NOTE *Surgery*

Cloning and expression analysis of prohibitin mRNA in canine mammary tumors

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ABSTRACT. Prohibitin is an antiproliferative protein that is a product of a putative tumor suppressor gene. However, there is little information on prohibitins in companion animals. In this study, we cloned canine prohibitin mRNA using RT-PCR and 3′-RACE (Rapid Amplification of cDNA Ends). The sequence was well conserved compared with those of other mammals, including human. The deduced amino acid sequence translated from the open reading frame completely corresponded to the human sequence. Canine prohibitin mRNA was expressed in all normal mammary and tumor samples examined. These results suggest that this protein plays a vital role in cell growth mechanisms and may be related to the occurrence of canine mammary tumors.
KEY WORDS: canine mammary tumor, diagnosis, expression, prohibitin mRNA, RT-PCR

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In cases of canine tumor, more than 40% of tumors in female dogs are mammary tumors [4], of which about half are malignant [7, 10]. We have shown frequent expression of the *c-kit* gene in malignant canine mammary tumors, and genetic analysis may be helpful for development of a novel diagnostic method [9]. However, information on genes with putative involvement in canine mammary tumorigenesis remains limited.

Prohibitin is a ubiquitous protein that is highly conserved among many organisms [11]. However, the physiological functions of prohibitin are not completely defined. The first mammalian prohibitin gene was isolated from normal rat liver [12], and the mRNA caused growth arrest when microinjected into HeLa cells [14]. Prohibitin binds to retinoblastoma (RB) protein in competition with E2F to inhibit transcriptional activity [16, 17], enhances p53-mediated transcriptional activity and is exported to perinuclear regions upon apoptotic stimulation [5, 7]. These results indicate the antiproliferative nature of prohibitin and have led to the proposal that prohibitin is a potential tumor suppressor gene. In this study, we cloned canine prohibitin mRNA using the 3′-rapid amplification of cDNA ends (3′-RACE) method. The deduced amino acid sequence was compared with those of other mammalian species. We also demonstrated expression of canine prohibitin mRNA in canine mammary tumor tissues using RT-PCR.

A sample of canine liver tissue was excised under anesthesia from a clinically normal female beagle. The tissue was separated from the connective tissue and cut into small pieces in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline [PBS(−);137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4] containing 0.8 mg/m*l* gentamycin sulfate and washed in PBS(−) to remove remaining blood. Total RNA samples were prepared using the acid-guanidium thiocyanate-phenol-chloroform method [2] and stored at −80°C, as described previously [8, 9]. Canine prohibitin cDNA was amplified by RT-PCR. The total RNA sample (5 μg) was incubated with an oligonucleotide containing poly d(T) (5′-GGCCACGCGTCCGACTAG-TACTTTTTTTTTTTTTTTTT-3′) and Superscript II (200 units, Invitrogen, Carlsbad, CA, U.S.A.) at 42°C for 1 hr. The reaction mixture was heated at 70°C for 15 min to inactivate the reverse transcriptase. Ten units of RNase H (Toyobo, Osaka, Japan) were added, and the mixture was incubated at 37°C for 20 min. This sample was used as a template for further PCR. Prohibitin cDNA was amplified by *Z-Taq* DNA polymerase (TaKaRa, Otsu, Japan) with prohibitin-specific sense (5′-CAGAGTGGAAGCAGGTGTGAG-3′) and antisense adaptor-containing (5′-GGCCACGCGTCCGAC-TAGTAC-3′) primers. PCR was performed at 98°C for 1 sec, 55°C for 1 sec and 72°C for 10 sec (30 cycles), followed by final extension at 72°C for 5 min. The PCR product was detected using 1.0% agarose gel electrophoresis and purified using a GFX^{TM} column (GE Healthcare Bioscience, Piscataway, NJ, U.S.A.). The DNA fragment was subcloned into a pCR2.1 plasmid vector using a TA cloning kit (Invitrogen). DNA sequencing of the PCR product was carried out using

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the fluorescence-labeled dideoxynucleotide termination cycle sequencing method with a PRISM310 genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence data obtained were analyzed by GENETYX-Mac ver.11.

Total RNAs were extracted from 2 canine normal mammary glands and 12 mammary tumor samples (Table 1). These samples were obtained from a tumor resected surgically at the Veterinary Teaching Hospital of Osaka Prefecture University. For histological examination, some tumor samples were fixed in 10% formalin and embedded in paraffin. Thin sections were, then, prepared and stained with hematoxylin-eosin. The histopathological diagnoses of these tumors are listed in Table 1.

All tumor tissues were separated from the connective tissue and cut into small pieces in Ca^{2+}- and Mg^{2+}-free PBS(−) containing 0.8 mg/m*l* gentamycin sulfate. The samples were then washed in PBS(−) to remove necrotic tissues.

