Osteopontin expression and its relationship with prognostic biomarkers in canine mammary carcinomas

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ABSTRACT.- Monteiro L.N., Salgado B.S., Oliveira D.E., Rivera-Calderon L.G, Montoya-Flórez L., Sanctis P. & Rocha N.S. 2020. Osteopontin expression and its relationship with prognostic biomarkers in canine mammary carcinomas. Pesquisa Veterinária Brasileira 40(3)210-219 Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Dr. Valter Maurício Corrêa s/n, Botucatu, SP 18618-681, Brazil. E-mail: maomontoya53@yahoo.es, lmontoyaf@unal.edu.co

Osteopontin is a glycoprophosphoprotein implicated in different physiologic and pathologic processes and is known to be involved in progression and metastasis of various cancers in humans, but this relation is still little explored in the veterinary. The aim was to evaluate the expression of osteopontin in canine mammary carcinomas and its relation with well-established canine mammary tumor biomarkers. For that, expression of OPN, EGFR, HER2, and c-Kit were evaluated along with Ki67 rate in 43 mammary carcinomas. Osteopontin was demonstrated to be expressed by neoplastic epithelial cells in all carcinomas as well as in stromal cells from the tumor microenvironment. Relation between high osteopontin expression and EGFR positivity (P<0.001) and HER2 overexpression (P=0.012) was demonstrated. In conclusion, high OPN expression seems to be related to poor prognosis and MAPK pathway activation, given the association with EGFR and HER2, members of the MAPK signaling pathway.

INDEX TERMS: Dogs, cancer, osteopontin expression, prognostic biomarkers, canine mammary carcinomas, immunohistochemistry, epidermal growth factor receptor, human epidermal growth factor receptor 2.
INTRODUCTION

Several studies have pointed cancer as the main cause of death for dogs in developed countries and 45% of dogs have over 10 years of age (Bronson 1982, Michell 1999, Proschowsky et al. 2003, Battisti et al. 2013, Dobson 2013, Daleck et al. 2016). In Brazil, the disease figures as the second most common cause of death for dogs (Fighera et al. 2008, Trapp et al. 2010, Andrade et al. 2012, Battisti et al. 2013, Daleck et al. 2016) and it is estimated that one in five dogs will develop cancer. The skin and subcutaneous tissue being the most prevalent followed by mammary, hematopoietic and bone tumors. Mammary tumors are the most common cancer diagnosed in women, likewise in female dogs (Dobson 2013, Pawlowski et al. 2013, Salas et al. 2015, Dias et al. 2016, Salas et al. 2016). Statistical surveys estimate that mammary neoplasms represent about 50% of all tumors afflicting female dogs, of which at least 40% are malignant (Brodley et al. 1983, Andrade et al. 2012, Feliciano et al. 2012, Battisti et al. 2013, De Nardi et al. 2013, Li et al. 2013, Pawlowski et al. 2013, Santos et al. 2013, Peña et al. 2014, Arias et al. 2015, Salas et al. 2015, Dias et al. 2016, Salas et al. 2016, Soler et al. 2016).

Advances in the diagnosis and therapy of the animals, the application of efficient measures in the prevention of infectious diseases through vaccination and deworming, in addition to improvements in the nutritional quality of dog food, have contributed towards a higher quality of life and longevity for dog (Dobson 2013, Salas et al. 2015), resulting in an increase in the diagnoses of neoplasms for the species (Pawlowski et al. 2013, Santos et al. 2013, 2014, Soler et al. 2016). Canine mammary tumours are highly heterogeneous in morphology and behavior and successful clinical management requires robust prognostic factors. The biological behavior of canine mammary neoplasms is widely variable in morphology and behavior, making validation and the use of tumor biomarkers to support the diagnosis and prognosis extremely important to successful clinical management (Graham & Myers 1999, Kandoler-Eckersberger et al. 2000, Arias et al. 2015, Damasceno et al. 2016b, Pysrri et al. 2017). Immunohistochemistry techniques may be useful to anticipate a diagnosis of cancer and to present prognostic information regarding the disease (Graham & Myers 1999, Kandoler-Eckersberger et al. 2000, Zacchetti et al. 2003, Peña et al. 2014, Santos et al. 2014, Arias et al. 2015, Damasceno et al. 2016a, Soler et al. 2016). Several biomarkers have been identified and associated with the survival rates of dogs afflicted with mammary neoplasms, such as the estrogen and progesterone hormone receptors (Sartin et al. 1992, Nieto et al. 2000), p53 (Lee et al. 2004) e-cadherin (Marmor et al. 2004), caspase-3 (West et al. 2008), cathepsin D (Lemmon & Schlessinger 2010) survivin (Bongiovanni et al. 2015), cell proliferation markers such as Ki-67 and PCNA (Zacchetti et al. 2003) epidermal growth factor receptor (EGFR) (Nieto et al. 2000, Rangaswami et al. 2006, Vollmann-Zwerner et al. 2010, Arias et al. 2015, Elebro et al. 2016) and human epidermal growth factor receptor 2 (HER-2) (Sartin et al. 1992, Zacchetti et al. 2003, Carvalho et al. 2013, Ferreira et al. 2014, Peña et al. 2014, Silva et al. 2014, Burrai et al. 2015, Theoharis et al. 2015, Damasceno et al. 2016a, 2016b).

