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Comparison of KIT patterns and infiltration of eosinophils in canine mast cell tumor

[*Comparação dos padrões de KIT e infiltração de eosinófilos em mastocitoma canino*]

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ABSTRACT

KIT protein is associated with the etiology of canine mast cell tumors (MCT); however, the expression patterns of KIT are highly variable. The aim of this study was to determine if KIT patterns are related with eosinophil count in MCT. Hematoxylin eosin and May Grünwald-Giemsa stain techniques were applied, histological grading and eosinophil counting were performed in 48 MCT samples. Immunohistochemical evaluation was performed with IL-5, VEGFr, and c-KIT antibodies. The percentage of immunolabeling with IL-5 and VEGFr was determined, and the samples incubated with c-KIT were graded according to the immunolabeling pattern. Comparison of the mean eosinophil count between the histological grades and the different KIT expression patterns demonstrated a significant difference between KIT pattern 1 and KIT pattern 3, KIT pattern 3 showed a higher mean of eosinophil count. There was no significant correlation between eosinophil count and KIT patterns (p = 0.2648). However, a positive correlation was observed between the KIT patterns and Patnaik and Kiupel grades (p = 0.0006 and p = 0.0267, respectively). There was no significant correlation between eosinophil count, IL-5, or VEGFr. Further studies should determine whether eosinophil counts are an independent predictor of clinical outcome or simply correlated with already known predictors.

Keywords: dogs, immunohistochemistry, eosinophil, mast cell, c-KIT

RESUMO

A proteína tirosina quinase está associada com a etiologia dos mastocitomas (MC) em cães; entretanto, a expressão dos padrões de KIT é extremamente variável. O objetivo deste estudo é determinar se os padrões de KIT estão relacionados com a contagem de eosinófilos em tumores de MC. As técnicas de hematoxilina-eosina e MayGrünwald-Giemsa foram utilizadas; as graduações histológicas e a contagem de eosinófilos foram realizadas em 48 amostras de MC. A avaliação imuno-histoquímica foi feita utilizando anticorpos anti-IL-5, VEGFr e c-KIT. Foi determinada a porcentagem de área imunomarcada com IL-5 e VEGFr, e as amostras incubadas com c-KIT foram classificadas de acordo com o padrão imuno-histoquímico observado. A comparação da média de eosinófilos entre as graduações histológicas e os padrões de KIT mostraram uma diferença significativa entre o padrão 1 e o 3 de KIT, tendo o padrão de KIT 3 apresentado uma média maior de eosinófilos. Não houve correlação significativa entre a contagem de eosinófilos das amostras e os diferentes padrões de KIT (P = 0,2648). Entretanto, uma correlação positiva foi observada entre os padrões de KIT e as graduações de Patnaik e Kiupel (P = 0,0006 e P = 0,0267, respectivamente). Não houve correlação significativa entre contagem de eosinófilos, IL-5 ou VEGFr. Estudos futuros devem ser feitos para determinar se o número de eosinófilos é um preditor independente de prognóstico ou se está correlacionado com outros preditores já estabelecidos.

Palavras-chave: cães, imunohistoquímica, eosinófilos, mastócito, c-Kit

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INTRODUCTION

Canine mast cell tumors (MCTs) represent approximately 20% of all the skin tumors in dogs and have unpredictable biological behavior (London and Thamm 2013, Kiupel 2017). KIT is a protein that is associated with the etiology of MCT, and the activation of the KIT signal transduction pathway affects the growth and development of normal mast cells. KIT is encoded by the *c-KIT* proto-oncogene, which is deregulated in several types of cancer (Dobson and Scase, 2007). In dogs, normal mast cells have KIT only in the cell membrane, while undifferentiated MCT expresses cytoplasmic pattern (Kiupel *et al*., 2004).

Current prognostic factors for MCT include clinical characterization, such as clinical staging, breed, anatomical site, and history of recurrence (Blackwood *et al*., 2012). The combined evaluation of tumor grade and sentinel lymph node histology can provide better information on the extent and behavior (Warland *et al*., 2014). Immunohistochemical evaluation of Ki 67, fibroblast activation protein and KIT immunostaining pattern showed to be prognostic markers for MCT (Kiupel *et al*., 2004, Strefezzi *et al*., 2010, Giuliano *et al*., 2017).

