Clinical significance of minimal residual disease (MRD) quantification in dogs with lymphoma

(犬リンパ腫における微小残存病変測定の臨床的有用性)

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CONTENTS

General Introduction

Lymphoma accounts for approximately 7 to 24% of all canine neoplasia and 83% of all canine hematopoietic and lymphoid malignancies (Jacobs et al., 2002; Kaiser, 1981). Combination chemotherapy is a standard care for lymphoma in dogs. It is generally accepted that CHOP-based protocols (cyclophosphamide, vincristine, doxorubicin, and predonisolone) are the first-line treatment for canine lymphoma, showing a high initial response rate (Garrett et al., 2002; Moore et al., 2001; Sorenmo et al., 2010). Although various CHOP-based protocols have been introduced during the last decade or two, majority of the dogs would eventually relapse and only approximately 20% of the cases would be alive for more than 2 years (Garrett et al., 2002; Sorenmo et al., 2010). To improve the treatment outcome in canine lymphoma, several treatment strategies, in addition to the CHOP-based protocols, were conducted. Such strategies included consolidation therapy with new drug combinations (Morrison-Collister et al., 2003; Rassnick et al., 2007), half-body radiation therapy (Rassnick et al., 2007; Williams et al., 2004), and high-dose chemotherapy with autologous bone marrow transplantation (Frimberger et al., 2006). However, optimal dose, schedule or number of cycles of maintenance or consolidation therapy for dogs after achievement of complete remission (CR) has not been established. Furthermore, apparent improvement in terms of survival or disease-free duration has not been obtained in these studies. Therefore, new approaches from different standpoints have been desired to overcome the standstill of the therapeutic outcome in canine lymphoma.

Estimation of the sizes of the lymph nodes and tumor masses after chemotherapy are used to indicate the treatment response. Since a high proportion of the dogs with lymphoma achieve CR in

a short period after initiation of chemotherapy, it is difficult to know the efficacy of the treatment protocols based on data from a standard clinical assessment such as sizing of lymph nodes, imaging tests, and blood tests during most of the treatment period. Therefore, a certain objective marker to measure the amount of tumor cells in the body is required.

Residual malignant cells that escape antitumor therapies are considered to be the source of tumor relapses (Pott et al., 2006). These cells are called minimal residual disease (MRD) and monitoring MRD is an essential tool as an objective marker to measure tumor cell burden for risk-group stratification in human hematopoietic and lymphoid malignancies (Bruggemann et al., 2006; Pott et al., 2006; Rambaldi et al., 2002). In addition, several reports documented the usefulness of MRD providing a molecular assessment of treatment efficacy for human diffuse large B-cell lymphoma (DLBCL) (Mitterbauer et al., 2001; Yashima et al, 2003), which is the disease that canine high-grade B-cell lymphoma, most common type in canine lymphoma, has historically been described as being similar to.

Yamazaki et al. recently reported an MRD detection system in canine lymphoma using real-time polymerase chain reaction (PCR), enabling the number of neoplastic lymphoid cells to be detected in dogs even in CR (Yamazaki et al., 2008). This method enabled to detect as little as 1 tumor cells per 10^4 peripheral blood mononuclear cells (PBMCs). Since MRD levels in the peripheral blood generally parallel changes in lymph node volumes in canine lymphoma patients, the number of neoplastic lymphoid cells in peripheral blood measured by real-time PCR may represent the total body tumor cell burden (Yamazaki et al., 2008). The authors also demonstrated

that the MRD level at the end of chemotherapy was correlated with outcome in dogs with lymphoma (Yamazaki et al., 2010).

Although MRD can be detected in dogs with lymphoma, information with respect to its clinical significance of MRD quantification is still limited in canine lymphoma. Therefore, a series of studies in this thesis were carried out for investigation of the clinical significance of MRD quantification in canine lymphoma. The study in Chapter 1 of the present thesis was carried out to examine the prognostic significance of MRD level at an early phase of multidrug chemotherapy in canine lymphoma. The study in Chapter 2 was carried out to evaluate the cytoreductive efficacy of vincristine, cyclophosphamide, and doxorubicin in dogs with lymphoma that received a multidrug combination chemotherapy by measuring the number of neoplastic lymphoid cells. The study in Chapter 3, I carried out prospective MRD monitoring in the PBMCs of dogs with lymphoma that had achieved CR after chemotherapy to detect early changes in tumor cell growth in the body prior to clinical relapse.

Chapter 1

Prognostic significance of minimal residual disease (MRD) level in canine lymphoma at an early phase of multidrug chemotherapy

Abstract

This study was performed to examine the prognostic significance the minimal residual disease (MRD) level at an early phase of chemotherapy protocol in dogs with lymphoma. Thirty-six dogs with multicentric high-grade B-cell lymphoma were enrolled. Based on the sequences of immunoglobulin heavy chain (IgH) gene fragments amplified from the lymphoma cells, allele-specific primers and probe for the real-time PCR were prepared in each patient. These patients were treated with a 6-month modified version of the University of Wisconsin-Madison chemotherapy protocol (UW-25) and monitored for MRD levels in the peripheral blood mononuclear cells (PBMCs) at weeks 6 and 11 of UW-25. Of the 31 dogs examined for MRD level at week 11, 14 were shown to be MRD-negative (detection limit: < 10 tumor cells in 10^5 PBMCs), whereas the other 17 were MRD-positive (≥ 10 tumor cells in 10^5 PBMCs). All of the MRD-negative dogs and 15 of the 17 MRD-positive dogs achieved clinical complete remission at week 11. Progression-free survival (PFS) in the MRD-negative dogs (median 337 days) was significantly longer than that in the MRD-positive dogs (median 196 days) (P=0.0002). The present study indicated clinical significance of MRD level at an early phase of chemotherapy protocol for prediction of the prognosis.

Introduction

Almost all dogs with high-grade lymphoma can achieve complete remission (CR) with multidrug chemotherapy protocol; however, remission duration and survival time are variable, some lymphoma patients live for more than 2 years but the others live for less than 6 months (Garrett et al., 2002; Moore et al., 2001; Sorenmo et al., 2010). Treatment efficacy is difficult to evaluate during chemotherapy protocol because tumor cells cannot be detected after achievement of CR. Therefore, a certain sensitive and objective marker to evaluate treatment efficacy is needed in canine lymphoma.

Identification of clinical and biological features associated with the poor outcome allows introduction of a new strategy to improve the treatment outcome. In human hematopoietic and lymphoid malignancies, quantity of minimal residual disease (MRD) is recognized to be an indicator for treatment outcome. It has been reported that high MRD after chemotherapy indicates poor prognosis in follicular lymphoma (Rambaldi et al., 2002), mantle cell lymphoma (Pott et al., 2006), adult acute lymphoblastic leukemia (Bruggemann et al., 2006), and acute promyelocytic leukemia (Miller et al., 1993).

Yamazaki et al. developed MRD detecting system in canine lymphoma by a real-time polymerase chain reaction (PCR) system to amplify the rearranged immunoglobulin or T-cell receptor gene fragments (Yamazaki et al., 2008). With this technology, the authors recently reported that MRD at the end of multidrug chemotherapy protocol correlated with the outcome in

canine lymphoma (Yamazaki et al., 2010). However, since multidrug chemotherapy protocol cannot be completed in approximately half of the dogs with lymphoma (Sorenmo et al., 2010), identification of a prognostic indicator at an early phase of chemotherapy protocol is warranted. In this prospective study, I examined the prognostic significance of MRD level at an early phase of a multidrug chemotherapy protocol in dogs with lymphoma.

Material and methods

Case selections

Dogs with cytologically confirmed high-grade lymphoma according to the updated Kiel classification (Fournel-Fleury et al., 1997) were eligible for this study at the Veterinary Medical Center of the University of Tokyo, 14 private animal hospitals, and 3 referral veterinary hospitals from December 2007 to April 2010. Further eligibility criteria were; (1) no prior treatment (except for administration of predonisolone for less than 7 days) had been conducted prior to diagnosis, (2) anatomic form of lymphoma was multicentric, (3) Allele-specific oligonucleotides (ASOs) for the primers and probes could be successfully generated for measuring lymphoma cells with the real-time PCR, and (4) a 6-month modified version of the University of Wisconsin-Madison chemotherapy protocol (UW-25) (Garrett et al., 2002) was chosen for the treatment of lymphoma.

Pretreatment Evaluation

Pretreatment evaluation for all dogs included a physical examination, complete blood count, serum biochemistry, thoracic and abdominal radiographs, and abdominal ultrasound. Clinical staging was determined as follows; stage I, involvement limited a single node; stage II, involvement of many lymph nodes in a regional area; stage III, generalized lymph node involvement; stage IV, liver and/or spleen involvement (suggested by imaging test and/or cytology); stage V, manifestation in the blood and/or bone marrow. B or T cell type was

determined by polymerase chain reaction for antigen receptor gene rearrangement using primers described in 3 previous reports (Burnett et al., 2003; Tamura et al., 2006; Valli et al., 2006).

Assessment of the response to treatment and adverse events after chemotherapy

Response evaluation criteria for peripheral nodal lymphoma in dogs (version 1.0) (Vail et al., 2010) was used to evaluate response to therapy. Treatment associated hematologic and gastrointestinal adverse events after chemotherapy were graded from 1 to 5 according to the ‘‘Veterinary Co-operative Oncology Group Common Terminology Criteria for Adverse Events (version 1.0) (Veterinary cooperative oncology group, 2004).

Quantification of neoplastic lymphoid cells at diagnosis and MRD levels after chemotherapy

Peripheral blood samples were collected at diagnosis and weeks 6 and 11 of UW-25 for quantification of the number of neoplastic lymphoid cells. Quantification of the number of tumor cells in peripheral blood was carried out as described previously (Yamazaki et al., 2008). In brief, to determine the sequence of the rearranged antigen receptor gene in each lymphoma case, PCR product of the clonally rearranged immunoglobulin heavy chain (IgH) fragment was inserted into a T/A cloning vector (pGEM-T Easy, Promega), and subjected to sequence analysis. In order to obtain the sequence of the 3’ flanking region of the rearranged IgH J segment, the sequence was searched in the dog genome data base using the BLAST search program provided by the National Center for Biotechnology Information. ASOs complementary to the complementarity-determining region 3

region of the IgH gene and adjacent regions were designed for use of the primers and probe using primer making software program (Primer Express software program version 2.0, Applied Biosystems).

To assess the number of neoplastic lymphoid cells in peripheral blood mononuclear cells (PBMCs), peripheral blood (3 ml) was collected in EDTA-treated tubes, followed by density gradient centrifugation using Ficoll/Hypaque (specific gravity, 1.077) (Lymphoprep, Nycomed Pharma AS). Blood samples collected at private animal hospitals were shipped to the University of Tokyo in a container kept at 4°C, and PBMCs were separated within 24 hours from collection. The genomic DNA of PBMCs was extracted by using a DNA extraction kit (QIAamp Blood mini kit, QIAGEN).

Real-time PCR was performed using Takara thermal cycler and data was analyzed by the software (Takara TP800, Takara Bio Inc). To normalize the amount of DNA samples, the albumin gene was used as an internal reference. The rearranged IgH gene copy number to half of the albumin gene copy number corresponds to the number of tumor cells in PBMCs used for the template. All measurements were conducted in triplicate.

Statistical analysis

Differences in presenting characteristics were compared using chi-square test and Mann-Whitney *U* test. Kruskal-Wallis test was performed to examine differences in the number of neoplastic lymphoid cells at diagnosis and MRD at weeks 6 and 11 of UW-25. Wilcoxon's

rank-sum test with Bonferroni's correction after Krsukal-Wallis was performed to examine significance difference of the number of neoplastic lymphoid cells at each time point. Progression free survival (PFS) was defined as the time from the initiation of treatment to the first date that criteria for progressive disease were met or the date of death from any cause. Dogs were censored in PFS analysis for the following reasons: still alive while progressive disease (PD) had not occurred before the end of the study or lost to follow-up. Overall survival (OS) was defined as the time from the first day of chemotherapy until death from any cause. Dogs were censored in OS analysis if alive at the end of chemotherapy or lost to follow-up. PFS and OS were assessed with high, intermediate and low number of neoplastic lymphoid cells at diagnosis, and MRD at week 6 and week 11 of UW-25. The Kaplan-Meier method was used to estimate the distribution of PFS and OS. Univariate associations between 3 groups were tested using log-rank tests. Multivariable regression analysis of PFS was conducted using Cox proportional hazard models, including MRD and several prognostic factors reported previsously such as gender (MacEwen et al., 1987), body weight (≤ 18kg) (Garrett et al., 2002), presence of anemia (PCV < 35%) (Miller et al., 2009), stage V (Hosoya et al., 2007), and substage b (Greenlee et al., 1990; Garrett et al., 2002). All tests conducted were 2-sided at 0.05 significance level.

Results

Lymphoma cases

Thirty-six dogs with lymphoma met inclusion criteria of this study (29 dogs at University of Tokyo, 7 dogs at 4 private animal hospitals). Three dogs had already received predonisolone therapy (0.2 – 0.5mg/kg/day) during 3-7 days before diagnosis.

The dogs included belonged to 21 breeds. Breeds commonly represented were Welish Corgi (6), Golden Retreiver (3), Miniature Schnauzer (3), and Miniature Dachshund (3). The other breeds were Shiba (2), Border Collie (2), Bernese Mountain Dog (2), Pug (2), Shetland Sheepdog (1), Scotish Terrier (1), Shih Tzu (1), Maltese (1), Australian Kelpie (1), Beagle (1), Polish Lowland Sheepdog (1), Yorkshire Terrier (1), Bulldog (1), Jack Russell Terrier (1), Standard Poodle (1), French Bulldog (1), and Mix breed (1). Median age was 8 years old (range: 2-14 years) and median body weight was 11.6 kg (range: 3-36.3 kg). Seventeen dogs were male (6 dogs were castrated) and 19 dogs were female (13 dogs were spayed).

Clonal IgH gene rearrangement was detected in all of the 36 cases, representing the all of the cases as B-cell lymphoma. Therefore, all cases were classified into B-cell high-grade lymphoma according to updated Kiel classification (Fig 1A, B).

Four dogs (11%) were classified as stage III, 19 dogs (53%) as stage IV, and 13 dogs (36 %) as stage V. Nineteen dogs (53%) were categorized as substage a and 17 dogs (47%) as substage b.

Response to treatment and adverse events after chemotherapy

Thirty-three dogs (92%) achieved a complete remission (CR) and 3 dogs (8%) achieved a partial remission after chemotherapy. Median duration from initiation of the treatment to CR was 16 days (range: 7-58 days). At week6, 31 (89%) of the 35 dogs achieved CR, and at week 11, 29 (94%) of the 31 dogs achieved a CR.

Four (11.1%) of the 36 dogs received dose reduction of the chemotherapeutic agents because of the severe adverse events (grade 3 or more).

One dog died because of acute tumor lysis syndrome after the first vincristine administration, therefore, 35 (97.2%) of the 36 dogs reached week 6 of UW-25. Four dogs quit UW-25 because of PD observed during week 7 – 10 and departed from UW-25, therefore, 31 (86.1%) of the 36 dogs could be assessed at week 11 of UW-25.

The number of neoplastic lymphoid cells at diagnosis and MRD levels at weeks 6 and 11 of UW-25.

