



RESEARCH ARTICLE

Loss of Tumor-Associated Macrophages and Vascular Endothelial Growth Factor Immunopexpression in Solid Mammary Carcinoma in Dogs

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ABSTRACT

Tumor-associated macrophages (TAM) are related to poor prognosis in canine mammary tumors (CMT). An association between TAM and sustained angiogenesis has been implicated with malignancy; however, comparison of the expression of TAM and vascular endothelial growth factor (VEGF) in different histologic types of CMT has not been completely studied. The objective of this study was to evaluate the immunostaining of TAM and VEGF in four different types of malignant CMT. Ninety-nine mammary carcinomas [tubular (29), tubulopapillary (22), mixed-type (28) and solid carcinoma (20)] were evaluated by immunohistochemical staining using MAC387 and VG-1 to detect TAM and VEGF immunopexpression, respectively. The total number of positive macrophages within the tumor was used for TAM immunolabeling and a score of four categories was used for VEGF immunolabeling. TAM immunopexpression was found to be statistically higher in tubular carcinomas than in solid carcinomas ($P=0.0015$). Differences between other types of carcinoma were not statistically significant. VEGF score was higher in tubular, papillary and mixed-type carcinomas than in solid carcinomas ($P<0.0001$). A positive relationship between the highest mean value of TAM and VEGF immunopexpression was also found ($P=0.0015$). TAM and VEGF expressions can be lost in more aggressive CMT, as in solid carcinoma. Furthermore, regardless of histologic type, higher numbers of TAM are associated with higher scores of VEGF, thus favoring the relationship between TAM and angiogenesis.

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INTRODUCTION

Macrophages are the most common immune cells that constitute tumor microenvironment and represent up to 50% of the tumor mass (Chanmee *et al.*, 2014). Macrophages and some other immune cells are important for their pro-tumoral and anti-tumoral effects on neoplastic cells. More specifically, macrophages can be activated into two different phenotypes, M1 (classical type) and M2 (alternate type) (Gordon and

Taylor, 2005). M1 macrophages present anti-tumoral functions like cell destruction because of activation of Th1 lymphocytes (cell-mediated immunity). In contrast, M2 macrophages or tumor-associated macrophages (TAM) have pro-tumoral effects, promoting proliferation, tumor cell survival and metastasis (Mantovani *et al.*, 2002). TAM activate Th2-cells (humoral mediated immunity) that suppress functionality of Th1-cells, thus endorsing immunosuppression (Allavena *et al.*, 2008).

TAM and angiogenesis appear to be well associated in different studies (Bingle *et al.*, 2006) and in human breast cancer their role in carcinogenesis has been documented (Zhao *et al.*, 2017). Apparently, TAM is related in tumor cell proliferation by secreting transcription factors like NF- κ B, HIF-1 and STAT3 (Karin, 2006; Diakos *et al.*, 2014) but several studies have associated TAM density to diverse negative prognostic factors in human breast cancer (Leek *et al.*, 2000). However, the role of TAM is still controversial. It contributes to carcinogenesis and poor prognosis in different type of human tumors: breast, urogenital, stomach, pancreas and prostate cancer (Galon *et al.*, 2006; Obeid *et al.*, 2013; Zhao *et al.*, 2017); however it is related to good prognosis in colorectal tumors and osteosarcoma (Buddingh *et al.*, 2011; Ong *et al.*, 2012) and its function is not completely defined in other tumors (Quatromoni and Eruslanov, 2012). This can be explained by the specific type of immunity that TAM activate, since Ong *et al.* (2012) found that high number of tumors infiltrating T-cells were correlated to the number of TAM, promoting a pro-inflammatory microenvironment that inhibited neoplastic cells in colorectal carcinomas.

In canine mammary tumors (CMT), TAM have been linked to metastasis, angiogenesis and poor prognosis (Krol *et al.*, 2011; Raposo *et al.*, 2012; Raposo *et al.*, 2013). As in human breast cancer, Raposo *et al.* (2013) found a significant association between TAM expression through MAC387 immunoreexpression and VEGF positive tumors and concluded that TAM could enhance VEGF expression promoting angiogenesis and subsequently tumor progression. They also found that TAM was associated with some clinicopathologic aggressive features in CMT and for this reason, constitute a new target in malignant CMT. In order to confirm TAM and VEGF association in malignant CMT in Brazilian dogs, this study was performed using immunohistochemistry for TAM and VEGF expression in four different histological types and grades of malignant CMT.

