

Expression of the *MDR1* Gene and P-Glycoprotein in Canine Mast Cell Tumor Cell Lines

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ABSTRACT. Cellular drug resistance to antineoplastic drugs is often due to the presence of a drug efflux pump that reduces intracellular drug accumulation and chemosensitivity. P-glycoprotein (P-gp), which is encoded by the *MDR1* gene, is considered to function as an ATP-driven membrane drug efflux pump and appears to play an important role in tumor cell resistance. In the present report, we assessed the expression of *MDR1* by RT-PCR in three canine mast cell tumor cell lines, TiMC, CoMS and LuMC, originating from a cutaneous tumor, an oral-mucosal tumor and a gastrointestinal tumor, respectively. P-gp expression was also examined by Western blot analysis, while the functional activity of P-gp was assessed by flowcytometric analysis of intracellular rhodamine-123 (Rhd-123) uptake. The results revealed that *MDR1* gene and P-gp were both expressed in CoMS and LuMC cells, whereas neither was present in TiMC cells. In CoMS and LuMC cells, intracellular uptake of Rhd-123 increased in the presence of verapamil, a functional modulator of P-gp. In contrast, TiMC cells did not show any changes in the intracellular accumulation of Rhd-123 after the verapamil addition. These findings suggest that the expressions of *MDR1* gene and P-gp probably contribute to cellular drug resistance in canine mast cell tumors.

KEY WORDS: canine mast cell tumor, cellular chemosensitivity, *MDR1*, P-glycoprotein.

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Mast cell tumors (MCTs) represent one of the most common cutaneous tumors found in dogs, accounting for up to 15% of all cutaneous tumors [3, 19]. Surgical excision, radiotherapy, chemotherapy or a combination of these modalities have been currently performed for canine MCTs. Among these treatment modalities, chemotherapy has played an important role, especially for presentation of local recurrence or metastases to other distant organs [24, 27].

One of the most important factors influencing the efficacy of chemotherapy has been believed to be cellular multidrug resistance. Reduced drug accumulation via functional proteins induced on the plasma membrane is generally recognized as a mechanism of multidrug resistance [1, 8]. One such protein with a molecular weight of 170-195 kD, designated P-glycoprotein (P-gp), can act as a drug efflux pump in an energy-dependent manner [6, 14, 25, 26]. Tumor cells overexpressing P-gp may survive exposure to antineoplastic drugs by decreasing the intracellular accumulation of these drugs to below their effective level via the P-gp drug efflux pump [4, 12, 17].

In veterinary medicine, multidrug resistance has been investigated especially in hematopoietic tumors such as leukemia and lymphoma, and the results have indicated that P-gp and its encoding gene of *MDR1* may be involved in multidrug resistance as reported in human medicine [2, 11, 14, 26]. In contrast to hematopoietic tumors, few reports have described multidrug resistance in canine MCTs. It is possible that MCT patients may exhibit chemotherapy-induced

acquired resistance to antineoplastic drugs by overexpressing of P-gp, similar to canine lymphoma cases.

In this paper, we examined the expression of *MDR1* gene in three MCT cell lines derived from spontaneous canine MCTs. The expression of P-gp encoded by *MDR1* was also assessed by Western blot analysis. Finally, the functional activity of P-gp as a drug efflux pump was investigated by flowcytometric analysis of rhodamine-123 efflux. The present study is the first report to investigate MDR development in canine MCTs.

MATERIALS AND METHODS

Cell lines: TiMC, CoMS and LuMC canine MCT cell lines were originated from a cutaneous MCT, an oral-mucosal MCT and a gastrointestinal MCT, respectively [10, 23]. CoMS cell line was kindly provided by Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University. The doxorubicin-resistant GL-DOX4000 cell line was used in this study as a P-gp-positive cell [15, 26].

TiMC cells were cultured in RPMI 1640 medium (Gibco BRL, MO, U.S.A.) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 10 U/ml penicillin G, 10 µg/ml streptomycin sulfate, 50 µM 2-mercaptoethanol and 0.2% sodium bicarbonate. CoMS and LuMC cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/ml gentamicin, 1.5 µg/l amphot-

ericin B and 0.2% sodium bicarbonate. GL-DOX4000 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10 U/ml penicillin G and 10 μ g/ml streptomycin sulfate. All the cells were stably maintained at 37°C in a humidified atmosphere of 5% CO₂.

