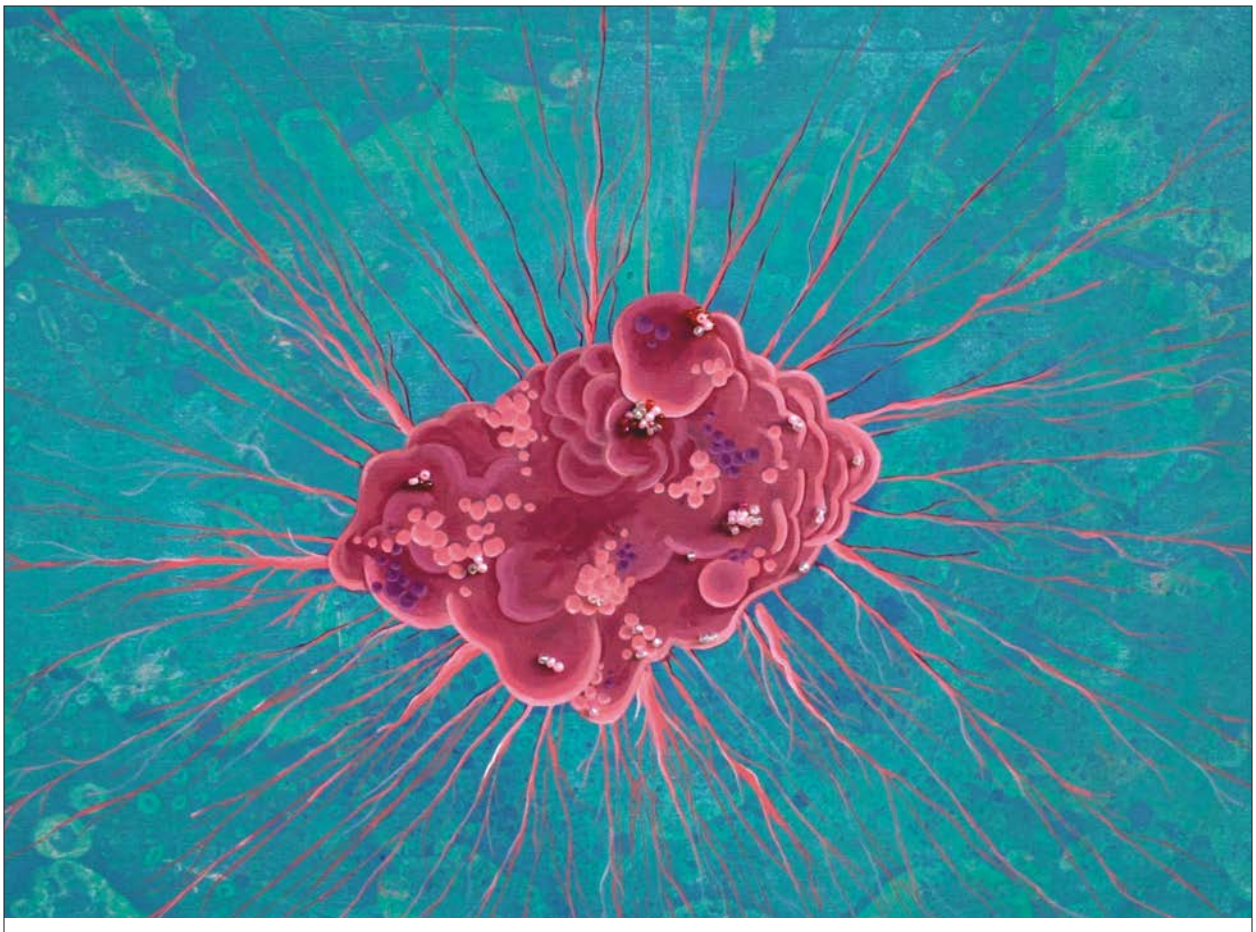

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Association of CD44+/CD24-Cells to More Aggressive Molecular Phenotypes in Canine Mammary Carcinomas

