Molecular analysis of multidrug resistance in feline lymphoma cells

Yoshiko Okai, DVM; Noriko Nakamura, DVM; Haruka Matsushiro, DVM; Hirotomo Kato, DVM, PhD; Asuka Setoguchi, DVM, PhD; Mitsuhiro Yazawa, DVM; Masaru Okuda, DVM, PhD; Toshihiro Watari, DVM, PhD; Atsuhiko Hasegawa, DVM, PhD; Hajime Tsujimoto, DVM, PhD

Objective—To evaluate the mechanism of multidrug resistance in feline lymphoma cell lines.

Sample Population—A feline lymphoma cell line (FT-1) and its adriamycin (ADM)-resistant subline (FT-1/ADM).

Procedures—The FT-1 cell line was cultivated in the presence of a gradually increasing concentration of ADM to generate its ADM-resistant subline (FT-1/ADM). Susceptibility of cells from the parental FT-1 cell line and the FT-1/ADM subline to antineoplastic drugs was determined. From the complementary DNA (cDNA) template of FT-1/ADM cells, feline *MDR1* cDNA was amplified by use of polymerase chain reaction (PCR) and sequenced. Reverse transcription (RT)-PCR and Western blot analyses were performed to assess expression of the *MDR1* gene and P-glycoprotein (P-gp) in FT-1/ADM cells, compared with that in FT-1 cells.

Results—A drug sensitivity assay revealed that FT-1/ADM cells were much more resistant to ADM and vincristine than the parental FT-1 cells. The feline *MDR1* cDNA amplified by use of PCR was 3,489 base pairs long, corresponding to approximately 90% of the whole open reading frame of human *MDR1* cDNA; its amino acid sequence was 91.5, 87.0, and 79.4% identical to that of human *MDR1*, mouse *mdr1a*, and *mdr1b* cDNA, respectively. By RT-PCR analysis, expression of *MDR1* messenger RNA was clearly detected in FT-1/ADM cells but not in the parental FT-1 cells. Western blot analysis also revealed the expression of P-gp encoded by the *MDR1* gene in FT-1/ADM cells but not in FT-1 cells.

Conclusions—The basic structure of the feline *MDR1* gene was essentially the same as that of multidrug-resistance genes of other species. Expression of P-gp appeared to be one of the mechanisms responsible for the development of multidrug resistance in feline lymphoma cell lines in vitro. (*Am J Vet Res* 2000;61:1122–1127)

Lymphoma is the most common neoplasm that develops in cats and accounts for one third of all tumors in cats.[1] Chemotherapy with antineoplastic drugs is generally used for the treatment of lymphoma in cats. Although remission rates for lymphoma in cats treated with chemotherapy are as high as 65 to 75%, many cats undergo a relapse several months after the initiation of chemotherapy, and it is generally difficult to reinduce a state of remission in these cats.[1]

Drug resistance observed during chemotherapy is a common obstacle in the treatment of neoplastic diseases in cats and dogs as well as in humans. Cross-resistance to multiple structurally unrelated drugs, such as vinca alkaloids, anthracyclines, etoposide, and actinomycin D, after exposure to one drug is known as multidrug resistance.[2,3] The most highly established mechanism contributing to multidrug resistance is mediated by **P-glycoprotein (P-gp)**, which is encoded by *MDR* genes. There are 2 *MDR* genes, termed *MDR1* and *MDR2*, in humans, whereas there are 3 *mdr* genes, termed *mdr1a*, *mdr1b*, and *mdr2*, in mice. The human *MDR1* gene and mouse *mdr1a* and *mdr1b* genes encode the P-gp responsible for multidrug resistance.[4-7] As for other mechanisms of multidrug resistance, there are several mechanisms reported thus far, such as active efflux of chemotherapeutic drugs mediated by multidrug resistance-associated protein[8] and lung cancer-associated protein,[9] inactivation of the chemotherapeutic drugs, and impairment of metabolic activation of the drugs.

