REVIEW ARTICLE

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Specific cellular biomarkers for canine's breast cancer: literature review

Biomarcadores celulares específicos para neoplasias mamárias caninas: revisão de literatura

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ABSTRACT: Although there are many studies about the cellular biomarkers' use in canine's mammary cancer, the results are not to compare. This paper offers orientations about the most useful immunohistochemical markers, which can help to treat canine's mammal tumors. The main biomarkers of the canines' mammal epithelia and of the myoepithelial cells were reviewed and based on that, some orientations about immunoexpression of cadherin (E cadherin and P cadherin), proliferation marker (Ki-67), Human Epidermal Growth Factor (HER2 and HER3), estrogen and progesterone receptors were made. The incorporation of these information in future studies, either they be prospective or retrospective, will offer mechanisms that allow the direct comparison of the studies, and therefore will help to determine if the biomarkers have prognostic significance. The use of these biomarkers can help to point out the most adequate treatment to malignant canine's mammal cancers.

RESUMO: *Embora existam muitos estudos sobre o uso de biomarcadores celulares em neoplasias mamárias caninas, os resultados são difíceis de comparar. Este artigo fornece orientações sobre os marcadores imuno-histoquímicos mais úteis e que podem auxiliar o tratamento de tumores mamários caninos. Os principais biomarcadores do epitélio mamário canino e das células mioepiteliais foram revisados e orientações sobre imunoexpressão das caderinas (E caderina e P caderina), marcador de proliferação (Ki-67), Factor de Crescimento Epidérmico Humano (HER2 e HER3), receptores de estrógeno e progesterona foram descritas. A incorporação dessas informações em estudos futuros, seja prospectivos ou retrospectivos fornecerão mecanismo para a comparação direta dos estudos e ajudará a determinar se estes biomarcadores celulares têm significância prognóstica. A utilização destes biomarcadores pode auxiliar e direcionar o tratamento mais adequado para as neoplasias mamárias caninas malignas.*

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1 Introduction

Mammary tumors correspond to the prevailing neoplasias in intact female dogs, with the rate of 40 to 50% of being histologically malignant according to the literature. These neoplasias have variable biological behavior and distant metastasis is an obit's common cause in these patients, which difficult the individual clinic results based only in its clinical and histological characteristics (Klopfleisch et al., 2011; Santos et al., 2013).

The prognostic value of clinicopathological characteristics in breast tumors in female dogs and cats led to an ongoing debate for more than 30 years between the veterinary oncologists. At the same time, the longevity of pets had increased significantly, leading a greater number of animals to develop neoplasias. Moreover, the conscious of needing health care of these animals and the improvement of veterinary clinical services have also increased (Matos et al., 2012).

Recent publications indicate many similarities between the mammary neoplasia in female dogs and cats and women, among them, age, incidence, risk factors, biological behavior, metastatic pattern, histopathological and molecular characteristics and responses to therapy (Rivera and von Euler, 2011; Matos et al., 2012). Thus, the first step was taken when a group of scientists in veterinary and human medicine gathered in 1966 to start a project together with the World Health Organization (WHO), which aimed to "reveal similarities and differences between human and domestic animals tumors, and thus provide a solid foundation for research in general comparative oncology and as a secondary objective of assisting the advancement of veterinary pathology" (Beveridge and Sobin, 1974). Eight years later, this group published the first International Histological Classification of Domestic Animals tumors (Hampe and Misdorp, 1974) and then another classification systems have been proposed since then (Sassi et al., 2010; Matos et al., 2012).

The second step was the proposal of clinical TNM classification system (T, tumor diameter, N regional lymph node involvement, M, distant metastasis) by a group of veterinary scientists experienced in oncology and / or pathology, one more time together with the WHO (Matos et al., 2012).

However, when trying to complete studies based on prognostic, the reviewer have faced a multiplicity of methodologies in all aspects of the work, making it difficult to compare results and conclusions. In contrast, the studies with human breast tumors are currently focused on specific groups of patients based on well-established prognostic and predictive factors and with similar prognosis. Lately, attention has been directed to the prognostic value of activity of various genes through profiling expression, which has been shown to be a powerful tool (Matos et al., 2012).