Median number of tumor cells at diagnosis and MRD levels at weeks 6 and 11 of UW-25 were 71,301 cells (range: 1,416 – 100,000 cells, n=36), 78 cells (range: <10 – 58,674 cells, n=35) and 12 cells (range: <10 – 2,555 cells, n=31) per 10^5 PBMCs, respectively (Fig 2). Nine dogs (26%) and 14 dogs (45%) were MRD-negative (detection limit: 10 cells in 10^5 PBMCs) at weeks 6 and 11, respectively. Significant difference between the number of neoplastic lymphoid cells at diagnosis and those at week 6 and week 11 was observed (Krsukal-Wallis test, $P<0.001$). MRD levels at week 6 and week 11 were significantly lower than the number of neoplastic lymphoid cells at

diagnosis (Wilcoxon's rank sum test with Bonferroni's correction, $P<0.001$).

Prognostic significance of the number of neoplastic lymphoid cells at diagnosis and MRD levels at weeks 6 and 11 of UW-25

Thirty-six dogs at diagnosis were assigned into 3 groups; high (75,000 – 100,000 cells, n=17), intermediate (10,000 – 74,999 cells, n=13), and low (≤ 9,999 cells, n=6) numbers of neoplastic lymphoid cells. Median PFS in the high, intermediate and low groups were 211, 295 and 407 days, respectively (Fig 3A). Median OS in the high, intermediate and low MRD groups were 336, 401 and 494 days, respectively (Fig 3B). This prognostic significance was not observed among the 3 groups at diagnosis (PFS: $P=0.54$, OS: $P=0.80$).

Thirty-five dogs at week 6 were assigned to 3 groups; high MRD (100 cells ≤, n=14), intermediate MRD (10 – 99 cells, n=12) and low MRD (MRD negative, n=9). Median PFS in the high, intermediate and low MRD groups were 211, 196 and 286 days, respectively (Fig 3C). Median OS in the high, intermediate and low MRD groups were 336, 271 and 432 days, respectively (Fig 3D). Significant difference of PFS ($P=0.98$) and OS ($P=0.90$) was not detected among 3 groups at week 6.

Thirty-one dogs at week 11 were assigned to 3 groups; high MRD (100 cells ≤, n=7), intermediate (10 – 99 cells, n=10) and low MRD (MRD negative, n=14). Median PFS in the high, intermediate and low MRD groups were 190, 232 and 337 days, respectively (Fig 3E). Median OS in the high, intermediate and low MRD groups were 221, 281 and 411 days, respectively (Fig 3F).

Significant difference of PFS (*P*=0.0008) and OS (*P*=0.0039) was observed in 3 groups at week 11. Furthermore, the 31 dogs at week 11 were reassigned to 2 groups; MRD-positive (n=17) and MRD negative (n=14). Prognostic significance was observed between the 2 groups (PFS: *P*=0.0002, OS: *P*=0.0033) (Fig 4A,B). Median PFS in MRD-positive and MRD-negative groups were 196 and 337 days, respectively. Median OS in MRD-positive and MRD-negative groups were 271 and 411 days, respectively. Distribution for age, gender, stage V, substage b, presence of anemia (PCV<35%), pretreatment of predonisolone, and body weight were not significantly different between MRD-positive and MRD-negative groups at week 11 (Table 1). MRD-negative group was more likely to complete UW-25 protocol compared to MRD-positive group (*P*=0.017) (Table 1). Cox regression analysis revealed MRD, substage b, and body weight had significant independent association with duration of OS (Table 2).

Discussion

Yamazaki et al. previously indicated that MRD at the end of a multidrug chemotherapy protocol (UW-25) was an important indicator of treatment outcome in dogs with lymphoma (Yamazaki et al., 2010). Here, I report a prognostic significance of MRD level at early phase of multidrug chemotherapy protocol in dogs with B-cell high-grade lymphoma. Eighty-six percent of dogs could be assessed at week 11 of UW-25 in this study. In most instances, combination chemotherapeutic protocol like UW-25 can be completed in approximately half of dogs enrolled in the chemotherapy regimens (Sorenmo et al., 2010). Therefore, for the prognostic significance, the MRD level at an early phase of chemotherapy (ex. week 11 of UW-25 in this study) would be a suitable practical indicator.

The present study included only dogs with B-cell lymphoma, therefore, prognostic significance of MRD in dogs with T-cell lymphoma is unknown. Recently, Brodsky et al. reported that dogs with T-cell lymphoma could be more favorably treated with MOPP protocol (mechlorethamine, vincristine, procarbazine, and prednisone) in comparison to CHOP protocols (Brodsky et al., 2009). Further studies are needed to identify clinical utility of MRD for dogs with T-cell lymphoma.

In the present study, the number of malignant cells at diagnosis did not provide significant information for prognosis. Several reports indicated that stage V, a condition in which neoplastic lymphoid cells emerge in peripheral blood, is not included in prognostic factors (Garrett et al., 2002; Simon et al., 2006; Sorenmo et al., 2010). Lana et al reported carried out a qualitative PCR

analysis with consensus primers to detect clonally rearranged cells in the peripheral blood of dogs with lymphoma at the initial diagnosis and found that PCR positivity did not influence prognosis (Lana et al., 2006). The present result that the number of tumor cells measured by real-time PCR at diagnosis did not have prognostic significance did not conflict those obtained in previous studies.

MRD level at week 6 of UW-25 also did not show prognostic information. Although the reason is not clear, MRD levels at week 6 were conceivably still affected the number of tumor cells at diagnosis because MRD was shown to reach nadir around week 9 of UW-25 (Yamazaki et al., 2010).

Considerable difference between the current study and a previous study (Yamazaki et al., 2010) was the number of dogs that achieved MRD-negative status. Although only 11% of the dogs treated with UW-25 achieved MRD-negative status in a previous study (Yamazaki et al., 2010), 45% of the dogs achieved MRD-negative status in the present study. This may be due to the fact that different characteristics of population included in the two studies: the current study included only multicentric, B-cell high-grade lymphoma, almost all cases were centroblastic type; however the previous study included not only typical lymphoma types in dogs, but also alimentary form, various cell types such as lymphoblastic and anaplastic types, and T-cell lymphoma.

I have demonstrated that MRD level at week 11 of UW-25 is an independent prognostic indicator in dogs with B-cell high-grade lymphoma. Dogs with MRD-positive at week 11 of UW-25 had a shorter time to progressive disease and a 7.1-hold higher risk of tumor progression than those with MRD-negative dogs.

Difference between the MRD-positive and MRD-negative dogs was not clear in this study. Several molecular markers associated with treatment response and overall survival time were identified in human diffuse large B-cell lymphoma (DLBCL) (Rosenwald et al., 2002) which is the disease that canine high-grade B-cell lymphoma has historically been described as being similar to. Further studies are needed to explain the difference of prognosis in canine lymphoma.

Treatment stratification for standard risk adult human ALL patients significantly improved the outcome when MRD information was included (Bruggemann et al., 2006). In children with B-cell ALL, at the end of induction therapy, the 5-year risk of relapse was 5% in patients with no detectable MRD and 44% in patients with detectable MRD, and they have changed their treatment approach, children with B-lineage ALL and high MRD at the end of induction therapy now receive treatment intensification to attempt alter the prognosis (Zhou et al., 2007). Moreover, MRD was shown to be further declined by postinductive consolidation therapy in acute promyelocytic leukemia (Miller et al., 1993) and mantle cell lymphoma (Brugger et al., 2004).

Based on the results of the current study, alternative treatment approach would be beneficial to attempt to decrease their risk of subsequent progressive disease for MRD-positive dogs with B-cell high-grade lymphoma at week 11 of UW-25. Further clinical studies are warranted to identify if consolidation therapy in dogs with B-cell high-grade lymphoma based on MRD would lead to improvement in treatment outcome.

Chapter 2

Evaluation of cytoreductive efficacy of vincristine, cyclophosphamide, and doxorubicin in dogs with lymphoma by measuring the number of neoplastic lymphoid cells with real-time polymerase chain reaction

Abstract

The cytoreductive efficacy of the individual components of multidrug chemotherapy for canine lymphoma is difficult to evaluate after complete remission. The purpose of this study was to compare the cytoreductive efficacy of vincristine (VCR), cyclophosphamide (CPA), and doxorubicin (DXR) in dogs that received a 6-month modified version of the University of Wisconsin-Madison chemotherapy protocol (UW-25).

Twenty nine dogs with high-grade B-cell lymphoma of multicentric form were used. Rearranged immunoglobulin heavy chain gene fragments from lymphoma cells were amplified by polymerase chain reaction (PCR) and sequenced to prepare clone-specific primers and probes for real-time PCR. The numbers of lymphoma cells in peripheral blood were measured from diagnosis to week 11 of UW-25. The number of lymphoma cells after the first administration of VCR, CPA, and DXR in weeks 1-4 was reduced in 29/29 (100%), 15/29 (51.7%), and 26/27 (96.3%) dogs, respectively; the cytoreductive efficacy of CPA was therefore less than that of VCR and DXR. The cytoreductive efficacy of VCR, CPA, and DXR administered in weeks 6-9 was observed in 5/26 (19.2%), 5/20 (25.0%), and 14/19 (73.7%) dogs, respectively, indicating the sustained cytoreductive efficacy of DXR. CPA non-responders were heavier and exhibited a shorter first remission than CPA responders.

When applying UW-25 for the treatment of canine lymphoma, CPA was found to have less cytoreductive efficacy than VCR and DXR. Real-time PCR-based quantification of tumor cells will be a good objective marker of the efficacy of chemotherapeutic agents.

Introduction

In Chapter 1, I demonstrated that minimal residual disease (MRD)-negative dogs at week 11 of a 6-month modified version of the University of Wisconsin-Madison chemotherapy protocol (UW-25) (Garrett et al., 2002) showed better prognosis than the MRD-positive dogs. UW-25, one of the standard chemotherapeutic protocols for canine lymphoma, employs 3 anticancer drugs administration, vincristine (VCR), cyclophosphamide (CPA), and doxorubicin (DXR). However, cytoreductive efficacy of each drug in multidrug protocol in canine lymphoma is unknown. I hypothesized that efficacy of each drug used in UW-25 might affect the MRD level at week 11 of UW-25.

Since a high proportion of the dogs with lymphoma respond well to these chemotherapeutic agents and achieve CR in a short period after initiation of chemotherapy (Simon et al., 2006), it is difficult to know the efficacy of each drug in the multidrug regimen based on data from a standard clinical assessment such as sizing of lymph nodes, imaging tests, and blood tests during most of the treatment period. Although a single agent protocol with DXR for the treatment of dogs with lymphoma has been reported (Carter et al., 1987; Mutsaers et al., 2002; Valerius et al., 1997), there has been no report on a single agent protocol with CPA or VCR. It is difficult to know how much each of these three drugs contributes to the reduction in tumor cell burden when multidrug chemotherapy is used.

Yamazaki et al. recently reported an MRD detection system in canine lymphoma using real-time

polymerase chain reaction (PCR), enabling the number of neoplastic lymphoid cells to be detected in dogs even in CR (Yamazaki et al., 2008). Since MRD levels in the peripheral blood generally parallel changes in lymph node volumes in canine lymphoma patients, the number of neoplastic lymphoid cells in peripheral blood measured by real-time PCR may represent the total body tumor cell burden (Yamazaki et al., 2008). In hematopoietic and lymphoid malignancies in humans, MRD level has been recently recognized to indicate the efficacy of treatment, representing an index marker of tumor burden in a patient's body, even in CR (Bruggemann et al., 2006; Miller et al., 1993; Pott et al., 2006; Rambaldi et al., 2002). Although UW-25 has been accepted as one of the most effective combination multidrug protocols in canine high-grade lymphoma, its modification was conceivably able to provide better treatment outcome. I hypothesized that some idea to improve the protocol could be obtained by introducing the MRD monitoring system in canine lymphoma patients that underwent remission induction chemotherapy with UW-25.

This study compared the cytoreductive efficacy of VCR, CPA, and DXR that constitute UW-25 in canine lymphoma patients treated with UW-25 by quantifying lymphoma cells in peripheral blood with real-time PCR.

Materials and Methods

Lymphoma cases

Dogs diagnosed with high-grade lymphoma according to the updated Kiel classification (Fournel-Fleury et al., 1997), based on the cytology of enlarged lymph nodes, were eligible for this study at the Veterinary Medical Center of the University of Tokyo, 5 private animal hospitals, and 2 referral veterinary hospitals. Further eligibility criteria included (1) no prior treatment including predonisolone, (2) multicentric lymphoma, (3) successful generation of allele-specific oligonucleotides (ASOs) for real-time PCR, and (4) UW-25 as the chosen therapy.

Pretreatment evaluation

Pretreatment evaluation for all dogs included a physical examination, complete blood count, serum chemistry profile, thoracic and abdominal radiographs, and abdominal ultrasound. Clinical staging was performed according to the World Health Organization criteria for canine lymphoma (Owen, 1980) except for the classification of stage V, because bone marrow aspiration is not routinely performed in patients with high-grade lymphoma. Judgment of the presence of circulating neoplastic lymphoid cells by microscopic observation of peripheral blood smear was used to classify as Stage V in this study. Stage IV was determined by an abnormal appearance of liver and spleen detected by ultrasound and radiography. B or T cell type was determined by PCR for antigen receptor gene rearrangement using primers described in 3 previous reports (Burnett et al., 2003;

Tamura et al., 2006; Valli et al., 2006).

Chemotherapy and assessment of treatment response

Dogs diagnosed with lymphoma were treated with VCR (NIPPON KAYAKU), CPA (SHIONOGI & CO, LTD), DXR (KYOWA KIRIN) and predonisolone according to UW-25 (Garrett et al., 2002). Administration of L-asparaginase was omitted from the original UW-25 protocol because its administration did not influence the therapeutic efficacy (MacDonald et al., 2005). DXR (30 mg/m^2 in dogs with body weight > 10kg, 1 mg/kg in dogs with body weight ≤ 10kg) was injected intravenously over 30 min. Response evaluation criteria for peripheral nodal lymphoma in dogs (version 1.0) (Vail et al., 2010) was used to evaluate the therapeutic response.

Assessment of the toxicity of chemotherapeutic agents

A complete blood count was performed at each admission prior to chemotherapy. Treatment-associated hematologic and gastrointestinal toxicity was graded from 1 to 5 according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events (version 1.0) (Veterinary Cooperative Oncology Group, 2004).

Measuring the number of tumor cells with real-time PCR

The number of lymphoma cells in the peripheral blood mononuclear cells (PBMCs) was measured as described previously (Yamazaki et al., 2008). In brief, to determine the antigen receptor

gene sequence in each lymphoma case, the PCR product amplified from the rearranged immunoglobulin heavy chain (IgH) gene fragment was inserted into a T/A cloning vector (Promega) and subjected to sequence analysis. To obtain sequences of the 3′ flanking region of the rearranged IgH J segments, their sequences were searched in the dog genome database using the BLAST search program provided by the National Center for Biotechnology Information. ASOs complementary to the complementarity-determining region 3 of the IgH gene in each case were used to design primers and probes for real-time PCR using a primer design software program (Applied Biosystems).

