博士論文

論文題目

Studies on the molecular mechanism of

multidrug resistance in canine lymphoid tumors

（犬のリンパ系腫瘍における多剤耐性の分子機構に関する研究）

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CONTENTS

General Introduction

Lymphoid tumors are known to be common hematopoietic tumor in dogs (Dobson et al., 2002). Among canine lymphoid tumors, lymphoma is most common and comprises 83% of all hematopoietic neoplasms (Vail and Young, 2007). Canine lymphoma has been considered to be a clinically heterogenous disease, because the prognosis of dogs with lymphoma varied among patients. Therefore, canine lymphoma is divided into a number of subtypes based on its anatomic form, immunophenotype, and cytological or histological characteristics (Marconato et al., 2013). Among these subtypes, multicentric B-cell high-grade lymphoma (diffuse large B-cell lymphoma) is the most common subtype in dogs (Vezzali et al., 2010).

As a treatment for dogs with lymphoma, combination chemotherapy is generally selected, and various chemotherapeutic protocols have been reported (Marconato et al., 2013). Among these protocols, CHOP-based protocol using cyclophosphamide, doxorubicin and vincrisitine is a representative protocol as treatment for canine lymphoma (Garrett et al., 2002). Such chemotherapy protocol yields complete responses in over 80% of dogs with lymphoma (Marconato et al., 2013). However, the duration of this remission is generally as short as 6 - 11 months, and post-remission chemotherapy (rescue protocol) is usually not effective for dogs with lymphoma after relapse although various rescue protocols have been proposed (Vail and Young, 2007). The progression free survival time when these rescue protocols were conducted was reported to be 61-70 days (Alvarez et al., 2006; Rassnick et al., 2002; Saba et al., 2009), and the treatment outcome for dogs with high-grade lymphoma is not satisfactory.

The most common cause of the failure in the treatment for canine lymphoma is the acquisition of the multidrug resistance (MDR) by tumor cells (Bergman, 2003). A

number of factors associated with MDR have been studied in human and veterinary medicines (Bergman, 2003; Lage, 2008). Among these factors, a number of molecules have been reported to contribute to acquire the MDR phenotype in humans (Gimenez-Bonafe et al., 2009; Harris and Hochhauser, 1992; Kaina and Christmann, 2002). Such molecules are known to mediate reduced drug accumulation, decreased apoptosis, increased detoxification and increased DNA repair. Based on these observations, the acquisition of MDR phenotype is thought to be multifactorial.

Of the factors associated with MDR, the overexpression of drug efflux pumps such as ATP-binding cassette (ABC) transporter family is one of the mechanisms that have been most extensively studied in both human and veterinary medicines (Bergman, 2003; Lage, 2008). The overexpression of drug efflux pumps leads to reduce the intracellular drug concentrations and confers MDR phenotype to tumor cells. Among ABC transporter family, P-glycoprotein (P-gp, the product of *ABCB1* gene, formerly called *MDR1* gene), multidrug resistance-associated protein 1 (MRP1) (the product of *ABCC1* gene) and breast cancer-resistant protein (BCRP) (the product of *ABCG2* gene) are representative ABC transporters associated with acquisition of MDR in human tumor cells (Modok et al., 2006). Besides ABC transporters, overexpression of lung resistance-associated protein (LRP) is also reported to be associated with MDR phenotype in human tumor cells (Izquierdo et al., 1996). LRP has different function from P-gp, MRP1 or BCRP and redistributes drugs from the nucleus to the cytoplasm because it is expressed on nuclear membrane (Gromicho et al., 2011).

In veterinary medicine, expression of P-gp was reported to be enhanced in cells of relapsed and chemotherapy-resistant dogs with lymphoma (Bergman et al., 1996; Lee et al., 1996; Moore et al., 1995), and expression of P-gp has been detected in a

chemotherapeutic agent-resistant canine lymphoma cell line (Uozurmi et al., 2005).

As mechanisms that induce the overexpression of genes coding drug efflux pumps, gene amplification, increase of amount or translocation of transcriptional factors, change in epigenetic regulation, and modulation of intracellular signaling have been suggested especially in *ABCB1* gene in human tumor cells (Chen and Sikic, 2012). However, the mechanisms that induce the overexpression of drug transporters have not been well understood. In addition, no study on the mechanisms of expression regulation of genes coding drug efflux pumps has been conducted in veterinary medicine.

Based on the results of studies that indicated the association of the overexpressions of drug transporters with MDR phenotype in tumor cells, it has been attempted to inhibit the function of drug transporters overexpressed in tumor cells. Mainly, two methodologies to overcome the MDR induced by overexpressions of drug efflux pumps have been proposed in human medicine; inhibition of drug transporters function such as competitive inhibition (Shukla et al., 2012) and reduction of the expression of drug transporters (Abbasi et al., 2013). However, both methodologies have not been successfully applied into clinical practice because of the adverse events after application of these strategies.

Although expression of P-gp or other drug efflux pumps has been demonstrated in a variety of MDR tumors in both humans and dogs, these expressions are not always linked to MDR *in vivo* and the inhibition of drug efflux pumps cannot always reestablish the sensitivity for drugs (Leonard et al., 2003). This might be due to the phenomena that acquisition of MDR is multifactorial, although the overexpression of drug transporters is one of the most important MDR factors. Therefore, other MDR factors than drug efflux pumps are needed to be identified.

From these backgrounds, I conducted a series of studies to elucidate the mechanisms of acquisition of MDR in canine lymphoma. In chapter 1, I conducted experiments focusing on 10 molecules known to be associated with MDR in human medicine, and the results indicated the overexpression of *ABCB1* gene in a proportion of dogs with lymphoma showing MDR. Therefore, I performed a series of studies to identify molecular mechanisms to induce the overexpression of *ABCB1* gene and to explore strategies to modulate the gene expression in chapters 2 to 4. Finally, for further development of these studies, comprehensive analysis of the gene expression profiles associated with MDR in canine lymphoma patients were conducted in chapter 5.

In chapter 1, I compared the mRNA expressions of 10 different factors that have been known to be associated with MDR in canine tumor cells between drug-sensitive and drug resistant patients. Based on the results obtained in chapter 1, epigenetic regulation of *ABCB1* gene expression through DNA methylation and histone H3 acetylation was explored in canine lymphoid tumor cell lines in chapter 2. In addition, analysis of DNA methylation statuses was also performed in primary tumor cells derived from dogs with multicentric B-cell high-grade lymphoma. In chapter 3, the regulation of *ABCB1* and *LRP* gene expressions through the intracellular signaling, MAPK/ERK pathway, was examined. Based on the results obtained from chapter 3, antitumor effect and modulation of *ABCB1* gene expression through modulation of intracellular signals by perifosine, an Akt inhibitor, were examined in canine lymphoid tumor cell lines in chapter 4. Finally, I investigated MDR mechanisms that have not been examined in canine tumor cells by comprehensive analysis of gene expression proflies using canine B-cell high-grade multicentric lymphoma patient samples collcted at drug-sensitive and MDR phases in chapter 5.

Chapter 1

Quantitative analysis of mRNA for 10 different drug resistance factors in dogs with lymphoma

Abstract

Expression levels of *ABCB1*, *ABCC1*, *Lung resistance-associated protein* (*LRP*), *ABCG2*, *p53*, *p21^{waf1}*, *Bcl-2*, *CD40L*, *glutathione S-transferase alpha* (*GSTα*), and *O^{6}-methylguanine-DNA-methyltransferase* (*MGMT*) genes, and mutation of *p53* gene were examined in 23 dogs with multicentric high-grade lymphoma to explore their association with drug resistance of the tumor cells. Dogs were divided into chemotherapy-sensitive (n=13) and -resistant (n=10) groups according to the response to a 6-month modified version of the University of Wisconsin (UW)-Madison chemotherapy protocol (UW-25), and expression levels of these factors and frequency of *p53* gene mutation were compared between the two groups. No significant difference was observed in expression levels of each factor between the groups. However, elevated expression level of *ABCB1* gene was observed in 3 dogs in the chemotherapy-resistant group. No significant difference was observed in the frequency of *p53* mutation between the two groups. A possible association of *ABCB1* with resistance to UW-25 was indicated, but no uniform mechanism associated with drug resistance could be identified in dogs with lymphoma.

Introduction

Drug resistance is the most common cause of failure in chemotherapy for dogs with lymphoma. A number of factors associated with drug resistance have been studied in human and veterinary medicines (Bergman, 2003; Lage, 2008). Among these factors, a number of molecules have been reported to contribute to the mechanisms of chemotherapy resistance of tumors in humans (Gimenez-Bonafe et al., 2009; Harris and Hochhauser, 1992; Kaina and Christmann, 2002).

Various studies have been conducted to elucidate drug resistance in various human tumors. Over-expressions of transport proteins including P-glycoprotein (P-gp) (the product of *ABCB1,* formerly called *MDR1* gene (O'Connor, 2007)), multidrug resistance-associated protein 1 (MRP1) (the product of *ABCC1* gene (O'Connor, 2007)), lung resistance-associated protein (LRP) (Izquierdo et al., 1996), and breast cancer-resistant protein (BCRP) (the product of *ABCG2* gene (Burger et al., 2003)) are known to be the major mechanism of reduced intracellular drug accumulation. Mechanisms of decreased apoptosis include aberrations of *p53* gene and over-expression of Bcl-2 (Buchholz et al., 2003; Khor et al., 2007; Kupryjanczyk et al., 2003; Nagamatsu et al., 2008). Wild-type P53 has many functions, such as induction of a transient (cell cycle arrest) or permanent (senescence) blockage of cell proliferation and the activation of cell death pathways in response to genotoxic stress (apoptosis). In particular, in the regulation of the cell cycle, wild-type P53 promotes the expression of $P21^{WAF1}$ (Kim et al., 2009). CD40L is expressed on the T lymphocytes and is known to promote the survival of the B-cell lymphoma cells through activation of CD40 on

cancer cells (Voorzanger-Rousselot and Blay, 2004). Meanwhile, one recent study (Voorzanger-Rousselot et al., 1998) reported coexpression of CD40 and CD40L on B lymphoma cells, suggesting its autocrine anti-apoptotic role. Increased detoxification is also considered to mediate drug resistance. Glutathione S-transferase (GST) acts to induce detoxification of cytotoxic drugs (Moscow and Dixon, 1993). DNA repair pathways are also known to be involved in the development of drug resistance. O^6-alkylguanine DNA alkyltransferase, which is encoded by *O^6-methylguanine DNA methyltransferase* (*MGMT*) gene, has been reported to provide resistance to treatment with alkylating agents (Brent et al., 1985; Gerson, 2004; Schold et al., 1989).

In veterinary medicine, expression of P-gp was reported to be enhanced in tumor cells of dogs with relapsed or chemotherapy-resistant lymphoma (Bergman et al., 1996; Lee et al., 1996; Moore et al., 1995), and expression of P-gp was detected in a chemotherapeutic agent-resistant canine lymphoma cell line (Uozurmi et al., 2005). However, association of *ABCC1*, *LRP*, *ABCG2*, *p53*, *Bcl-2*, *CD40L*, *GST* and *MGMT* gene with drug resistance has not been reported in canine lymphoma. The objective of the study in chapter 1 was to reveal the correlations between these drug resistance factors and multidrug resistance in dogs with lymphoma by measuring expressions of *ABCB1*, *ABCC1*, *LRP*, *ABCG2*, *p53*, *$p21^{waf1}$*, *Bcl-2*, *CD40L*, *GSTα*, and *MGMT* gene using a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) system and detecting *p53* gene mutations.

Materials and methods

Animals

Twenty-three dogs with age ranging from 2.7 to 11.8 years were examined. Of the 23 dogs, 13 were females (9 spayed) and 10 were males (3 castrated). Breeds of these dogs were Golden Retrievers (n=5), Welsh Corgi Pembroke (n=2), Shih Tzu (n=2), Miniature Schnauzer (n=2), Border Collie (n=2), Miniature Dachshund (n=2), Shiba Inu (n=1), Polish Lowland Sheepdog (n=1), Pug (n=1), Australian Kelpie (n=1), French Bulldog (n=1), Scottish Terrier (n=1), Shetland Sheepdog (n=1), and Bernese Mountain Dog (n=1). Twenty-one of the 23 dogs had been referred to the Veterinary Medical Center of the University of Tokyo. The remaining 2 dogs had been referred to private veterinary hospitals and the samples were sent to our laboratory. All dogs were diagnosed with multicentric lymphoma based on cytological examinations of enlarged lymph nodes. According to the cytological examinations, the 23 dogs were diagnosed as high-grade lymphoma based on the updated Kiel classification (Fournel-Fleury et al., 1997). Lymphoma cell samples from all 23 dogs were shown to be clonally expanded B-lymphoid cells by PCR amplification test for the antigen receptor gene rearrangements as described previously (Burnett et al., 2003).

Evaluation of response to treatment: Veterinarians measured the size of the lymph nodes with calipers at each admission, and response to treatment was evaluated based on the new Response Evaluation Criteria in Solid Tumors (RECIST) (Eisenhauer et al., 2009). In the evaluation of response to treatment, complete remission (CR) and partial remission (PR) were regarded as indicating chemotherapy-sensitive status, and stable

disease (SD) and progressive disease (PD) were regarded indicating as chemotherapy-resistant status.

Treatment

A cyclophosphamide, hydroxydaunorubicin (doxorubicin), vincristine, plus prednisolone (CHOP)-based chemotherapy, 6-month modified version of the University of Wisconsin (UW) - Madison chemotherapy protocol (UW-25) (Garrett et al., 2002), was used as a standard multidrug combination chemotherapy protocol. However, administration of L-asparaginase (L-asp) was omitted in most of the cases, because L-asp has been reported to have no influence on the efficacy of the UW-25 protocol (MacDonald et al., 2005). One dog of chemotherapy-resistant group received L-asp at week 1 of UW-25 protocol. All dogs that were enrolled in this study had not received any chemotherapeutic agent before the application of UW-25 protocol; however, three dogs in chemotherapy-sensitive group and two dogs in chemotherapy–resistant group had received corticosteroids for 1 day - 1 month before chemotherapy.

Definition of patient groups

Chemotherapy-sensitive dogs were defined as those achieving CR or PR by week 5 of the UW-25 protocol. Tumor cell samples were collected just prior to the chemotherapy at the initial consultation. Chemotherapy-resistant dogs were defined as those that had underwent a relapse after achieving remission by UW-25 protocol and did not respond to all of the three chemotherapeutic agents used for UW-25, cyclophosphamide, doxorubicin, and vincristine. The tumor cell samples of the chemotherapy-resistant group were collected after confirming the non-responsiveness to

these three drugs in conjunction with prednisolone after the relapse.

RNA extraction and reverse transcription

Total RNA was extracted from samples obtained by fine-needle aspiration (FNA) of neoplastic lymph nodes using RNAqueous (Ambion, Austin, TX) and treatment with DNase I (Invitrogen, Carlsbad, CA). To synthesize cDNA, total RNA was reverse transcribed using a PrimeScript^{TM} RT reagent Kit (Perfect Real Time) (Takara Bio, Shiga, Japan).

Real-time PCR

Oligonucleotides complementary to the sequences of *ABCB1*, *ABCC1*, *LRP*, *ABCG2*, *p53*, *p21^{waf1}*, *Bcl-2*, *CD40L*, *GSTα* and *MGMT* genes were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm). The mRNA sequences of factors other than the *LRP* gene were identified at Genbank, and that of *LRP* gene was identified at Ensembl (transcript ID ENSCAFT00000026871, partial).

For normalization of the amount of cDNA sample, selection of the internal reference was carried out prior to the setting of quantitative analysis of the factors possibly related to the drug resistance. Candidates for an internal reference included *TATA box binding protein* (*TBP*), *ribosomal protein L13a* (*RPL13A*) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) genes (Peters et al., 2007). Expressions of these three genes were quantified, and accuracies as internal control were analyzed using geNorm v 3.5 software (http://medgen.ugent.be/~jvdesomp/genorm) (Schlotter et al., 2009). From the result of this analysis, *TBP* gene was selected for use as an internal reference. The same primer pair for *TBP* gene was used as in a previous report (Peters et al., 2007).

Sequences of the primers are listed in Table 1-1. Amplification of products was confirmed by electrophoresis. Real-time PCR for quantification of mRNA for *ABCB1*, *ABCC1*, *LRP*, *ABCG2*, *p53*, *p21*^{waf1}, *Bcl-2*, *CD40L*, *GSTα* and *MGMT* genes was performed using a Thermal Cycler Dice Real Time System TP800 (Takara Bio). Twenty-five microliters of the PCR mixture contained 12.5 μl of Master Mix (SYBR® Premix Ex Taq™ (Perfect Real Time)) (Takara Bio), 200 nM of sense and reverse primers, and 50 ng of cDNA. The cDNA samples were subjected to activation at 95 °C for 10 s, then 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 62 °C for 30 s. After 40 cycles, samples were run using the dissociation protocol to verify the occurrence of a single melting peak. In this assay, 6-carboxyfluorescein (FAM) binds to double-stranded DNA, and increased fluorescence is detected. The results are expressed as the threshold cycle (C_T), that is, the cycle number at which increasing reporter fluorescence crossed a fixed threshold baseline.

For each factor and *TBP* gene, an assay-specific standard curve with serial (10×) dilution of tumor cell cDNA from a dog with multicentric high-grade lymphoma was prepared. In these standard curves, the relative quantity of cDNA for each factor and *TBP* gene which was included in 50 ng of this cDNA sample was defined 10^5. These standard curves were examined in triplicate. The relative quantity of each factor and *TBP* gene in lymphoma cells of dogs included in this study was calculated by plotting C_T in the prepared standard curve. To normalize the amount of cDNA sample, the ratio of the relative quantity of cDNA for each factor to that of *TBP* gene was adopted. All samples were examined in duplicate.

Examination of p53 gene mutation

The sequence of cDNA for *p53* gene was divided into three fragments (fragments A, B and C) for PCR amplification and sequence analysis and three pairs of primers were selected for each of fragment A (nucleotides 2 to 479), fragment B (nucleotides 341 to 778), and fragment C (735 to 1,178), as described previously (Setoguchi et al., 2001). Primer sequences are listed in Table 1-2. The cDNA of *p53* gene for each dog was amplified by PCR using TaKaRa Ex Taq® (Takara Bio) and primer pairs for each fragment. The cDNA samples were subjected to 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. The sequence of each fragment was analyzed directly from PCR products using a BigDye terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and Applied Biosystems 3130xl genetic analyzer (Applied Biosystems). When the sequence of cDNA of *p53* gene fragment could not be analyzed directly, PCR products were inserted into a T/A cloning vector (pGEM – T Easy) (Promega Corporation, Leiden, The Netherlands), and subjected to sequence analysis. Ten clones from each cDNA sample were sequenced. To confirm aberration in function of P53 protein, relative quantities of $p21^{waf1}$ cDNA were compared between dogs that had mutations in *p53* gene and those without mutations.

Statistical analysis

Relative quantities of cDNA for each factor were compared between chemotherapy-sensitive and -resistant groups using the Wilcoxon rank-sum test. Relative quantities of $p21^{waf1}$ cDNA between dogs with *p53* gene mutation and dogs with wild-type *p53* gene were compared using the same test. In addition, frequency of mutation in the *p53* gene was also compared between chemotherapy-sensitive and -resistant groups using Fisher's exact probability test. Value of $P < 0.05$ was considered

statistically significant. Statistical testing was performed using JMP version 5.0.1 (SAS Institute, Cary, NC).

Results

PCR amplification using primer pairs and accuracy of the real-time RT-PCR system

PCR amplification of cDNA samples with each primer pair yielded distinct bands of predicted size (Fig. 1-1). Analysis of dissociation curves revealed a single melting peak for each factor, indicating the specificity of primer pairs (data not shown). The accuracy of the real-time RT-PCR system developed in this study was examined using cDNAs that were used to make standard curves in triplicate. According to these examinations, differences ≤ 0.3 in C_T were regarded as intra-assay error. Ranges of *r* values and slopes of the standard curves for each drug resistance factors were 0.991 ~ 0.999 and -3.244 ~ -3.85, respectively. The variation in duplicate analyses was also ≤ 0.3 in C_T, and the results were reasonably repeatable and precise.

Comparison of expression levels of factors between groups

Of the 23 dogs included in this study, 13 were chemotherapy-sensitive and 10 were chemotherapy-resistant. No significant difference was observed in expression level of any factor between chemotherapy-sensitive and -resistant groups (Table 1-3 and Fig. 1-2). However, elevated expression of *ABCB1* was observed in 3 dogs in the chemotherapy-resistant group, with increases of 19- to 33-fold greater than the median relative quantity in the sensitive group.

Mutation in p53 gene

In 6 (26.1%) of 23 dogs, mutations in *p53* gene were observed. Of these 6 dogs, 2

were chemotherapy-sensitive, and 4 were chemotherapy-resistant. Five dogs showed a missense point mutation in *p53* gene (Cys^{163} to Tyr, Arg^{145} to Cys, Ser^{114} to Pro, Arg^{261} to Cys, and Arg^{237} to Trp, respectively), and 1 dog showed a 1-base deletion (G, nt 453) resulting in introduction of a stop codon at codon 156 (Table 1-4). No significant difference was observed in the frequency of *p53* gene mutation between chemotherapy-sensitive and -resistance groups. The relative quantity of $p21^{waf1}$ cDNA was significantly lower in dogs with *p53* gene mutation than in those with wild-type *p53* gene (P=0.02) (Fig. 1-3).

Discussion

In this study, no significant difference was observed between the chemotherapy-sensitive and –resistant groups in relative quantities of mRNA for various genes known to have possible associations with drug resistance. Reasons for this result may include the fact that the relative quantities of cDNA for each factor varied among dogs. However, 3 dogs in the chemotherapy-resistant group showed elevated expression of *ABCB1*. Although the *ABCB1* expression was conceivably associated with the resistance to UW-25, a possibility that the *ABCB1* expression was a result of exposure to P-gp substrates such as doxorubicin and vincristine could not be excluded. Similar results were obtained in a previous report on canine lymphoma (Moore et al., 1995), in which expression of P-gp protein was detected in 1 of 30 lymphoma tissues prior to chemotherapy, meanwhile it was detected in 3 of 8 lymphoma tissues at the chemotherapy resistance phase. In addition, two other studies on canine lymphoma (Bergman et al., 1996; Lee et al., 1996) showed that the rates of dogs with P-gp expression was higher after relapse than before treatment, and the dogs with P-gp expression prior to chemotherapy had low remission rate and short survival time. From the results in the present study in conjunction with those in previous reports, P-gp could conceivably be associated with resistance to chemotherapy in a subset of dogs with lymphoma. As shown in the study by Moore *et al.* (Moore et al., 1995), less than half of chemotherapy-resistance canine lymphoma cases showed positive results for P-gp expression in the tumor tissues. In the present study, 10 kinds of the associated molecules were examined for elucidation of the molecular mechanism that confers drug

resistance on canine lymphoma cells. However, any consistent change associated with the drug resistance could not be found; therefore, further effort is needed to clarify the drug resistance mechanism in canine lymphoma.

In this study, the quantity of *ABCB1* mRNA was measured by real-time RT-PCR, whereas in the previous three studies (Bergman et al., 1996; Lee et al., 1996; Moore et al., 1995) P-gp expression was examined at the protein level. Kourti *et al.* (Kourti et al., 2007) reported that high *ABCB1* mRNA expression was correlated with the worse prognosis in childhood acute lymphoblastic leukemia. However, there is also a report which indicated that *ABCB1* gene mRNA level in the lymphoma tissues did not correlate with the clinical response to doxorubicin in dogs (Steingold et al., 1998). Studies on the expression analysis in protein level are warranted with respect to a number of molecules possibly associated with drug resistance; however, it is problematic that antibodies cross-reacting with proteins of these canine counterparts are not easily available at a moment.

Although frequency of *p53* gene mutation was not significantly different between chemotherapy-sensitive and -resistant groups, relative quantity of $p21^{waf1}$ cDNA was significantly lower in dogs with *p53* gene mutations than in those with wild-type *p53* gene (P=0.02). This result indicates a loss of function of the product from the mutated *p53* gene compared to wild-type P53. However, the correlation between *p53* gene mutation and drug resistance in dogs with lymphoma could not be identified and expression levels of $p21^{waf1}$ were not significantly different between the chemotherapy-sensitive and –resistant groups. The *p53* gene mutations found in the 2 chemotherapy-sensitive lymphoma patients prior to chemotherapy were considered to be generated through the molecular events to develop lymphoma. The mutations

detected in 4 dogs in the chemotherapy-resistant group could be a result of exposure to chemotherapeutic agents or acquisition of drug resistance as well as the lymphomagenesis.

In the present study, the samples from chemotherapy-sensitive patients were obtained prior to chemotherapy, whereas those from chemotherapy-resistant patients were collected at the relapse when the patients had been already received chemotherapeutic agents. Therefore, the differences in the gene expression profile between the chemotherapy-sensitive and -resistant groups might be a result of the acquisition of chemotherapy-resistance or the exposure to chemotherapeutic agents. In a pretreatment phase, only a small proportion of dogs with lymphoma show resistance to chemotherapy. When it is possible, comparison of gene expression profile prior to chemotherapy between the chemotherapy-sensitive and –resistant groups would deny the influence of exposure to the drugs. In addition, the change of gene expression profile during the acquisition of drug resistance should be evaluated in individual dogs, because the variation of expression levels of each factor among dogs was large in this study. Considering these limitation in the present study, further effort is needed to accurately understand the molecular mechanism of drug resistance in dogs with lymphoma.

In conclusion, although a possible association of P-gp with chemotherapy resistance in canine lymphoma was indicated, no single factor could comprehensively explain chemotherapy-resistant in dogs with lymphoma. However, like P-gp in this study, chemotherapy resistance in individual cases might be associated with some factors. Besides the factors examined in this study, epigenetic changes in tumor cells (Lee et al., 2008) and cancer stem cell hypothesis have recently come into greater focus as mechanisms associated with drug resistance (Hirschmann-Jax et al., 2004). Further

studies employing such strategies would be warranted to elucidate the mechanisms of chemotherapy resistance in dogs with lymphoma.

Table 1-1.

Primer pairs used for real-time RT-PCR measurement of the relative quantity of each drug resistance factor in dogs with lymphoma.

	Sense primer	Reverse primer	Amplicon size (bp)	Genbank accession number
ABCB1	5'-ACTCGGGAGCAGAAGTTTGA-3' (2734-2753)	5'-AATGAGACCCCGAAGATGTG-3' (2809-2828)	95	NM001003215
ABCC1	5'-CACGTCGACCTGCTACAGAA-3' (3144-3163)	5'-CCGTGTCCAGCTCCTTAGAG-3' (3227-3246)	103	NM001002971
LRP	5'-ACAAGACCCGTGTGGTTAGC-3'	5'-AGAGGGACAACACGGTGAAC-3'	144	-
ABCG2	5'-AACTTCTGCCCAGGAGTCAA-3' (1768-1787)	5'-ATCATGCATCCCAAGGCTAC-3' (1891-1910)	143	DQ222459
p53	5'-GGACGTACTCCCCTCTCCTC-3' (332-351)	5'-ACAACCTCGGTCACGAACTC-3' (460-479)	148	AB020761
p21^{waf1}	5'-GCGATGGAACTTTGACTTCG-3'	5'-GCAGGGAGACCTTGGACAG-3'	95	AJ830019 (partial)
Bcl-2	5'-CTCCTGGCTGTCTCTGAAGG-3' (636-655)	5'-GTGGCAGGCCTACTGACTTC-3' (719-738)	103	AB154172
CD40L	5'-AAACAGTTGGCCGTGAAAAG-3' (472-491)	5'-GACTCTCTCCGTTCCACTCG-3' (590-609)	138	NM001002981
GSTα	5'-CAAGTTCAGTTGGGCAGACA-3' (519-538)	5'GCCTGTAGCAGACGGAAGTC-3' (595-614)	96	XM847777
MGMT	5'-GATGAGGAGCAATCCTGTGC-3' (411-430)	5'-GAGTCCTCCGGTGTAGTTGC-3' (479-498)	88	NM001003376
TBP	5'-CTATTTCTTGGTGTGCATGAGG-3' (-145 - -124)	5'-CCTCGGCATTCAGTCTTTTC-3'(-31 - -50)	96	-

Table 1-2.

Primer pairs used for sequence analysis of *p53* gene in dogs with lymphoma.

p53	Sense primer	Reverse primer	Amplicon size (bp)
Fragment A	**5'-TGCAAGAGCCACAGTCAGAG-3' (2-21)**	**5'-ACAACCTCGGTCACGAACTC-3' (460-479)**	**478**
Fragment B	**5'-CCCTCTCCTCAACAAGTTG-3' (341-359)**	**5'-CCTCAAAGCTGTTGCGTCCC-3' (759-778)**	**438**
Fragment C	**5'-GGAAGACTCCAGTGGAAACGTG-3' (735-756)**	**5'-GCGTGTTGGGGGAGGACAGG-3' (1159-1178)**	**444**

Table 1-3.

Relative quantities of cDNA of *ABCB1, ABCC1, LRP, ABCG2, p53* $p21^{waf1}$, *Bcl-2, CD40L, GSTα,* and *MGMT* genes in the chemotherapy-sensitive and -resistant groups.

