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The Regulation of the Expression of *ABCG2* Gene through Mitogen-Activated Protein Kinase Pathways in Canine Lymphoid Tumor Cell Lines

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ABSTRACT. Treatments for canine lymphoma often fail, because tumor cells acquire multidrug resistance (MDR). MDR can develop through several mechanisms, among which the overexpression of drug transporters in tumor cells is a well-studied mechanism. ATP-binding cassette sub-family G member 2 (ABCG2) belongs to the ABC-transporters, that are representative drug efflux pumps associated with MDR in human tumor cells. However, the regulation of *ABCG2* gene expression in canine tumors is not well understood. The purpose of the present study was to reveal the regulatory mechanism of *ABCG2* gene expression in 4 canine lymphoid tumor cell lines, GL-1, CLBL-1, UL-1 and Ema. Treatment with phorbol 12-myristate 13-acetate (PMA), the protein kinase C (PKC) activator, stimulated MAPK/ERK pathway in GL-1, UL-1 and Ema cells and JNK pathway in UL-1 and Ema cells. When GL-1 and UL-1 cells were treated with PMA and the MAPK/ERK kinase inhibitor U0126, *ABCG2* gene expression levels were elevated above those in untreated cells. Similarly, *ABCG2* gene expression increased above control levels in UL-1 and Ema cells treated with PMA and the JNK inhibitor SP600125. However, *ABCG2* gene expression was unaffected by U0126 exposure in CLBL-1 cells, in which activation of MAPK/ERK pathway was observed in non-treated cells. These results suggested that MAPK/ERK and JNK pathways downregulate *ABCG2* gene expression, which is upregulated by unidentified but possibly PKC-dependent pathways, in several types of canine lymphoid tumor cells.

KEY WORDS: ALL, BCRP, lymphoma, MAPK, multidrug resistance.

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The most important causes of failure in the chemotherapy for dogs with lymphoma include the acquisition of multidrug resistance (MDR) in tumor cells. Lots of mechanisms underlying the development of MDR have been studied in human and veterinary medicine [1, 4, 9]. Among these mechanisms, the overexpression of drug efflux pumps is one of the most studied ones. The overexpression of drug efflux pumps is known to induce the reduction of intracellular concentrations of chemotherapeutic agents in tumor cells [4].

Some ATP-binding cassette (ABC)-transporters have been shown to be associated with the acquisition of MDR phenotype [5]. Among ABC-transporters, *ABCB1*, *ABCC1* and *ABCG2* genes encode representative ABC-transporters associated with MDR, and the overexpression of these molecules was found in various tumor cells with MDR phenotype [11]. Association of the expression of *ABCB1* gene encoding P-glycoprotein (P-gp) with MDR has been well studied in veterinary medicine as well as human medicine. The expression of P-gp was shown to be enhanced in tumor cells after relapse or acquisition of MDR in canine lymphoma [2, 10, 12]. We previously reported that the expression level of *ABCB1* gene was high in some canine patients with drug-resistant lymphoma compared with dogs with drug-sensitive lymphoma [20]. However, the overexpression of *ABCB1* gene or P-gp was not always observed in dogs with MDR lymphoma, and the mechanisms by which tumor cells acquire MDR are thought to vary. Therefore, investigating the expression of other transporters can help elucidate the mechanisms of the acquisition of MDR in canine lymphoma.

Expression of ABCG2 is known to be one of the causes of the acquisition of MDR in various human tumor cells [14]. As to lymphoid tumors, the possible correlation of expression of ABCG2 with shorter disease-free survival was observed in human acute lymphoblastic leukemia [19], and the expression of ABCG2 was observed in 78% of human mature T/NK cell lymphoma cases [17]. Meanwhile, information of ABCG2 associated with MDR is scant in veterinary medicine, except for 2 reports on the expression of ABCG2 in canine mammary tumors [7, 15]. We previously showed that the relative quantity of *ABCG2* mRNA was not significantly different between chemotherapy-sensitive and chemotherapy-resistant lymphoma patients [20]. Therefore, the clinical significance of the overexpression of *ABCG2* gene or ABCG2 in MDR phenotype in tumor cells has been unclear in dogs.