MATERIALS AND METHODS

Case selection: Paraffin-embedded mammary tumors samples were selected from dogs attended in Franca Veterinary Hospital (Unifran). Specifically, four histologic types of mammary carcinomas were selected and were classified by a single pathologist in accordance to previous classification (Goldschmidt *et al.*, 2011). A total of ninety-nine mammary carcinomas samples were selected having different gradings [tubular (29), tubulopapillary (22), mixed-type (28) and solid carcinoma (20)] (Table 1).

Table 1: Mammary carcinomas from four histological types with different grading selected in this study

Type/grade	I	II	III	Total (n=99)
Tubular	10	10	9	29
Mixed-type	10	8	10	28
Tubulopapillary	8	10	4	22
Solid	0	10	10	20

Macrophage and VEGF immunohistochemistry: For immunohistochemistry, REVEAL Biotin-Free Detection System method (Spring Biosciences, Pleasanton, USA),

was used. Paraffin embedded samples were cut into 3 μ m sections and mounted into silane-coated glass slides (Knittel Starfrost®, Braunschweig, Germany). The slides were de-waxed and hydrated and subjected to heat-induced antigen retrieval by electric pressure cooker (Maxi-Matic EPC-808BL®, City of Industry, USA) using EDTA (ph=8.5) for 20 minutes. After this, endogenous peroxide was blocked through the blocking solution, according to the manufacturer recommendation. Unspecific reactions were blocked using protein block and subsequently, for macrophage and VEGF identification the sections were incubated overnight with anti-MAC (clone 387 – DBS, Pleasanton, USA) and anti-VEGF (clone VG-1, Abcam, Cambridge, USA), diluted at 1:400 and 1:500. Secondary antibody was used and sections were stained with the chromogenic substrate 3,3' diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, USA). After this, sections were counterstained with Harris hematoxylin.

Quantification of macrophage and VEGF immunohistochemistry: For macrophage immunohistochemical evaluation the total number of macrophages located within the tumor was counted. A single pathologist selected the areas with higher density of macrophages at 100X magnification. Then, typical morphological macrophages were counted at 400X magnification. Five high power fields (HPF) were analysed to calculate the mean value of positive cells.

VEGF immunolabeling was quantified by a score system according to the positive area of the cells. Five HPF were randomly selected and the areas were analyzed for quantification in percentage of positive cells. The score system consisted in 4 categories as described by Fujimoto *et al.* (2009): 1 (0-25% positive cells), 2 (25-50% positive cells), 3 (50-75% positive cells) and 4 (75-100% positive cells).

Statistical analysis: Kruskal-Wallis variance with Dunn's post-hoc tests were performed to compare groups in respect of histologic type or grade. Mann-Withney was performed when two groups were compared. The statistical program GraphPad Prism v.6.0 (GraphPad Software, La Jolla, USA) was used for analysis. Significance was considered at P<0.05.

RESULTS

Immunoreexpression by MAC 387 was evident in the cytoplasm of macrophages and in few granulocytic cells infiltrated in the mammary neoplastic tissue. Macrophages were identified manually by nuclear morphology. MAC 387 immunolabeling was found in all four types of CMT in different pattern of expression (Fig. 1). Interestingly, solid mammary tumors had the lowest mean value immunoreexpression (2.77 \pm 3.59) compared to other carcinomas, but this difference was significant just with tubular carcinomas (P=0.0015) (Fig. 2).

Mean values of MAC 387 among grades of malignancy were also compared independently from histological types (grade I: 8.62 \pm 9.11; grade II: 11.62 \pm 14.17; grade III: 5.00 \pm 4.84) showing no statistical difference (P=0.1115). We did not identify any order of MAC 387 immunoreexpression in grades I to III, although grade III had the lowest mean value compared to others.