Factors		*ABCB1*	*ABCC1*	*LRP*	*ABCG2*	*p53*	$p21^{waf1}$	*Bcl-2*	*CD40L*	*GSTα*	*MGMT*
Median		0.0323	1.9084	0.6822	0.4907	0.3692	0.3420	0.9406	2.0366	0.6005	0.3502
Sensitive	Dog 1	0.0131	1.9084	1.2727	0.0786	0.4492	1.3804	0.8782	2.2052	0.7830	0.4860
	Dog 2	0.0082	2.8207	0.7106	0.0079	0.4318	0.2326	0.4172	1.2587	0.5394	0.3617
	Dog 3	0.0199	3.9530	1.1427	1.4151	0.3479	3.5849	0.9626	2.9568	1.2317	0.6090
	Dog 4	0.0043	3.0239	0.6822	0.0078	0.2420	0.1180	0.4506	0.7371	0.4010	0.0970
	Dog 5	0.0336	1.6832	1.1204	0.2118	0.2262	0.9402	0.5722	2.9666	0.5353	0.4825
	Dog 6	0.0068	3.8931	0.9150	0.5884	0.6396	0.4145	1.6045	1.5179	1.2439	0.6269
	Dog 7	0.0576	0.7199	0.4524	0.1186	0.2897	0.8604	0.9406	1.3627	0.5297	0.1260
	Dog 8	0.0175	1.7561	0.5047	0.0162	0.2276	0.2227	1.0084	0.5984	0.5993	0.0557
	Dog 9	0.0874	6.0156	0.8119	0.7607	0.4437	1.0572	0.7265	2.9413	0.5968	0.1388
	Dog 10	0.0323	1.5592	0.5102	0.8259	0.4177	0.1584	2.1209	3.0211	0.6529	0.0203
	Dog 11	0.0703	1.3134	0.5433	0.7284	0.4886	0.3222	1.3470	3.3817	0.8412	0.3790
	Dog 12	0.0235	1.7439	0.8625	0.0434	0.4557	0.3420	1.1007	4.1984	0.2661	0.4785
	Dog 13	0.0412	1.3547	1.1191	0.7149	0.3692	0.9639	0.8906	1.8801	0.6489	0.3451
Resistant	Dog 14	0.4467	0.8153	0.8761	0.6480	0.1944	0.1449	0.4942	0.6175	0.6368	0.4992
	Dog 15	0.0053	2.9323	0.6072	1.1202	0.2246	0.1415	2.4540	2.8736	0.3847	0.0662
	Dog 16	0.0112	2.8919	0.6070	0.4550	0.0478	0.3270	0.9027	2.4785	0.6802	0.2204
	Dog 17	0.6464	1.2024	0.4728	0.6057	0.0498	0.4173	4.5011	0.7928	0.5270	0.0496
	Dog 18	0.0030	2.3511	0.6772	0.2426	0.3125	0.1178	0.7203	1.1899	0.6005	0.3777
	Dog 19	0.0295	1.4397	0.5387	0.0418	0.3821	0.4690	1.1740	4.2560	0.4597	0.3875
	Dog 20	0.0348	3.2411	0.5731	0.5370	0.3056	0.1640	0.7655	3.7518	0.7383	0.5863
	Dog 21	0.0342	2.1118	0.6007	0.0270	0.4492	0.1401	1.2154	2.0366	0.9364	0.1278
	Dog 22	0.6779	2.4296	0.7176	0.4907	0.5371	2.3811	0.4877	1.0524	0.5254	0.1494
	Dog 23	0.1544	1.7240	0.8656	1.0829	0.5901	1.5355	3.2253	1.1934	0.8457	0.3502

Table 1-4.

Mutations in *p53* gene detected in dogs with lymphoma.

Dog	Mutations
4	**TGC to TAC, Cys163 to Tyr**
8	**CGC to TGC, Arg145 to Cys**
16	**G (nt 453) deletion, stop codon156**
17	**TCC to CCC, Ser114 to Pro**
18	**CGC to TGC, Arg261 to Cys**
21	**CGG to TGG, Arg237 to Trp**

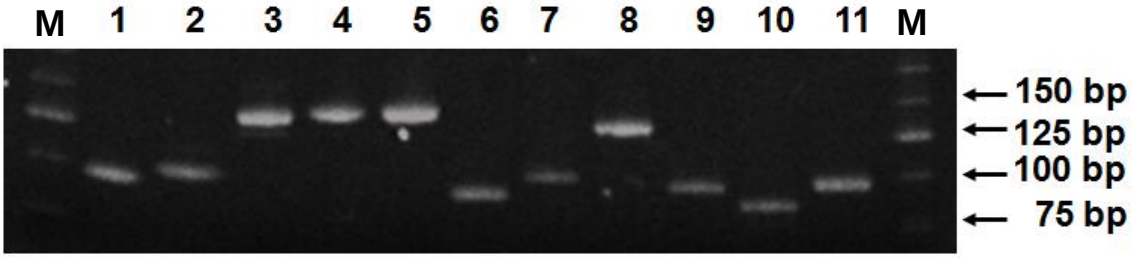

Fig. 1-1. Amplification of products using the primer pairs for real-time RT-PCR. RT-PCR using primer pairs for *ABCB1* (lane 1), *ABCC1* (lane 2), *LRP* (lane 3), *ABCG2* (lane 4), *p53* (lane 5), $p21^{waf1}$ (lane 6), *Bcl-2* (lane 7), *CD40L* (lane 8), *GSTα* (lane 9), *MGMT* (lane 10) and *TBP* (lane 11) genes gave distinct bands of predicted size. M; size marker.

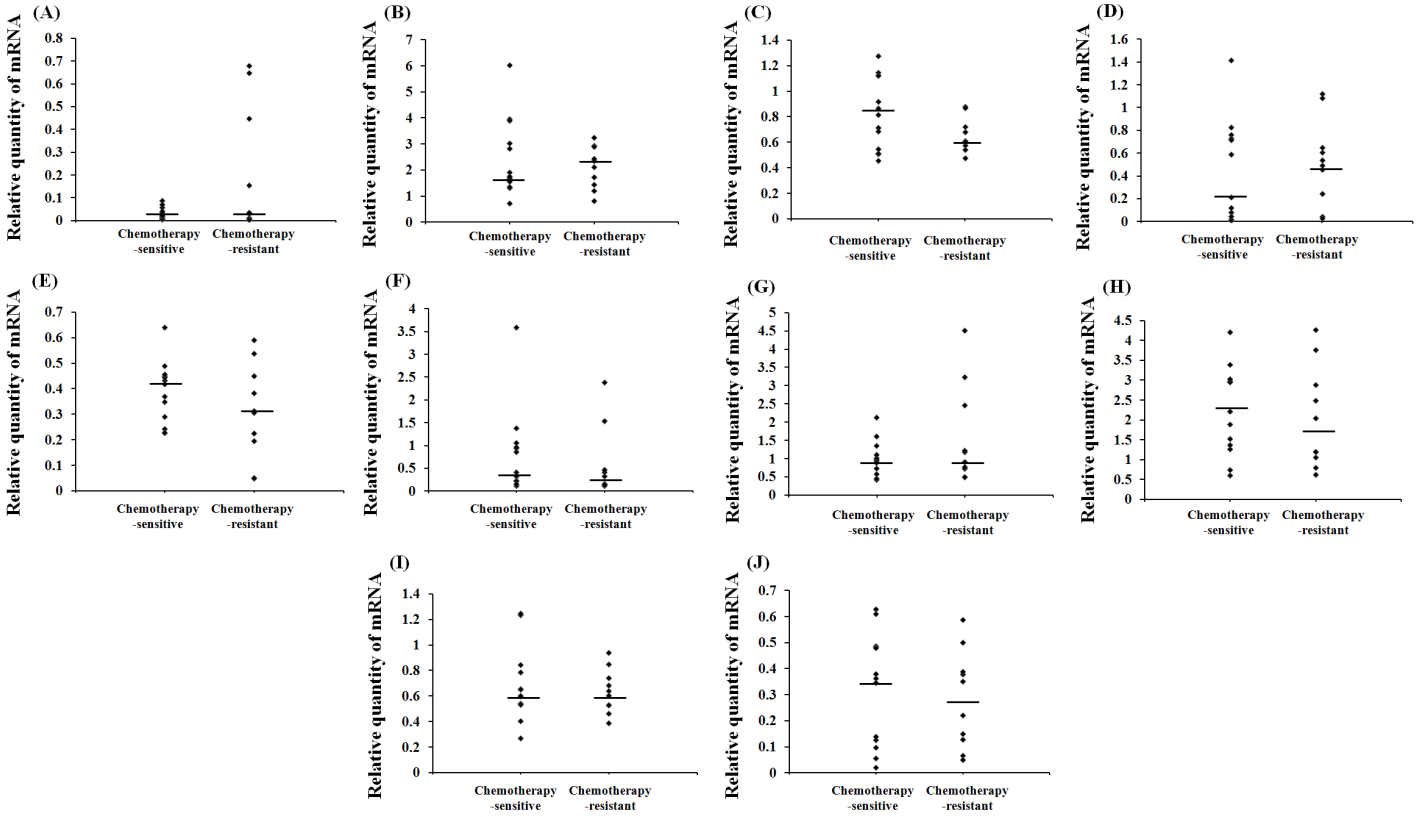

Fig.1-2. Comparison of relative quantities of cDNA of *ABCB1* (A), *ABCC1* (B), *LRP* (C), *ABCG2* (D), *p53* (E), *p21*^{wafl} (F), *Bcl-2* (G), *CD40L* (H), *GSTα* (I) and *MGMT* (J) genes between chemotherapy-sensitive and -resistant groups. The y axis indicates relative quantity of cDNA of the factor to that of *TBP*.

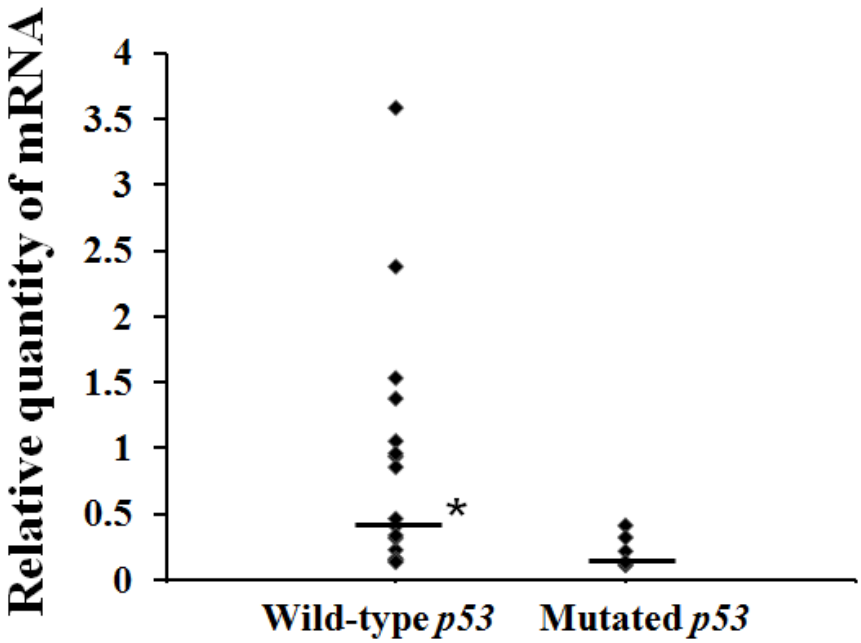

Fig.1-3. Comparison of relative quantities of cDNA for $p21^{waf1}$ between dogs with *p53* gene mutation and dogs with wild-type *p53* gene. In the mutated *p53* group, the relative quantity of cDNA of $p21^{waf1}$ was significantly lower than that of the wild-type *p53* gene group (P=0.02).

Chapter 2

Epigenetic regulation of *ABCB1* gene expression

in canine lymphoid tumor cells

Chapter 2-1

Epigenetic regulation of the *ABCB1* gene in drug-sensitive and drug-resistant lymphoid tumor cell lines obtained from canine patients

Abstract

Multidrug resistance (MDR) is a major obstacle in the treatment of cancer. Overexpression of P-gp, encoded by the *ABCB1* gene, is an important factor in determining the MDR phenotype of a tumor. Although recent studies have revealed the epigenetic transcriptional regulation of the human *ABCB1* gene, such regulation of this gene has not been examined in dogs. The aim of this study was to evaluate differences in epigenetic regulation of the *ABCB1* gene, between drug-sensitive and drug-resistant canine lymphoid tumor cell lines. In two drug-sensitive cell lines, GL-1 and CLBL-1, *ABCB1* mRNA expression was significantly lower than in two drug-resistant cell lines, UL-1 and Ema, using real-time quantitative polymerase chain reaction (QPCR). Bisulphite sequencing and real-time methylation-specific PCR revealed that the CpG island present in the upstream region of exon 2 was hypermethylated in GL-1 and CLBL-1, but hypomethylated in UL-1 and Ema. Chromatin immunoprecipitation and QPCR revealed that histone H3 acetylation in the same CpG island was significantly increased in UL-1 and Ema compared to GL-1 and CLBL-1. Treatment with 5-aza 2'-deoxycytidine or trichostatin A increased *ABCB1* mRNA expression in GL-1 and CLBL-1. Thus, DNA methylation and histone H3 acetylation were demonstrated to be involved in *ABCB1* gene expression and associated with an MDR phenotype in these canine lymphoid tumor cell lines.

Introduction

Lymphoma is a common hematologic neoplasm in dogs (Dobson et al., 2001) and a representative tumor that responds to conventional chemotherapy. However, lymphoma cells can acquire a multidrug resistance (MDR) phenotype after or during chemotherapy in many cases and long-term treatment tends to fail (Marconato, 2011). Therefore, the mechanisms that confer an MDR phenotype in tumor cells needs to be elucidated.

There are various molecules that have been shown to be associated with an MDR phenotype, including P-glycoprotein (P-gp), encoded by the *ABCB1* gene, which is one of the best characterized in human cancers (O'Connor, 2007) and veterinary oncology (Bergman, 2003). Overexpression of P-gp reduces the intracellular concentration of chemotherapeutic agents (Bergman et al., 1996) and transduction of the canine *ABCB1* gene has been reported to induce chemo-resistance in a canine culture cell line (Matsuura et al., 2007). Furthermore, an association between P-gp expression and the MDR phenotype has been reported in dogs with lymphoma (Bergman et al., 1996; Lee et al., 1996; Moore et al., 1995). In chapter 1 of this thesis, the association of MDR phenotype with the overexpression of *ABCB1* gene was suggested in a proportion of the dogs with lymphoma.

The mechanisms that induce overexpression of P-gp have not been well documented. Recent investigations have revealed epigenetic regulation that induces *ABCB1* mRNA and P-gp expression in human tumor cells. The predominant epigenetic mechanisms include DNA methylation, modifications to chromatin structure, loss of

imprinting, and non-coding RNA (Gibney and Nolan, 2010). In terms of *ABCB1*, an inverse correlation has been observed between DNA methylation in the promoter region and *ABCB1* mRNA expression in T-cell leukemia (El-Osta et al., 2002; Kantharidis et al., 1997), breast cancer (Chekhun et al., 2006; David et al., 2004), and other tumor cell lines (Desiderato et al., 1997; Lee et al., 2008) obtained from human patients. Similar negative correlation has been observed in primary tumor cells from human patients with acute myeloid leukemia (Nakayama et al., 1998) or bladder cancer (Tada et al., 2000). Histone modifications, such as acetylation of histone H3 or H4, have also been proposed to regulate *ABCB1* mRNA expression in various human cancer cell lines (Baker et al., 2005; David et al., 2004; Xiao et al., 2005). Since the epigenetic regulation of *ABCB1* gene expression has not been investigated in great depth in dogs, the study in this chapter was designed to examine this, via analysis of DNA methylation and histone H3 acetylation status in canine lymphoid tumor cell lines.

Materials and methods

Cell lines

Four canine lymphoid tumor cell lines were used in the current study: GL-1, a canine B-cell leukemia cell line (Nakaichi et al., 1996); CLBL-1, a canine B-cell lymphoma cell line (Rutgen et al., 2010); UL-1, a canine T-cell lymphoma cell line (Yamazaki et al., 2008); and Ema, a canine T-cell lymphoma cell line (Hiraoka et al., 2009). UL-1 and Ema cell lines were established from dogs with lymphoma showing drug resistance after chemotherapy; however, GL-1 and CLBL-1 cell lines were established from dogs with leukemia or lymphoma not showing drug resistance. All cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum (Biowest, Nuaillé, France) and cultured at 37 °C in a humidified atmosphere of 5% CO_2.

Drug sensitivity assay

The 50% inhibitory concentration (IC_{50}) values for vincristine were determined for the four cell lines. Cells (2×10^5 cells/mL) were cultured in a 96-well plate with various concentrations (0.1 – 100 ng/mL) of vincristine sulfate (Oncovin; Nippon Kayaku, Tokyo, Japan) for 48 h. After cultivation, cell viability was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Absorbance was measured using a Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA) and IC_{50} values were determined. In addition, changes in IC_{50} values for vincristine were determined in UL-1 and Ema cells, co-incubated with 2 μM ciclosporin (Cs; Wako, Osaka, Japan), an ATP-binding cassette (ABC) transporter inhibitor.

Quantification of ABCB1 mRNA by real-time quantitative polymerase chain reaction

Expression of *ABCB1* mRNA was quantified using real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) as described in chapter 1. For normalization purposes, *TATA box binding protein* (*TBP*) was selected as the internal reference (housekeeping) gene, based on the results obtained in chapter 1.

Detection of P-gp expression by Western blotting

Whole cell lysates were extracted from each cell line using RIPA buffer (Cell Signaling Technology, Danvers, MA), Complete protease inhibitor cocktail (Roche, Basel, Switzerland) and PhosSTOP phosphatase inhibitor cocktail (Roche). Protein concentrations were determined using the Lowry protein assay kit (Bio-rad Laboratories), and the equal amount of extracted proteins were separated by SDS-PAGE using 12.5% gel and blotted on a PVDF membrane (Immobilon-P membrane; Millipore, Billerica, MA). Membranes were blocked in 5% skimmed milk/Tris-buffered saline with Tween20 (MP Biomedicals, Solon, OH) then incubated with primary antibodies against P-gp (murine monoclonal: C219, Merck Calbiochem, Billerica, MA) diluted at 1:100, or β-actin (rabbit monoclonal: #4970, Cell Signaling Technology) diluted at 1:1000, for 12 h at 4 °C. After incubation with secondary antibodies, either horseradish peroxidase-labeled goat anti-mouse IgG (1:5000; GE Healthcare, Tokyo, Japan) or donkey anti-rabbit IgG (1:10000; GE Healthcare), for 1 h at room temperature, positive immunoreactivity was detected using Luminata Forte Western HRP Substrate (Millipore) and visualized using a ChemiDoc XRS Plus (Bio-Rad Laboratories).

Rhodamine 123 efflux test

One million cells of each cell line were incubated in triplicate with 200 ng/mL of Rhodamine 123 (Sigma-Aldrich, Zwijndrecht, Netherlands) in RPMI 1640 medium at 37 °C for 20 min. After washing in PBS, cells were incubated in Rhodamine 123-free medium at 37 °C for 60 min, either with or without 2 μM of Cs. After incubation, cells were washed with PBS, and subjected to flow cytometric analysis using a FACSCalibur (BD Biosciences, San Jose, CA). Cells that had not been exposed to Rhodamine 123 were used as negative controls, and the Rhodamine 123 efflux index (REI) was calculated as (mean fluorescence intensities of the sample incubated with Rh123 after Cs minus those of the negative control) / (those of the sample incubated with Rh123 minus those of the negative control).

Analysis of the methylation profile of ABCB1 gene

The methylation status of the CpG island within the canine *ABCB1* gene was analyzed by bisulfite sequencing and real-time methylation-specific PCR (MSP). Genomic DNA was extracted from cell lines using the QIAmp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Bisulfite conversion was performed on 2 μg of DNA using a MethylEasy *Xceed* Rapid DNA Bisulfite Modification Kit (Takara Bio, Shiga, Japan). A CpG island was identified in the canine *ABCB1* gene sequence (GenBank accession number; NC_006596) using Methyl Primer Express v1.0 software (Applied Biosystems, Foster City, CA). The sequences of the primer pairs are shown in Table 2-1 and the regions examined are shown in Fig. 2-1.

For bisulfite sequencing, 200 ng of converted DNA was amplified using Ampli Taq Gold DNA polymerase (Applied Biosystems) according to the manufacturer's

instructions. The PCR products were inserted into a T/A cloning vector (pGEM–T Easy; Promega Leiden, The Netherlands) and transferred into competent *Escherichia coli* (strain DH5α). Plasmid DNA was extracted with NucleoSpin Plasmid Quick Pure (Macherey-Nagel, Bethlehem, PA). Sequence analysis of the extracted plasmid DNA was conducted using a BigDye terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems). Fifteen clones from each PCR product were sequenced. The methylation rate was calculated for each clone examined by dividing the number of methylated sites by the total number of CpG sites examined. The mean methylation rate of total CpG sites in the examined regions was then calculated for all clones analyzed.

For real-time MSP, completely methylated and completely unmethylated DNA controls were prepared from the genomic DNA of UL-1 cells using the CpG methyltransferase *M.SssI* (New England Biolabs Japan, Tokyo, Japan) and an Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) according to the manufacturers' instructions. The control DNA were then inserted separately into a T/A cloning vector (pGEM-T Easy) and transferred into competent *Escherichia coli* (strain DH5α). Assay-specific standard curves for methylated and unmethylated DNA controls were prepared in triplicate with serial (10×) dilution of the plasmid DNA extracted from recombinant bacteria Real-time MSP was performed in duplicate using an Episcope MSP Kit (Takara Bio) with 100 ng DNA. The absolute quantity of methylated or unmethylated DNA was calculated from the standard curves. The methylation rate in real-time MSP was calculated by dividing the mean absolute quantity of methylated DNA by the sum of the mean absolute quantity of methylated and unmethylated DNA controls.

Analysis of the histone H3 acetylation profile of ABCB1 gene

Chromatin immunoprecipitation (ChIP) was performed using a SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology) and anti-acetyl histone H3 antibody (Upstate Biotechnology Lake Placid, NY) according to the manufacturer's instructions. Immunoprecipitated DNA and the 2% input sample DNA were subjected to real-time PCR. The real-time PCR was conducted using 200 ng genomic DNA by the same procedure used for *ABCB1* mRNA quantification. The sequences of the primer pairs are shown in Table 2-1 and region examined shown in Fig. 2-1. An assay-specific standard curve with serial (5×) dilution of genomic DNA from the 2% input sample was prepared in triplicate. The relative quantities to 2% input sample were calculated from the standard curves and the final relative values for enrichment of histone modifications were calculated as (anti-acetyl histone H3 antibody bound relative quantity - normal rabbit IgG bound relative quantity) ×2 / 2% input sample relative quantity. Real-time PCR was performed in triplicate and the means and standard deviations were calculated from three independent immunoprecipitated samples for each cell line.

Treatment with 5-aza 2'-deoxycytidine (5-aza dC) and Trichostatin A (TSA)

The effects of DNA demethylation induced by 5-aza dC, a DNA methyltransferase inhibitor, and histone H3 acetylation mediated by TSA, a histone deacetylase inhibitor, were examined as previously described (El-Osta et al., 2002). For DNA demethylation, cells were incubated with 1 μM 5-aza dC (Sigma-Aldrich) for 32 h followed by two additional doses for 16 h. For histone H3 acetylation, cells were incubated with 25

ng/mL TSA (Upstate Biotechnology) for 24 h. DNA demethylation and increased acetylation of histone H3 within CpG island of *ABCB1* gene and global acetylation of histone H3 were verified using real-time MSP, real-time PCR following ChIP and Western blotting, respectively. The extracted total RNA from these treated cells was subjected to real-time RT-PCR for the relative quantification of *ABCB1* mRNA as described previously.

Statistical analysis

The non-parametric Mann-Whitney *U* test was used to compare relative mRNA expression of *ABCB1* and REI. The same test was used to compare the histone H3 acetylation profile between cell lines. All statistical tests were two-sided and performed using JMP version 5.0.1 (SAS Institute). For all tests, $P < 0.05$ was used as the level of significance.

Results

Sensitivity to vincristine

The IC_{50} values for vincristine in GL-1, CLBL-1, UL-1 and Ema were 6.10, 6.07, 51.2 and 75.9 ng/mL, respectively (Fig. 2-2). When cells were treated with vincristine plus Cs, the IC_{50} values for vincristine markedly decreased in both UL-1 and Ema (0.735 and 0.846 ng/mL, respectively).

ABCB1 mRNA expression and expression of P-gp

The amount of *ABCB1* mRNA was relatively low in the two drug-sensitive cell lines, GL-1 and CLBL-1 (mean relative quantity; 2.08×10^{-5} and 7.77×10^{-5}, respectively), whereas the *ABCB1* mRNA values were significantly higher in the drug-resistant cell lines, UL-1 and Ema (mean relative quantity; 1.03 and 15.6, respectively; Fig. 2-3A). In Western blotting, P-gp (approximate size = 170 kD) was detected in UL-1 and Ema cells but not in GL-1 and CLBL-1 cells (Fig. 2-3B).

Rhodamine 123 efflux test

In the Rhodamine 123 efflux test, the dye was retained in GL-1 and CLBL-1 cells, whereas there was clear evidence of efflux in UL-1 and Ema cells (Fig. 2-3C). The REIs in GL-1, CLBL-1, UL-1 and Ema cells were 0.975 ± 0.0452, 1.02 ± 0.0303, 6.18 ± 1.14, and 18.5 ± 5.27, respectively. There were significant differences in REI between GL-1 and UL-1, CLBL-1 and UL-1, and UL-1 and Ema (P <0.01, P <0.01, and P = 0.03, respectively).

Methylation profile of ABCB1 gene

A CpG island was identified upstream of exon 2 in the canine *ABCB1* gene. In bisulfite sequencing, regions 1 and 2 were revealed to be hypermethylated in GL-1 and CLBL-1 cells, although those of the UL-1 and Ema cells were hypomethylated (Fig. 2-4). The mean (range) methylation rates of regions 1 and 2 in the GL-1 cells were 98.6% (95.7-100%) and 93.1% (81.3-100%), respectively. Those of the CLBL-1 cells were 93.2% (82.6-100%) and 84.0% (81.3-93.8%), respectively. Those of the UL-1 cells were 0 and 1.14% (0-6.25%), respectively. Those of the Ema cells were 1.63% (0-13.0%) and 0.417% (0-6.25%), respectively. Similar results were obtained in real-time MSP. The methylation rates of the CpG islands in *ABCB1* gene were 98.5%, 100%, 0.154%, 0.0651% in the GL-1, CLBL-1, UL-1, and Ema cells, respectively.

Histone H3 acetylation profile of ABCB1 gene

In ChIP assays, before the experiment, the specificity of the anti-acetyl histone H3 antibody was examined. In Western blotting, using lysate from UL-1 cells with or without treatment with TSA, bands of different intensities were observed at the expected position (Fig. 2-5A). The proportions of immunoprecipitated DNA to that of input DNA were 0.81%, 1.11%, 9.22%, and 6.26% in the GL-1, CLBL-1, UL-1, and Ema cells, respectively (Fig. 2-5B).

Effect of DNA demethylation and histone H3 acetylation

When GL-1 and CLBL-1 cells were incubated with 5-aza dC, their DNA methylation rates decreased to 42.0% and 29.1%, respectively, in real-time MSP (Table

2-2). In addition, TSA treatment of GL-1 and CLBL-1 cells increased the rate of histone H3 acetylation in CpG island of *ABCB1* gene in real-time PCR following ChIP by 1.6- and 1.2-fold, respectively (Table 2-2). Treatment with 5-aza dC significantly increased the amount of *ABCB1* mRNA in both of the GL-1 (15.9-fold) (Fig. 2-6A) and the CLBL-1 (161-fold) cells (Fig. 2-6B). TSA treatment also significantly increased the amount of *ABCB1* mRNA in both the GL-1 (120-fold; Fig. 2-6C) and the CLBL-1 (10.4-fold; Fig. 2-6D) cells.

Discussion

In the current study, epigenetic regulation of the *ABCB1* gene, through DNA methylation and histone H3 acetylation, was found to be different comparing two drug-sensitive and two drug-resistant canine lymphoid tumor cell lines.

The IC_{50} values for vincristine were considerably lower in GL-1 and CLBL-1 cells compared with UL-1 and Ema cells. Although there has been limited studies of the pharmacokinetics of vincristine in dogs, the C_{max} of vincristine was reported to be 21.9 and 28.6 ng/mL in two human patients when intravenously injected at a dosage of 0.05 mg/kg (Skolnik et al., 2006). Since the dosage of vincristine is 0.7 mg/m^2 in representative chemotherapeutic protocols for canine lymphoma (Garrett et al., 2002; Keller et al., 1993; Moore et al., 2001), this suggests that UL-1 and Ema could be drug-resistant from the IC_{50} values obtained in the present study (51.2, and 75.9 ng/mL, respectively).

In real-time RT-PCR, *ABCB1* mRNA expression was significantly greater in the two drug-resistant cell lines, UL-1 and Ema, than in the two drug-sensitive cell lines, GL-1 and CLBL-1. In addition, P-gp was detected by Western blotting in the UL-1 and Ema but not in GL-1 and CLBL-1 cells. Rhodamine 123 efflux occurred only in the UL-1 and Ema cells, indicating that functional P-gp was expressed in these cell lines but not in GL-1 and CLBL-1 cells and this functional P-gp might be associated with the MDR phenotype of the former.

Bisulfite sequencing and real-time MSP analysis of the CpG islands of *ABCB1* gene revealed the presence of hypermethylation in GL-1 and CLBL-1 cells and

hypomethylation in UL-1 and Ema cells. In addition, real-time PCR after ChIP assay revealed apparent histone H3 acetylation in the CpG island in UL-1 and Ema cells in contrast with GL-1 and CLBL-1 cells. DNA demethylation induced by 5-aza dC and histone H3 acetylation mediated by TSA resulted in a distinct increase of the amount of *ABCB1* mRNA in the drug-sensitive cell lines, GL-1 and CLBL-1 cells. These results indicated that DNA methylation is likely associated with the transcriptional silencing of *ABCB1* gene, and histone H3 acetylation upregulated the *ABCB1* gene expression in canine lymphoid tumor cell lines. Methylation of cytosine of CpG within CpG island in promoter region correlates with the transcriptional silencing of the associated gene (Suzuki and Bird, 2008). Whereas, histone acetylation is almost invariably associated with the activation of transcription (Kouzarides, 2007). An inverse correlation between DNA methylation of the promoter region of the *ABCB1* gene and mRNA expression has been observed in various tumor cells (Desiderato et al., 1997; El-Osta et al., 2002; Kantharidis et al., 1997). Regulation of *ABCB1* mRNA expression by histone H3 acetylation has been also reported (Baker et al., 2005). Further studies should be conducted in tumor cells to enable to suppress the *ABCB1* gene expression through these epigenetic regulations.

