of antigen receptor genes and to detect MRD from lymphoma-affected dogs in clinical remission following standardized chemotherapy.

CHAPTER II

LITERATURES REVIEW

Canine lymphoma; background and incidence

The lymphomas are a diverse group of neoplasms that have in common their origin from clonal expansion of malignant lymphoid cells. They usually arise from lymphoid tissues, such as lymph nodes, gut-associated lymphoid tissue (GALT), spleen and bone marrow; however, they may arise in almost any tissue in the body. Lymphomas which occur in any location outside the lymphatic system called primary extranodal lymphoma, such as eyes, central nervous system, bone, testes, urinary bladder, heart, and nasal cavity (Vail and Young, 2007). This distinguishes lymphomas from lymphoid leukemias, which originate in the bone marrow (Couto, 2003).

In most cases, lymphoma is characterized clinically as a disseminated disease (Vonderhaar and Morrison, 1998). Very rarely is a dogs diagnosed with single involved node, indicating clinical stage I disease. Specific clinical signs may related to dysfunction of an involved organ system; however, nonspecific clinical signs include lethargy, fever, anorexia, weight loss, abdominal distention, and polyuria/polydypsia (Vail et al., 2001).

Lymphoma accounts for approximately 7-24% of all canine neoplasm and 83% of all canine hematopoietic malignancies (Vail and Young, 2007). Canine lymphoma represents the third most common malignant neoplasm in dogs. The annual incidence rate is 13 to 24 per 100,000 dogs (Sueiro et al., 2004). In a review of the Veterinary Medical Data Base (VMDP) program, based at Purdue University, from 1987 to 1997, the frequency with which canine lymphoma patients were presented to 20 veterinary institutions increased from 0.75% to 2% of the total caseload and the frequency apparently continues to increase (Vail and Young, 2007). In Thailand, Rungsipipat et al. (2003) indicated that the incidence of hematopoietic and lymphatic tumors of dogs in Bangkok was 1.94% of all neoplasms and lymphoma was the most common hematopoietic neoplasm that had the incidence 76.60% of hematopoietic and

lymphatic tumors. Lymphoma affects primarily middle-aged to older dogs (Vail and Young, 2007). The mean age of dogs varies from 6.3 to 7.7 years (Madewell, 1985; Teske et al., 1994). A lower risk has been reported for intact females, but most reports show that gender is not an important risk factor (Haga et al., 1988; Vail and Young, 2007).

The etiology of canine lymphoma is largely unknown and likely multifactorial because no single etiologic agent has been identified. However, a genetic component is evident, in that the neoplasm is highly prevalent in certain bloodlines. There is also a distinct breed-related predisposition to lymphoma in dogs, with some breeds, such as Boxer, Bull Mastiff, Basset Hound, Saint Bernard, Scottish terrier, Bull dog, Rottweiler, Cocker Spaniel, Airedale Terrier, and Golden Retriever, being at high risk. In addition, several studies identified that chromosomal aberrations also have been reported in canine lymphoma. About the infectious factors, the hypothesis that a retrovirus may be involved in the pathogenesis of canine lymphoma has not been confirmed. In environmental factors, the risk of canine lymphoma was reported to rise twofold with four or more yearly application of herbicides, particularly 2,4-dichlorophenoxyacetic acid; 2,4-D (Vail and Young, 2007). Interestingly, canine lymphoma is proposed as animal models for their human counterparts (Owen et al., 1975; Hayes, 1978; MacEwen, 1990) due to the dogs have long lifetime enough for treatment (Greenlee et al., 1990) and they live in the same environment as the human (Fournel-Fleury et al., 1997).

Diagnosis and classification

Classification of canine lymphoma is similar to human lymphoma, by showing far less histological diversity than in human (Carter et al., 1986; Jaffe et al., 2001). Many attempts have been made to classify canine lymphoma according to similar histological criteria, but a clinically relevant classification system for this disease has yet to be universally accepted and adopted (Dobson et al., 2002). Lymphomas have been able to be classified into subgroups where the pathological appearance can be correlated with the clinical staging, anatomical appearance, anatomical locations, histological criteria and immunophenotypic characteristics. There are five

anatomical locations in dogs with lymphoma; multicentric, mediastinal, alimentary, cutaneous, and extranodal lymphoma. However, the classification by this mean is not appropriate for malignancy and prognosis indication due to their inconsistent correlation (Garrett et al., 2002). The histopathological classification in canine lymphoma is various and originates from human lymphoma. The World Health Organization (WHO) classification bases on morphological and immunophenotypical criteria (Valli et al., 2002) but this classification is as yet too establish to have formed the basis for further studies (Sueiro et al., 2004). Other two most widely used classification schemes are the National Cancer Institute (NCI) working formulation and the Kiel classification (Dobson et al., 2002). According to the NCI-working formulation system, lymphoma classification was based on lymph node architecture (diffuse or follicular) and morphological cell type including small lymphocytic, small cleaved cells, lymphoblastic and immunoblastic. The updated Kiel classification includes the grading of malignancy, morphology (centroblastic, centrocytic or immunoblastic), and immunophenotype (T cell or B cell) of the tumor cells (Page et al., 1992; Garrett et al., 2002; Vail and Young, 2007). In addition, the morphological classification of the Working Formulation is currently based on the histopatholocigal features alone. Therefore this reason Harris et al. (1994) suggested the updated Kiel classification, which also incorporates immunohistochemistry, to be a more appropriate classification system. An accurate histological diagnosis is an essential prerequisite not only for an accurate prognosis, but also a selection of a suitable therapeutic protocol. Nowadays, it is widely accepted that clinical staging and histopathological classification are the two most reliable variables in canine lymphoma for determining prognosis, response to treatment, remission periods and survival times (Carter et al., 1986; Greenlee at al., 1990; Teske et al., 1994). Use of the updated Kiel and Working Formulation classifications, have been implemented by several veterinary investigators (Carter et al., 1986; Greenlee at al., 1990; Fournel-Fleury et al., 1997), either individually or together, with success. However, opinion differ as far as the usefulness of the different classification systems are concerned. Greenlee et al (1990) was of the opinions that the Working Formulation was of limited use, mostly due to the

prominence accorded follicular lymphomas in this classification, and the scarcity there of in dogs (Greenlee et al.,1990). However, previous reports concluded in multivariate analysis of lymphomas in dogs, that both the Working Formulation and Kiel classification are readily applicable to canine lymphoma (Teske et al., 1994).

Lymphoma in dogs has several subtypes which show different biological behavior and response to treatment (Dobson, 2004). The important factor that effects to biological behavior of lymphoma is immunophenotype (Greenlee et al., 1990; Ruslander et al., 1997). The structure of immunophenotype is glycoprotein on the cell surface called cluster of differentiation (CD) antigens. Lymphocyte CD antigens serve multiple functions, including intracellular signaling, cell-to-cell communication, and lymphocyte trafficking that have been able to be investigated by detecting with specific antibody to those antigens (Morrison and Neuberger, 2001). Common CD antigens that use to differentiate type of lymphoma are CD3 and CD79a. CD3 is a complicate structure of polypeptide that associated with T cell receptor. CD3 is an excellent marker because its antigenicity is retained following neoplastic transformation. The expression of CD3 antigen on malignant lymphocytic cells indicates the malignant lymphocytes originating from T cell. On the other hand, CD79a is a heterodimer structure that associated with B cell receptor and their expression of on malignant lymphocytic cells indicates the malignancy originating from B cell. Several laboratory techniques have been used to investigate of CD3 and CD79a antigen on cell surface, such as flow cytometry, immunofluorescence, cytofluorographic, and immunohistochemistry (MacEven et al., 1987; Greenlee et al., 1990; Teske, 1994). The canine lymphomas were classified as either T cell (reacting with CD3 antibody) or B cell (reacting with CD79a antibody) by immunohistochemistry which enzyme-adhered antibody detected antigen on the tumor cell surface represent fluorescent agent. Interestingly, it has been demonstrated in patients suffering from B cell chronic lymphocytic leukemia (CLL) that the leukemic B cells commonly secreted small amounts of IgM (Qian et al.,1984).

Many attempts have been made to correlate the type of tumor and patient factors that may act as useful predictors of treatment response and prognosis for canine lymphoma. Immunophenotype is widely accepted as an important prognostic indicator. Immunopositive B cell lymphoma displayed a better prognosis than T cell lymphoma (Dobson et al., 2001; Ruslander et al., 1997). Moreover, the identification of the immunophenotype of canine lymphomas is important not only for a better understanding of the lymphoma biology but also for survival times. Some canine T-cell lymphomas showed a shorter survival times (Ferrer et al., 1993). However, as 60% of canine lymphomas are B cell and there is still considerable variation in tumor response and survival within this group, up-to-date knowledge of B cell type does not really provide a definitive prognosis to the practitioner (Dobson, 2004). In addition, dogs suffering from T cell lymphoma significantly gained a high risk to die in the early phase of disease as well as tumor relapsing occurred between and after therapy comparing with B cell lymphoma (Teske et al., 1994; Ruslander et al., 1997; Ponce et al., 2004). Therefore, both lymphomas are considerable variation in response to therapy. Some tumors show a rapid and sustained response; some relapse and become resistant to therapy within a short space of time whilst others do not respond at the outset (Dobson, 2004). The considerable range in survival times is associated with the fact that lymphoma subtypes have different prognoses (Ponce et al., 2004) and require different treatments. Knowledge of the specific lymphoma subtypes is therefore necessary to select an adequate treatment, to reduce potentially unnecessary suffering of the patient due to side effects of chemotherapy, and to save costs for the owner (Arespacochaga et al., 2007).

