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Single nucleotide variations of the canine *RAD51* domains, which directly binds PALB2 and *BRCA2*

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Abstract

Tumors of the mammary glands are the most common tumors to affect entire female dogs representing between 50–70% of all tumors types, which is three times higher rate of incidence than humans. No other animal species has such high probability of onset of mammary tumors. Homologous recombination (HR) is the most important double-strand breaks (DSBs) repair mechanisms of DNA and *RAD51* plays an important role in this repair mechanism. The tumor suppressors *RAD51*, *BRCA2* and *PALB2* act together to initiate the chromosomal lesions repair. *BRCA2* and *PALB2* mutations lead to destabilization of the genome and engender cancer risk. *PALB2* binds to DNA and associates with the *RAD51* recombinase. In this study we investigate the genetic variations in *RAD51* gene, which directly interactions with *PALB2* and *BRCA2* domains. From a total of 64 canine patients with mammary tumors, 31 mammary tumors with benign and malign carcinomas and the 3 normal mammary glands were used for the study. We have identified 2 SNPs (Single Nucleotide Polymorphisms) and 7 SNVs (Single Nucleotide Variants) in canine *RAD51* exon 7- intron 9 regions, among them 7 SNVs and 1 SNPs were detected for the first time in this study.

Key Words: Dog, *RAD51*, SNPs, Mammary Tumors

Introduction

Tumors of the mammary glands are the most common tumors to affect entire female dogs representing between 50–70% of all tumors types[4], which is three times higher rate of incidence than humans. No other animal species has such high probability of onset of mammary tumors[11]. In humans, heritable breast cancers have been found related to mutations in the breast cancer sensibility gene *BRCA2*[18]. The primary function of *BRCA2* is homologous recombination (HR), and it mediates the recruitment of recombinase *RAD51* to DNA double-strand breaks (DSBs)[15]. Previously, we have identified 28 somatic SNPs in canine *BRCA2* exon 11 region and we speculate

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that T1425P variation in *BRC3* to be the most probable disease associated nsSNP and may affect *RAD51* binding strength[12].

Homologous recombination is the most important DSBs repair mechanisms of DNA and *RAD51* plays an important role in this repair mechanism. Among different types of DNA damage in the mammalian cell, DSBs has been recognized as the most lethal. *RAD51* is correlated with the search for the process of homology and strand-pairing in homologous recombination. According to the literature information, *RAD51* contains an entire homologous template. This template is exactly the same to the sequence in the nucleoprotein complex and the following stage *RAD51* catalyzes the attack of single strand DNA (ssDNA) on the homologous double-stranded DNA fragment. *RAD51* has covered with a single strand DNA and a nucleoprotein filament is formed. This form makes pairs with a homologous region in duplex DNA and cause to the activation of strand exchange. Also, *RAD51* may be required for non-homologous end joining (NHEJ) pathway to DSB repair to interact with proteins (*BRCA2, PALB2* and *RAD52*) that bind to the single-strand DNA[2,9].

RAD51 contains a complete homologous template and it's identical to the sequence in the nucleoprotein complex. After this process *RAD51* catalysis attaches to the ssDNA on the homologous double-stranded DNA (dsDNA) fragment[9].

The tumor suppressors *RAD51, BRCA2* and *PALB2* act together to initiate the chromosomal lesions repair. *BRCA2* and *PALB2* mutations lead to destabilization of the genome and engender cancer risk. For this reason, *PALB2* binds DNA and associates with the *RAD51*[5]. *RAD51* is essential for HR and responsible for the tumor-suppressive function of this repair process[15]. Observation of the presence of increased amounts of *RAD51* in multiple tumor cell types suggests a role in the initiation or progression of tumorigenesis[14].

The canine *RAD51* gene comprises 10 exons on chromosome 30 and encodes a protein of about 339 amino acids. In canine *RAD51* gene sequence, exon 6–9 regions have been covered to encode the interaction domains with the *PALB2* and *BRCA2*. Also, the sequence similarity of canine and human *RAD51* proteins is 99%[11].

