GENERAL INDRODUCTION

Lymphoma is the most common hematopoietic malignancies in dogs, which account for 7 - 24 % of all canine cancers and 83 % of canine hematologic tumors [Vail *et al.*, 2007]. Chemotherapies are the first-line therapy for the canine lymphoma. Although the drugs used to the canine lymphoma are quite effective for B cell lymphomas, they finally acquire resistances to the chemotherapies [Dobson *et al.*, 2001; Moore et al., 1995]. On the other hand, these drugs are usually less effective to T-cell lymphomas even on the initial treatment [Dobson *et al.*, 2001]. In patients, who were burdened with resistance to the lymphoma, generally anti-cancer agents, which act via different mechanisms rather than common agents, are used as "rescue protocols". In spite of efforts to overcome the resistance, the rescue protocols do not be effective compared with the first-line protocols. In my opinion, there are two important things to improve a treatment outcome; 1) to analyze and overcome drug-resistance mechanism(s) in lymphoma cells 2) to discover key regulators for tumorigenesis, proliferation, and survival of the lymphoma cells.

The ATP-binding cassette (ABC) transporter such as P glycoprotein (P-gp) and multidrug resistance associated protein (MRP) is one of the common mechanisms of the resistance to the chemotherapeutics [Drain *et al.*, 2010]. Lomustine is not substrates of P-gp, which usually contributes to the multidrug resistance in the lymphoma cells [Tew *et al.*, 2001]. For that reason, lomustine is frequently used for the rescue protocol in lymphoma patients, and the efficacy has been shown. However, as lomustine was continued to use, the lymphoma cells acquired the resistance. It is known in human cancer, that the DNA repair protein, O^6-methylguanine-DNA methyltransferase (MGMT) have expressed and reversed O^6-methylguanine, which was altered forcibly by alkylating agents into guanine. As a result, the MGMT protein protects the cancer cells from a cell death by the alkylating agents. Therefore, in chapter 1, I focused on MGMT in the canine lymphoma cells, and analyzed the resistance against the alkylating agents, nitrosoureas by MGMT using canine lymphoma cell lines *in vitro*.

In terms of the development of the cancer treatment, recently the molecular targeted therapy, such as an anti-CD20 antibody pharmaceutical for the treatment of human B- cell lymphomas and

leukemias, has been progressed remarkably. These types of agents inhibit specific molecules, which are necessary for carcinogenesis, cancer proliferation or survival. In the veterinary medicine, although a tyrosine kinase inhibitor, imatinib have been shown to be applicable for canine mast cell tumor [Bonkobara *et al.*, 2008], studies to treat the lymphoma by molecular-targeted therapies are ongoing, so that discovering a novel therapeutic target is expected.

Hypoxia inducible factor (HIF) has been reported as the key regulator for poor prognostic factors in cancer tissues, which involve hypoxic regions [Semenza *et al.*, 2012]. Furthermore, HIF-1α transgenic mice developed lymphoproliferative diseases including the lymphoma at a high rate, and this protein is considered as an important factor for the survival and/or growth of lymphoma cells [Sueoka et al., 2013]. Notably, in murine T cell lymphoma, which was resulted from an insertional abrogation of an epilepsy, progressive myoclonus type 2A (Epm2a) gene, HIF-1α and Notch1 cooperates to maintain a cancer stem cell population, since silencing or inhibiting HIF-1α can lead to remission the lymphoma [Wang et al., 2011]. Based on these finding, in chapter 2, I examined whether HIF-1α expresses on the canine lymphoma cells, and HIF-1α inhibitors have therapeutic potential to canine lymphoma cell lines, clinical samples and tumors in xenograft models.

Chapter 1

O6-methylguanine DNA Methyltransferase Expression Causes Lomustine Resistance in Canine Lymphoma Cells

SUMMARY

O^6-methylguanine DNA methyltransferase (MGMT) is a DNA repair protein that causes resistance to nitrosoureas in various human cancers. In this study, I analysed the correlation between canine lymphomas and MGMT *in vitro*. Two of the five canine lymphoma cell lines that showed higher IC_{50} against lomustine regained their sensitivity when cultured with an MGMT inhibitor. Fluorometric oligonucleotide assay and real-time PCR on these cell lines revealed MGMT activity and high MGMT mRNA expression, respectively. I analysed the methylation status of the CpG islands of the canine MGMT gene by the bisulfite sequencing method. Unlike human cells, the canine lymphoma cell lines did not show significant correlation between the methylation status and the MGMT suppression levels. My results suggest that in canine lymphoma, the MGMT activity may influence sensitivity to nitrosoureas; thus, its inhibition would benefit nitrosoureas-resistant patients. Additional studies are necessary to elucidate the mechanism of regulation of MGMT expression.

Introduction

Lymphoma, the most common hematopoietic malignancy in dogs, is usually treated with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone)-based first-line therapy [Garrett *et al.*, 2002, Keller *et al.*, 1993]. While the remission rate and duration of these protocols is more than 80% and over 9 months respectively, most animal patients eventually develop chemotherapeutic resistance, one of the causes being an increased expression of the gene encoding P-glycoprotein [Moore *et al.*, 1995, Lee *et al.*, 1996].

Lomustine [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea], also called CCNU is an alkylating nitrosourea compound with a molecular weight of 233.7 Da, and it cannot act as a substrate for P-glycoprotein. Lomustine induces alkylation and cross-linking of DNA at the O^6-position of guanine, and thus inhibits DNA synthesis [Tew *et al.*, 2001]. Although lomustine is an effective rescue agent for relapsed canine lymphoma [Moore *et al.*, 1999, Saba *et al.*, 2007], the remission often lasts for less than 100 days, indicating that the tumor cells may also acquire resistance to lomustine.

The DNA repair protein O^6-methylguanine DNA methyltransferase (MGMT), expressed in human tumor cells, plays a significant role in the development of resistance to nitrosoureas like lomustine and carmustine (BCNU) [Pegg *et al.*, 1995]. MGMT reverses the formation of adducts at the O^6-position of guanine by transferring the alkyl adduct to a cysteine residue in itself [Ludlum *et al.*, 1990], thereby averting the formation of lethal DNA cross-links. Absence of MGMT activity has been detected in approximately 30% of the brain tumors [Silber *et al.*, 1993 and 1998] and may be associated with enhanced sensitivity to the action of alkylating agents [Beanich *et al.*, 1996, Jaeckle *et al.*, 1998, Silber *et al.*, 1999]. The inactivation of MGMT is most frequently caused by epigenetic changes, specifically, alterations in the methylation status of the promoter region. The methylation of the MGMT promoter region, which suppresses the expression of MGMT messenger RNA (mRNA), is a strong predictor of the cell response to carmustine and overall survival in human brain tumors [Esteller *et al.*, 2000]. Moreover, hypermethylation of the MGMT promoter is associated with a favorable prognosis of human diffuse large B-cell lymphoma (DLBCL) treated

with multidrug regimens, including an alkylating agent called cyclophosphamide [Esteller *et al.*, 2002]. However, the role of MGMT expression in canine lymphoma remains understudied.

In order to determine MGMT expression in canine lymphoma cells, a cytotoxicity assay with lomustine and an MGMT inhibitor, O^6-Benzylguanine (BG) was performed on canine lymphoma cell lines, and the MGMT activity was detected by a fluorometric oligonucleotide assay. Moreover, hypermethylation of the MGMT promoter region was examined by a bisulfite sequencing method.

MATERIALS AND METHODS

Cells

Five canine lymphoma cell lines, GL-1 (B cell type) [Nakaichi *et al.*, 1996], CL-1, Ema, Nody-1 and UL-1 (T cell type) [Momoi *et al.*, 1996, Yamazaki *et al.*, 2008, Hiraoka *et al.*, 2009] and immortalized human T lymphocytes, i.e., Jurkat cells were used in this study. All the cell lines were maintained in complete medium [RPMI-1640 containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (55 μM)] and grown at 37°C in an atmosphere containing 5% CO_2. Peripheral blood mononuclear cells (PBMC) from three healthy dogs were prepared using Lymphoprep (Fresenius Kabi Norge, Oslo, Norway) and were used as the control. Briefly, the heparinized whole blood was centrifuged and the buffy coat was suspended in phosphate-buffered saline (PBS) [2.7 mM KCl, 0.14 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$]. The PBMC were isolated by gradient centrifugation using Lymphoprep and further purified by overlaying on fatal bovine serum (FBS), followed by centrifugation, in order to remove the contaminating platelets.

Chemicals

BG (Sigma-Aldrich, St. Louis, MO) and lomustine (Bristol-Myers Squibb, NY) were dissolved in 200 proof ethanol at a concentration of 16.7 mM and 100 mM, respectively, and stored at -20°C until further use. 5-Aza-2′-deoxycytidine (Sigma-Aldrich), which causes DNA demethylation, was dissolved in distilled water at a concentration of 43.8 mM, diluted to 20 μM in the complete medium, and stored at -20°C until further use.

Cytotoxicity assay

Treatment conditions of BG and lomustine were determined by previously described protocols [Zaboikin *et al.*, 2004, Casorelli *et al.*, 2008]. The BG-treated [BG (+)] and BG-untreated [BG (-)] groups were pre-treated with BG (at a final concentration of 80 μM) and ethanol (equal amount)

respectively, for 2 hr at 37°C in 5% CO_2. The canine lymphoma cells were washed with PBS, and incubated for 1 hr with various concentrations of lomustine. The cells were washed with PBS, and the medium was replaced with a fresh medium without the drugs. The cells were then grown for five days, and an MTT-based assay was performed using the Cell Growth Determination Kit (Sigma-Aldrich). Each experiment was performed in triplicate and independently repeated three times. The concentrations of lomustine that inhibited the cell growth by 50% (IC_{50}) were calculated from the drug survival curves.

Fluorometric oligonucleotide assay for the measurement of MGMT activity

Measurement of MGMT activity was performed by a fluorometric oligonucleotide assay described by Kreklau *et al.* (2001). with minor modifications. Briefly, exponentially growing cultured cells or PBMC were suspended in an assay buffer [50 mM Tris (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol], pulse-sonicated in ice-cold water 25 times (for 10 s each) using BioRuptor UCD-200T (Thermo Fisher Scientific, Waltham, MA), and then centrifuged at 14,000 × g at 4°C for 30 min. Protein concentration of the supernatants was quantified using the Bradford protein assay (Thermo Fisher Scientific). The protein samples were snap-frozen in liquid nitrogen and stored at -80°C for further analysis. To obtain a double-stranded 45 bp oligonucleotide containing a single O^6-methylated guanine residue nested in a *Pvu*II restriction site and a fluorometric 5′-hexachloro-fluorescein phosphoramidite (HEX), two custom oligonucleotides were purchased from Sigma-Aldrich (Sense 45: 5′-GCAGTCCAGCTTCAG^{m}CTGCACGTCATCCTGTGCAGTCGTCTCGAC-3′ and Antisense 45: 5′-HEX-GTCGAGACGACTGCACAGGATGACGTGCAGCTGAAGCTGGACTGC-3′). These oligonucleotides were diluted to 2 μM with TE Buffer [50 mM Tris (pH 8.0) and 1 mM EDTA], mixed in a 1:1 molar ratio, denatured at 95°C for 10 min, and annealed at room temperature for 30 min. The resulting double-stranded oligonucleotide (25 nmol) was incubated with the extracted proteins (600 μg) at 37°C for 2 hr, followed by phenol-chloroform extraction and ethanol

precipitation of the oligonucleotide. The purified oligonucleotides were then digested with *Pvu*II and electrophoresed on a 20% denaturing polyacrylamide gel. The HEX-labelled oligo was detected using a green LED light (excitation wavelength of 530 nm, MeCan Imaging, Saitama, Japan) and a digital camera with FUJI FILTER SC54 (Fujifilm, Tokyo, Japan), and the fluorescence intensity was analysed using Multi gauge ver. 3.0 (Fujifilm).

5-Aza-2'-deoxycytidine treatment

Treatment conditions (incubation time and concentration) of 5-Aza-2′-deoxycytidine treatment were determined by the previously described protocols [Cario *et al.*, 2006, Fulda *et al.*, 2006]. The five lymphoma cell lines and PBMC were treated with 5-Aza-2′-deoxycytidine (at a final concentration of 0.5 μM) for 3 d at 37°C in 5%. The culture medium was replaced with fresh medium containing 5-Aza-2′-deoxycytidine every day. After 3 d of culture, the cells were washed with PBS and stored at -80°C until further use.

Real-time PCR

Total RNA was isolated from the five lymphoma cell lines and PBMC using the TRI reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions, and resolved with 30 μl of DEPC-treated water. Total RNA (2 μg) was treated with DNase I using Turbo DNA-free (Applied Biosystems, Carlsbad, CA), and transcribed into cDNA using Superscript II (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. An Oligo dT primer was used to prime the first-strand synthesis for each reaction. The single-stranded cDNA was subjected to real-time PCR amplification with a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. The primers used for assaying the expression of MGMT and the housekeeping gene ribosomal protein L32 (RPL32) [Peters *et al.*, 2007] were designed based on canine nucleotide sequences (Table 1). Each assay was performed in duplicate. Pre-denaturation at 95°C for 15 min was followed by 45 cycles of PCR

amplification consisting of denaturation at 94°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. PCR and fluorescence intensity detection were performed using the StepOne PCR system (Perkin–Elmer, Waltham, MA). The data were analysed using the StepOne software v.2.0. Briefly, the PCR cycle number at the threshold was represented as Ct and the difference between Cts for the target and internal control, i.e., ΔCt, was calculated.