Treatment of canine lymphoma

Therapeutic planning of canine lymphoma is considered with various prognostic factors, including clinical stages, histopathologic grade and subtype, immunophenotype and paraneoplastic's syndrome (Fan and Lorimier, 2005). Lymphoma is a disease that manifests at presentation with systemic dissemination. Systemic chemotherapy remains the treatment of

choice for canine lymphoma; without treatment, dogs with lymphoma usually survive only 4 to 12 weeks. Canine lymphoma is also reported to the most response to chemotherapy. Local treatment such as surgery and radiation are rarely applied to treat lymphoma. A variety of protocols with single agents or multidrug chemotherapy have been reported (Vail and Young, 2007). Combination chemotherapy is most widely used and efficacious which can be applied with concurrent administration. The most widely used cytotoxic drugs with efficacy against lymphoma include L-asparaginase, vincristine, cyclophosphamide, doxorubicin, methotrexate and prednisone (Bay et al., 2003). During the recent years, the results of combination chemotherapy in veterinary oncology have improved significantly. At present response rates of 80-90% and median survival times of 250 to 300 days are common (Jeglum, 1996; Vonderharr and Morrison, 1998). Almost all of the combination chemotherapy protocols are quite similar and vary slightly with regard to dose and scheduling of the same drugs. Thirthy-eight published protocols for canine lymphoma have been reported such as, ACOPI, CHOP, COAP and University of Madison-Wisconsin protocols and University of Madison-Wiscosin protocols (Jeglum, 1996). The latter protocol adds methotrexate to the other drugs and has the highest published proportion of patients with 2-years survival (Keller et al., 1993). Previously, a report of combination use of chemotherapy composing a high-dose of L- asparaginase and vincristine sulfate which is a modified from the ACOP I protocol has been successfully treated in a dog with cutaneous form of lymphoma with a few toxicities (Theewasutakul et al., 2007). Shorter treatment cycles are the goal of ongoing research which is attempting to minimize treatment-related toxicity and thus enhance patient quality-of-life (Marconato, 2010).

L- asparaginase is a bacteria-derived enzyme that degrades the amino acid asparagine to aspartic and ammonia depriving growing cells of that amino acid and inhibiting protein synthesis. The therapy is based on a metabolite defect in which many neoplastic cells are unable to synthesize an adequate amount of asparagines for protein synthesis and survival. The main potential adverse drugs reaction to L – asparaginase is a hypersensitivity reaction due to antibodies to the foreign bacterial protein develop. Affected animals may show vomiting,

diarrhea, urticaria, and rarely collape. Less common toxicities include pancreatitis and myelosuppression (Frazier and Hahn, 1995; Chun et al., 2007).

Vincristine is derived from periwinkle and also is cell cycle phase specific. Its mechanism of action is through inhibition of intracellular microtubule formation, an important part of the mitotic spindle formation. The major side effect of vincristine is severe perivascular tissue reaction with accidental extravasation. Other possible side effects include constipation and peripheral neuropathy. Although vincristine has been reported to be less myelosuppressive than other chemotherapeutic drugs, and significant myeosuppression may occur when L – asparaginase is combined with vincristine (Chun et al., 2007).

Minimal Residual disease (MRD)

Combination chemotherapy for dogs with lymphomas resulted in complete remission at high rates (Vonderhaar and Morrison, 1998). However, most dogs died as a consequence of tumor relapse. Despite having achieved a clinical complete remission, the patient may still a few up to 10^{10} leukemic cells that persist at levels undetectable by conventional cytomorphologic methods (Campana and Pui, 1995). Residual malignant cells that escape anti-neoplastic treatments are considered to be the source of tumor relapses (Kern el al., 2005; Pott et al., 2006). These cells are called minimal residual disease (MRD). A Variety of methods have been developed for the detection of MRD, including flow cytometry, immunologic studies, fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) (Faderl et al., 1999). Recent advances on the molecular detection of tumor cells in the peripheral blood and lymph nodes of canine lymphoma has been reported by using the PCR method with specific primers to detect rearranged antigen receptor genes (Keller et al., 2004; Calzolari et al., 2006; Lana et al., 2006). The sensitivity for detecting neoplastic lymphoid cells was shown to be a approximately 1 per 100 cells (Burnett et al., 2003).

Almost lymphomas and lymphocytic leukemias and myelomas are clonal expansions of malignant lymphocytes each particular neoplasm contains DNA regions that are unique in

length and sequence. The CDR3 (3^{rd} complentary determining region) (Figure 1) both immunoglobulin and T-cell receptor (TCR) genes encodes the antigen-binding region of the respective receptor. In B lymphocytes, CDR3 is produce through the recombination of V, D and J genes. In T lymphocytes, CDR3 of the TCRγ gene is produced through recombination of V and J genes (Figure 2) (Raulet, 1989; Schatz et al., 1992; Rezuke et al., 1997). During early lymphoid development, the different variable (V), diversity (D), and joining (J) gene segments of the Ig and TCR gene loci undergo recombination processes. The V(D)J rearrangements are mediated by recombinase enzyme complex (Rezuke et al., 1997; Jung et al., 2006). During the rearrangement process at the junction sites of the V, D, and J gene segments, deletion and random insertion of nucleotides occur, resulting in diverse junctional regions (Harris et al., 2001; Jung et al., 2006).

In human diagnostic pathology, a method commonly used for the detection of clonal expansions of malignant lymphocytes uses primers specific for conserved regions of V and J genes to amplify CDR3, followed by PCR technique (Rezuke et al., 1997). A clonal band indicates the presence of an expanded lymphocytes population. This method is most commonly used to distinguish reactive from neoplastic cells of lymphocytes and to detect minimal residual disease (Provan et al., 1996).

The assessment of clonal rearrangements of antigen receptor genes has also been introduced into veterinary medicine (Vernau and Moore, 1999; Burnett et al., 2003; Keller et al., 2004; Moore et al., 2005; Werner et al., 2005; Tamura et al., 2006; Yagihara et al., 2007; Thilakaratne et al., 2010). The methods described in some of these studies were reliably accurate which allows their use in clinical application. The assay may provide a useful aid for the detection of lymphoid neoplasia and can be a different to immunophenotyping. Other potential is detecting residual disease after treatment.

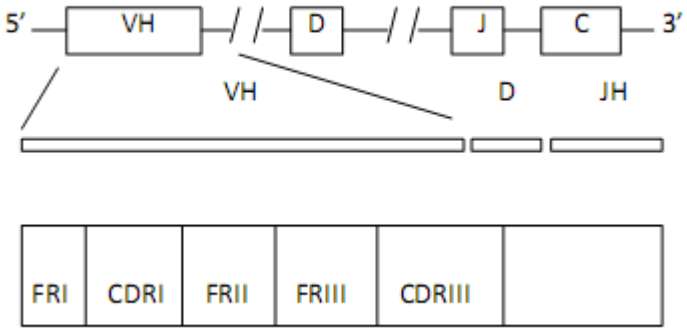


Figure 1 CDR3 region (3rd complentary determining region)

; Lymphoid neoplasia contain DNA regions unique

in length and sequence.

(Rezuke et al., 1997)

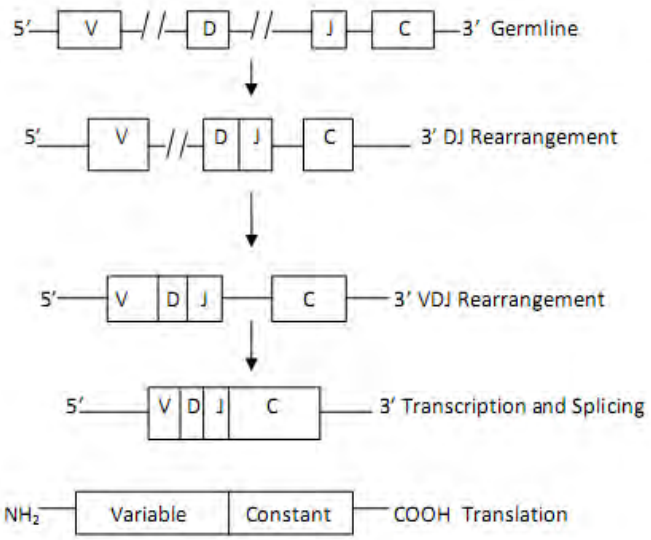


Figure 2 Schematic diagram; the sequential steps involved

in immunoglobulin and TCR gene rearrangements.

(Rezuke et al., 1997).

CHAPTER III

MATERIALS AND METHODS

1. Animals

Fifty dogs, clinically diagnosed as canine lymphoma, were referred from the Small animal Teaching Hospital, Faculty of Veterinary Science Chulalongkorn University, Bangkok Thailand during 2003-2010. All cases were evaluated by complete history taking and physical examination with thoracic and abdominal radiographs. Blood test including a complete blood count (CBC) and serum biochemical profile were performed. The definitive diagnosis of lymphoma was based on the cytologic and histopathologic assessment of lymph node samples obtained by fine needle aspiration (FNA) or biopsy specimens.

2. Clinical staging and anatomical classification

Clinical staging has categorized into 5 stages according to World Health Organization (WHO) V-stage criteria for canine lymphoma (Table 1). In addition, dogs were assigned to substage categories of a (without systemic sign) or b (with systemic signs) (Greenlee et al., 1990; Ettinger, 2003). The anatomical classification was classified as multicentric, mediastinal, alimentary, extranodal, and cutaneous form according to the results of physical examination, biopsy and necropsy reports (Table 2).

Table 1 Clinical stages of canine lymphoma (Greenlee et al., 1990; Ettinger, 2003)

Stage	Involvement
I	Single lymph node or lymphoid tissue in single organ involvement
II	Regional node involvement
III	Generalized node involvement
IV	Hepatic and/or splenic involvement
V	Blood, bone marrow and/or other organs involvement

Table 2 Anatomical classification of canine lymphoma (Vail and Young, 2007).

