SHORT COMMUNICATION

A study on VEGFR-2 Expression in Canine Mammary Tumors

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Abstract

Angiogenesis is one of the hallmarks of cancer, and VEGFR-2, the receptor for VEGF is said to be more responsible for tumor angiogenesis than the other receptors. The objective of the present study was to examine the VEGFR-2 expression in different grades of tumors with the help of quantitative PCR. Expression profiling was studied as part of the work in VEGFR-2 with HPRT-1 gene as endogenous control using qPCR. RNA extracted from different grades of Canine Mammary Tumors (CMTs) were used for the study. VEGFR-2 expression was found to be overexpressed as the grade of the tumor increase, which establishes the dependency of the high grade tumors on angiogenic factors, which in turn indicates that angiogenic factors are indispensable for angiogenesis for enhanced angiogenesis tumor tissue proliferation.

Keywords: Angiogenesis Canine Mammary Tumors, qPCR, VEGFR-2.

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Introduction

Self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis are regarded as the hallmarks of cancer (Hanahan and Weinberg, 2000). Among all the hallmarks of cancer, angiogenesis in tumors is a crucial factor associated with tumor growth, progression, and metastasis (Teleanu *et al.,* 2020). Embryogenesis, organ development, and wound healing involve the most critical process called angiogenesis, which is defined as the developmental process of new blood vessels from the existing ones (Sarabipour *et al.,* 2016 and Nessa *et al*., 2009). Angiogenesis is also associated with specific physiological and pathological compensations *viz*. in diabetic and age-related muscular degeneration in the growth of solid tumors, which recruit blood vessels to ensure adequate supply of oxygen and nutrients (Luo *et al*., 2008). Angiogenesis which is evident in neoplasms, is regulated by Ligands and receptors of the vascular endothelial growth factor (VEGF) signaling network (Koch *et al*., 2011). Till date three transmembrane tyrosine kinase receptors were identified as the VEGF receptors family *viz.,* vascular-endothelial growth factor receptor-1 (VEGFR-1), VEGFR-2, and VEGFR-3. These regulate the formation of blood and lymphatic vessels (Guo *et al.,* 2010). Among all the VEGF receptors, VEGFR-2 plays a pivotal role in regulating endothelial cell proliferation and migration (Takahashi *et al*., 2005). Keeping in view the critical role of VEGFR-2 in angiogenesis and tumor progress, the present study was attempted to determine the quantitative expression of VEGFR-2 in different grades of tumors.

Materials and Methods

Forty mammary tissues were collected from the canine mammary tumor cases presented at different clinics in Hyderabad, Bangalore, Visakhapatnam, and Gannavaram. Every sample was stored separately for Histopathological studies and RNA extraction. Histopathological grading was done for all the samples and could obtain n=10 each in Grades I, II, and III. The normal adjacent tissue of the canine mammary tumor was also collected, which were treated as a control group. Tumor grading was done as per the guidelines given by Goldschmidt *et al.* (2011). Total RNA extraction was done for all the 40 samples by Trizol method and assessed the quality of the RNA using Nanodrop™ 2000C (ThermoScientific™). cDNA was synthesized from all the RNA samples using Bio-Rad thermocycler C1000 Touch™ and Himedia Hi-cDNA Synthesis Kit, following the protocol

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Table 1: Primers used in the study

S.No.	Gene	Primer	Accession Number	Reference
1	VEGFR-2 Forward	TGTCATCCTTACCAACCCCATT	NM_001048024.1	Jais *et al.*,2011
	VEGFR-2 Reverse	CGG AGAGATCAGGGATTTCTCA		
2	HPRT-1 Forward	CAC TGGGAAAACAATGCAGA	NM_001003357.1	
	HPRT-1 Reverse	ACA AAGTCAGGTTTATAGCCAACA		

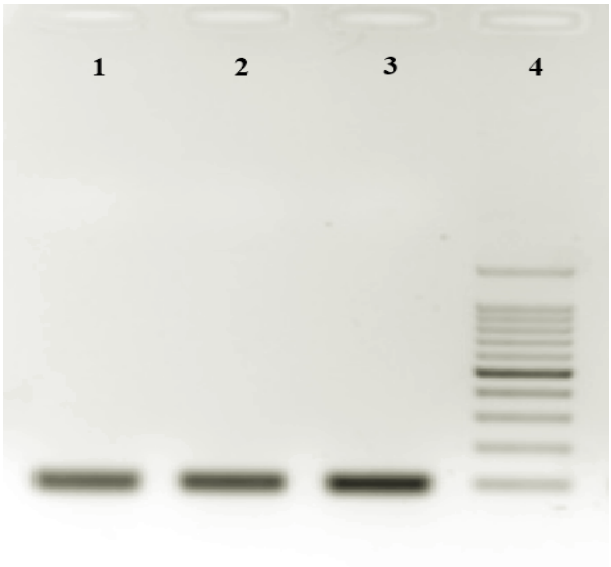

Fig. 1: Electrophoresis gel run of the qPCR product showing the VEGFR-2 bands (103 bps). qPCR product from tumor grades I, II and III in Lanes 1, 2 and 3 respectively, 100 bp ladder of Thermo scientific in Lane 4.