The objective of this study is to investigate the genetic variations in *RAD51* gene, which directly affect the interactions with *PALB2* and *BRCA2* (exon 6–9) in canine mammary tumors (CMTs).

Materials and Methods

Tissue samples, Histopathology and DNA isolation: Tumoral masses and/or biopsies taken from the mammary gland regions of 64 canine patients were examined histopathologically and after histopathological examination a total of 31 mammary tumors (benign n = 10 and malign n = 21) were used for the study. Radiological examination of the thorax was performed to reveal detectable pulmonary metastases. Mammary gland tumor samples were resected surgically at the Uludag, Istanbul and Firat Universities, Faculties of Veterinary Medicine, Department of Obstetrics and Gynecology. For histopathological examination, a representative part of the tumor samples were fixed in 10% buffered formalin for 48 hours then they were processed according to routine tissue dehydration and clearing procedures and embedded in paraffin. Serial tissue sections at a thickness of 5 μm were stained with hematoxylin-eosin and they were evaluated histopathologically according to the current CMTs grading and classification guide[6].

Clinical and demographic information of the dogs were recorded at the time of diagnosis for the following criteria, age, breed, clinical stage, macroscopic appearance of the tumoral masses, ovariohysterectomy history, hormonal treatments, and prior chemotherapy. Case 32 was excluded from the analysis, because of the lack of pathology result (Table 1). None of the animals had undergone chemotherapy or hormonal treatments.

Table 1. The characteristics, pathological features and histopathologic classification of CMTs

Case no	Breed	Age	Tumor type	Histological Grade	Exon7 c.586A>G (N196D)	Exon7 c.595C>G (H199D)	Exon7 c.625G>T (A209S)	Exon8 c.673A>T (T225S)	Exon8 c.681C>T	Exon8 c.766G>A	Intron8 g.7,876,099G>A	Intron9 g.7,876,450GCA>ATG	Intron9 g.7,876,453A>G
1	Golden retriever	13	Complex Adenocarcinoma	II	A	C	G	A/T	C	G	G	GCA	A
2	Mixed	9	Carcinosarcoma	II	A	C	G	A	C	A	G	GCA/ATG	A
3	Rottweiler	12	Carcinosarcoma	III	A	C	G	A	C	G	G	GCA	A
4	Mixed	4	Lipid-rich Carcinoma	I	A	C	G/T	A	C	A	G	GCA/ATG	A
5	Terrier	12	Solid Carcinoma	II	A	C	G	A	C	A	G	GCA/ATG	A
6	Pincher	15	Anaplastic Carcinoma	III	A	C	G	A	C	G	G	GCA	A
7	Rottweiler	8	Adenosquamous Carcinoma	III		C	G	A	C	G	G/A	GCA/ATG	A
8	Terrier	11	Comedocarcinoma	II	A	C	G	A	C	G	G	GCA	A
9	Terrier	11	Fibroblastic Hemangiomaperitoma	-	A	C	G	A	C	G	G	GCA	A
10	Terrier	13	Tubular Adenocarcinoma	I	A	C	G	A	T	A	G	ATG	A
11	Rottweiler	13	Solid Carcinoma	II	A	C	G	A	C	G	G	GCA	A
12	Terrier	7	Anaplastic Carcinoma	III	A	G	G	A	C	A	G	GCA/ATG	A
13	Golden retriever	11	Tubular Adenocarcinoma	I	A	C	G	A	C	G	G	GCA	A
14	Pit pull	14	Osteosarcoma	-	A	C	G	A	C	G	G	GCA/ATG	A
15	Terrier	15	Complex carcinoma	III	A	C	G	A	C	G	G	ATG	A
16	Mixed	13	Malign Mix carcinoma	II	A	C	G	A	C	A	G	GCA/ATG	A
17	English Setter	9	Malign Mix carcinoma	II	A	C	G	A	C	A	G	ATG	A
18	E. Cocker Spaniel	11	Malign Mix carcinoma	I	A	C	G	A	C	A	G	GCA/ATG	A
19	Boxer	11	Malign Mix carcinoma	II	A	C	G	A	C	A	G	GCA/ATG	A
20	Kangal	8	Malign Mix carcinoma	II	A/G	C/G	G	A	C	G	G	GCA/ATG	A
21	Golden retriever	12	Complex Adenocarcinoma	II	A	C	G	A	C	A	G	GCA/ATG	A
22	Boxer	6	Papillary cystic adenoma	-	A	C	G	A	C	G	G	GCA	G/A
23	Terrier	13	Papillary adenoma	-	A	C	G	A	C	A	G	GCA/ATG	A
24	Husky	10	Papillary cystic adenoma	-	A	C	G	A	C	A	G	ATG	A
25	Terrier	8	Fibroadenoma	-	A	C	G	A	C	A	G	GCA/ATG	A
26	Terrier	16	Benign Mix Tumor	-	A	C	G	A	C	A	G	ATG	A
27	E. Cocker Spaniel	14	Cystic Ducthal Granuloma	-	A	C	G	A	C	A	G	ATG	A
28	Mixed	7	Complex Adenoma	-	A	C	G	A	C	G	G	GCA	A
29	Mixed	7	Benign Mix Tumor	-	A	C	G	A	C	G	G	GCA	A
30	Terrier	9	Benign Mix Tumor	-	A	C	G	A	C	A	G	ATG	A
31	E. Cocker Spaniel	10	Fibroadenoma	-	A	C	G	A	C	G	G	GCA	A
33	Mixed	8	Normal Mammary Gland	-	A	C	G	A	C	G	G	GCA	A
34	Mixed	6	Normal Mammary Gland	-	A	C	G	A	C	G	G	GCA	A
35	Mixed	4	Normal Mammary Gland	-	A	C	G	A	C	G	G	GCA	A

