



Comparison of vascular endothelial growth factor/vascular endothelial growth factor receptor 2 expression and its relationship to tumor cell proliferation in canine epithelial and mesenchymal tumors

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ABSTRACT. The vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR2) signaling pathway plays an important role in tumor angiogenesis. VEGFR2 is expressed not only in vascular endothelial cells but also in tumor cells; however, the relationship of VEGF/VEGFR2 expression and tumor proliferation has yet to be elucidated. In addition, since several studies have reported that VEGFR2 inhibitors are more effective against epithelial tumors than mesenchymal tumors, there may be a difference in VEGF/VEGFR2 expression between epithelial and mesenchymal tumors. The purpose of this study was to elucidate differences in VEGF/VEGFR2 expression between epithelial and mesenchymal tumors and the relationship of VEGF/VEGFR2 expression and proliferation in canine tumor cells. We assessed 29 epithelial and 21 mesenchymal canine tumors for microvessel density (MVD), mRNA transcription levels of *von Willebrand Factor* (*vWF*) and *endoglin*, expression of VEGF, VEGFR2, and phosphorylated VEGFR2 (pVEGFR2), and proliferation index (PI) using real-time reverse transcription polymerase chain reaction and immunohistochemistry. VEGFR2 expression on vascular endothelial cells, MVD, and mRNA transcription levels of *vWF* and *endoglin* were not significantly different between the two groups. However, expression of VEGF, VEGFR2, and pVEGFR2 was higher in epithelial tumors ($P < 0.01$). Moreover, PI correlated with pVEGFR2 expression in only epithelial tumors ($P < 0.01$, $R_s = 0.543$). These results suggest that the activity of VEGF/VEGFR2 signaling in tumor cells is raised in epithelial tumors, and that this signaling pathway may be related to tumor cell proliferation in epithelial tumors.

KEY WORDS: angiogenesis, dog, proliferation index, phosphorylated vascular endothelial growth factor receptor 2, spontaneous tumor

J. Vet. Med. Sci.

84(1): 133–141, 2022

doi: 10.1292/jvms.21-0388

Received: 8 July 2021

Accepted: 6 November 2021

Advanced Epub:

25 November 2021

Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis [16, 30]. VEGF interacts with VEGF receptors (VEGFRs) such as VEGFR1, VEGFR2, and VEGFR3. Among them, VEGF/VEGFR2 signaling has been reported to mediate cellular responses involved in angiogenesis prominently [25]. VEGF binds to VEGFR2 expressed on vascular endothelial cells, causing phosphorylation of VEGFR2 and activation of VEGF/VEGFR2 signaling [16, 30]. Angiogenesis, which is mediated by this signaling pathway, is important for tumor growth and progression as it allows tumors to acquire oxygen and nutrients from new blood vessels [10]. VEGF/VEGFR2 signaling has thus become a target for molecular-targeted drugs, an anticancer therapeutic strategy.

It has been reported that VEGFR2 is expressed not only in vascular endothelial cells but also in tumor cells. In human medicine, it has been shown that VEGFR2 on tumor cells is directly involved in tumor cell proliferation, and that VEGFR2 inhibitors can suppress tumor cell proliferation and induce apoptosis in several types of tumors including non-small cell lung cancer, breast cancer, melanoma, ovarian carcinoma and pancreatic carcinoma [7, 20, 23]. The expression of VEGFR2 on tumor cells has also been reported in various canine tumors including nasal carcinoma, mammary gland tumor and transitional cell carcinoma [14, 18, 33]. Despite this, the relationship of VEGFR2 with tumor cell proliferation is still unclear.

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(Supplementary material: refer to PMC <https://www.ncbi.nlm.nih.gov/pmc/journals/2350/>)

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In human medicine, the inhibitors targeting VEGF/VEGFR2 signaling have been approved by the Food and Drug Administration mainly for treating epithelial tumors. An anti-VEGF monoclonal antibody, bevacizumab, has been approved for the treatment of colorectal cancer, non-small cell lung cancer, cervical cancer, and ovarian cancer. Sunitinib and sorafenib, of which VEGFR2 is one of their targets, have been approved for the treatment of renal cell carcinoma and hepatocellular carcinoma. Moreover, in veterinary medicine, the effects of VEGFR2 inhibitor toceranib are stronger for epithelial tumors than for mesenchymal tumors in dogs [21, 22]. Therefore, VEGF/VEGFR2 signaling in epithelial and mesenchymal tumors appear to be different, although it is not known to what extent.

The purpose of this study was to clarify differences in angiogenesis and VEGF/VEGFR2 expression on tumor cells of epithelial and mesenchymal tumors, and to investigate the relationship of VEGF/VEGFR2 expression and cell proliferation in canine tumors. To compare angiogenesis, we examined expression levels of VEGFR2 on vascular endothelial cells, microvessel density (MVD), and mRNA levels of pan-endothelial marker, *von Willebrand factor (vWF)* and an activated endothelial marker, *endoglin*, using immunohistochemistry and real-time reverse transcription polymerase chain reaction (RT-qPCR). Next, we examined the expression levels of VEGF, VEGFR2, and phosphorylated VEGFR2 (pVEGFR2) on tumor cells using immunohistochemistry. Finally, we examined correlations between the expression levels of VEGF, VEGFR2, and pVEGFR2, and the proliferation index (PI) by Ki-67 immunostaining. We also assessed the relationship of angiogenesis and PI.