In the current study, there were differences in epigenetic regulation of *ABCB1* gene through DNA methylation and histone H3 acetylation between two drug-sensitive and two drug-resistant canine lymphoid tumor cell lines. As a further study, it should be examined whether similar differences are observed between primary tumor cells of drug-sensitive and drug-resistant dogs with lymphoid tumors. One limitation of the present study was that it could not be clarified that the sensitivity for vincristine was altered by changing DNA methylation status or histone acetylation status, because 5-aza

dC and TSA are both cytotoxic and therefore any cytotoxic effect of vincristine could not be assessed after treatment. A further limitation was that some of the parameters for real-time RT-PCR that should be evaluated for quality assurance purposes, such as RNA integrity (Bustin et al., 2009), were not measured in the present study. The 3':5' assay was not performed, although the RT reaction was conducted using Oligo dT primers that bind to the polyA tail at the 3' end of mRNA. In addition, only one housekeeping gene was used for normalization, although the validity of using the *TBP* gene as a housekeeping gene had previously been confirmed by comparing with *RPL13A* and *GAPDH* genes using geNorm software in chapter 1. These deficiencies in quality control can potentially affect real-time RT-PCR results in studies using clinical samples, although are likely to have less impact when using material derived from cultured cell lines, where sample processing and analysis are consistent and standardized.

In conclusion, DNA methylation and histone H3 acetylation in the CpG island of *ABCB1* gene were associated with regulation of *ABCB1* mRNA expression in canine lymphoid tumor cell lines. Further studies should be conducted in primary tumor cells to investigate whether changes in these epigenetic regulations might be associated with the acquisition of MDR phenotype in canine lymphoid tumor patients.

Table 2-1.

Sequences of primer pairs used in bisulphite sequencing, real-time methylation specific polymerase chain reaction (MSP), and real-time polymerase chain reaction (PCR) after chromatin immunoprecipitation (ChIP). [a]The numbers in the parentheses indicate the nucleotide numbers registered in GenBank (NC_006596).

	Forward	Reverse	Amplicon size (bp)
Primer pair for bisulfite sequencing of region 1	5'-AGGAGGATATTTTTTTGGA-3' (13742947-13742966)[a]	5'-AAAAATCAAACTTTAAAAACCC-3' (13742715-13742736)[a]	252
Primer pair for bisulfite sequencing of region 2	5'-GGGTTTTTAAAGTTTGATTTTTATT-3' (13742712-13742736)[a]	5'-AACTCTAATTCTCACCCCACTA-3' (13742504-13742525)[a]	233
Methylated primer pair for real-time MSP	5'-AGGTTGCGAAGGAAAGTTC-3' (13742670-13742688)[a]	5'-AACCAAACGCGAAAAACTAA-3' (13742589-13742608)[a]	100
Unmethylated primer pair for real-time MSP	5'-GGAGGTTGTGAAGGAAAGTTT-3' (13742670-13742690)[a]	5'-AACCAAACACAAAAAACTAAAA-3' (13742589-13742610)[a]	102
Primer pair for real-time PCR following ChIP	5'-TGGAACTCCAACCTGTTTCG-3' (13742931-13742950)[a]	5'-GTCCACACCCTGGTGCTC-3' (13742839-13742856)[a]	112

Table 2-2.

Changes in the DNA methylation rates and histone H3 acetylation rates after treatment with 5-aza 2'-deoxycytidine (5-aza dC) or Trichostatin A (TSA) in two drug-sensitive cell lines, GL-1 and CLBL-1.

Cell line		No treatment	Treatment with	
			5-aza dC	TSA
GL-1	DNA methylation rate (%)	98.5	42.0	-
	histone H3 acetylation rate (fold change)	1	-	1.6
CLBL-1	DNA methylation rate (%)	100	29.1	-
	histone H3 acetylation rate (fold change)	1	-	1.2

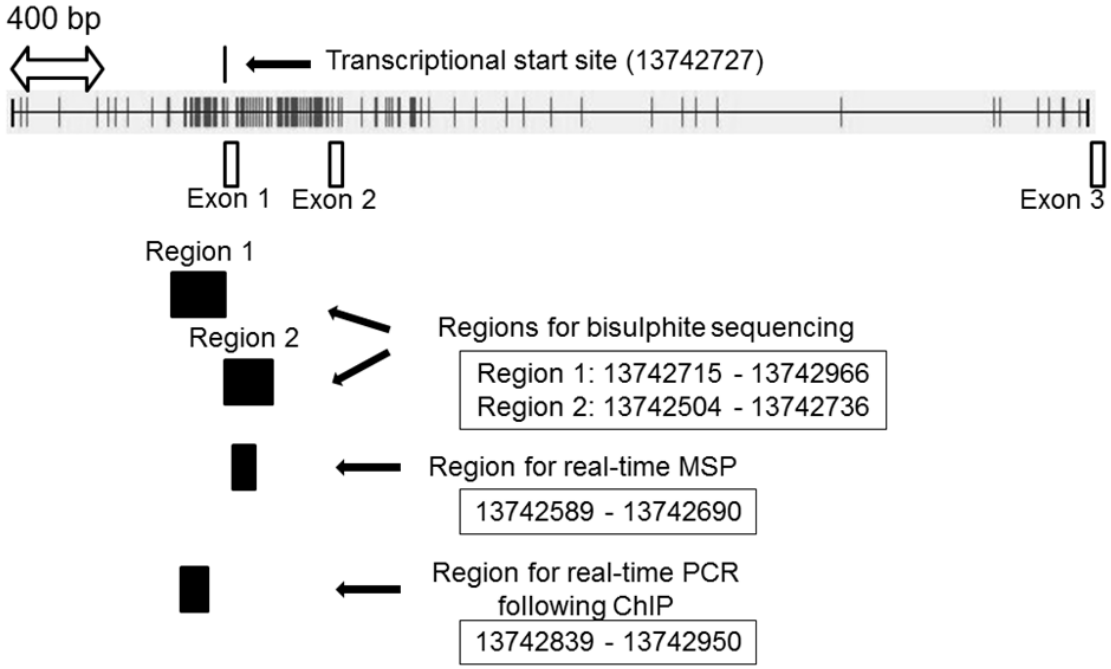

Fig. 2-1. CpG sites upstream of canine *ABCB1* exon 2 and the position of the regions examined by bisulfite sequencing, MSP and RT-PCR after ChIP assay. Each vertical line represents a CpG site. The numbers in the boxes and parenthesis indicate the nucleotide numbers registered in GenBank (NC_006596.).

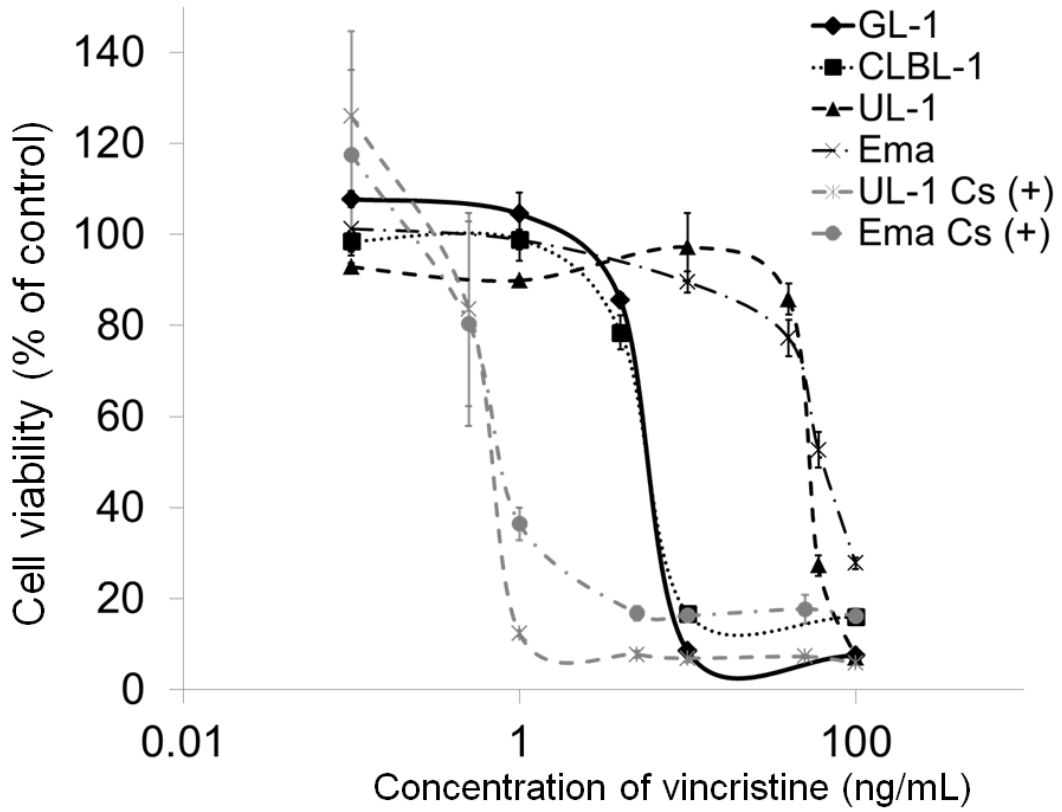

Fig. 2-2. Dose-response curve of the cytotoxic effect of vincristine in the GL-1, CLBL-1, UL-1, and Ema cells. Cell viability data were plotted against the log of vincristine concentrations. Data represent the mean ± SD of triplicate cultures. Cs: cyclosporine (ABC transporter inhibitor).

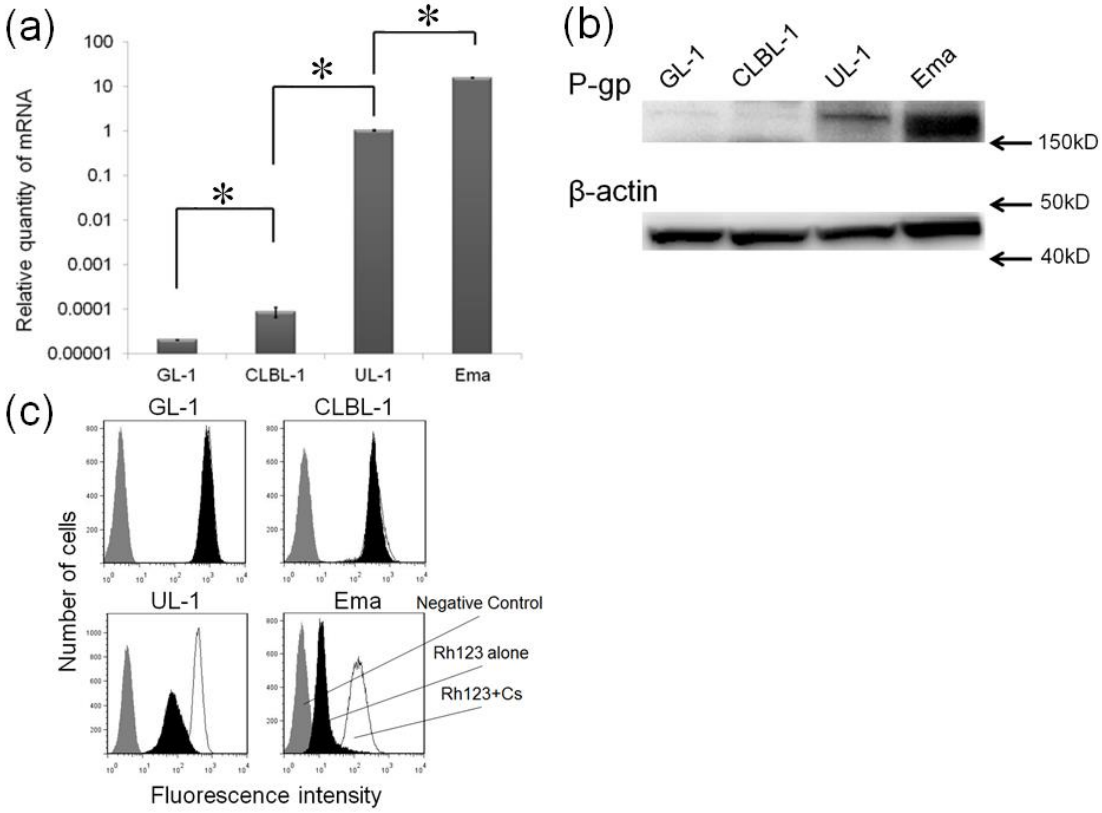

Fig. 2-3. *ABCB1* mRNA and P-gp expression and the functional analysis of P-gp in the four canine lymphoid tumor cell lines. (a) The expression level of *ABCB1* mRNA was significantly higher in the UL-1 and Ema cells than in the GL-1 and CLBL-1 cells. **P* <0.01. (b) In Western blotting, P-gp expression (approximate size = 170 kD) was detected in the UL-1 and Ema cells but not in the GL-1 and CLBL-1 cells. (c) Efflux of the Rhodamine 123 dye (Rh123) was scant in the GL-1 and CLBL-1 cells, whereas its efflux was obvious in the UL-1 and Ema cells. Inhibition of the efflux by the treatment with Cs indicates the action of functional P-gp.

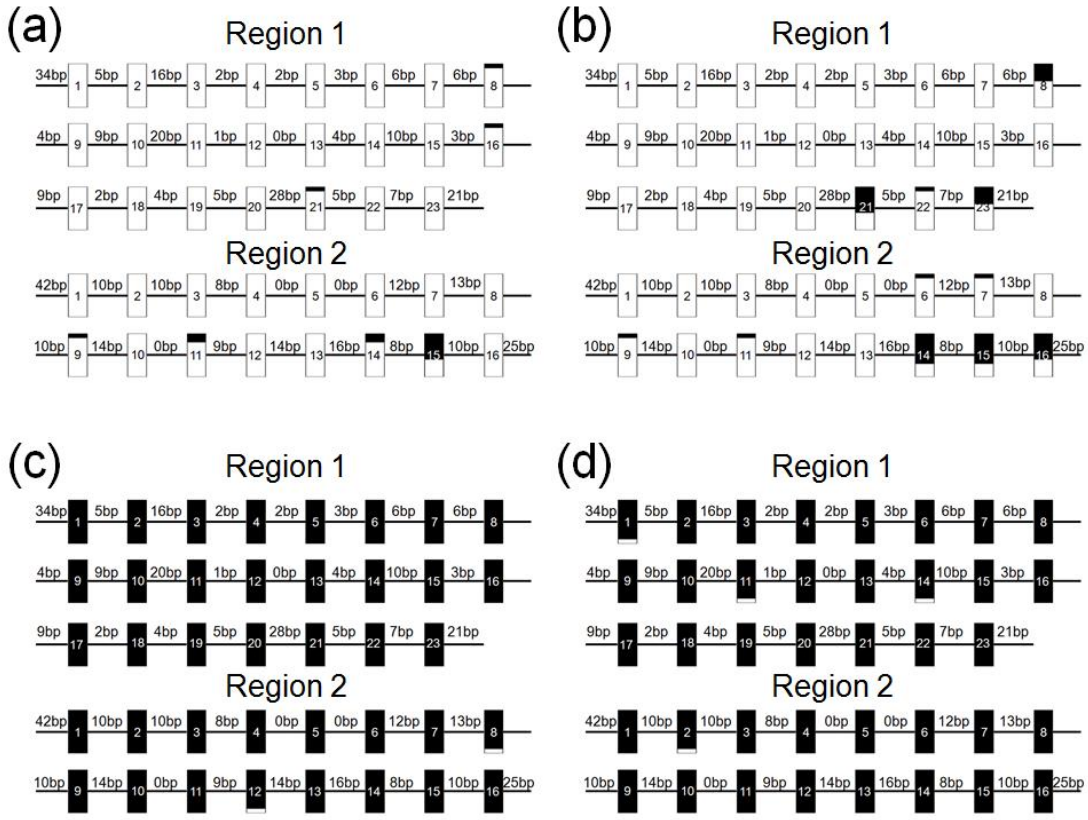

Fig. 2-4. Methylation status of the CpG sites in regions 1 and 2 within the *ABCB1* gene, examined by bisulfite sequencing analysis in the GL-1 (a), CLBL-1 (b), UL-1 (c), and Ema (d) cells. The CpG island of *ABCB1* gene was hypermethylated in the GL-1 and CLBL-1 cells, although those in the UL-1 and Ema cells were hypomethylated. Each box represents one CpG site. The white boxes represent methylated CpG sites, the black boxes represent unmethylated CpG sites, and the proportion of the white area in each box represents the methylated rate of each CpG site. The numbers in the box indicate the order of each CpG site within the regions. Numbers between boxes indicate the number of bases between CpG sites.

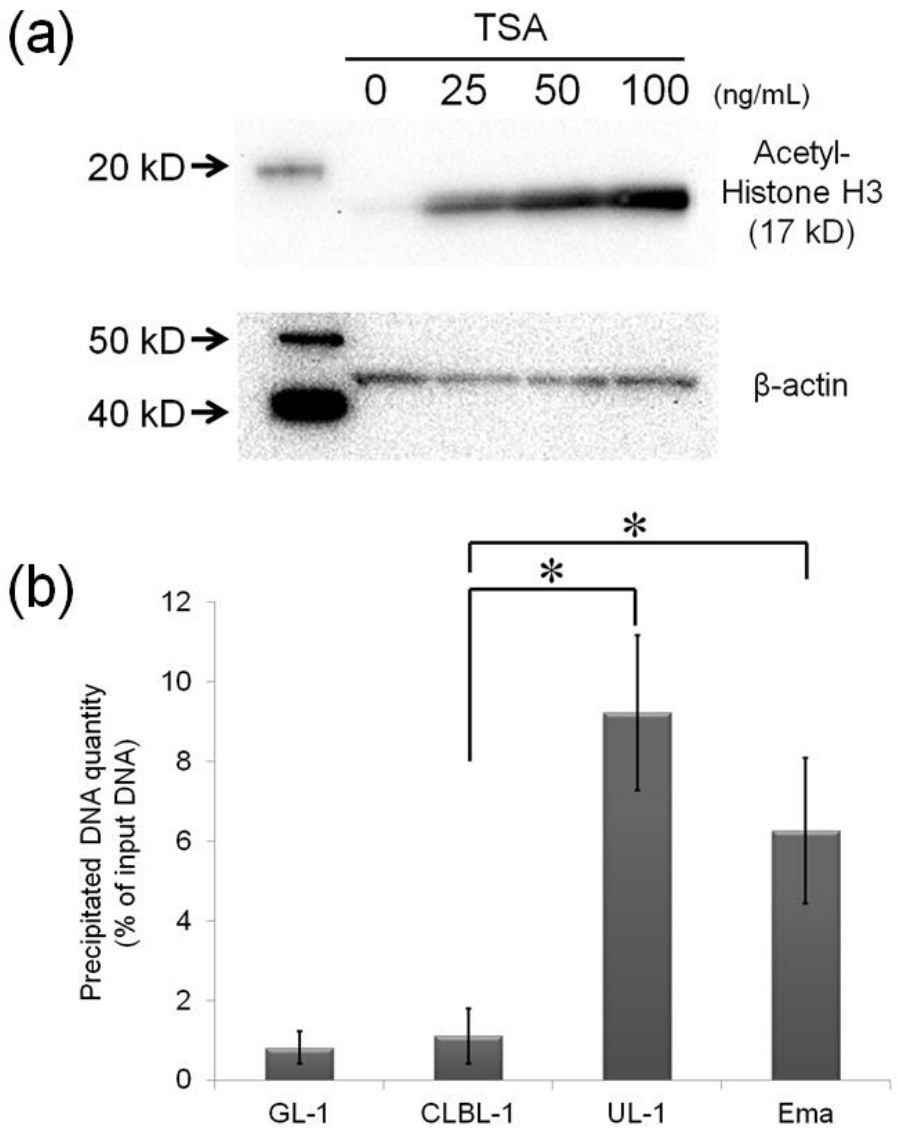

Fig. 2-5. (a) Results of Western blotting for acetyl-histone H3 and β-actin after treatment with trichostatin A (TSA) in UL-1. (b) Comparison of the levels of histone H3 acetylation upstream of *ABCB1* exon 1 using real-time PCR after ChIP assay of the four canine lymphoid tumor cell lines. Compared with the drug-resistant cell lines (UL-1 and Ema cells), the drug-sensitive cell lines (GL-1 and CLBL-1 cells) exhibited lower levels of histone H3 acetylation. **P* <0.01.

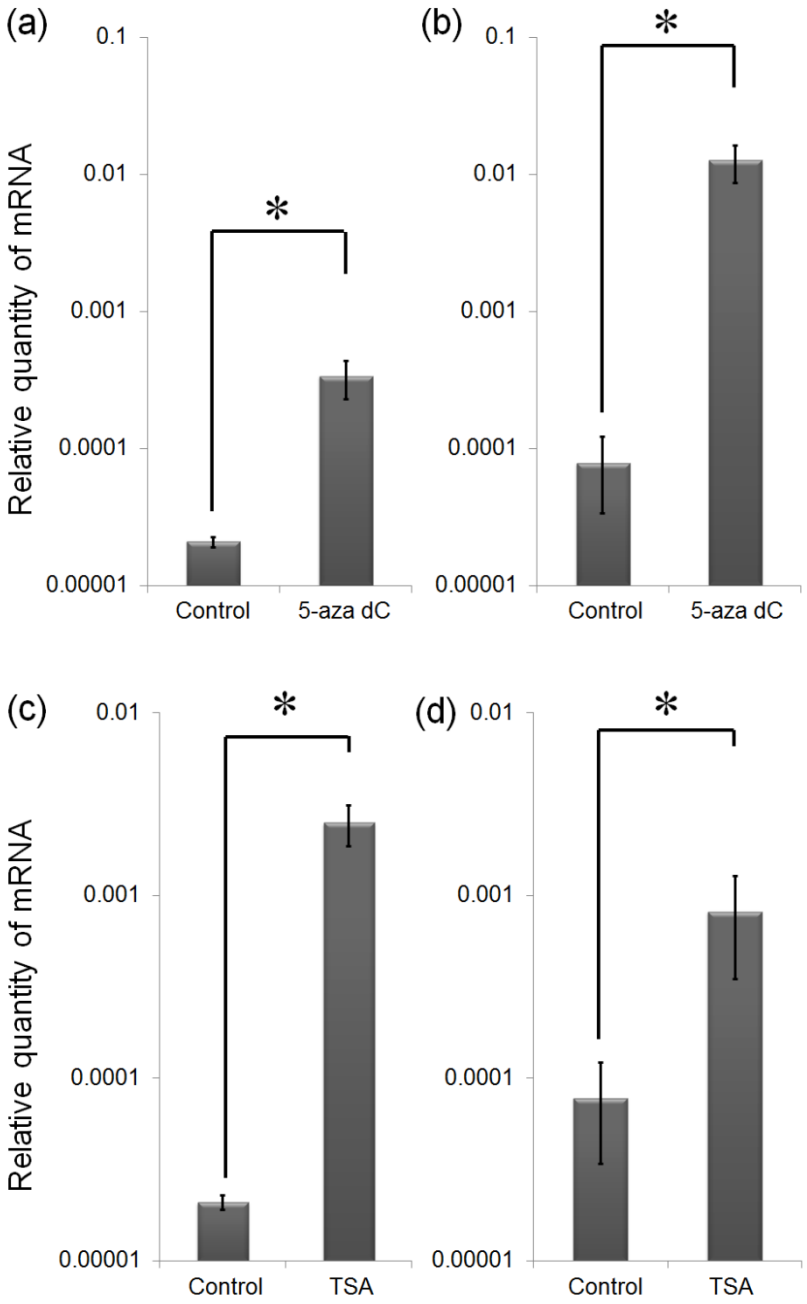

Fig. 2-6. Changes in the amounts of *ABCB1* mRNA after treatment with 5-aza dC in GL-1 (a) and CLBL-1 (b), and those after treatment with TSA in GL-1 (c) and CLBL-1 (d). Both of the treatments significantly increased the amount of *ABCB1* mRNA in GL-1 and CLBL-1 cells. **P* <0.01.

Chapter 2-2

DNA methylation profiles of the CpG island of

ABCB1 gene in dogs with lymphoma

Abstract

The study in this chapter examined the DNA methylation status of *ABCB1* gene in primary tumor cells of dogs with lymphoma. Twenty-seven dogs with multicentric B-cell high-grade lymphoma were included, and 19 were chemotherapy-sensitive and 8 were chemotherapy-resistant. DNA methylation profile of the CpG island of *ABCB1* gene was analyzed by bisulphite sequencing and real-time MSP in lymphoma cell samples. qRT-PCR for *ABCB1* gene was conducted to measure the amount of its mRNA. Correlation between the amount of *ABCB1* mRNA and the methylation rate was examined. As a result, the CpG island of *ABCB1* gene was shown to be hypomethylated in most dogs in both the chemotherapy-sensitive and -resistant groups by bisulphite sequencing and real-time MSP. There was no significant difference in the methylation rate between the two groups and no significant correlation was noted between the methylation rate and the mRNA expression level. In conclusion, the expression of *ABCB1* gene was not suppressed by hypermethylation of its CpG island in most dogs with lymphoma regardless of their chemotherapy sensitivity.

Introduction

Lymphoma is the most common hematologic neoplasm in dogs (Dobson et al., 2002) and a representative tumor that responds to chemotherapy. It was reported that treatment with chemotherapeutic agents for dogs with lymphoma resulted in high rates of complete remission (CR) ranging from 76.3% to 92.3% (Garrett et al., 2002; Simon et al., 2006). However, most of the patients with lymphoma relapse. Efficacy of rescue protocols is limited after acquisition of multidrug resistance (MDR) to the agents used in remission induction therapy. Therefore, the mechanisms that confer MDR phenotype to the tumor cells have been warranted to overcome the difficulties of dog lymphoma treatment.

Among molecules that have been shown to induce the MDR phenotype, P-glycoprotein (P-gp) (coded by *ABCB1* gene, and formerly called as *MDR1* gene) has been one of the most studied molecules in humans (O'Connor, 2007) and dogs (Bergman, 2003; Lee et al., 1996; Moore et al., 1995). This molecule is expressed on the cellular membrane and efflux drugs from cytoplasm to cell exteriors. Thus, the overexpression of P-gp reduces the intracellular concentration of the substrate chemotherapeutic agents. However, the mechanisms that induce overexpression of P-gp are not well understood.

Epigenetics has been intensively investigated to understand the regulation system of gene expression without changing the nucleotide sequence of the genome. The main epigenetic mechanisms include DNA methylation, modifications to chromatin structure, loss of imprinting, and non-coding RNA (Gibney and Nolan, 2010). Among these

mechanisms, DNA methylation in the CpG island is associated with silencing gene expression. In human medicine, an inverse correlation has been observed between DNA methylation in the CpG island of the promoter region of *ABCB1* and mRNA expression in various tumor cell lines (David et al., 2004; Desiderato et al., 1997; El-Osta et al., 2002; Kantharidis et al., 1997). In addition, similar reverse correlation was also observed in the primary tumor cells collected from patients with acute myeloid leukemia (AML) (Nakayama et al., 1998) and bladder cancer (Tada et al., 2000). These studies in human medicine suggested a possibility that the methylation status of the CpG island in its promoter region was associated with the expression level of *ABCB1* gene, thereby influencing the acquisition of the MDR phenotype in tumor cells.

I revealed that an inverse correlation was observed between DNA methylation of the CpG island of *ABCB1* and mRNA expression levels in canine lymphoid tumor cell lines in chapter 2-1. However, the epigenetic regulation of *ABCB1* has not been explored in primary tumor cells obtained from dogs with lymphoma. The purpose of the study in this chapter was to analyze the DNA methylation profile of *ABCB1* in the primary tumor cells collected from dogs with lymphoma with and without the MDR phenotype.

Materials and Methods

Dogs

Dogs referred to the Veterinary Medical Center of the University of Tokyo and diagnosed with multicentric lymphoma between July 1, 2008 and June 31, 2013 were enrolled in the present study. Diagnosis was made based on cytological examination of FNA samples obtained from lymph nodes using the updated Kiel classification (Fournel-Fleury et al., 1997). Discrimination between B-cell and T-cell lymphomas was defined by PCR amplification tests for the antigen receptor gene rearrangements as previously described (Burnett et al., 2003; Valli et al., 2006). Only dogs that were diagnosed with B-cell high-grade multicentric lymphoma and met the definition as described below were included in this study. The clinical stage was evaluated based on the results of physical examination, complete blood count, thoracic and abdominal radiographs and abdominal ultrasounds using the WHO staging criteria (Owen, 1980). Written consent was obtained from all dog owners prior to study enrollment.

Chemotherapy

Veterinarians measured the size of the enlarged lymph nodes with calipers at each admission, and the response to treatment was evaluated based on the response evaluation criteria for canine lymphoma (Vail et al., 2010). In the evaluation, complete response and partial response were seen as indicating chemotherapy-sensitive status, while stable disease and progressive disease were regarded as chemotherapy-resistant status.

A CHOP-based chemotherapy, UW-25 protocol (Garrett et al., 2002), was used as a standard multidrug combination chemotherapy protocol. However, administration of L-asparaginase was omitted in all cases, because L-asparaginase was reported to have no influence on the efficacy of the UW-25 protocol (MacDonald et al., 2005). Dogs that received a corticosteroid before chemotherapy were not excluded in the present study.

