

EpCAM Expression in Canine Malignant Mammary Tumors - Immunohistochemical Study

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ABSTRACT

Background: Epithelial cell adhesion molecule (EpCAM) is a glycoprotein responsible for multiple cellular functions that includes cell-to-cell adhesion and signal transduction. Studies in human breast cancer demonstrated that its overexpression has been linked to a more aggressive histological phenotype. However, in canine mammary tumors, its use is still scarce and its function, remains unknown. The main goal of the present study was to characterize the expression of EpCAM in canine mammary malignant tumors and associate its expression with clinicopathological features.

Materials, Methods & Results: Forty-eight canine mammary samples were analyzed in the present study and went through the Hematoxylin-Eosin (HE) routine technique for histopathological and immunohistochemical analysis. EpCAM expression was evaluated considering the total immunolabelling (TI) which consists of the addition of 2 parameters: proportion score (PS) and intensity score (IS). Thirteen (27.1%) cases showed weak expression, 9 (18.7%) were characterized by a moderate expression and 27 (54.2%) were classified as intense. EpCAM overexpression was described in 36 (72.9%) primary tumors and in 5 lymph node metastases (71.4%). Immunoreactivity was, mainly, characterized by a staining in the lateral cell membrane of luminal epithelial cells, both in primary tumors and lymph node metastases. The statistical analysis was performed using the SPSS Statistics, version 24. Clinicopathological features, such as histological grade ($P = 0.027$), intravascular emboli ($P = 0.004$) and lymph node metastases ($P = 0.016$) demonstrated to be associated with a more aggressive histological type. Intravascular emboli and lymph node metastases were also linked to a higher histological grade.

Discussion: EpCAM overexpression has been described in the literature both in canine mammary tumors and breast cancer. While the results in breast cancer could be ambiguous, depending on the tumor subtype, in the present study our findings demonstrated that overexpression was present in more than 50% of the total cases evaluated. In addition, immunoreactivity was also in accordance with the literature that describes membranous staining as the most prevalent pattern type. It was not possible to correlate overexpression with histological type and histological grade. However, those clinicopathological features are essential to evaluate tumor prognosis. For instance, our results demonstrated that histological type was statistically significant with histological grade ($P = 0.027$), intravascular emboli ($P = 0.004$) and lymph node metastases ($P = 0.016$). Additionally, the Nottingham system was statistically significant with intravascular emboli ($P = 0.033$) and lymph node metastases ($P = 0.006$). These findings have also been described in similar studies using canine mammary tissues in bitches. When it comes to pattern of expression in primary tumors and lymph nodes metastases, our findings described that neoplastic cell presented the same phenotype in these 2 different locations. In addition, loss of expression was also described in cluster cells within the subcapsular area which reflects the idea that the expression of EpCAM molecule changes according to time, which could be translated into the multistep process of metastases. Finally, it demonstrates that the development of neoplasia is not something static, but rather dynamic.

Keywords: EpCAM, canine, bitches, mammary tumors, neoplasia, adhesion cell molecule, immunohistochemistry.

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INTRODUCTION

Besides mediating cell-to-cell contact, EpCAM also participates in juxtacrine signal transduction [22]. In normal tissues, this molecule is expressed at varying levels, differently from what occurs in carcinomas, where its expression is, usually, higher [26]. However, recent discoveries have revealed a more versatile role for this molecule that is not exclusively limited to cell adhesion, but include other process, such as signaling, proliferating, migration, and tissue maintenance [23,26,28].

EpCAM is normally located in the luminal epithelia cells where its expression is mainly described as membranous and, sporadically, can also be described as cytoplasmic [11]. It has been well described in the literature that the process of metastasis is characterized by a series of steps that involves the detachment of epithelial cells from the primary tumor to distant sites [4]. As a result, the use of this molecule in human medicine, especially in human breast cancer, have demonstrated that its overexpression has been associated with a more aggressive histological subtype, poor disease free and overall survival, which suggest its use as a prognostic tool [24].