Table 2. Primers used for PCR amplification of the dog *RAD51* gene

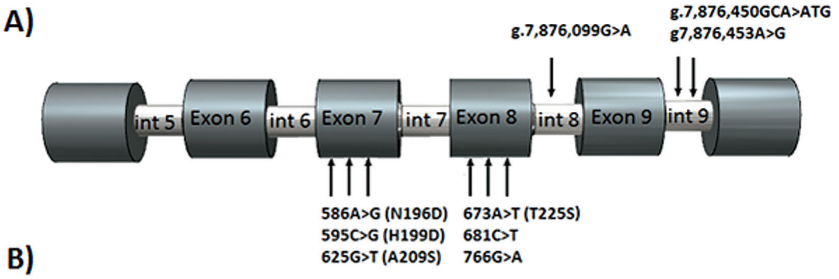
Primer Pair	Forward Primer 5'-3'	Reverse Primer 5'-3'	Annealing Temperatures (°C)	Amplicon Size (bp)	Covered Region
RAD51 _E6	GGTGGGTAGGGAAGTTGGTT	AACTTCGGGATACAGAGCATT	60	223	Exon 6, partial intron 6
RAD51 _E7	CCAGTCTGCCTGAGTTTTCA	AAGGGCTCTAGGCCATATTCA	55	221	Exon 7, intron 7
RAD51 _E8/9	GGAGCACAATCTGACACAGG	TCTGGTTTCCCCTCTTCCTT	55	984	Exon 8, intron 8, exon 9, intron 9

Genomic DNA was extracted from the 31 CMTs and 3 normal mammary glands tissue samples using the DNeasy blood and tissue isolation kit (Qiagen Cat No: 69504) according to the manufacturer's protocol.

PCR and DNA sequencing: For this study, according to the canine sequence (ENSCAFT00000014658) of *RAD51* gene, three pairs of primers were designed to amplify exons 6, exon 7, exon 8, intron 8, exon 9 and intron 9 of dog *RAD51* gene. Primer pairs and their annealing temperatures are shown in Table 2. For primer design, Primer3 software[17] was used. PCR reaction were carried out for each primers in 25 μL of total volume, containing 10 X PCR buffer (50 mM/L KCl, 10 mM/L Tris-HCl (pH 8.0), 0.1% Triton X–100), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pM/L of each primer, 50 ng dog genomic DNA and 1 U Taq DNA polymerase. PCR thermocycling conditions were as follows: denaturation at 94°C for 4 min, followed by 34 cycles of denaturation at 94°C for 45 sec, annealing at 60/55°C for 30 sec, extension at 72 °C for 40 sec and final extension at 72°C for 10 min. PCR products were analyzed by 2% agarose gel electrophoresis. Then, PCR samples were sequenced from both directions, following the purification of PCR products. Direct sequencing was performed on 3100 ABI PRISM sequencer (Applied Biosystems, USA), and sequenced by commercial services. Sequences were obtained with the same primers used for PCR amplification.

