Canine mdr1 Gene Mutation in Japan

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ABSTRACT. Frequency of the 4-bp deletion mutant in canine mdr1 gene was examined in 193 dogs of eight breeds in Japan. The mutant allele was found in Collies, Australian Shepherds, and Shetland Sheepdogs, where its respective frequencies were 58.3%, 33.3%, and 1.2%. The MDR1 protein was detected on peripheral blood mononuclear cells (PBMC) from a MDR1/MDR1 dog, but not on PBMC from a mdr1-1A/mdr1–1A Collie. Rhodamine 123 was extruded from MDR1/MDR1 lymphocytes. That excretion was inhibited by a MDR1 inhibitor, verapamil. On the other hand, Rh123 excretion was not observed from lymphocytes derived from a mdr1-1A/mdr1–1A Collie. These results indicated that the mutant mdr1 allele also existed in Collie-breed dogs in Japan at high rates and that mdr1-1A /mdr1–1A dogs have no functional MDR1.

KEY WORDS: canine, ivermectin, MDR1, mutation.

The MDR1 protein, also called P-glycoprotein, is a 170-kDa membrane protein, a member of ATP-binding cassette (ABC) transporters that is encoded by the mdr1 gene [11]. Regarding its role in multidrug resistance of tumor cells, MDR1 has been studied intensively [1, 2, 14, 17]. It is expressed not only in multidrug resistant tumor cells, but also in normal tissues such as the apical membrane of epithelial cells of the intestine and brain capillaries or in peripheral blood cells [3–6, 15, 32, 33]. A major physiological role of MDR1 is thought to be its function as an efflux pump of xenobiotics and cellular metabolites [3, 12].

Numerous single nucleotide polymorphisms (SNP) of mdr1 gene have been reported in humans. Some SNPs are inferred to be linked to its reduced function [8, 13, 22, 31]. In veterinary medicine, one polymorphism, 4-bp deletion mutation, was first reported in association with ivermectin sensitivity in Collies [21]. It has been found in several breeds among the herding group and sighthounds [18, 23]. Deletion generates a premature stop codon, thereby engendering prevention of synthesis of the complete MDR1 protein [21]. Frequency of the mutant mdr1 allele in Collies has been reported as 51–64% in the United States, United Kingdom and France [10, 23]. Ivermectin, a MDR1 substrate, is a widely used parasiticide of the avermectin family. Many empirical cases of high sensitivity to ivermectin and experimental ivermectin-induced neurotoxicity have been reported among Collie-breed dogs [7, 25, 27]; the mdr1 mutation was identified in some of these dogs [24]. It is believed that high sensitivity to ivermectin is at least partially attributable to the deletion of the mdr1 gene.

The MDR1 substrates include various drugs that are routinely used in veterinary medicine in addition to ivermectin. Possible toxicosis to other MDR1 substrate drugs including vincristine, vinblastine, doxorubicin, and loperamide, has been reported in relation to the mdr1 gene mutation [20, 29]. This study examined the frequency of the mdr1 mutant allele in a Japanese population of eight breeds including Collie. Using peripheral blood mononuclear cells from a Collie having homozygous mdr1 mutant allele, MDR1 protein expression and its function were assessed.

MATERIALS AND METHODS

DNA amplification of canine mdr1 gene: DNA samples were obtained from 192 client-owned dogs and a Collie that had been housed at our facility. The breeds included Collie, Australian Shepherd, Shetland Sheepdog and five other popular breeds in Japan: Labrador Retriever, Golden Retriever, Shih Tzu, Shib Inu, and Daichshund. A venous blood sample was collected from each dog for genomic DNA extraction. The DNA was extracted using a PAXgene Blood DNA Kit (Qiagen GmbH, Germany) or a QIAamp DNA Blood Mini Kit (Qiagen GmbH), depending on the amount of the blood sample. For PCR reactions, 500 nM of each primer at final concentration, 0.05–1.56 µg of extracted DNA, and 12.5 µl of PCR Master Mix (Promega Corp., Madison, WI) were used. The total volume was adjusted to 25 µl with nuclease-free water. The primers were designed to amplify 60-bp DNA fragments of the wild type allele, which encompasses the putative deletion site [28] (GenBank AF045016). Nucleotide sequences of the primers were 5′-CCTCTCATGATGCTGGT (forward primer) and 5′-TGAAAATCTGACATTGGTTG (reverse primer). The PCR condition was: initial denaturation at 94°C for 2 min; 30 cycles consisting of denaturation at 94°C for 1 min; annealing at 56.2°C for 1 min; and extension at 72°C for 1 min; then extra extension at 72°C for 1 min. The PCR products were electrophoresed in 5% agarose gels/0.5 µg of nucleic acid/5 ml of gel.
× Tris-Borate EDTA buffer. The DNA bands were stained with ethidium bromide following electrophoresis and were visualized using a UV-transilluminator.

