博士論文

Studies on the pathophysiological role of CD44 variant isoforms in canine lymphoma

（犬のリンパ腫における CD44 variant isoform の病態生理学的役割に関する研究）

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General Introduction

Lymphoma is one of the most common malignant tumors in dogs accounting for 7–24% of all canine tumors and approximately 83% of all hematopoietic tumors (Dobson *et al.*, 2002). Canine lymphoma has been considered as a heterogeneous disease showing different response to treatment and various outcome. To predict the outcome, various prognostic factors have been reported in canine lymphoma, including body weight (Garrett *et al.*, 2002), anatomic location (Withrow *et al.*, 2013), histopathological classification (Valli *et al.*, 2011), presence of anemia (Miller *et al.*, 2009), clinical stage (Hosoya *et al.*, 2007), clinical substage (Greenlee *et al.*, 1990; Garrett *et al.*, 2002), and immunophenotype (Greenlee *et al.*, 1990). Among various subtypes, multicentric B-cell high-grade lymphoma (diffuse large B-cell lymphoma, DLBCL) is the most common subtype in dogs (Fournel-Fleury *et al.*, 1997; Vezzali *et al.*, 2010).

Dogs with multicentric B-cell high-grade lymphoma respond favorably to anticancer drugs and combination chemotherapy is generally selected for the treatment. CHOP-based protocol (C: cyclophosphamide, H: hydroxydaunorubicin [doxorubicin], O: oncovin [vincristine], P: prednisolone) is one of the most commonly used treatments for canine high grade B-cell lymphoma (Moore *et al.*, 2001; Garrett *et al.*, 2002; MacDonald *et al.*, 2005; Simon *et al.*, 2006; Hosoya *et al.*, 2007; Burton *et al.*, 2013; Curran and Thamm, 2016), resulting in complete remission in 70–90% of dogs with a disease-free period of 9–11 months. However, relapse is observed in most of the lymphoma dogs, when the disease control becomes difficult (Flory *et al.*, 2011). As a result, the median survival times of dogs with multicentric B-cell high-grade lymphoma were reported to be 10-14 months (Garrett *et al.*, 2002; MacDonald *et al.*, 2005; Flory *et al.*, 2011; Marconato *et al.*, 2011; Zandvliet *et al.*, 2013).

The most common cause of the failure of treatment for canine lymphoma is the acquisition of drug resistance by tumor cells (Bergman *et al.*, 2003). A number of factors

associated with drug resistance have been studied in human and veterinary medicine (Bergman *et al.*, 2003; Lage, 2008). In canine lymphoma, P-glycoprotein (P-gp) (Lee *et al.*, 1996) and P53 (Dhaliwal *et al.*, 2013) were reported to be associated with drug resistance. Although P-gp was reported to enhance drug excretion from lymphoma cells (Lee *et al.*, 1996), the expression is not common in dogs suffered from drug-resistant lymphoma (Tomiyasu *et al.*, 2010). Mutated *TP53* also induces chemoresistance through the failure of apoptosis, but its mutation rate was not high in dogs with lymphoma (Koshino *et al.*, 2016). Although the drug resistance is commonly observed in dog patients with lymphoma, its molecular mechanism has not been identified in most cases.

In human studies, a number of molecules to contribute drug resistance in non-Hodgkin's lymphoma have been identified such as CDKN1A (Winter *et al.*, 2010), CD5 (Ennishi *et al.*, 2008), P53 (Sehn *et al.*, 2005), VEGFR2 (Gratzinger *et al.*, 2010), and CD44 (Stauder *et al.*, 1995). Among these molecules, CD44 is a hyaluronan-binding protein and has many physiological functions such as lymphocyte homing (Mackay *et al.*, 1988), migration (Stoolman, 1989), and cancer metastasis (Aruffo *et al.*, 1990; Ponta *et al.*, 2003). Moreover, CD44 is known to express on the cancer stem cell (CSC) (Al-Hajj *et al.*, 2003; Collins *et al.*, 2005; Dalerba *et al.*, 2007; Visvader and Lindeman, 2008). Various isoforms of CD44 generated through alternative mRNA splicing have been reported in human cells (Screaton *et al.*, 1992). The standard form of human *CD44* (*CD44s*), which consists of 10 exons, is expressed predominantly in hematopoietic cells as well as epithelial cells (Screaton *et al.*, 1992). On the other hand, variant isoforms of *CD44* (*CD44v*), which consists of 11 to 20 exons, with insertions of up to 10 exons at the membrane-proximal extracellular region, are expressed in many types of tissues such as epidermis, thyroid grand, tonsil, lymph node, and thymus in humans (Salles *et al.*, 1993; Mackay *et al.*, 1994). Physiological functions of CD44v are not well understood. Recent studies have shown that CD44v protein expression is

related to the resistance to anticancer agents in many types of human tumors including mammary gland tumor (Van Pham *et al.*, 2012), colorectal cancer (Ishimoto *et al.*, 2011), and ovarian cancer (Gao *et al.*, 2015). CD44v expression is also known to be a prognostic parameter in human non-Hodgkin's lymphoma (Stauder *et al.*, 1995) especially in DLBCL (Nagel *et al.*, 2010; Wei *et al.*, 2014).

In dogs, CD44 is also expressed in many tissues including macrophage, subsets of lymphocyte, epithelial cells, and thymus cells (Alldinger *et al.*, 1999) as well as in some tumors such as mammary gland tumor (Paltian *et al.*, 2009) and acute leukemia (Gelain *et al.*, 2014). Moreover, some reports indicated that CD44 was a marker for cancer stem cell (Ferletta *et al.*, 2011; Michishita *et al.*, 2012) and poor prognosis (Magalhaes *et al.*, 2013) in mammary gland tumor. Another study using microarray analysis indicated analysis that *CD44* mRNA expression was related to tumor pathogenesis and prognostic importance in canine B-cell lymphoma (Zamani-Ahmadmahmudi *et al.*, 2016). However, since there has been no report to distinguish CD44v from CD44s, association between CD44v expression and prognosis remains unclear in canine tumors.

From these backgrounds, for the purpose to understand the pathophysiological roles of CD44v in lymphoma, a series of studies from Chapter 1 to Chapter 3 were conducted in this thesis.

In Chapter 1, I investigated the influence of the expression level of *CD44v* on the prognosis of dogs with multicentric high-grade B-cell lymphoma. Based on the results obtained in Chapter 1, induction of drug resistance by the expression of *CD44v* in canine lymphoma cells was studied in Chapter 2. Finally, molecular mechanism for the induction of *CD44v* and prognostic role of its key molecule were investigated in canine lymphoma in Chapter 3.

Chapter 1

Influence of the expression of *CD44* variant isoforms

on the prognosis in canine multicentric high-grade B-cell lymphoma

Abstract

Expression of CD44 variant isoform (CD44v) is known to be one of the prognostic markers in human non-Hodgkin's lymphoma, especially in diffuse large B-cell lymphoma. In this study, I investigated an association of *CD44v* mRNA expression with the prognosis of canine multicentric high-grade B-cell lymphoma. Forty-five lymphoma dogs diagnosed as multicentric high-grade B-cell lymphoma were included in this study. I measured the amount of *CD44v3, CD44v6, and CD44v7* mRNAs in the lymph node FNA samples by using real-time RT-PCR and categorized into high- and low-expression groups according to the expression level of each variant isoforms. Dogs categorized into *CD44v3* ^{high}, *CD44v6* ^{high}, and *CD44v7* ^{high} groups showed worse prognosis compared with the low expression groups of corresponding isoform. In particular, overall response (OR) rate in *CD44v6* ^{high} group (9%) was significantly lower (P<0.01) than that in *CD44v6* ^{low} group (65%). Moreover, progression-free survival (PFS; median 76 days) and overall survival (OS; median 157 days) in *CD44v6* ^{high} group were significantly shorter (P<0.01) than those in *CD44v6* ^{low} group (271 days and 297 days, respectively). Similar results were obtained between *CD44v3* ^{high} and *CD44v7* ^{high} groups and *CD44v3* ^{low} and *CD44v7* ^{low} groups. Further study was considered to be needed to know how the CD44v expression influence the prognosis in canine lymphoma.

Introduction

Canine lymphoma is the most common hematopoietic malignancy in dogs. The classification of canine lymphoma utilizes anatomical form (Withrow *et al.*, 2013), cell morphology (Fournel-Fleury *et al.*, 1997), and immunophenotype (Greenlee *et al.*, 1990) to define the subtypes. Of these subtypes, multicentric high grade B-cell lymphoma is common and most of the cases initially respond to multi-agent chemotherapy (Ito *et al.*, 2014). Many of the dogs (70-90%) with multicentric high grade B-cell lymphoma can achieve complete remission and will survive 10–14 months by CHOP-based treatment (Garrett *et al.*, 2002; MacDonald *et al.*, 2005; Flory *et al.*, 2011; Marconato *et al.*, 2011; Zandvliet *et al.*, 2013). However, relapse is invariably observed in most of the lymphoma dogs, resulting in anticancer drug resistance. Moreover, a small number of cases die in the early of treatment because of the drug resistance (Marconato *et al.*, 2011). Many molecules to contribute to drug resistance have been identified such as CDKN1A (Winter *et al.*, 2010), CD5 (Ennishi *et al.*, 2008), P53 (Sehn *et al.*, 2005), VEGFR2 (Gratzinger *et al.*, 2010), and CD44 variant isoform (Stauder *et al.*, 1995) in human non-Hodgkin's lymphoma. Mutated *TP53* (Koshino *et al.*, 2016) and overexpression of P53 (Dhaliwal *et al.*, 2013) were also reported to induce chemoresistance in dogs with lymphoma, but their frequencies were not high. CD5 (Rao *et al.*, 2011) and VEGFR2 (Wolfesberger *et al.*, 2012) did not influence the disease outcome of canine lymphoma. With respect to CD44 variant isoform has no report to examine its association with the prognosis of canine lymphoma.

CD44 is expressed by a wide range of hematopoietic and non-hematopoietic cells (Gunthert *et al.*, 1991). CD44 has many physiological functions such as lymphocyte homing (Mackay *et al.*, 1988), migration (Stoolman, 1989), and cancer metastasis (Aruffo *et al.*, 1990; Ponta *et al.*, 2003) by cellular adhesion for hyaluronic acid. A variety isoforms of *CD44*

generated through alternative mRNA splicing of *CD44* precursor mRNA in humans (Screaton *et al.*, 1992) and dogs (Milde *et al.*, 1994). Whereas standard form of *CD44* (*CD44s*), which consists of 10 exons, is expressed predominantly in hematopoietic cells and epithelial cells (Screaton *et al.*, 1992), *CD44* variant isoforms (*CD44v*), which consist of 11 to 20 exons with insertions of up to 10 exons at the membrane-proximal extracellular region, are expressed in many tissues such as peripheral blood, lymph node, and thymus in humans (Salles *et al.*, 1993; Mackay *et al.*, 1994). In dogs, *CD44* was also expressed in many tissues including macrophage, lymph node, epithelial cells, spleen, bone marrow, and thymus cells (Alldinger *et al.*, 1999). In addition, thymus and lymph node were shown to express a variety *CD44v* mRNAs (up to 48 types).

Recent studies have shown that CD44v expression is related to the resistance to anticancer agents in many types of tumors including mammary tumor (Van Pham *et al.*, 2012), colorectal cancer (Ishimoto *et al.*, 2011), and ovarian cancer (Gao *et al.*, 2015) in humans. CD44v expression is also known to be a prognostic parameter in human non-Hodgkin's lymphoma (Stauder *et al.*, 1995) especially in diffuse large B-cell lymphoma (DLBCL) (Nagel *et al.*, 2010; Wei *et al.*, 2014). In dogs, one study is shown that *CD44* mRNA expression was related to tumor pathogenesis and prognostic importance in canine B-cell lymphoma (Zamani-Ahmadmahmudi *et al.*, 2016). However, since there has been no report to distinguish CD44v from CD44s, association between CD44v expression and prognosis remains unclear in canine tumors. However, since there has been no report to distinguish CD44v from CD44s, association between CD44v expression and prognosis remains unclear in canine tumors.

The purpose of this study is to detect the expression of the standard form of CD44 and its variant isoforms and to evaluate their influence on the prognosis of dog patients with multicentric high-grade B-cell lymphoma.

Materials and methods

Lymph node samples from healthy dogs and dogs with lymphoma

Lymph nodes were obtained from 10 healthy Beagles kept for experimental purposes. The procedure was conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo (Accession number P15-63).

Canine lymphoma cases from 2006 to 2016 years were referred to the Veterinary Medical Center of the University of Tokyo. Forty-five dogs diagnosed with multicentric high-grade B-cell lymphoma were included in this study. Cytology of the lymph node aspirates was evaluated according to the updated Kiel classification (Fournel-Fleury *et al.*, 1997), resulting in centroblastic type in 43 dogs and immunoblastic type in 2dogs. Five cases (3 cases of centroblastic type and 2 cases of immunoblastic type) were subjected to histopathological examination of after resection biopsy of the peripheral lymph nodes, revealing the histopathological characteristics as DLBCL in all the cases. Immunophenotype T or B cell lineage was analyzed by polymerase chain reaction for antigen receptor gene rearrangements (Burnett *et al.*, 2003; Goto-Koshino *et al.*, 2015). Clinical sub-stage was "a" in 25 dogs and "b" in 20 dogs.

Evaluation of the treatment efficacy and prognosis

All of the 45 lymphoma dogs with lymphoma were first treated with a modified CHOP-based protocol, UW-25 (Garrett *et al.*, 2002). I omitted L-asparaginase at week 1 of the original protocol because it was reported that L-asparaginase did not influence the outcome in dogs with lymphoma treated with CHOP-based chemotherapy (Valerius *et al.*, 1997; Piek *et al.*, 1999; MacDonald *et al.*, 2005).

Response to the treatment was evaluated by lymph node size according to the response evaluation criteria for peripheral nodal lymphoma v1.0 (Vail *et al.*, 2010). Progression-free survival (PFS) was defined as the time from the initiation of treatment to the first time that criteria for progressive disease (PD) were met, or the time of death from any cause. Dogs were censored in PFS analysis if they were still alive, if PD had not occurred before the end of the study, if they were euthanized by owner's wish, or if they were lost during follow-up. Overall survival time (OS) was defined as the time 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 chemotherapy, euthanized by owner's wish, or lost during follow-up.

Second line treatment after tumor relapse was a retreatment with UW-25 (without L-asparaginase). When the patient became not to respond to UW-25, they were treated with rescue protocols using L-asparaginase, LAP protocol (Saba *et al.*, 2007), DMAC protocol (Alvarez *et al.*, 2006), or nimustine (Takahashi *et al.*, 2014).

Real-time RT-PCR to quantity the amount of CD44 mRNA

Lymph node aspirate samples were stored in RNAlater (Life Technologies, Carlsbad, CA) at −80 °C immediately after collection. Total RNA was isolated using a commercial kit (illustra RNAspin, GE Healthcare, Little Chalfont, UK) and transcribed to cDNA by using ReverTra Ace ® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Genome DNA was doubly removed by DNase I in the total RNA extraction step and by gDNA remover in the cDNA synthesis step. Primers to amplify whole isoforms including a standard and variant isoforms of *CD44* (*CD44w*), variant exon 3 (*CD44v3*), variant exon 6 (*CD44v6*), or variant exon 7 (*CD44v7*) were designed by Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table 1-1). Primers for the internal control genes (*ACTB, GAPDH, TBP, and RPL32*) were synthesized as shown in

the previous reports (Peters *et al.*, 2007). Among these four control genes, TBP was selected as the most suitable reference gene in this study by geNorm software (Schlotter *et al.*, 2009). Twenty μL of real-time PCR mixture containing THUNDERBIRD SYBR qPCR Mix (TOYOBO), 100 nM of sense and reverse primers, and 25 ng of cDNA were subjected to Thermal Cycler Dice Real Time System TP800 (Takara Bio, Shiga, Japan). Cycle conditions consisted of an initial step at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s. After 40 cycles, dissociation protocol at 95°C for 60 s, at 60°C for 30 s, and 95°C for 15 s was performed to verify the occurrence of a single melting peak. The results were expressed as the threshold cycle (*Ct*), that is, the cycle number at which increasing reporter fluorescence crossed the fixed threshold baseline. Relative mRNA expression levels of a target gene in the lymph node samples from healthy dogs and those from dogs with lymphoma were calculated by $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001). The data were obtained as means of triplicated samples

Statistical analysis

Survival curves and median survival time were estimated using the Kaplan–Meier product limit method and were compared using the log-rank test. Fisher's exact test was used to compare OR rate. $P < 0.05$ was set significant. Statistical testing was performed using JMP version 11.2.0 (SAS Institute, Cary, NC, U.S.A.).

