Analysis of genetic polymorphisms in the intron 9–10/exon 10 region of the *BRCA1* gene in a population sample of dogs with mammary cancer from Uruguay

Análisis de polimorfismos genéticos en la región intrón 9–10/exón 10 del gen *BRCA1* en una muestra poblacional de perras con cáncer de mama de Uruguay

Alicia Decuadro[1]*, Micaela Sosa[1], Federico García[1], Nariné Balemian[2], María del Carmen Montenegro[2], Silvia Llambí[2]

[1]Universidad de la República, Facultad de Veterinaria, Unidad Académica de Clínica de Pequeños Animales. Montevideo, Uruguay.
[2]Universidad de la República, Facultad de Veterinaria, Unidad Académica de Genética y Mejora Animal. Montevideo, Uruguay.
**Correspondence author: oncologiafvetudelar@gmail.com*

ABSTRACT

This study involved clinical and genetic analysis of 15 female dogs with mammary tumors. Fourteen healthy female dogs were used as controls, and blood samples were collected from them for genetic analysis. Polymorphisms located in a splicing region of the largest exon of the *BRCA1* gene were studied, both at the population and evolutionary level, in a population of female dogs with different histopathological types of mammary tumors. In the intron 9–10/exon 10 initiation region, two SNP–type polymorphisms are described: SNP1 and SNP2. The SNP1 produces a non–synonymous change with unknown effect on the coding protein. Selected animals underwent surgery, and samples were sent for histopathological analysis. Peripheral blood was also collected for DNA extraction. A region corresponding to intron 9–10/exon 10 of the *BRCA1* gene (ENSCAFE00845051080) was amplified by endpoint PCR, with PCR results subsequently confirmed through agarose gel electrophoresis at 1%. PCR products were sequenced to study the polymorphisms identified within this region. No statistically significant differences were observed between the genotype frequencies in both populations (Chi^2 0.33, *P*>0.5), indicating that SNP1 is not linked to mammary tumors in the studied animals. Regarding SNP2, the mutation was not identified in the studied groups (females with mammary tumors and controls), being monomorphic. Although this SNP2 is described in the Ensembl database, there are no genotyping data in reference populations. The phylogenetic analysis of the amplified intron 9–10/exon 10 revealed an evolutionary homology with *Canis lupus familiaris*, and a more distant relationship with other genera such as *Vulpes* and *Nyctereutes* within the Canidae family. It can be concluded that mutations in this splicing region of the largest exon of *BRCA1* are not associated with the development of mammary tumors in canines within this group of animals.

Key words: Canine mammary tumors; canine *BRCA1* gene; SNP

RESUMEN

Se realizó el estudio clínico y genético en 15 perras con tumores de mama. El grupo control incluyó 14 perras sanas a las cuales se les extrajo sangre para el estudio genético. Se estudió a nivel poblacional y evolutivo, polimorfismos localizados en una región de corte y empalme del exón más grande del gen *BRCA1* en una población de perras con distintos tipos histopatológicos de tumores mamarios. En la región de inicio de intrón 9–10/exón10 hay descritos dos polimorfismos de tipo SNP: SNP1 y SNP2. El SNP1 produce un cambio no sinónimo y se desconoce el efecto del mismo a nivel de la proteína codificante. Los pacientes fueron intervenidos quirúrgicamente, con posterior estudio histopatologico, y obtención de muestras de sangre para la extracción de ADN. Se amplificó por PCR a tiempo final una región correspondiente al intrón 9–10/exón 10 del gen *BRCA1* (ENSCAFE00845051080), confirmándose los resultados por PCR a través de electroforesis en geles de agarosa al 1 %. Los productos de PCR se secuenciaron para estudiar los polimorfismos identificados en dicha región. No existieron diferencias estadísticamente significativas entre las frecuencias genotípicas en ambas poblaciones (Chi^2 0.33, *P*>0,5), por lo tanto, este SNP1 no estaría vinculado con los tumores mamarios en los animales estudiados. En referencia al SNP2, en ambos grupos estudiados, no fue identificada la mutación siendo monomórfico. Si bien este SNP2 se encuentra descrito en la base Ensembl, no existen datos de genotipado en poblaciones de referencia. La secuencia amplificada intrón 9–10/exón 10 a nivel filogenético mostró homología con la especie *Canis lupus familiaris* y relación más lejana con otros géneros como *Vulpes* y *Nyctereutes* de la familia *Canidae.* Concluimos que las mutaciones en esta región de splicing del mayor exón de *BRCA1* no estarían relacionadas con la aparición de tumores mamarios en caninos en este grupo de animales.