Definition of patient groups

Chemotherapy-sensitive dogs were defined as those who had not received any chemotherapeutic agent except for a corticosteroid before the application of the UW-25 protocol and achieved complete response or partial response by week 5 of the UW-25 protocol. Tumor cell samples were collected just prior to the chemotherapy at the initial consultation. Chemotherapy-resistant dogs were defined as who underwent a relapse after achieving a response by the UW-25 protocol and did not respond to any of the three chemotherapeutic agents used in the UW-25 protocol including cyclophosphamide, doxorubicin, and vincristine. Tumor cell samples of the chemotherapy-resistant dogs were collected after confirming the non-responsiveness to these three drugs.

Analysis of methylation profile of ABCB1 gene

DNA methylation profile of the CpG island in *ABCB1* was analyzed using bisulphite sequencing and real-time MSP as introduced in chapter 2-1. Genomic DNA was extracted from FNA samples using QIAmp DNA Blood Mini Kit (Qiagen). Bisulphite conversion was performed on 2 μg DNA using MethylEasy *Xceed* Rapid DNA Bisulphite Modification Kit (TaKaRa Bio) and converted DNA was stored at -80°C until batch analysis. Sequences of primer pairs used for bisulphite sequencing and

real-time MSP are shown in Table 2-3 and locations of the analyzed regions are shown in Fig. 2-7. In real-time MSP, two regions were examined; upstream and downstream regions.

In bisulphite sequencing, 200 ng of converted DNA was amplified using Ampli Taq Gold DNA polymerase (Applied Biosystems) according to the manufacturer's instructions. The PCR products were inserted into a T/A cloning vector (pGEM-T Easy Vector Systems, Promega), and transferred into competent *Escherichia coli* (strain DH5α). Plasmid DNA was extracted with NucleoSpin Plasmid Quick Pure (MACHEREY-NAGEL). Sequence determination of the extracted plasmid DNA was conducted using BigDye terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems) and Applied Biosystems 3130xl genetic analyzer (Applied Biosystems). Fifteen DNA clones derived from each PCR product were sequenced. Methylation rate was calculated for each DNA clone examined by dividing the number of methylated sites by the total number of CpG sites examined. The median methylation rate was then calculated for all DNA clones.

In real-time MSP, both the completely methylated DNA control and completely unmethylated DNA control were prepared from genomic DNA of canine lymphoma cell line, UL-1 (Yamazaki et al., 2008), using CpG methyltransferase *M.SssI* (New England Biolabs) and illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare), respectively. Complete methylation and demethylation were verified by bisulphite sequencing as described above. These control DNAs were then inserted into a T/A cloning vector and transferred into competent *Escherichia coli* (strain DH5α). Assay-specific standard curves for methylated and unmethylated DNAs were prepared in triplicate with serial (10x) dilution of plasmid extracted from the *Escherichia coli*.

Real-time MSP was performed in duplicate using an Episcope MSP Kit (TaKaRa Bio) and Thermal Cycler Dice Real Time System TP800 (TaKaRa Bio) with 100 ng of DNA. The absolute quantity of methylated or unmethylated DNA was calculated from the standard curves. Methylation rate in real-time MSP was calculated by dividing mean absolute quantity of methylated DNA by the sum of mean absolute quantity of methylated and unmethylated DNA.

qRT-PCR

For estimation of the relative quantity of mRNA of *ABCB1* gene, qRT-PCR was performed as described in chapter 1. Total RNA was extracted from each FNA samples using RNAqueous (Ambion) and stored at -80°C until batch analysis. After treatment with DNase (DNase I, Invitrogen), cDNA was synthesized with total RNA using PrimeScript RT reagent Kit (Perfect Real Time). The same primer pair for qPCR of *ABCB1* gene expression was used as in chapter 1 (Table 2-3). For normalization of the amount of cDNA sample, *TBP* gene was selected as the internal reference based on result of chapter 1. qPCR was performed using SYBR Premix Ex Taq II (Perfect Real Time) and Thermal Cycler Dice Real Time System TP800 (TaKaRa Bio) with cDNA synthesized from 10 ng of total RNA. For each gene, an assay-specific standard curve with serial (10x) dilution of cDNA from UL-1 cells was prepared in triplicate. The relative expression level of *ABCB1* calculated from the standard curve was divided by that of *TBP* gene for normalization. qRT-PCR was performed in triplicate.

Statistical analysis

Correlation of the methylation rate in the CpG island of *ABCB1* gene with the relative

quantity of mRNA was examined using the Spearman's rank correlation test. The Mann-Whitney *U*-test was used for comparison of the methylation rate between chemotherapy-sensitive and -resistant dogs. Values of $P < 0.05$ were considered to be significant. Statistical analysis was performed with a commercially available software (JMP version 5.0.1, SAS Institute).

Results

Dogs

Twenty-seven dogs with B-cell high-grade multicentric lymphoma were included in the study in this chapter. Breeds included Welsh Corgi Pembroke (4), Pug (3), Miniature Dachshund (3), Golden Retriever (2), Shiba Inu (2), French Bulldog (2), and Beagle (2), and 1 each of Shih Tzu, Doberman, mixed, Bulldog, Bernese Mountain Dog, Scottish Terrier, Pomeranian, West Highland White Terrier, and Australian Kelpie. The median age was 8 years (range, 2 to 13 years) and the median body weight was 10.9 kg (range, 4.2 to 33.0 kg). There were 8 female (4 spayed) and 19 male (7 castrated) dogs. Lymphoma was classified based on WHO clinical stage as stage II in 1 dog, stage III in 3 dogs, stage IV in 8 dogs, and stage V in 15 dogs, and WHO substage a in 17 dogs and substage b in 10 dogs. Based on the definition of patient groups, 19 dogs were included in the chemotherapy-sensitive group and 8 dogs were included in the chemotherapy-resistant group. Records of corticosteroid administration before chemotherapy were found in 3 of the 27 dogs with lymphoma. In these 3 dogs, prednisolone was orally administrated at 1 mg/kg/day for 6 days, 1mg/kg/day for 16 days, and 2mg/kg/day for 3 days, respectively.

Bisulphite sequencing- Among dogs included, 4 dogs were randomly selected from each group, and DNA methylation profiles of the CpG island of *ABCB1* gene in the tumor cells were analyzed in detail using bisulphite sequencing. In a chemotherapy-sensitive patient, dog 1, DNA methylation rate was consistently more than 50% in all of the 23 CpG sites of *ABCB1* gene (Fig. 2-8). On the contrary, most of the 23 CpG sites were hypomethylated in 2 chemotherapy-sensitive dogs (dogs 3 and 4).

Another chemotherapy-sensitive patient, dog 2, showed an intermediate DNA methylation pattern. In a chemotherapy-resistant patient, dog 5, degree of DNA methylation was variable among the 23 CpG sites of *ABCB1* gene. However, most of the 23 CpG sites were unmethylated in 3 chemotherapy-resistant patients (dogs 6, 7, and 8). There was no apparent difference in the methylation rates of CpG sites between the chemotherapy-sensitive and chemotherapy-resistant groups. Moreover, the methylation profile of each CpG site varied among 15 DNA clones derived from each patient of both groups.

Real-time MSP- DNA methylation profiles were also analyzed using real-time MSP in 19 chemotherapy-sensitive dogs and 8 chemotherapy-resistant dogs. In the upstream region of the CpG island of *ABCB1*, methylation rates were lower than 15% in 17 of 19 chemotherapy-sensitive dogs, and the median (range) of methylation rates was 0.409% (0-14.1%) in these dogs, although methylation rates in 2 dogs were more than 50% (57.2% and 90.2%) (Fig. 2-9A). In the chemotherapy-resistant group, methylation rates in the upstream region were lower than 1.5% in 6 of 8 dogs, and the median (range) of methylation rates was 0.635% (0-1.42%) in these dogs, although methylation rates in 2 dogs were more than 40% (41.7% and 59.4%).

Similarly, in the downstream region of the CpG island, methylation rates were lower than 10% in 18 of 19 chemotherapy-sensitive dogs, and the median (range) of methylation rates was 0.332% (0-7.79%) in these dogs, although methylation rate in 1 dog was 55.2% (Fig. 2-9B). In chemotherapy-resistant group, methylation rates in downstream region were lower than 2% in 6 of 8 dogs, and the median (range) of methylation rates was 0.276% (0-1.98%) in these dogs, although methylation rates in 2 dogs were more than 40% (41.5% and 66.0%). There was no significant difference in

the methylation rate between chemotherapy-sensitive and -resistant dogs in either the upstream or downstream region (P = 0.82 and 0.48, respectively).

Relative quantity of *ABCB1* gene mRNA- qRT-PCR was examined using FNA samples of 16 chemotherapy-sensitive dogs. The *ABCB1* mRNA was detected in all 16 dogs. However, there was no significant correlation between the methylation rate of the CpG island obtained in real-time MSP examination and the relative quantity of *ABCB1* mRNA in both the upstream and downstream regions (ρ^2 = 0.00038 and 0.022, and P = 0.94 and 0.57, respectively) (Fig. 2-10).

Discussion

In the present study, the DNA methylation status of *ABCB1* was analyzed by using bisulphite sequencing and real-time MSP in dogs with lymphoma.

In bisulphite sequencing, methylation rates were variable among 8 dogs examined, and the DNA methylation profile of each CpG site varied among 15 DNA clones derived from each patient. These results raised a possibility that canine lymphoma tissues might be composed of a heterogeneous population with various methylation profiles of CpG sites of *ABCB1*. In a previous study in humans, the DNA methylation profile of *ABCB1* gene was analyzed using bisulphite sequencing in tumor cells of AML patients before chemotherapy (Fryxell et al., 1999). The study indicated that the DNA methylation status was different among patients in a subset of CpG sites of the CpG island and agree with the results obtained from the present study. However, methylation patterns of the CpG sites were similar among DNA clones derived from human patients with AML. The disagreement compared with the results in the study of this chapter in canine lymphoma might be due to the difference in the number of DNA clones analyzed in bisulphite sequences, since only 4 to 5 DNA clones from each patient were analyzed in the previous study (Fryxell et al., 1999).

In real-time MSP, CpG sites of *ABCB1* gene examined in this study were not methylated in most of the tumor cells obtained from dogs with lymphoma, regardless of sensitivity for chemotherapy, and there was no significant difference in methylation rate between the chemotherapy-sensitive and -resistant dogs. It was reported that *ABCB1* was in a hypermethylated state in 66% of human AML patients who achieved CR by

chemotherapy when examined by PCR with methylation-sensitive restriction enzymes (Nakayama et al., 1998). Although the methylation rates of CpG island of *ABCB1* gene in chemotherapy-sensitive lymphoma dogs were lower compared to the rates in the human AML patients, the reason for the difference was not clear. Another study examined the methylation status of *ABCB1* in human hematopoietic malignancy patients including 5 patients with non-Hodgkin's lymphoma, but the methylation status of the gene in these non-Hodgkin's lymphoma patients was not described in detail (Shi et al., 2011). Since it is thought that canine lymphoma shares many similarities with human non-Hodgkin's lymphoma (Vail and MacEwen, 2000), further studies that compare the DNA methylation status of *ABCB1* gene between human and canine lymphoma cells might help to utilize canine lymphoma as a spontaneous animal model of human non-Hodgkin's lymphoma.

In this study, there was no significant correlation between the amount of *ABCB1* gene mRNA and methylation rate in real-time MSP. I revealed that DNA methylation in the CpG island regulated the expression of *ABCB1* gene mRNA in canine lymphoma cell lines in chapter 2-1, and the gene expression of *ABCB1* gene was silenced in cell lines with hypermethylated *ABCB1* gene. In the present study, qRT-PCR was conducted by the same method as used in chapter 2-1, and the expression levels of *ABCB1* gene in canine primary lymphoma cells in the present study were markedly higher than those in canine cell lines with hypermethylated *ABCB1* gene, including CLBL-1, derived from dogs with B-cell high-grade multicentric lymphoma. Therefore, it was possible that *ABCB1* gene was in a hypomethylated state and the expression of this gene was not silenced in tumor cells of chemotherapy-sensitive dogs. This might be the reason for no correlation between relative quantities of *ABCB1* gene mRNA and methylation rates

calculated from the results of real-time MSP.

As shown in chapter 2-1, DNA methylation is involved in the regulation of canine *ABCB1* gene expression. However, the expression of *ABCB1* gene was not silenced by DNA methylation in canine lymphoma patients before chemotherapy in the study in this chapter. This observation suggests that mechanisms other than DNA demethylation might play a major role when lymphoma cells acquire the increased expression of *ABCB1* gene.

One limitation in the present study was that the number of chemotherapy-resistant dogs was small because the resistance for all three chemotherapeutic agents could not be confirmed in a certain number of dogs due to the rapid progress of lymphoma.

As another limitation, the expression level of *ABCB1* gene was not examined in the primary tumor cells obtained from dogs with chemotherapy-resistant lymphoma. It was unclear whether overexpression of *ABCB1* gene occurred and what mechanisms induced the MDR phenotype in chemotherapy-resistant patients included in the present study.

In conclusion, the present study indicated that canine lymphoma tissues contained a heterogeneous population with various methylation profiles of CpG sites of *ABCB1* gene. Moreover, it was also shown that the CpG island of *ABCB1* gene was not in hypermethylated state and expression of this gene was not silenced in most of the dogs with lymphoma, regardless of their different chemotherapy sensitivity.

Table 2-3.

Primers used for bisulphite sequencing, real-time MSP and quantitative RT-PCR (qRT-PCR).

	Forward	Reverse	Amplicon size (bp)	Genbank accession number
Primer pair for bisulphite sequencing	5'-AGGAGGATATTTTTTTTGGA-3' (13742947-13742966)	5'-AAAAATCAAACTTTAAAAACCC-3' (13742715-13742736)	252	NC_006596
Methylated primer pair for real-time MSP in upstream region	5'-TAATTAGTATTCGGGCGAGC-3' (13742899-13742918)	5'-CCTACGTCCCGAAAAATAAA-3' (13742806-13742825)	113	
Unmethylated primer for real-time MSP in upstream region	5'-TTGTAATTAGTATTTGGGTGAGT-3' (13742899-13742921)	5'-TCCTACATCCCAAAAAATAAAAC-3' (13742805-13742827)	117	
Methylated primer pair for real-time MSP in downstream region	5'-AGGTTGCGAAGGAAAGTTC-3' (13742670-13742688)	5'-AACCAAACGCGAAAAACTAA-3' (13742589-13742608)	100	
Unmethylated primer for real-time MSP in downstream region	5'-GGAGGTTGTGAAGGAAAGTTT-3' (13742670-13742690)	5'-AACCAAACACAAAAAACTAAAA-3' (13742589-13742610)	102	
Primer pair for *ABCB1* gene in qRT-PCR	5'-ACTCGGGAGCAGAAGTTTGA-3' (2734-2753)	5'-AATGAGACCCCGAAGATGTG-3' (2809-2828)	95	NM001003215
Primer pair for *TBP* gene in qRT-PCR	5'-CTATTTCTTGGTGTGCATGAGG-3' (-145 - -124)	5'-CCTCGGCATTCAGTCTTTTC-3' (-31 - -50)	96	XM_849432

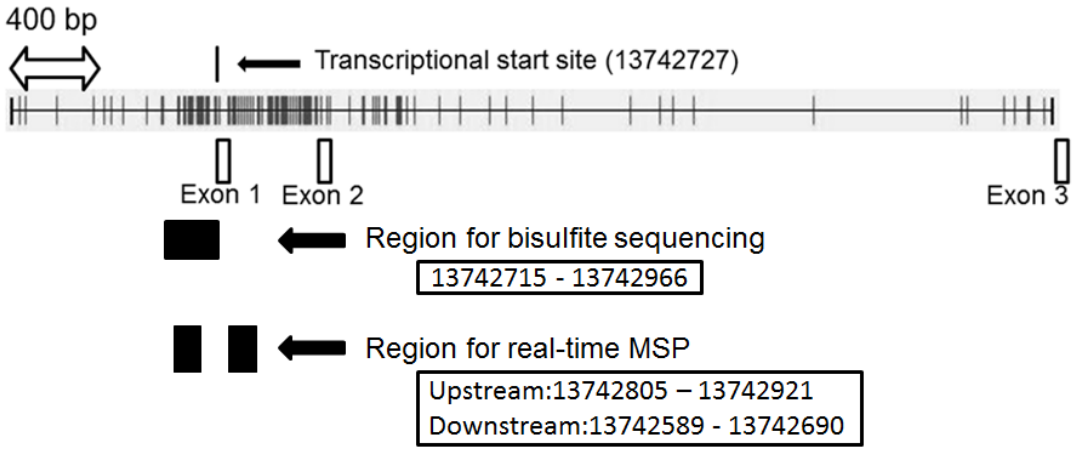

Fig. 2-7. CpG island in canine *ABCB1* gene and the locations of the regions examined by bisulphite sequencing and real-time MSP. Each gray vertical line represents a CpG site. The numbers in the boxes and parenthesis indicate the nucleotide numbers registered in GenBank (NC_006596).

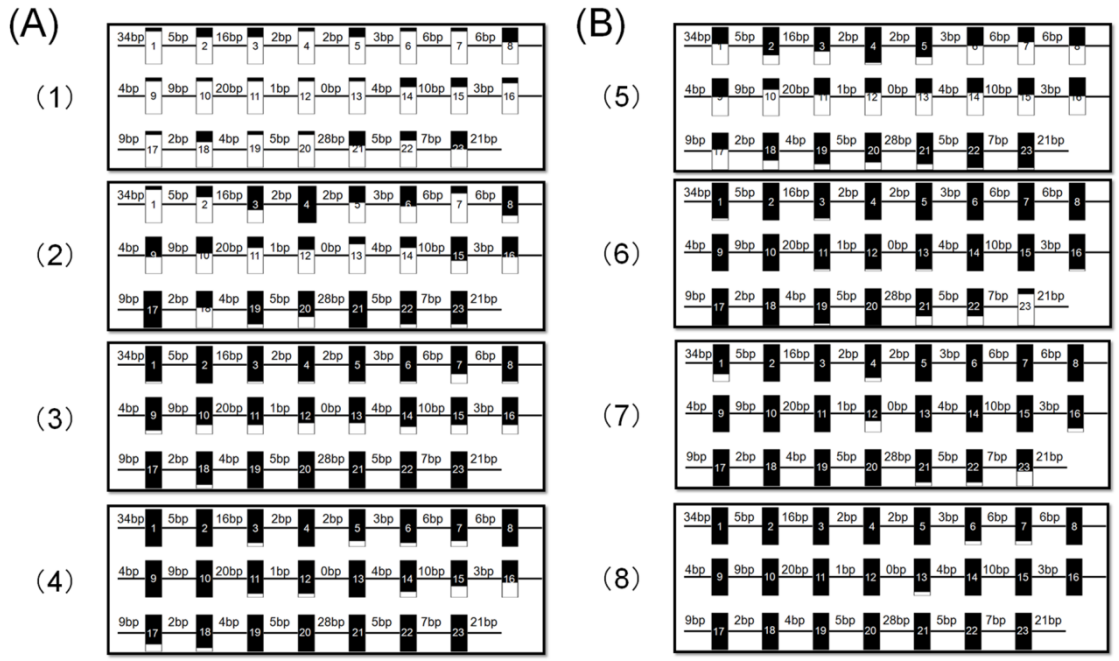

Fig. 2-8. DNA methylation profiles of CpG sites of *ABCB1* gene in chemotherapy-sensitive dogs (A, Dog Nos. 1-4) and -resistant dogs (B, Dog Nos. 5-8). Each box represents one CpG site. The white represents methylated CpG sites and black represents unmethylated CpG sites, and the rate of area of white in each box represents the rate of DNA clones with methylated cytosine in each CpG site. Numbers in the box indicate the order of each CpG site within regions. Numbers between boxes indicate the number of bases between CpG sites. The median (rages) of methylation rates in chemotherapy-sensitive dogs were 74.5% (0-100%) in dog 1, 46.6% (8.7-69.6%) in dog 2, 11.7% (0-65.2%) in dog 3 and 7.36% (0-17.4%) in dog 4, and those in chemotherapy-resistant dogs were 54.3% (0-87.0%) in dog 5, 7.36% (0-17.4%) in dog 6, 5.14% (0-13.0%) in dog 7 and 0.932% (0-4.35%) in dog 8, respectively.

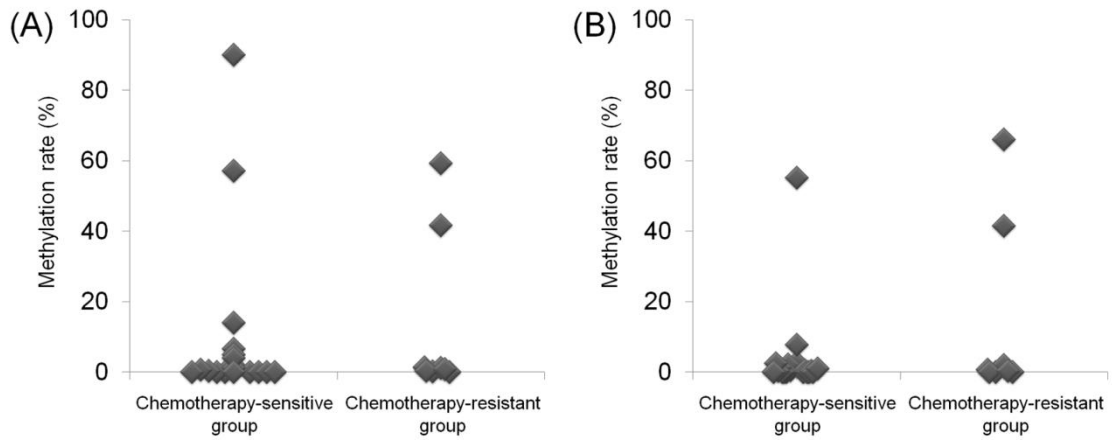

Fig. 2-9. Methylation rates of *ABCB1* gene in the upstream (A) and downstream (B) CpG motifs of *ABCB1* gene in chemotherapy-sensitive dogs (n=19) and -resistant dogs (n=8).

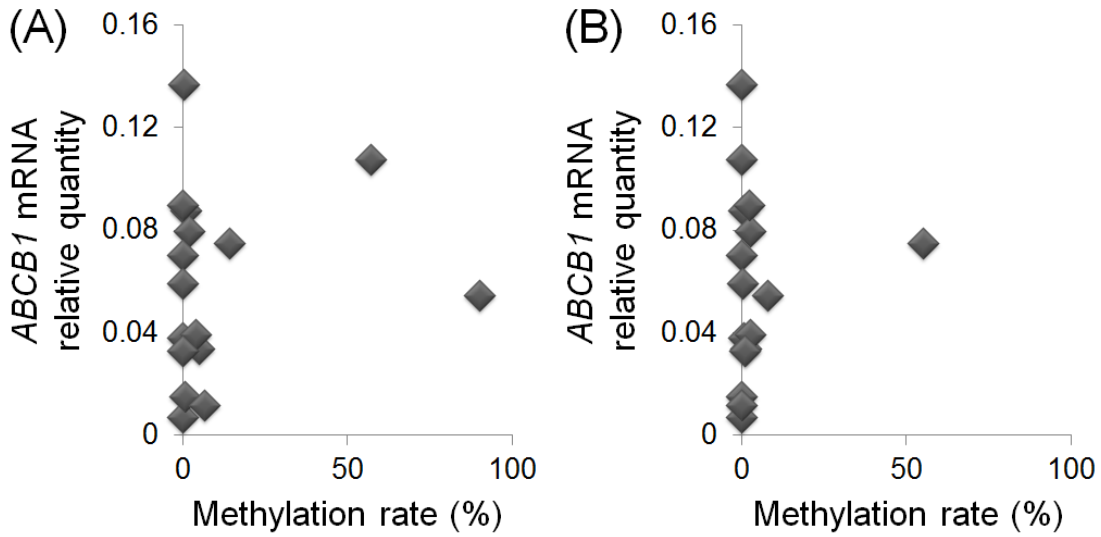

Fig. 2-10. Correlation of methylation rates of *ABCB1* gene with the relative quantities of *ABCB1* mRNA in chemotherapy-sensitive dogs. There was no significant correlation between these two parameters in both of the upstream (A) and downstream (B) regions of CpG island (ρ^2 = 0.00038 and 0.022, and P = 0.94 and 0.57, respectively).

Chapter 3

Regulation of *ABCB1* and *LRP* genes through

MAPK/ERK pathway in canine lymphoma cell lines

Abstract

Cancer stem cell (CSC) concept has been recognized as a key for elucidation of the mechanisms that confer the MDR phenotype to tumor cells, and side population (SP) fraction has been shown to be enriched by cells with CSC phenotype. The purpose of the study in this chapter was to indicate mechanism that induces the difference of phenotype between SP and other population (major population; MP) using 2 canine lymphoma cell lines. Expression levels of *ABCB1* and *LRP* genes, which encode efflux pumps, were significantly higher in SP than MP. Microarray analysis revealed the upregulation of expression of *TGF-β type II receptor* gene in SP compared with MP, and MAPK/ERK pathway was more activated in SP than MP. Stimulation of MAPK/ERK pathway significantly increased the mRNA expression of both of *ABCB1* and *LRP* genes. These results indicated the increased expression of the efflux pumps through MAPK/ERK pathway in SP cells.

Introduction

Multidrug resistance (MDR) is one of the major obstacles during the chemotherapy in the patients with various cancers. Although a lot of molecules that contribute to the development of MDR have been reported (Gimenez-Bonafe et al., 2009; Harris and Hochhauser, 1992), the mechanisms of acquisition of MDR in tumor cells are not well understood.

Cancer stem cell (CSC) has recently been considered as the key for elucidation of the mechanisms of acquisition of MDR in various tumors (Baguley, 2010). In this concept, it is thought that a small population with stem cell-like characteristics is initially resistant to conventional chemotherapy and these cells are cause of relapse and acquisition of MDR. Therefore, the clarification of the mechanisms that confer MDR phenotype to CSC is important for elucidation of the mechanisms of acquisition of MDR in tumor tissues.

The Hoechst 33342 dye exclusion test is one of the methods to detect stem cells from various normal and tumor tissues (Moserle et al., 2010). Although uptake of this reagent occurs universally in all cells, efflux is less permissive. Only a small population of cells shows high potency to exclude the dye. These cells are referred to as side population (SP) and are distinguished from the remaining major population (MP). SP has been identified and characterized in various tumors including non-Hodgkin's lymphoma such as human follicular lymphoma (Lee et al., 2012) and murine mantle cell lymphoma model (Vega et al., 2010), and SP in tumor cell lines and primary tumor cells have been shown to be enriched by CSC (Moserle et al., 2010). SP is considered to

efflux the dye using highly expressed ABC transporter family members such as P-glycoprotein (P-gp) and ABCG2 (Hadnagy et al., 2006; Wu and Alman, 2008). SP has been shown to have MDR phenotype and such high efflux potency is thought to be the mechanism to confer MDR phenotypes on SP (Zhang et al., 2010). Therefore, it might be important to study on mechanisms that activate the expression of these efflux pumps in SP. However, such mechanisms have not been clarified.

As in human medicine, SP has been identified in canine lung adenocarcinoma (Nemoto et al., 2011) and mammary tumor cells (Ferletta et al., 2011), and it was shown that these SP possessed stem cell-like characteristics. However, the mechanisms that confer MDR phenotype upon SP have been unclear. In addition, SP in canine lymphoma cells has not been identified.

In chapter 2, it was revealed that epigenetic regulation was associated with the regulation of *ABCB1* gene expression, but DNA methylation was not associated with the acquisition of MDR phenotype in canine lymphoma cells. Therefore, other mechanisms than epigenetic regulations should be investigated to elucidate the mechanisms that induce overexpression of drug efflux pumps in canine MDR lymphoma cells.

The purpose of the study in this chapter was to identify SP in canine lymphoma cell lines and to reveal the mechanisms that activate the expressions of efflux pumps in SP. Furthermore, more detailed mechanisms that activate the function of the efflux pumps were tried to be clarified.

Materials and Methods

Cell cultures

In the present study, 2 canine T-cell lymphoma cell lines, UL-1 (Yamazaki et al., 2008) and Ema (Hiraoka et al., 2009), were used. These cell lines were cloned from a single cell using the limit dilution method before using in the following experiments, and grown in RPMI 1640 supplemented with 10% fetal calf serum and cultured at 37 °C in a humidified atmosphere of 5% CO_2.

Hoechst 33342 dye efflux test

SP were detected as previously described (Arends et al., 2009). Briefly, cells were incubated in the presence of 5 μg/mL Hoechst 33342 (Sigma-Aldrich, Zwijndrecht, Netherlands) for 90 min. Another set of cells was pre-incubated with the transport blocker, reserpine (50 μM; Sigma-Aldrich), for 20 min before Hoechst 33342 incubation. After washing, Hoechst 33342-stained cells were resuspended in ice-cold phosphate buffered saline containing 2mM EDTA and 1μg/mL propidium iodide (PI; Sigma-Aldrich), and kept on ice before and during fluorescence-activated cell sorting (FACS) analysis using FACSAria (BD Biosciences, San Jose, CA, USA). After analysis, SP and MP were sorted separately.

Relative quantification of mRNA of efflux pumps

In the comparison of expression level of efflux pumps between sorted SP and MP, the expressions of *ABCB1*, *ABCC1*, *LRP* and *ABCG2* genes were analyzed in the present study. The efflux pumps coded by *ABCB1*, *ABCC1* and *ABCG2* genes are

known as representative ABC-transporters associated with acquisition of multidrug resistance in tumor cells (Modok et al., 2006). LRP has different function from these efflux pumps and redistributes drugs from the nucleus to the cytoplasm because it is expressed on nuclear membrane (Gromicho et al., 2011). For the comparison of the expressions of these efflux pumps between SP and MP, mRNA of *ABCB1* (coding P-gp), *ABCC1*, *LRP* and *ABCG2* were relatively quantified using real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) as described in chapter 1 using the same primer pairs (Table 3-1). For normalization of the amount of cDNA sample, *TATA box binding protein* (*TBP*) gene was used as an internal reference based on the result obtained in chapter 1. The fold-change in relative quantity of each gene mRNA of SP were calculated by comparing with that of MP. Real-time RT-PCR was performed in triplicate.