Results

Expression levels of CD44w and CD44v mRNAs in the lymph node samples from healthy dogs

To quantity the amount of mRNAs of *CD44w* and *CD44v3, CD44v6, CD44v7* in normal lymph node cells in dogs, I performed real-time RT-PCR using lymph node samples from 10 healthy dogs. Relative mRNA expression levels in normal lymph node samples when TBP was used as a reference gene were 4.83-10.7 (mean ± SD; 7.6±1.74) for *CD44w*, 0.18-1.09 (mean ± SD; 0.5±0.29) for *CD44v3*, 0.40-1.13 (mean ± SD; 0.21±0.08) for *CD44v6*, and 0.17-1.06 (mean ± SD; 0.45±0.26) for *CD44v7* (Fig. 1-1).

Expression level of CD44w and CD44v mRNAs in the lymph nodes samples from dogs with lymphoma

The mRNA expression levels of *CD44w* and each *CD44v* isoforms in lymph node samples from 45 dogs with high-grade B-cell lymphoma were measured by real-time RT-PCR. The relative expression levels in lymph node samples from dogs with lymphoma were 0.03-2.15 for *CD44v3*, 0.01-0.76 for *CD44v6*, 0.02-3.13 for *CD44v7*, and 0.47-53.45 for *CD44w* (Fig. 1-1). Although each *CD44* mRNA expression level was lower in lymphoma dogs compared to healthy dogs, no significant difference was observed between the lymph node samples from healthy dogs and those from dogs with lymphoma (*CD44v3*, P=0.10; *CD44v6*, P=0.16; *CD44v7*, P=0.58; *CD44w*, P=0.29; Fig.1-1). The dogs with lymphoma were divided into 2 groups, high and low mRNA expression groups, by setting the cut-off level of mean minus standard division (SD) the lymph node samples from healthy dogs. The lymph node samples from lymphoma dogs showing higher and lower levels than the cut-off level were grouped into high and low expression groups, respectively. With respect to the expression level of whole CD44 mRNA, 12 dogs and 33 dogs were categorized into

CD44w^{high} and *CD44w*^{low} dogs. As for *CD44v3*, there were 12 *CD44v3*^{high} dogs and 33 *CD44v3*^{low} dogs. As for *CD44v6*, there were 11 *CD44v6*^{high} dogs and 34 *CD44v6*^{low} dogs. As for *CD44v7*, there were 20 *CD44v7*^{high} dogs and 25 *CD44v7*^{low} dogs. (Supplementary Table 1-1).

Comparison of prognosis of dogs with lymphoma between groups showing high and low expression of CD44

To evaluate the relation between treatment response and the *CD44* expression level, I compared the OR rate, PFS, and OS groups showing high and low expression of whole *CD44* and each variant isoform of CD44. OR rates of the *CD44v3*^{high} groups was significantly lower compared to *CD44v3*^{low} group. OR rates of *CD44v6*^{high} groups was also significantly lower compared to *CD44v6*^{low} group but *CD44v7*^{high} group and *CD44w*^{high} were not significantly difference of OR rate (Table 1-2). Median of PFS were significantly shorter in all of the *CD44*^{high} groups compared to the corresponding *CD44*^{low} groups, respectively (Table 1-2). Median of OS were significantly shorter in of the *CD44*^{high} groups except *CD44v7*^{high} groups (Table 1-2).

No significant differences in the distribution of age, gender, WHO clinical stage, sub-stage, and the presence of anemia were observed between *CD44w*^{high} and *CD44w*^{low} group. Similar findings were observed between the high expressed groups of each isoforms (*CD44v3*, *CD44v6*, and *CD44v7*) and respective low expression groups. WHO clinical sub-stage and the presence of anemia significantly were also shown to influence the prognosis in the dogs with lymphoma (45 dogs) analyzed in this study (Table 1-3). However, I was not able to conduct multivariate analysis for the expression of *CD44* mRNA isoforms and these because of the under power of the analysis in this study.

In Kaplan-Meier analysis, PFS in the high expression groups of *CD44v3*, *CD44v6*, and *CD44v7* was shorter in the respective low expression groups of each isoform, and similar result was obtained between the high and low expression of *CD44w* (Fig 1-2). On the other hand, OS in the high expression groups of *CD44v3*, and *CD44v6* was significantly shorter in the respective low expression groups of each isoforms and similar result was obtained between the $CD44w^{high}$ and $CD44w^{low}$ groups (Fig 1-2A, B, and D). However, OS was not significantly different between $CD44v7^{high}$ and $CD44v7^{low}$ groups (Fig 1-2C).

Expression pattern of CD44v isoforms

In the forty-five lymphoma cases analyzed for *CD44* mRNA expression in this study, variable combination pattern of each CD44 isoforms were observed. The most common pattern was $CD44v3^{low}/\ v6^{low}/\ v7^{low}$ (n=25) and the second common pattern was $CD44v3^{high}/\ v6^{high}/\ v7^{high}$ (n=11). $CD44v3^{high}/\ v6^{low}/\ v7^{high}$ and $CD44v3^{low}/\ v6^{low}/\ v7^{high}$ patterns were observed in 1 and 8 dogs, respectively (Supplementary Table 1-1).

Discussion

In this study, I evaluated the mRNA expression of *CD44v3*, *CD44v6*, *CD44v7*, and *CD44w* in canine multicentric high-grade B-cell lymphoma. The median expression levels of mRNA of those *CD44* variant isoforms were lower in canine lymphoma cells compared to those in lymph node cells from healthy dogs. Similar results were reported in a previous study showing that *CD44w* expression in dogs with B-cell lymphoma was lower than that in healthy dogs (Liu *et al.*, 2015). In humans, compared to human healthy lymph node cells, the CD44w protein expression levels were shown to be lower in acute B-lymphoblastic leukemia/lymphoma, follicular lymphoma and Burkitt's lymphoma cells but equal to or higher in mantle zone lymphoma (Moller *et al.*, 1992). The population of dog patients with high-grade B-cell lymphoma analyzed in this study was heterogeneous with respect to *CD44* expression, showing both lower and higher expression levels of *CD44* in comparison to lymph node cells obtained from healthy dogs.

Because CD44 expression level has been reported to be related to prognosis in various human cancers, I investigated the relationship between *CD44* expression and prognosis in dogs with lymphoma. When the cut-off level was set at the mean minus the SD, calculated using data obtained from normal lymph node samples, the OR rate, PFS, and OS were lower in the $CD44^{high}$ group than in the $CD44^{low}$ group. In particular, the $CD44v6^{high}$ group showed the lowest OR rate and the shortest PFS and OS among the groups with high expression of the 3 *CD44* variant isoforms and whole *CD44*. In human NHL, CD44v6 protein expression is related to a shorter OS and was shown to be an independent prognostic marker (Stauder *et al.*, 1995). As in humans, in the present study, *CD44v6* expression was revealed to be a negative prognostic marker. I also revealed that high expression of *CD44v3* was related to shorter PFS and OS, although in human high-grade NHL, CD44v3 protein expression level did not

influence the prognosis (Stauder *et al.*, 1995). Further study is needed to make comparison between canine lymphoma and human NHL by the detailed histological classification of canine lymphoma's based on WHO classification system (Valli *et al.*, 2011).

CD44v6 protein expression was also reported to relate with resistance against CHOP-based chemotherapies in human DLBCL, resulting in poor prognosis (Nagel *et al.*, 2010; Wei *et al.*, 2014). The present study disclosed that expression of *CD44* isoforms, especially *CD44v3* and *CD44v6*, was shown to reduce the OR rate and shorter the PFS and OS. Expression of these molecules (*CD44v3* and *CD44v6*) are expected to induce chemoresistance to the agents for CHOP. Further study is needed to understand the role of CD44v3 and CD44v6 protein in the development of chemoresistance in canine lymphoma.

As the expression profile of CD44 variant, $CD44v3^{low}/\ v6^{low}/\ v7^{low}$ pattern was most common in dogs with lymphoma in this study, indicating that *CD44s* mRNA was a major *CD44* mRNA in lymphoma cells. Of the CD44 variant isoforms, $CD44v3^{high}/\ v6^{high}/\ v7^{high}$ pattern was the majority of variant high expression group. In a previous study, *CD44v* mRNA containing its variant exons 3, 6, and 7 was found in canine lymphoid tissue (Milde *et al.*, 1994). This *CD44v* mRNA consists variant exon 3 to 10 (*CD44v3-10*) and CD44v3-10 is common in rat (Gunthert *et al.*, 1991; Schwarzler *et al.*, 2001) and human (Koopman *et al.*, 1990; Jackson *et al.*, 1992). $CD44v3^{high}/\ v6^{high}/\ v7^{high}$ samples may indicate *CD44v3-10* mRNA is increased in tumor cells. $CD44v3^{high}/\ v6^{high}/\ v7^{high}$ samples may indicate *CD44v3-10* mRNA is more expressed in tumor cells. There were a small number of lymphoma cell samples with their CD44 variant isoform expression profiles, $CD44v3^{high}/\ v6^{low}/\ v7^{high}$ and $CD44v3^{low}/\ v6^{low}/\ v7^{high}$, in this study. A previous study (Milde *et al.*, 1994) showed the presence of various CD44 variant isoforms containing a variant exon 7 as well as both of variant exons 3 and 7 in normal dog lymph nodes. It is conceivable that these CD44 variant isoforms are generated in normal lymphoid tissues and increased in lymphoma tissues,

influencing the biological behaviors such as worse prognosis and drug resistance.

The mechanism of drug resistance induced by *CD44v* in tumor cells has not been elucidated in human. However, recent reports revealed that CD44v6 activated Akt pathway and induced inhibition of apoptosis (Jung *et al.*, 2011; Garouniatis *et al.*, 2013) and CD44v3 exerted interaction with Oct4, Sox2, and Nanog, resulting in cisplatin resistance (Bourguignon *et al.*, 2012). Development of canine lymphoma cell model with high *CD44v* expression would be also provide an useful animal model to analyze the relation between CD44v expression and drug resistance.

In conclusion, several type of *CD44* variant isoforms were expressed in canine multicentric high-grade B-cell lymphoma cells. High expression level of *CD44v3, CD44v6*, and *CD44v7* was related to poor prognosis in dogs with lymphoma.

Table 1-1

Primer pairs used for real-time RT-PCR.

Gene	Accession number	Forward primer (5'- 3')	Position	Reverse primer (5'- 3')	Position
CD44s	NM_001197022	CGCTCCTGGCCTTGGCTTTGATT	1020-1042	CCCCACTGCTCCATTGCCATTGTT	1106-1129
CD44v3	L28932	CAAGTATCATCTCAGCAGGC	260-279	GCTGGAGATAAAATCTTCATCATC	349-372
CD44v6	L28932	GCAGTGGGTTGAGAATGGAT	657-676	AGCTGTCCCTGCTGTTGAAT	716-735
CD44v7	L28932	CAAGACAGCCATCCAGATCA	745-764	TTGGATGTGAGATTGGGTCA	813-832
TBP[*]	XM849432	CTATTTCTTGGTGTGCATGAGG	1331-1352	CCTCGGCATTCAGTCTTTTC	1407-1426

* Primer sequences were reported prviously (Peters *et al.*, 2007)

Table 1-2

Comparison of overall response (OR) rate, median of progression-free survival (PFS), and median of overall survival (OS) between high and low expression groups each *CD44* variant isoforms mRNA and whole *CD44* mRNA.

Group	OR rate		Median PFS (days)		Median OS (days)	
CD44v3^{high}	17% (2/12)		76		157	
		P<0.01		*P*<0.01		*P*=0.01
CD44v3^{low}	64% (21/33)		271		297	
CD44v6^{high}	9% (1/11)		76		157	
		P<0.01		*P*<0.01		*P*<0.01
CD44v6^{low}	65% (22/34)		271		297	
CD44v7^{high}	35% (7/20)		98		157	
		P=0.07		*P*<0.01		*P*=0.06
CD44v7^{low}	64% (16/25)		275		228	
CD44w^{high}	25% (3/12)		76		157	
		P=0.05		*P*=0.04		*P*=0.01
CD44w^{low}	61% (20/33)		271		297	

Table 1-3

Evalutation of prognositc factors in 45 dogs with lymphoma analyzed in this study.

Prognostic factor (n)		Median PFS (days)		Median OS (days)	
Age					
	<7 yr (7)	164		204	
			P=0.25		*P*=0.24
	≥7 yr (38)	271		477	
Gender					
	Male (22)	139		204	
			P=0.07		*P*=0.17
	Female (23)	286		235	
Body weight					
	<18 kg (36)	76		159	
			P=0.40		*P*=0.08
	≥18 kg (9)	265		297	
WHO clinical stage					
	I-IV (22)	164		228	
			P=0.77		*P*=0.31
	V (23)	197		204	
WHO clinical sub-stage					
	a (25)	271		337	
			P=0.02		*P*<0.01
	b (20)	104		197	
Anemia					
	PCV<35% (12)	129		197	
			P=0.03		*P*=0.04
	PCV≥35% (33)	228		271	

Fig.1-1.

Expression levels of (A) *CD44w*, (B) *CD44v3*, (C) *CD44v6*, and (D) *CD44v7* mRNA examined in 10 normal canine lymph node samples (Healthy) and 45 multicentric high-grade B-cell lymphomas samples (Lymphoma). Scale indicates mean ± SD range in normal canine lymph node samples.

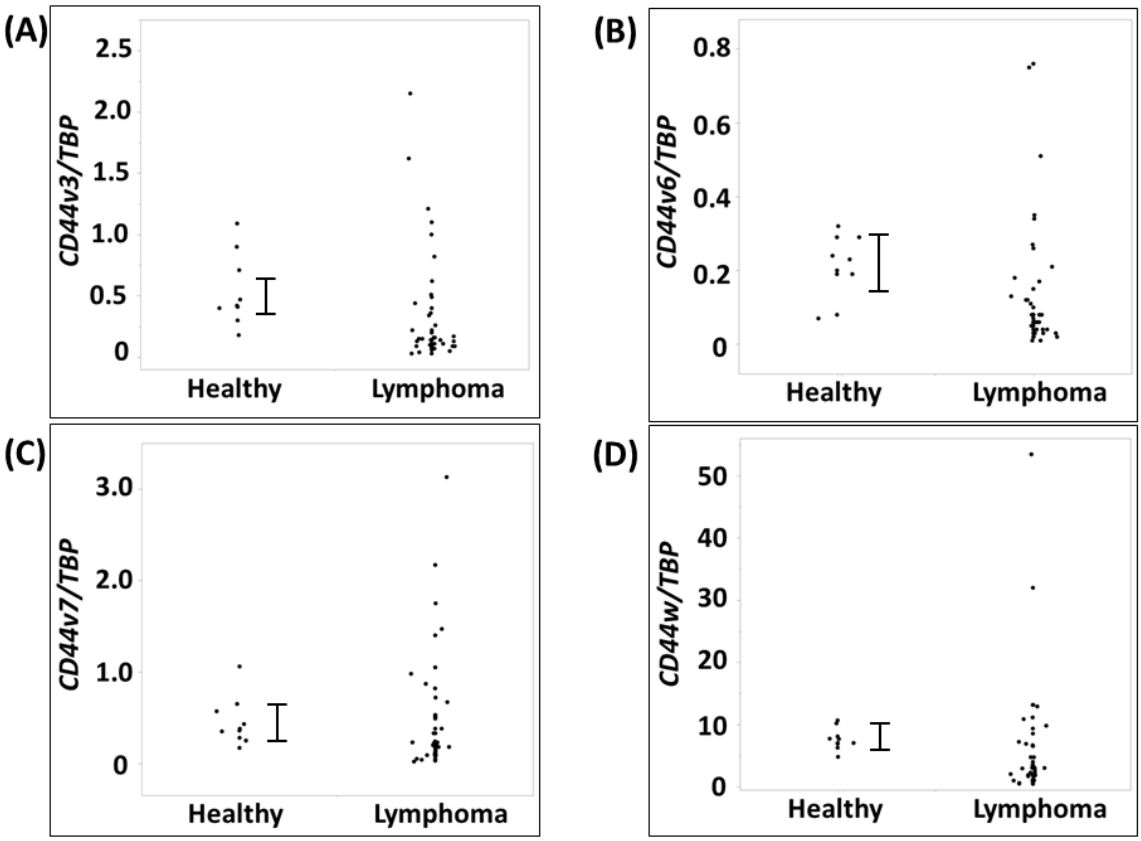
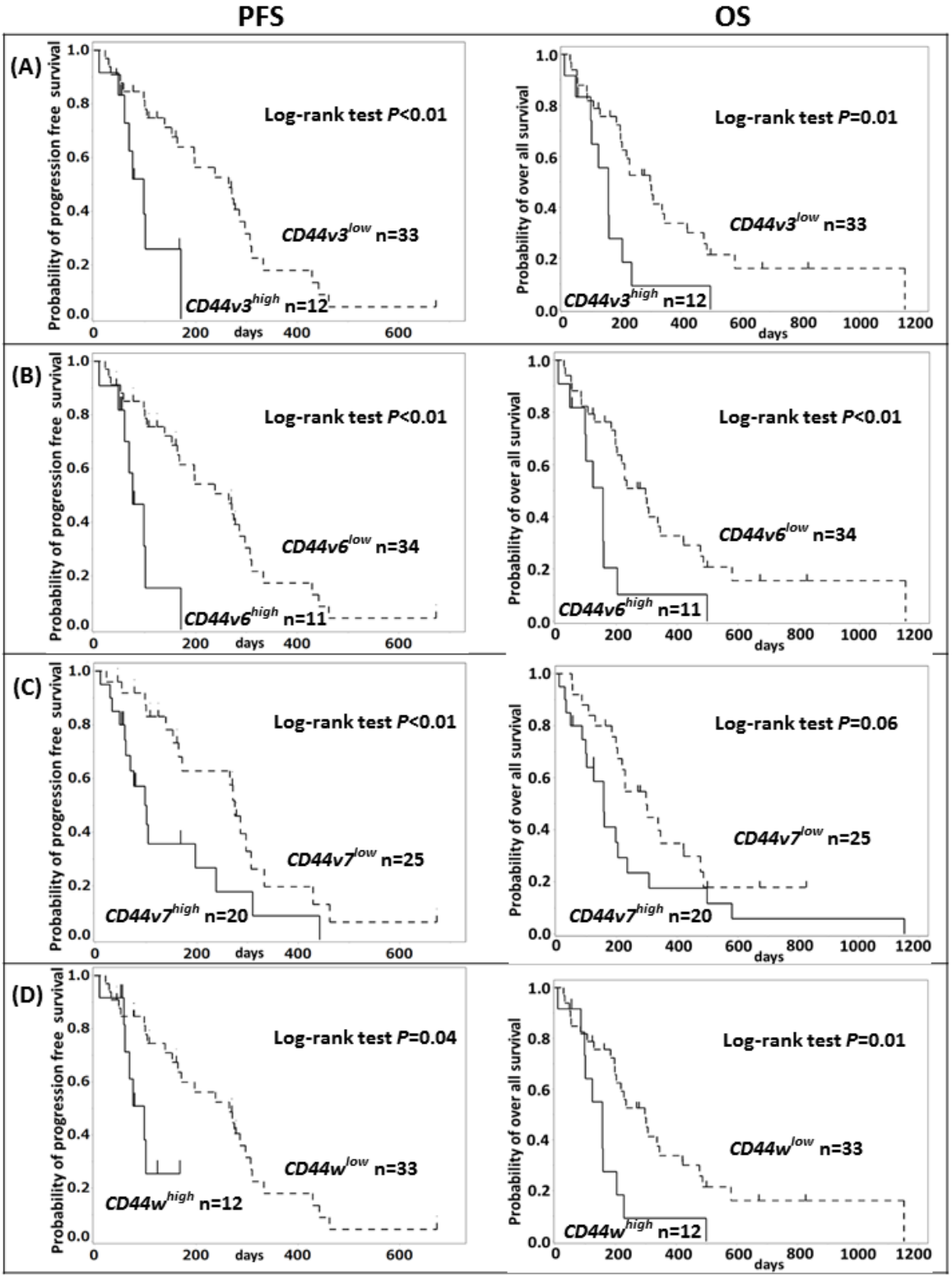


