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Investigation of the tumor microenvironment, hypoxia and angiogenesis by immunohistochemical and histopathological methods in canine mammary tumors

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ABSTRACT

The tumor microenvironment (TME) is an important component for studying tumor behavior in several cancers in human beings. However, little information regarding the role of the TME in canine mammary tumors (CMTs) is available compared to humans. In this study, the aim was to investigate the relationship between the TME, hypoxia and angiogenesis through CD31, VEGF, HIF-1a, CD68 and CD163 expression by using immunohistochemical (IHC) methods in formalin-fixed paraffin-embedded canine mammary tumor samples [(n=34: malignant (n=28) and benign (n=6)], to compare them with the clinicopathological features of tumors, and to analyze the relationship between them. There was no significant relationship between CD31, VEGF, HIF-1a, CD68 and CD163 expression in malignant tumors compared to benign tumors (P>0.05). There was an association between microvessel density and clinicopathological variables (the tumor size P=0.013, the presence of necrosis P=0.022) and individual histological grade (G2 vs. G3 P=0.028) in malignant tumors. While there was a positive correlation between CD68 and CD163 in malignant tumors in the dogs (P<0.01), no correlation was determined between other antibodies. Immunohistochemical determination of the level of angiogenesis in the TME may give further useful information about the angiogenic potential and grading of the clinical aggressiveness of some CMTs.

Key words: angiogenesis; canine mammary tumors; tumor-associated macrophages; tumor microenvironment

Introduction

Mammary tumors are considered to be the most common tumors in female dogs; they account for approximately 50–70% of all neoplasms in intact bitches (MISDORP, 2002; GOLDSCHMIDT et al., 2017). The tumor microenvironment (TME) contains cancer cells as well as cells of the immune system, mesenchymal stromal cells (fibroblasts, endothelial cells and others) that are frequently

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distinguished by cell specific markers and cell surface molecules (BALKWILL et al., 2012).

Tumor-associated macrophages (TAM), a major proportion of the leucocytic infiltrate in the TME, are classified as antitumoral (M1) or protumoral (M2) macrophages depending on their activation status (MURDOCH et al., 2004; MANTOVANI and LOCATI, 2013). CD68 is a pan-macrophage molecule and is expressed in all macrophages without M1 and M2 distinction, while CD163 is expressed only in M2 macrophages (MINAMI et al., 2018). TAMs play an important role in extracellular matrix destruction and reconstruction in the tumor microenvironment, tumor cell motility, triggering angiogenesis, metastasis, invasion of tumor cells, progression of cancer, and poor prognosis (KRÓL et al., 2011; BALKWILL et al., 2012; ZHANG et al., 2012; CHANMEE et al., 2014). Infiltration of TAMs is associated with VEGF expression (RAPOSO et al., 2014) and the ability to metastasize in canine mammary tumors (KRÓL et al., 2011). In veterinary medicine, only few studies have evaluated the presence of CD163 macrophages using immunohistochemical methods in tumors in canine and feline species (MIYAMOTO et al., 2018; KRANE et al., 2021; VÁZQUEZ et al., 2021).

Hypoxia inducible factor (HIF) is a key regulator of hypoxic adaptation for cells in TME (MCNEIL et al., 2017). This transcription factor enables the reprogramming of genes involved in angiogenesis, glycolysis metabolism, oxygen consumption, invasion and migration, for tumor cells to adapt and survive in hypoxic conditions in the tumor microenvironment (RAPISARDA and MELILLO et al., 2009; MCNEIL et al., 2017). HIF-1α expression has been shown to manifest a positive correlation with the density of microvessels in CMTs (MADEJ et al., 2013; SHIN et al., 2015).

Angiogenesis is defined as the formation of new capillaries from a pre-existing vasculature that facilitates tumor progression, metastasis and invasion, as well as providing the oxygen, growth factors and nutrients that tumor tissue needs (FOLKMAN, 1990; FERRARA, 2000; FERNANDEZ and RICKLES, 2002; TONINI et al., 2003). Many potential regulators play a role in angiogenesis. VEGF is the most important and most emphasized among angiogenic molecules (FERRARA and DAVIS-SMYTH, 1997; FERRARA, 2000). VEGF is a specific mitogen for vascular endothelial cells, and may contribute to increased angiogenesis and malignancy in CMTs (TAKAHASHI et al., 1995; FERRARA and DAVIS-SMYTH, 1997; RESTUCCI et al., 2002; QUEIROGA et al., 2011).

The assessment of angiogenesis may involve the measurement of microvessel density (MVD). Measurement of MVD can be achieved by using immunohistochemical (IHC) methods by counting vessels in the tumor tissue using antibodies such as CD31, CD34, CD105 and Von-Willebrand factor (Factor VIII) that are specific for vessel endothelium (ŞENER et al., 2016). It has been reported that MVD increases with the grade in malignant CMTs (RESTUCCI et al., 2000).

In this study, the aim was to investigate the relationship between the TME, hypoxia and angiogenesis through CD31, VEGF, HIF-1a, CD68 and CD163 expression by using immunohistochemical (IHC) methods in formalin-fixed paraffin-embedded CMTs. Additionally, the purpose was to compare these results with the clinicopathological features of tumors, such as the histological grade of malignancy, tumor size, mitotic counts, vascular lymphatic invasion, and necrosis of tumors, and to analyze the relationships between them.

