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Short Communication

Interactions between canine RAD51 and full length or truncated BRCA2 BRC repeats

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24 **Abstract**

25 In humans, mutations of the breast cancer susceptibility protein BRCA2 interact with
26 recombinase RAD51 and increase the risk of cancer. This interaction occurs via a series of
27 eight BRC repeat sequences of BRCA2. A mammalian two-hybrid assay using individual
28 BRC repeats demonstrated that all the repeats except BRC6 bind RAD51 strongly (BRC1, 2
29 and 4), with intermediate strength (BRC8), or weakly (BRC3, 5 and 7). In serial deletion
30 mutation experiments, the binding strengths were increased when the C-terminal BRC repeat
31 was removed from BRC1-8, BRC1-5 and BRC1-3. These results provide an understanding of
32 the basic function of canine BRCA2 and may be helpful to estimate the effect of missense or
33 truncation mutations in canine mammary tumours.

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35 *Key words:* Canine; BRCA2; RAD51

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In humans, mutations in the breast cancer susceptibility gene BRCA2 are associated with a predisposition to breast and ovarian cancers (Moynahan and Jasin, 2010). The BRCA2 protein is required for homologous recombination repair of double-stranded DNA breaks (DSBs) (Moynahan and Jasin, 2010) and has been shown to promote assembly of RAD51 recombinase onto single-stranded DNA (Jensen et al., 2010). To initiate DSB repair, BRCA2 and RAD51 bind directly at the highly conserved BRC repeats of BRCA2, which consist of 13 conserved amino acid (aa) residues (Bignell et al., 1997).

In female dogs, mammary tumours are the most frequently occurring neoplasm. It has been reported that mammary tumor development in dogs is associated with BRCA2 (Rivera et al., 2009).

To explore possible roles of canine BRCA2 and Rad51 in mammary tumours, we cloned their cDNAs, confirmed mRNA expression in mammary glands (Ochiai et al., 2001) and suggested the presence of interactions between the C-terminus of canine BRCA2, a region distinct from BRC repeats, and RAD51 in irradiation-induced DSBs (Ochiai et al., 2004). Although these studies suggest structural and functional similarities between canine and human BRCA2 proteins, their low sequence homology (68%) creates difficulties in the estimation of their tumour-suppressive roles across species.

We and others recently identified several variations in BRC repeats in canine mammary tumours (Yoshikawa et al., 2008; Hsu et al., 2010), but the functional significance of these variations remained largely unknown. We believe that studying the interaction

between canine BRC repeats and RAD51 will extend our knowledge of the DNA repair and tumour-suppressor functions of BRCA2. In the present study, we used yeast and mammalian two-hybrid assays to investigate the interaction between individual or deletion mutants of canine BRCA2 BRC repeats and RAD51.

cDNA fragments of BRC repeats (NM_001006653) and RAD51 (NM_001003043), encoding NCBI reference sequences (RefSeq¹) were obtained and cloned into vectors for yeast and mammalian two-hybrid assays. Methods were described in Ochiai et al., (2004). The list of oligonucleotide primers is provided in a Supplementary Table 1.

Canine BRC repeats were aligned with human BRC repeat 4 (BRC4) consensus motifs and sequence fingerprints (Fig. 1). The conservation of aa residues in these sequences and their homology with human and chicken sequences are summarised in Supplementary Table 2. The sequences of canine BRC1, 2, 4, 7 and 8 shared high sequence homology with human and chicken (Bignell et al., 1997). BRC3 and 5 had well-conserved sequence fingerprints, but low sequence homology with human and chicken. Little conservation of the BRC repeat features was observed in BRC6.

We constructed yeast two-hybrid assay vectors (Fig. 2A) and investigated the direct association of canine BRC repeats with canine RAD51, as shown in Fig. 2B. We also confirmed the binding of canine RAD51 to itself, as previously observed (Ochiai et al., 2004) (Fig. 2B). The mammalian two-hybrid assay using individual BRC repeats demonstrated that

¹ <http://www.ncbi.nlm.nih.gov/refseq/>

all the repeats except BRC6 bind RAD51 strongly (BRC1, 2 and 4), with intermediate strength (BRC8), or weakly (BRC3, 5 and 7) (Fig. 3A). BRC6 contains fewer consensus residues (6/13) (Bignell et al., 1997) and sequence fingerprints (3/8) (Lo et al., 2003) (Fig. 1) than do other repeats, so BRC6 may have lost RAD51 binding ability. BRC repeats sharing high homology between species tend to strongly bind RAD51 (Supplementary Table 2). Recently, a missense variation in BRC3 (K1435R) was identified in tumour-bearing dogs (Yoshikawa et al., 2008; Hsu et al., 2010), but the functional significance of this variation remained unknown. Although canine BRC3 has low homology to human (59%) and chicken (19.2%) repeats (Supplementary Table 2), it does bind to RAD51 (Fig. 3A); thus, K1435R could have some effect on binding. Further studies are needed to clarify the significance of K1435R in tumorigenesis.

