NOTE *Internal Medicine*

Apoptosis of Canine Mammary Tumor Cells Induced by Small Interfering RNA (siRNA) against *Bcl-xL* Gene

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ABSTRACT. The inhibition of Bcl-xL mRNA expression and the acceleration of apoptotic cell rates in canine mammary tumor cell line (CF33) by the small interfering RNA (siRNA) were analyzed. The level of *Bcl-xL* transcripts in CF33 was decreased when cultured with siRNA, suggesting that siRNA might inhibit the expression of *Bcl-xL* mRNA in the CF33. Apoptotic cell rates in CF33 cultured with siRNA in Oligofectamine medium, with double strand RNA in Oligofectamine medium, without siRNA in Oligofectamine medium and in DMEM alone were 60.9%, 30%, 28.7% and 11.6% at 48-hr incubation, respectively, when evaluated by TUNEL assay. From these results, it was suggested that canine *Bcl-xL* might be an anticancer target of canine tumors.

KEY WORDS: *Bcl-xL*, canine mammary tumor, small interfering RNA (siRNA).

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Inhibition of apoptosis is conferred as a survival advantage on cells harboring genetic alternations and may promote acquisition of further mutations that induce neoplastic progression and also promote resistance to chemotherapy [5, 9].

Up-regulated expression of the antiapoptotic proteins such as Bcl-xL and Bcl-2 was reported in various human tumors [4, 10]. Expression levels of these proteins in tumor cells were different depending on the degree of malignancy or the effectiveness of chemotherapy [1, 6]. As in the human malignancies, Bcl-2 and Bcl-xL would be related to the increased resistance to treatments in canine malignancies.

In a previous study, cDNA encoding canine Bcl-xL gene was identified and mRNA expression of Bcl-xL gene was investigated in tumor cell lines [14]. The canine Bcl-xL mRNA was constitutively expressed in tumor cell lines and might be an anticancer target of canine tumors [14].

Mammary neoplasm accounts for about half of all tumors in dogs and adenocarcinoma is common among canine and feline malignant mammary tumors [11]. The recurrence rate within 2 years after mastectomy in bitches with poorly differentiated (i.e. anaplastic) tumors was reported to be 90% versus rates of 68% and 24% in animals with moderately- and well-differentiated tumors, respectively [11]. Since the surgical excision of malignant mammary tumors is not curative, effective therapies are needed.

In the past few years RNA interference (RNAi) has become the most widely used technology for gene knockdown. When long double-stranded (ds) RNA molecules are introduced into plants and invertebrates, they are processed by the endonuclease direct into 21- to 23-nucleotide small RNAi. The small interfering RNA (siRNA) are then incorporated into the multicomponent for RNA-induced silencing complex (RISC), which unwinds the duplex and uses the antisense (AS) strand as a guide to seek and degrade homologous mRNAs [2]. Gene silencing in mammalian cells using siRNA was investigated to induce an effective RNAi [7]. The potentials of RNAi as a therapeutic procedure were investigated to specifically silence disease-related genes including oncogenes. When siRNA constructs were successfully utilized to silence expression of the *Bcl-xL* in tumor cells, apoptosis could be induced, offering a new strategy to treat canine tumors. However, the siRNA against canine antiapoptotic genes has not been investigated.

In this study, we investigated the inhibition of *Bcl-xL* mRNA expression and the acceleration of apoptotic cell rates in canine mammary tumor cell line (CF33) by oligonucleotides in Oligofectamine.

The oligonucleotides on canine *Bcl-xL* gene (GeneBank accession No. AB073983) used in this study were designed and purchased from Ambion (www.ambion.com/techlib/misc/siRNA_finder.html). Target position of canine Bcl-xL mRNA was 187–207 bp.

The sequence of oligonucleotide for canine *Bcl-xL* gene was as follows;

5'- CCA CAA AGA AAC CAG UUC Utt-3'

3' ttG GUG UUU CUU UGG UCA AGA-5'

The sequence of ds RNA (negative control for nonsilencing siRNA) was that reported in Lima *et al.* [8] with the following;

5'-UUC UCC GAA CGU GUC ACG Utt-3'

3'-ttA AGA GGC UUG CAC AGU GCA-5'.