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ABSTRACT

Leading the cause of death among female dogs, mammary gland tumor awakes the interest of the scientific community, mostly because the similarities in clinical presentation and evolution to breast cancer in women. Due to the importance of investigating canine mammary tumors, this research aims to identify the protein expression of CD44, CD24, MUC-1, CD10 and ESA by immuno-histochemistry. Thus, slides were prepared by the tissue microarray technique to assess the presence of these markers in different subtypes. Of the 112 tumors, 66.9% were positive for CD10 (75/112), 42.86% positive for CD24 (48/112), 21.43% for ESA (24/112), 20.54% for MUC-1 (23/112) and 5.35% for CD44 (6/112). Of these tumors, 3.57% showed the immuno-phenotype CD44+/CD24-(4/112). The results suggest that mammary gland tumors of female dogs were positives for stem cell markers, which may be associated with cancer stem cells. This association also may allow setting further models to study breast cancer in women.

Key words: Canine, mammary cancer, stem cells, tissue microarray

INTRODUCTION

Cancer is the most important public health problem in the western world, being breast neoplasia the most common malignancy in women (Siegel *et al*., 2015). Only in the United States, in 2015, breast cancer was expected to reach approximately 235,000 new cases (Siegel *et al*., 2015). In Brazil, mammary gland tumor is the most common carcinoma in female dogs. However, it is only associated majorly with metastasis because poor patient prognosis (Nerurkar *et al*., 1989). Due to similarity between mammary tumor in bitches and breast cancer in women, dogs represents a good model for studies in comparative oncology (Thuroczy *et al*., 2007). In both species, mammary tumor develops spontaneously and shares several epidemiological, clinical and biological characteristics (Muscatello *et al*., 2015).

Recently, a high attenuated Listeria monocytogenes-based vaccine was developed against Her2/neu-overexpressing breast cancer (Shahabi *et al*., 2011). Dr. Nicola Mason group-University of Pennsylvania used this vaccine successfully in dogs with osteosarcoma (unpublished data). Using dogs as a model of human cancer in comparative oncology is a unique opportunity to benefit both species by acquiring more accurate results in clinical trials (Fonseca-Alves *et al*., 2013).

In human medicine, the role of Cancer Stem Cells (CSC) is focus of many researches and their molecular markers have been well defined (Cocola *et al*., 2009). Nevertheless, in veterinary medicine, few papers characterize CSCs (Fonseca-Alves *et al*., 2013). The CSCs can be defined as a cell population presents in tumors and responsible for self-renewal and differentiation (Dunphy *et al*., 2013). Similar to normal stem cells, the tumor can give rise to all cell types (Piscitelli *et al*., 2015). Evidence supports this type of cellular vital role in the initiation and maintenance of tumor, besides to their ability to invade, metastasize and being resistant to therapy (Dunphy *et al*., 2013). The identification and isolation of these cells using immuno-histochemical surface markers has been a priority in cancer research (Jaggupilli and Elkord, 2012).

The most studied neoplastic cells with cell stem characteristics in human medicine are those strongly expressing CD44 which show low or absent expression of CD24 (CD44+/CD24-phenotype) (Abraham *et al*., 2005). Al-Hajj *et al*. (2003) were the first to distinguish tumorigenic cancer cells by using the combination of these two cell surface markers. Neoplastic cells with CD44+/CD24-phenotype are more tumorigenic and aggressive when compared with tumors without this phenotype (Al-Hajj *et al*., 2003). The cell phenotypes CD44+/CD24-are present from 22-31% of human breast carcinomas (Vieira *et al*., 2012). On the other hand, this information cannot be found in veterinary medicine. Breast carcinomas with cells showing this phenotype are aggressive with high rate of metastasis, particularly bone metastasis (Abraham *et al*., 2005).

