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Increased VEGF, COX-2 and CDH-1 Expression Favor TVT Implantation?

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ABSTRACT

***Background*:** The transmissible venereal tumor (TVT) is a round cell neoplasm that can be implanted by physical contact between the affected dog and a susceptible host, and can affect males and females. It mainly affects the genital areas, but can be observed in the oral cavity, nasal cavity, eyes and on the skin. Macroscopically, the tumor is characterized by a reddish color, with a "cauliflower" appearance, friable consistency and hemorrhagic exudate, with cytology and histopathology being performed to confirm the diagnosis. Despite rare or diagnosed cases, metastatic TVT is mainly described in adjacent lymph nodes (inguinal and popliteal), spleen, liver, eyes, and bone marrow. Due to the low metastatic rate and the high implantation ability of canine transmissible venereal tumor, the study of the factors that are possibly involved in this process is necessary. Among the genes that contribute to metastasis and reduction of cell adhesion, VEGF, COX-2 and E-cadherin (CDH-1) are mentioned, which may encode proteins involved in angiogenesis and neovascularization, reduction of cell adhesion, cell proliferation and inhibition of apoptosis. COX-2 has an important role in angiogenesis as well as cell proliferation and inhibition of apoptosis. The increase in COX-2 stimulates endothelial growth factors, such as VEGF, promoting angiogenesis. Furthermore, new blood or lymphatic vessels predispose the dissemination of neoplastic cells, facilitating metastasis. Another important factor is intercellular adhesion. For the loss of intercellular adhesion, it is necessary to reduce the gene or protein of E-Cadherin, promoting the disaggregation of neoplastic cells, thus facilitating distant dissemination. Furthermore, at the moment the cells find their adhesion site, the low expression of E-Cadherin promotes the release of proteins from the cell adhesion complex such as β-Catenin with the aim of aggregation and development of the metastatic focus. Therefore, the aim of this study was to evaluate the gene expression of COX-2, VEGF and CDH1 in transmissible venereal tumor, to verify the role of these genes in tumor progression and in the mechanism of implantation of neoplastic cells, characteristic of this neoplasm.

***Materials, Methods & Results*:** A total of 32 dogs from the Zoonoses Center (Botucatu, SP and Seropédica, RJ, Brazil) with transmissible venereal tumor were selected, 19 females and 13 males, with an average age of 4 years (from 2 to 6 years). Tumor samples were collected from the vulva or penis and immediately frozen in liquid nitrogen and kept at -80ºC in the freezer. The samples were subsequently subjected to cleavage, followed by RNA extraction using the Trizol® protocol. The material's mRNA was extracted for qRT-PCR. For statistical analysis, the normality of the data was evaluated and the gene expression values of COX-2, VEGF and CDH-1 of the TVT were compared with the control group using the Mann-Whitney test ($P < 0.05$). All analyzes were carried out using the GraphPrisma program, considering a significance level of 95%. The correlation between variables was performed using the Spearman test ($P < 0.05$). The control group was made up of 10 healthy dogs, with an average age of 2.5 years, of no defined breed, 6 females and 4 males, where blood was collected and subsequent leukocyte extraction. In the evaluation of COX-2, VEGF, CDH-1, a significant increase ($P < 0.0001$; $P < 0.0001$; $P = 0.04$, respectively) in the expression of gene transcripts in tumor tissue (TVT) was observed when compared with the control group. With this result, the biological behavior of the transmissible neoplasm can be explained, a fact that may favor the potential for implantation of this tumor in another animal.

Keywords: cell adhesion, genes, metastasis, oncology.

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INTRODUCTION

Transmissible venereal tumor (TVT) is a round cell neoplasm that can be implanted by simple physical contact between the affected dog and a susceptible host [11]. TVT has been reported in oral and nasal cavity and skin [13]. Despite the rare or underdiagnosed cases, metastatic TVT is described, mainly, in adjacent lymph nodes (inguinal and popliteal), spleen, liver, eyes and marrow bone [26].