Studies focusing on epithelial adhesion molecules - especially cadherins and catenins - have been extensively described both in canine and feline mammary tumors [3,6-9,19-21,26].

As regards EpCAM expression in dogs and cats, there is limited information in the literature [26]. Hence, this study aimed to characterize the expression of EpCAM in canine mammary malignant tumors and to associate its expression with clinicopathological parameters.

MATERIALS AND METHODS

Tissue samples

All 48 samples analyzed in the present study were retrieved from the repository of the Veterinary Pathology Laboratory at the Abel Salazar Biomedical Science Institute (ICBAS) - University of Porto (U.PORTO) and went through the Hematoxylin-Eosin (HE)¹ routine technique for histopathological and immunohistochemical analysis. The process of HE included fixation in 10% neutral buffer formalin for posteriorly embedding in paraffin wax. The sections were cut into 4 µm thickness and posteriorly were stained using the Hematoxylin-Eosin (HE)¹.

Histological classification

The samples were morphologically evaluated according to their histological type and histological grade. In order to evaluate the histological type, it was applied the histological classification criteria proposed by the World Health Organization for canine mammary tumors [15]. To evaluate the histological grade, the Nottingham Grading System, adapted from human breast carcinoma was used [16]. This grading system is based on the evaluation of 3 morphologic features: tubule formation, nuclear pleomorphism and mitotic counts. All these features are added up reaching a final score that can range from I to III, where grade I is classified as low, II grade as moderately differentiated and, finally, grade III, high grade.

Immunohistochemistry

The immunohistochemistry technique applied in the present study was performed using a polymer-based system (Novolink Max Polymer Detection System)² following the manufacturer's instructions. Water bath was chosen as the antigen retrieval where all the mammary tissues were put for 20 min. In the next steps samples were de-waxed in xylene and rehydrated through graded alcohols.

Endogenous peroxidase activity was blocked by treating the sections in methanol containing 3% hydrogen peroxide in methanol for 10 min and rinsing in Tris-buffered saline (TBS, pH 7.6, 0.5M). Sections were incubated overnight at 4°C in a humid chamber and labelling was performed using 3,3'-diaminobenzidine at room temperature. In the next step, sections were counterstained with Mayer's Hematoxylin, dehydrated and mounted. Normal canine sections were used as positive controls and PBS were used as negative controls.

EpCAM expression was evaluated according to an equation that consisted of 2 parameters: proportion score (PS) and intensity score (IS). The total immunolabelling score (TIS) is the addition between these 2 parameters. Proportion score (PS) is described by the fraction of stained cells of the tumor [0, none; 1, <10%; 2, 10-50%; 3, 51-80%; 4, >80%]. The intensity of the stained cells is represented by the intensity score (IS) [0, no staining; 1, weak; 2, moderate; 3, strong] and the total immunolabelling score (TIS) ranges from 0 to 12 [0, 1, 2, 3, 4, 6, 8, 9 and 12] where EpCAM overexpression is considered when TIS>4. TIS is also classified as 4 subgroups: no expression, TIS 0; weak

expression, TIS 1-4; moderate expression, TIS 6 and 8; intense expression, TIS 9 and 12 [24].

Statistical analysis

In order to determine the significance of the relationship between the expression of EpCAM and clinicopathological features from tumors, data were organized in contingency tables and the likelihood ratio Chi-square test of associations was used to determine its significance. Tables of contingency were built and Fisher's exact test was performed, whenever biologically consistent. The statistical analysis in the present was performed using SPSS version 24 (IBM)³ and in all instances, $P < 0.05$ was statistically significant.