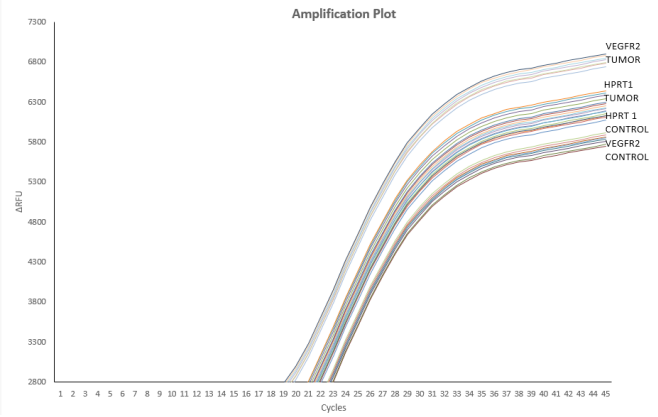

Fig. 2: Amplification plot of the qPCR data.

Table 2: Fold change of VEGFR-2 among tumor grades (Mean ± SE)

Tumor Grade	Grade-I	Grade-II	Grade-III
2-ΔΔCT of VEGFR-2	1.19 ± 0.04^{a}	2.28 ± 0.07^{b}	2.43 ± 0.05^{b}

Different superscripts ^{a,b} indicate significant differences at $p < 0.05$

given by the manufacturer of the kit. After the quality of the synthesized cDNA was assessed using Nanodrop, expression profiling was done for VEGFR-2 gene with HPRT-1 gene as endogenous control. Gene-specific primers for canine VEGFR-2 (Jais *et al.*,2011) with NCBI accession number NM_001048024.1 and canine-specific HPRT-1 primers were designed using the sequence from NCBI with accession No. NM_001003357.1 and used as endogenous control. The primers' details are given in Table 1.

QuantStudio™ 3 of Applied Biosystems was used to do the expression studies using the Bright Green qPCR Master mix-Low ROX. The final 20 µL qPCR reaction mixture consisted of with 1 µL template cDNA (100 ng), 0.25 µL each of forward and reverse primer (10 pmol/ µL), 10 µL of master mix and 8.5 µL of nuclease free water with qPCR cycling conditions as follows: Initial denaturation: 95°C for 10 mins; Denaturation: 95°C for 15 sec; Annealing: 60°C for 30 sec; Extension: 60°C for 40 sec x 40 cycles ; Dissociation step: 95°C for 60 sec with a ramp of 0.15°C/sec. The relative gene expression, *i.e.,* the qPCR data were expressed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), wherein the qPCR data were expressed as fold change in gene expression in the tumor sample, which was normalized to the geometric mean of reference gene relative to the normal mammary gland. To compare the expression of VEGFR-2 in different grades of tumors, a t-test was performed.

Results and Discussion

The qPCR data obtained for VEGFR-2 from the tissues with endogenous housekeeping control *i.e.,* HPRT-1 was subjected to $2^{-\Delta\Delta CT}$ method of expression, a method of relative expression of the gene of interest (VEGFR-2) with respect to endogenous housekeeping control, *i.e.,* HPRT-1. The amplification plot of VEGFR-2 and HPRT genes in normal and tumor conditions is given in Fig. 2. The calculated fold change values are presented in Table 2.

The Electrophoresis gel run of the qPCR product showing the VEGFR-2 bands is given in the Fig. 1. The amplification plot of VEGFR-2 and HPRT genes in normal and tumor conditions is given in Fig. 2

It was found that the VEGFR-2 was up-regulated in all the grades of the tumors. VEGFR-2 was 1.19±0.04 folds up-regulated in Grade-I tumors compared to the normal tissues. In Grade-II tumors, VEGFR-2 has up-regulated 2.28±0.07 folds, and in Grade-III, 2.43±0.05. The VEGFR-2 expression was significantly ($P<0.05$) higher in Grade-II than in Grade-I, and there is an increasing trend in Grade-III compared to Grade-II, but a significant difference could not

