Properties of the Tumor Suppressor Gene *Brca2* in the Cat

Toshina OONUMA¹⁾, Masami MORIMATSU^{1)*}, Kazuhiko OCHIAI¹⁾ and Bunei SYUTO¹⁾

¹⁾Laboratory of Veterinary Physiology, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

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ABSTRACT. Mammary tumors are common in cats. As mutations in human *Brca2* confer an increased risk of breast cancer, the full-length cDNA of the feline homologue of *Brca2* was sequenced to obtain a basis for studying the relationship between its function and susceptibility to mammary tumors. The feline *Brca2* cDNA is 10 kb long, and encodes 3,371 amino acids. The amino acid sequence of feline *Brca2* shares low homology with the *Brca2* of other mammals, *e.g.*, 53% homology with the murine protein. Analysis of the expression pattern of the feline *Brca2* gene revealed that, as previously reported for other mammals, it is transcribed in various tissues, including the mammary gland.

KEY WORDS: *Brca2*, feline, mammary tumor.

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Breast tumors are more common in the female cat than in any other domestic species except the dog [6], and account for 17% of the neoplasms in female cats [17]. The percentage of breast tumors that are malignant is higher in the cat (86%) than in the dog (42%). Adenocarcinoma predominates among malignant mammary tumors in cats [11], and in many biological and histological aspects (*e.g.*, rapid growth, high metastatic rates to distant organs at an early stage, and poor prognosis) it resembles adenocarcinoma in women more closely than does experimentally induced cancer in inbred laboratory animals [10, 24]. In both humans and cats, treatment of advanced breast cancer is often futile and disfiguring, making early detection a high priority in the management of this disease.

Germline mutation of the *Brca2* gene in humans carries a high risk of developing breast cancer [16]. The *Brca2* gene appears to act as a tumor-suppressor gene, in that carriers show loss or mutation of the wild-type allele within tumors (loss of heterozygosity). After sequencing the full-length cDNA, many reports have described mutational analyses of the gene in families and in primary tumors [9, 12, 21]. The *Brca2* cDNA sequence, which predicts a large protein of 3,418 amino acids, shows no obvious homology to any other protein [5, 26]. The tumor suppressor function of *Brca2* appears to be associated with its biochemical interaction with Rad51 protein, which plays a central role in maintaining chromosome stability and double-strand break repair [1, 14, 19, 25].

To establish a basis for diagnostic methods and for using as a prognostic factor, and to understand the role of *Brca2* in mammary tumors, we report the sequence of feline *Brca2* cDNA. We also studied the pattern of gene expression.

The polymerase chain reaction (PCR) and direct sequencing were performed using the primers shown in Table 1. The amplified fragments are shown in Fig. 1A. The primer sequences were designed from reported human (GenBank

accession No.: NM_000059), murine (U65594), and canine (AB043895) sequences, or designed from the feline sequence that we obtained using these primers. Total RNA was extracted from the testis of a mongrel male cat using TRIzol reagent (Invitrogen, Leek, The Netherlands). Total RNA (2 µg) was denatured at 70°C for 10 min, cooled immediately, and reverse transcribed using 200 units of M-MLV reverse transcriptase (Invitrogen), 25 pmol of random primer, and 10 nmol dNTPs in a total volume of 20 µl at 37°C for 50 min. After heating at 75°C for 15 min, PCR amplification was performed with 2.5 units of *Taq* polymerase or KOD Plus (TOYOBO, Osaka, Japan), 1.5 mM MgCl₂, 20 nmol of dNTPs, and 20 pmol of primers. PCR was conducted for 30 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, and extension at 68°C for 2.5 min. PCR amplification of the 5' and 3' cDNA ends of feline *Brca2* was performed with the RACE system (Invitrogen), and 10 pmol of the target-specific primers shown in Fig. 1. For the RACE system, PCR was conducted for 30 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 5 min. The sequences were determined by direct sequencing using BigDye Terminator Cycle sequencing reagents and an Applied Biosystem Sequencer 310 (Perkin Elmer, Tokyo, Japan).

