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Analysis of Genetic Variations in the Exon 27 Region of the Canine *BRCA2* Locus

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ABSTRACT. Mammary tumors are the most common tumor type in women as well as in female dogs. The *BRCA2* gene encodes a large nuclear protein that is involved in DNA repair, and mutations in the human *BRCA2* confer an increased risk of female mammary tumors. The BRCA2 protein acts as a tumor suppressor, and inactivation of BRCA2 by loss of heterozygosity is implicated in mammary carcinogenesis. In this study, to establish an appropriate polymorphic marker for loss of heterozygosity analysis of the canine BRCA2, we analyzed the genomic sequences of the exon 27 regions of 30 mammary-tumor-bearing and 21 tumor-free dogs. In addition to 10204ins/delAAA, which is the only polymorphism previously identified for the canine BRCA2 locus, we discovered four novel single nucleotide polymorphisms. The analysis of these five polymorphisms revealed the presence of four allele types. Since 10204ins/delAAA was the most common of the five polymorphisms identified, we developed a PCR-based assay method to assay for this polymorphism. We believe that this method is valuable for loss of heterozygosity analysis of the canine *BRCA2* gene in tumor pathogenesis.
KEY WORDS: ammary tumor, BRCA, breast cancer susceptibility, LOH, polymorphic marker.

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In women and in female dogs, mammary tumors are the most frequently found neoplasms [21]. They constitute 40% of all tumors in female dogs, which is three-times the incidence of mammary tumors in humans [5]. No other animal has been reported to exhibit such a high incidence of mammary tumors.

In the case of human breast cancer, approximately one-tenth of the cases exhibit a familial pattern of inheritance [29]. Mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* account for 50% and 30% of these familial cases, respectively [7]. For both *BRCA1* and *BRCA2*, patients from afflicted families have mutations in one allele of the gene, and the tumors in these patients frequently exhibit loss of the other intact copy of the gene, in an event that is termed 'loss of heterozygosity' (LOH) [2, 4, 10, 12, 19, 27]. These features suggest that these genes encode tumor suppressor proteins for breast cancer. The human *BRCA2* gene encodes a large nuclear protein of 3,418 amino acids (aa). The BRCA2 protein plays important roles in the maintenance of genomic stability in DNA recombination and double strand-break (DSB) repair *via* its interaction with the Rad51 recombinase, and it is generally believed that the genomic instability observed in BRCA2-deficient cells underlies the tumor suppressor function of this protein [23, 27]. The detection of polymorphic sequences that can be used as markers is essential for the analysis of LOH. In addition, many *BRCA2* sequence alterations are associated with breast cancer risk in human [7, 8, 29, 30]. In the case of the human *BRCA2*, 1893 distinct mutations, polymorphisms, and variants have been reported (the Breast Cancer Information Core: http://research.nhgri.nih.gov/projects/bic).

In dogs, certain breeds are highly susceptible to mammary tumors, which suggests that this cancer is inheritable [3, 14, 21]. Chromosomal aneuploidy is found in canine mammary tumors [22], and thus it is very possible that repair proteins, such as BRCA2 and Rad51, are involved in the etiology of canine mammary tumors. To explore this possibility, we previously cloned and sequenced the canine orthologs of *BRCA2* and *Rad51* [16]. Although some polymorphic markers for canine BRCA1 have been reported [9, 26], those for canine BRCA2 have not, with the exception of our recent finding of a single insertion/deletion polymorphism in nuclear localization signal 2 (NLS2), which is encoded by putative exon 27, the last exon of the *BRCA2* gene [32].

In this study, we determined the exon-intron structure of the exon 27 region, performed polymorphism analysis, and established an efficient method to detect the insertion/deletion polymorphic allele.