Human P-gp encoded by the *MDR1* gene is a 170-kd transmembrane protein that belongs to the ATP-binding cassette superfamily and acts as an energy-dependent pump that effluxes antineoplastic drugs to the extracellular portion of the interstitium.[10-12] Increased expression of P-gp can be detected in tumor cells from patients that are unresponsive to treatment with antineoplastic drugs in the initial course of chemotherapy or during a relapse after chemotherapy.[13] In lymphomas that develop in humans, expression of P-gp is a considerable prognostic factor during chemotherapy.[14-16]

Expression of P-gp has also been detected by use of Western blot analysis and immunohistochemistry in lymphoma cells in dogs during relapse of the disease.[17,18] Furthermore, it has been demonstrated that

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From the Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences (Okai, Nakamura, Setoguchi, Yazawa, Tsujimoto), and the Department of Molecular Neurobiology and Pharmacology, School of Medicine (Matsushiro), University of Tokyo, Tokyo 113-8657, Japan; Department of Microbiology, Immunobiology Vaccine Center, University of Alabama, Alabama 35294-2170 (Kato); Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0521 (Okuda); and the Department of Pathobiology, School of Veterinary Medicine, Nihon University, Kanagawa 252-8510, Japan (Watari, Hasegawa).

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increased expression of P-gp is a negative prognostic indicator for survival in dogs with lymphoma.[19]

The purposes of the study reported here were to evaluate the mechanism of multidrug resistance in feline lymphoma cell lines and to establish a technique for detection of P-gp. We isolated a drug-resistant feline lymphoma cell line and investigated the structure and expression of the feline *MDR1* gene that encodes for P-gp.

Materials and Methods

Cell culture and isolation of an adriamycin-resistant cell subline—A **feline T-cell lymphoma cell line (FT-1)**,[20,21] which was derived from an FeLV-positive thymic lymphoma from a cat, was cultured in RPMI-1640[a] supplemented with 10% fetal calf serum, 10m*M* glutamine, and gentamycin (20 µg/ml) at 37 C in a humidified atmosphere of 5% CO_2. The FT-1 cell line was first passaged in a medium that contained 0.001 µg of **adriamycin (ADM)**[b] per ml. The concentration of ADM in the medium was then gradually increased to 0.4 µg/ml. After passages every 2 or 3 days for approximately 6 months, an **ADM-resistant subline of FT-1 (FT-1/ADM)** was obtained.

Drug sensitivity assay—Sensitivities to antineoplastic drugs of cells from the FT-1 cell line and the FT-1/ADM subline were determined. The FT-1 and FT-1/ADM cells were allocated to wells of a 96-well flat-bottomed microtiter plate (1×10^5 cells/ml; 0.1 ml/well) and exposed to serially diluted ADM (0.3 to 5,000 ng/ml) or **vincristine**[c] **(VCR**; 0.02 to 500 ng/ml), then incubated at 37 C. Fifty microliters of solution containing 1 mg of sodium **3′-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT)**/ml and 25 m*M* PMS was added to each well 48 hours after the start of cultivation, and cells were cultured for an additional 4 hours. The amount of colored formazan product that was obtained from the metabolized XTT was determined from the absorbance at 492 nm with a microplate reader, as reported by Roehm et al.[22] The **50% inhibitory concentrations (IC_{50})** were determined from the viable cell counts at the various concentrations of ADM and VCR.

Polymerase chain reaction (PCR) amplification—Total RNA was extracted from FT-1/ADM cells by use of the acid guanidium-phenol-chloroform method.[d] Reverse transcription of total RNA was performed to generate single-stranded **complementary DNA (cDNA)** for use as a template for PCR analysis with a commercially available kit[e] and an oligo dT primer.[e]

The PCR primers h1S, h1R, h2S, h2R, h3R, and h4R were synthesized based on the sequences conserved between the sequences of human and mouse *MDR* genes **(Table 1; Fig 1)**. A 310-**base pair (bp)** fragment containing an ATP binding site was amplified with primers h1S and h1R. Primers f1S and f1R were then prepared based on the sequence of the h1S-h1R fragment obtained by use of PCR. Two fragments covering the 5' and central regions were amplified with 2 primer pairs, h2S/f1R and f1S/h2R. Furthermore, another fragment was amplified with f2S primer that was prepared based on the sequence of the f1S-h2R fragment and h3R primer. Finally, a 3' fragment was amplified with f3S primer that was prepared based on the sequence of the f2S-h3R fragment and h4R primer.