To assess the number of lymphoma cells in PBMCs, peripheral blood samples (3 ml) were collected in EDTA-treated tubes, followed by density gradient centrifugation with Ficoll/Hypaque (specific gravity, 1.077) (Nycomed Pharma AS). Blood samples collected at private animal hospitals were shipped to the University of Tokyo in a container kept at 4°C and subjected to separation of PBMCs within 24 h of collection. The genomic DNA of PBMCs was extracted with a DNA extraction kit (QIAGEN). The genomic DNA samples were stored at -20 °C for 5 – 18 weeks until the assay for the PCR measurement of the tumor cell number.

Real-time PCR was performed using a thermal cycler (Takara Bio Inc) and data were analyzed with a software supplied by the manufacturer. To normalize the amount of DNA samples, the albumin gene was used as an internal reference. The ratio of the rearranged IgH gene copy number to half of the albumin gene copy number corresponds to the number of tumor cells in the PBMCs sample. Samples in each patient for comparison of the cytoreductive efficacy of the chemotherapeutic agents were measured in the same run. All measurements were conducted in

triplicate.

Comparison of the cytoreductive efficacy of VCR, CPA, and DXR in reducing the number of tumor cells, as measured by real-time PCR

Peripheral blood samples were collected at weeks 1, 2, 3, 4, 6, 7, 8, 9, and 11 of UW-25 for quantification of tumor cells in PBMCs prior to injection of the chemotherapeutic agent at each admission.

Sampling was stopped if clinical progressive disease (PD) was observed. Each change in the number of lymphoma cells was categorized as “decrease”, “increase”, or “no change”. The judgment was made as follows. To quantify the measurement uncertainty level of the real-time PCR system, reference DNA specimens extracted from 10, 10^2, 10^3, 10^4, and 10^5 cells (measurable range with this method) from a canine lymphoma cell line[7] were measured. Six equivalent samples containing each amount of DNA were assayed by real-time PCR to calculate the mean and standard deviation (SD). The data were log-transformed to make it easy to compare the range of SD for samples containing amounts of different order. I tentatively defined an “increase” or “decrease” as a change of more than 2 SD in the number of tumor cells from the previous examination. When the change was less than 2 SD at each level, it was defined as “no change”. When the number of tumor cells decreased to an undetectable level, less than 1 as expressed by $\log_{10}$[number of tumor cells per 10^5 PBMCs], the change was also defined as a “decrease”. When the number of tumor cells increased from the undetectable level to the detectable range, the change was defined as an “increase” as well.

Statistical analysis

The frequency of a "decrease" in the number of tumor cells after each drug administration was compared by a Chi-square test.

CPA responders and non-responders were defined as groups that showed "decrease" and "increase/no change" 1 week after the first CPA administration, respectively. I carried out survival analyses for the 2 groups, CPA responders and CPA non-responders. Progression-free survival (PFS) was defined as the time elapsed from treatment initiation to the first date when criteria for progressive disease were met or the date of death from any cause. Dogs were censored in PFS analysis for the following reasons: still alive and did not undergo PD at the end of the study or lost to follow-up. Overall survival (OS) was defined as the time elapsed from the first day of chemotherapy until death from any cause. Dogs were censored in OS analysis if they were alive at the end of the study or lost to follow-up. Kaplan-Meier log-rank test was used to compare remission and survival times between responders and non-responders. Fisher's exact test was used to compare stage V, substage b, anemia (PCV < 35%), remission rate, and gender. The Mann-Whitney *U* test was used to compare age and body weight.

All statistical analyses were performed with a standard software (Statmate 3, ATMS). Significance of differences was Bonferroni-adjusted for multiple comparisons. Values of $P < 0.05$ (2 sided) were considered significant.

Results

Lymphoma cases

Forty-one dogs with high-grade lymphoma were newly diagnosed between December 2007 and August 2009 at the Veterinary Medical Center of the University of Tokyo, 5 private animal hospitals, and 2 referral veterinary hospitals. Twelve dogs were excluded from the study for the following reasons: (1) Prednisolone or antineoplastic agents had been already administered in 5 dogs, (2) ASOs could not be generated in 5 dogs (2 cases, B-cell lymphoma; 3 cases, T-cell lymphoma), (3) UW-25 was not chosen for treatment in 1 dog, and (4) Death after the first VCR administration occurred in 1 dog. Consequently, 29 dogs were enrolled (24 cases at the University of Tokyo and 5 cases at 3 private animal hospitals).

The dogs belonged to 18 breeds. Breeds most commonly represented were Golden Retriever (3), Welsh Corgi (3), and Miniature Schnauzer (3). The other breeds were Pug (2), Miniature Dachshund (2), Shiba (2), Bernese Mountain dog (2), Border collie (2), Bulldog (1), Shetland Sheepdog (1), Scottish terrier (1), Shih Tzu (1), Maltese (1), Australian Kelpie (1), Beagle (1), Polish Lowland Sheepdog (1), Yorkshire Terrier (1), and French Bulldog (1). Median age was 8 years (range: 2–14 years) and median body weight was 11.7 kg (range: 3.0–36.3 kg). Fourteen dogs were male (4 dogs were castrated) and 15 dogs were female (11 dogs were spayed).

Clonal IgH gene rearrangement was detected in all of the 29 dogs, representing B-cell lymphoma. Clonal T-cell receptor γ chain (TCRγ) gene rearrangement was not detected in any of the cases. All

dogs exhibited the multicentric form in pretreatment evaluation. Therefore, all dogs were classified with B-cell high-grade lymphoma of multicentric form (Figure 1A, B).

Of the 29 dogs with lymphoma, 2 dogs (7%) were classified as stage III, 16 dogs (55%) as clinical stage IV, and 11 dogs (38%) as stage V. Fourteen dogs were categorized as substage a (48%) and 15 (52%) as substage b.

Chemotherapy and treatment response

All 29 dogs received UW-25 for the initial induction therapy. In 28 of the 29 dogs, the initial doses of VCR, CPA, and DXR were 0.7 mg/m^2, 250 mg/m^2, and 30 mg/m^2 (BW >10 kg) or 1mg/kg (BW ≤ 10kg), respectively, as indicated in the original UW-25 protocol (Garrett et al., 2002). One dog was initially administered at lower doses (VCR 0.5 mg/m^2, CPA 200 mg/m^2, DXR 25 mg/m^2) at the clinician's discretion.

The dose of VCR was reduced by 20% at week 3 (1 dog) and week 6 (2 dogs) because Grade 3 neutropenia had occurred after the previous injection of VCR. A 20% reduction in CPA dose at week 7 was made because of Grade 3 neutropenia and Grade 3 thrombocytopenia after injection of CPA at week 2 in 1 dog. Chlorambucil was used instead of CPA at week 7 because of Grade 3 cystitis after CPA injection at week 2 in 1 dog.

Chemotherapy with UW-25 yielded complete remission in 27 dogs (93%) and 2 dogs (7%) showed partial remission. Median duration from the initiation of chemotherapy to CR was 15 days (range: 7–58 days) in the 27 dogs that achieved CR.

Cytoreductive efficacy of VCR, CPA, and DXR as measured by real-time PCR quantification of tumor cell number

Median durations from the dates of VCR, CPA, and DXR administration to those for the measurement of the number of tumor cells in peripheral blood were 7 days [range: 7–21 days, 7 days after injection in 84/104 dogs (80.7%)], 7 days [range: 7–14 days, 7 days after injection in 40/49 dogs (81.6%)], and 14 days [range: 11–21 days, 14 days after injection in 42/46 (91.2%)], respectively.

From the SD values obtained by real-time PCR of the reference canine lymphoma cell line, I tentatively classified the changes into "decrease", "increase", or "no change". When 10, 10^2, 10^3, 10^4, and 10^5 reference lymphoma cells were included in the templates, values of SD as expressed by $\log_{10}$[number of tumor cells] were 0.22, 0.1, 0.04, and 0.02, respectively. Consequently, when the number of lymphoma cells as expressed by $\log_{10}$[number of tumor cells] was $\geq 1 - < 2$, $\geq 2 - < 3$, $\geq 3 - < 4$, $\geq 4 - < 5$, "decrease" of the number of tumor cells was defined as decreases by more than 2 SD values at each level, namely, 0.44, 0.20, 0.08, and 0.04, respectively.

Changes in the number of tumor cells in PBMCs at weeks 1–11 of UW-25 in the 29 dogs were measured (Table 3). In case 1 for example, the number of lymphoma cells was reduced 1 week after the first injection of VCR; the number increased after the first CPA administration at week 2. Administrations of VCR at week 3 and DXR at week 4 reduced the number of tumor cells to the undetectable range at week 6. However, the number of tumor cells increased after administrations of

VCR at week 6 and CPA at week 7, and PD was observed at week 8. I examined the changes of the number of tumor cells in the other 28 dogs (Table 3) and categorized them into “decrease”, “no change”, or “increase”.

Decrease rates were calculated after each drug administration (Table 4). The tumor cell decrease rate after CPA administration at week 2 was significantly lower than that after administration of VCR at week 1 ($P < 0.0001$) and after administration of DXR at week 4 ($P < 0.0005$). Tumor cell reduction after DXR administration at week 9 was significantly higher than those of VCR administration at week 6 ($P < 0.01$) and CPA administration at week 7 ($P < 0.01$). Decrease rates after VCR administration at weeks 6 and 8 were significantly lower than those at weeks 1 and 3 ($P < 0.0001$). The reduction in tumor cells after CPA administration at week 2 was not significantly different from that after CPA administration at week 7. The decrease rate of lymphoma cells after DXR administration at week 9 was still as high as 73.7% with no significant difference from that after DXR administration at week 4.

Comparison of CPA responders and CPA non-responders

After administration of CPA at week 2, a tumor cell reduction was shown in 15 dogs (cases 15–29) (CPA responders) but not in the remaining 14 dogs (cases 1–14) (CPA non-responders). Clinical CR could be achieved in 9 (60%) of the 15 CPA responders and in 5 (36%) of the 14 CPA non-responders by week 3; however, the CR rate was not significantly different between the 2 groups ($P = 0.35$). Median follow-up duration from the initial diagnosis was 305 days (range:

35–663 days, 95% confidence interval (CI): 234–401 days). Median PFS duration in CPA responders was 305 days (range: 70–617 days, 95% CI: 204–undefined); meanwhile, that in CPA non-responders was 95 days (range: 22–475 days, 95% CI: 35–283 days). A significant difference in PFS was observed between the 2 groups ($P < 0.01$) (Fig 5A). Median OS in CPA responders was 442 days (range: 117–663 days, 95% CI: 300–undefined), significantly longer than that in CPA non-responders, 234 days (range: 35–634 days, 95% CI: 103–305 days) ($P < 0.005$) (Fig 5B). Breed imbalance was not observed in the 2 groups. Distributions for age, gender, stage V, substage b, anemia, and CR rate were not significantly different (Table 5). However, median body weights of the 2 patient groups were significantly different ($P < 0.01$): 9.0 kg (range: 3.0–22.9 kg, 95% CI: 8.6–11.5 kg) in CPA responders vs 23.5 kg (range: 5.2–36.3 kg, 95% CI: 11.7–26.2 kg) in CPA non-responders (Table 5). In CPA responders, 7 of the 15 dogs (47%) achieved MRD-negative at week 11 of UW-25. In CPA non-responders, 2 of the 14 dogs (14%) achieved MRD-negative at week 11 of UW-25. CPA responders tended to achieve MRD-negative at week 11 of UW-25 compared to CPA non-responders, although the different was not statistically significant ($P = 0.14$) (Table 5).

Disucussion

In the present study, I compared the cytoreductive efficacy of VCR, CPA, and DXR used in UW-25 by quantifying tumor cells with real-time PCR. Since the median duration from chemotherapy initiation to the achievement of CR was 15 days in this cohort, similar to the data in a previous study (Simon et al., 2006), the efficacy of each drug could not be evaluated from the lymph node sizes in these cases. Therefore, measurement of the number of tumor cells with real-time PCR seemed to be an appropriate approach for the judgment of the efficacy of each drug.

Administration of chemotherapeutic agent was sometimes delayed because of hematologic and/or gastrointestinal adverse events or owner's inconvenience in a proportion of patients. However, most of drug administration was on schedule (VCR, 80.7 %; CPA, 81.6 %; DXR, 91.2 %), therefore, the delay of the measurement did not seriously influence the overall results.

In this study, I regarded that the change of the tumor cell numbers in the peripheral blood reflected the activity of the most recently administered drug. Cumulative or synergistic effect by the previously administered drugs cannot be completely excluded in the study design of the present work. However, a recent pharmacokinetics study on VCR, CPA, and DXR in the dog revealed that serum concentrations of these agents were shown to be below the limit of detection 7 days after treatment (Knobloch et al., 2010). Therefore, I considered such cumulative or synergistic effect might not strongly influence the results. Moreover, when the cytoreductive efficacy was compared between CPA at week 2 and DXR at week 4, VCR had been administered 1 week before the

administration of both agents. In such similar situation, decrease rate of the number of tumor cells was significantly higher after DXR administration than after CPA administration. Single agent protocol with DXR generated in a reasonable efficacy in canine lymphoma (Carter et al., 1987; Mutsaers et al., 2002; Valerius et al., 1997); however, we do not have opportunity to conduct VCR- or CPA-alone treatment, being difficult to strictly compare the activity of each agent.

Efficacy of VCR was high at weeks 1 and 3 after injection, but found to be low after the injection at weeks 6 and 8. Reason for this difference is not clear; however, one explanation may lie in the pharmacological action of VCR as a cell cycle-dependant drug, acting in the M phase of the cell cycle. The proportion of tumor cells in the M phase may be larger at the initial stage of remission induction therapy. A report on a mouse model (Yefenof et al., 1993) indicated that a large population of dormant lymphoma cells was in the G_0/G_1 phase. For such tumor cell populations, VCR would be unsuitable. As another explanation, induction of P-glycoprotein (Moore et al., 1995) or other drug resistance-related molecules might be associated with the decreased efficacy of VCR at weeks 6 and 8 after receiving multiple administrations of antineolasitc agents. VCR has been adopted as a major drug and frequently used not only at initiation but also in later phases of most current protocols for the treatment of canine lymphoma. Based on the results on the cytoreductive efficacy of VCR in this study, modified CHOP protocols employing multiple VCR administration at the initial phase would be of value for testing in a clinical trial.

In the present study, the number of tumor cells did not decrease in approximately half of the dogs after the first CPA administration. The rate of achievement of CR by week 3 was 60% (9/15) and

36% (5/14) in CPA responders and CPA non-responders, respectively; however, significant difference was not observed between the 2 groups. CPA responders tended to achieve MRD-negative at week 11 of UW-25 compared to CPA non-responders; however, the difference was not statistically different. Therefore, it is presumable that several factors affect the chemotherapy response and prognosis in canine lymphoma. In human diffuse large B-cell lymphoma , which is the disease that canine high-grade B-cell lymphoma has historically been described as being similar to, several prognostic factors based on gene expression profile were reported (Rosenwald et al., 2002). Further studies are needed to elucidate the underlying causes of poor prognosis in canine lymphoma.