A canine mammary tumor cell line (CF33; ATCC CRL6227) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 10 mM glutamine, and gentamycin (20 μg/m*l*) at 37°C in a humidified atmosphere of 5% CO_2. The sample cells were allocated to wells of a 6-well flat-bottomed plate (1.5×10^5 cells/m*l*; 4 m*l*/well). The transfection procedure of siRNA was performed according to the user manual of OligofectamineTM Reagent (Invitrogen, Carlsbad, CA, U.S.A.). The cells were transfected with 0.2 mM of cationic liposome

(Oligofectamine) and 200 nM of oligonucleotides (siRNA) for canine Bcl-xL in the DMEM, then incubated at 37°C for 48 hr.

Control samples were obtained as follows: 1) CF33 cells were transfected with 0.2 mM of Oligofectamine and 200 nM control oligonucleotide (ds RNA) at 37°C for 48 hr, 2) the cells were incubated with no siRNA in 0.2 mM of Oligofectamine at 37°C for 48 hr, and 3) the cells were incubated in DMEM alone at 37°C at least 48 hr.

After 48-hr incubation, the cells were investigated by RT-analysis. All experiments were performed in triplicate. Total RNAs were extracted from CF33 cells and from the normal canine mammary gland of a healthy dog with a RNeasy Mini Kit (QIAGEN). Subsequently, total RNAs were treated to remove contaminating DNA with DNA-free™ kit (Ambion, Austin, TX, U.S.A.). Reverse transcription of the poly(A)$^+$ RNAs (using 0.5 mg) was performed with Oligo dT primer and Omniscript™ Reverse Transcriptase kit (QIAGEN).

Primer sequences used for amplification of canine *Bcl-xL* cDNA were sense 5'-GGC CTT TTT CTC CTT CGG TG-3' (Genbank accession number AB073983, 828–847bp) and reverse 5'-CTC TCG GCT GCT GCA TTG TT -3' (994–1013 bp).

The cDNA samples were amplified by PCR in 32 μl of reaction mixture containing a pair of primers (0.5 mM each), 1.5 unit of Taq polymerase (Takara, Kyoto, Japan) and the reagents as recommended by the manufacturer (Takara). The PCR amplification was carried out for 30 cycles consisting of template denaturation (94°C, 45 sec), primer annealing (69°C, 30 sec) and polymerization (72°C, 1 min). With these primers, 185 bp fragments consisting of canine *Bcl-xL* were expected to be amplified. The PCR products were analyzed on a 2% agarose gel.

After 35 cycles of amplification by RT-PCR, *Bcl-xL* mRNA were detected in CF33 cells but not in normal canine mammary gland tissue (Fig. 1). The level of *Bcl-xL* transcripts in CF33 decreased when cultured with the oligonucleotides on canine *Bcl-xL* gene rather than double strand RNA (Fig. 1), confirming the siRNA inhibition of *Bcl-xL* mRNA expression in mammary tumor cells.

After 48-hr incubation, the cells were investigated by apoptosis analysis. The CF33 cells were washed, collected and dropped on slides using cytospin. Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) stain. The TUNEL staining was performed according to the protocol of the DeadEnd™ Colorimetric Apoptosis Detection System (Promega Corp., Madison WI. U.S.A.). At least 500 cells were counted by light microscopy. Apoptotic cells were identified according to the criteria of Oguma [12]. All experiments were performed in triplicate. Data was expressed as means ± SE. Statistical significance ($p<0.05$) analysis was determined by Student's *t*-test in MS Excel.

Apoptotic cell rates of CF33 cells evaluated by TUNEL assay were given in Fig. 2. The average rates in CF33 cultured with siRNA in Oligofectamine medium, double strand RNA in Oligofectamine medium, without siRNA in Oligofectamine medium and without siRNA in DMEM with no Oligofectamine were 60.9%, 30%, 28.7% and 11.6% at 48 hr incubation, respectively. These siRNA were confirmed significantly to accelerate the apoptosis of CF33 at 48 hr.

Bcl-2 family proteins are key regulators of programmed cell death or apoptosis that are implicated in many human diseases, particularly in cancers [4]. High expression of anti-apoptotic genes such as *Bcl-2* and *Bcl-xL* commonly found in human cancers contributes to neoplastic cell expansion and inhibits the therapeutic action of many chemotherapeutic drugs [4].