Materials and methods

Animals and histopathological examination. The archival paraffin blocks of biopsy specimens from 34 female dogs with mammary tumors, submitted for diagnostic purposes to Selcuk University Veterinary Faculty and Bornova Veterinary Control Institute, Turkey, between the years 2015 and 2019, were studied. For the study, approval was obtained from the Selcuk University, Faculty of Veterinary Medicine, Experimental Animal Production and Research Center Ethics Committee (SUVDAMEK) (Date: 31.01.2019, Decision No:2019/04). The inclusion criteria of the 34 tumors were based on the quality of the tissues and the availability of a

documented medical history, including, age, breed, and macroscopical tumor size as indicated either by a clinician or a laboratory.

All the histological slides of each tumor were reviewed. Among 34 mammary tumors, 6 tumors were considered benign and 28 were malignant. The CMTs were reclassified according to GOLDSCHMIDT et al. (2011) (Table 1). The benign mammary gland tumors including the following subtypes: simple adenoma (n=2 tumors); intraductal papillary adenoma (n=2) and benign mixed tumor (n=2 tumors). The malignant mammary tumors included the following subtypes: carcinoma-mixed type (n=15), carcinoma-complex type (n=4), carcinoma-tubulopapillary (n=4), carcinosarcoma (n=3), carcinoma-tubular (n=1), carcinoma-solid (n=1), and intraductal papillary carcinoma (n=1) (Table 1). Additionally, the clinicopathological characteristics of tumors were recorded, such as: the tumor size, the presence of lymphatic invasion, necrosis, mitotic count, and the histological grade of malignancy (Table 5). Since the animals were not brought to the clinics by the owners for regular check ups after the operation, data on clinicopathological findings, such as lymph node metastases and clinical stage, could not be obtained. Mitotic counts were recorded in 10 high-power fields according to the recommended guidelines (GOLDSCHMIDT et al., 2011). Grading was performed according to the Elston and Ellis (Nottingham) method adapted to CMTs (PEÑA et al., 2013). The malign mammary tumors were classified as well-differentiated (Grade 1, n=6), moderately differentiated (Grade 2, n=11), and poorly differentiated (Grade 3, n=11).

Table 1. Classification and grading of canine mammary tumors used in this study

Histological type	Grade1	Grade2	Grade3	n
Benign tumors				
Adenoma-simple				2
Intraductal papillary adenoma				2
Benign mixed tumor				2
Total				6
Malignant tumors				
Carcinoma-tubular	1			1
Carcinoma-tubulopapillary	1	3		4
Carcinoma-solid	1			1
Carcinoma-complex type	2	2		4
Carcinoma-mixed type	1	5	9	15
Carcinosarcoma			2	2
Intraductal papillary carcinoma		1		1
Total	6	11	11	28

Immunohistochemical analysis. After a review of the tumor histological slides, one best representative slide was selected and stained for each tumor. 4 μm thick sections, placed on polylysine coated slides from the corresponding paraffin blocks, were stained according to the IHC Polymer kit procedure of the Novolink™ Polymer Detection System (RE7150-K- Leica Microsystems) after deparaffinization and rehydration. According to the kit's procedure, sections were heated in citrate buffer, pH 6.0, in a 600 W microwave oven for 15 min for antigen retrieval. After washing with deionized water and cooling the slides to room temperature, endogenous peroxidase was blocked, through incubation with 3% hydrogen peroxidase for 30 min. Between each step, 5 min washes were done 3 times by tris-buffered saline (TBS). In order to block non-specific antibody binding, the protein block solution was instilled and incubated for 30 min. Following this procedure, the slides were coated and incubated with primary antibodies HIF-1a, CD68, CD163, CD31 and VEGF. The clone numbers, dilution, and incubation times of the primary antibodies used are summarized in Table 2. Then, the post-primer block solution was added to the slides and incubated for 30 min, followed by the polymer solution for 30 min. Slides were stained by DAB (3,3'- diaminobenzidine tetrahydrochloride) for 5 min. After counter-staining with hematoxylin, the slides were closed by coverslips and evaluated under a light microscope. The primary antibody was replaced by TBS for negative controls. As the positive controls, canine lung sections with pneumonia were used for CD68, CD163 and HIF-1a, a canine liver tissue section for VEGF, and a canine hemangiosarcoma section for CD31.

Table 2. Primary antibodies used for immunohistochemistry

Primary antibody	Product Codes	Source	Dilution	Incubation
Monoclonal mouse anti-human CD31	H-3; sc-376764	Santa Cruz	1:400	Room temperature / 1 h
Monoclonal mouse anti-human CD68	3F103; sc-70761	Santa Cruz	1:50	+4°C/ Overnight
Monoclonal mouse anti-human CD163	GHI/61; sc-20066	Santa Cruz	1:50	+4°C/ Overnight
Monoclonal mouse anti-human VEGF	VG1; sc-53462	Santa Cruz	1:250	Room temperature / 1 h
Monoclonal mouse anti-human HIF-1a	28b; sc-13515	Santa Cruz	1:100	+4°C/ Overnight

Quantification of immunolabeling. For the evaluation of VEGF and HIF-1a expression, we used the Allred score previous described (CHOUDHURY et al., 2010; HAMEED et al., 2015), similar to the standard scoring system in which stain intensity and stain pattern are evaluated. Briefly, intensity score (IS) was determined as 0 (no stain), 1 (weak), 2 (moderate), or 3 (strong). Proportion score (PS) was determined on the basis of the ratio of stained cells to all cells in the area examined as: 0 (no stain) 1 (>0-1/100), 2 (>1/100-1/10), 3 (>1/10-1/3), 4 (>1/3-2/3), or 5 (>2/3-1). The sum of the two values (IS+PS) gave the total Allred score, and for each case the Allred score was determined between 0 and 8 (Table 7).

