



Diagnostic and Prognostic Immunostaining in the Mammary Gland of *Canis Lupus Familiaris* and *Felis Silvestris Catus* for Comparative Oncology

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Abstract

Cancer is the most common breast cancer in the domestic dog and very uncommon in the domestic cat. Currently, there are about 400 domestic dog breeds, which have a high prevalence of specific human diseases and the clinical manifestation is generally similar to that of humans. Thus, due to the relative long-life span, the size of the species and physiological similarity, canine diseases are of special interest to human medicine. In contrast, because this neoplasm is not so common in cats, it becomes an interesting model for the comparison and understanding of breast cancer. The objective of this work was to identify, quantify and compare specific biological markers in breast tissue of domestic dogs and cats in their different reproductive phases (spayed, young, adult and elderly), using immunohistochemistry technique. The tissue collected came from clinics, tumor banks and castration campaigns, and kept in 4% paraformaldehyde at room temperature, and then processed for histopathological analysis using Hematoxylin and Eosin stains, Masson's Trichrome, Picrosirius red and Alcian Blue. For immunohistochemistry analyzes, markers p53, Bcl2; VEGF and CD31. The ultrastructural analysis of the tissue was performed using the Scanning Electron Microscopy (SEM) technique for some tissues. The tissues were analyzed and classified according to the histological type and immunohistochemical marking. The results of Picrosirius staining, revealing the predominant presence of type I collagen fibers, and Alcian Blue, the presence of acid polysaccharides, demonstrate the importance for the neoplasia of these fibers and the energy supply. Immunohistochemistry reflects the need for neoformation blood supply, through signaling for neovascularization (VEGF), and cell perpetuation through the expression of mutated p53 and CD31 related to cell adhesion and inflammatory process. There is still not enough data to infer about the possible changes that interfere in the carcinogenesis of the different groups analyzed.

Keywords: Breast cancer; Immunohistochemistry; Molecular oncology; Prognosis; Mammary gland

Introduction

Breast cancer is the most frequent and malignant neoplasm, being the main cause of cancer-related death in dogs and cats (between 50% and 70% of cases) [1-4]. There is interest in the study and understanding of carcinogenesis, due to the great financial and emotional impact, and even as a study model for human breast cancer [5]. We currently have approximately 400 breeds of domestic dogs (*C. lupus familiaris*) and have a high prevalence of specific human diseases and the clinical manifestation is generally similar. Thus, due to the relative long-life span, the size of the species and physiological similarity, canine diseases are of special interest to human medicine [6], including cancer (OSTRANDER; GALIBERT; PATTERSON, 2000). Brazil has the second largest canine population in the world, with an average of 1.8 dogs and 1.9 cats per household. The states of Rio Grande do Sul, Santa Catarina and Paraná (southern region of the country) have an index of 58.6% of homes with the presence of dogs, followed by the central-west regions, with 49.8% and southeast with 42.4% of households [7]. In 2013, there were a total of 52.2 million animals [7] and, in 2018, there was an increase of approximately 4%, totaling 54.2 million dogs [8]. The economic importance of the pet market is undeniable and in 2018 it

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moved the amount of R \$ 20 billion reais and with a perspective of growth of 6.9% in 2019, with expected revenue above R \$ 22 billion [9]. In comparison to cats, there was an increase of 8.3% in relation to the 2013 census. The largest number of animals in the country is in the states of São Paulo (21.6%), Rio de Janeiro (9.1%), Minas Gerais (7.2%) and Rio Grande do Sul (7.2%) [8]. According to the literature, the incidence of breast cancer is different when age, species and sex are taken into account, so researching the events involved in the tumor microenvironment, as well as the biochemical elements related to the disease, may bring knowledge to the events that lead to cancer, being fundamental for the understanding of its general mechanism. Another point that needs clarification and a lot of information is missing is the characteristics of breast protein markers in healthy animals, of different ages and reproductive stages, compared to animals affected by breast cancer [10-14]. In the domestic cat (*F. silvestris catus*), breast cancer is not so common and corresponds to 17% of feline tumors, being the third that most affects them, after skin and hematopoietic tumors [11,15]. There are fewer epidemiological studies on the incidence of breast cancer in cats compared to dogs and, due to differences in veterinary care for cats, the available data may underestimate the true incidence of the disease [14]. Thus, studies and information are limited and apparently have no relation to early castration, as observed in female dogs, but demonstrate a relationship with chemical castration with the use of progestogens or an exaggerated response to prolactin [16]. Based on the literature exposed above, we assume that there are differences in the stromal environment of the mammary gland of healthy animals of different species and affected by neoplasia, and considering the urgency of the theme, this study aims to emphasize the importance of breast immunostaining without oncological disease taking into account taking into account the clinical data, risk factors and management of the different patients in question. In this context, it is intended to promote a greater discussion on comparative oncology, with unique health prevailing.