Palabras clave: Tumores mamarios caninos; *BRCA1* canino; SNP

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INTRODUCTION

Cancer stands as the leading cause of death in dogs, being responsible for 27–30% of deaths, and reaching 50% in certain breeds, such as the Golden Retriever and the Bernese Mountain Dog [1, 2].

In recognition of the importance of cancer research in companion animals, the National Cancer Institute established the Comparative Oncology Program in 2003. This initiative has facilitated numerous studies that have benefited human medicine [3].

Dogs develop spontaneous mammary tumors with clinical and molecular similarities to human breast cancer. In addition to the spontaneous presentation of the tumor, there are several clinical parallels between human breast cancer (HBC) and canine mammary tumors (CMT). These include age of onset (from 6 years in dogs and 40 years in women), hormonal etiology, disease progression, tumor size, stage, and invasion of regional lymph nodes. In situ ductal carcinomas in both human and canine mammary glands exhibit analogous pathological and molecular characteristics. The traits of CMT and their resemblances to HBC indicate that dogs could serve as models for studying the disease in humans [4].

Genetic components and hereditary risk factors for breast cancer are shared between humans and dogs. One notable example is the *BRCA1* tumor suppressor gene. Mutations in this gene have been linked to an increased likelihood of developing breast and ovarian cancer in women due to the accumulation of DNA damage [5]. *BRCA1* has also been identified as a contributing factor in the development of mammary tumors in certain breeds, including the English Springer Spaniel [4, 5] Mutations in the *BRCA1* tumor suppressor gene have been linked to a higher risk of mammary cancer in both humans and canines [6, 7]. In canines, this gene is located on chromosome CFA9, encompassing 23 exons and four transcripts (splice variants). In our country, Decuadro *et al.* (2022) [8], studied the smaller exons (exons 22 and 23) of the *BRCA1* gene through DNA sequencing, and found no significant differences in the molecular markers identified in a group of dogs with mammary tumors compared to the control group. Furthermore, exon 10 (3444 bp in transcript 202) is the largest exon of this gene in canines and corresponds, by sequence homology, to exon 11, the largest exon of the homologous gene in humans [9].

In the Ensembl Dog database (ROS_Cfam_1.0), two SNP–type polymorphisms are described in the intron 9–10/exon10 initiation region: SNP1 (missense) (R, G/A) c.715G>A, at protein position Gly239Ser 239 (aa change G/S) and SNP2 (synonymous variant) (W, A/T) c.738T>A, at protein position Thr246= 246 (aa T/T) [10]. The interest has focused on SNP1 as it produces a nonsynonymous change, and its effect on the coding protein remains unknown.

In humans, extensive evidence shows that various types of mutations (SNPs, InDels, and rearrangements in intron and exon splicing) ultimately affect the functionality of the encoded protein, thereby preventing DNA repair [4]. Over time, the progressive accumulation of DNA damage increases the likelihood of developing mammary tumors [5]. Since the cloning of *BRCA1* in 1994, it has been observed that it undergoes high alternative splicing, with more than 100 transcripts detected in humans via next–generation sequencing (NGS) [11].

The *BRCA1* gene encodes a protein that is homologous to the breast cancer type 1 susceptibility protein (Brc–1). This protein plays a crucial role in DNA repair and cell cycle regulation in the mammary gland [12].

Phylogenetically, *BRCA1* is an ancient gene, the precise origin of which remains uncertain. However, evidence suggests a common ancestor may have emerged approximately 1.6 billion years ago, at the time of the divergence of animals and plants. [13].

This study aimed to investigate population–level and evolutionary polymorphisms located in a splicing region of the largest exon of the *BRCA1* gene, with a specific focus on SNP1 and its impact on the amino acid change in the encoded protein in a population of female dogs with diverse histological types of mammary tumors.

The aim of this work was to compare the analysis of *BRCA1* gene sequence in a group of canines with and without mammary tumors. Since there are few studies in female dogs that study breast cancer at a molecular level. The study proposed in this work will collaborate in obtaining this knowledge in order to have more elements when facing these clinical cases.

MATERIALS AND METHODS

This study was conducted at the Veterinary Hospital and the Laboratory of the Genetics and Animal Improvement Unit of the Faculty of Veterinary Medicine at the University of the Republic (Montevideo, Uruguay). The study was approved by the Ethics and Animal Use Committee (CEUA) under the number 1383.

