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Research Article

The Correlation of Intratumoral Flt-4 Positive Lymphatic Vessels with the Expression of iNOS and VEGF-C in Canine Mammary Gland Tumor

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Abstract

Vascular endothelial growth factor-C (VEGF-C) and iNOS are considered to be a potentially lymphangiogenic and can selectively induce hyperplasia of the lymphatic vasculature. In this study, we aimed to clarify the relation between expression of VEGF-C and iNOS with Flt-4, lymphatic vessel marker, to investigate the intralymphatic tumor growth in canine mammary gland tumor by immunohistochemical method. Using the specific polyclonal antibodies, VEGF-C and iNOS immunolabeled cells were count in benign and malignant canine mammary gland neoplasm. All cases showed some tumor cell positive for VEGF-C and iNOS, and the staining pattern was seen predominantly cytoplasmic. The percentage of VEGF-C and iNOS positive cells ranged from 52.3% to 81.6% and 71.8% to 94.4%, respectively. Lymphatic vessel was investigated by the expression of Flt-4 in lymphatic endothelium and the result shows percentage number of lymph vessel among 15.5% - 37.7%. Although the percentage of Flt-4 positive lymph vessel, VEGF-C and iNOS positive cell in malignant neoplasm were produced in higher amounts than the benign neoplasm, there was highly significant difference ($p < 0.05$) among neoplastic group. Moreover, the numbers of VEGF-C, iNOS positive cells and tumor lymphatic vessels were positive correlated with each other for both benign and malignant tumor ($p \leq 0.01$). This present study suggests that the upregulation of VEGF-C, iNOS in canine mammary gland tumors was interconnected with the intralymphatic tumor growth..

Keywords: *VEGF-C; iNOS; Flt-4; Lymphatic Vessel Density; Canine Mammary Gland Tumor*

Introduction

Canine mammary tumor is the most common tumor in female dog. It can be diagnosed with benign and malignant neoplasm. Malignant neoplasm is found about 50% in this tumor and lymphatic metastasis is one of the most important metastatic routes. The most important factors in the initiation and progression of lymphangiogenesis are nitric oxide (NO) and the vascular endothelial growth factor (VEGF) family. NO is a product of the conversion of L-arginine to L-citrulline by the NO synthase. Three isoforms of NO synthase have been identified and inducible nitric oxide synthase (iNOS) is the most-active NOS isoform [2,18,25]. For the VEGF family, VEGF-C has been reported to induce hyperplasia and dilation of the pre-existing and tumour lymphatic vessels and to increase lymph flow rate, which may have an impact on lymph node metastasis [15,20]. Additionally, NO is thought to accelerate lymphangiogenesis via increasing the expression of VEGF-C and Flt-4 [9,10,23]. As we know, lymphatic invasion (LI) and lymph node metastasis are the most important steps of mammary carcinoma progression. However, in the literature of veterinary oncology, few studies have investigated the lymphogenic factor in canine mammary gland tumor. In this study, we employed immunohistochemical (IHC) staining to detect the expression of iNOS, VEGF-C and Flt-4 to examine the relationship between iNOS, VEGF-C and Flt-4 positive lymphatic vessel in canine mammary gland tumor.

