Research Article

VEGF-C and p53 Gene Expression in the Normal and Neoplastic Mammary Gland of Canines: A Pilot Study

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Abstract

The p53 is a tumor suppressor protein that plays an essential role in controlling the cell cycle. In addition, vascular endothelial growth factor (VEGF) is one of the most strong and specific angiogenic factors. The main objective of this study was to evaluate the impact of p53 and VEGF-C gene expression in the neoplastic and normal mammary glands of canines as an animal model. Eleven benign and malignant and five normal specimens were collected. After RNA extraction and cDNA synthesis, relative quantification of *p53* and *VEGF-C* genes was accomplished by real-time quantitative PCR (RT-qPCR), in which β-actin was used as a reference gene. The relative mRNA expression of the *p53* and *VEGF-C* genes was analyzed by GLM procedure of SAS software v9.2. The results indicated that the VEGF-C and p53 mRNA expression in neoplastic specimens was over-and down-expressed, respectively, compared with normal specimens. The p53 mRNA expression was significantly negatively associated with VEGF-C (~4 fold) in neoplastic specimens ($P < 0.01$). These findings emphasized that simultaneous evaluation of p53 and VEGF-C expression can be used as tumor biomarkers for the early diagnosis of malignancy in canines. Furthermore, RT-qPCR is a rapid and sensitive method for monitoring and investigating suspicious canines at the early stage of malignancy and may provide an alternative explanation for deregulated p53 signaling in breast cancer.

***Keywords*:** Canine mammary tumor, Breast cancer, p53, VEGF-C, Real-time PCR

Introduction

Breast cancer is one of the most prevalent types of human and canine neoplasia. Although there are numerous reports of mammary tumors in both man and male dogs (Li et al., 2012; Saba et al., 2007), it is rated the most common malignancy in women and female canines (Ghoncheh et al., 2016; Kaszak et al., 2018). The canine mammary tumor (CMT) is frequently diagnosed in dogs, accounting for 52% of all tumors, and is the most typical form of malignant neoplasia of the bitch (Kaszak et al., 2018). Due to the ethical issues and scarcity of human tissue sampling, various animals are used as human breast cancer models in current years (Abdelmegeed and Mohammed, 2018; Qiu et al., 2008a). Furthermore, recent studies showed clinical and molecular similarities between human breast cancer (HBC) and canine mammary tumors, including spontaneous tumor incidence, onset age, hormonal etiology, and molecular characteristics and gene expressions (Abdelmegeed and Mohammed, 2018; Queiroga et al., 2011; Visan et al., 2016). The etiology of BHC and CMT is multifactorial and includes factors such as genetic predisposition, the timing of onset of menarche and first pregnancy, and hormonal receptor activity in the mammary tissues (Abdelmegeed and Mohammed, 2018).

Meta-analysis studies revealed that gene expression of mammary tumor cells varies, and this can be used as a marker for early diagnosis of the disease that may help evaluate the cancer progression and increase the chance of a cure by chemotherapy (Bell et al., 2017). Two of the most common tumor biomarkers as proteins that can be measured in blood or cancer tissues to show the presence of the disease identified in humans and dogs are p53 and VEGF-C (Bell et al., 2017; Klopfleisch and Gruber, 2009; Santos et al., 2010).

