Cloning and Sequencing Full Length of Canine *Brca2* and *Rad51* cDNA

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ABSTRACT. Mammary tumors are the most common neoplasm in female dogs, *Canis canis*, and in women. Mutations in human *Brca2* confer an increased risk of female breast cancer. Previous studies have shown that the *Brca2* tumor suppressor protein interacts with the recombinational repair protein Rad51. We cloned the full-length cDNA of the canine homologues of *Brca2* and *Rad51* to obtain a basis for studying their relationship with susceptibility to mammary tumors. The canine *Brca2* and *Rad51* cDNAs are 11 and 1.5 kb long, encoding 3,471 and 339 amino acids, respectively. The amino acid sequence of canine *Brca2* showed 68% homology with the human protein, and 58% homology with a murine protein. There were highly conserved regions in the C-terminus of all three proteins, where the Rad51 interacting domain and putative nuclear localization signals are located. Comparing with the partial genomic sequence previously reported, we found possible nuclear polymorphisms in exon 11, some of which result in amino acid substitutions. On the other hand, canine Rad51 protein had extremely high homology (99%) to the human and murine proteins. Expression of both *Brca2* and *Rad51* was detected in the mammary gland, suggesting that these two genes interact in the canine mammary gland.

KEY WORDS: *Brca2*, genetic instability, mammary tumor, *Rad51*, tumor suppressor gene.

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In female canines and women, mammary tumors are the most frequent neoplasm [5, 15]. They constitute 40% of all tumors in female dogs, which is three times the incidence of mammary tumors in humans [4, 12, 20]. No other animal species has such high provability of onset of mammary tumors.

The treatment of advanced breast cancer is often futile and disfiguring, making early detection a high priority in management of the disease. Six years ago, the human breast cancer susceptibility gene *Brca2* was identified [22, 23]. After sequencing the full-length cDNA, many reports described mutational analyses of the gene in families and in primary tumors [7, 9, 19]. The *Brca2* gene consists of 27 exons, which encode an 11- to 12-kb transcript. The *Brca2* cDNA sequence, which predicts a 3,418 amino acid protein, shows no obvious homology to any other protein [3, 22]. The tumor suppressor function of *Brca2* appears to be associated with its biochemical interaction with Rad51 protein [11, 17, 21]. Rad51 plays a central role in mediating homologous recombination events, and can promote strand-exchange *in vitro* [13, 14]. A recent report suggested that alterations in the *Rad51* gene are involved in the development of hereditary mammary tumors [8].

To understand the role of tumor-related genes in canine mammary gland tumors, we describe the cloning and sequencing of canine *Brca2* and *Rad51* cDNA as a first step toward the functional analysis of these genes. We compared the canine sequences with those of other species, and studied the pattern of gene expression.

MATERIALS AND METHODS

cDNA cloning of canine *Brca2* and *Rad51*: The polymerase chain reaction (PCR) was performed using the primers shown in Fig. 1A. For *Brca2*, the primer sequences corresponding to exon 11 were designed from the sequence reported by Bignell *et al.* (GenBank accession number: Z75664), while those for other regions were designed from reported human (GenBank accession number: NM_000059) and murine (GenBank accession number: U65594) sequences. *Rad51* primers were designed using human (GenBank accession number: D14134) and murine (GenBank accession number: D13803) sequences.

Total RNA was extracted from the testis of a 3-year-old male beagle by the guanidine-isothiocyanate method [2]. The total RNA (4 µg) was denatured at 70°C for 10 min, cooled immediately, and reverse transcribed using 200 units of M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD, U.S.A.), 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 Extensor Long PCR Enzyme Mix (AB Gene, Surrey, U.K.) 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 canine *Brca2* and canine *Rad51* was performed with the RACE System (Gibco-BRL, Gaithersburg, MD, U.S.A.), and 10 pmol of the target-specific primers shown in Fig. 1A. PCR was conducted for 30 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 55°C, and extension at 72°C for 5 min. The PCR products were subcloned into pGEM-T Easy Vector.