Material and Method

Animal and tissue sample

Thirty samples of canine mammary tumor were obtained at the time of surgery from animal hospital. In all cases, the samples were fixed in 10% buffer formalin solution, then embedded in paraffin. All the tumors were histologically examined by hematoxylin-eosin staining. Tumors were histologically classified into benign and malignant neoplasm. One thousand neoplastic cells were count in each case to identified the VEGF-C and iNOS immunoreactive cell. Flt-4 positive lymph vessels were count in 10 random areas.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted using the immunohistochemistry kit according to the manufacturer's instructions. For investigated the expression of VEGF-C, iNOS and Flt-4 with we designed to use goat-anti rabbit polyclonal antibodies against VEGF-C (Santa Cruz), iNOS (Santa Cruz), and Flt-4 (Santa Cruz), respectively. Briefly, serial section slides of 5 mm were obtained from the paraffin embedded specimens. After regular de-paraffin and rehydration, the slides were placed in 10 mM tris-citrate buffer, pH 6.0 and heated three times for 5 minutes each at 750W in a microwave oven. Then, the slides were incubated with 3% hydrogen peroxide-methanol solution for 20 minutes to remove endogenous peroxidase. Next, nonspecific binding was blocked with 1.5% normal blocking serum for 60 min; the slide was incubated overnight with primary antibodies to VEGF-C or iNOS or Flt-4 at 1:30, 1:50 and 1:100 dilution, respectively. Followed by incubation for 30 min with a biotin-labelled secondary antibody for 30 min; and subsequently the slide was incubated for 30 min with avidin-biotinylated HRP. Color was developed using DAB for 10 minutes and the slide was counterstained with hematoxylin. The slides were observed under light microscope.

A count of immunoreactive positive cells was observed at 400× magnification. At least 10 fields per tumor were examined and a minimum of 1,000 neoplastic cells were counted [2]. VEGF-C, iNOS, and Flt-4 positive cells showed dark brown particles in their cytoplasm. VEGF-C and iNOS intensity was scored as follows: (+) = strong; (−) = weak or negative immunoreactive. The results were expressed as a percentage. For Flt-4-positive vessel density (FVD) determination. Ten fields per tumor were randomly chosen, and every immunolabeled intratumoral vessel was counted on a 400× field. VEGF-C, iNOS positive cell and FVD were determined as the median value of cells and vessel counts, respectively.

Statistical analysis

VEGF-C, iNOS positive cell number and FVD were compared with each other among the benign and malignant neoplasm by used Mann-Whitney test. The correlation of FVD with VEGF-C and iNOS was investigated by the Spearman test. A computer program IBM SPSS statistic 21 was used for all of the statistical testing.

Result

Thirty samples of canine mammary tumor were used in this experiment. There are 9 benign and 21 malignant tumor. 1,000 neoplastic cells were count in each case to identified the VEGF-C and iNOS immunoreactive cell. Flt-4 positive lymph vessels were count in 10 random areas. The classification of canine mammary tumor in our experiment is show in table 1.

Tumor Type	No. in Study
Benign	
Duct papilloma	6
Adenoma	2
Fibroadenoma	1
Malignant	
Papilla adenocarcinomas	8
Tubular adenocarcinomas	2
Solid adenocarcinomas	1
Malignant mix tumor	10
Total	30

Table 1: *The classification of canine mammary tumor in this experiment.*

VEGF-C and iNOS immunoreactivity were both observed as positive cytoplasmic staining in neoplastic cells (Figure 1A and 1B). The expression of VEGF-C, iNOS and Flt-4 positive lymph vessel in each type of canine mammary tumors are shows in table 2.