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p53 is an important tumor suppressor protein that plays an essential role in controlling the cell cycle by inducing apoptosis when cell damage cannot be repaired (Yang et al., 2013). It is located in the nucleus of the cell, which directly connects to the DNA. Following damage of DNA by various factors such as toxic chemicals, radiation, or ultraviolet (UV) rays from sunlight, p53, as a transcription factor, regulates the expression of genes involved in apoptosis (Levine, 2019). Mutations in the *p53* gene located in the chromosomes 17 and 5 of humans and dogs respectively seem to play a critical role as an oncogene in the carcinogenesis of mammary glands and tumor progression (Lee and Kweon, 2002). The vascular endothelial growth factor (VEGF) family is one of the most strong and specific angiogenic factors and is a well-known biomarker in oncologic studies [7]. VEGF proteins are encoded by four genes: *VEGF-A*, *VEGF-B*, *VEGF-C*, and *VEGF-D* (Kaszak et al., 2018). While *VEGF-A* and *VEGF-B* are responsible for angiogenesis, *VEGF-C* and *VEGF-D* have a key role in lymphangiogenesis (Karpanen et al., 2001). VEGFs mediates new vessel formation and regulates their functions and structures in healthy tissues (Karpanen et al., 2001; Millanta et al., 2010). Increased expression of VEGF in numerous human cancer cells is a main factor in the growth of malignant tumors and muscle destruction. Furthermore, overexpression of VEGF-C led to enhanced metastasis of regional lymph nodes and invasive lymphatic vessels in breast cancer in humans and canines (Karpanen et al., 2001; Qiu et al., 2008a).

In recent decades, real-time quantitative PCR (RT-qPCR) is one of the most useful biomolecular techniques that have been used for gene expression studies. In the present study, we established an RT-qPCR method to quantify the expression of *p53* and *VEGF-C* genes accurately and reproducibly in normal and neoplastic canine mammary glands.

Materials and Methods

Animals and Tissue Samples

A total of 11 adult intact bitches of various breeds bearing CMT that had not received any chemotherapy treatments before surgery were included in this study. All bitches were referred to the veterinary teaching hospital, the Ferdowsi University of Mashhad, for surgical excision of mammary tumors. Mammary tumor and normal mammary tissues were collected from the same bitch to avoid the different endocrine status among individual bitches.

Sample Collection and Histopathological Analysis

Both mammary tumors and contralateral normal mammary tissues from the same canines were obtained during the surgical procedure. Immediately after surgical excision, each tissue sample was divided into two parts. One part was maintained in liquid nitrogen for real-time PCR analysis. The other half of the sample was fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Tissues were sectioned in 4 μm slices for hematoxylin and eosin staining and send for histopathology analysis. Tumor characteristics such as degree of differentiation and the other associated tumor properties were analyzed (Goldschmidt et al., 2011)

RNA Extraction and cDNA Synthesis

Total RNA was extracted from mammary gland specimens using Trizol kit (Iso Gene Company, Moscow, Russia) and treated with RNase-free DNase I to remove any DNA contamination. RNAs were reverse transcribed and cDNAs synthesized using RevertAid™ H minus Reverse Transcriptase kit (Fermentas Company, Burlington, USA). The quantity of RNA and cDNA samples was determined by Nano-Drop ND 2000 spectrophotometer (Thermo, Wilmington, USA). cDNAs were diluted at 300 ng/μl concentration for uniformity by DNase-free diluted water.

Primer Design

Primers for *β-actin*, as the reference gene, *p53*, and *VEGF-C*, as target genes, were designed by the Primer premier software, version5 (Table 1). Primers were blasted in the primer database such as RT (http://rtprimerdb.org) to confirm the total gene specificity of the nucleotide sequences chosen for the primers and the structure of primers.

Table 1. The Specifications of the primers used in the Real-Time PCR reactions

Gene	Primer sequence	Application size	The accession number of related genes

p53	Forward 5' TGACAGTAGTGACGGTCTTGCC 3'	117	NM_001003210.
	Reverse 5' TCATAAGGCACCACCACACTG 3'		1
VEGF-C	Forward 5' GAGCAGCAACAAACACCTTCTT 3'	110	
	Reverse 5' GAGGTGGCTTGTGCTGGTG 3'		XM-540047
beta-Actin	Forward 5' CAAATGTGGATCAGCAAGCAG 3'	103	
	Reverse 5' GAAAGGGTGTAACGCAACTAAAG 3'		XM-544346

Real-time Quantitative Reverse Transcriptase PCR Assay

300 ng of cDNA were amplified in a real-time quantitative polymerase chain reaction (RT-qPCR) using TaqMan Universal Master Mix (PE Applied Biosystems), 0.8 ng primers for p53, β-actin, and VEGF-C. The RT-qPCRs were performed in an ABI PRISM Model 7300 sequence detector by using the fluorescent dye SYBR Green I. The optimum concentration of primers was determined in preliminary experiments. Thermal cycling conditions included initial denaturation in 1 cycle of 10 minutes at 95°C, followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C and melting curve in 1 cycle of 15 seconds at 95°C, one minute at 60°C, 15 seconds at 95°C and 15 seconds at 60°C. A melting curve was performed after qPCR cycles to verify amplification specificity. Reactions without reverse transcriptase or template served as controls for p53 and VEGF-C genomic DNA contamination. The specificity of the amplified products was confirmed by gel electrophoresis (1.5% agarose gels).