Bisulfite sequencing

Total DNA was isolated from the five lymphoma cell lines and PBMC using the QIAamp DNA Mini Kit (Qiagen). DNA (5 μg) was digested with *BamHI* for 1 hr. After phenol-chloroform extraction and ethanol precipitation, 1 μg of the DNA sample was subjected to bisulfite modification using the CpGenome DNA Modification Kit (Chemicon, Temecula, CA), according to the manufacturer's instructions, and stored at -80°C until further use. CpG islands around exon 1 of the canine MGMT gene, in which methylation is associated with the silencing of the MGMT expression in human tumor cells [Rollins *et al.*, 2006], were analysed using the Methyl Primer Express software v1.0 (http://www.appliedbiosystems.com/absite/us/en/home/support/software-community/free-ab-software.html) with the following parameters: a G+C content greater than 55% and observed CpG/expected CpG of greater than 0.65 [Takai *et al.*, 2002]. Based on the analyses, six sets of PCR primers were prepared (Table 1). PCR amplification was performed as follows: pre-denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50–61°C (Table 1) for 30 s, and extension at 72°C for 60 s, and then a final extension at 72°C for 7 min, using the bisulfite-modified DNA as the template. For direct sequencing, the gel-purified PCR product was sequenced with the same primers with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Perkin–Elmer) and analysed with an ABI377 automated DNA sequencer at the Yamaguchi University Center for Gene Research. Alternatively, the amplified DNA was cloned into a pCR2.1 plasmid vector using the TOPO TA Cloning Kit (Invitrogen), and five independent clones that were obtained were then subjected to sequence analysis (direct sequencing).

Statistical analyses

The cytotoxicity of lomustine with and without BG was analysed by the two-way ANOVA and Bonferroni post hoc tests. The association between the IC_{50} of lomustine and the real-time PCR values for the mRNA expression of MGMT was assessed by calculating the Spearman's rank correlation coefficient. Analyses were performed using the GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). A p-value of ≤ 0.05 was considered to be statistically significant.

RESULTS

BG treatment increased the sensitivity to lomustine in CL-1 and Nody-1 cells

To examine cell susceptibility to lomustine with and without BG, the cytotoxicity assay was performed on five canine lymphoma cell lines as well as Jurkat cells that are known to have MGMT activity [D'Atri *et al.*, 2000]. Fig. I-1 and Table 2 represent the rates of cell survival at various concentrations of lomustine and the mean IC_{50} values, respectively. All the cell lines showed a dose-dependent decrease in cell survival. Interestingly, pre-treatment with BG resulted in a significant decrease in the viability of the Jurkat, CL-1, and Nody-1 cells. In contrast, no difference in viability with or without BG was observed in the Ema, GL-1, and UL-1 cell lines. Moreover, the IC_{50} values of Jurkat, CL-1, and Nody-1 cells, in which the BG pre-treatment decreased the overall IC_{50} values, were relatively high compared to those in Ema, GL-1, and UL-1 cells.

CL-1 and Nody-1 cells contain MGMT activity by the fluorometric oligonucleotide assay

The difference in the MGMT activity between the cell lines was examined by the fluorometric oligonucleotide assay [Kreklau *et al.*, 2001]. A HEX-labeled oligonucleotide was incubated with cellular extracts obtained from the five canine lymphoma cell lines and Jurkat cells. If the extract contains MGMT activity, an O^6-methylated guanine residue nested within a *Pvu*II restriction site can be demethylated, and the oligonucleotide can be digested by *Pvu*II. Fig. I-2 represents the fluorometric oligonucleotide assay results. *Pvu*II digested band was observed in Jurkat as well as CL-1 and Nody-1 cells, indicating that these cells contain MGMT activity.

Methylation inhibitor induced the MGMT mRNA expression in Ema and GL-1 cells that did not originally express the MGMT gene

To examine the expression of MGMT mRNA in the canine lymphoma cells, real-time PCR analysis was performed on the five lymphoma cell lines and PBMC collected from healthy dogs. PBMC, CL-1, Nody-1, and UL-1 cells expressed MGMT in the absence of the methylation

inhibitor. In contrast, no MGMT mRNA was detected in the Ema and GL-1 cell lines (Fig. I-3). Since methylation of the MGMT promoter region suppresses the MGMT gene expression in some human cancer cells, the canine cells were pre-treated with 5-Aza-2′-deoxycytidine to inhibit DNA methylation. As shown in Fig. I-3, Ema and GL-1 cells gained the expression of the MGMT mRNA to some extent; however, the other cell lines and PBMC did not show any change in the MGMT mRNA expression after treatment with the methylation inhibitor, suggesting that the MGMT mRNA expression is suppressed, at least in part, by the DNA methylation in Ema and GL-1 cells, but not in the other cell lines and PBMC.

Search for hypermethylation of the putative MGMT promoter region

Hypermethylation of the CpG islands surrounding the human MGMT exon 1 can suppress the expression of MGMT mRNA [Qian *et al.*, 1997]. To investigate if this also occurs in the canine lymphoma cells, bisulfite sequencing was performed. Four CpG islands were identified in the sequences located approximately 10,000 bp upstream to 7,000 bp downstream of the canine MGMT exon 1 by using the Methyl Primer Express software v1.0 (Fig. I-4). I first examined Region 1, which contains 24 CpGs, by a direct bisulfite sequencing method. DNA isolated from the canine lymphoma cells was modified using the CpGenome DNA Modification Kit, and then subjected to PCR. The amplified DNA was purified from agarose gel and directly sequenced. As shown in Fig. I-5A, the CpGs in the Ema cells that do not express the MGMT activity and mRNA, were not methylated. In contrast, the CpGs in all the other cell lines were mostly methylated. In order to confirm the results obtained from this direct sequencing method, the amplified DNA was cloned into a plasmid vector, and then five independent clones were subjected to sequencing (Fig. I-5B-D). Almost all the CpGs in the Ema cell line were found to be unmethylated by this method. Approximately 70% of the CpGs in Nody-1 cells were methylated in more than 60% of the clones.

I next examined the Regions 2-1, 2-2, 3-1, and 3-2, which contain 29, 30, 20, and 27 CpGs, respectively, by the direct bisulfite sequencing method. The CpGs in the Ema cell line are

unmethylated, while those in Nody-1 and the canine PBMC are mostly methylated (Fig. I-6A-C). Since I was unable to determine the methylation status of Region 4 via the direct bisulfite sequencing method, I analyzed it by the bisulfite subcloning-sequencing method (Fig. I-6D-F). Almost all the CpGs in the Ema cells were found to be unmethylated, while approximately half of the CpGs in Nody-1 were methylated and almost all the CpGs in the canine PBMC were found to be methylated.

DISCUSSION

Tomiyasu *et al.* have reported that the expression of the MGMT mRNA is not associated with the chemosensitivity observed in canine B-cell lymphoma cases treated with the CHOP protocol (2010). However, these cases were never treated with the nitrosoureas. Lomustine is an effective rescue agent used to treat relapsed canine lymphoma [Moore *et al.*, 1999, Saba *et al.*, 2007]. Therefore, in this study, I examined whether MGMT affects the sensitivity of canine lymphomas to lomustine.

As shown in Fig. I-1 and Table 2, the BG treatment that inactivates the MGMT activity, significantly increased the cell toxicity of lomustine in CL-1 and Nody-1 cell lines. This increment was also observed in the Jurkat cells that express MGMT [D'Atri *et al.*, 2000]. In addition, the fluorometric oligonucleotide assay also revealed that CL-1 and Nody-1 cell lines have the MGMT activity as the Jurkat cells (Fig. I-2). Furthermore, the MGMT gene expression was also observed in these cell lines using real-time PCR (Fig. I-3). These results strongly demonstrate that CL-1 and Nody-1 cell lines with MGMT activity are resistant to toxicity caused by lomustine. In contrast, the other three cell lines, namely, Ema, GL-1, and UL-1, do not show a similar resistance to the cytotoxicity caused by lomustine, although real-time PCR could detect a weak MGMT expression in UL-1. These results indicate that MGMT strongly influences the sensitivity of the canine lymphoma cell lines to lomustine. Furthermore, as shown in Table 2 as IC_{50}, canine lymphoma cell lines like CL-1 and Nody-1 that have MGMT activity are resistant to lomustine, while cell lines like Ema, GL-1, and UL-1 that have no MGMT activity are sensitive to lomustine. Although it is unclear why the cells do or do not have the MGMT activity, I hypothesize that: normal lymphocytes have MGMT activity (Fig. I-3) [Briegert *et al.*, 2007]. MGMT may act as a tumor suppressor gene by preventing DNA mutations. When the MGMT expression is inhibited by an unknown mechanism, MGMT may fail to repair DNA and lead to mutations in oncogenes and/or tumor suppressor genes, resulting in tumorigenesis. If the suppression of MGMT occurs in the early stages of tumor development, gene mutations that are essential for tumorigenesis may remain unrepaired,

promoting neoplasticity. In contrast, cancer development in lymphoma cells that have MGMT activity may result from mechanisms other than the inactivation of MGMT. Although the suppression of MGMT may not occur, accumulation of various gene mutations may establish cancer cells that constitutively express MGMT.

In this study, the CL-1, Nody-1 and Jurkat group showed significantly lower IC_{50} than the Ema, GL-1 and UL-1 group after the BG treatment (data not shown). This indicate that other mechanisms besides MGMT contribute to drug resistance in the latter group. In addition to MGMT activity, mismatch repair system or WAF/Cip1 has been reported as cause of resistance to alkylating agents [Aquilina *et al.*, 2000, Ruan *et al.*, 1998]. The combination of these factors might result in complicated refractory mechanisms to anticancer agents.

In human gliomas and colorectal cancer, methylation of the MGMT exon 1 is strongly associated with the loss of the MGMT mRNA expression [Esteller *et al.*, 1999]. Therefore, I compared the expression level of the MGMT mRNA with and without a methylation inhibitor to investigate whether DNA methylation affects MGMT expression in canine cells. As shown in Fig. I-3, treatment with the methylation inhibitor induces the MGMT mRNA expression in Ema and GL-1 cells that did not originally express the MGMT gene. In contrast, the methylation inhibitor did not show any effect on the MGMT mRNA expression in the CL-1, Nody-1, and UL-1 cells and the healthy canine PBMC. The increase in the MGMT gene expression observed in this study indicates that the MGMT gene expression is inhibited by DNA methylation in the Ema and GL-1 cells.

To investigate the methylation status of the promoter region of the canine MGMT gene, I analysed Region 1, which corresponds to the promoter region in the human MGMT gene (Fig. I-4). Indeed, it has been demonstrated that the methylation of this region contributes to the suppression of MGMT in human cancers [Qian *et al.*, 1997]. Intriguingly, the CpGs of Region 1 of the Ema cells were completely unmethylated, and almost all the CpGs of the other four cell lines were methylated, as shown by the direct bisulfite sequencing analysis (Fig. I-5A). To confirm these results, I next performed a subcloning method followed by the bisulfite sequencing on the Ema and

Nody-1 cells that are without and with MGMT gene expression and activity, respectively. As shown in Fig. I-5B-D, Ema cells show 100% unmethylated status of almost all the CpGs, Nody-1 cells show more than 60% methylated status in 70% of the CpG regions, and the healthy canine PBMC show 100% methylated status in most of the CpGs. Taken together, the results of the subcloning-mediated bisulfite sequencing reflect the results of the direct bisulfite sequencing analysis in the Ema cells. Interestingly, the Nody-1 cells had many clones that were a mix of both methylated and unmethylated cytosines in the same CpGs, as seen by the subcloning-mediated bisulfite sequencing, and many of these regions showed a double wave of thymine and cytosine by the direct bisulfite sequencing method (data not shown). Therefore, the CpG regions defined as 'unknown' in the direct bisulfite sequencing analysis may have a mixture of methylated and unmethylated cytosines. Indeed, the human cell lines expressing MGMT have been shown to have almost 100% demethylation of the CpGs in the promoter region, while a variable methylation status of clones has been observed in the human cell lines that show no MGMT expression in the promoter region [Qian *et al.*, 1997]. These results indicate that if unmethylated cytosines are observed by direct bisulfite sequencing, most cells may not have CpG methylation. In contrast, if methylation is observed, the cells could contain a potential mix of methylated and unmethylated cytosines. Collectively, the results of the direct bisulfite sequencing may well correlate with the subcloning-mediated bisulfite sequencing. Although the expression of the MGMT gene is suppressed by the CpG methylation of the promoter region in human cells, Ema cells that do not express MGMT show complete CpG demethylation. Since the increase in the MGMT gene expression caused by the methylation inhibitor is observed in the Ema cells, Region 1 is unlikely to correlate with the regulation of the MGMT gene expression in canine cells.

In order to identify the CpG region whose methylation is fundamental to the expression of the MGMT gene, I further analysed the regions upstream of the canine MGMT gene. However, I could not identify the region (Fig. I-6). Interestingly, it can be speculated that CpG methylation or demethylation in these regions depend on the cells, rather than their association with the MGMT

expression; the CpGs in the Ema cells tend not to be methylated, while the Nody-1 cells and healthy canine PBMC are methylated.

Although I targeted and analysed the tentative promoter regions of the canine MGMT gene including the upstream 10,000 bp and downstream 7,000 bp of exon 1, I was unable to identify the regions in which methylation is essential to suppress the expression of the MGMT gene. I hypothesize that the canine MGMT promoter may include regions other than the ones analysed in this study, and the ones found in the human MGMT promoter. Alternatively, the expression of the canine MGMT gene may be regulated by a different mechanism rather than direct methylation of the MGMT promoter region. For example, promoter regions of other transcription factors that promote the MGMT expression could be methylated, resulting in the suppression of MGMT. Additional studies are necessary to elucidate this mechanism.

This study demonstrates that similar to human cells, MGMT is strongly correlated with the resistance of canine lymphoma cell lines to one of the nitrosoureas, lomustine. Furthermore, I show that there were two types of lymphoma cells; ones that possess the MGMT activity and show low sensitivity to lomustine, and others that do not possess the MGMT activity and show high sensitivity to lomustine. In future, investigating the expression of the MGMT gene and its activity in clinical samples may prove beneficial. If my *in vitro* results could be translated into *in vivo* results, they may help to predict the effectiveness of nitrosoureas and to overcome the resistance to nitrosoureas in lymphoma cases. The analysis of the effect of the methylation inhibitor suggests that the MGMT gene expression may be suppressed by DNA methylation in canine lymphomas. In future, it is important to identify such regulatory regions and develop methylation-specific PCR strategies for clinical application in terms of predicting the effectiveness of nitrosoureas.