Body weight differed significantly between the CPA responders and non-responders. CPA was more likely to be effective in small dogs than large dogs; moreover, CPA responders showed prolonged PFS and OS when compared with CPA non-responders. It is well known that chemotherapeutic drug dose calculated from an estimation of body surface area results in overdose in small dogs (Price et al., 1998). Therefore, current CPA dosing according to body surface area would be optimal for small breed dogs but insufficient for large breed dogs to exert its cytoreductive efficacy. It is reported that small dogs survived longer than larger dogs (Garrett et al., 2002). They suggested that the difference was due to the dose calculation based on body surface area; small dogs probably received more intensive chemotherapy than larger dogs. Our finding that the body weight of CPA responders was lighter than that of CPA-non responders coincided with their result on the difference of the survival with respect to the dog size. Recently, it is reported that addition of CPA to

DXR single-agent protocol did not result in the improvement of the treatment outcome in dogs with lymphoma (Lori et al., 2010). Mean body weight of the dogs enrolled in their study was more than 30 kg; therefore, it is conceivable that CPA was likely to be less effective for these dogs.

Dose of DXR was 1.0 mg/kg in small dogs (≤ 10 kg) and 30 mg/m^2 in medium-sized and large dogs (> 10 kg) in this study. Decrease rate of the number of tumor cells after administration of DXR was not different between small and medium-sized/large dogs, indicating that such dosing would be appropriate in both of the dog groups. There was no significant difference of the body weight between the responders and non-responders after VCR administration of any timing.

In the current study, DXR was found to be the most effective of the 3 agents for the reduction of tumor cells at week 1-11 of UW-25. This result corresponds with the fact that dogs treated with CHOP-based chemotherapy showed longer remission duration and survival time (Garrett et al., 2002; Greenlee et al., 1990; Simon et al., 2006) than those treated with COP-based chemotherapy (Carter et al., 1987). Dogs treated with multidrug chemotherapy without DXR were at approximately 2 times higher risk for relapse and death than dogs treated with UW-19 (Hosoya et al., 2007). Furthermore, in a recent report, there was no significant difference in the remission and survival durations in dogs treated with a continuous multiagent protocol including DXR and dogs treated with a short-term single DXR protocol (Simon et al., 2008). The results of these reports and the current study suggest that DXR should be considered as a main drug in the multiagent chemotherapeutic protocols for canine lymphoma.

No T-cell lymphoma case was included in this study. Therefore, the results obtained here can be

only applied to dogs with B-cell lymphoma. Immunophenotype is of prognostic importance, as dogs with the B-cell phenotype have better prognoses than dogs with the T-cell phenotype (Garrett et al., 2002; Greenlee et al., 1990; Simon et al., 2006). A recent study (Brodsky et al., 2009) reported the use of alkylating agents-rich protocol might improve the treatment outcome in dogs with T-cell lymphoma. Further studies are needed to identify differences in treatment response between B-cell lymphoma and T-cell lymphoma in dogs.

In this study, the quantitative MRD monitoring system was used to compare the cytoreductive efficacy of each agent used in one chemotherapeutic protocol, UW-25. Such strategy can be used to provide suggestions to modify the component agents included in a certain combined chemotherapeutic protocol. Moreover, quantitative MRD evaluation at the end of protocol would provide an objective evaluation to compare the efficacy of different protocols, especially to indicate an advantage of newly introduced modalities such as high-dose therapy with autologous stem cell transplantation (Pott et al., 2006) and immunotherapy with monoclonal antibodies (Rambaldi et al., 2002) adopted in human lymphohematopoietic malignancies. In these clinical trials, the MRD level at the end of the protocol was shown to be actually a good indicator to predict the remission duration following the therapy.

The present study provided several suggestions to modify the UW-25 protocol to advance the treatment efficacy in canine B-cell high-grade lymphoma. VCR use might be preferred in the early phase because the cytoreductive efficacy of VCR decreased in the later phase of the combination protocol. CPA administration can be reconsidered especially in large dogs (e.g., dose increase or

substitution with other agents). DXR at the dose used in the current UW-25 protocol is highly effective, thus, it can be recognized as a main drug in the combination protocol. Findings obtained by the novel molecular biological analysis in this study would be helpful to construct a new or modified chemotherapeutic protocol to obtain a better treatment outcome in canine lymphoma.

Chapter 3

Increase in minimal residual disease (MRD) in peripheral blood before clinical relapse in dogs with lymphoma that achieved complete remission after chemotherapy

Abstract

Relapse after achievement of complete remission (CR) is an inevitable event in most dogs with canine lymphoma. The present study was carried out to identify the change of the minimal residual disease (MRD) level before relapse in dogs with lymphoma that achieved CR after chemotherapy.

Twenty dogs with multicentric high-grade B-cell lymphoma were used. MRD levels in peripheral blood mononuclear cells (PBMCs) were measured by real-time polymerase chain reaction (PCR) amplifying the rearranged immunoglobulin heavy chain gene. MRD measurement and clinical assessment were performed every 2–4 weeks for 28–601 days after completion of chemotherapy. MRD elevation was defined as an increase by more than 0.5, calculated by $\log_{10}$[copy number of MRD per 10^5 PBMCs], based on the uncertainty level observed in a canine lymphoma cell line.

During the follow-up period, 15 dogs relapsed in 28–320 days (median, 120 days) after completion of chemotherapy. MRD elevation was detected 2 weeks or more before relapse in 14 of the 15 dogs; however, preceding MRD elevation could not be detected in the remaining 1 dog. The duration from the point of MRD elevation to the clinical relapse was between 0–63 days (median, 42 days). In contrast, no MRD elevation was detected in 5 dogs that did not experience relapse.

MRD elevation can be detected before clinical relapse in dogs with lymphoma. Application of early reinduction therapy based on MRD elevation prior to clinical relapse has the potential to improve the treatment outcomes of canine lymphoma.

Introduction

I found prognostic significance of minimal residual disease (MRD) quantification in canine lymphoma in Chapters 1 and 2. However, relapse is an inevitable event even in dogs with lymphoma that can be categorized into a better prognosis group from the MRD level. Early detection of tumor relapse and prompt therapy are conceivably a reasonable approach for managing recrudescent disease. Therefore, I carried out this study to identify whether MRD monitoring could detect early changes in tumor cell growth in the body prior to clinical relapse.

Canine lymphoma is one of the most chemoresponsive malignancies in dogs. Initial response rates of chemotherapy are reported to be as high as 69–94.2% (Carter et al., 1987; Garrett et al., 2002; Moore et al., 2001; Simon et al., 2006). However, nearly all treated dogs experience relapse and die due to disease progression. Despite the fact that various multidrug chemotherapeutic protocols have been introduced, the median survival time in a recent study was approximately 12 months (Sorenmo et al., 2010). In general, the treatment of relapsed lymphoma results in a lower response rate and shorter remission duration compared to untreated lymphoma (Alvarez et al., 2006; Saba et al., 2007). Early detection of tumor relapse and prompt therapy are conceivably a reasonable approach for managing recrudescent disease.

Monitoring MRD is an essential tool for risk-group stratification and appears to be useful for predicting relapse in some human hematopoietic and lymphoid malignancies (Bruggemann et al., 2006; Lane et al., 2008; Pott et al., 2006; Rambaldi et al., 2002). Yamazaki et al. previously

reported a highly sensitive MRD-detection system in canine lymphoma using a real-time polymerase chain reaction (PCR) that enabled the detection of as little as 1 tumor cell per 10^4 peripheral blood mononuclear cells (PBMCs) (Yamazaki et al., 2008). The authors recently revealed that the MRD level at the end of chemotherapy was correlated with outcome in dogs with lymphoma (Yamazaki et al., 2010).

Real-time PCR-based monitoring is considered useful for detecting early changes in tumor cell growth in the body prior to clinical relapse. In the present study, I carried out prospective PCR monitoring of tumor cell numbers in the PBMCs of 20 dogs with lymphoma that had achieved CR after chemotherapy, most of which subsequently experienced clinical relapse during the observation period.

Materials and Methods

Lymphoma cases

Between April 2006 and August 2009, 20 dogs diagnosed cytologically with high-grade B-cell lymphoma (Figure 1A, B) via fine needle aspiration of enlarged peripheral lymph nodes according to the updated Kiel classification (Fournel-Fleury et al., 1997) were enrolled in this study. B-cell lineage was determined by the detection of clonal immunoglobulin heavy chain (IgH) gene rearrangement by using PCR for antigen receptor gene rearrangement with primers described in 3 previous reports (Burnett et al., 2003; Tamura et al., 2006; Valli et al., 2006).

This study was performed at the Veterinary Medical Center of the University of Tokyo and 3 private animal hospitals. Further eligibility criteria were as follows: (1) anatomic form of lymphoma was multicentric, (2) clone-specific oligonucleotides/primers and probes could be successfully designed for measuring MRD, and (3) dogs achieved a complete remission (CR) and completed a 6-month modified version of the University of Wisconsin-Madison chemotherapy protocol (UW-25) (Garrett et al., 2002).

Pretreatment evaluation for all dogs included physical examination, complete blood count, serum chemistry profile, thoracic and abdominal radiographs, and abdominal ultrasound. Clinical staging was performed according to the World Health Organization criteria for canine lymphoma (Owen, 1980) except for the assessment of bone marrow involvement. Classification into stage V was determined by detection of neoplastic lymphoid cells in circulation by microscopy.

Follow-up evaluation and sampling for MRD measurement

Response evaluation was performed according to the Veterinary Cooperative Oncology Group consensus document (Vail et al., 2010) with a cytologic finding of enlarged lymph nodes FNA samples. Routine follow-up evaluation was conducted every 4 weeks after the completion of the chemotherapeutic protocols. Follow-up evaluation was conducted more frequently (every 2 weeks) after MRD elevation was detected until the identification of progressive disease. Blood samples were obtained at diagnosis, post-chemotherapy (2 weeks after the last injection of chemotherapeutic agent), and at each follow-up evaluation.

MRD measurement with real-time PCR

MRD measurement was performed as previously described (Yamazaki et al., 2008). Briefly, to determine the sequence of the rearranged antigen receptor gene in each lymphoma case, the PCR products amplified from the rearranged IgH gene fragments were ligated into a T/A cloning vector (Promega) and subjected to sequence analysis. In order to obtain the sequence of the 3′-flanking region of the rearranged IgH J gene segments, their sequences were searched in the dog genome data base using the BLAST search program (National Center for Biotechnology Information). Allele-specific oligonucleotides complementary to the complementarity-determining region 3 of the IgH genes of each dog were designed using a primer-designing software program (Applied Biosystems).

To assess the MRD level in PBMCs, peripheral blood samples (3 mL) were collected in EDTA-treated tubes, followed by density gradient centrifugation with Ficoll/Hypaque (specific gravity, 1.077) (Nycomed Pharma AS). Blood samples collected at private animal hospitals were shipped to the University of Tokyo in a container kept at 4°C, and PBMCs were separated within 24 hours from collection. The genomic DNA of the PBMCs was extracted using a DNA extraction kit (QIAGEN). Concentration of the extracted genomic DNA was calculated based on the absorbance at 260 nm measured with a spectrophotometer.

Real-time PCR was performed using a thermal cycler[e], and the data were analyzed using the software supplied by the manufacturer. To normalize the amount of DNA samples, the albumin gene was used as an internal reference (Yamazaki et al., 2008).

Two consecutive samples—a new sample to be evaluated plus a sample with a known MRD level from the last examination—were measured simultaneously. The accuracy of the calculated MRD level was assessed from the inter-run variability of the results from a control DNA derived from a canine lymphoma cell line (Yamazaki et al., 2008). All measurements were conducted in triplicate.

Measurement uncertainty level for MRD detection with real-time PCR

In order to determine the measurement uncertainty level of this assay, template DNAs derived from a canine lymphoma cell line (Yamazaki et al., 2008) corresponding to 10, 10^2, 10^3, 10^4, and 10^5 copies (the measurable range using this method) of the rearranged gene were subjected to

real-time PCR. Six samples of each number of copies were subjected to real-time PCR to calculate the mean and standard deviation (SD) of the copy number of the tumor cells. The copy number of the tumor cells was transformed into $\log_{10}$. As the number of reference lymphoma cells became smaller in this assay, the SD became larger. When 10 reference lymphoma cells were included in the template, the resultant SD showed the largest value (0.22) as calculated using the formula: $\log_{10}$[copy number of tumor cells]. Thus, I tentatively defined MRD elevation as an increase of more than 2 SD (0.44). Regarding the results of the control lymphoma cell line, if the MRD increased by more than 0.5 (calculated from $\log_{10}$[copy number of MRD per 10^5 PBMCs]) in comparison to that from the previous visit, I judged this as MRD elevation.

Statistical analysis

The number of tumor cells at diagnosis and subsequent MRD levels at post-chemotehrapy, admissions for follow-up, and clinical relapse were analyzed using the Krsukal-Wallis test to assess for differences. Differences of the MRD between the post-chemotherapy and other time points were evaluated using Dunnett's test. All statistics were calculated using statistical software (ATMS). Values of $P < 0.05$ were considered significant.

Results

Lymphoma cases

The 20 dogs in this study belonged to 13 breeds: the most commonly represented were Welsh corgi (3) and miniature schnauzer (3) followed by pug (2), miniature dachshund (2), mixed (2), standard poodle (1), bulldog (1), golden retriever (1), shih tzu (1), Australian kelpie (1), Polish lowland sheepdog (1), Yorkshire terrier (1), and French bulldog (1). Median age was 7 years (range, 2–13 years) and median body weight was 10.3 kg (range, 3.0–36.3 kg). Ten dogs were male (3 were castrated) and 10 were female (6 were spayed).

At diagnosis, 4 dogs (20%) were classified as clinical stage III, 10 (50%) as IV, and 6 (30%) as V. Six dogs (30%) were classified as substage b and 14 (70%) as substage a.

Eighteen (cases 1–5, 7, 9–20; Table 6, 7) out of the 20 dogs, who did not receive any treatment for lymphoma before entering this study, were treated with the UW-25 (Garrett et al. 2002) and achieved CR. Two dogs (cases 6 and 8; Table 6) had received the first round of UW-25 and achieved CR. Cases 6 and 8 experienced relapse 4 and 3 months later, respectively. After receiving a second round of UW-25, both cases achieved CR again and were enrolled in the present study.

Follow-up duration

Median follow-up duration after chemotherapy was 133 days (range, 28–601, 95% confidence interval (CI), 104–235). Fifteen out of the 20 dogs experienced relapses during the observation

period. The median duration from the end of chemotherapy to relapse in these 15 dogs was 120 days (range, 28–350, 95% CI, 82.5–203). The other 5 dogs did not experience relapse during their follow-up durations (92, 119, 173, 374, and 601 days).

Number of tumor cells in PBMCs at diagnosis, and the change of MRD levels in 15 dogs that experienced clinical relapse during the follow-up duration

In the 15 dogs that experienced clinical relapse during the follow-up, MRD levels were measured at several points prior to and at clinical relapse (Table 6, Fig 6).

The number of tumor cells indicated as $\log_{10}$[copy number of tumor cells per 10^5 PBMCs] at diagnosis ranged from 3.18–5.0 (median, 4.58) in the 15 dogs (Table 6, Fig 6). Post-chemotherapy (2 weeks after the last injection of chemotherapeutic agent) MRD levels decreased down to <1.0 – 1.83 (median, 1.19). In 6 out of the 15 dogs, the MRD level was lower than the detection limit in the real-time PCR assay used in this study. A significant difference was observed between the number of tumor cells at diagnosis and MRD level at post-chemotherapy ($P < 0.001$) (Fig 6).

