Do alterations in gene expressions influence tumorigenesis in the transmissible venereal tumor in dogs?

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ABSTRACT: Canine transmissible venereal tumor (CTVT) is a transmissible neoplasm, which spreads naturally between dogs through the halogentic transfer of tumour cells, mainly during coitus. It is the oldest known tumoral lineage in nature and reports on gene mutations have been extended. Also, this tumor shares several genetic mutations with some cancers in humans, among them lung carcinomas, melanoma, prostate, breast, among others. Thus, expression of tumour suppressor genes such as TP53, P21, and apoptosis-related genes such as BAX, BCL-2, and BCL-xL, both in vivo and in vitro (primary cell culture) were quantified. In the present study, the comparison of gene expression, the TP53 gene, in most cases, was shown to be high in the majority of tissues (65%) and primary cell culture (100%), while BCL-2, BCL-xL, and BAX presented variation among the animals analyzed. Moreover, in these situations, the results suggested that the apoptotic regulation of these genes did not occur for TP53. The P21 gene was shown to be mostly normal (70%); although, absence (6%) and underexpressions (24%) were also observed. Statistical analysis of the BCL-xL gene demonstrated significant differences between the tissues of the animals when compared to the cell cultures; however, to the other genes, no statistical difference was observed between the groups. Preliminarily, the results suggested the presence of alterations in the gene expressions of the TP53, P21, BAX, BCL-2 and BCL-xL leading to loss of function in these genes, which affect the tumorigenesis of CTVT.

Key words: cancer, apoptosis, genetic alterations, cell cultures.

INTRODUCTION

The Canine transmissible venereal tumor (CTVT) arouses great interest in oncology researchers due to several characteristics, among them its controversial origin (MUKARATIRWA et al., 2003; FLOREZ et al., 2016; DUZANSKI et al., 2017), questionable spontaneous regression
Characterization of the gene expression profile in tumors has been a powerful approach for the delineation of the molecular pathways and altered cellular processes in the neoplasias (Murchison et al., 2014; Decker et al., 2015). Regarding CTVT, reports on gene mutations have been increasing (Murgia et al., 2006; Fonseca et al., 2012; Murchison et al., 2014)

The TP53 gene is a key component of the cell cycle and one of the most studied tumor suppressor genes. This gene is inactivated, commonly by mutation, in more than half of the cancers in humans (Kusewitt, 2013). The p53 protein encoded by this gene can positively regulate p21 expression, which induces the expression of repair genes against DNA damage, as well as having an active role in cell cycle arrest (Abbas & Dutta, 2009).

In addition to p21, TP53 also participates in the regulation of Bcl-2 family members. The Bcl-2 family is a group of proteins that induce or inhibit cell death by apoptosis. Some members of the Bcl-2 family, including Bcl-2 and Bcl-xL, are anti-apoptotic regulators that inhibit apoptosis, preventing the release of cytochrome c from mitochondria. Other members of this family, Bax, Bid and Bak, are pro-apoptotic proteins (Stockmann et al., 2011).

To date, in CTVT, there have been no reports on BCL-xL expression; however, BCL-2 expression has been identified, as well as mutations in TP53 (Choi & Kim, 2002; Vázquez-Mota et al., 2008; Alzate et al., 2009; Stockmann et al., 2011; Flórez et al., 2017). However, although there is evidence of mutations of the TP53 gene in CTVT, the ability of this mutation to cause functional changes in the protein, as well as in the tumor evolution remains unknown (Vázquez-Mota et al., 2008).

According to Stockmann et al. (2011) overexpression of Bcl-2 occurs independent of the stage of tumor development and this overexpression would promote the acquisition of functions in the tumor, which are associated with progression and survival. Similarly, AMARAL et al. (2011) showed that CTVTs with a lower degree of aggressiveness have a high apoptotic index and may present a better prognosis.

Due to the importance of better understanding the evolution and biological behavior of CTVT, this study aimed to observe if there are differences in the expression of tumor suppressor genes (TP53 and P21) and genes related to the apoptosis process (BCL-2, BCL-xL, and BAX) in vivo and in vitro (primary cell culture) in CTVT.

MATERIALS AND METHODS

Tumor collection

Once the diagnosis of CTVT was confirmed by cytological and histological analysis, and after anesthesia of the animal with total asepsis at the location, 2 replicates of the tumor samples were collected through incisional biopsy of fragments of approximately 1 cm³. The clinical data of the animals with CTVT submitted to the present study like sex, age (years), breed and tumors’ location are specified in table 1.