Anatomical form	Association
Multicentric	Bilateral and/or symmetrical peripheral lymphadenopathy. Absent or present of tumor metastasis to liver, spleen, tonsil and bone marrow
Mediastinal or Thymic	Associated with abnormal mediastinal lymph nodes with or without tumor metastasis to bone marrow
Alimentary	Gut-associated lymphoid tissue involvement
Extranodal	Occur in any location outside the lymphatic system such as eyes, central nervous system, bone, testes, urinary bladder, heart, and nasal cavity
Cutaneous	Multifocal or generalized skin involvement

3. Histopathological study and classification

The specimens for light-microscopic examination and immunohistochemical examnination were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4

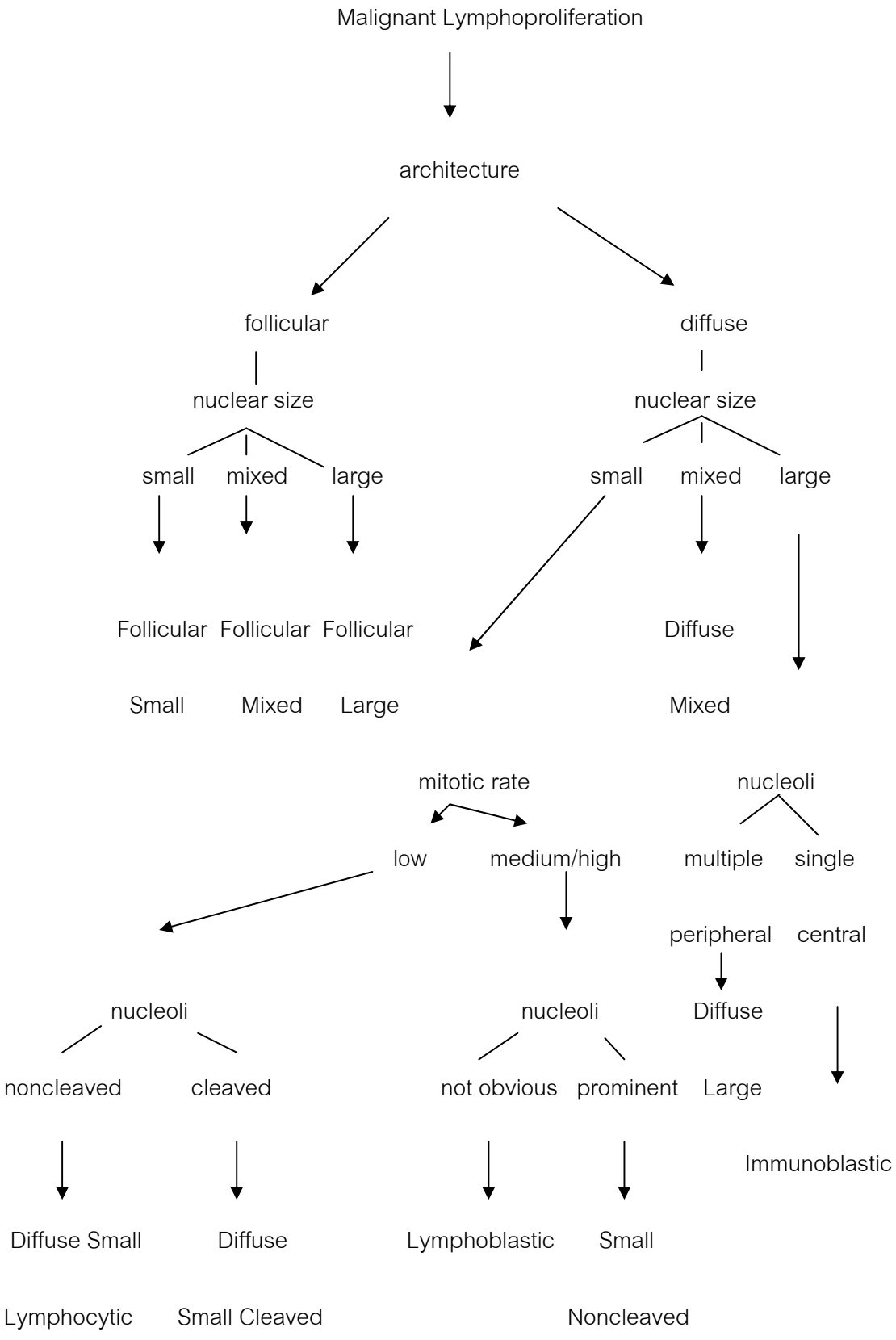
to 6 μm thick and stained with hematoxylin and eosin (H&E). The histopathological findings of canine lymphomas were classified according to the National Cancer Institute Working Formulation and the Updated Kiel classification (Carter et al., 1986; Fournel-fleury et al., 1997; Fournel-fleury et al., 2002 ; Arespacochaga et al., 2007).

Lymphoma classification criteria of NCI Working Formulation was based on the pattern of lymphomatous architecture characterized by follicular or diffuse pattern. The morphological classification criteria were relied on the cell's size and shape, the conformation of cytoplasm, nucleus's size and form, the chromatin pattern, the number and size of nucleoli as well as the the mitotic index. The mitotic rate was estimated in histological specimens by scanning 10 fields at 400x and counting mitotic figures. A low mitotic index was defined as 0–2 mitoses/field, medium as 3–5/field, and high as ≥ 6/field (Table 3 and Figure 3).

Table 3 Criteria for NCI Working Formulation Classification (Carter et al., 1986 ; Arespacochaga et al., 2007)

Lymphoma grading	Histomorphologic Classified
Low grade	- Small lymphocytic - Follicular, predominantly cleaved cell - Follicular, mixed small cleaved and large Cell
Intermediate	- Follicular, predominantly large cell - Diffuse, small cleaved cell - Diffuse, mixed small and large cell - Diffuse, large cell
High grade	- Large cell, immunoblastic - Lymphoblastic - Small non-cleaved cell

Figure 3 Step for NCI Working Formulation classification (Carter et al., 1986)



4. Immunohistochemical study and classification

Fifty cases were re-classified according to the updated Kiel classification which is based on the basic distinction between B-cell and T-cell lymphomas (Fournel-Fleury et al., 2002). The morphological classification criteria were based on cell size and shape, the conformation of cytoplasm, nucleus size and form, the density of chromatin, the number and size of nucleoli (Fournel-Fleury et al., 1997) as shown in table 4

Table 4 The updated Kiel Classification (Fournel-Fleury et al., 1997)

	B-cell	T-cell
Low grade	Lymphocytic Lymphoplasmacytic/cytoid Prolymphocytic Centroblastic / centrocytic Centrocytic Macronucleolated medium-sized cell	Lymphocytic clear cell Prolymphocytic Pleomorphic, small cell Mycosis fungoides
High grade	- Centroblastic - Immunoblastic - Burkitt's lymphoma - Large cell anaplasic - Lymphoblastic	- Pleomorphic, medium and large cell - Immunoblastic - Large cell anaplasic - Lymphoblastic

Canine lymphoma was Immunophenotyped for T and B cell lineages by immunohistochemical reaction to CD3 and IgM antibodies, respectively. Spleen and normal lymph node sections from healthy dogs were used as positive controls. For CD3 immunophenotyping, the modified avidin-biotin-peroxidase complex (EnVision™ polymer DAKO®, Denmark) technique was used. Briefly, 4-6 µm-thick paraffin-embedded samples were cut and placed on silane (3-aminopropyltriethoxy silane®, Sigma, USA)-coated slides. Section

were deparafinized in xylenes, rehydrated through a graded series of alcohols (95-100%), and rinsed in phosphate-buffered saline (PBS). An antigen retrieval method was done by autoclave for 5 minutes at 121ºC in Tris-EDTA pH 9 followed by PBS washing. Endogenous peroxidase enzyme was block with 0.3% H_2O_2 in a steamer for 30 min at room temperature. After 3 cycles of PBS washes, 1% bovine serum albumin (BSA) was added and incubated at 37ºC for 30 minutes to reduce non-specific background. Polyclonal rabbit anti-human CD3 (Dako A0452 Denmark) at dilution1: 200 was used as primary antibodies by incubating sections at 4°C 12-14 hrs. After washing with PBS, EnVision™ (Dako K5007 Denmark) was employed for 45 minutes at room temperature. After rinsed in PBS, 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, USA) as a chromogen was applied at room temperature. The sections were then washed with distilled water and counterstained with Mayer's hematoxylin. Slides were washed again with tap water until clear and then dehydrated. Slides were passed through 95%, 100% methanol and xylenes. The slides were coversliped with Permount mounting solution.

For IgM immunophenotyping, a standard avidin-biotin complex (peroxidase solution, Streptavidin, DAKO, Denmark) was performed. After pretreatment and blocking non specific backgrounds as previously described, polyclonal goat anti-dog IgM antibody (dilution 1:200, Bethyl Lab, USA) was applied, at 4°C, 12-14 hrs. After 3 rinses with PBS, a biotinylated secondary antibody (biotinylated anti-goat IgM antibody; Dako, Denmark) was applied to each slide for 60 minutes at 37ºC. After 3 washes in PBS, avidin-biotin complex peroxidase solution was applied for 30 minutes at 37ºC. After 3 rinses in PBS, DAB was applied as a chromogen. The sections were then washed with distilled water and counterstained with Mayer's hematoxylin. Slides were washed again with tap water until clear and then dehydrated. Slides were passed through 95%, 100% methanol until a final incubation in xylenes. Coversliped the slides with Permount mounting solution.

For immunohistochemical evaluation, T-cell lymphoma will be considered when at least 10% neoplastic cells reacted positively with anti-CD3 wherea B-cell lymphoma will be diagnosed when they were at least 10% of neoplasm cells positive with anti-IgM.

5. Detection of the rearrangements of antigen receptor genes by using polymerase chain reaction (PCR)

5.1 DNA extraction

DNA was extracted from cytologic sample (N=6) and peripheral blood (N=14). Cells or tissue specimens were collected in a 1.5 ml tube by repeated flushing of the needle with 200 µl PBS after fine needle aspiration (FNA). Whole blood samples were spun down (5,000 rpm) at room temperature for 5 minute and 200-µl buffy coat were collected. Genomic DNA was extracted using commercial kits (QIAamp DNA mini kit, Qiagen®, Germany) according to the maufacture's instruction. DNA was kept at -20°C until used.

5.2 PCR detection of antigen receptor genes rearrangement

To detect antigen receptor genes rearrangement, amplification of Immunoglobulin (Ig) and T-cell receptor gamma (TCRγ) sequences were performed using previously described methods and primers (Bunett et al., 2003) shown in Table 5. DNA sample was amplified by 4 sets of primers.Cµ primers complementary to the constant region gene of IgM were used as an internal control. Two primers pairs for the IgH (major and minor) genes and TCRγ gene were used to detect rearrangement of these antigen receptor genes. The reaction mixture containing 12.5 µl of a commercial master mix (Master Mix, Promega©, Madison, USA), 0.5 µl of each set of primers, 1 µl of DNA template and distilled water 9.5 µl to yield a final volume of 25 µl. PCR conditions were 1 cycle with initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 8 s, annealing at 60°C for 10 s and extension at 72°C for 10 s. PCR product (10 µl) were detected by electrophoresis 4% agarose gel, stained with 10% ethidium bromide and visualized under UV transluminator for bands of expected size. The Cµ product is

approximately 130 base pair (bp), the Ig products around 120 bp and the the TCR products around 90 bp.