Samples from human breast cancer often reveal BRCA2 truncation mutants that have lost some or all of their BRC repeats which were recorded in The Breast Cancer Information Core Database (BIC²) (Arai et al., 2004). To explore the importance of the arrangement of the eight BRC repeats in canine BRCA2, deletion analysis was performed (Fig. 3B). We speculated that RAD51 binding would be weakened when any of the eight BRC repeats were truncated and were surprised to find that removal of the C-terminal BRC repeat from some truncation mutants (e.g., BRC1-3, BRC1-5 and BRC1-8) increased Rad51 binding strength. These results indicate that BRC3, 5 and 8, in the context of the eight BRC repeats of BRCA2, may have novel suppressive roles in RAD51 binding. We have also identified an unrelated RAD51 interaction domain at the C-terminus of canine BRCA2 (Ochiai et al., 2004).

² <http://research.nhgri.nih.gov/bic/>

Although BRCA2 has eight BRC repeats and a C-terminal domain, a single human BRCA2 binds to six RAD51 molecules (Jensen et al., 2010). Thus, all eight BRC repeats may not bind to RAD51, or the binding may be regulated by the suppressive roles of the BRC repeats as shown here.

We hypothesise that the different strengths of RAD51 binding and the correct arrangement of the eight BRC repeats are necessary for proper execution of homologous recombination. Our findings suggest that BRCA2 mutants containing truncations of the BRC repeat sequences may cause fluctuations in RAD51 binding strength (Fig. 3B), which may be one of the causes for predisposition to mammary tumours.

In summary, we analysed the basic function of BRC repeats and RAD51 of normal dogs by using a two-hybrid assay. Our findings regarding the interactions between BRCA2 and RAD51 will be helpful for understanding BRCA2 polymorphisms or truncation mutations.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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128 **Appendix A. Supplementary material.**

129 Supplementary data associated with this article can be found in the online version at

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Table 1

Primer pairs for generating the eight BRC repeat constructs of canine BRCA2 and RAD51.

Primer		Sequence
Canine BRC1	1F	5'-GGGGATCCACATTACCTCAGATATAGTTAGG-3'
	1R	5'-GGATCCAGCTGTGTGACCACTTTCAC-3'
cBRC2	2F	5'-GGATCCCAAGAACCAGCTGTAACAGAAG-3'
	2R	5'-GGATCCAGCGTCAGTACATTGGTTAC-3'
cBRC3	3F	5'-GGATCCCTATGTCAAATAAACAGCAG-3'
	3R	5'-GGATCCTATTTTCAGTACCAATTAGG-3'
cBRC4	4F	5'-GGATCCCGAAAGAAAGTGACCTAATTGG-3'
	4R	5'-GGATCCGTCCCACAAGCTAATTCACG-3'
cBRC5	5F	5'-GGATCCTATCAGATCATGCCTCTCAG-3'
	5R	5'-GGATCCCCACATGAAGGATTTTCTAC-3'
cBRC6	6F	5'-GGATCCCATGCAAAAATAAAAATACAG-3'
	6R	5'-CGGATCCTAATCTGCCACAATTTCTGC-3'
cBRC7	7F	5'-GGATCCACCAAAGTATGTCTGGATTGG-3'
	7R	5'-GGATCCAATGTTCTTCATTATCTTTA-3'
cBRC8	8F	5'-GGATCCAACTCTTTCCTGAAGTATCAC-3'
	8R	5'-GGATCCTGGGGTTCTCTTACCAATAC-3'
cRAD51	F	5'-GAGAAGCTTCATGGCTATGCAAATGCAGCTTG-3'
	R	5'-GCTCTAGATCAGTCTTTGGCATCTCCCA-3'

E-only Supplementary Table 1

Conservation of consensus motifs and sequence fingerprints in canine BRC repeats.

