

博士論文

**Studies on the expression of interleukin-2 receptor alpha chain (IL-2r α)
and measurement of its soluble form in canine lymphoid tumours**

(犬のリンパ系腫瘍におけるインターロイキン2受容体 α 鎖(IL-2r α)の
発現および血清中可溶性 IL-2r α の測定に関する研究)

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General Introduction

Non-Hodgkin lymphomas are common in dogs, and their annual incidence has been estimated as 13–107 per 100,000 dogs per year (Dorn et al., 1968; Dobson et al., 2002; Mellanby et al., 2003). Lymphoma accounts for approximately 80% of hematopoietic malignancies in dogs (Dorn et al., 1968). In human medicine, World Health Organization (WHO) classification of lymphoid neoplasms has been used to distinguish the indolent subgroups from the major aggressive subgroups (The Non-Hodgkin's Lymphoma Classification Project., 1997). Clinical features in each patient are quite diverse; therefore, lymphoma is composed of multiple disease subtypes in humans. Therapeutic trials focusing on each subtype have been carried out to identify more effective treatment modalities (The Non-Hodgkin's Lymphoma Classification Project., 1997; Armitage., 2012). In veterinary medicine, canine lymphoma is also found to include diverse range of its subtypes and the World Health Organization (WHO) classification of hematopoietic tumours of domestic animals was published in 2002 (Valli et al., 2002). The clinical features of various lymphoma subtypes were found to be remarkably variable as with human lymphomas and a recent study on a large number of dogs with lymphoma showed the subtype categorization as well as their prognoses after treatment (Valli et al., 2013). Further efforts to determine the most effective therapeutic strategy in each subtype of lymphoma are considered to be necessary for obtaining better prognoses and outcomes in each subtype of canine lymphomas showing different histopathological characteristics.

Valli *et al* reported architectural features, immunophenotypes, and molecular clonality findings for canine indolent lymphoma and proposed a new subtype named nodal paracortical (T-zone) lymphoma (TZL), which showed clinically indolent behavior with long survival despite generalized lymphadenopathy and limited clinical response to chemotherapy (Valli et al., 2006). It was reported that the most common histopathological subtype in canine indolent lymphoma was TZL (62%), followed by marginal zone lymphoma (25%) in 60 dogs with indolent lymphoma (Flood-Knapik et al., 2013). Dogs with TZL had the longest median survival (622 days), although conceivably they were diagnosed in later stages of the disease because of the lack of signs associated with progression (Valli et al., 2013). Recently, Seelig et al. reported that immunophenotyping was an important tool for classification of canine lymphomas: all cases with CD45⁺ T-cell lymphoma could be classified as TZL (Seelig et al., 2014). Immunophenotypic features in conjunction with the histopathological findings would facilitate the subtype classification and understanding its pathophysiology.

In humans, Interleukin (IL)-2 receptor alpha chain (CD25) expression has been found in various types of lymphoproliferative diseases such as adult T-cell leukaemia/lymphoma (ATL), hairy-cell leukaemia (HCL), chronic lymphocytic leukaemia (CLL), acute lymphoblastic leukaemia (ALL), Hodgkin's lymphoma (HL), and non-Hodgkin's lymphoma (NHL) (Sheibani et al., 1987; Tesch et al., 1993; Burton et al., 1994; Nakase et al., 1994a, b; Horiuchi et al., 1997). CD25 is a component of interleukin-2 receptor and has two other

subunits, beta and gamma chains. Heterotrimerization of these subunits leads to formation of a receptor with high-affinity binding to IL-2, which induces proliferation and maturation of lymphocytes (Lowenthal et al., 1985). Therefore, it was hypothesized that CD25 on the tumour cells played some biological role in tumorigenesis or resistance to chemotherapeutic drugs (Kuhn et al., 2005). Moreover, CD25 could be a new diagnostic or prognostic marker as well as the therapeutic target in lymphoid malignancies with CD25 expression (Waldmann et al., 2007; Olejniczak et al., 2008; Fujiwara et al., 2013). As shown in humans, canine CD25 is known as an important marker of activated lymphocytes (Helfand et al., 1992; Galkowska et al., 1996). Although expression of CD25 messenger RNA (mRNA) has also been reported in canine lymphoma and leukaemia (Dickerson et al., 2002), the relationships between its expression and pathophysiology or prognosis have not been well understood.

In general, dogs with lymphoma are treated by combination chemotherapy, because many of the canine lymphoma subtypes respond to chemotherapy (Garrett et al., 2002; Ettinger et al., 2003). CHOP-based protocols, using cyclophosphamide, doxorubicin (hydroxydaunorubicin), vincristine (oncovin), and prednisolone, have been used as a standard therapy for canine lymphoma, and the complete response (CR) rates and initial remission durations were reported to be 60–90% and 8–12 months, respectively (Keller et al., 1993; Myers et al., 1997; Zemann et al., 1998). Various CHOP-based protocols have been introduced and remarkable improvement in terms of the response rate, survival time, and

disease-free duration has been obtained since the CHOP-based protocol had been introduced for the treatment of canine lymphoma. In one of the standard CHOP-based protocols, a 6-month modified version of the University of Wisconsin (UW)-Madison chemotherapy protocol (UW25), remission rate as high as 94.2% and median survival time as long as 397 days were reported (Garrett et al., 2002). Many of the canine lymphoma patients can achieve remission after combination chemotherapy; however, they eventually relapse or acquire drug resistance (Garrett et al., 2002; Sorenmo et al., 2010). Therefore, new monitoring systems to predict the relapse or drug resistance are warranted.

In veterinary medicine, measurement of the sizes of lymph nodes and tumor masses after chemotherapy has been used to indicate the response to the treatment. An objective marker to evaluate the amount of tumor cells burden in the body has been required to precisely compare the efficacy of different chemotherapeutic protocols. Recently, a minimal residual disease (MRD) quantification system for canine lymphoma to detect residual malignant cells that have escaped from anti-tumor therapies has been reported (Yamazaki et al., 2008). It was demonstrated that an increase in MRD could be detected before clinical relapse in dogs with lymphoma (Sato et al., 2011); however, sequencing of the rearranged antigen receptor genes and synthesis of patient-specific primers and probe were needed to measure the MRD in these studies (Yamazaki et al., 2008; Sato et al., 2011)

In lymphoid malignancies in humans, soluble form of CD25 is shown to be released

directly from the surface of neoplastic cells constitutively expressing CD25 and its serum level has been found to reflect the prognosis. The level of soluble interleukin-2 receptor (sIL-2r) has been measured in serum samples from the patients with various type of lymphoid malignancies such as HL, NHL, HCL, and ATL (Rubin et al., 1990; Tesch et al., 1993; Nakase et al., 1994a). Several reports revealed the relation between serum sIL-2r concentration and the clinical outcome (Kono et al., 2000; Niitsu et al., 2001; Oki et al., 2008; Ennishi et al., 2009; Morito et al., 2009). Therefore, serum sIL-2r concentration can be used as a biological marker to indicate clinical behavior and the prognosis in human NHL. However, there has been no study to examine the presence of serum sIL-2r in dogs with lymphoma so far.

A series of studies in this thesis were carried out to investigate the expression of CD25 and measurement of sIL-2r in relation to the prognosis and clinical behavior in canine lymphoid tumours. The study in Chapter 1 was carried out to understand the clinical and histopathological features of TZL in conjunction with the expression of CD25. The study in Chapter 2 was aimed to evaluate CD25 expression in various canine lymphoid tumour cells and its relationship with their prognosis. Based on the results from Chapters 1 and 2, Chapter 3 was carried out to detect the presence of sIL-2r in canine serum and quantify its concentration using a newly developed sandwich enzyme-linked immunosorbent assay (ELISA) system especially in dogs with high-grade B-cell lymphoma that underwent a

CHOP-based chemotherapy.

Chapter 1

Clinical and histopathological evaluation of
16 dogs with T-zone lymphoma

Abstract

Clinical and histopathological characteristics of 16 dogs with nodal paracortical (T-zone) lymphoma (TZL) were evaluated. At initial examination, generalized lymphadenopathy was found in all dogs, and peripheral lymphocytosis was found in 10 of the 16 dogs. At initial diagnosis or during the disease course, 8 dogs (50%) were affected with demodicosis. Immunohistochemical analysis for CD3, CD20, and CD25 was performed for 6 dogs with TZL; the tumor cells were positive for CD3 and CD25 and negative for CD20. Median overall survival time was 938 days. A watchful waiting approach was adopted for 6 cases (38%), and 5 of the 6 dogs were still alive at the end of follow-up. The clinical course of TZL in dogs is generally indolent; however, many cases develop a variety of infectious and other neoplastic diseases after the diagnosis of TZL.

Introduction

Non-Hodgkin lymphomas are common in dogs, and their annual incidence has been estimated as 13–107 per 100,000 dogs per year (Dorn et al., 1968; Dobson et al., 2002; Mellanby et al., 2003). In 2002, the World Health Organization (WHO) classification of hematopoietic tumors of domestic animals was published (Valli et al., 2002). In 2006, Valli *et al* reported architectural features, immunophenotypes, and molecular clonality findings for 66 cases of canine indolent lymphoma and proposed a new subtype named nodal paracortical (T-zone) lymphoma (TZL), which showed clinically indolent behavior with long survival despite generalized lymphadenopathy and limited clinical response to chemotherapy (Valli et al., 2006). In the Kiel classification, the lymphoma subtype corresponding to TZL was shown to have cytological characteristics of small clear cell (Fournel-Fleury et al., 1997).

It was reported that the most common histopathological subtype in canine indolent lymphoma was TZL (61.7%), followed by marginal zone lymphoma (25%) in 60 dogs with indolent lymphoma (Flood-Knapik et al., 2013). Dogs with TZL had the longest median survival (622 days), although conceivably they were diagnosed in later stages of the disease because of the lack of signs associated with progression (Valli et al., 2013). Recently, immunophenotyping has been considered an important tool for classification of canine T-cell lymphoma (TCL), which is clinically and histologically heterogeneous. Seelig *et al*

demonstrated immunophenotyping assessment for dogs with TCL by flow cytometric analyses using several types of antibodies and reported that all cases with CD45⁺ TCL could be classified as TZL (Seelig et al., 2014).

In human medicine, WHO classification of lymphoid neoplasms has been used to distinguish the indolent subgroups from the major aggressive subgroups (The Non-Hodgkin's lymphoma Classification Project., 1997). The clinical features of various lymphoma subtypes were found to be remarkably variable. Therapeutic trials focusing on these specific subgroups have been carried out to identify more effective treatment options (The Non-Hodgkin's lymphoma Classification Project., 1997; Armitage et al., 2012). In veterinary medicine, a recent study showed the subtype categorization of 456 dogs with lymphoma and reported their prognoses after a variety of treatment protocols (Valli et al., 2013). Further efforts to determine the most effective therapeutic strategy in each subtype of lymphoma are considered to be necessary for obtaining better prognoses and outcomes in canine lymphoma.

The aim of the present study is to understand the clinical and histopathological features in 16 dogs with TZL, which seems to have distinct clinical characteristics and disease course compared with the more common high-grade lymphomas in dogs.

Materials and methods

Criteria for selection of cases

A retrospective study was performed at the Veterinary Medical Center of the University of Tokyo (UTokyo-VMC) from May 2004 through November 2012. Medical records were reviewed to identify dogs with TZL that presented to the UTokyo-VMC during the period. Cases included in this study were those involving 25 dogs that were histologically diagnosed with multicentric TZL. Of the 25 cases, 9 were excluded for the following reasons: 6 cases for incomplete clinical data and 3 cases for inadequate biopsy procedures instead of the resection of a whole lymph node. All nodal biopsy specimens were reviewed by a single pathologist blinded to clinical information during the slide-review process. All cases were also required to have adequate follow-up information from initial diagnosis.

Immunological subtypes

To examine the clonal expansion of the cells and the cell lineage, polymerase chain reaction (PCR) for antigen receptor gene rearrangement (PARR) and immunohistochemistry were employed. For the detection of clonal rearrangement of *IgH* and *TCR γ* genes, PCR analyses were performed for 8 cases. DNA samples were extracted from primary lymphoma cells obtained by fine needle aspiration or biopsy. PARR was performed as described

previously (Burnet et al., 2003; Valli et al., 2006).

Immunohistochemistry for CD3 and CD20 was employed to determine the tumor phenotype for 16 cases in this study. Anti-CD3 (polyclonal rabbit anti-human A0452; DAKO, Glostrup, Denmark; 1:50 dilution) and anti-CD20 (polyclonal rabbit anti-human RB-9013-P; Thermo Fisher Scientific, Waltham, MA; 1:400 dilution) antibodies were used as primary antibodies. Moreover, immunohistochemical analysis using anti-CD25 (monoclonal mouse anti-human, clone 4C9; Thermo Fisher scientific, Waltham, MA; 1:40 dilution) antibody was performed for 6 dogs with TZL.

Routine protocols of immunohistochemistry were performed on 4- μ m sections of formalin-fixed and paraffin-embedded tissues. These sections were dewaxed and rehydrated through graded alcohols to water, and antigen retrieval was performed in 1% citrate buffer solution (pH 6.0) in the autoclave at 120 °C for 15 min. Endogenous peroxidase was inactivated by 1% hydrogen peroxide in methanol for 30 min, and blocking was processed by 8% skim milk in Tris-buffered saline (TBS) for 40 min. The slides then were incubated overnight at 4 °C with a panel of primary antibodies directed to CD3, CD20, and CD25. Negative and positive control sections also were incubated at this stage. After all slides were washed for 15 min in TBS, EnVision + Dual Link System-HRP for CD3, CD20, and CD25 was applied for 45 min at room temperature. After the slides were washed for 15 min, binding of the antibody was visualized using 3,3'-diaminobenzidine (DAB) as a chromogen, and

slides were counterstained in Mayer's hematoxylin.

Clinicopathological characteristics

Medical records of the cases were reviewed to study clinicopathological characteristics. Age, sex, breed, physical examination features, the complete blood cell count profile at the time of diagnosis, concomitant illness, and survival time were reviewed. Staging was based on the WHO clinical stage criteria for canine lymphomas (Withrow et al., 2007). In addition, dogs were assigned to substage categories "a" (without systemic signs of illness) or "b" (with systemic signs of illness) (Withrow et al., 2007).

Sizes of the peripheral lymph nodes were measured with slide calipers, and the sum of the longest diameter for the target lesions (up to 5 nodes) was calculated (Vail et al., 2010). All cases were divided into 4 groups according to treatment: dogs treated with (1) CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone)-based protocols; (2) MP (melphalan and prednisolone); (3) prednisolone alone; or (4) no chemotherapy. Owners or referring veterinarians were contacted when the information of the treatment or outcome in the medical record was incomplete.

Statistical analysis

Overall survival time (OST) and lymphoma-specific survival time (LSST) were

calculated by Kaplan-Meier statistics for 16 cases with TZL. For both OST and LSST calculations, dogs were censored if they were still alive or lost to follow up. OST was measured from the day on which dogs were newly diagnosed as having TZL to the date of death as a result of any cause. LSST was defined as the time from the day on which dogs were newly diagnosed with TZL to the date of death from TZL. Dogs that died due to causes not likely associated with lymphoma (e.g., progression of concomitant malignant tumors, cardiac disease) were censored.

Results

Characteristics of the dogs with TZL

Sixteen dogs diagnosed with TZL included the following breeds: 9 Golden Retrievers (56%), 3 Shih Tzu (19%), and 1 case each of Cavalier King Charles Spaniel, Maltese, Pembroke Welsh Corgi, and mixed breed. The median age was 8 years (range: 6–11 years), and the sexes were as follows: 2 males, castrated; 4 females, spayed; 7 males, intact; and 3 females, intact.

Clinical conditions and complete blood cell count at diagnosis

All of the 16 dogs had generalized lymphadenopathy, primarily including submandibular, superficial cervical, axillary, inguinal, and popliteal lymph nodes. Splenomegaly and/or hepatomegaly were found in 8 of the 16 dogs. Cutaneous lesions were found in 7 dogs: 3 cases with demodicosis, 2 with pyoderma, 1 with lipoma, and 1 with otitis externa. Four dogs were found to have pale mucous membranes. Of the 16 cases, only 3 cases showed systemic symptoms such as a reduction in activity and appetite (2 cases), and weight loss (1 case). In the WHO clinical staging, 10 cases (63%) were shown to have a large number of typical “clear cells” (as observed in fine-needle aspiration [FNA] samples of the lymph node) in peripheral blood, and thus were classified into stage V. Of the 16 cases, 13 cases (81%) were

assigned into substage “a.” Consequently, WHO clinical stages and substages of the 16 dogs were IIa (n = 1), IIIa (n = 3), IVa (n = 1), IVb (n = 1), Va (n = 8), and Vb (n = 2).

In complete blood cell count at initial diagnosis, of the 16 dogs, 2 (13%) had mild anemia (hematocrit [Hct] 0.30, 0.34). Ten cases (63%) had lymphocytosis (5,230–19,200/ μ l) containing lymphoid cells characterized as “clear cells” in Kiel classification, in the peripheral blood (Fournel-Fleury et al., 1997). Four cases had thrombocytosis (511,000–845,000/ μ l) above the laboratory’s reference range. Information regarding signalment, WHO clinical stages, substages, and clinical information is presented in Table 1–1.

Cytological and histopathological characteristics

The mature lymphoid cells similar to “clear cells” that increased in the peripheral blood in 10 cases had small- to medium-sized nuclei (an average size of 1 to 1.5 red blood cells) showing densely stained chromatin and relatively abundant clear cytoplasm (Fig 1–1. A, B). Such lymphoid cells were also found in the peripheral blood, even in cases without lymphocytosis, although these cases were not assigned to stage V in the WHO clinical staging system.

In the cytological specimens obtained from enlarged peripheral lymph nodes, TZL was composed of small- to medium-sized lymphocytes with clear cytoplasm sometimes showing extension to form hand-mirror or tear-drop image (Fig 1–2. A–D). The nuclei had little

internal detail and were small to medium in size, showed densely stained chromatin, and sometimes had sharp shallow indentations. Mitotic figures were quite rare.

