Short interspersed CAN SINE elements as prognostic markers in canine mammary neoplasia

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Abstract. The genome of mammals is characterized by a large number of non-LTR retrotransposons, and among them, the CAN SINEs are characteristics of the canine species. Small amounts of DNA freely circulate in normal blood serum and high amounts are found in human patients with cancer, characterizing it as a candidate tumor-biomarker. The aim of this study was to estimate, through its absolute expression, the number of copies of CAN SINE sequences present in free circulating DNA of female dogs with mammary cancer, in order to correlate with the clinical and pathological characteristics and the follow-up period. The copy number of CAN SINE sequences was estimated by qPCR in 28 female dogs with mammary neoplasia. The univariate analysis showed an increased number of copies in female dogs with mammary tumor in female dogs >10 years old (p=0.02) and tumor time >18 months (p<0.05). The Kaplan-Meier test demonstrated a negative correlation between an increased number of copies and survival time (p=0.03). High amounts of CAN SINE fragments can be good markers for the detection of tumor DNA in blood and may characterize it as a marker of poor prognosis, being related to female dogs with shorter survival times. This estimate can be used as a prognostic marker in non-invasive breast cancer research and is useful in predicting tumor progression and patient monitoring.

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Introduction

The canine mammary gland tumor is considered as an appropriate model for the study of human mammary cancer (1,2), since these tumors share histopathological and biological characteristics and it is also useful for testing therapeutic agents (3,4). The current growing interest of cancer researchers in these models and the increasing number of publications in veterinary oncology make this comparison of great importance, allowing the rapid and valid transfer of data for clinical application in human (5).

The genetic analysis of the dog *(Canis familiaris)* is easier nowadays due to a variety of resources which were pointed out by Bentolila *et al* (6), that include a standard canine karyotype (7), physical mapping tools (8-11), highly polymorphic genetic markers (12,13), canine-rodent hybrid cell lines (11), linkage groups (14) and a linkage map (13). Since the domestic dog is a good alternative as a mammalian model for a number of diseases and phenotypes, Bentolila *et al* (6) reinforce that it is important to have further studies once the canine genome is more clearly understood.

The mammalian genomes are characterized by a large number of repetitive DNA sequences, many of which are remnants of transposable elements (TEs) (6,15-17). These repetitive sequences can be classified, according to their transposition mechanism, into two classes: i) Class I or retrotransposons, which are sequences that move via an RNA intermediate. They can be divided into five orders, the LTR retrotransposons, DIRS-like elements, Penelope-like elements (PLEs), LINEs (long interspersed nuclear elements) and SINEs (short interspersed nuclear elements); and ii) Class II elements, which replicate without an RNA intermediate by a mechanism called 'cut-and-paste' and can be classified as Subclass 1 and 2 (18).

The SINEs, especially CAN SINEs, the focus of this study, are repetitions with genomic DNA 80-500 bp in length, often enriched in CpG, apparently arising from accidental retrotransposition of multiple transcripts from polymerase III

(Pol III) (tRNA, 7SL RNA and 5S RNA). SINE sequences were found first in primates and rodents, but now it is generally accepted that several SINE families are present in higher eukaryotes (6). In carnivores, CAN short retroposons are typical tRNA related highly repeated SINEs [~400.000 bp in the haploid genome of the dog (6,19)].

The best known SINE is Alu, that began to amplify early in primate evolution and is one of the most successful mobile elements. The human genome (>10%) is represented by these elements, which comprise approximately one million copies. This high copy number creates a large number of insertion mutations resulting in human disease or genome evolution and the high level of genetic instability contributes to a significant number of germ-line mutations and is an important factor in cancer and/or aging (20).

Present understanding of the molecular mechanisms involved in cancer means that there is an increasing use of diagnostic strategies. Many of the genetic changes involved in tumorigenesis are now time-sequenced, as also occurs with tumors which liberate nucleic acids into various biological fluids, including blood. Thus, the detection of such genetic alterations (those associated with tumor development) in circulating DNA (ctDNA) is a logical new approach as a non-invasive, diagnostic or theranostic test for cancer (21). ctDNA has been observed and described in the plasma, serum and body fluids of pregnant patients, those patients with severe trauma and other patients with different diseases, such as autoimmune disease and cancer (22). It was first observed in plasma samples in 1948 by Mandel and Metais (23) and, only later, Stroun *et al* (24) determined that ctDNA in the plasma of cancer patients originated from tumor cells.

