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Analysis of polymorphisms in *BRCA1* and *BRCA2* genes in a population sample of canines from Uruguay

Análisis de polimorfismos en los genes *BRCA1* y *BRCA2* en una muestra poblacional de caninos de Uruguay

Alicia Decuadro*[1], *Silvia Llambí*[2], *Alejandro Benech*[1] *and Rosa Gagliardi*[2]

[1]*University of the Republic, Faculty of Veterinary Medicine Hospital. Montevideo, Uruguay.*
[2]*University of the Republic, Faculty of Veterinary Medicine, Genetics and Animal Improvement Unit. Montevideo, Uruguay.*
**Email: rgagliar@gmail.com*

ABSTRACT

In non-ovariectomized female dogs, breast tumors are the most frequent neoplasms. There are several points where canine and human breast tumors have clinical and molecular similarities. *BRCA1* and *BRCA2* genes have been extensively studied in both species. Regarding dogs, alterations in *BRCA1* and *BRCA2* have been identified in the development of breast tumors in different breeds. In this work, it was proposed to study exons 22 and 23 of the *BRCA1* gene and exons 11 and 27 of the *BRCA2* gene, in female dogs. It was studied two groups of female dogs, with or without mammary tumors. Regarding the genetic study of 15 loci, six were polymorphic, all of them were singles nucleotides polymorphisms (SNPs), while the other nine were monomorphic. It was obtained a low allelic variability, but at the population level, the tumor group has greater variability than the control group. On the other hand, the different analyses of possible groupings were negative, and it was not possible to clearly define groups with the parameters it was used. The foregoing may be a consequence of numerous factors such as characteristics inherent to the populations studied, such as the size of both populations; the breeds studied; tumor diversity. As it was mentioned before, the genes studied in this work have been widely related to breast cancer, both in humans and in dogs. In the former, they have been highly implicated in hereditary tumors. In dogs, it do not have that information. In the present case, it was founded no relationship between each of the markers studied and the occurrence of mammary tumor between the problem group and the control group.

Key words: Canine *BRCA1* gene; canine *BRCA2* gene; mammary tumours in dogs

RESUMEN

En perras no castradas, los tumores de mama son las neoplasias más frecuentes. Hay diversos puntos en que los tumores en humanos y caninos presentan similitudes, tanto clínicas como moleculares. Los genes *BRCA1* y *BRCA2* han sido ampliamente estudiados en ambas especies. En lo que respecta a perros, las alteraciones en *BRCA1* y *BRCA2* se han identificado en el desarrollo de tumores mamarios en diferentes razas. En este trabajo, se procedió a estudiar los exones 22 y 23 del gen *BRCA1* y los exones 11 y 27 del gen *BRCA2* en perros. Se estudiaron dos grupos de perras, con y sin tumores mamarios. De los 15 loci analizados, seis fueron polimórficos, todos polimorfismos de nucleótidos únicos (SNPs), mientras que, los nueve restantes fueron monomórficos. Se obtuvo una baja variabilidad alélica, aunque a nivel poblacional, el grupo con tumores tuvo mayor variabilidad que el grupo control. Por otra parte, los diferentes análisis de posibles agrupamientos fueron negativos, no fue posible diferenciar ambos grupos con los parámetros empleados. Estos resultados pueden ser debidos a numerosos factores como: características inherentes a las poblaciones estudiadas como ser su tamaño; las razas estudiadas; la diversidad tumoral. Como ya se mencionó, los genes analizados en este trabajo han sido ampliamente relacionados a los tumores mamarios, tanto en humanos como en caninos. En los primeros tienen una gran implicación en los tumores de base hereditaria, dato con el que no se contó en el presente caso. Aquí no se encontró relación entre ambos marcadores estudiados y la ocurrencia de tumores mamarios entre el grupo problema y el grupo control.

Palabras clave: Gen *BRCA1* canino; gen *BRCA2* canino; tumores mamarios en perras

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INTRODUCTION

In dogs (*Canis lupus familiaris),* particularly in non-ovariectomized (spayed) females, breast tumors are the most frequent neoplasms, accounting for about half of cancer cases worldwide described [7, 15, 21]. On the contrary, less than 1% of mammary gland neoplasms occur in male dogs [15, 23]. Tumor incidence correlates with life expectancy, and is significantly reduced by ovariohysterectomy, before the third estrus, in young female dogs. The incidence is increased by the use of estrous-inhibiting-progestagens [9, 12].