Histopathologically, neoplastic cells were small to intermediate in size, and they expanded from the paracortex to the cortex and medullary cords. The capsule of the lymph node was at least focally thinned, although perinodal tissue was not involved. Fading follicles and germinal centers were found and tended to be peripheralized by the expanding population of neoplastic T cells (Fig 1–3. A, B).

PARR and immunohistochemistry

Of 16 dogs, 8 underwent genetic clonality analyses. Four of the 8 dogs with T-zone lymphoma showed monoclonal rearrangement of *TCR γ* . One dog showed clonal rearrangement of both *IgH* and *TCR γ* . In the other 3 dogs, clonal rearrangement of *IgH* or *TCR γ* was not detected by PARR.

Immunohistochemical analysis for CD3 and CD20 was performed for 16 dogs. In 15 dogs (94%), the majority of the lymphoid cells on the nodes were immunopositive for CD3. In 1 dog (6%), the tumor cells were double-positive for CD3 and CD20. The results regarding immunohistological phenotype and PARR are presented in Table 1–1.

Immunohistochemical analysis for the CD25 antigen was performed for 6 dogs with TZL that were positive for CD3, as confirmed by immunohistochemistry. In all 6 cases,

CD25-positive cells were found in accordance with CD3-positive and CD20-negative neoplastic cellular localization. Among the expanding population of neoplastic cells in the paracortical regions of nodes, almost all the cells were strongly immunopositive for CD25 (Fig 1–4. A–F).

Complications observed in the 16 dogs with TZL

Eleven (69%) of 16 cases had complications during the disease course by follow-up (181–1,662 days; median, 841 days): demodicosis (8 cases), cystitis (4 cases), pyoderma (4 cases), otitis externa (3 cases), corneal ulcer (2 cases), conjunctivitis (2 cases), epulis (2 cases), mast cell tumor (2 cases), sebaceous carcinoma (1 case), pyelonephritis (1 case), chronic renal failure (1 case), melanoma (1 case), hemangiosarcoma (1 case), and meningioma (1 case). Three cases (19%) had a benign skin tumor (lipoma, papilloma, and cutaneous plasmacytoma) and there were no complications in 5 cases (31%) (Table 1–2).

Treatment and response to treatment

All cases were divided into 4 groups according to the treatment: dogs treated with [1] CHOP-based protocols (cyclophosphamide, doxorubicin, vincristine and prednisolone) (2 cases); [2] MP (melphalan and prednisolone) (6 cases); [3] prednisolone alone (2 cases); and [4] no chemotherapy (6 cases) (Table 1–1).

Chemotherapy with a CHOP-based protocol was conducted for 2 cases, because these dogs were found at the initial visit to have respiratory distress due to the lymphadenopathy. A follow-up of response to therapy was conducted for the 2 cases. After the treatment, 1 case attained a partial remission (PR); however, the other case did not respond to CHOP-based protocol.

Treatment with MP protocol was conducted for 6 cases, because of respiratory distress from enlargement of nodes (2 cases) and progressive lymphocytosis in the peripheral blood (4 cases). Of the 6 cases, 5 attained PR and 1 achieved CR. Prednisolone alone was administered for 2 cases. One case achieved PR, but the other did not show any response.

Watchful waiting was selected for 6 cases, because they did not show any symptoms caused by lymphoma during the follow-up period. Although these dogs did not receive any chemotherapy, the lymph node sizes were found to wax and wane for the observation period (10.7–37.1 months, median 26.2 months). One case developed diffuse large B-cell lymphoma (DLBCL) 37.1 months after the diagnosis of TZL.

Prognostic analyses

The median OST was 938 days, and the median LSST was not calculated because lymphoma-related death was found in only 3 dogs, and the number did not exceed half (Fig 1–5). One of the 3 dogs had respiratory distress from enlargement of nodes, and the other 2

dogs had disseminated intravascular coagulation (DIC) associated with progression of lymphoma. All 3 dogs that died of the progression of lymphoma received chemotherapy with CHOP-based protocol or MP protocol. Of the 16 dogs with TZL, 8 dogs died of any reason by the end of the follow-up duration. Cause of death other than lymphoma included metastasis of sebaceous carcinoma (1 dog), hemangiosarcoma (1), IMHA plus IMT (1), congestive heart failure (1) and melanoma (1).

A case that developed DLBCL after the onset of TZL died of the progression of DLBCL 45.8 months after the initial diagnosis of TZL. The dog was treated with CHOP protocol but was unresponsive to the chemotherapeutic agents. In the prognostic analyses, the case was included in the OST group and censored at onset of DLBCL.

Discussion

In this study, I evaluated the clinical and histological characteristics of 16 dogs with TZL and demonstrated that 10 cases with TZL were found to be in advanced clinical stage (stage V) at time of the initial diagnosis; however, 8 of these dogs retained good general conditions and 6 of 16 dogs with TZL showed long-term survival (10.7–37.1 months, median 26.2 months) even without any chemotherapy.

At the initial diagnosis or during the disease course, many cases were found to be affected with demodicosis or experienced the infectious diseases associated with mucocutaneous lesions. The peripheral lymph nodes biopsied from the 16 cases in this study showed characteristic histological appearances as reported by Valli et al (Valli et al., 2006; 2011). Moreover, immunohistochemistry revealed that the tumor cells of canine TZL were strongly positive for CD25.

Golden Retrievers and Shih Tzu accounted for three-fourths of all breeds evaluated, and other breeds included in the study were Cavalier King Charles Spaniel, Maltese, Pembroke Welsh Corgi, and mixed breed. It might be possible that TZL occurs with elevated frequency in Golden retrievers and Shih Tzu, as described in the previous reports (Flood-Knapik et al., 2013; Seelig et al., 2014). In this study, the median age at the time of diagnosis was 8 years (range: 6–11years). A previous study showed that the median age of 10 dogs with TZL was

8.9 years (Valli et al., 2006). Age of the dogs that developed TZL is conceivably similar or slightly older than that of dogs with lymphomas in general (Valli et al., 2013).

At the initial visit, generalized lymphadenopathies in more than 4 regions of the peripheral lymph nodes were found in all of the 16 cases in this study. Demodicosis was found frequently, occurring in 8 cases (50%) throughout the observation period. A previous report showed that almost 10% of canine cases with TZL had demodex infection at diagnosis (Flood-Knapik et al., 2013); however, many more cases with TZL in the study developed an infection of demodex at time of initial diagnosis (3 cases, 19 %) and during the treatment (8 cases, 50%). *Demodex canis* is normally present in dog skin but it has been assumed that immune dysfunctions and genetic disorders cause overgrowth of demodex in canine patients (Barriga et al., 1992; Sarkar et al., 2004). Humans undergoing chemotherapy have been shown to have an increased incidence of demodex infestation (Seyhan et al., 2004; Herron et al., 2005). Considering that many dogs with TZL developed demodicosis, canine TZL could be one of the important underlying diseases for the development of canine demodicosis. The immune abnormalities might be associated with the development of TZL and lead to a frequent occurrence of demodicosis. When peripheral lymphadenopathy is found in the presence of dermatologic problems, differentiation between hyperplastic/inflammatory and neoplastic changes should be made. Diagnosis as TZL should be made not just by cytology but by histopathological examination in order to discriminate accurately between neoplastic

proliferation and hyperplasia by cutaneous lesions. PARR also could be used for the demonstration of the clonal antigen of the lymphoma cells.

Immunohistochemically, the neoplastic cells of 6 cases with TZL examined were intensely positive for CD25. It has been known that heterotrimerization of interleukin-2 receptor α , β , and γ chains leads to high-affinity binding for IL-2 and IL-2/IL-2R complex and has an important role in controlling proliferation and differentiation of immune system cells (Lowenthal et al., 1985). In humans, previous reports have shown that CD25 expression is found in several types of leukemia or malignant lymphoma, including Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL), which express CD25 antigen in a great proportion of cases (Tesch et al., 1993; Nakase et al., 1994a). The complete biological role of IL-2R α expression in tumors is unclear, although IL-2R α can stimulate tumor cell proliferation (Strauchen et al., 1987; Peuchmaur et al., 1990; Tsilivakos et al., 1994). It has been reported that particular hematological tumors highly express the membranous CD25 forming an important phenotypic trait that could be a therapeutic target molecule (Sheibani et al., 1987; Waldmann et al., 1993; Horiuchi et al., 1997). A recent study revealed that canine TZL was associated with loss of CD45 expression, and CD45-negative cells expressed CD25 by flow cytometry analysis (Seelig et al., 2014). A large amount of CD25 expression on canine TZL cells might have some influence on proliferation of lymphoma cells or status of host immunity that leads to demodicosis in the dogs with TZL. It is important to learn more

about aberrant phenotypes in canine lymphomas, not only for accurate diagnostic information but also for the study of the pathophysiology of canine lymphoma.

In our study, of the 16 dogs, only 3 dogs died of lymphoma (TZL) at the end of the follow-up duration. All of them were treated with CHOP-based chemotherapy or MP protocol. Although the number of dogs treated with chemotherapy was small, response rates for dogs treated with CHOP-based chemotherapy or MP protocol were 50% and 100%, respectively. In dogs treated with prednisolone alone or those who did not undergo chemotherapy, there were no cases of death from progression of TZL. A previous report concluded that combination chemotherapy seemed to be less effective in dogs with TZL, compared with that of more aggressive lymphomas (Valli et al., 2007). It was also reported that systemic chemotherapy such as CHOP-based chemotherapy did not make a difference in improvement of outcome, and thus watchful waiting may be a reasonable approach in the early phase of the disease (Flood-Knapik et al., 2013). The findings from this study also concluded that therapeutic intervention based on aggressive chemotherapy was not necessarily required for all dogs with TZL. Moreover, MP protocol could be an effective and reasonable approach in terms of response rate and fewer side effects, if chemotherapy is required.

Clinical features associated with TZL were characterized in most cases by systemic generalized lymphadenopathy, splenomegaly and/or hepatomegaly, lymphocytosis, or reduction in activity. There have been no criteria for initiation of treatment and therapy

protocols, therefore chemotherapy was started for the cases with conceivable disease-related symptoms such as extreme fatigue, occurrence of anemia and/or thrombocytopenia, progressive lymphocytosis, or massive or progressive splenomegaly or lymphadenopathy in this study. This study included only a small number of cases. Therefore, further studies are required to clarify the optimal timing for the initiation of chemotherapy for canine TZL.

A watchful waiting approach was made for 6 (38%) of 16 cases in the study. During the clinical course, most cases retained a good health performance with repeated regression and swelling of lymph nodes for a long time, and no cases experienced the lymphoma-related death by the end of follow-up. It could be suggested that watchful waiting may be a reasonable approach for a proportion of dogs with TZL, especially for dogs without problems in general conditions and blood cytopenias.

In conclusion, TZL is a characteristic subgroup of lymphoma in dogs. Dogs with TZL show an indolent clinical course; however, it may be difficult to achieve a complete remission even if using chemotherapy. Special attention is needed to consider the diagnosis and treatment modalities when a dog is suspected to have TZL.

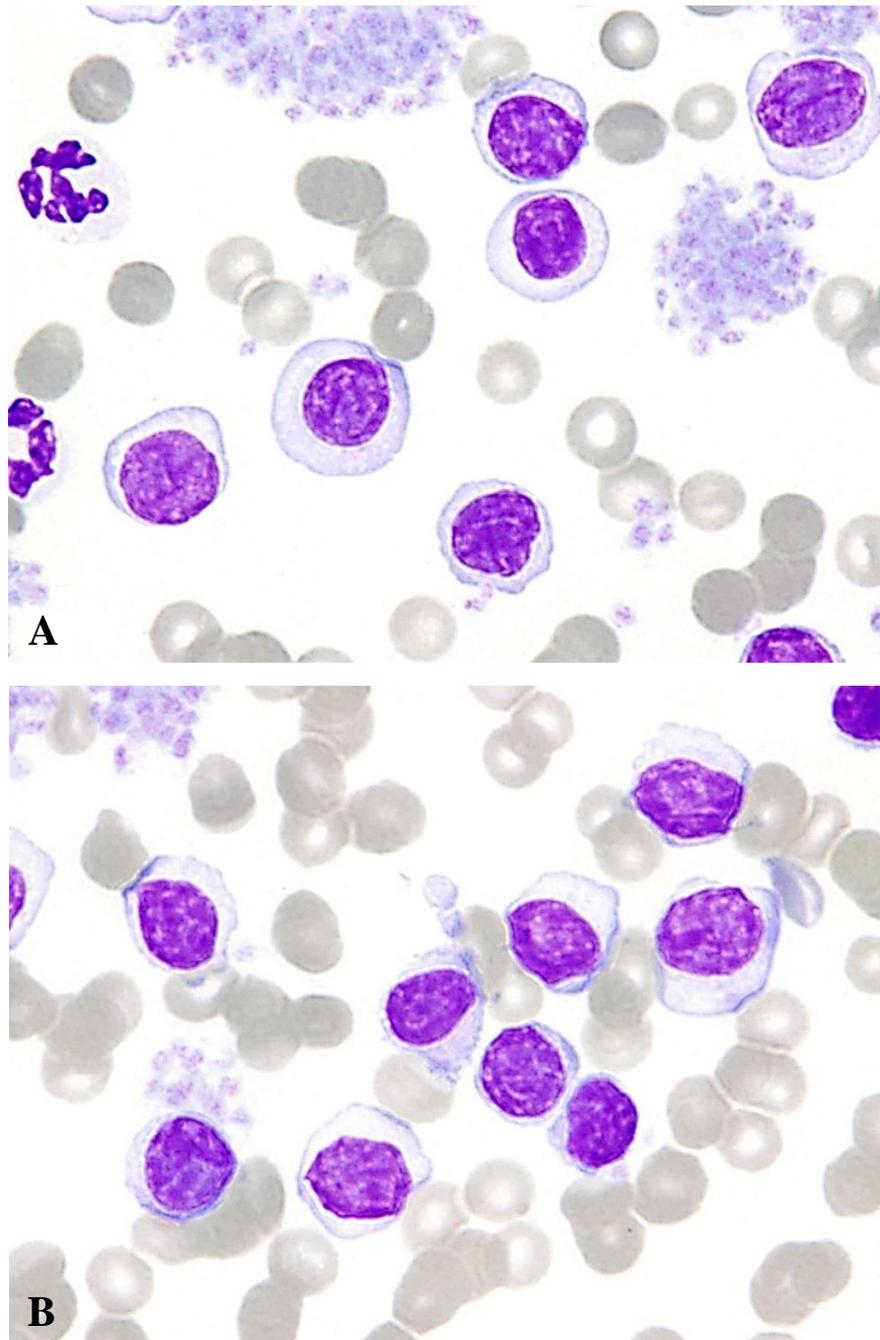


Fig 1-1. Neoplastic cells in PBMC.

(A) TZL cells are small or intermediate lymphocytes, and the cytoplasm is more abundant than that in normal lymphocytes and very lightly stained. $\times 1000$.

(B) The nuclei are relatively uniform in size and about 1 to 1.5 red blood cells in diameter. The cytoplasm is abundant and has indentations. Wright-Giemsa stain, $\times 1000$.

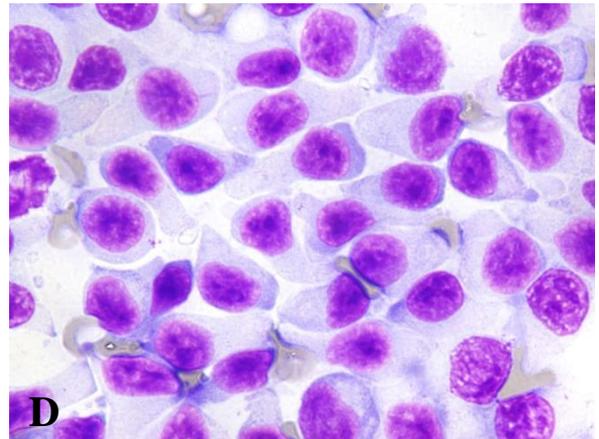
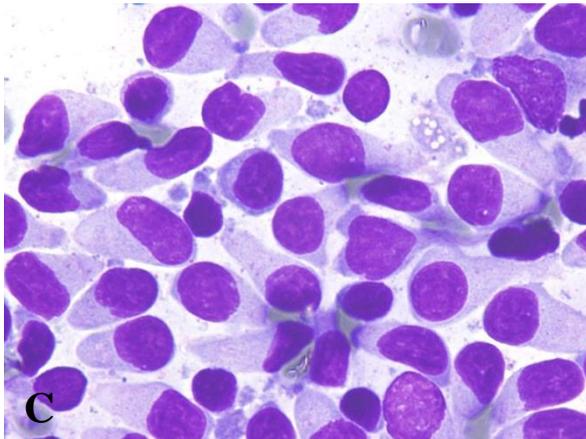
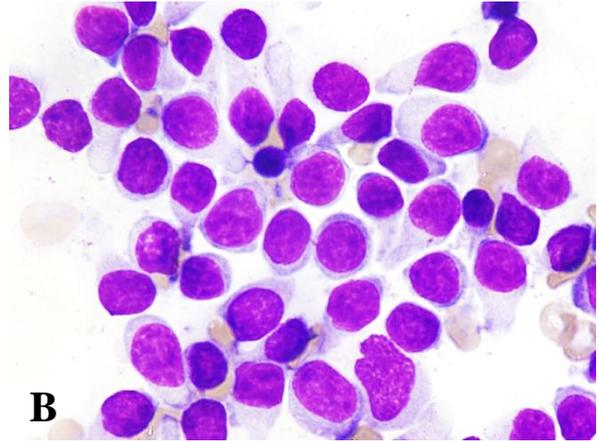
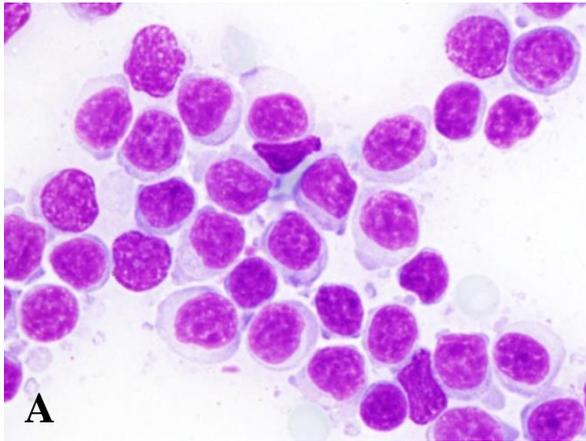


Fig 1–2. Neoplastic cells in lymph node.