Data analysis: Sequences were analyzed using the BIOEDIT ver 7.2.5 software[8] for sequence alignment. All chromatogram peaks were confirmed by visual inspection and compared to canine *RAD51* reference sequence (Ensemble accession number ENSCAFT00000014658). The DnaSP software 5.10.01[15] was used to calculate polymorphic sites, average number of nucleotide differences (k), number of haplotypes (h), nucleotide diversity (π) haplotype diversity (Hd).

To analyse the effect of amino acid variations on protein structures we used SIFT (Sorting Intolerant From Tolerant) web-based tool. It is predicting a substitution value according to the degree of affecting the protein function. If the SIFT score is less than 0.05, the amino acid change is predicted as deleterious[10]. PROVEAN (Protein Variation Effect Analyzer) is another web-based tool, which estimates the effect of amino acid variation on protein function. It gives a PROVEAN score and the variant is considered deleterious when it is < -2.5 and is predicted to be "Neutral"[3,7]. The PolyPhen server was used to analyse the functional assessment of nsSNPs (non-synonymous Single Nucleotide Polymorphisms) and its calculates position-specific independent count (PSIC) scores for every variant. PolyPhen PSIC score >0.5 indicates that the variant is possibly damaging and probably damaging and PSIC <0.5 score is

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10         20         30         40         50
MAMQMQLEAN ADTSVEEESF GPQPISRLEQ CGINANDVKK LEEAGFHTVE
        60         70         80         90        100
AVAYAPKKEL ISIKGISEAK ADKILTEAAK LVPMGFTTAT EFHQRRSEII
       110        120        130        140        150
QITTGSKELD KLLQGGIETG SITEMFGEFR TGKTQICHTL AVTCQLPIDR
       160        170        180        190        200
GGGEGKAMYI DTEGTFRPER LLAVAERYGL SGSDVLDNVA YARGFNTDHQ
       210        220        230        240        250
TQLLYQASAM MVESRYALLI VDSATALYRT DYSGRGELSA RQMHLARFLR
       260        270        280        290        300
MLLRLADEFG VAVVITNQVV AQVDGAAMFA ADPKKPIGGN IIAHASTTRL
       310        320        330
YLRKGRGETR ICKIYDSPCL PEAEAMFAIN ADGVGDAKD
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Fig. 1. Positions of genetic variations within exon 7–9 and intron 9 regions of canine *RAD51* gene. A) The positions of point mutations identified in this study are indicated with arrows. Nucleotides are numbered using AB043896 for ORF regions. B) Canine *RAD51* protein sequence (UniProt ID: Q8MKI8). Sequence amino acids in the binding domain to *BRCA2* are shown in red. Shaded amino acids represent the interaction domain with *PALB2*.

considered as benign[1].

Results

To characterize the normal mammary gland sequence, the genomic DNA of canine *RAD51* exon 6–9 and intron 9 regions of three normal mammary gland samples were amplified and sequenced. ENSCAFT00000014658 Ensemble sequence was used as a reference sequence for *RAD51* exon 6–9 and intron 9 regions. Results showed that the sequences of exon 6–9 were identical and no SNPs/SNVs were detected in exon 6- intron 9 regions in all sampled normal mammary glands.

Compared with the sequence in healthy mammary gland, 2 SNPs and 7 SNVs were identified. Five of these SNVs were in coding regions (exon 7 and exon 8) of the *RAD51* gene, on the other hand two of these SNVs were detected in intronic regions (intron 8 and intron 9), however no SNPs/SNVs were detected in exon 6 and exon 9 regions (Fig. 1). Three mutation points were determined in exon 7, which are non-synonymous mutation: c.586A>G (N196D), c.595C>G (H199D) and c.625G>T (A209S); three mutation points were determined in exon 8, one of them was non-synonymous substitutions: c.673A>T (T225S), c.681C>T and c.766G>A (Fig. 2). All these non-synonymous substitutions were found only in the malign tumor samples. Three variation positions are only found in intron 8 (g.7,876,099G>A) and intron 9 (g.7,876,450GCA >ATG and g.7,876,453A>G) regions.