Approximately half of canine mammary tumors are malignant, and half of them have metastasized by the time of the initial diagnosis. Two-thirds of these tumors occur in the caudal abdominal, and inguinal breasts. More than 50% of cases involve multiple glands. They may be attached to the skin, but they usually do not adhere to the underlying body wall, this being a more frequent feature in malignant tumors; in addition, they may also be covered by ulcerated skin [12, 23]. Dog mammary neoplasms usually appear as circumscribed nodules with variable size, consistency, and mobility and they can also be associated with ulceration and local inflammatory reactions. Multiple tumors are often seen in a single mammary gland, or they may simultaneously involve multiple glands, and may be of different histological types [8, 9]. The tumor with the worst prognosis is the one that will always determine the clinical course of the patient [3, 4]. The inguinal and caudal abdominal mammary glands are more frequently affected than the thoracic glands, which is due to the greater volume of breast tissue in these glands [5].

In this species, fibroadenomas are among the most benign breast tumors, while solid carcinomas are among the most common malignant tumors, followed by tubular adenocarcinomas [18]. The average age of affected female dogs is 10 to 11 years old (YO) (range 2 to 16 YO). In younger animals, benign tumors are usually more frequent than malignant ones [15, 17].

On the other hand, the development of mammary tumors in female dogs is known to be hormone-dependent. Compared with intact female dogs, the risk for malignant tumors in female dogs spayed before the first estrus is 0.5%, if spaying is after the first estrus it increases to 8%, and it increases to 26% if spaying is after the second estrus [2, 10, 16, 20]. Spaying after the latter estrus does not reduce the risk for malignant tumors, although it does appear to decrease the risk for benign tumors [9, 17, 18].

There are several points where canine and human breast tumors have both clinical and molecular similarities [1, 22, 24]. In addition to spontaneous tumor presentation, clinical similarities between human breast tumors (HBC) and canine mammary tumors (CMT) include age of onset, hormonal etiology, and disease evolution. In addition, factors affecting the outcome of the disease, including tumor size, stage, and lymph node invasion, are similar in both species. In particular, *in situ* ductal carcinomas in both HBC and CMT mammary glands are particularly similar in their pathological, molecular, and visual characteristics.

One of these elements that has led to different studies is the hereditary risk of breast cancer, as well as the alterations that may take place at the genetic level. In these aspects, both *BRCA1* and *BRCA2* genes, as well as tumor suppressor genes, have been extensively studied [10, 16, 17, 22, 24]. These studies have been mostly performed in humans, a species where mutations in these genes leads to the accumulation of Deoxyribonucleic acid (DNA) damage and an increased possibility of developing breast and ovarian cancer [17, 22, 24]. Most mutations in these genes were shown to cause protein truncation through indels, nonsense mutations, cut-and-splice variants, or rearrangements [17].

Regarding dogs, alterations in *BRCA1* and *BRCA2* have been identified in the development of breast tumors in different breeds [17]. In particular, the *BRCA1* gene is involved in tumor etiopathogenesis, loss of BRCA1 protein function results in defective DNA due to the lack of DNA repair, leading to a decreased expression, which is a critical step in the development of breast cancer [13, 23]. With regard to the *BRCA2* gene, its mutations are associated with the development of CMT [14, 22, 24]. It should also be mentioned that given the characteristics of CMTs and their similarities with HBCs, dogs could be an excellent model for the study of the disease in humans [1, 11].

In this work, it was proposed to study exons 22 and 23 of the *BRCA1* gene and exons 11 and 27 of the *BRCA2* gene in dogs.

MATERIALS AND METHODS

This work was carried out in the Department of Clinics and Veterinary Hospital, and in the Department of Genetics and Animal Improvement of the Faculty of Veterinary Medicine of the University of the Republic (UdelaR), Uruguay. This study was approved by the Ethics and Animal Use Committee (number 518).

Case selection criteria

CMT cases were selected according to the clinical characteristics previously described in the species. It was worked with animals with nodules/tumors in their mammary region with clinical characteristics consistent with tumors. It was selected females between six and 12 YO, all were clinically examined and classified as suitable for subsequent work with them. For the control cases, it was selected females within the same age range, without oncological pathologies, that have been admitted to the Hospital of the Faculty of Veterinary Medicine for other reasons. All patients were admitted through the general medicine section, where the first clinical examination was performed and the corresponding routine management was indicated. Female dogs without tumors, which would be part of the control group, underwent abdominal ultrasound (ultrasound equipment: TOSHIBA Nemio MX, Japan) and chest X-ray (x-ray equipment: Carestream, España), in order to rule out any oncologic pathology.