BRC repeat	Number of conserved amino acids		Sequence homology (%)	
	Consensus motifs	Sequence fingerprints	Human	Chicken
	(total <i>n</i> = 13)	(total <i>n</i> = 8)	(GenBank U43746)	(GenBank AB066374)
BRC1	10	7	80.8	65.4
BRC2	8	8	84.6	50.0
BRC3	11	7	56.0	19.2
BRC4	12	8	84.6	53.8
BRC5	9	8	52.7	7.7
BRC6	6	3	42.3	30.8
BRC7	11	8	84.0	69.2
BRC8	13	8	73.1	61.5

Figure legends

Fig. 1. Canine BRC repeats were compared with human BRC4 consensus sequences (Bignell et al., 1997) and RAD51-binding sequence fingerprints (Lo et al., 2003). (A) Human BRC4. (B) Consensus motifs of BRC repeats. (C) Sequence fingerprints of BRC repeats, with the eight critical residues indicated in italics. Notations are as follows: o, polar; I, hydrophobic; i, slightly hydrophobic; +, positively charged; –, negatively charged. (D) Sequence alignment of canine BRC repeats: underlined, conserved consensus motifs; bold, conserved sequence fingerprints; dark grey box, amino acid (aa) substitutions unfavourable for RAD51 binding; grey box, aa substitutions producing residues with significantly different properties.

Fig. 2. (A) Two-hybrid constructs were introduced into SFY526 yeast cells. The left panel depicts the two constructs containing overlapping canine BRC repeats and the right panel shows the full-length canine RAD51 used in this assay. Numbers correspond to the amino acids in canine BRCA2 or RAD51. (B) The yeast two-hybrid assay was conducted using the plasmids pACT (TA) and pAS1 (DBD) (Ochiai et al., 2004). Empty vectors were used as negative controls (emp). To identify interactions between BRC repeats and RAD51, colonies were picked from DO plates (-Leu, -Trp) to assay β -galactosidase reporter gene expression. Grey colonies indicate protein interactions; white colonies indicate no interaction. β -Galactosidase activity was measured using the Gal-Screen System (Applied Biosystems). The results are given as the mean (standard error) ($n = 10$).

Fig. 3. The left panel depicts the individual canine BRC repeat constructs (A) and BRC

205 deletion mutants (B), using the primers listed in the Supplementary Table 1. These constructs
206 were introduced into HeLa cells to determine their interaction with RAD51 in a mammalian
207 two-hybrid assay measuring luciferase activity (right panel). Numbers correspond to the
208 amino acid residues in canine BRCA2. HeLa cells were co-transfected with the canine BRC
209 repeat constructs or canine RAD51 expression vector constructs and with the reporter
210 plasmids pG5luc and pRL-TK. The pRL-TK construct was used to normalise transfection
211 efficiency (Ochiai et al., 2004). Lysate luciferase activity was determined 48 h after
212 transfection. DBD, GAL4-DNA-binding domain fusion protein; VP16, VP16 transactivation
213 domain fusion protein. The results are given as the mean (standard error) ($n = 4$).