It is accepted that solid malignant tumors release a significant amount of genomic DNA into the systemic circulation (25). The source of ctDNA is still not fully understood, but it has been suggested that necrosis, apoptosis, phagocytosis, direct release and release from macrophages/scavengers following absorption of necrotic cells may play a role (26-31).

The general diagnostic value of simple quantitative measures of circulating DNA is controversial. Further work on the use of ctDNA as a diagnostic marker for neoplasia included the detection of qualitative rather than quantitative differences, such as specific oncogene mutations, loss of heterozygosity, specific Alu amplicons, and methylation patterns found in plasma or serum, and matching them with DNA characteristics in primary tumors (32).

Studies involving dogs with this approach are not found in the literature therefore the aim of this study was to estimate, through its absolute expression, the number of copies of CAN SINE sequences present in free circulating DNA of female dogs with mammary cancer in order to correlate this with the clinical and pathological characteristics and the follow-up period.

Materials and methods

Sample characterization. Peripheral blood samples and tumor fragments from 28 female dogs were collected. The control group consisted of 50 non-affected female dogs. After tumor excision and blood collection, the animals were examined at intervals of one, six and 12 months, by new blood sampling. The age of the animals varied from 6 to 16 years

(mean, 10 years). Most of the tumors were classified as malignant (89%) and consisted mostly of the mixed carcinoma form. The local recurrence rate was 28%, metastasis 18% and death 32% (Table I). The exclusion criteria for the control group were rigorously followed and included females outside the estrus period, with no history of tumors and no detectable disease inflammation/infections in the period before the sampling. We included animals with variable age and breed, besides that, it was verified that the animals were not subjected to surgery in the previous period.

Tumor fragments were fixed in buffered 10% formaldehyde for 24 h and paraffin-embedded. Histological sections (5 μm) were obtained and stained with hematoxylin and eosin (H&E) for histopathology. Tumor grading was done according to Misdorp *et al* (33) by the AFIP (Armed Forces Institute of Pathology). This study was approved by the Comite de Ética no Uso de Animal (CEUA) - Animal Ethics Committee/ FAMERP (protocol no. 3945/2009).

Female dogs from the test group were evaluated with respect to physical (breed and age), pathological (time course - interval between tumor diagnosis and surgical removal, tumor nodules location, lymph node involvement, tumor size, clinical staging, ulceration and vascularization) and clinical (metastasis, tumor recurrence and death) characteristics. The parameters employed for the classification of clinical tumor grading were in accordance with the TNM system (size, lymph node involvement and metastasis) established by the World Health Organization (WHO) for canine mammary gland tumors [modified from Cassali *et al* (34)]. Clinical grading was assigned as I, II, III or IV according to the tumor size and established prognosis.

Plasma sample collection. Blood (3 ml) was collected in a CORVAC serum separator tube (Labor Import Co., Sao Paulo, Brazil) containing clot activation additive and barrier gel, stored at 4˚C and processed within 6 h: the tubes remained in a water bath for 15 min at 37˚C and the blood was separated by centrifugation (1000 x g, 25 min). The serum was immediately cryopreserved at -80˚C.

Cloning and estimation of CAN SINE copy number. Serum samples selected for cloning were submitted to a pre-treatment to deactivate or eliminate proteins binding to template DNA. For this, 10 μl of serum sample was mixed with 0.1 ml of DNAzol® Direct (Molecular Research Center Inc., Cincinnati, OH, USA) according to manufacturer's conditions. CAN SINE sequences were amplified by PCR reaction using specific primers CAN SINE: 5'-CTGGGTGGCTCAGTCRGT-3' and 5'-AGCACAGAGCCYGAYGYG-3' (19) that amplify 174 bp. The sequences amplified were purified with the GFX kit-Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare), measured through a 1% agarose gel stained with ethidium bromide and subsequently used for cloning.

The CAN SINE sequence was cloned into the Topo TA vector® plasmid, using the TOPO® TA Cloning kit (Invitrogen) according to manufacturer's conditions in competent cells (One Shot® TOP10 Chemically Competent *E. coli*). The presence of insert using primers M13F and M13R was investigated in colonies. Subsequently extraction was performed using the plasmid phenol-chloroform. The presence of the CAN SINE sequence in the plasmid was confirmed by restriction profile

(restriction enzymes in different combinations were used with enzyme cuts at different positions of the plasmid with the enzymes *Hin*dIII, *Eco*R1, *Xma*I + *Eco*R1 and *Xma*I + *Kpn*I) (Invitrogen) and sequencing.