Animal study groups

Group 1: female dogs with mammary tumors: 32 females of the above mentioned ages, of breeds: Poodle, Labrador Retriever, Cimarron, Cocker Spaniel, German Shepherd, Boxer, Pitbull, Rottweiler, Bernese Mountain Dog, Golden Retriever, Dogo, American Staffordshire Terrier, Chihuahua, and mixed breeds. All the female dogs had blood values within the reference parameters. It was selected intact (not spayed) female dogs. Eleven of them had given birth. It was performed a clinical examination that included complete case history: age, breed, reproductive status, and tumor characteristics, mainly: location, size, evolution, whether single or multiple. It was registered the location of the tumors in the mammary chain, number, size, consistency, color, adhesions, presence of pain and ulceration.

In each patient, a thorough physical examination was carried out, including individual inspection and palpation of each breast of both chains, and of the regional lymph nodes (axillary and inguinal).

None of the female dogs had lymph node metastases or abdominal metastases. This was ruled out by palpation of satellite lymph nodes and abdominal ultrasound. Two female dogs had lung metastases.

The guardians of the selected patients received information explaining the objectives of the study, the associated risks and benefits, the design of the activities to be carried out, and required interventions, as well as any possible conflict of interest of the personnel involved in the study, and had to provide their signed consent before each surgery (treatment used).

Group 2: Control cases: 13 adult canine females from six to 12 YO, intact (not spayed) who were admitted into the Hospital of the Faculty of Veterinary Medicine (UdelaR), with other non-oncologic pathologies. As in the previous group, there were different breeds: cross breeds, Border Collie, Blood Hound, Poodle, Great Dane. All animals were examined by a Veterinary Doctor by means of a general and particular objective examination in order to determine their health status.

Blood sampling and DNA extraction

Blood was collected from all the female dogs under study under aseptic conditions and considering animal welfare standards. Collection was carried out using tubes with Ethylenediaminetetraacetic acid (EDTA) as anticoagulant, and samples were kept at -18°C in freezer Panavox (China) until further processing. DNA extraction was carried out in the Genetics Laboratory of the UdelaR, with the Qiagen kit (DNeasy Blood & Tissue Kits in spin column). All DNA samples were then quantified using NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, USA) with total spectrum (220-750 nanometers (nm)).

The DNA samples were sent to GeneSeek, ink. in the USA, to study the genotypes of mutations in the *BRCA1* and *BRCA2* genes in both tumor and control female dogs. 25 nanograms per microliter ($ng \cdot \mu l^{-1}$) per sample were sent in solution.

It was analyzed exon 22 and 23 of the *BRCA1* gene, and exon 11 and 27 of the *BRCA2* gene (ENSCAFG00000014600 and ENSCAFG00000006383 respectively).

Statistical analysis

For calculations of population variability and genetic structure statistics: allele frequencies of polymorphisms, Hardy-Weinberg equilibrium, Wright's F statisticians (F_{IS}, F_{IT}, F_{ST}), and Correspondence Factorial Analysis (CFA), it was used the GENETIX V 4.05 freeware [3]. Cluster analysis of the identified polymorphisms by singles nucleotides polymorphisms (SNPs, indels) was performed according to the clinical characteristics of the two populations (animals with tumors/animals without tumors), tumor stage, and breeds; for this it was used bioinformatic tools such as cluster analysis or hierarchical clustering (Hclust function of the Stats package in R programming code, freely distributed).

RESULTS AND DISCUSSION

Allele frequencies

The studied samples were sent to GeneSeek, Lincoln-Nebraska, USA, to be genotyped for 15 different markers (SNPs or indels). This was done in both groups, in the group of female dogs with tumors and in the control group (female dogs without tumors). The data were analyzed using the freely available Genetix software. From these analyses, it appears that of the 15 loci studied, six were polymorphic (all of them SNPs), and the other nine were monomorphic (no variation). Polymorphic loci were: EXON 11 A>C (23125: rs851104585), EXON 11 A>G (22986: rs851293339), EXON 11 A>C3 (23382: rs23244160), EXON 11 A>G1 (23203: rs851048998), EXON 11 A>G3 (23469: rs851757509) and EXON 23 C>T (64182: rs850652146). It can be seen that polymorphic loci are the same in both populations (TABLE I). It was found five of these SNPs in exon 11 of the *BRCA2* gene and one SNP in exon 23 of the *BRCA1* gene in both tumor and control female dog groups. This is equivalent to 40% of the SNPs studied.