Differently from the human, very little is known about the mechanisms and cellular signaling pathways that contribute to the canine neoplasias' development. This deficiency is aggravated by the limited or even unknown specificity of molecular tools used in studies about canine neoplasias, either because it is used by small groups or for the lack of patient follow-up, which is necessary to the assessment of the clinical meaning of individual molecular data (Klopfleisch et al., 2011).

During the mammary carcinogenesis, the specific differentiation markers have normally kept in the cell. The morphologic evaluation, together with these immunophenotypic differences can be used to establish the definitive diagnosis. Cellular differentiation markers are used to study the origin of the canine mammary tumors, mainly the epithelial and myoepithelial cell's role in the genesis of mixed tumors, based on its immunophenotypic characteristics (Peña et al., 2014). In veterinary science, some potential prognostic cellular biomarkers were investigated for canine's mammary neoplasias, as the proliferation markers and hormonal and oncogenes receptors (Santos et al., 2013).

In this context, the present review intends to summarize the current knowledge about the most used cellular biomarkers, such as human epidermal growth factor, progesterone and estrogen receptors, E-cadherin and P-cadherin, cyclooxygenase 2, proliferation markers, and vascular endothelial growth factor, in order to identify the independent prognostic factors that can be adopted in the routine and constitute potential targets for adjuvant therapies.

2 Development

2.1 Human epidermal growth factor (c-ERB-2 and c-ERB-3)

The receptor of the epidermal growth factor (Epidermal Growth Factor Receptor - EGFR) is coded by the proto-oncogene c-erbB-1 and it belongs to the same family as the gene Human Epidermal Growth Factor Receptor-type 2 coded by the gene c-ErbB2. The receptors of the EGFR family include the HER-1/ErbB-1, HER-2/ErbB-2, HER-3/ErbB-3 and HER-4/ErbB-4, generally located in the cellular membranes. The family consists in transmembrane receptors that contain three domains: (1) extracellular ligand-binding domain, (2) transmembrane domain and (3) intracellular domain with tyrosine kinase activity (Kim et al., 2011).

Other genes and proteins expressed by epithelial and myoepithelial mammary cells are explored with very variable expression and prognostic/predictive importance little clarified. The expression of the EGFR and c-ERB-2 epidermal growth factors in the mammary carcinomas of the female dog are possibly related to the tumor's development and progression, but the results are controversial (Horta et al., 2012).

The biggest expression of EGFR was identified in the malignant components of carcinoma in mixed tumors, suggesting the participation of this receptor in the acquirement of the malignant epithelial phenotype. The same result was not observed for the c-ERB-2 receptors, as it occurs with the human species (Perou et al., 2000; Viale et al., 2009; Bertagnolli et al., 2011; Horta et al., 2012). The super expression of c-ERB-2 occurs in about 15% of the invasive female dogs' mammary neoplasias and it allows the activation of the growth factor and signaling of surviving, proliferation and tumor cells' invasion (Kim et al., 2011).

The expression of estrogen and progesterone receptors is clearly related to the best prognostic of the canine patients with mammary carcinomas (Sorenmo, 2003). The inhibition

of the estrogen receptors can influence the survival of these patients, through anti estrogenic drugs, as tamoxifen and ovarian hysterectomy (Sorenmo, 2003; Tavares et al., 2010). Nevertheless, despite of the therapeutic potential, the tamoxifen can promote serious collateral effects in the female dog, related to the agonist effect in other tissues, as increasing the risk of pyometra development in the uterus, for example (Tavares et al., 2010; Horta et al., 2012).