The receptors tyrosine kinase (RTK) of the ErbB family, known as EGFR/HER-1, erbB-2/HER-2, erbB-3/HER-3 and erbB-4/HER-4, play an important molecular control role as a signal for the development and maintenance of several organs and systems (Graham & Myers 1999, Nieto et al. 2000, Lee et al. 2004, Marmor et al. 2004, West et al. 2008, Damasceno et al. 2016b) since they are a group of primary mediators for the fundamental cell responses (Lemmon & Schlessinger 2010). Furthermore, it has been shown to have an important role to contribute to a better understanding of the progression mechanisms in malignant mammary tumors (Graham & Myers 1999, Bongiovanni et al. 2015, Damasceno et al. 2016b). Its role appears to be associated with increased angiogenesis and metastasis (Bongiovanni et al. 2015). In addition, another receptor tyrosine kinase (c-Kit) also plays an important role in cell proliferation and differentiation (Li et al. 2001). These receptors have been widely studied due to recent discoveries regarding their involvement in the pathogenesis of hyperproliferative diseases such as cancer (Vollmann-Zwerner et al. 2010, Liang et al. 2013, Elebro et al. 2016).

Recently, osteopontin (OPN), an adhesive glycoprotein found in tissues and body fluids that is involved in both physiological and pathological processes (Rangaswami et al. 2006, Vollmann-Zwerner et al. 2010, Liang et al. 2013, Shevde & Samant 2014, Elebro et al. 2016) has been widely pointed as a biomarker with potential prognostic implications for cancer due to its functional role over the tumor progression and metastasis control pathways (Rangaswami et al. 2006, Weber et al. 2010, Anborgh et al. 2011, Shevde & Samant 2014, Burrai et al. 2015, Damasceno et al. 2016a, Li et al. 2016, Pysrri et al. 2017, Wei et al. 2017). OPN belongs to the family of small integrin-binding glycoproteins related to N playing a key role in cell-matrix and cell-cell communication and interaction, modulating cellular behavior through autocrine and paracrine mechanisms (Fisher et al. 2001, Bellahcène et al. 2008). Studies have described its role in several development and differentiation processes in tissues, including bone (Yamate et al. 1997), skin (Chang et al. 2008) and mammary glands (Rittling & Novick 1997, Carvalho et al. 2013, Silva et al. 2014, Pysrri et al. 2017). In addition, it plays a key role in immune and inflammatory responses (Tuck & Chambers 2001, Rangaswami et al. 2006, Rittling & Singh 2015), including the healing process of wounds (Liaw et al. 1998).

The expression of OPN is up-regulated by many factors such as epidermal growth factor (EGF), transforming growth factor-Beta (TGF-β), tumor necrosis factor α (TNFα), interferon gamma (IFN-γ) and interleukin-1 β (IL-1β) (Rangaswami et al. 2006). Regarding pathological events, studies have shown that OPN is overexpressed in sepsis (Hirano et al. 2015), autoimmune diseases, cardiovascular diseases (Waller et al. 2010), neurodegenerative diseases (Carecchio & Comi 2011) and several tumors, especially carcinomas (Coppola et al. 2004, Anborgh et al. 2011, Shevde & Samant 2014). In the case of tumors, OPN’s potential for predicting the prognosis was initially reported by Chambers et al. (1996), later, Tuck et al. (1997) have described the relationship between OPN overexpression and tumor progression in human mammary neoplasms, suggesting that OPN could be employed as a tumor prognostic marker both in tumor cells and in plasma.