Microvessel density (MVD) was evaluated immunohistochemically by CD31 staining, according to a modification of the method of

WEIDNER et al. (1991). Briefly, the areas of the highest vascular density (hot spots) in the CD31-immunostained sections were marked under x40 and x100 magnification. After individualization of hot spots, five adjacent, non-overlapping fields from each section were selected at x100 (x10 objective and x10 ocular) magnification. The microvessel count was performed in one field (x200) per each of the five different hot spot areas. MVD was quantified as the mean vessel count obtained from the average vascular density of five fields in each tumor section (Table 7).

The TAMs were evaluated immunohistochemically by CD68 and CD163 staining, according to the method of MONTEIRO et al. (2018). Briefly, TAMs were counted for each tumor in five hot spot areas (areas of maximum macrophage infiltration) observed first at low magnification (x10) and then captured in one field (x400) per each of the five different hot spot areas. The total number of macrophage cells stained with CD68 and CD163 for each tumor was estimated by adding up the counts in all areas (Table 7).

Statistical analysis. SPSS 25 for Windows was employed for all statistical analyses, the Chi-square test for comparing groupable clinicopathological data with each other and IHC data, the Mann-Whitney U test for comparison of mean values and other clinicopathological data (comparison between two groups), and the Kruskal Wallis test (comparison between more than two groups). The cut-off applied to split the TAMs into two groups (low and high counting) was defined by the median value for CD68 (low≤50, high>50) and for CD163 (low≤61, high>61) in malignant CMTs, since the samples studied did not follow a Gaussian distribution.

We investigated whether there was a significant correlation between markers in benign and malignant tumors using Sperman's Correlation test. Values for quantitative variables were given as mean ± standard deviation. Values for $p < 0.05$ were considered significant.

Results

Immunoexpression of CD31, VEGF, HIF-1a, CD68 and CD163 in canine mammary tumors. A positive immune reaction was observed in all sections stained with CD31, VEGF and HIF-1a. CD31 staining was noticed in the cytoplasm of endothelial cells from large and small blood vessels, and rarely in macrophages (Fig. 1A, 1B, 1C).

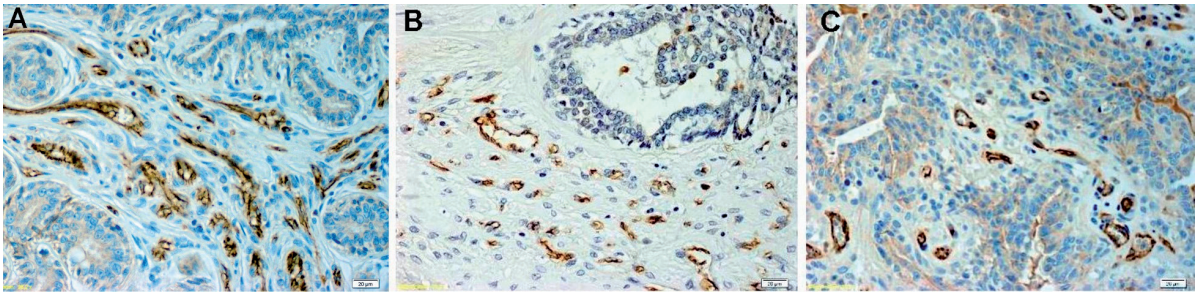

Fig. 1. Expression of CD31 in vascular endothelium A) Tubulopapillary carcinoma (case no. 11), IHC, Bar=20 µm, B) Benign mixed tumor, (case no. 4), IHC, Bar=20 µm, C) Complex carcinoma, (case no. 34), IHC, Bar=20 µm

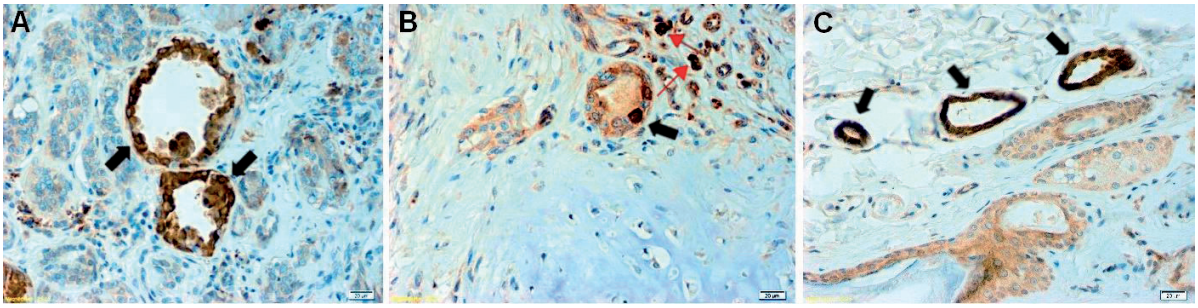

Fig. 2. Expression of VEGF in tumor cells (thick arrows) and vascular endothelium (red arrows), A) Mixed carcinoma, (case no. 19), IHC, Bar=20 µm, B) Mixed carcinoma, (case no. 3), IHC, Bar=20 µm, C) Mixed carcinoma (case no.7), IHC, Bar=20 µm

VEGF expression was detected as diffuse or focal granules in the cytoplasm of tumor cells, and weakly in endothelial cells (Fig. 2A, 2B, 2C).