Cell-cycle analysis

Cell cycles of sorted SP and MP cells were analyzed by calculating DNA content. Cells were fixed and permeabilized using BD cytofix/Cytoperm Kit (BD biosciences), and stained with 7-amino-actinomycin D (BD Biosciences). Flow cytometric analysis was performed with FACSCalibur (BD Biosciences). The cell-cycle analysis was conducted using Flowjo software (TreeStar, Ashland, OR, USA).

Microarray analysis

Total RNA was isolated from sorted SP and MP using 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, USA) and RNA integrity numbers were confirmed as above 9.0. The synthesis of cDNA and Cy3 or Cy5 labelled cRNA were conducted using Quick Amp Labeling Kit (Agilent Technologies) and RNA spike-In two-color Kit (Agilent Technologies). The cRNA derived from SP were labelled with Cy3, and that derived from MP were labelled with Cy5. The labelled cRNAs were purified using RNeasy Mini Kit (QIAGEN), and fragmented and hybridized to Canine ver.2 (4×44K) array (Agilent Technologies) using Gene Expression Hybridization Kit (Agilent Technologies). After hybridization, slide was washed with Gene Expression Wash (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). Spots of compromised quality and with low intensity were eliminated from the analysis. The data was normalized by LOWESS method, and Cy5:Cy3 ratios and $\log_2$ values of those were calculated. Filtering and complication of data were conducted using Microsoft Excel. Only those genes, which showed $\log_2$ values greater than 1 (0.5-fold downregulated in SP) or less than -1 (2-fold upregulated in SP) and consistent regulation in UL-1 and Ema, were included in analysis. Among such genes, the fold change of *TGF-β type II receptor* gene was confirmed using real-time RT-PCR with primer pair listed in Table 3-1 as described above.

Western blotting for Smad pathway and MAPK/ERK pathway

For comparison of activation of TGF-β signalling between SP and MP, Western blotting for Smad pathway and MAPK/ERK pathway were examined. SP and MP of Ema were sorted as described above. Then, whole cell lysates were extracted from each

cells, and equal amounts of protein were separated by SDS-PAGE and blotted onto a PVDF membrane as in chapter 2-1. Membranes were blocked in 5%-skimmed milk/tris-buffered saline with Tween20, and then incubated with primary antibody against phospho-Smad2/Smad3 (diluted at 1:500), Smad2 (1:1000), Smad3 (1:1000), Smad4 (1:1000), phospho-p44/42 (ERK1/2) (1:2000), p44/p42 (ERK1/2) (1:1000) and β-actin (1:1000) (Cell Signaling Technology) overnight at 4 °C. After incubation with the secondary antibody of horseradish peroxidase-labeled anti-rabbit IgG (1:3000; Bio-rad Laboratories) 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).

Activation of MAPK/ERK pathway

For activation and MAPK/ERK pathway and its inhibition, phorbol 12-myristate 13-acetate (PMA) (Wako, Osaka, Japan), protein kinase C (PKC) activator, and U0126 (Promega, Leiden, Netherlands), MAPK/ERK kinase (MEK) inhibitor, were used as previously described (Yang et al., 2001; Zhang et al., 2011). Briefly, UL-1 and Ema and these cells pre-incubated with 10 μM U0126 for 1 h were incubated with 200 nM PMA for 8 h, and the relative quantity of *ABCB1* and *LRP* genes mRNA of these cells were compared with non-treated cells using real-time RT-PCR as described above. The fold-change in relative quantity of each gene mRNA of treated cells were calculated by comparing with that of non-treated cells. The increase of phospho-p44/p42 (ERK1/2) by PMA and its inhibition by U0126 were verified using Western blotting as described above.

Changes in rates of SP through activation of MAPK/ERK pathway

To examine the regulation of SP phenotype through activation of MAPK/ERK pathway, the rates of SP were analyzed after incubation with PMA or both of PMA and U0126. Briefly, UL-1 and Ema cells (pretreated with 10 μM U0126 for 1 h or non-treated) were cultured in the presence of 200 nM PMA for 24 h. After wash with appropriate culture medium, the rates of SP cells were analyzed as described above, and these rates were compared with those in non-treated cells. These analysis were examined in three independent assays.

Statistical analysis

The nonparametric Mann-Whitney *U*-test was used for comparison of relative quantities of genes. The same test was used for comparison of the rate of cells that were in G0/G1 phase and comparison for the rates of SP. All statistical tests were two-sided and performed using JMP version 5.0.1 (SAS Institute, Cary, NC, USA). For all tests, $p < 0.05$ was used as the level of significance.

Results

Identification of SP

In the Hoechst 33342 dye exclusion test, a distinct population that apparently effluxed the dye was observed in both of UL-1 and Ema (Fig. 3-1A and B, respectively). This population was identified to be SP, as preincubation with reserpine resulted in the loss of this population of cells in both of UL-1 and Ema (Fig. 3-1C and D, respectively). The percentage of SP in UL-1 and Ema ranged from 0.79 to 1.23% and 10.5 to 16.1%, respectively, in three independent assays.

ABCB1 and LRP genes were upregulated in SP

Among the 4 genes encoding efflux pumps, the amounts of *ABCB1* and *LRP* gene transcripts were shown to be significantly higher in SP than MP in both of UL-1 and Ema ($p < 0.01$) (Table 3-2). No significant difference was observed in the amount of *ABCC1* gene transcript in both of UL-1 and Ema. The expression of *ABCG2* gene was not detected in Ema and its amount was not different between SP and MP of UL-1.

Most of SP were in G0/G1 phase

Although 61.0±2.7% of MP were in G0/G1 phase, 93.5±4.9% of SP were in the G0/G1 phase in UL-1 (Fig. 3-2A) in cell cycle analysis. Also in Ema, 97.1±0.2% of SP were in the G0/G1 phase, whereas 54.1±3.0% of MP were in G0/G1 phase (Fig. 3-2B). In both of two cell lines, the rates of cells that were in G0/G1 phase were significantly larger in SP than MP ($p < 0.01$).

TGF-β type II receptor gene expression was upregulated in SP than MP

In microarray analysis, 86 spots (82 genes) whose expressions were upregulated in SP (Table 3-3) and 33 spots (31 genes) whose expressions were downregulated in SP (Table 3-4) were sorted. Among these sorted genes, *TGF-β type II receptor* gene, which was highly expressed in SP than MP in both of UL-1 and Ema, was focused because TGF-β signaling was reported to be correlated with SP phenotype in human tumor cell lines (Kabashima et al., 2009; Yin et al., 2008). Therefore, the upregulation of *TGF-β type II receptor* gene expression was validated using real-time RT-PCR. Also in real-time RT-PCR, the relative mRNA quantity of the gene in SP was significantly higher than MP in both of UL-1 and Ema ($p < 0.01$) (Table 3-2).

MAPK/ERK pathway was more activated in SP than MP

As downstream of TGF-β signaling, Smad pathway and MAPK/ERK pathway were focused, and the activation states of these pathways were compared between SP and MP of Ema. When Western blotting for Smad pathway was performed, the quantities of phospho-Smad2 and Smad4 of SP were not different compared with MP (Fig. 3-3A). Smad3 and phospho-Smad3 were not detected in both of SP and MP. However, in Western blotting for MAPK/ERK pathway, the quantities of phopho-p44/p42 (ERK1/2) were found to be larger in SP than MP (Fig. 3-3B).

MAPK/ERK pathway regulated the expression of ABCB1 and LRP genes

When UL-1 and Ema were incubated with PMA, the quantities of phosphor-p44/p42 (ERK1/2) were increased compared with non-treated cells (Fig. 3-4). This effect of PMA was inhibited by pre-incubation with U0126. After incubation with

PMA for 8 h, the relative quantity of *ABCB1* (Fig. 3-5A) and *LRP* (Fig. 3-5B) mRNAs were significantly increased compared with non-treated cells in both of UL-1 and Ema ($p < 0.01$). When cells were pre-incubated with U0126 before incubation with PMA for 8 h, the relative quantity of *ABCB1* and *LRP* genes mRNA were significantly smaller compared with cells incubated with PMA alone in both of UL-1 and Ema ($p = 0.02$ in *ABCB1* genes of UL-1 and $p < 0.01$ in others).

PMA stimulation increased the rates of SP

Finally, changes of the rates of SP by PMA stimulation were examined. The rates of SP were significantly increased through PMA stimulation in both of UL-1 and Ema (44.7±6.40%; $p < 0.01$ and 56.7±4.36%; $p < 0.01$) compared with non-treated cells (Fig. 3-6). When the cells were pretreated with U0126, a MAPK/ERK inhibitor, this increase in rates of SP was significantly inhibited in Ema (28.8±6.42%; $p < 0.01$), although such inhibition by the treatment with U0126 was not observed in UL-1 (38.6±9.74%; $p = 0.37$).

Discussion

For elucidation of mechanisms which confer the MDR phenotype on cancer cells, two major models have been proposed; one is ‘activation model’ (Hu et al., 1999), and another is ‘selection and expansion model’ (Goldie and Coldman, 1984). The former proposes that chemotherapeutic drugs directly activate the mechanisms which confer the MDR phenotype. The latter describes that small MDR population expands by drug-induced selection, and eventually overgrows residual drug-sensitive population. However, which model truly reflects the mechanisms of MDR acquisition has been unclear. CSC concept, in which a small population is initially resistant to conventional chemotherapy, supports the latter model. In the present study, SP fraction that has been shown to be enriched with cells with CSC phenotype in various tumor cells was identified in clonal canine lymphoma cell lines. In addition, the regulation of the expression of *ABCB1* and *LRP* genes through MAPK/ERK pathway was revealed, and the possibility was shown that SP possessed more drug-resistant phenotype compared with MP by activating MAPK/ERK pathway in a certain type of canine lymphoma cells.

Real-time RT-PCR showed that the expression levels of *ABCB1* and *LRP* genes were significantly higher in SP than MP in both of UL-1 and Ema. These results indicated that P-gp and LRP might have an important role in the efflux of Hoechst 33342 dye. In previous studies, SP was suggested to efflux the dye using highly expressed ABC transporter family members, such as P-gp and ABCG2 (Hadnagy et al., 2006; Wu and Alman, 2008). However, there has been no study that indicates high expression level of LRP in SP. LRP, also named as major vault protein (MVP), is expressed on nuclear membrane, and associated with redistribution of the drug from the

nucleus to the cytoplasm (Gromicho et al., 2011). The results in the present study indicated that LRP played a key role in efflux of Hoechst dye from nuclear to cytoplasm, because ABC transporter such as P-gp and ABCG2 are expressed in cell membrane. In addition, it is possible that SP were more drug-resistant compared with MP, as P-gp and LRP have been reported to be important factors associated with drug resistance in human lymphoma patients (Valera et al., 2004).

Besides the expression level of efflux pump, the distribution of cell-cycle of SP was different from that of MP. In both of UL-1 and Ema, most of SP was in G0/G1 phase compared with MP. These results indicated that SP are generally in the quiescent state while MP are actively proliferating. It can also be assumed that SP was more resistant to chemotherapy than MP, because the chemotherapeutic agents generally have a more cytotoxic effect in actively proliferating cells than stable cells. Indeed, a previous study on lung cancer cell line showed that SP, which were mainly in a quiescent state, showed apparent resistance to chemotherapeutic agents such as doxorubicin and cisplatin (Ho et al., 2007).

To clarify the mechanisms that induced the difference of phenotype between SP and MP, comprehensive gene expression analysis using microarray and verification with real-time RT-PCR were examined, and upregulation of *TGF-β type II receptor* gene expression in SP was focused. In addition, activations of Smad and MAPK/ERK pathways, which are downstream of TGF-β signaling, were compared between SP and MP of Ema. As a result, the quantity of phopho-p44/p42 (ERK1/2) was larger in SP than MP, although quantities of proteins involved in Smad pathway were not different between SP and MP. Furthermore, MAPK/ERK signaling was found to upregulate the expression of *ABCB1* and *LRP* genes in the present study. These indicated the presence

of upregulated expression of *ABCB1* and *LRP* genes through MAPK/ERK pathway in SP. To examine the role of MAPK/ERK in regulation of SP phenotype, changes in the rates of SP by PMA stimulation were analyzed. As a result, PMA stimulation increased the percentages of SP cells and treatment with an inhibitor of MAPK/ERK pathway, U0126, inhibited this increase in Ema cells. In UL-1 cells, although the percentages of SP cells increased by PMA stimulation, this increase was not inhibited after treatment with U0126. From these findings, MAPK/ERK pathway was thought to play the central role in regulation of SP phenotype in Ema, although other pathways activated by PMA stimulation might be involved in regulation of SP phenotype in UL-1. Previous studies showed that treatment with doxorubicin upregulated the expression of *ABCB1* gene through MAPK/ERK pathway in human tumor cell lines (Shen et al., 2011; Yang et al., 2001). The results in the present study were consistent with those of these previous studies (Shen et al., 2011; Yang et al., 2001). However, there has been no study on activation of MAPK/ERK pathway in SP or the correlation of this pathway with regulation of *LRP* gene expression. The results obtained in the present study suggested the possibility that MAPK/ERK pathway has a crucial role in the regulation of SP phenotype in a certain type of canine lymphoma cells, and MAPK/ERK pathway could be the target of therapy to overcome the tumor cells with MDR phetnotype conferred by the upregulation of efflux pumps such as P-gp and LRP.

Although SP fraction in tumor cell population has been thought to be enriched by cells with CSC phenotype (Moserle et al., 2010), functions as the stem cells were not compared between SP and MP in the present study. The phenotype such as tumorigenesis should be examined in the future study. However, the results in the present study suggested that small population with enhanced MDR phenotype existed in

clonal cell lines. These findings might support the ‘selection and expansion model’. Further studies are needed to clarify whether such heterogeneous phenotypes exist of tumor tissue in patients and ‘selection and expansion model’ truly reflects the mechanisms of acquisition of MDR *in vivo*.

In conclusion, the present study revealed that MAPK/ERK pathway upregulated the expressions *ABCB1* and *LRP* genes in SP cells of canine lymphoma cell lines. The possibility should be examined in the future that the modulating of intracellular signalings such as MAPK/ERK pathway could decrease the expression of *ABCB1* and *LRP* genes and reconstitute the sensitivity of MDR lymphoma cells to anticancer drugs.

Table 3-1

Primer pairs used for real-time RT-PCR measurement of the relative quantity of each gene.

	Sense primer	Reverse primer	Amplicon size (bp)	Genbank accession number
ABCB1	5'-ACTCGGGAGCAGAAGTTTGA-3' (2734-2753)	5'-AATGAGACCCCGAAGATGTG-3' (2809-2828)	95	NM001003215
ABCC1	5'-CACGTCGACCTGCTACAGAA-3' (3144-3163)	5'-CCGTGTCCAGCTCCTTAGAG-3' (3227-3246)	103	NM001002971
LRP	5'-ACAAGACCCGTGTGGTTAGC-3' (1358-1377)	5'-AGAGGGACAACACGGTGAAC-3' (1482-1501)	144	XM_536910
ABCG2	5'-AACTTCTGCCCAGGAGTCAA-3' (1768-1787)	5'-ATCATGCATCCCAAGGCTAC-3' (1891-1910)	143	DQ222459
TGF-β type II receptor	5'-GCCACATGAAGTCTGTCTGG-3' (243-262)	5'-GAGGCAGCATCTTCGAGAAC-3' (340-359)	117	XM_534237
TBP	5'-CTATTTCTTGGTGTGCATGAGG-3' (-145 - -124)	5'-CCTCGGCATTCAGTCTTTTC-3'(-31 - -50)	96	XM_849432

Table 3-2

The fold-changes of relative amount of cDNA of each gene in side population (SP) cells compared with major population (MP) cells

Gene	UL-1	Ema
ABCB1	1.60-fold ($p < 0.01$)	2.19-fold ($p < 0.01$)
ABCC1	1.48-fold ($p = 0.11$)	0.69-fold ($p = 0.08$)
LRP	2.61-fold ($p < 0.01$)	3.57-fold ($p < 0.01$)
ABCG2	1.00-fold ($p = 0.86$)	-
ABCG2	2.46-fold ($p < 0.01$)	2.65-fold ($p < 0.01$)

Table 3-3

The fold-changes of relative amount of cDNA of each gene whose expression was upregulated in side population (SP) cells compared with major population (MP) cells.

gene name	Fold-change	
	UL-1	Ema
Beta-defensin 140	7.177997159	2.408147909
macrophage expressed gene 1	5.82597984	3.038061713
molybdenum cofactor synthesis	5.193571332	2.436546596
prostaglandin I2 (prostacyclin) synthase	5.088448931	3.830239758
H2B histone family, member F	4.78790378	2.197703863
TGF-beta type II receptor isoform B precursor	4.407346084	4.186169445
KIAA1370	4.388386513	3.367057736
G protein-coupled receptor 177	4.078213158	2.626933498
CG9005-PA, transcript variant 1	4.027323175	2.496339921
KIAA1370	4.004825199	3.503506842
regulator of G-protein signaling 1	3.880043606	3.463442107
HMG-box transcription factor 1	3.826761634	3.222065715
clone VDJ53 immunoglobulin IgH heavy chain VDJ region	3.785528734	2.562137094
lipin 1, transcript variant 3	3.659475808	6.23433414
SYCP3_MOUSE (P70281) Synaptonemal complex protein 3 (SCP-3)	3.643974778	2.127208886
solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3)	3.412496657	11.64418358
KIAA1370	3.26675325	7.130131882
HMG-box transcription factor 1, transcript variant 8	3.151946462	2.804212538
Heat shock protein beta-8 (HspB8)(Protein kinase H11)	3.061486389	2.024232286
solute carrier family 12 (potassium/chloride transporters), member 4	2.944680835	3.043481165
lipin 1	2.943914121	3.48093992

zinc finger and BTB domain containing 20	2.937240652	2.221294978
Retinoic acid receptor RXR-beta (Retinoid X receptor beta)(Nuclear receptor subfamily 2 group B member 2)	2.867486726	2.30548846
phosphatidylinositol 4-kinase type 2 alpha	2.861886174	2.017060061
Canis familiaris similar to phosphodiesterase 7A isoform a, transcript variant 1	2.836178206	3.637248525
testis-specific kinase 2	2.832031998	3.532026434
MID1 interacting G12-like protein	2.822747707	2.061215097
cortactin, like, transcript variant 6	2.768643418	2.165043073
Probable ATP-dependent RNA helicase DDX6 (DEAD-box protein 6) (ATP-dependent RNA helicase p54) (Oncogene RCK), transcript variant 2	2.740847272	2.02085533
MORN repeat containing 4	2.728428733	2.469519896
Rho GTPase activating protein 1 (ARHGAP1)	2.665395011	2.079474696
Potassium channel subfamily K member 5 (Acid-sensitive potassium channel protein TASK-2) (TWIK-related acid-sensitive K(+) channel 2)	2.659558783	3.915750024
ATG16 autophagy related 16-like 1 (S. cerevisiae)	2.652034566	2.194823974
F-box and leucine-rich repeat protein 20	2.616778991	7.506140746
regulator of G-protein signaling 1	2.609351715	2.771618186
Bardet-Biedl syndrome 4	2.585749323	2.479325452
F-box and leucine-rich repeat protein 11, transcript variant 1	2.573154673	2.051097476
Putative GTP-binding protein PTD004, transcript variant 2	2.561397895	2.107904712
NudC domain containing 3	2.561301157	2.188631514
Q833M3_ENTFA (Q833M3) Inosine-uridine preferring nucleoside hydrolase	2.537983347	2.007164922
valosin-containing protein	2.514733029	2.318264864
HSU37436 AICAR formyltransferase/IMP cyclohydrolase bifunctional enzyme {Homo sapiens} (exp=-1; wgp=0; cg=0)	2.508039508	3.055592267
PREDICTED: Canis familiaris similar to testis expressed sequence 27	2.499193744	4.494960991
TBC1 domain family, member 14	2.497230057	2.634611931
mitogen-activated protein kinase kinase 3	2.480738188	3.405153387
zinc finger protein 577	2.422396309	2.190529846
HMG box domain containing 3	2.416385231	2.821409091
perforin 1 (PRF1)	2.300278805	2.283052695
Dingo protein isoform 1	2.290335131	2.757642047
CG8233-PC, isoform C, transcript variant 1	2.262737306	3.13546085

thioredoxin interacting protein	2.257494182	3.953105453
dystroglycan 1 (dystrophin-associated glycoprotein 1) (DAG1)	2.249823765	2.634736504
CD200 receptor 1 isoform a	2.245020111	4.695546771
WD repeat domain 55	2.236829697	2.360066505
ring finger protein 111	2.227655029	2.131695008
phosphatase and actin regulator 4	2.213644973	2.692772129
Potassium channel tetramerisation domain containing protein 5	2.213456341	2.513789221
ubiquitin specific peptidase 37	2.209773372	2.359616652
phosphatidylserine decarboxylase	2.195277876	3.031684546
prosaposin	2.186348835	2.739186774
DEP domain containing 5	2.164791948	2.182098878
ubiquitin associated and SH3 domain containing, B	2.153811498	2.594376661
6-phosphofructo-2-kinase	2.14126113	7.222881924
neuroligin 3, transcript variant 2	2.128487061	4.220944441
NGFI-A binding protein 2 (EGR1 binding protein 2)	2.112186167	3.428313066
TAO kinase 1	2.106840991	4.90387507
adiponectin receptor 2, transcript variant 2	2.10605446	2.147066034
potassium channel tetramerisation domain containing 5	2.104881756	2.271759955
UPF0474 protein C5orf41	2.101308358	2.200432021
neighbor of BRCA1 gene 1	2.100448228	3.295487932
DRE1 protein	2.094454277	3.217265715
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	2.073032189	2.378740907
dual specificity phosphatase 5	2.055500212	2.238965995
Nuclear receptor co-repressor 2 Fragment	2.05467892	2.146390699
fucokinase	2.052565849	2.347336583
zinc finger protein 133	2.051873234	4.8409085
v-raf-1 murine leukemia viral oncogene homolog 1	2.043537588	2.403738294
FA36A_MOUSE (Q9D7J4) Protein FAM36A	2.039809875	3.075565286
sphingosine-1-phosphate lyase 1	2.033662923	2.312190825

MAP-kinase activating death domain	2.018896481	2.659067222
prosaposin, transcript variant 6	2.010108071	2.698349871
ring finger protein 145	2.009359784	2.322147468
human immunodeficiency virus type I enhancer binding protein 2, transcript variant 1	2.00297006	2.866028056
acetyl-Coenzyme A carboxylase alpha	2.00178217	2.776618962
phosphodiesterase 7A	2.000842618	2.389489039

Table 3-4

The fold-changes of relative amount of cDNA of each gene whose expression was downregulated in side population (SP) cells compared with major population (MP) cells.

gene name	Fold-change	
	UL-1	Ema
Q29VI1_GOSHI (Q29VI1) Patatin-like protein	0.125202922	0.130683355
DEP domain containing 1	0.188007289	0.208739022
topoisomerase (DNA) II alpha 170kDa	0.211815873	0.373163468
ubiquitin-conjugating enzyme E2C	0.253897562	0.181965683
Abnormal spindle-like microcephaly-associated protein homolog Fragment	0.28767783	0.129504272
aurora kinase B	0.304152362	0.251501513
Q8MLV2_DROME (Q8MLV2) CG30398-PA	0.332262639	0.448895923
tubulin, beta, 2, transcript variant 2	0.337684524	0.226847164
family with sequence similarity 166, member A	0.338740487	0.113965275
DEP domain containing 1B	0.351434142	0.402110831
Tubulin alpha-2 chain (Alpha-tubulin 2), transcript variant 2	0.352011404	0.17168418
Mitosin Fragment	0.360028512	0.263028672
Importin alpha-2 subunit (Karyopherin alpha-2 subunit) (SRP1-alpha) (RAG cohort protein 1), transcript variant 6	0.369136721	0.34667397
cyclin-dependent kinase 1	0.372430929	0.208691068
aurora kinase B	0.385916909	0.282529045
Canis lupus familiaris Toll-like receptor 7 (TLR7), mRNA [NM_001048124]	0.421205117	0.383500583
ZW10 interactor	0.424181762	0.393137556
trophinin associated protein (tastin)	0.437235685	0.268585526
Canis lupus familiaris cyclin B3 (CCNB3)	0.440181045	0.368148176
melanoma inhibitory activity	0.44168097	0.478811092

cyclin B2	0.446337835	0.262691001
ADP-ribosylation factor-like 6 interacting protein 1	0.447620413	0.435296992
Opa interacting protein 5	0.45212784	0.298365923
Q3WDZ3_9ACTO (Q3WDZ3) Forkhead-associated (FHA)	0.45714755	0.288373728
SHC SH2-domain binding protein 1	0.460352168	0.495211691
leucine rich repeat and coiled-coil domain containing 1	0.46720885	0.339789137
Holliday junction recognition protein	0.470863539	0.449220979
NIMA (never in mitosis gene a)-related kinase 2	0.476489782	0.360516357
thyroid hormone receptor interactor 6	0.484122659	0.412130899
cancer susceptibility candidate 5 isoform 2	0.485812514	0.437664734
SLBP2_XENLA (Q9YGP6) Oocyte-specific histone RNA stem-loop-binding protein 2	0.489707121	0.352368293
NIMA (never in mitosis gene a)-related kinase 2	0.497651543	0.493814128

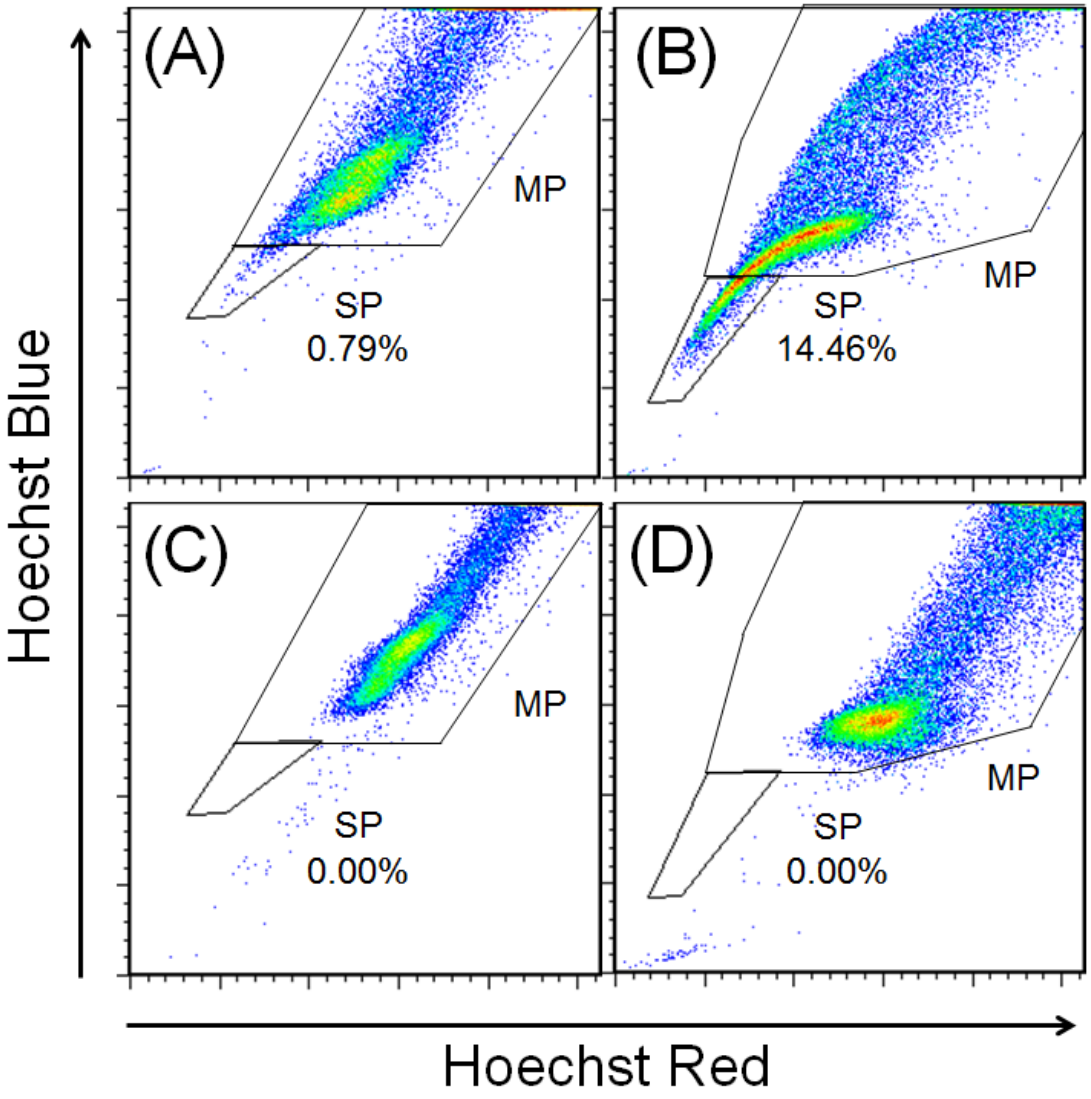

Fig. 3-1. Identification of SP and MP cells in canine lymphoma cell lines, UL-1 and Ema. SP and MP of UL-1 (A) and Ema (B) were gated. SP disappeared when UL-1 (C) and Ema (D) were incubated with reserpine.