There are few studies in the field of canine immunohistochemistry assessing the role of biomarkers in general, and OPN specifically, in tumor initiation and progression, as well as in the identification of patients with high disease recurrence risks. This study has analyzed the immunoexpression of OPN, EGFR, HER2, c-Kit...
and Ki67 in ex vivo canine mammary carcinomas to obtain data supporting a better understanding of the role OPN plays and its relationship with other established biomarkers for this particular tumor.

**MATERIALS AND METHODS**

**Tissue samples.** The Brazilian Ethics Commission for the Use of Animals (protocol no. 88/2011 - CEUA) approved the study and the owners of all animals involved in the study have signed a Free and Clarified Consent Term authorizing the collection of data and the use of the data in research papers. Canine mammary carcinomas specimens (n=43) were collected at the time of surgical excision by research of the Investigative and Comparative Pathology Laboratory, "Universidade Estadual de São Paulo", Botucatu, Brazil, and it were used for the study. Samples were fixed in 10% neutral formalin and embedded in paraffin wax. Sections (4μm thick) were obtained and stained with hematoxylin and eosin for histological examination in order to confirm the diagnosis of mammary carcinoma. Tumor classification was defined according to the WHO classification of canine mammary tumors (Misdorp et al. 1999). Tumor sections were examined in an optical microscope (Zeiss® Axio Lab. A1) by three independent pathologists. For each slide, 10 fields were read with 400x magnification. Inter-observer variation was resolved by simultaneous re-evaluation.

**Immunohistochemistry.** For the immunohistochemistry assays, 3μm thick sections were obtained from paraffinized tissue blocks and subsequently deparaffinized and rehydrated. The primary antibodies used in this study are summarized in Table 1. A polymer-based labeling system kit (NovoLink Polymer System, Novocastra Laboratories, Newcastle, UK) was used for detecting the antigen-antibody reaction and peroxidase and protein blockages. Antigen retrieval was carried out by heat treatment in 10mM citrate buffer, pH 6.0. After cooling (20 minutes at room temperature), sections were sequentially immersed in solutions provided in the kit according to manufacturer’s instructions in order to block the endogenous activity of peroxidase and unspecific proteins. The slides were incubated overnight at 4°C with the specific antibodies. Subsequently, 3,3’ diaminobenzidine (DAB) tetrahydrochloride was used as a chromogen in order to allow the visualization of antigen-antibody reaction. The slides were then counterstained using Harris’s hematoxylin, dehydrated, and mounted for microscopic assessment.

**Immunohistochemical results evaluation.** The evaluation of the immunohistochemistry results was performed by three pathologists. OPN was considered positive whenever cytoplasmic staining was observed in the neoplastic and stromal cells. The assessment of OPN expression in neoplastic cells was performed semi-quantitatively using the Allred 8-unit system (Allred et al. 1993). In this scoring system, the tumor epithelial cells proportion score and intensity score were determined for each tumor, represented by one slide. The proportion score included the fraction of positively stained tumor cells and was as follows: 0 = none; 1 = <1/ 100th; 2 = 1/100th to 1/100th; 3 = 1/100th to 1/3; 4 = 1/3 to 2/3; 5 = >2/3. The estimated average staining intensity of positive tumor cells was expressed as follows: 0 = none; 1 = weak; 2 = intermediate; 3 = strong. For statistical purposes, an OPN score of 1–3 was considered low (+1), an OPN score of 4–6 was considered intermediate (+2), and an OPN score of 7–8 was considered high (+3). The expression of OPN was also evaluated in peritumoral inflammatory cells and tumor stromal cells.

The expression of HER2 was evaluated according to the Dako Cytomation Hercep Test scoring system: 0 = no staining or membrane staining in less than 10% of tumor cells; 1+ = faint, barely perceptible membrane staining in more than 10% of tumor cells, the cells are stained only in part of the membrane; 2+ = weak to moderate, complete membrane staining observed in more than 10% of tumor cells; and 3+ = strong, complete membrane staining in more than 10% of tumor cells. Cases were considered positive (overexpressed) for HER2 when immunostaining was characterized as 2+ or 3+. EGFR staining was classified in positive or negative, according to Dako’s EGFR pharm Dx interpretation manual.

For Ki67, four categories were defined as follows: <10%, 10-25%, 26-50% and >50% of stained nuclei. For c-kit expression, the reaction product was evaluated along the cell membrane and in the cytoplasm. The quantity of immunoreactive cells was estimated according to the classification adopted by Biemann et al. (2007) none; <10%; 10–75%; and >75% of the cells. The level of immunoreactivity was assessed based on its predominant intensity: weak (+); moderate (++); and strong (+++).