HIF-1a expression was mostly observed as cytoplasmic and nuclear staining in tumor cells. It was evident mostly in the central parts and invasive margins of the tumor, in the tips of the papillary extensions of the gland and duct lumens, and in the perinecrotic areas (Fig. 3A, 3B, 3C). Because HIF-1a is a transcription factor and its active form involved in tumorigenesis is localized in the nucleus, only nuclear staining was evaluated.

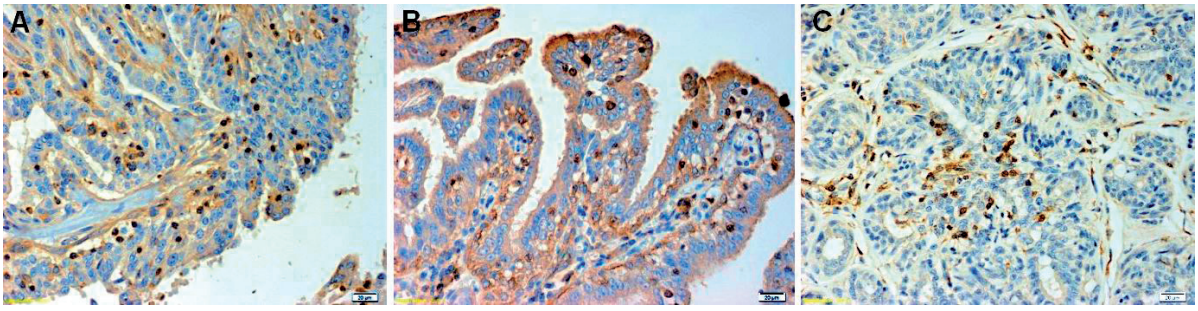

Fig. 3. Expression of HIF-1a in tumor cells, A) Complex carcinoma, (case no. 34), IHC, Bar=20 µm, B) Simple adenoma, (case no. 25), IHC, Bar=20 µm, C) Tubulopapillary carcinoma, (case no. 11), IHC, Bar=20

A positive immune reaction was observed in 33 of 34 sections stained with both CD68 and CD163. Macrophages labeled with CD68 (Fig. 4A, 4B, 4C) and CD163 antibodies (Figures 5A, 5B, 5C) were usually seen in individual or in clusters of tumor stroma, locally in the periphery and lumens of the gland and ducts.

Differences in MVD, VEGF and HIF-1a immunoexpression and TAM (CD68 and CD163) count between benign and malignant CMT:

MVD and HIF-1a expression in benign tumors were lower than malignant tumors, while VEGF expression, CD68 and CD163 TAM counts were higher compared to malignant tumors. However, there was no statistically significant difference in their immunoexpressions between benign and malignant tumors ($p>0.05$) (Table 3). There was also no statistically significant difference between benign and malignant tumors according to the number of CD68+ and CD163+ cells ($p> 0.05$) (Table 4).

Table 3. Association between immunoreactivities of TAM (CD68 and CD163), HIF-1a, VEGF, MVD between benign and malignant CMT and clinicopathological factors in malignant tumors (Mean±sd)

	n	MVD	VEGF	HIF-1a	CD68	CD163
Benign	6	48.23±19.99	5.50±2.07	4.50±1.97	69.00±46.93	74.00±51.95
Malignant	28	58.01±43.14	5.29±1.65	5.11±1.50	46.89±36.65	58.75±40.43
P		0.843	0.809	0.522	0.341	0.494
Clinicopathological variables						
Histological grade						
1	6	59.63±35.10^{ab}	5.67±1.75	5.17±0.98	52.17±31.95	54.33±39.66
2	11	38.27±16.75 ^{b}	4.55±0.93	5.09±1.58	45.00±40.93	52.09±45.66
3	11	76.87±57.50 ^{a}	5.82±1.99	5.09±1.76	45.91±37.69	67.82±37.30
P		0.090	0.208	0.985	0.771	0.817
Tumor size						
0-3 cm	9	40.80±15.62 ^{a}	5.56±1.51	4.89±1.45	34.89±26.11	33.89±31.39
3-5 cm	7	34.60±10.32 ^{a}	4.86±1.35	5.71±0.95	44.57±39.86	76.29±44.74
>5 cm	12	84.58±54.50 ^{b}	5.33±1.97	4.92±1.78	57.25±41.20	67.17±37.74
P		0.013*	0.720	0.525	0.439	0.092
Mitotic counts						
0-9	5	52.96±26.17	5.40±1.82	6.00±0.00	47.80±39.73	46.20±40.06
10-19	9	44.27±28.62	4.78±1.20	4.56±1.33	39.11±28.05	54.78±43.17
≥20	14	68.66±53.73	5.57±1.87	5.14±1.75	51.57±41.89	65.79±40.36
P		0.339	0.602	0.196	0.866	0.622
LVI						
Present	2	55.00±13.29	7.00±1.41	6.50±0.71	69.50±44.55	104±19.80
Absent	26	58.25±44.74	5.15±1.62	5.00±1.50	49.63±38.93	58.78±41.94
P		0.529	0.159	0.190	0.339	0.132
Necrosis						
Present	13	76.35±54.78	5.31±1.70	4.62±1.80	40.54±33.54	57.23±38.94
Absent	15	42.12±20.81	5.27±1.67	5.53±1.06	52.40±39.46	60.07±42.99
P		0.022*	0.964	0.201	0.440	0.751