MATERIALS AND METHODS

Structure of the exon 27 region: Intron 26 of canine *BRCA2* was amplified using the forward primer 5'-ATAGTCCTTGGCATAGGAAA-3', which is located in putative exon 26 at nucleotides (nt) 9884 to 9903, and the

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reverse primer 5'-TGGCTGAAATGCCTTCTG-3', which is located in putative exon 27 complementary to nt 10151 to 10168 (GenBank accession number AB043895.4). The exon 27 downstream region listed in the published sequence was amplified using the LA-PCR *in vitro* Cloning Kit (Takara) using the 5'-GTAAACACACTTTTTCTTTA-3' and 5'-GTGTCCCTAATTAAATGAAAG-3' primers. Total RNA was isolated from the testis of a 3-year-old male beagle using the TRIzol Reagent (Invitrogen), and the RNA was reverse-transcribed using SuperScript II (Invitrogen). 3'-RACE was performed using the 5'-GTAAACACACTTTTTCTTTA-3' and 5'-GTGTCCCTAATTAAATGAAAG-3' primers. The products were sequenced using the ABI PRISM BigDye Terminator v. 3.1 Ready Reaction Cycle Sequencing Kit and the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

DNA extraction from tissue and blood samples: Blood samples from 30 mammary-tumor-bearing dogs and blood or tissue samples from 21 tumor-free dogs were obtained from the veterinary hospitals at Iwate University, Hokkaido University, and Tokyo University of Agriculture and Technology. These samples were stored at –80°C until use. The formalin-fixed tissue samples obtained from Hokkaido University had been stored in 10% formalin at room temperature for 10–13 years. Genomic DNA was extracted from the blood or tissue samples using the PUREGENE DNA Isolation Kit (Gentra Systems).

Analysis of sequence variations: The primer pair was designed based on the sequences of intron 26 and the 3'-end of exon 27. The 1079-bp exon 27 region of the canine *BRCA2* was amplified by PCR using the forward primer 5'-CATTAGTGCATAGTTACCTTG-3', which lies 24–44 nt upstream from the intron 26-exon 27 boundary, and the reverse primer 5'-CAACTATAAGAGAACCAACAC-3', which is located in exon 27 complementary to nt 10970 to 10990. The PCR products were sequenced as described above. For the analysis of allele sequences, the PCR products were cloned in the pGEM-T Easy vector (Promega).

PCR detection of 10204ins/delAAA: Six primers were designed based on the 10204ins/delAAA sequence in the canine *BRCA2* (GenBank accession numbers AB043895 and AB210823). The 10204delAAA-specific primer (5'-GCACCAAATATGAAACACTGATG-3') and 10204insAAA-specific primer (5'-GTGGAGAATTCAACTCTTTCTTCTTT-3') were designed. Also designed were common primer pairs for normal samples (5'-GTTTCTCCAGCTGCACAGAA-3' and 5'-TGAACCCAACAAAAGGGCTTG-3') and for formalin-fixed samples (5'-GCTGCACAGAAGGCATTTCA-3' and 5'-GTCTGCTATTGAATCACTTTCCAA-3'). PCR amplification was performed with the Eppendorf Mastercycler gradient (Eppendorf) in a volume of 10 μl that contained 10–50 *n*g of template, 0.2 pmol of each of the primers, 0.2 pmol of each of the dNTPs, 1.5 mmol $MgCl_2$, 0.25 U AmpliTaq Gold (Applied Biosystems), and 1 × PCR buffer, which was supplied together with the enzyme. The following PCR conditions were used: one cycle of 10 min at 95°C, followed by 30–35 cycles of 30 sec at 94°C, 30 sec at 61.3°C, 30 sec at 72°C, and a final extension step of 2 min at 72°C. The PCR products were analyzed on 3% or 4% agarose gels, which were prepared with 1 × TAE and 0.2 μg/m*l* ethidium bromide.

RESULTS

Exon-intron structure of the exon 27 region: In a previous study, we identified the 10204ins/delAAA variation in NLS2 sequence within the exon 27 region, and we expected that more variations would be found in this region. Since, in both the human and mouse, exon 27 is the third-largest exon and encodes essential domains, such as NLSs and Rad51-binding region, it is important to determine the structure of exon 27 in the canine *BRCA2* locus [6, 15, 23–25, 27, 31]. We sequenced the cDNA and genomic DNA obtained by 3'-RACE and LA-PCR, respectively, and determined the complete structure of exon 27, including the exon-intron boundary and poly-adenylation signal (Fig. 1). Although Ochiai *et al*. [16] did not report the poly-adenylation signal (AATAAA), it starts at 10963b. Thereafter, we designed the primer pair to amplify the exon 27 region.