cDNA sequencing and analysis: The sequences were

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A

Clone number	Primer	Nucleotides	Sequence (5' to 3')	Product size
Canine Brca2				
No. 1	Reverse	453-472	AGGAGATTGTTGGTATATTG	473
No. 2	Forward	287-306	ATAAGCCTCAATTGGTTTGA	2092
	Reverse	2359-2378	TTGCGGCCGCGCAGGGATGACAGACAATCA	
No. 3	Forward	2176-2196	TTGCGGCCGCTGTTTACAGAATGACTCAGA	2266
	Reverse	4422-4441	AGCTTTCATAACTTCCAAAC	
No. 4	Forward	4273-4292	TGATTCCAGTAAAAATGATA	2396
	Reverse	6649-6668	TGACGTCGACAGTGTCTGGGGTTCTCTTA	
No. 5	Forward	6459-6478	TTGCGGCCGCATTTACTATCATCTGCTTTC	1809
	Reverse	8246-8267	TTAGGCATCTATTAGCAAATTC	
No. 6	Forward	8112-8133	GAAAAGAAGAATTTTATAGGGC	2117
	Reverse	10211-10228	TGGCTGAAATGCCTTCTG	
No. 7	Forward	9974-9993	ATGTCTTCTCCCAATAATGA	985
Canine Rad51				
No. 8	Reverse	473-492	TTTGTTCAGCTTTGGCTTCAC	493
No. 9	Forward	274-295	ATGGCWATGCGATGCAGCTTG	1020
	Reverse	1274-1293	TCAGTCTTTGGCATCKCCCA	
No. 10	Forward	1173-1192	GTATCTGAGGAAAGGAAGAG	341

Fig. 1. Cloning canine *Brca2* (A) and *Rad51* (B) cDNA. (A) Profiles of cloning primers and PCR products of canine *Brca2* and *Rad51*. For RACE clones (clones 1, 7, 8 and 10) only specific primers are shown. Primers containing the restriction site are: No. 2 reverse, No. 3 forward, No. 4 reverse, and No. 5 forward. (B) Seven cDNA clones were amplified from canine testis RNA. Shown here are the seven clones representing the full-length canine *Brca2* cDNA. The upper bar shows the canine *Brca2* protein (3,471 amino acids). (C) Canine *Rad51* cDNA was amplified in the same way. The three clones represent the full-length canine *Rad51* cDNA. The upper bar shows the canine *Rad51* protein (339 amino acids).

determined for at least three independent clones using Big Dye Terminator Cycle sequencing reagents and an Applied Biosystem Sequencer 310 (Perkin, Elmer, Tokyo, Japan). The complete nucleotide sequences and the predicted amino acid sequences were analyzed and compared with the GenBank databases using the personal computer-based sequence-analyzing software Clustal X 1.81 and GENE-TYX-MAC (Software Development, Co., Tokyo, Japan).

The tissue distribution of canine Brca2 and Rad51: RT-reaction was performed for RNA extracted from testis, ovary, mammary gland, spleen, skeletal muscle, and brain.

PCR amplification was performed with the following primers (the nucleotides in parentheses indicate mixtures): canine *Brca2*, 5'-CCGCTCGAGTCACCAATTCATTGTCATTTTGTG-3' and 5'-CAGAGCCACACACAGCTCAA-3'; canine *Rad51*, 5'-ATGGC(AT)ATGCA(AG)ATGCAGCTTG-3' and 5'-TCAGTCTTTGGCATC(GT)CCCA-3'. RT-PCR was performed as described for cDNA cloning, except that 1 μ g of 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.