Chapter 2

Hypoxia Inducible Factor 1α Expression and Effects of its Inhibitors in Canine Lymphoma

SUMMARY

Hypoxic conditions in various cancers are believed to relate with their malignancy, and hypoxia inducible factor-1α (HIF-1α) has been shown to be a major regulator of the response to low oxygen. In this study, I examined HIF-1α expression in canine lymphoma using cell lines and clinical samples and found that these cells expressed HIF-1α. Moreover, the HIF-1α inhibitors, echinomycin, YC-1 and 2-methoxyestradiol, suppressed the proliferation of canine lymphoma cell lines. In a xenograft model using NOD/scid mice, echinomycin treatment resulted in a dose-dependent regression of the tumor. My results suggest that HIF-1α contributes to the proliferation and/or survival of canine lymphoma cells. Therefore, HIF-1α inhibitors may be potential agents to treat canine lymphoma.

INTRODUCTION

Hypoxia inducible factor (HIF) is a transcription factor that induces the expression of various genes to resist tissue hypoxia. HIF forms heterodimers, which consist of an alpha and beta subunit. There are three HIF alpha subunits (1α, 2α, and 3α) and a single beta subunit (1β) [Semenza, 1999]. The HIF-1 (1α and 1β) and HIF-2 (2α and 1β) proteins are constitutively expressed; however, under normoxic conditions, a specific proline residue in the HIF alpha subunit is hydroxylated by prolylhydroxylase 2 (PHD2). This allows the von Hippel-Lindau (VHL) protein to recognize it and recruit ubiquitin ligase [Epstein *et al.*, 2001, Kaelin *et al.*, 2008]. As a result, the HIF-1α and -2α proteins are degraded under well-oxygenated conditions. This degradation does not occur under hypoxic conditions, because PHD2 requires oxygen to combine the hydroxyl group to the HIF α subunit. Undegraded, the HIF-1α and -2α proteins are able to migrate to the nucleus and act as transcription factors via heterodimerization with HIF-1β [Jiang *et al.*, 1996], resulting in the expression of several genes, such as vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT1) and pyruvate dehydrogenase isozyme 1 (PDK1), which relates with angiogenesis and metabolism [Gleadle *et al.*, 1997, Hickey *et al.*, 2006].

Cancer tissue has hypoxic regions that express the HIF-1 protein, which causes resistance to chemotherapy, angiogenesis, increasing metastasis and maintenance of cancer stem cells [Huang *et al.*, 2014, Keith *et al.*, 2007, Liao *et al.*, 2007]. Human hematopoietic malignancies, such as leukemia and lymphoma, also express high levels of HIF-1, the expression of which is considered a poor prognostic factor [Deeb *et al.*, 2011, Evens *et al.*, 2010, Wellmann *et al.*, 2004]. Recently, Wang *et al.* (2011) reported that treatment with an HIF-1α inhibitor resulted in tumor regression in murine lymphoma, which is caused by an abrogation of the epilepsy, progressive myoclonus type 2A (*Epm2a)* gene, and also showed that HIF-1α knockdown with small hairpin RNA (shRNA) resulted in the growth suppression of lymphoma cells isolated from the transgenic mice.

Lymphoma is the most common hematopoietic malignancy in dogs. Generally, lymphoma patients are treated with multidrug chemotherapies. The remission rate and duration have been

reported as 80% and more than 9 months, respectively [Garrett *et al.*, 2002, Keller *et al.*, 1993]. However, almost all lymphoma patients experience a recurrence and develop drug resistance. Therefore, a novel treatment is strongly desired.

Here, I examined whether HIF-1α contributes to tumorigenesis and/or the survival of canine lymphoma, and investigated whether HIF-1α inhibitors could suppress the proliferation of canine lymphoma cells *in vitro* and *in vivo.*

MATERIALS AND METHODS

Cells

Seven canine lymphoma cell lines; the CLBL-1 [Rütgen *et al.*, 2010], GL-1 [Nakaichi *et al.*, 1996] and 17-71 [Rosales *et al.*, 1988] B cell lines; the CL-1 [Momoi *et al.*, 1997], CLC [Umeki *et al.*, 2013], CLGL90 [Suter *et al.*, 2005] and Nody-1 [Umeki *et al.*, 2013] T cell lines; and a Raji human lymphoma cell line were used. CLBL-1, GL-1, CL-1, CLC, Nody-1 and Raji cell lines were cultured in RPMI1640 medium, and CLGL90 and 17-71 cell lines were cultured in Dulbecco's Modified Eagle medium, which were supplemented 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 2-mercaptoethanol (55 μM). Cells were incubated at 37 ºC in a humidified incubator containing 21% O_2 and 5% CO_2.

Peripheral blood mononuclear cells (PBMCs), which served as a control, were collected from healthy beagle dogs using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Briefly, heparinized whole blood was centrifuged, and the buffy coat was suspended in PBS. The diluted cells were gently overlaid on Lymphoprep and then centrifuged at 800 × *g* for 30 min. The PBMCs layer was collected and diluted with PBS. The isolated PBMCs were overlaid on whipped fetal bovine serum in order to remove the contaminating platelets. After a centrifugation at 1,000 × *g* for 10 min, the purified PBMCs were obtained as the cell pellet and were washed with PBS.

Healthy dog tissue samples and clinical samples

Tissues were obtained from a healthy beagle, which was euthanized by anesthesia. Clinical canine lymphoma samples were collected from patients at the Yamaguchi University Animal Medical Center with a written informed consent from dog owners. Lymphoma cells were collected from swelled lymph nodes by fine needle aspiration using a 21-23 gauge needle and 5 ml disposable syringe. In patient 5, which was used for immunoblotting, lymphoma cells also existed in the peripheral blood; thus, blood was collected by the routine blood collection method.

Reagents

Echinomycin (Calbiochem, San Diego, CA, USA), YC-1 (Cayman Chemicals, Ann Arbor, MI, USA) and 2-methoxyestradiol (Merck Millipore, Billerica, MA, USA) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and stored at -20 ºC until use.

Quantitative real-time PCR

Total RNA was isolated from each cell line, PBMCs, canine normal tissues and clinical samples (patients 1-4) using the ISOGEN II (Nippon Gene, Tokyo, Japan) and resolved with nuclease free water. One μg of total RNA was treated with DNase I using a Turbo DNA-free kit (Applied Biosystems, Carlsbad, CA, USA) and transcribed into cDNA with Superscript III (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The single-stranded cDNA was subjected to real-time PCR amplification with a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Real-time PCR was performed with StepOne PCR system (Perkin-Elmer, Waltham, MA, USA). The data were analyzed using StepOne software v.2.2.2. The following primers were used: canine HIF-1α forward 5′-TGACGGTTCACTTTTTCAAGC-3′ and reverse 5′-TTGCTCCATTCCATTCTGTTC-3′; canine GLUT forward 5′-ACTGCCCTGGATGTCGTATC-3′ and reverse 5′-GGACCCTGGCTGAAGAGTTC-3′; and RPL32 forward 5′-TGGTTACAGGAGCAACAAGAAA-3′ and reverse 5′-GCACATCAGCAGCACTTCA-3′.

The relative expression levels of HIF-1α, GLUT1 and PDK1 were calculated as ΔCt from the difference between expression of the internal control, RPL32 [Peters *et al.*, 2007], and $2^{-\Delta Ct}$ were calculated to represent the expression levels of each gene. Mean cycle threshold (Ct) value less than 35 was included in this study. After the PCR, all products were analyzed by the melting curves confirming no other genes were amplified.

Immunoblotting

Cultured cells, clinical samples (cases 1-5) and PBMCs were washed twice with ice cold PBS. Tissue samples were homogenized in liquid nitrogen before cell lysis. Samples were lysed with a lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1% sodium lauryl sulfate, protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), 1 mM Na_3VO_4 and 50 mM NaF] and then sonicated for 15 min (30 cycles of 10 sec on/20 sec off). Then, lysates were boiled at 100 ºC by heat block for 5 min. The cell suspension was centrifuged at 15,000 × *g* at 4 ºC for 15 min, and the supernatant was transferred into a new tube as the whole cell lysate. The amount of protein in the cell lysate was measured with a Micro BCA™ Protein Assay Reagent Kit (Thermo Fischer Scientific, Waltham, MA, USA).

The lysate was subjected to SDS-PAGE on a polyacrylamide gel containing 5.5–13.2% acrylamide. After SDS-PAGE, the proteins were transferred to Immobilon® Membranes (Merck Millipore). The membrane was blocked with a blocking buffer (TBS-T; Tris-buffered saline with 0.05% Tween 20 and 5% skimmed milk or 5% bovine serum albumin) for 1 hr at room temperature and then incubated with a primary antibody overnight at 4 ºC. Rabbit polyclonal anti-HIF-1α (NB100-449) was purchased from Novus Biologicals (Littleton, CO, USA) and used at a 1:500 dilution [Petty et al., 2008]. Mouse monoclonal antibody for β-actin (AC-15) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and used at a 1:2,000 dilution. Rabbit polyclonal anti-Lamin B1 was purchased from Abcam (Cambridge, UK) and used at a 1:1,000 dilution. The membranes were washed twice in TBS-T and then incubated with a secondary antibody for 1 hr at room temperature. An antibody for horseradish peroxidase-conjugated mouse IgG (1:4,000 dilution) and rabbit IgG (1:4,000 dilution) were from Thermo Fischer Scientific. Then, the chemiluminescence was detected by using Western Lightning® Plus-ECL (Perkin-Elmer) and LAS-3000 mini (FUJIFILM, Tokyo, Japan).

Cytotoxicity assay

Canine lymphoma cell lines were treated with echinomycin, YC-1 or 2-methoxyestradiol at various concentrations in 96-well plates. After 48-hr incubation at 37 ºC, 10 µl 5 mg/ml 3-(4,5-di-methylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was added. After further 4 hr incubation, 100 µl MTT-lysis buffer [20% SDS and 40% N,N-dimethylformamide (Nacalai Tesque)] was added. After 1 hr, the absorbance was measured at 570 nm. Each experiment was performed in triplicate and independently repeated three times. The concentration of each drug that inhibited the cell growth by 50% (IC_{50}) was calculated from the drug survival curves.

Flow cytometry

The CLBL-1 cells were treated with echinomycin or DMSO as the negative control, for 12 hr. Apoptosis was detected using the MEBCYTO® Apoptosis Kit (MBL, Nagoya, Japan) according to manufacturer's protocol. The fluorescence intensity of 10,000 cells was measured with BD Accuri™ C6 Flowcytometer (BD Bioscience, San Jose, CA, USA). Data were analyzed using the FlowJoX v10 software (FLOWJO, LLC, Ashland, OR, USA).

Xenograft models

NOD/scid mice (CLEA Japan, Tokyo, Japan) were maintained under pathogen-free conditions, and studies were conducted in accordance with the Yamaguchi University Animal Care and Use Committee (approval number 220). CLBL-1 cells (5×10^6 cells in 50 µl PBS) were implanted subcutaneously into the right hind limb of 7- to 8-week-old female mice under general anesthesia. When the tumor volume reached 100 mm^3, as calculated from tumor width and length, echinomycin or DMSO was injected intraperitoneally every other day five times. Tumor size was measured every other day. When the tumor size exceeded 4,500 mm^3, the mouse was euthanized with diethyl ether anesthesia. Statistical analysis was performed using the Student's *t*-test. A P value < 0.05 was considered statistically significant.

RESULTS

Canine HIF-1α gene and protein expressed in canine lymphoma cell lines under normoxic conditions

At first, I examined gene expression levels of canine HIF-1α (cHIF-1α) using normal tissue samples obtained from a healthy beagle dog and seven canine lymphoma cell lines incubated under normoxic conditions (21% O_2), by real-time PCR. Although the expression levels varied in the tissues, the cHIF-1α gene was expressed in all canine tissues (Fig. II-1A, black bars). On the other hand, one of the representative target genes of HIF-1α, GLUT1, was expressed without correlation to the expression pattern to HIF-1α (Fig. II-1A, white bars). In contrast, all canine lymphoma cell lines expressed the cHIF-1α gene, and the expression levels of cHIF-1α were similar to those of the GLUT1 gene (Fig. II-1B).

Next, I performed the immunoblotting for cHIF-1α to compare protein expression levels. While it has been reported that the adrenal, kidney, pancreas, spleen and tonsil tissues weakly express HIF-1α protein in human normal tissue samples [Zhong *et al.*, 1999], only brain tissue expressed cHIF-1α in dogs (Fig. II-1C). On the other hand, all canine lymphoma cell lines demonstrated cHIF-1α expression (Fig. II-1D).

Canine lymphoma cells obtained from clinical cases also express cHIF-1α

To examine whether the protein expression of cHIF-1α is limited to the cell lines, I next investigated whether clinical samples obtained from canine lymphoma cases express the cHIF-1α gene and protein using real-time PCR and immunoblotting, respectively. The gene expression level was at a similar level in PBMCs isolated from the healthy dog (Fig. II-2A). However, all tested lymphoma samples expressed the cHIF-1α protein (Fig. II-2B). Therefore, the mRNA and cHIF-1α protein were expressed not only in the cell lines, but also in the primary cells obtained from the clinical cases.