Materials and Methods

Samples

Breast tissue from animals that died or underwent mastectomy as a result of breast cancer was collected. No animals were submitted to surgery due to or as a result of the project. Only the breast tissue resulting from mastectomy or death was collected, and thus, the work was subject to a fortuitous occurrence of the generating event and having no control over the generating event in its validity period. Twelve breast tissues / satellite lymph nodes were collected from the dog and one from the domestic cat. The mammary chain was collected and maintained at 4% paraformaldehyde, for fixation, according to Caputo, et al. [17] and standardization in Borghesi et al. [18]. Animal data were collected: Race, species, sex, age, clinical history], concomitant disease, location / origin; nutritional management; sanitary management.

Histopathological analysis

Fragments of normal mammary gland and carcinoma were fixed in a 4% paraformaldehyde solution. After fixation, the material was washed in phosphate buffered saline (PBS). Then, dehydration was performed in a series of ethanol in increasing concentrations (from 70 to 100%), followed by diaphanization in xylol and inclusion in paraffin (Histosec) [19]. Then, the paraffin blocks were subjected to microtomy in an automatic microtome (Leica, RM2165, Germany), obtaining cuts of 5µm. The fragments were adhered to histological slides and left in an oven at 60° C for 2 hours. After being deparaffinized, the cuts were stained following routine tissue staining techniques, using Hematoxylin

and Eosin staining (H / E), Masson's Trichrome, Picrocosirius red and Alcian Blue (EasyPath Diagnostics kit).

Scanning Electron Microscopy (SEM)

For this technique, the samples collected were fixed in 2.5% glutaraldehyde. Subsequently, they were subjected to digestion of the cellular matrix to maintain the architecture of the extracellular matrix. The maceration was carried out in 10% NaOH aqueous solution at room temperature for 25 days. Then, the material was washed for 10 days in distilled water at room temperature [20]. The material was impregnated with 1% tannic acid for 2 hours and post-fixed with 1% osmium tetroxide for 3 hours. The samples were then frozen in liquid nitrogen and fractured after continuous dehydration in ethyl alcohol (70%, 80%, 90%, 95% and 100%). Finally, they were dehydrated to dry at the critical point (Balzers CPD 020). Subsequently, the material was placed in a golden metallic support ("sputtering" Emitech K550). Data were collected using an ME Leo 435 VP electron microscope at CADI-FMVZ USP.

Immunohistochemical analysis

For immunohistochemistry, the Envision Flex (Dako) immunostaining kit, described below, was used, according to protocol by Shi et al. [21-23]. The initial samples were collected and preserved in a 4% buffered paraformaldehyde solution and, later, processed with the paraffin embedding technique, as previously described. The histological sections from the blocks and the control samples were deparaffinized, hydrated and placed in an antigenic recovery procedure in a 10mM citric acid solution, pH 6.0, through moist heat for 3 minutes under pressure. The samples were removed and kept in a 60°C oven with hydrogen peroxide (20 volumes) to block endogenous peroxidase for 15 minutes. The slides were incubated with their respective antibodies (Table 1) and left to rest in the refrigerator for 16 hours for later washing with phosphate saline solution (PBS). Then, they were incubated again using the Reveal HRP Polymer (Spring-Cod kit - SPD-125 - Reveal - Biotin-Free Polyvalent DAB). To reveal the reaction, diaminobenzidine (DAB - Sigma D5637) was used, forming a brown, insoluble and stable polymer at the site of the target antigen. After the end of the reaction, the slides were counterstained in hematoxylin, and subjected to routine histological processing to preserve the cuts. The breast of a healthy animal was used as a control. In addition, a negative control was prepared for each of the animals analyzed in this initial stage of the work.