A

1 MPVGCKERTPTFFEIFKTRCNQADLGPISLWFEELSLEAPPYNSEPTTEESGYKISYEPNLFKTPQRKPYNQLASTPIVREPIYQOSPLKELDKYRLDSG
101 KDITDSKHKSCCTMKSKMDRANVTSPPLNSYLSSESVLRCTHVTTPQREKSVVCGSLFHTPRLMKGTQPKRISESLGAEVDSMDSSSSSLATPPTLSSRI
201 SESLGAEVDSMDSSSSSLTVLIVRDEEVSAAVFPNDTATFIFKSCFSNHDESLLKKNDRFIPCGPGKENKNQREAKSQSLGNSFGKVNSTKDFHVKSTPNVL
301 EGEVHEKVLVDVSEEDSFLCVPKYKTRNLQIKTSKTRKNIFNETKTSCEEAQKQMKENKHSVSEMEPNDSHPLDWNVTHEKPFNGTDKISKEIVL
401 SSASGCSDLTLSSLNGAQMEKTPLLHTSYDQNNSEKDLIITDKECTNFITLNSWPQISNVPKYSEKTLNEEIVVNKINEGQCLESHEDSVSVKQAIYE
501 TTLIASPLQGIKRSIFRIRESPEGMSNAMFSNNMTNPNFKEPEASESGLEKHTICSQKEDSLCTSSIDDGSPATIKHTSVALKNLGLISSLKKTCKFI
601 YVINDETSNQLKTKQKQESRLINLSTQFEANAFEGPLTFTNADSGLLHSSSIKKNC**LQNDSEK**PALSITSSFGTILRKVSSNGASSPNKRIISQDPDYK
701 **EAKINKKLESF**ITTE**TDCLSSLQEKHEDDAKQ**RVSD**IK**EKVLP**TVSEHPVPHSEVEGSDIHFQSPESFSFDCDNTSLLTPSSRDS**SPSSLVVMSRGKE
801 **SYKISEKLCKNHETGFELTKNIPMEKNQDIHVLNADSKNAKLST**EKHITVASSSVKVQFNQANLTTIQKDKQKETTISKITVNPNSEELFPDDENN
901 **VLKITNESNTPVLGNTKELHDSNLCCVRDSVPKNS**TMVVC**TDLD**DQK**TAKVSI**MKDCYSSSIDDLTERNRSTIKQQLKMTLDQDSKSDITSDIVRKSNNG
1001 **SDYMDNWARLSDPISNHSFENGFKTASNKEIKLSENNIRKSKMLFKDIEEHYPTNLACLEIVNTSSLESQKKPSKSHALDPQ**SINIISGFVQNSTYVSDS
1101 **ESGHTAPPTLSLQKQDFSNRNLTPSQKAEITELSTILEESG**SQFE**TQFRKPSHIIQKNPFEMPENQ**LITLNSTSKENKDDDLHLLTNAPSISQVDSKKS
1201 **EGII**GGKQ**KFACLSRTSCNRSASGYSTDKNEVEFRG****FYSARGTKLVNGSEALQAKKL****FS**DLENINEETSVEVDRSFSSSKYNDSVSMIQIEDCNKLN
1301 **EKNK**CRLILQNNIEMTTDIFVEEYTESRRHTENEG**QC**TDAGRNTCNSES**SDGSDSSKNDTVYI**HEEENGLPCIDQHNIDLKLFSQFMKEGNTQIK**EGL**
1401 **SDLTCL**EVMAEETS**SVTMSNKQ**LTA**NTGQNI**KDFDT**YILSFQ**TAS**RKNI**RVSR**ESLN**KARS**LLNQK**WTEELNNFSDSLNSELLPGIDIKKTDISNHE
1501 **VIENTERKDKIT**KESDLIGTENILLIQOR**PESKIKKIK**ESA**VLGFHTASG**KIEIT**KESL**DKV**KNLF**EKEQDNSEITNFSHRGAKMSKDRECKD**GRE**
1601 **LACGTEITIT**PEYETHSLEKKLV**SNEIAAL**RP**LLSDNL**YKQ**TENL**KISDHASQKVDVHENTEKETAKKPTMTYTNQSTYSIAENSPLT**FTYTGGRK**
1701 **ISVSEASL**FEAK**KWL**REG**WDDQ**SE**RINA**AKVNC**LKEY**PDY**VENP**SCGSSNS**SAIT**ENDKNHSEKQGSTYLSNSTMSNSYSYHPGCHSSEVYNKSEY
1801 **LSRSKIDNSGIEPVIK**NI**RERKNIGF**SEIMSPG**READTPQ**SVNEDICVEKLATNSCKNKNTA**IKVAIS**DSNNF**TIQKL**NSDSNNSVPA**YSTVNSKRV**