**MDR1 protein expression on canine PBMC: Anti-human MDR1 monoclonal antibody JSB-1, which has been shown to react to canine MDR1 [5], was used to assess the expression of MDR1 protein on canine PBMC. Peripheral venous blood samples were obtained from a mdr1–1Δ/MDR1 mdr1–1Δ Collie and a mixed-breed dog that was homozygous for the normal mdr1 allele (a MDR1/MDR1 dog). The PBMC were collected through density centrifugation with a Histopaque®-1077 (Sigma-Aldrich Corp., St. Louis, MO). The cells were washed twice with cold phosphate buffered saline (PBS)/1% fetal bovine serum (FBS). Then the cells were suspended in cold PBS/1%FBS; then, fluorescence intensity of the cells was analyzed using flow cytometry. Lymphocyte populations were selected for analysis using forward scatter (FSC) and side scatter (SSC) dot plots. Thereby, approximately 5,000 lymphocytes were assessed.

**RESULTS**

**Frequency of the mutant mdr1 allele: DNA samples obtained from 193 dogs of eight breeds were tested for the mdr1 gene mutation. The two different bands—one of which was 60-bp fragment of the normal mdr1 allele and the other of which was 56-bp fragment of the mutant mdr1 allele—were comparably distinguished on 5% agarose gels (Fig. 1). The nucleotide sequence of a mdr1–1Δ allele from a Collie predicted to be mdr1–1Δ/MDR1 showed an identical mutation to one that was already reported to be associated with ivermectin sensitivity in Collies (data not shown).

The 4-bp deletion mutation of mdr1 gene was detected in three collie-breeds: Collie, Australian Shepherd, and Shetland Sheepdog. The deletion was not found in five other breeds (Table 1). Of the 12 Collies, 5 (41.7%) were homozygous for the mutant allele (mdr1–1Δ/MDR1–1Δ), and 4 (33.3%) were heterozygous (mdr1–1Δ/MDR1). Of the 9 Australian Shepherds, 1 (11.1%) was mdr1–1Δ/MDR1–1Δ, and 4 (44.4%) were mdr1–1Δ/MDR1. Among Shetland Sheepdogs, 1 (2.4%) was mdr1–1Δ/MDR1.

**MDR1 protein expression on canine PBMC: Expression of MDR1 protein on canine PBMC was analyzed using anti–human P-gp monoclonal antibody, JSB-1 (Fig. 2).**

- **Table 1. Frequency of the mdr1 mutation in 193 dogs of 8 breeds in Japan**

<table>
<thead>
<tr>
<th>Breed</th>
<th>n</th>
<th>MDR1/MDR1</th>
<th>mdr1–1Δ/MDR1</th>
<th>mdr1–1Δ/MDR1</th>
<th>Mutant allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collie</td>
<td>12</td>
<td>3 (25.0%)</td>
<td>4 (33.3%)</td>
<td>5 (41.7%)</td>
<td>58.3%</td>
</tr>
<tr>
<td>Australian Shepherd</td>
<td>9</td>
<td>4 (44.4%)</td>
<td>4 (44.4%)</td>
<td>1 (11.1%)</td>
<td>33.3%</td>
</tr>
<tr>
<td>Shetland Sheepdog</td>
<td>42</td>
<td>41 (97.6%)</td>
<td>1 (2.4%)</td>
<td>0 (0.0%)</td>
<td>1.2%</td>
</tr>
<tr>
<td>Labrador Retriever</td>
<td>37</td>
<td>37 (100.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0.0%</td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>28</td>
<td>28 (100.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0.0%</td>
</tr>
<tr>
<td>Shih Tzu</td>
<td>37</td>
<td>37 (100.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0.0%</td>
</tr>
<tr>
<td>Shiba Inu</td>
<td>21</td>
<td>21 (100.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0.0%</td>
</tr>
<tr>
<td>Dachshund</td>
<td>7</td>
<td>7 (100.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

![Fig. 1. Canine mdr1 gene was amplified from genomic DNA from 193 dogs. PCR products were electrophoresed in 5% agarose gel. Representative cases are shown. Lane A, a 56-bp band was detected from a mdr1–1Δ/MDR1–1Δ Collie; B, a 60-bp band was detected from a MDR1/MDR1 Shih Tzu; C, a 60-bp band and a 56-bp band were detected from a mdr1–1Δ/MDR1 Australian Shepherd.](image)
PBMC from a mdr1–1D/mdr1–1D Collie.