HIF-1α inhibitors showed a cytotoxic effect to canine lymphoma cell lines

To examine the effects of HIF-1α inhibition on canine lymphoma cells, the lymphoma cell lines were incubated with a HIF-1α inhibitor, echinomycin, and I performed the MTT assay to evaluate the proliferating capacity. Echinomycin treatment resulted in the inhibition of cell proliferation on all the lymphoma cell lines in a dose dependent manner (Fig. II-3A). Notably, CL-1, CLBL-1, GL-1 and 17-71 cells, in which the IC_{50} were 0.80 nM, 0.54 nM, 0.88 nM and 0.52 nM, respectively, showed high sensitivity to echinomycin (Fig. II-3B). When the Annexin V staining using a FACS analysis was performed, the Annexin V positive cells were 25.6% in the DMSO control and 45.4% in the echinomycin treated cells, respectively (Fig. II-3C). Therefore, it is considered that this effect results from apoptosis. To confirm the inhibitory effect of echinomycin against HIF-1α, the expression of the downstream target genes, GLUT1 and PKD1, was examined by quantitative real-time PCR using the CLBL-1 and 17-71 cells, which showed lower IC_{50} to echinomycin than those of the others. As depicted in Fig. II-3D, CLBL-1 and 17-71 cells incubated with 2 nM echinomycin for 12 to 24 hr exhibited suppressed GLUT1 and PDK1 gene expression.

Other HIF-1α inhibitors, YC-1 and 2-methoxyestradiol, also inhibited lymphoma-cell proliferation; however, the sensitivity of this inhibition evaluated from the IC_{50} varied from the results of echinomycin treatment cells (Fig. II-4). For example, 17-71 cells showed the lowest IC_{50} against echinomycin, but cell lines showing the lowest IC_{50} to YC-1 and 2-methoxyestradiol were CLBL-1 and CLC, respectively.

Engrafted lymphoma was regressed by echinomycin

Finally, I tested the effect of echinomycin in the mouse xenograft model using NOD/ShiJic-scidJcl mice, which lack not only functional B and T lymphocytes, but also have a lower activity of NK cells, complements and macrophages. The tumor volume reached 100 mm^3 by days 8–10 after the CLBL-1 cell line was inoculated. Compared to the DMSO control treatment, the echinomycin

treatment did not affect the tumor at a dosage of 10 μg/kg for a total of five times. When the dosage was increased to 20 μg/kg five times every other day, the tumor volume of the xenografted canine lymphoma significantly decreased. Especially, five treatments of 40 μg/kg echinomycin resulted in complete tumor regression, after which the tumor was macroscopically undetectable (Fig. II-5).

DISCUSSION

In human medicine, reports that mention the importance of angiogenesis and metabolic changes in cancer metastasis or proliferation have increased [Chen *et al.*, 2012, Liao *et al.*, 2007, Zhong *et al.*, 1999]. Hypoxia is one of the most significant factors that promote angiogenesis or metabolic changes, and HIF-1 is a major regulator in the presence of low O_2 levels [Semenza, 1998]. However, there are few reports exploring the relationship between cancers and HIF in the veterinary field [Madej *et al.*, 2013, Mees *et al.*, 2011, Petty et al., 2008].

Canine lymphoma has a high recurrence rate, and thus, new treatments against this disease are desired. In this study, I showed that HIF-1α is expressed in the canine lymphoma cells, both in cell lines and clinical samples, and that its inhibition leads to suppression of cell proliferation both *in vitro* and *in vivo*.

The quantitative real-time PCR showed that the HIF-1α gene was expressed at a various levels in normal canine tissues, whereas the expression of HIF-1α in canine lymphoma cell lines was at similar levels (Fig. II-1). However, HIF-1α is regulated by protein degradation, but not gene expression [Semenza, 1999]; therefore, the analysis of protein levels is more important. Interestingly, all seven canine lymphoma cell lines included in this study expressed the HIF-1α protein at the 21% normoxic condition. These results suggest that canine lymphoma cells can use the HIF-1α protein, which induces tumor proliferation, survival and an anti-apoptotic effect like human cancer cells [Vaupel *et al.*, 2007], even under normal oxygen concentrations through oxygen independent pathways. Previous studies using murine lymphoma models also supported my idea. In murine lymphoma, treatment with an HIF-1α inhibitor and HIF-1α knockdown resulted in the suppression of lymphoma cell proliferation *in vitro* and *in vivo* [Wang *et al.*, 2011]. Furthermore, lymphocytes from HIF-1α transgenic mice exhibited prolonged survival duration and formed lymphoma [Sueoka *et al.*, 2013].

As demonstrated in Fig. II-2, all canine lymphoma clinical samples expressed HIF-1α, similar to the cell lines. The cHIF-1α expression data support the idea that HIF-1α has a role in cancer cell

proliferation and/or survival in canine lymphoma. However, it is still unclear how canine HIF-1α is stabilized in canine lymphoma cells. In human cells, phosphorylation of the mammalian target of rapamycin (mTOR) and/or the p70 S6 kinase (S6K1) contributes to the oxygen independent stabilization of HIF-1 [Jiang *et al.*, 2001, Wounters *et al.*, 2008]. Although I analyzed the phosphorylation of these two pathways and Akt by immunoblotting, the HIF-1α expression levels seem to be unrelated (data not shown).

The known drugs that have an inhibitory potential of HIF-1α are highly diverse, and there is no specific HIF-1α inhibitor [Xia *et al.*, 2012]. Therefore, I used three HIF-1α inhibitors, echinomycin, YC-1 and 2-methoxyestradiol. These inhibitors have different mechanisms by which they inhibit HIF-1α transcriptional activity. It is reported that echinomycin binds to the hypoxia responsible element (HRE) on DNA [Van Dyke *et al.*, 1984] instead of HIF-1; moreover, YC-1 inhibits HIF-1 protein accumulation and promotes its degradation [Yeo *et al.*, 2004], and 2-methoxyestradiol inhibits HIF-1α translation and translocation into nucleus [Mabjeesh *et al.*, 2003]. Here, I showed that these agents inhibited the proliferation of canine lymphoma cell lines in a dose-dependent manner (Figs. II-3 and II-4). These data suggest that the expression of HIF-1α in canine lymphoma cells is important for their proliferation and survival, and therefore, HIF-1α inhibitors may be potential agents for canine lymphoma treatment. In the *Epm2a* gene-targeted mouse lymphoma model, either echinomycin or HIF-1α shRNA suppressed the expression of the downstream genes of HIF-1α, such as GLUT1 and Hes1, and resulted in prolonged mouse survival [Wang *et al.*, 2011]. Although I also found the HIF-1α inhibitors suppressed the proliferation of canine lymphoma cells, it is necessary to confirm the specificity of their HIF-1α inhibition in this experiment. However, because of a technical issue that genes are not easily introduced into canine lymphoma cells by retrovirus or lipofection, I could not successfully silence HIF-1α expression. When a new method that can induce expression of exogenous genes in canine lymphoma cells is developed, I will try the gene silencing of HIF-1α in the future.

I show that echinomycin has the same robust effect on canine lymphoma cells as in the mouse

xenograft model (Fig. II-5). Past studies using the *Epm2a* gene-targeted mice lymphoma and human leukemia cells reported that five injections of 10 μg/kg echinomycin could treat those tumors [Wang *et al.*, 2011]. On the other hand, this dose had no effect on canine lymphoma in my models. Although the mice strain used in this study is different from the previous report, my result suggests that canine lymphoma cells have a higher resistance potential. Since the dose increment leads to tumor regression, I should administer echinomycin at a high dosage or in combination with other chemotherapy if using this drug in clinics.

In this study, I revealed that the HIF-1α protein is ubiquitously expressed in canine lymphoma cell lines and clinical samples. Because these cell lines were incubated at normoxic conditions, it was suggested that HIF-1α is stabilized on oxygen independent pathways. Moreover, three different types of HIF-1α inhibitors suppressed the cell proliferation of canine lymphoma cell lines. Whether this effect was specific to the HIF-1α inhibition is still unknown; it is necessary to perform gene silencing of the HIF-1α gene. However, I guess echinomycin, which showed an anti-lymphoma effect *in vivo*, can be used as an anti-cancer agent clinically, although its side effects to dogs should be explored before the use. This study is the first report to demonstrate a relationship between HIF-1α and canine lymphoma. Although additional research is needed, the HIF-1α inhibitors may be potential agents to treat the canine lymphoma.

CONCLUSION

Canine lymphomas has clinical problems due to their high incidence and their recurrence rate. In patients with the lymphomas, which have multidrug resistance or low sensitivity for chemotherapeutics, a wide variety of the anticancer agents was administrated as rescue therapies. However, these cases also have low sensitivity to the agents used for rescue protocols. Thus, it is extremely important to overcome the chemo-resistance or to develop novel treatments.

In chapter 1, I analysed O^6-methylguanine DNA methyltransferase (MGMT), which involved in the chemo-resistance of lomustine, one of the major nitrosoureas for the rescue protocols. As a result, when cells were exposed to O^6-benzylguanine, the MGMT inactivator, inhibition of the cell proliferation was observed in some canine lymphoma cell lines. Additionally, these cell lines also have the MGMT activity, which was examined by fluorometric oligonucleotide assay. These results suggest that at least some lymphoma cells acquired the lomustine resistance through MGMT. In contrast, the other lymphoma cell lines had low or no MGMT activity and tended to have a high sensitivity against lomustine. In other words, the canine lymphoma cell lines divided into two types; cells having a high MGMT activity or not. Therefore, perhaps there are two ways for lymphomagenesis, i.e. tumorigenesis from aberrant DNA repair capacity resulted from the absence of the MGMT activity, or from mechanisms unrelated to the MGMT activity (ex. through abberations of important genes for tumorigenesis). If this hypothesis is correct, since the aberrant DNA repair capacity would be occured in an initial stage of the neoplastic transformation, one can predict either the tumor cells having the MGMT resistance at the diagnosis. Although it must be investigated whether these observations could apply to clinical cases and in vivo usages, the studies examined for the mechanisms by what regulates MGMT expression are important for the prediction of effectiveness of nitrosoureas and overcoming the chemo-resistance.

In chapter 2, I examined hypoxia inducible factor (HIF)-1α as a candidate for a novel target of the canine lymphoma treatment. It is reported that the HIF-1α transgenic mice highly developed lymphoproliferative diseases [Sueoka et al., 2013]. Furthermore, HIF-1α also contributes to maintain cancer stem cells of a murine lymphoma, and its inhibition by the agents or the gene

silencing with short-hairpin RNA was effective to regress the tumor [Wang et al., 2011]. Therefore, I guessed that HIF-1α and the downstream target genes/proteins could be targets for the treatment of the canine lymphoma. The immunoblotting revealed that the canine HIF-1α protein did not expressed in normal dog tissues other than brain. In contrast, all the canine lymphoma cell lines cultured in a normooxic condition, and lymphoma clinical samples used in this study expressed the HIF-1α protein. Moreover, three HIF-1α inhibitors could suppress the proliferation of the canine lymphoma cell lines *in vitro*. Consistent with these results, an *in vivo* canine lymphoma xenograft model also showed that one of the HIF-1α inhibitors, echinomycin, could reduce the transplanted tumor volume. Although a more specific inhibition of HIF-1α using a gene silencing method have to be explored, it is suggested that the HIF-1α protein related to the pathophysiology of the canine lymphoma cells, and its inhibition could be a potential target for the canine lymphoma treatment.

The HIF protein expression in solid cancers is believed to make cancer cells more malignant. Notably, HIF-1α contributes to the anaerobic glycolysis in cancer cells even under a normooxic condition, which is called the Warburg effect [Warburg, 1956]. Although oxidative phosphorylation can produce more ATPs than the glycolysis, the anaerobic glycolysis can rapidly supply nucleotides, amino acids, and lipids. For that reason, this phenomenon is believed to have an advantage for cancer cells [Vander Heiden *et al.*, 2009]. HIF-1α upregulates a lot of genes related with glycolytic pathway such as GLUT1 and GLUT3, the accelerator of uptake of glucose into cells, hexokinases, which are trigger of glycolysis, and lactate dehydrogenases (LDH), which catalyzes a reaction changing pyruvates into lactates [Semenza, 2012]. Especially, LDH is known to be increased in blood of the human and canine lymphoma patients. Overall, these facts suggest that HIF-1α changes cell metabolisms of the lymphoma cells. In this study, all the canine lymphoma cell lines expressed the HIF-1α protein under the normoxic condition. I considered that the canine lymphoma cells may also have an altered metabolic system, and further studies such as metabolome analysis might reveal the mechanisms, which can be targeted for the lymphoma treatment. Interestingly, in the rescue treatment for the lymphoma, the combination with lomustine and L-

asparaginase achieved a better therapeutic outcome on the relapsed canine lymphoma [Saba *et al.*, 2007]. Asparagine is not an essential amino acid for mammals, however, the lymphoma cells usually required it. Therefore, a starvation of asparagine in the blood by L-asparaginase results in the lymphoma cell death [Cooney *et al.*, 1970]. Since this feature is almost specific for lymphoid tumors, it is also suggested that the lymphoma cells have an original metabolic style. Although more studies concerning a nutrient utilization are necessary, I considered that the combination of the overcoming nitrosourea resistance and the inhibition of the lymphoma specific metabolisms have great potentials for the treatment of the lymphoma.

These two lines of strategies, namely the analyses of mechanisms for the chemo-resistance and the factors involved in tumorigenesis or cancer survival, are greatly important for the cancer therapy. In the future, we could utilize these researches not only for targeted therapies, but also for choosing anticancer agents to overcome drug resistance. I hope this paradigm that enables selection of the treatments matched for a patient may leads to the better of the treatments matched for a patient may leads to the better prognosis.

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REFERENCES

Aquilina G, Ceccotti S, Martinelli S, Soddu S, Crescenzi M, Branch P, Karran P and Bignami M. 2000. Mismatch repair and p53 independently affect sensitivity to N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea. *Clin. Cancer. Res.* **6**: 671-680.

Belanich M, Pastor M, Randall T, Guerra D, Kibitel J, Alas L, Li B, Citron M, Wasserman P, White A, Eyre H, Jaeckle K, Schulman S, Rector D, Prados M, Coons S, Shapiro W and Yarosh D. 1996. Retrospective study of the correlation between the DNA repair protein alkyltransferase and survival of brain tumor patients treated with carmustine. *Cancer Res.* **56**: 783-788.