To screen the markers, the tumor environment was taken into account, such as vascularization, tumor suppressor genes, inflammatory mediators, T and B lymphocyte immunophenotype; cell and histological adhesion molecules. To quantify the immunohistochemistry result, the classification of 0, 1+, 2+, 3+ was used, where for 3+, there must be strong staining of the cell membrane in more than 10% of the tumor cells; 2+ is a moderate stain, while 1+ is weaker and the cell is not completely marked.

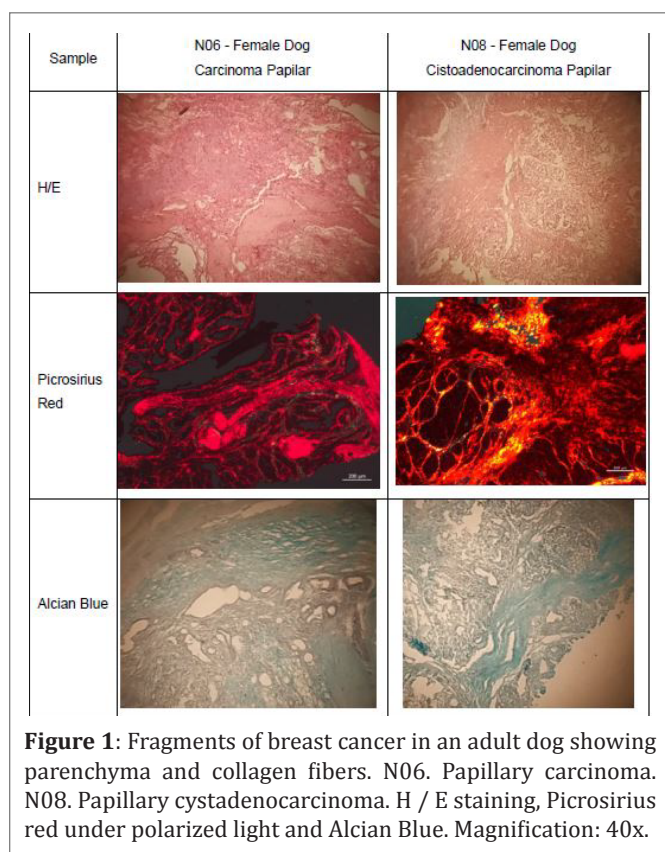
Results

In the first stage of the work, aiming at the standardization

Marker	Antibody	Company	Dilution
Anti-CD31	Rabbit policlonal	Abcam	1:50
Anti-p53	Rabbit policlonal	Dako	1:50
Anti-Bcl2	Rabbit policlonal	Dako	1:50
Anti-VEGF-A	Rabbit policlonal	Santa Cruz	2:40