(A) Cells with nuclei only slightly larger than red blood cells and sharp shallow nuclear indentation. The cytoplasm in most neoplastic cells is more abundant than normal small lymphocytes. $\times 1000$.

(B) Cells with small nuclei and dense chromatin pattern. The cytoplasm is small or moderate in volume and clear. $\times 1000$.

(C) Cells with dense chromatin pattern and highly extended cytoplasm. The nuclei have sharp shallow indentations and little internal detail. The nucleoli are inapparent. $\times 1000$.

(D) Cells with small central nucleoli and abundant clear cytoplasm. Mitoses are not present in most fields. Wright-Giemsa stain, $\times 1000$.

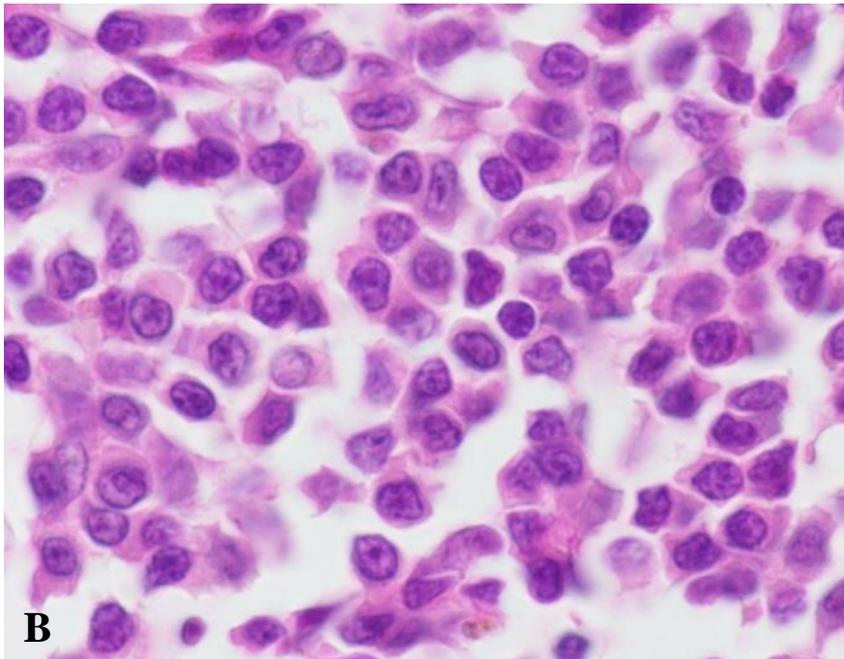
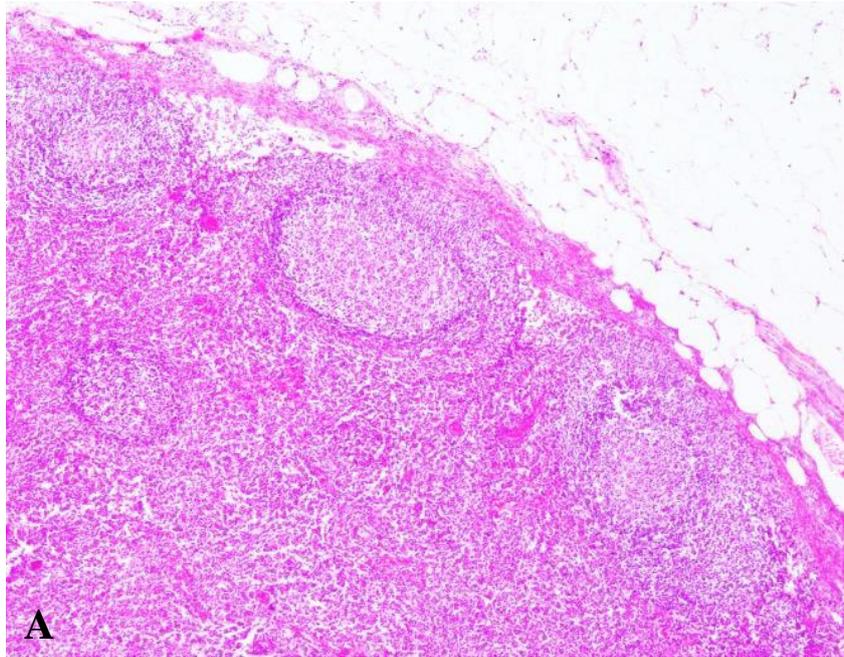


Fig 1–3. The histological features of canine TZL.

(A) Neoplastic lymphocytes expand in the paracortex and are peripheralizing the fading lymphoid follicles. Hematoxylin and eosin (H&E) stain. $\times 40$.

(B) Neoplastic cells are small to intermediate in size. The nuclei are small and have sharp shallow indentations. Some nuclei have small central nucleoli. H&E stain. $\times 1000$.

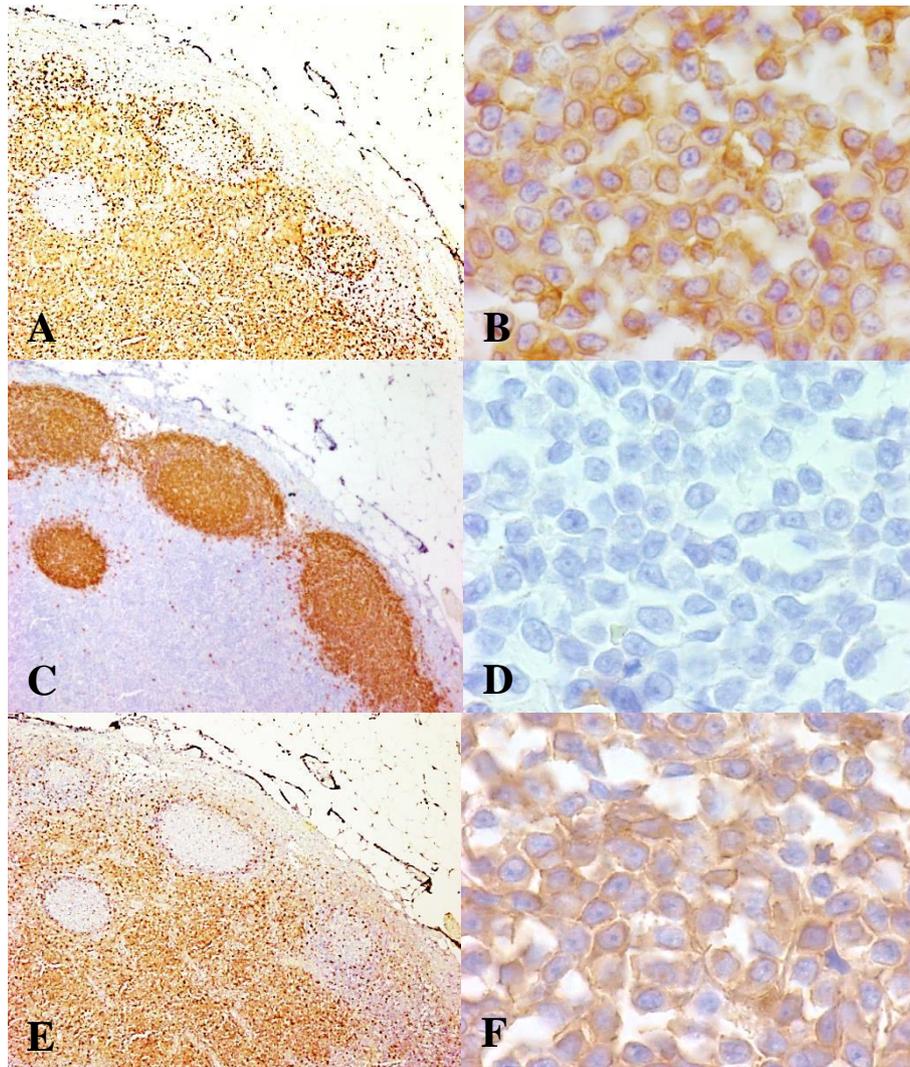


Fig 1–4. The immunohistochemical features of canine TZL.

(A) Neoplastic T cells in the paracortex are uniformly immunopositive for CD3. Immunolabeling with anti-CD3. $\times 40$.

(B) Intense labeling of neoplastic T cells with CD3. The neoplastic T cells are small or intermediate in size. Immunolabeling with anti-CD3. $\times 1000$.

(C) B cells in fading lymphoid follicles are immunopositive for CD20. Follicular structures are pressed against the outer sinus by the expanding population of neoplastic T cells. Immunolabeling with anti-CD20. $\times 40$.

(D) Neoplastic cells in the paracortex are negative for CD20. Immunolabeling with anti-CD20. $\times 1000$.

(E) Neoplastic cells in the paracortex are strongly immunopositive for CD25. B cells in the lymphoid follicles are negative for CD25. Immunolabeling with anti-CD25. $\times 40$.

(F) CD25-positive neoplastic cells are small to medium in size. Some cells have sharp shallow nuclear indentations and abundant cytoplasm. Immunolabeling with anti-CD25. $\times 1000$.

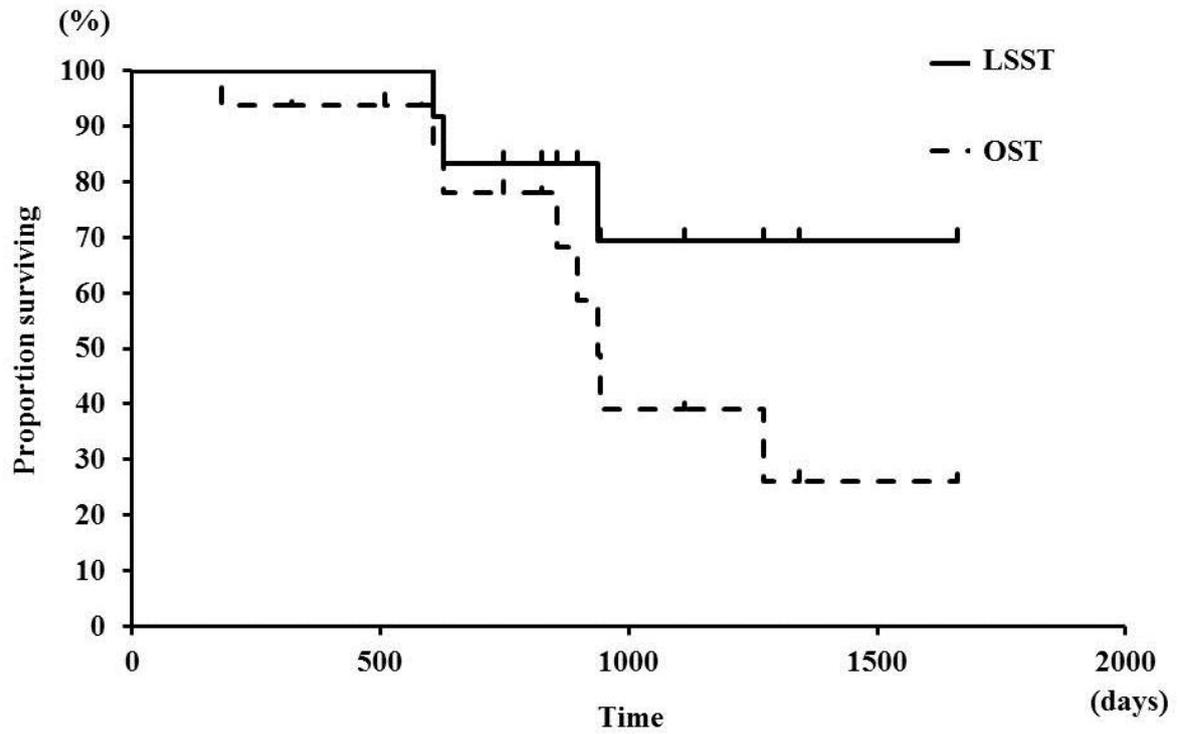


Fig 1–5. Survival curves for overall survival and lymphoma-specific survival in 16 dogs with TZL.

The median OST was 938 days and the median LSST wasn't calculated.

Table 1–1. Profiles of 16 dogs with T-zone lymphoma.

	Median	Range
Age (years)	8	6-11
Body weight (kg)	26.4	3.9-39.2
	No of Cases	%
Sex		
Male castrated	2	13
Female spayed	4	25
Male intact	7	44
Female intact	3	19
Breed		
Golden Retriever	9	56
Shih Tzu	3	19
Others	4	25
Stage		
I	0	0
II	1	6
III	3	19
IV	2	13
V	10	63
Substage		
a	13	81
b	3	19
Immunological phenotype		
T	15	94
T, B	1	6
PARR		
Clonal rearrangement for <i>TCRγ</i> gene	4	25
Clonal rearrangement for <i>IgH</i> gene	0	0
Clonal rearrangement for <i>TCRγ</i> & <i>IgH</i> genes	1	6
Negative	3	19
Not determined	8	50
Enlarged lymph node		
Systemic	16	100
Local	0	0
Clinical symptom		
Enlargement of spleen or liver	8	50
Cutaneous lesion	7	44
Pale mucous membranes	4	25
Reduction in activity and appetite	2	13
Respiratory distress	2	13
Hematological abnormality		
Anemia	2	13
Leukocytosis	6	38
Lymphocytosis	10	63
Thrombocytosis	4	25
Chemotherapy		
CHOP-based protocol ^{a)}	2	13
M-P protocol ^{b)}	6	38
Prednisolone alone	2	13
No treatment	6	38

a) CHOP-based protocol: cyclophosphamide, doxorubicin, vincristine, and prednisolone.

b) M-P protocol: melphalan and prednisolone.

Table 1–2. Concomitant illness that occurred during the observation period in 16 dogs with TZL.

	No of cases	%
Demodicosis	8	50
Cystitis	4	25
Pyoderma	4	25
Otitis externa	3	19
Corneal ulcer	2	13
Conjunctivitis	2	13
Epulis	2	13
Mast cell tumor	2	13
Sebaceous carcinoma	1	6
Pyelonephritis	1	6
Chronic renal failure	1	6
Melanoma	1	6
Hemangiosarcoma	1	6
Meningioma	1	6
IMHA ^{a)}	1	6
IMT ^{b)}	1	6
Congestive heart failure	1	6
Benign skin tumors ^{c)}	3	19

a) IMHA: immune-mediated hemolytic anemia.

b) IMT: immune-mediated thrombocytopenia.

c) Benign skin tumors: lipoma, papilloma, and plasmacytoma.

Chapter 2

Expression of interleukin-2 receptor alpha chain
(IL-2 α) in canine lymphoid tumour cells
and its relationship with prognosis

Abstract

Interleukin-2 receptor alpha (CD25) expression has been reported in human lymphoid tumours and suggested to correlate with the prognosis. In this study, I evaluated the expression of CD25 in various canine lymphoid malignancies. Immunohistochemical analyses of the tissues from diffuse large B-cell lymphoma (DLBCL) ($n=6$), T-zone lymphoma (TZL) ($n=5$), and follicular lymphoma (FL) ($n=2$) revealed that intense CD25 expression was observed in accordance with lymphoma cell localization. CD25 expression was detected in all of the TZL and FL cases, while it was variable among DLBCL cases. Furthermore, I evaluated CD25 expression in tumour cells by flow cytometric analysis in 29 dogs with lymphoid malignancies, including high-grade B-cell lymphoma ($n=17$), TZL ($n=5$), FL ($n=2$), cutaneous lymphoma ($n=2$), and acute lymphoblastic leukaemia (ALL) ($n=3$). CD25 expression was significantly higher in dogs with high-grade B-cell lymphoma ($n=17$, mean \pm SD, 49.6 \pm 31.3%) or TZL ($n=5$, mean \pm SD, 80.2 \pm 10.0%), but significantly lower in dogs with ALL ($n=3$, mean \pm SD, 3.3 \pm 2.2%) than that in healthy dogs. In prognostic analysis of 15 cases with high-grade B-cell lymphoma, the progression-free survival was significantly shorter in CD25-high group than that in CD25-low group. The results obtained in this study are useful for subtype differentiation and prognostic analysis of canine lymphomas and future development of molecular-targeted therapy directed at CD25.

Introduction

Interleukin (IL)-2 receptor alpha chain (CD25) is a component of IL-2 receptor and has two other subunits, beta and gamma chains. Heterotrimerization of these subunits leads to high-affinity binding to IL-2, which induces proliferation and maturation of lymphocytes (Lowenthal et al., 1985).

In human medicine, CD25 expression has been reported in activated normal T cells and B- and T-cell neoplasms, whereas only <5% of unstimulated peripheral blood T cells were CD25-positive (Robb et al., 1981; Sheibani et al., 1987). Particularly intense expression of CD25 was observed in blast cells in adult T-cell leukaemia (ATL) or hairy-cell leukaemia patients (Sheibani et al., 1987; Horiuchi et al., 1997). Expression of CD25 has also been demonstrated in B-cell chronic lymphocytic leukaemia, acute lymphoblastic leukaemia (ALL) (Burton et al., 1994; Nakase et al., 1994a, b), Hodgkin's lymphoma, and non-Hodgkin's lymphoma (Tesch et al., 1993; Nakase et al., 1994a). Recently, it was reported that CD25 expression was associated with poor response rates and inferior progression-free survival (PFS) in diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma (FL), and are expected to constitute a new prognostic marker (Fujiwara et al., 2013, 2014).

Similar to humans, canine CD25 is known as an important marker of activated

lymphocytes (Helfand et al., 1992; Galkowska et al., 1996). Although expression of CD25 messenger RNA (mRNA) has also been reported in canine lymphoma and leukaemia (Dickerson et al., 2002), the relationship between its expression level and the prognosis has not been investigated.

In the present study, I evaluated CD25 expression in various subtypes of canine lymphoma and ALL by using immunohistochemistry (IHC) and flow cytometry (FCM). Furthermore, I analysed the relationship between CD25 expression and the prognoses for dogs with high-grade B-cell lymphoma.