The final immunoreactivity score was calculated as strong (4), when at least 75% of cells exhibited at least moderate immunoreactivity; in cases of weak immunoreactivity in <10% of all tumor cells, the final score was considered as (1). The score (0) was considered negative. The score (3) was assigned if weak immunoreactivity was present in >75% of tumor cells or if strong or moderate staining was observed in 10-75% tumor cells. All other cases were given a score of (2).

Positive and negative controls were included in each run in order to guarantee the reliability of the assay. For OPN, canine kidney was used. Additionally, a canine mammary carcinoma already recognized as HER-2 positive, a canine cutaneous mast cell tumor positive for c-Kit, and a canine cutaneous squamous cell carcinoma recognized as positive for EGFR and Ki67 were used. Rabbit and mouse IgGs (Dako, Carpinteria/CA) were used in tumor samples for negative control purposes.

**Statistical analysis.** Differences in the expression of proteins were compared using Fisher's exact test or Pearson's X2 test for qualitative variables. All statistical tests were two sided, and statistical significance was accepted at P<0.05. All analyzes were performed using the Prism GraphPad software version 5.0 (San Diego/CA).

**RESULTS**

**Immunohistochemistry**

Cytoplasmic OPN staining was observed consistently in neoplastic cells of all mammary tumors evaluated (43/43), always presenting an intermediate or high classification score (Fig.1); 12 of 43 (28%) samples presented intermediate score and 31 of 43 (72%) presented high score. We observed that all samples presented more than 50% of positive neoplastic cells and presented a high score variation due to the staining intensity. OPN positivity was observed mainly within the

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**Table 1. Antibodies used in the immunohistochemical study**

| Antibody | Clone | Dilution | Origin | Source |
|----------|-------|----------|--------|--------|
| Osteopontin | LFMb-14 | 1:50 | Novocastra Laboratories, UK | Mouse |
| Ki67 | MIB1 | 1:50 | Dako | Mouse |
| CD117 | 104D2 | 1:400 | Dako | Mouse |
| HER-2 | policlonal | 1:2000 | Dako | Rabbit |
| EGFR | NCL-EGFR | 1:100 | Novocastra Laboratories, UK | Mouse |
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Cytoplasm, sometimes perinuclearly or, less commonly, in a cell surface distribution in the neoplastic cells. No sample was immunohistochemically negative for osteopontin.

Strong OPN expression was observed consistently in the epithelial component and in the myoepithelial cell layer, with cells presenting a cytoplasmic expression pattern. The surrounding stroma was usually OPN positive, since the OPN immunoeexpression was also observed in the tumor stromal matrix component, stromal fibroblasts, vascular endothelial cells, muscular cells, macrophages and other inflammatory cells in all samples (Fig.2). Consistent OPN immunoeexpression was also observed in areas with necrosis (Fig.3), chronic inflammation (Fig.4) and on invasive tumor borders (Fig.5). In addition, the weak OPN immunoeexpression was observed in luminal mammary cells from the normal mammary tissue adjacent to neoplastic foci (Fig.5).

**Relationship between osteopontin and other markers**

From the 43 samples of mammary carcinoma evaluated, 34 (79.07%) were positive for HER2 and 36 (83.72%) were positive for EGFR. Additionally, 28 tumors with OPN high expression (90.3%) also presented HER2 overexpression, revealing a statistically significant association ($P=0.012$). For EGFR, 29 (93.5%) of the positive cases also presented high OPN expression, revealing a statistically significant relationship ($P<0.001$). All cases (43/43) were positive for c-Kit, presenting score variation between 1 and 4. There was no significant association with c-Kit ($P>0.05$). Likewise, 27 from the 31 cases with OPN overexpression (87%) revealed less than 25% neoplastic cells positive for Ki67. However, no statistically significant relationship could be noted between OPN and Ki67.

