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Global DNA hypomethylation in canine mammary tumors

Hipometilación genómica global en tumores de mama canino

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ABSTRACT

Due to its influence in transcriptional potential of genes, genetic regulation by means of epigenetic mechanisms is essential for normal growth and development. In mammary cancer, epigenetic modifications play a key role for its development and progression. In early carcinogenesis stages, due to genetic alterations or environmental factors, chromatin structure alterations due to DNA methylation and post-translational modifications of DNA-bound proteins may appear. As with other types of tumor, genome-wide hypomethylation and hypermethylation of specific genes, particularly in CpG islands that normally are not methylated, are observed. In order to compare global DNA methylation levels between tumor tissue and normal mammary tissue, we studied 11 intact female dogs with mammary tumors. Both types of tissue were collected during surgery, with subsequent clinical staging and histopathological classification of tumors. For each animal, DNA was extracted from paired samples of tumor tissue and normal mammary tissue. Global genomic methylation levels were calculated by relative quantitation of 5-methyl-2'-deoxycytidine (5mdC) with HPLC. Results showed that tumoral tissue had a global DNA hypomethylation when compared with normal mammary tissue ($P < 0.05$). This difference was greater in high histopathological grade tumors, characterized by their aggressive clinical behavior and high metastatic rate. These findings underscore the importance of additional studies in this line of research, with greater sample sizes. In the future, global DNA methylation may be used as a prognosis biomarker for mammary cancer in dogs.

Key words: Canine mammary tumors; epigenetics; global DNA methylation

RESUMEN

La regulación génica mediante mecanismos epigenéticos es esencial para el crecimiento y el desarrollo normal, ya que influye en el potencial transcripcional de los genes. En el cáncer de mama, las modificaciones epigenéticas desempeñan un papel clave en su desarrollo y progresión. En etapas tempranas de la carcinogénesis, como consecuencia de lesiones genéticas o factores ambientales, se producen alteraciones en la estructura de la cromatina a través de la metilación del ADN y de modificaciones postraduccionales de proteínas unidas al ADN. Al igual que varios tumores, se observa una hipometilación a nivel genómico y la hipermetilación de ciertos genes, especialmente en islas CpG que normalmente no se encuentran metiladas. Con el objetivo de comparar el nivel de metilación global del ADN entre tejido tumoral y tejido mamario sano, se estudiaron 11 perras enteras con tumores de mama. Ambos tipos de tejido fueron extraídos durante la cirugía, y se realizó la estadificación clínica y clasificación histopatológica de los tumores. A partir de muestras pareadas de tejido tumoral y tejido mamario sano de cada animal, se extrajo ADN y se determinaron los niveles de metilación genómica global mediante cuantificación relativa de 5-metil 2-desoxicitidina (5mdC) utilizando HPLC. Los resultados revelaron una hipometilación global del ADN en el tejido tumoral en comparación con el tejido mamario sano ($P < 0.05$). Esta diferencia fue más pronunciada en tumores de alto grado histopatológico, caracterizados por un comportamiento clínico agresivo y una elevada tasa de metástasis. Estos hallazgos subrayan la importancia de continuar con esta línea de investigación, ampliando el tamaño muestral. En el futuro, la metilación global del ADN podría utilizarse como un biomarcador pronóstico del cáncer de mama en perros.

Palabras clave: Tumores de mama en caninos; epigenética; metilación global del ADN

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INTRODUCTION

Epigenetics refers to changes in genetic expression impacting on phenotype and that involve no modifications of the DNA sequence [1 , 2]. Epigenetic processes include DNA methylation, histone post-translational modifications, non-coding RNA functions, and co-transcriptional modification of different RNA molecules (mRNA, rRNA, tRNA, RNAmi or lncRNA) [3]. Genetic regulation by means of epigenetic mechanisms is essential for normal growth and development, conditioning transcriptional potential of genes. In normal cells, DNA methylation in necessary for maintenance of cellular growth and metabolism, while abnormal DNA methylation may cause different diseases, like cancer [1]. In cancer, DNA methylation is one of the most frequently altered epigenetic processes [4 , 5].