MATERIALS AND METHODS

Samples

This study included 50 dogs with various spontaneous tumors that had undergone surgery at Iwate University Veterinary Teaching Hospital between 2017 and 2021. Informed consent was obtained from the owners of the participating dogs. The general characteristics of dogs such as breed, age and sex were collected from the medical records. From the 50 dogs, 29 samples from epithelial tumors and 21 samples from mesenchymal tumors were obtained. The types of tumors are summarized in Table 1. A histopathological diagnosis was made using hematoxylin and eosin-stained sections according to the WHO classification for each specimen. Each surgical sample was divided for histological study and RNA extraction. The samples for histological study were kept in 10% neutral buffered formalin and samples for RNA extraction were stored at -80°C .

Immunohistochemical staining of *vWF*, *Ki-67*, *VEGF*, *VEGFR2*, and *pVEGFR2*

Immunohistochemistry was performed using the EnVisionTM+System (Dako, Glostrup, Denmark) detection methods according to the manufacturer's protocol. The samples were embedded in paraffin and sliced into 3 μm sections. Tissue sections were deparaffinized and rehydrated, and antigen retrieval was performed by autoclaving at 120°C for 20 min in Target Retrieval Solution (Dako) for Ki-67 and room temperature for 6 min in proteinase K (Dako) for VEGFR2 and pVEGFR2. Antigen retrieval was not performed for vWF and VEGF. Endogenous peroxidase activity was blocked with peroxidase block (Dako) for 10 min at room temperature. After washing with phosphate buffer saline (PBS, pH 7.4), the sections were incubated with rabbit anti-vWF polyclonal antibody (1:4,000, ab6994; Abcam, Cambridge, UK), mouse anti-Ki-67 monoclonal antibody (1:150, M7240; Dako), mouse anti-VEGF monoclonal antibody (1:100, ab1316; Abcam), rabbit anti-VEGFR2 polyclonal antibody (1:100, ab2349; Abcam), and rabbit anti-phospho-VEGFR2 antibody (1:250, MBS462155; MyBiosource, Nagoya, Japan) diluted with Antibody Diluent with Background-Reducing Components (Dako) overnight at 4°C . The cross-reactivities of anti-vWF, Ki-67, VEGF, and VEGFR2 antibody to dogs were previously reported [3, 6, 11, 12, 29]. We investigated the cross-reactivity of anti-phospho-VEGFR2 antibody to dogs using western blot (Supplementary Fig. 1). For negative controls, the primary antibody was replaced with normal rabbit IgG and mouse IgG1. The sections were then washed with PBS and incubated with Envision polymer reagent for 30 min at room temperature. Next, after washing with PBS, the sections were stained with 3,3'-diaminobenzidine tetrachloride. After a final wash in distilled water, the sections were counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene, and mounted with coverslips.

Table 1. Types of tumors

Organ	Diagnosis	n
Epithelial tumors		
Lung	Adenocarcinoma	3
	Squamous cell carcinoma	1
Paranasal sinus	Adenocarcinoma	1
Oral	Squamous cell carcinoma	2
Salivary gland	Squamous cell carcinoma	1
Intestine	Adenocarcinoma	4
Liver	Hepatocellular carcinoma	7
Kidney	Renal adenocarcinoma	3
Prostate	Adenocarcinoma	1
Subcutaneous	Perianal adenoma	4
	Trichoepithelioma	1
	Sebaceous adenocarcinoma	1
Mesenchymal tumors		
Oral	Fibrosarcoma	1
Intestine	Gastrointestinal stromal tumor	2
Spleen	Stromal tumor	2
Kidney	Sarcoma of unknown origin	1
Bladder	Sarcoma of unknown origin	1
Vagina	Fibroma	1
	Leiomyoma	1
Uterus	Leiomyoma	2
Abdominal cavity	Fibrosarcoma	1
Limb	Osteosarcoma	4
	Undifferentiated sarcoma	1
Subcutaneous	Soft tissue sarcoma	3
	Canine hemangiopericytoma	1

n=number of tumors.

Determination of MVD and PI

MVD determinations were performed according to the procedure described by Weidner *et al* [34]. Briefly, each slide with immunohistochemically stained vWF was first scanned at low magnification (40×) to identify regions of highest vascular density (vascular ‘hot spots’). Then, microvessels were counted in 10 microscopic fields of these areas at high magnification (400×). Any positive endothelial cell or cluster of endothelial cells with a recognizable lumen was counted as an individual microvessel. The mean microvessel count was calculated for each specimen and recorded as the MVD. The PI was determined using a similar method, referring to the previous report [32]. First, areas with high numbers of Ki-67-positive cells (‘hot spots’) were identified at low magnification (40×). In these areas, 200 tumor cells were counted at high magnification (400×). A total of 1,000 tumor cells were counted, and the percentage of Ki-67-positive cells was calculated.

Immunohistochemical scoring of VEGF, VEGFR2, and pVEGFR2

The immunoreactivities of VEGF, VEGFR2, and pVEGFR2 were evaluated on the basis of a semi-quantitative immunoreactivity score (IRS), as previously published [13, 24, 35]. Briefly, to evaluate immunoreactivity of VEGFR2 in endothelial cells, the intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong) was scored (range 0–3). For VEGF, VEGFR2, and pVEGFR2 in tumor cells, scores for the percentage of positive cells (0, 0%; 1, <10%; 2, 10–50%; 3, 51–80%; 4, >80%) and staining intensities (0, negative; 1, weak; 2, moderate; 3, strong) were multiplied to obtain the final IRS (range 0–12).