Table5 Primers used for amplification of Cμ , TCRγ and IgH (Burnett et al., 2003)

Product	Primer Names	Primer Specificity	Primer Sequence (5'-->3')
Cμ	Sigmf1	Cμ	TTC CCC CTC ATC ACC TGT GA
	Srμ3	Cμ	GGT TGT TGA TTG CAC TGA GG
IgH major	CB1	VH	CAG CCT GAG AGC CGA GGA CAC
	CB2	JH	TGA GGA GAC GGT GAC CAG GGT
IgH minor	CB1	VH	CAG CCT GAG AGC CGA GGA CAC
	CB3	JH	TGA GGA CAC AAA GAG TGA GG
TCRγ	TCRγ1	JH	ACC CTG AGA ATT GTG CCA GG
	TCRγ2	JH	GTT ACT ATA AAC CTG GTA AC
	TCRγ3	VH	TCT GGG A/GTG TAC/T TAC TGT GCT GTC TGG

6. Chemotherapeutic protocols

The combination chemotherapy utilized L-asparaginase (Leunase® Kyowa Hakko Kouo, Japan) dosage 10,000 IU/m^2 intravenous infusion in normal saline and Vincristine sulfate (Vincristin® Gadeon Richter, Hungary) with 0.75 mg/m^2 at weeks 1, 2, 3, 4 for a cycle of treatment was modified from ACOP I protocol (Theewasutakul et al., 2007). Supportive and antibiotic treatment were given based on the patient condition such as hematotonic, hepatotonic and multivitamins.

7. Assessment of chemotherapeutic response

The response to chemotherapy was evaluated one week after first treatment. Complete response (CR) was defined as a regression of tumor size (75-100%) of lymph node enlargement and related clinical signs. Partial response (PR) was defined as 50-75% resolution and

improvement of clinical signs. Stable disease (SD) was defined as 0-50% decrease in measurable lymphadenopathy. Progressive disease (PD) was defined as a enlarged lymph node led to a deteriorating quality of life (Lucroy et al., 1998). The patients were clinically evaluated every week during therapy.

8. PCR detection of MRD before chemotherapy and during remission

PCR assay was performed on DNA extracted from peripheral blood (N=14) from patients with before onset of treatment and repeated in complete or partial remission. The primers and PCR condition was described above.

9. Data analysis

The clinical data and histopathological classification of canine lymphomas have analyzed and shown as descriptive analysis form. Otherwise analysis of difference of clonal band of MRD-positive group and MRD-negative group to tumor relapse and their prognosis.

CHAPTER IV

RESULTS

Clinical feathers

The age of the dogs ranged from 1-16 years (mean 7.5 years, median 7 years and mode 8 years). The prevalence of canine lymphomas in pure breeds and mixed breeds were 67.5% (27/40) and 32.5% (13/40), respectively, therefore the pure breeds had more prevalence than mixed breeds. The canine lymphomas were not apparently sex related. Anatomical classification was multicentric 64% (32/50), mediastinal 6% (3/50), alimentary 10% (5/50), extranodal 12% (6 /50) and cutaneous 8% (4/50) (Table 6). Clinical stage was divided to 5 stages. That was the most prevalence both T and B cell in stage IV (Table 7).

Table 6 Anatomical classification of 50 canine lymphomas

Anatomical classification	Number of cases (%)
Multicentric	32 (64.0%)
Mediastinal	3 (6.0%)
Alimentary	5 (10.0%)
Extranodal	6 (12.0%)
Cutaneous	4 (8.0%)

Table 7 Clinical stage and immunophenotype of 50 canine lymphomas

Clinical stage	Immunophenotype	
	T cell (%)	B cell (%)
1	6 (3/50)	-
2	6 (3/50)	6 (3/50)
3	8 (4/50)	8 (4/50)
4	30 (15/50)	22 (11/50)
5	10 (5/50)	6 (3/50)

Hematological results

Lymphoma dogs showed some significant changes in hematology such as anemia and thrombocytopenia and leukocytosis had a tendency to relative with lymphocytosis. An involvement of the peripheral blood (circulating lymphoblasts) was found in 7 cases (Figure 4). Paraneoplastic hypercalcemia was observed in two cases of T-cell lymphoma. Serum protein was generally lower than the reference values, except for evidence of hyperproteinemia, which was shown in a case compatible with lesion of renal amyloidosis (Figure 5). The percentage of hematological changes lymphomas dogs is summarized in Table 8.

Table 8 The hematological results of canine lymphomas

Biological prognostic factors	Cut off value*	$\bar{x} \pm S.D.$	Percentage of total
Anemia		33.55±6.28	46 (23/50)
mild	30 ≤ %PCV ≤ 37		24 (12/50)
moderate	20 ≤ %PCV ≤ 29		14 (7/50)
severe	13 ≤ %PCV ≤ 19		8 (4/50)
Thrombocytopenia	Platelets< 200 × 10^3cells/µl	154±5.13	40 (20/50)
Leukocytosis	Total WBC >170× 10^3cells/µl	19.6±13.19	30 (15/50)
Circulating lymphoblasts	Presence of lymphoblast in peripheral blood		35 (7/20)
Hypoproteinemia	Serum protein < 5.4 g/dl	3.6±4.57	40 (8/20)
Hyperproteinemia	Serum protein > 7.1 g/dl	12.4	5 (1/20)
Hypercalcemia	Serum calcium >12.9 mg/dl	14.5±0.63	10 (2/20)

*(Madewell, 1985; Vail, 2000)

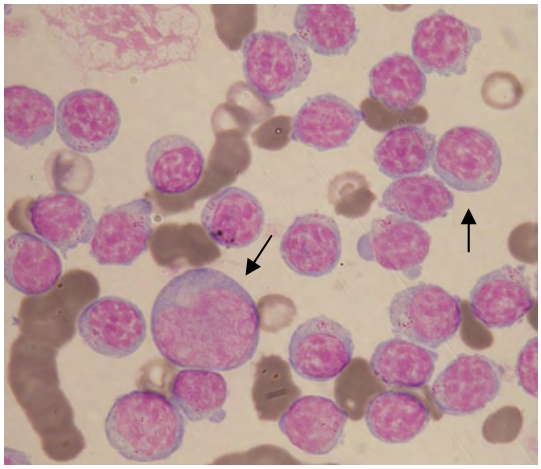


Figure 4 Circulating lymphoblasts; Anisocytotic neoplastic cells with scant cytoplasm an large nuclei with coarse chromatin pattern (arrow)

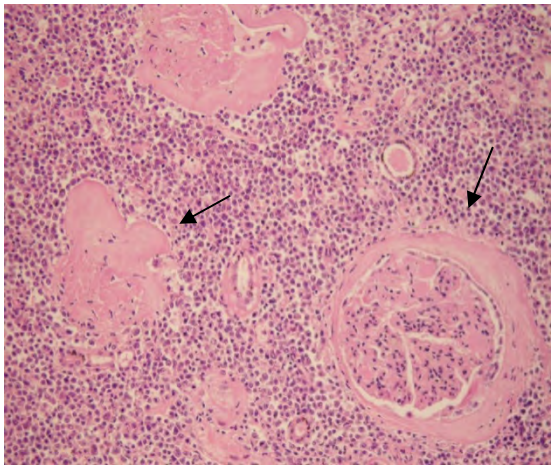


Figure 5 Histopathology of renal amyloidosis ; Homogeneous pale eosinophilic substance deposited at glomerulus and perimembranous (arrow; HE)

Histopathological and immunophenotyping results

The prevalence of the different lymphoma subtype that classified by the National Cancer Institute (NCI) Working Formulation and updated Kiel classification is shown in Table 9 and 10

NCI- Working Formulation Classification

For the NCI-Working Formulation, fifty lymphomas were classified as low, intermediate and high grade. All cases of lymphomas are diffusely (100%). Intermediated-grade lymphoma were the most frequent type (54%), followed by high grade lymphomas (26%) and low-grade (20%).

Low grade

The diffuse small cell lymphomas accounted for 20% of all lymphomas. The architecture is diffuse, the mitotic index is low. The nuclei are uniformly round with an even periphery. Nucleoli are absent. The cytoplasm is scantly.

Intermediated grade

There were 10% diffuse small cleaved cell lymphomas. The diffuse mixed cell lymphoma form 12% of the total . The diffuse large cell lymphoma was the most common subtype, with 16 cases (32%). Diffused mixed cells was the third of subtype and compose of small cleaved cells and large cells. The large nuclei are almost non-cleaved and has vesicular chromatin pattern and multiple prominent nucleoli.The cytoplasm of large cells is abundant.

High grade

For all high grade lymphoma is diffuse, (26%) immunoblastic lymphoma (Figure 11) accounted for 8% (N=4) of all lymphomas. The characteristics of immunoblastic are highly mitoses with vesicular nuclei. The prominent central nucleolous is present. The cytoplasm is basophilic and clearly (plasmacytoid) In the present study a minority of cases were classified as lymphoblastic lymphomas (18%) (Figure 15). The architecture is diffuse, and the mitotic rate is very high, lymphoblastic cells have large round to angular nuclei. Uniform chromatin pattern is presented by obscure nucleoli. Cytoplasm in the lymphoblastic cell types is scant and lightly stained.

Updated Kiel Classification

About the different lymphoma subtype, thirty lymphomas (60%) were classified as B cell subtype and twenty (40%) as T cell subtype. High-grade lymphomas were the most frequent type of T cell and B cell lymphoma (70%, 53.4%), followed by low-grade lymphomas (30% of T cell and 46.6% of B cell). Within the both low-grade B cell and T cell groups, small cell

lymphomas predominated, while centroblastic lymphoma predominated in high-grade B cell and pleomorphic large cell lymphoma predominated in high-grade T cell.

Low grade

The small cell lymphomas accounted for 34% of all lymphomas. Of these, six dogs were classified as T cell subtype (12%) (Figure 16) and eleven were classified as B cell subtype (22%) (Figure 17). All of six small T cell were classified as prolymphocytic lymphoma (Figure 7). In small B cell group, three were classified as lymphocytic lymphomas (6%) (Figure 6), five were classified as prolymphocytic lymphomas (10%), and three were classified as centrocytic lymphomas (6%)(Figure 14). In addition, three were classified as macronucleolated medium-sized B cell lymphomas (6%) (Figure 12).

High grade

For B cell lymphoma (Figure 20, 21), centroblastic lymphoma accounted for 24% (n=12) of all lymphomas, two dogs was classified as monomorphic lymphoma (4%) (Figure 8) and ten were classified as polymorphic lymphomas (20%) (Figure 9). In addition, four were classified as immunoblastic B cell lymphomas (8%) (Figure 11). In T cell subtype (Figure 18, 19), pleomorphic large cell (Figure 10) accounted for 20% (N=10) of all lymphomas, pleomorphic, mixed, small and large cell (Figure 13) accounted for 6% (N=3) and lymphoblastic (Figure 11) accounted for 2% (N=1).