DNA methylation consists in a covalent chemical modification that adds a methyl group (CH3) to the fifth carbon of the cytosine pyrimidine ring, leading to the formation of 5-methylcytosine (5-me-C). This process is catalyzed by the DNA methyltransferases (DNMT), a family of enzymes that may carry out de novo DNA methylation or establish methylation of hemimethylated DNA [2 , 6].

In mammary cancer, epigenetic modifications are a key element in tumor development and progression. In early carcinogenesis stages, due to genetic lesions or environmental impacts, alterations in chromatin structure take place through DNA methylation and post-translational modifications of DNA-bound proteins [7]. These alterations impact cellular plasticity and favor oncogenic reprogramming of tumor progenitor cells, promoting the acquisition of uncontrolled self-renewal properties. In later stages of cancer growth, further epigenetic modifications, combined with subclonal mutations and microenvironmental signals, modulate tumor cell phenotype and influence its metastatic propensity [8].

As for the role of DNA methylation in mammary cancer, it has been demonstrated, similarly to cancer in general, both hypomethylation at the genomic level and hypermethylation of certain genes, particularly in usually unmethylated CpG islands [2]. More than 100 genes with hypermethylated promoters have been identified; many of them are critical for tumor suppression, cell cycle regulation, apoptosis, angiogenesis, tissue invasion, and metastasis [9].

On the other hand, DNA methylation has been associated with clinical and pathological characteristics of women with breast cancer, such as tumor clinical stage and grade. Additionally, specific methylation profiles associated with different breast cancer subtypes in women have been identified, suggesting they could play an important role in their development and progression [10].

Cancer is the best-characterized complex human disease associated with epigenetic defects [11 , 12], with epigenetic modifications observed in human tumors being shared with canines. This supports the possibility of using dogs (*Canis lupus familiaris*) as a model for studying such alterations for diagnosis, prognosis and development of new therapies. Canines are an excellent study model, as they share the same environment with humans [13].

Humans and dogs share similar aspects of the disease, including spontaneous tumor development, hormonal components, age of incidence, and disease progression. Tumor size and lymph node invasion show no differences between both species. Likewise, humans and dogs share expression of certain factors such as steroid receptors, epidermal growth factors, proliferation factors, and P53 mutations [14].

DNA methylation studies related to canine mammary tumor (CMT) development are scarce. Research includes the analysis of genome-wide methylation profiles (methylome) [15] and quantification of global DNA methylation [16]. Brandão *et al.* [17] analyzed the DNA methylation status of the ESR1 gene encoding estrogen receptor alpha in canine mammary tumors. Beetch *et al.* [18] analyzed samples from different tumor stages, as well as from healthy donors, describing hypermethylation patterns of cancer-related genes.

In view of the above, this study aims to compare global DNA methylation levels between tumor tissues and normal mammary tissue of female dogs with mammary tumors.

MATERIALS AND METHODS

The research was carried out at the Faculty of Veterinary Medicine Hospital, the Genetics and Animal Improvement Unit of the Faculty of Veterinary Medicine, and the Genetics Department of the Faculty of Medicine (University of the Republic). All procedures were approved under number 1383 by the Honorary Commission for Animal Experimentation (CHEA).

Eleven intact female dogs, with mammary tumors and ages ranging from 6 to 12 years of old that had been admitted to the Hospital, were selected. Mammary tumor cases were selected according to the clinical characteristics previously described in the species. The animals included in the study exhibited an acceptable general clinical condition and underwent pre-surgical studies (blood biochemistry and urinalysis) within reference ranges, which, combined with disease staging, allowed for the selection of an appropriate surgical approach (anesthetic risk classification: ASA I or II).