At clinical relapse, the MRD level was increased up to 2.82–4.97 (median, 3.29), showing a significant increase compared to the MRD level at post-chemotherapy ($P < 0.001$) (Table 6, Fig 6).

In these 15 cases, the MRD level was evaluated prior to the identification of clinical relapse at 2–4 week intervals. The MRD levels 4 weeks prior to clinical relapse ranged from <1.0 to 3.17 (median, 2.19), which showed an increasing tendency compared to those at post-chemotherapy; however, the difference between the data of the 2 groups was not significant. Meanwhile, the MRD

levels 2 weeks prior to clinical relapse (range, 2.2–3.68, median, 2.67) were significantly greater than those at post-chemotherapy ($P < 0.01$) (Fig 6).

Time points of MRD elevation in dogs that experienced clinical relapse

During the follow-up duration in CR, both monitoring of MRD level and clinical assessment via physical examination, hematological analysis, and imaging tests were carried out every 4 weeks during the initial phase. However, when the MRD level increased by more than 0.5 in comparison to the previous admission, the interval of admission was shortened to 2 weeks.

In case 1 for example, the MRD level was 1.21 at post-chemotherapy; after 4 weeks (6 weeks prior to clinical relapse), it increased to 1.74 (Table 6). The MRD level in this case progressively increased to 1.95 and 2.22 after 2 and 4 weeks respectively. At these time points, our clinical assessment determined the dog was still in CR. However, relapse was detected 6 weeks later, and the MRD level at this point was as high as 3.47. The changes of MRD level indicated that an MRD elevation of more than 0.5 could be detected 6 weeks prior to clinical relapse. Similar results were obtained in the other 13 cases (cases 2–4 and 6–15).

In contrast, MRD elevation prior to relapse could not be detected in 1 dog (case 5). This dog had a low post-chemotherapy MRD level that was less than 1.0; however, it exhibited an apparent clinical relapse 4 weeks later with a distinct increase of its MRD level (2.82).

The median duration from the point of MRD elevation to clinical relapse in the 15 dogs was 42 days (range, 0–63, 95% CI, 33–50.5). When the MRD elevation was defined as an increase of more

than 0.5, the sensitivity for predicting relapse using the MRD elevation in consecutive samples obtained at 4-week intervals was as high as 93.3% (14/15).

MRD levels in dogs that did not experience clinical relapse during the follow-up duration

In 5 of the 20 dogs, no clinical relapse was detected during the follow-up. MRD levels in these dogs were evaluated at 4-week intervals during the follow-up period (Table 7).

In case 16 for example, the post-chemotherapy MRD level decreased to a value below the detection limit (1.0) of the assay. The MRD levels 4, 8, 12, and 16 weeks from the post-chemotherapy point remained low—less than 1.0 in this case. Similarly, MRD elevation of more than 0.5 was not observed during the follow-up period (92–601 days) in the other 4 dogs. The specificity for predicting relapse using the MRD elevation (>0.5) was 100% (5/5) in the consecutive samples obtained at 4-week intervals.

Discussion

Based on previous studies describing quantitative (Yamazaki et al., 2008) and qualitative PCR (Lana et al., 2006) assays to detect neoplastic lymphoid cells in the peripheral blood of dogs both before chemotherapy and after achieving CR, I decided to examine the clinical usefulness of MRD monitoring to predict clinical relapse in cases of canine lymphoma. The number of tumor cells and MRD levels in the peripheral blood were correlated with the clinical status of patients. There were statistically significant differences in MRD levels between the samples at the following points: diagnosis and post-chemotherapy, post-chemotherapy and clinical relapse, and post-chemotherapy and 2 weeks before clinical relapse.

In the present study, MRD elevation was tentatively defined as an increase of more than 0.5 as calculated from the value of $\log_{10}$[copy number of MRD per 10^5 PBMCs], based on the measured uncertainty level obtained from the data generated using a canine lymphoma cell line. This threshold value appeared appropriate since it provided a high sensitivity (93.3%) and specificity (100%) for predicting relapse in canine lymphoma. Moreover, we could infer tumor cell growth from the elevation of the MRD value 42 days (median) prior to the actual clinical relapse. Therefore, MRD monitoring of peripheral blood after the completion of chemotherapy is useful for detecting early growth of tumor cells that eventually lead to clinical relapse. One dog relapsed without preceding MRD elevation 4 weeks prior. At the time of apparent clinical relapse, this dog exhibited MRD elevation. In this case, it is conceivable that the progression from CR to clinical

relapse was rapid; therefore, I could not detect the MRD elevation before relapse. There is a possibility that consecutive MRD monitoring at 4-week intervals can overlook such rapid tumor cell growth leading to clinical relapse. Therefore, more frequent monitoring may be required to detect such rapid tumor cell growth. However, such cases are considered to be infrequent in dogs with B-cell high-grade lymphoma.

I judged the presence of MRD elevation from the relative increase compared to the value 4 weeks prior for predicting the clinical relapse. Alternatively, an absolute value of MRD level can be also helpful for detecting tumor cell growth leading to clinical relapse. When I found MRD increasing over the absolute value of 1.5 as MRD elevation, I could detect the tumor cell growth in 11 of the 15 dogs (73.3%) that experienced clinical relapse in our case series. By using this criterion, duration from the point of MRD elevation to clinical relapse was 0–63 days (median 37 days). When it was defined as an increase over 2.0, tumor cell growth before clinical relapse could be detected in 14 of the 15 dogs (93.3%) that experienced clinical relapse. However duration from the point of the MRD elevation to clinical relapse was shortened to 0–43 days (median 28 days). Therefore, in terms of the sensitivity and duration to clinical relapse, MRD elevation defined as a relative increase by more than 0.5 in comparison to the value at the previous visit would be more useful to predict subsequent clinical relapse.

In all of the 15 dogs that exhibited a progression of relapse in this study, the real-time PCR system at diagnosis was able to amplify the IgH gene of the lymphoma cells proliferating prior to and during clinical relapse. Elevation of the MRD was shown to be in accordance with the

progression from CR to relapse in these cases. These results indicate that the lymphoma cells proliferating at relapse were derived from the same clones that formed the initial lymphoma lesions at diagnosis. Therefore, it is conceivable that clonal change of the tumor cells is an infrequent phenomenon in canine B-cell high-grade lymphoma.

In hematopoietic and lymphoid malignancies in humans, recent studies reported that some patients with rising MRD levels were successfully treated with antineoplastic agents to avoid overt relapse (Doubek et al., 2005; Ladetto et al., 2006). Preemptive therapy is shown to be effective for preventing morphologic or cytogenetic relapse following molecular relapse in acute promyelocytic leukemia (Doubek et al., 2005). In another report, rituximab induced the effective clearance of MRD detected in molecular relapses of mantle cell lymphoma, resulting in long remission durations (Ladetto et al., 2006). Studies on MRD with clinical significance in humans have been reported predominantly in mantle cell lymphoma and follicular lymphoma in which disease-specific genetic markers derived from chromosomal translocation are applicable in many of the patients. In contrast, in human DLBCL, such genetic marker cannot be used. Hence, to set up an MRD monitoring system in human DLBCL, preparation of patient-specific IgH gene primers/probe is needed as described in the present study in dogs. For these reasons, there have been only a small number of studies on the MRD monitoring in human DLBCL (Mitterbauer et al., 2001; Yashima et al., 2003). Therefore, I believe our canine study can provide beneficial information for human DLBCL.

In conclusion, I successfully detected MRD elevation prior to relapse in canine high-grade

lymphoma, indicating that molecular relapse is a warning sign of impending clinical relapse. The present study provides a basis for conducting a novel therapeutic strategy of early reinduction therapy based on the MRD level in canine lymphoma.

Conclusion

A series of studies in this thesis were carried out to reveal clinical significance of minimal residual disease (MRD) quantification in canine lymphoma.

The study in Chapter 1 of the present thesis was carried out to examine the prognostic significance of MRD level in canine lymphoma at an early phase of multidrug chemotherapy. I revealed that MRD level at week 11 of a combination chemotherapy protocol (UW-25) (Garrett et al., 2002) was the most important prognostic indicator determining subsequent relapse in dogs with B-cell high-grade lymphoma. MRD-positive dogs at week 11 of UW-25 had shorter progression-free survival and overall survival times than the MRD-negative dogs. Based on the results, alternative treatment approach would be beneficial to attempt to decrease their risk of subsequent progressive disease for MRD-positive dogs at week 11 of UW-25. Further clinical studies are warranted to identify if consolidation therapy based on MRD monitoring would lead to improvement in treatment outcome.

Since I hypothesized that efficacy of each drug used in UW-25 might affect the MRD level at week 11 of UW-25, I conducted the study in Chapter 2 of this thesis to compare the cytoreductive efficacy of vincristine (VCR), cyclophosphamide (CPA), and doxsorubicin (DXR) that constitute UW-25 by quantifying lymphoma cells in peripheral blood with real-time PCR. The study revealed that the number of tumor cells did not decrease in approximately half of the dogs after the first CPA administration. CPA responders tended to achieve MRD-negative at week 11 of UW-25 compared to CPA non-responders; however, the difference was not statistically different. Body weight differed significantly between the CPA responders and non-responders. CPA was more likely to be

effective in small dogs than large dogs. Efficacy of VCR was high at weeks 1 and 3 after injection, but found to be low after the injection at weeks 6 and 8. DXR was found to be the most effective of the 3 agents for the reduction of tumor cells at week 1 – 11 of UW-25. The present study indicated several suggestions to modify the UW-25 protocol to improve its treatment outcome. VCR use might be preferred in the early phase because the cytoreductive efficacy of VCR decreased in the later phase of the combination protocol. CPA administration can be reconsidered especially in large dogs (e.g., dose increase or substitution with other agents). DXR at the dose used in the current UW-25 protocol is highly effective, thus, it can be recognized as a main drug in the combination protocol.

Canine lymphoma is a fundamentally fatal disease and cure of the disease is hardly achieved by the current combined chemotherapeutic protocols. Early detection of tumor relapse and prompt therapy are conceivably a reasonable approach for managing recrudescent disease. Therefore, I carried out the study in Chapter 3 of this thesis to identify whether MRD monitoring could detect early changes in tumor cell growth in the body prior to clinical relapse. The study revealed that MRD monitoring of peripheral blood after the completion of chemotherapy was useful for detecting early growth of tumor cells that eventually lead to clinical relapse. The result of this study provides a basis for conducting a novel therapeutic strategy of early reinduction therapy based on the MRD level in canine lymphoma.

A series of studies in this thesis revealed several aspects with respect to the clinical significance of MRD quantification in dogs with lymphoma. MRD quantification was considered to be very

useful as a prognostic indicator, an objective marker showing the treatment efficacy, and a predictor of clinical relapse in canine lymphoma. MRD measurement can be used to develop a tailor-made antitumor therapy for each patient. Treatment strategy based on the result of MRD level will lead to remarkable improvement of the treatment outcome in dog patients with lymphoma.

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References

Alvarez, F.J., Kisseberth, W.C., Gallant, S.L., Couto, C.G., 2006, Dexamethasone, melphalan, actinomycin D, cytosine arabinoside (DMAC) protocol for dogs with relapsed lymphoma. J Vet Intern Med 20, 1178–1183.

Brodsky, E.M., Maudlin, G.N., Lachowicz, J.L., Post, G.S., 2009, Asparaginase and MOPP treatment of dogs with lymphoma. J Vet Intern Med 23, 578–584.

Bruggemann, M., Raff, T., Flohr, T., Gokbuget, N., Nakao, M., Droese, J., Luschen, S., Pott, C., Ritgen, M., Scheuring, U., Horst, H.A., Thiel, E., Hoelzer, D., Bartram, C.R., Kneba, M., 2006, Clinical significance of minimal residual disease quantification in adult patients with standard-risk acute lymphoblastic leukemia. Blood 107, 1116–1123.

Brugger, W., Hirsch, J., Grunebach, F., Repp, R., Brossart, P., Vogel, W., Kopp, H.G., Manz, M.G., Bitzer, M., Schlimok, G., Kaufmann, M., Ganser, A., Fehnle, K., Gramatzki, M., Kanz, L., 2004, Rituximab consolidation after high-dose chemotherapy and autologous blood stem celltransplantation in follicular and mantle cell lymphoma: a prospective, multicenter phase II study. Ann Oncol 15, 1691–1698.

Burnett, R.C., Vernau, W., Modiano, J.F., Olver, C.S., Moore, P.F., Avery, A.C., 2003, Diagnosis of canine lymphoid neoplasia using clonal rearrangements of antigen receptor genes. Vet Pathol 40, 32–41.

Carter, R.F., Harris, C.K., Withrow, S.J., Valli, V.E.O., Susaneck, S.J, 1987, Chemotherapy of canine lymphoma with histopathological correlation: doxorubicin alone compared to COP as first treatment regimen. J Am Anim Hosp Assoc 23, 587–596.

Doubek, M., Brychtova, Y., Berkovcova, J., Krejci, M., Palasek, I., Dvorakova, D., Mayer, J., 2005, Acute myeloid leukemias with recurrent genetic abnormalities: frequent assessment of minimal residual disease and treatment of molecular relapse with chemotherapy. Leukemia 19, 885–888.

Fournel-Fleury, C., Magnol, J.P., Bricaire, P., Marchal, T., Chabanne, L., Delverdier, A., Bryon, P.A., Felman, P., 1997, Cytohistological and immunological classification of canine malignant lymphomas: comparison with human non-Hodgkin's lymphomas. J Comp Pathol 117, 35–59.

Frimberger, A.E., Moore, A.S., Rassnick, K.M., Cotter, S.M., O'Sullivan, J.L., Quesenberry, P.J., 2006, A combination chemotherapy protocol with dose intensification and autologous bone marrow transplant (VELCAP-HDC) for canine lymphoma. J Vet Intern Med 20, 355–364.

Garrett, L.D., Thamm, D.H., Chun, R., Dudley, R., Vail, D.M., 2002, Evaluation of a 6-month chemotherapy protocol with no maintenance therapy for dogs with lymphoma. J Vet Intern Med 16, 704–709.

Greenlee, P.G., Filippa, D.A., Quimby, F.W., Patnaik, A.K., Calvano, S.E., Matus, R.E., Kimmel, M., Hurvitz, A.I., Lieberman, P.H., 1990, Lymphomas in dogs. A morphologic, immunologic, and clinical study. Cancer 66, 480–490.

Hosoya, K., Kisseberth, W.C., Lord, L.K., Alvarez, F.J., Lara-Garcia, A., Kosarek, C.E., London, C.A., Couto, C.G., 2007, Comparison of COAP and UW-19 protocols for dogs with multicentric lymphoma. J Vet Intern Med 21, 1355–1363.

Jacobs, R.M., Messick, J.B., Valli, V.E., 2002, Tumors of the hemolymphatic system. In: Tumors of Domestic Animals, 4th edn., Meuten, D.J. (Eds.), State Press, Iowa, pp.138.

Kaiser, H.E., 1981, Animal neoplasms: a systemic review. In: Neoplasms–Comparative Pathology in Animals,Plants and Man, Kaiser, H.E. (Eds.), Williams and Wilkins, Baltimore MD, USA, pp.747–812.

Knobloch, A., Mohring, S.A.I., Eberle, N., Nolte, I., Hamscher, G., Simon, D., 2010, Drug residues in serum of dogs receiving anticancer chemotherapy. J Vet Intern Med 24, 379–383.