Materials and methods

Samples for IHC

Thirteen dogs with enlarged peripheral lymph nodes who were referred to the Veterinary Medical Center of the University of Tokyo, or whose tissue samples were subjected to histopathological examination at North Lab, were diagnosed as DLBCL ($n=6$), TZL ($n=5$), and FL ($n=2$) based on the histopathological findings of the biopsy specimens by referring World Health Organization (WHO) histological classification system for haematopoietic tumours of domestic animals (Valli et al., 2011). These tissue samples were further subjected to IHC to examine the expression of CD25. As controls, lymph node specimens from seven healthy dogs and six dogs with reactive lymphadenopathy were also subjected to this analysis.

IHC

Sections of formalin-fixed paraffin-embedded tissues (4 μm) were prepared using the following protocol. The sections were de-waxed, rehydrated, and subjected to antigen retrieval in 1% citrate buffer solution (pH 6.0) in an autoclave at 120°C for 15 min. Endogenous peroxidase was inactivated by 1% hydrogen peroxide in methanol for 30 min, and blocking was processed by 8% skim milk in Tris-buffered saline (TBS) for 40 min. The

slides were then incubated overnight at 4°C with the panel of anti-CD3 (polyclonal rabbit anti-human A0452; DAKO, Glostrup, Denmark; 1:50 dilution), anti-CD20 (polyclonal rabbit anti-human RB-9013-P; Thermo Fisher Scientific, Waltham, MA, USA; 1:400 dilution), or anti-CD25 (monoclonal mouse anti-human, clone 4C9; Thermo Fisher Scientific; 1:40 dilution). After washing for 15 min in TBS, slides underwent reactions with EnVision + Dual Link System-HRP (DAKO) for 45 min at room temperature, were washed for 15 min, and visualized using 3,3'-diaminobenzidine (DAB) as a chromogen. Slides were counterstained in Mayer's hematoxylin.

In six DLBCL cases, the number of CD25-positive lymphoma cells was counted and classified into three groups: group 1 with <20% positive tumour cells, group 2 with 20–60% positive tumour cells, or group 3 with >60% positive tumour cells.

Samples for FCM analysis

Neoplastic cells were obtained from 29 dogs with lymphoid malignancies who were referred to the Veterinary Medical Center of the University of Tokyo. Samples were obtained from peripheral lymph nodes in 24 nodal lymphoma cases, cutaneous masses in two cutaneous lymphoma cases, and peripheral blood separated by Ficoll-Paque gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare, Amersham Place, England) from three ALL cases at the initial diagnoses. Diagnosis was made based on clinical data and

cytological/histological examination of lymph-node or bone marrow in dogs with lymphoma and leukaemia, respectively. PCR for antigen-receptor gene rearrangement (PARR) was performed to determine the clonality and cell lineage of 26 lymphoma and three ALL cases. DNA samples of all cases were extracted from fresh samples from the lymph node, peripheral blood, or bone marrow. Primers and protocols for PCR analyses were described previously (Burnett et al., 2003; Valli et al., 2006). Twenty six dogs with lymphomas were further classified into subtypes of high-grade B-cell lymphoma ($n=17$), TZL ($n=5$), FL ($n=2$), or cutaneous lymphomas ($n=2$) according to the cytology and/or histopathology (Fournel-Fleury et al., 1997, 2002; Valli et al., 2006, 2011). For comparison, samples were also obtained from peripheral lymph nodes from seven healthy dogs and six dogs with reactive lymphadenopathy.

FCM

Single-color FCM was performed for the cell samples obtained from dogs with lymphoid malignancies. Cell suspensions were washed with staining medium (phosphate-buffered saline supplemented with 5% foetal calf serum) and stained with fluorescein isothiocyanate (FITC)-conjugated anti-canine CD25 mAb (P4A10; eBioscience, San Diego, CA, USA) for 30 min at 4°C. Cells were washed twice and analysed with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA). An FITC-conjugated mouse IgG1 was used to analyse

the background fluorescence intensity during each analysis. Lymphoma or leukaemic cells were gated from the forward- and side-scatter properties. A minimum of 10,000 events in the gated region was collected for each sample. The obtained data were analysed using FlowJo software (Tree Star, Ashland, OR, USA). The percentages of CD25-positive cells were calculated from the isotype-matched control for each sample.

Statistical analysis

The Mann-Whitney U test was used to compare the prognosis in dogs with DLBCL showing different CD25 expression. PFS and overall survival (OS) were calculated according to the criteria proposed by the veterinary cooperative oncology group (Vail et al., 2010). PFS was calculated from the date of initial treatment to disease progression or death from any cause. OS was calculated from the date of initial treatment to death from any cause. Dogs that were still alive or lost to follow-up were censored at the date on which they were last known to be alive. PFS and OS were estimated by the Kaplan-Meier method, and the groups were compared by the log-rank test. The p values were two-sided and regarded as significant if $p < 0.05$. Data were analysed using StatMate software version IV for windows (ATMS, Tokyo, Japan).

Results

IHC for CD25 expression

Six dogs with DLBCL, five dogs with TZL, two dogs with FL, six dogs with reactive lymphadenopathy, and seven healthy dogs were subjected to immunohistochemical analyses to examine CD25 expression.

In seven healthy dogs and six dogs with reactive lymphadenopathy, CD25 expression was generally weak in both CD3-positive and CD20-positive cells, regardless of lymph node regions (Fig 2–1. A–D). Less than 10% of the cells in the entire lymph node field were CD25-positive in these dog groups.

Next, CD25 expression was examined in the lymph nodes derived from dogs with TZL. In the lymph nodes from five dogs with TZL, neoplastic T cells, which are small or intermediate in size, expanded in the paracortex, pressing the atrophic germinal centres against the outer sinus. Majority of the neoplastic CD3-positive cells were shown to be strongly immunopositive for CD25 (Fig 2–2. A–D).

Further, I evaluated CD25 expression in the lymph nodes of FL cases. In the two FL cases included in this study, the majority of CD20-positive centrocytes in the follicles were shown to be strongly immunopositive for CD25 (Fig. 2–3. A–D).

Six dogs with DLBCL were also examined for CD25 expression. In all the six cases,

neoplastic large B cells with uniformly large nuclei and scanty cytoplasm diffusely proliferated in the lymph nodes as a mixture of centroblastic cells with multiple nucleoli and immunoblastic cells with a single central prominent nucleolus. Unlike in TZL and FL, CD25 expression in the lymph nodes of DLBCL was variable. Two of the six cases showed relatively weak CD25 expression and were categorized as group 1 (<20% positive tumour cells; Fig 2–4. A, B). Two cases showed intermediate CD25 expression and were judged as group 2 (20–60% positive tumour cells; Fig 2–4. C, D), and the remaining two cases strongly expressed CD25 and were categorized as group 3 (>60% positive tumour cells; Fig 2–4. E, F). Morphological features were not different among the six DLBCL cases exhibiting various degrees of CD25 expression.

FCM for CD25 expression

Samples from 26 dogs with lymphoma, three dogs with ALL, six dogs with reactive lymphadenopathy, and seven healthy dogs were subjected to FCM. Immunological subtypes of tumour samples were determined by PARR. Information regarding signalment, staging at the initial diagnosis, and immunological phenotype is presented in Table 2–1.

Single-color FCM was performed to evaluate CD25 expression in the lymphoid neoplastic cells. The percentages (mean±SD) of lymph node-derived lymphocytes that were CD25-positive in healthy dogs and dogs with reactive lymphadenopathy were 9.8±2.8% ($n=7$,

range: 6.4–13.8%) and $13.7 \pm 5.0\%$ ($n=6$, range: 8.3–22.2%), respectively. CD25 expression was $49.6 \pm 31.3\%$ ($n=17$, range: 0.4–97.1%) in high-grade B-cell lymphoma, $80.2 \pm 10.0\%$ ($n=5$, range: 64.6–95.4%) in TZL, $69.4 \pm 22.3\%$ ($n=2$, range: 47.1–91.7%) in FL, $73.7 \pm 16.5\%$ ($n=2$, range: 57.2–90.2%) in cutaneous lymphoma, and $3.3 \pm 2.2\%$ ($n=3$, range: 1.5–6.4%) in ALL. The percentages of CD25-positive cells were significantly higher in high-grade B-cell lymphoma and TZL, but significantly lower in ALL than those in healthy dogs (Fig 2–5).

Therapeutic response and prognosis of dogs with high-grade B-cell lymphoma

In order to examine the relationship between CD25 expression and prognosis, the clinical course of 15 dogs with high-grade B-cell lymphoma, who received CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone)-based protocols (Garret et al., 2002), were followed for up to 919 days. Since the mean value of CD25-positive tumours among the 15 cases was 58.9%, the cut-off value was set to 60% in this study. Fifteen dogs were divided into two groups: CD25-positive cells $> 60\%$ (CD25-high) ($n=7$) and $<60\%$ (CD25-low) ($n=8$). Three of seven CD25-high dogs (42.9%) responded to the treatment, while seven of eight CD25-low dogs (87.5%) showed the response to treatment. The response rate was not significantly different between the CD25-high and CD25-low dogs. The median value of PFS was 28 days in CD25-high dogs, which was significantly shorter than that observed for CD25-low dogs (median: 140 days; Fig 2–6. A). Kaplan-Meier curves also

showed an apparently inferior OS in the CD25-high group (median: 115 days) relative to the CD25-low group (median: 244 days). However, there was no significant difference between the two groups (Fig 2–6. B).

Discussion

IHC revealed that neoplastic lymphocytes strongly expressed CD25 in all cases of TZL and FL, while lymphocytes of healthy dogs and dogs with reactive lymphadenopathy weakly expressed CD25. However, CD25 expression was variable among DLBCL cases. In all of these types of lymphoma, CD25 expression was predominantly detected in malignant cells. The surrounding reactive lymphoid cells were negative or weakly immunopositive for CD25. In agreement with IHC results, FCM revealed that non-neoplastic lymphocytes of healthy dogs and dogs with reactive lymphadenopathy showed low CD25 expression as compared to lymphoma cells at the initial diagnosis.

In several types of leukaemia or malignant lymphoma in humans, CD25 was shown to be expressed mainly in tumour cells (Waldmann, 1986; Sheibani et al., 1987). Participation of the α subunit of the IL-2R complex was shown to be necessary for the formation of the high-affinity IL-2 receptor in human lymphocytes (Gutgsell et al., 1994; Cassell et al., 2002). Since it was reported that CD25-positive lymphoma cells also produce IL-2 (Sheibani et al., 1987; Peuchmaur et al., 1990), IL-2/IL-2R signalling might activate the proliferation of lymphoma cells (Olejniczak et al., 2008). In dogs, it was shown that multicentric lymphoma cells expressed mRNA of interleukin-2 receptor α , β , and γ chains (Dickerson et al., 2002). The resulting surface IL-2R on lymphoma cells had binding affinity for IL-2. Moreover, it

has been suggested that canine lymphoma cells expressing IL-2R might maintain their proliferative state through an IL-2-dependent autocrine-growth pathway (Helfand et al., 1995). The expression of CD25 in lymphoma cells shown in this study might result in a high-affinity binding to IL-2 leading to lymphoma cell proliferation; however, it remains unknown whether IL-2/IL-2R signalling on each type of canine lymphoma is functional.

Notably, neoplastic lymphocytes from three ALL cases (mean±SD: 3.3±2.2%) showed weak CD25 expression. In human ALL, CD25 expression has been demonstrated (Nakase et al., 1994c), with expression levels significantly lower in T-lineage ALL relative to B-lineage ALL (Nakase et al., 2007). In this study, the three cases of ALL were found to be of T-cell origin based on results of CD3 expression from FCM and clonal rearrangement of the TCR γ gene in PARR. Although the number of ALL dogs analysed in our study was only three, the results showing that canine T-ALL expressed low levels of CD25 was consistent with a previous study, which showed no expression of IL-2R α mRNA in a dog with T-ALL (Dickerson et al., 2002). The result obtained in this study may indicate that expression of CD25 in canine T-ALL is different from that in canine T-cell lymphoma.

In agreement with IHC results, FCM analysis also demonstrated that neoplastic lymphocytes of TZL and FL cases showed high CD25 expression. Canine cutaneous lymphomas also expressed high levels of CD25. In humans, high CD25 expression in lymphoma cells was also reported in cutaneous T-cell lymphomas (CTCL) and FL (Talpur et

al., 2006; Fujiwara et al., 2014). Moreover, in human CTCL, CD25 expression was more commonly observed in lesions from advanced patients, and high CD25 expression was associated with clinical tumour-node metastasis stage and histologic grade (Talpur et al., 2006). In this study, all cases of TZL and FL were found to have systemic lymphadenopathy at their initial diagnosis. Therefore, CD25-positivity might be associated with tumour-node metastasis stage, as is the case with human lymphoma.

However, CD25 expression levels were variable in high-grade B-cell lymphoma (mean±SD: 49.6±31.3%, range: 0.4–97.1%). In human DLBCL, the rate of CD25-positive neoplastic cells was significantly higher than that observed for reactive lymphadenopathy. Similar to our results, the rates of CD25-positive cells detected by single-color FCM varied among human DLBCL cases ($n=123$; mean±SD: 27.8±30.6%) (Fujiwara et al., 2013). In view of the prognosis, human DLBCL was shown to be a heterogeneous group based on the pattern of gene expression or particular protein expression (Alizadeh et al., 2000; Hans et al., 2004). As with human DLBCL, canine high-grade B-cell lymphoma might comprise several different subtypes of lymphoma. It is most likely that canine high-grade B-cell lymphoma could be subdivided on the basis of gene or protein expression pattern in tumour cells.

I investigated the relationship between CD25 expression and the prognosis in dogs with high-grade B-cell lymphoma. PFS was significantly shorter in the CD25-high group (median: 28 days) than that in the CD25-low group (median: 140 days). Although not significant, the

response rate tended to be higher in the CD25-low group than that in the CD25-high group. In human DLBCL patients with high CD25 expression, response rate and PFS were significantly inferior to those with low CD25 expression (Fujiwara et al., 2013). While the biological role of CD25 expression or IL-2/IL-2R signalling in canine lymphoma cells is not clear, the tumour cells express large amounts of CD25, which most likely induces proliferation through IL-2/IL-2R binding, as shown in human lymphoma. In canine high-grade B-cell lymphoma, strong CD25 expression might be associated with proliferation rates and result in a shorter PFS in CD25-high groups. Moreover, CD25 might be an important phenotypic trait and a novel therapeutic target molecule in particular lymphoma subtypes.

In summary, I demonstrated the expression of CD25 in canine lymphoid tumours and that its phenotype varied between subtypes. Moreover, CD25 expression was related with the prognoses in high-grade B-cell lymphoma cases. The results obtained in this study contribute to the subtype differentiation, prognostic analysis, and future development of molecular-targeted therapy directed at CD25.

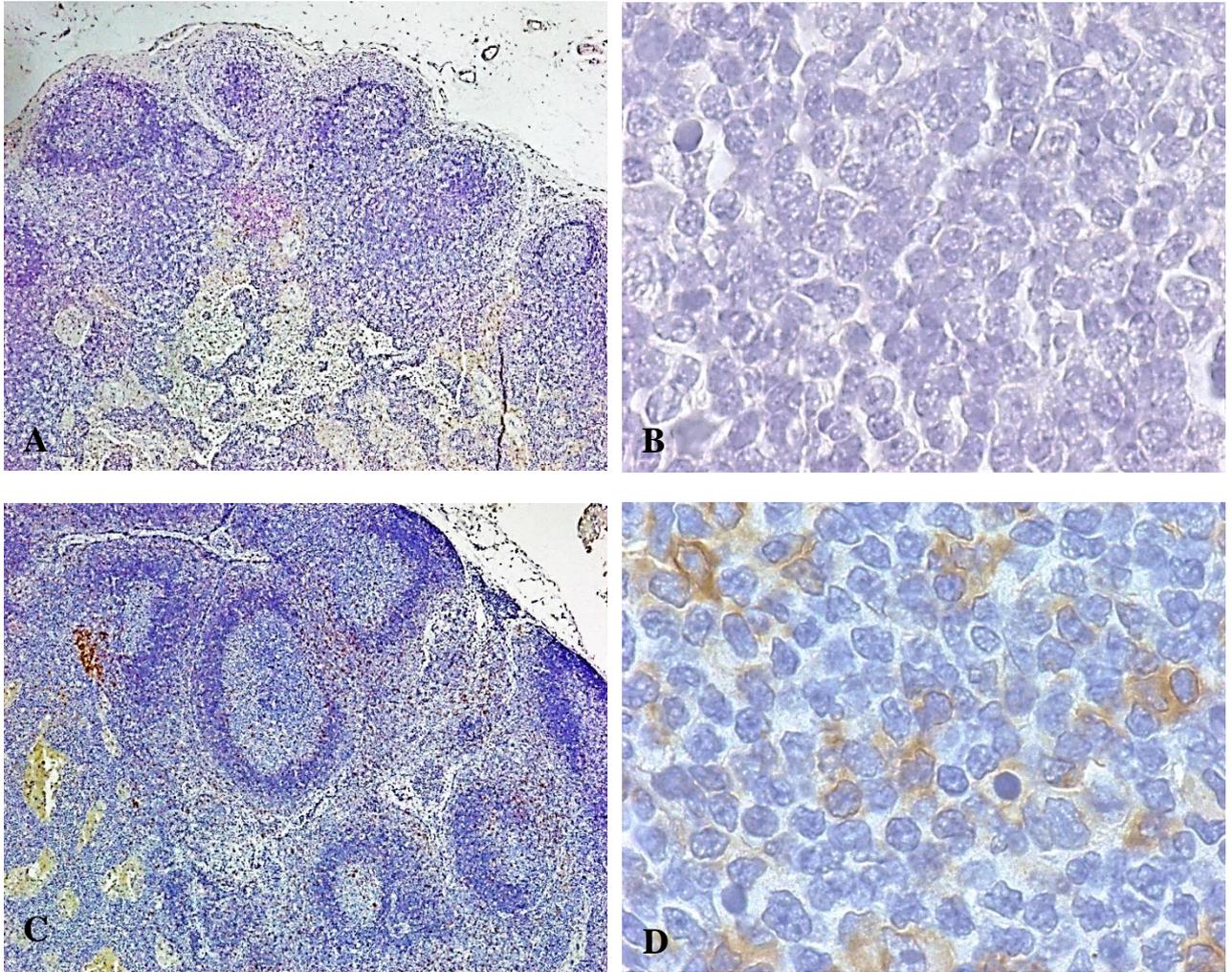


Fig 2-1.
Immunohistochemistry to detect CD25 expression in the lymph nodes of (A, B) a healthy dog and (C, D) a dog with reactive lymphadenopathy. Magnification for A and C: $\times 40$; B and D: $\times 1,000$.