RT-qPCR

Total RNA was extracted from the tumor tissue samples using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s protocol. The quantity and quality of total RNA were measured spectrophotometrically at 260 nm using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). Samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) according to the manufacturer’s protocol in order to prevent genomic DNA contamination. The samples were incubated at 37°C for 30 min, and pure total RNA was re-extracted using Trizol Reagent. cDNA was synthesized from 2 µg of total RNA from each sample using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. The resultant cDNA was amplified by RT-qPCR with the QuantiNova™ SYBR® Green PCR Kit (Qiagen, Hilden, Germany). The amplification mixture contained 1 × SYBR Green PCR Master Mix, 0.4 µM forward and reverse primers, template (2.5 µl), 1 × QN ROX Reference Dye, and nuclease-free water (up to 25 µl). The reactions were performed on a Step One Plus™ Real-Time PCR System (Applied Biosystems). Amplification conditions were 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 60 sec. Each sample was tested in duplicate, and the results were analyzed using the standard curve method. The primer pairs used in this study are shown in Table 2. The sequences of the primer sets for vWF, endoglin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein S18 (RPS18) and ribosomal protein L32 (RPL32) have been described previously [5, 15, 17, 26, 27]. For determination of stable reference genes, Pearson’s correlation coefficient (r) of GAPDH, RPS18 and RPL32 was calculated using Bestkeeper software.

Statistical analysis

The differences in general characteristics of dogs in the two groups were compared using the Mann-Whitney *U* test for categorical variables, and Fisher’s exact test for categorical variables. The Mann-Whitney *U* test was used to assess differences in MVD, IRS, and mRNA transcription levels of vWF and endoglin between epithelial and mesenchymal tumors. Correlations between IRS and PI were examined by the Spearman rank correlation test, with correlation coefficients defined as “poor” (below 0.2), “fair” (from 0.2 to 0.4), “moderate” (from 0.4 to 0.6), “strong” (from 0.6 to 0.8), and “very strong” (from 0.8 to 1.0). All statistical analyses were performed using the GraphPad Prism® program (version 8.0; GraphPad Software, Inc., San Diego, CA, USA) with a significance level of 0.05.

Table 2. Primer pairs used for quantitative real-time PCR

Gene	Accession number	Direction	Sequence (5'-3')	Product size (bp)
vWF	NM_001002932	Forward	CCC AGT GCT CCC AGA AGC CCT	130
		Reverse	TCG CCC CGT GGT GAA CCG AT	
Endoglin	XM_005625330	Forward	TCC AAG CAA AAT GGC ACT CG	122
		Reverse	ATC AGT TTG GGG TTG AAG GC	
GAPDH	NM_001003142.2	Forward	AGT ATG ATT CTA CCC ACG GCA AA	146
		Reverse	CAC AAC ATA CTC AGC ACC AGC AT	
RPS18	NM_001048082.1	Forward	ATA GCC TTT GCC ATC ACA GCA ATT A	86
		Reverse	TTG GTG AGA TCG ATG TCT GCT TTC	
RPL32	XM848016	Forward	TGG TTA CAG GAG CAA CAA GAA A	100
		Reverse	GCA CAT CAG CAG CAC TTC A	

vWF, von Willebrand Factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPS18, ribosomal protein S18; RPL32, ribosomal protein L32.

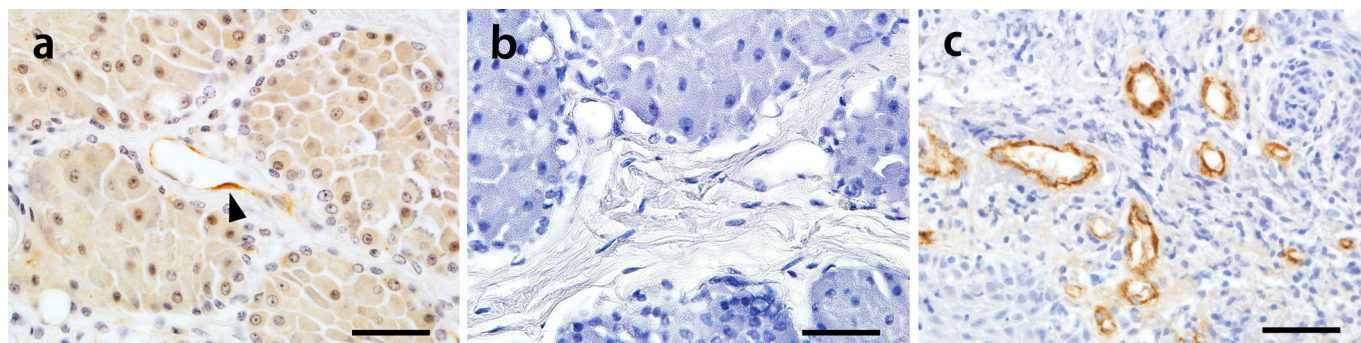


Fig. 1. Immunohistochemical staining of vascular endothelial cells for vascular endothelial growth factor receptor 2 (VEGFR2) and *von Willebrand Factor* (vWF). (a) VEGFR2 (brown) in perianal adenoma (immunoreactivity score; 3). Positive reactions were observed in vascular endothelial and tumor cells. Arrowhead indicates VEGFR2-positive vascular endothelial cells. (b) Negative staining for VEGFR2 in the same tissue as (a). (c) vWF (brown) in sebaceous adenocarcinoma. A positive reaction was observed in vascular endothelial cells. The sections were counterstained with hematoxylin (blue). Bar=40 μ m.

