Is there a relationship between the expression of β-catenin and Ki-67 in canine melanocytic neoplasms?

Existe relação entre a expressão de β-catenina e Ki-67 em neoplasias melanocíticas caninas?

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ABSTRACT

An immunohistochemical evaluation of 26 cutaneous and oral benign and malignant canine melanocytic neoplasms was performed to identify a possible relationship between the expression of the β-catenin molecule with cell proliferation using Ki-67 expression. This molecule is a component of the Wnt/β-catenin signalling pathway, which causes a

cascade of intracellular events that activate transduction genes and nuclear transcription. The microscopic evaluation was performed considering the β-catenin labelling site (cytoplasmic, nuclear or mixed). The mitotic index was evaluated by the expression of Ki-67 in 10 high power fields (HPF) (400x). Statistically significant difference was neither detected between the β-catenin labelling sites and the various neoplasms, nor a correlation between the β-catenin molecule and the cell proliferation marker Ki-67 in the oral or cutaneous, benign or malignant neoplasms. Our study brings interesting findings and points to future research on this topic, especially with established variables in the clinical, pathological and immunohistochemical fields.

Keywords: melanocytic neoplasms, melanoma, β-catenin, Ki-67, immunohistochemistry.

RESUMO

Foi realizada uma avaliação imuno-histoquímica de 26 neoplasias melanocíticas caninas benignas e malignas, cutâneas e orais para identificar uma possível relação entre a expressão da molécula de β-catenina com a proliferação celular através da expressão do Ki-67. Essa molécula é um componente da via de sinalização Wnt/β-catenina, que causa uma cascata de eventos intracelulares que ativam genes de transdução e transcrição nuclear. A avaliação microscópica foi efetuada considerando o sítio de marcação da β-catenina (citoplasmática, nuclear ou mista). O índice mitótico foi avaliado através da expressão do Ki-67 em 10 campos de alta potência (HPF) (400x). Não foi detectada diferença estatisticamente significativa entre os sítios de marcação de β-catenina, tampouco correlação entre a molécula de β-catenina e o marcador de proliferação celular Ki-67 nas neoplasias orais ou cutâneas, benignas ou malignas. Nosso estudo traz descobertas interessantes e aponta para futuras pesquisas nessa temática, sobretudo com variáveis estabelecidas no campo clínico, patológico e imuno-histoquímico.

Palavras-chaves: neoplasias melanocíticas, melanoma, β-catenina, Ki-67.

1 INTRODUCTION

Melanocytic tumours in dogs are known as melanocytomas when benign, and as melanomas when malignant. The prognosis for melanomas, which on average represent less than 10% of malignant tumours in dogs, is usually bleak (Millanta et al., 2002; Sulaimon and Kitchell, 2003; Spangler and Kass, 2006; Lacroux et al., 2012; Nishiya et al., 2016; Goldschmidt and Goldschmidt, 2017). Some studies demonstrate the important role of clinical and pathological variables, especially the measurement of mitotic activity, in the prognostic significance of these neoplasms (Laprie et al., 2001; Millanta et al., 2002; Sulaimon and Kitchell, 2003; Spangler and Kass, 2006; Bergin et al., 2011; Sinnberg et al., 2011; Lacroux et al., 2012; Goldschmidt and Goldschmidt, 2017). Thus, the Ki-67 immunohistochemical marker is used to make up the prognostic panel of dogs with melanoma (Ramos-Vara et al., 2000; MacDonald et al., 2009; Lacroux et al., 2012;

Rolim et al., 2012; Han et al., 2013). Beta-catenin protein is known as a multifunctional molecule. The most widely studied functions of this protein are its participation in cell adhesion together with the transmembrane glycoprotein E-cadherin (epithelial cadherin), and its importance as a component of the Wnt/β-catenin signalling pathway (Valenta et al., 2012).

Research in the fields of human and veterinary medicine indicates that β-catenin performs important functions in Wnt/β-catenin signalling, given that it is involved in the nuclear transcription mechanism and plays an important role in the stimulation of genes involved in cell transduction and transcription (Smith et al., 2002; MacDonald et al., 2009; Han et al., 2010; 2013; Valenta et al., 2012).

Immunohistochemical (IHC) studies have demonstrated that deregulation of the E-cadherin/β-catenin complex is closely involved in both canine and human oral and cutaneous melanomas, because it causes β-catenin, which is no longer bound to E-cadherin, to accumulate in the cytoplasm. Due to as yet unknown factors of failure in the degradation process, as well as a cascade of intracellular events, β-catenin then enters the nucleus, binding to TCF/LEF receptors. This process activates transduction and nuclear transcription genes, triggering tumour progression (Smith et al., 2002; Han et al., 2010; 2013; Valenta et al., 2012).