Fig. 1-2.

Kaplan–Meier curves of progression-free survival (PFS, left) and overall survival (OS, right) for lymphoma dogs with high (solid line) and low (dashed line) mRNA expression groups of (A) *CD44v3*, (B) *CD44v6*, (C) *CD44v7*, and (D) *CD44w*.



Supplementary Table 1-1

Individual data for all dogs with high-grade B-cell lymphoma (n = 45) in the present study

Case	Breed	Sex	Age (years old)	Body weight (kg)	WHO clinical stage	WHO clinical sub-stage	*CD44v3* expression	*CD44v6* expression	*CD44v7* expression	*CD44w* expression	PFS (days)	OS (days)
1	Labrador retriever	S	5y5m	34.5	V	a	L	L	L	L	108	163
2	Shih Tzu	F	8y1m	4.4	V	b	H	L	H	L	78	235
3	Miniature dachshund	M	12y1m	8.7	V	a	H	H	H	H	69	124
4	Welsh corgi	M	6y8m	11.3	III	b	L	L	H	L	310	581
5	Welsh corgi	M	8y7m	12.0	V	a	L	L	H	L	238	307
6	Jack Russell terrier	S	13y3m	6.0	V	a	L	L	L	L	77	85
7	Maltese	S	10y2m	3.9	II	a	L	L	L	L	286	827
8	Pomeranian	S	12y4m	5.9	V	b	L	L	H	L	29	29
9	Welsh corgi	S	6y7m	10.8	IV	a	L	L	L	L	673	673
10	Welsh corgi	M	12y11m	15.9	IV	a	L	L	L	L	164	184
11	Miniature Schnauzer	F	10y11m	7.4	V	a	L	L	L	L	270	270
12	French bulldog	M	2y9m	10.3	IV	a	L	L	L	L	271	477
13	Doberman pinscher	M	8y8m	25.7	IV	a	H	H	H	H	76	159
14	Shih Tzu	M	9y10m	4.4	V	a	H	H	H	H	98	157
15	French bulldog	S	4y4m	12.8	IV	a	L	L	L	L	265	345
16	Miniature dachshund	S	12y3m	4.3	IV	a	H	H	H	H	60	157
17	Golden retriever	S	9y5m	35.0	III	b	L	L	L	L	52	52
18	Welsh corgi	F	12y11m	9.6	III	b	L	L	L	L	161	203
19	Miniature dachshund	M	8y11m	4.2	IV	b	L	L	L	H	124	228
20	Welsh corgi	F	9y3m	16.3	IV	a	L	L	L	L	333	487
21	Beagle	M	13y4m	14.5	V	b	L	L	L	L	297	297
22	Border collie	S	9y0m	14.9	V	a	L	L	L	L	307	421

23	Mix	M	9y7m	13.6	III	a	L	L	H	L	442	1151
24	West Highland White Terrier	M	9y6m	7.1	V	b	L	L	L	L	100	218
25	Shih Tzu	F	12y0m	3.5	V	b	L	L	L	L	198	198
26	Miniature dachshund	M	10y8m	3.3	V	b	L	L	H	L	104	124
27	Golden retriever	M	12y6m	31.3	V	b	L	L	H	L	197	197
28	American cocker spaniel	F	9y4m	13.8	V	a	L	L	L	L	462	500
29	Beagle	S	12y0m	9.6	IV	a	H	H	H	H	24	499
30	Yorkshire terrier	F	10y8m	6.2	II	a	L	L	L	L	153	228
31	Golden retriever	M	11y11m	40.0	V	b	H	H	H	L	48	48
32	Mix	M	14y1m	24.0	V	a	H	H	H	H	52	204
33	American Eskimo dog	M	10y4m	15.4	IV	a	L	L	L	L	275	337
34	Shih Tzu	M	11y8m	6.2	V	a	H	H	H	H	10	10
35	Labrador retriever	M	12y7m	25.4	IV	b	L	L	H	L	33	33
36	Shih Tzu	S	12y3m	6.5	V	b	H	H	H	H	79	98
37	Toy poodle	C	6y11m	7.6	IV	b	H	H	H	H	55	55
38	Beagle	C	8y0m	17.2	IV	b	L	L	H	H	58	86
39	Doberman pinscher	S	10y10m	30.5	IV	b	L	L	L	L	22	54
40	Pug	C	3y2m	11.6	V	b	L	L	L	L	98	107
41	Golden retriever	F	2y7m	30.3	V	b	L	L	L	L	44	129
42	Dandie Dinmont terrier	S	9y8m	10.0	III	a	H	H	H	H	101	101
43	Mix	C	10y3m	5.0	IV	b	L	L	L	L	139	301
44	Welsh corgi	F	9y8m	14.2	V	a	L	L	L	L	422	422
45	Shih Tzu	S	12y5m	8.0	V	a	L	L	L	L	278	278

Sex: C, castrated; M, male, S, spayed; F, Female

CD44w v3, v6, v7 expression: L, low expression level; H, high expression level

Chapter 2

Characterization of *CD44* variant isoforms in dogs and their association with drug resistance in canine lymphoma

Abstract

Expression of *CD44* variant isoforms (*CD44v*) was shown to be a prognostic marker in canine lymphoma in Chapter 1. However, association between the CD44v expression and clinical outcome was not well understood in canine lymphoma. In this study, full-length cDNA sequences of *CD44* variant isoforms in canine lymphoma cells were obtained and their association with drug resistance in canine lymphoma was explored. Lymph node samples from four dogs with multicentric high-grade B-cell lymphoma showing high expression levels of *CD44* variant exons 3, 6, and 7 were used. I detected the full-length cDNA sequencing *CD44v* in lymph node samples using *CD44* variant exon-specific primers. Eight types of *CD44v* mRNA were obtained and *CD44v3-5, 7*, *CD44v3-5*, and *CD44v6* were frequently observed. I generated *CD44v3-5, 7*- and *CD44v6*-overexpressing cells using a retroviral vector expression system in the canine lymphoid cell lines, CLBL-1 and CL-1. Sensitivity to antineoplastic agents, vincristine and doxorubicin, was significantly decreased by *CD44v6* expression but not by *CD44v3-5, 7* expression. Moreover, I evaluated Akt signalling proteins using HGF because CD44v6 activated the Akt pathway via HGF stimulation in human tumors. *CD44v6* transduction in cells increased p-PDK1 and p-Akt protein levels via HGF stimulation. Viability of LY294002 (Akt inhibitor) treated cells was significantly decreased compared to that of untreated cells. Studies in this Chapter disclosed the presence of 8 types of *CD44v* in canine lymphoma cells and a common form of *CD44v*, *CD44v6*, was found to induce drug resistance via activating of Akt signaling.

Introduction

CD44 is a hyaluronan-binding protein and has many physiological functions such as lymphocyte homing (Mackay *et al.*, 1988), migration (Stoolman, 1989), and cancer metastasis (Aruffo *et al.*, 1990; Ponta *et al.*, 2003). Numerous isoforms of *CD44* are generated through alternative mRNA splicing. Standard form of *CD44* (*CD44s*) is expressed predominantly in hematopoietic cells and epithelial cells (Screaton *et al.*, 1992). On the other hand, variant isoforms (*CD44v*), which consist of 11 to 20 exons with insertions of up to 10 exons at the membrane-proximal extracellular region, are expressed in many tissues or organs including epidermis, thyroid gland, tonsil, lymph node, and thymus (Salles *et al.*, 1993; Mackay *et al.*, 1994). In dogs, *CD44* was also expressed in many tissues including macrophage, lymph node, epithelial cells, spleen, bone marrow, and thymus cells (Alldinger *et al.*, 1999). In addition, thymus and lymph node were shown to express a variety *CD44v* mRNAs (up to 48 types).

The physiological function of CD44v has not been well understood, but several function of some variants are known. The CD44v3 contain some specific post-translational modifications that include a heparan sulphate site, which binds heparin-binding proteins such as FGF2 (Ruiz *et al.*, 1995). CD44v6 expressing cells activated the Akt pathway by HGF stimulation (Jung *et al.*, 2011; Ghatak *et al.*, 2014). CD44v8-10 interacts with and stabilizes SLC7A11, and thereby promotes cystine uptake for GSH synthesis. Then, CD44v8-10 contributes to ROS defense through upregulation of the synthesis of reduced glutathione, the primary intracellular antioxidant (Ishimoto *et al.*, 2011).

CD44v are also expressed in many types of tumors in human including head and neck squamous cell carcinoma (Herold-Mende *et al.*, 1996), colorectal cancer (Yamaguchi *et al.*, 1998), and breast cancer (Iida and Bourguignon, 1995). Recent studies have shown that CD44v protein expression is related to an anticancer agent resistance in these human tumors

including head and neck squamous cell carcinoma (Wang *et al.*, 2009), colorectal cancer (Ishimoto *et al.*, 2011), and ovarian cancer (Tjhay *et al.*, 2015). In dogs, CD44 protein was shown to express in tumor cells of mammary gland tumor (Paltian *et al.*, 2009) and acute leukemia (Gelain *et al.*, 2014). Moreover, some reports indicated that CD44 was a cancer stem cell marker (Ferletta *et al.*, 2011; Michishita *et al.*, 2012) and a poor prognostic marker in canine mammary gland tumor (Magalhaes *et al.*, 2013). Another study using microarray analysis indicated that *CD44* mRNA expression was related to tumor pathogenesis and prognostic importance in canine B-cell lymphoma (Zamani-Ahmadmahmudi *et al.*, 2016). However, in these studies, there was not discrimination between *CD44s* and *CD44v*.

In Chapter 1, I revealed high expression level of *CD44v3* and *CD44v6* mRNA was a poor prognostic parameter in canine multicentric high-grade B-cell lymphoma. In both of human and canine lymphomas, expression of *CD44v* were shown to reduce the overall response (OR) rate and shorten the progression-free survival (PFS). However, the relation between the *CD44v* expression and prognosis of lymphoma was not clear.

The purpose of the study in this Chapter was to reveal the mRNA sequence of full-length *CD44* variant isoforms and evaluate their function in canine lymphoma cells.

Materials and methods

Lymph node samples from lymphoma-affected dogs and cell cultures

Canine lymphoma cases were referred to the Veterinary Medical Center of the University of Tokyo. Lymph node samples from four dogs diagnosed with multicentric high-grade B-cell lymphoma showing high expression levels of *CD44v3*, *CD44v6*, and *CD44v7* mRNA were used.

Canine lymphoma cell lines, CLBL-1 which is lymphoma of B-cell origin (Rutgen *et al.*, 2010) and CL-1 which is lymphoma of T-cell origin (Momoi *et al.*, 1997) were used in this study. These cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum under 5% CO_2 and 100% humidity at 37°C.

Detection of CD44v exons in lymph node samples

Total RNA was isolated using a commercial kit (Illustra RNAspin, GE Healthcare UK Ltd., Little Chalfont, UK), and transcribed to cDNA by using ReverTra Ace ® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Primers to amplify all isoforms including a standard form and variant isoforms of *CD44* (*CD44w*) and specific CD44 variant exons were designed by Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table 2-1). First, 20 μL of PCR mixture containing PrimeSTAR HS premix (Takara Bio, Shiga, Japan), 200 nM of *CD44w* primers, and 25 ng of cDNA was subjected to Thermal Cycler Dice (Takara Bio). Second, PCR was carried out using 2 μL of the initial PCR products and each exon specific primer. Cycle conditions consisted of an initial step at 98°C for 60 s, followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 5 s, and extension at 72°C for 150 s. These PCR products were

purified by agarose gel electrophoresis, inserted into a TA cloning vector (pGEM-T Easy) (Promega Corporation, Leiden, The Netherlands), and subjected to sequence analysis.

Generation of CD44v3-5, 7 and CD44v6 expressing cell lines

To generate the CD44v expression vector, CD44s, nested PCR product and pQCXIN Retroviral Vector (Takara Bio) were annealed using In-Fusion HD Cloning Kit (Takara Bio). All of the constructs were verified by sequence analysis. Twenty μg of pQCXIN-CD44vx and pVSV-G were cotransfected in GP2-293 cell lines to generate retrovirus using the cationic lipid method (Lipofectamine 3000, Thermo Fisher Scientific, Waltham, MA). The culture supernatants containing retrovirus were harvested 72 h after cotransfection and filtrated through a 0.45 μm-pore size filter.

CLBL-1 and CL-1 cells were inoculated with pQCXIN-CD44vx retrovirus and 8 μg/ml polybrene. After incubation for 2 h at 37°C, the cells were washed in RPMI 1640 and cultured for 48 h. The cells were selected using 700 μg/ml G418 reagent (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 14 days.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. The cells were treated for 48 h with doxorubicin (DXR) or vincristine (VCR). The cells were incubated with 10 μl of WST-8 for 3 h. The absorbance of the coloured formazan product that was produced by mitochondrial dehydrogenases in metabolically active cells was recorded at 450 nm as the background value. Cell proliferation was expressed as the percentage of absorbance obtained in the treated wells relative to that in the untreated control wells.

LY249002 (Cell Signaling Technology) was used to inhibit Akt/PI3k signalling. Concentration of LY249002 used in CLBL-1 and CL-1 cells were 1.5 and 10 μg/ml, respectively.