RESULTS

Forty-eight malignant mammary tumors where, morphologically, reevaluated where, according to the histological classification (WHO), 5 subtypes were described in order of occurrence: complex carcinoma (20/41.6%), solid carcinoma (13/27.1%), tubulopapillary simple carcinoma (9/18.7%), carcinosarcoma (3/6.3%), and micropapillary carcinoma (3/6.3%). Additionally, all the carcinomas were graded according to the Nottingham system where 19 (39.6%) tumors were graded as grade I (low grade), 22 (45.8%) as grade II (moderately differentiated) and 7 (14.6%) as grade III (high grade). Furthermore, 22 lymph nodes were available for inspection where 7 were compromised with metastases.

In primary tumors, all the cases analyzed in the present study demonstrated immunoreactivity for the EpCAM molecule, where 13 (27.1%) cases showed weak expression, nine (18.7%) demonstrated moderate expression and 27 (54.2%) were described as strong expression. Overexpression (TIS > 4) was observed in 36 (72.9%) cases (Table 1).

When it comes to immunoreactivity, EpCAM was characterized by a membranous staining that was located in the lateral cell membrane of luminal epithelial cells (Figure 1). Five clinicopathological parameters were also available in the present study: mode of growth, ulceration, intravascular emboli, lymph node status and necrosis. In lymph node metastases, all the cases showed immunoreactivity for the EpCAM molecule where 2 (28.6%) cases showed weak expression, 1 case was classified as moderate (14.3%) and 4 cases were classified as strong expression (57.1%). Overexpression (TIS > 4) was observed in 5 cases (71.4%) [Table 2].

In lymph node metastases, the pattern of immunoreactivity was similar to the primary tumors where EpCAM was mainly located in the lateral cell membrane of epithelial cells (Figure 2).

Infiltrative growth was described in 25 (52.1%) tumors and 21 (41.7%) were classified as expansive. Intravascular emboli were found in 6 cases (12.5%) and skin ulceration was found in 1 case. Necrosis was present in 29 (60.4%) cases.

Three clinicopathological parameters demonstrated to be statistically significant with histological type: histological grade ($P = 0.027$), intravascular emboli ($P = 0.004$) and lymph node metastasis ($P = 0.016$), while the Nottingham system was associated with 2 clinicopathological parameters that includes intravascular emboli ($P = 0.033$) and lymph node metastases ($P = 0.006$). In addition, intravascular emboli were associated with lymph node metastases ($P = 0.005$). The total immunostaining score (TIS) was statistically significant with 2 clinicopathological parameters: mode of growth ($P = 0.041$) and lymph node metastasis ($P = 0.042$).

Table 1. EpCAM expression in canine mammary malignant tumors.

Histological type	N	No EpCAM overexpression		EpCAM overexpression	
		No expression (TIS 0)	Weak (TIS 1-4)	Moderate (TIS 6,8)	Strong (TIS 9,12)
Complex Carcinoma	20		6	3	11
Micropapillary Carcinoma	3		0	1	2
Solid Carcinoma	13		4	1	8
Tubulopapillary Carcinoma	9		2	3	4
Carcinosarcoma	3		1	1	1
Total	48		13 (27.1%)	9 (18.7%)	27 (54.2%)
					36 (72.9%)

Table 2. EpCAM expression in lymph node metastasis in canine malignant mammary tumors.

Histological type	N	No EpCAM overexpression		EpCAM overexpression	
		No expression (TIS 0)	Weak (TIS 1-4)	Moderate (TIS 6,8)	Strong (TIS 9,12)
Micropapillary Carcinoma	2		1		1
Solid Carcinoma	3		1		2
Tubulopapillary Carcinoma	1			1	
Carcinosarcoma	1				1
Total	7		2 (28.6%)	1 (14.3%)	4 (57.1%)
					5 (71.4%)