Table 9 The histological classification according NCI Working Formulation Classification

Grade of malignancy	No. of cases
Low-grade	20% (10)
Diffuse small cell lymphocytic	20% (10)
Follicular small cleaved	-
Follicular mixed	-
Intermediate grade	54% (27)
Diffuse small cleaved	10% (5)
Diffuse Mixed	12% (6)
Diffuse large	32% (16)
High-grade	26% (13)
Immunoblastic	8% (4)
Lymphoblastic	18% (9)
Small cell non-cleaved	-
Total	50

Table 10 The Histological and immunophenotyping classification according to the updated Kiel classification

B cell	No. of cases	T cell	No. of cases
Low-grade malignancy	46.6% (14)	**Low-grade malignancy**	30% (6)
Small cell	*36.6% (11)*	*Small cell*	*30% (6)*
- *Lymphocytic*	10% (3)		
- *Lymphoplasmacytic*	-	- *Clear cell*	-
- *Prolymphocytic*	16.6% (5)	- *Prolymphocytic*	30% (6)
- *Centrocytic*	10% (3)	- *Pleomorphic small cell*	-
Centroblastic/ Centrocytic	-		
Macronucleolated medium-sized cell	10% (3)	*Mycosis fungoides*	
High-grade malignancy	53.4% (16)	**High-grade malignancy**	70% (14)
Centroblastic	40% (12)	Pleomorphic, mixed, small and large cell	15% (3)
- *Monomorphic*	6.6% (2)	Pleomorphic large cell	50% (10)
- *Polymorphic*	33.3% (10)		
Immunoblastic	13.4% (4)	Immunoblastic	-
Lymphoblastic	-	Lymphoblastic	5% (1)
Total	30		20

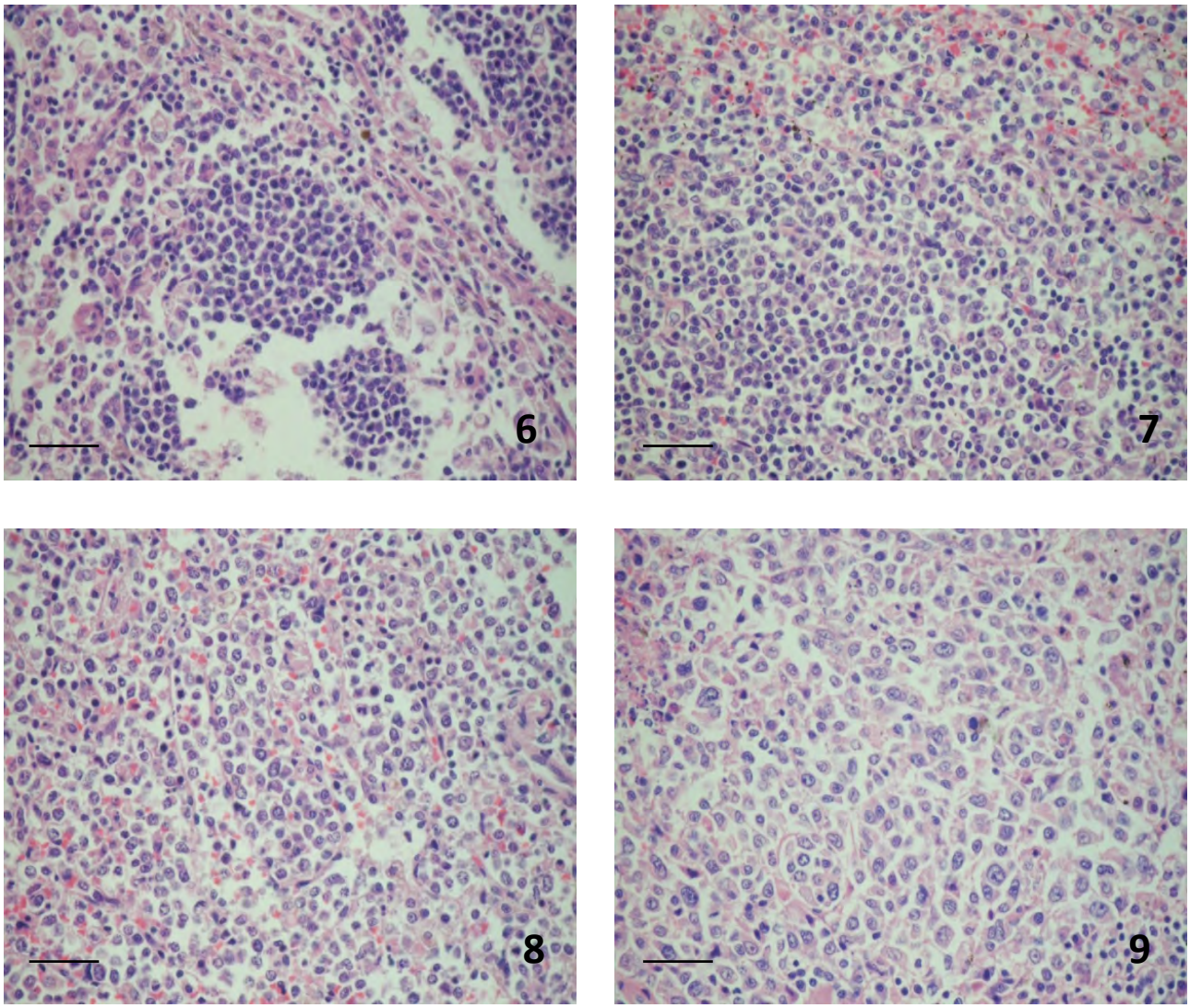


Figure 6 Small cell lymphocytic lymphoma (HE, bar = 25 μm)

Figure 7 Small cell prolymphocytic lymphoma (HE, bar = 25 μm)

Figure 8 Centroblastic monomorphic lymphoma (HE, bar = 25 μm)

Figure 9 Centroblastic polymorphic lymphoma (HE, bar = 25 μm)

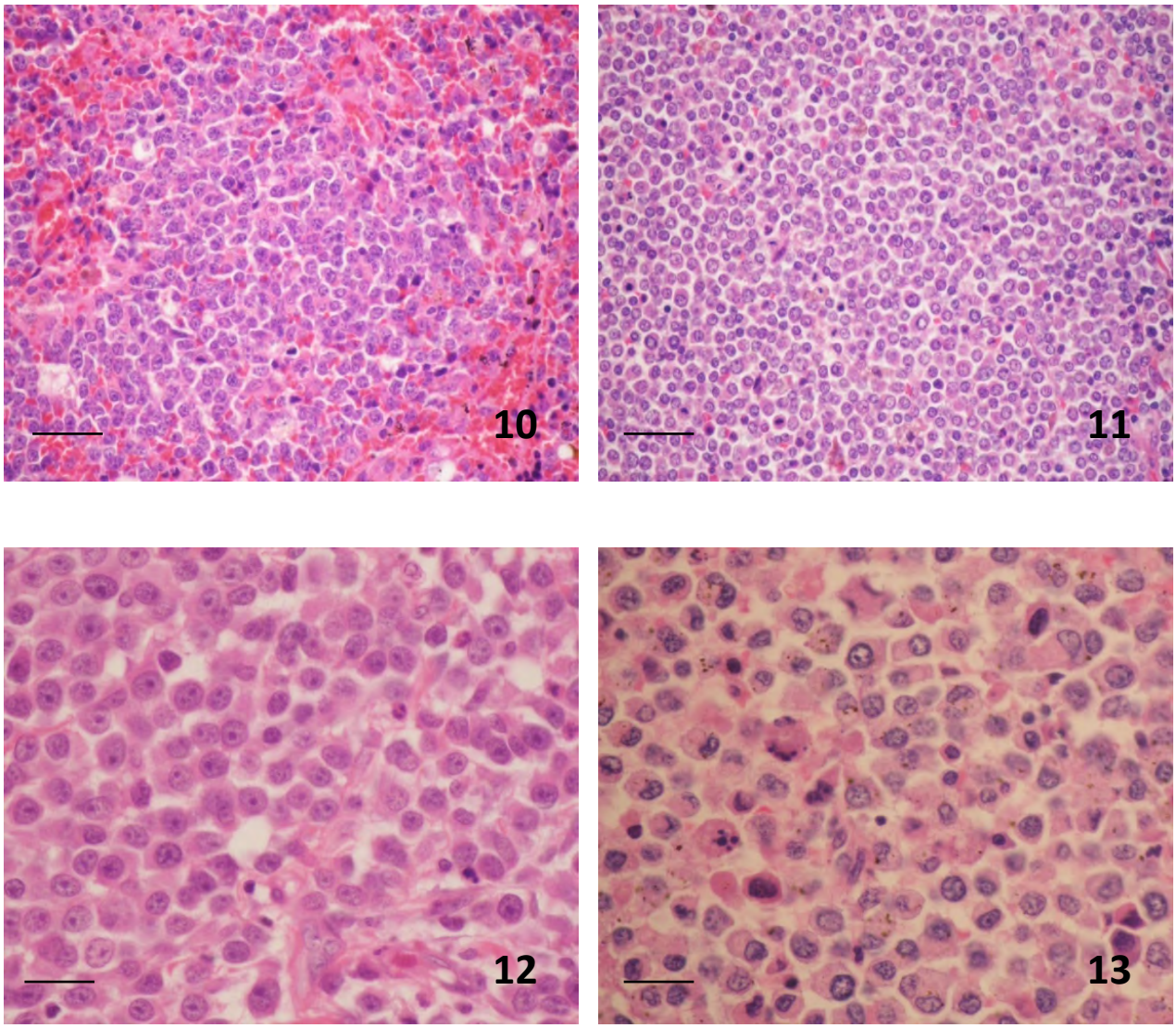
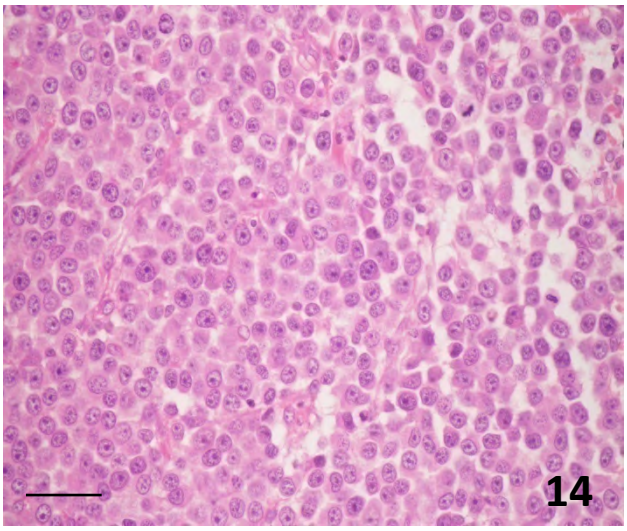