Western blotting for proteins related to the Akt pathway

To compare the activation of the Akt pathway after HGF stimulation between pQCXIN- and pQCXIN-CD44v6-transfected cells, Western blot analysis was carried out. After starving in RPMI 1640 without fetal bovine serum for 3 h, the cells were treated with 5 ng/ml HGF for 15 min. After washing with PBS, whole cell lysates were extracted from each cell using RIPA buffer with protease inhibitor cocktail (Protease and Phosphatase Inhibitor Cocktail, EDTA-free x100 (Thermo Fisher Scientific). Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific), and extracted proteins were separated by SDS-PAGE using 12.5% polyacrylamide gel and blotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P membrane; Millipore, Billerica, MA). Membranes were blocked in 1% skimmed milk /tris-buffered saline with Tween20, and then incubated with primary antibodies against CD44 whole variant isoform (CD44w, diluted at 1:2000) (IM7 clone, Becton, Dickinson and Company), p-PDK1 (1:1000), pan Akt (1:1000), p-Akt ser 437 (1:1000), and β-actin (1:1000) (Cell Signaling Technology) overnight at 4°C. After incubation with the HRP-labelled anti-rabbit IgG (1:2000, Bio-Rad Laboratories) or HRP-labelled anti-rat IgG (1:2000, Becton, Dickinson and Company) for 1 h at room temperature, positive immunoreactivity was detected using Luminata Forte Western HRP Substrate (Millipore) and visualized using ChemiDoc XRS Plus (Bio-Rad Laboratories).

Statistical analysis

One-way ANOVA followed and the Dunnett test was performed for the cell proliferation assay. $P < 0.05$ was considered significant. Statistical testing was performed using JMP version 11.2.0 (SAS Institute).

Results

Expression pattern of CD44v mRNA in lymphoma-affected canine lymph node samples

To determine the sequence of *CD44* variant isoforms in lymphoma cells, I performed nested PCR using four lymph node samples from dogs with multicentric high grade B-cell lymphoma. I obtained eight types of *CD44v* mRNA (Table 2-2). Transcripts containing *CD44* variant exon 3 were commonly observed. Three types of *CD44v* isoform mRNAs containing variant exons 3, 4, 5, and 7 (*CD44v3-5, 7*), exons 3, 4, and 5 (*CD44v3-5*), and exon 6 (*CD44v6*) were observed in all cases.

Sensitivity to doxorubicin (DXR) and vincristine (VCR) in canine lymphoma cell lines transduced with CD44v3-5, 7 and CD44v6.

To evaluate the influence of the expression of *CD44* variant isoforms on the sensitivity of anticancer drugs, I examined sensitivity to DXR and VCR in canine lymphoma cell lines which were transduced with *CD44v3-5, 7* or *CD44v6*. The expression levels of *CD44v3-5, 7* and *CD44v6* were examined by RT-qPCR and Western blotting in CLBL-1 and CL-1. Relative mRNA expression level of *CD44v* exon was increased in both cell lines. Further, the expression of *CD44v* protein were evaluated by Western blotting. Whereas CD44v protein was observed as a 100-120 kDa band in *CD44v3-5, 7*-transduced cells, CD44v6 protein were observed as 80-100 kDa broad band slightly larger than CD44s protein (Fig. 2-2).

Sensitivity of CLBL-1 and CL-1 to DXR and VCR was significantly decreased by *CD44v6* expression when compared to mock transfected cells, but did not significantly change by *CD44v3-5, 7* expression (Fig. 2-1). The 50% inhibitory concentration of cell viability (IC_{50}) values for DXR and VCR were also increased by CD44v6 transduction. The

IC_{50} values in *CD44v6* transduced cells were approximately twice as high as mock transfected cells for each drugs (Table 2-3).

Akt pathway signalling in CD44v6 overexpressed cell lines

To reveal the change of Akt pathway signalling in *CD44v6*-overexpressed cells, I evaluated the expression level of proteins related to the Akt pathway by Western blotting after HGF stimulation. The amounts of p-PDK1 and p-Akt proteins increased in *CD44v6*-overexpressed CLBL-1 and CL-1 cells when compared to mock transfected cells, indicating the activation of the Akt pathway in these cells (Fig. 2-2).

Effect of Akt inhibition on drug sensitivity of CD44v6 overexpressed cells

To evaluate the relationship between activation of the Akt pathway and drug resistance in *CD44v6*-overexpressed cells, I examined their sensitivity to DXR and VCR with or without treatment of an Akt inhibitor, LY294002. Viability of the cells treated with LY294002 was significantly decreased compared to untreated *CD44v6*-overexpressed cells (Table 2-3). The drug sensitivity in *CD44v6*-overexpressed cell lines was recovered to that of mock transfected cells by the treatment with LY294002 (Fig. 2-3).

Discussion

In this study, I evaluated the expression pattern of CD44 variant exons in dogs with multicentric high-grade B-cell lymphoma. The common variant isoforms were *CD44v3-5, 7*, *CD44v3-5*, and *CD44v6*. The results were consistent with the results in Chapter 1 showing that expression of *CD44* variant exons 3 and 7 was often accompanied by its variant exon 6 in canine B-cell lymphoma. Seven of eight variant isoforms identified in this study were reported previously in normal canine lymphoid tissues (Milde *et al.*, 1994); however, *CD44v3, 9-10* mRNA was found as a new variant from lymphoma tissues in this study. Although this variant was not common in lymphoma tissues, *CD44v3, 9-10* might have a particular function in lymphoma cells, because CD44 variant exon 9 provided anticancer drug resistance, which prevents the generation of reactive oxygen species in human colorectal cancer (Ishimoto *et al.*, 2011).

Since *CD44v3-5, 7* and *CD44v6* mRNAs were common in canine lymphoma samples, I investigated the relationship between CD44v expression and anticancer drug sensitivity. Sensitivities to DXR and VCR were significantly decreased in *CD44v6*-overexpressed cells. Previous studies reported that CD44v6 expression was related to resistance against CHOP-based treatment in human DLBCL resulting in poor prognosis (Nagel *et al.*, 2010; Wei *et al.*, 2014). The results obtained in Chapter 1 in this thesis also suggested that a high expression level of *CD44v6* mRNA was a prognostic marker in canine multicentric high-grade B-cell lymphoma. This study indicates that similarly to humans CD44v6 expression in canine lymphoma is related to poor prognosis by inducing DXR and VCR resistance.

On the other hand, sensitivity to these chemotherapeutic agents did not significantly change in *CD44v3-5, 7*-overexpressed cells in this study. CD44v3 expression levels did not correlate with OS in human non-Hodgkin's (Stauder *et al.*, 1995), but the study carried out in

Chapter 1 in this study revealed that a high expression level of *CD44v3* mRNA was a poor prognostic marker in lymphoma dogs. It could be suggested that expression of *CD44v3-5, 7* mRNA did not induce drug resistance, but might play other roles associated with a poor prognosis for canine multicentric high-grade B-cell lymphoma. In human pancreatic tumors, CD44v6 and CD44v9 expressions were associated with the progression of pathological stages and CD44v2 was associated with vascular invasion (Li *et al.*, 2014). Therefore, further study is warranted to know the mechanism to be association with the poor prognosis in canine lymphoma by the expression of CD44v3-5, 7.

The mechanism of drug resistance induced by *CD44v6* was not clear before starting the present study. However, some reports indicated that CD44v6 formed coreceptors with Met, ITGα6β4, EGFR, and VEGFR in other tumors (Jung *et al.*, 2011; Garouniatis *et al.*, 2013). This complexes enhanced the activity of Akt signalling through HGF stimulation, resulting in the escape from apoptosis. Results in this study indicated that the activation of the Akt pathway using HGF was enhanced by *CD44v6* induction in canine lymphoid cell lines. Moreover, the sensitivity to DXR and VCR was recovered by the treatment with an Akt/PI3k inhibitor, LY249002. It might be possible that CD44v6 also formed a coreceptor with above mentioned molecules and induced the activation of Akt signalling in canine lymphoma. Colocalization of CD44v6 with Met, ITGα6β4, EGFR, and VEGFR should be evaluated in *CD44v6*-overexpressed cells. Furthermore, inhibition of the Akt pathway might be a new strategy of treatment in canine lymphoma with *CD44v6* expression. Acalabrutinib, a BTK inhibitor repressing p-Akt and p-ERK, was reported to inhibit proliferation in a subset of canine DLBCL (Harrington *et al.*, 2016). Dogs affected with lymphoma showing high *CD44v6* expression might be useful as a spontaneous tumor animal model for the treatment with acalabrutinib.

In conclusion, various types of *CD44* variant isoforms were shown to express in canine multicentric high-grade B-cell lymphoma. Cells showing *CD44v6* overexpression developed doxorubicin and vincristine resistance conceivably through the activation of Akt signalling.

Table 2-1

Primer pairs used for nested PCR.

Target Gene	Accession number	Forward primer (Position)	Reverse primer (Position)
CD44w	NM_001197022	5’-CTCGCACCATGGACAAGTT- 3’ (exon1)	5’-TGCCATTTCTCTCCAAGGTC- 3’ (exon20)
CD44v3	L28932	5’-ATACCCCCATTACCAGTACGGATTC- 3’ (exon5 and v3 fusion)	
CD44v4	L28932	5’-ATACCCCCATTACCATTCCAACCACAC- 3’ (exon5 and v4 fusion)	
CD44v5	L28932	5’-ATACCCCCATTACCAGATGTGGAC- 3’ (exon5 and v5 fusion)	
CD44v6	L28932	5’-ATACCCCCATTACCAACCGAGG- 3’ (exon5 and v6 fusion)	
CD44v7	L28932	5’-ATACCCCCATTACCACCACAGCCCAA- 3’ (exon5 and v7 fusion)	
CD44v8	L28932	5’-ATACCCCCATTACCAGATATGGACTCCA- 3’ (exon5 and v8 fusion)	
CD44v10	L28932	5’-ATACCCCCATTACCAAATAGAACTGATG- 3’ (exon5 and v10 fusion)	
CD44e19	NM_001197022		5’-CCCACTGCTCCATTGCCATTGTT- 3’ (exon19)

Table 2-2

Expression patterns of *CD44v* mRNA

CD44v name	Variant exon	*3*	4	*5*	6	*7*	*8*	9	*10*	number of cases
CD44v3		●								1/4
CD44v3-5, 7		●	●	●		●				4/4
CD44v3-5		●	●	●						4/4
CD44v3-4		●	●							1/4
CD44v3, 9-10		●						●	●	2/4
CD44v4-5			●	●						2/4
CD44v6					●					4/4
CD44v8-10							●	●	●	1/4

Table 2-3

The IC_{50} values for each drugs in CD44v transduced cells

	IC_{50} for DXR (ng/ml)	
	CLBL-1	CL-1
mock	6.8	24.7
CD44v3-5, 7	8.0	29.3
CD44v6	14.7	43.5
CD44v6 with LY294002	7.4	21.4

	IC_{50} for VCR (ng/ml)	
	CLBL-1	CL-1
mock	0.21	0.32
CD44v3-5, 7	0.32	0.35
CD44v6	0.40	0.64
CD44v6 with LY294002	0.26	0.33

Fig.2-1.

Comparison of cell viability after treatment with doxorubicin (DXR) and vincristine (VCR) between cells transfected with empty vector (mock; dotted line), CD44v6 (solid line), and CD44v3-5, 7 (chain line). *; p <0.05 when compared with mock transfected cells.

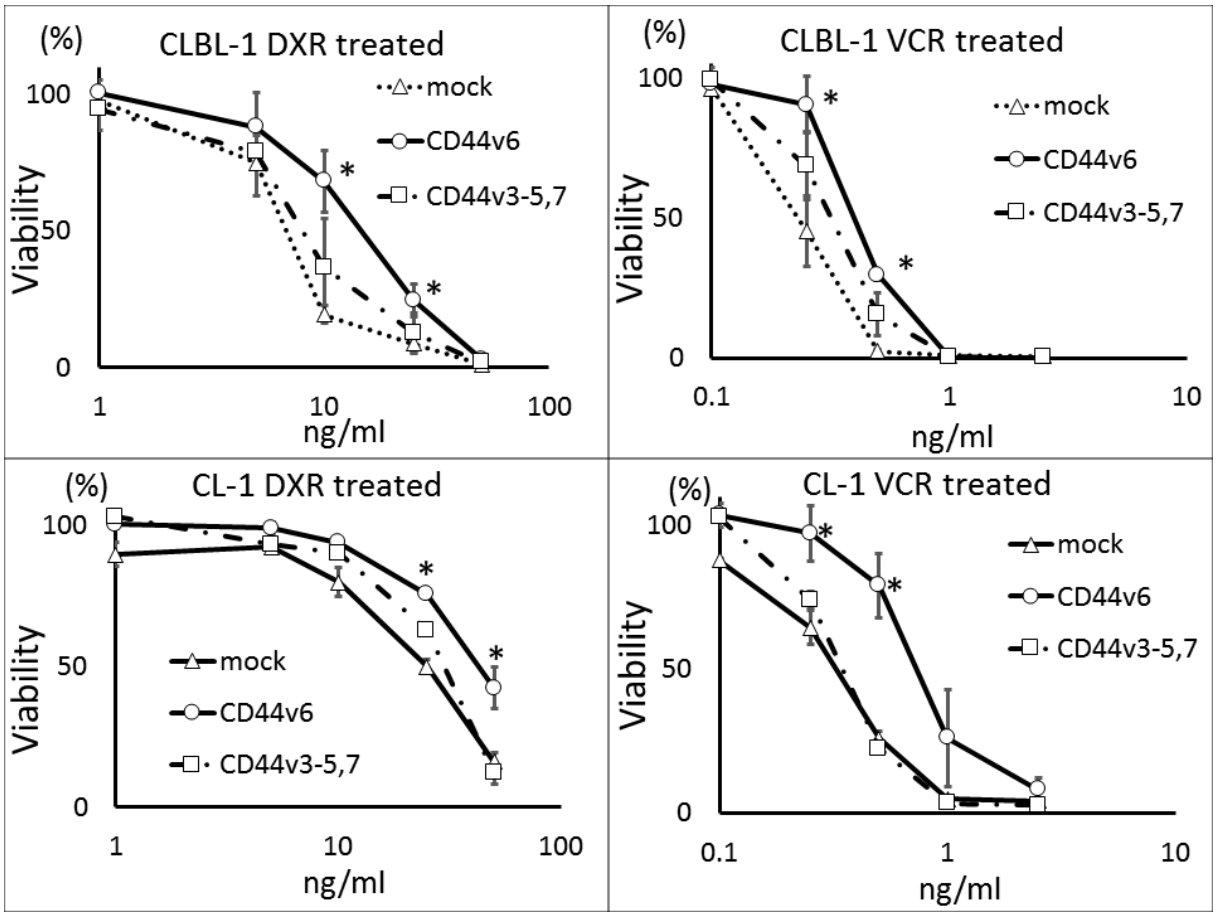


Fig. 2-2.

Comparison of the amounts of proteins related to the Akt pathway after HGF stimulation between cells transfected with empty vector (mock) or CD44v6.

