Evaluation of DNA methylation profiles of the CpG island of the ABCB1 gene in dogs with lymphoma

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Objective—To examine the DNA methylation status of the ABCB1 gene in tumor cells of dogs with lymphoma.

Animals—27 dogs with multicentric B-cell high-grade lymphoma (19 chemotherapy-sensitive dogs and 8 chemotherapy-resistant dogs).

Procedures—The DNA methylation profile of the CpG island of the ABCB1 gene was analyzed by use of bisulphite sequencing and real-time methylation-specific PCR assay in lymphoma cells. Quantitative reverse transcriptase PCR assay of the ABCB1 gene was conducted to measure the amount of mRNA. Correlation between the amount of ABCB1 mRNA and the methylation rate was examined.

Results—The CpG island of the ABCB1 gene was hypomethylated in most dogs in both the chemotherapy-sensitive and -resistant groups. No significant difference was detected in the methylation rate between the 2 groups, and no significant correlation was detected between the methylation rate and the mRNA expression level.

Conclusions and Clinical Relevance—Expression of the ABCB1 gene was not suppressed by hypermethylation of its CpG island in most dogs with lymphoma regardless of their chemotherapy sensitivity status. (Am J Vet Res 2014;75:835–841)

Lymphoma is the most common hematologic neoplasm in dogs and is a representative neoplasm that responds to conventional chemotherapy. It has been reported that treatment with chemotherapeutic agents for dogs with lymphoma results in high rates of complete response, ranging from 76.3% to 92.3%. However, most canine patients with lymphoma relapse. Efficacy of rescue protocols is limited after acquisition of MDR to the agents used in remission induction therapy. The overall response rate and median response duration after initiation of rescue protocols are reported to be 31% to 44% and 2 months, respectively. Therefore, the mechanisms that confer the MDR phenotype to the tumor cells have been targeted in efforts to develop successful treatments for dogs with lymphoma, because most current treatments for canine lymphoma end in failure.

Among molecules that induce the MDR phenotype, P-gp (coded by the ABCB1 gene [formerly called the MDR1 gene]) is one of the most studied molecules in humans and dogs. This molecule is expressed on the cellular membrane, and it facilitates the efflux of drugs from the cytoplasm to the cell exterior. Thus, the overexpression of P-gp reduces the intracellular concentration of chemotherapeutic agents. However, the mechanisms that induce overexpression of P-gp are not well understood.

Epigenetics is the study of heritable changes in gene expression that occur without changing the nucleotide sequence of the genome; regulation of this phenomenon has been intensively investigated. The main epigenetic mechanisms include DNA methylation, modifications to chromatin structure, loss of imprinting, and noncoding RNA. 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. In addition, a similar inverse correlation has been observed...
in the primary tumor cells of patients with AML\textsuperscript{15} and urinary bladder cancer.\textsuperscript{16} These studies in human medicine suggest a possibility that the methylation status of the CpG island in its promoter region is associated with the expression level of \(\text{ABCB1}\), thereby influencing the acquisition of the MDR phenotype in tumor cells.

An inverse correlation between DNA methylation of the CpG island of \(\text{ABCB1}\) and mRNA expression levels in canine lymphoid tumor cell lines has been reported.\textsuperscript{17} However, the epigenetic regulation of \(\text{ABCB1}\) has not been explored in primary tumor cells obtained from dogs with lymphoma. The purpose of the study reported here was to analyze the DNA methylation profile of \(\text{ABCB1}\) in the primary tumor cells collected from dogs with lymphoma with and without the MDR phenotype.

**Materials and Methods**

Dogs—Dogs with multicentric lymphoma evaluated at the Veterinary Medical Center of the University of Tokyo between July 1, 2008, and June 31, 2013, were enrolled in the study. Diagnosis was made on the basis of cytologic examination of FNA samples obtained from lymph nodes by use of the updated Kiel classification.\textsuperscript{17} Discrimination between B-cell and T-cell lymphomas was defined by PCR amplification tests for the antigen receptor gene rearrangements, as described.\textsuperscript{19,20} Dogs that had the multicentric anatomic form with cytologic characteristics indicating a centroblastic polymorphic type that has been categorized into B-cell high-grade malignancy in the updated Kiel classification\textsuperscript{18} were included. When the cytologic findings suggested a possibility of low-grade lymphoma such as the small clear cell type in the updated Kiel classification,\textsuperscript{18} resection biopsy of the lymph node was performed. Because these cases were categorized into T-zone lymphoma,\textsuperscript{21} or lymphoid hyperplasia, they were excluded from the study. In all dogs, the clinical stage was evaluated on the basis of the results of physical examination, CBC, thoracic and abdominal radiography, and abdominal ultrasonography by use of the WHO staging criteria.\textsuperscript{22} Written consent was obtained from all dog owners prior to study enrollment.