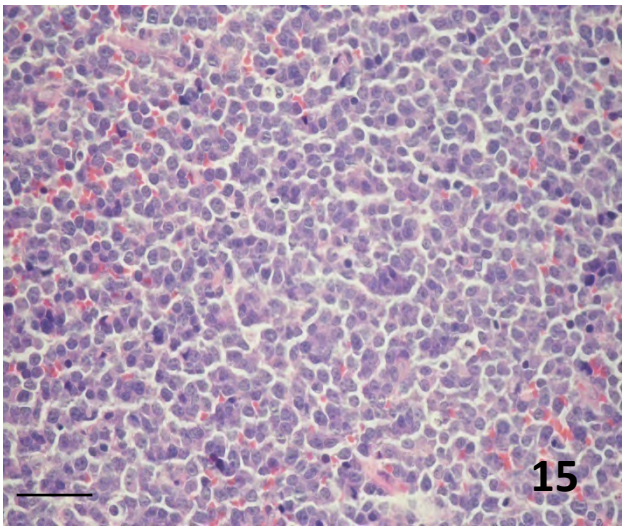
Figure 10 Pleomorphic large cell lymphoma (HE, bar = 25 μm)

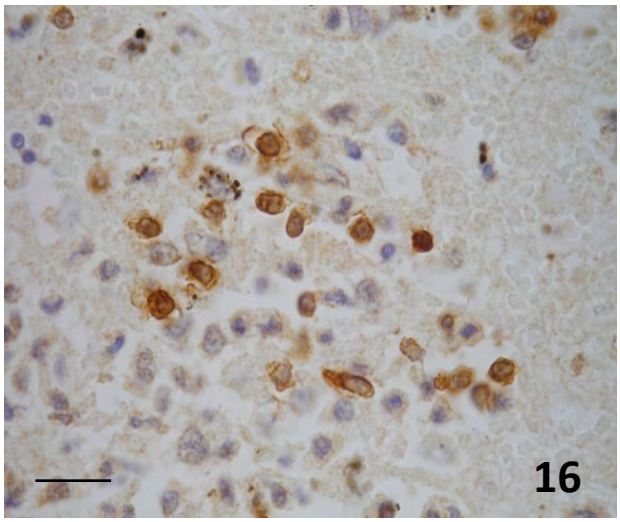
Figure 11 Immunoblastic lymphoma (HE, bar = 25 μm)

Figure 12 Macronucleolated medium-sized cell (HE, bar = 15 μm)

Figure 13 Pleomorphic, mixed, small and large cell (HE, bar = 15 μm)







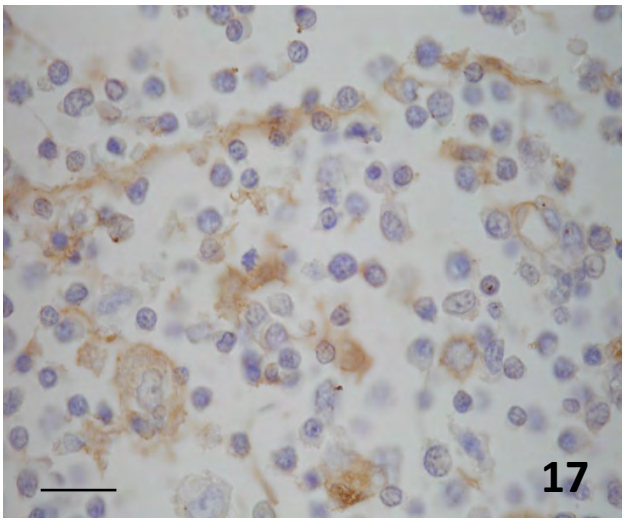


Figure 14 Centrocytic lymphoma (HE, bar = 25 µm)

Figure 15 Lymphoblastic lymphoma (HE, bar = 25 µm)

Figure 16 T cell lymphoma immunolabelled with CD3 antibody, low grade (IHC, bar = 10 µm)

Figure 17 B cell lymphoma immunolabelled with IgM antibody, low grade (IHC, bar = 10 µm)

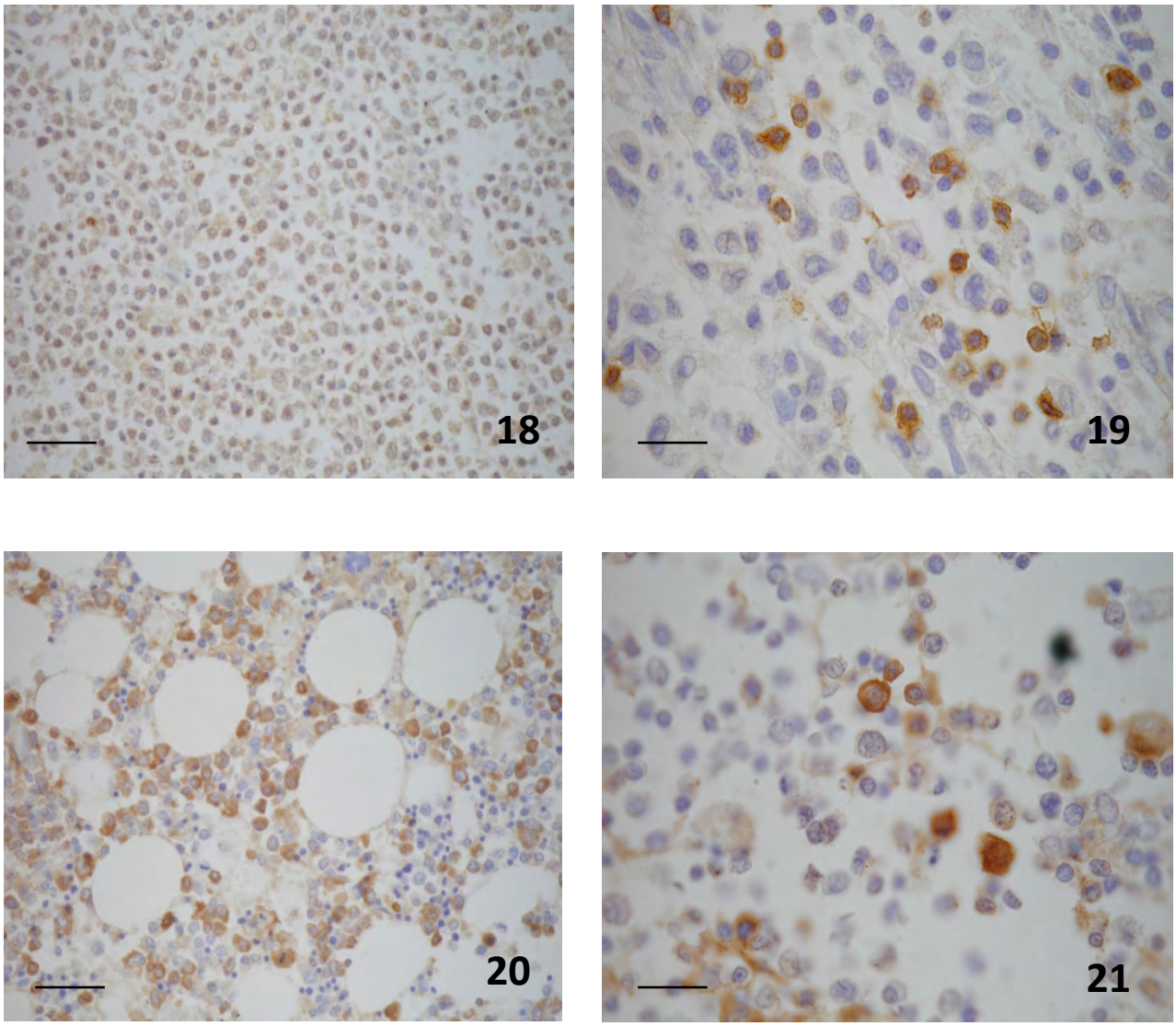


Figure 18 T cell lymphoma immunolabelled with CD3 antibody, high grade (IHC, bar = 25 µm)

Figure 19 T cell lymphoma immunolabelled with CD3 antibody, high grade (IHC, bar = 10 µm)

Figure 20 B cell lymphoma immunolabelled with IgM antibody, high grade (IHC, bar = 25 µm)

Figure 21 B cell lymphoma immunolabelled with IgM antibody, high grade (IHC, bar = 10 µm)

Detection of the rearrangements of antigen receptor genes by using polymerase chain reaction (PCR)

The results of PCR assay detected clonal rearrangements of antigen receptor genes in 6 cytologic and 14 peripheral blood samples of lymphoma cases and was summarized in Table 11. For B-cell lymphoma dogs were positively 9 cases for IgH genes (Figure 22). In addition. T-cell lymphoma dogs were positive 4 cases for TCRγ genes (Figure 23). Only one case of T-cell lymphoma had no amplification product identified in either samples. Of the one dog demonstrated clonality of IgH genes but negative to standardized immunohistochemistry.

PCR detection of MRD before chemotherapy and during remission

PCR assay was positive for 35.7% (5/14) of sample collected in complete remission. Interestingly, Two cases of T-cell lymphoma with pleomorphic mixed and large cell subtypes had partial response which, was the presence of persistence the clonal TCRγ band. The majority of PCR positive sample of MRD during remission were TCR clonal rearrangements (Table 12, 13).

Morphological subtype associated tumor response

For 14 of prospective cases were classified according to the updated Kiel system. The results of prognostic significance of morphological and immunophenotypes subtypes and MRD of 14 cases of canine lymphoma are summarized in Table 13 Eight different morphological subtypes of canine lymphoma, five subtypes of the B-cell, and three of the T-cell phenotype, entered in this study. Almost dogs had a complete response to chemotherapy. Only two cases (L11, L14) had a partial response which were T-cell pleomorphic mixed and large cell types.