The CD44 is a transmembrane glycoprotein having several isoforms. Normally, it regulates the cell-cell and cell-matrix, as well as cell migration. This glycoprotein binds primarily to hyaluronic acid, as well as to collagen, fibronectin, laminin, chondroitin sulfate and other important components of the extracellular matrix (Vieira *et al*., 2012). In addition, it binds to the cytokine osteopontin (Orian-Rousseau, 2010). Some variants, especially CD44v6, are associated with tumors with more aggressive behavior and its expression correlates with a worse prognosis in several human tumors, including breast cancer (Afify *et al*., 2009).

The CD24 is a small heavily glycosylated mucin-like cell surface protein, which is bound to the cell membrane via GPI anchor (glycosyl) (Abraham *et al*., 2005). This molecule is involved in the regulation of cell proliferation and cell-cell interaction (Aigner *et al*., 1998). Moreover, CD24 also is expressed in pre-B lymphocytes not only in normal tissues but also in various hematological cancers and solid organ tumors (Lim, 2005). The CD24 is a ligand for P-selectin also expressing adhesion receptors on activated endothelial cells and platelets. This suggests that this protein may play an important role in the metastasis process (Aigner *et al*., 1998; Kim *et al*., 2007).

The MUC-1 is a member of the mucin family of Glycoproteins, being a glycosylated membrane protein presents on the apical surface of cells of normal epithelial tissue (Nassar *et al*., 2004). This protein participates in cell signaling, inhibition of cell-cell and cell-matrix, immune regulation, apoptosis, proliferation and transcription (Rakha *et al*., 2010; Abba *et al*., 2006; Hattrup and Gendler, 2006). In breast cancer, MUC-1 accumulates in the cytoplasm around the nucleus, interacting with estrogen receptor. The MUC-1 may be aberrantly overexpressed by 90% of human breast tumors (Brayman *et al*., 2007).

The CD10 is a cell membrane known for regulating the biological activity of peptide substrates and reducing the local concentration available for binding and signal transduction. The CD10 may also play an important role in maintaining homeostasis, neoplastic transformation and tumor progression (Iwaya *et al*., 2002). The loss of CD10 methylation allows increased migration, cell growth and survival, contributing to neoplastic development and progression (Papandreou and Nanus, 2010).

The ESA has been considered a cancer stem cell marker from various neoplasms, including carcinomas of breast, prostate and pancreas (Li *et al*., 2007). In breast cancer, ESA is a stem cell marker in tumor positive for estrogen receptors. It may be associated with lymph node metastasis and tumor recurrence (Liu *et al*., 2009).

The ESA over expression in breast cancer often correlates with poor prognosis (Spizzo *et al*., 2004). Osta *et al*. (2004) showed that the ESA was highly overexpressed in primary and metastatic breast cancer up to 100 times when compared to normal breast tissue. On the other hand, when silencing ESA gene expression, proliferation, migration and invasiveness of cancer cells in vitro breast decreases (Spizzo *et al*., 2004).

This study aims to evaluate the expression of stem cell markers using the antibodies CD44, CD24, MUC-1, CD10 and ESA through immuno-histochemistry in different subtypes of canine mammary tumors.

MATERIAL AND METHODS

Samples and histology analysis: One hundred twelve paraffin blocks of mammary gland tumors from female dogs were used and classified in simple and complex according to Goldschmidt *et al*. (2011). Biopsy sample was acquired from archives of Pathology Service, Univ. Estadual Paulista (UNESP). Individuals age ranged from 4-15 years and all of them were intact. Unfortunately, information on the survival time of patients was not obtained. Tissue microarray block was constructed with triplicate samples, according to criteria established by Muscatello *et al*. (2015).

Immuno-histochemical analysis: Immuno-histochemistry expression of Estrogen Receptors (ER), HER-2, p63 and CK5 was performed to identify molecular phenotype of canine mammary tumors according to Muscatello *et al*. (2015). Based on these results the tumors were classified as luminal A ($ER^+/HER2^-$), luminal B ($ER^+/HER2^+$), overexpression of HER2 ($ER^-/HER2^+$) and basal ($RE^-/HER2^-/CK5^+$and/or $p63^+$).