1901 **FVAHQTKVT**EG**FTDNC**SMVTKQ**NTKS**SDT**CHAE**IVADY**PKALD**SEA**IFPNS**LGAIECSP**SEKVFADIQ**SE**QTSQ**LNQ**MSG**LEKVSE**TPPCQ**INSKTS
2001 **DRCELPRGKLP**KS**SVSYTN**ACG**IFST**ASGKS**VQVSDAAIQKAREVF**SKLE**SAKQLF**EVSLKDNEEHSEKFTNEENTV**IYTSQ**NLL**SAFSG**FTASG**KQ**
2101 **VVPVSE**ALCKV**KGML**EEF**NLIR**TESCLQ**BSSTSRQ**DSV**KMP**PPSCIGK**RTP**EHSRNSKLDKACNKEFRLSSNCNNQSGSSENHHSIKVSPCPSQLKRD**KP**
2201 QLLVSGSGSLVENI**HLPG**KEQALPKNI**KTEIG**KAETFPNL**PVK**TNIEFCSTYKDPENYFETETVEIAKAFMEDGELTDS**ELL**SHAKHFV**FTC**QNTKEMV
2301 LLNSRIGKRRGDALSVSGEPPIK**RNLL**NEFGRII**KNQ**ETSLKASKSTPDGILKDRSLFMHHISLEPI**SCGP**FRTEERQEIQ**NP**FTAPGQ**EF**LPSKSHFY
2401 EHLASEKSSSNLSVS**RQPF**CMVPATGNEKRRHLI**APGK**PVKV**FVPP**FKTSKSHFRDEQCISKNTKLEKNKQNSKIDELGSGDSEKNINDSGIHQ**LK**NN
2501 SNQAA**TI**IFTKNEKEPLDLITNLQ**NARDIQ**DMRIKKQ**RQHIF**PQGS**LYLAKT**STLPRISLREAVEGRVPSAC**SHQ**LYMYGVSKHC**VK**INSKNAES**FQ**
2601 PHAQDY**PGKE**LWSGEG**IQ**LPDGGW**LIP**SN**DGKI**GKEEFY**RALCD**TPGVDPNCISRVWVY**NHYRWII**WKLAAEF**AF**PK**E**FANRCLSP**ERV**LLQ**LK**YRCD
2701 VEIDKSR**SAIK**KIMERDDTA**AKT**LVLCISEI**ISS**ADIS**ETSS**SKTSSVG**TKKVGII**IALTDGWYAIKAQ**LD**PPLALV**KNG**RLTVGQKITIHGAELVGS
2801 PDACTPLEAP**ESL**MKISANSTR**PACWY**TKLGFSPDPRFP**PL**LSL**FS**DGGNGVCVDVVQ**RAYPIQ**WMERTPSGLC**IFR**NEEEKEATKYAEIQ**Q**KK
2901 LEVLFNKIQAEFEKNDENITKQ**CIP**SCAL**TRQ**QIC**ALQ**DGAELYEAVTNAPDPSDLEGYFSE**EQ**L**RAL**NNH**RQ**MLNDK**KQ**AIQLEFFK**AMES**AEQGEQ**I**
3001 LPRDVT**TVW**KLRIISYRKKEKDSV**LSI**WRSPDLYSL**IEG**KRYRIY**HLA**ASQSKSGKANTQ**L**TATK**TQYQ**QLPASDEILSQVYQ**PRE**PLYFNK**LL**
3101 DPDPQ**PPC**SEVDLIGFVVSVVKKIGLAPVVYLSDECHN**LLA**IK**FW**TD**FN**EDI**IKPY**T**LIA**SNLQWR**PEAK**SGIPT**L**FAGD**FSR**FSAS**PKE**EHFQ**ET**FKH
3201 MKNTVENIGMFYND**AE**NKL**VH**ILN**AND**PKLSTPTKDYASE**PHTA**Q**IV**LG**IGN**FLMSSPN**NEM**NYQ**SPL**SLCK**PK**ESV**PI**PGSTQ**MT**SKSYCKE**KE**MD
3301 **DPKTKCKRR**ALDFLSRV**PL**PPSV**SPIC**TFVSPAAQ**AFQ**PPRSCGT**KYE****TLMKKEL**NSPQ**MT**PRK**FN**DL**SL**ESDS**IA**DEELAMINTQ**ALL**L**GSP**GEH**Q**L
3401 VSVSDSTR**TAPT**SSKDY**LGL**KRHSTAPGVRG**PESQ**ACTRKREPRVQ**NTS****DLKRTI**SETAEATNTKMT**MNW***

