

Genetic variations of *BRCA1* and *BRCA2* genes in dogs with mammary tumours

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Abstract Mammary tumours are the most common tumour type in female dogs. The formation of the mammary tumours is multifactorial but the high incidence of tumour disease in certain canine breeds suggests a strong genetic component. *BRCA1* and *BRCA2* are the most important genes significantly associated with mammary tumours. The aim of this study was to determine the association between the variations of these two genes and canine mammary tumours. 5'-untranslated region, intron 8 and exon 9 of *BRCA1* and exons 12, 24, 27 of *BRCA2* were sequenced in order to detect the genetic variations. In addition to six previously identified polymorphisms, six novel single nucleotide polymorphisms (SNPs) were detected. Five of the coding SNPs were synonymous and three of them were non-synonymous. The comparison of the sequences from 25 mammary tumour bearing and 10 tumour free dogs suggested that the two SNPs in intron 8 and exon 9 of *BRCA1* and two SNPs in exon 24 and exon 27 of *BRCA2*, which are firstly identified in this study, might be associated with mammary tumour development in dogs. Especially one SNP in exon 9 of *BRCA1* and one SNP in exon 24 of *BRCA2* were found to be significantly associated with canine mammary tumours.

Keywords Dog · Mammary tumour · *BRCA1* · *BRCA2* · Single nucleotide polymorphism

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Introduction

Mammary tumours are the most common neoplasm observed in female dogs (Arnesen et al. 2001; Moe 2001; Egenvall et al. 2005). Canine mammary tumours (CMT) develop with age and they occur usually after 5 years of age (Cohen et al. 1974; Egenvall et al. 2005). Approximately half of the CMT's are reported to be malignant (Moulton et al. 1970). The development of the CMT's is multifactorial, but the higher incidence of mammary tumours in certain dog breeds supports the important role of the genetic factors (Borge et al. 2011). There are several candidate genes, which have been evaluated for association to CMT risk, like *BRCA1*, *BRCA2*, *TP53*, *PTEN*, *CHEK2*, *TOX3*, *ERBB2*, *BRIP1*, *STK11* (Rivera et al. 2009; Borge et al. 2011). Two of these genes, *BRCA1* and *BRCA2*, are significantly associated with mammary tumours in dogs. These genes encode proteins participating in DNA damage—recognition and repair pathways and act as tumour suppressors. *BRCA1*-deficient cells are hypersensitive to ionizing radiation (IR) and characterized by defects in the repair of both oxidative DNA damage by transcription-coupled processes and chromosomal double-strand breaks by homologous recombination (Gowen et al. 1998). *BRCA2* protein has a key role in the maintenance of genomic stability in DNA recombination and double strand-break repair through interaction of with Rad51 recombinase. The genomic instability of *BRCA2* deficient cells points to the tumour suppressor function of this protein (Scully and Livingston 2000; Ventikaraman 2002). The mutations in *BRCA1* and 2 genes include point mutations which cause termination codons, indel's leading frame-shift mutations and mutations that affect regulation of the transcription. Most of the mutations observed in *BRCA1* and 2 genes result protein truncations, representing loss of function alterations. There are also many sequence variants detected in these genes, whose effects are not yet identified (Peto et al. 1999; Easton et al. 2007; Rivera et al.

2009; Borge et al. 2011). The results of the studies on both coding and non-coding SNP's in BRCA genes indicated that these genes are involved in the development of CMTs (Rivera et al. 2009; Borge et al. 2011). The coding SNPs might change protein structure or function, whereas some non-coding SNPs might have effects on transcription (Borge et al. 2011). The aim of this study was to determine the association between the variations of these two genes and CMTs. 5'-UTR, intron 8 and exon 9 of *BRCA1* and exons 12, 24, 27 of *BRCA2* were sequenced in order to detect the genetic variations.

Materials and methods

Animals

In this study 25 female dogs with mammary tumours and 10 female dogs with healthy mammary glands were used. All animals in the study were presented in Istanbul University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology. The characteristics of the dogs were given in Table 1.