After classification into subtypes immuno-histochemical staining was performed to identify tumor stem cells with antibodies against to CD44, CD24, MUC-1, CD10 and ESA. Antibodies, dilutions, used clone, manufacturers and cutoff values are summarized in Table 1.

Immunohistochemical staining was performed using peroxidase method and 3,3' diaminobenzidine tetrachloride (DAB). Briefly, slides were dewaxed in xylol and rehydrated in graded ethanol. For antigen retrieval, slides were incubated in citrate buffer (pH 6.0) into a pressure cooker (Pascal®; Dako, Carpinteria, CA, USA).

Normal canine mammary gland and normal adjacent tissue in tumor samples were used as positive controls for p63 and CK5. Canine uterus block was used for ER positive control. In the case of breast carcinoma with HER2, FISH-proved amplification was used for the same purpose. For negative control, primary antibody was replaced by PBS.

Table 1: Antibodies used in immunohistochemical markers: dilution, clone used, manufacturer and cut off values to assess the positivity of each marker

Antibody	Dilution	Clone	Manufacturer	Positives
ER	1:40	LH2	Novocastra, UK	>10%
HER-2	1:50	NCL-CB11	Novocastra, UK	0 e 1+ = negative 2+ e 3+ = positive
p63	1:150	4A4	Neomarkers, USA	>10%
CK5	1:50	XM26	Novocastra, UK	>10%
CD44	1:50	DF1485	Novocastra, UK	>10%
CD24	1:50	SN3b	Thermo Scientific, USA	>10%
MUC-1	1:50	695	Biocare Medical, USA	>10%
CD10	1:50	56C6	Santa Cruz, USA	>10%
ESA	1:50	H-90	Santa Cruz, USA	>10%

Table 2: Distribution of CD10.CD24.CD44.ESA and MUC-1 immuno-histochemical scores in molecular phenotype of canine mammary carcinomas

Parameters	Luminal A	Luminal B	HER2-overexpressing	Basal (%)
CD10	86. 2% (n = 25)	81.7% (n = 31)	50% (n = 8)	51.7 (n = 15)
CD24	48.2% (n = 14)	65.7% (n = 25)	12.5% (n = 2)	17.2% (n = 5)
CD44	-	2.6% (n = 1)	25% (n = 4)	3 4% (n = 1)
ESA	-	7.9% (n = 3)	12.5% (n = 2)	20.6% (n = 6)
MUC-1	-	7.9% (n = 3)	31.25% (n = 5)	17.2% (n = 5)
CD44+/CD24-	-	-	75% (n = 3)	2.5% (n = 1)

Immuno-histochemistry analysis: For ER, CD10, CD44, CD24, ESA, MUC-1, CK5 and p63 antibodies, the scores were attributed as follow: 0 = No. positive staining, <10%, score 1, 10-50% of positive cells, score 2, 51-75% of positive cases, score 3, >75%, score 4. Samples with scores 0 and 1 were considered negatives. Those scoring 2, 3 and 4 were considered positives. For HER2 evaluation, Herceptest scoring system was applied (0 = No. membrane staining or <10% of cells stained, 1+ = Incomplete membrane staining in >10% of cells, 2+ = >10% of cells with weak to moderate complete membrane staining and 3+ = Strongly and complete membranous staining in >10% of cells. All cases 2+ and 3+ was considered positive (Table 2).

Statistical analysis: Statistical analysis was performed using Kruskal-Wallis test for analyses between subtypes tumor groups and histopathological variables (tumors with simple or complex pattern). Fisher's exact test was used to evaluate immunohistochemical scores between molecular subtypes tumors. All tests were performed using the computational program graph pad prism®. The results were considered statistically significant when $p<0.05$.