Although the association of the expression of ABCG2 with MDR has been revealed in human medicine, regulation of the expression of ABCG2 has not been well understood. Recent studies in human breast and colon cancer revealed that the expression of *ABCG2* gene was downregulated through mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway [8] and upregulated through c-Jun NH_2-terminal kinase (JNK) pathway

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[25]. In veterinary medicine, we previously revealed that the expression of *ABCB1* gene was regulated through MAPK/ERK pathway, which might contribute to elucidate the mechanisms that induce overexpression of *ABCB1* gene in canine lymphoma cells [21], but the associations of MAPK/ERK and JNK pathways with the regulation of *ABCG2* gene have not been examined in canine tumor cells.

The purpose of the present study was to investigate the *ABCG2* gene expression and its regulatory mechanisms in canine lymphoid tumor cell lines to elucidate the mechanisms that induce overexpression of *ABCG2* gene in canine lymphoid tumor cells.

MATERIALS AND METHODS

Cell cultures: In the present study, 4 canine lymphoid tumor cell lines were used: GL-1, a canine B-cell leukemia cell line [13]; CLBL-1, a canine B-cell lymphoma cell line [16]; UL-1, a canine T-cell lymphoma cell line [22]; and Ema, a canine T-cell lymphoma cell line [6]. All cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% fetal calf serum (Biowest, Nuaillé, France) and penicillin-streptomycin solution (Nacalai tesque, Kyoto, Japan) and cultured at 37°C in a humidified atmosphere of 5% CO_2.

Relative quantification of ABCG2 mRNA: The expression level of *ABCG2* gene was relatively quantified using real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) as reported in the previous study [20]. For normalization of the amount of cDNA sample, *TATA box binding protein* (*TBP*) gene was used as an internal reference based on the previous study [20]. Total RNA was extracted from cultured cell lines using RNAqueous (Ambion, Austin, TX, U.S.A.) and treated with DNase I (Invitrogen). To synthesize cDNA, total RNA was reverse transcribed using a 1 μg of total RNA and PrimeScript^{TM} RT reagent Kit (Perfect Real Time) (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. Real-time PCR for quantification of mRNA for *ABCG2* and *TBP* genes was performed using twenty-five microliters of the PCR mixture that contained 12.5 μl of Master Mix (SYBR Premix Ex Taq [Perfect Real Time)] (Takara Bio), 200 nM of sense and reverse primers and 50 *n*g of cDNA according to manufacturer's instructions. After PCR reaction, samples were run using the dissociation protocol to verify the occurrence of a single melting peak. Amplification of products was confirmed by electrophoresis. The results are expressed as the quantification cycle (C_q), that is, the cycle number at which increasing reporter fluorescence crossed a fixed threshold baseline. For *ABCG2* and *TBP* genes, assay-specific standard curves with serial (10×) dilution of UL-1 cells were prepared. In these standard curves, the relative quantity of cDNA for *ABCG2* and *TBP* genes which was included in 50 *n*g of this cDNA sample was defined 10^5. These standard curves were examined in triplicate. The relative quantities of *ABCG2* and *TBP* genes were calculated by plotting C_q in the prepared standard curves. To normalize the amount of cDNA sample, the ratio of the relative quantity of cDNA for *ABCG2* gene to that of *TBP* gene was adopted. Real-time RT-PCR assays were performed in triplicates.

Western blot for MAPK/ERK and JNK pathways: Western blot analysis was carried out to examine the activation of MAPK/ERK and JNK pathways in the 4 canine lymphoid tumor cell lines as described in a previous study [21]. In brief, whole cell lysates were extracted from each cell line using RIPA buffer (Cell Signaling Technology, Danvers, MA, U.S.A.), and the 10 μg of extracted proteins were separated by SDS-PAGE. After separated proteins were blotted on a PVDF membrane (Millipore, Billerica, MA, U.S.A.) by wet transfer method using tris (Wako, Osaka, Japan)-glycine (Wako) buffer supplemented with 20% methanol (Wako), the membrane was blocked using 5%-skimmed milk (Wako)/tris-buffered saline with Tween20 (MP Biomedicals, Solon, OH, U.S.A.) and then incubated overnight at 4°C with primary antibodies against phospho-ERK1/2 (1:2,000), ERK1/2 (1:1,000), phospho-JNK (1:1,000), JNK (1:1,000), phospho-c-jun (1:1,000), c-jun (1:1,000) and β-actin (1:1,000) (Cell Signaling Technology) as indicated in the previous studies [18, 21]. The membrane was washed in tris-buffered saline with Tween20 (TBS-T) at 3 times using shaker, and it was incubated with a secondary antibody of horseradish peroxidase-labeled anti-rabbit IgG (1:3,000; Bio-rad Laboratories, Hercules, CA, U.S.A.) for 1 hr at room temperature. After the membrane was washed in TBS-T as described above, positive immunoreactivity was detected using Luminata Forte Western HRP Substrate (Millipore) and visualized using ChemiDoc XRS Plus (Bio-rad Laboratories).