Clinical staging of patients with mammary tumors was performed according to the TNM mammary tumor classification criteria of the World Health Organization (WHO) by means of triple view chest X-ray and abdomen ultrasound. Normal and tumoral tissue samples were collected the same day of the surgery, and were immediately stored in Eppendorf tubes with no solution at -80°C in a freezer Thermo Fisher Scientific, TSX Series (USA) until their epigenetic and histological and pathological analysis (TABLE I).

Normal tissue samples were obtained from the mammary glands adjacent to those with tumors, using standardized sampling site criteria. This procedure was followed because regional excisions or radical mastectomies generally involve removal of normal mammary glands in the same surgical procedure, thus eliminating the need for additional biopsies for collection of these types of samples.

The DNA extraction from tumoral and normal mammary tissue samples was performed with the DNeasy Blood & Tissue kit (Qiagen). Extracted DNA concentration and purity was measured with a DeNovix DS-11 spectrophotometer USA. Global genomic methylation levels were measured by relative

quantification of 5-methyl-2'-deoxycytidine (5mdC) with HPLC as described by Berdasco *et al.* [11] and Cappetta *et al*. [12]. For enzymatic hydrolysis, 1 µg of genomic DNA (in 10 µg of ultra-pure water) of each sample was denatured for 10 minutes (min) at 94 °C, followed by cooling on ice for 5 min. Subsequently, 1.5 µL of nuclease P1 (New England BioLabs), with its corresponding buffer was added, with subsequent incubation at 37 °C for 15 hours (h) using a Multigene II machine (Labnet International, Inc. USA Hydrolyzed DNA was incubated with alkaline phosphatase (Thermo Scientific™ USA) for an additional hour at 37 °C and 10 min at 75 °C, according to the manufacturer›s instructions, in a reaction with a final volume of 50 µL.

Nucleoside separation following genomic hydrolysis was performed with an HPLC Agilent series 1100 equipment (USA) with UV detection. 50 µL of hydrolyzed DNA were injected into a dC18 Atlantis (Waters) reverse-phase column (2.1 × 20 mm; 5 mm particle size), protected by a guard column (2.1 x 20 mm; 5 mm particle size; Agilent), at a 0.150 mL/min constant flow rate. The following buffers were used: 0.1 % formic acid in water (solvent A), and 0.1 % formic acid in water:methanol (40 %) (solvent B); in an isocratic gradient of 95 % solvent A and 5 % solvent B for 30 min. As standards, a 5 mM mixture of the following mononucleosides were used: deoxyadenosine (dA), deoxythymidine (dT), deoxyguanosine (dG), deoxycytidine (dC), 5-methyl-2'-deoxycytidine (5mdC), and deoxyuridine (dU) (Sigma-Aldrich).

Quantification of 5mdC and dC percentages was directly calculated from the area of their respective peaks in the HPLC chromatogram. Samples were analyzed in duplicate, and those with a 5mdC content differences between duplicates greater than 3 % were excluded [12]. Global genomic DNA methylation level is shown as the relative amount of 5mdC in total cytidine residues; thereby, global genomic DNA methylation percentage is: [mdC / (mdC + dC)] × 100.

Statistical analysis

The Shapiro-Wilk test was performed to assess normality of global methylation percentage data distribution. Upon confirmation of normality assumptions, Student's t-test was performed. Cohen's d effect size was calculated to assess the magnitude of the difference between both groups [19].

RESULTS AND DISCUSSION

Global genomic DNA methylation was assessed in 11 female dogs of various breeds diagnosed with mammary cancer, comparing tumor tissue with normal mammary tissue from each animal studied. TABLE I summarizes age, breed, disease stage, histopathological classification and grade of tumors following surgery, as classified according to Goldshmidt *et al.* [20], as well as the DNA methylation percentage of normal and tumor tissues.