To explore the tissue distribution of feline *Brca2*, the RT-reaction was performed for total RNA extracted from testis, prostate, ovary, mammary gland, spleen, thymus, lymph node, heart, liver, kidney, and brain. PCR amplification was performed with the primers shown in Fig. 1, with forward primer No. 8 and reverse primer No. 9. RT-PCR was performed as described for cDNA sequencing, except that 1 µg of total RNA was used. The PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. Glyceraldehyde-phosphate dehydrogenase (GPDH) was used as a control.

The open reading frame of the feline *Brca2* cDNA was 10 kb long, encoding a large protein of 3,371 amino acids (accession No. AB107955). The feline *Brca2* protein

*CORRESPONDENCE TO: MORIMATSU, M., Laboratory of Veterinary Physiology, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan.

Table 1. Profiles of the PCR products and primers used in PCR and sequencing of feline *Brca2*

Fragment number	Primer	Nucleotides	Sequence (5' to 3')	Fragments size
I	Reverse	148–167	<u>TAACTGATTTTATATCCAGA</u>	168
II	Forward	79–98	ATAAGCCTCAATTGGTTGA	586
	Reverse	645–664	AGCTTTTAAAAATAGCAGTA	
III	Forward	462–481	GCTTATTCATACACCGAGGC	945
	Reverse	1387–1406	<u>TCATGAGATTCAAGACACTG</u>	
IV	Forward	1254–1273	CCTCATAATCACGGATAAAG	638
	Reverse	1872–1891	GCAATTAACCTGAATCAGCA	
V	Forward	1620–1239	AGAGGATTCTTTATGTACAA	1346
	Reverse	2946–2965	ACAGTCTTGCCCAATTATCC	
VI	Forward	2814–2833	<u>GAACATAGATGATCTTACAG</u>	659
	Reverse	3453–3472	<u>CATCTTTCTGTTCCCTCAGGA</u>	
VII	Forward	3283–3302	TCAAATCGTAATTTAACTCC	891
	Reverse	4154–4173	AGCTTTCATAACTTCCAAAC	
VIII	Forward	4034–4053	<u>TTCATAAAGAAGAAAATGGC</u>	2040
	Reverse	6054–6073	<u>CTAGTATTCCCTTAACTTTG</u>	
IX	Forward	5978–5997	<u>ATTTACTATCATCTGCTTTC</u>	1682
	Reverse	7640–7659	<u>ACCTGGGGTGTCACACAGAG</u>	
X	Forward	7619–7640	<u>GAAAAGAAGAATTTTATAGGGC</u>	1512
	Reverse	9111–9130	AATTATGGCATTTCATCTGAC	
XI	Forward	8917–8936	<u>GCAACAAAGAAAACCTCAGTA</u>	595
	Reverse	9492–9511	GATAATTCATCTCATTATTG	
XII	Forward	9423–9442	<u>CTATGCTTCAGAGCCACACA</u>	1058
Sequencing primer				
No. 1	Forward	2029–2048	<u>GATCTTGATTATAAAGAAGC</u>	
No. 2	Forward	4437–4456	<u>TCATGGAGAAATGGAAATAC</u>	
No. 3	Reverse	5323–5342	<u>GAAACCGTCACTTTAATGGC</u>	
No. 4	Forward	6406–6425	<u>TGGAAATTGGAAACTGAAGC</u>	
No. 5	Reverse	7099–7118	<u>TCTTCCACATGAGTATTCTT</u>	
No. 6	Forward	8000–8019	<u>ATGGATGGTATGCCATTAAAG</u>	
No. 7	Forward	8307–8326	<u>ATACCCCATACAGTGGATGG</u>	
No. 8	Forward	9747–9766	<u>TGGACCAAATATGAAACAC</u>	
No. 9	Reverse	10250–10269	<u>ATCAAAGCACAGAGGCAGAC</u>	