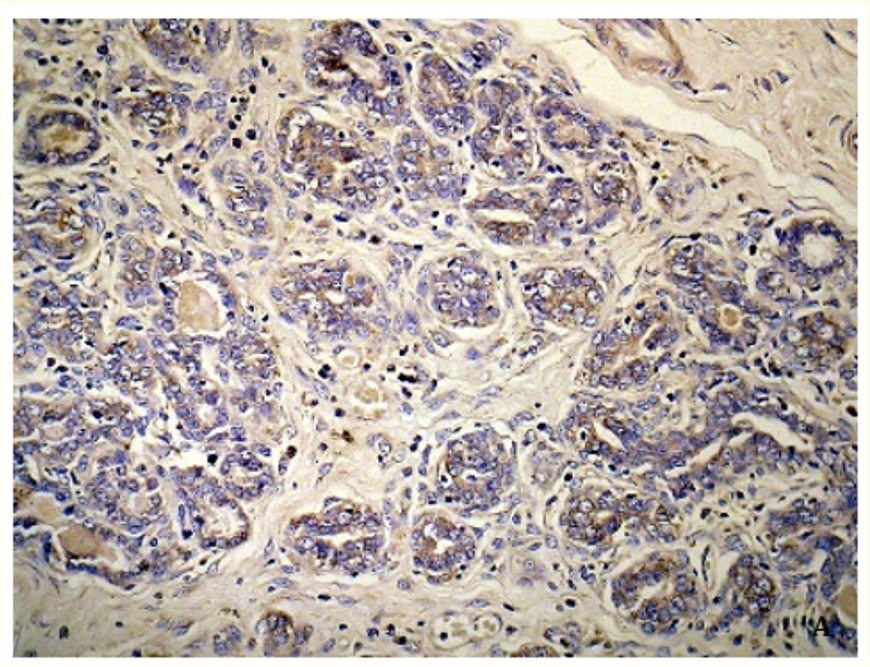
Histologic type	VEGF-C Positive Cells (%) Median ± SD	iNOS Positive Cells (%) Median ± SD	Flt-4 Positive lymph vessel (%) Median ± SD
Adenoma	54 ± 4.72	73.5 ± 3.86	15.5 ± 2.12
Duct papilloma	52 ± 6.00	72 ± 6.44	15.5 ± 1.47
Fibroadenoma	53 ± 5.00	72 ± 5.53	16 ± 0.69
Papilla adenocarcinomas	73 ± 6.70	79 ± 5.56	38.5 ± 3.09
Tubular adenocarcinomas	70.5 ± 4.97	80 ± 4.74	36.5 ± 0.70
Solid adenocarcinomas	80 ± 4.97	95 ± 2.76	34 ± 1.50
Malignant mix tumor	79 ± 5.88	84 ± 6.40	38.5 ± 2.16

Table 2: *The expression of VEGF-C, iNOS and Flt-4 positive lymph vessel in each type of canine mammary tumor.*

The percentage of VEGF-C and iNOS positive cells ranged from 52.3% to 81.6% and 71.8% to 94.4%, respectively. The expression of VEGF-C and iNOS were significantly higher in malignant than the benign mammary gland neoplasm. ($P < 0.05$; Table 3). The number of lymph vessel was investigated by the expression of Flt-4 positive vessel (Figure 1C) and the result shows percentage number of lymph vessel among 15.5% - 37.7%. The number of lymph vessel was significant greater in malignant than in benign tumors ($P < 0.05$; Table 3).

Parameter (%)	Benign	Malignant	P-value
VEGF-C positive cell	52.35 ± 0.39*	76.13 ± 5.36*	0.05
iNOS positive cell	72.10 ± 0.79*	81.37 ± 7.26*	0.05
Flt-4 positive lymph vessel	15.83 ± 0.25*	36.81 ± 1.63*	0.05

Table 3: *The expression of VEGF-C, iNOS and Flt-4 positive lymph vessel in benign and malignant canine mammary tumor.*