The linearized plasmid was obtained by digestion by the enzyme *Hin*dIII (Invitrogen) according to manufacturer conditions. After digestion, the material was purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and quantified by NanoDrop 1000 Spectrophotometer (Thermo Scientific). A final concentration of 83.6 ng/μl for the linearized plasmid with the CAN SINE sequence was established. The estimated number of copies of the sequence of interest was calculated using the formula, according to Zhang and Fang (35) and Liang *et al* (36):

$$\frac{X \ g/\mu l \ DNA}{size \ of \ the \ oligo \ bp \ x \ 649} \ x \ 6.022 x 10^{23}$$

Absolute quantification by real-time (qRT-PCR). The number of copies of the CAN SINE sequence for each sample was calculated using the absolute quantification by real-time PCR. To deactivate or eliminate proteins binding to template DNA that might invalidate qRT-PCR results, a pre-treatment of samples was necessary following the protocol of Umetani *et al* (37) modified, 20 μl of each serum sample was mixed with 20 μl of a preparation buffer that contained 2.5% of Tween-20, 50 mmol/l Tris and 1 mmol/l EDTA. This was digested with 16 μg of proteinase K solution (Qiagen, Valencia, CA, USA) at 37°C overnight, followed by 5 min of heat deactivation and insolubilization at 95°C. After subsequent centrifugation (10000 x g, 5 min), the supernatant was stored at -20°C for later quantification in NanoDrop 1000 Spectrophotometer (Thermo Scientific).

The reaction mixture for each qRT-PCR consisted of a template sequence 10 pmol/μl of the forward and reverse CAN SINE primers, the same used for cloning, 100 ng/μl of sample and system detection Master Mix SYBR™ Green (Applied Biosystems). The absolute equivalent amount of DNA in each sample and the initial concentration of genomic DNA from the constructed plasmid were determined by a standard curve with serial dilutions. In each reaction, a standard curve was included corresponding to a baseline distinguishing the number of copies and five serial dilutions and analyses where performed to estimate the number of copies of the target sequence in each sample.

The amplification was performed with pre-cycling heat activation of DNA polymerase at 95°C for 10 min, followed for initial denaturation at 95°C for 15 min and 60°C for 1 min. For the melt-stage curve, the program continued with 40 cycles of 15 sec at 95°C, 1 min at 60°C for annealing of primers and extension of the chains, followed by 15 sec at 95°C to collect the signal using Real-Time PCR System Step One Plus (Applied Biosystems). A negative control (without template) was performed for each plate. All qRT-PCR assays were performed in a blinded fashion without knowledge of specimen identity. Mean values were calculated from triplicate reactions.

Statistical analysis. The results were first submitted to descriptive analyses for the determination of normality and were considered to have a normal distribution. The different clinicopathological characteristics and quantifications were
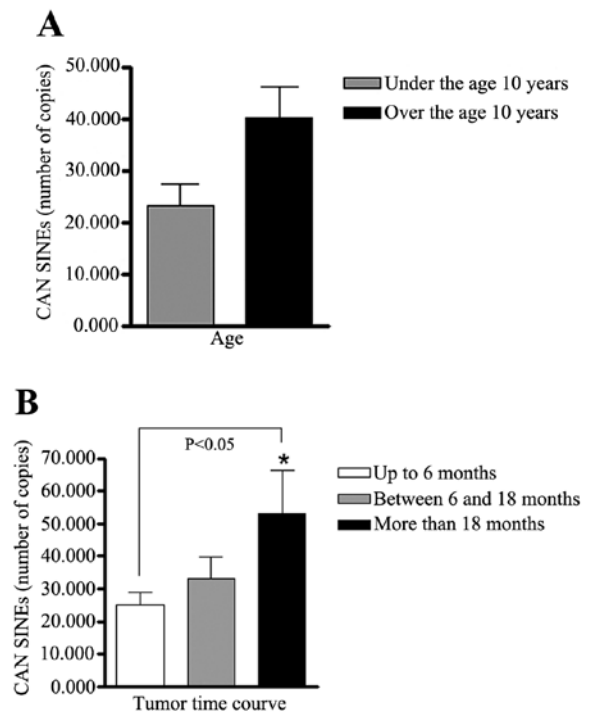