Quantification of Target Gene Expression

PCR efficiency and data analysis were performed using the Pfaffle method (Pfaffl, 2001). The standard curve simplifies calculations and avoids practical and theoretical problems currently associated with relative real-time PCR-efficiency assessment. p53 and VEGF-C standard curve by real-time PCR was plotted by serial dilution of Ct values vs. log of input cDNAs. A standard curve slope of –3.32 indicates a PCR reaction with 100% efficiency. The slope of this curve was –3.1, and it was in the expected range. In this study, PCR efficiency was noted 95% for p53, VEGF-C, and β-actin genes. After determining the expression content of the *VEGF-C* and *p53* genes for each cancerous sample, the obtained value is divided into the mean internal control of normal samples (β-actin) and the relative expression of these genes obtained according to mean ± SD for each cancerous sample.

Statistical Analysis

All samples were analyzed in triplicate. Statistical analysis was performed using the SDS software (v1.4). Fisher's exact test was used for categorical variables. Student t-test procedure was performed in SAS (v9.2) and Microsoft Excel to determine statistical significance. The level of significance was 5% ($P < 0.05$).

Results

Histopathological Analysis

Of eleven dogs, four had benign mammary gland tumors, including two benign mixed-type tumors and two fibroadenomas (Table 2). From seven malignant mammary gland tumors (Figure.1. A-D), one showed carcinosarcoma features with malignant epithelial and myoepithelial cells with connective tissues (Figure.1A). Another case showed complex carcinoma features with proliferated myoepithelial cells and abundant chondromucinous substance (Figure.1B). Among the cases, extensive necrosis was noted in one with features of a solid tumor (Figure.1C). Tubulopapillary subtype was also noted in one case (Figure.1D), which showed papillary projections with hyperchromatic nuclei.

Table 2. Histopathological classification of tumor

Histological diagnosis	Number of cases
Benign tumor	
Benign mixed tumor	2
Fibroadenoma	2
Malignant tumor	
Tubulopapillary carcinoma	1
Simple carcinoma	2
Cystic papillary carcinoma	1
Complex-type carcinoma	3