Among the genes involved in the neoplastic process COX-2, VEGF and E-cadherin (CDH-1) can be cited. COX-2 has an important role in angiogenesis, as well as in the cell proliferation and apoptosis inhibition [7]. The increase of COX-2 stimulates endothelial growth factors such as VEGF, which in turn, promotes angiogenesis [11,32]. Furthermore, the new blood or lymphatic vessels predispose neoplastic cell dissemination leading to metastasis [10,27].

Another important factor of metastasis formation is the loss of intercellular adhesion. The low expression of CDH-1 promotes disaggregation of neoplastic cells and leads to its dissemination [38]. Once these cells find a new distant site, the release of cell adhesion proteins complex, such as β-catenin, occurs to aggregate and develop the metastatic foci [4,6,25].

The aim of this study was to evaluate COX-2, VEGF and CDH1 expression in canine transmissible venereal tumor to understand the role of these genes in tumoral progression and implantation mechanisms of TVT.

MATERIALS AND METHODS

Specimen

A total of 32 dogs with TVT from the Zoonosis Center (Botucatu, SP and Seropédica, RJ, Brazil) were selected, of which 19 females and 13 males, averaged 4 years old (range 2-6 years). From the total, 29 dogs were mongrel dogs, 1 Pit Bull and 2 Poodles. Primary diagnosis was made by cytology and the lesion was collected for histopathology in 10% buffer formalin and frozen in liquid nitrogen for gene expression. The lesions were collected from vulva or penis.

The control group consisted of leukocytes extracted from peripheral blood of 10 healthy dogs. The subjects were 2 to 3-year-old mixed breed female or male dogs (average 2.5 years old) selected based on clinical examination to confirm that they were healthy.

Collecting material

To collect TVT fragments, the dogs were pre-medicated with midazolam[1] [0.3 mg/kg - IM] associated with tramadol[2] [TRAmadon® - 3 mg/kg, IM]. After 15 min fluid therapy with 0.9% saline solution [10 mL/kg/h, IV] was instituted intravenously. Anesthetic induction was obtained with propofol[2] [PROpovan® - 4 mg/kg, IV]. Subsequently, the tumor samples were collected. Approximately 40 mg of tumor were removed with a scalpel and separated into 2 fragments of 20 mg that were immediately placed in sterile cryotubes and kept in liquid nitrogen until stored in a freezer at -80ºC. The TVT frozen samples were sequentially cut in a cryostat microtome[3] into 5 µm histological sections. The slides were stained with hematoxylin and eosin (HE) and analyzed under a light microscope[4] in order to verify the presence of the tumor in the frozen samples. All samples had 95% of neoplastic cells.

Subsequently, these samples were subjected to cleavage through pipes with magnetic bids in the equipment Precellys[4], followed by RNA extraction using TRIzol®[5] protocol, following the manufacturer's instructions.

To compare TVT COX-2, VEGF and CDH-1 expression, blood sample was drawn from 10 healthy dogs and conditioned in tubes with anticoagulant and immediately refrigerated. The blood samples were centrifuged at 4°C for 10 min at 123RCF. Subsequently, the isolated leukocytes were transferred to a new tube containing 1 mL of red cell lysis buffer (RCLB - pH 7.6 1X: 10 mM Tris, 5 mM $MgCl_2$ and 10 mM NaCl) and centrifuged at 4°C for 10 min at 123RCF to obtain a leukocyte pellet free of red blood cells. The leukocyte pellet was re-suspended in 1 mL TRIzol®[5] reagent for total RNA extraction.