CMT cases were selected based on the presence of nodules or tumors in the mammary region with clinical characteristics consistent with those observed in mammary tumors. Fifteen female dogs. aged 6 to 12 years, were selected, as this is a prime age for this pathology. Pre–surgical studies, including blood chemistry, chest X–rays, and urine analysis.

The Clinical Analysis Laboratory has the necessary instruments for blood tests (CB 31 Oi Biotecnica Instruments S.p.A.). Thoracic radiographs were obtained in a Vetter Rems 100 device (Argentina) and digitalized in a Kodak DirectView, Sistem Classic CR Caresream (Japan) and a TOSHIBA Nemio MX.(Japan) fixed hospital ultrasound scanner.

As well as disease staging according to WHO standards enabled the implementation of appropriate surgical techniques, with anesthetic risk classified as ASA I or II. Sedation was performed with acepromazine 0.05 $mg \cdot kg^{-1}$ and morphine 0.5 $mg \cdot kg^{-1}$ intramuscularly, anesthesia was induced with propofol 5 $mg \cdot kg^{-1}$ intravenously for placement of the tracheotube and inhalational anesthesia was maintained with isoflurane.

Post–surgery tissue samples were sent for histopathological study with an Olympus BX51 microscope using hematoxylin and eosin staining at 40× and 10× magnification

Tumors were grouped according to the histopathological classification of Goldschmidt *et al*. (2011) [14] (TABLE I).

The control group consisted of fourteen female dogs, within the same age range and with no oncological pathologies. These dogs, admitted to the Veterinary Faculty Hospital for other reasons. To dismiss any oncological pathology all dogs underwent clinical examination and collateral studies. Blood samples were collected to perform laboratory analysis (as with dogs with breast tumors), abdominal ultrasound and thorax radiography. An aliquot of the blood sample preserved with EDTA was reserved for genetic analysis.

DNA Molecular Studies

Peripheral blood samples were collected from selected animals under aseptic conditions in accordance with animal welfare protocols. Blood samples were obtained (3 mL) from the cephalic vein with a 21 G butterfly by the veterinary nurse. DNA extraction was conducted using the Quick_DNA™ Miniprep Plus Kit (Zymo Research). DNA samples were quantified using a NanoDrop ND 1000 spectrophotometer, (Thermo Fisher Scientific, USA) with full spectrum (220–750 nm).

TABLE I
Description of patients, clinical staging (according to the WHO), histopathological diagnostic and classification

Case	Age (years)	Breed	Stage	Histopathological Grade	Histopathology
1	6	Poodle	I	II	Mixed Carcinoma
2	8	Rottweiler	III	III	Simple Tubular Carcinoma
3	12	American Staffordshire Terrier	III	III	Tubulopapillary Carcinoma
4	6	Cocker	III	II	Simple Tubular Carcinoma
5	10	Mixed Breed	II	Benign	Complex Adenoma
6	12	Mixed Breed	V	III	Poorly Differentiated Complex Carcinoma
7	10	Cimarrón	III	III	Anaplastic Carcinoma
8	8	Cocker	I	III	Solid Carcinoma
10	12	Cimarrón	III	II	Tubulopapillary Carcinoma
11	6	Poodle	I	Benign	Complex Adenoma with Intraductal Lobular Hyperplasia
12	12	Poodle	I	Benign	Benign Mixed Tumor
13	12	Cimarrón	III	II	Tubulopapillary Carcinoma
14	12	Boxer	II	II	Complex Carcinoma
15	11	Pitbull	I	Benign	Complex Adenoma

A 281 bp fragment corresponding to the intron 9–10/exon 10 region of the *BRCA1* gene (ENSCAFE00845051080) was amplified by endpoint PCR using a Multigene II machine (Labnet International, Inc., USA). The following primers were utilized: F 5’→3’ AGGTGCTTATTTCCACTCCCC and R 5’→3’ TCATGCTGTAATGAGCTGGCA. Amplification conditions comprised an initial denaturation at 95°C for 5 min, followed by 35 cycles of 1) denaturation at 95°C for 30 s, 2) annealing at 56°C for 30 s, and 3) extension at 72°C for 30 s, with a final extension at 72°C for 5 min.