Thus, our aim was to verify the expression of β-catenin, as well as its labeling site, in cutaneous and oral canine melanocytic tumors, and to relate the expression of this molecule with the Ki-67 expression.

2 MATERIAL AND METHODS

The study included tissue samples of primary, cutaneous and oral melanocytic neoplasms (n=26) from 24 dogs obtained by necropsy and/or surgical removal, diagnosed in a veterinary pathology laboratory.

The tissue samples were embedded in paraffin blocks, which were then cut into 3-µm thick histological sections for immunohistochemical analysis, using the avidin-biotin-peroxidase (ABC) method. In cases of strong pigmentation, the brown pigment formed by DAB chromogen is not easily distinguishable from the brown granules of melanin pigment. To correct this problem, the melanin was removed by soaking the most strongly pigmented sections in 5% oxalic acid solution for 10 min, and the less pigmented ones for 5 min. Tissue sections were then incubated in 10% hydrogen peroxide solution (30 vol.) In TBS buffer (Tris with sodium chloride, pH 7.6) for 20 minutes. For heat-

induced antigen retrieval, citrate buffer, pH 6, was used for 40 min in a steamer with a 500W power supply. Beta-catenin (14, Cell Marque) and Ki-67 monoclonal antibodies (MIB-1, DakoCytomation) were used, both diluted 1:100 in phosphate buffered saline (PBS). The slides were then incubated with the primary antibody in a wet-chamber for 14-16 hours (overnight) at 4ºC, followed by incubation with streptavidin peroxidase conjugated biotinylated secondary antibody (LSAB+System-HRP kit, KO690, DakoCytomation), taking 10 min per step. Counterstaining was performed using DAB chromogen and Harris hematoxylin solution, followed by mounting in synthetic Canada balsam (Pró-Cito, Brasil). Positive controls were used.

The following pattern was used to evaluate the markers: - (negative) = 0, + (up to 25% positive cells) = 1, ++ (26-50% positive cells) = 2, +++ (51-75% of positive cells) = 3, and ++++ (<75% of positive cells) = 4.

The mitotic rate (MR) was evaluated by Ki-67 expression viewing it in 10 high-power fields (HPF) (400x). In addition, the cytoplasm, nucleus or mixed (cytoplasm and nucleus) labelling sites were considered for the β-catenin marker. The variables of Ki-67 and β-catenin expression, and β-catenin labelling pattern of tumour location and marker location were organised in a contingency table, and their relative and/or absolute frequencies were obtained from descriptive statistics. The likelihood ratio test was used in 5 × 2 and 5 × 3 tables to determine whether there was an association between the categorical variables, since the expected frequencies were lower than 5. The β-catenin labelling pattern and the histological and immunohistochemical variables were correlated, using Spearman's correlation. Data were considered to differ significantly when the probability was less than 5% ($p < 0.05$). The analysis was performed using SPSS® version 20.0 software.

3 RESULTS

Of the 26 canine melanocytic neoplasms studied here, 4 were melanocytomas (all cutaneous), 18 were melanotic melanomas (14 cutaneous and 4 oral) (Figura 1A), and 4 were amelanotic melanomas (all oral). The MR was significantly altered in the amelanotic melanomas, but varied in the melanotic melanomas and was negligible in the melanocytomas. As for cell proliferation, which was evaluated by Ki-67 expression in the melanocytomas (Table 1), labelling was absent in two neoplasms and positive in up to 25% of the cells in the other two. Among the melanotic melanomas, 9 cases showed up to 25% of labelled cells, 4 showed 26 to 50% of positive cells, 3 presented 51 to 75% of

positive cells, 1 had more than 75% of labelled cells, and only 1 showed no positive labelling of any cell. As for the amelanotic melanomas, 1 neoplasm tested positive for each pattern.

The labelling sites for β-catenin molecule were cytoplasmic in 16 tumours (4 melanocytomas, 2 amelanotic, and 10 melanocytic), and mixed in 9 tumours (2 amelanotic and 7 melanocytic), while only one melanocytoma showed no labelling for this molecule (Table 1). Eight neoplasms showed weak multifocal labelling patterns (1 melanocytoma, 6 melanotic melanomas, and 1 amelanotic melanoma), 5 showed weakly diffuse patterns (all melanotic melanomas), 4 showed strongly diffuse patterns (1 melanocytoma, 2 melanotic melanomas and 1 amelanotic melanoma), 3 were moderately multifocal (1 melanocytoma and 2 melanotic melanomas), 3 moderately diffuse (1 melanocytoma, 1 melanotic melanoma and 1 amelanotic melanoma), and 2 showed a strongly multifocal pattern (1 melanotic melanoma and 1 amelanotic melanoma). No neoplasm showed single nuclear labelling.