RNA extraction

Total RNA from blood and TVT samples were extracted using the TRIzol®[5] according to the manufacturer's protocol. The samples were incubated for 5 min at room temperature, 200 µL of chloroform was added to the tubes. After a 3 min homogenization followed by 3 min incubation at room temperature, the samples were centrifuged at 12.000 x *g* for 15 min at 4°C. The supernatant was transferred to a new tube using a micropipette and added 500 µL of isopropyl alcohol. After incubation at room temperature for 10 min and centrifugation at 12.000 x *g* for 10 min at 4°C, the supernatant was removed, and the sediment washed with 70% ethanol. After another centrifugation at 7.500 x *g* for 5 min at 4°C, excess ethanol was removed by vacuum drying and the sediment re-suspended with water was free of DNAses and RNAses.

The RNA concentration and purity were assessed by spectrophotometer Nanodrop™ ND-8000[6],

while RNA integrity was evaluated by the Bionalyzer equipment (kit for RNA 6000 Nano Series)[7].

To eliminate any contamination with genomic DNA, the extracted total RNA was treated with DNAse. Reverse transcription from RNA to cDNA was performed using 1 µg of total RNA and the enzyme Super Script III™ Reverse Transcriptase[6]. The protocol uses 1 µL oligo(dT) (500 µg/mL), 1 µL random primers (100 µg/mL) and 1 µL dNTP. The mixture was heated at 65°C for 5 min, to which 4 µL of transcription buffer 5x, 1 µL of 0.1 M DTT and finally, 1 µL of enzyme Super Script III (200 U/µL) were added. Subsequently, the mixture was incubated at 25°C for 5 min; at 50°C for 1½ h; followed by 70°C for 15 min. After transcription, the cDNA was kept at -20°C.

COX-2, VEGF e CDH-1 primers were designed using the Primer Express software[7] and based on the works [5,17] (Table 1). The tested reference genes (endogenous) were RPS5, RPS19, RPL8, ACTB, HPRT, and the primers of these genes were obtained according a study [3]. The specificity of the primers of each gene was assessed by the BLAST software for both forward and reverse sequences.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The RT-qPCR reactions were standardized for each primer while the efficiencies of the reactions for each primer pair were also determined, using the standard curves from leukocytes cDNA.

The amplification reactions were performed in 96-well plates containing duplicates of each TVT and leukocyte sample that did not have cDNA, considered as a negative control. An 11.5 µL aliquot of a solution consisting of 6.25 µL of the reagent Power SYBR Green PCR MasterMix[7], 0.3 µL of each primer (reverse and forward sequences) at 10 Mm and 1µL of cDNA (dilution 1:10) was added to each well.

Amplification reaction conditions for all primers were 40 cycles of 15 s at 94°C and 1 min at 60°C. Dissociation curves were included at the end of each run to determine the specificity of the PCR products, visualized by a single peak of the amplified product.

The analysis of each reaction was performed by first determining the baseline, which is the fluorescence quality read during the PCR initial cycles. Subsequently, the threshold was determined according to Livak e Schmittgen [21].

Statistical analysis

For the statistical analysis, data normality was evaluated and the values from TVT COX-2, VEGF and CDH-1 gene expression were compared with blood samples (control) using the Mann-Whitney test. The correlation between TVT variables was performed by the Spearman test. All analyses were made using the GraphPrisma software, at 95% significance level.

RESULTS

It was observed a significant increase of COX-2, VEGF, CDH-1 ($P < 0.0001$; $P < 0.0001$; $P = 0.04$, respectively) in tumoral samples (TVT) when compared with control group (Figure 1A, 1B and Figure 2).

Table 1. Primers used for genic expression with aid of Primer Express program*.

Gene	FORWARD PRIMER (5' - 3')	Reverse PRIMER (5' - 3')
VEGFA	GCAACTCCGGCAGAAGCAT	TGGCGATCCAATTCCAAGAG
CDH1	CAGCATGGACTCAGAAGACAGAAG	TTCCGGGCAGCTGATAGG
PTGS2	GCTTCGATTGACCAGAGCAG	ACCATAAAGGGCCTCCAAG

*Primer Express program [5,17].

Table 2. Sequence of primers used in genic expression analyzes using software Primer Express 2.0*.