B

1 MAMQMQL**EAN**ADTSVEEESFG**QP**IPSR**LQ**CGIN**AND**VKKLE**EAG**FHTVEAVAYAP**KKEL**ISIKGISEAKADKILTEAAKL**VPM**GFTT**ATE**PHQRSE**II**
101 QITTSK**EL**DKLLQGG**IT**GS**IT**EMFGE**FR**TGKTQ**IC**HT**LA**VT**CQLP**IDRGGGEGK**AM**YID**TE**G**FR**PER**LLA**VAERYLGSGSDVLD**N**VAYARG**FN**TD**HQ**
201 TQLLYQASAM**ME**SR**YAL**LIVDSAT**ALY**RTDYSGRGEL**SAR**Q**M**HLARFL**RL**LADEFGVAVVIT**NQ**VVAQVDGA**AM**FAADPK**NP**IGG**NI**IAHAST**RL**
301 YLRKGRGETRICKIYDS**PL**PEAE**AM**FAINADGVGDA**KD***

Fig. 2. The full-length canine Brca2 and Rad51 amino acid sequences. (A) The deduced amino acid sequence of canine Brca2 (GenBank accession number: AB043895). The region in bold face is exon11, and the eight underlined areas are the BRC repeats. Three sequences in bold italics are the predicted NLSs. (B) Deduced amino acid sequence of canine Rad51 (GenBank accession number: AB043896).

RESULTS

The full-length canine *Brca2* cDNA was cloned in seven fragments, while the *Rad51* cDNA was cloned in three, as shown in Fig. 1. The open reading frames encoded by the canine *Brca2* and *Rad51* cDNA were 3,471 and 339 amino acids long, respectively (Fig. 2).

The canine *Brca2* protein is longer than the human and murine proteins by 53 and 143 amino acids, respectively.

The canine *Brca2* protein shows 68 and 58% homology with the human and murine *Brca2* proteins, respectively. At the nucleotide level, homology between the canine and human *Brca2* cDNA is 76%, whereas that between canine and murine cDNA is 59%. Canine *Brca2* includes the BRC repeats that are conserved among the species so far reported [1]. In addition, the nuclear localization signals (NLS) identified in the human protein [18] exist in the C-terminal region of the canine protein (Fig. 3). Comparison of our

Fig. 4. Polymorphisms in canine *Brca2* exon 11. The sequence of canine *Brca2* that we cloned was compared with that determined by Bignell *et al.* (GenBank accession number: Z75664).

cDNA sequence with the partial genomic sequence reported by Bignell *et al.* [1] identified several nuclear polymorphisms in exon 11. These involve the following nucleotide substitutions: 2,305 A to G, 3,904–3,906 CCC to AAA, 4,071 C to A, 5,077 insert T, and 5,102 delete T. Some of these result in amino acid substitutions (Fig. 4). In contrast to *Brca2*, the canine *Rad51* protein has extremely high homology (99%) with the human and mouse proteins.