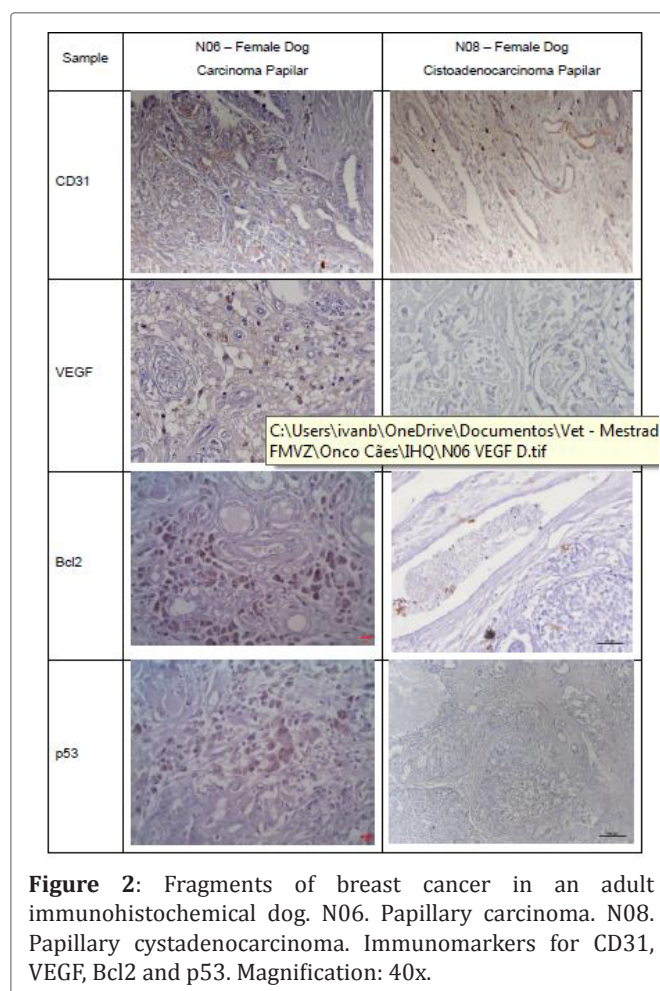
Table 1: Antibodies used in immunohistochemistry

Sample	Specie	Histopathological Classification TRADUZIR	VEGF	CD31	Bcl2	p53
N01	Female Dog	Carcinoma Acinar Intraductal	2+	2+	0	0
H02	Male Cat	Hígido	0	0	0	1+
N03	Female Dog	Adenocarcinoma Acinar	1+	0	0	2+
N04	Female Dog	Hiperplasia Mamária	0	1+	0	1+
N05	Female Dog	Adenocarcinoma Complexo Mucinoso	2+	2+	1+	1+
N06	Female Dog	Carcinoma Papila	1+	1+	1+	2+
N07	Female Dog	Carcinoma Tubular	0	0	0	2+
N08	Female Dog	Cistoadenocarcinoma Papilifero Ou Papilar	0	1+	1+	1+
N09	Female Dog	Carcinoma Sólido	0	0	1+	1+
N10	Female Dog	Carcinoma Acinar Sólido	1+	2+	0	2+
N11	Female Dog	Carcinoma Misto Sólido	1+	2+	2+	0
N12	Female Dog	Carcinoma Acinar Solido	0	0	2+	1+
N14	Female Dog	Adenocarcinoma Complexo	0	0	1+	0

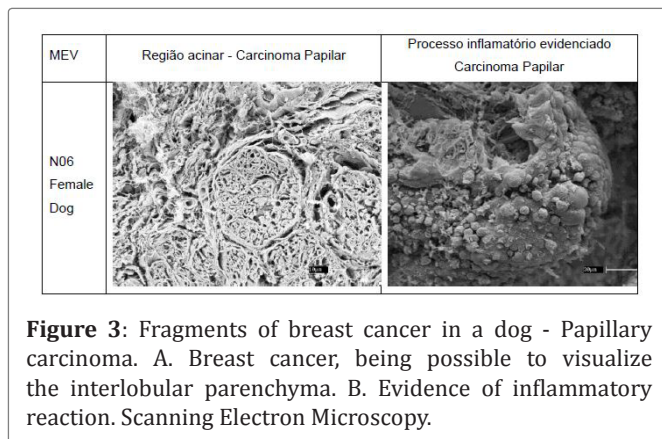
Table 2: Immunohistochemistry result for VEGF, CD31, Bcl2 and p53 markers in breast tissue

Figure 1: Fragments of breast cancer in an adult dog showing parenchyma and collagen fibers. N06. Papillary carcinoma. N08. Papillary cystadenocarcinoma. H / E staining, Picrosirius red under polarized light and Alcian Blue. Magnification: 40x.

of techniques, breast tissues of male domestic feline and female neoplastic tissue were used. The collected materials were kept in 4% paraformaldehyde 4% at room temperature, and then processed for histological analysis stained in Hematoxylin and Eosin staining of Picrosirius red, and staining of Alcian blue, which allowed to classify and evidence the tissues initially analyzed (Table 2).