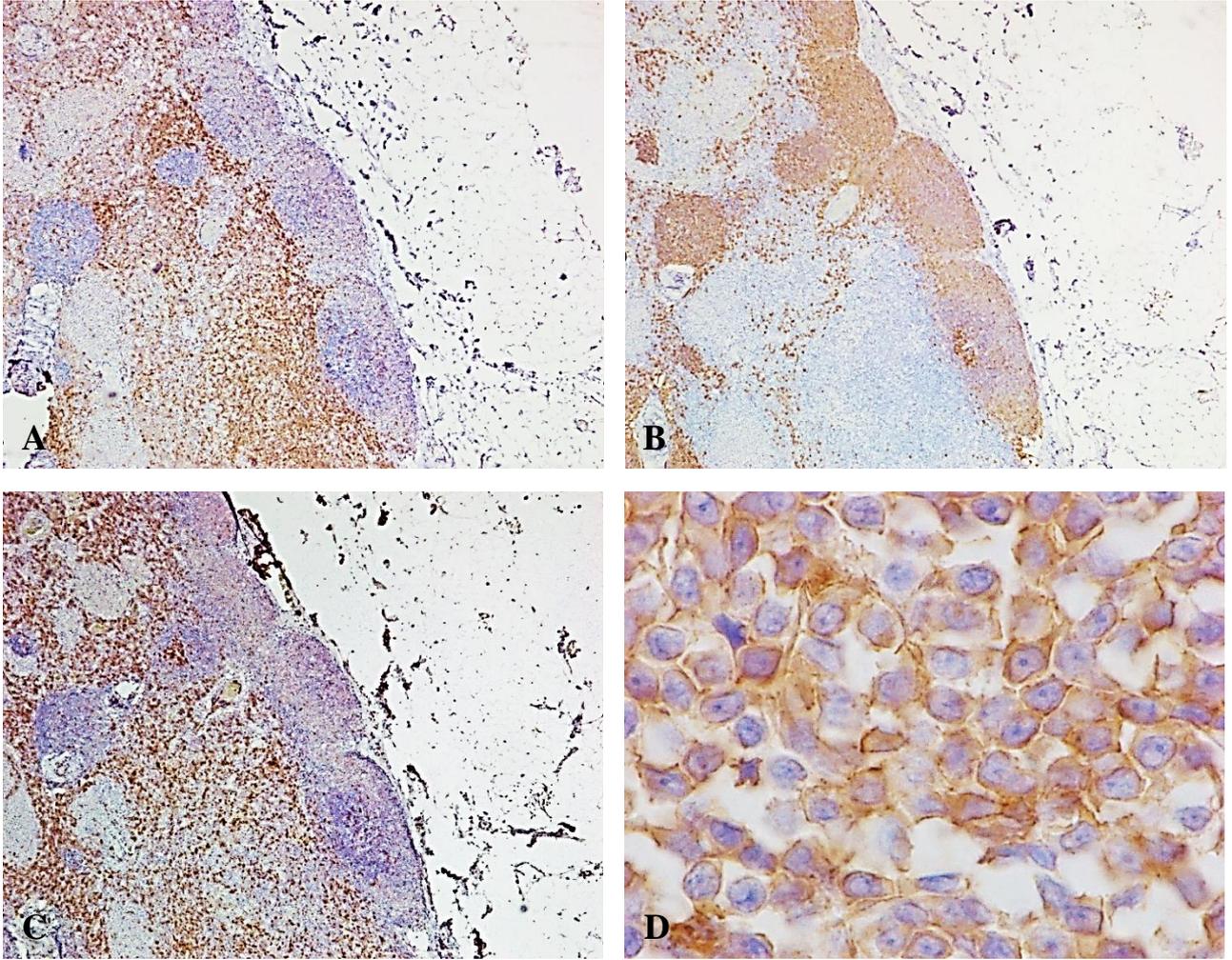


Fig 2–2. Immunohistochemistry of a lymph node in a dog with T-zone lymphoma. Immunolabeled with (A) anti-CD3, (B) anti-CD20, or (C, D) anti-CD25. Magnification for A–C: ×40; D: ×1,000.

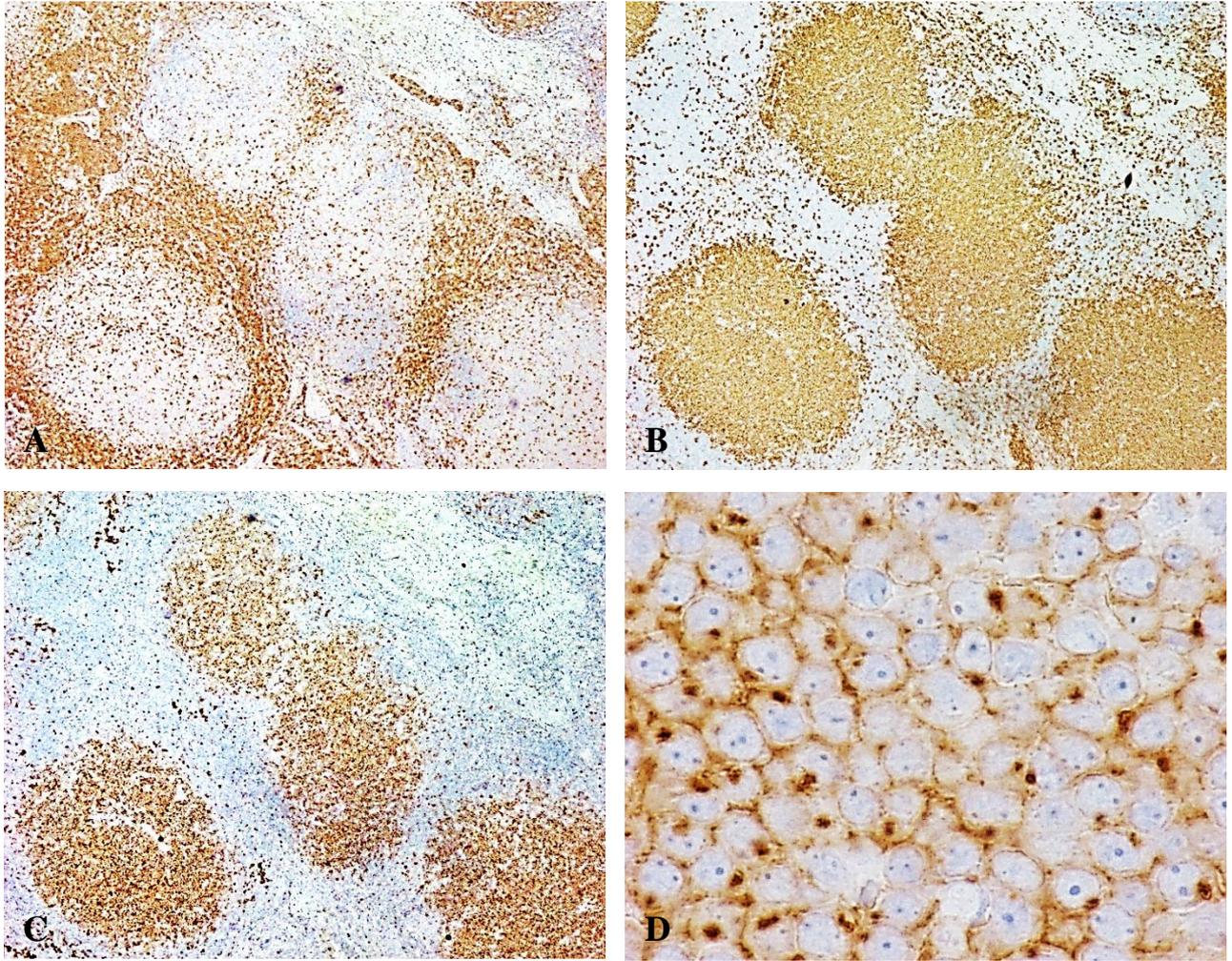


Fig 2-3. Immunohistochemistry of a lymph node in a dog with follicular lymphoma. Immunolabeled with (A) anti-CD3, (B) anti-CD20, or (C, D) anti-CD25. Magnification for A-C: $\times 40$; D: $\times 1,000$.

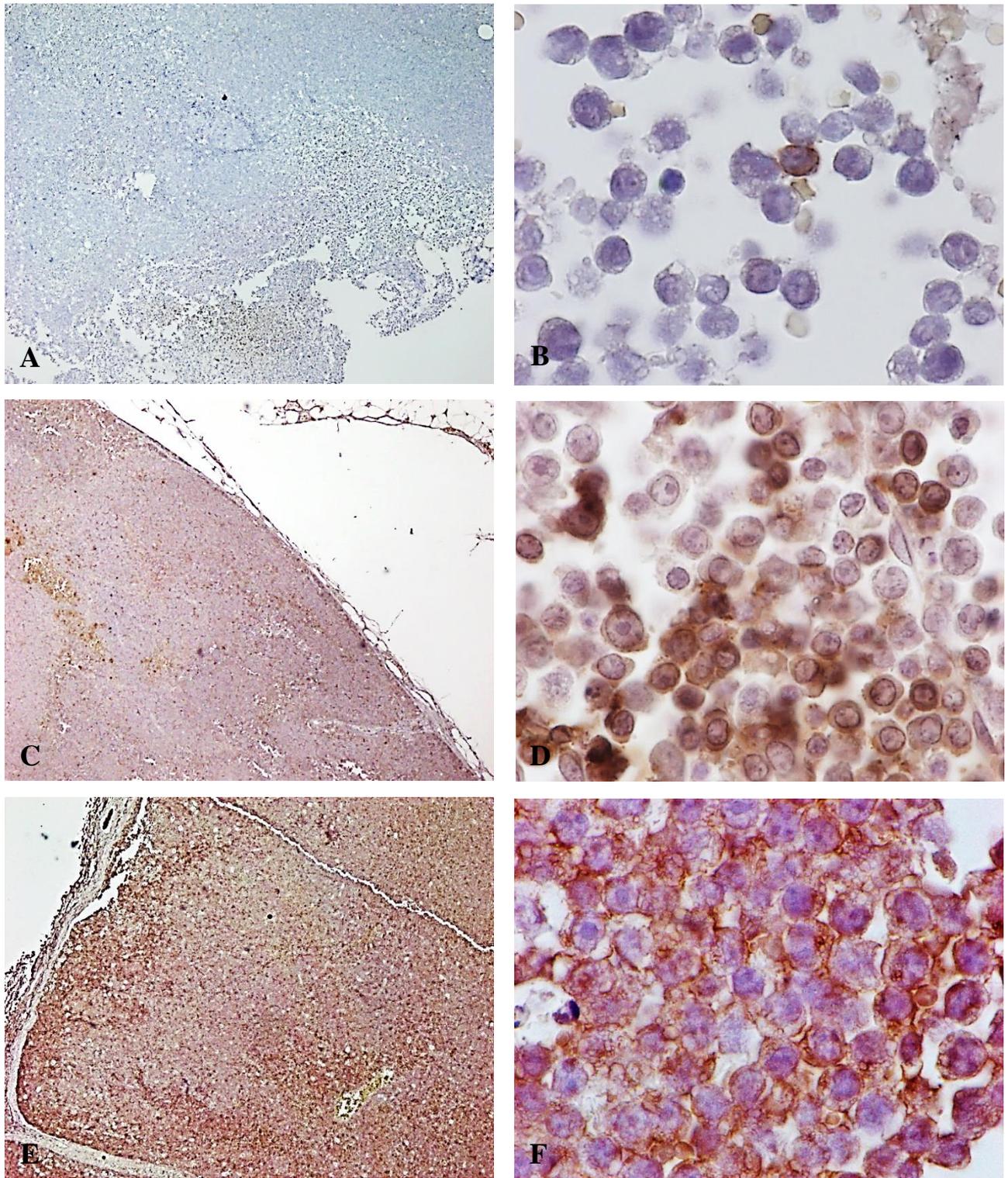


Fig 2–4. Immunohistochemistry of three lymph node samples from dogs with diffuse large B-cell lymphoma.

(A) Group 1 (CD25-positive tumour cells < 20%), (B) Group 2 (expression 20–60%), and (C) Group 3 (expression > 60%). Magnification for A, C, and E: ×40; B, D, and F: ×1,000.

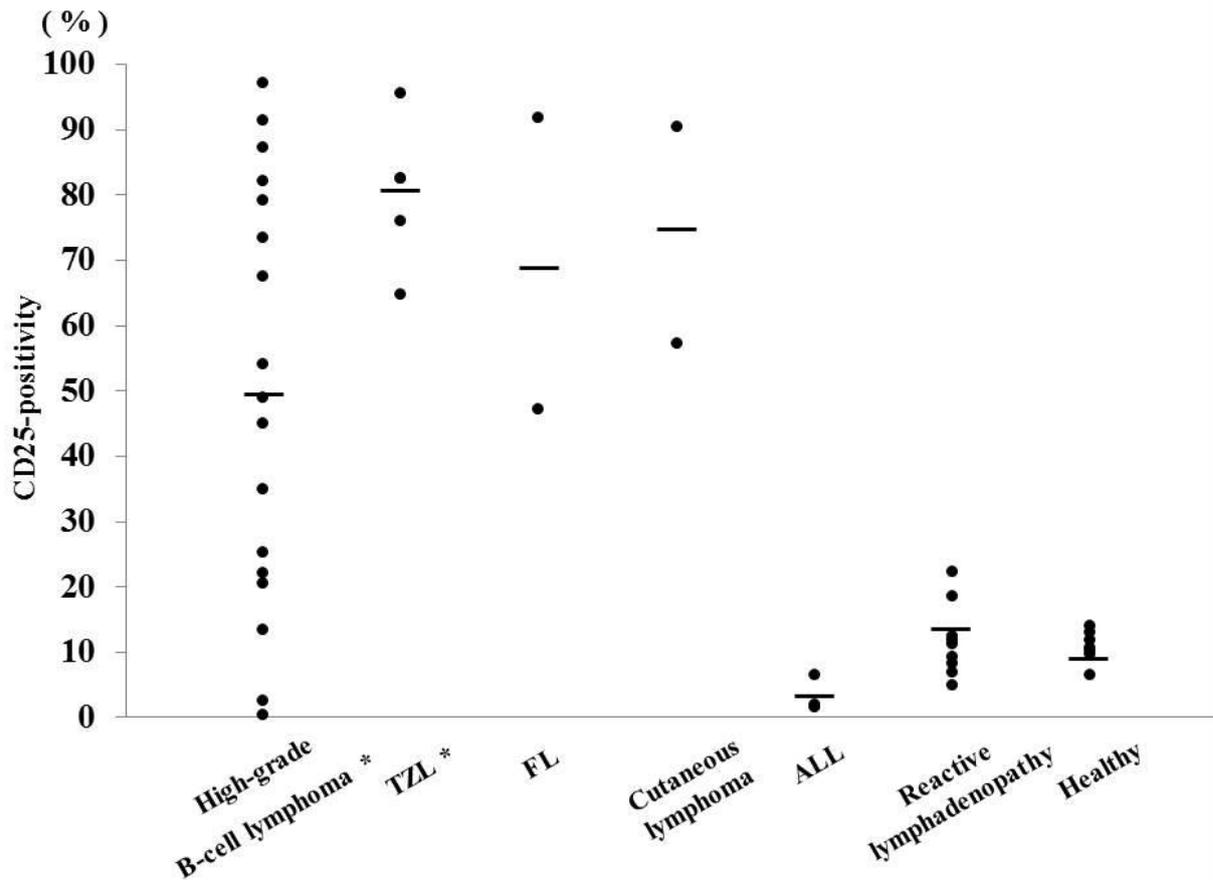


Fig 2–5. Flow cytometric analysis of the lymphoid cell samples from dogs with lymphoid tumours and reactive lymphadenopathy and healthy dogs for CD25.

Lymph-node aspirates were obtained from 17 dogs with high-grade B-cell lymphoma, five dogs with T-zone lymphoma (TZL), two dogs with follicular lymphoma (FL), six dogs with reactive lymphadenopathy, and seven healthy dogs.

Aspirates of the cutaneous masses were obtained from two dogs with cutaneous lymphoma. Peripheral blood mononuclear cells were obtained from three dogs with acute lymphoblastic leukaemia (ALL).

Horizontal bars indicate the mean value of each group.

* CD25 expression is significantly higher as compared to healthy dogs.

$p < 0.05$, Mann-Whitney U test.