The mediators stored or synthesized by mast cells attract leukocytes (eosinophils, basophils, Th2 lymphocytes, and neutrophils) and amplify the inflammatory response. Cytokines produced by mast cells include interleukins (ILs), IL-3, IL-5, and granulocyte macrophage colony stimulating factor (GM-CSF), which are essential for eosinophil development and survival (Stone *et al*., 2010). Mast cells can release tumor cytokines and growth factors such as fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), IL-8, and IL-10, which stimulate tumor cell expansion (Ribatti and Ranieri 2015).

Eosinophils and the remaining myeloid blood lineage cells develop in the bone marrow microenvironment from pluripotent hematopoietic stem cells, which originate from a population of eosinophil progenitors (EoPs) that are capable of terminally differentiating into mature eosinophils (Klion *et al*., 2020). The molecular steps involved in eosinophil development and trafficking have been described, with special attention to the important role played by the transcription factor GATA-1, the eosinophil selective cytokine IL-5, and the eotaxin subfamily of chemokines (Rothenberg and Hogan 2006).

The remarkable relationship between mast cells and eosinophils is well known, and both participate in a complex, self-perpetuating cycle. Eosinophils produce mediators responsible for mast cell differentiation, activation, proliferation, and survival. In turn, activated mast cells release mediators that improve eosinophil recruitment and activation, as well as IL-5 and GM-CSF (Piazuelo *et al*., 2008). A study by Caruso *et al*. (2007) reported morphological evidence of crosstalk between activated mast cells and eosinophils. These authors identified ultrastructural signs of activation, including changes in the size and number of granules, cytoplasmic vacuoles, and numerous free granules in the extracellular space.

The three cytokines, IL-3, IL-5, and GM-CSF are particularly important in the regulation of eosinophil development, with IL-5 being the most specific for the eosinophil lineage and responsible for its selective differentiation (Rothenberg & Hogan 2006).

Both cell types release profibrogenic (transforming growth factor-ß) and pro-angiogenic (VEGF) factors. Angiogenesis contributes to the perpetuation of the inflammatory process by promoting the migration of inflammatory cells (Piazuelo *et al*., 2008).

Despite the large number of MCT cases, there are no studies correlating the grade assigned to the MCT with the number of eosinophils and the factors that may influence the eosinophil count in the tumor tissue. The biological behavior of MCT is highly variable, and an improved understanding of prognostic indicators of MCT will result in better clinical management of canine patients with MCT.

MATERIAL AND METHODS

This experiment was approved by the Animal Ethics Committee, under protocol number 01171/2017.

A retrospective study was performed using 48 MCT samples that were previously sent to the Histopathology Laboratory. The animals were selected regardless of sex, breed, or age. The samples were processed using routine tissue processing techniques, with paraffin embedding and microtome sectioning into 4-μm thick sections. The slides prepared from the samples were stained with hematoxylin–eosin (HE) and May Grünwald–Giemsa (MGG). Histological grading was performed in a double-blinded manner by three veterinary pathologists following the criteria established by Patnaik *et al.* (1984) and Kiupel *et al.* (2011) on the HE stained slides. The histopathologic grade was established by the consensus of two or more observers (Strefezzi *et al*., 2003). The MGG-stained slides were scanned and digitalized using an Axio Scanner Z1 microscope (Carl Zeiss, Germany). A study made by Behjati *et al*. (2009) shown no statistically significant difference in a comparison of the mean number of eosinophils in 3 HPFs with 10 HPFs. Eosinophil counts were performed on scanned images using the ZEN 2.0 Image software (Blue ed., Carl Zeiss Microscopy GmbH, 2011), in three randomly delimited areas of each sample. The area was delimited in HPF, and the image software provides de area number in mm². Eosinophil counts were measured within each area. The mean of each area and the cells counted in each sample were calculated, and these values were converted into the number of cells per square millimeter.