Ladetto, M., Magni, M., Pagliano, G., De Marco, F., Drandi, D., Ricca, I., Astolfi, M., Matteucci, P., Guidetti, A., Mantoan, B., Bodoni, C.L., Zanni, M., Boccadoro, M., Gianni, A.M., Tarella, C., 2006, Rituximab induces effective clearance of minimal residual disease in molecular relapses of mantle cell lymphoma. Biol Blood Marrow Transplant 12, 1270–1276.

Lana, S.E., Jackson, T.L., Burnett, R.C., Morley, P.S., Avery, A.C., 2006, Utility of polymerase chain reaction for analysis of antigen receptor rearrangement in staging and predicting prognosis in dogs with lymphoma. J Vet Intern Med 20, 329–334.

Lane, S., Saal, R., Mollee, P., Jones, M., Grigg, A., Taylor, K., Seymour, J., Kennedy, G., Williams, B., Grimmett, K., Griffiths, V., Gill, D., Hourigan, M., Marlton, P., 2008, A ≥ 1 log rise in RQ-PCR transcript levels defines molecular relapse in core binding factor acute myeloid leukemia and predicts subsequent morphologic relapse. Leuk Lymphoma 49, 517–523.

Lori, J.C., Stein, T.J., Thamm, D.H., 2010, Doxorubicin and cyclophosphamide for the treatment of canine lymphoma: a randomized, placebo-controlled study. Vet Comp Oncol 8, 188–195.

MacDonald, V.S., Thamm, D.H., Kurzman, I.D., Turek, M.M., Vail, D.M., 2005, Does L-asparaginase influence efficacy or toxicity when added to standard CHOP protocol for dogs with lymphoma?. J Vet Intern Med 19, 732–736.

MacEwen, E.G., Hayes, A.A., Matus, R.E., Kurzman, I., 1987, Evaluation of some prognostic factors for advanced multicentric lymphosarcoma in the dog: 147 cases (1978-1981). J Am Vet Med Assoc 190, 564–568.

Miller, W.H., Jr., Levine, K., DeBlasio, A., Frankel, S.R., Dmitrovsky, E., Warrell, R.P., Jr., 1993, Detection of minimal residual disease in acute promyelocytic leukemia by a reverse transcription polymerase chain reaction assay for the PML/RAR-alpha fusion mRNA. Blood 82, 1689–1694.

Mitterbauer, M., Neumeister, P., Kalhs, P., Brugger, S., Fischer, G., Dieckmann, K., Hoecker, P., Hinterberger, W., Linkesch, W., Simonitsch, I., Jaeger, U., Lechner, K., Mannhalter, C., Mitterbauer, G., Greinix, H.T., 2001, Long-term clinical and molecular remission after

allogenic stem cell transplantation (SCT) in patients with poor prognosis non-Hodgkin's lymphoma. Leukemia 15, 635–641.

Moore, A.S., Leveille, C.R., Reimann, K.A., Shu, H., Arias, I.M., 1995, The expression of P-glycoprotein in canine lymphoma and its association with multidrug resistance. Cancer Invest 13, 475–479.

Moore, A.S., Cotter, S.M., Rand, W.M., Wood, C.A., Williams, L.E., London, C.A., Frimberger, A.E., L'Heureux, D.A., 2001, Evaluation of a discontinuous treatment protocol (VELCAP-S) for canine lymphoma. J Vet Intern Med 15, 348–354.

Morrison-Collister, K.E., Rassnick, K.M., Northrup, N.C., Kristal, O., Chretin, J.D., Williams, L.E., Cotter, S.M., Moore, A.S., 2003, A combination chemotherapy protocol with MOPP and CCNU consolidation (Tufts VELCAP-SC) for the treatment of canine lymphoma. Vet Comp Oncol 1, 180–190.

Mutsaers, A.J., Glickman, N.W., DeNicola, D.B., Widmer, W.R., Bonney, P.L., Hahn, K.A., Knapp, D.W., 2002, Evaluation of treatment with doxorubicin and piroxicam or doxorubicin alone for multicentric lymphoma in dogs. J Am Vet Med Assoc 220, 1813–1817.

Owen, L.N., 1980, TNM Classification of tumours in domestic animals. World Health Organization, Geneva, Switzerland, pp.46–47.

Pott, C., Schrader, C., Gesk, S., Harder, L., Tiemann, M., Raff, T., Bruggemann, M., Ritgen, M., Gahn, B., Unterhalt, M., Dreyling, M., Hiddemann, W., Siebert, R., Dreger, P., Kneba, M., 2006, Quantitative assessment of molecular remission after high-dose therapy with autologous stem cell transplantation predicts long-term remission in mantle cell lymphoma. Blood 107, 2271–2278.

Price, G.S., Frazier, D.L., 1998, Use of body surface area (BSA)-based dosages to calculate chemotherapeutic drug dose in dogs: I. Potential problems with current BSA formulae. J Vet Intern Med 12, 267–271.

Rambaldi, A., Lazzari, M., Manzoni, C., Carlotti, E., Arcaini, L., Baccarani, M., Barbui, T., Bernasconi, C., Dastoli, G., Fuga, G., Gamba, E., Gargantini, L., Gattei, V., Lauria, F., Lazzarino, M., Mandelli, F., Morra, E., Pulsoni, A., Ribersani, M., Rossi-Ferrini, P.L., Rupolo, M., Tura, S., Zagonel, V., Zaja, F., Zinzani, P., Reato, G., Foa, R., 2002, Monitoring of minimal residual disease after CHOP and rituximab in previously untreated patients with follicular lymphoma. Blood 99, 856–862.

Rassnick, K.M., McEntee, M.C., Erb, H.N., Burke, B.P., Balkman, C.E., Flory, A.B., Kiselow, M.A., Autio, K., Gieger, T.L., 2007, Comparison of 3 protocols for treatment after induction of remission in dogs with lymphoma. J Vet Intern Med 21, 1364–1373.

Rosenwald, A., Wright, G., Chan, W.C., Connors, J.M., Campo, E., Fisher, R.I., Gascoyne, M.D., Muller-Hermelink, H.K., Smeland, E.B., Staudt, L.M., 2002, The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med 346, 1937–1947.

Saba, C.F., Thamm, D.H., Vail, D.M., 2007, Combination chemotherapy with L-asparaginase, lomustine, and prednisone for relapsed or refractory canine lymphoma. J Vet Intern Med 21, 127–132.

Simon, D., Nolte, I., Eberle, N., Abbrederis, N., Killich, M., Hirschberger, J., 2006, Treatment of dogs with lymphoma using a 12-week, maintenance-free combination chemotherapy protocol. J Vet Intern Med 20, 948–954.

Simon, D., Moreno, S.N., Hirschberger, J., Moritz, A., Kohn, B., Neumann, S., Jurina, K., Scharvogel, S., Schwedes, C., Reinacher, M., Beyerbach, M., Nolte, I., 2008, Efficacy of a continuous, multiagent chemotherapeutic protocol versus a short-term single-agent protocol in dogs with lymphoma. J Am Vet Med Assoc 232, 879–885.

Sorenmo, K., Overley, B., Krick, E., Ferrara, T., LaBlanc, A., Shofer, F., 2010, Outcome and toxicity associated with a dose-intensified, maintenance-free CHOP-based chemotherapy protocol in canine lymphoma: 130 cases. Vet Comp Oncol 8, 196–208.

Tamura, K., Yagihara, H., Isotani, M., Ono, K., Washizu, T., Bonkobara, M., 2006, Development of the polymerase chain reaction assay based on the canine genome database for detection of monoclonality in B cell lymphoma. Vet Immunol Immunopathol 110, 163–167.

Vail, D.M., Michels, G.M., Khanna, C., Selting, K.A., London, C.A., 2010, Response evaluation criteria for peripheral nodal lymphoma in dogs (v1.0)--a Veterinary Cooperative Oncology Group (VCOG) consensus document. Vet Comp Oncol 8, 28–37.

Valerius, K.D., Ogilvie, G.K., Mallinckrodt, C.H., Getzy, D.M., 1997, Doxorubicin alone or in combination with asparaginase, followed by cyclophosphamide, vincristine, and prednisone for treatment of multicentric lymphoma in dogs: 121 cases (1987-1995). J Am Vet Med Assoc 210, 512–516.

Valli, V.E., Vernau, W., de Lorimier, L.P., Graham, P.S., Moore, P.F., 2006, Canine indolent nodular lymphoma. Vet Pathol 43, 241–256.

Veterinary Cooperative Oncology Group., 2004, Common Terminology Criteria for Adverse Events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.0. Vet Comp Oncol 2, 195–213.

Williams, L.E., Johnson, J.L., Hauck, M.L., Ruslander, D.M., Price, G.S., Thrall, D.E., 2004, Chemotherapy followed by half-body radiation therapy for canine lymphoma. J Vet Intern Med 18, 703–709.

Yamazaki, J., Baba, K., Goto-Koshino, Y., Setoguchi-Mukai, A., Fujino, Y., Ohno, K., Tsujimoto, H., 2008, Quantitative assessment of minimal residual disease (MRD) in canine lymphoma by using real-time polymerase chain reaction. Vet Immunol Immunopathol 126, 321–331.

Yamazaki, J., Takahashi, M., Setoguchi, A., Fujino, Y., Ohno, K., Tsujimoto, H., 2010, Monitoring of Minimal Residual Disease (MRD) after Multidrug Chemotherapy and Its Correlation to Outcome in Dogs with Lymphoma: A Proof-of-Concept Pilot Study. J Vet Intern Med 24, 897–903.

Yashima, A., Maesawa, C., Uchiyama, M., Tarusawa, M., Satoh, T., Satoh, M., Enomoto, S.,

Sugawara, K., Numaoka, H., Murai, K., Utsugisawa, T., Ishida, Y., Masuda, T., 2003, Quantitative assessment of contaminating tumor cells in autologous peripherl blood stem cells of B-cell non-Hodgkin lymphomas using immunoglobulin heavy chain gene allele-sapecific oligonucleotide real-time quantitative-polymerase chain reaction. Leuk Res 27, 925–934.

Yefenof, E., Picker, L.J., Scheuermann, R.H., Tucker, T.F., Vitetta, E.S., Uhr, J.W., 1993, Cancer dormancy: isolation and characterization of dormant lymphoma cells. Proc Natl Acad Sci U.S.A. 90, 1829–1833.

Zhou, J., Goldwasser, M.A., Li, A., Dahlberg, S.E., Neuberg, D., Wang, H., Dalton, V., McBride, K.D., Sallan, S.E., Silverman, L.B., Gribben, J.G., 2007, Quantitative analysis of minimal residual disease predicts relapse in children with B-lineage acute lymphoblastic leukemia in DFCI ALL Consortium Protocol 95-01. Blood 110, 1607–1611.

Table 1. Comparison of characteristics between MRD-negative (n=14) and MRD-positive (n=17) dogs with lymphoma at week 11 of UW-25

Variable	MRD-negative	MRD-positive	*P* value
Gender[a]			0.87
Male	7 (50%)	8 (47%)	
Female	7 (50%)	9 (53%)	
Clinical stage V[a]	4 (29%)	9 (53%)	0.17
Clinical substage b[a]	5 (36%)	11 (65%)	0.11
Anemia[a]	3 (21%)	4 (24%)	0.89
Predonison pretreatment[a]	2 (14%)	1 (6%)	0.43
Age[b]	7.5 (6.0-9.0)	8.0 (6.5-9.0)	0.98
Body Weight[b]	10.5 (7.8-19.3)	11.5 (9.8-15.7)	0.47
Achievement of UW-25[a]	13 (93%)	8 (47%)	0.017

[a] Gender, clinical stage V, clinical substage b, anemia (PCV < 35%), predonison pretreatment, and achievement of UW-25 show the number of dogs and percentage.

[b]Age (years) and Body Weight (kg) shows median and 95% CI.

Table 2. Estimates from the Cox model of OS with MRD-negative versus MRD-positive at week 11 of UW-25, substage, anemia, stage V, gender, and body weight included as covariates

Cox model estimates				
Predictors	Hazard ratio	95% hazard ratio confidence limits		*P*
MRD (positive vs negative)	7.1	2.5	22.8	0.0001
Substage (b vs a)	4.5	1.5	14.7	0.008
Body weight (18kg < vs ≤ 18kg)	2.9	1.0	8.0	0.042
Gender (male vs female)	0.5	0.2	1.4	0.19
Clinical Stage (5 vs 3 and 4)	0.5	0.2	1.7	0.27
Anemia (PCV < 35% vs PCV 35% ≤)	1.1	0.3	3.5	0.88

Table 3. Changes in the number of tumor cells in PBMCs from week 1 to 11 of UW-25 in 29 dogs with lymphoma

	Weeks of UW-25								
	1	2	3	4	6	7	8	9	
	VCR	CPA	VCR	DXR	VCR	CPA	VCR	DXR	
Case No.									
1	4.91[a]	***3.37***	4.03	***2.40***	***<1***	1.24	2.82		
2	4.98	***4.26***	4.56	***4.01***	***1.59***	3.21			
3	4.97	***3.94***	4.26	***3.70***	***<1***	1.61	2.53	2.57	***1.98***
4	4.92	***4.21***	4.52	4.54	***3.92***	4.32			
5	3.81	***1.93***	2.11	***1.21***	***<1***	<1	<1	1.27	1.28
6	4.95	***4.15***	4.79	***3.98***	***<1***	1.12[b]	1.30	1.35	***<1***
7	3.18	***2.75***	2.85	***2.57***	***1.88***	2.01	1.89	1.96	1.87
8	4.30	***2.97***	3.32	***2.89***	***1.87***	***1.32***	1.41	2.21	***1.42***
9	4.58	***3.39***	3.62	***2.79***	***<1***	<1	<1	<1	<1
10	4.98	***4.60***	4.59	***4.11***	***1.93***	1.82	1.88	1.79	***1.19***
11	3.80	***3.10***	3.10	3.25	***1.89***	3.32			
12	4.71	***4.48***	4.64	***3.98***	***2.22***	2.13	2.28	***1.81***	***1.02***
13	4.11	***3.98***	4.12	***3.88***	***2.73***	3.92			
14	4.79	***4.44***	4.75	4.88					
15	4.24	***2.67***	***1.82***	***<1***	<1	1.08	1.22	1.31	1.26
16	3.97	***3.59***	***2.95***	***2.30***	***2.00***	***1.41***	1.58	1.88	***1.09***
17	4.92	***4.60***	***4.42***	***4.14***	***2.02***	2.01	***1.49***	1.66	***<1***
18	3.73	***2.17***	***1.41***	1.55	***<1***	2.21			
19	4.28	***2.57***	***1.82***	***1.21***	***<1***	1.18	1.21	1.16	1.23
20	4.38	***1.60***	***<1***	<1	1.52	***1.01***	2.10	***1.52***	***<1***
21	4.99	***4.78***	***4.60***	***4.29***	***<1***	1.18	***<1***	1.32	***<1***
22	4.86	***4.44***	***4.06***	***3.52***	***1.81***	***1.01***	1.21	1.15	1.14
23	4.92	***3.18***	***2.85***	***1.40***	***<1***	1.37	2.22	2.82	***1.14***
24	4.75	***3.81***	***2.86***	***2.54***	***1.07***	1.21	1.13	***<1***	<1
25	4.97	***4.77***	***4.50***	***4.32***	***3.17***	***2.84***	2.99	3.01	***1.82***
26	4.98	***4.81***	***4.52***	***4.31***	***1.71***	1.62	***1.04***	1.91	***1.10***
27	3.12	***2.49***	***2.22***	***1.98***	***<1***	1.21	***<1***	1.52	***<1***
28	4.71	***3.11***	***2.67***	***2.33***	***<1***	1.09	***<1***	1.41	***<1***
29	4.78	***2.76***	***2.26***	***1.85***	***1.01***	1.12	1.09	***<1***	<1

[a] Data are represented as $\log_{10}$ [the number of tumor cells per 10^5 PBMCs].