RESULTS

Patient characteristics

In the epithelial tumor groups, there were 12 males (6 intact, 6 castrated) and 17 females (1 intact, 16 spayed), and their median age was 11 years (range 5–14 years). In the mesenchymal groups, there were 10 males (4 intact, 6 castrated) and 11 females (5 intact, 6 spayed), and their median age was 10 years (range 6–14 years). There was no statistical difference in these general characteristics between the two groups. The general characteristics of dogs and IRS for each tumor type are shown in [Supplementary Table](#).

VEGFR2 scores in vascular endothelial cells, MVD, and mRNA transcription levels of vWF and endoglin

Vascular endothelial cells were positive for anti-VEGFR2 (Fig. 1a) and anti-vWF (Fig. 1c) antibodies. Tumor cells of undifferentiated sarcomas were negative for anti-vWF, indicating that they were not derived from vascular endothelial cells. The results of the Bestkeeper analysis showed that *RPS18* ($r=0.985$, $P=0.001$) was more stable genes than *GAPDH* ($r=0.922$, $P=0.001$) and *RPL32* ($r=0.970$, $P=0.001$). Therefore, mRNA level of each sample was normalized to that of *RPS18*. The following indices were not significantly different between epithelial and mesenchymal tumors: VEGFR2 scores of vascular endothelial cells ($P=0.721$; Fig. 2a), MVD ($P=0.536$; Fig. 2b), mRNA transcription levels of vWF ($P=0.905$; Fig. 2c) and endoglin ($P=0.164$; Fig. 2d).

VEGF, VEGFR2, and pVEGFR2 scores in tumor cells

Granular staining of VEGF was observed in the cytoplasm of tumor cells, fibroblasts, and endothelial cells (Fig. 3a). For most of the tumors, stronger staining was seen in tumor cells than stromal cells. VEGFR2 staining was strong in the nucleus, moderate in the cytoplasm and membrane of tumor cells, and weak in stromal cells (Fig. 3c, 3d). pVEGFR2 staining was strong in the membrane and cytoplasm of tumor cells and weak in stromal cells (Fig. 3f, 3g). pVEGFR2 staining was concentrated on the cell membrane. Compared with mesenchymal tumor cells, epithelial tumor cells showed higher VEGF ($P<0.01$; Fig. 4a), VEGFR2 ($P<0.01$; Fig. 4b), and pVEGFR2 ($P<0.01$; Fig. 4c) median scores.

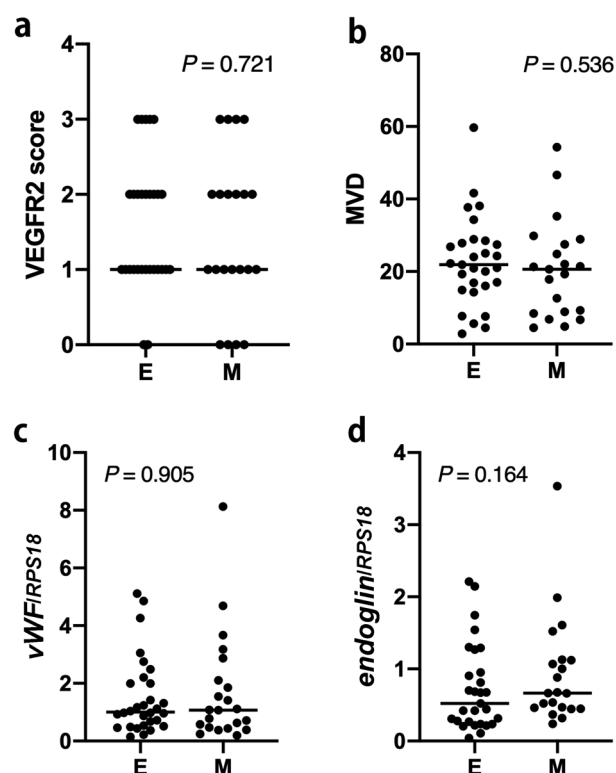


Fig. 2. Comparisons of (a) vascular endothelial growth factor receptor 2 (VEGFR2) scores in vascular endothelial cells ($P=0.721$), (b) microvessel density (MVD) ($P=0.536$), and mRNA transcription levels of (c) *von Willebrand Factor* (vWF) ($P=0.905$) and (d) *endoglin* ($P=0.164$) between epithelial and mesenchymal tumors. Relative mRNA transcription levels were normalized to *ribosomal protein S18* (*RPS18*). E=epithelial tumor, M=mesenchymal tumor.