Total	11

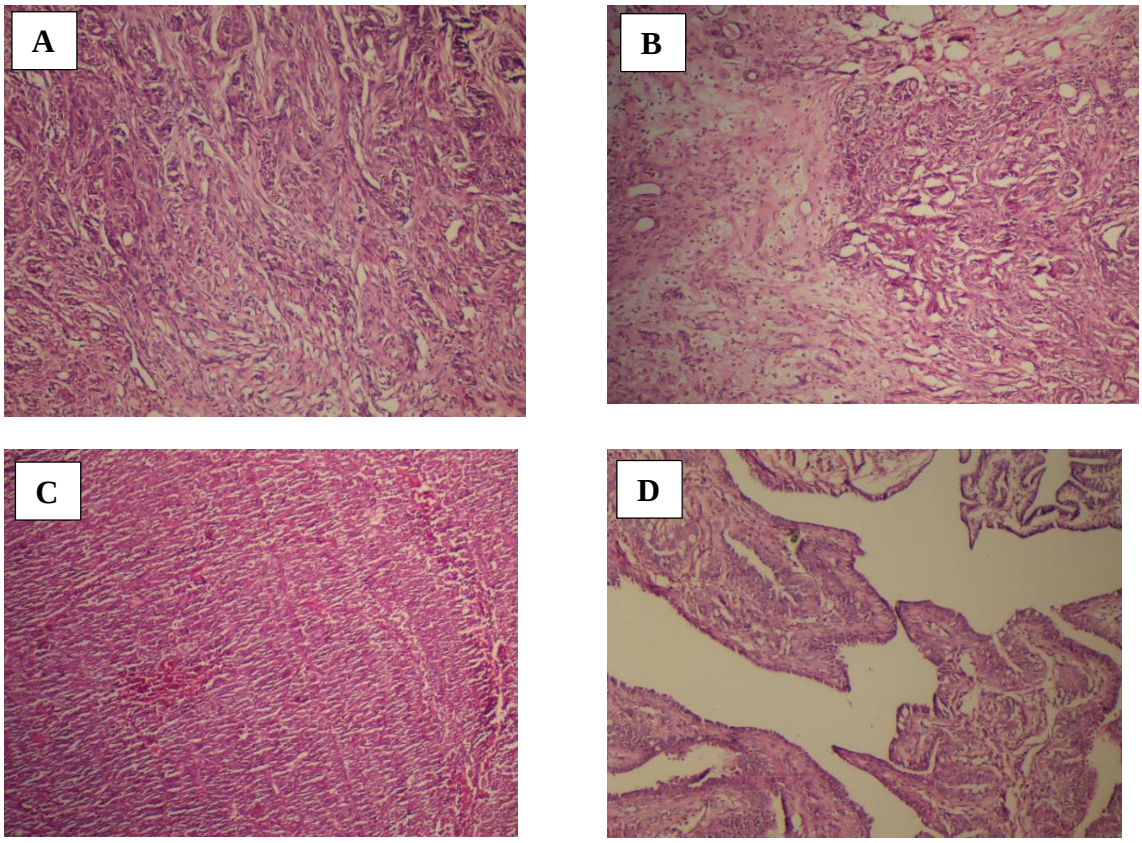

Figure 1. Histopathology of different types of malignant canine mammary glands.
A: Carcinosarcoma, a tumor composed of cells morphologically resembling malignant epithelial and myoepithelial cells with malignant connective tissue B: Complex carcinoma showed proliferation of luminal epithelial cells with pleomorphic and hyperchromatic nuclei and also the proliferation of spindle-shaped myoepithelial cells arranged in a stellate pattern with chondromucinous substance C: Caudal mammary gland as Solid carcinoma, tumor cells were arranged in solid sheets. Some tumor cells showed vacuolated cytoplasm. There were scattered necrotic foci. D: Large mammary gland, Tubulopapillary carcinoma. There are tubules with papillary projections consist of tumor cells with hyperchromatic nuclei. Mitotic figures were 8 per 10 HPF.

Gene Expression

To test the VEGF-C and p53 expression in CMT compared to the normal mammary gland of the same dogs, mRNA copy numbers (Ct) of VEGF-C per mRNA the reference gene was determined using RT-qPCR. The results showed that VEGF-C was overexpressed significantly (approximately 4-fold change) in the neoplastic tissue compared to normal tissues (Figure 2). On the other hand, tumor suppressor p53 gene expression in cancerous tissues was significantly lower than normal mammary glands.