Detection of *MDR1* expression: *MDR1* expression was examined in all three canine MCT cell lines by RT-PCR. Total RNA was extracted from the cultured cells using a QIAamp® RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription (RT) of the RNA was performed with a Sensi-script® RT Kit (Qiagen) using an oligo-(dT)₁₂₋₁₈ primer (Invitrogen, CA, U.S.A.), according to the manufacturer's instructions.

The cDNA products of the RT reaction were amplified with Takara Ex® Taq (hot start version; Takara, Shiga, Japan) using primers 1F (5'-CCCATCATTGCAATAG-CAGG-3'; corresponding to nucleotides 2596-2615) and 1R (5'-GTTCAAACCTTCTGCTCCTGA-3'; corresponding to nucleotides 2733-2752) to produce a 156 bp product extending from exons 19 to 22 according to the human *MDR1* sequence [16, 21]. Initial denaturation was carried out at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, and a final extension at 72°C for 10 min. The PCR products were then mixed with loading buffer (Wako, Osaka, Japan) and separated by electrophoresis in Tris-borate-EDTA 1.5% agarose gels. The gels were stained with SYBER® Green I Nucleic Acid Gel Stain (Takara Shuzo, Tokyo, Japan) and photographed under UV illumination using a CCD camera. To verify that the PCR products were *MDR1*, they were extracted from the Tris-borate-EDTA 1.5% agarose gels using a QIA Quick® Gel Extraction Kit (Qiagen), purified and sequenced using a Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, U.S.A.). The sequence data obtained were analyzed for their homology with the *MDR1* gene sequence in the database (DDBJ Accession No.: *MDR1*, AY582533) using the computer program CLUSTAL W version 1.8 (DNA Data Bank of Japan (DDBJ), Shizuoka, Japan).

Detection of *P-gp* expression: *P-gp* expression was investigated in all three MCT cell lines by Western blotting analysis using a murine anti-human *P-gp* monoclonal antibody (C219; DAKO, CA, U.S.A.). Briefly, the cultured cells were washed twice with phosphate-buffered saline (PBS) and then centrifuged into pellets. The cell pellets were homogenized in NP-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% SDS, 2 μ g/ml aprotinin and 2 mg/ml leupeptin), sonicated for 5 min, boiled for 5 min and centrifuged at 160 g at 4°C for 10 min. The soluble proteins in the supernatants were diluted with NP-40 lysis buffer to adjust the samples to equal concentrations and then mixed with gel-loading sample buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue and 0.2% glycerol) at 1:1. The samples were boiled at 100°C for 5 min and then electrophoresed in an 8% SDS-polyacrylamide gel. Following transfer of the separated proteins to a polyvinylidene difluoride membrane

using a semi-dry electroblotter, the membrane was blocked with 5% skimmed milk powder in TBST (0.2% Tween 20, 10 mM Tris-HCl pH 7.4 and 150 mM NaCl) for 1 hr, washed 3 times with TBST and incubated with the primary antibody C219 (1:50) overnight at 4°C. Next, the membrane was washed 5 times with TBST and incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (1:1,000; Invitrogen) at room temperature for 1 hr. Following 5 washes in TBST, the membrane was developed by incubation in Western Lightning® Chemiluminescence Reagent PLUS (Perkin Elmer, MA, U.S.A.), in which HRP catalyzes light emission from the oxidation of luminol. The resultant bands on the membrane were photographed with a CCD camera. Finally, the membrane was stained with Coomassie Brilliant Blue (CBB) solution (0.1% CBB R-250, 45% ethanol and 10% acetic acid), a nonspecific protein dye, to verify the presence of equal amounts of protein in each lane.

Flowcytometric analyses of *P-gp* function: *P-gp* function was investigated in all three MCT cell lines. For detection of *P-gp* function as a transporter, Rhd-123 (Sigma, Taufkirchen, Germany) was used as a fluorescent [9, 18, 22]. Verapamil hydrochloride (Sigma) was also used in this study to reverse *P-gp*-mediated drug resistance. Briefly, the cells were incubated in RPMI 1640 medium containing 1.0 μ g/l Rhd-123 at 37°C in a humidified atmosphere of 5% CO₂ for 40 min to allow uptake. The cells were then centrifuged into pellets, washed twice with PBS and resuspended in fresh medium with or without verapamil hydrochloride (20 mg/l) at 37°C in a humidified atmosphere of 5% CO₂ for 45 min. The cells were then centrifuged into pellets, washed twice with PBS, resuspended in PBS and fixed with 2% (w/v) paraformaldehyde. Green fluorescence, indicating cellular Rhd-123 accumulation, was analyzed using a flowcytometer (EPICS XL/XL-MCL System II v.3.0; Beckman Coulter, CA, U.S.A.).