Changes of expression levels of ABCG2 gene by activation or inhibition of MAPK/ERK and JNK pathways: For activation of MAPK/ERK and JNK pathways and their inhibition, phorbol 12-myristate 13-acetate [(PMA), a protein kinase C (PKC) activator, (Wako)], U0126 [MAPK/ERK kinase (MEK) inhibitor, (Promega, Leiden, Netherlands)] and SP600125 [JNK inhibitor, (Wako)] were used as previously described [23–25]. In this experiment, 4 cell groups were prepared using each of 4 cell lines; untreated, PMA treated, PMA and U0126 treated and PMA and SP600125 treated. PMA treated group was prepared by incubating with 200 nM PMA for 8 hr to activate MAPK/ERK and JNK pathways. As to PMA and U0126 treated group or PMA and SP600125 treated group, cells were incubated with 10 μM U0126 for 1 hr or 50 μM SP600125 for 40 min before treatment with PMA, respectively. The relative quantities of *ABCG2* gene mRNA of these 3 treated cell groups were compared with untreated cells using real-time RT-PCR as described above, and the fold-changes in relative quantity of *ABCG2* gene mRNA were calculated. For confirmation of the activation and inhibition of MAPK/ERK and JNK pathways, the 4 cell lines and those pre-incubated with U0126 or SP600125 as described above were incubated with 200 nM PMA for 30 min, and their whole cell lysates were subjected to Western blot as described above.

Statistical analysis: The nonparametric Mann-Whitney *U*-test was used for comparison of relative quantities of genes. All statistical tests were two-sided and performed using JMP

version 5.0.1 (SAS Institute, Cary, NC, U.S.A.). For all tests, $P<0.05$ was used as the level of significance.

RESULTS

Comparison of relative quantities of ABCG2 mRNA: In real-time RT-PCR, the expression of *ABCG2* gene was detected in all 4 cell lines (Fig. 1). The relative quantity of mRNA of this gene was the highest in GL-1, followed by UL-1, CLBL-1 and Ema showing less mRNA (relative quantities; 0.54, 0.15, 0.0057 and 0.0020, respectively).

Comparison of intrinsic activation of MAPK/ERK and JNK pathways: In Western blot analysis for MAPK/ERK pathway, the quantities of phospho-ERK were larger in CLBL-1 and Ema compared with those of GL-1 and UL-1 (Fig. 2). As to JNK pathway, the quantities of phospho-JNK and phospho-c-jun were larger in UL-1 compared with those of GL-1, CLBL-1 and Ema. There was no visible band of c-jun in CLBL-1, and there was no visible band of p-c-jun in GL-1, CLBL-1 and Ema.

Activation or inhibition of MAPK/ERK and JNK pathways by PMA, U0126 and SP600125: In GL-1, UL-1 and Ema, the quantity of phospho-ERK1/2 apparently increased by treatment with PMA, and this increase was inhibited by the treatment with a MEK inhibitor, U0126 (Fig. 3). The extent of the decrease of the quantity of phospho-ERK1/2 was smaller in UL-1 compared with those of GL-1 and Ema. However, the quantity of phospho-ERK1/2 was already high before treatment and it did not change after treatment with PMA in CLBL-1, although its quantity slightly decreased after treatment with the combination of PMA and U0126. Therefore, this cell line was next treated with U0126 alone, and the quantity of phospho-ERK1/2 was compared with that of non-treated cells. As a result, the quantity of phospho-ERK1/2 was markedly decreased by treatment with U0126 alone. As to JNK pathway, the quantity of p-JNK increased after treatment with PMA in CLBL-1, UL-1 and Ema, although there was no obvious change in the quantity of p-JNK after treatment with PMA in GL-1. In addition, the quantity of p-JNK decreased after treatment with PMA and SP600125 compared with after treatment with PMA alone in all of the 4 cell lines. However, these changes in the quantity of p-JNK were relatively small. The phosphorylation status of c-jun, which is the target molecule of JNK, did not change by the treatment with PMA or SP600125 in GL-1 and CLBL-1, and no visible band could be detected in these cell lines (Fig. 4). However, the quantity of phospho-c-jun markedly increased after treatment with PMA in UL-1 and Ema, and this increase was inhibited by treatment with the combination of PMA and SP600125 in both cell lines, indicating that the activation of JNK pathway and its inhibition successfully occurred in these cell lines.