Reverse transcription using 5 μg of each total RNA sample was performed with Superscript II and the poly d(T)-containing oligonucleotide described above. Using the cDNA samples as templates, expression of canine prohibitin mRNA was detected by PCR with a prohibitin-specific primer set (sense, 5′-CGCTCTCGACCACGTAATGT-3′; antisense, 5′-TCGCCCTCGGGGAGATGAT-3′; 443 bp) [11] and recombinant *Taq* DNA polymerase (Toyobo). PCR was performed at 94°C for 1 min, 55°C for 2 min and 72°C for 2 min (30 cycles), followed by final extension at 72°C for 5 min. An α-tubulin primer set (sense, 5′-TCCATCCTCACCACCCACAC-3′; antisense, 5′-CGCTTGGTCTTGATGGTGGC-3′; 458 bp) was used to detect α-tubulin as an internal standard [9]. After 1.0% agarose gel electrophoresis, PCR products were stained with ethidium bromide and analyzed by NIH Image.

Canine prohibitin mRNA and its deduced protein sequence were compared to the human, mouse and rat sequences (Fig. 1 and Table 2). The predicted length of prohibitin mRNA and the number of amino acids were 1,003 bp and 273 aa, respectively. The sequence was completely homologous to the human sequence (Fig. 1), and only a single amino acid differed between the canine sequence and those of rat and mouse (Table 2). Expression of prohibitin mRNA in canine mammary normal and tumor tissues was examined by RT-PCR. The products of RT-PCR were confirmed by direct sequencing. As shown in Fig. 2A, all tested tissues expressed prohibitin mRNA. The fluorescence intensity of the RT-PCR product amplified from canine prohibitin mRNA was normalized using the intensity of the product amplified from α-tubulin mRNA as an internal standard. The quantified data are depicted in Fig. 2B. Samples from three cases (3, 4 and 5) had the same expression level as that in normal mammary glands; slightly elevated expression was

Table 1. Histological classification of canine mammary tumor samples

Sample No.	Diagnosis	Age (years)
1	Adenocarcinoma	8
2	Malignant mixed mammary tumor	10
3	Benign mixed mammary tumor	7
4	Adenocarcinoma	11
5	Malignant mixed mammary tumor	9
6	Adenocarcinoma	12
7	Malignant mixed mammary tumor	9
8	Malignant mixed mammary tumor	14
9	Malignant mixed mammary tumor	9
10	Malignant mixed mammary tumor	7
11	Malignant mixed mammary tumor	6
12	Malignant mixed mammary tumor	6

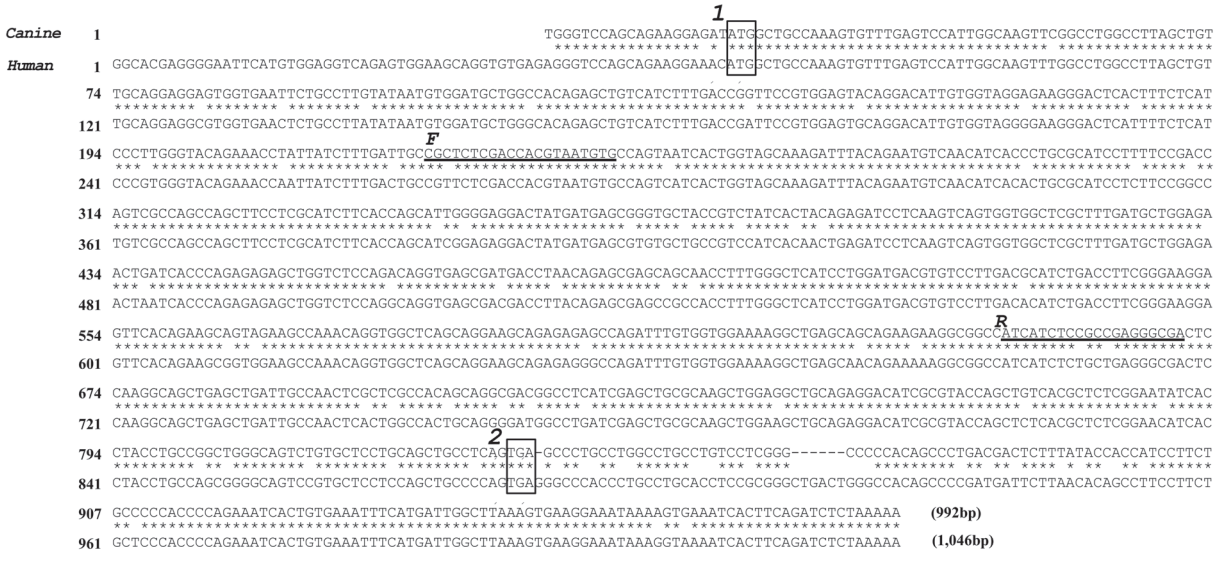

Fig. 1. Sequence of canine prohibitin cDNA compared with the human sequence. Asterisks show corresponding nucleotides; "F" and "R" indicate the annealing sites of the prohibitin-specific primer set; and "1" and "2" show the initiation and termination codons, respectively.