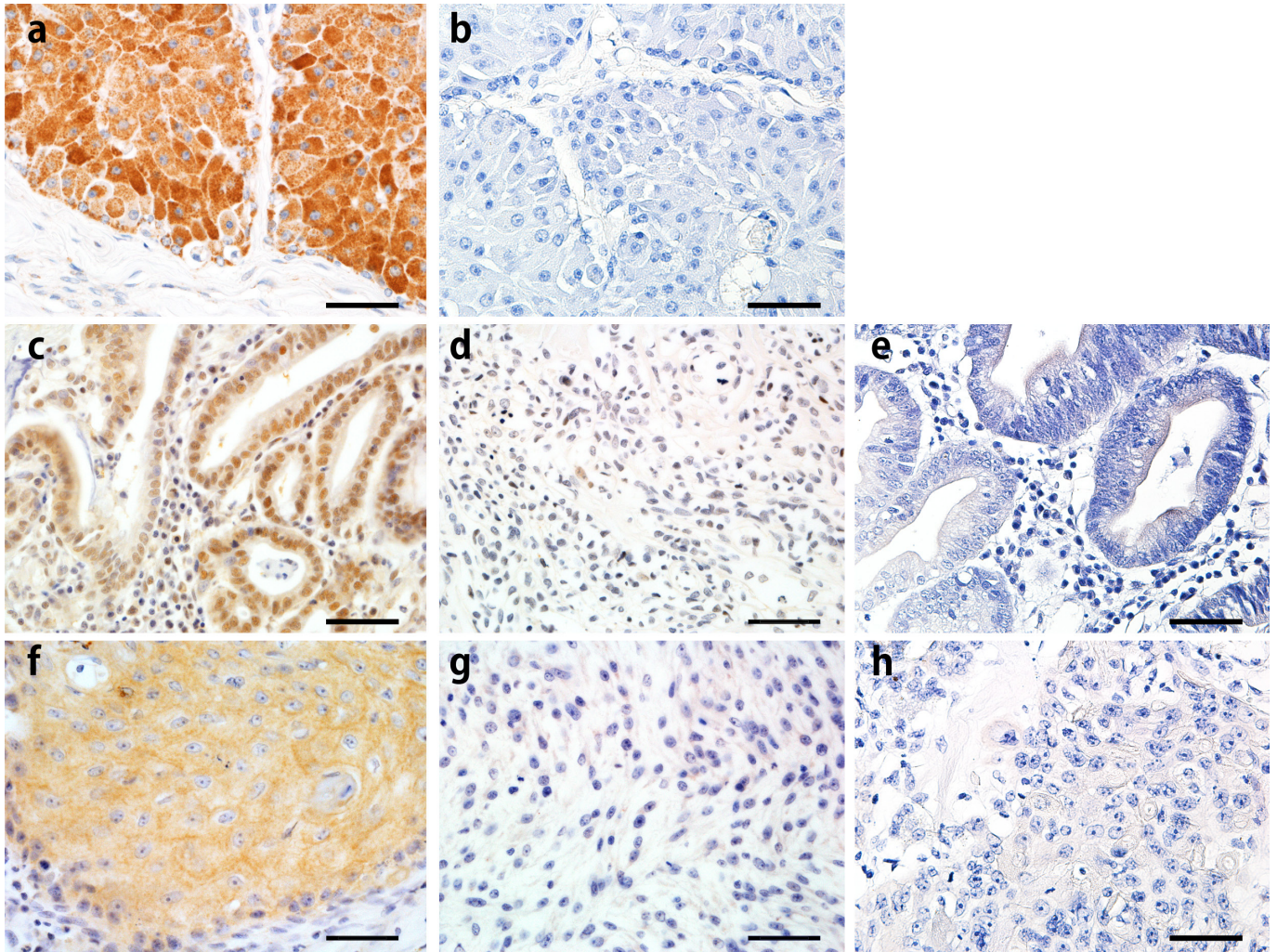


Fig. 3. Immunohistochemical staining for vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR2), and phosphorylated VEGFR2 (pVEGFR2). VEGF staining was strong in the cytoplasm of tumor cells and weak in stromal cells. Tumor cell nuclei showed stronger staining for VEGFR2 than tumor cell membranes, cytoplasm, or stromal cells. pVEGFR2 was detected in the membranes and cytoplasm of tumor cells. (a) VEGF expression in perianal adenoma cells (immunoreactivity score, IRS; 12). (b) Negative staining for VEGF in the same tumor as (a). (c) VEGFR2 in intestinal adenocarcinoma cells (IRS; 12) (d) VEGFR2 in renal sarcoma cells of unknown origin (IRS; 4) (e) Negative staining for VEGFR2 in the same tumor as (c). (f) pVEGFR2 in trichoepithelioma cells (IRS; 12) (g) pVEGFR2 in bladder sarcoma cells of unknown origin (IRS; 3). (h) Negative staining for pVEGFR2 in the same tumor as (f). Sections were counterstained with hematoxylin (blue). Bar=40 μ m.