**DISCUSSION**

This study aimed at determining the expression level of the glycoprotein OPN in the stroma and neoplastic cells of canine mammary tumors, as well as its association with other proteins by means of immunohistochemistry assays. In humans, there are well documented reports showing the overexpression of OPN in malignant tissues and in the plasma, as well as the correlation with the tumor stage in the brain (Zakaria et al. 2016), mouth, esophagus, stomach, large intestine, liver,
pancreas, kidney (Coppola et al. 2004), ovaries, prostate (Tilli et al. 2014), lungs (Yan et al. 2015) and breasts (Bramwell et al. 2014). These studies clearly point towards the importance of this glycoprotein and its multidimensional ability to influence biological events associated with tumorigenesis and tumor progression, including the possibility of employing it as a molecular parameter in the prognostic evaluation of cancer patients. Since there are no similar reported studies in dogs, a clarification regarding its significance in canine mammary tumors was required. OPN-producing cells and OPN deposition in extracellular matrix were previously identified through IHC in canine bone and cartilage (Schnapper & Meyer 2004). Additionally, a quantitative real-time reverse transcription polymerase chain reaction array was established to quantify the expression levels of 49 genes relevant to carcinogenesis in laser-microdissected tumor cells of 10 benign and 13 metastatic canine mammary tumors (Klopfleisch et al. 2010). The study in question detected OPN in normal tissues, but observed no significant differences in the expression levels of osteopontin among any of the groups tested.

Presently, the scientific community fully accepts that OPN is expressed by tumor cells both in humans and animals and that it affects the malignant properties of neoplastic cells, specifically by affecting their ability to grow, invade, and metastasize. In addition, it is also known that OPN is expressed in both normal and malignant tissues (Shevde & Samant 2014, Ng et al. 2015) and effectively mediates many physiological and pathological events (Kahles et al. 2014).

In this study, we have found that all carcinomas were stained positively for OPN and, according to the immunohistochemical grading system employed; tumors presented consistently high expression levels for the protein. This finding is in accordance to studies that focused on OPN levels in women breast tumor tissue (Tuck & Chambers 2001, Coppola et al. 2004, Rodrigues et al. 2009, Bramwell et al. 2014), since authors report a high frequency of OPN-positive samples. For example, some authors reported a positivity of 88.4% for OPN in metastatic human brain tumors (Zakaria et al. 2016) and a positivity of 100% in human hepatocellular carcinomas (Tsai et al. 2012) similarly to what was observed in this study. Although canine mammary tumors may occasionally be histologically and biologically different than human breast cancer, the results regarding OPN immunoexpression seems to be in concordance. This study and that of others (Tuck & Chambers 2001, Rodrigues et al. 2009, Luo et al. 2011) reported that OPN is expressed both in the epithelial and stromal components of neoplastic wounds. In humans, OPN immunoexpression is also observed outside neoplastic cells and reveals a variable staining pattern in which luminal cells from normal mammary tissue, luminal tumor cells, stromal fibroblasts, macrophages, lymphocytes, and blood vessels are weakly to highly stained (Kim et al. 1998, Tuck et al. 1998, Tuck & Chambers 2001, Rodrigues et al. 2009, Luo et al. 2011).

In this study, it was observed that cells from the tumor microenvironment and cells adjacent to the tumor presented OPN positivity in all samples. Studies have shown that, aside from being expressed in different cell types, including immune system cells, the expression of OPN is highly increased during the inflammatory process (Rittling & Singh 2015) and regulated positively through several growth factors and cytokines, including LPS, Ang II, NO, IL-1β, IL-2, IL-3, IFN-γ, TNF-α and TGF-β (Denhardt et al. 2003). In our study, we observed a more intense OPN immunoexpression in cells near necrotic or inflammatory foci, as well as in invasive tumor areas, in
agreement with others reports (Brown et al. 1994, Hirota et al. 1995, Tuck et al. 1998). These findings suggest that those cell types may contribute to OPN production levels. However, our understanding regarding the molecular mechanisms involved in the regulation of OPN expression remains incomplete (Kahles et al. 2014). The presence of OPN in the tumor stroma and on the surface of tumor cells interfacing with the stroma suggests that this glycoprotein may participate in adhesive interactions at the tumor/normal tissue interface. Studies have shown that OPN overexpression, especially the OPNb and OPNc variants, at esophageal adenocarcinoma enhances tumor cell invasion and metastasis (Lin et al. 2015) and also contribute towards macrophage adhesion and migration (Brown et al. 1994).

In a previously reported study (Rodrigues et al. 2009) using invasive human breast cancer cases, no statistically significant association was reported between stromal OPN expression, major clinical and pathological parameters, and some of the most commonly used molecular markers for those tumors. Whether epithelial and stromal OPN has distinct roles during neoplastic development and progression is an important question to be further addressed, but it seems to be related to metastasis in various neoplasms (Brown et al. 1994).