For immunohistochemical evaluation, the tissue microarray (TMA) technique described by Mattioli *et al.* (2011) was applied to the embedded samples using a 3 mm metal drill, and new paraffin blocks were made. TMA blocks were sectioned on a microtome with 4-μm thick sections, deparaffinized with xylene (twice for 10 min), and rehydrated with absolute ethyl alcohol (thrice for 1 min) and 80% ethyl alcohol (once for 1 min). Endogenous peroxidase activity was inhibited using H_2O_2 with 5% methanol for 15 min. Antigen retrieval was performed using a pH 6 Immuno Retriever (BioSB, Santa Barbara, USA), induced by heating the TMA blocks in a water bath at 99°C for 25 min. The samples were incubated with polyclonal anti-IL-5 antibodies (Bioss, USA) at 1:200 dilution, polyclonal anti-VEGFr (Thermo Fisher Scientific, Waltham, USA) at 1:50 dilution, and polyclonal c-KIT antibody (Dako, Glostrup, Denmark) at 1:100 dilution. For the negative control, the antibody was replaced by the addition of phosphate buffered saline, positive control was performed with injured skin and injured artery for IL-5 and VEGFr respectively. For c-KIT positive control a high grade MCT was used. A Reveal Polyvalent Detection Kit (Spring, Pleasanton, USA) was used according to the manufacturer's instructions. The immunoreactions were incubated with DAB chromogen 1.1 (OriGene, Rockville, USA), and then counterstained with Harris hematoxylin for 3 min.

IL-5 and VEGFr slides were scanned using an Axio Scanner Z1 microscope (Carl Zeiss, Germany), and the percentage of immunolabeling area of each antibody was calculated with Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA) using a semi-automated color segmentation method, in which the immunopositive area was delimited and quantified expressed in square millimeters (mm²) (Witkowski *et al*., 2016, Faria *et al*., 2018). The slides labeled with *c-KIT* were observed under a light microscope at 40x and 100x magnifications to classify the immunolabeling pattern as described by Webster *et al*. (2006).

The results were statistically analyzed using Spearman's correlation and Levene's test to verify the homogeneity of variance, followed by ANOVA and Tukey's test ($p \leq 0.05$). The kappa statistic was used to analyze the agreement between the KIT patterns and histological grading.

RESULTS

According to the classification by Patnaik *et al*. (1984), 24/48 (50%) samples were classified as grade I, 23/48 (47.9%) as grade II, and 1/48 (2.1%) as grade III. The classification by Kiupel *et al*. (2011) resulted in 11/48 (22.9%) classified as high-grade tumors and 37/48 (77.15%) as low-grade tumors.

Sex and age showed no correlation with histological grading (Patnaik: $p = 0.0572$ for age, $p = 0.1822$, for sex; Kiupel: $P = 0.1032$ for age, $p = 0.148$ for sex).

Positive immunolabeling for both IL-5 and VEGFr was observed in the analyzed samples

(Fig. 1). There was no correlation between histological grading and eosinophil count (p = 0.0939; p = 0.0772). There was also no significant correlation between eosinophil count and IL-5 and VEGF percentages (p = 0.1554 for IL-5; p = 0.3635 for VEGFr).