a,b Different letters in the same column for the same parameter/variables are statistically significant; *P value <0.05 considered to be statistically significant; LVI (lymphovascular invasion); MVD was determined by counting the average vessels in five different hot spot areas (200x); TAM (CD68 and CD163) counting was the total number of staining cells counted in five different hot spot areas (400x); The evaluations of VEGF and HIF-1a immunoexpressions were made according to the allred scoring method, based on staining density and staining ratio.

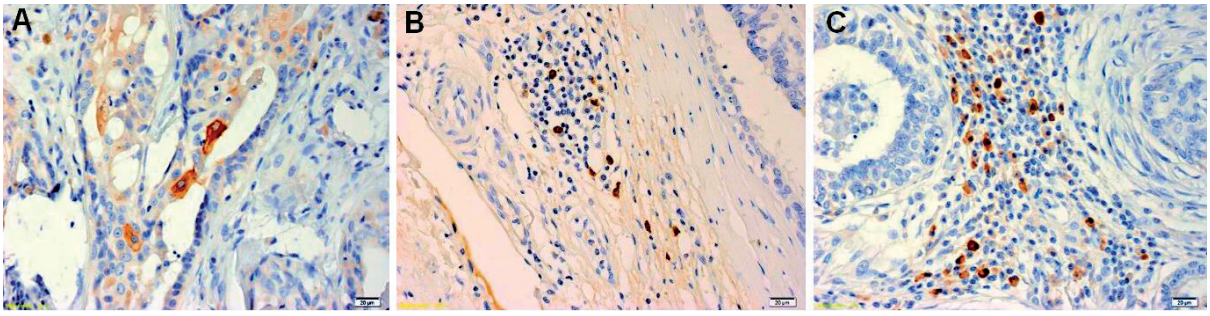

Fig. 4. Expression of CD68 in macrophage cytoplasms, A) Mixed carcinoma, (case no. 16), IHC, Bar=20 µm, B) Mixed carcinoma, IHC, (case no. 13), Bar=20 µm, C) Mixed carcinoma, (case no. 15), IHC, Bar=20 µm

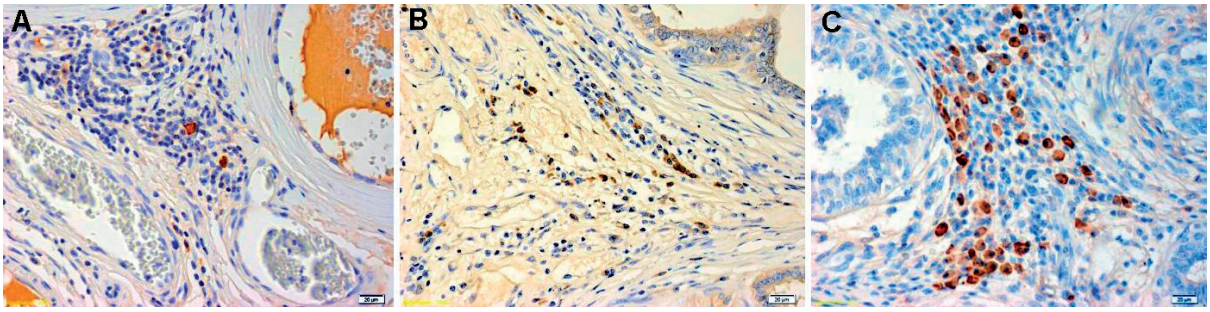

Fig. 5. Expression of CD163 in macrophage cytoplasms, A) Intraductal papillary carcinoma, IHC, (case no. 9), Bar=20 µm, B) Solid carcinoma, (case no. 21), IHC, Bar=20 µm, C) Mixed carcinoma, (case no. 15), IHC, Bar=20 µm

Table 4. Comparison of benign and malignant tumors according to the numbers of $CD68^+$ and $CD163^+$ cells

	CD68			CD163		
	<50	>50	P	<61	>61	P
Benign	2 (33.3%)	4 (66.7%)	0.370	2 (33.3%)	4 (66.7%)	0.660
Malignant	17 (60.7%)	11 (39.3%)		14 (50.0%)	14 (50.0%)	

Associations between MVD, VEGF, HIF-1a immunoexpression and TAM (CD68 and CD163) count and clinicopathological variables in malignant CMTs:

In the malignant tumors, the MVD was not found to show a statistically significant association with mitotic count (P=0.339), lymphatic involvement (P=0.529) or tumor grading (P=0.090) (Table 3). However, the Mann–Whitney test revealed significant differences in MVD between histological grades of G2 and G3 tumors (P=0.028) (Fig. 6). Also a statistically significant association was found between MVD and clinicopathological features, such as tumor size (P=0.013) and the presence of necrosis (P=0.022) (Table 3). However, there were no statistically significant differences between VEGF, HIF-1a immunoexpressions and TAM (CD68 and CD163) counts, and clinicopathological variables (Table 3; Table 5).