The mammary tumors with positive amplification and/or the super expression of c-ERB-2 are an indicative of poor clinical prognostics, which signalizes an aggressive behavior of the tumor and especially the resistance to endocrine, clinical and experimental therapy (Kim et al., 2011, Burrai et al., 2015; Campos et al., 2015). The current patient's treatment with positive c-ERB-2 is represented mainly by the chemotherapy, combined with a therapy targeted to c-ERB-2, being both adjuvant and in the presence of metastasis. This approach has changed the natural history of c-ERB-2 positive mammary tumors, being normally indicated as the first option for treatment of patients with metastatic disease (Montemurro et al., 2013). The c-ERB-2 role in dogs is miscomprehended and studies with molecular, histopathologic and clinical data are necessary to promote a wider understanding of the real role of HER-2 in the canine mammary tumors (Kim et al., 2011; Campos et al., 2015).

In women, studies examining the patterns of gene and molecular expression suggests the classification of four subtypes in breast cancer: luminal-like (further sub-classified as type A and B), basal-like HER-positive and Normal-like. The main purpose of applying this classification system is to demonstrate its role in prognosis (Sassi et al., 2010).

In veterinary medicine, the canine mammary tumors morphologically classified have good indications of prognosis that can be improved by other tools based on molecular classification, for example. However, canine mammary tumors are a heterogeneous group of tumors that can benefit from molecular classification taking into account these differences (SASSI et al., 2010). However, caution must be present when using this classification system in dogs. In this specie, the most useful information for the prediction is obtained by histological grade and invasion power (Sassi et al., 2010).

As well as the c-ERB-2, other studies have been suggesting that the nuclear expression of c-ERB-3 can influence the tumor's progression, but the prognostic meaning of the c-ERB-3 expression in tumors remains controversial (Mujoo et al., 2014; Burrai et al., 2015; Campos et al., 2015), possibly related to the location of the tumor (Koutras et al., 2010). The non-nuclear expression of c-ERB-3 was reduced in the malignant canine's mammary neoplasias and it was increased in the benign canine's mammary neoplasias. In contrast, c-ERB-3 was expressed robustly in the nucleus of other malignant tumors. Moreover, its nuclear expression was higher in malignant tumors of high histological grade and lymphatic invasion when compared to tumors without these features. Therefore, the nuclear expression of EGFR can play a role in the tumor's progression and metastasis, in addition to representing a cell biomarker very useful for prognostics in malignant canine's mammary tumors (Kim et al., 2011; Burrai et al., 2015; Campos et al., 2015).

2.2 Progesteron and Estrogen receptors (PR and ER)

The progesterone and estrogen have a crucial role in the control and formation of the mammary gland. In the other hand, they can raise the risk of developing mammary neoplasias. The female dog's prolonged exposure to progesterone – either due to the administration of exogenous progesterone in order to interrupt the ovarian cycle or to the action of endogenous progesterone from the corpus luteum during the luteal phase – stimulates the proliferation of the mammary epithelium (Thuróczy et al. 2007). This element is metabolized within the mammary gland and the physiological effect is primarily mediated by receptors expressed in normal and neoplastic breast's tissue found in the cell's nucleus (Queiroga et al., 2005; Guillen-Luna et al., 2014.).

Immunohistochemistry technique studies have identified that, at the moment of the diagnosis, about two thirds of canine mammary carcinomas have positive progesterone receptor (PR) (Guillen-Luna et al., 2014). As in humans, the canine progesterone receptors have two isoforms (PRA and PRB), which are transcribed from a single gene under the control of different promoters. Under physiological conditions, normal human breast tissue can express PRA and PRB in equimolar levels. However, an altered ratio of PRA / PRB is often associated to mammary carcinogenesis, with the PRA being predominant over PRB in benign and malignant human mammary tumors. These findings in dogs remain controversial, due to the lack of research and the limited number of samples (Guil-Luna et al., 2014).

Ninety-five percent of normal canine mammary tissues contain PR and/or ER. More than 50% of canine's mammary tumors and 65-70% of human breast cancers express PR and/or ER (Thuróczy et al., 2007). The lack of PR expression in breast tumors is strongly associated with poor prognosis (Purdie et al., 2014). The role of these receptors in breast tumors is still controversial. Some researchers suggest that they can be discarded, because patients with positive PR and negative ER that respond to endocrine therapy are very rare, limiting their usefulness. However, other researchers believe that, as a prognostic indicator, it is still appropriate to evaluate the PR expression (Purdie et al., 2014).