Change of the relative quantity of ABCG2 mRNA by treatment with PMA, U0126 and SP600125: We examined whether the expression level of *ABCG2* gene was influenced when MAPK/ERK and JNK pathways were activated or inhibited by treatment with PMA, U0126 or SP600125 in each cell line. When GL-1 was treated with PMA, the relative quantity of *ABCG2* gene increased (1.9-fold) compared with that of non-treated cells, and relative quantity of this gene was more largely increased (2.8-fold) after treatment with the combination of PMA and U0126 (Fig. 5A). In CLBL-1, the expression level of *ABCG2* gene did not significantly change after treatment with U0126 (Fig. 5B). In UL-1, although the relative quantity of *ABCG2* mRNA did not significantly change after treatment with PMA, it significantly increased after treatment with the combination of PMA and U0126 (2.2-fold) or the combination of PMA and

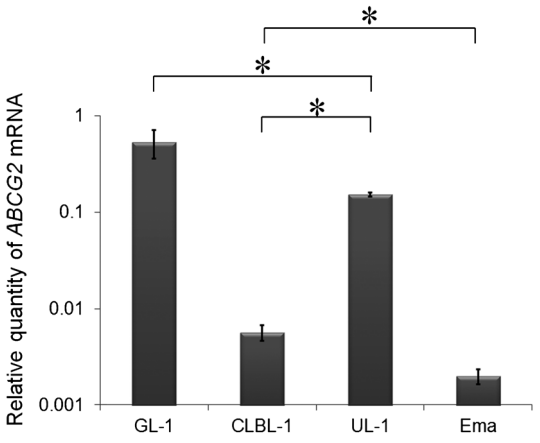

Fig. 1. The relative quantities of *ABCG2* gene mRNA calculated by using *TBP* gene as housekeeping gene in GL-1, CLBL-1, UL-1 and Ema. The graphs showed mean ± SD values. *$P<0.01$.

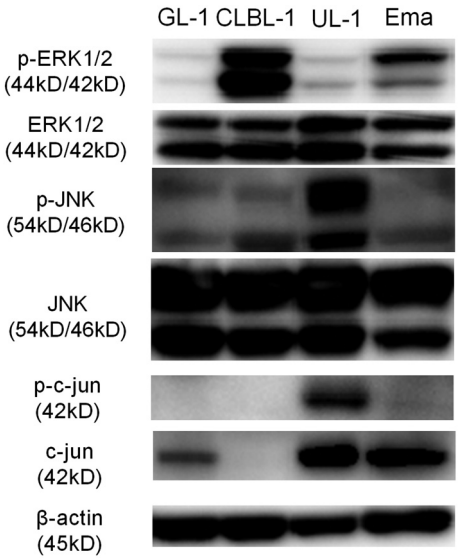

Fig. 2. The results of Western blot for phospho-ERK, ERK, phospho-JNK, JNK, phospho-c-jun, c-jun and β-actin in GL-1, CLBL-1, UL-1 and Ema. The figure shows representative results of 3 independent experiments.

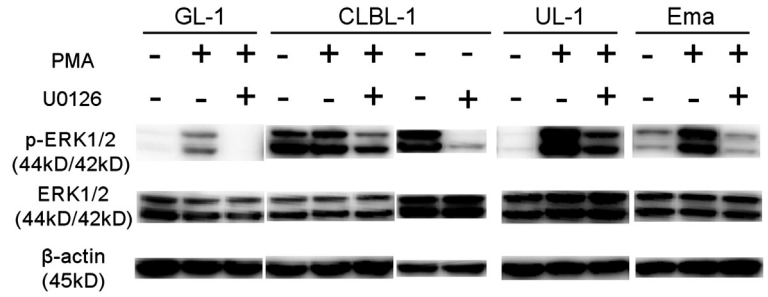

Fig. 3. The results of Western blot for phospho-ERK, ERK and β-actin after treatment with PMA or the combination of PMA and U0126 in GL-1, CLBL-1, UL-1 and Ema. CLBL-1 cells were also treated with U0126 alone and the quantity of phospho-ERK1/2 was compared with that of non-treated cells, because the quantity of phospho-ERK1/2 was already high before treatment and it did not change after treatment with PMA in this cell line. The figure shows representative results of 3 independent experiments.