The tissue distribution of canine *Brca2* and *Rad51* mRNA was investigated by RT-PCR, using RNA from testis, ovary, mammary gland, spleen, skeletal muscle, and brain. As previously reported in human and mouse [3, 14, 16, 19], *Brca2* and *Rad51* mRNA were expressed in mammary gland, testis, ovary, and brain (Fig. 5).

DISCUSSION

We cloned and sequenced the full-length cDNA of canine *Brca2* and *Rad51*. Although Bignell *et al.* partially sequenced exon 11 of canine *Brca2* in a pioneering work [1], no information is available concerning its cDNA. Since human *Brca2* mutations that cause mammary tumor exist in the stretch of overall sequence (<http://archive.uwcm.ac.uk/uwcm/mg/ns/1/387848.html>), it seems necessary to obtain the entire sequence of canine *Brca2*. Thus, we amplified the full-length canine *Brca2* and *Rad51* cDNA by RT-PCR. This should permit the genetic analysis of breast cancer and analysis of the relationships between these genes, to determine whether mutations of these genes in canines are also responsible for a predisposition to mammary tumors.

The overall amino acid sequence homology of *Brca2* proteins, 68% between canine and human and 58% between canine and mouse, is lower than values reported for other mammalian tumor suppressor genes, such as *p53*, *RBI* and *APC*, which show over 80% homology [3, 16]. However, this degree of homology is similar to values reported previously for mouse and human *Brca2* proteins. Thus, the degree of homology is sufficient to deem that the clone obtained in this study is canine *Brca2*. In fact, we have not produced any other homologous clones using our cloning procedure. In spite of the low overall homology of *Brca2*,

Fig. 5. Expression of canine *Brca2* and *Rad51*. Total RNA from various tissues from an adult beagle was used for RT-PCR. Lane 1: testis, lane 2: ovary, lane 3: mammary gland, lane 4: spleen, lane 5: skeletal muscle, and lane 6: brain. *GPDH* was used as a control.

we can recognize highly homologous regions. Such evolutionarily conserved regions are expected to be of functional importance. A region in the N-terminus that interacts with a transcriptional co-activator protein p300/CBP-associated factor (P/CAF) is highly conserved in the canine sequence [6, 10]. In the middle region corresponding to exon 11, we confirmed the presence of BRC repeats that participate in the interaction between *Brca2* and *Rad51*, as revealed in a human study [21]. Another homologous region is part of the C-terminal region that was also identified to interact with *Rad51* in the mouse [11, 17]. Additionally, the C-terminal region also includes the NLSs [18]. Of the three NLSs identified in human *Brca2*, NLS1 is well conserved, but NLS2 and 3 are not as well conserved. These results support the previous findings that NLS1 is the most important for the appropriate localization of *Brca2* protein [18]. In addition to these domains, there should be some functional domains in the C-terminus, because observed subregions in this area show homology exceeding 80%. Alternatively, it is possible that the regions with low homology are responsible for canine specific function of *Brca2*. However, we could not recognize any motifs or putative functional domains specific for canine *Brca2*. Further experimental researches are required to examine this possibility.

Comparison of our sequence with that by Bignell *et al.*

[1] suggests the existence of nucleotide polymorphisms in canine *Brca2*. Some of these contribute to amino acid polymorphisms. It is intriguing that one polymorphism alters BRC repeat 5, which is important for the interaction of human *Brca2* with Rad51 [21].

In contrast to *Brca2*, the canine Rad51 protein has extremely high homology (99%) with the human and mouse proteins. Rad51 is a homologue of *Escherichia coli* recombination protein RecA [13], and the conservation of the sequence indicates its importance throughout evolution [14].

Analysis of the expression pattern of the canine *Brca2* and *Rad51* genes revealed that, like the human [19] and mouse [3] genes, the canine genes are widely transcribed. Both are also expressed in the testis, ovary and brain. The expression of both genes in the mammary gland suggests that these two molecules interact in the canine mammary gland.

In summary, we cloned and sequenced the full-length cDNA of canine *Brca2* and *Rad51* to provide a basis for study of their relationship to the onset of mammary tumors.

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