TABLE I
Observed allele frequencies

SNP	Allele	Sick population	Healthy population
EXON 11 A>C	C	0.0469	0.0769
	A	0.9531	0.9231
EXON 11 A>G	A	0.6094	0.6154
	G	0.3906	0.3846
EXON 11 A>indel	A	1.0000	1.0000
	-	00000	00000
EXON 11 A>C1	A	1.0000	1.0000
	C1	00000	00000
EXON 11 A>C2	A	1.0000	1.0000
	C2	00000	00000
EXON 11 A>C3	C3	0.2813	0.1923
	A	0.7188	0.8077
EXON 11 A>G1	A	0.9688	0.8846
	G1	0.0313	0.1154
EXON 11 A>G2	G2	1.0000	1.0000
	A	00000	00000
EXON 11 A>G3	A	0.9688	0.8846
	G3	0.0313	0.1154
EXON 11 C>T	T	1.0000	1.0000
	C	00000	00000
EXON 22 GAGA>indel	GAGA	1.0000	1.0000
	-	00000	00000
EXON 23 AG>indel	AG	1.0000	1.0000
	-	00000	00000
EXON 23 C>T	C	0.0313	0.0769
	T	0.9688	0.9231
EXON 27 A>G	G	1.0000	1.0000
	A	00000	00000
EXON 27 TG>indel	TG	1.0000	1.0000
	-	00000	00000

SNPs with polymorphisms are shown in red

Chi-Square test for analysis of Hardy Weinberg equilibrium (H&W)

In order to know whether the analyzed loci were or not in H&W equilibrium, it was performed a chi-square test for each of them in both populations. TABLE II shows these results. In the case of monomorphic loci, all of them were in disequilibrium, with chi-square values greater than 10.83; that is, $P \leq 0.001$. Of the loci with polymorphism, two were in disequilibrium [19].

TABLE II
Chi-square values obtained for loci with observed polymorphisms.

Locus	With tumors		No tumors	
	X^2	*P*	X^2	*P*
EXON 11 A>C	0.08	≥ 0.70	0.09	≥ 0.70
EXON 11 A>G	0.69	≥ 0.30	0.01	≥ 0.90
EXON 11 A>C3	0.22	≥ 0.50	0.75	≥ 0.30
EXON 11 A>G1	0.04	≥ 0.80	4.94	< 0.01
EXON 11 A>G3	0.04	≥ 0.80	4.94	< 0.01
EXON 23 C>T	0.04	≥ 0.80	0.09	≥ 0.70

The two loci with no H&W equilibrium are shown in red

F_{IS}, F_{IT} and F_{ST} values

It was calculated F_{IS}, F_{IT} and F_{ST}. The results obtained were 0.07681, 0.06800 and 0.00954, respectively, it can be seen that there is no subdivision among the animals studied, which is another way to see what was previously mentioned.

In particular, with regard to the F_{IS} values, positive values were found in both populations, which would indicate a deficit of heterozygotes or excess of homozygotes, which again agrees with the genotyping results obtained.

Correspondence factor analysis

It was performed a correspondence factor analysis in both populations (with and without tumors) for the six polymorphic markers. It was used the GENETIX V 4.05 program to construct the figure (FIG. 1).

In summary, regarding the genetic study of the 15 loci, six were polymorphic (40%), all of them of the SNP type, while the other nine were monomorphic (with no variation). It was obtained a low allelic variability, but at the population level the tumor group has greater variability than the control group. On the other hand, the different analyses of possible groupings (dendrograms, correspondence factorial analysis) were negative, and it was not possible to clearly define groups with the parameters it was used.

In dogs, there is evidence that changes in the *BRCA1* and *BRCA2* genes are associated with mammary tumors, just like in humans. Mutations in these genes can be used both for diagnosis and treatment of breast tumors and to further advance cancer treatment in Veterinary Oncology. In this work, it was performed different analyzes with polymorphisms of the *BRCA1* and *BRCA2* genes. In first place, the fact that most of them are in H&W disequilibrium may be due to different elements, among them the fact that they are part of the sequence of the same exon in each of the studied genes (*BRCA1* and *BRCA2*).