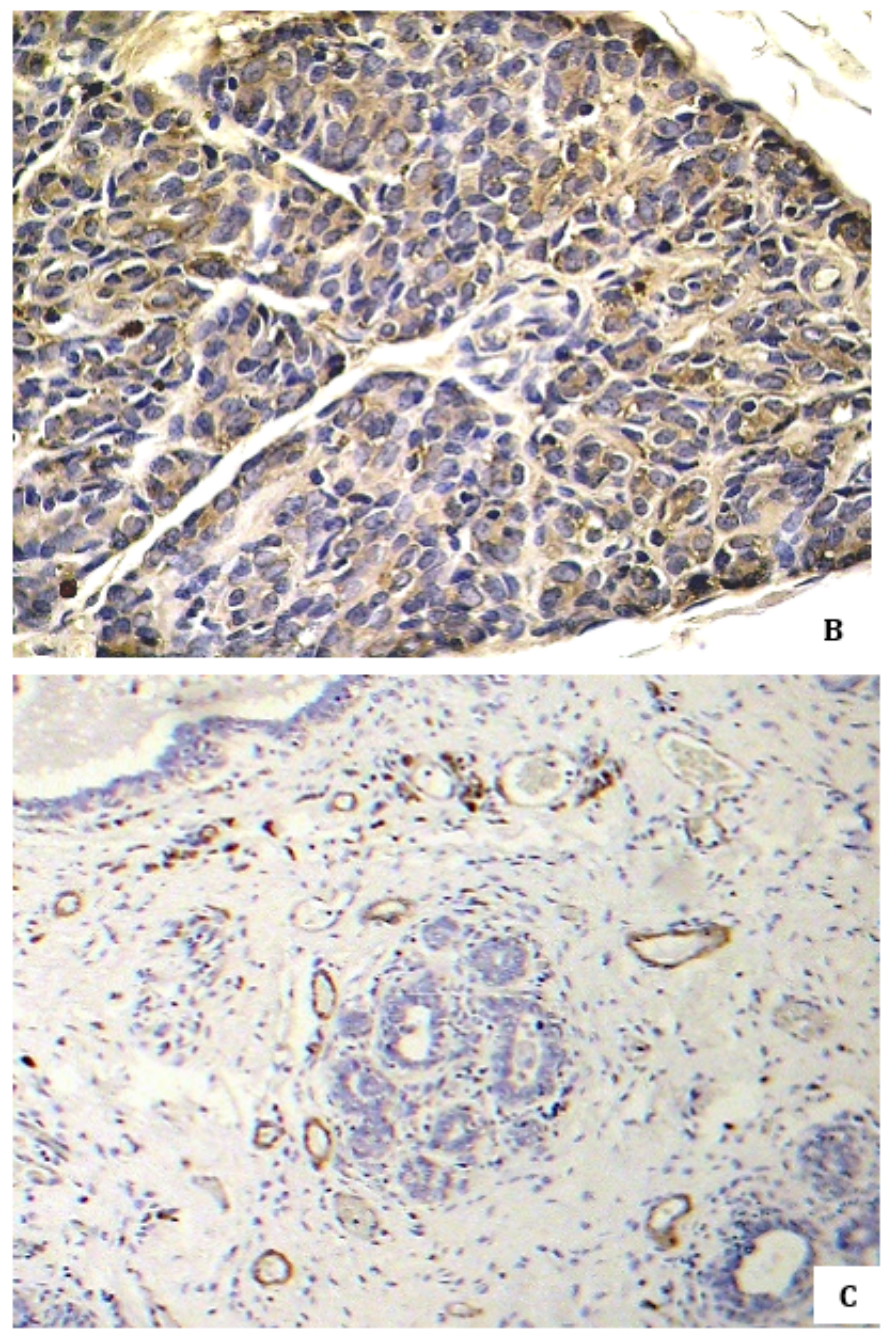


Figure 1: *The expression of VEGF-C (A), iNOS (B), and Flt-4 (C) in canine mammary carcinoma tissues. A. IHC detection of VEGF-C (×100); B. IHC detection of iNOS (×400); and C. IHC detection of Flt-4 positive lymph vessel (×100).*

The immunoreactive of VEGF-C, iNOS and FVD were positive correlated with each other for both benign and malignant tumors (P<0.01, Table 4). The result showed that VEGF-C positive cell has correlated significantly with iNOS (r = 0.65) and Flt-4 positive vessel. (r = 0.36). Additionally, iNOS positive cells also has correlated with Flt-4 positive vessel (r = 0.49).