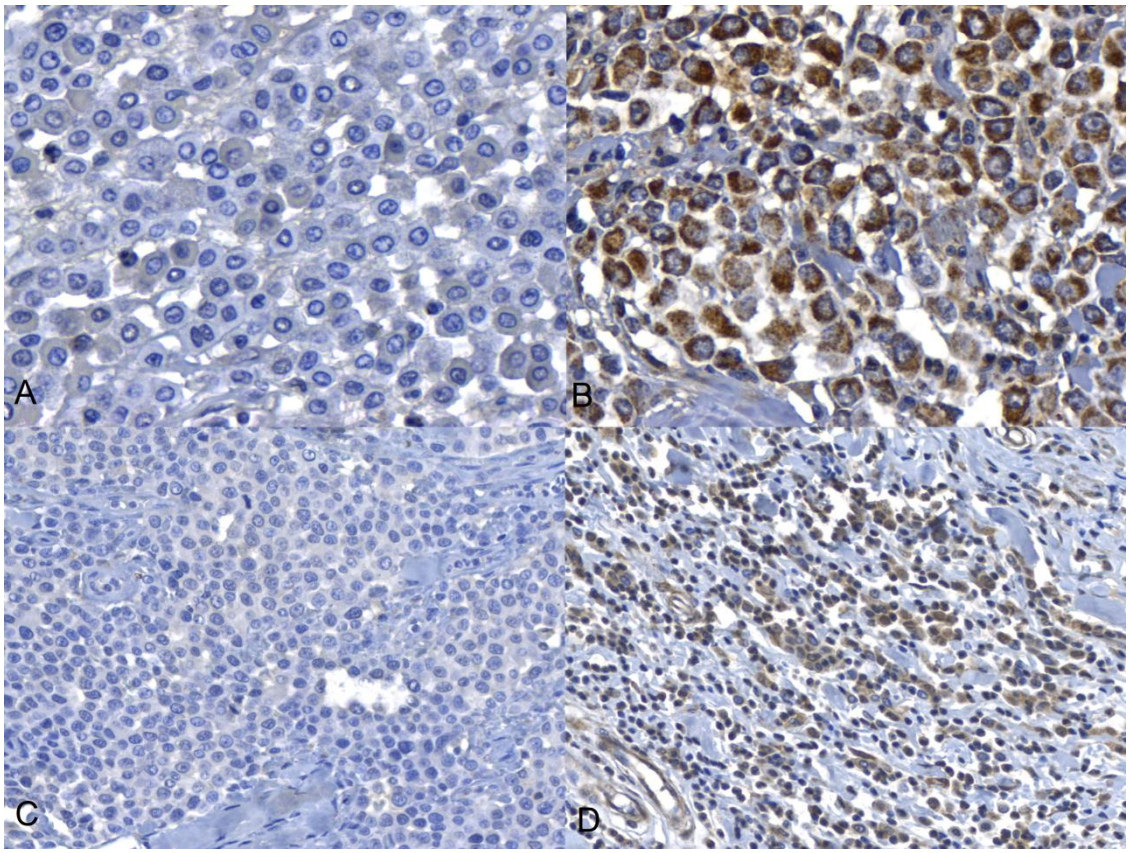

Figure 1. Canine mast cell tumor, histology sample. **A** Canine mast cell tumor sample negative for IL-5, obj.40x. **B** Canine mast cell tumor showing mast cells with intense cytoplasmic immunolabeling (brownish staining) of IL-5 obj.40x. **C** Canine mast cell tumor sample negative for VEGFr, obj.20x. **D** Canine mast cell tumor, mast cells showing cytoplasmic immunoexpression (brownish staining) for VEGFr obj.20x.

The percentages of IL-5 and VEGFr in the samples were not correlated with the grading classification by Patnaik *et al*. (1984) (p = −0.2879; p = 0.1392) and Kiupel *et al*. (2011) (p = 0.2075; p = 0.1234), and KIT patterns (p = 0,5935). Levene's test followed by ANOVA and Tukey's test were used to compare the means of the eosinophil, IL-5, and VEGRr counts for the different histological grading classifications (Table 1). KIT patterns also showed no correlation with IL-5 and VEGFr (p = 0,5935).

The comparison between eosinophil count and KIT standards showed no significant correlation (p = 0.2648) (Fig. 2).

There was a positive correlation between KIT patterns and the gradings assigned by Patnaik *et al*. (1984) and Kiupel *et al*. (2011) classifications (p = 0.0006, p = 0.0267, Spearman's correlation).

The mean eosinophil count for the MCT samples was compared between histological grading classifications and different KIT standards and showed a significant difference between KIT patterns 1 and 3 (Table 2).