Analysis of variations in exon 27: The canine *BRCA2* exon 27 was amplified successfully from the blood samples of 30 mammary-tumor-bearing dogs and from the blood or tissue samples of 21 tumor-free dogs. All of the samples were checked for PCR products of the expected sizes, which indicated the absence of large deletions/insertions. We identified four novel single nucleotide polymorphisms (SNPs) in the ORF of exon 27: at nt 10180C>T, 10398A>G, 10421A>C, and in the 3'-UTR at nt 10704C>T (Fig. 1).

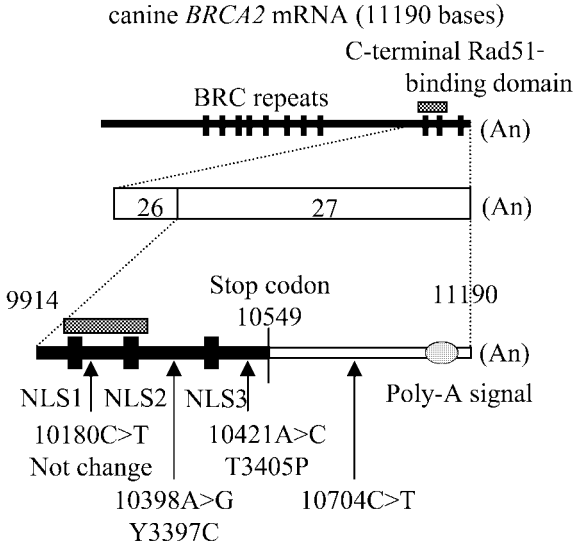

Fig. 1. The exon-intron structure of the exon 27 region of canine *BRCA2* and four newly identified variations. The coding regions of eight BRC repeats, the C-terminal Rad51-binding region, three NLSs, and the poly-adenylation [poly(A)] signal are also shown.

Table 1. Identified allele types and frequencies

Allele type[a)]	Base sequence					Allele frequency	
	10180	10204	10398	10421	10704	Tumor-bearing (%)	Tumor free (%)
A type	C	——	A	A	C	4 (6.7%)	15 (35.7%)
B type	C	AAA	A	A	C	52 (86.7%)	26 (61.9%)
C type	C	AAA	G	A	C	–	1 (2.4%)
D type	T	AAA	A	C	T	4 (6.7%)	–

a) The A- and B-type sequences have been reported previously (GenBank accession numbers AB043895 and AB210823, respectively). The C type contains variations 10204insAAA and 10398A>G. The D type has variations 10203insAAA, 10180C>T, 10421A>C, and 10704C>T.

Table 2. Relationship between genotype and tumor status

Genotype[a)]	AA	AB	AC	BB	BD	DD
Tumor-bearing dogs	–	2/30	–	25/30	2/30	1/30
Tumor free dogs	4/21	6/21	1/21	10/21	–	–

a) AA indicates homozygosity of the A-type allele; AB, heterozygosity of the A- and B-type alleles; AC, heterozygosity of the A- and C-type alleles; BB, homozygosity for B-type allele; BC, heterozygosity of the B- and C-type alleles; BD, heterozygosity of the B- and D-type alleles; DD, homozygosity for the D-type allele.

