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BRIEF COMMUNICATION

Methylation pattern and mutational status of *BRCA1* in canine mammary tumors in a Brazilian population

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Abstract

In female dogs, mammary tumors are the most common neoplasia representing about 50% of the tumors affecting this species. In women, the importance of mutations in *BRCA1* and mammary tumors development is well established. However, little information is available on the molecular mechanisms that contribute to canine mammary tumors. In this work, we evaluated the mutational and methylation status of the *BRCA1* gene, in tumoral and non-tumoral tissues of canine mammary glands in order to characterize its influence in mammary carcinogenesis on this species. Samples of 16 animals were collected and two hotspot regions (intron 8-exon 9 and 5′UTR) were sequenced. For methylation analysis, the bisulfite sequencing PCR approach was used. No evidence of hypermethylation was observed in the *BRCA1* promoter region, suggesting this mechanism may not be involved in *BRCA1* silencing in canine mammary tumorigenesis. No alteration was observed in intron 8-exon 9 region. On the other hand, two polymorphisms in the 5′UTR region were observed: a transition (T > C) that has not been previously described in the literature, and observed in one patient with an unfavorable prognosis, and the previously described transversion (C > G). We suggest that methylation is not the main *BRCA1* inactivation mechanism in sporadic CMTs. Regarding the genetic alterations, two variations were detected in our population, and we were able to detect regional allele frequency differences in our population.

Keywords *BRCA1* · Dog · Mammary cancer · Mutation

Introduction

Mammary tumors (CMTs) are the most common neoplasm in female dogs, with an incidence two to three times superior to that observed in humans, and approximately half of them are reported to be malignant (Arnesen et al. 2001; Brodey et al. 1983).

The development of CMTs is multifactorial with intrinsic and extrinsic factors are involved in its pathogenesis, such as hormonal and genetic influences, breed, age, and pregnancy, are already reported (Borge et al. 2011; Cassali et al. 2014; Rivera et al. 2009).

In women, mutations in *BRCA1* are responsible for 5–10% of all familial breast and ovarian cancers (Hopper et al. 1999; Szabo et al. 1996). This gene codifies a protein that participates in the DNA repair pathways, especially in the repair of double-strand DNA breaks by homologous recombination (Foulkes and Shuen 2013).

Several studies indicated that molecular alterations in *BRCA1*, such as point mutations and indels, are involved in the development of CMTs as they can affect the transcriptional regulation of *BRCA1* (Borge et al. 2011; Easton et al. 2007; Rivera et al. 2009). Besides, tumor suppressor genes, including *BRCA1*, may also be affected by epigenetic mechanisms such as DNA methylation (Jones and Baylin 2007; Sharma et al. 2010).

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The aim of this study was to evaluate the methylation pattern and two mutational hotspot regions (5′UTR and intron 8-exon 9) of the *BRCA1* gene in tumoral and non-tumoral tissues of canine mammary glands in a population of Northern Brazil.

Material and methods

Paired samples of 16 animals subjected to mastectomy were collected in the Veterinary Hospital at Universidade Federal Rural da Amazônia (Belém, Pará, Brazil). Information about breed, age, reproductive history, histopathological type, and survival rate were collected when available from the hospital database. All procedures were approved by the Ethics Committee on Animal Use from Universidade Federal Rural da Amazônia (Protocol 23,084.000265/2013-53) and all animals' owners signed a written informed consent.

The DNA was obtained by phenolic extraction (Sambrook and Russell 2000) and its concentration measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific), while its integrity was assessed by electrophoresis on 1% agarose gels stained with ethidium bromide.