The RNA was isolated from 17 tumor tissues and 3 cell cultures from tumor tissues. The sample size followed the criteria established by Gaspar et al., (2009; 2010), Dooho et al., (2009) and Stockman et al., (2011). Samples were stored in Phosphate Buffered Saline (PBS) pH 7.4, and RNA later (Qiagen) in the Department of Pathology, until the material was processed. The relative concentration of the studied genes was normalized according to Larionov et al. (2005) through the most stable endogenous control among the three endogenous tested (RP55, RPS19, and ACTB) according to Brinkhof et al. (2006) and all reactions were performed in duplicate.

Tissue samples and insulations of CTVT cultures had the number of chromosomes analyzed at the Animal Genetics Laboratory of the Biosciences Institute (Flórez et al., 2016).

Primary culture of CTVT

Insulations of CTVT cultures from the tumor collections (Table 1) were performed according to the protocol described by HSiao et al. (2008) and Flórez et al. (2016). For this purpose, tumor fragments were placed in PBS pH 7.4 and taken to the In vitro Fertilization and Cell Culture Laboratory of the Animal Reproduction and Veterinary Radiology Department of Veterinary Hospital.

Subsequently, the fragments were transferred to trypsin solution (TrypLESelect - Invitrogen) at 37.5 °C for 40 min in a magnetic
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RNA extraction was performed with the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. After purification of the RNA, it was treated with RNase-free DNase (Promega) for 30 min at 37 °C to avoid false-positive results from genomic DNA amplification. The quality of RNA extracted was evaluated in 2% agarose gel stained with ethidium bromide in NanoVue (GE Healthcare) equipment, and the samples were subsequently stored at -80 °C.

For the cDNA synthesis, 1 μg of RNA and the High Capacity kit (Applied Biosystems) were used. The reaction was performed with 6 μL Random Primer (10x), 6 μL RT buffer (10x), 2.5 μL dNTPs (25x), 3 μL Multiscribe (50 U/μL) and RNasefree water, according to the manufacturer’s protocol. Subsequently, incubation was performed at 25°C for 10 min and at 37 °C for 120 min with the automatic thermal cycler (ABI Prism 7500 FAST Sequence Detection System, Applied Biosystems). The samples remained at 4 °C until amplification by RT-qPCR.

For amplification of primer sequences (Table 2), SYBR Green and the universal PCR Master Mix (Promega) were used according to the manufacturer’s instructions. The qPCR reaction was performed with the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. After purification of the RNA, it was treated with RNase-free DNase (Promega) for 30 min at 37 °C to avoid false-positive results from genomic DNA amplification. The quality of RNA extracted was evaluated in 2% agarose gel stained with ethidium bromide in NanoVue (GE Healthcare) equipment, and the samples were subsequently stored at -80 °C.

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consisted of 4 μL of the cDNA sample, 200 nM of each primer, 10 μL of GoTaq qPCR Master Mix (Promega), and nuclease-free water, with a final volume of 20 μL.

The reaction conditions for all genes were: initial denaturation at 95 °C for 2 minutes, followed by 40 cycles of amplification (95 °C for 15 s for denaturation, 60 °C for 1 min for annealing and extension) and the dissociation curve (95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s). As a negative control, nuclease-free water was used. The relative standard curve for each gene was generated by serial dilutions of cDNA from a reference sample. The lowest dilution of the standard was considered as the relative value of 100, followed by 1/10 serial dilutions. The 3 points were 10, 1, and 0.1.

The relative concentration of the studied genes was normalized according to LARIONOV et al. (2005) through the most stable endogenous control among the three endogenous tested (RPS5, RPS19, and ACTB) according to BRINKHOF et al. (2006). All reactions were performed in duplicate and the value of QR < 0.5 was considered as a reduction in the expression and QR > 2.0 as an increase in the expression.

Statistical analysis
For statistical analysis, non-parametric tests of Wilcoxon (2 independent samples) were used. Results of the gene expressions obtained from tissue were compared to the expression of the same genes obtained from in vitro assays. The medians for each group were compared and statistically significant differences were indicated by the acronym QR, considered when the test P-value was below the significance level of 0.05 (P < 0.05). Statistical analyses were performed in the SAS statistical program.

RESULTS
The RNA was isolated from 17 tumor tissues and 3 cell cultures from tumor tissues (Table 3). In agarose gel they presented good integrity of ribosomal RNA 18s and 28s. Cell culture of 3 samples from tumor tissues was also performed.

Analyzes of the TP53 gene revealed high expression in 65% of tissues (QR = 2.7 to 7.6), except for 29% that lacked expression and one 6% that presented low expression (QR = 0.2). The cultured cells also presented high expression for this gene (100%) (QR = 2.6 to 3.5). For the gene encoding the p21 protein, 70% presented normal expression (QR = 0.5 to 1.4), except for 30% of the samples, where 6% demonstrated an absence and the others were underexpressed (24%) (QR = 0.2 to 0.4). The cells also presented normal expression (67%) (QR = 0.5 and 1.6) and underexpression (33%) (QR = 0.4). Statistical analyzes of TP53 and P21 genes did not demonstrate significant differences between tissues when compared to cell cultures (P > 0.05).