Table 1. Mean comparison of samples between histological classifications by Patnaik *et al*. (1984) and Kiupel *et al*. (2011) with IL-5 and VEGFr. Results are expressed as mean ± standard deviation

		IL-5 %	VEGFr %	Number of eosinophils/mm^2
Patnaik *et al*. (1984)	I	2.83±2.2	15.55±10.68	1.43±0.59
	II	1.92±2.11	22.99±18.61	1.82±0.87
	III	0.66±	5.55±	1±
P		0.2678	0.1709	0.1581
Kiupel *et al*. (2011)	Low	2.52±2.14	19.39±15.66	1.65±0.79
	High	1.73±2.26	16.29±13.31	1.49±0.64
P		0.2965	0.5515	0.5582

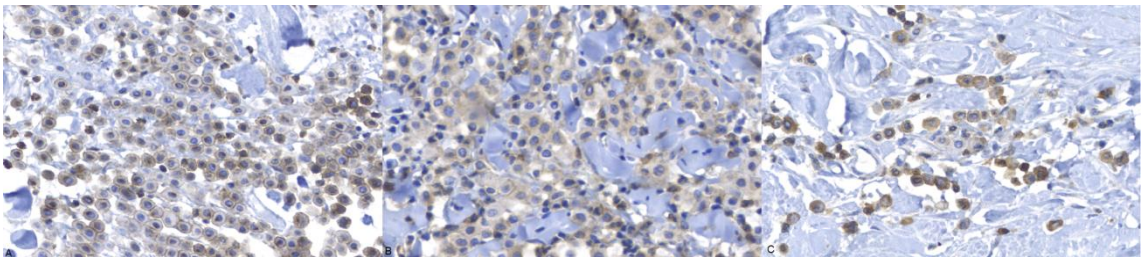

Figure 2. KIT immunolabeling patterns in canine mast cell tumor. **A** Pattern I or perimembranous pattern: showing only cell membrane staining. **B** Pattern II: focal cytoplasm. **C** Pattern III: diffuse cytoplasmic.

Table 2. Comparison of the mean eosinophil counts of the samples between the histological classifications by Patnaik *et al*. (1984). Kiupel *et al*. (2011). and KIT standards. Results are expressed as mean and standard deviation

		Number of samples	Number of eosinophils/mm^2
Patnaik *et al*. (1984)	I	24	1.43 ± 0.59
	II	23	1.82 ± 0.87
	III	1	1±
P			0.1581
Kiupel *et al*. (2011)	Low	37	1.65 ± 0.79
	High	11	1.49 ± 0.64
P			0.5582
KIT	I	15	1.41 ± 0.57b
	II	23	1.49 ± 0.66ab
	III	9	2.16 ± 1.03a
P			0.0345

DISCUSSION

The present study correlates the number of eosinophils in MCT samples with factors that may be associated with their migration to neoplastic tissue.

The comparison of the mean eosinophil count between KIT patterns showed a significant difference between patterns of grades I and III, indicating that MCT patients with higher cytoplasmic expression presented higher mean eosinophils. A recent study by Galietta *et al*. (2023) compared tumor-associated tissue eosinophilia (TATE) and VEGF expression between themselves, their results associated high grades of TATE with less differentiated tumors, higher recurrence rates and aberrant KIT expressions.

Kiupel *et al*. (2004) evaluated KIT and tryptase expression patterns as prognostic tools for MCT, noting that the higher the cytoplasmic KIT expression, worse the prognosis. Gross *et al*. (2005) described how eosinophilic infiltration decreases as cell anaplasia increases; however, the evaluation of the relationship between KIT patterns presented a higher mean eosinophil count for KIT grade III pattern, in which cells tend to be more anaplastic. Therefore, we cannot state that as histological grading increases, the eosinophils lose their chemotactic effect. Pardanani *et al*. (2003) identified a mutation in the *c-KIT* of eosinophils in human patients with eosinophilia associated with systemic mastocytosis, proving the mutation to be the clonal origin and not the reactional origin of the high numbers of eosinophils.