Generally, the PR expression can be used to identify patients with a good prognosis and that may get benefits from additional adjuvant chemotherapy, endocrine therapy and other treatments (Purdie et al., 2014).

2.3 E-cadherin and P-cadherin

Cadherin is a superfamily of trans membrane's glycoproteins binding to calcium with extracellular domain responsible for cell-cell interactions, a trans membrane's domain and a cytoplasmic domain that is often associated with the cytoskeleton. They are located on the basolateral membrane in the adherent junctions (Klopfleisch et al., 2011).

When E-cadherin immunostainings are reduced, it can be affirmed that they are associated with the malignant phenotype of cancer and that the loss of expression is related to cell detachment, favoring the tumor dissemination. However, when this protein's overexpression is observed in metastatic cores

and inflammatory carcinomas, it suggests a reversible dynamic in the production of E-cadherin, with its re-expression being important for the tumor's growth (Horta et al., 2012).

Most studies of cadherin in tumorgenesis focused on E-cadherin, once it is the main cadherin expressed by epithelial cells (Klopfleisch et al., 2011). In normal mammary glands, the polarized epithelial cells lining the ducts and alveolus express E-cadherin, which keeps them together. In contrast, myoepithelial cells may express P-cadherin, but not E-cadherin. Several mechanisms have been disclosed and they describe the association of the regulation or inactivation of E-cadherin with the tumor's development and progression. These mechanisms include gene mutation, epigenetic and gene silencing that modulates and regulates the functionality of E-cadherin (Klopfleisch et al., 2011).

The undifferentiated canine's mammary carcinomas, both invasive and metastatic, may present loss of E-cadherin expression in some subpopulations of tumor cells, when compared to well differentiated carcinoma, suggesting that altered expression of E-cadherin could be an important process for malignant transformation. This hypothesis is supported by the observation that loss of E-cadherin expression is correlated with a shorter overall survival and free of diseases (Klopfleisch et al., 2011; Asproni et al., 2015).

P-cadherin is a glycoprotein with a structure similar to cadherin. P-cadherin is expressed on the myoepithelial cells of adult and normal mammary tissue. The selective expression of E and P-cadherin seems to be important for differentiation of the mammary gland. E-cadherin is expressed on the luminal epithelial cells, while P-cadherin is restricted to myoepithelial cells. However, during pregnancy and late lactation in humans and dogs, P-cadherin is not found in the cell-cell junctions as expected in an adhesion molecule, but is secreted in epithelial cells (Walls et al., 2007, Klopfleisch et al., 2011).

In human's breast tumors, the P-cadherin is frequently overexpressed in invasive carcinoma of high degree and it is a good indicator of poor prognosis. In dogs, there is a significant correlation between the expression of P-cadherin and the high degree of the tumor, suggesting that their aberrant expression is a marker of biologic aggressiveness of these tumors. In addition, aberrant expression of P-cadherin in these tumors is associated with the infiltrative growth pattern of the tumor (Walls et al., 2007; Klopfleisch et al., 2011; Asproni et al., 2015).

2.4 Cyclooxygenase 2 (COX-2)

The cyclooxygenase enzyme (COX) has the function of catalyzing the biosynthesis of prostanoids, and two isoforms of COX have been identified: COX-1 and COX-2. Both isoenzymes are similar in protein structure, but they are produced by different genes and have distinct biological functions (Queiroga et al., 2007). The COX-1 isoform is only expressed constitutively and it is related to homeostasis of the individual. COX-2 can be expressed in an inducible form in the inflammatory site and it is related to the formation of inflammatory mediators (Costa et al., 2002).