**Evaluation of response to treatment**—Veterinarians measured the size of the enlarged lymph nodes with calipers at each admission, and the response to treatment was evaluated on the basis of the response evaluation criteria for canine lymphoma.\textsuperscript{23} In the evaluation, complete response and partial response were seen as indicating chemotherapy-sensitive status, whereas stable disease and progressive disease were regarded as chemotherapy-resistant status.

**Chemotherapy**—A cyclophosphamide, doxorubicin, vincristine, and prednisone-based chemotherapy, UW-25 protocol,\textsuperscript{2} was used as a standard multidrug combination chemotherapy protocol. However, administration of L-asparaginase was omitted in all cases because L-asparaginase is reported to have no influence on the efficacy of the UW-25 protocol.\textsuperscript{24} Dogs that received a corticosteroid before chemotherapy were not excluded.

**Definition of patient groups**—Chemotherapy-sensitive dogs were defined as those that had not received any chemotherapeutic agent except for a corticosteroid before the application of the UW-25 protocol and that had complete response or partial response by week 5 of the UW-25 protocol. Tumor cell samples were collected just prior to administration of chemotherapy at the initial consultation. Chemotherapy-resistant dogs were defined as those that underwent the UW-25 protocol, had a relapse after achieving a response, and did not respond to any of the 3 chemotherapeutic agents used in the UW-25 protocol (cyclophosphamide, doxorubicin, and vincristine). Tumor cell samples of the chemotherapy-resistant dogs were collected after confirming the nonresponsiveness to these 3 drugs. For dogs that underwent UW-25 protocol, achieved remission, but acquired resistance to the 3 chemotherapeutic agents and relapsed, the tumor cell samples of the drug-resistant phase were included in the study.

**Analysis of the methylation profile of \(\text{ABCB1}\) gene**—The DNA methylation profile of the CpG island in \(\text{ABCB1}\) was analyzed by use of bisulphite sequencing and real-time MSP assay as in a previous study.\textsuperscript{17} Genomic DNA was extracted from FNA samples with a DNA extraction kit.\textsuperscript{2} Bisulphite conversion was performed on 2 \(\mu\)g of DNA with a commercially available kit,\textsuperscript{2} and converted DNA was stored at \(-80^\circ\)C until batch analysis. Sequences of primer pairs used for bisulphite sequencing and real-time MSP assay were listed (Appendix), and locations of the analyzed regions were illustrated (Figure 1). In the real-time MSP assay, upstream and downstream regions were examined.

In bisulphite sequencing, 200 ng of converted DNA was amplified with DNA polymerase\textsuperscript{2} according to the manufacturer’s instructions. The PCR products were inserted into a T-A cloning vector\textsuperscript{2} and transferred into competent *Escherichia coli* (strain DH5\(\alpha\)). Plasmid DNA was extracted with a DNA purification kit.\textsuperscript{2} Sequence determination of the extracted plasmid DNA was conducted with a sequencing kit\textsuperscript{2} and capillary sequencer.\textsuperscript{2} 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 the real-time MSP assay, both the completely methylated DNA control and completely unmethylated DNA control were prepared from genomic DNA of a canine lymphoma cell line, UL-1, by use of CpG methyltransferase and a DNA amplification kit, respectively. Complete methylation and demethylation were verified by bisulphite sequencing as described. These control DNAs were then inserted into a T-A cloning vector and transferred into competent E coli (strain DH5α). Assay-specific standard curves for methylated and unmethylated DNAs were prepared in triplicate with serial (10×) dilutions of plasmid extracted from the E coli. Real-time MSP assay was performed in duplicate with an MSP kit and a thermal cycler with 100 ng of DNA. The absolute quantity of methylated or unmethylated DNA was calculated from the standard curves. Methylation rate in the real-time MSP assay was calculated by dividing the mean absolute quantity of methylated DNA by the sum of the mean absolute quantity of methylated and unmethylated DNA.