Table 1—Primers for polymerase chain reaction (PCR) amplification of feline *MDR1* complementary DNA fragments

Primers	Nucleotide positions in human *MDR1*	Primer sequences
h1S*	1216–1237	5′-GAAGTTAAGATCTTGAAGGGCC-3′
h1R*	1525–1507	5′-CATTGGCTTCCTTGACAGC-3′
h2S*	159–180	5′-GGGAACTTTGGCTGCCATCATC-3′
f1R†	1260–1239	5′-CCCACTCTGAACCTTCAGGTTG-3′
f1S†	1485–1506	5′-CACCATGGAGGAGATTGAGAAA-3′
h2R*	2430–2408	5′-GGTGTTTTTAGGGTCATCAAACC-3′
f2S†	2385–2406	5′-CATGCTAAGACAGGATGTGAGC-3′
h3R*	3196–3175	5′-GCGTCTGGCCCTTCTTCACCTC-3′
f3S†	3149–3168	5′-CCCAGTGCTTCAAGGGACTG-3′
h4R*	3687–3667	5′-CACAATGCAGGTGCGGCCTTC-3′

*Sequences of the primers were based on the sequence of the human *MDR1* gene.

†Sequences of the primers were based on the sequence of the PCR fragment of feline *MDR1* gene obtained in this study.

A template cDNA of FT-1/ADM cells was amplified by use of PCR in a volume of 50 µl containing a pair of primers (10 ng/µl each), 1.5 units of *Taq* polymerase,[f] and reagents, as recommended by the manufacturer.[f] The PCR amplification was performed by 30 cycles of denaturation at 94 C for 1 minute, annealing at 55 C for 1 minute, and polymerization at 72 C for 1 minute, followed by extension at 72 C for 10 minutes. The resulting products were electrophoresed in 2% agarose gel and extracted from the gel. These DNA fragments were cloned into a plasmid vector.[e] *Escherichia coli* competent cells[e] were transformed with the ligation mixture and plated onto 2 × TY agar plates containing ampicillin (50 µg/ml), 5-bromo-4-choloro-3-indolyll-D-galactoside (36 µg/ml), and isopropyl-D-thiogalactoside (40 µg/ml). Plasmid DNA was prepared, according to manufacturer's instructions, by use of a commercially available kit.[g]

Nucleotide sequence analysis—The PCR products that were cloned into plasmids were sequenced by the dideoxy chain termination method.[h] Nucleotide sequences of cloned fragments were determined on both DNA strands in opposite directions.

Detection of transcripts by RT-PCR—Total RNA samples prepared from FT-1 and FT-1/ADM cells were reverse-transcribed with a commercially available kit[e] and then amplified by use of PCR. The primer pairs used to amplify a

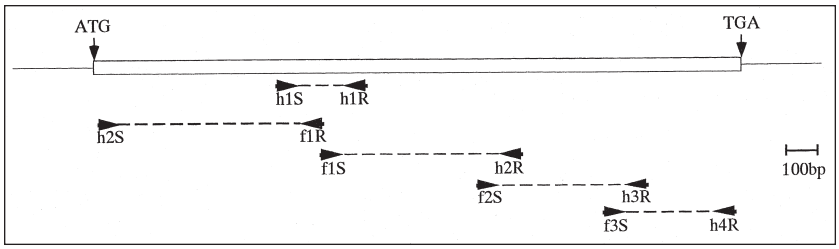

Figure 1—Location of primers used for polymerase chain reaction amplification of feline *MDR1* complimentary DNA fragments within the full-length human *MDR1* gene. Bp = Base pairs.

cDNA fragment of the feline *MDR1* gene were h1S and h1R. As a control, primers used to amplify the feline **glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)** gene were prepared based on sequences of the human *GAPDH* gene.[23] The PCR amplification consisted of 30 cycles of denaturation at 94 C for 1 minute, annealing at 55 C for 1 minute, and polymerization at 72 C for 1 minute, followed by extension at 72 C for 10 minutes. The PCR products were subjected to elecrophoresis in 3% agarose gel.