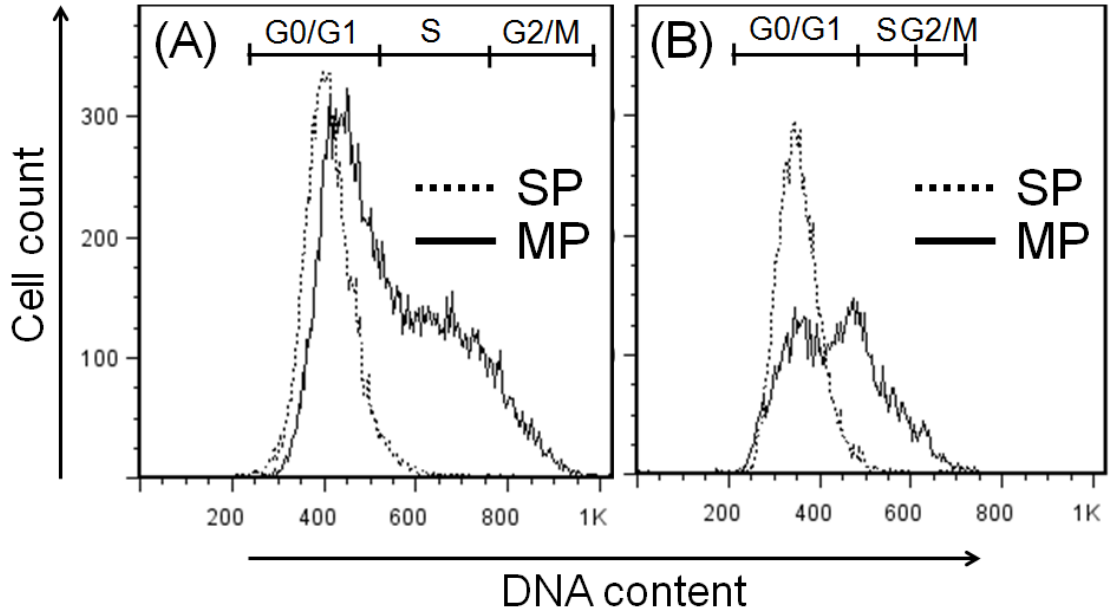

Fig. 3-2. The comparison of cell-cycle distribution between SP and MP in UL-1 (A) and Ema (B). In both of two cell lines, the rates of cells that were in G0/G1 phase were significantly larger in SP than MP ($p < 0.01$).

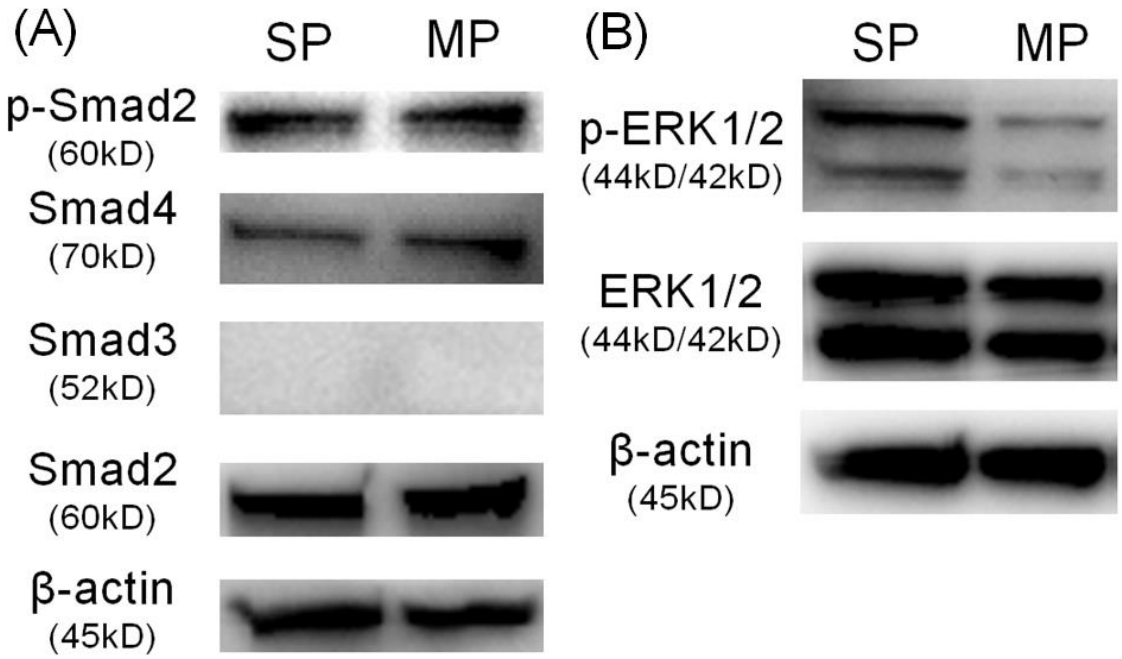

Fig. 3-3. The comparison of quantities of proteins involved in Smad (A) and MAPK/ERK pathway (B) between SP and MP of Ema. In Smad pathway, the quantities of phospho-Smad2, Smad4 and Smad2 were not different between SP and MP. Smad3 was not detected in Ema. As to MAPK/ERK pathway, the quantity of phospho-p44 (ERK1) was found to be larger in SP than MP. Western blotting analysis was carried out in triplicate.

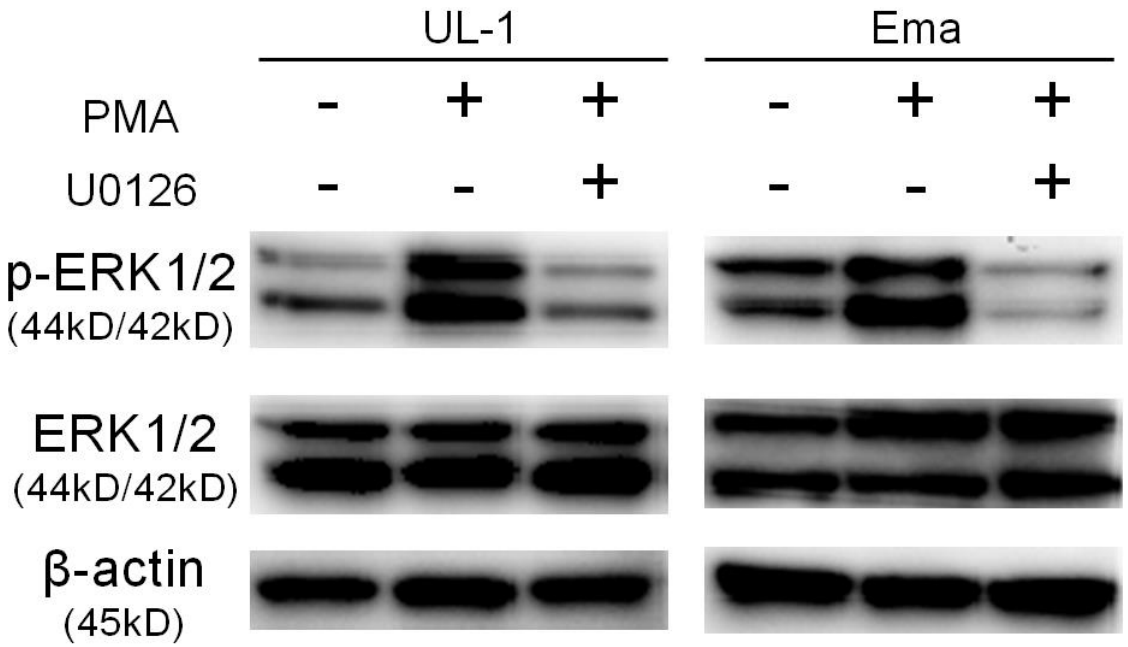

Fig. 3-4. The confirmation of activation of MAPK/ERK pathway with PMA and its inhibition with U0126. The quantity of phosho-p44 (ERK1) was markedly increased by incubation with PMA, and this increase was inhibited with U0126 in both of UL-1 and Ema. Western blotting analysis was carried out in triplicate.

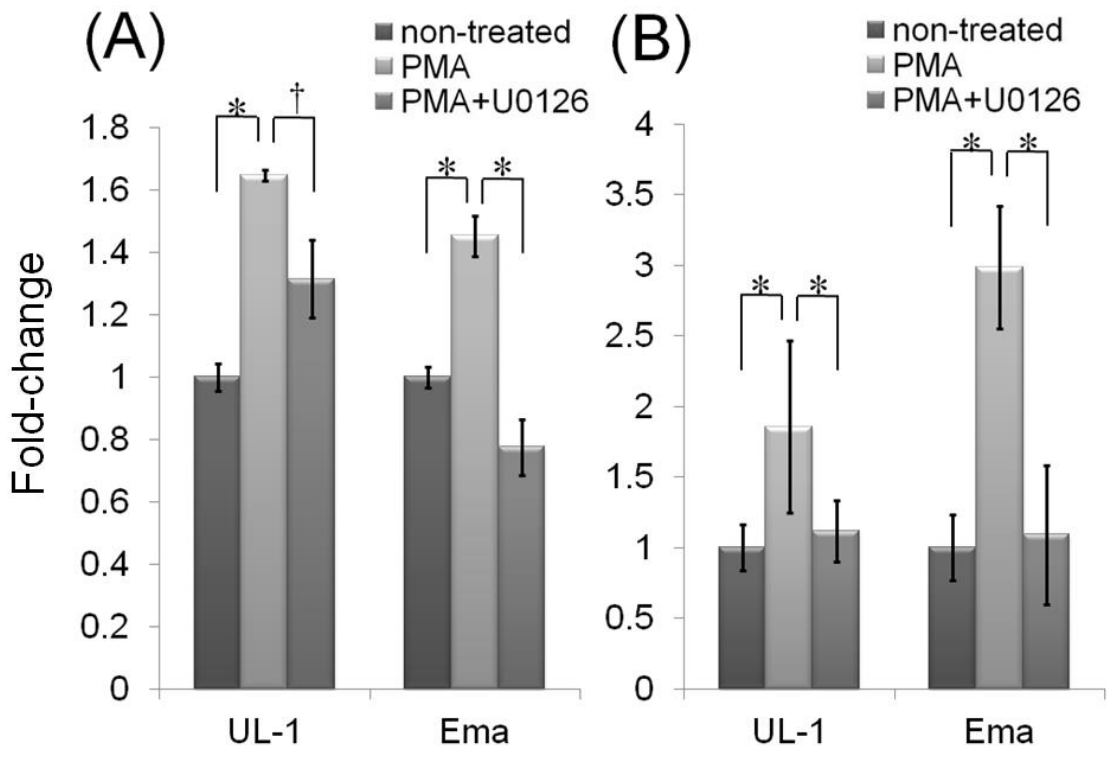

Fig. 3-5. The fold-change of relative quantities of *ABCB1* (A) and *LRP* (B) genes in UL-1 and Ema by PMA stimulation and its inhibition with U0126. In both of two cell lines, the relative quantities of *ABCB1* and *LRP* genes mRNA were significantly increased by incubation with PMA compared with non-treated cells. In addition, when UL-1 and Ema were incubated with PMA and U0126, the relative quantities of *ABCB1* and *LRP* genes mRNA were significantly decreased compared with those when incubated with PMA alone. *$p < 0.01$ and †$p = 0.02$.

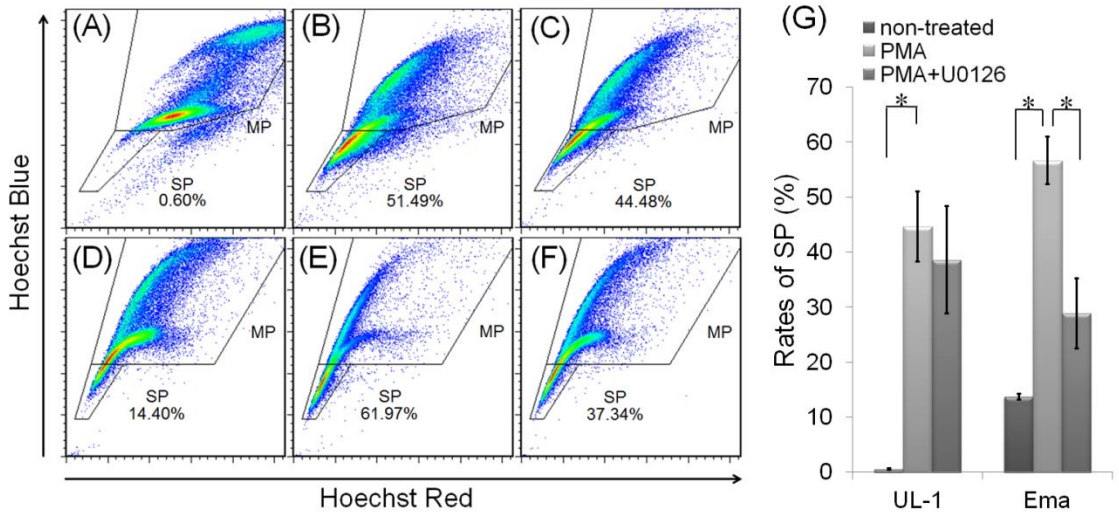

Fig. 3-6. Changes of the rates of SP in UL-1 and Ema after treatment with PMA and PMA+U0126. SP and MP were gated in each scattergram. The rates of SP were significantly increased through PMA stimulation in both of UL-1 (B) and Ema (E) compared with non-treated UL-1 (A) and Ema (D), respectively. When the cells were pretreated with a MAPK/ERK pathway inhibitor, U0126, the increase in rates of SP was significantly inhibited in Ema (F), but not in UL-1 (C). Changes of the percentages of SP in three independent assays were shown in (G). *$p < 0.01$.

Chapter 4

Antitumor effect and modulation of *ABCB1* gene expression through intracellular signalings by perifosine in canine lymphoid tumor cell lines

Abstract

Acquisition of multidrug resistance (MDR) is a common cause of failure during chemotherapy for dogs with lymphoma. The overexpression of P-glycoprotein (P-gp), coded by *ABCB1* gene, is known to be associated with MDR. Perifosine, an Akt inhibitor, was recently reported to downregulate the expression of P-gp in human medicine. However, antitumor effect or modulation of the expression of drug efflux pumps by perifosine has not been examined in canine tumor cells. The purpose of the present study was to examine the antitumor effect and the modulation of the expression of *ABCB1* and *LRP* genes following perifosine treatment in canine lymphoid tumor cell lines. In the study in this chapter, 4 different canine lymphoid tumor cell lines, GL-1, CLBL-1, UL-1 and Ema were used: the former 2 cell lines were negative for P-gp and the latter 2 were positive for P-gp. GL-1 and UL-1 were sensitive to perifosine whereas CLBL-1 and Ema were resistant. The amount of *ABCB1* mRNA significantly decreased after treatment with perifosine through activation of c-jun NH_2-terminal kinase (JNK) pathway in UL-1, but such an effect was not observed in Ema. The amount of *LRP* mRNA did not change after perifosine treatment in either UL-1 or Ema. In UL-1, the efflux of rhodamine 123 dye and the IC_{50} value for vincristine were reduced by perifosine, but such effects were not observed in Ema. In conclusion, perifosine produced an antitumor effect, downregulated *ABCB1* gene expression, and increased the sensitivity for vincristine in a certain type of canine lymphoid tumor cells.

Introduction

Lymphoma is the most common hematologic neoplasm in dogs (Dobson et al., 2001). In most cases, chemotherapy for lymphoma is initially effective; however, lymphoma cells acquire multidrug resistance (MDR) phenotype during or after chemotherapy, and the treatment tends to fail (Marconato, 2011). Therefore, it is important to elucidate the mechanisms that confer MDR phenotype to tumor cells and to establish an effective treatment for tumor cells with MDR.

Various molecules have been shown to induce MDR phenotype in human (O'Connor, 2007) and veterinary medicine (Bergman, 2003). Acquisition of MDR phenotype is thought to be multifactorial, and the overexpression of efflux pumps such as ATP-binding cassette (ABC) transporter family is one of the important MDR factors (Bergman, 2003). Among ABC transporters, the efflux pumps coded by *ABCB1*, *ABCC1* and *ABCG2* genes are known to be representative ones associated with the acquisition of MDR (Modok et al., 2006). In addition to these ABC transporters, the overexpression of lung resistance-related protein (LRP) has also been reported to be associated with acquisition of MDR (Gromicho et al., 2011). The overexpression of such transporters reduces the intracellular concentration of chemotherapeutic agents. In veterinary medicine, the expression of P-glycoprotein, coded by *ABCB1* gene, has been reported to associate with the MDR phenotype in dogs (Bergman et al., 1996; Lee et al., 1996; Moore et al., 1995). However, the mechanisms that induce P-gp overexpression are not fully understood, and a successful treatment has not yet been established to overcome

the MDR phenotype acquired as a result of P-gp overexpression.

Perifosine is an anticancer drug that inhibits phosphoinositide 3-kinase (PI3K)/Akt pathway, and it is under clinical trial as a treatment for human patients with colorectal cancer and multiple myeloma (Srivastava and Cho, 2013). It was reported that perifosine inhibited the activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway and activated c-jun NH_2-terminal kinase (JNK) pathway. In addition, perifosine downregulated the expression of P-gp through activation of JNK pathway (Chiarini et al., 2008).

In veterinary medicine, I revealed that *ABCB1* and *LRP* genes were upregulated by MAPK/ERK pathway in canine lymphoma cell lines in chapter 3. However, there has been no report on the antitumor effect or the effect on modulation of the expression of drug efflux pumps of perifosine in canine tumor cells.

The purpose of the study in this chapter was to examine the antitumor effect and change in the expressions of *ABCB1* and *LRP* genes by the treatment with perifosine in canine lymphoid tumor cell lines.

Materials and methods

Cell lines

In the present study, 4 canine lymphoid tumor cell lines were used: GL-1, a canine B-cell leukemia cell line (Nakaichi et al., 1996); CLBL-1, a canine B-cell lymphoma cell line (Rutgen et al., 2010); UL-1, a canine T-cell lymphoma cell line (Yamazaki et al., 2008); and Ema, a canine T-cell lymphoma cell line (Hiraoka et al., 2009). GL-1 and CLBL-1 do not express *ABCB1* gene and P-gp, whereas UL-1 and Ema express them as shown in chapter 2-1. All the cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum and cultured at 37 °C in a humidified atmosphere of 5% CO_2.

Drug sensitivity assay

The 50% inhibitory concentration (IC_{50}) values for perifosine were determined in the 4 cell lines. The cells (2×10^5 cell/mL) were cultured in a 96-well plate in media containing various concentrations (0.1 – 100 μM) of perifosine (Selleck) for 48 h. After cultivation, the viability of the cells was measured using Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions. Absorbance was measured using a Model 680 Microplate Reader (Bio-Rad Laboratories), and IC_{50} values were determined. The induction of apoptosis after cultivation with 20 μM of perifosine for 24 h was also detected using ApoAlert Annexin V-FITC Apoptosis Kit (TaKaRa Bio) and FACSCalibur (Becton Dickinson) according to the manufacturer's instructions. In addition, IC_{50} values for vincristine (Nippon Kayaku) following simultaneous

incubation of cells with perifosine were examined in UL-1 and Ema. Cell viabilities were measured in cells after simultaneous treatment with 5 μM (in UL-1) or 20 μM (in Ema) of perifosine and various concentrations of vincristine (1 - 500 ng/ml) for 48 h. The cell viabilities relative to cells treated with perifosine alone were measured as described above. To examine the IC_{50} values for vincristine in UL-1 and Ema without treatment with perifosine, cells were incubated with various concentrations of vincristine (1 - 500 ng/ml) for 48 h, and the cell viabilities were measured as described above.

Western blotting for PI3K/Akt, MAPK/ERK and JNK pathways

Western blotting was used to examine the activation statuses of PI3K/Akt, MAPK/ERK and JNK pathways in cell lines after treatment with 20 μM perifosine as in chapter 3. In brief, whole cell lysates were extracted from each cell line using RIPA buffer (Cell Signaling Technology), and equal amounts of total protein were separated by SDS-PAGE. After blotting on a PVDF membrane, 5% skimmed milk/tris-buffered saline with Tween20 was used to block unspecific binding before the membrane was incubated with primary antibodies against phospho-Akt (Ser473), phospho-Akt (Thr308), Akt (pan), phospho-ERK1/2, ERK1/2, phospho-SAPK/JNK, SAPK/JNK, phospho-c-jun, c-jun, and β-actin (Cell Signaling Technology) overnight at 4 °C. After incubation with the secondary antibody of horseradish peroxidase-labeled anti-rabbit IgG (1:3000; Bio-rad Laboratories) 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).

Quantification of the amount of ABCB1 and LRP mRNA

Changes in the expression levels of *ABCB1* and *LRP* genes after treatment with 20 μM perifosine for 12 h were examined in UL-1 and Ema using real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) as described in chapter 1 using the same primer pair used in the study of chapter 1. To normalize the amount of the cDNA samples, *TATA box binding protein* (*TBP*) was selected as an internal reference based on the study in chapter 1. Real-time RT-PCR was performed in triplicate.

Inhibition of JNK or PI3K/Akt pathway

To determine the association between *ABCB1* and *LRP* gene expression and signaling pathway activation in UL-1 and Ema cell lines, SP600125 (Wako) and LY294002 (Cell Signaling Technology) were used to inhibit the JNK and PI3K/Akt pathways respectively. For inhibition of JNK pathway, cells were treated with 50 μM SP600125 for 40 min before incubation with 20 μM perifosine for 8 h or 12 h for extraction of total RNA or total protein, respectively. The amounts of *ABCB1* and *LRP* mRNAs and the amounts of phospho-SAPK/JNK, SAPK/JNK, phospho-c-jun and c-jun were compared among non-treated cells, cells treated with perifosine alone, and cells treated with both perifosine and SP600125 using real-time RT-PCR and Western blotting, as described above. For inhibition of PI3K/Akt pathway, cells were treated with 20 μM LY294002 for 3 h or 12 h for extraction of total RNA or total protein, respectively. The amounts of *ABCB1* and *LRP* mRNAs and the amounts of phospho-Akt (Ser473), phospho-Akt (Thr308) and Akt were compared between non-treated cells and cells treated with LY294002 using real-time RT-PCR and Western blotting as described above.

Rhodamine 123 (Rh) efflux test

One million UL-1 or Ema cells, with or without pretreatment with 20 μM perifosine for 12 h, were incubated with 200 ng/mL of Rh (Sigma-Aldrich) in RPMI 1640 medium at 37 °C for 20 min. After washing in PBS, the cells were incubated in Rh-free medium at 37 °C for 60 min, either with or without 2 μM of cyclosporine (Cs). Following incubation, the cells were washed with PBS, and subjected to flow cytometric analysis using FACSCalibur (Becton Dickinson). The cells that had not been exposed to Rh were used as negative controls, and the Rh efflux index (REI) was calculated as (mean fluorescence intensities of the sample incubated with Rh and Cs - those of the negative control) / (those of the sample incubated with Rh - those of the negative control). The Rh efflux test was performed 5 times.

Statistical analysis

The nonparametric Mann-Whitney *U* test was used to compare the relative mRNA expression level of *ABCB1* and *LRP*, and REI. The repeated-measures two-way ANOVA and Bonferroni post tests were used to compare the cell viabilities after incubation with vincristine between cells with and without treatment of perifosine. All statistical tests were 2-sided and performed using Prism 5 (GraphPad). For all tests, $P < 0.05$ was used as the level of significance.

Results

Cytotoxic effect and modulation of intracellular signaling by perifosine

The IC_{50} values for perifosine in GL-1, CLBL-1, UL-1 and Ema were 9.91, 33.0, 7.01 and 58.7 μM, respectively (Fig. 4-1A). After cultivation with 20 μM of perifosine for 24 h, large increase of Annexin-V positive cells was observed in GL-1 and UL-1 (Fig. 4-1B). Western blotting detected increased amounts of phospho-SAPK/JNK, phospho-c-jun, and c-jun following treatment with perifosine in GL-1 and UL-1, although these effects were not observed in CLBL-1 and Ema (Fig. 4-2). As for MAPK/ERK pathway, a decrease in the amount of phospho-ERK1/2 was observed by treatment with perifosine in GL-1, UL-1, and Ema, but this effect was not observed in CLBL-1. In addition, decreases in the amounts of phospho-Akt (Ser473) and phospho-Akt (Thr308) were observed in GL-1 and UL-1; however, these effects were not observed in CLBL-1 and Ema.

Changes in quantities of ABCB1and LRP mRNAs by perifosine

Treatment with perifosine significantly decreased the amounts of *ABCB1* (0.538-fold) and *LRP* (0.823-fold) mRNAs in UL-1 (Fig. 4-3A and B); however, the extent of the decrease of *LRP* gene expression in this cell line was within the intra-assay error (≥ 0.81-fold in difference). In contrast, the amounts of both *ABCB1* and *LRP* mRNAs did not significantly change after treatment with perifosine in Ema (1.03-fold and 1.09-fold, respectively; Fig. 4-3C and D).

Effects of inhibition of JNK or PI3K/AKT pathway

In UL-1, the increase of the amounts of phospho-SAPK/JNK, phospho-c-jun, and c-jun that was previously observed following treatment with perifosine was inhibited by treatment with SP600125 prior to the treatment with perifosine (Fig. 4-4A). In addition, the amount of *ABCB1* mRNA was significantly increased when cells were incubated with both perifosine and SP600125 (0.698-fold) compared with incubation with perifosine alone (Fig. 4-4B). However, the amounts of *LRP* mRNA were not significantly different between the cells treated with both perifosine and SP600125 (0.873-fold) and those treated with perifosine alone (Fig. 4-4C).

When the inhibition of PI3K/Akt pathway was examined using LY294002, decreased amounts of phospho-Akt (Ser473) and phospho-Akt (Thr308) were observed in both UL-1 and Ema (Fig. 4-5A). However, the amounts of *ABCB1* or *LRP* mRNAs were not significantly different between the cells treated with LY294002 and control cells in both UL-1 and Ema (Fig. 4-5B-E). The fold changes in the amounts of *ABCB1* and *LRP* mRNAs were 1.25- and 1.17-fold in UL-1 and 1.10- and 1.31-fold in Ema, respectively.

Changes in efflux function by treatment with perifosine

To examine the changes in efflux function by treatment with perifosine, Rh efflux test was performed. Although the intracellular fluorescence intensities of Rh in cells incubated with Rh and Cs were not different between control cells (Fig. 4-6A) and cells treated with perifosine (Fig. 4-6B), fluorescence intensities in cells incubated with Rh alone was increased in cells treated with perifosine compared with control cells in UL-1. However, such a difference was not observed in Ema (Fig. 4-6C and D). The REI value

was significantly decreased by treatment with perifosine (4.65) compared with control cells (7.29) in UL-1 (Fig. 4-6E), although the REI values were not significantly different between control cells (9.73) and cells treated with perifosine (8.36) in Ema (Fig. 4-6F).

Changes in sensitivity for vincristine by treatment with perifosine

Cell viabilities were examined after treatment of vincristine with or without simultaneous perifosine exposure in order to determine whether perifosine affected the sensitivity of cells to vincristine. As shown in Fig. 4-7A, in UL-1, the cell viabilities when treated with 20 or 30 ng/ml of vincristine were significantly lower in cells exposed to perifosine than in cells not exposed to perifosine. The IC_{50} values for vincristine were 15.6 or 29.6 ng/ml in cells with or without treatment with perifosine, respectively. In Ema, there was no significant difference in cell viabilities at any concentration of vincristine (Fig. 4-7B) between control cells and cells treated with perifosine. The IC_{50} values for vincristine were 77.8 or 81.9 ng/ml in cells with or without treatment with perifosine, respectively.

Discussion

The present study revealed that perifosine produced an antitumor effect by inducing apoptosis and modulated the expression of *ABCB1* gene in a certain type of canine lymphoid tumor cells.

IC_{50} values for perifosine in GL-1, CLBL-1, UL-1 and Ema were revealed to be 9.91, 33.0, 7.01 and 58.7 μM, respectively. In clinical trials in human medicine, the trough levels of perifosine obtained on dosage of 200 mg/day were reported as 2.6 - 8.2 μg/ml (5.6 – 17.8 μM) (Crul et al., 2002). Considering these data, it might be conceivable that 2 of the 4 cell lines used in the present study were sensitive to perifosine.

The results obtained in this study showed that the treatment with perifosine inhibited PI3K/Akt and MAPK/ERK pathways, and activated JNK pathway. These results were in agreement with the results obtained in a previous study (Chiarini et al., 2008). In addition, it was revealed in the present study that the modulation of these intracellular signaling by perifosine differed among cell lines. All 3 pathways were modulated in GL-1 and UL-1, which were sensitive to perifosine, but in the perifosine-resistant cell lines CLBL-1 and Ema, none of these pathways and only MAPK/ERK pathway was modulated, respectively. Perifosine was reported to exert an antitumor effect mainly owing to its ability to the inhibit PI3K/Akt pathway in human medicine (Schmidt-Hieber et al., 2012). Considering these observations in human medicine and the results of the present study, it was suggested that it might determine the sensitivity for perifosine whether inhibition of PI3K/Akt pathway occur or not in

tumor cells.

The ability of perifosine to modulate the expression of *ABCB1* and *LRP* genes as well as the function of efflux pumps was examined in UL-1 and Ema, cell liens previously identified as being positive for the *ABCB1* gene and P-gp in chapter 2-1. The amount of *ABCB1* mRNA and the REI value significantly decreased by treatment with perifosine in UL-1. In addition, the downregulation of *ABCB1* gene expression by perifosine was significantly inhibited by SP600125, a JNK inhibitor, although treatment with LY294002, a PI3K inhibitor, had no effect on *ABCB1* mRNA levels. These observations suggested that perifosine downregulated *ABCB1* gene expression at least in part through the activation of JNK pathway, and inhibition of PI3K/Akt pathway might not be involved in the downregulation of the expression of this gene. The downregulation of the expression of P-gp through the activation of JNK pathway by perifosine was also reported in human medicine (Chiarini et al., 2008). The study in chapter 3 showed that MAPK/ERK pathway upregulated the expression of *ABCB1* and *LRP* genes in UL-1 and Ema. However, treatment with perifosine did not significantly alter the amounts of *ABCB1* or *LRP* mRNAs in Ema, despite an inhibition of MAPK/ERK pathway by perifosine being observed. Considering these results, the inhibition of MAPK/ERK pathway might be insufficient whereas the activation of JNK pathway might be important for the downregulation of the *ABCB1* gene by perifosine.