The MR of melanocytomas followed a pattern of less than 3 mitotic cells in 10 HPF. The melanotic melanomas showed no statistically significant difference with respect to the pattern of approximately 3 mitotic cells per 10 HPF, while all the amelanotic melanomas presented more than 3 mitotic cells per 10 HPF. The cellular pattern of the melanocytic neoplasms did not differ statistically. Beta-catenin expression did not differ significantly in the different melanocytic tumours (Table 1), and no correlation was found between the expression of this molecule and that of Ki-67 ($r = 0.27$; $p = 0.18$). As for the labelling site of the β-catenin molecule, no significant difference was found between the labelling sites (cytoplasm/nucleus, cytoplasm and nucleus) in melanocytic tumours, amelanotic melanomas and melanocytomas (Table 1).

Table 1. Expression of Ki-67 and β-catenin, based on the histological classification of canine melanocytic tumors, as well as β-catenin expression for tumor localization and labeling site.

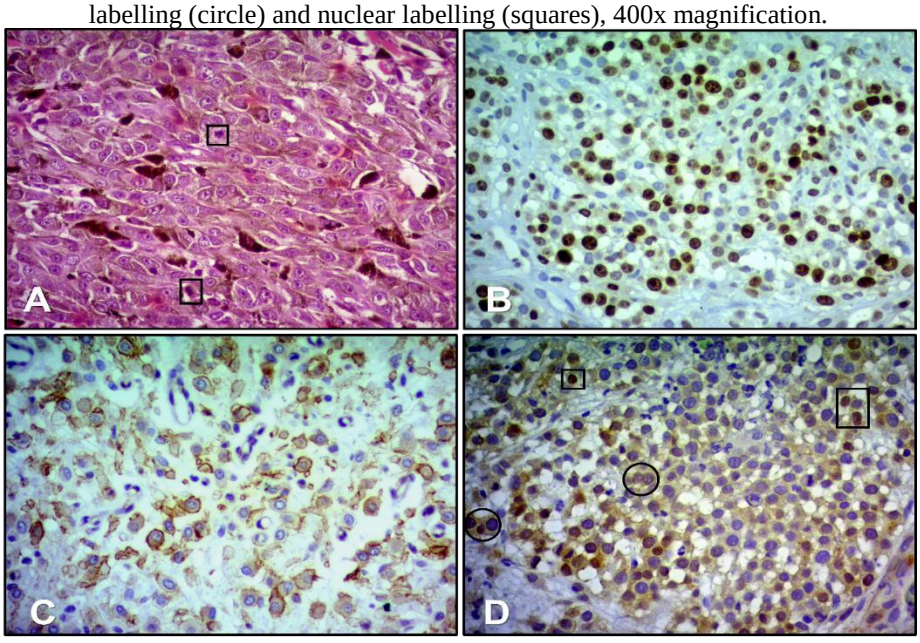
	Melanocytoma N (%)	Melanoma melanotic N (%)	Melanoma amelanotic N (%)	Significance (*p*)
Mitotic rate				
< 3/10 HPF	4 (15,40%)	8 (30,80%)	0 (0,00%)	0,01*
> 3/10 HPF	0 (0,00%)	10 (38,40%)	4 (15,40%)	
Total	**4 (15,4%)**	**18 (69,2%)**	**4 (15,4%)**	
KI-67				
Negative	2 (7,70%)	1 (3,85%)	0 (0,00%)	
< 25% positive cells	2 (7,70%)	9 (34,60%)	1 (3,85%)	
26-50% positive cells	0 (0,00%)	4 (15,40%)	1 (3,85%)	0,40
51-75% positive cells	0 (0,00%)	3 (11,50%)	1 (3,85%)	
> 75% positive cells	0 (0,00%)	1 (3,85%)	1 (3,85%)	
Total	**4 (15,4%)**	**18 (69,2%)**	**4 (15,4%)**	
β-catenin				
Negative	0 (0,00%)	1 (3,85%)	0 (0,00%)	
< 25% positive cells	1 (3,85%)	7 (26,90%)	1 (3,85%)	
26-50% positive cells	0 (0,00%)	1 (3,80%)	0 (0,00%)	0,92
51-75% positive cells	1 (3,85%)	5 (19,20%)	1 (3,85%)	
> 75% positive cells	2 (7,70%)	4 (15,40%)	2 (7,70%)	
Total	**4 (15,4%)**	**18 (69,2%)**	**4 (15,4%)**	