RESULTS

***MDR1* expression:** One major band was found at approximately 150 bp by RT-PCR in both CoMS and LuMC cells following Tris-borate-EDTA 1.5% agarose gel electrophoresis (Fig. 1). GL-DOX4000 cells, as a positive control for *MDR1*, also showed one major band in the same region. In contrast, no bands were detected in TiMC cells. The PCR products extracted from CoMS, LuMC and GL-DOX4000 revealed 100% homologies to nucleotides 2629-2735 bp of canine wild-type *MDR1*.

***P-gp* expression:** Figure 2 shows the results of Western blot analysis of a *P-gp* expression in the MCT cell lines. One major protein band was detected by Western blot analysis at approximately 190 kD in LuMC and CoMS cells. This finding was obviously consistent with that in the previous report on canine *P-gp* [26]. On the other hand, a protein band of this molecular weight was not clearly identified in TiMC cells. Stain with CBB solution of membrane proteins after the Western blot analysis confirmed that equal

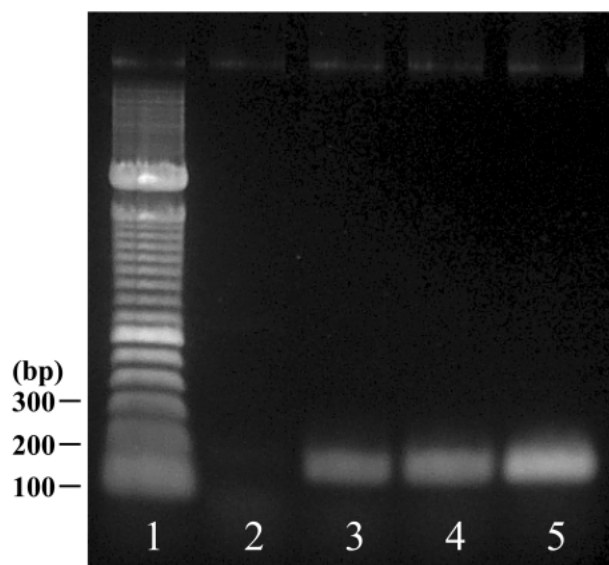


Fig. 1. *MDR1* gene expression in TiMC, CoMS, LuMC and GLDOX-4000 cells evaluated by RT-PCR. Lane 1: Markers; lane 2: TiMC cells; lane 3: CoMS cells; lane 4: LuMC cells; lane 5: GL-DOX4000 cells (positive control).

amounts of protein were loaded in each lane.

P-gp function: Figure 3 shows flowcytometric histograms of P-gp function, i.e. the populations of cells sorted in accordance with their intracellular Rhod-123 accumulation. As shown in this figure, there was almost no difference in the histograms of TiMC cells, regardless of the presence of verapamil. On the other hand, the histograms of CoMS and LuMC cells in the presence of verapamil shifted from low levels of Rhod-123 uptake to high levels of uptake, compared to the histograms in the absence of verapamil.

DISCUSSION

The purposes of the present study were to assess the expressions of *MDR1* gene and P-gp in three canine MCT cell lines, and further to assess the functional activity of the P-gp expressed on the cell lines.

As the results of the PCR study, one major band was clearly detected at the 150 bp region in CoMS and LuMC cells, which was considered as *MDR1*, but not detected in TiMC cells. This band was also detected in GL-DOX4000 cells as a positive control for *MDR1* [26], and the PCR products showed complete homologies to the nucleotides of the parts of canine wild-type *MDR1*. These data strongly suggest that *MDR1* is expressed in CoMS and LuMC cell lines.