Table 5. Characteristics of malignant tumors according to the $CD68^+$ and $CD163^+$cell count

	TAMs counting					
	CD68			CD163		
Clinicopathological variables	<50	>50	P	<61	>61	P
Histological grade						
1	3 (10.7%)	3 (10.7%)	0.832	3 (10.7%)	3 (10.7%)	0.441
2	7 (25.0%)	4 (14.3%)		7 (25.0%)	4 (14.3%)	
3	7 (25.0%)	4 (14.3%)		4 (14.3%)	7 (25.0%)	
Tumor size						
0-3 cm	6 (21.4%)	3 (10.7%)	0.592	2 (7.1%)	5 (17.9%)	0.111
3-5 cm	5 (17.9%)	2 (7.1%)		5 (17.9%)	7 (25.0%)	
>5 cm	6 (21.4%)	6 (21.4%)		3 (10.7%)	2 (7.1%)	
Mitotic counts						
0-9	6 (21.4%)	3 (10.7%)	0.900	5 (17.9%)	4 (14.3%)	0.742
10-19	8 (28.6%)	6 (21.4%)		6 (21.4%)	8 (28.6%)	
≥20	1 (3.6%)	1 (3.6%)		0 (0.0%)	2 (7.1%)	
LVI						
Present	1 (3.6%)	1 (3.6%)	0.747	0 (0.0%)	2 (7.1%)	0.142
Absent	16 (57.1%)	10 (35.7%)		14 (50.0%)	12 (42.9%)	
Necrosis						
Present	10 (35.7%)	3 (10.7%)	0.102	6 (21.4%)	7 (25.0%)	0.705
Absent	7 (25.0%)	8 (28.6%)		8 (28.6%)	7 (25.0%)	

LVI-lymphovascular invasion

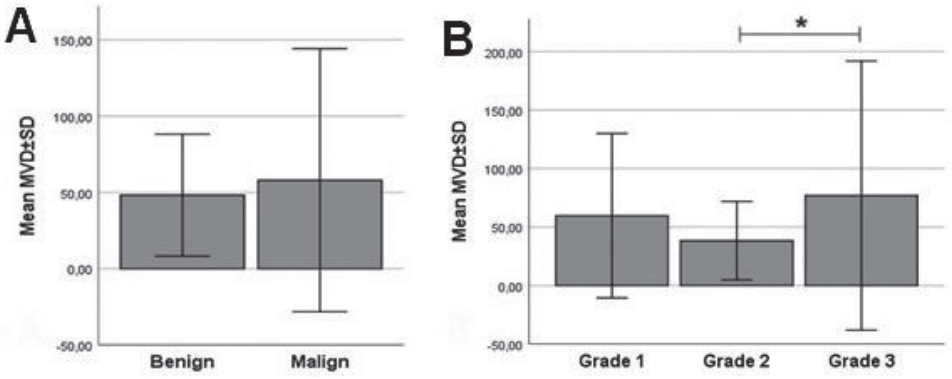

Fig. 6. MVD in benign and malignant tumors (A) and in relation to the grade of malignancy of the studied malignant mammary tumors of dogs (B).*P<0.05, Mann–Whitney U test

Correlation between MVD, VEGF, HIF-1A, CD68 and CD163 levels:

While a positive correlation was found between CD68 and CD163 immunoreactivities in malignant tumors in dogs (P<0.01), no correlation was found between other data. Correlations in benign tumors were not statistically significant. (Table 6).

Table 6. Correlation between MVD, VEGF, HIF-1a, CD68 and CD163 levels in benign and malignant tumors

	BENING (n=6)					
		MVD	VEGF	HIF-1a	CD68	CD163
MALIGNANT (n=28)	MVD	---	0.059	-0.568	-0.543	-0.600
	VEGF	-0.011	---	0.127	-0.353	-0.559
	HIF-1a	-0.091	0.321	---	0.031	0.494
	CD68	0.105	0.171	0.271	---	0.714
	CD163	-0.079	-0.123	0.127	0.497**	---

(**) shows a positive and significant <0.01 correlation

Table 7. IHC scores of canine mammary tumors used in the study

No	DIAGNOSIS	MVD (CD31)	VEGF (TS)	HIF-1A (TS)	CD68	CD163
1	Intraductal papillary adenoma	45.6	8	5	103	95
2	Carcinoma-complex type	108.2	5	4	30	24
3	Carcinoma-mixed type	37.6	8	6	30	66

Table 7. IHC scores of canine mammary tumors used in the study (continued)