Figure 1. Representation of CAN SINE copies in female dogs with mammary tumor - Correlation between (A) age (p=0.02) and (B) tumor time course (p<0.05) *p = significant difference after the Student's t-test or ANOVA.

separated in groups and compared by the Student's t-test or ANOVA, followed by the Bonferroni test. The association between the characteristics and quantifications with animal death was evaluated by Fisher's exact test. All values were expressed as the mean ± standard deviation (SD).

The survival curve was constructed following the Kaplan-Meier method. The cut-off points for the CAN SINE measurements were established by the receiver operating characteristic (ROC) curve. Survival curves were plotted using the Kaplan-Meier method and the differences between the curves were evaluated by a log-rank test and hazard function. A multivariate logistic regression analysis was employed to evaluate the simultaneous influence of the prognostic factors on animal death. A p<0.05 value was considered statistically significant. All the analyses were done using the GraphPad Prism4 and Stats Direct software.

Results

The estimated value of CAN SINE sequences in the serum was $1.89 x 10^{13}$ on 100 ng/μl plasmid. The estimated mean number of CAN SINE copies was 3,197,000 number/μl serum of female dogs with mammary tumors and 3,379,300 number/μl serum in the control samples with no statistically significant difference (p=0.7) and the mean estimate of the number of copies of female dogs with mammary tumors immediately after surgery and at intervals of one, six and 12 months did not show any statistically significant difference (p>0.05) (Table I). The univariate analysis showed an increase in the number of copies in female dogs with mammary tumor over the age of 10 years (p=0.02) and tumor time of >18 months (p<0.05) (Table I) (Fig. 1).

Table I. Relationship between CAN SINEs copies of dogs with mammary cancer and the clinicopathological parameters.

Clinicopathological parameters	No. of dogs	CAN SINEs
Clinical feature[a]		
Control	50	3,197,000±4.089
Samples	28	3,379,300±3.423
p-value		0.7
Age[a]		
≤10 years	15 (54%)	2,326,000±4.082*
>10 years	13 (46%)	4,024,000±6.006*
p-value		0.02
Histological type[a]		
Malignant	25 (89%)	2,990,000±4.143
Benign	3 (11%)	4,923,000±1.494
p-value		0.2
Tumor nodules location[a]		
Multiple	11 (39%)	3,698,000±7.007
Single	17 (61%)	2,821,000±4.845
p-value		0.2
Tumor size[a]		
T1/T2	14 (50%)	3,061,000±5.610
T3/T4	14 (50%)	3,333,000±6.140
p-value		0.7
Lymph node involvement[a]		
N0	16 (57%)	2,826,000±4.877
N1/N2	12 (43%)	3,529,000±5.991
p-value		0.3
Metastasis[a]		
MO	23 (82%)	3,099,000±4.488
M1	5 (18%)	4,136,000±1.055
p-value		0.3
Time course[b]		
Up to 6 months	13 (46%)	2,506,000±3.711**
Between 6 and 18 months	10 (36%)	3,302,000±6.913
More than 18 months	5 (18%)	5,312,000±1.330**
p-value		<0.05
Tumor recurrence[a]		
Yes	8 (28%)	2,438,000±3.715
No	20 (72%)	3,501,000±5.435
p-value		0.2
Ulceration[a]		
Yes	10 (36%)	2,140,000±3.004
No	18 (64%)	3,784,000±5.737
p-value		0.05
Vascularization[a]		
Abundant	15 (53%)	2,978,000±4.773
Moderate	13 (47%)	3,629,000±6.951
p-value		0.4
Clinical staging[a]		
I/II	13 (46%)	3,049,000±6.058
III/IV	15 (54%)	3,516,000±5.788
p-value		0.5
Censorship[a]		
Death	9 (32%)	3,250,000±6.747
Alive	19 (68%)	3,172,000±5.232
p-value		0.9