Expressions of P-gp on these cell lines were further investigated by Western blot analysis. P-gp is 170–195 kD in molecular weight and reacts with the anti-human P-gp monoclonal antibody C219 [25, 26]. Several studies have been conducted to verify the presence of P-gp in canine lymphoma tissues or cells using C219 [2, 11, 14, 26]. In our experiments using the C219 antibody, one major protein

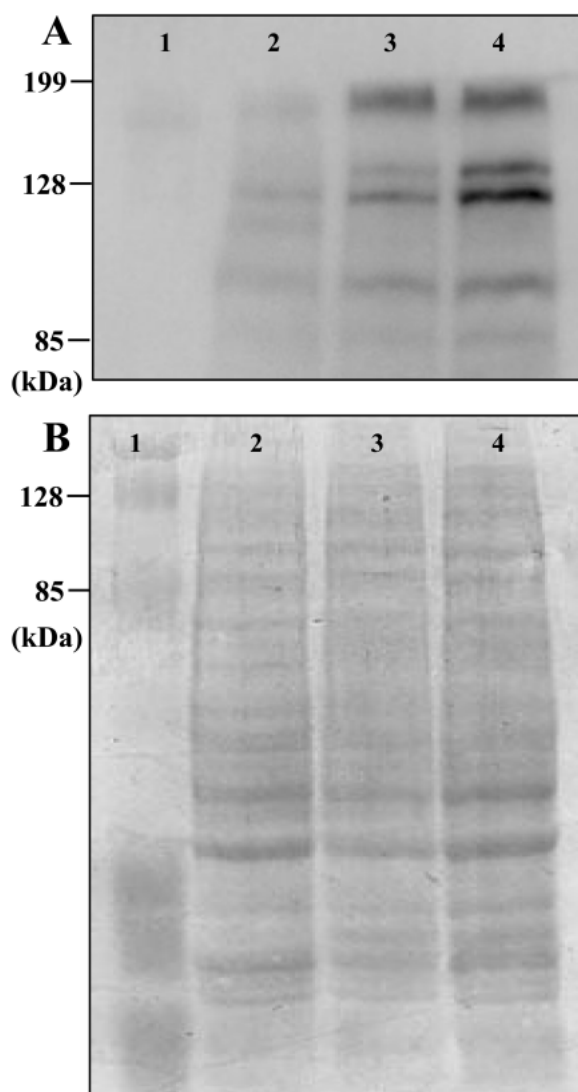


Fig. 2. Western blot analysis with C219 (A) and Coomassie brilliant blue (CBB) staining of the membrane (B). Lane 1: Markers; lane 2: TiMC cells; lane 3: CoMS cells; lane 4: LuMC cells. CoMS and LuMC cells show a major band of about 199 kD in molecular weight, which is not present in TiMC cells (A). The presence of equal amounts of protein in each lane is confirmed by CBB staining (B).

band with a molecular weight of approximately 190 kD was confirmed in both CoMS and LuMC cells. However, this protein band could not be confirmed in TiMC cells, which also did not express *MDR1*. In addition, the differences in the band intensities at approximately 190 kD among the three cell lines were not considered to be caused by differences in the protein amounts applied to the gel, based on the uniformity observed in the amounts of CBB-stained membrane proteins. This protein band is considered to be a rational candidate for canine P-gp. Our data indicate that some canine MCT cells may express *MDR1* constitutively or inductively, and that P-gp has functionally expressed on