RESULTS

Of all analyzed tumors, 43.75% (49/112) were classified as the simple carcinoma type and 56.25% (63/112) as the complex type. These tumors are also categorized into distinct molecular subtypes, being 33.93% (38/112) considered as luminal B, 25.89% of tumors (29/112) as luminal A, 25.89% of tumors (29/112) as basal subtype and 14.29% (16/112) as overexpression of HER2. There was no statistical difference between histopathological types and molecular subtypes ($p = 0.986$). The distribution of simple and complex tumors was homogenous in each group.

For the luminal B tumors, 81.7% (31/38) resulted positive for CD10 (Fig. 1a), 65.7% (25/38) for CD24 (Fig. 1a), 7.9% (3/38) for ESA, 7.9% (3/38) for MUC-1 and 2.6% (1/38) for CD44 (Fig. 1b). About luminal A subtype, 86.2% (25/29) tumors had positive outcomes for CD10 (Fig. 1b), 48.2% (14/29) for CD24 and 34.4% (10/29) coexpression of CD10/CD24. Luminal A positive tumors for CD44, MUC-1 and ESA antibodies were not found.

For the Basal subtype, 51.7% (15/29) showed positivity for CD10, 31% (9/29) for ESA, 20.6% (6/29) for MUC-1, 17.2% (5/29) for ESA (Fig. 1c), 17.2% (5/29) for CD24 and only 3.4% (1/29) was positive for CD44. About tumors with overexpression of HER-2. 50% (8/16) had positive reaction for CD10, 31.25% (5/16) for MUC-1 (Fig. 1d), 25% (4/16) for CD44 (Fig. 1d), 12.5% (2/16) for CD24 and 12.5% (2/16) for ESA.

Four tumors (3.57%) showed positive staining for CD44, negative staining for CD24 and also CD44+/CD24-phenotype. The CD44+/CD24-tumors were positive for ESA and negative for CD10 and MUC-1. An interesting finding is related to the fact that 75% (3/4) for CD44+/CD24-tumors had basal-like subtype and 25% (1/4) had overexpression of HER2 molecular subtype.

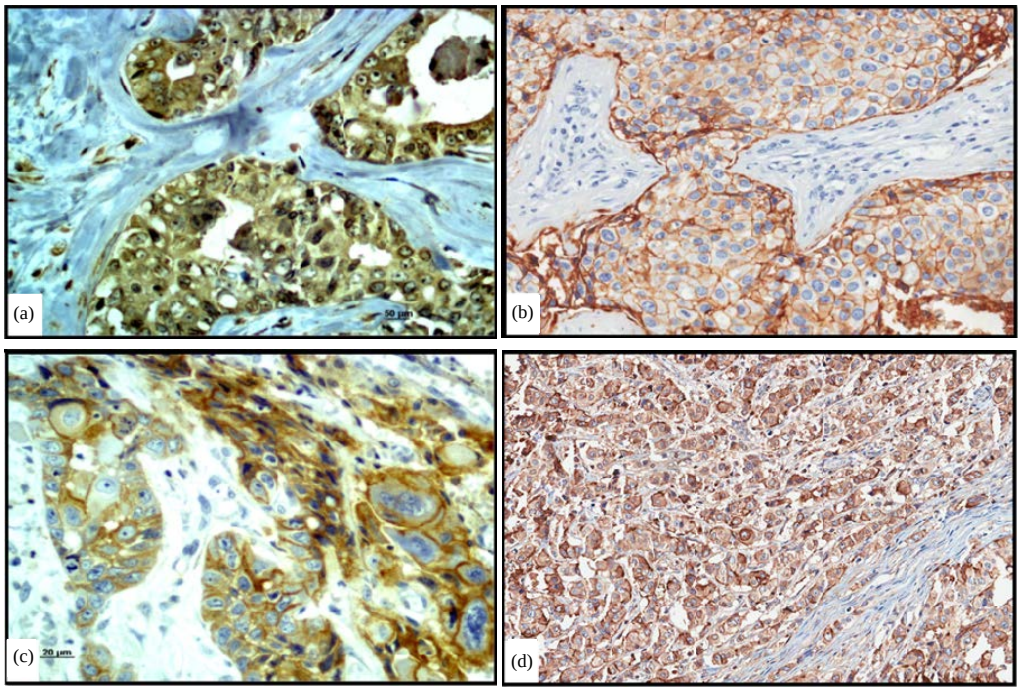

Fig. 1(a-d): Immuno-histochemistry of mammary carcinoma, (a) Cytoplasmic staining for CD24, 400x, (b) Membranous staining for ESA, 400x, (c) Membranous staining for CD44, 400x and (d) Cytoplasmic staining for MUC-1, 200x