qRT-PCR assay—For estimation of the relative quantity of mRNA of the ABCB1 gene, the qRT-PCR assay was performed as in a previous study. Total RNA was extracted from each FNA sample with an extraction kit and stored at –80°C until batch analysis. After treatment with DNase, cDNA was synthesized with total RNA by use of a reverse transcription kit. The same primer pair for the qPCR assay of ABCB1 expression was used as in a previous study. For normalization of the amount of cDNA sample, the TBP gene was selected as the internal reference on the basis of previous results. The qPCR assay was performed with a master premix and a thermal cycler, with cDNA synthesized from 10 ng of total RNA. For each gene, an assay-specific standard curve with serial (10×) 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 the TBP gene for normalization. The qRT-PCR assay was performed in triplicate.

Statistical analysis—Correlation of the methylation rate in the CpG island of the ABCB1 gene with the relative quantity of mRNA was examined by use of the Spearman 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 significant. Statistical analysis was performed with commercially available software.

Results

Dogs—Twenty-seven dogs with B-cell high-grade multicentric lymphoma were included in the study. Breeds included Welsh Corgi Pembroke (n = 4), Pug (3), Miniature Dachshund (3), Golden Retriever (2), Shiba Inu (2), French Bulldog (2), Beagle (2), and Shih

Figure 2—The DNA methylation profiles of CpG sites of ABCB1 in chemotherapy-sensitive dogs (A; dogs 1 to 4) and chemotherapy-resistant dogs (B; dogs 20 to 23) with lymphoma. Each box represents 1 CpG site. The white portion represents methylated CpG sites, and the black portion represents unmethylated CpG sites; the ratio of the white portion to the black portion of each box represents the ratio of DNA clones with methylated cytosine in each CpG site. Numbers in the boxes indicate the order of each CpG site within these specific regions. Numbers between boxes indicate the number of bp between CpG sites.
Tzu, Doberman Pinscher, mixed breed, English Bulldog, Bernese Mountain Dog, Scottish Terrier, Pomeranian, West Highland White Terrier, and Australian Kelpie (1 each). Median age was 8 years (range, 2 to 13 years), and 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 on the basis of the WHO clinical staging system: 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. On the basis of 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/d for 6 days, 1 mg/kg/d for 16 days, and 2 mg/kg/d for 3 days. Of the 3 dogs that had received the corticosteroid, 2 were in the chemotherapy-sensitive group and 1 was in the chemotherapy-resistant group. The duration to remission was 7 days in all 27 dogs. The median remission duration was 247 days (range, 7 to 436 days), and the median overall survival time was 310 days (range, 50 to 1,146 days). Median number of days when samples were collected was 262 days (range, 50 to 1,387 days) in the chemotherapy-resistant group.

**Bisulfite sequencing**—Four dogs were randomly selected from each group, and DNA methylation profiles of the CpG island of ABCB1 in the tumor cells were analyzed in detail by use of bisulfite sequencing. In 1 chemotherapy-sensitive dog, the DNA methylation rate was consistently >50% in all 23 CpG sites of ABCB1 (Figure 2). On the contrary, most of the 23 CpG sites were hypomethylated in 2 other chemotherapy-sensitive dogs. Another chemotherapy-sensitive dog had an intermediate DNA methylation pattern. The median methylation rates in 4 chemotherapy-sensitive dogs were 74.9% (range, 0% to 100%), 46.6% (range, 8.7% to 69.6%), 11.7% (range, 0% to 63.2%), and 7.36% (range, 0% to 17.4%).

In 1 chemotherapy-resistant dog, the degree of DNA methylation was variable among the 23 CpG sites of ABCB1. However, most of the 23 CpG sites were unmethylated in 3 other chemotherapy-resistant dogs. The median methylation rates in 4 chemotherapy-resistant dogs were 54.3% (range, 0% to 87.0%), 7.36% (range, 0% to 17.4%), 5.14% (range, 0% to 13.0%), and 0.932% (range, 0% to 4.35%).

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 dog in both groups.