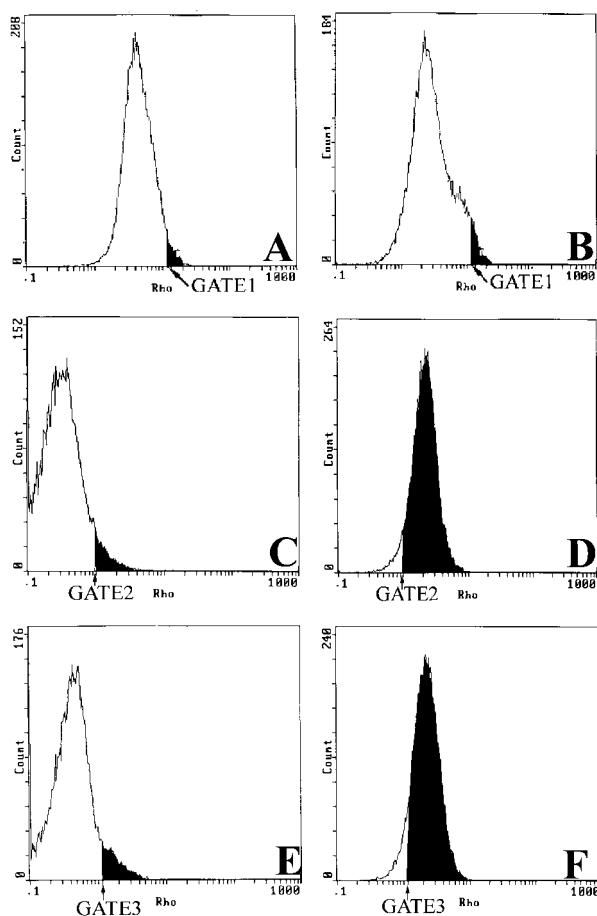


Fig. 3. Flow cytometric detection of P-gp function by rhodamine-123 (Rhd-123) efflux. Flow cytometric histograms of P-gp function in the canine mast cell tumor cell lines TiMC (A, B), CoMS (C, D) and LuMC (E, F) evaluated using the Rhd-123 efflux test in the absence (A, C, E) or presence (B, D, F) of verapamil, a P-gp modulator. Each cell population was gated at the indicated point (GATE1 in A, GATE2 in C and GATE3 in E), over which the cumulative cell count shown as the black area contained 5% of the cells in the absence of verapamil.

some MCT cells as reported in other canine tumors such as lymphomas and leukemias.

In cancerous tissues, the degree of spontaneous P-gp expression is usually highest in epithelial tumors derived from the tissues which have P-gp even in intact tissues, resulting in the potential for resistance to some toxic agents even before chemotherapy is initiated. According to a previous report on *MDR1* mRNA distribution in dogs, *MDR1* mRNA expressed highly in jejunum and colon [5]. In other tumors with negligible P-gp expression at the time of diagnosis, *MDR1* is reported to be induced and then P-gp has expressed after the exposure to antineoplastic agents [7]. The differences in the expressions of *MDR1* gene and P-gp among TiMC, LuMC and CoMS cells may be associated with their original locations to some extent.

Furthermore, *MDR1* expression may be influenced by the

chemotherapy previously given to host animals. Frequency and intensity of the chemotherapy may potentially give influences on *MDR1* expression, however, none of these factors correlated with *MDR1* status of the cell lines investigated in this study [10, 23]. Alternatively, TiMC cells may not show induction of *MDR1* gene expression in response to some factors, similar to the previously reported *MDR1*-deficient genotype [13, 20].

The substrates of P-gp are known to include various structural substances, such as anthracyclines, vinca alkaloids and Rhd-123 [25]. Rhd-123 has been used for imaging and in surrogate marker assays of P-gp function in normal and malignant human cells [9, 18, 22]. The present results of the Rhd-123 efflux tests revealed that P-gp was functionally active as a drug efflux pump in CoMS and LuMC cells, but not in TiMC cells. These results coincided with the results of P-gp assay. Furthermore, the verapamil addition increased the level of Rhd-123 uptake in P-gp-positive MCT cells, possibly because of functional inhibition of P-gp. Therefore, P-gp-positive MCT cells may be able to realize lower intracellular accumulation of Rhd-123, and also some antineoplastic drugs which are included in P-gp substrates, unless some functional inhibitors such as verapamil are present.

The present results suggested that the expression of *MDR1* was observed in some canine MCT cell lines, and functional P-gp proteins might be induced. The new findings reported here, although limited to *in vitro* findings, might indicate that canine MCT cases with *MDR1* gene and P-gp expression may potentially exist, and may be refractory to chemotherapy. We should consider *MDR1* and P-gp involvements in clinical cases of canine MCTs which show refractory behaviors to chemotherapies using some antineoplastic drug such as vinca alkaloids, which have been believed the effective chemotherapeutic agents against canine MCTs. Furthermore, an improper usage of the antineoplastic drugs against canine MCT may have the possibility to induce P-gp expression.

Further detailed investigations of *MDR1* gene and P-gp expressions in canine MCT cases should be conducted. Expressions of the *MDR1* gene and/or the functional P-gp protein in spontaneous canine MCT tissues, and time-sequential changes in these gene and/or protein expressions during chemotherapy may be the issues to be dissolved. These investigations can raise profound issues for experimental and clinical oncology in canine MCTs.

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