Two of these SNPs were missense variations (10398A>G and 10421A>C) and one was a silent variation (10398A>G) (Fig. 1). It was deduced that the 10398A>G SNP substitutes tyrosine (polar amino acid) for cysteine (nonpolar amino acid) at amino acid 3397 (Y3397C), and that the 10421A>C SNP substitutes threonine (uncharged polar amino acid) for proline (nonpolar amino acid) at amino acid 3405 (T3405P). Each missense variation occurred at a conserved residue in the human, cat, and dog sequences [16, 18], which suggests the importance of these amino acids from the comparative viewpoint. In the analysis of allele sequences, the SNPs 10180C>T, 10204insAAA, 10421A>C, and 10704C>T were found together, and 10204insAAA and the 10398A>G SNP were also found together, as summarized in Table 1. Therefore, in this study, we identified four allele types in exon 27. Based on the results of the χ^2 test of allele frequency, the mammary tumor morbidity rate of dogs was significantly higher with the AAA insertion (sum of allele type B, C and D, tumor-bearing dogs: 56/60, tumor-free dogs: 27/42) than with the AAA deletion (allele type A, tumor-bearing dogs: 4/60, tumor-free dogs: 15/42) ($p<0.001$). The relationships between genotype and mammary tumor status are shown in Table 2. Although the sample number was limited, the D-type (10180C>T, 10204insAAA, 10421A>C, and 10704C>T) allele was detected only in tumor-bearing dogs.

The AAA insertion/deletion variation marker: Of the five variations in exon 27, 10204ins/delAAA was determined to be the most suitable for LOH analysis, based on the fact that it represents the most dynamic change and the existence of numerous heterozygosities (Table 2). We designed four PCR primers, to amplify specific DNA fragments for each allele (Fig. 2A). The sizes of the products amplified by this primer set were 195 bp or 198 bp, 152 bp, and 94 bp, as shown in Fig. 2B. This primer set was able to amplify targets from frozen tissue samples, but not from formalin-fixed tissue samples. We then attempted to solve this problem using the second primer set. The sizes of the products amplified by this second primer set were 141 bp or 144 bp, 104 bp, and 85 bp, as shown in Fig. 2C. These primer sets distinguished three genotypes: insAAA/insAAA homozygous, insAAA/delAAA heterozygous, and delAAA/delAAA homozygous. The second primer set was useful for the amplification of the target sequences in formalin-fixed samples.

DISCUSSION

Exon 27 is located mainly downstream of the *BRCA2* gene. This exon contains sequences that code for the C-terminus of *BRCA2* and the 3'-UTR. The C-terminal sequence is highly conserved and plays some important roles in mammals. For example, the NLSs at the C-terminus are essential for the nuclear localization of human BRCA2 [25, 31]. Deletion of the C-terminal NLSs prevents BRCA2 localization to the nucleus, which results in dysfunction. This may also be true, at least in part, for the canine BRCA2. Recently, we discovered that, in the canine BRCA2, the insAAA-type NLS2 and NLS3 co-operate as functional localization signals [32]. Additionally, we recently identified the NLS2 variation, 10204insAAA causing amino acid change M3332IK, which enhanced nuclear localization [32]. Since the tumor morbidity rate of dogs was revealed to be higher with AAA insertion than with AAA deletion, it is possible that translocation efficiency of BRCA2 protein may be associated with mammary tumor morbidity in dogs. In this study, we found two novel missense variations, 10398A>G (Y3397C) and 10421A>C (T3405P), which resulted in alterations to the functions of the amino acid side-chains (from polar to nonpolar). Interestingly, these two residues are located close to NLS3 (aa 3400–3404). Because not only core basic residues (lysine and arginine)