Detection of P-gp by Western blot analysis—Cells were lysed in a buffer solution (20 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 1% TritonX, 10% glycerol, 0.5 m*M* dithiothreitol, and 0.5 m*M* phenylmethylsulfonyl fluoride) while on ice for 30 minutes. The reaction mixture was then centrifuged at 20,000 × g for 30 minutes. Aliquots of the supernatant protein samples were boiled for 5 minutes and subjected to electrophoresis in 8% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with a monoclonal antibody against human P-gp[i] for 4 hours. The membrane was then incubated with horseradish peroxidase-labeled goat anti-mouse IgG for 1 hour. Peroxidase activity was detected by use of an enhanced chemiluminescence Western blot analysis.[j] A human myelogeneous leukemia cell line, K562, and its adriamycin-resistant subline, K562/ADM,[24] were used as negative and positive controls, respectively.

Results

Establishment of an ADM-resistant subline—From the IC_{50} values of ADM and VCR, the FT-1/ADM cells were found to be 196- and 978-fold more resistant to ADM and VCR, respectively, compared with parental FT-1 cells **(Table 2)**.

Molecular cloning and sequencing of the feline *MDR1* gene—The PCR amplification for feline *MDR1* cDNA with 5 primer pairs yielded bands of DNA fragments of expected sizes (h1S-h1R, 310 bp; h2S-f1R, 1,102 bp; f1S-h2R, 946 bp; f2S-h3R, 812 bp; f3S-h4R, 539 bp). These PCR products were inserted into the PCR2 vector and sequenced. By combining these 5 overlapping fragments, h1S-h1R, h2S-f1R, f1S-h2R, f2S-h3R, and f3S-h4R, a nucleotide sequence of a putative feline *MDR1* cDNA of 3,489 bp was determined.[k] The sequence of the feline *MDR1* cDNA obtained from our study corresponded to approximately 90% of the length of the whole coding region of human *MDR1* cDNA. The predicted amino acid sequence of the feline *MDR1* gene was 91.5, 76.5, 87.0, 79.4, and 75.8% identical to those of human *MDR1* and *MDR2*, mouse *mdr1a*, *mdr1b,* and *mdr2* genes, respectively **(Table 3)**. Especially in the regions corresponding to the 5' and 3' ATP binding sites, the sequence of feline *MDR1* cDNA was nearly identical to those binding sites on the human *MDR1*, mouse *mdr1a*, and *mdr1b* genes. In a hydropathy analysis,[25] the amino acid sequence of feline *MDR1* cDNA obtained here had 12 hydrophobic domains, as does human *MDR1* cDNA (data not shown).

Table 2—Growth inhibition of a feline lymphoma cell line (FT-1) and its adriamycin (ADM)-resistant subline (FT-1/ADM) with antineoplastic drugs

Drug	IC_{50} (ng/ml) FT-1	IC_{50} (ng/ml) FT-1/ADM	Relative resistance
Adriamycin	4.90	960	196
Vincristine	0.225	220	978

IC_{50} = 50% inhibitory concentration.
Relative resistance = $\frac{IC_{50}\ ([FT\text{-}1]/ADM)}{IC_{50}\ (FT\text{-}1)}$.

Expression of the *MDR1* gene in FT-1 and FT-1/ADM cells—To examine the expression of the *MDR1* gene in FT-1 and FT-1/ADM cells, we performed RT-PCR analysis, using h1S and h1R primers to amplify the sequence of the h1S-h1R portion of the gene. In the RT-PCR analysis, *MDR1* **messenger RNA (mRNA)** was detected in FT-1/ADM cells but not in the parental FT-1 cells **(Fig 2)**.

Expression of P-gp in FT-1 and FT-1/ADM cells—Western blot analysis was performed, using a monoclonal antibody against human P-gp to evaluate

Table 3—Nucleotide and amino acid sequence similarities of *MDR* genes among species

Gene	Feline *MDR1*	Human *MDR1*	Human *MDR2*	Mouse *mdr1a*	Mouse *mdr1b*	Mouse *mdr2*
Feline *MDR1*						
Nucleotide	—	90.7	75	85	81.5	74.1
Amino acid	—	91.5	76.5	87	79.4	75.8
Human *MDR1*						
Nucleotide	90.7	—	75.5	83.3	79.7	72.5
Amino acid	91.5	—	75.8	87	80.3	74.4
Human *MDR2*						
Nucleotide	75	75.5	—	72.7	71.3	86.7
Amino acid	76.5	75.8	—	74.2	70.7	90.6
Mouse *mdr1a*						
Nucleotide	85	83.3	72.7	—	84.5	74
Amino acid	87	87	74.2	—	83.7	73.2
Mouse *mdr1b*						
Nucleotide	81.5	79.7	71.3	84.5	—	71.7
Amino acid	79.4	80.3	70.7	83.7	—	70
Mouse *mdr2*						
Nucleotide	74.1	72.5	86.7	74	71.7	—
Amino acid	75.8	74.4	90.6	73.2	70	—

— = Not applicable.