In each experiment, as analytical quality control a non–template PCR control tube was added using sterile deionizad water (negative PCR)

PCR results were confirmed by electrophoresis on 1% agarose gels in 1× TBE buffer. Electrophoresis was conducted using an HU13 MIDI horizontal gel electrophoresis system (Scie–plas, Great Britain) and a POWER PAC 3000 power supply (Bio–Rad, USA). The resulting bands were visualized under UV light using a BIOSENS SC805–BIOTOP instrument (Shanghai Bio–Tech Co., Ltd., China).

PCR products (F and R chains) were submitted to Sanger sequencing on an ABI 3500 instrument (Thermofisher, USA) at GENEXA, Uruguay, to study the polymorphisms identified in this region. Before the sequencing process, the PCR products are purified using the Quiagen column kit, in order to obtain good quality in the sequencing process (elimination of dNTSs, oligonucleotides, by–products).

Statistical and *in silico* analysis

The free Bioedit software [15] was used to align 29 sequences in order to identify SNP polymorphisms in the amplified fragment and to compare them with the reference canine genome sequence (Ensembl Dog, ROS_Cfam_1.0)

Genetic variability calculations, Chi–square test for allelic frequencies of polymorphisms and Hardy–Weinberg Equilibrium (HWE) were performed using the free POPGENE software version 1.32 [16].

To investigate the impact of SNP1’s amino acid change effect on the brc1 protein, two online bioinformatics tools were employed: a) SIFT Sorting Intolerant From Tolerant, [https://sift.bii.a–star.edu.sg/] [17], and b) PolyPhen–2 (Polymorphism Phenotyping v2) [18]. A sequence alignment and genetic distance Neighbor–Joining tree of the amplified sequence with available genomic data from Canidae species was constructed using the online BLAST algorithm (Basic Local Alignment Search Tool) [19, 20]. Furthermore, reference population data for 216 canine genotypes reported for SNP1 (204 homozygotes G/G, 10 heterozygotes G/A, and 2 homozygotes A/A) were downloaded from Ensembl Dog, ROS_Cfam_1.0, for the purpose of performing a comparative allele frequency and HWE analysis with the two aforementioned populations.

RESULTS AND DISCUSSION

The presence of the biallelic polymorphism SNP1 was identified in both populations of female canines (CMT group and C group) through the use of PCR–sequencing and subsequent sequence alignment. In the CMT group, two females exhibited the heterozygous genotype GA, while the remaining 13 were

GG homozygous. In the C group, three females exhibited the heterozygous genotype GA, while the remaining 11 were GG homozygous. The homozygous AA genotype for the mutant allele was not observed in either group (FIG. 1). No statistically significant differences were observed between the genotype frequencies in both populations (Chi–square 0.33, *P*>0.5), indicating that SNP1 is not linked to mammary tumors in the studied animals. Both populations were in HWE, whereas the reference population was not (TABLE II). This deviation from HWE in the reference population may be attributed to the lack of knowledge regarding the genotyping method employed, as Iniesta *et al*, 2005 [21] have indicated that such a method can introduce biases in interpreting genotype results. Additionally, the authors have proposed the biological possibility of inbreeding in the population or selection for a specific allele. With regard to SNP2 (synonymous variant, W, A/T) c.738T>A), the mutation was not identified in the studied groups (CMT and control), exhibiting monomorphic characteristics. Although this SNP2 is described in the Ensembl database,, there are no genotyping data in reference populations. Di Giacomo *et al*. (2022) [22], using an AmpliSeq panel for *BRCA1* variant identification in a population sample of 22 dogs, identified 12 SNPs (10 with nonsynonymous changes). These authors identified two SNPs in exon 10 that differed from the one identified in this work.