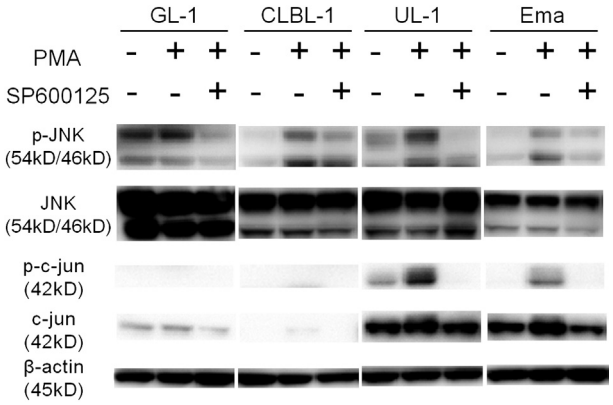

Fig. 4. The results of Western blot for phospho-JNK, JNK, phospho-c-jun, c-jun and β-actin after treatment with PMA or the combination of PMA and SP600125 in GL-1, CLBL-1, UL-1 and Ema. The figure shows representative results of 3 independent experiments.

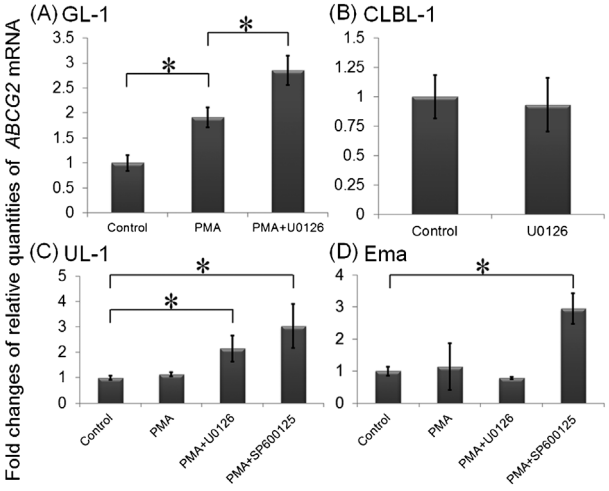

Fig. 5. The fold-changes of relative quantities of *ABCG2* gene in GL-1 (A) CLBL-1 (B), UL-1 (C) and Ema (D) after treatment with PMA, U0126 or SP60012. The graphs show mean ± SD values. **P*<0.01.

SP600125 (3.0-fold) compared with non-treated cells (Fig. 5C). In Ema, the expression level of *ABCG2* gene did not significantly change after treatment with PMA or the combination of PMA and U0126, but it significantly increased after treatment with the combination of PMA and SP600125 (3.0-fold) compared with non-treated cells (Fig. 5D).

In summary, the heterogeneities in intrinsic activation of MAPK/ERK and JNK pathways and the effect of treatment with PMA, U0126 and SP600125 among cell lines were observed. In GL-1, the intrinsic activation statuses of MAPK/ERK and JNK pathways were relatively low, and PMA stimulation and the inhibition of MAPK/ERK pathway significantly increased the *ABCG2* gene expression. In CLBL-1, the intrinsic activation status of MAPK /ERK pathway was relatively high, but that of JNK pathway was relatively low. The amounts of *ABCG2* mRNA did not change after inhibition of MAPK/ERK pathway in this cell line. In UL-1, MAPK /ERK pathway was not intrinsically activated, but the intrinsic activation of JNK pathway was observed. The amount of *ABCG2* mRNA increased after PMA stimulation and inhibition of MAPK/ERK or JNK pathways. In Ema, MAPK/ERK pathway was intrinsically activated, but JNK pathway was not. The amount of *ABCG2* mRNA increased after PMA stimulation and inhibition of JNK pathway, but it did not increase after PMA stimulation and inhibition of MAPK/ERK pathway.

DISCUSSION

The present study reveals that the expression of *ABCG2* gene is downregulated through MAPK/ERK and JNK pathways in several canine lymphoid tumor cell lines.

The expression of *ABCG2* gene was observed in all 4

cell lines used in the present study, GL-1, CLBL-1, UL-1 and Ema. Among these 4 cell lines, the relative quantity of *ABCG2* mRNA was larger in GL-1 and UL-1 compared to lower *ABCG2* mRNA levels in CLBL-1 and Ema cell lines.