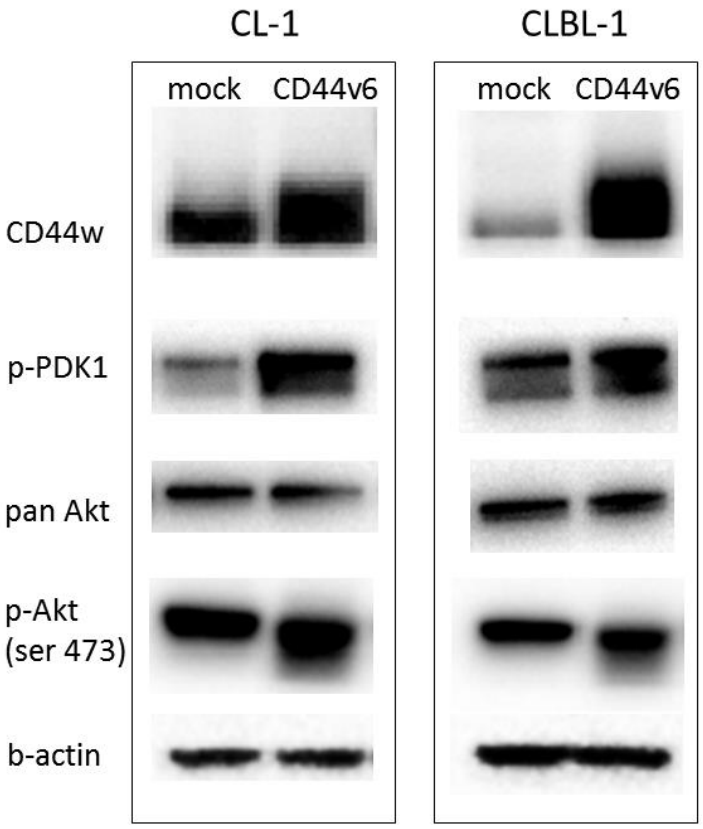
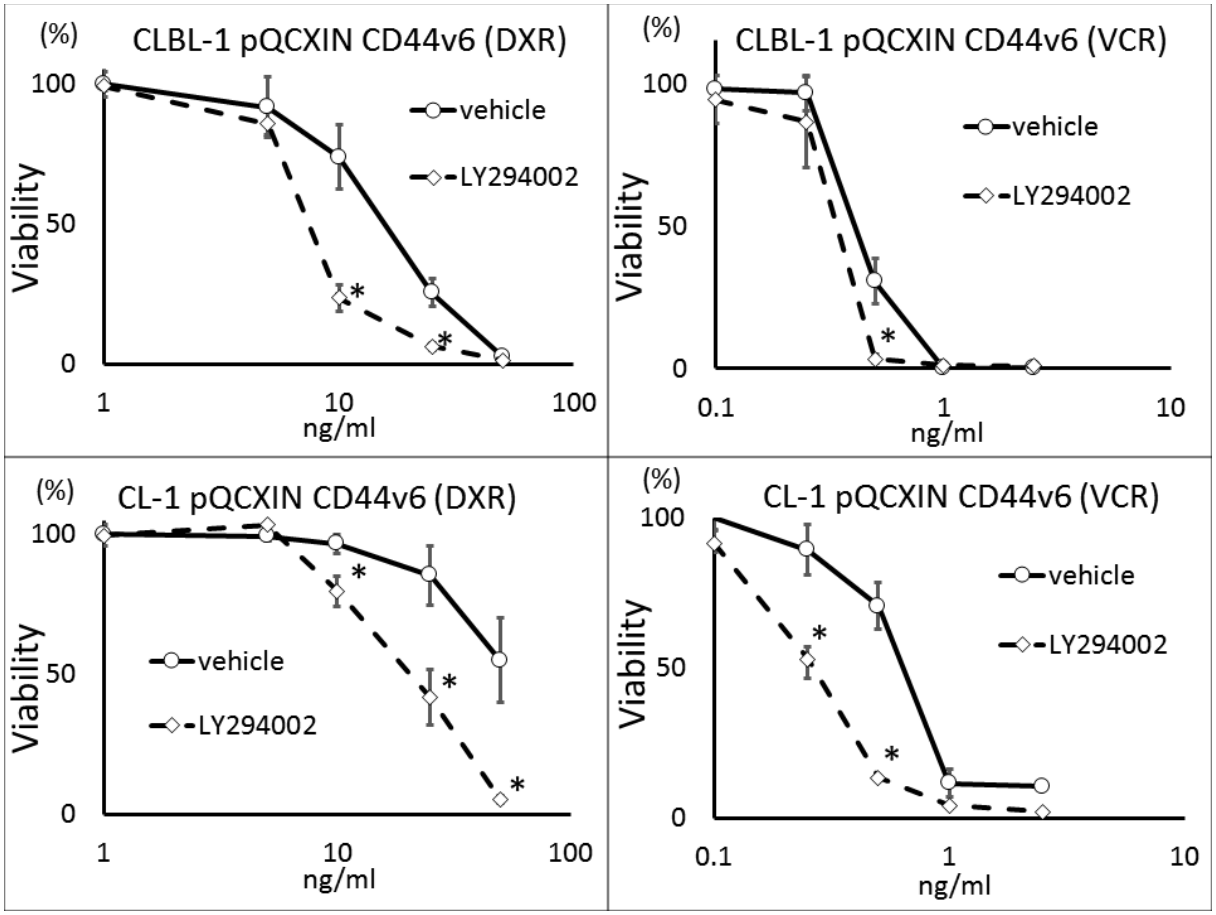


Fig. 2-3.

Cell viability after doxorubicin (DXR) or vincristine (VCR) treatment in cells transfected with CD44v6. Cells were treated with LY249002 (dash line) or vehicle (solid line). *; $p < 0.05$



Chapter 3

Identification of *ESRP1* as a regulator to induce

CD44 variant isoforms expression associated with clinical outcome

in dogs with high-grade B-cell lymphoma

Abstract

Since expression of *CD44* variant isoforms (*CD44v)* was shown to influence the prognosis of canine lymphoma, I conducted a comprehensive analysis of changes in gene expression profiles using canine lymphoma samples with high and low expression of *CD44v*. Lymph node samples from 9 dogs with lymphoma were used in microarray analysis. A total of 1249 differentially expressed genes (DEGs) showing at least 2-fold differences with significant level ($P < 0.05$) were extracted between 4 dogs with high *CD44v* expression and 5 dogs with low *CD44v* expression. Six hundred twelve DEGs were upregulated and 637 DEGs were downregulated in lymphoma dogs with high *CD44v* expression. Among top 5 upregulated and downregulated genes, the expression levels of *SCML2* and *ESRP1* were higher in dogs with high expression of *CD44v* than any of the 5 dogs with low expression of *CD44v*. ESRP1 was further investigated because of its conceivable regulator of CD44 variant isoforms. *ESRP1*-overexpressing cells were generated in the canine lymphoid cell lines to evaluate the expression of CD44v and the sensitivity to doxorubicin and vincristine. *ESRP1* was transduced into a canine lymphoma cell line (CL-1), resulting in induction of CD44v expression and drug resistance to antineoplastic agents. Moreover, progression-free survival in lymphoma dogs with high expression of *ESRP1* was significantly shorter compared with those with its low expression, indicating that the expression of *ESRP1* can be used as a negative prognostic marker.

Introduction

Many molecules which contribute to drug resistance have been investigated to predict poor outcome in dogs with lymphoma. In Chapter 1, I found that high expression levels of *CD44* variant exons 3 and 6 were related to poor prognosis in dogs with multicentric high-grade B-cell lymphoma. Moreover, I revealed in Chapter 2 that *CD44v6*-overexpressed cells activated Akt pathway and induced doxorubicin and vincristine resistance in canine lymphoma cell lines. These *CD44v* were considered to be useful to predict poor outcome and develop a new strategy of treatment in canine lymphoma. However, the mechanism to regulate the expression of *CD44* variant isoforms remains unclear in dogs.

Although the regulation for the alternative splicing of *CD44* was not clearly understood, recent studies have revealed the mechanism for maturation of *CD44v* mRNAs mediated by ESRP1 (Yae *et al.*, 2012; Preca *et al.*, 2015), TRA2B (Takeo *et al.*, 2009), and SC35 (Loh *et al.*, 2014; Wang *et al.*, 2016) in immortalized normal epithelial cells from human, mouse, and rat. These molecules are known to be well conserved in other species (Warzecha *et al.*, 2009). Therefore, I suspect generation of *CD44v* were also regulated by such proteins in dogs.

One of the effective methods provide a broad view of the molecular components in tumor cells is comprehensive analysis of gene expression profiles using microarray. There have been a small number of studies have been investigated using cDNA microarray to find molecules which contribute poor outcome in canine high-grade B-cell lymphoma (Mudaliar *et al.*, 2013; Zamani-Ahmadmahmudi *et al.*, 2016)..

Here I explored to understand the mechanism of regulation for the expression of CD44 variant isoforms using comprehensive gene expression profiling. Among these differentially expressed genes (DEGs), *ESRP1* was further examined for its function on the regulation of

CD44v protein expression and its influence on the prognosis of dogs with multicentric high-grade B-cell lymphoma.

Materials and methods

Dogs and lymph node samples

Lymph node samples were obtained from six healthy Beagles. The procedure was conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo (Accession number P15-63).

Dogs with canine lymphoma were referred to the Veterinary Medical Center of the University of Tokyo between November 2005 and November 2015. Forty-seven dogs diagnosed with multicentric high-grade B-cell lymphoma were included in this study. Lymphoma samples were obtained by fine needle aspiration from dogs. The cytology of these samples was evaluated according to the updated Kiel classification (Fournel-Fleury *et al.*, 1997). T or B cell linage was analyzed by PCR for antigen receptor gene rearrangements (Burnett *et al.*, 2003; Goto-Koshino *et al.*, 2015). Expression levels of *CD44v* was evaluated by RT-qPCR.

cDNA microarray analysis

Of the 47 dogs with lymphoma, 9 dogs were selected for microarray analysis (Supplementary Table 3-1). Total RNA was isolated from lymph node aspiration samples using RNA extration kit (RNeasy Mini Kit, QIAGEN, Hilden, Germany) according to the manufacturer's instruction. The RNA quantity and qualtiy were assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and RNA integrity numbers were confirmed as above 9.0. The synthesis of cDNA and Cy3 labelled cRNA were conducted using Low Input Quick Amp Labeling Kit (Agilent Technologies) and One-Color RNA Spike-In Kit (Agilent Technologies). The labelled cRNAs were purified using RNA extration

kit, and fragmented and hybridized to Canine oligo DNA microarray ver.2 (4×44K) (Agilent Technologies) using Gene Expression Hybridization Kit (Agilent Technologies). After hybridization, slide was washed with Gene Expression Wash Buffer Kit (Agilent Technologies), and was scanned using High-Resolution Microarray Scanner (Agilent Technologies). All image and data analysis were conducted using Feature Extraction software (Agilent Technologies). Three probe sets were used for each sample.

Microarray analysis was performed as previously described (Tomiyasu *et al.*, 2013). Spots with low intensity were eliminated from the analysis. The data were normalized by MAS5, filtered and complicated using analysis software (Gene Spring GX software, Agilent Technologies). The hierarchical clustering analysis was calculated by Ward's method using Manhattan distance. Genes that showed significant differences in expression level between samples with high and low expression of *CD44v* were extracted as the DEGs. Data have been annotated and deposited according to Minimum Information About a Microarray Gene Experiment guidelines with Gene Expression Omnibus (GEO) accession number GSE54744 and GSE83274.

ESRP1 transduction in canine lymphoma cell lines

Full-length *ESRP1* cDNA (GenBank LC201745) was cloned into PQCXIN retroviral vector (Retro-X Universal Packaging System Takara Bio, Shiga, Japan). Twenty μg of pQCXIN-ESRP1 and pVSV-G were cotransfected into GP2-293 cells to generate culture supernatants containing retrovirus particles according to the manufacture’s instruction.

CLBL-1 and CL-1 cells were inoculated with pQCXIN-ESRP1 retrovirus and 8 μg/ml polybrene. After incubation for 2 h at 37°C, the cells were washed in RPMI 1640 and cultured for 48 h. The cells were selected using 700 μg/ml G418 reagent (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 14 days.

Chemotherapy and prognosis in dogs with high-grade lymphoma

All 47 dogs included in this study were first treated using a modified CHOP-based protocol, UW-25 (Garrett *et al.*, 2002). Administration of L-asparaginase at week 1 was omitted because it was reported that L-asparaginase does not influence the outcome in dogs with lymphoma treated with CHOP-based chemotherapy (Valerius *et al.*, 1997; Piek *et al.*, 1999; MacDonald *et al.*, 2005).

The response to the treatment was evaluated by lymph node size, according to the response evaluation criteria for peripheral nodal lymphoma v1.0 (Vail *et al.*, 2010). PFS was defined as the time from the initiation of treatment to the first time that the criteria for progressive disease (PD) were met, or the time of death from any cause. Dogs were censored from PFS analysis if they were still alive, if PD had not occurred before the end of the study, if they were euthanized at the owner's request, or if they were lost during follow-up. Overall survival (OS) was defined as the time from the first day of chemotherapy until death from any cause. Dogs were censored from OS analysis if they were alive at the end of chemotherapy, euthanized at the owner's request, or lost during follow-up.

Second line treatment after tumor relapse was a retreatment with UW-25 (without L-asparaginase). If the dogs ceased to respond to UW-25, they were treated with rescue protocols using L-asparaginase, the LAP protocol (Saba *et al.*, 2007), the DMAC protocol (Alvarez *et al.*, 2006), or nimustine (Takahashi *et al.*, 2014).

RT-qPCR

Total RNA was isolated using an RNA extraction kit (Illustra RNAspin, GE Healthcare, Buckinghamshire, UK) and transcribed to cDNA (ReverTra Ace qPCR RT Master Mix with g DNA Remover, TOYOBO, Osaka, Japan). The primers for amplification

of *ESRP1* (forward 5'-GCCACCATTGAAGACATCCTAGAC-3'; nt.1615-1640 in XM_005638159, and reverse 5'-AATGCTCTGTCCGCAGACTTC-3'; nt.1732-1754 in XM_005638159) were designed for this study, and primer pairs for whole CD44 variant isoforms (*CD44w*), *CD44v3*, *CD44v6*, *CD44v7*, and *TBP* gene as internal control were described in chapter 1. qPCR was performed using SYBR green qPCR kit (TOYOBO) using the following cycling conditions: an initial step at 95 °C for 60 s, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s. After 40 cycles, a dissociation step, consisting of 95 °C for 60 s, 60 °C for 30 s, and 95 °C for 15 s, was performed to verify the presence of a single melting peak. The relative mRNA expression levels of the target gene were calculated using $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Results are shown as the mean of duplicate samples.

Western blotting

The cells were treated with RIPA buffer with protease inhibitor (Protease and Phosphatase Inhibitor Cocktail, EDTA-free x100, Thermo Fisher Scientific, Waltham, MA) for protein extraction. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). The extracted protein was separated by SDS-PAGE using 12.5% polyacrylamide gel and blotted on a PVDF membrane (Immobilon-P membrane, Millipore, Billerica, MA). Membranes were blocked in 1% skimmed milk, and then incubated with primary antibody against CD44 whole variant (IM7 clone, Purified Rat Anti-Mouse CD44, Clone IM7, Becton, Dickinson and Company, Franklin NJ; diluted at 1:2000) overnight at 4°C. After incubation with the HRP-labeled anti-rat IgG (HRP-labeled anti-rat IgG, Becton, Dickinson and Company; 1:2000) for 1 h at room temperature and positive immunoreactivity was detected using a chemiluminescence (Luminata Forte Western HRP Substrate, Merck KGaA, Darmstadt, Germany).

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and with or without doxorubicin (DXR) or vincristine (VCR). After incubation with 10 μl of WST-8 (Cell Counting Kit, Dojindo, Kumamoto, Japan) for 3 h, the absorbance of the colored formazan product derived from active cells was recorded at 450 nm. Cell viability was expressed as the percentage of absorbance obtained in the treated wells relative to that in the untreated control wells.

Statistical analysis

The relative intensities of each probe in the microarray analysis were compared between samples with high and low *CD44v* expression using moderated t-test. The comparisons of the relative quantities of each gene in the RT-qPCR were conducted by Wilcoxon singed-rank test. One-way ANOVA followed and Dunnet test was performed for cell proliferation assay. Receiver operating characteristic (ROC) curve were plotted to predict the cut-off point of *CD44v* expression level. Survival curves and median survival time were estimated using the Kaplan–Meier product limit method and were compared using the log-rank test. $P < 0.05$ was considered significant. Statistical testing was performed using statistics software (JMP version 11.2.0, SAS Institute, Cary, NC).

Results

cDNA expression pattern analysis of lymphoma samples with high and low expression of CD44v3, CD44v6, and *CD44v7*

To evaluate the mRNA expression pattern between lymphoma dogs with high and low *CD44v* expression, I performed microarray analysis using nine lymph node samples from multicentric high grade B-cell lymphoma dogs. Five dogs with *CD44v* expression level comparable to or higher than that of healthy control dogs (*CD44v* high expression group), and four dogs with *CD44v* expression level lower than control dogs (*CD44v* low expression group) were included in this study. Comparing these two groups, 1249 DEGs showing at least 2-fold differences with significant level ($P < 0.05$) were obtained. Six hundred twelve DEGs were upregulated (Supplementary Table 3-2) and 637 DEGs (Supplementary Table 3-3) were downregulated in lymphoma dogs with high *CD44v* expression. Top 5 upregulated genes in lymphoma dogs with high *CD44v* expression were *SCML2, ESRP1, LOC612180, LECT1,* and *CA2*. TOP 5 downregulated genes in lymphoma dogs with high *CD44v* expression were *LOC612553, GSTA3, SMPX, OLFM1,* and *MAGEE2*. The expression level of *SCML2* and *ESRP1* were higher in dogs with high expression of *CD44v* than any of the 4 dogs with low expression of *CD44v*. The hierarchical clustering using the 1249 DEGs divided the 9 samples into 2 clusters. One cluster was composed only of lymphoma dogs with high *CD44v* expression and another was composed only of lymphoma dogs with low *CD44v* expression (Fig. 3-1).

Induction of CD44v transcripts in canine lymphoma cells by transduction with ESRP1

Two canine lymphoid cell lines, CL-1 and CLBL-1, were transfected with *ESRP1*-expressing retrovirus vector. First, mRNA expression levels of *ESRP1* and *CD44* were

examined by RT-qPCR in ESRP1 overexpressing CLBL-1 and CL-1. Relative mRNA expression level of *ESRP1* was increased, whereas expression levels of *CD44v* and *CD44w* did not change in *ESRP1*-overexpressing CLBL-1 cells compared to mock transfected CLBL-1 cells (Fig. 3-2A). On the other hand, CL-1 overexpressing ESRP1 showed higher mRNA expression level of both *ESRP1* and *CD44* (Fig. 3-2A).

Further, the expression of CD44v protein in *ESRP1*-overexpressing cells was evaluated by Western blotting. CD44v proteins observed as 150-170 kDa broad bands were increased in *ESRP1*-overexpressing CL-1 cells, while they were not increased in ESRP1 overexpressing CLBL-1 cells (Fig. 3-2B).