[b] Chlorambucil was used instead of CPA. The result was excluded from further analysis.

Bold and italic fonts indicate the numbers of tumor cells that has decreased after administration of a chemotherapeutic agent at the previous admission.

Table 4. Evaluation of the changes in the number of tumor cells after administration of VCR, CPA, and DXR at weeks 1 to 9 of UW -25 in dogs with lymphoma

	Decrease (%)	No change (%)	Increase (%)
Week 1: VCR[a, c]	29/29 (100)	0/29 (0)	0/29 (0)
Week 2: CPA[a, d]	15/29 (51.7)	4/29 (13.8)	10/29 (34.5)
Week 3: VCR[c]	24/28 (85.8)	2/28 (7.1)	2/28 (7.1)
Week 4: DXR[a, e]	26/27 (96.3)	0/27 (0)	1/27 (3.7)
Week 6: VCR[b, c]	5/26 (19.2)	7/26 (26.9)	14/26 (53.9)
Week 7: CPA[b,d]	5/20 (25.0)	11/20 (55.0)	4/20 (20.0)
Week 8: VCR [c]	4/21 (19.4)	10/21 (47.6)	7/21 (33.0)
Week 9: DXR[b, e]	14/19 (73.7)	5/19 (26.3)	0/19 (0)

[a] Decrease rate of the number of tumor cells after administration of CPA at week 2 was significantly lower than those after administration of VCR at week1 ($P < 0.0001$) and DXR at week 4 ($P < 0.0005$).

[b] Decrease rate of the number of tumor cells after administration of DXR at week 9 was significantly higher than those after administration of VCR at week 6 ($P < 0.001$) and CPA at week 7 ($P < 0.01$).

[c] Decrease rate of the number of tumor cells after administration of VCR at weeks 6 and 8 was significantly lower than that after administration of VCR at weeks 1 and 3 ($P < 0.0001$).

[d] Decrease rate of the number of tumor cells after administration of CPA was not significantly different between weeks 2 and 7 ($P = 0.12$).

[e] Decrease rate of lymphoma cells after administration of DXR was not significantly different between weeks 4 and 9 ($P = 0.07$).

Table 5. Comparison of the CPA responders and CPA non-responders with respect to 7 variables

Variable	CPA responders (n=15)	CPA non-responders (n=14)	*P* value
Gender*			0.85
Male	7 (47%)	7 (50%)	
Female	8 (53%)	7 (50%)	
Clinical stage V*	7 (47%)	4 (29%)	0.45
Clinical substage b*	8 (53%)	7 (50%)	0.86
Anemia*	4 (27%)	3 (21%)	0.78
CR*	15 (100%)	12 (86%)	0.45
MRD-negative at week 11*	7 (47%)	2 (14%)	0.14
Age (years) (95% CI)**	7.0 (5.0–9.0)	8.5 (7.0–11.0)	0.22
Body weight (kg) (95% CI)**	9.0 (8.6–11.5)	23.5 (11.7–26.2)	0.007

* For the gender, clinical stage V, clinical substage b, anemia (PCV<35%), CR, and MRD-negative at week 11, the numbers of dogs and their percentages in parentheses are shown.

**For age and body weight, median and 95% CI in parentheses are shown.

Table 6. The number of tumor cells at diagnosis and MRD levels prior to and at the clinical relapse of 15 dogs that experienced clinical relapse

Case no.	Number of tumor cells at diagnosis	MRD level							Number of tumor cells at clinical relapse	Duration from chemotherapy to relapse (days)
		Post-chemotherapy	Before clinical relapse (weeks)							
			14–16	10–12	8	6	4	2		
1	4.96[a]	1.21		1.21	NT[b]	***1.74***[c]	1.95	2.22	3.47	71
2	4.71	<1.0	<1.0	<1.0	<1.0	NT	***2.71***	3.68	4.6	120
3	3.2	<1.0		<1.0	***1.51***	1.78	1.91	2.2	3.2	79
4	4.27	1.19			1.19	NT	***1.86***	2.42	3.01	56
5	4.58	<1.0					<1.0	NT	***2.82***	28
6[d]	4.21	1.22	1.31	1.28	***1.81***	2.11	2.38	2.79	2.97	161
7	5.0	1.83	1.41	1.42	***1.95***	2.16	2.54	2.94	4.97	189
8[d]	3.97	1.21	1.21	1.03	NT	***1.62***	1.9	2.34	3.1	108
9	3.18	1.52	1.81	1.62	1.83	***2.9***	3.17	3.21	3.29	244
10	4.92	<1.0			<1.0	NT	***1.76***	2.41	3.65	59
11	4.26	<1.0	1.08	<1.0	1.11	NT	***2.0***	3.1	4.83	301
12	4.96	<1.0	<1.0	1.01	***1.87***	2.12	2.51	2.71	3.01	350
13	3.81	1.03	<1.0	1.1	NT	<1.0	NT	***2.62***	3.25	231
14	4.97	1.31		1.31	***1.84***	2.2	3.14	3.51	4.11	73
15	4.75	1.43	1.81	1.62	1.55	NT	***2.44***	2.61	4.1	133

[a] The numbers of tumor cells at diagnosis and clinical relapse and MRD levels after chemotherapy are shown by the value of $\log_{10}$[copy number of tumor cells or MRD per 10^5 PBMCs].

[b] NT: Not tested for the MRD level.

[c] Bold and italic face indicate MRD elevation by more than 0.5 (as calculated from $\log_{10}$[copy number of MRD per 10^5 PBMCs]) in comparison to the MRD level at the previous admission.

[d] These 2 dogs were treated with a second round of UW-25 after clinical relapse.

Table 7. The number of tumor cells at diagnosis and MRD level after chemotherapy in 5 dogs that did not experience clinical relapse during the follow-up

Case no.	Number of tumor cells at diagnosis	MRD level at post-chemotherapy	MRD level after the point of post-chemotherapy (weeks)						
			4	8	12	16	20	40	80
16	4.99[a]	<1.0	<1.0	<1.0	<1.0	<1.0			
17	4.37	1.22	1.18	1.01	1.45	1.32	1.22	1.1	1.21
18	4.97	<1.0	<1.0	<1.0	1.04	1.09	<1.0	<1.0	
19	4.98	1.04	1.12	1.21	1.33	1.09	1.21		
20	4.96	1.13	1.08	1.22	1.06				

[a] The numbers of tumor cells at diagnosis and MRD levels after chemotherapy are shown as the value of $\log_{10}$[copy number of tumor cells (MRD) per 10^5 PBMCs].

Figure captions

Fig 1. Morphologic features of canine B-cell high-grade lymphoma in cytology. In the present thesis, almost all lymphoma cases were classified into centroblastic type according to updated Kiel classification; homogeneous proliferation of lymphoma cells (A), medium to large cells with round nuclei, fine chromatin, several prominent nucleoli, and narrow basophilic cytoplasm (B).

Fig 2. Number of neoplastic lymphoid cells at diagnosis and MRD at week 6, 11 of UW-25. Median number of neoplastic lymphoid cells at diagnosis and MRD at week 6, 11 were 71301.2, 77.5, and 12.1 cell in 10^5 PBMCs, respectively. Nine dogs (26%) at week 6 and 14 dogs (45%) at week 11 were MRD-negative. *: $P<0.001$.

Fig 3. Prognostic significance of the number of neoplastic lymphoid cells at diagnosis and MRD at week 6, 11. PFS (A) and OS (B) analysis at diagnosis; dogs were assigned to 3 groups; High (75000 – 100000 cells, n=17, median PFS: 211 days, median median OS: 336 days, the dotted line), intermediate (10000 – 74999 cells, n=13, median PFS: 295 days, median OS: 401 days, the solid line) and low (≤ 9999 cells, n=6, median PFS 407 days, median OS: 494 days, dashed line) number of neoplastic lymphoid cells. PFS (C) and OS (D) analysis at week 6; dogs were assigned to 3 groups; High (100 cells ≤, n=14, median PFS: 211 days, median median OS: 336 days, the dotted line), intermediate (10 - 99 cells, n=12, median PFS: 196 days, median OS: 271 days, the solid line) and low (MRD-negative, n=9, median PFS 286 days, median OS: 432 days, dashed line) MRD. PFS (E) and OS (F) analysis at week 11; dogs were assigned to 3 groups; High (100 cells ≤, n=7, median PFS: 190 days, median median OS: 221 days, the dotted line), intermediate (10 - 99 cells, n=10, median PFS: 232 days, median OS: 281 days, the solid line) and low (MRD-negative, n=14, median PFS 337 days, median OS: 411 days, dashed line) MRD.

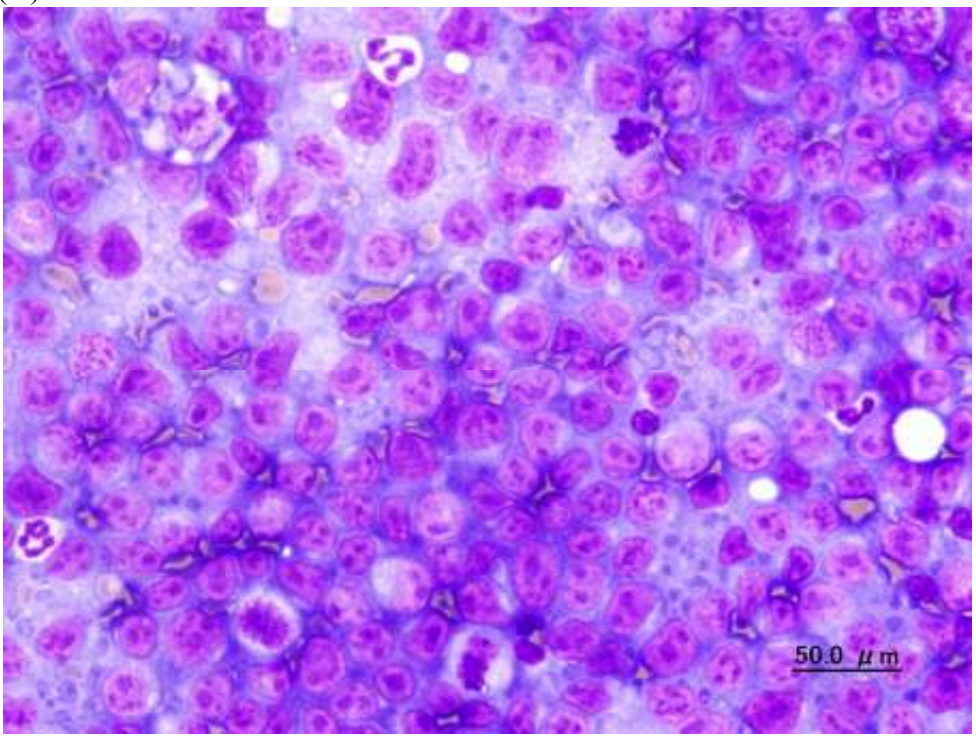
Fig 4. Prognostic significance of MRD at week 11; dogs were assigned to 2 groups; MRD-positive (n=17, solid line) and MRD-negative (n=14, dashed line). Median PFS of MRD-positive and MRD-negative were 196 days and 337 days, respectively (A). Median OS of MRD-positive and MRD-negative were 271 days and 411 days, respectively (B).

Fig 5. Kaplan-Meier curves of PFS (A) and OS (B) for dogs with lymphoma in CPA responders (solid line: n = 15) and CPA non-responders (dotted line: n = 14). Significant differences were observed between the 2 groups (PFS, $P < 0.01$; OS, $P < 0.005$)

Fig 6. The number of tumor cells at diagnosis and MRD levels after chemotherapy until relapse in the PBMCs of 15 dogs that experienced clinical relapse. Lines and numbers indicate medians at different time points. Group pairs showing significant differences are tied with horizontal lines with *P* values. All statistics were conducted using Dunnett's test.

Figure 1

(A)



(B)