Different symbols indicate different statistical analysis. [a]Student's t-test, [b]ANOVA. *For statistical significant (p<0.05).
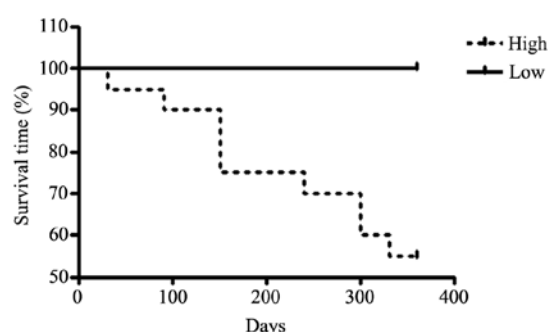

Figure 2. Global survival of female dogs with high and low CAN SINE copies (cut-off value: 19132.00 copies) (OR, 0.1; 95% CI=0.468104-0.876925; p=0.03).

Table II. Fisher's exact test in female dogs with mammary tumor and clinicopathological characteristics and CAN SINE copies.

Variables	OR	CI 95%	p-value
Tumor recurrence	18	1.79159-231.114723	0.004
Metastasis	Infinity	2.799376 to infinity	0.001
Ulceration	7.5	0.966769-64.221665	0.03
CAN SINEs	0	0-0.989268	0.03

OR, odds ratio; CI, confidence interval.

Table III. Multivariate logistic regression analysis in female dogs with mammary tumor and clinicopathological parameters.

Variables	OR	CI 95%	p-value
Tumor recurrence	13.6	1.580583-116.364655	0.01
Ulceration	8.5	1.211601-59.656459	0.03

OR, odds ratio; CI, confidence interval.

The female dogs were followed for 30-360 days for the survival curve. They were divided according to the last sample measured before death and, using the ROC curve, the best cut-off point of CAN SINEs to discriminate the high risk of death was 1,913,200 number/μl serum [sensitivity (95% CI)=100%, specificity (95% CI)=42%]. The Kaplan-Meier test demonstrated a negative correlation between an increased number of copies and survival time (OR, 0.1; 95% CI=0.468104-0.876925; p=0.03) (Fig. 2) where all female dogs with increased number of CAN SINE copies died.

When each clinical pathological characteristic of the test group was associated with higher risk of death, tumor recurrence (OR, 18; p=0.004), metastasis (OR, infinity; p=0.001), ulceration (OR, 7.5; p=0.03) and a higher number of CAN SINE copies (OR, 0; p=0.03) were statistically significant (Table II). Moreover, the simultaneous influence of prognostic factors and the number of copies on risk of death in female dogs with mammary tumors was correlated with

tumor recurrence (OR, 13.6; p=0.01) and ulceration (OR, 8.5; p=0.03) (Table III).

Discussion

Technical progress in the detection and the quantification of specific RNAs or DNAs has made disease diagnosis and follow-up possible. Specifically, ctDNA was found to carry tumor-associated genetic alterations and, thus, for more than a decade, it has been considered as a potential biomarker for cancer diagnostics and non-invasive testing (26,38,39), a predictor of the existence, progression and prognosis of breast cancer (40) proportional to the tumoral mass (41) as well as a way to monitor the effect of cancer therapy (30).

Only a few studies have been published on the analysis of the sequences that make up total circulating DNA and the estimation of CAN SINE sequences in dogs does not exist in the literature. PCR and real-time PCR revealed the presence of a number of genes such as p53, the Ras family, Ig Heavy chain, TP53, SRY and DYS1 (for fetal DNA), lamin B2, leptin, β-Globin, β-actin and Alu repeats, and a number of other polymorphic markers were used to detect alterations that are similar in tumor and plasma DNA (42). The composition of circulating DNA with regard to the sequence content and the variation in the amount of all or only specific sequences in malignant and other diseases compared to controls is largely unknown.

Elucidating the sequence contents of total circulating DNA may contribute to determining the source and function of circulating DNA (42). Continuing expansion of retrotransposons in humans and its consequences are responsible for several different human genetic disorders. Since the mobility of SINEs in the dogs is even higher than in humans, they may also be the cause of many genetic disorders in dogs. Retrotransposons can disrupt ORFs, alter splicing, or modulate gene expression and may also create genomic deletions upon insertion into host genomes and have a postinsertional impact through unequal homologous recombination (43). Results of Clark et al (44) further suggest that SINE insertions, present in the canine D2 dopamine receptor gene, the dystrophin gene and the PTPLA gene, implicated in centronuclear myopathy, may also play a significant role in the phenotypic diversity of dog breeds.