Through the coloring of Picrosirius red, it is possible to identify the predominant presence of type I collagen fibers. It is known that in the development process of the neoplasia, there may be an increase in collagen deposition, mainly of types I and III [22]. Continuing with tumor development, the main reactions of the organism, together with collagen deposition, are the vascular neoformation and the immune response [23] in line with the cells present in the inflammatory reaction observed under microscopy scanning electronics (Figure 1).


Figure 2: Fragments of breast cancer in an adult immunohistochemical dog. N06. Papillary carcinoma. N08. Papillary cystadenocarcinoma. Immunomarkers for CD31, VEGF, Bcl2 and p53. Magnification: 40x.

We also observed, through Alcian Blue staining, by marking acid polysaccharides, the presence of it in the extracellular matrix of carcinomas and with little expression in the healthy animal. Polysaccharides have a structural and energy supply function and it will be important to observe, during the project, variations in the presence of different species, phases and reproductive status of the animals. Different protocols were tested for immunohistochemistry, however, due to the characteristics of the breast tissue, several fragments were lost from the slides during the process. After standardizing the protocol, preliminary tests were performed on 13 samples collected using the markers for



the anti-VEGF, anti-CD31, anti-Bcl-2 and anti-p53 markers (Table 2 and Figure 2).

Scanning electron microscopy was performed on fragments of healthy neoplasm of a healthy dog and cat to assess the architecture of collagen fibers, which is important for understanding tumor development [24,25] and to know the ultrastructural changes in the breast tumor [26] (Figure 3).

Discussion

A point that needs to be clarified are the characteristics of breast protein markers in healthy animals, of different ages and reproductive phases, compared to animals affected by breast cancer [11-14], this knowledge brings light to the events that lead to cancer, being fundamental for the understanding of its general mechanism. The identification of the predominant presence of type I collagen fibers corroborates the report that in the development process of the neoplasia there is an increase in collagen deposition, mainly of types I and III [22]. As a result of tumor development, one of the main reactions of the organism, together with collagen deposition, is the vascular neoformation and the immune response [23,24] and is in line with the cells present in the inflammatory reaction observed in scanning electron microscopy and 50% of the affected animals expressed the vascular endothelial growth factor (VEGF), which is recognized as a factor responsible for encouraging neovascularization [27,28] 58 % expressed CD31 and Bcl2 and 83% expressed p53. CD 31 being related to cell adhesion and inflammatory processes [29,30]; Bcl2 favoring or preventing apoptosis, with suspicion that they are regulated by estrogen receptors [31,32]; p53 responsible for the control of the cell cycle and apoptosis, and high concentrations of altered p53 is related to a higher mitotic index, more invasive growth and necrosis [33,34]. However, we recognize that the sample number is still not significant at this stage of the project. The Alcian Blue stain, used for labeling acid polysaccharides, revealed its presence in the extracellular matrix of carcinomas and with little expression in the healthy animal, this leads us to infer about the structural and energy supply function in the tumor development process and it will be important to observe, during the project, the variations of the presence in the different species, phases and reproductive status of the animals.

Conclusion

The results of Picrosirius staining, revealing the predominant presence of type I collagen fibers, and Alcian Blue, the presence of acid polysaccharides, demonstrate the importance for the neoplasia of these fibers and the energy supply. Immunohistochemistry reflects the need for neoformation blood supply, through signaling for neovascularization (VEGF), and cell perpetuation through the expression of mutated p53 and CD31

related to cell adhesion and inflammatory process. There is still not enough data to infer about the possible changes that interfere in the carcinogenesis of the different groups analyzed.

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