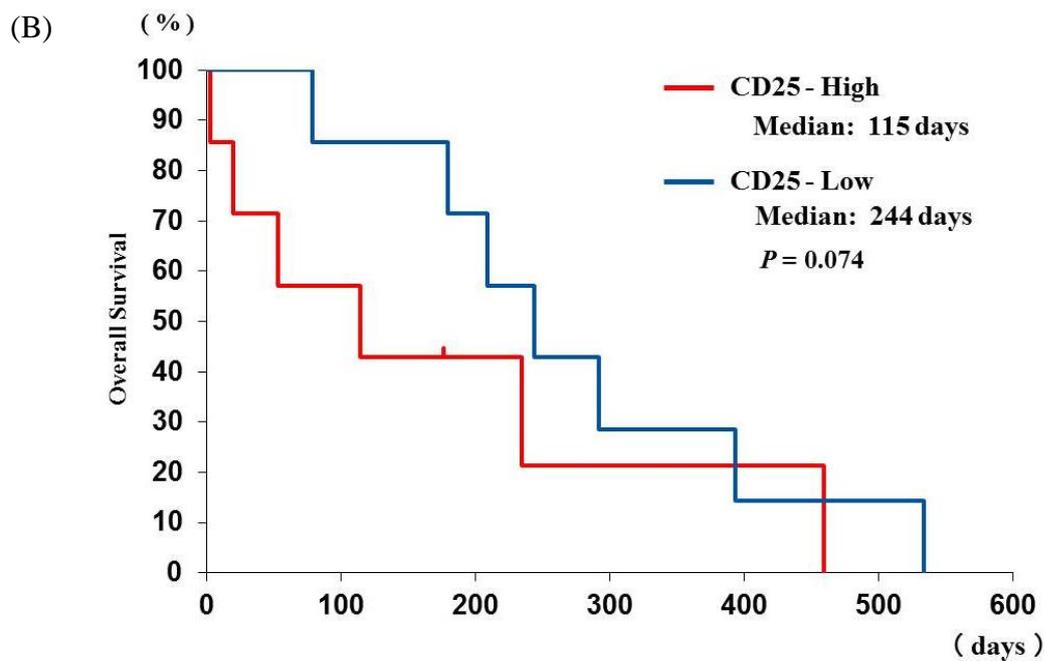
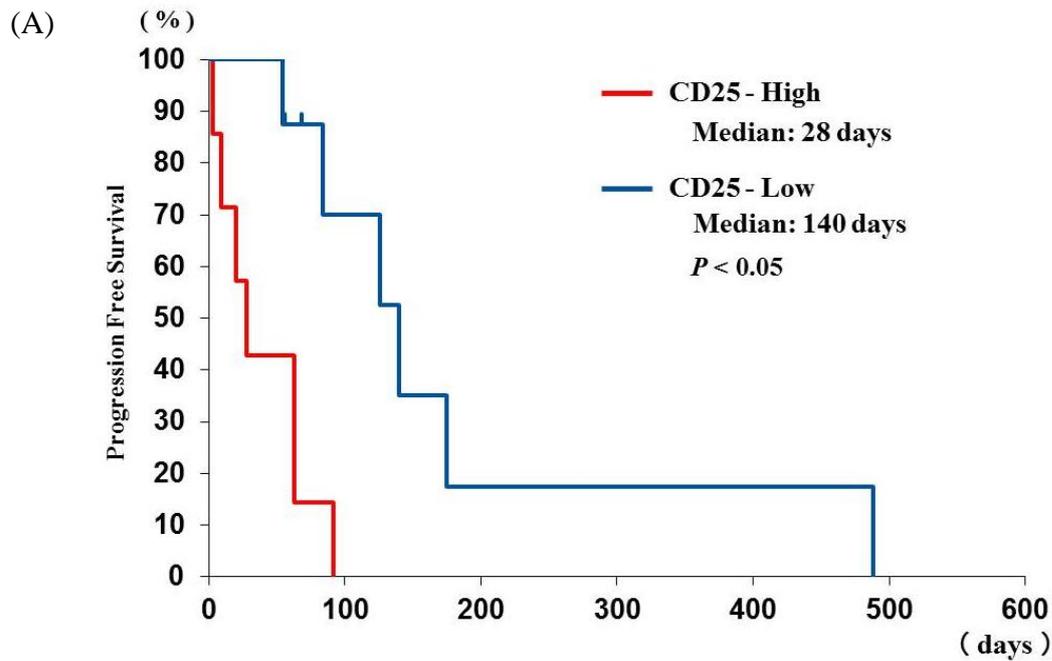


Fig 2–6. Kaplan-Meier plot of (A) progression-free survival and (B) overall survival in the CD25-high and CD25-low groups of high-grade B-cell lymphoma.

Dogs with CD25-positive cells $> 60\%$ were categorized as the CD25-high group ($n=7$), and those with CD25-positive cells $< 60\%$ as the CD25-low group ($n=8$). The two groups were compared using the log-rank test.

Table 2–1. Twenty-six dogs with lymphoma used for flow cytometric analysis.

	Cases	%
Sex		
Male, castrated	7	27
Female, spayed	9	35
Male, intact	6	23
Female, intact	4	15
Breed		
Pembroke Welsh Corgi	7	27
Miniature Dachshund	4	15
Shih Tzu	4	15
French Bulldog	2	8
Pomeranian	2	8
Mixed breed	2	8
Others	5	19
WHO clinical stage		
I	1	4
II	0	0
III	3	12
IV	2	8
V	20	77
Substage		
a	19	73
b	7	27
Histological/cytological subtype		
High-grade B-cell lymphoma	17	65
TZL ^b	5	19
FL ^c	2	8
Cutaneous lymphoma	2	8

^a FCM, flow cytometry.

^b TZL, T-zone lymphoma.

^c FL, follicular lymphoma.

Chapter 3

Measurement of the concentration of serum
soluble interleukin-2 receptor alpha chain
in dogs with lymphoma

Abstract

Soluble interleukin 2 receptor (sIL-2r) is released directly from the surface of lymphocytes expressing interleukin 2 receptor alpha chain (CD25) and its serum concentration has been found to reflect the prognosis in human lymphoproliferative malignancies. In this study, I demonstrated the existence of sIL-2r in canine serum and developed a specific sandwich enzyme-linked immunosorbent assay (ELISA) to quantify the concentration of canine serum sIL-2r. Using immunoprecipitation (IP) assay, CD25 protein weighing approximately 45 KDa was detected in canine serum, smaller than the membrane-bound CD25 (approximately 55 KDa). To measure the concentration of serum sIL-2r in dogs, ELISA system was developed. Serum sIL-2r level was significantly higher in dogs with multicentric high-grade B-cell lymphoma before therapy than that in healthy dogs and dogs with inflammatory diseases. Serum sIL-2r concentration was also found to be elevated in a proportion of dogs with other lymphoma types and immune-mediated diseases. Changes in serum sIL-2r levels generally paralleled with the changes of lymph node size in dogs with high-grade B-cell lymphoma. This study demonstrated that serum sIL-2r level would be a marker to monitor the tumor growth and regression in canine lymphoma.

Introduction

Interleukin (IL)-2 receptor alpha chain (CD25) is a component of IL-2 receptor (IL-2r) together with other subunits, beta and gamma chains. Heterotrimerization of these three subunits leads to high-affinity binding to IL-2, which induces proliferation and maturation of lymphocytes (Lowenthal et al., 1985). CD25 is shown to be a membrane-bound protein of approximately 55 kDa in humans and dogs (Leonard et al., 1983; Abrams et al., 2010). In humans, soluble IL-2 receptor (sIL-2r), which is smaller than its membrane-bound form (45 kDa vs. 55 kDa), can be detected in the serum of healthy individuals (Rubin et al. 1985, 1990). Elevation of the serum sIL-2r concentration has been shown in human patients with various lymphoid malignancies including Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NHL), hairy cell leukemia (HCL) and adult T-cell leukemia/lymphoma (ATL) (Rubin et al., 1990; Tesch et al., 1993; Nakase et al., 1994a). Several reports demonstrated the relation between serum sIL-2r concentration and the clinical outcome in these diseases (Kono et al., 2000; Niitsu et al., 2001; Oki et al., 2008; Morito et al., 2009). Therefore, serum sIL-2r concentration has been used as a biological marker to indicate the growth of tumour cells as well as the prognosis in lymphoid malignancies in humans, especially in NHL.

NHLs are common tumours in dogs, and their incidence has been estimated as 13–107

per 100,000 dogs per year (Dorn et al., 1968; Dobson et al., 2002; Mellanby et al., 2003). Lymphoma accounts for approximately 80% of hematopoietic malignancies in dogs (Dorn et al., 1968). Similar to humans, CD25 is expressed on the cell surface of T-lymphocytes during activation and proliferation in dogs (Helfand et al., 1992; Galkowska et al., 1996). Expression of CD25 messenger RNA (mRNA) has also been detected in canine lymphoma and leukaemia cells (Dickerson et al., 2002). Moreover, it has been suggested that canine lymphoma cells expressing CD25 maintain their proliferative state through an IL-2-dependent autocrine growth pathway (Helfand et al., 1995). In Chapter 1 in this thesis, strong expression of CD25 was revealed in the tumour cells derived from T-zone lymphoma (TZL) in dogs. Next, in Chapter 2, CD25 expression was detected in the tumour cells derived from diffuse large B-cell lymphoma, follicular lymphoma (FL), and cutaneous lymphoma. However, there has been no study to evaluate the existence of sIL-2r in dogs so far.

In this chapter, I carried out a study to examine the presence of sIL-2r in canine serum and quantify its concentration in canine serum using a newly developed sandwich enzyme-linked immunosorbent assay (ELISA) system. Moreover, sequential change of the serum sIL-2r concentration was examined in 4 dogs with lymphoma that had relatively high serum sIL-2r concentrations before chemotherapy.

Materials and methods

Antibody production

A rabbit polyclonal antibody (PAb) directed to a 20-amino acid peptide, CISESQFPDDEELQASTDAP, corresponding to the extracellular domain of canine CD25 (Dickerson et al., 2002) was synthesized. Rabbits were immunized with the synthesized peptide conjugated to keyhole limpet hemocyanin together with complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). Antiserum was subjected to an affinity purification column (Sigma-Aldrich) with the peptide used for the immunization. The purified polyclonal antibody was biotinylated as indicated by the manufacturer (EZ-Link NHS-Biotin; Pierce, Rockford, IL) for sandwich ELISA.

Sandwich ELISA

For the sandwich ELISA, microplates (96-well) (Immuno Plate Maxisorp C96; Nunc, Roskilde, Denmark) were coated with 2 µg/ml mouse anti-canine CD25 mAb (P4A10; AbD Serotec, Kidlington, UK) diluted in 50mM carbonate buffer (pH 9.6) and incubated overnight at 4°C. The antibody-coated plates were then washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T) three times. PBS containing 5% rabbit serum (Sigma-Aldrich), 2% block ace (DS Pharma Biomedical, Osaka, Japan) and 0.4 M NaCl was added to each well as a

blocking buffer. The plates were incubated for 1 hr at room temperature, and then washed three times with PBS-T. Recombinant canine IL-2 receptor α (rcCD25) (R&D Systems, Inc, Minneapolis, MN) was serially diluted 2-fold from 800 to 12.5 ng/ml in dilution buffer (PBS-T containing 5% rabbit serum, 0.5% block ace and 0.4 M NaCl) and used as a standard. Tenfold diluted canine sera (100 μ l) with dilution buffer were used as samples. Plates were incubated at room temperature for 2 hr and then washed three times with PBS-T. Following the wash steps, 100 μ l of 800 ng/ml biotin-conjugated rabbit anti-canine CD25 PAb diluted with dilution buffer was added to each well and reacted in the well at room temperature for 1 hr. After washing three times with PBS-T, 100 μ l of peroxidase-conjugated streptavidin (Thermo Fisher Scientific, Waltham, MA) diluted at 1:10,000 in PBS-T containing 5% rabbit serum was added to each well and incubated at room temperature for 1 hr in the absence of light. After washing three times with PBS-T, 100 μ l of 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Nacalai Tesque, Kyoto, Japan) substrate was added to each well and incubated at room temperature for 20 min in the absence of light. Finally, to stop the color development, 1 N H₂SO₄ was added and absorbance readings at 450 nm were measured using a microplate reader (Bio-Rad, Hercules, CA) to determine OD values.

The assay was validated in determination of working range detection limit, dilution parallelism, spike and recovery test, and intra-assay and inter-assay variability tests. The detection limit and working range were determined by comparing 10 independent results of

blank well and wells with various concentrations of rcCD25. The concentration was determined as detectable if mean \pm 3 SD value calculated from 10 independent results did not overlap with that of blank well. The dilutional parallelism was defined by the linearity of the serially diluted rcCD25 within the working concentration range as determined above. Spiking recovery was determined by adding 29.21 ng/ml and 66.59 ng/ml of rcCD25 to 3 different serum samples of tenfold dilution. Intra-assay variability was determined by evaluating 3 different serum samples with different concentrations of sIL-2r in 5 independent wells. Inter-assay variability was determined by repeating the assay for 3 times.

Serum samples for sandwich ELISA

Canine sera were obtained from 51 dogs with lymphoma referred to the Veterinary Medical Center of the University of Tokyo at initial diagnosis. The dogs with lymphoma were classified from the anatomic forms: multicentric (n=34), alimentary (n=8), cutaneous (n=4), splenic (n=2), mediastinal (n=2), and skeletal (n=1) forms. Diagnosis and anatomic form classification were made based on the physical examination findings, diagnostic imaging, and cytological/histological findings of the lesions. Thirty-four dogs with multicentric lymphoma were divided into high-grade (n=27) or low-grade (n=7) lymphoma. Twenty-seven dogs with multicentric lymphoma were diagnosed as having high-grade lymphoma from the cytological finding of the peripheral lymph node samples obtained by fine-needle aspiration (FNA)

according to the updated Kiel classification (Fournel-Fleury et al., 1997). In 7 dogs with generalized lymphadenopathy suspected as having low-grade lymphoma from the cytology of the FNA aspirates showing a homogeneous population of small clear cell in the updated Kiel classification, resection of a peripheral lymph node was performed for the histopathological examination, resulting in T-zone lymphoma (TZL) in the World Health Organization (WHO) histological classification system for haematopoietic tumours of domestic animals (Valli et al., 2011). PCR for antigen receptor gene rearrangement (PARR) was performed to indicate the clonal origin and the immunological subtype as the previous reports (Burnett et al., 2003; Valli et al., 2006). For comparison, sera were also acquired from 27 healthy dogs, 10 dogs with inflammatory diseases: bacterial gastroenteritis (4 cases) and dermatitis (6 cases) and 3 dogs with immune-mediated diseases: polyarthritis (1 case), myasthenia gravis (1 case) and immune-mediated hemolytic anemia (1 case). All samples were stored at -80°C until analysis.

Flow cytometry (FCM)

Neoplastic cells were obtained from peripheral lymph nodes of 16 dogs with multicentric high-grade B-cell lymphoma by fine-needle aspiration. According to the updated Kiel classification (Fournel-Fleury et al., 1997), all of the 16 dogs were classified as high-grade B-cell centroblastic type.

Single-color FCM was performed for 16 dogs with high-grade B-cell lymphoma. Cell suspensions were washed with staining medium (PBS supplemented with 5% FCS) and incubated with FITC-conjugated anti-canine CD25 mAb (P4A10; eBioscience, San Diego, CA) for 30 min at 4°C. Cells were washed twice and analyzed with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA). A control FITC-conjugated mouse IgG₁ was used to show the background fluorescence intensity during each analysis. Lymphoma cells were gated from the forward and side scatter properties. A minimum of 10,000 events in the gated region was collected for each sample. The obtained data were analyzed using FlowJo software (Tree Star, Ashland, OR). The percentages of CD25-positive cells were calculated in comparison to the isotype matched control for each sample.

Immunoprecipitation

For detection of sIL-2r, 2 µg of anti-canine CD25 monoclonal antibody (mAb) (P4A10, AbD Serotec) was incubated overnight at 4°C with 50 µl of 50% Protein G Sepharose beads (GE Healthcare UK, Amersham Place, UK). After incubating serum samples 250 µl with the Protein G Sepharose beads overnight at 4°C, the IgG-depleted serum samples were incubated with the beads-bound anti-canine CD25 mAb at 4°C for 1 hr. After washing, the beads were boiled with 30 µl of sample buffer (Laemmli sample buffer with 2-mercaptoethanol) and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Beads

without mAb were served as a negative control.

Three canine lymphoid tumour cell lines, UL-1 (Yamazaki et al., 2008), CL-1 (Momoi et al., 1997), and Nody-1 (Hiraoka et al., 2009) were used for immunoprecipitation. Two of these cell lines (UL-1 and CL-1) were cultivated in RPMI-1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Sigma-Aldrich). Nody-1 was cultivated in RPMI-1640 supplemented with 20% fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). All cell lines were cultivated at 37°C in a humidified atmosphere of 5% CO₂. Total protein samples were extracted from UL-1, CL-1, and Nody-1 using lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1.0% NP-40, 10% glycerol, 1 mM EDTA).

These samples were subjected to SDS-PAGE using 12.5% gels, and blotted onto a PVDF membrane (Amersham Hybond-P PVDF membrane, GE Healthcare, Little Chalfont, UK). The membrane was blocked with 1% skim milk in Tris-HCl buffer saline with 0.1% Tween 20 (TBS-T). Immunoblotting was carried out with a rabbit polyclonal antibody directed to human CD25 (sc-664, Santa Cruz Biotechnology, Santa Cruz, CA) (Fellman et al., 2011), which was labeled with horseradish peroxidase (HRP) by using a Peroxidase Labeling kit-SH (Dojindo, Kumamoto, Japan) and diluted at 1:250. After incubating overnight at 4°C and washing with TBS-T, immunodetection was performed by chemiluminescence (SuperSignal

West Dura, Thermo Fisher Scientific) according to the manufacturer's protocol.

Follow-up evaluation during chemotherapy

Follow-up evaluation of the serum sIL-2r concentration during treatment was carried out for 1 dog (Dog 1) with skeletal lymphoma and 3 dogs with multicentric high-grade B-cell lymphoma (Dog 2-4) who received a CHOP (cyclophosphamide, hydroxydaunorubicin [doxorubicin], vincristine [oncovin], and prednisolone)-based protocol, UW-25 (Garret et al., 2002) without administration of L-asparaginase at week 1 because it did not significantly influence the treatment outcome (MacDonald et al., 2005). Serum samples from these 4 cases were obtained at initial diagnosis and during the follow-up period. Sizes of the tumour masses or peripheral lymph nodes were measured with slide calipers, and the sum of the longest diameters of the target lesions (up to 5 nodes) was calculated. Responses to the treatments were evaluated according to the previous report (Vail et al., 2010).

Statistical analysis

Fitting of the curves were analyzed by linear regression analysis. The Mann-Whitney rank sum test was used to compare the sIL-2r levels between the different dog groups. Correlation between cell surface CD25 expression rate and sIL-2r concentration was calculated by the Spearman's rank correlation coefficient. Two-sided *P*-values were regarded

as significant if less than 0.05. Data were analyzed using StatMate software version IV for Windows (ATMS, Tokyo, Japan).

Results

Detection of sIL-2r in canine serum by immunoblotting

Detection of sIL-2r in canine serum was examined by IP analysis and compared with membrane-bound CD25 detected in the cell lines. A single band of CD25 weighing approximately 55 kDa was observed by immunoblotting in CL-1 and Nody-1 but not in UL-1. In a canine serum sample immunoprecipitated with anti-CD25 monoclonal antibody (P4A10)-conjugated beads, a single band of approximately 45 kDa corresponding to sIL-2r was detected, which was smaller than the membrane-bound CD25 (55 kDa). No band was immunoprecipitated by Protein G Sepharose beads without mAb (negative control) (Fig 3–1).