Parameter	Correlation coefficient	P-value
VEGF-C/iNOS	0.65*	0.01
VEGF-C/Flt-4	0.49*	0.01
iNOS/Flt-4	0.36*	0.01

Table 4: *Spearman's correlation test of VEGF-C, iNOS, Flt-4 in canine mammary gland tumor.*

Discussion

For basically, the central molecule that directs proliferation and migration of lymphatic endothelial cells (LEC) during embryogenesis is vascular endothelial growth factor C (VEGF-C). VEGF-C is therefore an important ingredient for LEC culture and attempts to (re) generate lymphatic vessels and networks [1,16]. In neoplasm, the previous studies have shown that VEGF-C expression is first considered to lymphangiogenic and may play the role in the route of lymphatic metastasis [5-7,22,24].

Furthermore, iNOS is also reported to involve the lymphangiogenesis and produced poor prognosis in various tumor [8,23]. Additionally, Tascene reported that serum nitric oxide level in dogs with mammary tumors is significantly higher than in healthy dogs [22]. Other studies have shown that iNOS expression in human colorectal and gallbladder cancer is associated with increased disease-specific fatality and/or malignancy degree [12,22]. Moreover, it has also been determined that iNOS expression is related to different stages of human breast carcinoma [14]. However, Anadol determined the expression of iNOS by qRT-PCR analysis in malignant mammary tumors in dogs, and the findings of this work show that the expression of iNOS depends on tumor grade [1].

Both iNOS and VEGF-C are the most importance factors in lymphogenic tumor growth [4,11]. However, there are few studies focusing on iNOS, VEGF-C expression and its correlation with lymphangiogenesis in CMT. Our results showed that percentage number of iNOS and VEGF-C expression was higher in malignant tumor compared with benign tumor. Moreover, we also identified a significant positive correlation (P < 0.01) between iNOS and VEGF-C expression in both benign and malignant neoplastic cells. This mean that when iNOS up regulated it will also raise the expression of VEGF-C. This agree with previous study in human that showed that stimulation of the iNOS pathway will lead to up-regulation of VEGF-C [4,9,10,23]. Additionally, our results also demonstrated the significant correlation between iNOS, VEGF-C and Flt-4 positive lymphatic vessels. This results inferred that the increase of the Flt-4 positive lymphatic vessels could produce by up regulated of iNOS and VEGF-C of canine mammary cancer cells. The value of iNOS, VEGF-C and Flt-4 positive lymph vessel in each type of canine mammary tumors in figure 2 showed that Flt-4 positive vessel has higher value according to iNOS and VEGF-C. This is the first study on the relationship between the expression of iNOS, VEGF-C and Flt-4 positive lymphatic vessels in canine mammary tumors. The present study demonstrated that iNOS and VEGF-C expression in canine mammary tumor cells was found to be closely related to FVD.