For the RACE fragments (fragments I and XII) only the specific primers are shown. Under lined sequences are obtained from feline *Brca2* sequence.

showed 68, 53, 78, and 32% homology with the human [21], murine [18], canine [15], and chicken *Brca2* proteins [20, 23], respectively. The degree of homology for feline and other mammals is low, but similar to values reported previously for mouse and human *Brca2* proteins [18]. Thus, the degree of homology is sufficient to deem that the PCR product obtained in this study is feline *Brca2*. In fact, we have not produced any other homologous PCR products using our PCR procedure. Feline *Brca2* includes predicted functional domains, such as BRC repeats [3], P/CAF interacting motif [8], three of NLSs [27], BLAT domain [23], and C-terminus Rad51 binding domain [14, 19], that are conserved in the species reported to date (Fig. 1B). The tissue distribution of feline *Brca2* was investigated by RT-PCR, using RNA from brain, lymph node, thymus, heart, liver, kidney, testis, mammary gland, prostate, ovary, and uterus (Fig. 2). As previously reported for the human and mouse, *Brca2* was expressed in the mammary gland, testis, ovary, uterus, thymus, and brain. Because the functional domains are important for the tumor suppressor function of human *Brca2* [4, 8, 23, 27], and because feline *Brca2* was expressed in mam-

mary gland, it may be possible that feline *Brca2* also acts as tumor suppressor in the cat.

Since the human *Brca2* mutations that cause mammary tumors occur throughout its sequence [22], it was deemed necessary to obtain the entire feline *Brca2* sequence. Therefore, we amplified the full-length feline *Brca2* cDNA by RT-PCR. This should permit the genetic analysis of feline breast cancer, to determine whether mutations of this gene are also responsible for a predisposition to mammary tumors in felines. If so, this technique will be useful for genetic diagnosis of hereditary mammary tumors in the cat. Since it has been reported that some feline breeds have twice the risk of developing mammary carcinoma as all breeds combined [11], the presence of a mammary tumor susceptibility gene in the cat, such as *Brca2*, may be possible. Loss of *Brca2* expression in the cat may cause tumors, since the human homologue is established as a tumor suppressor. Conversely, overexpression of *Brca2* in human sporadic breast cancer is reported to predict a poor prognosis [2, 7]. In addition to several proposed prognostic factors for feline mammary tumors [13], such as the age of onset and the diameter

Fig. 1. The schema of cDNA or protein of feline Brca2. (A) Amplification products of feline Brca2 cDNA. Twelve cDNA fragments were amplified from feline testis RNA. The Roman numerals I to XII represent the fragments covering the full-length of the feline Brca2 cDNA; the Arabic numerals 1 to 9 and the triangles represent the sequencing primers. The respective numbers correspond to those in Table 1. (B) The upper open bar represents feline Brca2 protein, and the solid bars show the predicted functional domain of feline Brca2, P/CAF interacting motif (a), eight of BRC repeats (b), BLAT domain (c), three of nuclear localization signals (NLSS, d) and C-terminus Rad51 binding domain (e). The middle bar is the human Brca2 protein and the lower one is the murine Brca2 protein. The shaded boxes represent the degree of homology between these proteins, which were divided into 100 amino-acid blocks and compared. AA: amino acids.

Fig. 2. Expression of feline Brca2. Total RNA from various tissues was used for RT-PCR. Lane 1: brain, lane 2: thymus, lane 3: lymph node, lane 4: heart, lane 5: liver, lane 6: kidney, lane 7: testis, lane 8: mammary gland, lane 9: prostate, lane 10: ovary, lane 11: uterus.

of the primary tumor, feline *Brca2* mRNA expression might also be a prognostic factor in feline mammary tumors.

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