Construction and validation of sandwich ELISA for sIL-2r

In the present sandwich ELISA system, anti-canine CD25 mAb (P4A10) and biotinylated rabbit anti-canine CD25 PAb were used as capture and detection antibodies, respectively. The concentrations of capture and detection antibodies were set to 2 µg/ml and 0.8 µg/ml, respectively. High linearity was confirmed within a concentration range from 493.8 ng/ml to 18.3 ng/ml ($R^2 > 0.99$) and no dilution effects were observed (Fig 3–2). From the 10 independent standard curves, the lower detection limit was set to be 25 ng/ml based on comparison to blank wells. Adding 29.21 and 66.59 ng/ml of rcCD25 to tenfold diluted

canine serum from 3 dogs with various native sIL-2r concentrations, the recovery rate for this assay was determined to be 97–112% (Table 3–1). Intra-assay and inter-assay variabilities were assessed using 3 serum samples with different concentrations (mean±SD; 148.09±5.44, 74.86±1.99, and 33.66±0.26). The coefficients of variations for 5 independent wells to assess intra-assay variability were 4.75, 5.39, and 12.9% in the 3 samples. Inter-assay variability assessed by the coefficients of variations calculated from 3 independent assays was 3.67, 2.66, and 0.76%. A relatively higher coefficient of variation for intra-assay variability was shown for the sample containing a lower concentration of sIL-2r. In contrast, inter-assay variability indicated that this assay had a high reproducibility (Table 3–2). These results showed that the sandwich ELISA developed in this study was reliable for measurement of canine serum sIL-2r ranging from 25 ng/ml to 493 ng/ml.

Serum sIL-2r levels in 51 dogs with lymphoma

Serum sIL-2r concentration was measured in 27 healthy dogs, 10 dogs with inflammatory diseases, 3 dogs with immune-mediated diseases and 51 dogs with lymphoma at initial diagnosis. Serum sIL-2r could be detected in 1 of 27 healthy dogs, 2 of 3 immune-mediated disease and 19 of 51 dogs with lymphoma. In the anatomic forms, the sIL-2r concentration was detectable in dogs with multicentric high-grade lymphoma (12/27, 44%), multicentric low-grade lymphoma (1/7, 14%), alimentary lymphoma (1/8, 13%), cutaneous lymphoma

(1/4, 25%), splenic lymphoma (2/2, 100%), mediastinal lymphoma (1/2, 50%), and skeletal lymphoma (1/1, 100%). The sIL-2r concentrations were significantly higher in dogs with multicentric high-grade lymphoma compared to healthy dogs and dogs with inflammatory disease (Fig 3–3).

Correlation between sIL-2r and CD25 expression

I further examined whether serum sIL-2r concentrations correlated with CD25 expression rate of the lymphoma cells. FNA samples from 16 dogs with high-grade B-cell lymphoma were subjected to single color FCM.

Seven of the 16 dogs with high-grade B-cell lymphoma examined had serum sIL-2r concentrations detectable by the present ELISA system (n=7, range: 41.8–256.9 ng/ml). In the remaining 9 dogs, serum sIL-2r concentration was below the detection limit (25 ng/ml). The rates of CD25 expression (mean±SD) in the FNA samples obtained from the 16 dogs with high-grade B-cell lymphoma were 47.0±30.4% (n=16, range: 0.4–97.1%). No correlation was observed between the serum concentration of sIL-2r and CD25-positive rate of lymphoma cells ($p=0.68$) in dogs with high-grade B-cell lymphoma (Fig 3–4).

Changes of the serum sIL-2r concentrations during the clinical course in dogs with lymphoma

In order to examine the relationship between the serum sIL-2r concentrations and the clinical outcome, I measured the sIL-2r concentrations in comparison to the lymph node sizes in 4 dogs with lymphoma that underwent multi-drug chemotherapy (UW-25) (Fig 3–5).

Case 1 was a 9-year-old Miniature Dachshund with an invasive mass of approximately 10 cm in diameter in the dorsal muscles which was histopathologically diagnosed as skeletal high-grade B-cell lymphoma. The dog was treated with UW-25 protocol and achieved complete response (CR) at week 9. CR could be maintained until week 22 (Fig 3–5. A). The serum sIL-2r concentration was 60.0 ng/ml before treatment (week 1). In parallel to the remission induction, serum sIL-2r level was found to be decreased at week 3 (26.5 ng/ml) and week 9 (<25 ng/ml). Low serum sIL-2r concentration under detection limit was maintained from week 9 to week 22.

A 12-year-old Pembroke Welsh Corgi (Case 2) was diagnosed as multicentric high-grade B-cell lymphoma and was treated with UW-25 protocol. Response to chemotherapy was observed, resulting in CR from week 3 to week 65. At the week 69, relapse with the peripheral lymph node enlargement was observed and the 2nd-round UW-25 protocol was started (Fig 3–5. B). The sIL-2r level was 31.6 ng/ml before chemotherapy and 31.9 ng/ml at week 3. The serum sIL-2r concentration was continuously low below the detection limit from week 4 to week 45; however, it increased again at week 56 (34.0 ng/ml) and continued to increase until week 69 when the relapse became apparent.

A 9-year-old West Highland White terrier (Case 3) was diagnosed as multicentric high-grade B-cell lymphoma. After initiation of treatment with UW-25 protocol, CR was achieved at week 10; however, relapse was observed during the treatment protocol at week 11. Due to the unresponsiveness to the chemotherapeutic agents used in UW-25 protocol, rescue protocols using lomustine and then D-MAC (dexamethasone, melphalan, actinomycin-D, and cytosine arabinoside) (Alvarez et al., 2006) were applied, resulting in limited efficacy (Fig 3–5. C). Because of the resistance to any of the chemotherapies used, Case 3 was euthanized at week 30. Serum sIL-2r concentration was 256.9 ng/ml at week 1 before UW-25 and was kept at relatively high level ranging from 80.9 to 254.0 ng/ml throughout the observation period. Serum sIL-2r concentration was shown to increase at week 3 and week 15 prior to the lymph node enlargement at week 5, and week 17, respectively.

Case 4 was a 9-year-old Border Collie and was diagnosed as multicentric high-grade B-cell lymphoma. After starting UW-25 protocol, this case showed CR at week 5. However, from week 5 the lymph node size was found to wax and wane until week 15 followed by the second CR period from week 17 to week 37. Because progressive disease (PD) was clinically apparent at week 41, the 2nd-round UW-25 protocol was started, resulting in PR from week 43 (Fig 3–5. D). Serum sIL-2r concentration in this case was 68.0 ng/ml before UW-25 and gradually increased until week 17. High sIL-2r level was maintained from week 5 to 17 in conflict with the small size of lymph node. At week 23 in the CR period, serum sIL-2r

concentration was found to decrease to undetectable level (<25 ng/ml). Before the clinical relapse at week 41, the serum sIL-2r was found to be increased (77.8 ng/ml) at week 32. Serum sIL-2r concentration decreased in parallel to the induction of PR at week 43.

Discussion

In dogs, previous studies reported that sIL-2r in bronchoalveolar lavage fluid might serve as a significant biological marker in monitoring the immunological status of dogs after lung transplantation (Chang et al., 1990, 1991). However, there has been no study to evaluate serum sIL-2r concentration in dogs with lymphoma so far because of the lack of specific ELISA system. The sandwich ELISA system developed in this study would enable us to monitor serum sIL-2r concentration in dogs and evaluate its correlation not only with lymphomas evaluated in this study but also other diseases such as various autoimmune and inflammatory disorders.

The data obtained in this study revealed that the sIL-2r concentrations of dogs with multicentric high-grade lymphoma at the initial diagnosis were significantly higher than those of healthy dogs and dogs with inflammatory disease. Moreover, it was found that sIL-2r level was also elevated in a proportion of dogs with other lymphoma types and immune-mediated disease. In humans, elevated serum sIL-2r concentration was observed at diagnosis of the several lymphoid malignancies such as HCL, ATL, HL, NHL, cutaneous T-cell lymphoma (CTCL), acute lymphoblastic leukaemia (ALL), and chronic lymphocytic leukaemia (CLL) (Bien et al., 2008). Serum sIL-2r concentration was reported to range approximately 100-500 U/ml (about 330-1,650 pg/ml) in healthy human individuals (Bien et al., 2008), whereas more

than 10 times higher in patients with aggressive NHL compared to healthy controls (Stasi et al., 1994). Moreover, in human NHL, some studies demonstrated that serum sIL-2r level could be an important prognostic factor for NHL (Niitsu et al., 2001; Goto et al., 2005). In our ELISA system for canine sIL-2r developed here, the detection limit was at 25 ng/ml, which was approximately 100-fold higher than the detection limit in the ELISA system for human sIL-2r (Bien et al., 2008). The ELISA system for canine sIL-2r could detect serum sIL-2r in only 19 of 51 dogs with lymphoma before chemotherapy and could not be used to monitor the change of sIL-2r concentration less than 25 ng/ml. Further study is needed to develop a sandwich ELISA system with higher sensitivity by using more appropriate sets of capture and detection antibodies through production of mAbs directed to canine CD25.

In this study, CD25 expression levels on lymphoma cells were variable in high-grade B-cell lymphoma. No correlation between serum level of sIL-2r and CD25 positive rate of lymphoma cells was observed in dogs with high-grade B-cell lymphoma. In humans, the correlation between CD25 expression level on the tumour cells and serum sIL-2r concentration has been reported in ATL or T lymphoblastic lymphoma (Yasuda et al., 1988; Toji et al., 2015); however, such correlation was not observed in the patients with DLBCL (Fujiwara et al., 2013; Toji et al., 2015). Elevated levels of sIL-2r in the serum have been also found in some autoimmune or inflammatory diseases (Rubin et al., 1990; Bien et al., 2008). Moreover, sIL-2r was reported to be most probably released from activated lymphoid cells

infiltrated in the neoplastic tissues (Frydecka et al., 1996). In the present study, serum sIL-2r concentration was below the detection limit in most of the dogs with TZL, in which high level of CD25 expression was confirmed on lymphoma cells in Chapters 1 and 2 in this thesis. These findings indicate the complex mechanism to generate sIL-2r depending upon the lymphoma subtypes and other possibilities derived from non-neoplastic lymphoid cells in various disorders.

The precise biological role of sIL-2r in lymphoid malignancies remains controversial. Several studies demonstrated suppressed IL-2 level through overexpression of sIL-2r in human patients in advanced clinical stages malignancies (De Vita et al., 1998; Olejniczak et al., 2008). It has been shown that sIL-2r released from the cell membrane keeps an ability to bind IL-2 in humans (Rubin et al., 1985, 1986, 1990). Therefore, some studies suggested that disease-related immunosuppression may be mediated by the increased concentration of sIL-2r (Heaney et al., 1998). Moreover, some studies in human lymphomas demonstrated that patients with higher serum sIL-2r concentration had poorer prognoses or response to therapy (Goto et al., 2005; Bien et al., 2008; Hashimoto et al., 2013). Unfavorable influence from the sIL-2r remains to be elucidated in the dogs with lymphoma in this study, therefore further works to evaluate sIL-2r will be able to disclose its role in various disorders in dogs.

In monitoring serum sIL-2r concentrations during the chemotherapy in high-grade B-cell lymphoma cases, serum sIL-2r concentration was shown to change in association with the

disease course in the 4 dogs examined. In Cases 2 and 4, sIL-2r level was found to increase 13 and 9 weeks before relapse, respectively, when clinical CR was still maintained. In humans, serial determinations of serum sIL-2r were shown to be useful to predict the relapse of malignant lymphoma more than 10 months preceding the relapse (Wakao et al., 2002). Moreover, half-life of sIL-2r was estimated to be 0.62 hr (Bien et al., 2008). Kinetics of canine sIL-2r could be similar to those of human sIL-2r because of the structural similarities of CD25 between the 2 species. The results obtained in this study suggest that serum sIL-2r concentration can be used as an early indicator of relapse in canine lymphoma patients at least which have high concentration of sIL-2r before treatment.

In Case 4, high levels of sIL-2r were continuously observed until week 17 despite regression of lymph nodes after initiation of chemotherapy. In this case, it might be possible that lymphoma cells in several organs in the body other than peripheral lymph nodes caused the increase in sIL-2r during the period. Although Case 4 had peripheral lymphadenopathy as detected by physical examination, there were enlarged intrathoracic, hepatic and subiliac lymph nodes as well as intrasplenic lesions revealed by ultrasonography before chemotherapy. Sizes of hepatic and subiliac lymph nodes and splenic lesion were found to increase at week 5 in spite of the decrease in sizes of the peripheral lymph nodes. Increase in serum sIL-2r concentrations during the period might reflect the lymphoma lesions in several organs in the body.

The sandwich ELISA assay developed in this study demonstrated the changes of serum sIL-2r concentrations reflecting the growth/regression of lymphoma in dogs as previously shown in humans (Wakao et al., 2002). Monitor of sIL-2r levels during the chemotherapy would enable us to acquire the predictive information of biological behavior for dogs with lymphoma. Using the system developed here, it would be possible to use sIL-2r as a novel biological marker of activity of neoplasms in the progression and regression of lymphoma. The sIL-2r level can be a candidate marker as an indicator for predicting early relapse and assessing treatment efficacy in canine lymphoma.

In conclusion, I demonstrated the presence of sIL-2r protein of 45KDa in canine serum and constructed a specific sandwich ELISA system for its quantification. Moreover, dogs with multicentric high-grade B-cell lymphoma were shown to have high serum sIL-2r level before treatment and serum sIL-2r level was found to reflect the tumour cell burden in canine lymphoma cases. Although improvement of the sensitivity of the ELISA system is required, the present study revealed the possible clinical usefulness to monitor the disease course in canine lymphoma. Further studies on the biological role of sIL-2r will contribute to advance the clinical monitoring and elucidation of pathophysiology in dogs with lymphoma.

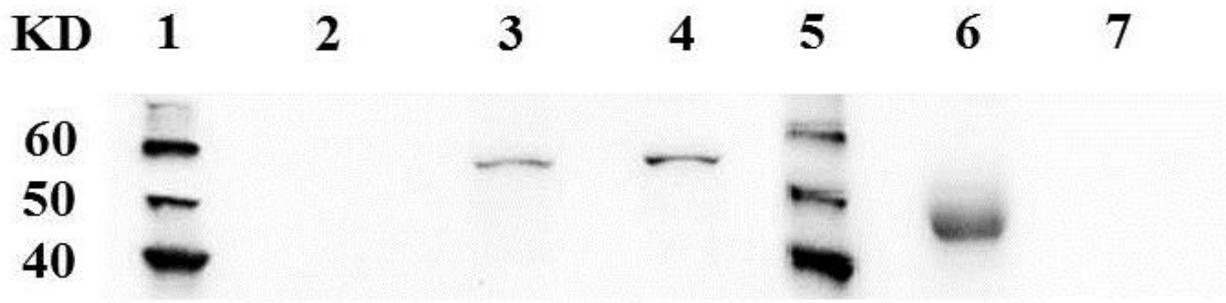


Fig 3-1. Immunoblot for detection of CD25 in 3 canine lymphoid cell lines and a serum sample from healthy dog.

Lanes 1 and 5: marker protein; Lanes 2, 3, and 4: cell lysates of canine lymphoid tumour cell lines, UL-1, CL-1, and Nody-1, respectively; Lanes 6 and 7: normal dog serum samples immunoprecipitated with and without P4A10, respectively.

Immunoblot was performed by rabbit anti-CD25 antibody (sc-664).

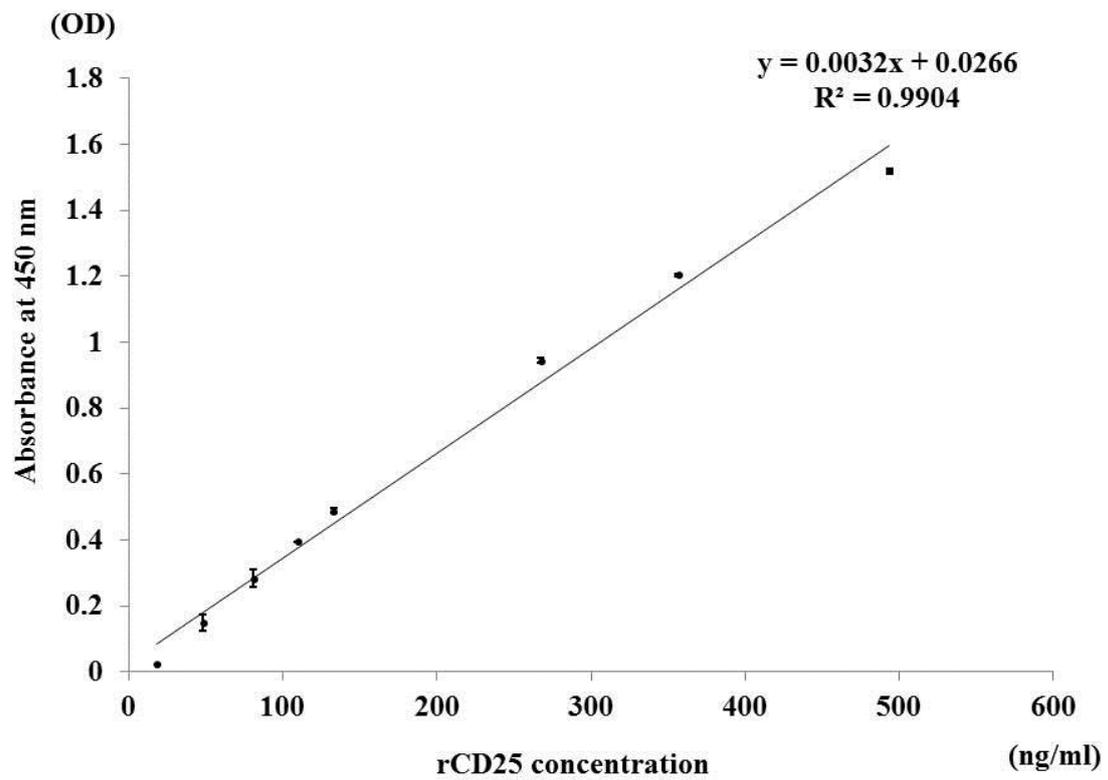


Fig 3–2. Linearity of recombinant canine CD25 measured by the sandwich ELISA. The dots and error bars represent mean absorbance values and standard deviations, respectively.