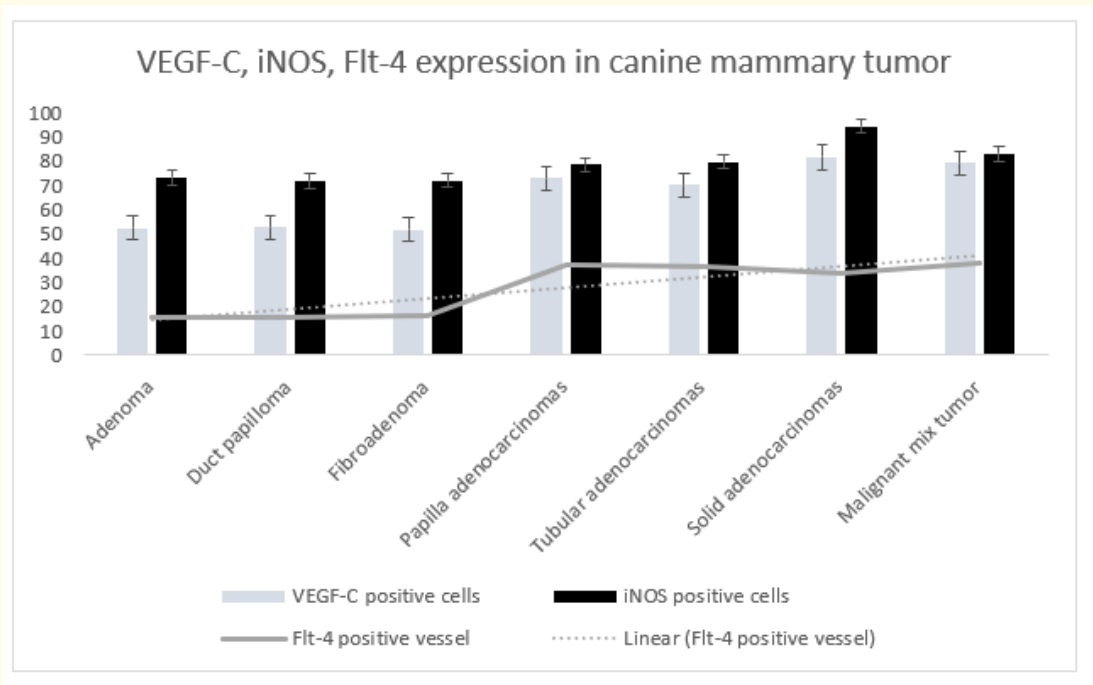


Figure 2: *The expression of VEGF-C, iNOS and Flt-4 positive lymph vessel in each type of canine mammary tumors.*

Therefore, high NO production with up regulation of VEGF-C by tumor cells, finally resulting in increased lymphangiogenesis and tumor spread through the lymphatic route. In previous study, they presented that tumor cell-derived vascular endothelial growth factor (VEGF)-C in promoting lymphangiogenesis by activating Flt-4 expressed on lymphatic endothelial cells via a paracrine or autocrine mechanism [3,19,24]. They are support our work that also showed higher value of Flt-4 positive vessel according to VEGF-C expression. However, the mechanisms responsible for the acquisition of the capacity of carcinomas to metastasize via lymphatic vessels are largely unexplored. In this study, we focused our attention on the possible role of iNOS and VEGF-C as lymphangiogenic factor in CMT.

The density of lymphatic invasion as well as intratumoral and peritumoral lymphatic vascular density were positively correlated with lymph node metastasis in CMT and other cancer [12,13].

Conclusion

In conclusion, our work suggests that VEGF-C expression with iNOS activity and NO production in malignant tumors and the tendency increase of LVD, indicating the existence of a link between this factors and tumor lymphangiogenesis are involved in inducing aggressiveness in canine mammary tumors. Although confirmation in further experiment is necessary, our findings suggest a possibility for therapeutic modalities against lymphangiogenesis and lymphatic borne metastasis by targeting iNOS and VEGF-C.

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Bibliography

1. Anadol E., *et al*. "Expression of iNOS, COX-2 and VEGF in canine mammary tumours and non-neoplastic mammary glands: Association with clinicopathological features and tumour grade". *Acta Veterinaria Hungarica* 65.3 (2017): 382-393.

2. Burke AJ., *et al*. "The yin and yang of nitric oxide in cancer progression". *Carcinogenesis* 34.3 (2013): 503-512.

3. Decio A., *et al*. "Vascular endothelial growth factor c promotes ovarian carcinoma progression through paracrine and autocrine mechanisms". *The American Journal of Pathology* 184 (2014): 1050-1061.

4. Franchi A., *et al*. "Inducible nitric oxide synthase activity correlates with lymphangiogenesis and vascular endothelial growth factor-C expression in head and neck squamous cell carcinoma". *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland* 208.3 (2006): 439-445.

5. Garcia EA., *et al*. "Lymphatic vessel density and VEGF-C expression are significantly different among benign and malignant thyroid lesions". *Endocrine Pathology* 21.2 (2010): 101-107.

6. Gomes FG., *et al*. "Tumor angiogenesis and lymphangiogenesis: tumor/endothelial crosstalk and cellular/ microenvironmental signaling mechanisms". *Life Sciences* 92 (2013): 101-107.