Induction of the drug resistance in canine lymphoma cells by transduction with ESRP1

To evaluate the effect of *ESRP1* overexpression on the sensitivity to antineoplastic agents, I examined the sensitivity to doxorubicin and vincristine in CL-1 cells transduced with *ESRP1*. The sensitivities of both doxorubicin and vincristine were significantly decreased by *ESRP1* overexpression when compared to mock transfected CL-1 cells (Fig. 3-2C). The 50% inhibitory concentration (IC_{50}) for doxorubicin was 38.3±1.1 ng/ml in *ESRP1* transfected CL-1, while it was 24.7 ±1.5 ng/ml in mock transfected CL-1. IC_{50} of vincristine was 0.48±0.05 ng/ml for *ESRP1* transfected CL-1 and 0.32 ±0.02 ng/ml in mock transfected CL-1.

Expression levels of ESRP1 in lymph node samples of dogs with lymphoma

The mRNA expression levels of *ESRP1* in lymph node samples from 47 dogs with multicentric high-grade B-cell lymphoma were measured by RT-qPCR. The dogs with lymphoma were divided into 2 groups with high and low expression of *CD44* variant exons 3, 6 and 7. The relative expression levels of *ESRP1* were 0.00-3.92 in lymphoma dogs with low *CD44v* expression, while it was 0.14-17.7 in lymphoma dogs with high *CD44v* expression

(Fig. 3-3A). The cut-off point of ESRP1 to predict high expression of *CD44* variant exons 3, 6 and 7 was estimated as 2.0-fold from ROC curve. Using 2.0 as the cut-off point, the area under curve (AUC) was 0.86 ($P < 0.001$) with minimized total prediction errors and it was considered optimal, assuming equal costs (Fig. 3-3B).

Prognosis of dogs with lymphoma showing high and low expression of ESPR1

To evaluate the relationship between prognosis and *ESRP1* expression levels, I compared progression-free survival (PFS) and overall survival (OS) between dogs with lymphoma showing high and low expression of *ESRP1*. The lymph node samples from dogs with lymphoma were grouped into the high and low *ESRP1* expression groups based on the cut-off point. High *ESRP1* expression group contained 18 dogs, while low *ESRP1* expression group contained 29 dogs. The median PFS was shorter in high *ESRP1* expression group (98 days) than in low *ESRP1* expression group (235 days). The median OS was also shorter in high *ESRP1* expression group (184 days) than in low *ESRP1* expression group (265 days). In the Kaplan–Meier analysis, the PFS was significantly shorter in the group with high expression of *ESRP1* than in the group with low expression of *ESRP1* (Fig. 3-3C). OS was not significantly different between the two groups (Fig. 3-3D)

Discussion

In this study, mRNA expression profiles were compared using microarray analysis between lymphoma dogs with high and low expression of *CD44* variant exons 3, 6 and 7. Hierarchical clustering analysis using the extracted 1249 DEGs clearly divided the dogs with different expression profiles of *CD44v* into two independent clusters showing high or low CD44 expression. This result suggested that canine multicentric B-cell high-grade lymphoma could be composed of heterogeneous subgroups. In humans, DLBCL were divided into germinal center B-cell like (GC) DLBCL and activated B-cell like (ABC) DLBCL using microarray analysis (Alizadeh *et al.*, 2000). ABC DLBCL showed poor prognosis compared to GC DLBCL. Similar subtypes were also observed in canine lymphoma using selected gene panel, although overall survival was not significantly different between the 2 groups (Richards *et al.*, 2013). Further study focusing on prospective prognostic factors such as CD44v might help discovering subtypes in canine lymphoma.

Among the 1249 DEGs, *ESRP1* mRNA was upregulated in all 4 dogs with high expression of *CD44* variant exons 3, 6 and 7 used in microarray analysis. ESRP1 is known as a regulator of CD44v in humans (Yae *et al.*, 2012; Preca *et al.*, 2015) and mice (Warzecha *et al.*, 2009). Using the *ESRP1*-transduced cells, I revealed that the upregulation of *ESRP1* induced the expression of CD44v protein in CL-1. However, *ESRP1* transduction did not increase the expression of CD44v in CLBL-1. In human cells, *CD44v* spliceosome require ESRP1 together with other proteins, such as TRA2B and SC35 (Takeo *et al.*, 2009; Ishimoto *et al.*, 2011; Loh *et al.*, 2014). The present result might suggest that CLBL-1 had insufficient proteins to regulate *CD44v* spliceosome. Alternatively, CLBL-1 might possess the expression of ESRP1-suppressive protein, such as ZEB1 (Preca *et al.*, 2015).

The sensitivities to doxorubicin and vincristine were shown to decrease by *ESRP1*

overexpression. In Chapter 2, Akt pathway was activated in CD44v6 transduced cells together with developing drug resistance to doxorubicin and vincristine resistance. Because the overexpression of *ESRP1* showed increase of CD44v protein, the reduced sensitivity to anticancer drugs might be related to the anti-apoptotic effect of activated Akt pathway.

ESRP1 expression was shown to influence the prognosis in human lung cancer (Yae *et al.*, 2012) and gastric cancer (Wang *et al.*, 2016). I investigated the relationship between *ESRP1* expression and the prognosis in dogs with lymphoma. When the cut-off level was set at 2.0-fold from normal lymph node samples, PFS and OS were shorter in the high *ESRP1* expression group than in the low *ESRP1* expression group. The result suggested that the expression level of *ESRP1* influenced the prognosis of canine lymphoma possibly by *CD44v* mRNA induction. However, OS was not significantly different between the two groups, although the reason was not well understood. Further study is needed to examine ESRP1 and ESRP1 regulating proteins such as TRA2B, SC35 and ZEB1 by immunohistochemistry in canine lymphoma.

In conclusion, the expression level of *ESRP1* was higher in lymphoma samples with high expression level of *CD44* variant exons 3, 6 and 7. *In vitro* study showed that expression of CD44v was regulated by ESRP1. Since *ESRP*1 expression level was related to prognosis, ESRP1 might be another prognostic marker in canine multicentric high-grade B-cell lymphoma.

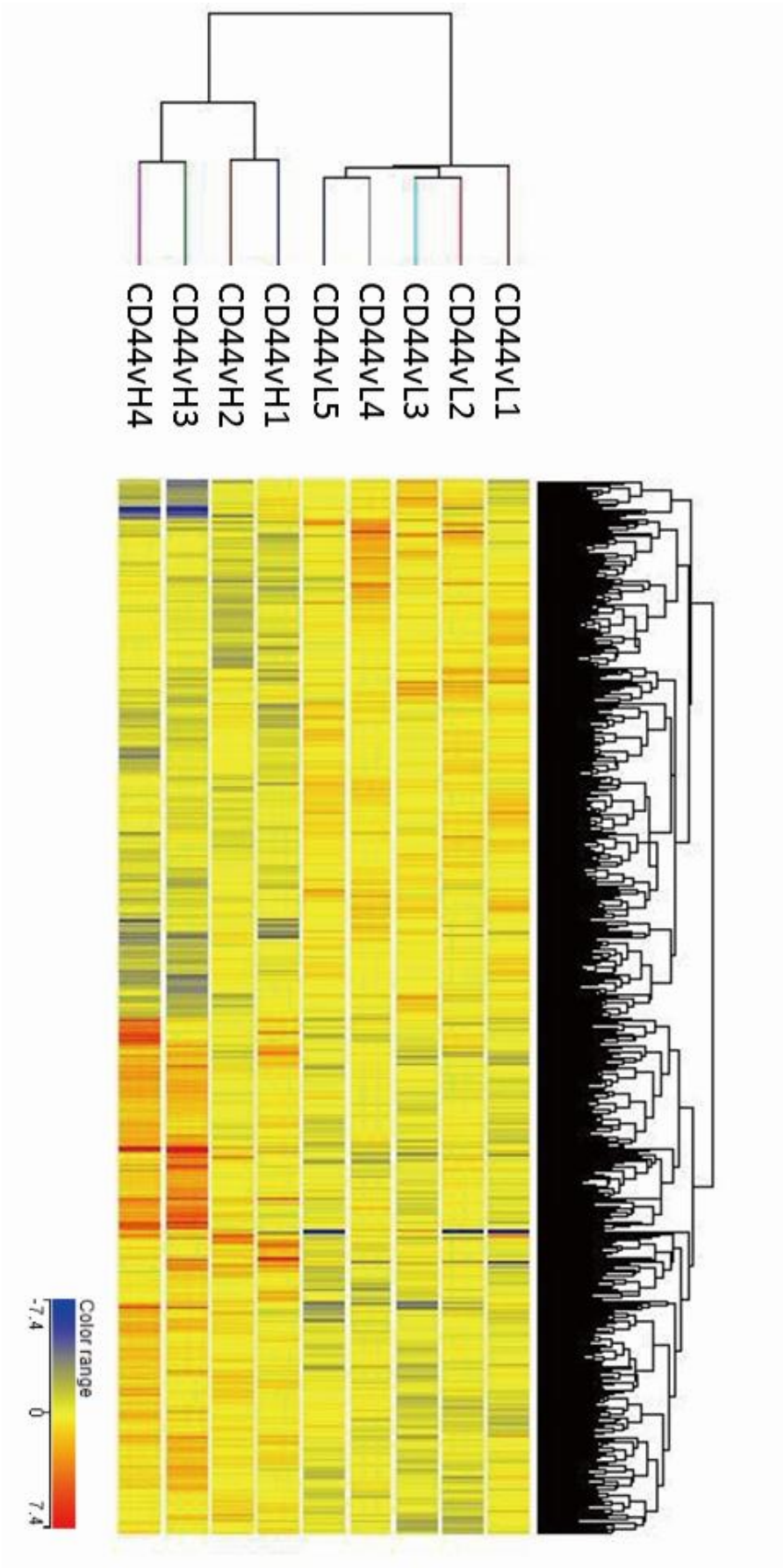
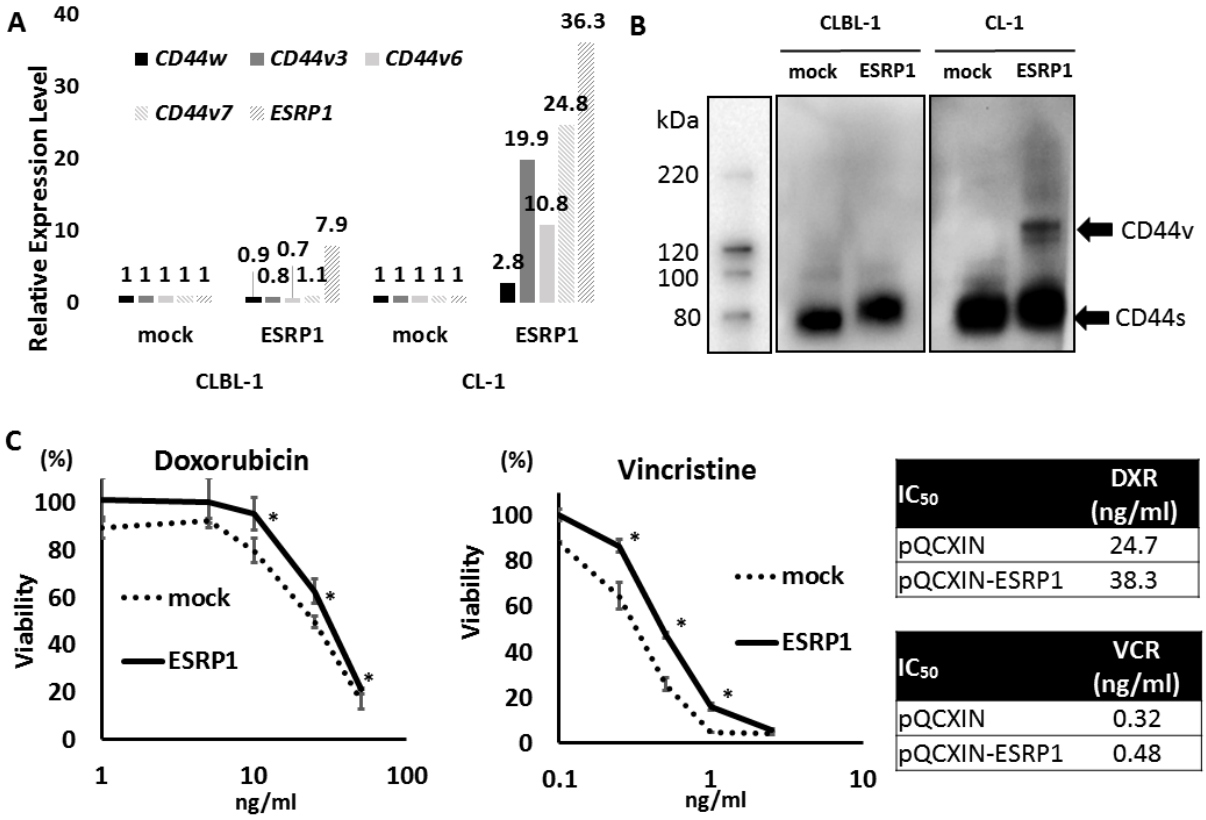

CD44vL1
CD44vL2
CD44vL3
CD44vL4
CD44vL5
CD44vH1
CD44vH2
CD44vH3
CD44vH4
Color range
-7.4
0
7.4

Fig. 3-1

The cDNA microarray of the hierarchical clustering for 9 lymphoma dogs with high and low expression of *CD44* variant exons 3, 6 and 7. This analysis yielded the smallest clusters composed of high expression and low expression of these *CD44v* mRNA. ‘CD44vLx’ indicates lymphoma samples with low expression of *CD44* variant exons 3, 6 and 7 and ‘CD44vHx’ indicates lymphoma samples with high expression of these *CD44v*.



IC_{50}	DXR (ng/ml)
pQCXIN	24.7
pQCXIN-ESRP1	38.3

IC_{50}	VCR (ng/ml)
pQCXIN	0.32
pQCXIN-ESRP1	0.48

Fig. 3-2

Changing of CD44v expression and anticancer agent sensitivity by ESRP1 overexpression. A, Expression levels of *ESRP1*, *CD44w*, *CD44v3*, *CD44v6*, and *CD44v7*. mRNA examined between cells transfected with empty vector (mock) and ESRP1 in CLBL-1 and CL-1. B, Expression of CD44v proteins in mock or ESRP1. C, Cell viability after doxorubicin or vincristine treatment in CL-1 cells transfected with mock (dotted line) or ESRP1 (solid line).IC_{50} for doxorubicin and vincristine are listed in the right panel *; $P<0.05$

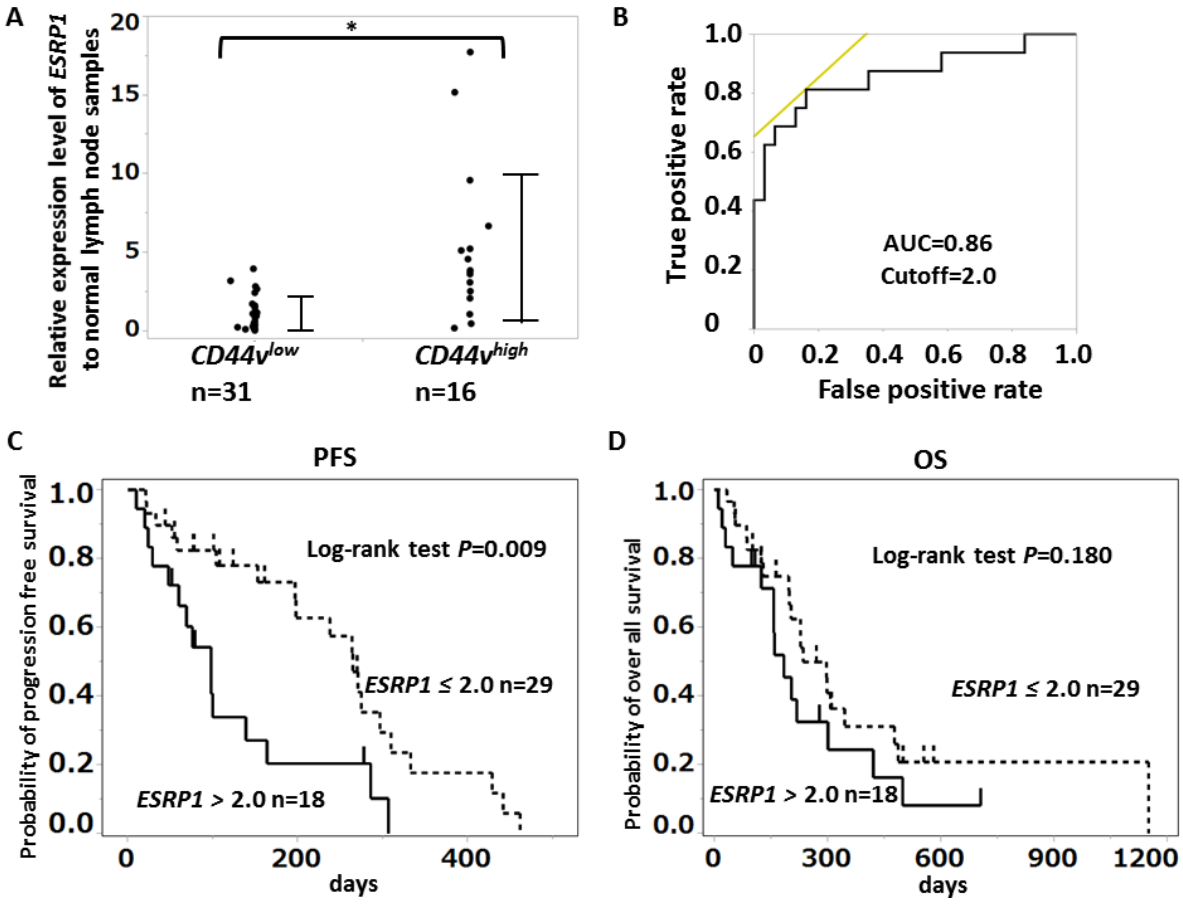


Fig. 3-3

Influence of the expression of ESRP1 on the prognosis in canine multicentric high-grade B-cell lymphoma. A, Expression levels of *ESRP1* mRNA examined in 31 canine lymph node samples with low expression of *CD44* variant exons 3, 6 and 7 ($CD44v^{low}$) and 16 canine lymph node samples with high expression of these *CD44v* ($CD44v^{high}$). Scale indicates mean ± SD range in each lymph node samples. B, ROC curve of ESRP1 and cut-off value for prediction of $CD44v^{high}$. C, Kaplan–Meier curves of PFS and D, OS for lymphoma dogs. Lymphoma samples were divided into 2 groups; cases with expression level of *ESRP1* more than 2-fold for normal lymph node samples (*ESRP1* ≤ 2.0; solid line) and less than 2-fold for normal lymph node samples (ESRP1 > 2.0; dashed line). *; $P<0.05$