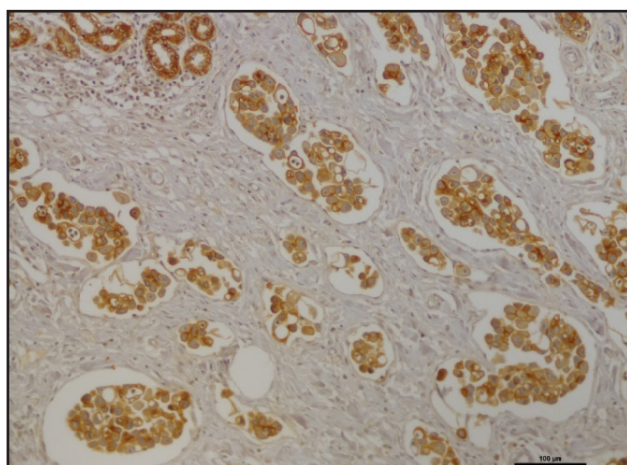


Figure 1. Canine mammary micropapillary carcinoma, primary tumor. Immunohistochemistry for EpCAM. Strong expression of EpCAM in the membrane of epithelial luminal cells (intravascular emboli). [100x; Bar= 100 µm].

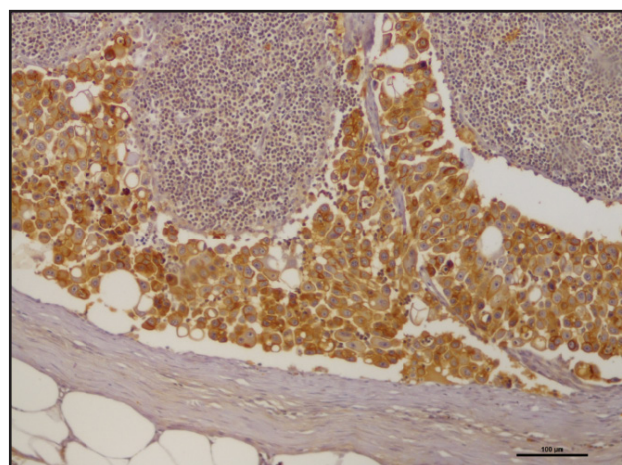


Figure 2. Canine mammary micropapillary carcinoma, lymph node. Immunohistochemistry for EpCAM. Strong expression of EpCAM in the membrane of epithelial luminal cells located in the subcapsular area of the lymph node. [200x; Bar= 100 µm].

DISCUSSION

EpCAM is a glycoprotein that mediates cell-to-cell contact and has a variety of roles, including signaling, proliferation, adhesion, migration, differentiation, and tissue maintenance [28]. In normal tissues, this molecule is expressed in low levels in epithelial luminal cells and is overexpressed in breast carcinomas, which suggests that its overexpression correlates with a more aggressive behavior [24]. This molecule has been a matter of great interest in human breast cancer, but its use in veterinary oncology is still scarce.

While studies in human have described its overexpression in many types of carcinomas [24], studies in cats and dogs comprising this molecule are still scarce [26]. For instance, the same group of researchers described the expression of EpCAM

in a series of epithelial and mesenchymal derived tumors which the results demonstrated that EpCAM is strongly expressed in most epithelial tumors, except those tumors derived from the adrenal gland. Studies in human have demonstrated that EpCAM immunoreactivity labelling was mainly membranous [10,23,24], which corroborates with our findings in the present study (Figure 1).

When evaluating the immunohistochemistry technique results, the literature describes a variety of protocols for the same technique and, it is advised to follow the manufacture's recommendation sheet [16]. For instance, adhesion molecules are, essentially, evaluated by the percentage of stained cells within the tumor and the staining pattern of the same cells [17,20]. In most cases, adhesion molecules are evaluated by the presence of labelled luminal epithelial cells, that

are classified as it follows: 0, < 25%; 1, 25-50%; 2, 50-75%; 3, > 75% [6,19]. However, there are different criteria to evaluate its immunoreactivity and, consequently, the comparison between 2 different criteria could be a challenge making it necessary to create a standardize guideline for the immunohistochemistry technique in canine mammary tumors [16].