Briegart M, Enk AH and KainaB. 2007. Change in expression of MGMT during maturation of human monocytes into dendritic cells. *DNA Repair* (Amst.) **6**: 1255-1263.

Cario S, Bodo B, Tanja M, Helga S and Frank L. 2006. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res.* **66**: 2794-2800.

Casorelli I, Russo MT and Bignami M. 2008. Role of mismatch repair and MGMT in response to anticancer therapies. *Anti-Cancer Agents Med. Chem.* **8**: 368-380.

Chen JQ and Russo J. 2012. Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells. *Biochem. Biophys. Acta* **1826**: 370-384.

Cooney DA, Capizzi RL and Handschumacher RE. 1970. Evaluation of L-asparagine metabolism in animals and man. *Cancer Res.* **30**: 929-935.

D'Atri S, Graziani G, Lacal PM, Nistico V, Gilberti S, Faraoni I, Watson AJ, Bonmassar E and Margison GP. 2000. Attenuation of O(6)-methylguanine-DNA methyltransferase activity and mRNA levels by cisplatin and temozolomide in jurkat cells. *J.Pharmacol. Exp. Ther.* **294**: 664-671.

Deeb G, Vaughan MM, McInnis I, Ford LA, Sait SNJ, Starostik P, Wetzler M, Mashtare T and Wang ES. 2011. Hypoxia-inducible factor-1α protein expression is associated with poor survival in normal karyotype adult acute myeloid leukemia. *Leukemia Res.* **35**: 579-584.

Drain S, Catherwood MA and Alexander HD. 2010. Multidrug resistance in the chronic lymphoproliferative disorders. *Leuk. Lymphoma* **51**: 1793-1804.

Epstein ACR, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukhenrji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ and Ratcliffe PJ. 2001. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**: 43-54.

Esteller M, Gaidano G, Goodman SN, Zagonel V, Capello D, Botto B, Rossi D, Gloghini A, Vitolo U, Carbone A, Baylin SB and Herman JG. 2002. Hypermethylation of the DNA repair gene O^6-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. *J. Nat. Cancer Inst.* **94**: 26-32.

Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB and Herman JG. 2000. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N. Engl. J. Med.* **343**: 1350-1354.

Esteller M, Hamilton SR, Burger PC, Baylin SB and Herman JG. 1999. Inactivation of the DNA-repair gene O^6-methylguanine DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res.* **59**: 793-797.

Evens AM, Sehn LH, Farinha P, Nelson BP, Raji A, Lu Y, Brakman A, Parimi V, Winter JN, Schumacker PT, Gascoyne RD and Gordon LI. 2010. Hypoxia-inducible factor-1 expression predicts superior survival in patients with diffuse large B-cell lymphoma treated with R-CHOP. *J.Clin. Oncol.* **28**: 1017-1024.

Fulda S and Debatin KM. 2006. 5-Aza-2'-deoxycytidine and IFN-gamma cooperate to sensitize for TRAIL-induced apoptosis by upregulating caspase-8. *Oncogene* **25**: 5125-5133.

Garrett LD, Thamm DH, Chun R, Dudley R and Vail DM. 2002. Evaluation of a 6-month chemotherapy protocol with no maintenance therapy for dogs with lymphoma. *J. Vet. Int. Med.* **16**: 704-709.

Gleadle JM and Ratcliffe PJ. 1997. Induction of hypoxia-inducible factor-1, erythropoietin, vascular endothelial growth factor, and glucose transporter-1 by hypoxia: evidence against a regulatory role for Src kinase. *Blood* **89**: 503-509.

Hickey MM and Simon MC. 2006. Regulation of angiogenesis by hypoxia and hypoxia-inducible factors. *Curr. Dev. Biol.* **76**:217-257.

Hiraoka H, Minami K, Kaneko N, Shimokawa Miyama T, Okamura Y, Mizuno T and Okuda M. 2009. Aberrations of the FHIT gene and Fhit protein in canine lymphoma cell lines. *J. Vet. Med. Sci.* **71**: 769-777.

Isotani M, Ishida N, Tominaga M, Tamura K, Yagihara H, Ochi S, Kato R, Kobayashi T, Fujita M, Fujino Y, Setoguchi A, Ono K, Washizu T and Bonkobara M. 2008. Effect of tyrosine kinase inhibition by imatinib mesylate on mast cell tumors in dogs. *J. Vet. Int. Med.* **22**: 985-988.

Jaeckle KA, Eyre HJ, Townsend JJ, Shulman S, Knudson HM, Belanich M, Yarosh DB, Bearman SI, Giroux DJ and Schold SC. 1998. Correlation of tumor O6-methylguanine-DNA methyltransferase levels with survival of malignant astrocytoma patients treated with bischloroethylnitrosourea: a Southwest Oncology Group study. *J. Clin. Oncol.* **16**: 3310-3315.

Jiang BH, Jiang G, Zheng JZ, Lu Z, Hunter T and Vogt PK. 2001. Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. *Cell Grow. Diff.* **12**: 363-369.

Jiang BH, Rue E, Wang GL, Roe R and Semenza GL. 1996. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J.Biol. Chem.* **271**: 17771-17778.

Kaelin WG, and Ratcliffe PJ. 2008. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol. Cell* **30**: 393-402.

Keith B and Simon MC. 2007. Hypoxia-inducible factors, stem cells, and cancer. *Cell* **129**: 465-472.

Keller ET, MacEwen EG, Rosenthal RC, Helfand SC and Fox LE. 1993. Evaluation of prognostic factors and sequential combination chemotherapy with doxorubicin for canine lymphoma. *J. Vet. Int. Med.* **7**: 289-295.

Kreklau EL, Liu N, Li Z, Cornetta K and Erickson LC. 2001. Comparison of single-versus double-bolus treatment of O^6-benzylguanine for depletion of O^6-methylguanine DNA methyltransferase (MGMT) activity in vivo: development of a novel fluorometric oligonucleotide assay for measurement of MGMT activity. *J. Pharmacol. Exp. Ther.* **297**: 524-530.

Lee JJ, Hunghes CS, Fine RL and Page RL. 1996. P-glycoprotein expression in canine lymphoma: a relevant, intermediate model of multidrug resistance. *Cancer* **77**: 1892-1898.

Liao D, Corle C, Seagroves TN and Johnson RS. 2007. Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. *Cancer Res.* **67**: 563-572.

Liao D and Johnson RS. 2007. Hypoxia: a key regulator of angiogenesis in cancer. *Cancer Meta. Rev.* **26**: 281-290.

Ludlum DB. 1990. DNA alkylation by the haloethylnitrosoureas: nature of modifications produced and their enzymatic repair or removal. *Mutant Res.* **233**: 117-126.

Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW and Giannakakou P. 2003. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. *Cancer Cell* **3**: 363-375.

Madej JA, Madej JP, Dziegiel P, Pula B and Nowak M. 2013. Expression of hypoxia-inducible factor-1α and vascular density in mammary adenomas and adenocarcinomas in bicthes. *Acta Vet. Scand.* **55**: 73.

Mees G, Vangestel C, Dierckx R, Loomans S, Van Damme N, Peremans K, De Rooster H, Van Goethem B, Pauwels P, Ducatelle R and Van de Wiele C. 2011. Metabolic correlates of tumour hypoxia in malignant canine mammary carcinoma. *Res. Vet. Sci.* **91**: e125-128.

Momoi Y, Okai Y, Watari T, Goitsuka R, Tsujimoto H and Hasegawa A. 1997. Establishment and characterization of a canine T-lymphoblastoid cell line derived from malignant lymphoma. *Vet. Immunol. Immunopathol.* **59**:11-20.

Moore AS, Leveille CR, Reimann KA, Shu H and Arias IM. 1995. The expression of P-glycoprotein in canine lymphoma and its association with multidrug resistance. *Cancer Invest.* **13**: 475-479.

Moore AS, London CA, Wood CA, Williams LE, Cotter SM, L' Heureux DA and Frimberger AE. 1999. Lomustine (CCNU) for the treatment of resistant lymphoma in dogs. *J. Vet. Int. Med.* **13**: 395-398.

Nakaichi M, Taura Y, Kanki M, Mamba K, Mom Y, Tsujimoto H and Nakama S. 1996. Establishment and characterization of a new canine B-cell leukemia cell line. *J. Vet. Med. Sci.* **58**: 469-471.

Pegg AE, Doran ME and Moschel RC. 1995. Structure, function and inhibition of O^6-alkylguanine-DNA alkyltransferase. Prog. Nuc. *Acid Res. Mol. Biol.* **51**: 167-223.

Peters IR, Peeters D, Helps CR and Day MJ. 2007. Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of canine gene expression studies. *Vet. Immunol. Immunopathol.* **117**: 55-66.

Petty JC, Lana SE, Thamm DH, Charles JB, Bachand AM, Bush JM and Ehrhart EJ. 2008. Glucose transporter 1 expression in canine osteosarcoma. *Vet. Comp. Oncol.* **6**: 133-140.

Qian XC and Brent TP. 1997. Methylation hot spots in the 5' flanking region denote silencing of the O^6-methylguanine-DNA methyltransferase gene. *Cancer Res.* **57**: 3672-3677.

Rollins RA, Haghighi F, Edwards JR, Das R, Zhang MQ, Ju J and Bestor TH. 2006. Large-scale structure of genome methylation patterns. *Genome Res*. **16**: 157-163.

Rosales C, Jeglum KA, Obrocka M and Steplewski Z. 1988. Cytolytic activity of murine anti-dog lymphoma monoclonal antibodies with canine effector cells and complement. *Cell. Immunol.* **115**: 420-428.

Ruan S, Ocku MF, Ren JP, Chiao P, Andreeff M, Levin V and Zhang W. 1998. Overexpressed WAF1/Cip1 renders glioblastoma cells resistant to chemotherapy agents 1,3-bis(2-chloroethyl)-1-nitrosourea and cisplatin. *Cancer Res.* **58**: 1538-1543.

Rütgen BC, Hammer SE, Gerner W, Christian M, de Arespacochaga AG, Willmann M, Kleiter M, Schwendenwein I and Saalmüller A. 2010. Establishment and characterization of a novel canine B-cell line derived from a spontaneously occuring diffuse large cell lymphoma. *Leukemia Res.* **34**: 932-938.

Saba CF, Thamm DH and Vail DM. 2007. Combination chemotherapy with L-asparaginase, lomustine, and prednisone for relapsed or refractory canine lymphoma. *J. Vet. Int. Med.* **21**: 127-132.

Semenza GL. 1998. Hypoxia-inducible factor 1: master regulator of O^2 homeostasis. *Curr. Opin. Gen. Dev.* **8**: 588-594.

Semenza GL. 1999. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. *Cell Dev. Biol.* **15**: 551-578.

Semenza GL. 2012. Hypoxia-inducible factors in physiology and medicine. *Cell* **148**: 399-408.

Silber JR, Blank A, Bobola MS, Ghatan S, Kolston DD and Berger MS. 1999. *O*^{6}-methylguanine DNA methyltransferase-deficient phenotype in human gliomas: frequency and time to tumor progression after alkylating agent-based chemotherapy. *J. Clin. Cancer Res.* **5**: 807-814.

Silber JR, Mueller BA, Ewers TG and Berger MS. 1993. Comparison of *O*^{6}-methylguanine-DNA methyltransferase activity in brain tumors and adjacent normal brain. *Mol. Carcinogenesis* **24**: 90-98.

Silber JR, Mueller BA, Ewers TG and Berger MS. 1998. *O*^{6}-methylguanine-DNA methyltransferase activity in adult gliomas: relation to patient and tumor characteristics. *Cancer Res.* **58**: 1068-1073.

Sueoka E, Sueoka-Aragane N, Sato A, Ide M, Nakamura H, Sotomaru Y, Taya C, Yonekawa H, Kitagawa T, Kubota Y, Kimura S, Nakachi K and Tanimoto K. 2013. Development of

lymphoproliferative diseases by hypoxia inducible factor-1alpha is associated with prolonged lymphocyte survival. *PLOS One* **8**: e57833.

Suter SE, Chein MB, von Messling V, Yip B, Cattaneo R, Vernau W, Madewell BR and London CA. 200. *In vitro* canine distemper virus infection of canine lymphoid cells: a prelude to oncolytic therapy for lymphoma. *Clin. Cancer Res.* **11**: 1579-1587.

Takai D and Jones PA. 2002. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *PNAS* **99**: 3740-3745.

Tew KD, Colvin OM and Chabner BA. 2001. Alkylating agents. Chabner BA, Longo DL, eds. Cancer Chemotherapy and Biotherapy: Principles and Practice, 3rd ed.: 374-414.

Tomiyasu H, Goto-Koshino Y, Takahashi M, Fujino Y, Ohno K and Tsujimoto H. 2010. Quantitative analysis of mRNA of 10 different drug resistance factors in dogs with lymphoma. *J. Vet. Med. Sci.* **72**: 1165-1172.

Umeki S, Ema Y, Suzuki R, Kubo M, Hayashi T, Okamura Y, Yamazaki J, Tsujimoto H, Tani K, Hiraoka H, Okuda M and Mizuno T. 2013. Establishment of five canine lymphoma cell lines and tumor formation in a xenotransplantation model. *J. Vet. Med. Sci.* **75**: 467-474.

Vander Heiden MG, Cantlye LC and Thompson CB. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**: 1029-1033.

Van Dyke MM and Dervan PB. 1984. Echinomycin binding sites on DNA. *Science* **225**: 1122-1127.

Vaupel P and Mayer A. 2007. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Meta. Rev.* **26**: 225-239.

Wang Y, Liu Y, Malek SN, Zheng P and Liu Y. 2011. Targeting HIF1α eliminates cancer stem cells in hematological malignancies. *Cell Stem Cell* **8**: 399-411.

Warburg O. 1956. On the origin of cancer cells. *Science.* **123**: 309-314.