Supplementary Table 3-1

Signalment data for all dogs with high-grade lymphoma (n=47) in this study

Case	Breed	Sex	Age (years old)	Body weight (kg)	WHO clinical stage	WHO clinical sub-stage	PFS (days)	OS (days)	Experiment
1	Shih Tzu	F	8y1m	4.4	V	b	78	235	M, R
2	Miniature dachshund	M	12y1m	8.7	V	a	69	124	M, R
3	Welsh corgi	M	8y7m	12.0	V	a	238	307	M, R
4	Jack russell terrier	S	13y3m	6.0	V	a	77	85	M, R
5	French bulldog	M	2y9m	10.3	IV	a	271	477	M, R
6	Doberman pinscher	M	8y8m	25.7	IV	a	76	159	M, R
7	Shih Tzu	M	9y10m	4.4	V	a	98	157	M, R
8	French bulldog	S	4y4m	12.8	IV	a	265	345	M, R
9	Mix	C	10y3m	5.0	IV	b	139	301	M, R
10	Labrador retriever	S	5y5m	34.5	V	a	108	163	R
11	Welsh corgi	C	6y8m	11.3	III	b	310	581	R
12	Welsh corgi	M	6y8m	11.3	III	b	310	581	R
13	Maltese	S	10y2m	3.9	II	a	286	827	R
14	Pomeranian	S	12y4m	5.9	V	b	29	29	R
15	Welsh corgi	S	6y7m	10.8	IV	a	673	673	R
16	Welsh corgi	M	12y11m	15.9	IV	a	164	184	R
17	Miniature Schnauzer	F	10y11m	7.4	V	a	270	270	R
18	Miniature dachshund	S	12y3m	4.3	IV	a	60	157	R
19	Golden retriever	S	9y5m	35.0	III	b	52	52	R
20	Welsh corgi	F	12y11m	9.6	III	b	161	203	R
21	Miniature dachshund	M	8y11m	4.2	IV	b	124	228	R
22	Welsh corgi	F	9y3m	16.3	IV	a	333	487	R
23	Beagle	M	13y4m	14.5	V	b	297	297	R
24	Border collie	S	9y0m	14.9	V	a	307	421	R
25	Mix	M	9y7m	13.6	III	a	442	1151	R
26	West Highland White Terrier	M	9y6m	7.1	V	b	100	218	R
27	Shih Tzu	F	12y0m	3.5	V	b	198	198	R
28	Miniature dachshund	M	10y8m	3.3	V	b	104	124	R
29	Golden retriever	M	12y6m	31.3	V	b	197	197	R

30	American cocker spaniel	F	9y4m	13.8	V	a	462	500	R
31	Beagle	S	12y0m	9.6	IV	a	24	499	R
32	Yorkshire terrier	F	10y8m	6.2	II	a	153	228	R
33	Golden retriever	M	11y11m	40.0	V	b	48	48	R
34	Mix	M	14y1m	24.0	V	a	52	204	R
35	American eskimo dog	M	10y4m	15.4	IV	a	275	337	R
36	Shih Tzu	M	11y8m	6.2	V	a	10	10	R
37	Labrador retriever	M	12y7m	25.4	IV	b	33	33	R
38	Shih Tzu	S	12y3m	6.5	V	b	79	98	R
39	Toy poodle	C	6y11m	7.6	IV	b	55	55	R
40	Beagle	C	8y0m	17.2	IV	b	58	86	R
41	Toy poodle	C	7y0m	7.6	IV	b	55	55	R
42	Pug	C	3y2m	11.6	V	b	98	107	R
43	Golden retriever	F	2y7m	30.3	V	b	44	129	R
44	Dandie dinmont terrier	S	9y8m	10.0	III	a	101	101	R
45	Beagle	C	8y7m	17.2	IV	b	58	86	R
46	Welsh corgi	F	9y8m	14.2	V	a	422	422	R
47	Shih Tzu	S	12y5m	8.0	V	a	278	278	R

Sex: C, castrated; M, male, S, spayed; F, Female
Experiment: M, Micorarray anasysis; R, RT-qPCR

Supplementary Table 3-2

Comparison of upregulated DEGs between high and low expression of *CD44v*.

Probe name	Fold change	Gene symbol	Genbank Accession
A_11_P114161	60.05	SCML2	XM_537972
A_11_P0000014122	56.56		
A_11_P0000041803	48.02		
A_11_P0000030726	35.33	ESRP1	XM_005638159
A_11_P054606	32.66	LOC612180	EU305406
A_11_P153463	30.63	LECT1	XM_846901
A_11_P064456	28.01	CA2	NM_001145170
A_11_P0000015547	27.21		DT539304
A_11_P0000029380	26.44	LECT1	XM_846901
A_11_P0000023190	26.27	CA2	NM_001145170
A_11_P071086	24.04	TOPAZ1	XM_003433152
A_11_P060716	22.85		DN363849
A_11_P0000022914	20.49	GTSF1	DN755704
A_11_P107176	20.18	RAVER2	XM_005620332
A_11_P088751	19.85		XM_005625660
A_11_P166713	17.75		JX964864
A_11_P115901	17.12	LOC102153030	XM_005641860
A_11_P0000025427	16.09	LOC480600	NM_001253735
A_11_P206623	15.41		
A_11_P0000019282	14.25		
A_11_P218018	13.68		
A_11_P069381	13.22	TUB	XM_005633625
A_11_P0000017200	12.39		XM_548922
A_11_P0000039633	12.30	ELMOD1	XM_003639043
A_11_P167983	12.01	TSPAN8	XM_531678
A_11_P0000040798	11.96		XM_538461
A_11_P093796	11.92	CLMN	XM_005623928
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A_11_P170028	11.49		
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A_11_P186763	2.47		
A_11_P0000025081	2.47	ENOSF1	XM_843532
A_11_P0000040361	2.46	GGA2	
A_11_P131036	2.46		CO587026
A_11_P0000018793	2.46		DN876848
A_11_P080371	2.45	LOC100855512	XM_005634064
A_11_P181158	2.45		
A_11_P074801	2.45	SLC41A3	XM_003639719
A_11_P0000029029	2.45		XM_542129
A_11_P116361	2.44	TGFBR2	XM_534237
A_11_P000003481	2.44		BU749628
A_11_P000008153	2.44		CO601388

A_11_P181793	2.43	ARHGEF40	XM_532621
A_11_P105826	2.43	ARHGEF12	XM_536546
A_11_P100841	2.43	SUPT7L	XM_005642266
A_11_P065026	2.42	BCL6	NM_001195404
A_11_P214908	2.42		XM_005638258
A_11_P0000041646	2.42	FOS	XM_547914
A_11_P0000014359	2.41		DN266440
A_11_P129361	2.41	C8H14orf37	XM_537458
A_11_P000003424	2.41		BU749272
A_11_P0000019920	2.41	TJP3	NM_001003202
A_11_P210608	2.40	TNNI3	NM_001003041
A_11_P156048	2.40		XM_848388
A_11_P0000017073	2.40		DN752696
A_11_P0000012798	2.39		CO704124
A_11_P000004462	2.38	NXT2	CF408700
A_11_P000009567	2.37		
A_11_P0000041229	2.37		
A_11_P0000040812	2.37	BMX	XM_005641132
A_11_P0000014859	2.36	ARHGEF10L	XM_003433866
A_11_P067626	2.36	L3MBTL1	
A_11_P0000031117	2.35	ELL3	XM_544650
A_11_P0000040599	2.35	SMPD1	XM_542452
A_11_P121401	2.34		CO591652
A_11_P0000020870	2.34	YIPF7	XM_003639455
A_11_P0000031800	2.34	PRKRA	XM_545545
A_11_P0000020146	2.34	DIO1	NM_001007126
A_11_P0000029516	2.33	THRB	XM_857597
A_11_P0000011413	2.33		CO682309
A_11_P0000032278	2.33	STOX1	XM_005618958
A_11_P066301	2.32	A2M	XM_534893
A_11_P00000914	2.32	C3	XM_533932
A_11_P117916	2.32	ADAMTS6	XM_005617441
A_11_P156358	2.31		
A_11_P059696	2.31	PIWIL1	XM_534638
A_11_P136826	2.30	CPEB2	XM_857623
A_11_P102801	2.30	CPNE2	XM_535289
A_11_P0000031045	2.30	TNFRSF1B	XM_005617982
A_11_P186593	2.30		
A_11_P070891	2.30	PER2	XM_005635925
A_11_P105546	2.29	APLP2	XM_536530
A_11_P086661	2.29	ATP6V1C2	XM_851829
A_11_P0000023504	2.29	DMXL2	XM_535481
A_11_P0000029045	2.29	HSD11B1L	XM_542145
A_11_P000003759	2.29	PLCB1	XM_542896
A_11_P187583	2.29	SLC39A14	XM_543250
A_11_P0000012019	2.29	CBD139	CO690100
A_11_P0000024020	2.28		
A_11_P050211	2.28	RBM47	NM_001002955
A_11_P0000023904	2.28	ITGA6	XM_003640176
A_11_P074356	2.27	LOC612044	XM_849772
A_11_P194363	2.27	SERPINA1	NM_001080109
A_11_P208413	2.27	TGFBR3	XM_547284
A_11_P000006419	2.27		CO584436
A_11_P0000024367	2.27	TTC23L	XM_536506
A_11_P054046	2.27	ABCC3	DQ225112
A_11_P137116	2.26	FAM188B	XM_532503
A_11_P0000040352	2.26		
A_11_P0000014251	2.26		CX014880
A_11_P0000034960	2.26		XM_549401
A_11_P000005191	2.25	ASB11	XM_843803

A_11_P0000031575	2.25	TMEM41A	XM_545235
A_11_P000005395	2.25	C8H14orf37	XM_537458
A_11_P0000024455	2.24	CHRNE	XM_536608
A_11_P188048	2.24		
A_11_P0000040997	2.24		
A_11_P0000031922	2.24	CTSE	XM_545694
A_11_P0000016260	2.24		XM_533390
A_11_P071956	2.23	MLF1	XM_534319
A_11_P0000016699	2.23	PIH1D2	XM_536575
A_11_P127506	2.23	VMP1	XM_548240
A_11_P0000016706	2.22		XM_005640324
A_11_P000008098	2.22		CO600911
A_11_P104386	2.22		XM_005619077
A_11_P125521	2.22	PPP1R1B	XM_845423
A_11_P0000018363	2.22	TGFBR2	XM_534237
A_11_P164213	2.22		XM_005623393
A_11_P000008795	2.22		CO606546
A_11_P000005897	2.21		CK996383
A_11_P0000029151	2.21	RAB38	XM_845119
A_11_P0000011404	2.21		CO682201
A_11_P0000020021	2.21	TGFB1	NM_001003309
A_11_P180698	2.20	FMO4	XM_547466
A_11_P113876	2.20	DPY19L3	XM_005616779
A_11_P0000017749	2.20		DN868127
A_11_P000003203	2.20		BU747967
A_11_P0000022473	2.20		XM_846647
A_11_P0000016222	2.20	P4HA1	DN744347
A_11_P000003655	2.19		BU750527
A_11_P150708	2.19	ITGA6	XM_003640176
A_11_P000002439	2.19		
A_11_P139176	2.19	LOC482880	XM_539995
A_11_P195238	2.19		
A_11_P113556	2.18		XM_005616637
A_11_P0000026209	2.17	CA9	NM_001145174
A_11_P0000011699	2.17		CO685852
A_11_P066761	2.17	RRBP1	NM_001003179
A_11_P158328	2.17	ARHGEF28	XM_005617472
A_11_P126146	2.16	RAB38	XM_845119
A_11_P0000015584	2.16	RYR1	XM_003638835
A_11_P0000023512	2.16	MNS1	XM_535489
A_11_P089701	2.16	ALDH1L2	XM_531763
A_11_P092141	2.16	WISP3	XM_005627789
A_11_P0000027221	2.16	HGSNAT	XM_539948
A_11_P00000177	2.16	ANLN	XM_539518
A_11_P0000021750	2.15	LMAN1	XM_533390
A_11_P0000029632	2.15	PRNP	NM_001013423
A_11_P197298	2.15	FAM149A	XM_005629966
A_11_P0000030074	2.15	VSIG10	XM_543418
A_11_P219108	2.15	C1H19orf47	XM_861894
A_11_P0000017188	2.15		DN754667
A_11_P097536	2.15	KCTD5	XM_547178
A_11_P157283	2.15		
A_11_P0000019878	2.15	DHDH	NM_001003160
A_11_P211698	2.15		
A_11_P171293	2.14	TYRO3	XM_544633
A_11_P000001054	2.14		
A_11_P0000017629	2.14		DN866755
A_11_P0000028659	2.14	IGFLR1	XM_005616897
A_11_P205048	2.13	MYBPC2	XM_533608
A_11_P0000028940	2.13	NACC1	XM_005632813

A_11_P0000032061	2.13	FES	XM_846743
A_11_P0000038748	2.12		
A_11_P124201	2.12		CX988986
A_11_P0000035141	2.12		
A_11_P0000035206	2.11	APLP2	XM_536530
A_11_P107926	2.11	KIAA0513	XM_005620637
A_11_P000008671	2.11		CO605311
A_11_P139266	2.11	RAPGEF6	XM_005626500
A_11_P102846	2.11	LPCAT2	XM_848987
A_11_P0000019897	2.11	RRBP1	NM_001003179
A_11_P085481	2.10		XR_139950
A_11_P000009878	2.10		
A_11_P114591	2.10		
A_11_P000005100	2.10		CF411184
A_11_P163078	2.09	PFKFB3	XM_005617170
A_11_P179978	2.09	IL4R	XM_547077
A_11_P102316	2.09	ARHGAP26	XM_005617330
A_11_P0000020079	2.09	C5AR1	NM_001003373
A_11_P000003301	2.09		BU748628
A_11_P061951	2.08	DLL4	XM_852991
A_11_P103791	2.08	PTCHD2	XM_845529
A_11_P0000025656	2.08	PHF16	XM_538010
A_11_P0000029883	2.08	CTSB	XM_543203
A_11_P0000034795	2.08	TSC22D3	XM_549177
A_11_P000004876	2.08		CF410349
A_11_P085666	2.08		XM_539648
A_11_P198008	2.07	TNFAIP3	XM_541123
A_11_P062536	2.07	ANKDD1A	XM_005638515
A_11_P0000033762	2.07		XM_547868
A_11_P101211	2.07		
A_11_P000003065	2.07		BU747071
A_11_P0000028648	2.07	WDR62	XM_003638839
A_11_P0000023255	2.06	AKR1E2	XM_845943
A_11_P0000029924	2.06	SLC39A14	XM_543250
A_11_P0000015356	2.06		DN393425
A_11_P115461	2.05	TSC22D3	XM_549177
A_11_P155523	2.05	RGS2	XM_545701
A_11_P156893	2.05	SEC31B	
A_11_P000002961	2.05		BU746461
A_11_P0000029545	2.05	SLC35G2	XM_005634465
A_11_P0000031926	2.04	RGS2	XM_545701
A_11_P0000041784	2.04		
A_11_P212948	2.04		
A_11_P215648	2.04	UCHL1	XM_536245
A_11_P208673	2.04		
A_11_P110246	2.03		
A_11_P000002472	2.03	CNTN1	BQ234878
A_11_P0000017650	2.03		DN867020
A_11_P0000016143	2.03		XM_005629382
A_11_P0000032775	2.03	TNFRSF4	XM_546720
A_11_P0000027799	2.03	OR5J2	XM_540650
A_11_P0000016152	2.03		DN443404
A_11_P000008547	2.03		
A_11_P0000011272	2.03		CO680109
A_11_P121196	2.03	MAPRE3	XM_532901
A_11_P0000031942	2.03	TLR5	NM_001197176
A_11_P0000033622	2.03	NLRP3	XM_843284
A_11_P127906	2.02		XM_531808
A_11_P068331	2.02	PANX1	XM_844236
A_11_P107266	2.02	JUN	XM_005620245

A_11_P177048	2.02		XM_533191
A_11_P0000026306	2.02	IER3	XM_538829
A_11_P0000016626	2.02	NEURL	DN747112
A_11_P0000017901	2.02	CAPN5	DN869784
A_11_P0000021552	2.01	CD59	XM_533156
A_11_P000004769	2.01		CF409891
A_11_P000003923	2.01	SLC7A5	CF406107
A_11_P194173	2.01		
A_11_P0000032292	2.01	SGPL1	XM_546150
A_11_P0000031004	2.01	PINK1	XM_003433795
A_11_P0000028872	2.01	PGPEP1	DN874309
A_11_P0000026485	2.00	LYRM2	XM_539041
A_11_P0000034079	2.00	VMP1	XM_548240
A_11_P212033	2.00	MAN2A1	XM_545995
A_11_P0000018648	2.00		DN876051
A_11_P062356	2.00		XM_846330
A_11_P0000014536	2.00		DN272157
A_11_P000008559	2.00		CO604386

Supplementary Table 3-3

Comparison of downregulated DEGs between high and low expression of *CD44v*.

