

Relationship between three novel SNPs of BRCA1 and canine mammary tumors

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ABSTRACT. The BRCA1 gene plays an important role in the development of human breast cancer, and recent research indicated that genetic variations of BRCA1 are also related to canine mammary tumors (CMTs). Here, using rapid amplification of cDNA ends (RACE), we cloned the 5'- and 3'-UTRs of BRCA1. By direct sequencing of the flanking sequences of the 5'- and 3'-UTRs of BRCA1, three previously unreported single-nucleotide polymorphisms (SNPs) were identified, two (-1228T >C, -1173C >T) in the putative promoter regions and one non-synonymous SNP (63449G >A) in exon 23. Compared with 16 normal samples, the sequences from 34 CMTs suggested that SNP (-1173C >T) was associated with the development of CMTs (odds ratio (OR)=2.57, 95% confidence interval (CI): 1.07–6.15).

KEY WORDS: breast cancer susceptibility gene 1 (BRCA1), canine mammary tumors (CMTs), rapid amplification of cDNA ends (RACE), single-nucleotide polymorphisms (SNPs), untranslated region (UTR)

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BRCA1 (breast cancer 1, early onset) is one of the most important breast cancer susceptibility genes in humans and is involved in many key cellular functions, including DNA repair, cell cycle regulation and transcriptional regulation [9, 13]. It is reported that genetic variations of BRCA1 have been significantly associated with canine mammary tumors (CMTs) [2, 5, 8]. Genetic variations mainly consist of single-nucleotide polymorphisms (SNPs); both the coding and noncoding SNPs might affect the development of tumors.

The 5'- and 3'-UTRs are important regions of mRNA: 5'-UTRs are significantly important for gene expression, and 3'-UTRs are widely recognized as important posttranscriptional regulatory regions of mRNA. In human BRCA1, some genetic variations of 5'- and 3'-UTRs are related to breast cancer [7, 10]. In the present study, our aim was to explore the relationship between SNPs of BRCA1 5'- and 3'-UTRs and CMTs. However, there are few reports on the sequences of canine BRCA1 5'- and 3'-UTRs. Hence, canine BRCA1 5'- and 3'-UTRs were firstly cloned and sequenced, and then, polymorphism analysis was conducted in CMTs and healthy controls.

Cloning of the 5'- and 3'-UTRs of canine BRCA1: Four ovary samples collected from client-owned dogs undergoing spay surgery at the China Agricultural University Veterinary Teaching Hospital (CAUVTH) were subjected to RNA extraction using RNAiso Plus (Takara, Dalian, P. R. China). For 5' and 3' rapid amplification of cDNA ends (RACE) and RACE-PCR, a SMARTer™ RACE cDNA Amplification Kit (Clontech, Takara) and Takara Ex Taq™ Hot Start Version (Takara) were used according to the manufacturer's instructions. Two canine BRCA1 cDNA gene-specific primers, a 5'

RACE primer and 3'RACE primer, were designed according to the manufacturer's guidelines (GenBank Accession No. U50709, Table 1). Gel-purified RACE products were cloned into pMD™18-T vector (Takara), and 10 different independent clones were picked as recommended. PCR products were sequenced by Beijing Genomics Institute (BGI), and alignment analysis was carried out with the DNAMAN software.

The only observed 5'-UTR of BRCA1 was determined to be 162 base pairs (bp) and to be interrupted by an intron of 983 bp; the 3'-UTR consisted of 258 nucleotides and contained a putative polyadenylation signal (AAATAA) 39 nucleotides upstream from the poly (A) tail (Fig. 1). Two important regulatory promoter elements, one CREB binding domain and one CAAT box, which are conserved in the human and murine BRCA1 promoters [1, 3, 11], were also identified in the flanking sequences of the 5'-UTR of canine BRCA1, indicating the possible location of the promoter region. The sequences have been submitted to GenBank and are available under Accession No. KM458140.

A search of the GenBank database for the 5'-UTR sequence of canine BRCA1 resulted in matches with XM_005624313, one of the 7 predicted transcript variants of canine BRCA1. In this study, no alternative first exon of canine BRCA1 gene was detected from the four ovary samples. Mouse and rat BRCA1 only have one first exon [1, 3], while human BRCA1 has two alternative first exons, 1a and 1b, which are controlled by promoters α and β , respectively; both transcripts were expressed in many primary or tumor tissues and normal or cancer cell lines [11, 12]. This difference may result from the fact that the human exon 1b contains the primate-specific ALU sequence.