Several proinflammatory cytokines, growth factors, oncogenes and carcinogens can induce COX-2 activity (Queiroga et al., 2010). The expression of this enzyme in neoplastic processes, both in humans and in other animal species, is related to tumor

progression, being considered a bad prognostic factor for various histological types (Queiroga et al., 2010; Hung et al., 2015). COX-2 and its products inhibit apoptosis, increasing the expression of anti-apoptotic proteins; decrease cellular adhesion; destroy basement membrane; induce cell proliferation; promote angiogenesis (increasing the production of VEGF) and also maintain the inflammatory response, which contributes to carcinogenesis (Costa et al., 2002; Cassali et al., 2011; Queiroga et al., 2007; Queiroga et al., 2011).

The immunohistochemical expression of COX-2 is higher in mast cell tumors and in breast tumors in dogs that have higher histological grade (Cassali et al., 2011; Hung et al., 2015)), and it represents a potential predictive value, once it is possible to associate selective inhibitors of COX-2, as firocoxib in adjuvant treatments (Lavalle et al., 2009; Queiroga et al., 2010; Cassali et al., 2011; Horta et al., 2012.). Furthermore, such an element is particularly evident in histological types of malignant tumors classically defined as aggressive, as squamous cell carcinomas and carcinosarcomas, for example, which are highly aggressive in dogs and has poor prognosis. This may indicate a link between elevated COX-2 expression in malignant and canine mammary tumors, as it has been observed in humans (Queiroga et al., 2007; Hung et al., 2015).

COX-1 and COX-2 are the primary targets of anti-inflammatory non-steroidal drugs (NSAID). Clinical trials in dogs with invasive bladder tumors showed that piroxicam assists in inducing remission of the tumor, by apoptosis and the decrease of the tumor's angiogenesis. It seems possible that NSAIDs, in particular COX-2 inhibitors, will also prove to be useful in the treatment of canine mammary tumors (Queiroga et al., 2010).

In animal models, treatment with selective or specific inhibitors of COX-2 reduced the growth and formation of metastasis in experimental tumors, indicating an emerging role for COX-2 (Queiroga et al., 2010). Thus, it can be used as an additional tool in the clinical treatment of canine's mammary tumors, delaying the progression of malignant breast tumors by blocking the COX-2 pathways related to angiogenesis (Queiroga et al., 2011: Hung et al., 2015).

2.5 Proliferation markers (Ki-67)

The proliferation of tumor cells is related to prognosis in several types of tumors, including breast tumors (Matsumoto et al., 2015; Ranzi et al., 2015.). The most studied cellular proliferation marker in tumors is the Ki-67, a nuclear protein with a molecular mass of 345 and 395 kD (double band), expressed in all the phases of the cellular cycle, except G0 (Zuccari et al., 2004; Matsumoto et al., 2015; Ranzi et al., 2015). This oncogenesis can be evaluated by a variety of methods. Currently, the most common method is the evaluation of Ki-67 monoclonal antibodies for immunohistochemical technique (Matsumoto et al., 2015; Ranzi et al., 2015.). The expression of Ki-67 nuclear antigen is used to determine cell proliferation, in order to research breast tumors in humans (Geraldes et al., 2000). Researchers noted that the prognostic value of this marker in predicting overall survival and time of disease-free as an independent prognostic factor (Peña et al., 1998).

The expression of Ki-67 in human breast tumors is positively correlated with tumor's size, metastasis, expression of estrogen receptors, death due to cancer and low survival rate (Zuccari et al.,

2004). Benign tumors have a low number of Ki-67 positive cells, while the malignant tumors have the opposite phenotype. In a study of 118 breast tumors in dogs, the Ki-67 index had prognostic value regarding the probability of metastasis, disease-free survival and overall survival. In addition, the high rate of Ki-67 was positively correlated with metastasis, death from cancer, lower overall survival rate and low disease-free interval (Thuróczy et al., 2007). These observations support the theory that the dependency of the tumor's hormonal receptor increases the rate of cell proliferation, promoting the tumor's progression to malignancy (Geraldes et al., 2000; Thuróczy et al., 2007).