Probe name	Fold change	Gene symbol	Genbank Accession
A_11_P0000014358	-28.64		DN266360
A_11_P069786	-17.87	LOC612553	XM_005633819
A_11_P052721	-16.07	GSTA3	XM_532173
A_11_P205443	-16.03	GSTA3	KJ651954
A_11_P0000041617	-14.01		
A_11_P114211	-13.00	SMPX	XM_849474
A_11_P211903	-12.05		
A_11_P203023	-10.11	OLFM1	
A_11_P0000013975	-9.60		
A_11_P0000041508	-8.52		
A_11_P115076	-8.46	MAGEE2	XM_538082
A_11_P050926	-8.32	CD1A8	NM_001128838
A_11_P105431	-8.15	FAM134B	XM_536520
A_11_P066181	-8.11	GPRC5D	XM_005637127
A_11_P000004627	-8.07		
A_11_P000002445	-7.82		BQ233981
A_11_P0000039677	-7.80	FXYD2	NM_001252337
A_11_P127996	-7.57		DT539070
A_11_P190023	-7.24	MAGEE2	XM_538082
A_11_P0000021281	-7.22	NEIL3	XM_532852
A_11_P070216	-7.03		
A_11_P146158	-6.97	DCN	NM_001003228
A_11_P0000035123	-6.95		XM_003433712
A_11_P000003578	-6.84		
A_11_P0000029122	-6.82	CNTN5	XM_005633312
A_11_P0000041059	-6.77		XR_134388
A_11_P153813	-6.64	EXPH5	XM_005619818
A_11_P076626	-6.58	TMEM205	XM_533912
A_11_P192748	-6.48		
A_11_P0000032229	-6.48	CHRM3	AF056305
A_11_P078411	-6.19		XM_843419
A_11_P185163	-6.18		
A_11_P0000015499	-6.03		DN400659
A_11_P163413	-5.90	MAGEE2	XM_538082
A_11_P0000041156	-5.78		
A_11_P0000018641	-5.67	ATRNL1	XM_544031
A_11_P00000499	-5.65	IFIT1	XM_843271
A_11_P0000026686	-5.63	TMPRSS11D	XM_849377
A_11_P095036	-5.39	PTPLAD2	XM_848993
A_11_P0000032609	-5.38		XM_005619796
A_11_P0000019833	-5.28	UACA	NM_001003112
A_11_P095041	-5.25	PTPLAD2	XM_848993
A_11_P129176	-5.19		BI430519
A_11_P110531	-5.18	NMBR	XM_849337
A_11_P159363	-5.12	ESRRB	XM_547920
A_11_P0000031585	-5.10		XM_005639817
A_11_P0000024216	-5.06	IFIT2	XM_005618758
A_11_P179198	-5.05		
A_11_P110196	-4.93	SERPINB2	XM_846892
A_11_P000002285	-4.88		BM538679
A_11_P0000019946	-4.82	DCN	NM_001003228
A_11_P205168	-4.79		XM_005629758
A_11_P199713	-4.77	SCN2A	XM_535939
A_11_P0000018304	-4.60	EXPH5	DN873237

A_11_P050116	-4.59	UACA	NM_001003112
A_11_P0000020441	-4.59	EFEMP1	XM_531834
A_11_P134076	-4.59		DR103650
A_11_P060006	-4.48	OAS1	NM_001048131
A_11_P206243	-4.48		
A_11_P083951	-4.45	PKHD1L1	XM_845403
A_11_P086586	-4.43	RSAD2	XM_846183
A_11_P055506	-4.42	LOC478952	XM_536110
A_11_P0000030484	-4.37	USP18	XM_005637402
A_11_P0000024462	-4.31		
A_11_P0000030602	-4.29	ATRNL1	XM_544031
A_11_P0000020237	-4.26	GUCY1B3	NM_001018034
A_11_P0000032226	-4.18	IFIT3	XM_005618759
A_11_P214488	-4.17		
A_11_P139376	-4.14	LRRC9	XM_005623459
A_11_P000002820	-4.11		BU745939
A_11_P0000040788	-4.07	GUCY1B3	
A_11_P206128	-4.06		XM_003432242
A_11_P152673	-4.03	PRELP	XM_545678
A_11_P0000033348	-3.97	ATP6V1G3	XM_547375
A_11_P083366	-3.95	QRFP	XM_845658
A_11_P088666	-3.92	C10H12orf56	XM_005625581
A_11_P052891	-3.88	SELP	NM_001287149
A_11_P053371	-3.87	PGB	NM_001003028
A_11_P0000010692	-3.86		CO665302
A_11_P0000024553	-3.84	ISG15	XM_003639053
A_11_P0000041743	-3.82		
A_11_P0000016698	-3.81		DN747554
A_11_P171553	-3.81	PCP4L1	XM_003434315
A_11_P206823	-3.78	CLIP4	XM_845695
A_11_P0000040371	-3.77		
A_11_P0000031603	-3.75	LOC488146	XM_545270
A_11_P051006	-3.74	CD1A6	NM_001128837
A_11_P174753	-3.74	PPAPDC3	XM_548410
A_11_P0000015277	-3.72	PCP4L1	DN391816
A_11_P123816	-3.70		DN264773
A_11_P055781	-3.68	PCP4L1	DN391816
A_11_P099181	-3.66	SELP	NM_001287149
A_11_P0000027364	-3.60	CLIP4	XM_845695
A_11_P0000022503	-3.58	PFN2	XM_003433114
A_11_P0000015208	-3.55		DN270838
A_11_P0000029017	-3.54	CLEC4G	XM_542117
A_11_P051016	-3.52	IL13RA2	NM_001003075
A_11_P0000033264	-3.52	FRRS1	XM_005621873
A_11_P0000027524	-3.52	RPTN	XM_003432279
A_11_P206003	-3.50	RYR2	XM_536330
A_11_P000006975	-3.46		CO590941
A_11_P0000033490	-3.44	RAB25	XM_547540
A_11_P0000039043	-3.41	RANBP17	XM_536433
A_11_P0000033436	-3.39	DUSP27	XM_547482
A_11_P0000025428	-3.39	LOC480601	XM_537721
A_11_P050411	-3.39	MGMT	NM_001003376
A_11_P0000033118	-3.35	DNAH3	XM_005621463
A_11_P0000040903	-3.35		
A_11_P065236	-3.35	SPATA16	DN747554
A_11_P000002417	-3.33		
A_11_P082876	-3.31	SERPINF2	XM_005624935
A_11_P074706	-3.31	FADS2	XM_540913
A_11_P0000034769	-3.30	ARMCX4	XM_005641571
A_11_P136856	-3.29	LOC102157036	XM_005616975

A_11_P172568	-3.29		
A_11_P0000031465	-3.27	LSAMP	XM_003434069
A_11_P125586	-3.25		XM_005619952
A_11_P073018	-3.25		DN270248
A_11_P129986	-3.25		CF411278
A_11_P0000026184	-3.24	IFNK	NM_001284464
A_11_P0000015550	-3.24		DN409158
A_11_P0000018997	-3.23	XAF1	XM_843450
A_11_P155203	-3.21	ARHGEF37	XM_546309
A_11_P0000031945	-3.20	LOC610699	XM_848242
A_11_P0000029252	-3.20	TRIM22	XM_542402
A_11_P0000011094	-3.20		
A_11_P0000023442	-3.19		XM_535413
A_11_P158553	-3.18	SH3GL3	XM_847205
A_11_P174043	-3.18	TNNT2	NM_001003012
A_11_P000001019	-3.18		AB117112
A_11_P202158	-3.18		
A_11_P150868	-3.17		
A_11_P124676	-3.15	TRIM22	XM_542402
A_11_P0000031850	-3.12	ABI2	XM_545606
A_11_P0000020164	-3.10	EDNRB	NM_001010943
A_11_P052146	-3.08	LOC610512	XM_848033
A_11_P0000023629	-3.08	LOC100685470	DR103595
A_11_P0000022939	-3.07	RND1	XM_534817
A_11_P0000015505	-3.06		
A_11_P0000040714	-3.06		XM_003432871
A_11_P0000016862	-3.06	SPOCK1	DN749111
A_11_P195178	-3.06	PMEPA1	XM_543070
A_11_P0000038822	-3.04	TIMP3	
A_11_P0000029777	-3.04		XM_849539
A_11_P0000026401	-3.04	CRIP3	XM_538933
A_11_P0000023162	-3.03	SDR16C5	XM_535080
A_11_P072925	-3.03		
A_11_P0000020676	-3.03		XM_005627349
A_11_P155268	-3.03		
A_11_P090191	-3.03	EFEMP1	XM_531834
A_11_P050601	-3.02	SSPO	NM_001081710
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Conclusion

Lymphoma is a malignant disease characterized by a clonal proliferation of lymphoid cells and is known to be the most common hematopoietic malignancy in dogs. It is classified using anatomical location (Withrow et al., 2013), cell morphology (Fournel-Fleury et al., 1997), and immunophenotype (Greenlee et al., 1990) to define subtypes. In these subtypes, multicentric high grade B-cell lymphoma is common and initially responsive to multidrug chemotherapy, resulting in complete remission in 70–90% of dogs with a disease-free period of 9–11 months (Ito et al., 2014). However, some cases in the same subtype are poorly responsive to the treatment. From these backgrounds, multicentric high grade B-cell lymphoma should further be stratified to predict outcome in the same subtypes.

In humans, many molecules to stratify the same types of lymphoma have been identified such as CDKN1A (Winter *et al.*, 2010), CD5 (Ennishi *et al.*, 2008), P53 (Sehn *et al.*, 2005), VEGFR2 (Gratzinger *et al.*, 2010), and CD44 (Stauder *et al.*, 1995). Mutated *TP53* (Koshino *et al.*, 2016) and overexpression of P53 (Dhaliwal *et al.*, 2013) were also reported to induce chemoresistance in dogs with lymphoma, but their frequencies were not high. CD5, CD21 (Rao *et al.*, 2011), and VEGFR2 (Wolfesberger *et al.*, 2012) did not influence the disease outcome of canine lymphoma. With respect to CD44 has not report to examine its association with the prognosis of canine lymphoma. The present thesis was carried out to evaluate the influence of prognosis and elucidate the molecular mechanisms of drug resistance in canine lymphoma cells focusing on CD44v

In Chapter 1, the expression levels of *CD44* variant exons 3, 6, and 7 were evaluated in dogs with multicentric high-grade B-cell lymphoma and compared with their prognosis. When the cut-off level was set at the mean minus 1 SD value calculated from normal lymph node samples, the overall response (OR) rate, progression-free survival (PFS), and overall survival (OS) were lower in the $CD44v^{high}$ group than in the $CD44v^{low}$ group. In particular, the $CD44v3^{high}$ and $CD44v6^{high}$ group showed lower OR rate and shorter PFS and OS compared

to *CD44v3*^{high} and *CD44v6*^{high} group, respectively. Therefore, expression of these molecules (*CD44v3* and *CD44v6*) were expected to induce chemoresistance to the agents for CHOP.

To clarify the mechanism of drug resistance induced by *CD44v* in tumor cells, the CD44 variant isoforms predominantly expressed in canine lymphoma samples were transduced to canine lymphoma cell lines in the study in a Chapter 2. The anticancer drug sensitivity was investigated using canine lymphoma cell lines transduced with representative *CD44v*, *CD44v3-5, 7* and *CD44v6*. The sensitivities to DXR and VCR were significantly decreased in CD44v6-overexpressed cells, while not changed in CD44v3-5, 7-overexpressed cells. Reduced drug sensitivity observed in CD44v6-overexpressed cells were possibly due to activation in Akt signaling since the sensitivity to DXR and VCR was recovered by Akt/PI3k inhibitor, LY249002.

In order to find a regulator of the expression of *CD44v* in canine lymphoma, comprehensive gene expression profiles was compared between cases with high and low expression of CD44v. ESRP1 was found to be highly expressed in cases with high expression of *CD44v* mRNA. Cell line overexpressing *ESRP1* showed increased level of CD44v protein together with reduced sensitivity to DXR and VCR. Moreover, expression of *ESRP1* was correlated with poor prognosis in dogs with multicentric high-grade B-cell lymphoma possibly through *CD44v* mRNA expression.

In conclusion, a series of studies in the present thesis indicated pathophysiological roles of CD44 variant isoforms in canine multicentric high-grade B-cell lymphoma. Higher expression of *CD44* variant isoforms resulted in poor prognosis clinical outcomes and reduced sensitivity to CHOP-treatment in canine multicentric high-grade B-cell lymphoma. *ESRP1* was shown to induce expression of CD44v, possible by regulating the *CD44* alternative splicing. Further, in lymphoma cells with higher expression of *ESRP1*, sensitivity to DXR and VCR decreased together with the activation of Akt signaling. Moreover,

inhibition of Akt signaling or ESRP1 protein might be a new strategy of treatment in canine lymphoma with high expression of *CD44v6*. Acalabrutinib, a BTK inhibitor repressing p-Akt, has been recently reported to inhibit proliferation in a subset of canine DLBCL (Harrington *et al.*, 2016). Lymphoma cases with higher CD44v6 expression might be a candidate for the treatment for acalabrutinib.

I think that canine lymphoma cells with CD44v6 expression might be cancer stem cells because some reports show that CD44 positive and chemoresistance tumor cells is cancer stem cell in several tumors. For example, $CD44^+$, $CD24^-$, and ESA^+ cells of mammary tumor are slow growth and drive tumor formation in a minority (Al-Hajj *et al.*, 2003). In human head and neck carcinoma, CD44v8-10 positive cells also contribute to tumor formation (Prince *et al.*, 2007) and these cell have chemoresistance (Yoshikawa *et al.*, 2013). Thus, this thesis show canine lymphoma cases with high expression level of CD44v6 might have more cancer stem cells than cases with low expression level of CD44v6 and show poor prognosis by chemoresistance of cancer stem cell. A key challenge remaining for anticancer therapy is the selective killing of cancer cells on the basis of cancer-specific features. Human head and neck carcinomas and lung cancer show that CD44v8-10 positive tumor cells selectively survive and increase in number after chemotherapy (Yae *et al.*, 2012; Yoshikawa *et al.*, 2013). These studies suggest that definitive treatment should target the highly CD44-expressing cell subpopulation. I believe that the results obtained in this thesis disclosed a part of the molecular mechanisms of drug resistance in canine spontaneous lymphoma cases and would be helpful to establish novel treatment strategy in canine lymphoma. Molecule target therapies have been introduced to canine medicine such as JAK1 inhibitor for atopic dermatitis and c-kit inhibitor for mast cell tumor. Therefore, the strategies employed in this thesis can be also adapted to other diseases to provide a new idea to understand the

pathophysiology of the disease and to develop new therapeutic modalities, leading to "Precision medicine" in veterinary field.

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