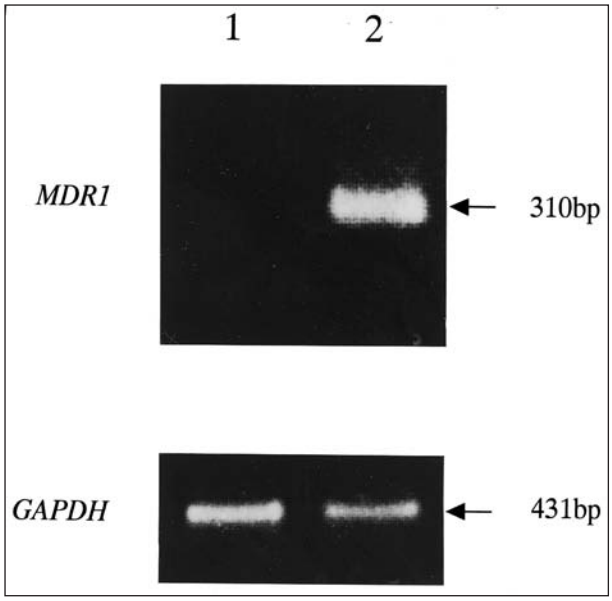

Figure 2—Ethidium bromide-stained agarose gel of the band amplified from *MDR1* messenger RNA in a feline lymphoma cell line (FT-1) and its adriamycin (ADM)-resistant (FT-1/ADM) subline. Total RNA samples extracted from the cell lines (lane 1, FT-1; lane 2, FT-1/ADM) were analyzed by use of reverse transcription and polymerase chain reaction (RT-PCR); primers for PCR were prepared from the sequence of human *MDR1* gene. bp = Base pairs. *GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase.

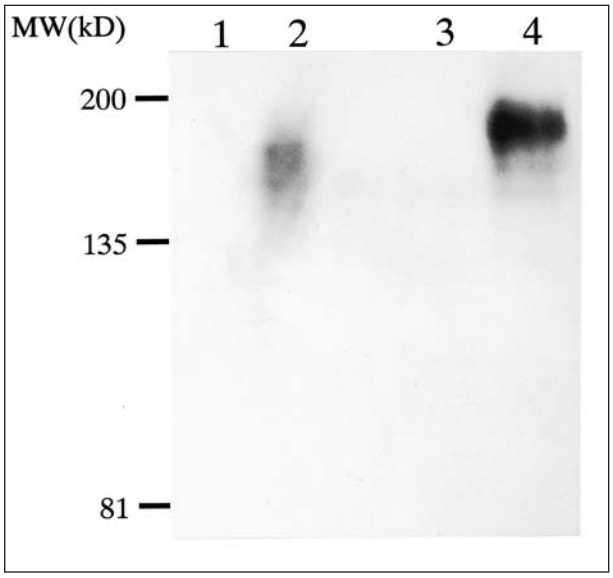

Figure 3—Western blot analysis of feline P-glycoprotein in FT-1 and FT-1/ADM cells. The protein samples extracted from the cell lines (lane 1, FT-1; lane 2, FT-1/ADM; lane 3, K562; lane 4, K562/ADM) were analyzed by use of a monoclonal antibody. MW = Molecular weight. kD = Kilodalton.

the expression of P-gp in FT-1 and FT-1/ADM cells. In the Western blot analysis, a broad band (approximately 150 to 175 kd) was detected in FT-1/ADM cells, but not in parental FT-1 cells (**Fig 3**). The molecular weight of the band that corresponded to feline P-gp in the FT-1/ADM cells was slightly smaller than that of the band of human P-gp in the K562/ADM cells.