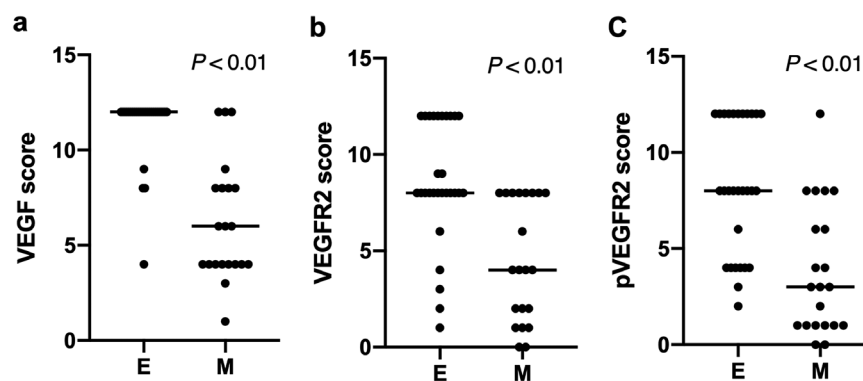


Fig. 4. Comparisons of (a) vascular endothelial growth factor (VEGF) ($P < 0.01$), (b) VEGF receptor 2 (VEGFR2) ($P < 0.01$), and (c) phosphorylated VEGFR2 (pVEGFR2) scores ($P < 0.01$) in tumor cells between epithelial and mesenchymal tumors. E=epithelial tumor, M=mesenchymal tumor.

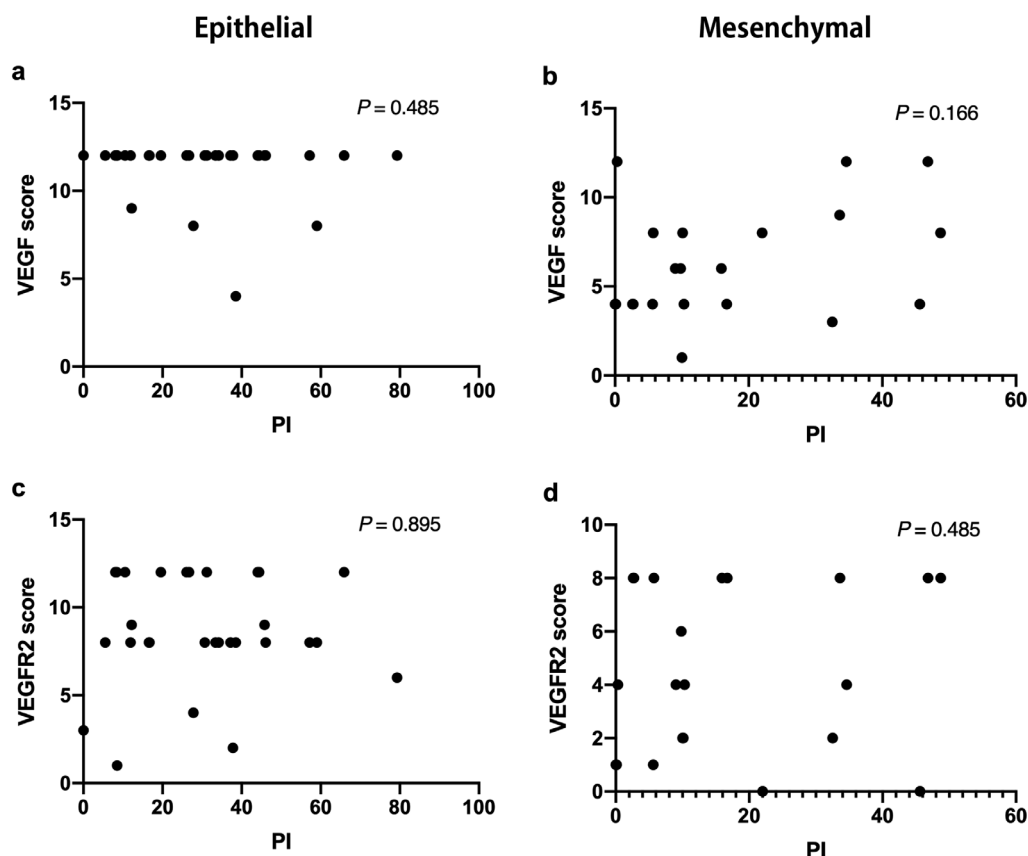


Fig. 5. Correlations between vascular endothelial growth factor (VEGF) scores and proliferation index (PI) in (a) epithelial ($P=0.485$) and (b) mesenchymal ($P=0.166$) tumors and VEGF receptor 2 (VEGFR2) scores and PI in (c) epithelial ($P=0.895$) and (d) mesenchymal ($P=0.485$) tumors.

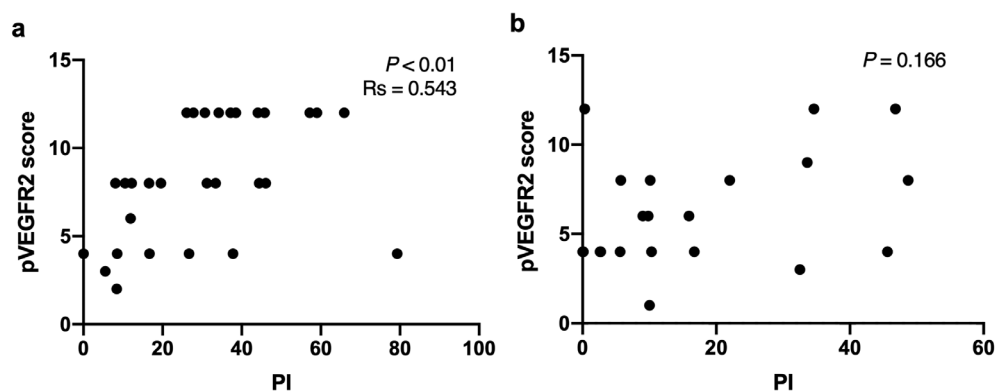


Fig. 6. Correlations between phosphorylated vascular endothelial growth factor receptor 2 (pVEGFR2) scores and proliferation index (PI) in (a) epithelial ($P < 0.01$, $R_s = 0.543$) and (b) mesenchymal ($P = 0.278$) tumors.