At the first examination a complete history and physical examination including the vaginal cytology was performed to all bitches. Only the bitches in anoestrus according to their vaginal cytology were included in this study and female dogs which have been spayed 1 year before the examination were accepted as in anoestrus, too. All control group dogs ($n=10$) and 15 dogs from the tumoural group were all intact during the study, 10 dogs from the tumoural group were spayed previously. Four of them were spayed at their 10th age 1 year ago. Two of them were spayed at their 8th age 1 year ago, two dogs were spayed at 11th age, 3 years ago. One dog was spayed 3 months ago which was 6 years old and one dog was spayed 4 years ago who was 18 years old when included to the study.

Specimen collections

For DNA extraction 5 ml blood samples were collected into vacuumed tubes containing EDTA before the surgeries. Twenty-five female dogs with mammary lesions were surgically treated and 10 normal mammary glands were obtained by surgical biopsy from left inguinal mammary lobes of the control group dogs ($n=10$) with the absence of any tumoural lesion at the palpation of all mammary glands and without a history of any mammary or endocrine disease. Immediately after the surgery, all the obtained tissue samples were sent to Pathology Department for histopathological evaluation. The mammary tissue samples were fixed in 10 % buffered formalin solution for 24 h, embedded in paraffin and cut in 5 μ m sections. Histopathological diagnosis was evaluated on haemotoxylin and eosin (H&E) stained sections by the

WHO's classification for canine mammary tumours, dysplasias and normal mammary glands.

DNA extraction, PCR and sequencing

Genomic DNA samples were isolated from whole blood by using the standard salt-out method (Miller et al. 1988). 5' UTR, intron 8 and exon 9 of *BRCA1* gene and 5' UTR, exon 12, exon 24 and exon 27 of *BRCA2* gene were amplified. Primers, annealing temperatures and product sizes were given in Table 2. PCR amplifications were performed in a reaction volume of 25 μ l using 1 U Taq polymerase, 2–2.5 μ l 10XPCR buffer (100 mM KCl, 20 mM Tris HCl (pH 8.0), 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 50 % glycerol), 2.5 mM $MgCl_2$, 50–100 ng genomic DNA, 100 μ M dNTP and 10 pmol of each primer. Amplification was carried out for 95 °C for 2 min; 34 cycles of 95 °C for 30 s, annealing temperature for 40 s, 72 °C for 50 s; and a final extension at 72 °C for 10 min. PCR products were run through 2 % agarose gel to check the results of amplification. Sequencing was performed by using an ABI-3100 sequencer (PE Biosystems, Germany) and the BigDye™ terminator cycle sequencing kit, after the purification of the PCR products. Forward primer was used to sequence the PCR products.

Identification of sequence variations

Nucleotide sequences of all amplified regions were aligned by using Clustal W program in the MEGA 4 software program (Tamura et al. 2007). The previous identified SNPs were obtained from GenBank for comparison. The correct reading frame for each region was determined on the protein sequence at Ensemble (Release 70, 2013) and the possible changes in amino acid sequence caused by coding SNPs were identified.

For this study permission from "Istanbul University Animal Researches and Ethic Committee" was taken with the verdict number 126.

Statistical analysis

The cases versus the controls were analyzed to examine if a SNP has a significantly higher frequency in dogs with CMTs compared with the dogs in control group. Pearson's two-sided chi-square test was performed to calculate the significance of the association by using SPSS 13.0 program. Odds ratios (OR) with 95 % confidence intervals (CI) were estimated by using unconditional logistic regression.

Results

The 5'-UTR, Intron 8, Exon 9 of the *BRCA1* gene and Exons 12, 24 and 27 of the *BRCA2* gene were sequenced. In addition

Table 1 The histopathological features and genetic variations of BRCA1 and BRCA2 in dogs with mammary tumours and normal mammary gland