Gene	FORWARD PRIMER (5' - 3')	Reverse PRIMER (5' - 3')
RPS5	TCACTGGTGAGAACCCCCT	CCTGATTCACACGGCGTAG
RPS19	CCTTCCTCAAAAGTCTGGG'	GTTCTCATCGTAGGGAGCAAG
RPL8	CCATGAATCCTGTGGAGC	GTAGAGGGTTTGCCGATG'
ACTB	GGCATCCTGACCCTCAAGTA	CTTCTCCATGTCGTCCCAGT
HPRT	AGCTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC

*Software Primer Express 2.0 [3].

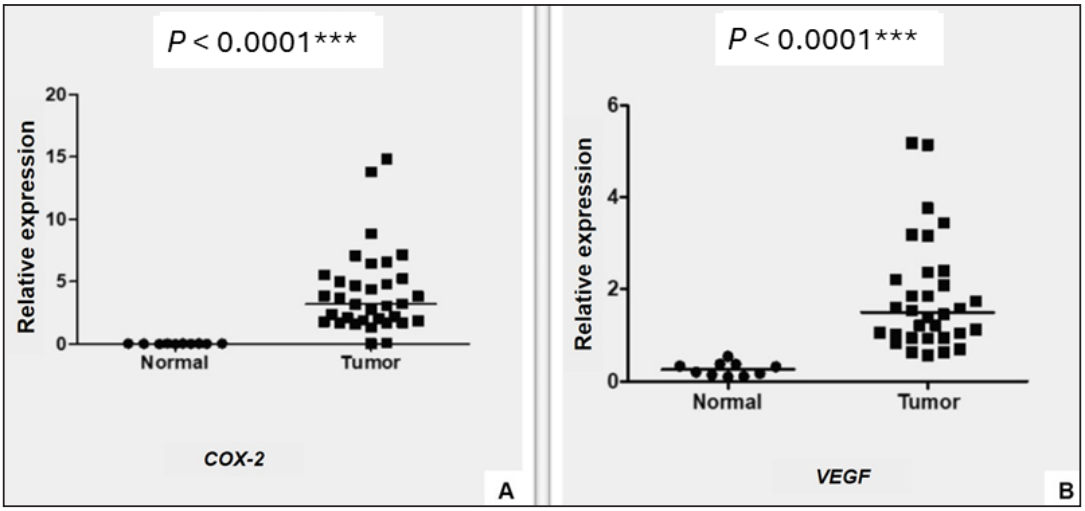

Figure 1. Graphic representation of median QR ± standard deviation of blood markers (Control group: normal; TVT: tumor). A- COX-2; B- VEGF. [Mann-Whitney test].

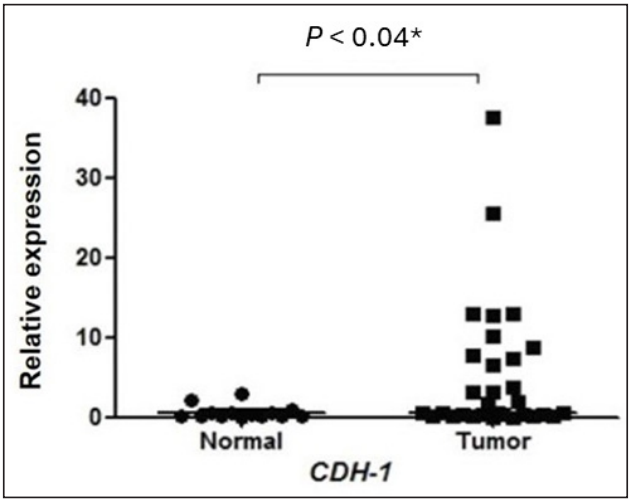

Figure 2. Graphic representation of median QR ± standard deviation of blood marker (CDH1) (Control group: normal; TVT: tumor). [Mann-Whitney test].