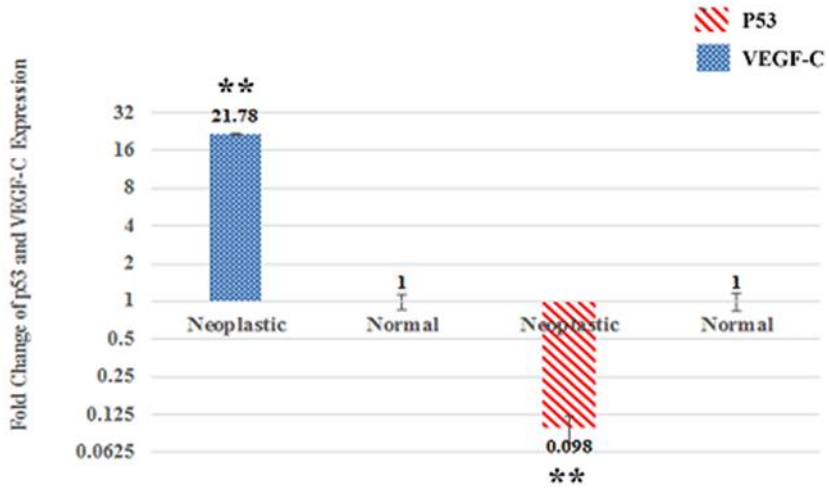

Figure 2. Normalized expression fold change indicates the mean expression of VEGF-C and p53 in the normal and neoplastic mammary glands. While VEGF-C was overexpressed in CMT samples, p53 was down-expressed in neoplastic tissues (* $P < 0.05$, ** $P < 0.01$).

Discussion

Canine mammary tumors (CMT) are usually reported in female elderly dogs (8-10 years old) and may vary depending on different breeds and lifestyles (Raposo et al., 2017; Sorenmo et al., 2011). The second global leading cause of death due to cancer among women is breast cancer. Common clinical and epidemiological features between HBC and CMT, including incidence rate and mortality, age of onset and an identical course of the disease, histopathological symptoms, hormonal etiology, as well as molecular markers, have been described in recent years (Visan et al., 2016; Garcia et al., 2021). These common clinical and epidemiological features make canine a suitable animal model to investigate different diagnoses and therapies of breast neoplasia, leading to comparative oncological studies (Abdelmegeed and Mohammed, 2018; Raposo et al., 2017; Visan et al., 2016). So far, surgery and removal of the affected glands are the main available treatment for HBC and CMT. In most malignant cases, follow-up chemotherapy or radiotherapy is performed, which is costly and might not be efficient (Kaszak et al., 2018). Therefore, the early detection of neoplasia seems essential in the disease prognosis in humans and dogs. Nowadays, biomarker investigation is suggested as a suitable way for the early diagnosis and evaluation of the risk assessments and prognosis of HBC and CMT (Chen et al., 2017; Ma et al., 2010).

Immunohistochemistry (IHS) analysis has been vastly used to investigate and evaluate the expression of proteins as biomarkers in breast cancer, either in humans or canines. However, quantification of biomarker expression by IHS is difficult. The fluorescence-based detection methods, like real-time quantitative PCR, have emerged as an accurate and sensitive technique to investigate the mRNA expression of different genes, including tumor biomarkers. The expression of p53 and VEGFs, two of the most studied common biomarkers, in dog normal and neoplastic mammary glands have been evaluated. Here, we conducted RT-qPCR as a rapid and precise method targeting mRNA using the fluorescent dye SYBR Green I.

Some specific protein biomarkers expressed by cancerous cells can be detected in serum or tissues and are reported to be common in humans and dogs (Kaszak et al., 2018; Pena et al., 2014; Qiu et al., 2008c; Raposo et al., 2017). VEGFs and p53 have an essential role in HBC and CMT (Howard et al., 2004; Karpanen et al., 2001; Levine, 2019). The *p53* is a tumor suppressor gene that acts as a transcription factor and plays a vital role in genome stability by regulating cell proliferation, cellular death, and repairing damaged DNA (Wijnhoven et al., 2005). The amino acid sequence of the p53 protein in dogs is approximately 87% homologous to the human one. Like humans, it is mutated in different types of canine tumors, including CMT (Zhang et al., 2009).

Several studies showed numerous mutations leading to a different level of p53 expression in HBC as well as CMT and its direct correlation with tumor prognosis (Bae et al., 2018; Gasco et al., 2002; Howard et al., 2004; Lee et al., 2004; Levine, 2019;