Eight variations of *RAD51* in CMTs in this study were detected for the first time when compared to available nucleotide sequences in GenBank. The *RAD51* gene exon 6-intron 9 variations were deposited in GenBank database under the access number: KX906967-KX906968.

A total of 4 non-synonymous substitutions for

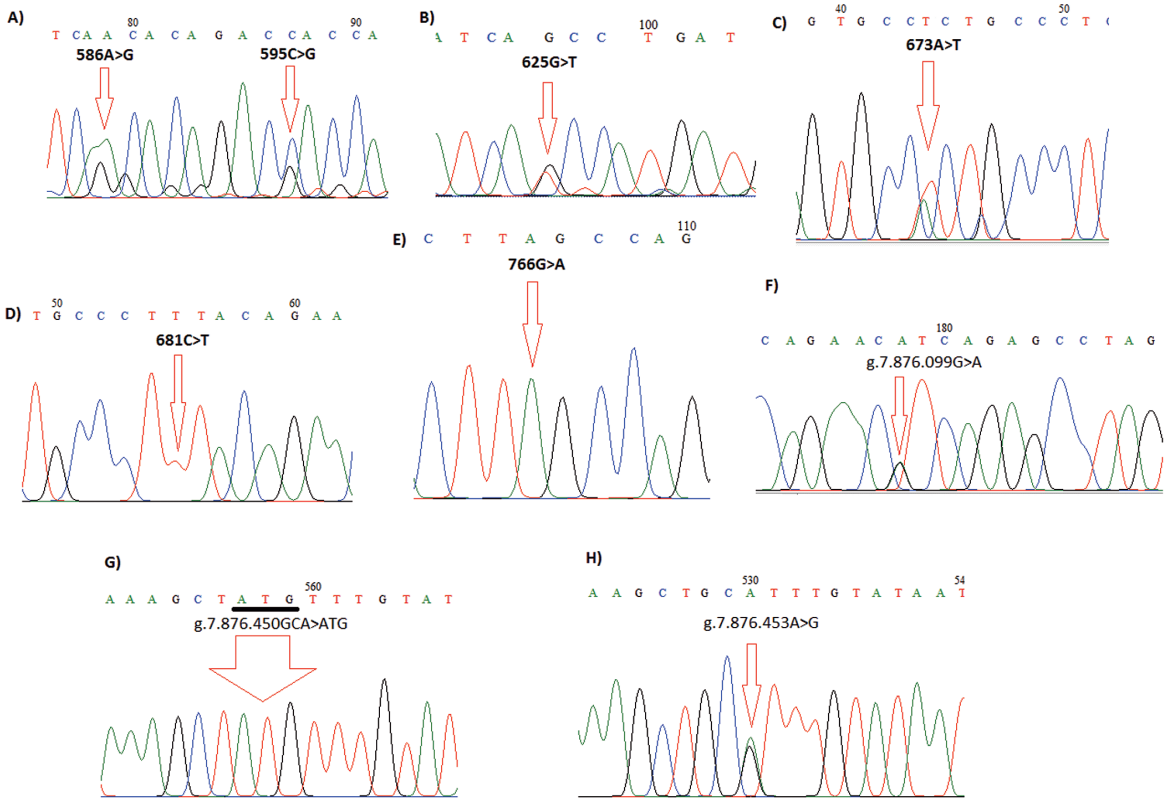

Fig. 2. Identified mutations by sequence analysis of the canine *RAD51* gene. The electropherogram shows nucleotide substitutions at positions c.586A>G, c.595C>G (A); c.625G>T (B); c.673A>T (C); c.681C>T (D); c.766G>A (E); g.7,876,099G>A (F); g.7,876,450GCA>ATG (G); g.7,876,453A>G (H) of the canine *RAD51* gene.