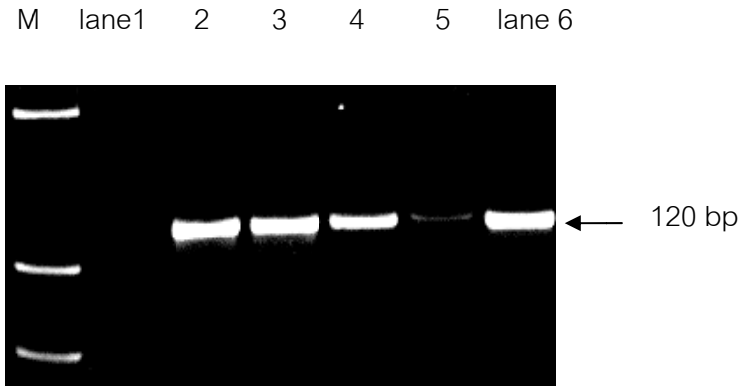


Figure 22 PCR detection of antigen receptor gene rearrangement.

of IgH gene (lane 2-6)

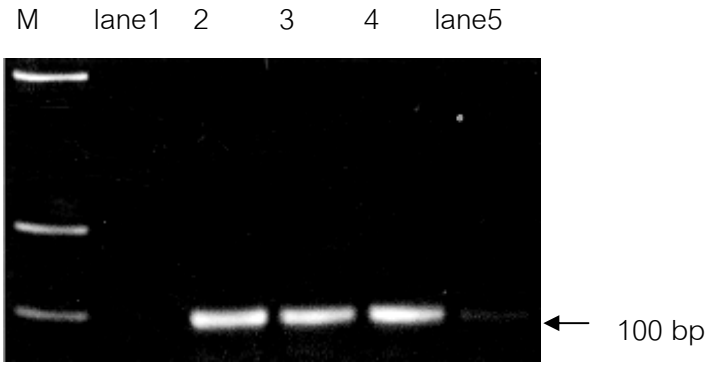


Figure 23 PCR detection of antigen receptor gene rearrangement

of TCRγ gene (lane 2-5)

Table 11 Canine lymphoma:PCR results of rearrangement antigen receptor genes compared with immunophenotyping

Number of case	Immunophenotype	IgH gene	TCRγ genes
L1	B	+	-
L2	B	+	-
L3	B	+	-
L4	T	-	+
L5	B	+	-
L6	B	+	-
L7	T	-	-
L8	T	-	+
L9	B	+	-
L10	B	+	-
L11	T	-	+
L12	B	+	-
L13	-[a]	+	-
L14	T	-	+

+: Positive, -: Negative, B: positive to IgM antibody, T: positive to CD3 antibody

-[a]: negative to IgM and CD3 antibody

Table 12 PCR results of MRD pretreatment and during remission and clinical feathers of prospective cases of canine lymphoma treated with L-asparaginase and Vincristine sulfate

Case Number	Anatomical classification	Clinical stage	Immunophenotype	Histological subtype	PCR result in pre-treatment	PCR result in post-treatment
L1	Multicentric	3	B	CB	+	+
L2	Extranodal	4	B	PL	+	-
L3	Multicentric	4	B	CB	+	-
L4	Multricentric	4	T	LB	+	+
L5	Multicentric	3	B	MMC	+	-
L6	Multicentric	3	B	SL	+	+
L7	Alimentary	5	T	PL	-	-
L8	Multicentric	4	T	Plm	+	+
L9	Muticentric	4	B	CB	+	-
L10	Multicentric	3	B	SL	+	-
L11	Cutaneous	3	T	Pll	+	+
L12	Extranodal	4	B	IB	+	-
L13	Alimentary	3	B	CB	+	+
L14	Multicentric	5	T	Plm	+	+

CB: Centroblastic, PL: Prolymphocytic, LB: Lynphoblastic, MMC: Macronucleated medium-sized-cell, SL: Small cell lymphocytic, Plm: Pleomorphic mixed cell, Pll: Pleomorphic large cell IB: Immunoblastic

Table 13 Tumor response and survival data of the 14 cases according to morphological, Immunophenotype subtypes of canine lymphoma during chemotherapy

Case Number	Morphological subtype	Immunophenotype	Tumor response	MRD	Relapsing Disease	Survival time(days)
L1	Centroblastic	B	CR	+	Yes	104
L2	Prolymphocytic	B	CR	-	Yes	160
L3	Centroblastic	B	CR	-	No	225
L4	Lymphoblastic	T	CR	+	Yes	42
L5	MMC	B	CR	-	No	alive
L6	Smallcell lymphocytic	B	CR	+	Yes	MD
L7	Prolymphocytic	T	CR	-	Yes	70
L8	Pleomorphic mixed cell	T	CR	+	Yes	MD
L9	Centroblastic	B	CR	-	MD	MD
L10	Small cell lymphocytic	B	CR	-	No	alive
L11	Pleomorphic large cell	T	PR	+	Yes	22
L12	Immunoblastic	B	CR	-	MD	MD
L13	Centroblastic	B	CR	+	No	230
L14	Pleomorphic mixed cell	T	PR	+	Yes	35

+: clonal band is present, -: clonal band is absent, CR: Complete remission, PR: Partial response, MD: Missing data, MMC: Maceonucleated medium cell-sized

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Canine lymphomas in this study found that the age of affected dogs were between 1-16 years (mean 7.6 years) which correlated to previous reports. Rungsipipat et al. (2003) found the dogs affected by hematopoietic and lymphatic tumors aged between 1.5-17 years (mean 7.10 years). Madewell (1985) and Teske (1994) found that the age of affected dogs were 6.3-7.7 years. The exact classification of clinical signs of lymphomas is useful to prognosis and treatment plans. The most clinical stage was stage 4 (45%) correlated to Jaffe et al. (2001) that found stage 3-5 were mostly stage of affected dogs. In addition, Ponce et al. (2004) also found that 70% of B-cell lymphoma were in stage 3-5 and these are an advanced stage of lymphoma affected dogs. The anatomical classification in this study was multicentric type mostly (64.0%) due to the presence of predominant lymphadenopathy. The affected cases showed diffuse metastatic lymphoma in various visceral organs that caused more severity in clinical signs and staging (Sueiro et al,. 2004; Teske et al., 1994). However, this classification could not completely apply for indication of malignancy and prognosis (Garrett et al., 2002).

Hematological studies showed the anemia in the present study is close to the percentages of 38% found in canine non-Hodgkin's lymphomas (NHLS) (Teske, 1994), and in human NHL (Moullet et al., 1998). Minor hemopathology was thrombocytopenia, that has been reported in 30-50% of dogs and cats with lymphoproliferative tumors in particular (Jordan et al., 1993; Ruslander and Page, 1995). Pathogenesis of anemia and thrombocytopenia was caused by the neoplastic cells infiltrated the bone marrow resulting myelophthisic anemia reduced number of erythroid and myeloid precursor and reduced the number of megakaryocytes (Madewell, 1986). Lymphocytosis with atypical lymphocytes in blood was recognized in 7 dogs. This present is useful for predict disseminated disease however, the previous reported Keller et

al., (2004) found neoplastic lymphocytes in the peripheral blood of dogs with lymphoma more frequently than this study. This result suggested that the microscopic evidence of peripheral blood involvement was low sensitivity and difficult to detection. Interestingly, the hyperproteinemia was observed in a case of B-cell lymphoma associated with renal amyloidosis. The hyperproteinemia especially hypergammaglobulinemia seen as a paraneoplastic syndrome is due to the excessive production of proteins from immunoglobulin-producing plasma cells or B-lymphocytes. In addition to the hypergammarglobulinemia was reported in multiple myelomas, lymphomas and lymphocytic leukemias (Forrester and Relford, 1992). However, this study did not examine the globulin protein. Paraneoplastic hypercalcemia was observed in this study which is associated with T-cell lymphoma cases (Greenlee et al., 1990; Teske 1994; Ruslander et al., 1997). It is particularly described for human NHL induced by the human T-cell lymphotropic virus (HTLV-I) (Grossman et al., 1981; Bazarbachi et al., 1997). Given that hypercalcemia can predict to immunophenotype, indicating T-phenotype. In addition, the assessment of serum calcium are useful in identifying dogs at increase risk of tumor lysis syndrome which can be triggered by chemotherapy (Altman, 2001).

The morphological aspect of canine lymphoma according to NCI-Working Formulation, all finding were diffuse lymphoma and intermediate grade and high grade over low-grade malignancy. The high prevalence of intermediate is similar to previous reports by Greenlee et al. (1990), Teske et al. (1994), Fournel-Fleury et al. (1997) and Arespacochaga et al. (2007), but differ of Carter et al. (1986), in which high grade lymphoma had the highest prevalence (66.8%). The most common lymphoma subtype was the diffuse large cell lymphoma. These results largely accord with those of other published studies (Greenlee et al., 1990; Fournel-Fleury et al., 1997; Arespacochaga et al. 2007). For the NCI-Working Formulation is not based on immunophenotype, a biological factor of important prognostic significance in relation to overall survival time (Kiupel et al., 1999; Dobson et al., 2001). In addition, it has been demonstrated that all endangering descriptive accuracy (Valli et al., 2000) and that the NCI-Working Formulation can be easily compared with other classification for lymphomas.

Determination of the immunophenotype of malignant lymphoma in human had become an essential step in their classification because of the relation to biological behavior and therapeutic response. Consequently, the updated Kiel, and WHO classifications all differentiate lymphomas based on immunophenotype (Fournel-Fleury et al., 2002). In the present study, the B-cell subtype represented 60% of lymphomas, while the T-cell subtype accounted for 40%. Closed to previous reports showed high proportion in B cell. (Fournel-Fleury et al., 1997; Valli et al., 2006). Sueiro et al. (2004) found that 72.7% were B cell neoplasia and 21.8% were T cell neoplasia. Furthermore, Modiano et al. (2005) reported the prevalence of B cell and T cell lymphoproliferative disease found that 61.4% were B cell tumors and 38.6% were T cell tumors associated with the study of NHLs in human that found B cell lymphoma mostly other than in Japan found adult T cell lymphoma more than B cell lymphoma (Intragumtornchai et al., 1996).