Case no	Breed	Age (year)	Body weight (kg)	Tumor type	BRCA1					BRCA2						
					5' UTR ^a C/G	5' UTR ^a A/G	18 A/C	18 A/G	E9 A/G	E9 A/T	E12 GTT/-	E12 A/G	E24 A/G	E24 A/G	E27 A/G	E27 AAA/-
1	Terrier	16	9	Complex carcinoma-complex adenoma	G	A	C	A	G	T	GTT	G	A	A	A	AAA
2	Terrier	9	7	Ductal carcinoma	G	A	A	A	A	T	GTT	A	A	A	G	AAA
3	Terrier	14	5.5	Dermatofibrosarcoma	G	A	C	A	G	A	GTT	G	A	A	G	AAA
4	Terrier	11	4.5	Ductal carcinoma-benign mixed tumour	G	A	A	A	A	T	Del	G	A	A	A	AAA
5	Terrier	14	7	Tubulopapillary carcinoma	G	A	C	G	G	A	GTT	G	A	A	G	AAA
6	Pitbull	6	22	Fibroadenoma-benign tumour	G	A	A	A	A	T	GTT	G	A	A	A	AAA
7	Mixed	13	22	Complex carcinoma	G	A	A	A	A	T	Del	G	A	A	G	AAA
8	Mixed	13	23	Complex adenocarcinoma	G	A	A	A	A	T	GTT	G	A	A	G	AAA
9	Irish Setter	10	13	Carcinosarcoma	G	A	C	A	A	T	GTT	G	A	A	A	AAA
10	Mixed	7	18	Complex adenocarcinoma	G	A	A	A	A	T	GTT	G	A	A	A	AAA
11	Cocker	10	25	Simple adenocarcinoma	C	A	A	A	A	A	Del	G	A	A	A	AAA
12	Terrier	14	25	Tubulopapillary adenocarcinoma	G	A	A	A	A	T	GTT	G	G	A	G	AAA
13	Terrier	11	12	Complex carcinoma	G	A	A	A	A	T	GTT	G	A	A	G	AAA
14	Terrier	15	10	Complex adenocarcinoma	G	A	A	A	A	T	GTT	G	A	A	G	Del
15	Terrier	18	3	Adenosquamous carcinoma	C	A	C	A	G	A	GTT	G	A	A	A	AAA
26	Terrier	11	25	Complex carcinoma	G	A	A	G	A	T	GTT	G	A	A	G	AAA
17	Terrier	13	7	Tubulopapillary carcinoma	G	A	A	G	A	T	GTT	G	A	A	G	AAA
18	Terrier	14	5	Complex adenocarcinoma	G	A	A	A	G	T	Del	G	A	A	G	AAA
19	German Shepherd	11	31.5	Simple carcinoma	G	A	A	A	G	T	GTT	A	A	A	G	AAA
20	Irish Setter	11	16	Complex carcinoma	G	A	A	A	A	T	GTT	G	A	A	G	AAA
21	German Shepherd mixed	10	9.5	Simple tubulopapillary adenocarcinoma	C	A	A	A	A	T	GTT	G	A	A	G	AAA
22	Welsh Corgi	9	12	Solid adenocarcinoma	G	A	A	A	A	T	GTT	G	A	A	G	AAA
23	Welsh Corgi	13	10	Tadenocarcinoma	G	A	A	A	A	T	Del	G	A	A	G	AAA
24	Terrier	9	8	Tubulopapillary adenocarcinoma	G	A	A	G	A	A	GTT	G	A	A	G	AAA
25	Terrier	12	7	Complex adenocarcinoma	G	A	A	A	A	T	GTT	G	A	A	G	AAA
C1 ^b	Mixed	12	22	NMG ^c	G	A	A	A	A	T	Del	G	G	A	G	AAA

Table 1 (continued)

Case no	Breed	Age (year)	Body weight (kg)	Tumor type	BRCA1				BRCA2								
					5' UTR ^a C/G	5' UTR ^a A/G	18 A/C	18 A/G	E9 A/G	E9 A/T	E12 GTT/-	E12 A/G	E24 A/G	E24 A/G	E27 A/G	E27 AAA/-	
C2	Mixed	10	18	NMG	G	A	A	A	A	T	Del	G	A	A	A	G	AAA
C3	Collie	11	18	NMG	G	A	A	A	A	T	GTT	G	G	A	A	G	AAA
C4	Mixed	13	32	NMG	G	A	A	A	A	T	GTT	G	G	A	A	A	Del
C5	Kangal	10	28	NMG	C	A	A	A	A	T	GTT	G	G	A	A	G	AAA
C6	Akbaş	10	35	NMG	G	A	A	A	A	T	GTT	G	G	A	A	G	AAA
C7	Kangal	14	38	NMG	G	A	A	A	A	T	GTT	G	G	A	A	G	AAA
C8	German Shepherd	10	23	NMG	G	A	A	A	A	T	GTT	G	A	A	A	G	AAA
C9	German Shepherd	9	23	NMG	C	A	A	A	A	T	GTT	G	G	A	A	G	AAA
C10	Mixed	11	17	NMG	G	A	A	A	A	T	GTT	G	G	A	A	G	AAA