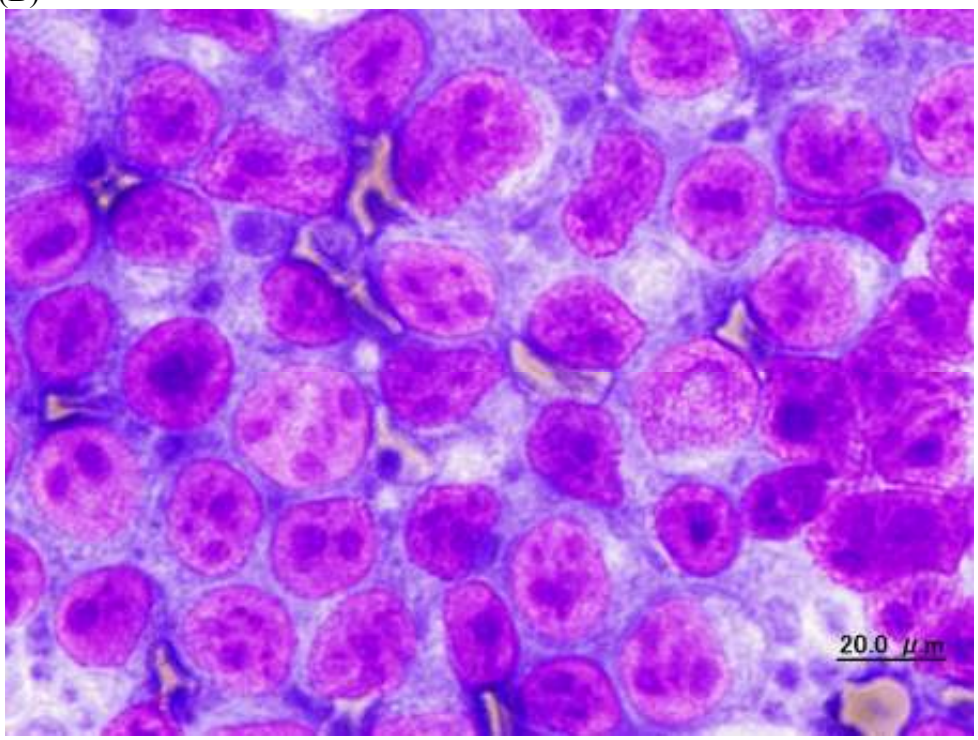


Figure 2

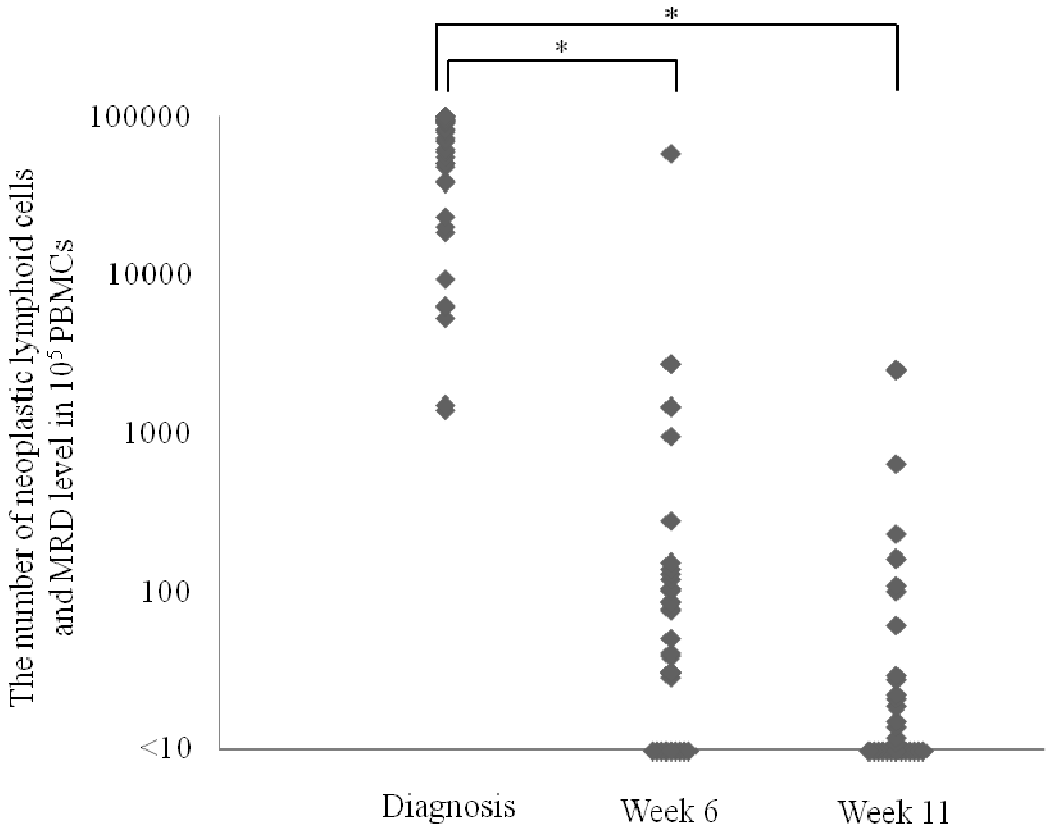

*
*
100000
10000
1000
100
<10
The number of neoplastic lymphoid cells and MRD level in 10^5 PBMCs
Diagnosis
Week 6
Week 11

Figure 3

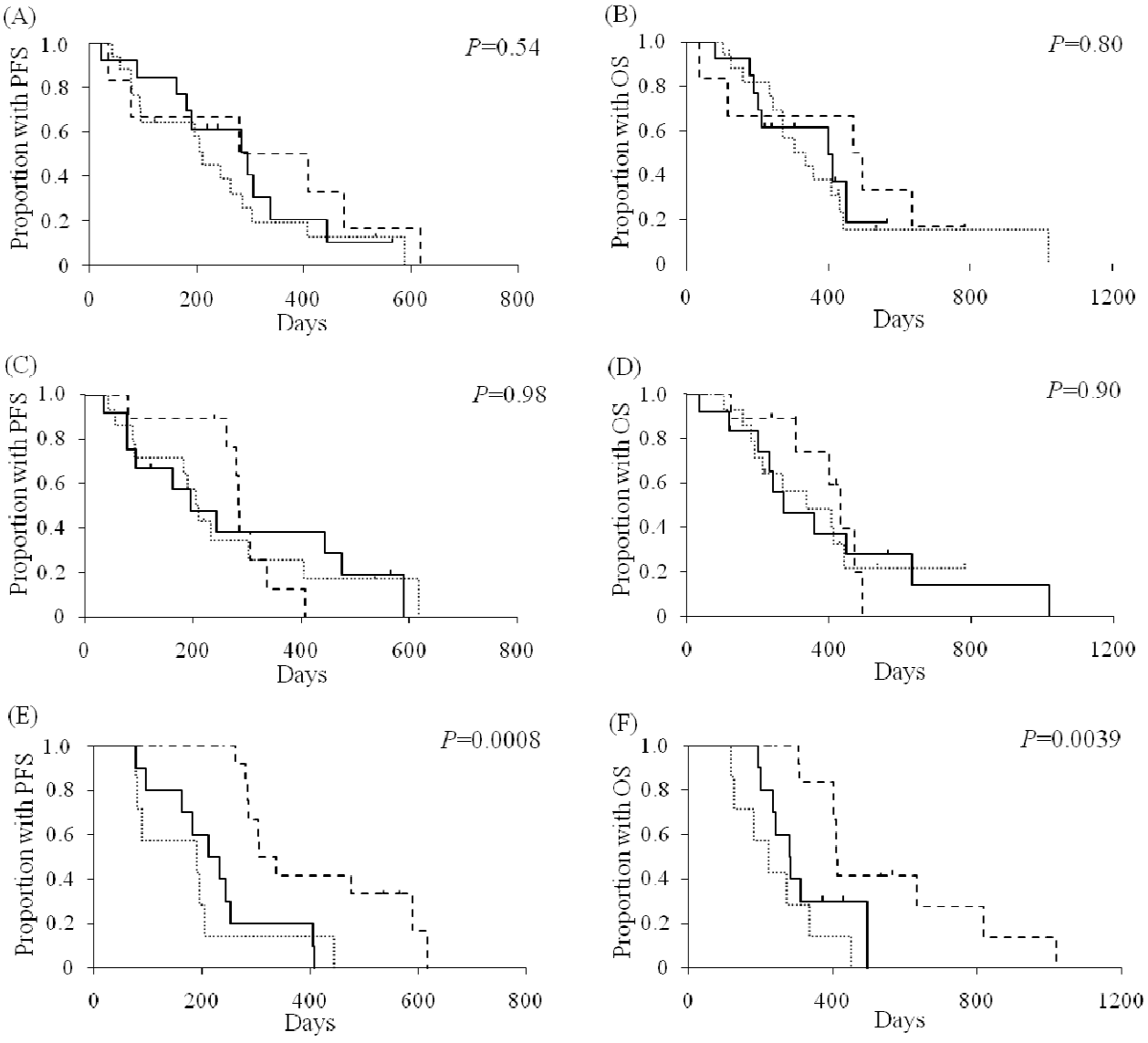

(A)
P=0.54
Proportion with PFS
1.0
0.8
0.6
0.4
0.2
0
0
200
400
600
800
Days
(B)
P=0.80
Proportion with OS
1.0
0.8
0.6
0.4
0.2
0
0
400
800
1200
Days
(C)
P=0.98
Proportion with PFS
1.0
0.8
0.6
0.4
0.2
0
0
200
400
600
800
Days
(D)
P=0.90
Proportion with OS
1.0
0.8
0.6
0.4
0.2
0
0
400
800
1200
Days
(E)
P=0.0008
Proportion with PFS
1.0
0.8
0.6
0.4
0.2
0
0
200
400
600
800
Days
(F)
P=0.0039
Proportion with OS
1.0
0.8
0.6
0.4
0.2
0
0
400
800
1200
Days

Figure 4

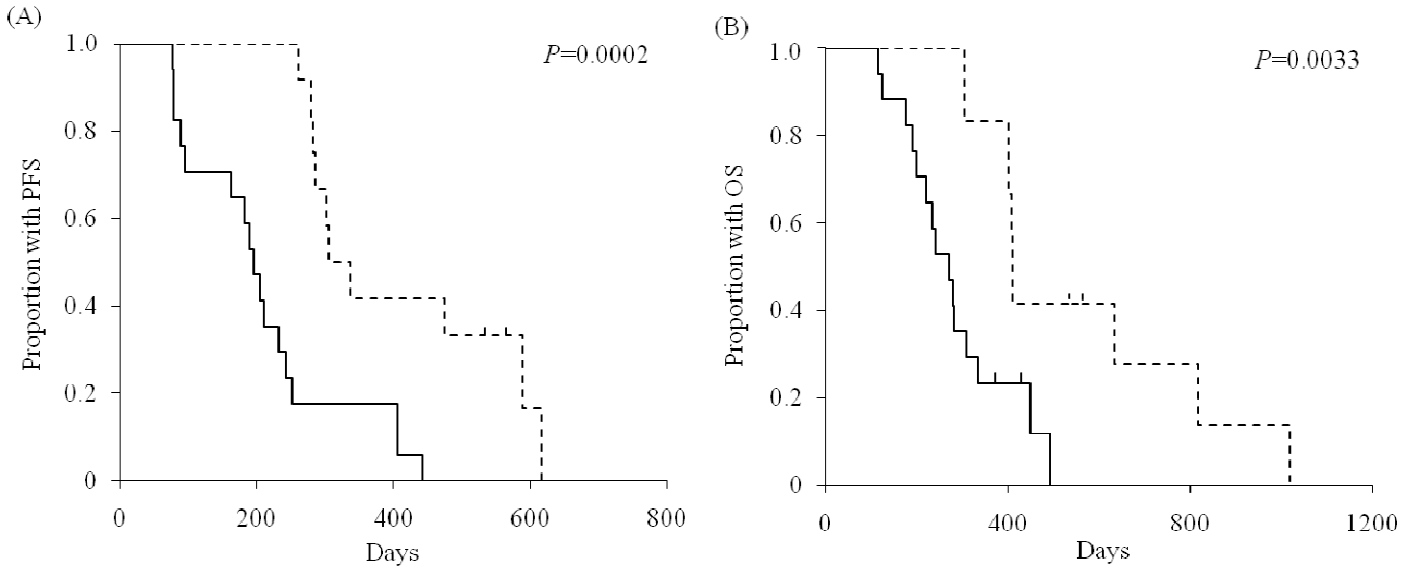

(A)
1.0
0.8
0.6
0.4
0.2
0
Proportion with PFS
P=0.0002
0
200
400
600
800
Days
(B)
1.0
0.8
0.6
0.4
0.2
0
Proportion with OS
P=0.0033
0
400
800
1200
Days

Figure 5

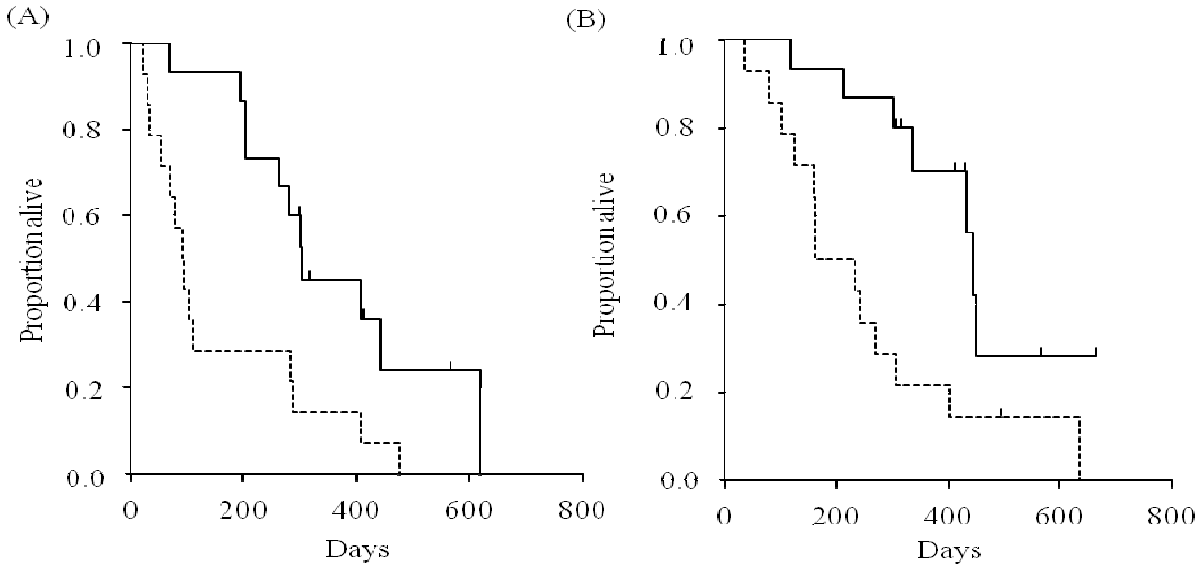
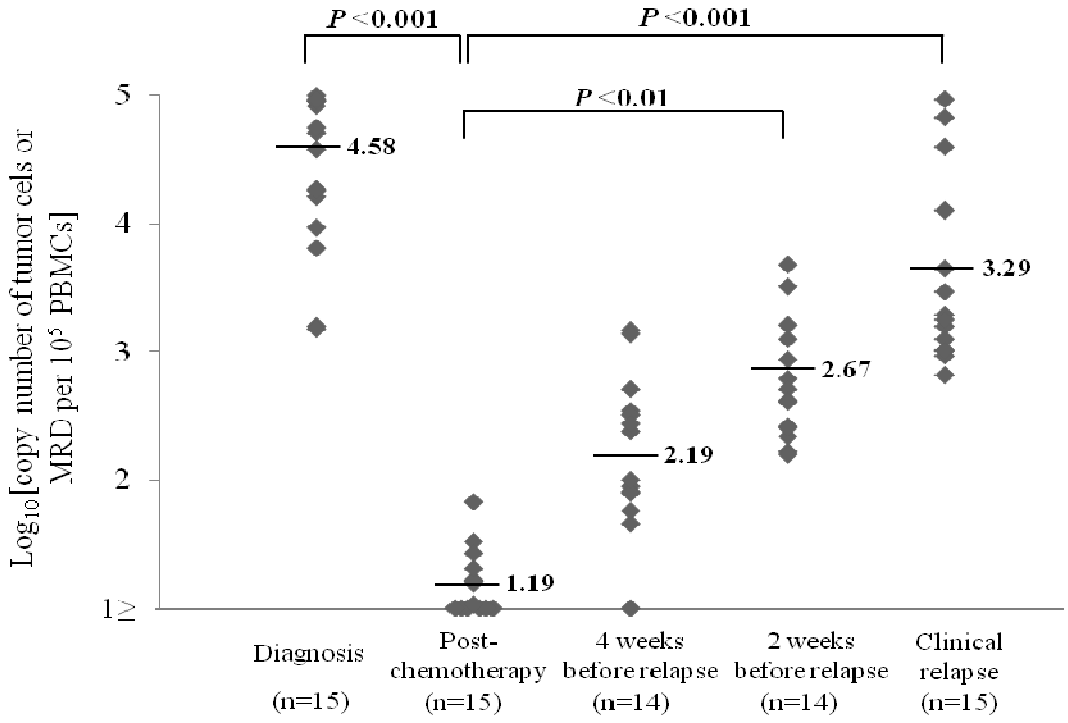

(A)
1.0
0.8
0.6
0.4
0.2
0.0
Proportion alive
0
200
400
600
800
Days
(B)
1.0
0.8
0.6
0.4
0.2
0.0
Proportion alive
0
200
400
600
800
Days

Figure 6


P <0.001
P <0.001
P <0.01
4.58
1.19
2.19
2.67
3.29
5
4
3
2
1≥
Log10[copy number of tumor cels or MRD per 10^5 PBMCs]
Diagnosis (n=15)
Post-chemotherapy (n=15)
4 weeks before relapse (n=14)
2 weeks before relapse (n=14)
Clinical relapse (n=15)