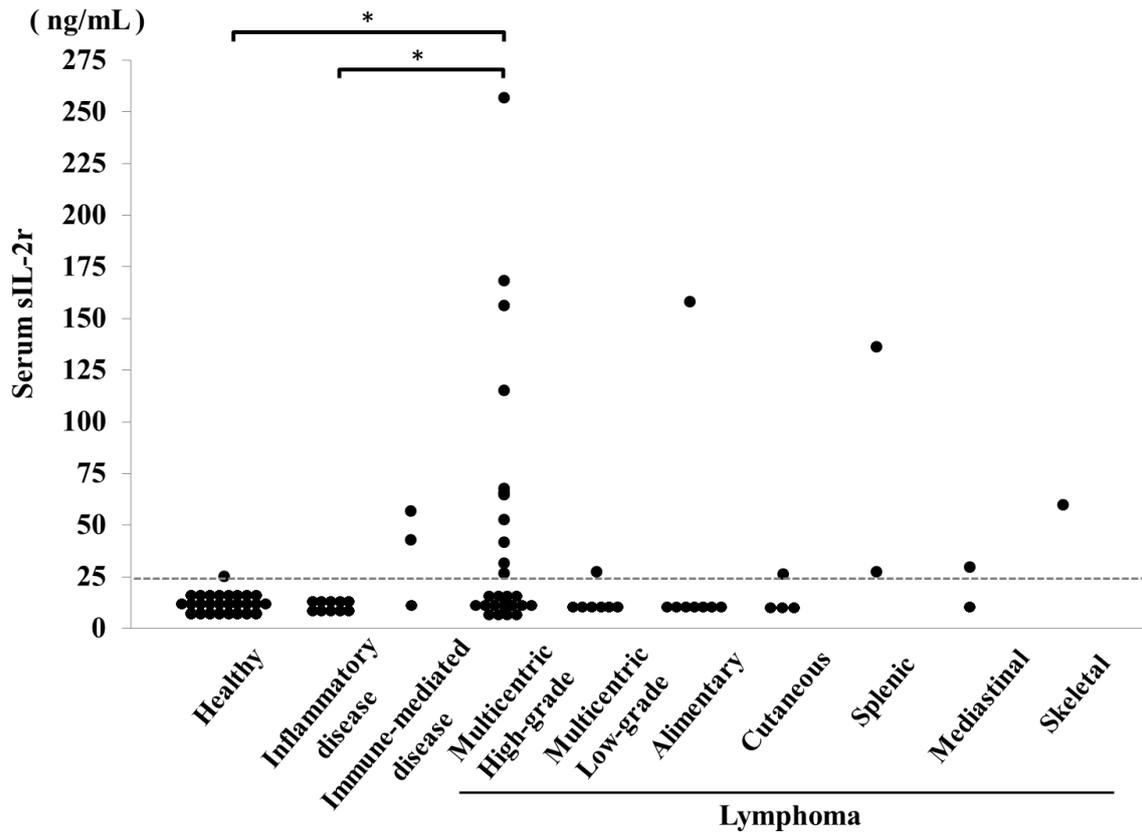


Fig 3–3. Serum sIL-2r levels in healthy dogs, dogs with inflammatory disease, dogs with immune-mediated disease and dogs with multicentric high-grade lymphoma, multicentric low-grade lymphoma, alimentary lymphoma, cutaneous lymphoma, splenic lymphoma, mediastinal lymphoma, and skeletal lymphoma before chemotherapy.

The dotted line indicates detection limit for sIL-2r in the ELISA (25 ng/ml).

*: $p < 0.05$, The Mann-Whitney rank sum test.

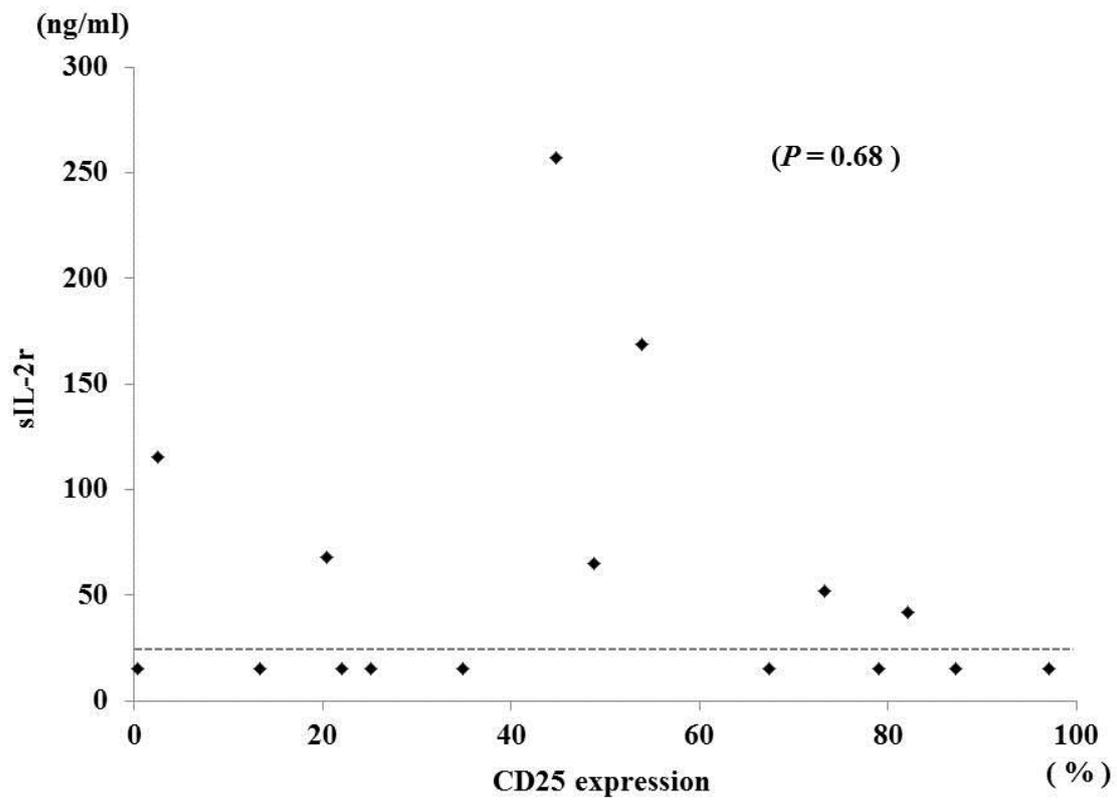


Fig 3-4. Correlation between serum sIL-2r concentration and CD25-positive rate of lymphoma cells in FNA samples from dogs with multicentric high-grade B-cell lymphoma. The dotted line indicates detection limit for sIL-2r in this ELISA (25 ng/ml). Spearman's rank correlation coefficient.

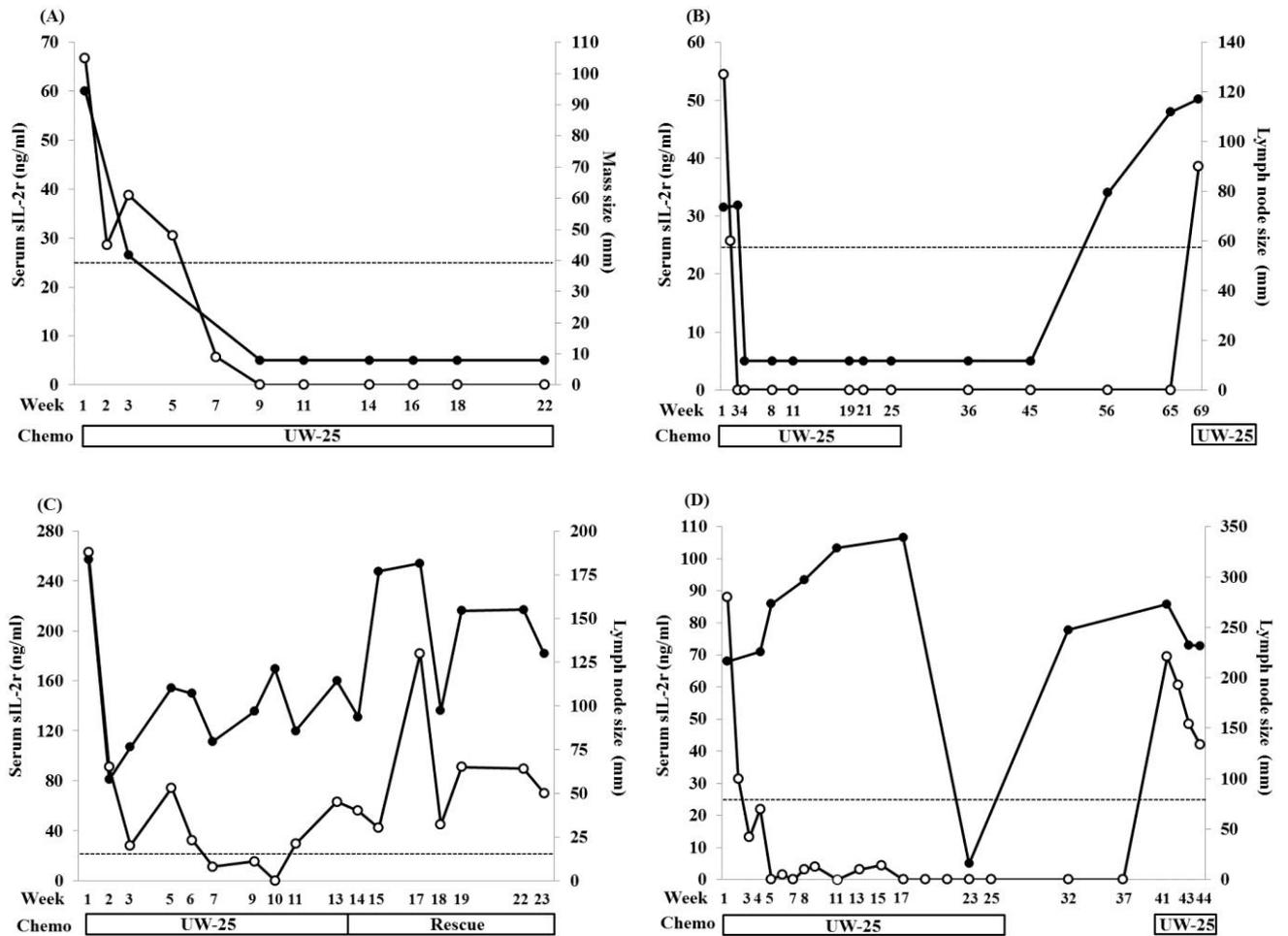


Fig 3–5. Changes in the serum sIL-2r concentrations and total lymph node volumes at several time points in 4 dogs with high-grade lymphoma. The closed and open circles indicate serum sIL-2r concentration and mass or lymph node sizes, respectively, in Case 1 (A), Case 2 (B), Case 3 (C), and Case 4 (D).

The dotted line indicates detection limit for sIL-2r (25 ng/ml). The period of chemotherapy protocol is indicated with the open columns below the graphs. The numbers of weeks after the initiation of chemotherapy are indicated below the graphs.

UW-25: University of Wisconsin 25-week chemotherapy protocol, Rescue: rescue protocols including the administration of lomustine, dexamethasone, melphalan, actinomycin-D, and cytosine arabinoside.

Table 3–1. Spiking recovery of the sandwich ELISA for canine sIL-2r using 3 dog serum samples.

Sample No.	Added rcCD25 (ng/ml)	Observed sIL-2r (ng/ml)	Recovery (%)
1	0	35.19	
	29.21	62.63	97
	66.59	107.85	106
2	0	99.86	
	29.21	130.56	101
	66.59	169.88	102
3	0	102.12	
	29.21	138.05	105
	66.59	188.69	112

Table 3–2. Intra-assay and inter-assay variability in the sandwich ELISA for canine sIL-2r for 3 serum samples.

Variability	Sample 1	Sample 2	Sample 3
Mean (ng/ml)	148.09	74.86	33.66
Standard deviation (ng/ml)	5.44	1.99	0.26
Intra-assay variability			
Number of repeats	5	5	5
Coefficient of variation (%)	4.75	5.39	12.9
Inter-assay variability			
Number of repeats	3	3	3
Coefficient of variation (%)	3.67	2.66	0.76

Conclusion

A series of studies in this thesis were carried out to investigate the biological role of the expression of interleukin-2 receptor alpha chain (CD25) in tumour cells and serum soluble interleukin-2 receptor (sIL-2r) in dogs with lymphoma for the future development of the clinically applicable diagnostic and therapeutic modalities.

T-zone lymphoma (TZL) was found to be a new subtype in canine lymphoma, which had an indolent clinical course, after publication of the World Health Organization (WHO) classification of hematopoietic tumours of domestic animals in 2002 (Valli et al., 2002). There has been no criteria to indicate optimal treatment approach to this specific subtype of canine lymphoma. First, the study in Chapter 1 of the present thesis was conducted to understand the clinical and histopathological features and evaluate CD25 expression in 16 dogs with TZL, which is a common histopathological subtype of canine indolent lymphoma. TZL was commonly diagnosed in Golden Retrievers and Shih Tzu of relatively older age. At initial examination, generalized lymphadenopathy was found in all dogs, and peripheral lymphocytosis was found in 10 of the 16 dogs. At initial diagnosis or during the disease course, 8 dogs (50%) were affected with demodicosis. Immunohistochemical analysis for CD3, CD20, and CD25 was performed for 6 dogs with TZL; the tumor cells were positive for CD3 and CD25 and negative for CD20. Median overall survival time was 938 days and only 3 cases (19%) with TZL experienced the death from lymphoma at the end of the follow-up duration. A watchful waiting approach was adopted for 6 cases (38%), and 5 of the 6 dogs

were still alive at the end of follow-up. The occurrence of infectious diseases including demodicosis might be due to the immunosuppression mediated by TZL with abundant expression of CD25.

CD25 expression has been reported in various human lymphoid tumours (Sheibani et al., 1987; Tesch et al., 1993; Burton et al., 1994; Nakase et al., 1994a, b; Horiuchi et al., 1997; Fujiwara et al., 2013, 2014) and suggested to correlate with their prognoses. Based on the results obtained in the study in Chapter 1, I investigated the expression of CD25 in various types of canine lymphoid tumours and explored its influence on the prognosis in dogs with high-grade B-cell lymphoma in Chapter 2. Immunohistochemical analyses of the tissues from diffuse large B-cell lymphoma (DLBCL) ($n=6$), T-zone lymphoma (TZL) ($n=5$), and follicular lymphoma (FL) ($n=2$) revealed that intense CD25 expression was observed in accordance with lymphoma cell localization. CD25 expression was detected in all of the TZL and FL cases, while it was variable among DLBCL cases. Furthermore, I evaluated CD25 expression in tumour cells by flow cytometric analysis in 29 dogs with lymphoid malignancies, including high-grade B-cell lymphoma ($n=17$), TZL ($n=5$), FL ($n=2$), cutaneous lymphoma ($n=2$), and acute lymphoblastic leukaemia (ALL) ($n=3$). CD25 expression was significantly higher in dogs with high-grade B-cell lymphoma or TZL than that in healthy dogs. In prognostic analysis of 15 cases with high-grade B-cell lymphoma, the progression-free survival was significantly shorter in CD25-high group than that in

CD25-low group. The results obtained in this study would be useful for subtype differentiation and future development of molecular-targeted therapy directed to CD25. From the results obtained in Chapters 1 and 2, CD25 expression was suspected to have an impact on the biological behaviour of the lymphoid malignancy and immune response of the dog patients. Based on the results obtained in Chapters 1 and 2, I speculated that soluble form of CD25 could be released from the canine lymphoma cells as with the lymphoid malignancies in humans. Thus, in Chapter 3, I examined the presence of soluble interleukin-2 receptor (sIL-2r) in canine serum and quantified its concentration using a newly developed sandwich enzyme-linked immunosorbent assay (ELISA). Using immunoprecipitation (IP) assay, CD25 protein weighing approximately 45 KDa was detected in canine serum, which was obviously smaller than the membrane-bound CD25 (approximately 55KDa). I constructed a new sandwich ELISA system to quantify the level of sIL-2r in dog sera. The serum sIL-2r concentration was significantly higher in dogs with multicentric high-grade B-cell lymphoma before treatment than that in healthy dogs and dogs with inflammatory disease at the initial diagnosis. Serum sIL-2r concentration was also found to be elevated in a small proportion of the dogs affected with other lymphoma types and dogs with immune-mediated disease. The present study indicated no correlation between sIL-2r concentration in serum and CD25-positive rate of lymphoma cells in dogs with high-grade B-cell lymphoma. Changes in serum sIL-2r level during the clinical course generally paralleled with the changes of lymph

node size in dogs with high-grade B-cell lymphoma. The sIL-2r level decreased along with the remission induction therapy by CHOP-based protocol. Increase in sIL-2r concentration was found 9 to 13 weeks before the relapse. Increase of sIL-2r concentration was considered to be an indication for the restart of the remission induction therapy even in the clinical remission. The results in Chapter 3 indicated that the sIL-2r concentration could be a marker as an indicator for predicting early relapse, and assessment of treatment efficacy in canine lymphoma.

Lymphoma is one of the most common neoplastic diseases in dogs and it arises in various anatomical locations. Combination chemotherapy is generally used as a standard therapeutic strategy for canine lymphoma and can induce remission in a large proportion of the cases; however, most dogs eventually experience relapse with frequent acquisition of drug resistance. Findings obtained through a series of the studies in this thesis will contribute to an adjunct to the diagnosis, subtype differentiation, and assessment of tumour progression in canine lymphomas. Moreover, in a future prospect, development of molecular-targeted therapy directed to CD25 and more effective chemotherapy protocol by employing the sIL-2r monitoring system will lead to the improvement of the treatment outcome in canine lymphoma.

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