^a Exon/intron position, SNP variants, genome position obtained from Dog BLAT Search on UCSC Genome Bioinformatics

^b C control

^c Normal mammary gland

to six previously identified polymorphisms (two of them were 3 bp indels in *BRCA2*), four novel SNPs in *BRCA1* and two novel SNPs in *BRCA2* were detected (Table 3). Animals were genotyped for 12 SNPs in total. Eight of these SNPs were in coding regions of the BRCA genes. Three coding SNPs were found to be non-synonymous, whereas the other five coding SNPs were synonymous (Table 4). Of the three non-synonymous SNPs, one of them was located in the second and two were located in the third position of the codon. The non-synonymous SNP (A/G) in exon 24 of BRCA2 gene resulted to lysine/arginine substitution. Two non-synonymous polymorphisms were determined in exon 27 of *BRCA2*. One of them was a A/G transition causing serine/asparagines variation (identified in this study) and the second one was an indel polymorphism (AAA), which substitutes methionine to isoleucine and lysine. The genetic variations in BRCA1 and 2 genes and the clinicopathological features of dogs with mammary tumours and dogs in control group are given in Table 1. For the genetic variations, which occurred more frequently in dogs with mammary tumours, no specificity of breed or tumour type were observed. The certain alleles of 4 SNPs in intron 8 and exon 9 of *BRCA1* were only observed in mammary tumour bearing dogs. The A allele of the SNP A/G in exon 27 of *BRCA2* was found in 7 of the 25 dogs with mammary tumour and only in one individual from the control group. The G allele of the SNP A/G in exon 24 of *BRCA2* was only present in 7 from the 10 dogs in control group.

Association analysis was performed for 25 cases with CMTs and 10 controls. Two monomorphic SNPs (ss244244320 and ss244244324) were excluded from statistical analysis. MAF, OR and *p*-values were given in Table 5. The most significant association (*p*=0.0001) was observed for the SNP A/G in exon 24 of *BRCA2* (ss748770619). The SNP A/G in exon 9 of *BRCA1* (ss748770617) was also found to be suggestively (*p*=0.089) associated with CMTs. Both of these SNPs were firstly identified in this study.

Discussion

BRCA1 and BRCA2 are two main candidate genes, which are associated with mammary tumours. Both of these genes belong to the granin family, which acts as tumour suppressor genes (Phillips et al. 1999). The genetic variations of these genes in dogs are under focus in recent years, to shed light on the pathogenesis, the clinicopathological status and prognosis of CMTs (Szabo et al. 1996; Judkins et al. 2005; Rivera et al. 2009; Borge et al. 2011). The genetic variations mainly consist of SNPs, but insertion and deletions were also found. Both the coding and non-coding SNPs might have effects on the tumour development. While the coding SNPs can change the protein structure or function, some non-coding SNPs may affect transcription. The missense mutations in the coding

Table 2 Primer sequences, annealing temperatures and product size of the amplified regions

Gene	CFA ^a	Exon	Primer sequence: 5'–3'	T _m (°C)	Product size (bp)
BRCA1	9	5' UTR	F: CGTGGCTTTCTTTTCTTGG R: CTGGATTCCAAAGCTCCTG	53	574
		Intron8-Exon 9	F: TCCTCAGGATTCTTGGTGGT R: GTGCTGGGAATCTGCCTATC	55	475
BRCA2	25	Exon 12	F: TCTTTCAAATAGGATGGTTGC R: CCCAATTACAAAGAACCCTCCA	52	567
		Exon 24	F: AGTATCTGGCGTCCATCACC R: CCTCCTTCTGTCCATGTCC	56	540
		Exon 27	F: CCCCATTCTCTCTCTCTC R: CAAAAGGGCTTGGGTGTTA	55	535

^a Chromosome, *Canis familiaris*

regions may cause loss of protein function due to the structural changes (Borge et al. 2011). The silent mutations in these regions do not result in amino acid alterations but they might affect protein folding. There are recent studies suggesting that silent mutations can have impact on the kinetics of protein synthesis leading to different final protein conformations (Kimchi-Safarty et al. 2007; Komar 2007).