Wang, 2017). Dolka et al. reported that expression of p53 was positive in only 30% of CMTs, depending on the tumor malignancy and the breed of the dogs (Dolka et al., 2016). Klopfleisch and Gruber showed the heterogeneous expression of p53 in lymph nodes metastasizing canine mammary adenocarcinoma and normal gland using real-time PCR and questioned the prognostic significance of p53 (Klopfleisch and Gruber, 2009). They only found a few significantly increased expressions of p53 in a low sample size (20% of adenomas and 10% of adenocarcinomas). Our results confirmed the previous findings by Ripoli et al. in 2016, which showed the lower expression of p53 in malignant tissues compared to normal tissues (Lüder Ripoli, 2016). The controversial reports of the p53 expression levels in canines might be due to a correlation between its expression and differences in dog breed (Veldhoen et al., 1999). It is demonstrated that expression of p53 is mainly associated with the weight of breed dogs as it was found in 67% of large breed dogs with CMTs in the study of Dolka et al. (Dolka et al., 2016). Since p53 plays as a tumor suppressor protein, its lower expression in cancerous tissues found in our study might lead to uncontrolled cell cycles and hyperplasia in mammary glands. It is reported that the less expression or inactivation of p53 in neoplastic tissues is due to numerous mechanisms mainly caused by mutations in the gene (Gasco et al., 2002; Muto et al., 2000).

The vascular endothelial growth factor (VEGF) family, which includes VEGF-A - D, in many human tumor types, plays an essential role in the induction of angiogenesis and uses as the most frequent biomarker in human clinical medicine (Kaszak et al., 2018). VEGF-C is believed to be a critical factor in lymph angiogenesis, leading to a poor prognosis of aggressive breast cancer (Karpanen et al., 2001). Overexpression of VEGF-C in HBC and CMT is associated with malignant tumors and a bad prognosis. It can be detected in serum and tissue, making it a useful biomarker in early HBC and CMT (Santos et al., 2010; Zajkowska et al., 2016). Higher expression of VEGF-C in malignant cases of both HBC and CMT is demonstrated (Mohammed et al., 2007; Qiu et al., 2008a). Furthermore, Thammineni et al. recommend VEGF-C evaluation as a diagnostic biomarker of lymph node metastasis in patients with breast cancer (Thammineni et al., 2019). The VEGF expression was significantly higher in malignant CMT cases than benign using IHS and RT-qPCR (Anadol et al., 2017; Qiu et al., 2008a; Queiroga et al., 2011). The use of immunohistochemistry showed that VEGFs increased in cancer tissues, serum, and plasma of animals with cancer compared to normal (Kato et al., 2007). Our results confirm that previous studies showed significant VEGF-C overexpression in malignant CMT compared to benign CMT using RT-qPCR. Furthermore, high VEGF-C expression was observed in CMTs with lymph node metastasis compared to the tumors without one (Qiu et al., 2008b). The correlation between the higher expression of VEGF-C and lymph node metastasis and its prognosis was also observed in human breast cancer (Chen et al., 2017; Li et al., 2012; Liang and Li, 2014; Saba et al., 2007). In our results, the expression of VEGF-C was more (4-fold overexpression) in tumor tissues than normal.

In HBC, the correlation between the expression of p53 and VEGF was controversial. While Lu et al. and Howard et al. found no correlation between the expression of p53 and VEGF in invasive breast cancer and primary breast tumor respectively (Howard et al., 2004; Lu et al., 2008), some studies found a positive correlation in patients with breast cancer and suggested it as the higher risk factor. (Linderholm et al., 2000; Noranizah et al., 2010). Iovino et al. showed a significant positive correlation between VEGF serum level and p53 overexpression in primary endocrine-positive breast cancer patients (Iovino et al., 2008). To the best of our knowledge, it seems that this is the first study investigating the simultaneous expression of p53 and VEGF in canines. Our findings showed the correlation between the higher expression of VEGF-C and lower expression of p53 in canine neoplastic mammary glands, which might be due to the mutation in p53 and its effect on VEGF and can cause poor prognosis (Linderholm et al., 2000; Linderholm et al., 2001).

In summary, our results showed that quantitative real-time PCR could be used as a sensitive and rapid method to investigate the quantification of biomarker expression, including p53 and VEFG-C, in different types of CMT. Furthermore, our finding suggested that overexpression of VEGF-C and down-expression of p53 may contribute to the malignancy of CMT and help the researchers for early diagnosis of malignant tumors, which help to prevent the metastasis of CMTs.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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