The IC_{50} value for vincristine decreased by treatment with perifosine (15.6 ng/ml) compared with non-treated cells (29.6 ng/ml) in UL-1, although IC_{50} values in Ema did not change, whether perifosine was added or not (77.8 or 81.9 ng/ml, respectively). There has been no study on the detailed pharmacokinetics of vincristine in dogs, but the C_{max} of vincristine was reported to be 21.9 and 28.6 ng/mL, respectively, in 2 human

patients when administrated at a dosage of 0.05 mg/kg (Skolnik et al., 2006). Combined with the dosage of 0.7 mg/m^2 in representative chemotherapeutic protocols for canine lymphoma patients (Garrett et al., 2002; Keller et al., 1993; Moore et al., 2001), UL-1 and Ema could be assumed to be resistant to vincristine, and UL-1 might become sensitive to vincristine by treatment with perifosine considering the change in IC_{50} values for vincristine when incubated with perifosine. In the study in chapter 2-1, treatment with cyclosporine (Cs), an ABC-transporter inhibitor, increased the sensitivity for vincristine in UL-1 and Ema, and it was suggested that some transporters might be associated with vincristine resistance that is seen in these cell lines. On the basis of these observations, the use of perifosine might increase the sensitivity of anticancer drugs that was substrate for P-gp through downregulation of *ABCB1* gene expression in a certain type of canine lymphoid tumor cells.

In conclusion, perifosine downregulated *ABCB1* gene expression through activation of JNK pathway and increased the sensitivity for vincristine in addition to exerting an antitumor effect in a certain type of canine lymphoid tumor cells. Combination chemotherapy of perifosine and anticancer drugs that is substrate for P-gp may represent an effective treatment for certain types of canine lymphoid tumor cells.

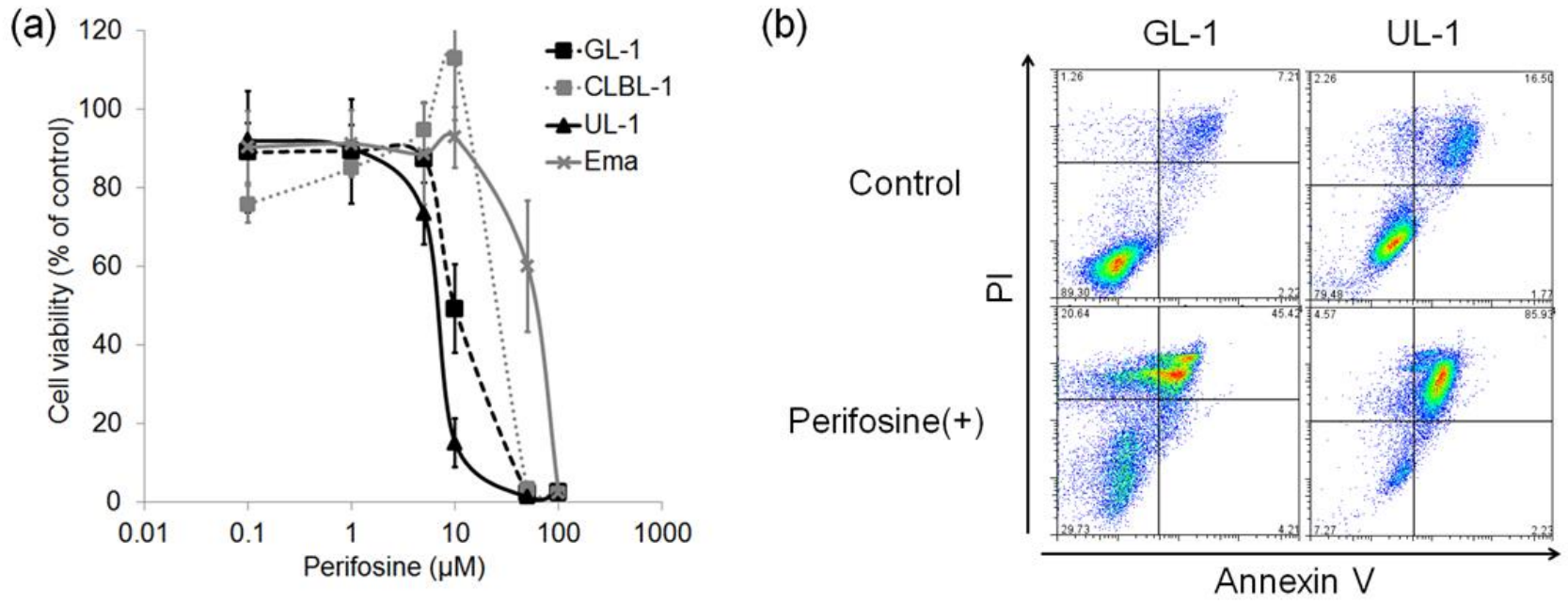

Fig. 4-1. (a) The dose-response curves of the cytotoxic effect of perifosine in the GL-1, CLBL-1, UL-1 and Ema. The cell viability data were plotted against the log of perifosine concentrations. The data represent the mean ± SD of triplicate cultures. (b) The detection of Annexin V and propidium iodide (PI) positive cells after cultivation with perifosine in GL-1 and UL-1.

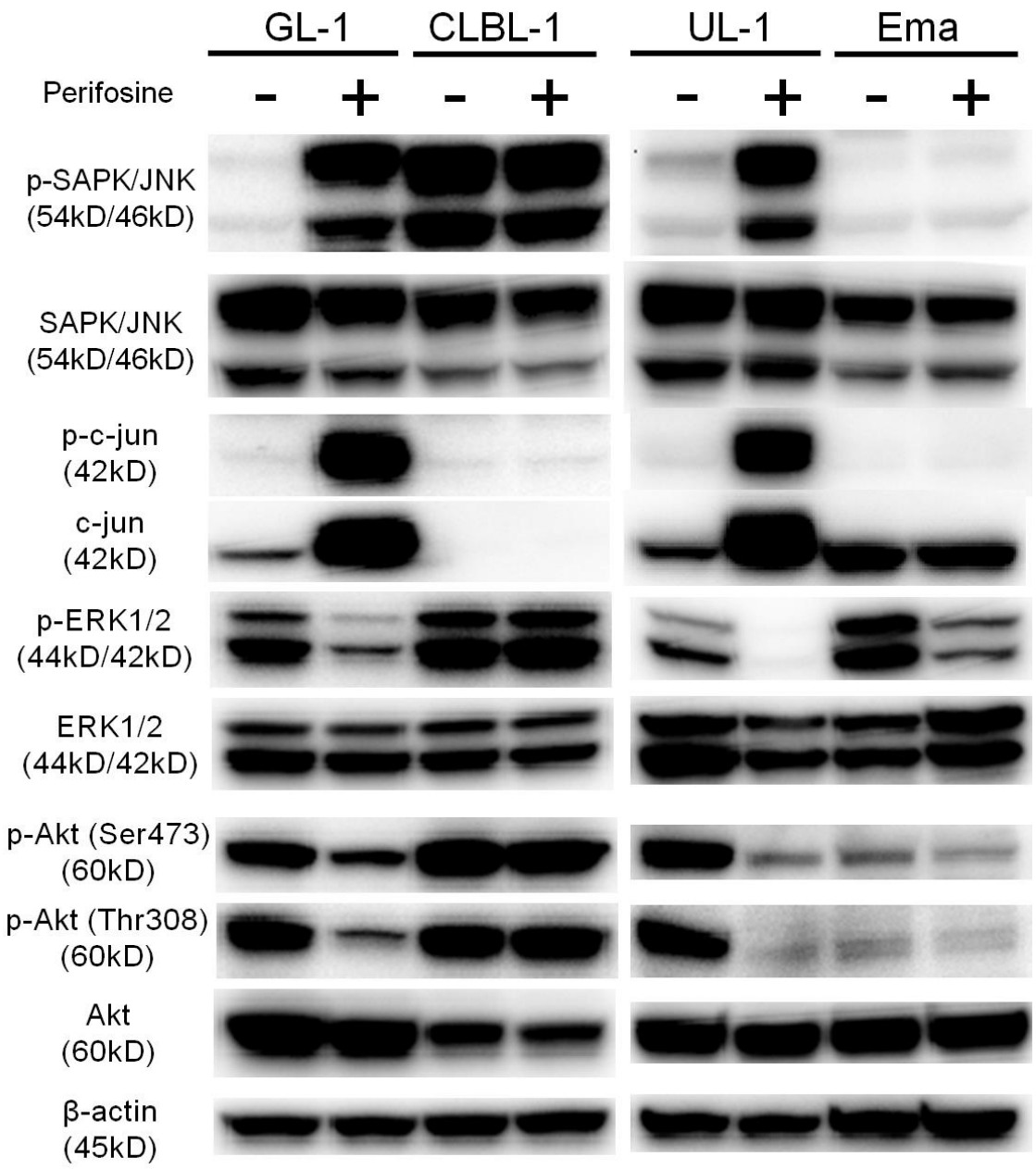

Fig. 4-2. The comparisons of quantities of proteins involved in JNK, MAPK/ERK and PI3K/Akt pathways between cells with or without treatment with perifosine. All of the three pathways were modulated in GL-1 and UL-1 by perifosine, but none of these pathways and only MAPK/ERK pathway was modulated in CLBL-1 and Ema, respectively.

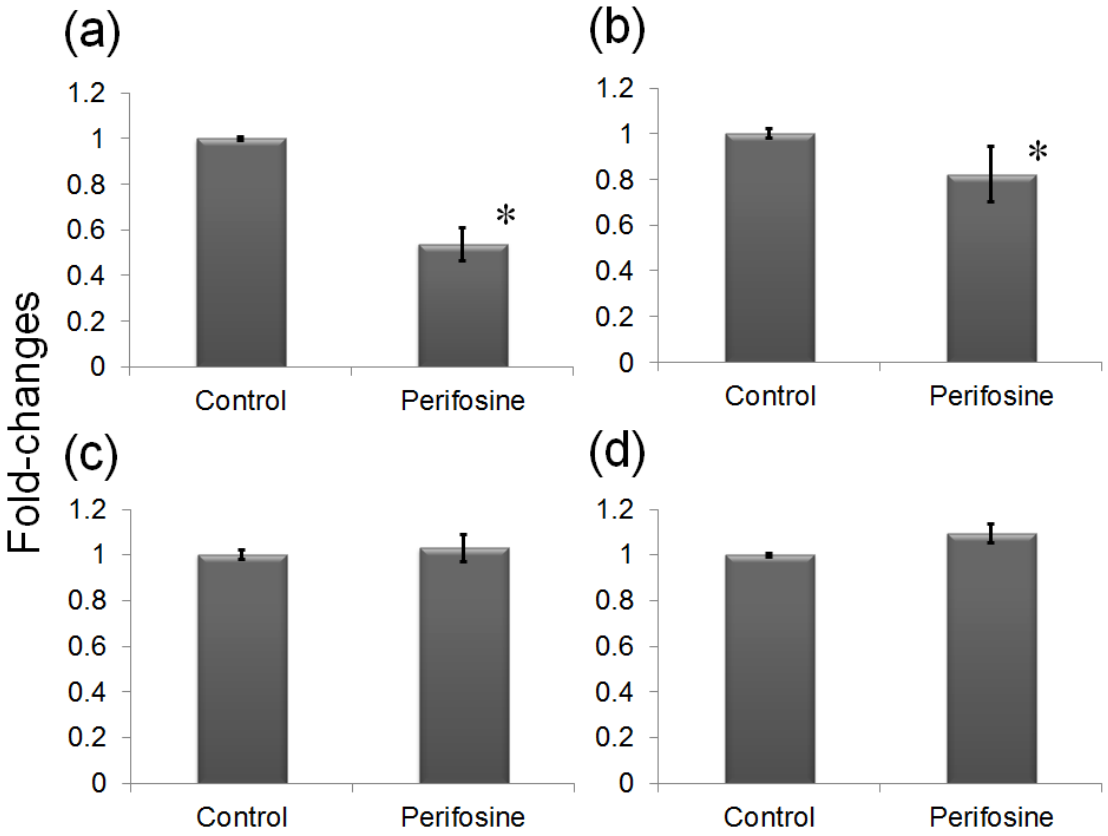

Fig. 4-3.

The fold-changes of relative quantities of *ABCB1* and *LRP* genes in UL-1 (a and b, respectively) and Ema (c and d, respectively) by treatment with perifosine. In UL-1, the treatment with perifosine significantly decreased the amounts of *ABCB1* and *LRP* mRNAs, although the extent of the decrease of *LRP* gene expression was within the intra-assay error. However, those of Ema did not significantly change after treatment with perifosine. *$p < 0.05$.

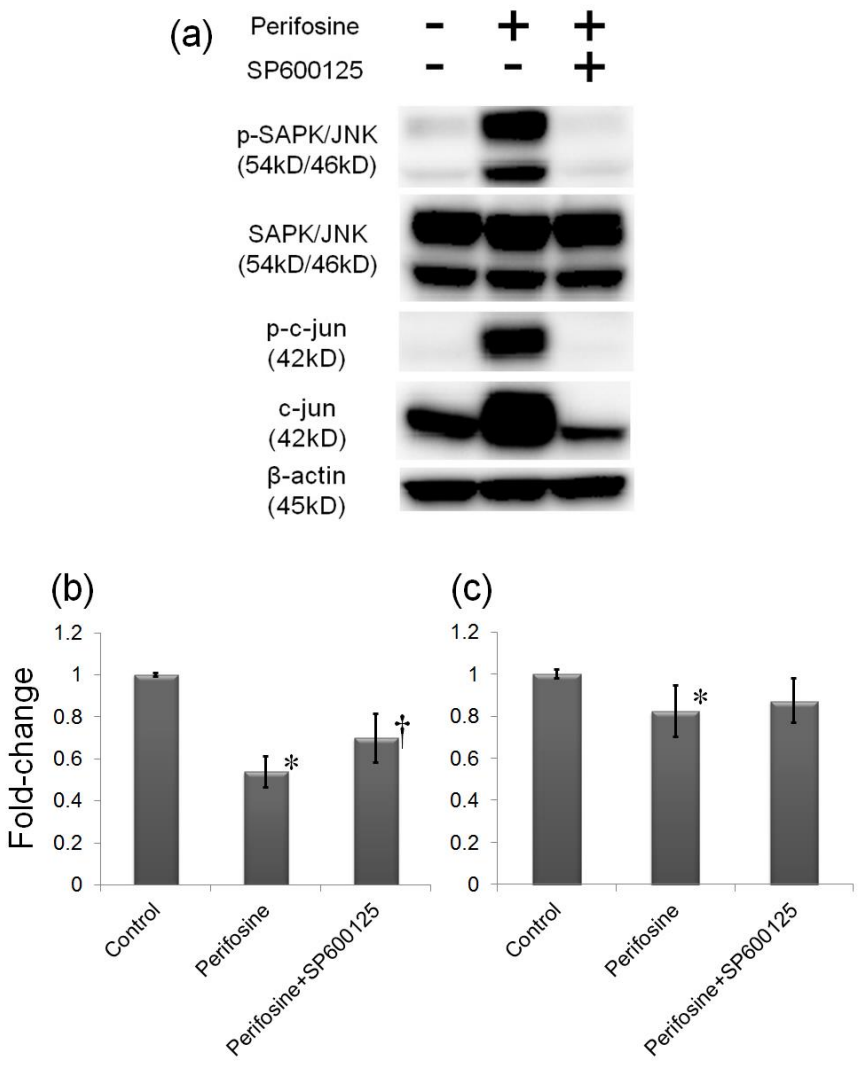

Fig. 4-4. Analysis on the activation of JNK pathway by perifosine and its inhibition by SP600125 in UL-1 (a). The quantities of phosho-SAPK/JNK, phospho-c-jun and c-jun increased by incubation with perifosine, and this increase was inhibited by SP600125. The fold-changes of relative quantities of *ABCB1* (b) and *LRP* (c) genes in UL-1 by the treatments with perifosine alone and perifosine plus SP600125. Decrease of the amount of *ABCB1* mRNA by the treatment with perifosine was restored by the treatment with SP600125, but the amounts of *LRP* mRNA were not significantly different between cells treated with both perifosine and SP600125 and cells treated with perifosine alone. *vs. control; $p < 0.05$, and †vs. cells treated with perifosine; $p < 0.05$.

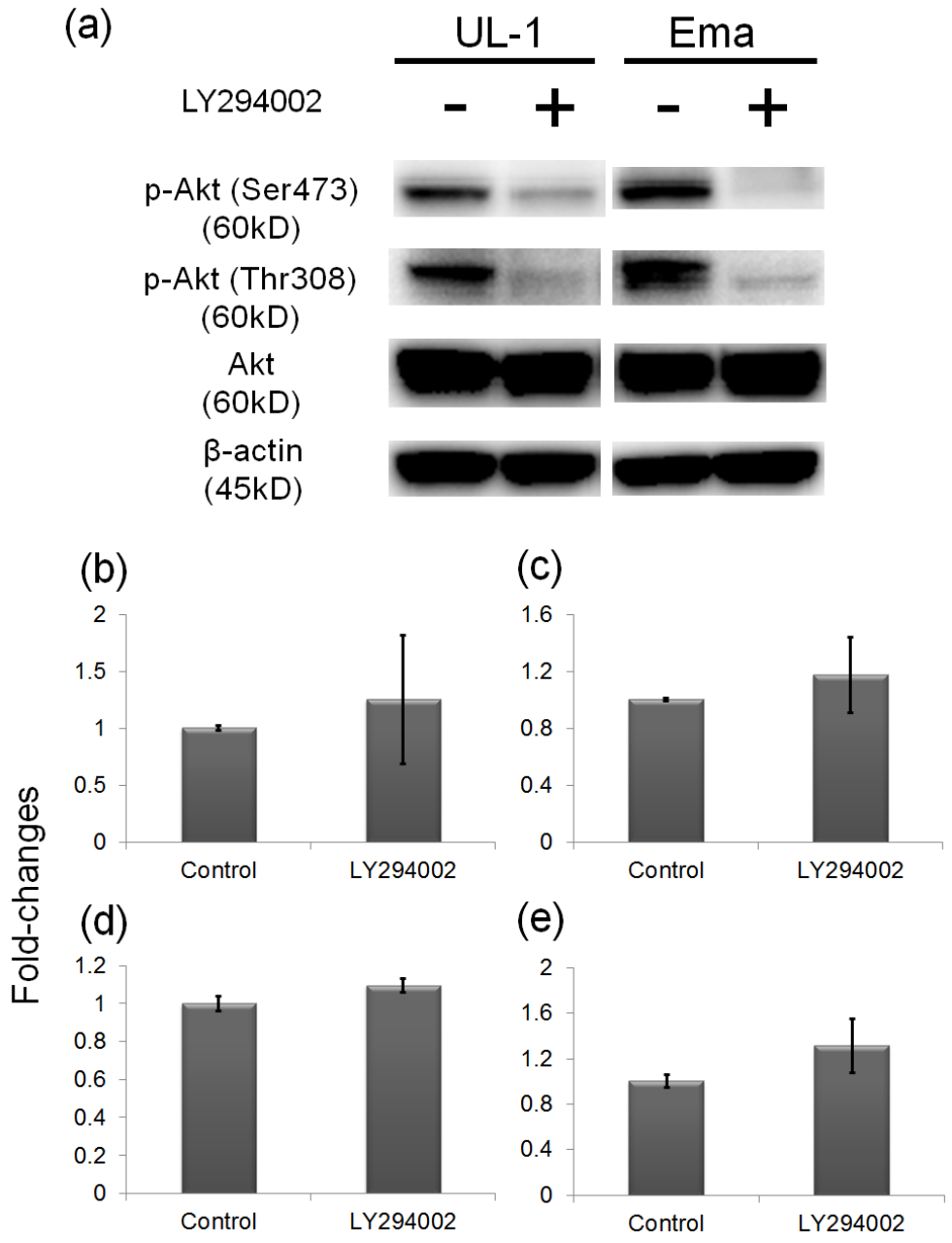

Fig. 4-5. Analysis on the inhibition of PI3K/Akt pathway with LY294002 in UL-1 and Ema (a). The quantities of phosho-Akt (Ser473) and phospho-Akt (Thr308) decreased by incubation with LY294002. The fold-changes of relative quantities of *ABCB1* and *LRP* genes in UL-1 (b and c, respectively) and Ema (d and e, respectively) by treatment with LY294002. The amount of *ABCB1* or *LRP* mRNA did not significantly change by treatment with LY294002 in both of UL-1 and Ema.

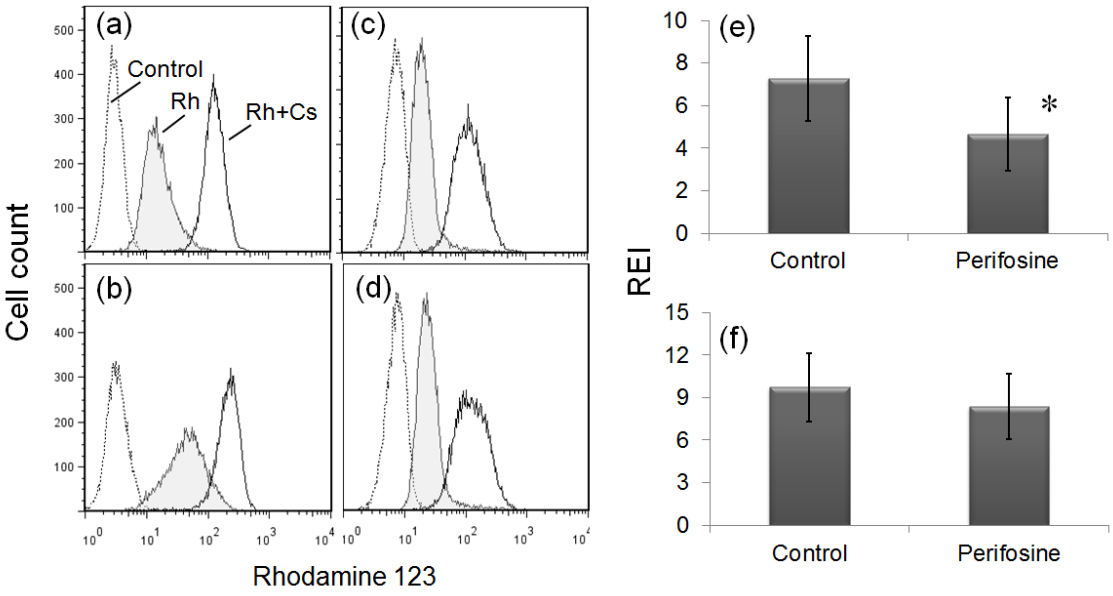

Fig. 4-6. The results of rhodamine 123 (Rh) efflux test in control cells and cells treated with perifosine in UL-1 (a and b, respectively) and Ema (c and d, respectively). Although the intracellular fluorescence intensities of Rh in the cells incubated with Rh and cyclosporine (Cs) were not different between the control cells (a) and those treated with perifosine (b), that of cells incubated with Rh alone was increased in cells treated with perifosine compared with control cells in UL-1. However, such a difference was not observed in Ema (c and d). The REI value significantly decreased when treated with perifosine compared with control cells in UL-1 (e), but those of Ema did not significantly change by treatment with perifosine (f). *$p < 0.05$.

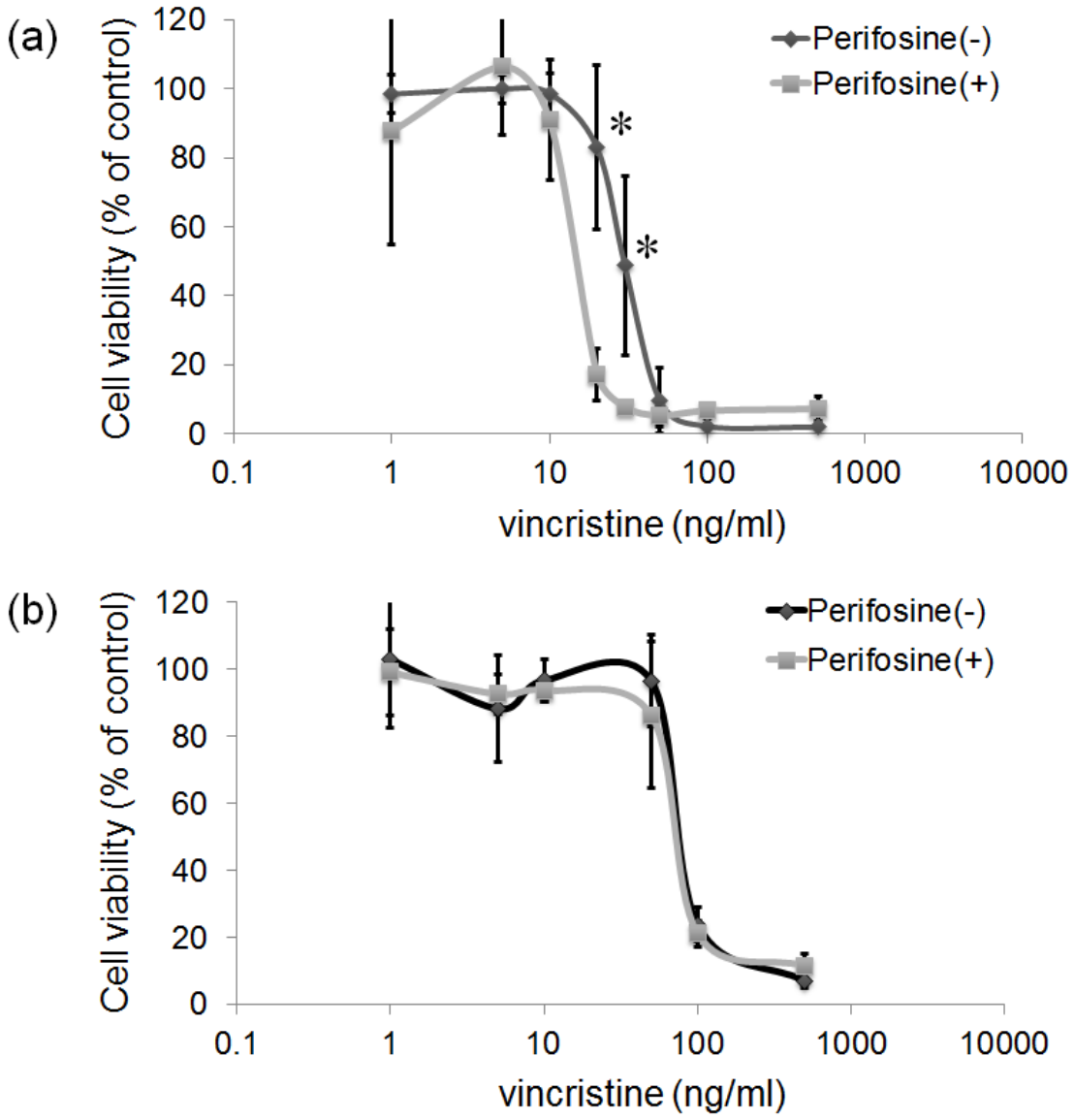

Fig. 4-7. The dose-response curves of the cytotoxic effect of vincrisine in UL-1 (a), and Ema (b) with or without treatment with perifosine. The cell viability data were plotted against the log of vincristine concentrations. The data represent the mean ± SD of triplicate cultures. *$p < 0.01$.

Chapter 5

Comprehensive comparison of gene expression profiles between drug-sensitive and drug-resistant phases in canine lymphoma patients

Abstract

Multidrug resistance (MDR) is one of the major obstacles during the chemotherapy in canine lymphoma patients. Although there have been various molecules that were shown to be associated with acquisition of MDR phenotype in human and canine tumor cells, the mechanisms that confer MDR phenotype to tumor cells have not been well understood. Therefore, new MDR factor(s) should be identified to overcome the MDR phenotype of canine lymphoma cells. In the present study, a comprehensive analysis of changes in gene expression profiles of lymphoma cells was conducted by microarray analysis using tumor cell samples at drug-sensitive and drug-resistant phases in 4 dogs. By gene ontology analysis and gene set enrichment assay, it was revealed that downregulation of genes that were associated with immunological reaction such as chemotaxis and inflammation was found to be a common change in the drug-resistant phase of the 4 dogs. Further studies are needed to examine the association of such change of the gene expression profiles with the acquisition of MDR phenotype in canine lymphoma cells.

Introduction

Multidrug resistance (MDR) is one of the major obstacles during the chemotherapy in the patients with various cancers including lymphoma. A lot of molecules that contribute to the development of MDR have been reported in human neoplastic cells (Gimenez-Bonafe et al., 2009; Harris and Hochhauser, 1992). Several mechanisms including overexpression of drug transporter (O'Connor, 2007), aberrations in molecules associated with apoptosis such as *p53* and *bcl-2* gene (Kupryjanczyk et al., 2003), increased detoxification of anticancer drug (Moscow and Dixon, 1993) and enhancement of DNA repair function (Gerson, 2004) have been shown to be associated with the acquisition of MDR in tumor cells of human origin. However, the expression levels of genes encoding these molecules were not significantly different between dogs with chemotherapy-sensitive and -resistant lymphoma, although overexpression of *ABCB1* gene was observed in 3 of 10 dogs with chemotherapy-resistant lymphoma in chapter 1. Therefore, it was indicated that novel MDR factor(s) should be identified to elucidate the mechanisms of MDR acquisition in canine lymphoma cells.

In human medicine, comprehensive analysis of gene expression profile has been conducted to identify significant MDR factors in tumor cells. As to the lymphoid tumor, comprehensive comparison of gene expression profile of diffuse large B-cell lymphoma (DLBCL) cells between patients that achieved continuous complete remission by treatment and patients that developed progressive disease during treatment was conducted using microarray analysis in one study (Linderoth et al., 2008). In their study, the enhanced immunological reaction in the tumor microenvironment of tissues was

demonstrated in the tumor cells obtained from patients that achieved continuous complete remission. However, there has been no study that analyzed novel MDR factors by comprehensively analyzing gene expression profiles in tumor cells obtained from patients in dogs.