In this study, we investigated on inhibition of *Bcl-xL* mRNA expression and the acceleration of apoptotic cell rates in CF33 by the oligonucleotides in Oligofectamine.

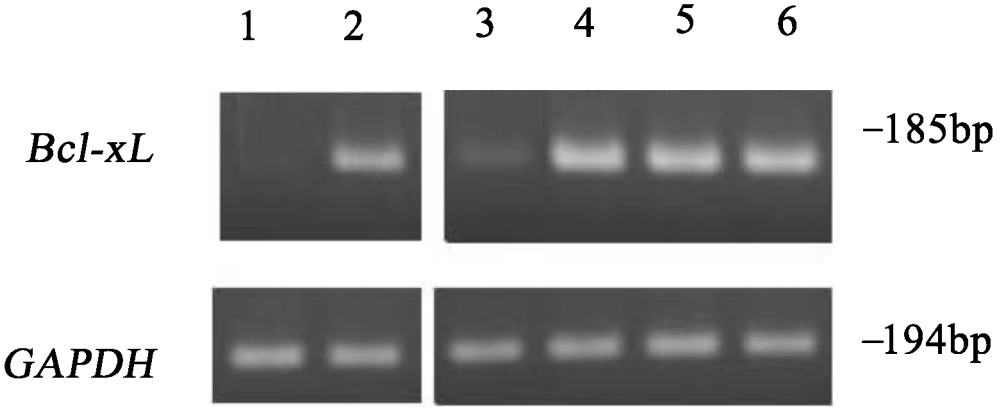

Fig. 1. *Bcl-xL* mRNA expression in normal canine mammary gland tissue and CF-33. PCR products from canine cDNAs were analyzed by electrophoresis in a 2% agarose gel. GAPDH was used as the internal control [3]. Lanes: 1, normal canine mammary gland tissue; 2, CF33 cultured in DMEM; 3, CF33 cultured with siRNA in Oligofectamine medium; 4, CF33 cultured with dsRNA in Oligofectamine medium; 5, CF33 cultured without siRNA in Oligofectamine medium; 6, CF33 cultured without siRNA in DMEM with no Oligofectamine.

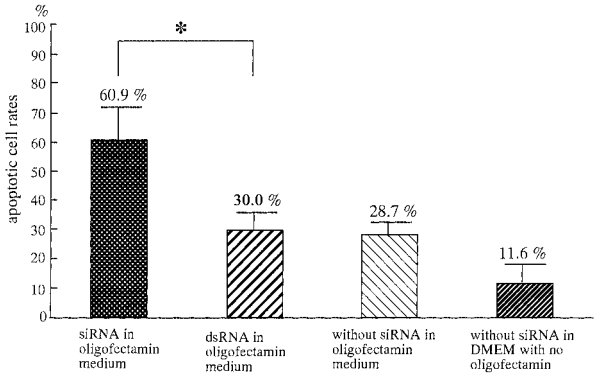

Fig. 2. Average of apoptotic cell rates in CF33 cultured with siRNA in Oligofectamine medium, with dsRNA in Oligofectamine medium, without siRNA in Oligofectamine medium and without siRNA in DMEM with no ologofectamin were assayed at 48-hr incubation, respectively. After incubation, the percentages of apoptotic cells were determined by TUNEL analysis. Results represent the mean ± SE. * $p<0.05$, when compared to siRNA with dsRNA in Oligofectamine medium.

This study on siRNA method against canine *Bcl-xL* mRNA in CF33 suggested that canine *Bcl-x*L might be an anticancer target of canine tumors and that it might be possible to design small molecules or even RNAi regulators to control the apoptosis or cell death of cancer tissues [13].

Average apoptotic cell rates in CF33 cultured in Oligofectamine medium without siRNA and without siRNA in DMEM were 28.7% and 11.6% at 48-hr incubation, indicating that Oligofectamine had weak cytotoxicity. Therefore, it is difficult to maintain the long-term effect and selective RNAi delivery against cancer tissues *in vivo*, requiring a simple inoculation of dsRNA-expressing vector.

Further, stimulation mechanisms of *Bcl-2* and *Bcl-xL* production in canine tumor cells should be elucidated to establish new strategies for the treatment of these disorders.

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