Figure 1

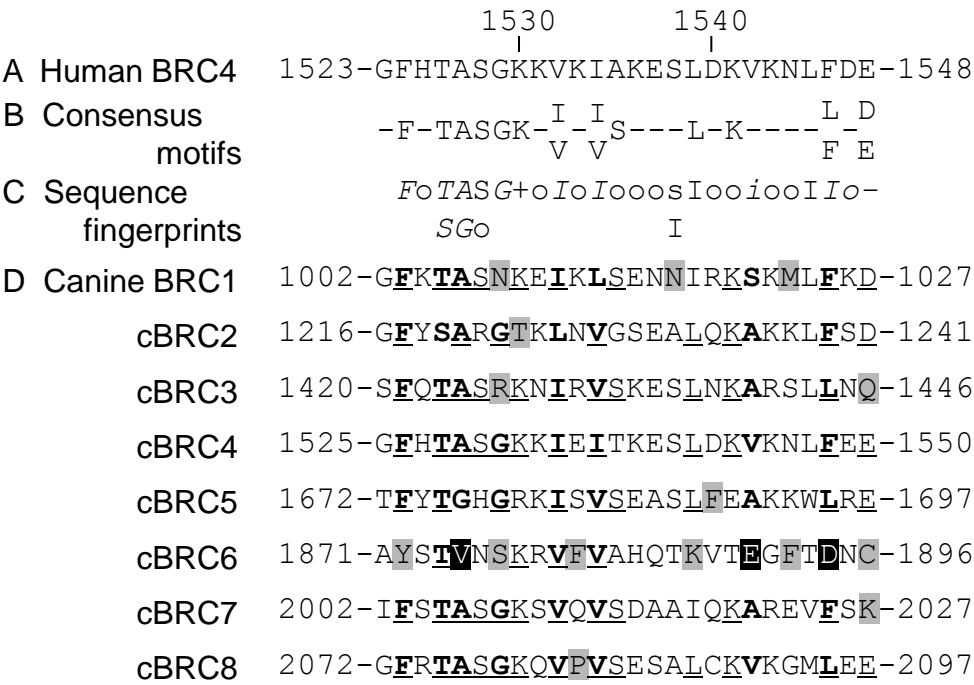


Figure 2

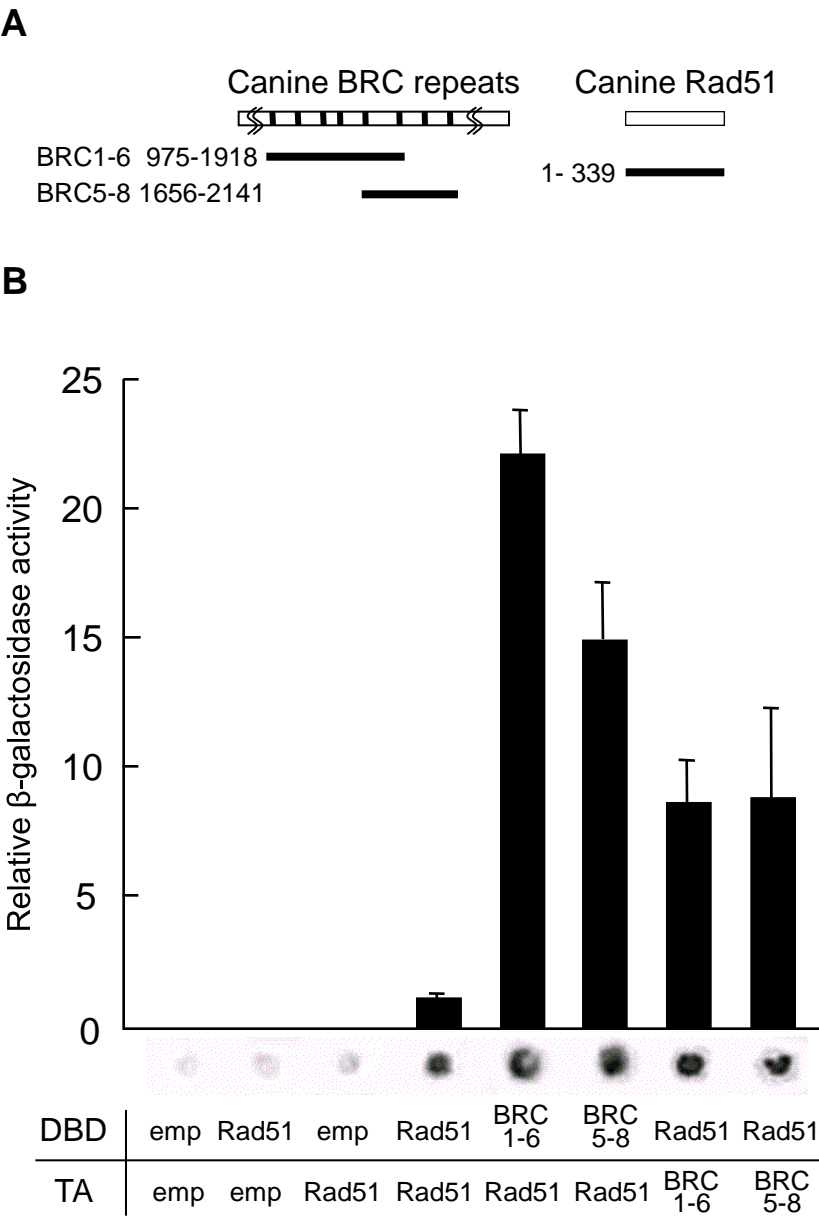


Figure 3