Polymorphism analysis of flanking sequences of BRCA1 5'- and 3'-UTRs: Thirty-four mammary tumor samples from 34 dogs bearing CMTs and 16 normal mammary gland samples from 16 healthy dogs were surgically obtained at the CAUVTH, and the procedures were approved by the Animal Welfare Committee of the Department of Clinical Veterinary Medicine of China Agricultural University. All of the

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Table 1. Primer sequences and annealing temperatures of the amplified regions

Primer	Primer sequence: 5'-3'	Chr Pos ^a
5'RACE	TCTTTTGGCACGGTTTCGATAGCCCATA	19980357–19980384
3'RACE	GAAATCACCAGGGTCCGAAGCGAGCAAG	20014492–20014519
5'UTR	5'F: CACTATCTTGCCTCATATTCG 5'R: CCCACACTCCACACCTGTTT	19959483–19959504 19959974–19959993
3'UTR	3'F: GGTACTGGACAGTGTAGCC 3'R: AAGTCACGTCACGGATC	20024288–20024306 20024824–20024841

a) Position in canine chromosome 9 (CanFam3.1).

A.

-1387 cagccatctctgtgacgtgataagcccagagtggaaaccaagtgaggg 19959572
-1337 ggcagggcctaataagaaaacttgggaaagattctttcttccgtctct 19959622
-1287 ttctaattacgttactcagggaagaattgatgaccaatcgagagccgg 19959672
-1237 agagactcttggctctttctgctecgaccttccgcaactgtttcgatct 19959722
-1187 ctgattctgagagggctcttctgctgagtgctgctgagccagtttccGTGGCAA 19959772
-1137 CAGGAAAGCGCGGAATTACAGGCAAATTGAAAGTGCAGCTGCGCGGCGC 19959822
-1087 GAGCTCGCTAAGGCTTCTTGAGTGAGGCGAGTCCGGGGGTTTTTCATAG 19959872
-1037 AACTGGGCTCTGGGCTGGGAAACCCGACGCTTCGCTCTGGGTGAAGg 19959922
-987 tgagagactccacaggaaggagagaccacggggtgctccgagggcag 19959972
-937 gaa.... (intron1).... tctttaaattcattggaacagGAAAA 19960909
1 ATG 19960912

B.

63380 GTACTGGACAGTGTAGCCCTCTACCAGTGCCAGGAGCTGGACACCTACCT 20024338
63430 GATCCCGCAGATTCACAGAGCTGCTGCAGACTCCAGCCAGCCATGCGTGT 20024388
63480 AAcatgtaggcctgtctctgcaggacccacacatgatcctaagaagga 20024438
63530 gccttttccagccctgagagctccttccacagtcccagttactgaata 20024488
63580 tttatgtacatcagcctgagaagaaattctggctatgcaagtgtccctt 20024538
63630 aaagatttctgcttcaagctcctttgaaatctaccatgagcacaata 20024588
63680 ttgtgtaattttcacctgaaaataatttaaacatataaacccacc 20024638
63730 aattgagcaa(a)n

Fig. 1. The flanking sequences of the 5'- and 3'-UTRs of canine BRCA1 gene. The first codon is defined as 1 on the left side, and right side is numbered base on the position in canine chromosome 9 (CanFam3.1). (A) The flanking sequences of the 5'-UTR of canine BRCA1. Exon sequences are given in capital letters. SNPs are shown by double-underlined bold text. ATG in bold is the start codon. The putative CREB binding domain and CAAT box are indicated by solid and dotted boxes, respectively. (B) The flanking sequences of the 3'-UTR of canine BRCA1. TAA in bold is the last codon. The double-underlined bold text represents the SNP (63449G >A). The putative polyadenylation signal is shown by underlined italicized text. The poly (A) tail is indicated by a (n).

mammary tissue samples were verified by histopathological diagnosis based on the classification and grading of CMTs [6]. In the 34 tumor cases, 9 were confirmed as benign, and 25 cases were malignant.