Seventeen tumors (15.18%) displayed positive staining for ESA while were negative for MUC-1. About tumors with ESA+/MUC1-phenotype, 41,1% (7/17) presented positivity for basal-like molecular subtype, 35.2% (6/17) for Luminal A subtype, 17.6% (3/17) had overexpression of HER2 and 11.7% (2/17)for Luminal B.

High immuno-expression of CD10 in Luminal A and Luminal B subtypes was established when compared toHER2-over expressing subtype ($p<0.05$) and basal subtype ($p<0.05$). Luminal B subtypes showed more CD24 positive cells when compared to Luminal A, HER2-overexpressing and basal subtypes ($p<0.05$). Statistical differences between CD44, ESA and MUC-1 expression and molecular subtypes were not proven. However, a tendency of higher expression of these markers in HER2-over expressing and basal subtypes was identified when compared to Luminal A and Luminal B subtypes.

DISCUSSION

Human breast cancer is a distressing disease. Only in the United States, 235,030 new cases were expected in 2014 (Siegel *et al*., 2015). Molecular phenotypes in human breast cancer is very important to predict patient survival and treatment with trastuzumab(Herceptin) (Khodarev *et al*., 2010). Female dogs represent a good model for comparative study due to genomic proximity to humans. In addition, both species share the same environment and, sometimes, the same sources of food and water (Fonseca-Alves *et al*., 2012).

In Brazil, early surgical castration of bitches is uncommon. This fact reflects in a higher number of mammary gland tumors in older patients. Despite canine mammary neoplasia represents an

opportunity to conduct researches in the benefit of human, there are few studies on it. Thus, studies showing the molecular subtypes of mammary gland tumors in bitches and their correlation with some features such as tumor aggressiveness, patient survival time and response to trastuzumab are very scarce.

In this study, mammary carcinomas with simple or complex subtypes were utilized and divided according to the molecular phenotype proposed by Gama *et al*. (2008). Statistical difference in the distribution of tumors, even simple or complex, in each molecular phenotypes is nonexistent (Luminal A, Luminal B, HER2-overexpressing and basal). Therefore, a similar division between simple and complex tumors exists for each molecular phenotypes in canine mammary carcinoma. The CD44 was positive in six samples, one for Luminal B, another for HER2-overexpressing subtype and four for basal subtype. The pattern of CD44 expression has been associated to the aid to the breast cancer progression by altering the characteristics of tumor cell adhesion and thereby facilitating vascular invasion in the tissue (Zheng *et al*., 2011).

Although the relationship between CD44 expression and other clinicopathological characteristics of breast cancer have been the subject of debate, many studies suggest that CD44 expression correlates with tumor metastasis. This may be useful to indicate prognosis (Lopez *et al*., 2005). Linking previous literature to the results (higher positive cases in basal subtype tumors), a poor prognosis provoked by the expression of CD44 can be inferred.

The CD24, strongly expressed in ovarian, breast, prostate, bladder and kidney carcinomas (Zheng *et al*., 2011), is also involved in cell adhesion and metastasis (Lee *et al*., 2010). These features may indicate an important function as a marker for tumor diagnosis and prognosis. Functionally, CD24 is identified to perform an alternative connection to P-selectin, an adhesion receptor of platelets and endothelial cells. Their interaction can facilitate the passage of tumor cells into the blood stream, promoting metastasis (Aigner *et al*., 1998).