The MAPK/ERK pathway was more activated in CLBL-1 and Ema compared with GL-1 and UL-1. In GL-1, where the MAPK/ERK pathway was activated by PMA and this activation was inhibited by treatment with PMA and U0126, the relative quantity of *ABCG2* gene was increased after treatment with PMA compared with that of non-treated cells, and relative quantity of this gene markedly increased after treatment with the combination of PMA and U0126. In UL-1, where the MAPK/ERK pathway was activated by PMA and this activation was inhibited by treatment with PMA and U0126, the expression level of *ABCG2* gene significantly increased after treatment with the combination of PMA and U0126 compared with non-treated cells, although it did not significantly change after treatment with PMA. These results indicate that MAPK/ERK pathway downregulates the expression of *ABCG2* gene and unidentified pathways that are activated by treatment with PMA might upregulate the expression of this gene in these cell lines. As to the difference in the effect of treatment with PMA alone between GL-1 and UL-1, it is possible that it might be due to the difference in the balance of activation of MAPK/ERK pathway and unidentified pathways that upregulated the *ABCG2* gene expression. A previous study [8] showed that *ABCG2* gene expression was downregulated through MAPK/ERK pathway in human breast cancer cell lines, and the findings in the present study agreed with those in this previous study [8]. In CLBL-1, the intrinsic activation of MAPK/ERK pathway was observed, but the treatment with U0126 successfully inhibited the activation of this pathway. In Ema, the MAPK/ERK pathway was activated by PMA, and this activation was inhibited by treatment with PMA and U0126. However, MAPK/ERK pathway was not associated with the regulation of *ABCG2* gene expression in CLBL-1 and Ema, where the expression level of *ABCG2* gene did not change after treatment with U0126 or the combination of PMA and U0126. The causes of this difference in role of MAPK/ERK pathway in regulation of *ABCG2* gene are unclear. However, one possibility is that MAPK/ERK pathway might downregulate the expression of *ABCG2* gene only in tumor cells with high expression of this gene.

The intrinsic activation of JNK pathway was observed only in UL-1, where the JNK pathway was activated by PMA and this activation was inhibited by treatment with PMA and SP600125, and the expression level of *ABCG2* gene was significantly increased after treatment with the combination of PMA and SP600125 compared with non-treated cells in this cell line. Also, in Ema, where the JNK pathway was activated by PMA and this activation was inhibited by treatment with PMA and SP600125, the expression level of *ABCG2* gene significantly increased after treatment with the combination of PMA and SP600125 compared with non-treated cells. These results indicate that JNK pathway downregulates the expression of *ABCG2* gene and unidentified pathways that are activated by treatment with PMA might upregulate the expression of this gene in these cell lines. It was previously reported that JNK1 signaling upregulated the expression of *ABCG2* gene in human medicine [25]. Reasons for this difference are unclear, but the difference of tumor cells might be one reason, because colon cancer cells were utilized in a previous study and the present study utilized lymphoid tumor cells. In GL-1 and CLBL-1, the activation of JNK pathway was not observed despite treatment with PMA. These results indicate that JNK pathway might not be responsive to PMA exposure in these cell lines. Among cell lines utilized in the present study, GL-1 and CLBL-1 were B-cell lineage, and UL-1 and Ema were T-cell lineage. Although it was reported that JNK pathway was activated through heat stress in human B-cell lymphoma cell line [3], it is possible that JNK pathway is not responsive in certain type of canine tumor cell lines. Further studies should be conducted to examine this possibility using canine lymphoma cells and normal lymphocytes.

In conclusion, the present study revealed the downregulation of *ABCG2* gene expression through MAPK/ERK and JNK pathways in certain type of canine lymphoid tumor cells. In the present study, the heterogeneity in intrinsic activation of MAPK/ERK and JNK pathways and the various activation patterns of these pathways by treatment with PMA, U0126 and SP600125 among cell lines were observed. In addition, these 2 pathways downregulated *ABCG2* gene expression in some cell lines, but not in other cell lines. Therefore, further studies are needed to identify the factors that determine the differences in intrinsic activation of MAPK/ERK and JNK pathways and the regulation of *ABCG2* gene expression by these 2 pathways. Although the association of the overexpression of ABCG2 with acquisition of MDR has been shown in human medicine, the clinical significance of the expression of ABCG2 in canine tumor cells is unclear. In the future, changes in sensitivity for drugs by induction of the expression of ABCG2 in cell lines or the comparison of the expression of ABCG2 between drug-sensitive and drug-resistant dogs with lymphoma should be examined to demonstrate more precisely the association of the expression of ABCG2 with the acquisition of MDR in canine tumor cells.

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