Genomic DNA was extracted from mammary tissue samples with an EasyPure[®] Genomic DNA Kit (TransGen Biotech, Beijing, P. R. China). The primers used in detecting the variations in the flanking sequences of the BRCA1 5'- and 3'-UTRs (GenBank Accession No. NC_006591) are shown in Table 1, and they were designed with the Oligo Primer Analysis Software (v. 7). PCR amplification was performed with 2×EasyTaq[®] PCR SuperMix (TransGen Biotech) according to the manufacturer's protocol. After PCRs were completed, direct sequencing of the PCR prod-

ucts was performed by BGI. The 5'F and 3'R primers were used to sequence the flanking sequences of 5'-UTR and 3'-UTR, respectively. SNPs were queried using the Dog BLAT Search tool on the UCSC Genome Bioinformatics website (<http://www.genome.ucsc.edu/cgi-bin/hgBlat>). The statistical significance of differences was determined by Pearson's two-sided chi-square test. The odds ratios (ORs) are shown with 95% confidence intervals (CIs). Differences were considered statistically significant for $P < 0.05$. Genetic variations in BRCA1 of canine mammary tissues with or without CMTs are listed in the Supplementary Table 1 (online only).

Two novel SNPs in the putative promoter region (-1228T >C, -1173C >T) and one novel non-synonymous SNP in the coding exon (63449G >A) were detected in the flanking se-

Table 2. Analysis of BRCA1 polymorphisms -1228T >C, -1173C >T and 63449G >A for breast cancer risk association in dogs

SNPs	Allele	Number (%)		P-value	OR (95% CI)
		CMTs	Control		
-1228T >C	T allele	39 (57.35)	24 (75.00)	0.09	1.00 (reference)
	C allele	29 (42.65)	8 (25.00)		2.23 (0.88–5.67)
-1173C >T	C allele	29 (42.65)	21 (65.63)	0.03	1.00 (reference)
	T allele	39 (57.35)	11 (34.37)		2.57 (1.07–6.15)
63449G >A	G allele	66 (97.06)	32 (100.00)	1.00	1.00 (reference)
	A allele	2 (2.94)	0 (0.00)		0.97 (0.93–1.01)

quences of the 5'- and 3'-UTRs of the BRCA1 gene (Table 2).

Two noncoding SNPs were observed in the putative promoter region of BRCA1. An in silico search for putative transcription factor binding elements harbored by these two SNP sites in the sense strand sequence was done using the AliBaba 2.1 software (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html>). One SNP (-1173C >T) was predicted within a C/EBP α (CAAT-enhancer-binding protein α) binding site, and no transcription factor binding sites (TFBSs) were predicted for the other SNP (-1228T >C). Statistically, no significant association was detected for the second SNP (-1228T >C), however, the first SNP (-1173C >T) was significantly prevalent in CMTs ($P=0.03$, OR=2.57, CI: 1.07–6.15), indicating that it might be a functional SNP and might cause an increased risk of CMTs in carriers. *In vitro* assays of human BRCA1 haplotypes showed that common promoter polymorphisms can influence transcription, as nucleotide changes may alter the binding affinity of transcriptional factors [4]. Further functional analysis might warranted to determine whether or not the first SNP (-1173C >T) might have an effect on transcription efficiency.

The coding SNP (63449G >A), located in the putative codon 1851, is a non-synonymous mutation (Ala1851Thr). The replacement of a hydrophobic non-polar amino acid (alanine) with a polar uncharged amino acid (threonine) might introduce a conformational change in the protein affecting its function. However, this SNP is not within the BRCT (BRCA1 C-terminal) domain, and homologous comparison indicated that this codon is not conserved among the dog, mouse, rat and human species. No significance was detected statistically, but this SNP was only identified in dogs with CMTs (one benign and one malignant). An increased number of cases may further reveal its relationship with CMTs.

In summary, we cloned and sequenced the 5'- and 3'-UTRs of the BRCA1 gene and detected three novel SNPs in canine BRCA1; one SNP (-1173C >T) is statistically associated with CMTs. A larger number of samples might be helpful to strengthen the evidence, and further studies of functional analysis are warranted to investigate the underlying mechanism for this association.

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