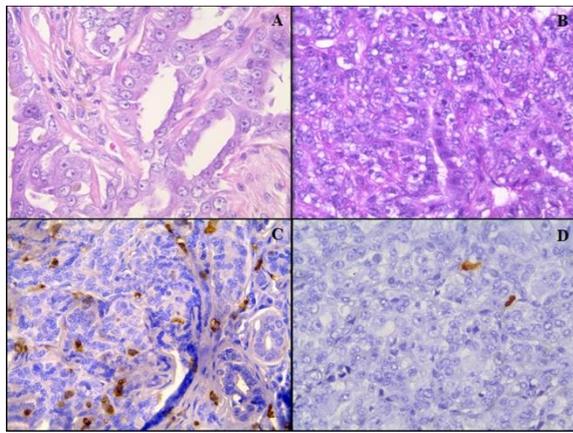


Fig. 1: Photomicrography of tubular (A) and solid carcinoma (B) in H&E stain. Tubular carcinoma presents neoplastic cells arranged in a linear fashion while in solid carcinoma this feature is lost, as neoplastic cells are disorganized through the mammary tissue. Photomicrography of MAC387 immunohistochemistry in tubular carcinoma (C) and in solid carcinoma (D). Note the increased number of positive cells in tubular carcinoma when compared to solid carcinoma.

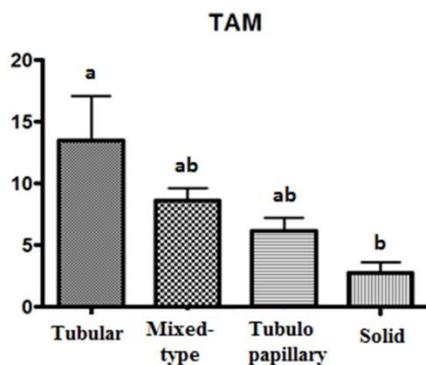


Fig. 2: MAC 387 mean value in four different histological types of CMT. Note the difference of mean value of all carcinomas compared with solid carcinomas. Different letters indicate statistically significant differences by Dunn’s test (P=0.0015).

VEGF immunohistochemistry was also cytoplasmic and neoplastic cells were positive in all the four histological types of CMT (Fig. 3). Score 4 was the most prevalent in tubular (20.41%) followed by tubulopapillary (15.31%) and mixed-type (15.31%). On the other hand, score 2 was the most commonly found in solid carcinomas representing 11.22% of cases. All of these scores totalized 62.25% of all analyzed samples. VEGF mean score in tubular, tubulopapillary and mixed-type carcinomas were significantly higher than in solid carcinomas (P<0.0001) (Fig. 4). In all three histological grades of malignancy VEGF immunohistochemistry was also studied. Score 4 were most commonly presented in all three grades, however with no statistical difference (P=0.1223).

We also wanted to know if there is any association between MAC 387 and VEGF immunohistochemistry in the four histologic types studied. For this, we identified and compared the mean value of MAC 387 for every score of VEGF (1-4). We found a higher mean value of MAC 387 immunohistochemistry in scores 3 and 4, but this association was not significantly different from scores 1 and 2 (P>0.05). Furthermore, we dichotomized VEGF groups in VEGF <50% (scores 1 and 2) and VEGF >50% (scores 3 and 4) and compared then with MAC 387 values. We found a statistical difference in MAC 387 immunohistochemistry between them (P=0.0015) (Fig. 5).

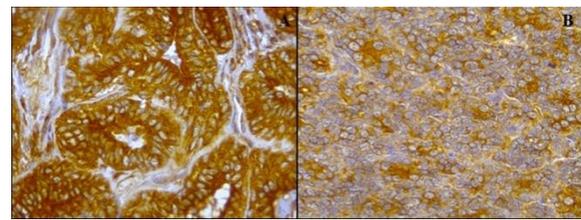


Fig. 3: Photomicrography of VEGF immunohistochemistry in tubular (A) and solid carcinoma (B). Increased cytoplasmic immunohistochemistry was found in tubular carcinoma when compared to solid carcinomas.

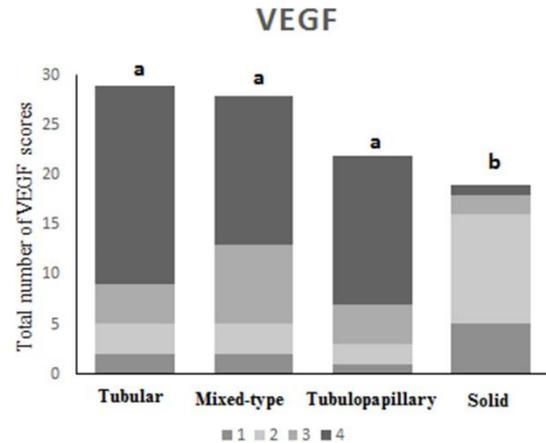


Fig. 4: VEGF score in 4 different tumor types of CMT. Tubular, mixed-type and tubulopapillary carcinomas presented more cases of VEGF score 4 over when compared to solid carcinomas. Different letters indicate statistically significant differences by Dunn test (P<0.0001).