Through absolute quantification by qPCR it was possible to estimate, in arbitrary units, the number of copies of CAN SINE sequences in free circulating DNA from cells of mammary tumors of female dogs and it is possible to correlate this data with the clinical and pathological features and survival. In addition, non-invasive monitoring of animals at different sampling times during the onset of the disease is able to help predict and describe accounts of death and/or tumor recurrence and, thus, can be used in establishing the prognosis in breast cancer. Besides that, according to Schwarzenbach et al (29), minimally invasive blood analyses of cell-free nucleic acid allow repetitive real-time monitoring of these events and treatment effectiveness.

No difference was found between the release of CAN SINE copies in DNA in the cancer and the control group, despite the exclusion criteria of the control group being strictly followed. A likely explanation for this is the different categories of ctDNA: tumor ctDNA from microenvironmental, neoplastic cells and ctDNA from normal extra tumoral cells which arise from several cell types from apoptosis, such as morphogenesis, the immune system, intestinal, mammary differentiation and dedifferentiation, or from necrosis induced by infection, infarction, injury, inflammation or poison, as pointed out by Mouliere et al (26). However, through the univariate and multivariate analysis it was possible to associate the predictive factors of tumor development and poor prognosis with the increased release of CAN SINE copies in free circulating DNA, characterizing it as a potential prognostic marker in breast cancer.

The genetic and epigenetic alterations in plasma DNA have been associated with worse prognosis in several tumor types (45,46). The presence of circulating free DNA in the blood is considered to be a derivative of increased and abnormal apoptotic pathways in the cancerous lesions (27). The abnormal DNA degradation or secretion leads to increased DNA levels and differing DNA fragmentation, readily identifiable with standardized and affordable molecular biology techniques. Necrosis is associated with tumor development, while apoptosis and phagocytosis are associated with defense mechanisms and both lead to the destruction of tumor cells and also the adjacent non-tumor tissues and to DNA fragmentation (24). A study of Schaefer et al (47) found that dogs with lymphoid neoplasia have increased plasma DNA, characterizing it as an indicator of poor prognosis.

The increased amount of copies of the CAN SINEs observed in female dogs with longer tumor times is in agreement with Thierry et al (31) indicating that increased amount of plasma ctDNA is observed as the tumor progresses and high ctDNA levels are found in patients with advanced disease, metastasis and this correlates with shorter survival. The integrity of circulating DNA could have a significant prognostic utility in the detection of breast cancer progression and axillary lymph node metastasis. It is assumed that the DNA released from malignant tumors into the bloodstream is enhanced by lymphovascular invasion and, as a result, circulating DNA may be directly related to tumor progression and rate of tumor cell turnover, representing biologic tumor aggressiveness (37). Furthermore, many benign tumors and preneoplastic regions, a higher rate of cell proliferation is accompanied by a commensurate increase in apoptotic cell death (48). Genetic changes due to apoptosis regulation are usually a fairly late event in tumor development and are involved in tumor progression rather than the start of the process. Mostly, tumor volume is small in the early stages of development but the rate of apoptotic cell death is relatively high. During later stages of tumor development, even if the rate of apoptosis decreases, the total number of apoptotic cells is still high due to the large size of the tumor. Two additional factors, which can affect the level of nucleic acids in circulation, are the degree of tissue vascularization and the clearance of circulating nucleic acids into urine and other compartments (48).

Through the survival curve, it was observed that all the female dogs that died showed an increase in the number of copies of the CAN SINE element, allowing us to propose the hypothesis that the increased release of free circulating DNA is associated with malignant cellular changes that occur during

the neoplasia process. Moreover, this estimate was considered an independent prognostic marker for survival and can be used to control the staging of breast cancer.

The finding that nuclear DNA of patients with cancer can release the nucleic acids for circulating tumor cells is of crucial importance in the diagnosis of these patients (49). Moreover, the absence of studies focused on markers that define the prognosis for the routine clinical practice for dogs with mammary cancer make this study of fundamental importance (50) especially when the targets are DNA fragments never previously studied in this species.

The wide circulation of CAN SINE copies observed in canine mammary cancer and its relationship to factors of tumor progression and increased risk of death makes it possible to characterize the free circulating DNA, derived from non-LTR retrotransposon, as a potential prognostic marker.

Acknowledgements

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