For mutational analysis, we amplified and sequenced two hotspot regions in the *BRCA1* (5′UTR and intron 8-exon 9), as previously described (Enginler et al. 2014). For methylation analysis, the primers for bisulfite sequencing PCR (BSP) (*BRCA1*metF: 5′-TTTAGGGAAAGAATTGATGATTAAT-3′ and *BRCA1*metR: 5′-TCCTCTCCCTTCCTATAAAA TCTCT-3′) were designed for the promoter region of *BRCA1* using the software Methyl Primer Express® v1.0 (Applied Biosystems). DNA was converted using sodium bisulfite treatment using the EZ Methylation Kit (Zymo Research) and submitted to PCR amplification performed in a final volume of 25 μL using 0.3 U/μL of Taq DNA polymerase, 1 × reaction buffer, 1.5 mM of $MgCl_2$, 100 ng of genomic DNA, 10 μM of each dNTP, 0.5 mM of each primer, and ultrapure water up to the final volume. Amplification was carried out with an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 40 s, 46 °C for 40 s, 72 °C for 40 s, and a final extension at 72 °C for 7 min. PCR products were visualized in a 3% agarose gel stained with ethidium bromide and purified using the EZ-10 Spin Column PCR Product Purification kit (Bio Basic/Ludwig Biotec), following the manufacturer's instructions.

All samples were subject to DNA sequencing using an ABI 3130 sequencer (Thermo Fischer Scientific) and the obtained sequences were aligned using BioEdit v. 5.0.6 (Hall 1999). The positions of the genetic alterations found were determined by comparing the sequence with the canine genome assembly (Broad CanFam3.1) using the BLAT tool (Kent 2002) available at Genome Browser. Sequences derived from BSP reactions were analyzed using the software BiQ Analyzer (Bock et al. 2005). In this study, we considered a sample hypermethylated when > 15% of the CpG islands examined were methylated (Ferreira et al. 2015).

Correlations between nucleotide variations and clinicopathological features were tested with the Fisher exact test

Table 1 Clinical and histopathological features and genetic variations and genome position according to Broad CanFam3.1 of *BRCA1* in dogs with mammary tumors

Case *n*	Age	Breed	Tumor type	BRCA1		
				5′UTR C/G 19,960,743	5′UTR T/C 19,961,117	I 8—E 9 G/A 19,985,052
1	15	Mixed	Carcinosarcoma	C/G	T	G
2	10	Mixed	Simple adenocarcinoma	C/G	T	G
3	7	Mixed	Carcinosarcoma	C/G	T	G
4	4	Poodle toy	Fibroadenocarcinoma	C/G	T	G
5	12	Mixed	Carcinosarcoma	C	T	G
6	12	Mixed	Complex adenocarcinoma	C/G	T	G
7	10	Pinscher	Complex adenocarcinoma	C	T	G
8	10	Mixed	Carcinoma	G	T	G
9	15	Mixed	Complex adenocarcinoma	C/G	T	G
10	15	Mixed	Osteochondrosarcoma	G	T/C	G
11	15	Mixed	Carcinosarcoma	C/G	T	G
12	8	Mixed	Simple adenocarcinoma	C	T	G
13	NI	Poodle toy	Adenoma—benign tumor	C/G	T	G
14	8	Yorkshire	Tubulopapillary carcinoma	C	T	G
15	NI	Poodle toy	Simple adenocarcinoma	C	T	G
16	5	Rottweiler	Tubulopapillary carcinoma	C/G	T	G