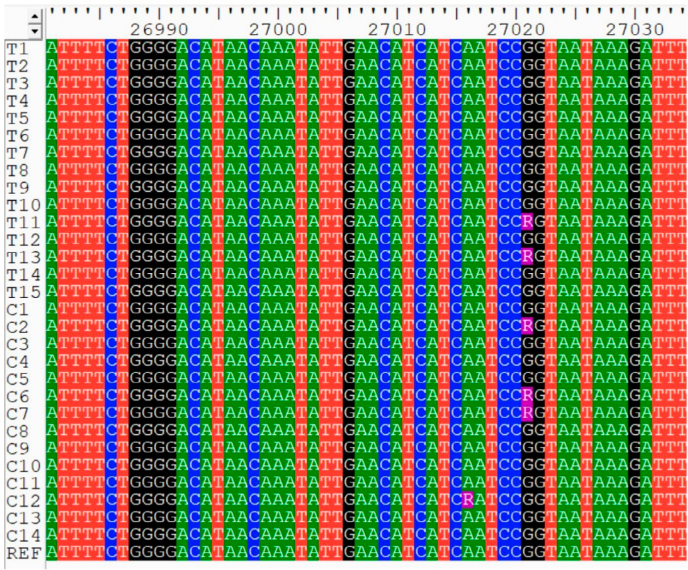

FIGURE 1. Partial multiple alignment of the amplified intron 9–10/exon 10 sequence of the *BRCA1* gene from 15 female canines with tumors (T1–T15) and control females (C1–C14). The REF sample corresponds to the reference sequence ENSCAFG00845006070 from Ensembl (*Canis Lupus familiaris*). At position 27021, the SNP1 polymorphism (letter R, in violet) is observed

With regard to the impact of the amino acid substitution resulting from SNP1 on the brc1 protein (Gly/Ser at position 239), the SIFT analysis returned a score of 0.08 (indicating a tolerated amino acid substitution), whereas substitutions with scores < 0.05 are regarded as "detrimental," signifying a potential alteration in protein function. Similarly, PolyPhen–2 analysis classified the substitution as benign with a score of 0.007 (sensitivity 0.96, specificity 0.75). In this program, values closer to 1 are predicted with higher confidence to be detrimental (in contrast to the SIFT scale).

TABLE II
Allelic Frequencies: Observed allelic frequencies of SNP1 and Chi–square (X^2) results for HWE

Exon 10–SNP1 c.715G>A Allelic Frequencies and X^2 for HWE	With tumors (CMT)	No tumors (C)	Reference Population
Allele G	0,933	0,893	0,968
Allele A	0,067	0,107	0,031
X^2 and *P* (HWE)	0.0765 $P \geq 0.70$	0,202 $P \geq 0.50$	16.03 $P<0.001$

Phylogenetically, the amplified sequence demonstrated greater homology with *Canis lupus dingo* and *Canis lupus familiaris* indicating an evolutionary divergence from the genus *Vulpes* and *Nyctereutes* as expected (FIG. 2). Recent taxonomic revisions propose that the dingo should be classified as *Canis familiaris*, which is the appropriate taxonomic designation for ancient, modern dog breeds and their hybrids [23].

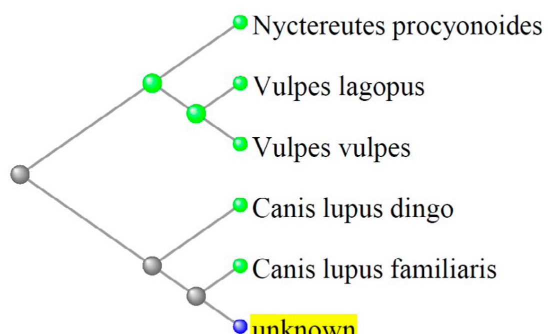

FIGURE 2 Neighbor–joining genetic distance tree, generated using the amplified sequence with the tBlastn multiple alignment program within the *Canidae* family. The consensus sequence amplified in this study is represented by the blue circle

In primates, there is evidence that *BRCA1* evolves through positive selection, whereby mutations confer resistance advantages to viral infections. This could explain its evolution and the transmission of cancer–related mutations [24].

This study confirmed that the splice initiation region of the largest exon of *BRCA1* in canines, despite presenting SNP–type polymorphisms, does not affect the functionality of the coding protein and is not associated with tumors in female canines.

CONCLUSIONS

It has been demonstrated that distinct mutations in the *BRCA1* gene are associated with mammary tumors in female canines. In this study, no statistically significant relationship was found between the SNP1 marker and mammary tumors in the problem and control groups. Furthermore, the amino acid substitution resulting from this mutation is regarded as being benign to the brc–1 protein. From a phylogenetic perspective, the amplified intron 9–10/exon 10 sequence demonstrated homology with *Canis lupus familiaris* and a more distant relationship with other

genera, such as *Vulpes* and *Nyctereutes*, within the *Canidae* family. Consequently, it can be concluded that mutations in this splicing region of the largest exon of *BRCA1* are not associated with the occurrence of mammary tumors in this group of animals.

This is the beginning of a line research at DNA in female dogs with mammary tumors, and the next step to deepen this work can be achieved by increasing samples number.

During the course of this work, two sample banks were created, one of blood and the other of DNA from female dogs with mammary tumors. These samples will be use in future studies. We also intend to study other gene sequences and their variants in a larger number of animals. Likewise, we hope to include the study of epigenetics in female dogs with mammary tumors compared to healthy animals in the future.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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