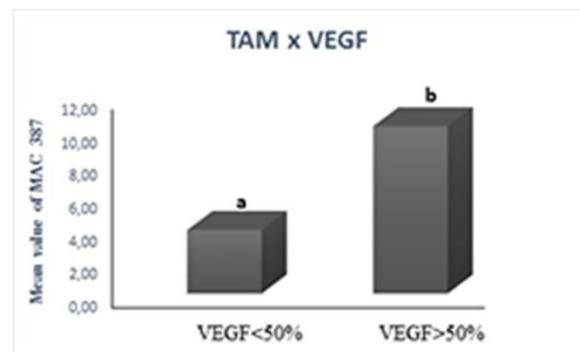


Fig. 5: Graphic representation of the relationship between VEGF immunohistochemistry <50% and >50% with TAM values. Cases with scores of VEGF >50% showed higher levels of TAM values when compared to cases score VEGF <50%. This difference was statistically significant by Mann-Whitney test (P=0.0015).

DISCUSSION

Macrophage tumor infiltration has been thought to be related to tumor progression and development. Though some studies suggest their relationship with a more malignant behavior in some cancers (Galon *et al.*, 2006; Obeid *et al.*, 2013; Zhao *et al.*, 2017), some other studies correlated macrophage infiltration with better prognosis (Buddingh *et al.*, 2011; Ong *et al.*, 2012). Briefly, monocytes are recruited from tumor cells during first step of carcinogenesis and then polarized to either M1 or M2 phenotypes. It is believed that tumor microenvironment influenced naive macrophages to activate in M2 phenotype, which have pro-tumoral functions (Chanmee *et al.*, 2014). However, a pro-inflammatory role of

macrophages can also be activated leading to M1 phenotype polarization which suppress tumor growth (Sica *et al.*, 2006). A recent study in canine mammary carcinomas (Monteiro *et al.*, 2018) showed how M1 macrophages are more evident in benign tumors while M2 macrophages are higher in malignant lesions, its expression was proved via specific antibodies to M1 or M2 phenotypes (NOS2 and CD68, respectively) and not only MAC 387 immunolabeling. Some studies in veterinary medicine have associated TAM with tumor progression, metastasis and poor prognosis in CMT and melanomas (Krol *et al.*, 2011; Pires *et al.*, 2011; Raposo *et al.*, 2013). Results of our study are in contrast with Raposo *et al.* (2013) since it was expected that solid carcinomas [26], a more aggressive form of CMT, express a higher number of macrophages within the tumor than other less malignant carcinomas.

Low number of macrophages in solid carcinomas observed in our study can be explained by two mechanisms. A possible loss of TAM may exist due to a dynamic change of the tumor microenvironment along tumor progression in advanced tumors like solid carcinomas (Chanmee *et al.*, 2014). In this context, Buddingh *et al.* (2011) found that macrophage polarization can be turned from M2 and M1 profile in osteosarcomas and this was explained because they found positive macrophages to M1 and M2 antibodies. Eventually, a tumor can start being infiltrated by M2 macrophages, but turned M1 later on or even loss its macrophage expression for unknown reasons.

Secondly, since MAC387 is not a specific M2 marker, less aggressive carcinomas (like tubular, mixed-type and tubulopapillary) that presented high MAC 387 immunorexpression may reveal an M1 phenotype, which have anti-tumoral rather than pro-tumoral effects (Chanmee *et al.*, 2014) thus justifying its less aggressive behavior. TAM had been correlated with good prognosis in some tumors in humans (Buddingh *et al.*, 2011; Ong *et al.*, 2012) and its anti-tumoral effects are explained by the activation of T-cells and consequently a pro-inflammatory microenvironment. Some of these effects are the production of reactive oxygen intermediators, serine proteases and lytic factors and improvement on antigen presentation to T-cells, which finally will destroy cancer cells (Buddingh *et al.*, 2011; Chanmee *et al.*, 2014).