The association between metastasis and increased CD24 expression is an important prognostic factor, in addition to being a marker for cancer stem cells (Lee *et al*., 2010). The results of this study demonstrate a higher expression of CD24 in Luminal B subtype when compared to Luminal A, overexpression HER2 and basal ones.

Many studies on tumor stem cells are related to transmembrane proteins CD44 and CD24. A subpopulation of tumor cells expressing strongly CD44 but not CD24 (CD44+/CD24-phenotype), has been identified as tumor stem cells by Kim *et al*. (2007). Other authors (Lopez *et al*., 2005) have subsequently confirmed this fact. Neoplastic cells with CD44+/CD24-phenotype was up to 100 times more tumorigenic than those that not exhibited this immunophenotype (Al-Hajj *et al*., 2003).

In a study of mammary carcinogenesis conducted by Al-Hajj *et al*. (2003), cell lines of CD44+/CD24-injected into the mammary fat pad of non-obese mice (NOD/SCID) were able to create macroscopic tumors, despite the implantation of few cells. Furthermore, the research revealed that implanted cells from CD44+CD24-lineage giving rise not only to cells of the same cell phenotype, but also to cells with a different one. This suggests that tumor stem cells of the breast could generate a homogeneous population of non-tumorigenic cells, besides generating new tumor stem cells. Cells with CD44+/CD24-phenotype are present in 22-31% of human breast carcinomas.

An increasing interest in the role of mucins in breast cancer is due to its potential as a prognostic indicator and its involvement in cancer therapy (Rakha *et al*., 2010). Patients with MUC1 positive tumors develop both humoral and cellular immunity as a response against MUC-1 antigens from malignant cells (Heuser *et al*., 2003). As a result, several vaccines based on MUC-1 are currently developed for the treatment of this disease. The MUC-1 is usually present on the

apical surface of secretory epithelial cells. In malignant tissues, it may be overexpressed in up to 90% of cases of breast cancer (Hattrup and Gendler, 2006). Overexpressing tumor formation is allowed since MUC-1 interacts with several transcription factors, such as p53, hormone receptors (ER and PR), β-catenin, among others (Brayman *et al*., 2007).

The MUC-1 is also involved in the modulation of several important cell signaling pathways (Hattrup and Gendler, 2006). Likewise, the overexpression of MUC1 leads to loss of cell-cell and cell-matrix, which favors metastasis (Abba *et al*., 2006; Giatromanolaki *et al*., 2000). In this study, a statistical correlation exits between more aggressive subtype (HER2 overexpression and Basal) and less aggressive subtype groups (Luminal A and B) ($p = 0.03389$). A higher expression in more aggressive subtypes was estimated.

The CD10, also known as the common acute lymphoblastic leukemia antigen, is a membrane-type-1 matrix metalloproteinase expressed in many tissues including myoepithelial cells (Moritani *et al*., 2002). In this study, significant difference in CD10 expression was recognized when compared Luminal A ($p = 0.0019$) and B ($p = 0.0021$) to HER2-over expression subtype. Several reports indicate that the expression of CD10 is associated with aggressive biological behavior in various epithelial neoplasms (Chen *et al*., 2000). This outcome agrees with the findings of the present study since the subtype HER2-over expression is considered one of the most aggressive.

On the other hand, the literature describes ESA as a marker of tumor stem cells of breast cancer positive for estrogen receptors (Spizzo *et al*., 2004). In this work, however, subtypes were also positive without being estrogen receptor positive. Basal subtype had more ESA positive cells when compared to others subtypes ($p = 0.0005$).

CONCLUSION

The findings show that mammary gland tumors with more aggressive molecular subtypes are associated with cells showing cancer stem cell phenotype. Tumors with Basal subtype had more CD44+/CD24-cells, which confirms their worst prognosis. Moreover, the outcomes indicate an association between mammary gland tumors of female dogs with cancer stem cells (CD44, CD14, CD10, ESA and MUC-1). These associations may conform a good model to study breast cancer in women.

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