However, the prognostic role of Ki-67 is controversial because most of the studies include heterogeneous populations of patients, also being retrospective studies and using several methods for assessing Ki-67 (Matsumoto et al., 2015). Thus, the combination of complementary and independent biomarkers enables a more accurate diagnosis or prognosis than an isolated marker (Ranzi et al., 2015).

2.6 Vascular Endothelial Growth Factor (VEGF)

Nowadays, it is now known that angiogenesis plays a critical role in tumor growth and metastatic process. Among the various pro-angiogenic factors known, vascular endothelial growth factor (VEGF - Vascular Endothelial Growth Factor), is one of the most potent inducers and the most widely distributed in tissues (Queiroga et al., 2011).

VEGF is a family of proteins encoded by four different genes (VEGF-A to VEGF-D). VEGF-A and VEGF-B forms are related to the process of angiogenesis, whereas VEGF-C and VEGF D are more related to lymphangiogenesis (Millanta et al., 2010).

Physiologically, VEGF stimulates the formation of new blood vessels and regulates their structures and functions. However, during tumorigenesis, the increased VEGF signaling results in loss of this regulatory effect and in formation of disordered vessels, tortuous and fragile, leading to increased vascular permeability, lesser perfusion and an increase of hypoxia in the tumor that further stimulate the production of VEGF (Borgatti et al., 2014).

Several studies have explored the relationship between VEGF expression and breast cancer in canines. Although still controversial, considering that some studies found no significant correlation between clinicopathological parameters and VEGF expression (Millanta et al., 2006; Santos et al., 2010; Fox et al., 2016), the prognostic value of the expression VEGF has been well described in literature.

The diagnostic value of the increase in VEGF expression was observed in several studies in which a significant difference was detected between malignant and benign tumors (Restucci et al., 2002; Kato et al., 2007; Qiu et al., 2008a, b; Queiroga et al., 2011; Feliciano et al., 2012; Moschetta et al., 2015; Raposo et al., 2015).

Besides, the overexpression of VEGF has also been correlated with histological type (Queiroga et al., 2011; Carvalho et al., 2016), presence of metastasis (Qiu et al., 2008a,b; Carvalho et al., 2015, 2016), tumor size (Kato et al., 2007; Carvalho et al., 2016), decreased survival (Carvalho et al., 2015, 2016), degree of tumor differentiation (Restucci et al., 2002; Carvalho et al., 2015), necrosis (Queiroga et al., 2011) and tumor microvessel density

(Restucci et al., 2002; Queiroga et al., 2011; Feliciano et al., 2012; Moschetta et al., 2015).

Due to its relationship with tumorigenesis, VEGF appears as a good target in the treatment of breast cancer in female dogs. Recently, Adelfinger et al. (2015) developed a viral oncolytic vaccine (GLV-5b451), capable of expressing an anti-VEGF antibody (GLAF-2) and infected different cell lines derived from canine tumors, including breast, in order to report its effectiveness in treating and preventing tumor. The authors observed viral replication occurs preferentially in tumor tissue and resulting in a decrease of angiogenesis and vascular density, suggesting that it has therapeutic potential in the treatment of malignancy.

3 Final Considerations

The possibilities for using cell biomarkers in veterinary oncology are limited. The assessment of cell proliferation, angiogenesis, COX-2 expression and other cell biomarkers allow establishing prognostic and predictive factors for many different neoplasias. Determining the correct treatment and establishing an accurate prognostic can only be realized with a thorough diagnosis, sometimes being also necessary to identify the neoplasia's immunophenotype. In veterinary medicine, few biomarkers are routinely used for the treatment of breast neoplasms, despite the wide variety of labels used in human medicine, none of these markers have been adopted for routine use. The expectation that increasingly immunohistochemistry for prognostic markers be used as a routine procedure for canine mammary tumors and contribute to provide more accurate and informative diagnostic to the veterinarian. Further investigation of molecular biomarkers with prognostic and predictive value is still necessary; in order to recognize that animals also have needs for adjuvant therapy, as well as to identify new therapeutic targets.

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