Wellmann S, Guschmann M, Griethe W, Eckert C, Stackelberg A, Lottaz C, Moderegger E, Einsiedel HG, Eckardt KU, Henze G and Seeger K. 2004. Activation of the HIF pathway in childhood ALL, prognostic implications of VEGF. *Leukemia* **18**: 926-933.

Wouters BG and Koritzinsky M. 2008. Hypoxia signaling through mTOR and the unfolded protein response in cancer. *Nat. Rev. Cancer* **8**: 851-864.

Xia Y, Choi HK and Lee K. 2012. Recent advances in hypoxia-inducible factor (HIF)-1 inhibitors. *Euro. J. Med. Chem.* **49**: 24-40.

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

Yeo EJ, Chun YS and Park JW. 2004. New anticancer strategies targeting HIF-1. *Biochem. Phar.* **68**: 1061-1069.

Zaboikin M, Srinivasakumar N, Zaboikina T and Shuening F. 2004. Cloning and expression of canine O^6-methylguanine-DNA methyltransferase in target cells, using gammaretroviral and lentiviral vectors. *Human Gene Ther.* **15**: 383-392.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL and Simons JW. 1999. Overexpression of hypoxia-inducible factor 1 alpha in common human cancers and their metastases. *Cancer Res.* **59**: 5830-5835.

Tables

Table 1. Primers used in this study.

Target	Forward primer	Reverse primer	Ta* [°C]	use
canine MGMT	5′ - TGCTCGGGAGGATGGACAAG - 3′	5′ - AGTCACTCAGATGTTTACTCCC - 3′		RT-PCR
MGMT region 1	5′ - GGGGATAGATTTTGGAAAATG - 3′	5′ - CAAAATCAACCCACTACTCCATC - 3′	55	Bisulfite
MGMT region 2-1	5′ - AGGGAAGTGGAGGTAGTAGG - 3′	5′ - CCCCTAACCCCTAATCCTAT - 3′	53	Bisulfite
MGMT region 2-2	5′ - ATAGGATTAGGGGTTAGGGG - 3′	5′ - ACCAACTTCCTTCACCAACAAA - 3′	54	Bisulfite
MGMT region 3-1	5′ - GTTTTTGTTGAGGAGGAGTA - 3′	5′ - CCTACTACCACCACAATAAC - 3′	50	Bisulfite
MGMT region 3-2	5′ - GGTTATTGTGGTGGTAGTAGG - 3′	5′ - CCCAAAACAACAATCTTACC - 3′	52	Bisulfite
MGMT region 4	5′ - GGGTATTGGGGGAAGGTGTT - 3′	5′ - CCAAAACCATCTCTCCAATCCC - 3′	61	Bisulfite
canine MGMT	5′ - CCTGGCTGGATGCCTATTTCC - 3′	5′ - CTGCTGGTAGGAAACCGTGT - 3′		real-time PCR
canine RPL32	5′ - TGGTTACAGGAGCAACAAGAAA - 3′	5′ - GCACATCAGCAGCACTTCA - 3′		real-time PCR
canine PRL13A	5′ - GCCGGAAGGTTGTAGTCGT - 3′	5′ - GGAGGAAGGCCAGGTAATTC - 3′		real-time PCR

*Annealing temperature

Table 2. Mean 50% inhibitory concentration (IC_{50}) by CCNU in each cell line.

	Jurkat	CL-1	Ema	GL-1	Nody-1	UL-1
BG (-) *	45.0 (±5.1)***	>50.0	14.0 (±1.7)	17.0 (±0.7)	36.5 (±3.5)	23.5 (±0.6)
BG (+) **	18.5 (±0.9)	11.5 (±4.0)	13.5 (±2.3)	16.0 (±0.5)	7.5 (±4.7)	19.0 (±0.7)

*BG (-); treated without *O*^{6}-Benzylguanine
**BG(+); treated with *O*^{6}-Benzylguanine
***Mean (±SD) μM

Figures

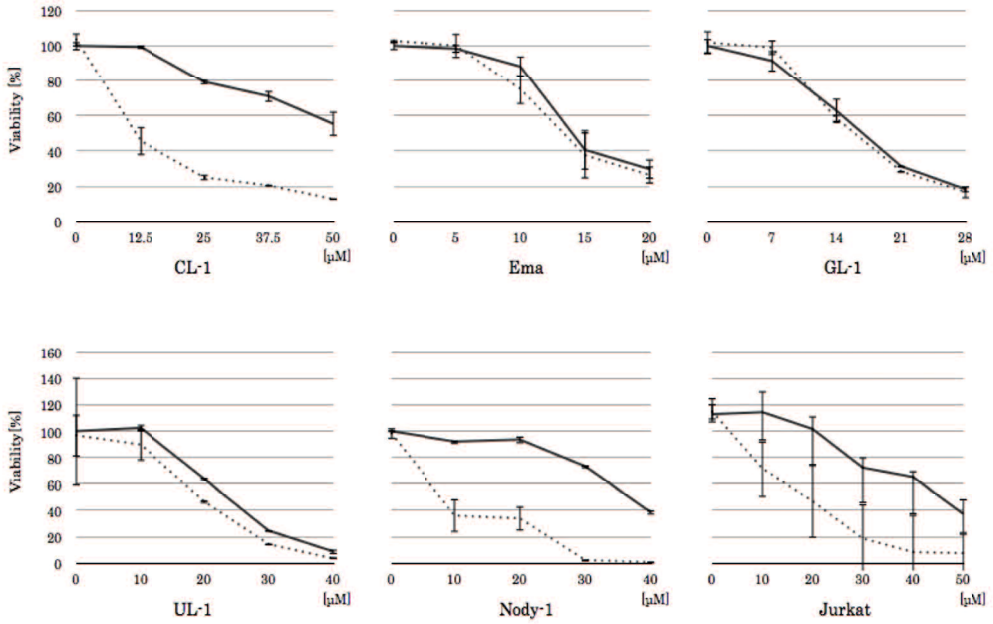


Fig. I-1. Viability of lymphoma cell lines treated with lomustine.

Cells pretreated with or without *O*^{6}-Benzylguanine (BG) were incubated with various concentrations of lomustine, and the cell viabilities were assessed. Solid lines, without BG; dotted lines, with BG. The experiment was performed in triplicate, mean ±SD was shown. Data are representative for three independent experiments.

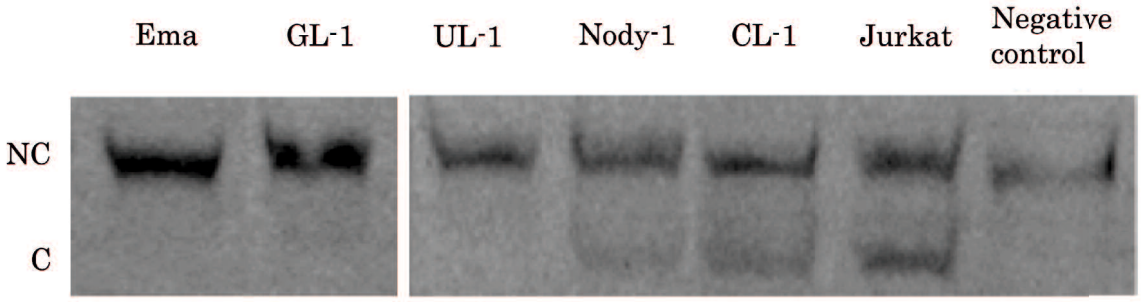


Fig. I-2. Fluorometric oligonucleotide assay for the MGMT activity.

A double-stranded 45 bp oligonucleotide containing a single *O*^{6}-methylated guanine residue nested in a *Pvu*II restriction site and a fluorometric 5′-hexachloro-fluorescein phosphoramidite (HEX) was incubated with the cell extracts, and then treated with *Pvu*II restriction enzyme, followed by electrophoresis. If the extract contains MGMT activity, an O^6-methylated guanine residue nested within the *Pvu*II restriction site can be demethylated, and the oligonucleotide can be digested by *Pvu*II.
NC, uncleaved oligonucleotide; C, cleaved oligonucleotide.

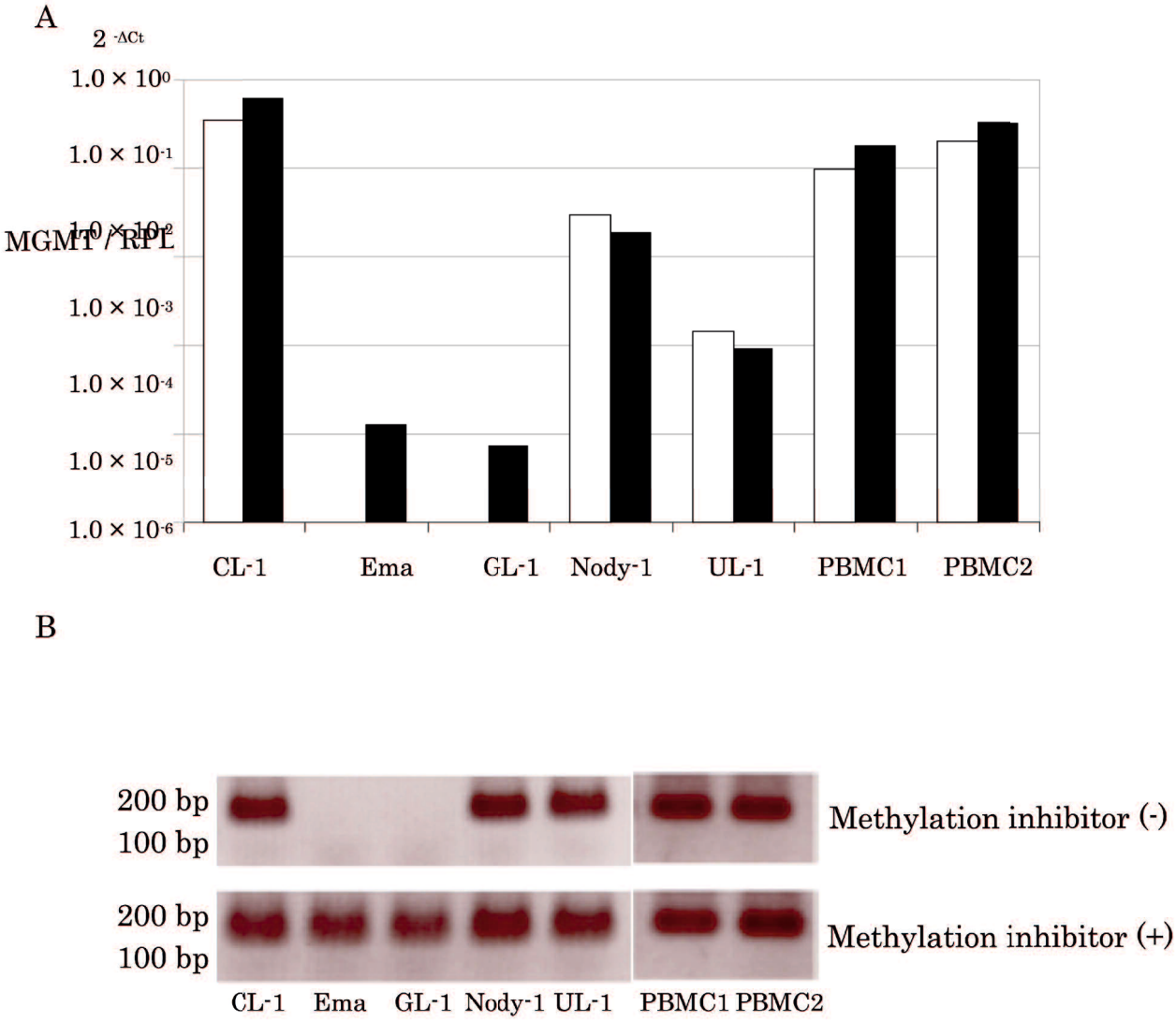
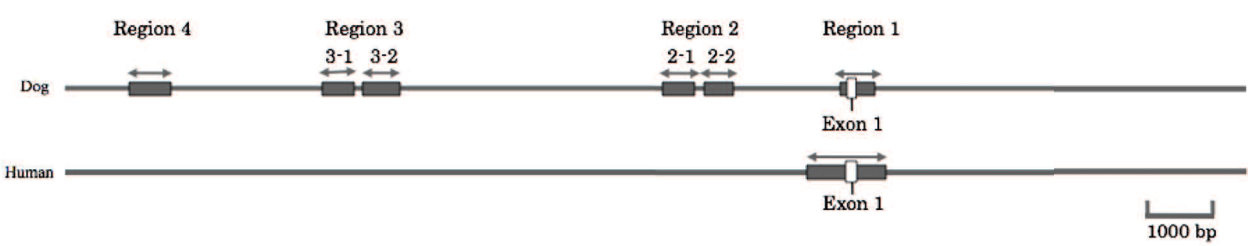


Fig. I-3. The MGMT gene expression with or without the methylation inhibitor.

(A) MGMT mRNA expression compared to the reference gene, RPL32, in the absence (white bar) or presence (black bar) of 0.5 μM methylation inhibitor 5-Aza-2′-deoxycytidine with real-time PCR is shown by the $2^{-\Delta Ct}$.

(B) Agarose gel electrophoresis of real-time PCR products obtained in (A).

A



B

	Exon 1	Region 1	Region 2-1	Region 2-2	Region 3-1	Region 3-2	Region 4
Nucleotide number	41,202,021 - 41,202,158	41,201,907 - 41,202,403	41,200,835 - 41,201,294	41,201,275 - 41,201,694	41,196,562 - 41,197,022	41,197,002 - 41,197,552	41,195,336 - 41,195,933

Canis lupus familiaris chromosome 28 (NC_006610.2)

Fig. I-4. Scheme of CpG islands surrounding the canine MGMT gene exon 1 and the regions analysed in this study.