	Oral N (%)	Cutaneous N (%)	Significance (*p*)
β-catenin			
Negative	0 (0,00%)	1 (3,85%)	
< 25% positive cells	3 (11,55%)	6 (23,10%)	
26-50% positive cells	0 (0,00%)	1 (3,85%)	1,0
51-75% positive cells	2 (7,70%)	5 (19,20%)	
> 75% positive cells	3 (11,55%)	5 (19,20%)	
Total	**8 (30,8%)**	**18 (69,2%)**	

	Cytoplasm N (%)	Mixed N (%)	
β-catenin			
Negative	0 (0,00%)	0 (0,0%)	
< 25% positive cells	6 (24,00%)	3 (12,0%)	
26-50% positive cells	1 (4,0%)	0 (0,0%)	1,0
51-75% positive cells	4 (16,0%)	3 (12,0%)	
> 75% positive cells	5 (20,0%)	3 (12,0%)	
Total	**16 (64,0%)**	**9 (36,0%)**	

* indicates significant difference (Likelihood ratio test, $p < 0.05$).

Figura 1. A) Cutaneous epithelioid melanotic melanoma (note the marked pleomorphism of the cells and the frequent mitotic figures (squares), H&E, 400x magnification. B) Oral epithelioid amelanotic melanoma, IHC, Ki-67, 400x magnification. C) Epithelioid melanocytoma, IHC, β-catenin (cytoplasmic labelling), 400x magnification. D) Oral epithelioid amelanotic melanoma, IHC, β-catenina (cytoplasmic labelling (circle) and nuclear labelling (squares), 400x magnification.



4 DISCUSSION

Similarly to another study (Smedley et al., 2011), we found that the plethora of methods for evaluating the MR render it difficult to make a reliable comparison. We believe that the method used here to evaluate MR is as simple, practical and easily applicable in routine histopathology as all the other methods described in the literature (Bergin et al., 2011; Sinnberg et al., 2011; Lacroux et al., 2012; Goldschmidt and Goldschmidt, 2017).

The canine malignant melanocytic neoplasms we evaluated showed a high proliferative index, which was revealed by IHC using Ki-67 (Figura 1B), while the benign neoplasms, the melanocytomas, showed a low index. In addition, the amelanotic melanomas showed homogeneous cell positivity. However, most of these neoplasms exhibited more than 51% of positive cells, unlike the melanocytes, which, for the most part, contained up to 25% of positive cells, as expected (Rolim et al., 2012; Nishiya et al., 2016), since these neoplasms tend to be more aggressive than melanocytes.

Despite the existence of numerous data on canine melanocytic neoplasms, doubts about the mechanisms of tumour proliferation and invasion still abound. Thus, like other studies on human and canine melanomas that have revealed changes associated with β-catenin, particularly internalization of this molecule in the cell nucleus (Smith et al., 2002; Han et al., 2010; 2013; Lucero et al., 2010; Liu et al., 2014), we aimed to make an immunohistological evaluation of its expression in canine melanocytic neoplasms, seeking to confirm the probable correlation between β-catenin and the invasive and metastatic potential of these tumours investigated by means of Ki-67 expression. We observed the translocation of β-catenin through the cell cytoplasm (Figura 1C) (abnormal accumulations of this protein in the cytoplasm) to the nucleus (Figura 1D) (abnormal accumulations of this protein in the nucleus) in the different melanocytic neoplasms.

We also found that the accumulation of β-catenin protein, predominantly in the cytoplasm, occurred in both malignant and benign neoplasms (Han et al., 2010), proving that this molecule undergoes deregulation and translocation, but this process does not express its potential contribution to cell proliferation, as was proposed in another study (Chon et al., 2013).

Although we found different distribution patterns of β-catenin labelling among the different tumours, we were unable to correlate these patterns with the degree of malignancy of the neoplasm. Hence, we cannot, at this time, determine whether these distribution patterns can be used as prognostic markers for melanocytic neoplasms.

No correlation was found between cytoplasmic or mixed β-catenin labelling and the mitotic rate, assessed by means of Ki-67 expression, suggesting the absence of a direct relationship between β-catenin protein translocation and tumour proliferative capacity. This also indicates the controversial nature of the correlation between β-catenin and the invasive and metastatic potential of melanomas (Chon et al., 2013). Thus, further studies are needed to establish the prognostic and therapeutic potential of this molecule in canine melanocytic tumors.

5 FINAL COMMENTS

Currently, there is a growing concern about the health of pets. Multiple cancer research has been using animal models, such as for the canine melanocytic neoplasms, due to the similarities between the biological behavior of some neoplasms, especially in the human and canine species. In this context, we consider that the present study brings interesting findings and points to future research on this topic, especially with variables established in the clinical, pathological and immunohistochemical fields.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no potential conflicts of interest with respect to the research, authorship or publication of this manuscript.

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