No	DIAGNOSIS	MVD (CD31)	VEGF (TS)	HIF-1A (TS)	CD68	CD163
4	Benign mixed tumor	69.2	3	2	93	102
5	Adenoma-simple	53.2	6	2	17	7
6	Carcinoma-mixed type	43.4	4	6	103	115
7	Carcinoma-mixed type	56.8	8	6	80	20
8	Carcinoma-tubulopapillary	28.4	5	4	13	11
9	Intraductal papillary carcinoma	33	4	6	62	35
10	Carcinoma-tubulopapillary	62.6	4	2	40	25
11	Carcinoma-tubulopapillary	61.8	4	5	10	30
12	Carcinoma-mixed type	103.8	3	2	0	90
13	Carcinoma-mixed type	177.4	3	4	26	80
14	Carcinoma-complex type	40	5	6	8	13
15	Carcinoma-mixed type	28.8	4	7	61	85
16	Carcinoma-mixed type	20.8	3	4	12	92
17	Carcinoma-mixed type	32.4	4	7	6	11
18	Benign mixed tumor	67.2	7	6	5	9
19	Carcinoma-mixed type	37.4	6	5	36	10
20	Carcinosarcoma	192.2	6	4	93	0
21	Carcinoma-solid	45.6	6	6	38	90
22	Carcinoma-tubular	95.6	3	6	98	90
23	Carcinoma-mixed type	46	7	6	42	83
24	Carcinoma-mixed type	28.2	7	4	12	20
25	Adenoma-simple	15.4	3	6	118	123
26	Carcinoma-mixed type	27.2	5	5	96	125
27	Intraductal papillary adenoma	38.8	6	6	78	108
28	Carcinosarcoma	64.4	8	7	101	118
29	Carcinoma-mixed type	58.2	8	7	14	41
30	Carcinoma-mixed type	26.8	5	6	15	18
31	Carcinoma-complex type	23.2	7	5	54	91
32	Carcinoma-mixed type	65.6	6	3	23	48
33	Carcinoma-tubulopapillary	16.8	4	3	90	96
34	Carcinoma-complex type	62.2	6	7	120	118

TS:total score= proportion score (PS)+ intensity score (IS); MVD was determined by counting average vessels in five different hot spot areas (200x); TAM (CD68 and CD163) counting was the total number of staining cells counted in a five different hot spot areas (400x).

Discussion

Several studies in veterinary oncology have revealed a link between angiogenesis and malignancy, as well as prognosis in different tumor types, including mammary tumors in dogs and cats (GRIFFEY et al., 1998; GRAHAM and MYERS, 1999; RESTUCCI et al., 2000; MAIOLINO et al., 2001; MILLANTA et al., 2002; RESTUCCI et al., 2003; MARTANO et al., 2004; PREZIOSI et al., 2004; LUONG et al., 2006; LAVALLE et al., 2009; ISLAM et al., 2012), similar to human breast cancers (WEIDNER et al., 1991; UZZAN et al., 2004; VAN DER AUWERA et al., 2004). In addition, many studies have reported that there is a correlation between lymph node metastasis, histological grade, necrosis and MVD (WEIDNER et al., 1991; HORAC et al., 1992; VALKOVIĆ et al., 2002; JAKAB et al., 2008; RAPOSO et al., 2014). In mammary tumor investigations, there is agreement on which MVDs are higher in malignant tumors compared to benign tumors (RESTUCCI et al., 2000; JAKAB et al., 2008; SLEECKX et al., 2014; RAPOSO et al., 2015). Similarly, the present study demonstrated that MVD was higher in malignant tumors than in benign tumors, but the difference did not reach the level of statistical significance ($P>0.05$). In addition, we found that there was a statistically significant difference in MVD between malignant tumors with histological grades G2 and G3 ($P<0.05$). This finding may be due to the high individual angiogenic potential of canine mixed carcinomas, or the fact that the tumor group used in this study mostly consisted of mixed carcinomas (n=15) and most of these were classified as grade 3 tumors (n=9) (Table 1). The evaluation of MVD by CD31 immunostaining could be used for grading the clinical aggressiveness of CMT, or the level of angiogenesis could be used as an indicator of the aggressiveness of CMTs.

In malignant tumors, there was a statistically significant association between MVD and the presence of necrosis and tumor size ($P<0.05$). This can be explained as an indication of the increased metabolic needs in response to tumor growth.

There are many hormones and/or growth factors that regulate angiogenesis. Vascular endothelial growth factor (VEGF) is one of the leading factors involved in angiogenesis (FERRARA et al., 1997). Some studies in CMTs have reported that VEGF protein expression was higher in malignant tumors compared to benign tumors, and gradually increased during canine mammary carcinogenesis (RESTUCCI et al., 2002; QIU et al., 2008). We could not detect such a relationship in this study. This may be related to the number of tumors examined in this study.

Some studies have indicated that there is a correlation between VEGF and MVD (RESTUCCI et al., 2002; QUEIROGA et al., 2011; ISLAM et al., 2012). In our study, no significant correlation was found between VEGF and MVD ($P>0.05$). Most studies state that the evaluation of MVD is subjective and difficult to standardize (JAKAB et al., 2008), because the scoring methodology may change according to the observer.

Some studies have shown that HIF-1a is effective in aggressive tumor development, and a significant correlation was found between HIF-1a and histological grade (MADEJ et al., 2013; SHIN et al., 2015). Furthermore, it was also reported that there is a positive correlation between HIF-1a and VEGF (SHIN et al., 2015). Because the majority of tumors covered in that study were of epithelial origin, the relationship between HIF-1a and VEGF and histological grade ($P>0.05$) might not have been demonstrated. Our study included tumors of both epithelial origin and mesenchymal origin. Studies on histological type and HIF-1a protein expression are limited and may be the subject of research.