(A) CpG islands in the upstream region of the canine MGMT gene. CpG islands around exon 1 of the canine MGMT gene were analysed by Methyl Primer Express software v1.0 (http://www.appliedbiosystems.com/absite/us/en/home/support/software-community/free-ab-software.html) with parameters, a G+C content greater than 55% and observed CpG/expected CpG greater than 0.65. The arrowed regions were identified from an approximately 10,000-base upstream through 7,000-base downstream region of the canine MGMT exon 1, and analyzed in this study.

(B) Nucleotide numbers of the analysed regions. The numbers are based on the sequence of Canis lupus familiaris chromosome 28 (Genbank accession No. is NC_006610.2).

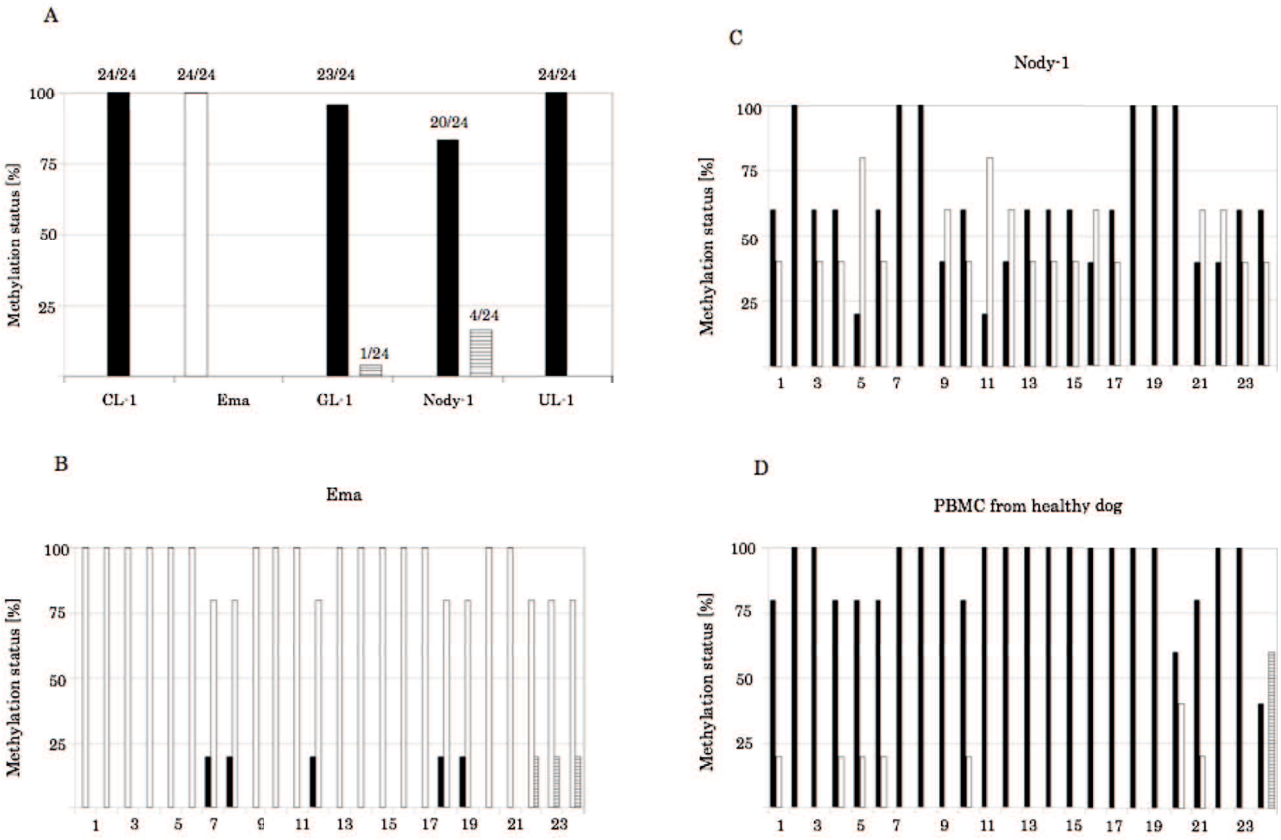


Fig. I-5. Methylation status of Region 1 obtained by bisulfite sequencing.

(A) DNA extracted from each cell line was treated with bisulfite, amplified by PCR, and assessed for its methylation status followed by the direct sequencing method. Methylation status in 24 CpGs in Region 1 is represented by numbers above the corresponding bars. Black bar, methylated cytosine; white bar, unmethylated cytosine; and striped bar, undetermined.
(B-D) Amplified DNA from (B) Ema cell line, (C) Nody-1 cell line, and (D) PBMC was inserted into the pCR2.1 plasmid vector and transformed into bacteria. The sequences of five independent clones were analysed as well as directly sequenced. The methylated or unmethylated pattern of each CpG is expressed in the form of percentages.

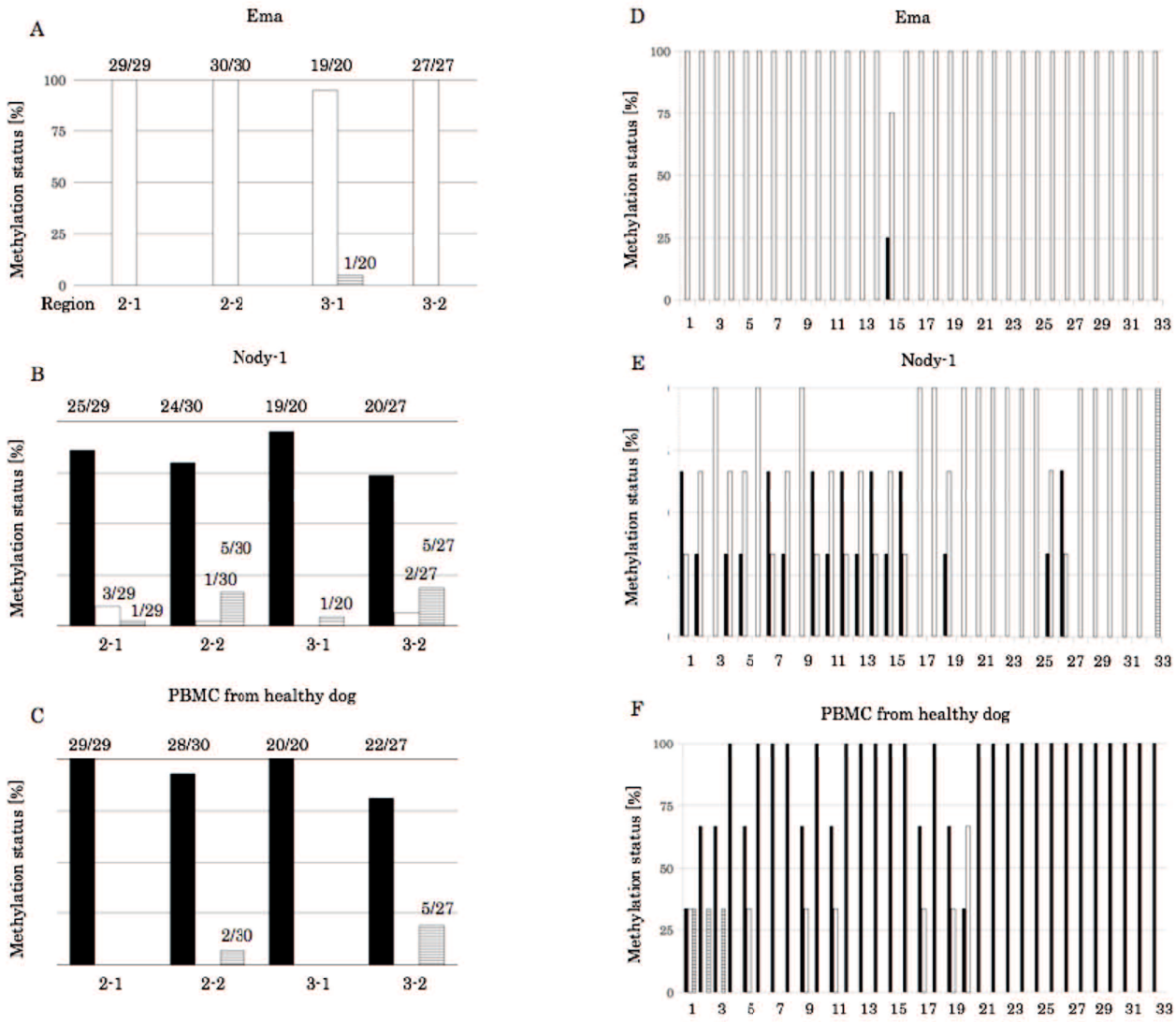


Fig. I-6. Methylation status in Regions 2-1, 2-2, 3-1, 3-2 and 4.

(A-C) DNA extracted from each cell line was treated with bisulfite, amplified by PCR, and assessed for its methylation status followed by the direct sequencing method. The methylation rate in each Region is represented by the numbers above the corresponding bars. Black bar, methylated cytosine; white bar, unmethylated cytosine; and striped bar, undetermined.
(D-F) DNA extracted from each cell line was treated with bisulfite, amplified by PCR, and then cloned into the plasmid vector. These clones were assessed for their methylation status by the direct sequencing method. This experiment was performed using at least five clones per cell line. The individual CpGs are shown on the X-axis, and the methylation status is shown on the Y-axis. Black bar, methylated cytosine; white bar, unmethylated cytosine; and striped bar, undetermined.

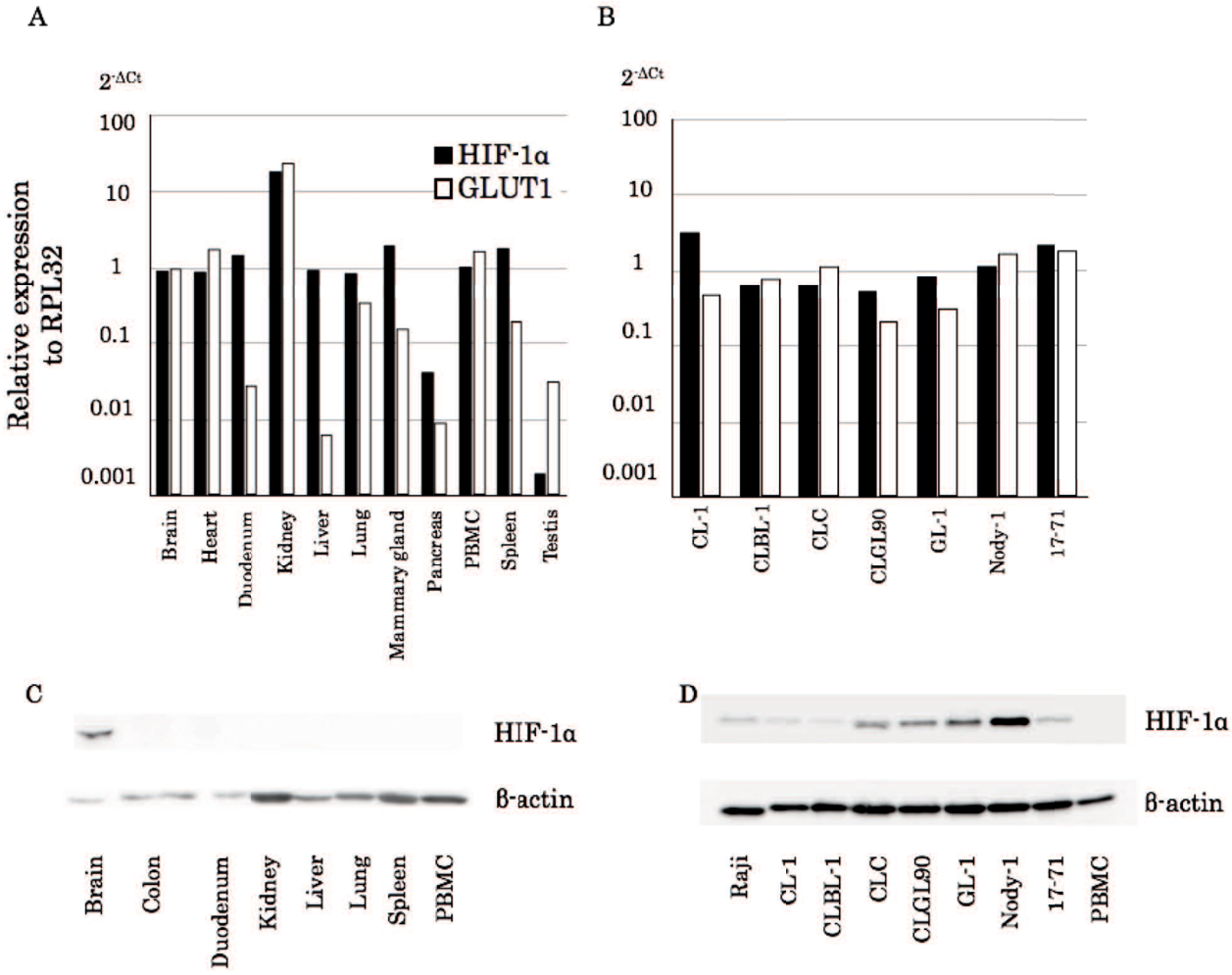


Fig. II-1. cHIF-1α mRNA and protein expression in canine normal tissues and lymphoma cell lines.

(A and B) The cHIF-1α mRNA expression levels in healthy dog tissues (A) and canine lymphoma cell lines (B) were examined by real-time PCR. Expression levels of cHIF-1α and GLUT1 were calculated as ΔCt in comparison to the reference gene, RPL32. The 2-ΔCt values represent relative expression levels.
(C and D) The cHIF-1α protein expression levels in healthy dog tissues (C) and the canine lymphoma cell lines (D) were examined by western blotting.