DISCUSSION

COX-2 has an important role in the development and invasion of cancer cells. Its high expression is directly related to endothelial growth factors, angiogenesis, and apoptosis inhibition [8,14,39]. In our evaluation of COX-2 expression, only TVT cells showed gene expression. This data corroborates what is described in the literature in which there is increased expression of COX-2 in mammary neoplasms in dogs [33], meningiomas [2] and in lung cancer in humans [9], evaluated using PCR.

Other studies evaluated COX-2 expression through immunohistochemistry, also found overexpression [20,29,31], who demonstrated that COX-2 expression is increased in several locally recurrent and metastatic neoplasms, such as carcinomas in the nasal cavity, prostate and breast in both humans and dogs, and these data are similar to our study.

Additionally, COX-2 is related to immune system and may occasion suppression of B and T lymphocytes, as well as inhibit natural killers' cells allowing tumor progression [16,37].

TVT is a highly vascularized tumor that presents rapid growth. Moreover, the COX-2 protein favors endothelial growth and angiogenesis providing nutrition and oxygen to the tissue [18]. Therefore, the high expression of COX-2 in our study may explain the growth rate of this tumor.

However, it is noteworthy that the rapid growth of TVT leads to regional hypoxia, ischemia and, consequently, necrosis, which represents the aggressive feature of malignancies. The low oxygen rate in the tissue stimulates the endothelial growth factor (VEGF) in order to form new blood vessels [11]. Thus, the peripheral cells of necrotic regions may express VEGF [12]. In fact, this event was observed in breast carcinomas in women, which can be related to our results, in view of the high levels of VEGF in the TVT group [24]. High immunomarking levels of VEGF was reported in breast carcinomas when compared with breast adenomas in women [1]. Studies also correlated higher expression

of VEGF in malignant mammary neoplasms than in the benign tumors in women [30,34].

Besides the angiogenic factors involved in tumoral progression, the loss of intercellular adhesion is necessary to trigger metastasis and cancer invasion. The decrease or absence of CDH-1 is an important step in this process [28]. In our study, there was statistical difference between TVT samples and the control group. The TVT group presented CDH-1 gene expression. In contrast, previous research reports different results, in which levels of CDH-1 presented no difference between benign mammary neoplasm and malignant mammary neoplasm in bitches [35]. On the other hand, in another study it was observed decreased CDH-1 expression along with increase of tumoral progression [25]. Also, Knudsen e Wheelock [19] compared 2 histological types of breast carcinomas in women and noted that invasive ductal carcinoma, a non-aggressive tumor, expressed CDH-1. Matos *et al*. [22] associated the expression of CDH-1, detected by immunohistochemical methods, with neoplastic histological types and verified that solid mammary carcinomas had low enzyme expression. This may suggest the high rate of metastasis in bitches with solid carcinoma.

In a study evaluating 24 oral melanomas in dogs, 4 samples (17%) presented CDH-1 expression, whereas in 20 (83%) the expression was low or absent [15]. This may elucidate the metastatic potential of melanoma. Mackowiak *et al*. [23] also observed a relationship between low expression of CDH-1 and malignity in a study with mastocytomas where those classified as grade III presented low CDH-1 when compared with grade I and II. Vara-Ramos *et al*. [36] also verified the opposite situation or, in other words, the relation of high expression and benignity, when evaluated 13 histiocytomas with strong CDH-1 immunomarking. Although the TVT is considered a malignant tumor, the incidence of metastasis is rare, possibly due to the presence of CHD-1.

CONCLUSION

There is a strong positive correlation between canine TVT and VEGF, COX-2 and CDH-1 gene expression when compared with leukocytes of healthy dogs. This relationship may explain the great implantation ability of this tumor.

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***Ethical approval*.** This experiment was ethical approved under protocol number 011303/11.

***Declaration of interest*.** The authors declare no conflicts of interest. Only the authors are responsible for the content and writing of this article.

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