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Relationship Between the Immunodetection of Alpha-Smooth Muscle Actin and the Aggressiveness of Mammary Papillar Tumors in Female Dog

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Summary

Papillary carcinoma is a mammary neoplasia of women and female dogs characterized by papillary fibrovascular projections lined by epithelial cells. Evaluation on the biology of these tumors can be done by immunohistochemistry through detection of alpha-smooth muscle actin protein in the papillary myoepithelium, which lacks such a molecule during malignant proliferations. Thus, this study aimed at determining the malignancy degree of papillary mammary tumors of female dogs by immunohistochemistry. Twenty samples of mammary neoplastic tissues collected from female dogs treated in the Veterinary Hospital at FCAV were evaluated by Hematoxylin and Eosin staining (H&E) and tumor cells were immunolabelled with monoclonal antibody to alpha-smooth muscle actin (α-SMA). Five out of 20 cases showed positive immunolabeling greater than 10% of the total immunolabeling. The remaining fourteen cases presented immunostaining lesser than 10% showing decrease or absence of α-SMA labeling in the myoepithelium of the papilla tumors. All those cases in which immunostained cell was over 10% of the neoplasm (5 immunostains of 20 total cases) were classified as benign whereas those below 10% of immunostained in the slid were considered as malignant. Therefore, immunohistochemistry played an essential role in differentiating benign and malignant papillary tumors of bitches as already described for female. Tumor classification by conventional methods, such as H&E staining, can lead to erroneous interpretations on the real biological behavior of the papillary mammary tumor.

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Introduction

Characteristic lesions of a neoplastic papillary growth negatively impact on the mammary tissue of women and female dogs. Tumor classification based on the histological method displays this neoplasia as a heterogeneous group of lesions. All of which share a growth pattern characterized by the presence of arborescent fibrovascular stalks, containing a central fibrovascular stroma, and lined by epithelial cells [1,6]. A high quantity of myoepithelium can be detected by histopathology in benign papillary mammary tumors whereas malignant variants present discrete myoepithelial cells (MC) in the center of the papillary formation, which may be even absent in some cases [6]. This cell type is often difficult to be distinguished by the routine Hematoxylin and Eosin (HE) staining. Therefore, the immunohistochemistry technique would play an important role in its identification [4,7,8,9]

The immunohistochemical technique facilitates the evaluation of MC presence and distribution in mammary papillary tumors of both women and female dogs [1,4]. In general, the malignant variant presents little or no expression of MC antigens within the papillae. Thus, the use of specific antibodies targeting the myoepithelium, such as the p63 protein, calponin, CD10 and the smooth muscle alpha-actin protein, to determine the biological behavior of mammary gland papillary tumors turns out to be essential for choosing the most appropriate therapy and to establish the prognosis for cancer patients (UENG, MEZZETTI, TAVASSOLI, 2009). Thus, the present study aimed at investigating the presence of MC into papillary mammary tumors of female dogs by immunohistochemistry and to compare the results of malignancy with those obtained from H&E classification.

Material and Methods

Mammary neoplastic tissue samples (20) were obtained from female dogs, which were cared in the Veterinary Oncology Service and the Obstetrics and Animal Reproduction Service of the Veterinary Hospital "Governador Laudo Natel", placed at the Faculty of Agrarian and Veterinary Sciences (FCAV-UNESP), Jaboticabal city (São Paulo State, Brazil). There was no predilection for race or age and all animals had gone through clinical screening and unilateral radical mastectomy.

Tissue samples were fixed in 10% neutral buffered formalin solution (pH 7.4) for 24 hours and then they were embedded in paraffin wax, sectioned at 5µm and HE stained. Canine mammary neoplasms were classified according to the World Health Organization [5] and the second consensus guidelines for diagnosis, prognosis and treatment of canine mammary tumors [1]. These criteria rendered the selection of 20 breast tumor samples classified as papillary mammary carcinoma, which assembled the experimental group for this research. The experimental procedures performed in this study were approved by the Ethical Committee on Animal Experimentation from the Faculty of Agrarian and Veterinary Sciences (license number 014526/12; approved on 03/07/2012).

Immunohistochemistry

Immunohistochemistry was performed by using the monoclonal antibody alpha-smooth muscle actin (1:600 dilution, clone 1A4, Mob 001, DBS) to visualize the myoepithelium present in the tumor papillae. The immunostaining protocol consisted of dewaxing the paraffin-containing tissues in oven (60ºC / one hour) followed by their hydration in solutions of decreasing alcohol concentrations. Endogenous peroxidase blockade (hydrogen peroxide and methanol, 8%, 30 minutes) was performed in a dark chamber, followed by blocking of nonspecific reactions / one hour (Protein Block serum-free, Dako, ref. X0909) at room temperature. Primary antibody was diluted as 1:600 and then added to the tissue for 18 hours at 4 °C. Peroxidase-bound Polymer Complex (Advance Kit, DAKO, code K4067) was used as substrate and DAB (3.3-diaminobenzidine, Dako, code K3468-1) was the chromogen of choice. Finally, tissues were counter-stained with Harris Hematoxylin and assembled on the slides with Permount (Fisher Scientific, cod.70104). All steps were preceded by washes with TRIS-HCl buffer solution (pH 7.4).