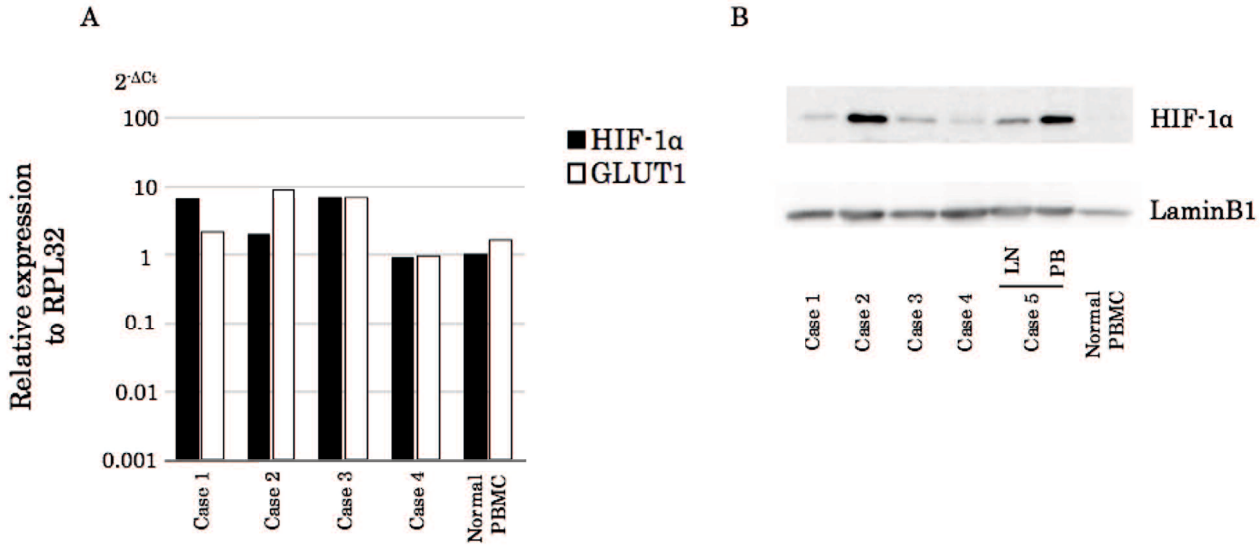


Fig. II-2. Analysis of the cHIF-1α expression levels in clinical samples from canine lymphoma patients.

(A) mRNA expression levels in canine lymphoma. The samples were obtained by fine needle aspiration from the affected lymph nodes. Expression levels of cHIF-1α and GLUT1 were calculated as ΔCt in comparison to the reference gene, RPL32. The 2-ΔCt values represent relative expression levels.
(B) The cHIF-1α protein levels in canine lymphoma. In patient 5, the cancer cells were in multiple lymph nodes (LN) and the peripheral blood (PB). Normal PBMC, PBMCs obtained from a healthy beagle.

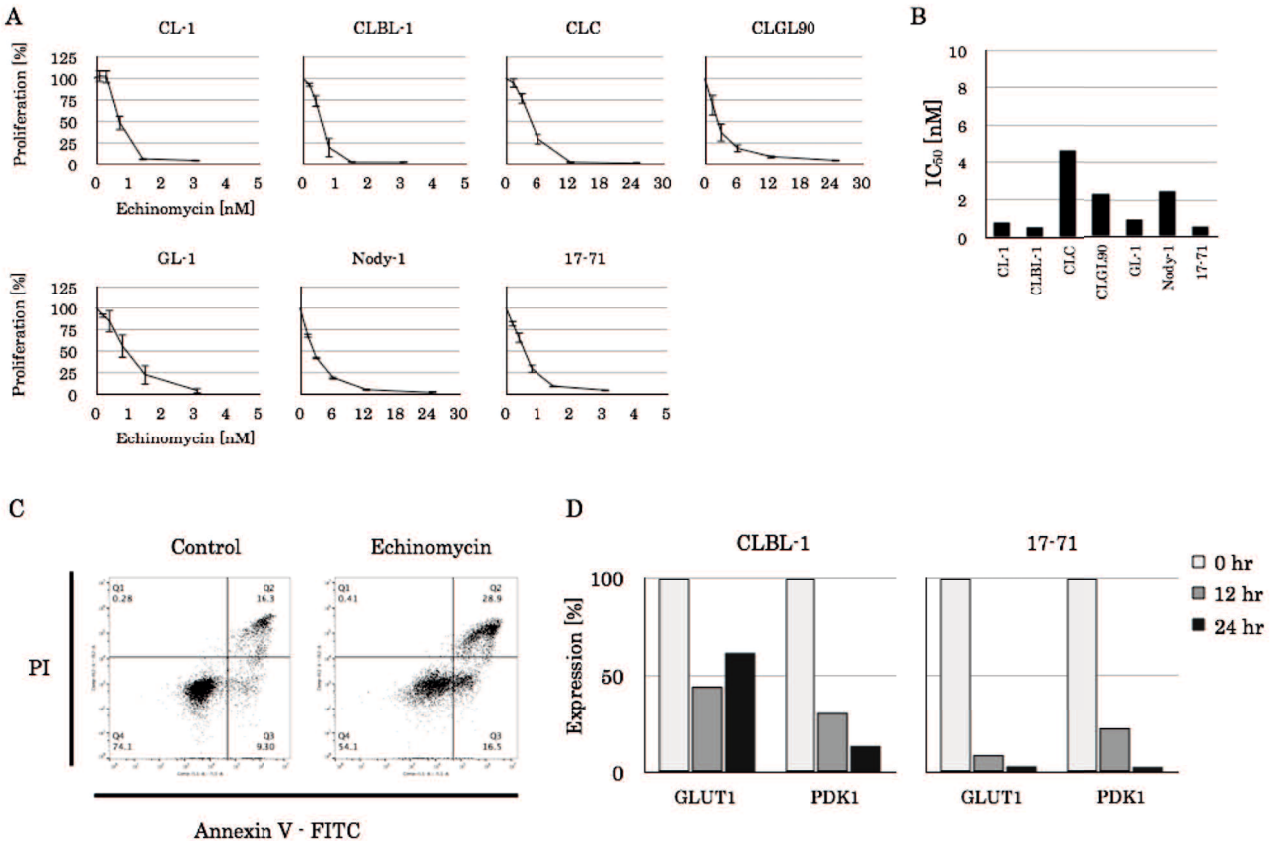


Fig. II-3. The effect of echinomycin on canine lymphoma cell lines.

(A) The proliferation rate of canine lymphoma cell lines in the absence or presence of echinomycin. Cell lines were incubated with each concentration of echinomycin for 48 hr at 37 ºC and subjected to MTT assay. The experiment was performed in triplicate, and data are the mean ± standard deviation (SD) of three independent experiments.
(B) The echinomycin IC_{50} were calculated from (A).
(C) The apoptosis rate in the presence of DMSO control or 2 nM echinomycin in CLBL-1 cell line. Cells were incubated with agents for 12 hr at 37 ºC and then stained with Annexin V-FITC. The fluorescence intensity was analyzed using flow cytometry.
(D) The CLBL-1 and 17-71 cells were incubated with 2 nM echinomycin for 12 or 24 hr and subjected to real-time PCR to examine the expression levels of GLUT1 and PDK1, the downstream genes of HIF-1α. The relative expression levels were calculated as ΔCt from the difference between expression of the internal control, RPL32. Data are reported as expression rates compared to the initial point.

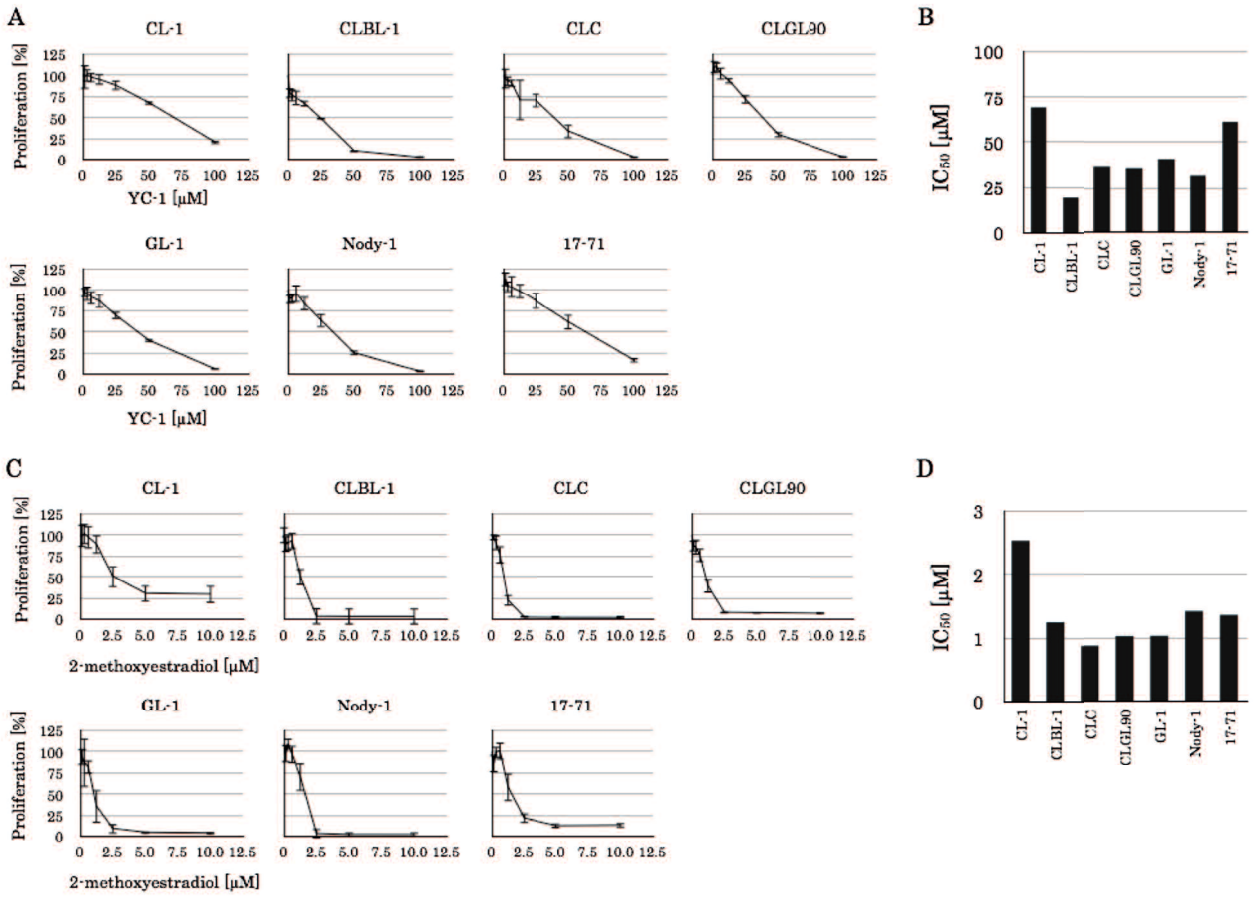


Fig. II-4. The effect of YC-1 and 2-methoxyestradiol on canine lymphoma cell lines.

(A) The proliferation rate of canine lymphoma cell lines in the absence or presence of the agent. Cell lines were incubated with each concentration of YC-1 for 48 hr at 37 ℃ and subjected to MTT assay and data are the mean ± standard deviation (SD) of three independent experiments.
(B) The YC-1 IC_{50} were calculated from (A).
(C) The proliferation rate of canine lymphoma cell lines in the absence or presence of the agent. Cell lines were incubated with each concentration of 2-methoxyestradiol for 48 hr at 37 ℃ and subjected to MTT assay and data are the mean ± standard deviation (SD) of three independent experiments.
(D) The 2-methoxyestradiol IC_{50} were calculated from (C).

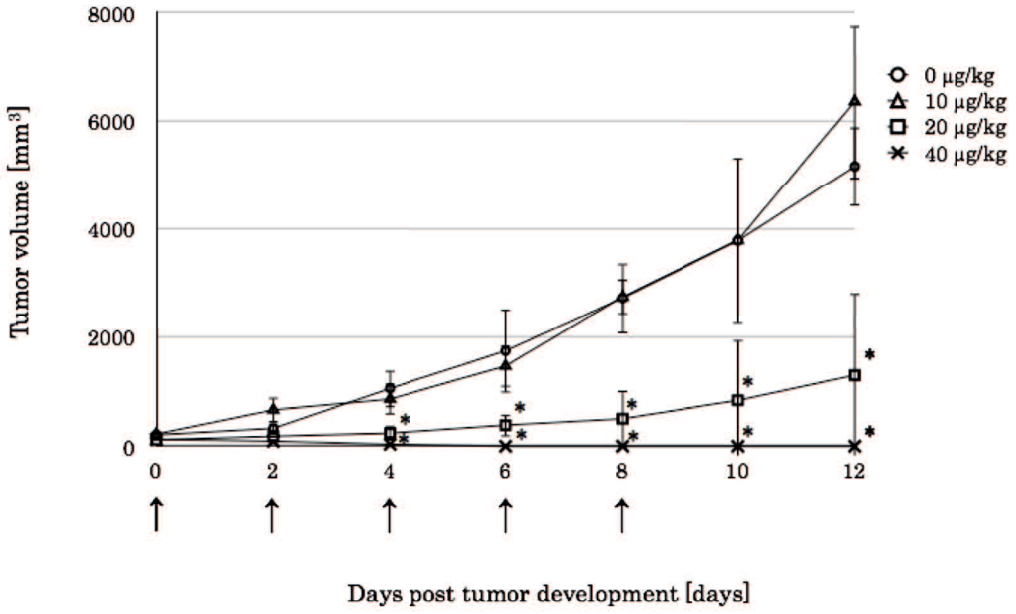


Fig. II-5. The engrafted CLBL-1 tumor size, which inoculated to NOD/scid mice.

When the tumor size exceeded 100 mm3, it was considered as day 0. Any concentration of echinomycin or DMSO was injected intraperitoneally at days 0, 2, 4, 6 and 8 (arrow). The tumor size was measured every other day. Data represents the median and SD of each group (n=4). The * corresponds to a $p < 0.05$ between the 20 or 40 μg/kg echinomycin treated groups and the untreated group.