Correlations between VEGF, VEGFR2, and pVEGFR2 scores in tumor cells and PI

VEGF scores were not correlated with PI in either epithelial ($P=0.485$; Fig. 5a) or mesenchymal ($P=0.166$; Fig. 5b) tumors. VEGFR2 scores were also not correlated with PI in either epithelial ($P=0.895$; Fig. 5c) or mesenchymal ($P=0.485$; Fig. 5d) tumors. pVEGFR2 scores correlated moderately with PI in epithelial tumors ($P < 0.01$, $R_s = 0.543$; Fig. 6a) but not in mesenchymal tumors ($P=0.278$; Fig. 6b).

Correlation between degree of angiogenesis and PI

In epithelial tumors, there was no correlation between MVD and PI ($P=0.125$; Fig. 7a), while moderate positive correlation was

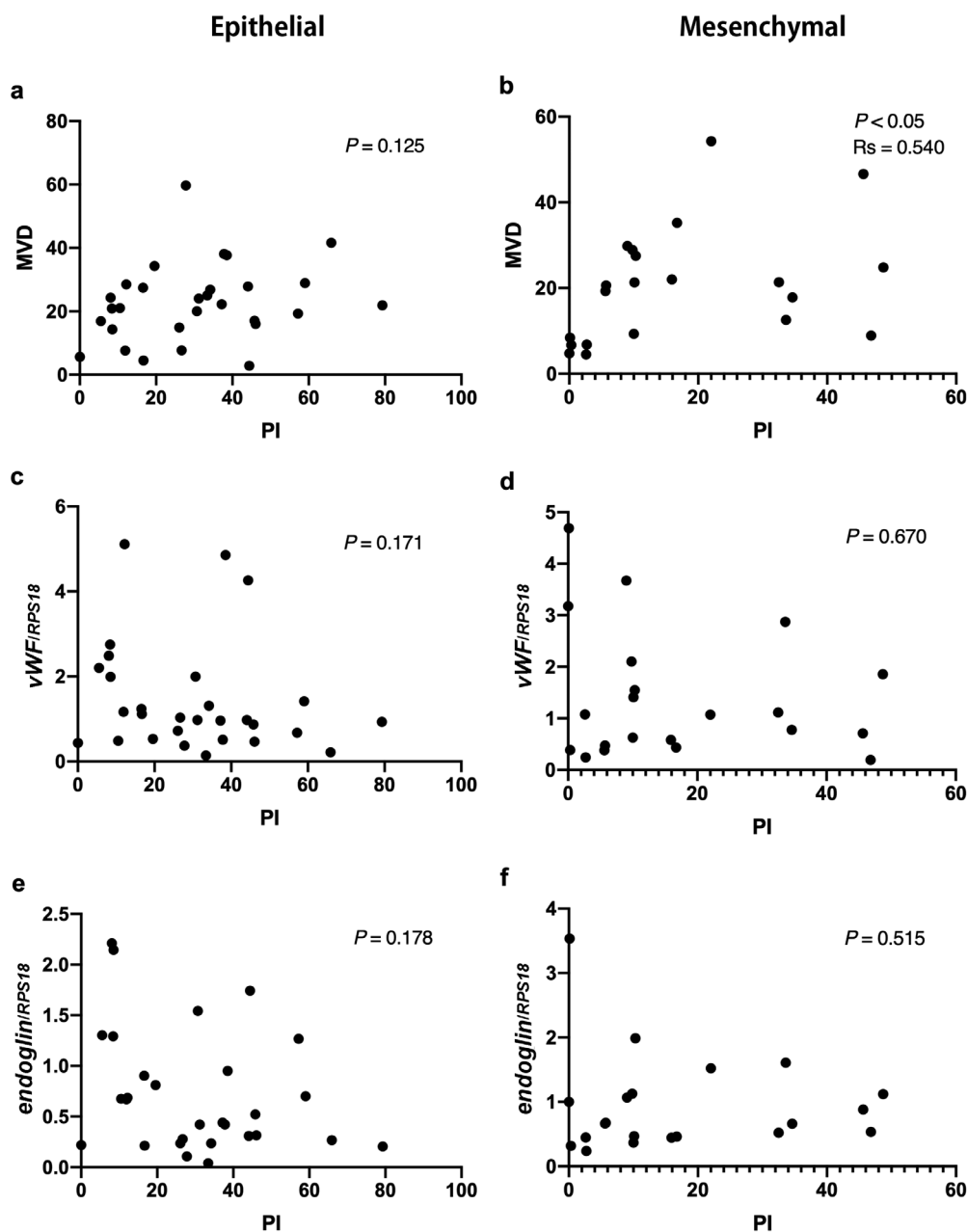


Fig. 7. Correlations between degree of angiogenesis and proliferation index (PI). (a, b) microvessel density (MVD) and PI in (a) epithelial ($P=0.125$) and (b) mesenchymal ($P<0.05$, $R_s=0.540$) tumors. (c, d) mRNA transcription levels of *von Willebrand Factor* (*vWF*) and PI in (c) epithelial ($P=0.171$) and (d) mesenchymal ($P=0.670$) tumors. (e, f) mRNA transcription levels of *endoglin* and PI in (e) epithelial ($P=0.178$) and (f) mesenchymal ($P=0.515$) tumors.