The histopathological analysis showed that 8 (72.7) and 3 (27.3 %) of the 11 female dogs analyzed had malignant and benign tumors respectively. Malignant tumors comprised: 3 simple carcinomas, 2 mixed carcinomas, 1 papillary cystic carcinoma, 1 complex adenocarcinoma, and 1 solid carcinoma. Benign tumors corresponded to 2 mixed adenomas and 1 complex adenoma. This distribution of tumor types is consistent with the most frequent tumors in CMT reported by Goldschmidt *et al*. [20].

TABLE I
Description of patients by age, breed, disease stage, histopathological classification, and methylation percentage in normal tissue and tumor tissue.

Case	Age	Breed	Stage	Histopathological Grade		Normal Tissue Methylation	Tumor Tissue Methylation
1	10	Cimarrón	III	II	Papillary cystic carcinoma	4.791892158	2.725887465
2	8	Mixed Breed	III	II	Simple adenocarcinoma	2.144284287	2.336448598
3	12	Cocker	III	II	Mixed carcinoma	2.413530977	2.285336856
4	8	Mixed Breed	III	III	Simple adenocarcinoma	4.342321813	2.766662856
5	7	Mixed Breed	III	Benign	Mixed adenoma	2.884515795	3.053574218
6	7	German Shepherd	III	II	Complex adenocarcinoma	2.46986528	1.924168464
7	8	Pug	I	I	Mixed carcinoma	3.143661972	2.307747267
8	8	German Shepherd	I	Benign	Complex Adenoma	3.750999886	3.754702599
9	6	Poodle	I	Benign	Mixed adenoma	3.879251734	3.617225363
10	8	Mixed Breed	III	II	Simple Carcinoma	4.002230611	2.622711446
11	10	Poodle	I	II	Solid Carcinoma	4.027690371	3.458671344

The breeds studied included 4 mixed breeds, 2 Poodles, 2 German Shepherds, 1 Cocker Spaniel, 1 Cimarrón, and 1 Pug. The prevalence of these breeds in the sample may reflect their overrepresentation in the canine population of our country, which may vary according to geographical location [21].

Comparison of global genomic methylation levels between normal tissue and tumor tissue from dogs histopathologically diagnosed with mammary tumors revealed a significant difference ($P < 0.05$). Specifically, tumor tissues had lower methylation levels than normal tissues animals (FIG. 1), indicating global hypomethylation in tumor tissues of the analyzed This finding is consistent with existing literature, where hypomethylation is reported as a common epigenetic alteration in different cancer types, including mammary cancer in women [12] and dogs [15 , 16], as well as other cancer types [15].

Cases 1, 4, and 10 showed the greatest differences in genomic methylation percentages between tumor and normal tissue. These three female dogs (two mixed breeds and one

Cimarrón) were diagnosed with high histopathological grade tumors (grade III): one cystic papillary carcinoma and two simple adenocarcinomas. These tumors are characterized by their clinically aggressive behavior and high metastatic rates.

These results are consistent with those of Biondi *et al.* [16], who also observed significant differences in global DNA methylation patterns in CMT, where hypomethylation was more frequent in malignant tumors. Although these authors used a different detection approach (5-methylcytosine immunostaining) and a larger sample size, the similarity of findings supports the existence of a relationship between global DNA hypomethylation and malignancy in canine mammary tumors, thus suggesting its potential as a prognostic biomarker, particularly in tumors with a more aggressive behavior and a higher relapse risk.