The T cell marker CD3 and the B cell marker IgM were used on all sections in the present study. The CD3 antibody is used for grouping T cell lymphomas because it is a highly specific marker for T cell and it is retained following neoplastic transformation. No other cells are known to express the CD3 antigen, with the possible exception of Purkinje cells in the cerebellum (Ferrer et al., 1993). The CD79α antibody (Dako JCB117 Denmark) recognized an extracellular epitope expressed on the CD79α molecule, and detects the full spectrum of B cell neoplasms in routine biopsy material. It is useful in mostly human studies. While CD79αcy antibody (Dako HM57 Denmark) is used for identifying canine B cell lymphoma because it labels normal and neoplastic B cells of mammalian species and it reacts with an intracytoplasmic epitope. At first, we tried to apply the CD79α antibody in this study but it could not express B cell at all. Whatever, it might be the result from the monoclonal type of CD79α raised from human being. Accordingly, we changed to used IgM antibody instead. The IgM antibody is very high specificity and good performances of the antibody have been ascertained in immunohistochemistry as well as in indirect ELISA and immunoblotting. Along with B leukemic lymphocytes do secrete a small amount of IgM, therefore IgM was represented for B cell in this study. However, not all B cells express detectable surface immunoglobulin. Progenitor and pre-

B cells and plasma cells lack surface immunoglobulin (Greenlee et al., 1990). Some reports found the co-expression of CD79α and CD3 Thomas et al., (2001). No co-expression was found in the present study and Sueiro et al. (2004).

Any lymphoma classifications could give information relevant to prognosis and therapy. Most classifications are based on cellular morphology and phenotype, both of which are relevant to therapy and overall survival times (Arespacochaga et al., 2007). The updated Kiel classification is helpful for description of cell types and prognosticating relapse. Most studies showed that high grade tumors response better to chemotherapy than low grade tumors (Valli et al., 2002). The other studies usually used two or three nomenclatures for co-classifying canine lymphoma. It is easy to relate relationship between anatomical classification, clinical staging, prognosis, and cells type. The high grade lymphomas (e.g., immunoblastic, centroblastic, pleomorphic large cell) are common seen in the dog similar to previous studies (Greenlee et al., 1990; Fournel-Fleury et al., 1997; Sueiro et al., 2004; Valli et al., 2006). The large B cell lymphoma was the most encountered type in Greenlee et al., (1990) and Sueiro et al., (2004) studies. In the current study, the high grade T cell lymphoma was the type most frequently encountered. In a study of 46 T cell canine lymphoma (Fournel-Fleury et al., 2002) found that 23.9 % were the pleomorphic, mixed, small and large cell and other 23% were pleomorphic large cell. In the present study 15.0 % (3/40) were the pleomorphic, mixed, small and large cell, 50% (10/40) were pleomorphic large cell, and 5% (1/40) were lymphoblastic. T cell phenotype showed more high malignancy than B cell phenotype and found in all clinical stage according to Table 7.

Cytologic and histopathological assessment of sample obtained by FNA and biopsy are often the first step of morphological diagnosis of lymphoma. However it can be difficult to differentiate reactive and neoplasia, particularly early stage of disease. In this study, have applied an assay that can be used to detect clonally rearranged antigen receptor genes in tissue and blood of dogs with lymphoid neoplasia. The presence of a clonally rearranged IgH

gene indicates a B-cell phenotype and a clonally rearranged TCR gene indicates a T-cell phenotype (Thilakaratne et al., 2010). In the present study, clonal rearrangement of either Ig or TCR genes were detected in 6/6 cytologic and 13/14 peripheral blood sample histopathologically confirmed lymphomas before treatment. The one sample that gave a negative result, despite the presence of histopathologically and immunophenotypically comfirmed lymphoma, Burnett et al. (2003) and van Dongen et al. (2003) explained sample that contained nucleotide sequences in their variable (V) or join (J) region genes to which our primers did not bind. The limitation may be overcome by using newly designed PCR primers that are directed to the conserved nucleotide sequence (Tamura et al., 2006; Yagihara et al., 2007).

One more another aim of this study was to evaluate usefulness of the PCR clonality assay for identification of MRD for determination of remission status of canine lymphomas. The PCR assay was positive for 7/14 of dogs in clinical remission (Table 12 and 13). Interestingly, six dogs especially, that had clonal results during remission had relapse of lymphoma especially 4 cases as T-cell lymphoma. This evidence could be predicted for chemo-resistance of T-cell lymphoma. Thilakaratne et al. (2010) suggest that physical examination and imaging diagnosis may not be sufficient for accurately determining remission, and that PCR is a useful tool for detecting minimal residual disease after chemotherapy.

In addition, Two cases of pleomorphic (mixed and large cell type) T-cell lymphomas were partial response to chemotherapy which had positive for MRD after treatment and short survival time (Table 13). In this study, Pleomorphic T-cell lymphoma is poor prognostic significant of morphological subtype in canine lymphoma. Similar to the study of Ponce et al. (2004), the centroblastic polymorphic B-cell lymphoma and the pleomorphic mixed T-cell lymphoma showed that the first remission and survival time were significantly shorter for the latter.

Conclusion

In conclusion, this study demonstrated important of prognostic differences between the clinical morphological subtypes and phenotypes. The combination of morphological and immunophenotypic data (updated Kiel) proved valuable as a useful for diagnostic classification and prognosis. No any the follicular lymphoma, the value of the Working Formulation for the canine lymphoma classified appears limited. The significant of morpho-immunologic subtype as Pleomorphic T-cell lymphoma is poor prognosis. This result demonstrated that the immunophenotype of lymphoma as a prognostic factor has been consistently confirmed and T-cell lymphoma has a poorer prognosis than B-cell. In addition molecular technique as a PCR is sensitive and applicable to a small amount of DNA obtained from cytologic specimen and peripheral blood. This assay is an effective value of detecting MRD in canine lymphoma, determining phenotype, monitoring therapy and possibly for assessing prognosis. In summary, MRD could be a useful marker to indicate the neoplastic lymphocytes in dogs with lymphoma even in complete response. MRD at the end of chemotherapy was found to be a prognostic marker to predict the relapse subsequence to treatment.

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Appendix

Appendix A

Table A1 Breed, sex, age, anatomical classification, clinical stage and immunophenotype of 50 canine lymphomas

No.	Breed (P/M)	Sex (F/M)	Age (year)	Anatomical classification	Clinical stage	Immunophenotype (T cell/B cell)
1	P	F	1	Multicentric	4	B cell
2	P	F	10	Extranodal	4	B cell
3	M	F	10	Extranodal	4	B cell
4	M	F	5	Multicentric	3	T cell
5	P	F	13	Multicentric	5	T cell
6	M	F	5	Multicentric	4	B cell
7	P	M	1.7	Extranodal	4	B cell
8	P	M	6	Extranodal	4	T cell
9	M	F	4	Mediastinal	5	T cell
10	P	M	4	Multicentric	5	B cell
11	M	F	10	Mediastinal	4	B cell
12	P	M	8	Cutaneous	3	T cell
13	P	F	5	Multicentric	3	B cell
14	P	F	8.5	Cutaneous	2	T cell
15	P	M	7	Multicentric	4	B cell
16	P	M	7	Multicentric	3	B cell
17	M	M	5	Multicentric	4	B cell
18	M	M	2	Multicentric	4	B cell
19	P	M	4	Extranodal	4	B cell
20	P	M	8	Extranodal	4	T cell
21	P	M	11	Multicentric	5	T cell
22	M	F	6	Multicentric	4	T cell
23	P	M	7	Multicentric	2	B cell
24	M	M	7	Alimentarv	3	T cell
25	P	M	6	Alimentarv	2	B cell
26	P	M	16	Multicentric	2	T cell
27	P	F	9	Cutaneous	2	T cell
28	P	M	8	Multicentric	4	B cell
29	P	F	7	Multicentric	5	T cell
30	P	M	7	Multicentric	4	T cell
31	P	M	13	Multicentric	3	T cell
32	P	M	10	Extranodal	2	T cell
33	M	F	8.5	Cutaneous	2	B cell
34	M	M	8	Multicentric	1	T cell
35	P	F	4	Multicentric	3	B cell
36	M	M	8	Multicentric	1	T cell
37	P	F	8	Extranodal	4	T cell
38	P	M	11	Multicentric	1	T cell
39	P	F	14	Multicentric	4	B cell
40	M	M	11	Multicentric	4	T cell

No.	Breed (P/M)*	Sex (F/M)**	Age (year)	Anatomical classification	Clinical stage	Immunophenotype
41	P	F	1	Multicentric	4	B cell
42	P	F	10	Multicentric	4	B cell
43	M	F	10	Alimentary	4	B cell
44	M	F	5	Multicentric	3	B cell
45	P	F	13	Multicentric	5	B cell
46	M	F	5	Multicentric	4	B cell
47	P	M	1.7	Multicentric	4	B cell
48	P	M	6	Alimentary	4	T cell
49	M	F	4	Alimentary	5	B cell
50	P	M	4	Multicentric	5	T cell

TableA2 PCR results of MRD pre-chemotherapeutic treatment and during remission

	Pre-treatment		During remission	
Case number	Peripheral blood	Cytology	Peripheral blood	Cytology
L1	+(IgH)	+(IgH)	+(IgH)	NA
L2	+(IgH)	NA	-	NA
L3	+(IgH)	NA	-	NA
L4	+(TCRG)	+(TCRG)	+(TCRG)	NA
L5	+(IgH)	NA	-	NA
L6	+(IgH)	+(IgH)	+(IgH)	NA
L7	-	NA	-	NA
L8	+(TCRG)	NA	+(TCRG)	NA
L9	+(IgH)	NA	-	NA
L10	+(IgH)	+(IgH)	-	NA
L11	+(TCRG)	+(TCRG)	+(TCR)	NA
L12	+(IgH)	NA	-	NA
L13	+(IgH)	NA	+(IgH)	NA
L14	+(TCRG)	+(TCRG)	+(TCRG)	+(TCRG)

+:Positive, -:Negative, NA:Not available, TCRG: T-cell receptor gene, IgH: Immunoglobulin heavy chain

BIOGRAPHY

Mr. Nawin Manachai was born in 1982 at Kanchanaburi, Thailand. He received Doctor of Veterinary Medicine from Chulalongkorn University in 2006. His major interest is small animal diagnostic pathology. He worked at Department of Pathology and Small Animal Teaching Hospital, Faculty of Veterinary Science Chulalongkorn University, Bangkok, Thailand for two years. At present, he works at Samsen Private Small Animal Hospital, Bangkok, Thailand