While there are studies reporting that there is a correlation between MVD and macrophage infiltration in many types of cancer in humans (LEEK et al., 1996; NISHIE et al., 1999; ORRE and ROGERS, 1999; HANADA et al., 2000; LISSBRANT et al., 2000; MÄKITIE et al., 2001; VALKOVIĆ et al., 2002; CHEN et al., 2005; TSUTSUI et al., 2005), there are also studies stating that that relationship does not exist (KOUKOURAKIS et al., 1998; SALVESEN and AKSLEN, 1999). Similar to the latter studies, we also could not find a significant correlation between CD68 and CD163 macrophage counts and MVD in benign and malignant tumors. In our study, MVD

evaluation was performed without distinguishing intratumoral or peritumoral regions. This suggests the necessity of standardizing the counting methods in CMTs.

A previous study comparing VEGF and TAMs in CMTs reported that there was a correlation between them (RAPOSO et al., 2014). In this study, VEGF immunoexpression was not correlated with CD68 and CD163 macrophage counts in benign and malignant tumors. This can be explained by the fact that VEGF acts synergistically with numerous factors and the oncogenes that take part in angiogenesis. Furthermore, it may be an indication that VEGF is not the only factor in macrophages being recruited to the tumor site and participating in the angiogenic process, as stated in other studies (CROWTHER et al., 2001; DIRKX et al., 2006; RIABOV et al., 2014).

It has been reported that the number of macrophages in malignant tumors was significantly higher compared to benign tumors in CMTs (RAPOSO et al., 2014; RAPOSO et al., 2015). However, we could not find any statistically significant relationship in the numbers of CD68 and CD163 macrophages between benign and malignant tumors ($P>0.05$). Contrary to these studies using only the MAC387 antibody which labels M1 macrophages, we evaluated TAMs by both antibodies of anti-CD68 which labels all macrophages, and anti-CD163 which labels M2 macrophages. In a different study conducted by MONTEIRO et al. (2018) on canine mammary tumors, they used MAC387 and CD206 antibodies and found that M1 macrophages were more numerous in benign tumors and M2 macrophages in malignant tumors. In the present study, we found that there was a significant correlation between the macrophage counts marked with CD68 and CD163 in malignant CMTs ($P<0.01$) (Table 6). This may be due to the dual pro-tumorigenic and anti-tumorigenic effect of TAMs. Since the effects of TAMs between tumorigenic and anti-tumorigenic mechanisms are not fully elucidated, more studies are needed to explain their effects in CMTs. To our best knowledge, this is the first study in which CD163 was demonstrated immunohistochemically in CMTs. With our study, we contribute to a better understanding of TAM infiltration in the tumor microenvironment and its effect on histopathological factors.

Finally, it is important to note that one of the main limitations of this study was the small size of the study material and its retrospective nature.

Conclusions

In conclusion, we found statistically significant differences between MVD and some of the clinicopathological variables of tumors, such as individual histological grade, tumor size and the presence of necrosis. This suggests that immunohistochemical determination of the level of angiogenesis in the tumor microenvironment may give further useful information for grading the clinical aggressiveness of some CMTs. Additionally, the evidence discussed in this study emphasizes the role of the tumor microenvironment and this will help a better understanding of the molecular pathways involving TAMs, angiogenesis and hypoxia in CMTs.

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Author contributions

All authors contributed to the study conception and design. Erdinc Guner: Investigation, Resources. Formal analysis, Writing - Original Draft. Resources, Fatih Hatipoglu: Conceptualization, Methodology, Writing - Review & Editing, Validation

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SAŽETAK

Mikrookruženje tumora (TME) važna je komponenta u proučavanju ponašanja tumora kod nekoliko vrsta karcinoma u ljudi. U usporedbi s ljudima, malo je informacija o ulozi TME-a kod tumora mliječne žlijezde u pasa (CMTs). Cilj je rada bio istražiti odnos između TME-a, hipoksije i angiogeneze putem ekspresije CD31, VEGF, HIF-1a, CD68 i CD163 upotrebom imunohistokemijskih (IHC) metoda. U uzorcima tumora mliječne žlijezde pasa (n=34) koji su fiksirani formalinom i ugrađeni u parafin (maligni n=28 i benigni n=6), provedena je usporedba s kliničkopatološkim svojstvima tumora i analiziran odnos među njima. Nije bilo znakovite povezanosti između ekspresije CD31, VEGF, HIF-1a, CD68 i CD163 kod malignih tumora u usporedbi s benignim tumorima (P>0,05). Uočena je povezanost između gustoće mikrožila i kliničkopatoloških pokazatelja (veličina tumora P=0,013; prisutnost nekroze P=0,022) te pojedinačne histološke ocjene malignosti (G2 prema G3 P=0,028) kod malignih tumora. Iako je postojala pozitivna korelacija između CD68 i CD163 kod malignih tumora u pasa (P<0,01), ista nije uočena između drugih protutijela. Imunohistokemijsko određivanje razine angiogeneze kod TME-a može pružiti korisne informacije o angiogenom potencijalu i stupnjevanju kliničke agresivnosti pojedinih tumora mliječne žlijezde u pasa.

Ključne riječi: angiogeneza; tumori mliječne žlijezde pasa; makrofagi povezani s tumorima; mikrookruženje tumora