In this study, we focused on the genetic variations in some coding and non-coding regions of the *BRCA1* and *BRCA2*, which have been pointed out by recent studies (Yoshikawa et al. 2005b; Hsu et al. 2009; Borge et al. 2011). In total 12 variations were identified in dogs with mammary tumours and compared to the variations in dogs with healthy mammary glands. Six of the variations were novel SNPs and the distribution of them among dogs with CMT and dogs in control group, were remarkable.

The two SNPs (A/C and A/G) in intron 8 of the *BRCA1* gene might be associated to CMTs due to their allele distributions. The C allele of the first SNP was observed in 5/25 dogs with mammary tumours and none of the dogs in the control group have allele C. The absence of the G allele of the second

SNP in tumour-free dogs also might point to a relationship with allele G and CMTs. These non-coding SNPs may show their effects through influence on the transcription. For example, a genetic variation in intron 8 of the human *BRCA1* was reported to induce an aberrant transcript, which deletes exon 8 (Pyne 2000). But statistically, no significant association was detected. This might be a result of the limited number of cases.

Two novel SNPs (A/G and A/T) identified in exon 9 of *BRCA1* were synonymous mutations. The allele distributions of both SNPs suggest that there may be a relation between these polymorphisms and CMTs. The G allele of the first SNP and the A allele of the second were only observed in dogs with mammary tumours. These silent mutations may have an influence on the conformation of the *BRCA1* protein (Kimchi-Safarty et al. 2007; Komar 2007). The SNP A/G showed suggestive association ($p=0.089$) with CMTs, whereas no significant association was found for the SNP A/T.

One synonymous (identified in this study) and one non-synonymous A/G transitions were observed in exon 24 of *BRCA2*. The non-synonymous SNP leads to a Lysine/Arginine change but no correlation was found between this SNP and CMT, because only A allele was present in all the dogs used in this study. The G variant of the synonymous mutation was detected in 7/10 dogs with normal mammary gland and in only one individual with CMT. The high frequency of the A allele in dogs with CMT is remarkable, considering the association between this allele and mammary tumour development. According to the statistical analysis, the association of this SNP was found to be highly significant ($p=0.0001$). This synonymous SNP may have a role other than the effect on the protein function, which may be related with CMT's (Mooney 2005). It has been reported that synonymous variations in exonic splicing enhancers can affect mRNA splicing and exon skipping (Blencowe 2000). Disease associated polymorphisms which effect exonic splicing enhancers, were also detected in human *BRCA1* and *BRCA2* genes (Liu et al. 2001; Orban and Olah 2001; Fackenthal et al. 2002).

Exon 27, the last exon of the *BRCA2*, encodes the C-terminus of *BRCA2* and the 3' UTR The C-terminal

Table 3 SNPs and indels in coding and non-coding regions of *BRCA1* and *BRCA2* genes

Gene	Exon/intron position	Allele	NCBI RefSNP/Assay ID
BRCA1	5' UTR	G/C	ss244244319
	5' UTR	A/G	ss244244320
	INT8	A/C	ss748770615 (This study)
	INT8	A/G	ss748770616 (This study)
	EX9	A/G	ss748770617 (This study)
BRCA2	EX9	A/T	ss748770618 (This study)
	EX12	GTT/-	ss244244371
	EX12	A/G	rs23255542
	EX24	A/G	ss748770619 (This study)
	EX24	A/G	ss244244324
	EX27	A/G	ss748770620 (This study)
	EX27	AAA/-	AB210823.1

Table 4 Coding SNPs and amino acid variations

Gene	CFA ^a	Region	Allele	Function ^b	Codon position	AA variation	AA position
BRCA1	9	EX9	A/G	Syn	3	Lysine	242
		EX9	A/T	Syn	3	Threonine	247
BRCA2	25	EX12	GTT/-	Syn	3	Leucine	2305
		EX12	A/G	Syn	3	Phenylalanine	2310
		EX24	A/G	Syn	2	Leucine	3045
		EX24	A/G	Ns	2	Lysine/Arginine	3103
		EX27	A/G	Ns	3	Serine/Asparagine	3322
		EX27	AAA/-	Ns	3	Methionine/Isoleucine and Lysine	3331–3332