NI, not informed

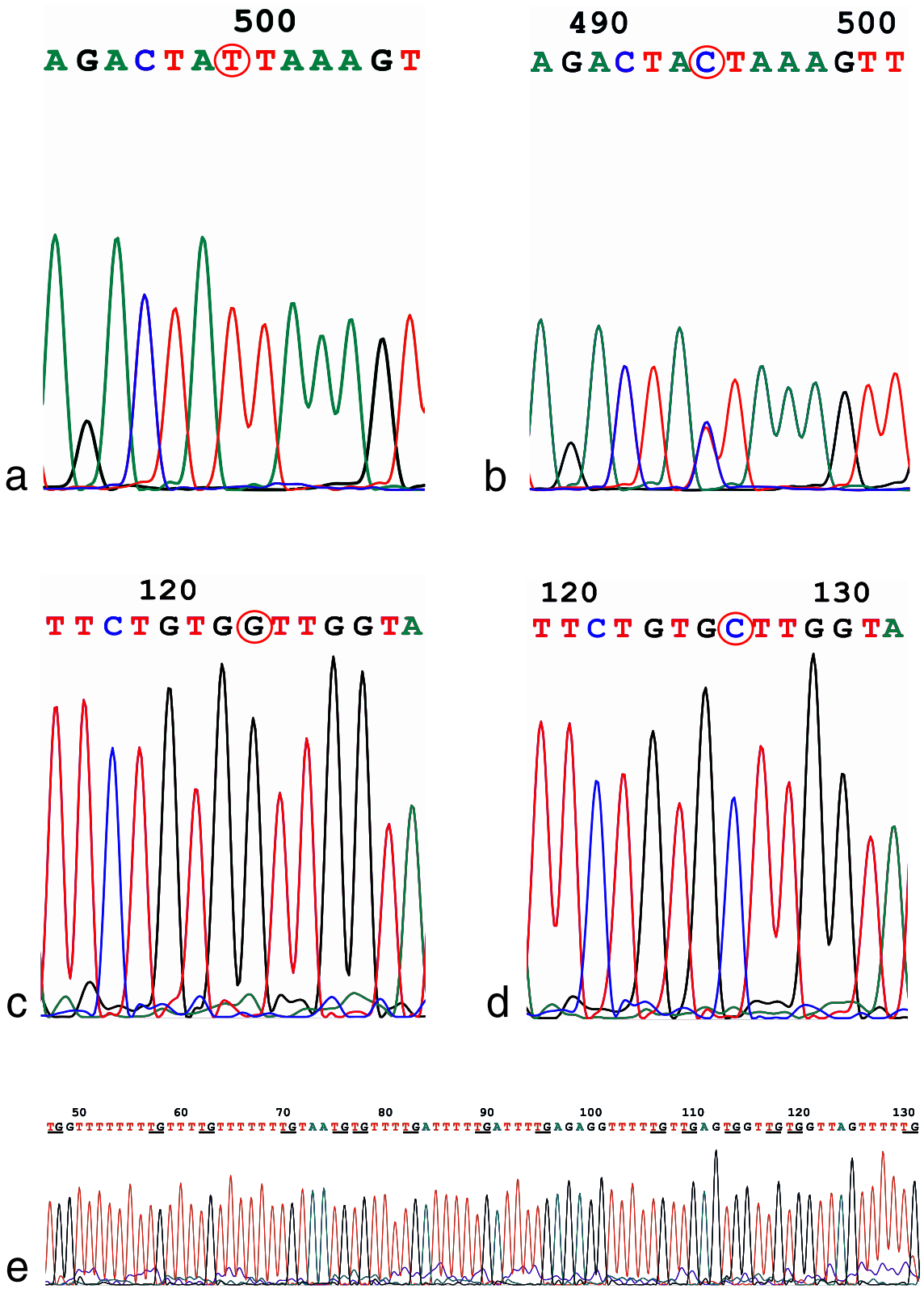

Fig. 1 Alterations found at the 5′UTR region of *BRCA1*. **a**. Homozygous sample for the T allele at genomic position 19,961,117. Red circle denotes the position of the alteration. **b**. Heterozygous sample (T/C) at genomic position 19,961,117. Red circle denotes the position of the alteration. **c**. Homozygous sample for the C allele at genomic position 19,960,743. Red circle denotes the position of the alteration. **d**. Homozygous sample for the G allele at genomic position 19,960,743. Red circle denotes the position of the alteration. **e**. Methylation pattern of the promoter region of the canine *BRCA1* gene. The observed pattern showed the absence of methylation (underlined regions) in the analyzed fragment

and odds ratios (OR) with a confidence interval of 95%. Hardy-Weinberg equilibrium was also tested. A significance level (α) of 0.05 was adopted for all statistical analyses calculated in GraphPad Prism® 5 version 5.01 (GraphPad Software, Inc., USA).