The purpose of the present study was to investigate the novel common MDR factor(s) in canine lymphoma by comprehensive analysis of changes in gene expression profile using tumor cell samples obtained at drug-sensitive and drug-resistant phases of the same patients.

Materials and Methods

Patient samples

Four dogs that were referred to the Veterinary Medical Center of the University of Tokyo and diagnosed as multicentric high-grade lymphoma according to cytological examinations of enlarged lymph nodes based on updated Kiel classification (Fournel-Fleury et al., 1997) were included in the present study (Table 5-1). Lymphoma cell samples from all 4 dogs were shown to be clonally expanded B-lymphoid cells by PCR amplification test for the antigen receptor gene rearrangements as described previously (Burnett et al., 2003).

A cyclophosphamide, hydroxydaunorubicin (doxorubicin), vincristine, plus prednisolone (CHOP)-based chemotherapy, 6-month modified version of the University of Wisconsin (UW) - Madison chemotherapy protocol (UW-25) (Garrett et al., 2002), was used as a standard multidrug combination chemotherapy protocol. Veterinarians measured the size of the lymph nodes with calipers at each admission, and response to treatment was evaluated based on the Response Evaluation Criteria in Solid Tumors (RECIST) (Eisenhauer et al., 2009). In the evaluation of response to treatment, complete response (CR) and partial response (PR) were regarded as indicating chemotherapy-sensitive status, and stable disease (SD) and progressive disease (PD) were regarded indicating as chemotherapy-resistant status.

Dogs were regarded as chemotherapy-sensitive if they achieved CR or PR by week 5 of the UW-25 protocol. Tumor cell samples were collected just prior to the chemotherapy at the initial consultation by fine needle aspiration (FNA). Dogs were regarded as chemotherapy-resistant if they had underwent a relapse after achieving

remission by UW-25 protocol and did not respond to all of the three chemotherapeutic agents used for UW-25, cyclophosphamide, doxorubicin, and vincristine. The tumor cell samples in the chemotherapy-resistant group were collected after confirming the non-responsiveness to these three drugs in conjunction with prednisolone after the relapse by FNA. Tumor cell samples at both of chemotherapy-sensitive and -resistant phases were collected in each patient, and a total of 8 tumor cell samples were used in the present study.

Microarray analysis

Total RNA was isolated from tumor cell samples that had been stored in RNA later (Ambion, Austin, TX, USA) at -20 °C using 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, USA) 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 RNeasy Mini Kit (QIAGEN), 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. Spots with low intensity were eliminated from the analysis. The data was normalized, filtered and complicated

using Gene Spring GX software (Agilent Technologies). Only those genes, which showed $\log_2$ values greater than 1 (2-fold upregulated in drug-resistant samples) or less than -1 (2-fold upregulated in drug-sensitive samples) were included in analysis. The comparison of gene expression profiles between the drug-sensitive and drug-resistant phases was conducted by principal component analysis (PCA) and gene ontology (GO) analysis using Gene Spring GX software (Agilent Technologies) and gene set enrichment analysis (GSEA) using GSEA software (Broadinstitute, Cambridge, MA, USA). $P < 0.05$ was defined as significant.

Results

At first, PCA analysis using all 24 expression profiles extracted from 8 samples were conducted. As a result, it was revealed that the differences of gene expression profiles among patients were larger than those between drug-sensitive phase samples and drug-resistant phase samples (Figure 5-1). Therefore, the comparisons of expression profiles between drug-sensitive phase sample and drug-resistant phase sample of the same patient were conducted. In each of the 4 dogs analyzed, 511, 659, 157 and 338 genes were significantly upregulated in their drug-resistant phase; meanwhile, 1165, 2754, 1366 and 306 genes were significantly downregulated in their drug-resistant phase (Table 5-2).

In GO analysis, the upregulated genes in drug-resistant phase in dog 1 were significantly associated with 16 ontologies, although those in other 3 dogs were not associated with any ontology (Table 5-2). The downregulated genes in drug-resistant phase were significantly associated with 100, 364, 234 and 8 ontologies in 4 dogs, respectively (Table 5-2). Among these ontologies, 70 ontologies and 1 ontology were common among 3 dogs, respectively, and 2 ontologies were common among all of the 4 dogs (Figure 5-2). The 2 ontologies that were common among 4 dogs were ‘extracellular region’ and ‘extracellular region part’, and a common ontology among dog 1, 3 and 4 was ‘binding’. The 70 ontologies that were common among dog 1, 2 and 3 included ontologies that were associated with cell chemotaxis and cell migration and immune response (Table 5-3).

In GSEA, the upregulated genes in drug-resistant phase were significantly associated with 11, 5, 5 and 9 terms in each of the 4 dogs (Table 5-2 and 5-4), and no terms were common among 3 or 4 dogs. The downregulated genes in drug-resistant phase were significantly associated with 49, 102, 72 and 23 terms in each of the 4 dogs (Table 5-2). Among these terms, 21, 3 and 1 term were common among the 3 dogs, and 10 terms were common among all of the 4 dogs (Figure 5-3). The terms that were common among 4 dogs included “chemokine signaling pathway”, “leukocyte transendothelial migration” and “NOD like receptor signaling pathway” (Table 5-5). The 21 terms that were common among dog 1, 2 and 3 included terms that were associated with immune response such as “cytokine cytokine receptor interaction”, “natural killer cell mediated cytotoxicity”, “T-cell receptor signaling pathway” and “Toll-like receptor signaling pathway” (Table 5-5).

Discussion

It was revealed that downregulation of genes associated with immunological reaction including inflammation and chemotaxis was found to be a common change in drug-resistance phase of 4 dogs analyzed in the study of this chapter.

In PCA analysis, it was shown that the differences of gene expression profiles among patients were larger than those between drug-sensitive phase samples and drug-resistant phase samples. As shown in the study in chapter 1, the expression levels of genes that were associated with MDR were variable among patients that acquired MDR. These observations indicated that it was needed to detect the changes of gene expression profiles during the acquisition of MDR phenotype of lymphoma cells using tumor cell samples derived from the same patients. Therefore, the comparisons of gene expression profiles between drug-sensitive phase sample and -resistant sample of each patient were conducted, and common changes in gene expressions among 4 dogs were investigated in the study of this chapter.

In GO analysis, 70 ontologies that were associated with downregulated genes in drug-resistant phase were common among dogs 1, 2 and 3, although only 2 ontologies were common among all of the 4 dogs. These findings indicated that the changes in gene expression profiles during the acquisition of MDR were more similar among dogs 1, 2 and 3 than among all of 4 dogs. In dog 4, upregulation of ABC transporters, which was already shown to be associated with the acquisition of MDR phenotype, was noted in GSEA, although similar result was not observed in dog 1, 2 and 3. It was suggested that the upregulation of ABC transporters was one of the causes of the acquisition of

MDR in lymphoma cells of dog 4, although similar mechanisms was not associated with the acquisition of MDR in other 3 dogs.

In GSEA, downregulation of genes associated with immunological reaction including chemotaxis and inflammation, which was common change among dogs 1, 2 and 3 in GO analysis, was common change among all of 4 dogs. These results suggested that the downregulation of immunological reaction such as chemotaxis and inflammation was a common event associated with the acquisition of MDR phenotype in all of the 4 dogs analyzed here. In human medicine, it was revealed that a mammary adenocarcinoma cell line treated with tamoxifen upregulated the expression of macrophage migration inhibitory factor, which is a pro-inflammatory cytokine and key modulator of immune responses, in macrophages when the cell line was cocultured with macrophages (Burnett et al., 2008). This result suggested that the tumor cells that were subjected to anticancer drug had the function to modulate the immunological reaction of cells such as macrophages. In addition, I revealed that M2 macrophages had antitumor effect against canine lymphoma cells by inducing apoptosis (data not shown). Based on these observations, it is reasonable to hypothesize that the loss of immunological reaction such as chemotaxis and inflammation in tumor cells might be associated with the loss of antitumor effect induced by chemotherapeutic agents.

Further studies are needed to validate the decrease of molecules that were associated with chemotaxis and inflammation in protein level in MDR lymphoma tissues. It is also needed to examine the cell types that express these molecules in lymphoma tissues by using immunohistochemistry. However, it was reported that FNA samples of canine multicentric lymphoma contained few cells other than tumor cells (Sato et al., 2012), and it might be suggested that the results in microarray analysis in

the present study might reflect the changes in gene expression profiles of lymphoma cells.

In conclusion, the study of this chapter revealed that the downregulation of genes that were associated with immunological reaction such as chemotaxis and inflammation might be MDR factor in canine lymphoma. Further studies are needed to examine the association of this change and the acquisition of MDR phenotype in lymphoma cells.

Table 5-1.

Signalments of 4 dogs with lymphoma that underwent microarray analysis for the tumor cells.

Dog	Age (years old)	Sex	Breed	BW (kg)
1	9.9	Male	Shih Tzu	6.2
2	8.8	Male	Doberman	25.7
3	2.8	Male (castrated)	French bulldog	10.3
4	4.5	Female (spayed)	French bulldog	12.8

Table 5-2.

The results of comparison of gene expression profiles between drug-sensitive and -resistant phases, gene ontology (GO) analysis and gene set enrichment assay (GSEA).

Dog	Upregulated genes in drug-resistant phase	Downregulated genes in drug-resistant phase	Ontologies associated with upregulated genes in drug-resistant phase in GO analysis	Ontologies associated with downregulated genes in drug-resistant phase in GO analysis	Terms associated with upregulated genes in drug-resistant phase in GSEA	Terms associated with downregulated genes in drug-resistant phase in GSEA
1	511	1165	16	100	11	49
2	659	2754	0	364	5	102
3	157	1366	0	234	5	72
4	338	306	0	8	9	23

Table 5-3.

The 70 ontologies that were commonly associated with downregulated genes in drug-resistant phase samples of dog 1, 2 and 3 in gene ontology analysis

Cell chemotaxis and cell migration	cell chemotaxis cell migration cellular component movement chemokine activity chemotaxis locomotion positive regulation of cell migration positive regulation of cell motility positive regulation of cellular component movement positive regulation of locomotion regulation of cell migration regulation of cell motility regulation of cellular component movement regulation of chemotaxis regulation of localization regulation of locomotion taxis
Response to stimulus	cellular response to chemical stimulus positive regulation of response to external stimulus positive regulation of response to stimulus regulation of response to external stimulus regulation of response to stimulus response to chemical stimulus response to external stimulus response to organic substance response to oxygen-containing compound response to stimulus
Binding	calcium ion binding carbohydrate derivative binding glycosaminoglycan binding growth factor binding heparin binding receptor binding sulfur compound binding
Antomical structure development	anatomical structure development

	cardiovascular system development
	circulatory system development
	developmental process
	multicellular organismal development
	organ development
	system development
	tissue development
Cellular component	cell surface
	external side of plasma membrane
	extracellular membrane-bounded organelle
	extracellular organelle
	extracellular space
	extracellular vesicular exosome
	plasma membrane part
Immune response	immune response
	immune system process
	inflammatory response
	positive regulation of immune system process
	positive regulation of inflammatory response
	regulation of immune system process
Defense response	defense response
	response to stress
	response to wounding
Regulaltion of cell death	regulation of apoptotic process
	regulation of cell death
	regulation of programmed cell death
Others	biological regulation
	extracellular matrix
	positive regulation of biological process
	positive regulation of transport
	regulation of biological process
	regulation of biological quality
	regulation of cell adhesion
	regulation of multicellular organismal process
	single-multicellular organism process

Table 5-4.

The terms that were associated with upregulated genes in drug-resistant phase samples of each patient.

Terms	Dog
Base excision repair	1
Cell cycle	1
Citrate cycle TCA cycle	1
DNA replication	1
Homologous recombination	1
Mismatch repair	1
Neurotrophon signaling pathway	1
Nucleotide excision repair	1
Parkinsons disease	1
Proteasome	1
RIG I like receptor signaling pathway	1
Base excision repair	2
Propanoate metabolism	2
Spliceosome	2
Steroid biosynthesis	2
Valine leucine and isoleucine degradation	2
Basal transcription factors	3
Butanoate metabolism	3
Selenoamino acid metabolism	3
Steroid biosynthesis	3
Tyrosine metabolism	3
ABC transporters	4
Alanine aspartate and glutamate metabolism	4
Ether lipid metabolism	4
Glycerophospholipid metabolism	4
Glycine serine and threonine metabolism	4
Glycosaminoglycan biosynthesis chondroitin sulfate	4
Hematopoietic cell lineage	4
Porphyrin and chlorophyll metabolism	4
RNA polymerase	4

Table 5-5.

The terms that were commonly associated with downregulated genes in drug-resistant phase samples of 3 or 4 dogs in gene set enrichment assay.

Common terms among 4 dogs	Adherens junction
	Arrhythmogenic right ventricular cardiomyopathy arvc
	Chemokine signaling pathway
	Cytosolic DNA sensing pathway
	Focal adhesion
	Leishmania infection
	Leukocyte transendothelial migration
	NOD like receptor signaling pathway
	Pathways in cancer
	Systemic lupus erythematosus
Common terms among dog 1, 2 and 3	Arginine and proline metabolism
	Axon guidance
	Bladder cancer
	Calcium signaling pathway
	Colorectal cancer
	Complement and coagulation cascades
	Cytokine cytokine receptor interaction
	Dilated cardiomyopathy
	Drug metabolism other enzymes
	ECM receptor interaction
	Epithelial cell signaling in helicobacter pylori infection
	Gap junction
	Hematopoietic cell lineage
	Hypertrophic cardiomyopathy HCM
	Melanoma
	Natural killer cell mediated cytotoxicity
	Prion diseases
	Regulation of actin cytoskeleton
	Small cell lung cancer
	T cell receptor signaling pathway
	Toll like receptor signaling pathway
Common terms among dog 2, 3 and 4	Fatty acid metabolism
	TGFβ signaling pathway
	Tight junction
Common terms among dog 1, 2 and 4	Pancreatic cancer

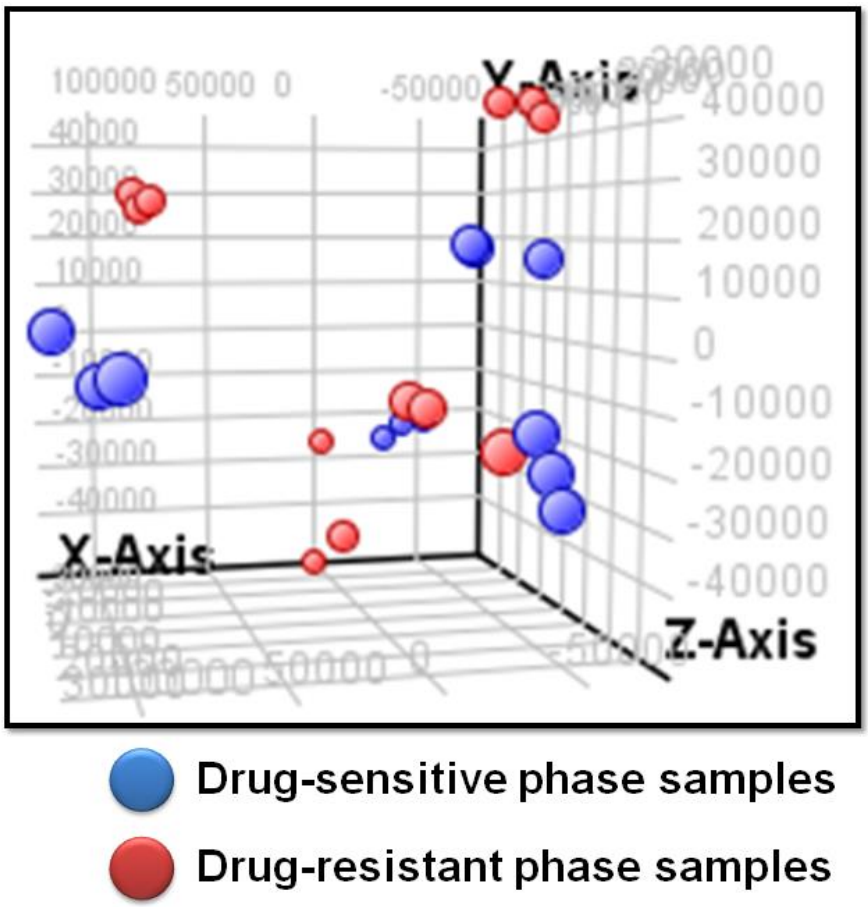

Fig. 5-1. The results of PCA analysis using all 24 expression profiles extracted from 8 samples were conducted. It was revealed that the differences of gene expression profiles among patients were larger than those between drug-sensitive phase samples and drug-resistant phase samples

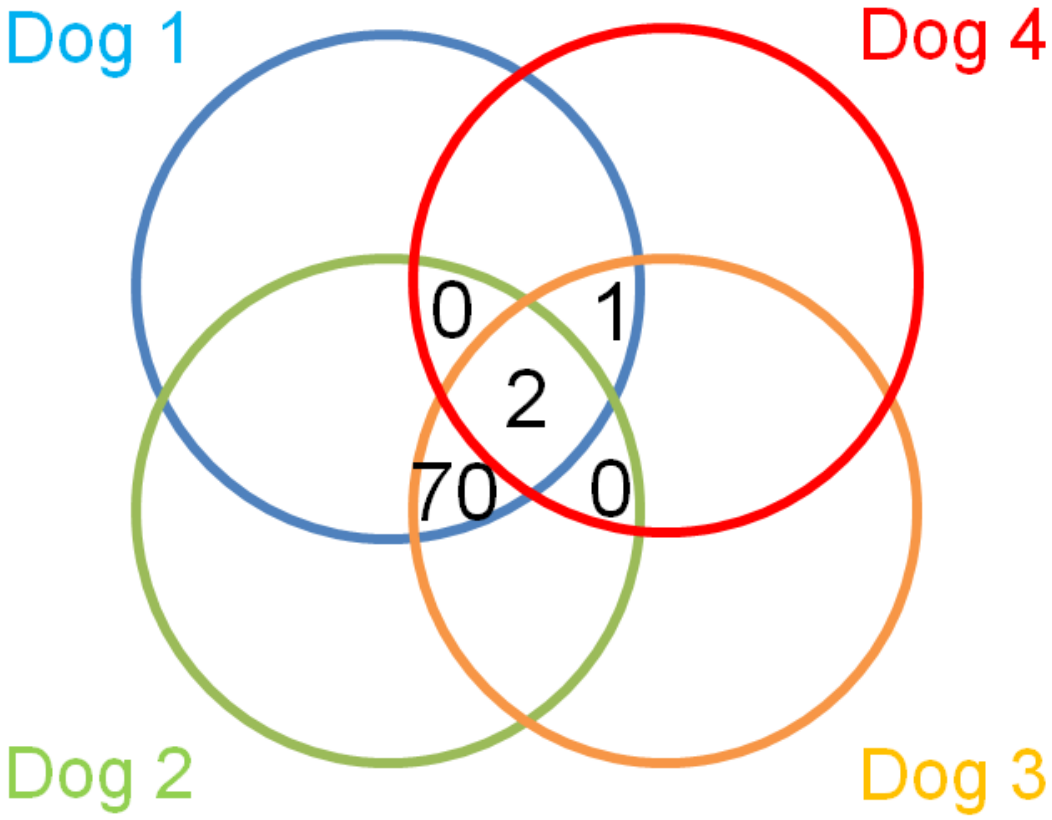

Fig. 5-2. The numbers of common ontologies that were associated with downregulated genes in drug-resistant phase samples among 3 or 4 dogs in gene ontology analysis. Although 70 ontologies were common among dog 1, 2 and 3, only 2 ontologies were common among all of the 4 dogs.

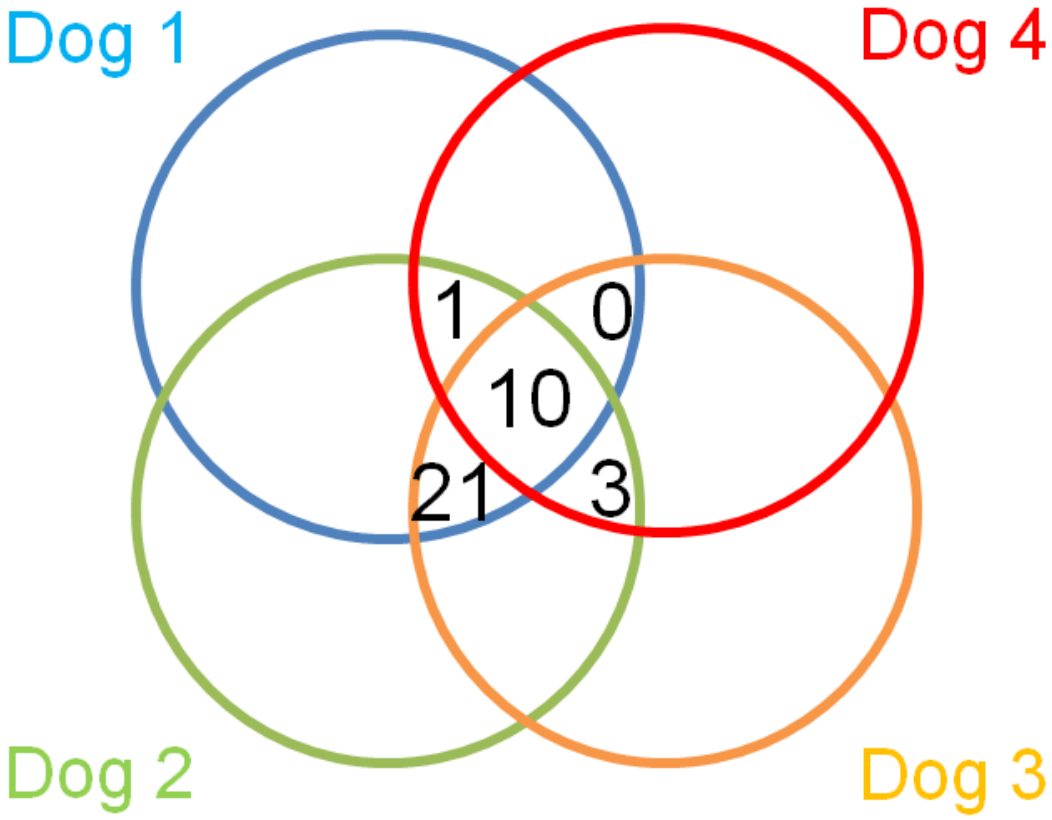

Fig. 5-3. The numbers of common terms that were associated with downregulated genes in drug-resistant phase samples among 3 or 4 dogs in gene set enrichment assay. The 21 terms were common among dog 1, 2 and 3, and 10 terms were common among all of the 4 dogs.

Conclusion

Acquisition of multidrug resistance (MDR) is one of the major causes of the failure in chemotherapy for various tumors including lymphoma. Several mechanisms have been proposed as the mechanisms that confer MDR phenotype to tumor cells, and various molecules were shown to be associated with MDR phenotype. However, the mechanisms of the acquisition of MDR in tumor cells have not been well understood. A series of studies in the present thesis were carried out to elucidate the molecular mechanisms of the acquisition of MDR phenotype in canine lymphoma.

In chapter 1, expression levels of 10 genes that were associated with MDR in human medicine and mutation of *p53* gene were examined in dogs with multicentric high-grade lymphoma to explore their association with drug resistance of the tumor cells. The expression levels of these genes and frequency of *p53* gene mutation were compared between drug-sensitive and drug-resistant groups. As a result, no significant differences were observed in expression levels of each gene between groups. However, 3 dogs in the chemotherapy-resistant group showed high expression of *ATP-binding cassette, sub-family B, member 1* (*ABCB1*). In addition, no significant difference was observed in the frequency of *p53* mutation between groups. Therefore, a possible association of *ABCB1* with resistance to 6-month modified version of the University of Wisconsin (UW)-Madison chemotherapy protocol (UW-25) was shown in certain types of canine lymphoma patients.

Next, epigenetic regulation of canine *ABCB1* gene was examined in canine lymphoid tumor cell lines and primary tumor cells derived from multicentric high-grade lymphoma patients to elucidate the mechanisms that induce overexpression of *ABCB1*

gene in chapter 2. In canine lymphoid tumor cell lines, 2 vincristine-sensitive cell lines, GL-1 and CLBL-1, expressed low levels of *ABCB1* gene possibly through hypermethylation of the CpG motif and histone H3 hypoacetylation compared with 2 vincristine-resistant cell lines, UL-1 and Ema. In addition, treatments with a DNA methyltransferase inhibitor (5-aza 2'-deoxycytidine; 5-aza dC) and a histone deacetyltransferase inhibitor (trichostatin A; TSA) significantly increased the expression of *ABCB1* gene in GL-1 and CLBL-1. These results indicated that DNA methylation and histone H3 acetylation were associated with the regulation of *ABCB1* gene expression. However, in primary tumor cells obtained from canine lymphoma patients, *ABCB1* gene expression was not silenced by DNA methylation before chemotherapy, and it was indicated that expression regulation through DNA methylation was not associated with the overexpression of *ABCB1* gene in MDR lymphoma cells.

Based on the results in chapter 2, other mechanisms that induced overexpression of *ABCB1* gene were investigated based on cancer cell concept in chapter 3. In 2 lymphoma cells, UL-1 and Ema, side population (SP) fraction, which is thought to contain cancer stem cells, was observed, and SP was shown to express larger amount of *ABCB1* and *LRP* mRNA compared with other cell fraction (major population, MP). In addition, SP was found to express larger amount of *TGF-β type II receptor* mRNA compared with MP in microarray analysis, and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, which is downstreamt of TGF-β signaling, was more activated in SP than MP. Finally, it was revealed that MAPK/ERK pathway upregulated the expression of *ABCB1* and *LRP* genes in these 2 cell lines. From the results of chapter 3, it was indicated that activation of a intracellular signaling, MAPK/ERK pathway, was involved in regulation of *ABCB1* gene expression.

In chapter 4, the decrease of the expression of *ABCB1* gene by modulation of intracellular signalings including MAPK/ERK pathway was examined based on the results in chapter 3. As a result, perifosine, an Akt inhibitor, had antitumor effect in 2 of 4 canine lymphoid tumor cell lines, GL-1 and UL-1. The amount of *ABCB1* mRNA significantly decreased after treatment with perifosine through activation of c-jun NH_2-terminal kinase (JNK) pathway in UL-1. In this cell line, the efflux of rhodamine 123 dye and the IC_{50} value for vincristine were reduced by perifosine, but such effects were not observed in Ema. In summary, perifosine had an antitumor effect, downregulated *ABCB1* gene expression, and increased the sensitivity for vincristine in a certain type of canine lymphoid tumor cells.

Finally in chapter 5, novel MDR factor(s) in canine lymphoma were investigated by comprehensive analysis of changes in gene expression profiles at acquisition of MDR phenotype using microarray analysis. In gene ontology analysis and gene set enrichment assay, the downregulation of genes that were associated with immunological reaction such as chemotaxis and inflammation was commonly observed among all of the 4 dogs examined, and it was suggested that these changes may correspond to the new MDR factors in canine lymphoma.

In this thesis, the overexpression of *ABCB1* was focused based on the results in chapter 1. Then, the molecular mechanisms that induce overexpression of *ABCB1* gene was investigated in chapters 2 and 3. Moreover, the reduction of the expression of *ABCB1* gene and reconstitution of the sensitivity to vincristine by treatment with perifosine was shown in certain types of canine lymphoma cells in chapter 4. In these chapters, the changes in molecular mechanisms occurring within tumor cells were focused to elucidate the mechanisms of the acquisition of MDR phenotype. In addition

to these studies, it was suggested that decrease of immunological reaction might be new MDR factor in canine lymphoma from the results of chapter 5. In human medicine, it has been indicated that changes in interactions of tumor cells with stromal cells in tumor tissues had important effects in acquisition of MDR phenotype of tumor cells (Rebucci and Michiels, 2013). Based on the results in chapter 5 and previous studies in human medicine, the changes in tumor microenvironments might be one of the key mechanisms to elucidate and overcome the acquisition of MDR of tumor cells.

The overexpression of ABC-transporters is one of the most extensively studied mechanisms that are associated with MDR phenotype in human (O'Connor, 2007) and veterinary medicines (Bergman, 2003). However, it was reported that expression of P-gp is not always linked to MDR *in vivo* and inhibition of the function of P-gp cannot always re-establish sensitivity for chemotherapeutic agents (Leonard et al., 2003). In the results of chapter 1 in this thesis, larger amount of *ABCB1* mRNA was expressed in 3 of 10 dogs with MDR lymphoma, but not in other 7 dogs. These observations suggested that the acquisition of MDR might be multifactorial, and the mechanisms of MDR might be different among patients. Therefore, it was needed to identify more new MDR mechanisms and to classify the mechanisms that were associated with acquisition of MDR mechanisms in each patient by analyzing large number of patients. In addition to the results obtained by comprehensive analysis of changes in gene expression profiles in chapter 5, it might also help to identify new MDR mechanisms to analyze the changes in genomic DNA sequence during acquisition of MDR phenotype by whole sequence analysis using next generation sequencing as conducted in human medicine (Tzoneva et al., 2013).

In conclusion, the association of overexpression of *ABCB1* gene with MDR in

canine lymphoma, the molecular mechanisms that induced overexpression of *ABCB1* gene, and the effect of perifosine to re-establish the sensitivity to substrate of P-gp were shown in a series of the studies in this thesis. In addition, it was also suggested that decrease of the immunological reaction in lymphoma cells might be new MDR factors. I believe that the results obtained in this thesis advanced the research to elucidate the molecular mechanisms and will contribute to develop effective therapy for tumor cells with MDR phenotype in both humans and dogs.

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