In this study, different tumor markers were tested for their association with OPN in canine mammary carcinomas, but only EGFR and HER2 showed a statistically significant relationship with OPN-positive immunostaining scores. These components are part of the MAPK signaling pathway, which is recognized as an important pathway for carcinogenesis, particularly for the epithelia (Sebott-Leopold & Herrena 2004). The findings suggest an osteopontin-associated activation of the MAPK pathway in canine mammary neoplasms, which is in agreement with the findings presented by other authors (Brown et al. 1994, Frey et al. 2007), revealing a possible relationship between OPN and the MAPK pathway in breast cancer (Tuck et al. 2003, Rodrigues et al. 2009), lung adenocarcinomas (Frey et al. 2007), hepatocellular carcinoma (Tsi et al. 2012) and in actinic keratosis/cutaneous squamous cell carcinomas (Luo et al. 2011) in humans. However, this finding was reported to be absent in other tumor histotypes such as mesothelioma (Frey et al. 2007), suggesting that this alteration may not be a distinctive feature for all tumor types.

In dogs, the expression of the erbB family components was previously evaluated in canine mammary tumors. A relatively high expression of the ERBB1 and ERBB2 genes suggests an important contribution to carcinogenesis in canine mammary tumors (Matsuyama et al. 2001, Singer et al. 2012). The overexpression of tyrosine kinase receptors EGFR and HER2 - proteins derived from ERBB1 and ERBB2 genes, respectively - is observed in many human cancers including bladder, breast, colon, and lung cancers (Eccles et al. 1995). HER2 overexpression is usually associated with poor prognosis indicators in canine mammary tumors such as tumor size, high histological grade, invasion, and high proliferation rates (De las Mulas et al. 2003).

Regarding the relationship between HER2 and OPN, Rodrigues et al. (2009) also did not find any association in women. However, this association was statistically significant in the tumors studied by our group. This finding may indicate the existence of a co-regulator expressed differently in human and canine mammary neoplasms, leading to the activation of this specific RTK in the canine counterpart.

In this study, 83.72% of the samples were positive for EGFR, a protein previously described to be related to reduced cure and overall survival rates in canine mammary tumors (Gama et al. 2009). We have also identified a statistically significant relationship between high OPN expression and EGFR positivity, which we believe to be a synergistic and complementary relationship between the molecules, in accordance to what was reported by other authors in human breast cancer (Tuck et al. 2003, Rodrigues et al. 2009) and hepatocellular carcinomas (Tsi et al. 2012).

For the latter, higher OPN and EGFR expression were significantly associated with advanced histological grades, advanced pathological stages, and poor survival rates (Hirota et al. 1995). Cell migration regulated by OPN is said to be dependent on the epidermal growth factor (EGF) and hepatocyte growth factor (HGF). OPN induces EGF receptor (EGFR) mRNA expression, EGFR tyrosine kinase activity, HGF receptor (Met) mRNA and protein expression, as well as increasing Met kinase activity during tumor cell migration in human mammary cancer cell lines (Tuck et al. 2003).

Previous reports indicated that ligation of OPN with integrin leads to c-Src-dependent transactivation of EGFR, resulting in the activation of downstream signaling pathways, including PI3-k, Ras–MAPK, phospholipase C, and protein kinase C (PKC) in cancer cells (Tuck et al. 2003). The transformation of epithelial cells induced by tissue-specific overexpression of EGFR in vivo provides direct evidence of the role EGFR plays in carcinogenesis (Yarden & Sliwkowski 2001).

These features which indicate a relationship between EGFR/HER2 and OPN overexpression are interesting since an arsenal of antibodies and tyrosine kinase inhibitors for growth factor receptors targeted to the MAPK pathway are currently either in development or already in clinical use, consequently being expected to be effective against tumors overexpressing OPN. Various therapeutic agents directed against EGFR and HER2 have provided promising alternatives to traditional chemotherapy in the search for better treatments for cancers overexpressing these tyrosine kinase receptors (Kamath & Boulamwini 2006). This could represent an evolution in the conventional treatment of canine mammary tumors focused in highly OPN expressing neoplasms.

**CONCLUSION**

Osteopontin overexpression is related to EGFR and HER2 expression in canine mammary tumors, probably by the activation of the MAPK signaling pathway. Although the mechanisms involving OPN and the progression of canine mammary carcinomas remain unknown, this study suggests that OPN, EGFR, and HER2 play important roles in canine mammary tumors carcinogenesis. These findings raise the question of whether is possible to use specific drugs to block the signaling pathway in OPN overexpressing tumors.

**Conflict of interest.** - The authors do not have any conflicts of interest to declare.

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