**Real-time MSP assay**—The DNA methylation profiles were analyzed by use of a real-time MSP assay in all dogs. In the upstream region of the CpG island of ABCB1 in 17 of 19 chemotherapy-sensitive dogs, methy-
methylation profiles of CpG sites of ABCB1. In a study, composed of a heterogeneous population with various possibilities, canine lymphoma tissues might be DNA clones derived from each dog. These results raised the methylation profile of each CpG site varied among 15 cases, and the DNA methylation rates were variable among 8 dogs examined, and the DNA methylation rate in 2 dogs were > 40% (41.7% and 59.4%). Similarly, in the downstream region of the CpG island in 18 of 19 chemotherapeutic-sensitive dogs, methylation rates were < 10% and the median methylation rate was 0.332% (range, 0% to 7.79%), although methylation rate in 1 dog was 55.2% (Figure 3). In 6 of 8 dogs in the chemotherapeutic-resistant group, methylation rates in the downstream region were < 2% and the median methylation rate was 0.276% (range, 0% to 1.98%), although the methylation rates in 2 dogs were > 40% (41.3% and 66.0%). There was no significant difference in methylation rate between chemotherapeutic-sensitive and -resistant dogs in either the upstream or downstream region (P = 0.82 and 0.48, respectively).

Relative quantity of ABCB1 mRNA—The FNA samples of 16 chemotherapeutic-sensitive dogs were examined by use of the qRT-PCR assay. ABCB1 mRNA was detected in all 16 dogs. However, there was no significant correlation between the methylation rate of the CpG island obtained in the real-time MSP assay and the relative quantity of ABCB1 mRNA in both the upstream and downstream regions (ρ < 0.001 and 0.022, and P = 0.94 and 0.57, respectively; Figure 4).

Discussion

In the present study, the DNA methylation status of ABCB1 was analyzed by use of bisulphite sequencing and a real-time MSP assay in dogs with lymphoma. By use of 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 dog. These results raised the possibility that canine lymphoma tissues might be composed of a heterogeneous population with various methylation profiles of CpG sites of ABCB1. In a study, in humans, the DNA methylation profile of the ABCB1 gene was analyzed via bisulphite sequencing in tumor cells ofAML patients before chemotherapy. Results indicated that the DNA methylation status was different among patients in a subset of CpG sites of the CpG island and agreed with the results obtained from the present study.

In the real-time MSP assay, CpG sites of ABCB1 were not methylated in most of the tumor cells, regardless of sensitivity for chemotherapy, and there was no significant difference in methylation rate between the chemotherapeutic-sensitive and -resistant dogs. It has been reported that ABCB1 is in a hypermethylated state in 66% of human AML patients who achieve complete response to chemotherapy when examined by PCR assay with methylation-sensitive restriction enzymes. Although the methylation rates of the CpG island of ABCB1 in the chemotherapeutic-sensitive dogs were lower, compared with the rates in humans with AML, the reason for the difference was not clear. Another study examined the methylation status of ABCB1 in humans with hematopoietic malignancy, including 5 patients with non-Hodgkin’s lymphoma, but the methylation status of the gene in those non-Hodgkin’s lymphoma patients was not described in detail. Because it is thought that canine lymphoma shares many similarities with human non-Hodgkin’s lymphoma, further studies that compare the DNA methylation status of ABCB1 between human and canine lymphoma cells might allow canine lymphoma to be used as a spontaneous animal model of human non-Hodgkin’s lymphoma.

In this study, there was no significant correlation between the amount of ABCB1 mRNA and methylation rate as determined by use of the real-time MSP assay. We previously reported that DNA methylation in the CpG island regulated the expression of ABCB1 mRNA in canine lymphoma cell lines, and the gene expression of ABCB1 was silenced in cell lines with hypermethylated ABCB1. In the present study, the qRT-PCR assay was conducted by the same method as used in the previous study, and the expression levels of ABCB1 in canine primary lymphoma cells were markedly higher than those in canine cell lines with hypermethylated ABCB1, including CLBL-1, which is a cell line established from dogs with B-cell high-grade multicentric lymphoma. Therefore, it is possible that ABCB1 was in a hypomethylated state and the expression of this gene was not silenced in tumor cells of chemotherapeutic-sensitive dogs. This might be the reason for the lack of correlation between relative quantities of ABCB1 mRNA and methylation rates calculated from the results of real-time MSP assay.

One limitation in the present study was that the number of chemotherapeutic-resistant dogs was small because the resistance for all 3 chemotherapeutic agents could not be confirmed in a certain number of dogs because of the rapid progress of lymphoma. In the future, DNA methylation profiles of ABCB1 should be examined in a larger number of dogs with chemotherapeutic-resistant lymphoma.