The correlation between the KIT receptor expression and the histological grading of MCTs was established in both well-differentiated tumors showing cell membrane KIT expression and poorly differentiated ones demonstrating cytoplasmic expression (Reguera *et al*., 2000, Welle *et al*., 2008). A study by Carvalho *et al.* (2017) reported a strong association between the KIT patterns and histological grading. The data obtained in the present study also indicated a correlation between immunohistochemical patterns and histological grading, in which tumors showing greater malignancy were associated with cytoplasmic immunohistochemical patterns.

There was no remarkable correlation between the eosinophil counts and IL-5 and VEGFr. Eosinophil adhesion is one of the necessary steps in the migration of these cells to target tissues (Ângulo *et al*., 2019), but only factors that influence the regulation of eosinophils and not their adhesions to the tumor tissues were evaluated. To date, there have been no studies associating IL-5 with MCT, and since no link was found in this study between IL-5 and KIT patterns, further studies are needed to better elucidate the action of this interleukin in mast cell tumor tissues.

In veterinary medicine, different studies have been conducted on the relationship between VEGF expression and the malignancy in canine and feline breast tumors (Restucci *et al*., 2002; Oliveira 2008; Camacho *et al*., 2014). VEGF is an important regulator of endothelial cell proliferation, vasculogenesis, angiogenesis, and vascular permeability, mediated mainly by the tyrosine kinase receptor VEGFr. Both cutaneous and subcutaneous MCT express VEGFr (VEGF ligand and VEGF receptors) (Silva *et al*., 2017). Angiogenic/lymphangiogenic molecules produced by mast cells at the sites of inflammation or tumor growth may play a dual role by directly influencing inflammation and tumor angiogenesis/lymphangiogenesis, contributing to chronic inflammation due to the recruitment of more mast cells and other cells of the immune system (Marone *et al*., 2016). However, this study showed no correlation between VEGFr and the number of eosinophils in the analyzed samples. Although several eosinophil chemotactic substances are well characterized, little is known about their mechanisms or the presence of regulatory molecules that negatively influence eosinophil migration, which may be responsible for a few eosinophils in tissues (Bournazou *et al*., 2009). Factors suppressing the migration of eosinophils to tumor tissue may be considered as a topic for future studies.

Most samples (50%) analyzed were classified as Patnaik *et al*. (1984) grade I, 47.9% as grade II, and 2.1% (n = 1) as grade III. Other studies showed a predominance of grade II tumors (Kiupel *et al*., 2004; Sabattini *et al*., 2015; Carvalho *et al*., 2017). However, the grading suggested by Patnaik *et al*. (1984) may be influenced by subjectivity of the observers. By improving the agreement between pathologists and reducing uncertain intermediate prognosis grading, the histological grading proposed by Kiupel *et al*. (2011), that classifies a MCT as high or low grade, demonstrated 96.8% consistency amongst pathologists (Sabattini *et al*., 2015). Of the 23grade II samples according to the classification by Kiupel *et al*. (2011), thirteen were classified as low grade and ten as high grade. There was a 77% agreement between the evaluated pathologists.

No remarkable correlation was observed between the number of eosinophils and histological grading, and this does not corroborate the findings of Gross *et al*. (2005). Moreover, Oliveira (2008) and Carvalho *et al*. (2017) reported no correlation between histological grading and eosinophil concentration,

corroborating the results of this study. Therefore, the eosinophil count cannot be used to determine the degree of malignancy in MCT.

CONCLUSION

The relationship between eosinophils and tumor cells is still obscure, and there is not enough data in the veterinary literature about eosinophil chemotactic substances, which lead to reactional eosinophilia that influences the number of eosinophils associated with tumor tissue. This research found a correlation between eosinophil infiltration and KIT cytoplasmatic pattern, corroborating with published literature. Therefore, eosinophil infiltration could be useful as prognostic marker.

The next step is to compare the data from this study with clinical progression and prognosis of animals to determine whether eosinophil counts are an independent predictor of clinical outcome or simply correlated with already known predictors. In addition, a comparison with other factors, such as tumor size, is necessary for future studies to further elucidate the biology of this neoplasm and to determine if there is any influence on the amount and action of eosinophils associated with mast cell tumors.

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