Rabbit cardiac muscle tissue was used as positive control whereas the antibody diluent with background reducing components (Dako, code S3022), in replacement of primary antibody, was used as negative control. Count of immunolabelled cells was performed on the light microscope coupled to digital photomicrograph equipment (Nikon E200). For the

quantification of the immunostained cells, 10 large-magnification fields (40 x obj. lens) were randomly chosen, where the counts of immunolabellated cells were measured in an area of 0.19625 μm^2. Counting criteria were those used by [3], who used the semi-quantitative evaluation method. Thus, samples containing less than 10% of immunodetected cells were considered as negative and those containing more than 10% were interpreted as positive.

Results

Regarding the number of cells positive for α-SMA, five of the 20 samples had MC labeling greater than 10% of the total (Figures 1A and 1B). In the remaining samples, α-SMA detection was less than 10% or absent in the papillary formations of the mammary tumor (Figures 1C and 1D). Samples with α-SMA positivity greater than 10% were classified as benign papillary tumors, while those with markings below 10% were considered malignant papillary tumors (Table 1).

Discussion

Data generated in this study showed that the immunodetection of MC played an important role on the aggressiveness classification of the papillary mammary tumor of female dogs. There are currently few reports evaluating this neoplasia in female dogs. However, in the latest consensus on diagnosis, prognosis and treatment of mammary gland tumors in female dogs, it was proposed that papillary tumors in this species should also be differentiated between benign or malignant behaviors by techniques such as immunohistochemistry [1]

HE staining is the most commonly used technique in the routine diagnosis and classification of