Table 2. Homology of canine prohibitin mRNA and its putative amino acid sequence with those of human, rat and mouse

Species	Homology (%)		NCBI Number
	cDNA	Amino acids	
Dog	/ (992 bp)	/ (272 aa)	EU188843 (mRNA)
Human	91.6% (1,009 bp)	100% (272 aa)	BC013401 (mRNA)
Rat	86.6% (992 bp)	99.6% (272 aa*)	NM031851 (mRNA)
Mouse	87.7% (999 bp)	99.6% (272 aa*)	NM008831 (mRNA)

*Putative amino acid sequences were translated from the open reading frame for each mRNA.

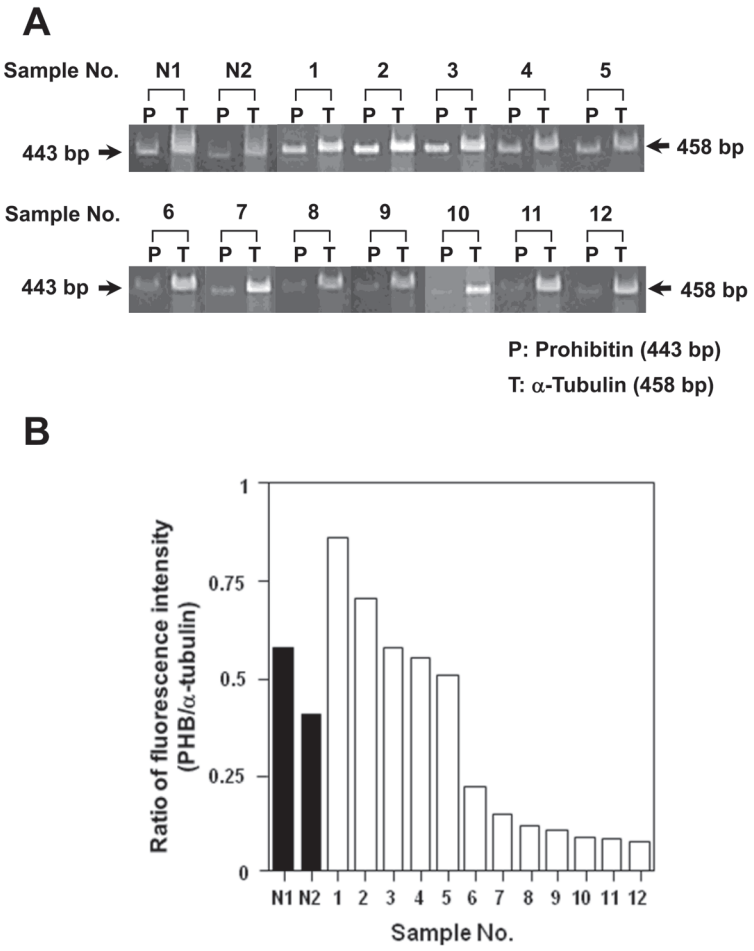

Fig. 2. A: Agarose electrophoresis of the products of RT-PCR using a canine prohibitin-specific primer set. Sample numbers are shown in Table 1. N1 and N2 indicate canine normal mammary gland samples. B: Relative expression level of canine prohibitin mRNA against α-tubulin mRNA. Fluorescence intensities of RT-PCR products of canine prohibitin mRNA relative to those of α-tubulin mRNA were measured using NIH Image.

found in 2 cases (1 and 2) compared with normal samples; and reduced expression of less than half that of prohibitin mRNA in normal mammary glands was apparent in 7 cases, including 6 malignant mixed mammary tumors and 1 adenocarcinoma. Thus, a clear relationship between the expression level of prohibitin mRNA and the pathological diagnosis was not observed, but reduced expression of prohibitin may be of importance and this requires validation in a further study.

Prohibitin is a ubiquitous and conserved protein from bacteria to human. In addition to the roles described above, prohibitin is involved in signal transduction pathways of estrogen and androgen receptors, vitamin D receptor and

IgM antigen receptor [1, 6, 15, 18]. These findings suggest that prohibitin is an essential protein for cells in normal and tumor tissues, since the canine homolog was expressed in all tumor samples examined in this study. In human mammary tumors, the expression level of prohibitin protein and point mutations in the prohibitin gene are correlated with malignancy [13]. These results suggest that a further study is needed on the relationship between the expression level of prohibitin mRNA and the prognosis in canine mammary tumor. It is well known that many proteins related to cell proliferation and cell cycle regulation, such as Myc family members, have relatively conserved amino acid sequences and structures [3]. Thus, the highly conserved amino acid sequence of canine prohibitin and the consistent tumor expression of prohibitin mRNA found in this study suggest vital and irreplaceable roles of prohibitin in maintenance of cellular integrity.

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