Rhodamine 123 efflux assay: MDR1 function of peripheral blood lymphocytes was assessed using fluorescence dye efflux assays. The PBMC were incubated with Rh123 in the presence or absence of VRP; fluorescence intensity in lymphocytes was measured (Fig. 3). The respective median fluorescence intensities after efflux in MDR1/MDR1 lymphocytes incubated with or without VRP were 78.8% and 67.4% of that before efflux, whereas the respective median fluorescence intensities in mdr1–1D/mdr1–1D lymphocytes incubated with or without VPR were 99.5% and 95.9% of that before efflux.

**DISCUSSION**

This study found 4-bp deletion of mdr1 gene in a Japanese population of three collie-breeds that are reported to have the mutant allele [23]. In Collies, the frequency of the mutant allele was 58.3%; about three-fourths of the Collie population were either homozygous or heterozygous for the mutant allele. Australian Shepherds showed a high frequency of the mutant allele. Among Shetland Sheepdogs, only one dog had the mutant allele as heterozygous. Although few dogs were examined in this study, these results correspond to previous reports about the frequency of the mutant allele in United States, United Kingdom and
Australia [10, 23]. The mutant allele was not found in this study in the other five breeds including Labrador Retriever, Golden Retriever, Shih Tzu, Shiba Inu, and Dachshund. The mdr1 mutation has not been reported in those breeds so far. For that reason, the frequency of the mutant allele is inferred to be low in those five breeds [23].

The MDR1 expression on human peripheral blood cells, including lymphocytes, has been demonstrated [3, 4, 6, 15]. In this study, expression of MDR1 was detected on PBMC from a MDR1/MDR1 dog, but not on PBMC from a mdr1–1/Δmdr1–1Δ Collie. Messenger RNA expression of the deleted mdr1–1 gene was detected in PBMC from a mdr1–1/Δ mdr1–1Δ Collie(data not shown). The functional MDR1 protein might not be expressed on any type of cells in mdr1–1/Δmdr1–1Δ dogs because the 4-bp deletion generates an early stop codon.

Fluorescence dye efflux assays were carried out to assess MDR1 functions of canine PBMC. The Rh123 excretion from MDR1/MDR1 lymphocytes was inhibited by 1 mg/ml of VRP. In mdr1–1/Δmdr1–1Δ lymphocytes, excretion of Rh123 was negligible both in the presence and absence of VRP. These results indicated that MDR1 that is expressed on canine PBMC functions in excretion of xenobiotics, which is consistent with a previous report describing overcoming of vincristine resistance by 1.1 μg/ml of VRP in leukaemic cells [34].

Not only is MDR1 related to a multidrug resistance state in cancer patients; it is possibly related to adverse effects of anticancer drugs in canine cancer patients [20]. Some anticancer drugs, including vincristine, vinblastine, and doxorubicin, are substrates of MDR1 protein [30]. Sensitivity to vincristine was examined using phytohemagglutinin-stimulated PBMC from a mdr1–1/Δmdr1–1Δ dog (data not shown). Although the PBMC showed no high sensitivity to vincristine compared to MDR1/MDR1 PBMC, anticancer drugs should be administered carefully to mdr1–1/Δmdr1–1Δ dogs [19, 21].

The MDR1 genotypes also give an indication for the use of avermectins including ivermectin and doramectin. Its administration to Collie-breed-dogs should be avoided, especially to Collies and Australian Shepherds [35]. Nevertheless, much information regarding the relationship between drug sensitivity and mdr1 genotypes remains unknown. Further investigation of other mutations of mdr1 gene or drug sensitivity of mdr1–1/ΔMDR1 dogs will be pharmacogenetically instructive for the adequate use of MDR1-substrate drugs.

REFERENCES