Discussion

Many cell lines selected for resistance to ADM have been obtained from tumor cell lines derived from humans, mice, and dogs.[26,27] A representative ADM-resistant human myelogeneous leukemia cell line, K562/ADM, was 134-fold more resistant to ADM than the parental K562 cell line.[24] The FT-1/ADM cell line in our study was 196-fold more resistant to ADM than its parental FT-1 cell line, a value similar to that in human K562/ADM cells. Furthermore, multidrug resistance of the FT-1/ADM cells was associated with the expression of *MDR1* mRNA and P-gp. To further understand the involvement of P-gp in the drug resistance of lymphomas, it is necessary to examine specific clinical cases from cats with drug-resistant lymphomas for the expression of P-gp. The FT-1/ADM cell line obtained for our study should be useful for further analysis of the mechanisms of drug resistance and for evaluating the efficacy of antimultidrug resistance agents.

The feline cDNA obtained in this study had a high number of amino acid sequence similarities to the human *MDR1*[28] gene and mouse *mdr1a*[29] and *mdr1b*[30] genes known to be related to multidrug resistance in these species, but had a lower number of amino acid sequence similarities to human *MDR2*[31] and mouse *mdr2*[32] genes, which have no association with multidrug resistance. Especially in regions of ATP binding sites, the amino acid sequence of the feline *MDR1* gene was almost completely identical to those of human *MDR1* and mouse *mdr1a* and *mdr1b* genes. An epitope of the monoclonal antibody against human P-gp was also conserved in the feline *MDR1* gene. Human P-gp is known to be an integral plasma membrane protein that contains 12 transmembrane domains and 2 ATP binding sites in the intracellular domains,[12] and its first extracellular loop carries 3 consensus N-linked glycosylation sites.[33,34] Similarly, the predicted amino acid sequence of the feline *MDR1* gene had a hydropathy profile containing 12 putative transmembrane domains, sequences of 2 ATP binding sites, and 3 N-linked glycosylation sites in the first extracellular loop. From these data, the basic structure of the feline *MDR1* gene had essentially the same structure as that for the human *MDR1* and mouse *mdr1a* and *mdr1b* genes, indicating a common function of the products of these genes in the 3 species.

There are various monoclonal antibodies against human P-gp. It has been demonstrated that C219,[35] C494,[35] and JSB-1[36] recognize epitopes that are located in the intracellular domains, whereas MRK-16,[37] 4E3,[38] and UIC-2[39] recognize epitopes located in the extracellular domains. Of these antibodies, the C219 antibody, which binds to an intracellular epitope near the ATP-binding site of human P-gp,[40-42] cross-reacted with P-gp from other species, including hamster and canine.[17,18,43] With the C219 antibody, a broad band of approximately 150 to 175 kd was detected in FT-1/ADM cells but not in FT-1 cells in our study. This finding (from Western blot analysis) correlated with the data on RT-PCR analysis for FT-1/ADM and FT-1 cells. These findings indicated that

the expression of P-gp is associated with the induction of multidrug resistance in the feline lymphoma cell line. Because the present study was performed using only one feline lymphoma cell line, further investigation on other feline lymphoma cell lines and primary lymphoma cells from tumors in vivo are required.

In this study, we demonstrated that induction of P-gp was one of the mechanisms for the development of multidrug resistance in a feline lymphoma cell line. Because multidrug resistance is a major obstacle when using chemotherapy to treat lymphomas in cats, the present study may help contribute to the improvement in chemotherapies for lymphomas and other neoplastic diseases in cats.

[a]RPMI 1640, Gibco, Grand Island, NY.
[b]Adriamycin, Kyowa Hakko, Tokyo, Japan.
[c]Vincristine, Shionogi, Osaka, Japan.
[d]RNAzol, Biotecx, Houston, Tex.
[e]cDNA cycle kit, TA-cloning kit and One shot, Invitrogen, San Diego, Calif.
[f]TaKaRa Taq, Takara, Kyoto, Japan.
[g]QIAGEN plasmid kit, Qiagen, Studio City, Calif.
[h]Thermo Sequenase Cycle Sequencing kit, Shimazu, Kyoto, Japan.
[i]C219, Signet Laboratories, Dedham, Mass.
[j]ECL, Amersham, Buckinghamshire, UK.
[k]DDBJ/EMBL/GenBank nucleotide sequence database accession number AB029153.

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