Results and discussion

The average age at diagnosis was 10.7 years old and mixed breed animals were the most prevalent (62.5%; 10/16), followed by Poodle (18.75%; 3/16), Yorkshire, Pinscher and Rottweiler (6.25% or 1/16 each). The most prevalent histopathological type was from tumoral epithelial mammary tissues, accounting for 68.75% (11/16), followed by tumoral mesenchymal tissues (31.25%; 5/16).

Three genetic alterations were found in the hotspot regions analyzed (5′UTR and intron 8-exon 9) on the studied population (Table 1).

No alterations were observed in the intron 8-exon 9 region. In the 5′UTR region, two alterations were observed: a novel mutation (T > C) at position 19,961,117 of canine chromosome 9 detected in one heterozygous animal with poor prognosis histopathological types (osteochondrosarcoma) and a previously reported variation (C > G) (Enginler et al. 2014; Rivera et al. 2009) observed at position 19,960,743 of chromosome 9 (Fig. 1a–d).

No differences were observed between tumoral and non-tumoral sequences from the same patient, suggesting these nucleotide variations are already present in the mammary tissue. No statistically significant risk was observed when we correlated the clinical history and histopathological types with the patients' genotypes/alleles.

We also checked for the methylation status of the promoter region of the *BRCA1* gene. A fragment of 340 base pairs was amplified, which contained 19 CpG sites. No methylation was observed in any of the analyzed samples (Fig. 1e).

It is known that alterations in *BRCA1* are commonly associated with mammary tumors in humans. In dogs, those variations have recently been focused upon as the key for understanding the pathogenesis, clinicopathological status, and prognosis of CMTs (Borge et al. 2011; Rivera et al. 2009; Sun et al. 2015; Qiu and Li 2016; Qiu et al. 2015). In this study, two hotspot regions and the promoter methylation pattern were assessed in order to detect genetic and epigenetic alterations that might influence the development of CMTs.

While in women, hypermethylation of *BRCA1* promoter region is observed in 11–30% and in 42–51% of spontaneous mammary tumors and familial breast cancer, respectively (Tapia et al. 2008). No methylation was observed to all analyzed samples, which is in agreement with previous studies, suggesting that hypermethylation was not the main cause of *BRCA1* inactivation in spontaneous CMTs (Qiu and Lin 2016).

Of the two genetic alterations (SNV) observed, one of them was previously described in literature (Enginler et al. 2014; Rivera et al. 2009). The transversion C > G, observed at position 19,960,743 of chromosome 9 (RefSNP/Assay ID: ss244244319; 5′UTR region), is described as related with the mammary carcinogenesis in dogs as the risk allele (G) has an elevate frequency in Swedish and Turkish populations (0.91 and 0.86, respectively in tumoral samples) (Enginler et al. 2014; Rivera et al. 2009). However, in our population, the observed frequency was remarkably lower (0.4). This difference may be a regional variation due to the higher proportion of mixed breeds in our population, which is in contrast with other populations where a larger number of pure breeds compose the sample groups.

A novel genetic variation was observed in the 5′UTR region (T > C at position 19,961,117 of chromosome 9). Although this mutation was observed in a patient with poor prognosis and a survival rate inferior to 6 months, due to the low frequency, it was not possible to confirm whether this variation could increase the risk of CMTs in female dogs.

Even though the functional meaning of these mutations in female dogs is not clear, studies of spontaneous mammary tumors in women suggest that modifications in the 5′UTR region of *BRCA1* could lead to low mRNA expression as it decreases the efficiency of the transcriptional process (Signori et al. 2001; Wang et al. 2007).

In conclusion, we suggest that methylation is not the main *BRCA1* inactivation mechanism in sporadic CMTs, and also report two genetic variations in our population. Although it was not possible to assess the molecular significance of those variations in canine mammary carcinogenesis, we were able to detect regional allele differences for the previously describe SNV, suggesting a populational variation in *BRCA1* polymorphisms.

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Compliance with ethical standards

Conflicting interests The authors declare that they have no conflict of interest.

Ethics statement All procedures were approved by the Ethics Committee on Animal Use from Universidade Federal Rural da Amazônia (Protocol 23,084.000265/2013-53) and all animals' owners signed a written informed consent

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