RAD51 exon 7 and exon 8 regions were analyzed to identify the deleterious mutations. N196D, H199D and T225S residues were predicted to be damaging by SIFT and deleterious by PROVEAN and probably damaging by PolyPhen. These results were summarized in Table 3.

Discussion

Many studies have indicated a link between DSB and cancer[5,13]. *RAD51* is one of the key protein for HR of DNA DSB and cell cycle control, also interacts directly or indirectly with tumor suppressor proteins. *BRCA2* and *PALB2* proteins act together to deliver *RAD51* to initiate repair[5]. *RAD51* is required for DNA repair by HR but its specific roles is not well characterized in CMTs. To shed light on this issue, we focused on *RAD51* gene variation, which directly binds *PALB2* and *BRCA2* binding domains in dogs with mammary tumors and nine variations were identified.

In this study, 7 SNVs (c.586A>G, c.595C>G, c.625G>T, c.673A>T, c.681C>T, g.7,876,099G>A and g.7,876,453A>G) and 1 SNPs (g.7,876,450 GCA>ATG) were novel when compared to available nucleotides in Ensemble (ENSCAFT00000014658) and GenBank (AB043896) database. Ochiai *et al.*[11] reported that c.766G>A SNPs in canine *RAD51* gene exon 8 region, which is similar to results presented in this study.

All these substitutions were classified as SNVs whereas two mutation points (c.766G>A and g.7,876,450 GCA>ATG) were classed as polymorphisms, which were identified in over 50% of the cases. Among these SNVs c.586A>G (N196D), c.595C>G (H199D), c.625G>T (A209S), c.673A>T (T225S) and c.681C>T were found in the *RAD51* gene *PALB2* interaction domain. No

Table 3. Prediction scores from SIFT, PROVEAN and Polyphen tools of the nsSNPs in *RAD51* gene exon 6–9

		SIFT		PROVEAN		PolyPhen			
						HumDiv		HumVar	
Amino acid change	Number of seqs at position	Prediction	Score	Prediction	Score	Prediction	Score	Prediction	Score
N196D	586	Affect protein function	0.00	Deleterious	−4.851	Probably Damaging	1.000	Probably Damaging	0.991
H199D	595	Affect protein function	0.00	Deleterious	−7.681	Probably Damaging	0.989	Probably Damaging	0.897
A209S	625	Tolerated	0.08	Neutral	−1.411	Benign	0.126	Benign	0.060
T225S	673	Affect protein function	0.02	Deleterious	−3.132	Probably Damaging	0.894	Probably Damaging	0.940

variation has been determined for *RAD51* gene *BRCA2* interaction domain.

According to results three amino acid substitutions predicted to have damaging/deleterious effect by SIFT, PROVEAN and PolyPhen tools. When results of all the tools were used to detect high-risk variations the functional SNVs at positions N196D, H199D and T225S showed damaging effects in the three servers (SIFT, PROVEAN and PolyPhen).

In conclusion, we have identified 7 SNVs and 2 SNPs in canine *RAD51* exon 7, exon 8, intron 8 and intron 9 regions, among them 1 SNPs (g.7,876,450 GCA>ATG) and 7 SNVs (c.586A>G, c.595C>G, c.625G>T, c.673A>T, c.681C>T, g.7,876,099G>A, g.7,876,450GCA>ATG and g.7,876,453A>G) were detected for the first time in this study. Four were non-synonymous substitutions that might cause altered protein functions, three of which were predicted to have damaging effects on the protein. According to the results, three amino acid substitutions (N196D, H199D and T225S) predicted to have damaging effect and these amino acid substitutions were located in the *RAD51* gene *PALB2* interaction domain. Interestingly, we were not determined any variations in the *BRCA2* interaction domain in this study. However, number of dogs in this study is too limited for exploring the association between tumor types and dog breeds. Although, our results suggest that N196D, H199D and T225S may be the most probable damaging variation, its association with *PALB2* requires further validation on a larger number of animals.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

The study was approved by the "Firat University Animal Researches and Ethic Committee" (Verdict number: 2012/06/63).

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