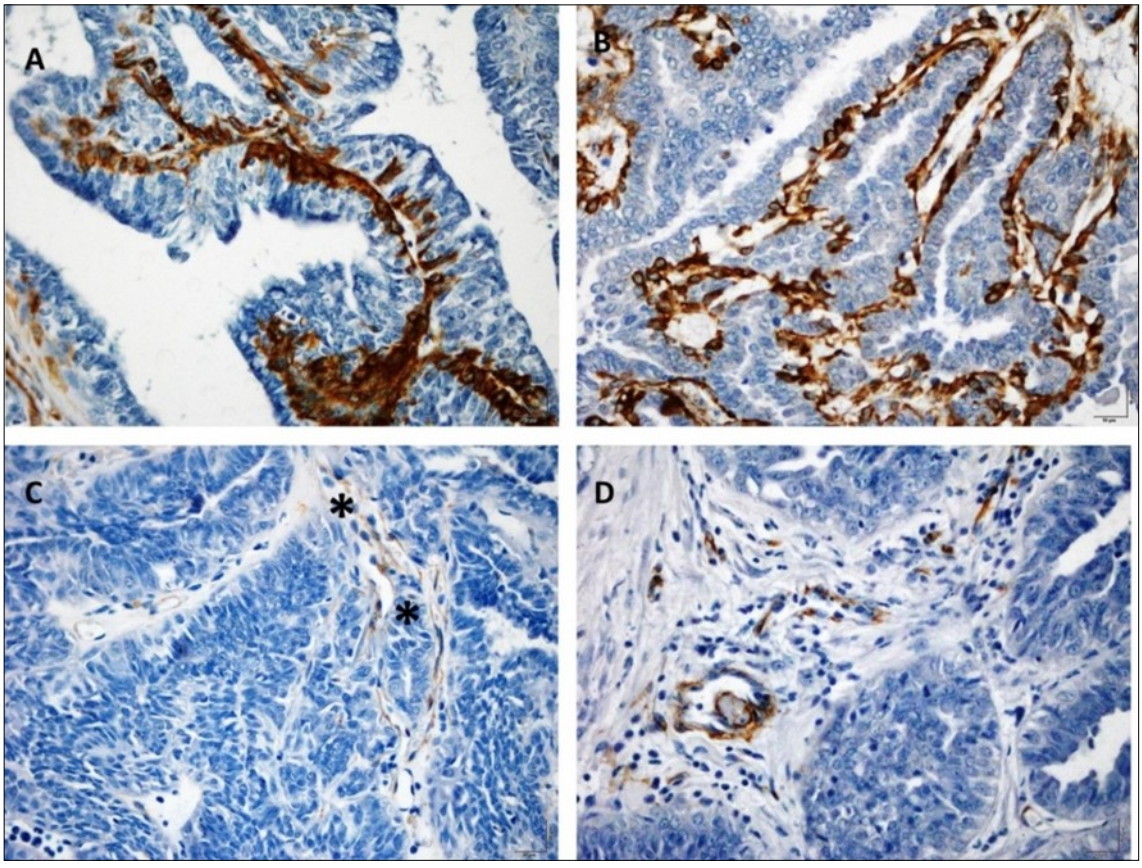

Figure 1. Photomicrographs of papillary tumors in mammary gland of female dogs. (A) Papillary tumor with intense positive marking for the α-SMA within the papillae (bar = 10 μm). (B) Positive to moderate positive papillary tumor for α-SMA (bar = 10 μm). Papillary tumors with reduced (C) or scarce immunolabeling (*) (D) for the same antibody (bar = 10 μm). All samples were classified by HE analysis as papillary carcinoma. Complex of Peroxidase bound polymers.

Table 1. Percentage of immunostained cells with α-SMA in mammary papillary tumors in female dogs. Five cases were detected in which the immunostaining was greater than 10% of the tumor and 15 cases where the detection of immunostained cells was less than 10%, according to CHU et al. (2012)[3].

Samples	HE	IHC (%)	Result of IHQ	Final classification
1	PC	46,51	> 10%	Benign
2	PC	7,47	< 10%	Malignant
3	PC	74,39	> 10%	Benign
4	PC	13,06	> 10%	Benign
5	PC	0,82	> 10%	Malignant
6	PC	2,82	> 10%	Malignant
7	PC	8,38	< 10%	Malignant
8	PC	2,01	> 10%	Malignant
9	PC	0,97	> 10%	Malignant
10	PC	4,29	> 10%	Malignant
11	PC	29,08	> 10%	Benign
12	PC	8,9	< 10%	Malignant
13	PC	5,92	< 10%	Malignant
14	PC	1,49	> 10%	Malignant
15	PC	3,56	< 10%	Malignant
16	PC	4,68	> 10%	Malignant
17	PC	0,44	> 10%	Malignant
18	PC	11,09	> 10%	Benign
19	PC	16,8	< 10%	Malignant
20	PC	9,05	> 10%	Malignant

Caption: HE (hematoxylin and eosin); IHC (immunohistochemistry); PC (papillary carcinoma);

mammary neoplasia of female dogs, nevertheless this method is not suitable to accurately identify the proportion of MC present in papillary tumors, especially for those cases in which the sample is very small or was not completely forwarded to the lab for analysis [4,910,6]. In the present study, the HE-based microscopic analysis was fundamental for distinguishing the histological tumor pattern (papillary), for determination of the mitotic index and invasion capacity to adjacent tissue, among other aspects. However, this method was not sufficient to show the biological behavior of the tumor, depending on the presence or absence of MC. In the present study, 25% of the samples (5/20) were classified as malignant in HE analysis, but by immunohistochemistry they were benign, since they showed a high percentage of α-SMA antibody positive cells.

Antibodies to detect myoepithelial tissue into mammary tumors samples have already been utilized for diagnostic purposes in humans, and the alpha smooth muscle antibody is one of those recommended for detection of MC in papillary tumors of both women and bitches [1,2,4,6,9]

The results from a study with tubulo-papillary carcinomas in female dogs demonstrated that there is an overestimation on the malignancy of neoplasia diagnosed by HE technique [3]. These authors reported that by using p63 and calponin antibodies nine tumors that had been classified as malignant by HE method were actually a benign process. Our data also supports the idea of an overestimation of malignancy when using HE staining to evaluate tumor samples. By using this criteria set up by [3], in which benign tumors had a positive mark greater than 10% of the entire tumor extension whereas malignant tumors should have positive MC below 10% of the total neoplastic cells, we

showed that five out of 20 predetermined malignant process by HE technique were in fact benign neoplasia with preservation of MC.

There are many specific antibodies available to detect myoepithelium in papillary tumors such as smooth muscle myosin heavy chain, CD10, calponin, maspine, p63, cytokeratins 5, 14 and 17, and Wilms-1 tumor. For some authors the target tissue should be tested with at least two of these markers to assure the prognosis. In the present study, we opted to use the smooth muscle alpha-actin protein alone because it was demonstrated to be sufficient to identify MC in tumors of humans [4,10] and it was possible to observe this aspect in dogs.

Conclusion this study, the immunohistochemical technique was essential for the differentiation between benign and malignant papillary neoplasia in bitches, as it had already been established for papillary tumors in women. Histological classification of papillary mammary tumors based on HE staining alone can bring erroneous interpretations of the true biological behavior of this tumor type and increases the diagnoses of malignancy, in turn, changing the directions of the treatments.

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Conflict of Interest

The authors declare that there were no conflicts of interest.

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