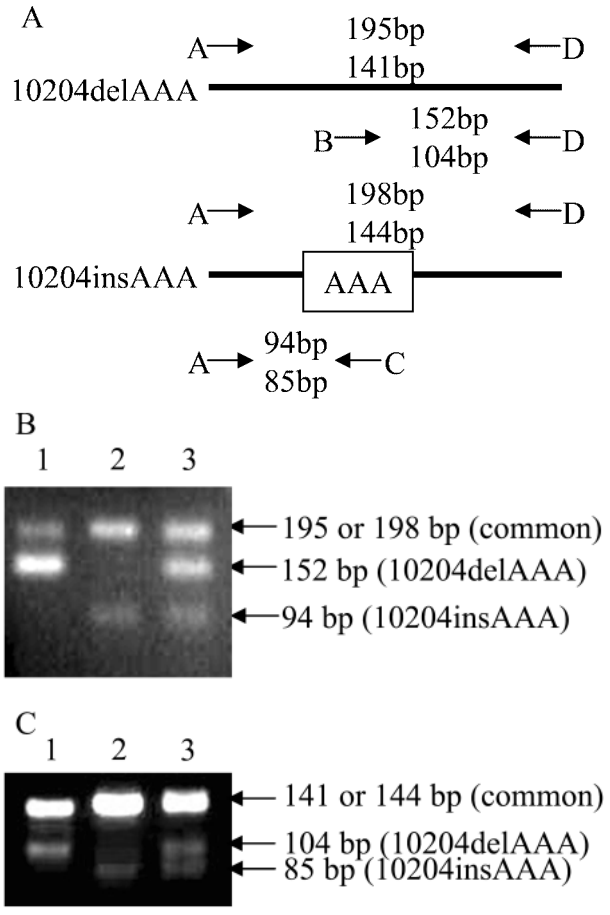

Fig. 2. Detection of 10204ins/delAAA. (A) This diagram indicates two primer sets for the detection of 10204ins/delAAA. Primers A and D are common primers, while primers B and C are 10204delAAA-specific and 10204insAAA-specific, respectively. (B) The PCR products amplified by the primer set for the normal samples. Lane 1, homozygous for 10204delAAA; lane 2, homozygous for 10204insAAA; lane 3, heterozygous. (C) The PCR products amplified by the primer set for the formalin-fixed samples. Lane 1, homozygous for 10204delAAA; lane 2, homozygous for 10204insAAA; lane 3, heterozygous.

but also surrounding amino acid residues can attenuate translocation function of NLSs, it is possible that these alterations affect nuclear localization of BRCA2 [11]. The 10421A>C variation was found in the D-type allele as the only missense variation. Although the sample number was limited, the fact that the D-type allele was detected exclusively in tumor-bearing dogs implies an association between the 10421A>C variation and mammary tumor pathogenesis. Another important role of the BRCA2 C-terminus is its interaction with the Rad51 recombinase [13, 15, 24]. This interaction was believed to be mouse-specific [1], but recently Esashi *et al.* [6] reported an interaction between the human counterparts. Recently, we also demonstrated this interaction in the canine system [17]. Missense variations, such as those found in this study, may influence this interaction. However, further studies, such as those that define the sequences essential for the interaction of canine proteins, are required to explore these possibilities.

The number of variations found in this study was fewer than expected. It is generally accepted that insertion/deletion variations are much rarer than substitutions. Indeed, many substitution mutations have been reported for the human *BRCA2* [7, 8, 28], which are listed in the database (Breast Cancer Information Core: http://research.nhgri.nih.gov/projects/bic). Since 10204insAAA was identified in many canine samples, we expected that more variations would be found in exon 27 of the canine *BRCA2*. One possible explanation is that the breeding of domestic dogs is under the control of humans, so artificial selection has accelerated the unexpected pattern of genetic variation [20].

In human, LOH of *BRCA2* was detected in both familial and sporadic breast cancer [2, 4, 10, 12, 19, 27]. *BRCA2* is a tumor suppressor gene and it is believed that LOH is a mechanism of inactivation in tumors from patients carrying a germline deleterious mutation in *BRCA2*. So, LOH analysis is a method to elucidate the relationship between breast cancer pathogenesis and *BRCA2* mutations. Using the ins/delAAA variation as a marker, we established a simple and rapid PCR-based method for the detection of LOH. The frequencies of heterozygous dogs were 7/21 (sum of genotype AB (6/21) and AC (1/21)) and 2/30 (genotype AB) for tumor-free and tumor-bearing dogs, respectively. The low frequency of tumor-bearing dogs may present an obstacle to the application of this method, although the use of formalin-fixed tissue samples may improve this situation.

In order to study tumor susceptibility genes, familial case histories are important. Unfortunately, family histories were not available for the dogs tested in this study. Due to the small sample size, breed-dependent variables were not analyzed. The novel variation markers and the assay method to determine ins/delAAA established in this study will be utilized in the future to address these important issues.

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