When it comes to evaluating EpCAM expression, studies have been using a score that provides information regarding the proportion of the expression and the intensity of the expression, which is translated into an equation: proportion score (PS) + intensity score (IS) = total immunolabelling score (TIS) [11,24].

According to the literature, epithelial derived tumors have demonstrated a strong expression of EpCAM and colorectal tumors showed the highest expression [24]. This fact could be explained due to the fact that epithelial cells in this organ are intensively going through the process of renovation [12]. When it comes to breast tumors, the results could be ambiguous, where depending on the mammary subtype, its expression could be different [24]. In the present study, only canine mammary tissues were evaluated and, EpCAM was expressed positively in all primary tumors. Immunoreactivity was described as it follows: 13 cases (27.1%) showed weak expression, 9 (18.7%) demonstrated moderate expression and 27 (54.2%) were classified as intense (Table 1). Taking into account all the primary tumors, overexpression was described in 36 samples, reaching 72.9% of the cases. These findings are in accordance with the literature, where EpCAM overexpression has been described both in breast cancer and in canine mammary carcinomas [24,26]. Moreover, overexpression has been linked to histological type and histological grade in breast cancer [11]. In the present study it was not possible to correlate overexpression with histological type and histological grade. However, our results demonstrated that clinicopathological parameters are important to take into consideration when evaluating prognosis. For instance, this study, TIS (total immunolabelling score) was statistically significant when compared with mode of growth ($P = 0.041$) and lymph node metastases ($P = 0.042$).

Studies have demonstrated that the histological assessment provides valuable information on tumors and, hopefully, histological type and histological grade could be used as a prognostic tool, especially

regarding tumor morphology [2,16,27]. In fact, our results demonstrated that the histological type was statistically significant when compared with 3 clinicopathological parameters: histological grade ($P = 0.027$), intravascular emboli ($P = 0.004$) and lymph node metastasis ($P = 0.016$). In addition, the Nottingham system was statistically significant with 2 clinicopathological parameters: intravascular emboli ($P = 0.033$) and lymph node metastases ($P = 0.006$). Presence of intravascular emboli was statistically significant with lymph node metastases ($P = 0.005$). These findings are in accordance with similar studies performed in canine mammary tissues in female dogs [13,24,25].

When it comes to lymph nodes metastases, 2 cases (28.6%) showed weak expression, 1 case (14.3%) was classified as moderate, and 4 cases (57.1%) showed strong expression. Overexpression was observed in 5 cases, which is similar to results in human breast cancer [24]. Lymph node is the main organ affected by metastasis from mammary tumors, both in human and small animals, which makes its assessment part of the routine practice of mammary tumors [4,5,24,25]. In bitches, most of the cases are diagnosed at a late stage which means that the lymph node has been already compromised with metastases [1,25]. Our results demonstrated that neoplastic cells were primarily located in the subcapsular area and presented the same phenotype as the primary tumor. In human breast carcinoma, the literature describes that the expression of metastases phenotype usually reflects the expression of the primary tumors [24]. In addition, loss of expression was also described in cluster cells within the subcapsular area, which reflects the idea that the expression of the EpCAM molecule could vary according to time - then it is well established that metastases is a multistep process [14]. Finally, it also demonstrates that the development of neoplasia is not something static, but rather dynamic [18].

CONCLUSION

In conclusion, all the primary tumors studied showed immunoreactivity for EpCAM. Additionally, overexpression was found both in primary tumors and lymph nodes metastases. In fact, lymph node metastases exhibited the same pattern of expression as observed in the primary tumors. The Nottingham

System classification was statistically significant with intravascular emboli and lymph node metastases, demonstrating that histological type and histological grade must be taken into consideration when evaluating canine mammary tumors. Further studies will be needed with a larger number of cases, as well as a follow up study to assure the applicability of the EpCAM molecule as a diagnostic and prognostic tool in canine mammary tumors.

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