seen between them in mesenchymal tumors ($P<0.05$, $R_s=0.540$; Fig. 7b). mRNA transcription levels of *vWF* were not correlated with PI in either epithelial ($P=0.171$; Fig. 7c) or mesenchymal ($P=0.670$, Fig. 7d) tumors. Likewise, mRNA transcription levels of *endoglin* were not correlated with PI in epithelial ($P=0.178$; Fig. 7e) or mesenchymal ($P=0.515$; Fig. 7f) tumors.

DISCUSSION

In this study, the expression of VEGF, VEGFR2, and pVEGFR2 was higher in epithelial tumor cells than in mesenchymal tumor cells. On the other hand, epithelial and mesenchymal tumors showed no differences in angiogenesis or VEGFR2 expression on vascular endothelial cells. Tumor cell proliferation was associated with pVEGFR2 expression in epithelial tumors and with MVD in mesenchymal tumors.

We found that tumor cells produce VEGF regardless of the type of tumor, and VEGFR2 is phosphorylated on many types of

canine tumor cells. In this study, there was discrepancy in the localization of expression of VEGFR2 and pVEGFR2. It has been reported that when VEGFR2 is phosphorylated by binding to VEGF, it translocates to the cytoplasm and nucleus to regulate its own transcription [8]. There have also been reports in which VEGFR2 and pVEGFR2 were immunohistochemically detected in the cell membrane, cytoplasm, and nucleus in human umbilical vein endothelial cells and a wide variety of normal and tumor tissues [8, 31]. The localization of expression of VEGFR2 and pVEGFR2 in this study were consistent with previous reports.

Correlations between VEGF and VEGFR2 expression and PI has been reported previously in some canine tumors. In squamous cell carcinomas, VEGF and VEGFR2 scores correlated positively with PI [1]. In trichoepitheliomas and mammary gland adenocarcinomas, VEGF scores correlated positively with PI [1, 2]. To the best of our knowledge, this study is the first to demonstrate a correlation between pVEGFR2 expression and tumor cell proliferation activity in canine tumors. In this study, only pVEGFR2 expression correlated with tumor cell proliferation in epithelial tumors, suggests that pVEGFR2 expression may be a better indicator of tumor proliferation in dogs than VEGF or VEGFR2, and that VEGF/VEGFR2 signaling is involved in tumor cell proliferation in epithelial tumors. However, the reactivity of anti-pVEGFR2 antibody used in this study to dogs has not been reported. Therefore, careful interpretation of the results is required.

In contrast, in mesenchymal tumors, PI correlated with MVD, but not with pVEGFR2 expression on tumor cells. These results suggest that the proliferation of mesenchymal tumor cells is dependent on supply of oxygen and nutrients from neovascularization. However, mRNA transcription levels of endothelial markers did not correlate with PI; further studies will be required to address these discrepancies. As VEGF is important for many cellular functions such as migration and viability, in addition to proliferation [9], VEGF/VEGFR2 signaling in mesenchymal tumor cells may be involved in these other functions.

In this study, the clinical characteristics of the samples were heterogeneous. In particular, benign and malignant tumors were randomly included, and the number of malignant tumors was clearly larger in epithelial tumors. It has been reported that the expression of VEGF and VEGFR2 is high in malignant tumors [4, 19], and in fact, our data also showed that malignant tumors had a significantly higher VEGF score than benign tumors in mixture of epithelial and mesenchymal tumors (data not shown). However, since there was no correlation between pVEGFR2 score and PI in malignant tumors, we believe that epithelial-mesenchymal differences influenced the results of this study. We need to increase the number of each tumor type and homogenize the clinical features in the further study.

We evaluated the pVEGFR2 score by using formalin-fixed paraffin-embedded (FFPE) samples. Since the phosphorylation state of VEGFR2 could be changed in a short time, the staining intensity and pattern might be artificially altered from the original expression in the tumor tissues during formalin fixation. Considering that evaluation of pVEGFR2 using FFPE samples in human lymphoma has been reported previously [28] and that the results of this study showed that pVEGFR2 score correlated with another clinical indicator, our results may reflect the actual values; still, alternative methods of analysis, such as frozen section or western blotting using snap-frozen samples would be more appropriate. Moreover, we did not conduct experiments to analyze VEGF/VEGFR2 signaling function. Additional studies will be required to confirm whether VEGF/VEGFR2 signaling in tumor cells is involved in tumor cell proliferation using canine tumor cell lines.

In conclusion, epithelial tumor cells had higher expression of VEGF, VEGFR2, and pVEGFR2 than mesenchymal ones. VEGF/VEGFR2 signaling on tumor cells may contribute to proliferation in canine epithelial tumors. These differences between epithelial and mesenchymal tumors may affect the efficacy of inhibitors targeting the VEGF/VEGFR2 signaling pathway and should be investigated further.

CONFLICT OF INTEREST STATEMENT. The authors declare no conflicts of interest.

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