As another limitation, the expression level of ABCB1 was not examined in the primary tumor cells obtained from dogs with chemotherapeutic-resistant lymphoma. In addition, the expression and function of P-gp were not examined in all 27 dogs. It was unclear whether overexpression of ABCB1 and P-gp occurred and what mechanisms induced the MDR phenotype.

The diagnosis of canine lymphoma in dogs examined for the DNA methylation profile of the CpG island of ABCB1 was made by use of cytologic but not by histologic examination in the present study. This can be considered as another limitation because recent studies have indicated the importance of subclassification of canine lymphoma according to the histopathologic classification (based on the WHO classification proposed in 2002). However, all of the dogs had centroblastic polymorphic–type lymphoma categorized into high-grade malignancy in the updated Kiel classification and it is reported that most dogs with this type of tumor have diffuse large B-cell lymphoma according to the modified WHO classification. Therefore, it is possible that lymphoma in the dogs of the present study could be assigned to diffuse large B-cell lymphoma (high-grade and inter-
mediate-grade) according to the recently reported WHO classification-based subclassification of canine lymphoma. Although it is not practical to perform resection biopsy for all dogs that are expected to die of lymphoma, diagnosis and subtype classification based on the histopathologic findings would be fundamentally warranted for developing more effective therapeutic strategies and prognostic stratification.

Finally, it was an important limitation in the present study that the proportion of tumor cells in each FNA sample was not quantified. Although the cytologic examination was conducted in each FNA sample and it was confirmed that almost all the cells in the samples were tumor cells, the results of bisulfite sequencing, real-time MSP assay, and qRT-PCR assay could be confounded by the presence of normal cells in the samples.

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

a. QiAmp DNA Blood Mini Kit, Qiagen Inc, Valencia, Calif.
b. MethylEasy Aced Rapid DNA Bisulphite Modification Kit, Takara Bio Inc, Shiga, Japan.
c. Ampli Taq Gold DNA polymerase, Applied Biosystems, Darmstadt, Germany.
e. Nucleo Spin Plasmid Quick Pure, MACHEREY-NAGEL, Duren, Germany.
g. Applied Biosystems 3130xl genetic analyzer, Applied Biosystems, Darmstadt, Germany.
i. Illustra GenomiPhi V2 DNA Amplification Kit, GE Healthcare, Princeton, NJ.
j. Epicope MSP Kit, Takara Bio Inc, Shiga, Japan.
k. Thermal Cycler Dice Real Time System TP800, Takara Bio Inc, Shiga, Japan.
l. RNAqueous, Ambion Diagnostics Inc, Austin, Tex.
m. DNase I, Invitrogen Corp, Carlsbad, Calif.
i. PrimeScript RT reagent Kit (Perfect Real Time), Takara Bio Inc, Shiga, Japan.
o. SYBR Premix Ex Taq II (Perfect Real Time), Takara Bio Inc, Shiga, Japan.
p. JMP version 5.0.1, SAS Institute Inc, Cary, NC.

References


### Appendix

Primers used for bisulfite sequencing, real-time MSP assay, and qRT-PCR assay in a study of dogs (*n* = 27) with lymphoma.

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<th>Assay number</th>
<th>Forward</th>
<th>Reverse</th>
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<th>Genbank accession No.</th>
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<td>5'-AGGAGGATATTTTTTGGA-3' (13,742,947 to 13,742,966)</td>
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<td>Methylated primer pair for real-time MSP in upstream region</td>
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<td>5'-CCTACGTCCCGAAAAATAAA-3' (13,742,806 to 13,742,825)</td>
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<td>Unmethylated primer pair for real-time MSP in upstream region</td>
<td>5'-TTGTAATTAGTATTTGGGTGAGT-3' (13,742,670 to 13,742,688)</td>
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<td>Methylated primer pair for real-time MSP in downstream region</td>
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<td>Unmethylated primer pair for real-time MSP in downstream region</td>
<td>5'-ACTCGGAGACAAAGTTTTA-3' (2,734 to 2,753)</td>
<td>5'-AATGAGACCCCCCAAAGATGTG-3' (2,809 to 2,828)</td>
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<td>Primer pair for ABCB1 gene in qRT-PCR</td>
<td>5'-CTATTTCTTCTTGTATAGGGGAG-3' (~145 to –124)</td>
<td>5'-CCTGGCATTCAAGTCTTTTC-3' (~31 to –30)</td>
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*Numbers in parenthesis indicate the nucleotide numbers registered in GenBank.*