^aChromosome number, *Canis familiaris*

^bSynonymous (Syn) and non-synonymous (Ns) SNPs

sequence is highly conserved in mammals and the nuclear localization signals (NLSs) in this region play an essential role in nuclear localization of human BRCA2 (Yano et al. 2000; Spain et al. 1999). An indel polymorphism of AAA, which results to an amino acid change of Methionine/Isoleucine and Lysine in NLS2, was indentified in exon 27 by Yoshikawa et al. (2005a). The researchers suggested that this amino acid alteration enhances nuclear localization. They also found higher tumour morbidity rate in dogs with AAA insertion than with AAA deletion, which might be a result of the association between translocation efficiency of BRCA2 protein and CMTs (Yoshikawa et al. 2005b). In a study on germline genetic variations in mammary tumour associated gene in dogs, the AAA insertion was found to occur more frequently than AAA deletion, but no significant difference between dog breeds with high- and low-risk, were identified (Borge et al. 2011). In a recent study of Yoshikawa et al. (2012), the researchers reported that the AAA insertion is consensus sequence in dogs and is probably a neutral variation. In this study only two individuals (one with CMT and one with normal mammary gland) with AAA deletion were observed. The presence of the AAA insertion observed in the rest of the 35 dogs showed no evidence for any association between this insertion and mammary tumours in dogs.

A novel non-synonymous SNP A/G in exon 27 of *BRCA2*, identified in this study, causes an amino acid change of Serine/Asparagine. The frequency of A allele is higher in dogs with CMT than in tumour free dogs, but no significant association was detected. An increased number of cases may reveal a possible relationship between this SNP and CMTs, which might be explained by another function of the C terminus of BRCA2, which is its interaction with Rad51 recombinase (Mizuta et al. 1997). This interaction was first reported in mouse (Sharan et al. 1997), human (Esashi et al. 2005) and dogs (Ochiai et al. 2004). Non-synonymous variations may have an impact on this interaction and can lead to uncontrolled cell growth and tumour formation via the loss of Rad51 activity (Cotroneo et al. 2007).

In conclusion, in this study four novel SNPs in *BRCA1* and two novel SNPs in *BRCA2* were identified. The distribution of the novel SNP variants in dogs with mammary tumour and normal mammary gland suggested that these genetic variations might be associated with CMTs. The effects of the two novel SNPs (one in BRCA1 gene and the other one in BRCA2 gene) which are significantly associated with mammary tumours should be investigated through functional analysis. Further studies on large number of animals from particular breeds with family histories would be helpful to strengthen the

Table 5 Association of the SNPs in BRCA1 and BRCA2 genes to CMTs

SNP	Gene-Exon/Intron	Allele ^a	MAF ^b	OR ^c (95 % CI)	p ^d
ss244244319	BRCA1-5' UTR	G/C	0.14	1.83(0.26–13.06)	0.541
ss748770615	BRCA1-Intron 8	A/C	0.14	0.18 (0.01–3.53)	0.127
ss748770616	BRCA1-Intron 8	A/G	0.11	0.23 (0.01–4.63)	0.179
ss748770617	BRCA1-Exon 9	A/G	0.17	0.14 (0.01–2.79)	0.089*
ss748770618	BRCA1-Exon 9	T/A	0.14	0.18 (0.01–3.53)	0.127
ss244244371	BRCA2-Exon12	GTT/-	0.20	1.00 (0.16–6.26)	1.00
rs23255542	BRCA2-Exon12	G/A	0.06	0.45 (0.02–10.16)	0.357
ss748770619	BRCA2-Exon24	A/G	0.23	56.00 (5.01–626.47)	0.0001***
ss748770620	BRCA2-Exon27	G/A	0.23	0.29 (0.03–2.69)	0.252
AB210823.1	BRCA2-Exon27	AAA/-	0.06	2.67 (0.15–47.30)	0.490

* $p < 0,1$

*** $p < 0,001$

^aMajor/minor allele

^bMinor allele frequency

^cOdds ratios

^dTest for allele frequency differences between dogs with and without CMTs using Pearson's two sided chi-square test

evidence and also would shed more light on the function of these two candidate genes, BRCA1 and BRCA2, in dogs.

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Conflict of interest The authors confirm that all co-authors have given their permission to be listed.

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