On the other hand, there is the possibility that there is some evolutionary force on them. It should be taken into account that pure bred animals have been subjected to an intense selection by man, which could indirectly generate imbalances in allelic frequencies. In the case of dogs, there is a stronger concern regarding whether there is sufficient mating within breeds, because they have developed over the past 150 years by selective breeding of animals with desirable phenotypic traits.

Breed development begins with a small founder population, usually two or four dogs that have such traits. The population grows from mattings of related animals, this leads to dogs of a given breed having a common genetic base. Selection by desirable phenotypic traits has resulted in latent selection of hereditary and genetically-based diseases, as well as predisposition to certain pathologies. Today, some

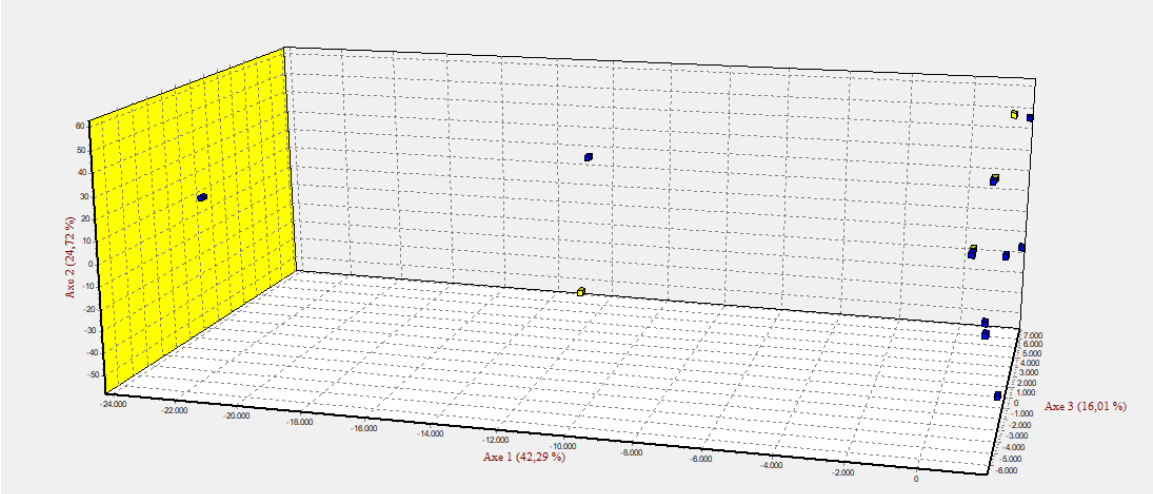

FIGURE 1. Three-dimensional graphical representation of the correspondence factor analysis. In this image, once more it is seen how individuals from both populations studied are admixed, with no subdivisions between them. The results are shown in yellow (dogs with tumours) and in blue (dogs without tumours)

breeds have a high incidence of certain diseases. Breed specificity in this regard makes dogs ideal candidates for comparative genetic association studies; however, it should be considered that most of the breeds would be too inbred to be used in this type of case-control association studies [18].

In this study, population's F_{IS} values were positive but low, thus indicating a deficit of heterozygotes or an excess of homozygotes, although there was allelic variability, which once more agrees with the genotyping results obtained where only six of the 15 markers studied showed polymorphism. In any case, it is interesting that, in the population with tumors, the F_{IS} is lower than in the control population (0.03876, which is a positive value but nevertheless low, that is, towards heterozygosis, while 0.15353 is positive and high, towards homozygosis), which means that there is greater genetic variability in the population with tumors. In this regard, one of the elements to be considered is the number of animals in each group (32 with tumors, and 13 without tumors), where it would be important to form groups with more similar sizes. Besides this, another fact that we must consider is the admixture of breeds in each group [6, 12].

CONCLUSIONS

The data presented in the analysis carried out here may be due to numerous factors such as inherent characteristics of the populations studied, as the size of both populations; the breeds studied; tumor diversity. For this reason, it was intended to homogenize the working groups in these aspects.

The genes studied in this work have been widely related to breast cancer, both in humans and in dogs. In the former, they have been highly implicated in hereditary tumors. In the present case, it was no found relationship between each of the markers studied and the occurrence of mammary tumor between the problem group and the control group. For this reason, in addition to the above, and because it do not have hereditary/non-hereditary data of this species, it was intend to increase the spectrum of genes studied in order to find markers useful in different aspects of clinical oncology, as well as starting to carry out family studies with the aim of analyzing the hereditary component.

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