7. Hao Yu., *et al*. "The role of VEGF-C-D and Flt-4 in the lymphatic metastasis of early stage invasive cervical carcinoma". *Journal of Experimental and Clinical Cancer Research* 28 (2009): 98.

8. Karadayı N., *et al*. "Inducible nitric oxide synthase expression in gastric adenocarcinoma: impact on lymphangiogenesis and lymphatic metastasis". *Diagnostic Pathology* 8.1 (2013): 151.

9. Lahdenranta J., *et al*. "Endothelial nitric oxide synthase mediates lymphangiogenesis and lymphatic metastasis". *Cancer Research* 69.7 (2009): 2801-2808.

10. Massi D., *et al*. "Inducible nitric oxide synthase expression in melanoma: implications in lymphangiogenesis". *Modern Pathology* 22.1 (2009): 21.

11. Nakamura Y., *et al*. "Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis". *Clinical Cancer Research* 12.4 (2006): 1201-1207.

12. Niu XJ., *et al*. "Relationship between inducible nitric oxide synthase expression and angiogenesis in primary gallbladder carcinoma tissue". *World Journal of Gastroenterology* 10 (2004): 725-728.

13. Pan Z., *et al*. "VEGF-C is positively associated with lymphangiogenesis and lymphatic metastasis in rectal cancer". *International Journal of Clinical and Experimental Pathology* 11.3 (2018): 1777-1783.

14. Ranganathan S., *et al*. "Significance of twist and iNOS expression in human breast carcinoma". *Molecular and Cellular Biochemistry* 412 (2016): 41-47.

15. Raposo-Ferreira TM., *et al*. "Evaluation of vascular endothelial growth factor gene and protein expression in canine metastatic mammary carcinomas". *Microscopy Research and Technique* 79.11 (2016): 1097-1104.

16. Rauniyar K., *et al*. "Biology of vascular endothelial growth factor C in the morphogenesis of lymphatic vessels". *Frontiers in Bioengineering and Biotechnology* 6 (2018): 7.

17. Shigetomi S., *et al*. "VEGF-C/Flt-4 axis in tumor cells contributes to the progression of oral squamous cell carcinoma via upregulating VEGF-C itself and contactin-1 in an autocrine manner". *American Journal of Cancer Research* 8.10 (2018): 2046.

18. Singh PP., *et al*. "Intratumoural and peritumoural lymphangiogenesis in canine mammary tumour linked to tumour spread and poor survival". *Current Science* 115.7 (2018): 1312.

19. Singh S and Gupta AK. "Nitric oxide: role in tumour biology and iNOS/NObased anticancer therapies". *Cancer Chemotherapy and Pharmacology* 67.6 (2011): 1211-1224.

20. Sleeckx N. Angiogenesis and lymphangiogenesis in canine mammary tumours (Doctoral dissertation, University of Antwerp) (2013).

21. Tascene N., *et al*. "Serum neopterin, sialic acid and nitric oxide levels in dogs with malignant mammary tumours". *Veterinary and Comparative Oncology* 10 (2012): 155-159.

22. Zafirellis K., *et al*. "Inducible nitric oxide synthase expression and its prognostic significance in colorectal cancer". *Acta Pathologica, Microbiologica, Et Immunologica Scandinavica* 118 (2010): 115-124.

23. Zhang W., *et al*. "Inducible nitric oxide synthase expression correlates with angiogenesis,lymphangiogenesis, and poor prognosis in gastric cancer patients". *Human Pathology* 42.9 (2011): 1275-1282.

24. Zhao YC., *et al*. "Peritumoral lymphangiogenesis induced by vascular endothelial growth factor C and D promotes lymph node metastasis in breast cancer patients". *World Journal of Surgical Oncology* 10.1 (2012): 165.

25. Ziche M and Morbidelli L. "Molecular regulation of tumour angiogenesis by nitric oxide". *European Cytokine Network* 20.4 (2009): 164-170.

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