We also wanted to establish an association between macrophage values and grades of malignancy, however no relationship was found. This result is in accordance with previous results (Krol *et al.*, 2011; Raposo *et al.*, 2012; Raposo *et al.*, 2013) in which despite to establish associations with other malignant clinicopathological features, did not find a difference of TAM immunorexpression in the three histological grades of malignancy.

MAC 387 immunolabelling was principally cytoplasmic with no nuclear staining and no other epithelial cells were stained with the antibody used. Some authors in veterinary medicine found the same macrophage immunolabeling (Pires *et al.*, 2011), but others (Raposo *et al.*, 2012; Raposo *et al.*, 2013) found cytoplasmic and nuclear immunostaining. We are uncertain to explain this phenomenon; however different antibody choice and fixation methods can interfere with immunohistochemical staining (Green *et al.*, 2012).

As MAC 387 was found low in solid carcinomas, VEGF immunorexpression was also lower in this CMT when compared to the other histologic types. Here, we found a decreased number of macrophages in a more aggressive mammary carcinoma. In accordance to Mahmoud *et al.* (2012), macrophages are a heterogeneous network of cells that infiltrate mammary tumors and not only its number may represent how these population of cells interact with tissue micro-environment, but its function. MAC387 antibody might not be expressed in the whole population of macrophages within the tumors analyzed in this study. Therefore, more specialized biomarkers than MAC387 antibody should be used in order to distinguished macrophage function and its role in prognosis (Mahmoud *et al.*, 2012). Our results are in contrast with previous results which showed an association between high VEGF immunolabeling and malignant behaviour (Millanta *et al.*, 2006; Raposo *et al.*, 2013). Other authors (Santos *et al.*, 2015) found similar results to us, they explained that in CMT, VEGF would be required at initial steps of the carcinogenesis process, while at final stages, tumors would do angiogenesis through other factors like fibroblast growth factor (FGF) or transforming growth factor (TGF) – β , as in women. Other possible explanation is the fact that tumoral neovascularization promoted by VEGF is structural and functionally abnormal, forming tortuous and feeble vessels, which will develop certain hypoxia conditions within the tumor microenvironment. These conditions can decrease VEGF expression in order to avoid the continuous growing of erratic vessels in the tumor (Santos *et al.*, 2016).

VEGF immunorexpression was not statically different among the grades of malignancy of the CMT evaluated in this study. This fact has been already confirmed in other study about CMT (Raposo *et al.*, 2013). However, it was expected that higher malignant tumors expressed more VEGF as seen in human tumors (Al-Dissi *et al.*, 2010) because as tumor grows, more blood inflow is required through new vessels, however angiogenesis is a more complex process than a dual association between VEGF and macrophages, as different chemokines and cells might stimuli blood proliferation in different ways (Raposo *et al.*, 2013). Some other factors like FGF and IL-8 are released by TAM and finally will stimulate the production of endothelial cells to permit new blood inflow (Chanmee *et al.*, 2014).

We could confirm the association between TAM and VEGF immunorexpression through the cutoff value of VEGF at 50% and solid carcinomas did not show increased levels of TAM immunolabeling and VEGF. In contrast, less aggressive carcinomas which expressed higher TAM had also higher VEGF immunorexpression. TAM and angiogenesis association has been described in several studies in women (Bingle *et al.*, 2006) and in one previous study in veterinary medicine which also correlated its findings with microvascular density estimated by the immunorexpression of CD31 in endothelial cells of malignant CMT (Raposo *et al.*, 2013).

In face of these results, we recommend to better determine the role of macrophages M1 and M2 in solid carcinomas, as further studies need to be conducted to confirm our results. Some limitations are inherent to the

immunohistochemical technique, as MAC 387 is not a M2 specific antibody, so we suggest that studies evaluating gene expression could determine better the role of TAM in CMT.

Conclusions: Solid carcinomas did not express higher levels of TAM – identified by MAC 387 antibody- or VEGF when compared to less aggressive carcinomas. A relationship between macrophages through MAC 387 immunostaining and VEGF immunoexpression was confirmed in this study. Treatment approaches using TAM as therapeutic target in solid carcinomas should be further evaluated.

Authors contribution: ORS, JS, MAA and SGC conceived and designed the study. JS, MAA LFM and CEF executed the experiment, GMM, CEF and ABN collected tissue samples. ORS and SGC analyzed the data and wrote the manuscript, all authors critically revised the manuscript for important intellectual contents and approved the final version.

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