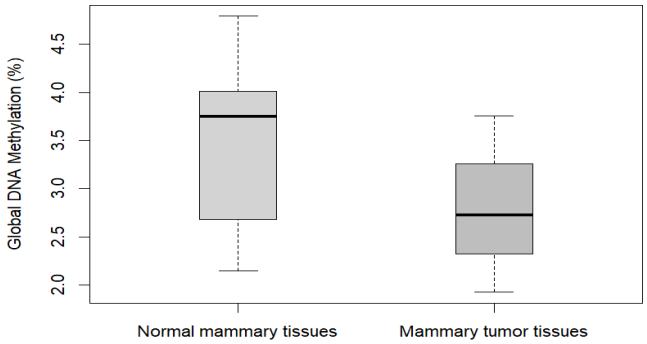

FIGURE 1. Comparison of global DNA methylation level percentages between normal mammary tissues and mammary tumor tissues. Boxes represent interquartile ranges, and lines across boxes the median values. Statistically significant differences between normal mammary tissues and mammary tumors were calculated using Student's t test ($P < 0.05$)

The observed difference in methylation between normal and tumor tissue in 11 of the samples, besides being statistically significant, was also of considerable magnitude according to the calculated Cohen's d effect value of 0.84 (values above 0.8 indicate a large effect).

Although research on global genomic methylation in female dogs with CMT is limited, there are studies focusing on methylation in specific genes. For example, Brandão *et al.* [17] investigated DNA methylation status of the ESR1 gene encoding estrogen receptor alpha in CMT. Their findings showed no significantly differences in ESR1 methylation status between different types of tumors, suggesting DNA methylation would not be involved in ESR1 regulation in canine mammary tumors.

This contrasts with findings in human breast cancer, where DNA methylation may have a significant impact on ESR1 expression. Although this study focused in global genomic methylation and not in specific genes such as ESR1, it is worth mentioning the differential effect that methylation may have at the global level, as well as the fact that modifications in specific genes do not necessarily reflect global epigenetic: modifications.

In addition, Jeong *et al.* [15] used bisulfite sequencing to investigate whole genome methylation profiles in both canine mammary tumors and peripheral blood mononuclear cells. Interestingly, they found unique methylation signatures enriched in CMT, where genes associated with apoptotic pathways and transmembrane ion transport exhibited hypermethylation, while genes involved in cellular proliferation and oncogenes were hypermethylated in tumor tissues. The inclusion of peripheral blood samples by Jeong *et al.* [15] constitutes a precedent for their use in CMT research. This poses a future line of research for our team, as these are less invasive samples that may be used as a suitable tool for diagnosis, prognosis and treatment monitoring.

Beetch *et al.* [18] examined samples from various tumor stages alongside normal donor controls, characterizing hypermethylation patterns in cancer-associated genes. Their findings demonstrated that genes involved in transcriptional regulation, apoptotic processes, signal transduction pathways, and cellular migration correlate with gene expression patterns and disease progression in canine patients.

This suggests that methylation profiling holds clinical potential for distinguishing between different stages of tumor progression. Although their approach differs from ours, they agree that aggressive canine mammary tumors and benign tumors have different methylation patterns. Thus, DNA methylation modifications could guide disease progression monitoring, which is linked to prognosis.

Human research focuses on achieving deeper understanding of epigenetic mechanisms to obtain better therapies, as well as leveraging therapies that promote global epigenetic normalization to counteract epigenetic aberrations. Such approaches promise to enhance the clinical utility of epigenetic therapeutics, optimizing research investment returns while improving patient outcomes [22]. Given the paucity of comparable studies in canine oncology, this findings contribute valuable data toward better understanding the epigenetic landscape of mammary tumor biology in dogs.

CONCLUSIONS

This study investigated global DNA methylation levels in female dogs with mammary tumors and normal mammary tissue (control samples). Lower DNA methylation percentages were observed in tumor tissues, being this effect more pronounced in high histopathological grade tumors when compared to normal tissue. Based on these results, we can conclude that tumor tissue DNA was hypomethylated when compared to normal tissue. This work highlights the importance of continuing this line of research with larger sample sizes, mainly so that in the future global DNA methylation can be used as a biomarker to provide more accurate prognosis for this disease in dogs.

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Conflict of interest

The authors declare no conflicts of interest related to this report.

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