be found between Grade II and Grade III. However, VEGFR-2 expression showed a significantly ($P<0.05$) higher value than Grade-I. In the present study, it was noticed that the VEGFR-2 expression was increasing as the grades of the tumor became higher from Grade-I to III. A similar trend was reported by Dos Anjos *et al.* (2019). They studied the expression of VEGFR-2 by immunohistochemistry and reported that higher-grade tumors show higher expression of VEGFR-2 as there will be more requirements of the receptor and its ligand for the more significant angiogenesis and cell proliferation. Santos *et al.* (2016) studied the VEGFR-2 expression with immunohistological studies and observed that VEGFR-2 was a determinant for the activation and mitogenesis of endothelial cells during the angiogenic shift. Diessler *et al.* (2016) observed an increase in VEGFR2 expression in histological grade tumors from I to III and attributed the same to the angiogenic behavior of the canine mammary tumors. VEGF/VEGFR2 signaling pathway has been well-established to promote neighboring vessel formation, thereby facilitating the delivery of nutrients for cancer cell survival (Lian *et al.,* 2019), and Prado *et al.* (2019) observed that vascular mimicry was a common finding in tumors overexpressing VEGFR-2. By far, all the above references are in accordance to the present finding, which suggests that higher grade tumors, as they require more blood supply and cell proliferation, need more angiogenesis supported by the over expression of VEGFR-2.

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References

Diessler, M.E., Castellano, M.C., Portiansky, E.L., Burns, S. & Idiart, J.R. (2017). Canine mammary carcinomas: influence of histological grade, vascular invasion, proliferation, microvessel density and VEGFR2 expression on lymph node status and survival time. *Veterinary and comparative oncology*, *15 (2):*450-461.

Dos Anjos, D.S., Vital, A.F., Lainetti, P.D.F., Leis-Filho, A.F., Dalmolin, F., Elias, F., Calazans, S.G. & Fonseca-Alves, C.E. (2019). Deregulation of VEGFR-2 and PDGFR expression and microvascular density in a triple-negative model of canine malignant mammary tumors with lymph node or lung metastasis. *Veterinary sciences*, *6(1):*3.

Goldschmidt, M., Pen˜a, L., Rasotto, R. & Zappulli V. (2011). Classification and Grading of Canine Mammary Tumors. *Veterinary Pathology*, *48(1*): 117-131

Guo, S., Colbert, L.S., Fuller, M., Zhang, Y. & Gonzalez-Perez, R.R. (2010). Vascular endothelial growthfactor receptor-2 in breast cancer. *BiochimicaetBiophysicaActa*. *1806*:108-1221.

Hanahan, D. & Weinberg, R.A. (2000). The hallmarks of cancer, *Cell*,*100*:57-70.

Jais, A., Klein, D., Wolfesberger, B. & Walter, I. (2011). Gene expression profile of vascular endothelial growth factor (VEGF) and its receptors in various cell types of the canine lymph node using laser capture microdissection (LCM). *Veterinary immunology and immunopathology*, *140(3-4*), 207-214.

Koch, S., Tugues, S., Li, X., Gualandi, L. & Claesson-Welsh, L. (2011). Signal transduction by vascular endothelial growth factor receptors.*Biochemical Journal*, *437*, 169–183.

Lian, L., Li, X.L., Xu, M.D., Li, X.M., Wu, M.Y., Zhang, Y., Tao, M., Li, W., Shen, X.M., Zhou, C. & Jiang, M. (2019). VEGFR2 promotes tumorigenesis and metastasis in apro-angiogenic-independent way in gastric cancer. *BMC cancer*, *19*:183

Luo, H., Jiang, B-H., King, S.M. & Chen, Y.C. (2008). Inhibition of cell growth and VEGF expression in ovarian cancer cells by flavonoids. *Nutrition and Cancer*. 60:800-809.

Nessa. A., Latif, S.A., Siddiqui, N.I., Hussain, M.A., Bhuiyan, M.R., Hossain, M.A., Akther, A. and Rahman, M. (2009). Angiogenesis. A novel therapeutic approach for ischemic heart disease. *Mymensingh Medical Journal: MMJ*.,*18*:264-272.

Prado, M.C.M., Macedo, S.D.A.L., Guiraldelli, G.G., De FariaLainetti, P., Leis-Filho, A.F., Kobayashi, P.E., Laufer-Amorim, R. & Fonseca-Alves, C.E. (2019). Investigation of the Prognostic Significance of Vasculogenic Mimicry and Its Inhibition by Sorafenib in Canine Mammary Gland Tumors. *Frontiers in Oncology, 9*:1445.

Santos, A., Lopes, C., Gärtner, F. & Matos, A.J.F. (2016). VEGFR-2 expression in malignant tumours of the canine mammary gland: a prospective survival study. *Veterinary and comparative oncology*, *14(3):*e83-e92.

Sarabipour, S., Ballmer-Hofer, K., *and* Hristova, K. (2016). VEGFR-2 conformational switch in response to ligand binding. eLife, *5*, e13876

Schmittgen, T. D. & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, *3*: 1101-1108

Takahashi, H. *and* Shibuya, M. (2005). The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clinical scienc*e, *109(3)*:227-241

Teleanu, R.I., Chircov, C., Grumezescu, A.M. & Teleanu, D.M. (2020). Tumor Angiogenesis and Anti-Angiogenic Strategiesfor Cancer Treatment. *Journal of Clinical Medicine*, *9*:84.