For BCL-2, only 6% of the samples presented high expression (QR = 2.3), 35% no expression, and 18% low expression (QR = 0.44 to 0.46), the others were considered normal (41%) (QR = 0.5 to 1.9). In the culture, one sample presented overexpression (33%) (QR = 2.5) and the others presented normal expression (67%) (QR = 0.7 and 0.8). Statistical analyzes of BCL-2 gene did not demonstrate significant differences between tissues and cell cultures (P > 0.05).

### Table 2 - Sequence of primers of the genes used in the RT-qPCR.

| Gene   | Sense (5' → 3')          | Antisense (3' → 5')          |
|-------|--------------------------|-----------------------------|
| RPS5  | GAGGCGTCAGGCTGTCGAT      | AGCCAAATGGCGGTGATTCAC       |
| RPS19 | GGGCGTCGAGCCCTGAGGAG     | CGGCCCTCAACTTGTTTCAAGTA     |
| ACTB  | GCCATCTGACCTTCTCAAGTA    | CTCTCAGTGCCTGCCAGCTTCTCAAGTA |
| TP53  | CCGAAAAAGAAAGAGACCACTA   | TCCACCTGGGCACTCCTCAAGTA     |
| P21   | ACCCTCGAGGCGCAGAAAC      | TGGCGCTCTCCTCGGAGAA         |
| BCL-2 | TGGATGACTGAGTACCTGAA     | GGGCTACTGACTTACTTAT         |
| BCL-xL| GCCTTTTTCTCCTCCGGTG      | CTCTCGCGGTGCTGATTGTT        |
| BAX   | GGTTGTGGCCCTCCTCTCAT     | GTAAGCAGCTCCAGCCACAAA       |

1 KLOPFLEISCH et al., 2009.
2 KLOPFLEISCH et al., 2010.
3 SANO et al., 2005.
The gene $BCL-xL$ was normal in 12% of the samples (QR = 0.7 and 0.9), underexpressed in 76% (0.005 to 0.3), and absent in other 12%. In the culture, 33% presented underexpression (QR = 0.1) and normal expression in 67% (QR = 0.5 and 0.7). $BAX$ was normal in 29% of the samples (QR = 0.9 to 1.6), overexpressed in 42% (QR = 2.3 to 3.7), and absent in other 29%. The cultured cells presented normal expression for this gene (100%) (QR = 0.8 to 1.2). Statistical analyzes of $BAX$ gene did not demonstrate significant differences between tissues and cell cultures, whereas $BCL-xL$ expression demonstrate significant differences in tissue samples in relation to samples from cell cultures ($P < 0.05$) (Figure 1).

**DISCUSSION**

The $TP53$ gene revealed high expression in tissues and the cultured cells also presented high expression for this gene. Overexpression of this gene has been considered a marker of the presence of mutations in several types of cancers (MORO et al., 2010) and its expression has been linked to an unfavorable prognosis in lymphomas (VELDHOEN et al., 1998) and breast tumors (LEE et al., 2004).

However, in other types of tumors, variable behavior has been described mainly due to the functions that this gene exerts in the cellular micro-environment. $TP53$ encodes the protein p53, responsible for inducing cell death via apoptosis (SUI et al., 2011), besides participating in different phases of the cell cycle, such as in the G1-S phase through the transactivation of p21, and in the G2-M phase blocking the entrance of the cell to mitosis (SUZUKI & MATSUBARA, 2011). Thus, p53, when functional, exhibits protective cellular behavior, preventing abnormalities from being transmitted further.

In our study, five tissues presented no expression of $TP53$ and one tissue presented $TP53$ underexpression, respectively. As previously described, WOLF & ROTTER already reported, back in 1984, that the gene encoding p53 in mice was inactivated by retroviral insertion into a cell line transformed into Abelson’s murine leukemia. Likewise, COLLAVIN et al., in 2010, also reported that functional loss or unregulated expression of p53 is common in primary tumors and that these
alterations may have a major impact on the regulatory effect common to proteins in the p53 family.

Conversely, our data differed from MORO et al. (2010) that identified a greater amount of p53 protein expression in CTVTs in the regression phase, describing a direct relationship between TP53 and the apoptotic index in CTVTs that are in this phase. Additionally, CHOI & KIM (2002), SÁNCHEZ-SERVÍN et al. (2004), and DECKER et al. (2015) also described the presence of mutations of this gene in CTFT. The same animal in which the TP53 expression was low, P21 under expression was also observed. As previously mentioned, TP53 participates in the G1-S phase through p21 transactivation. So P21 expression can be positively regulated by a p53-dependent mechanism, which induces the expression of repair genes in the face of damaged DNA (CHANG et al., 2006). Likewise, the other three samples that also showed decreased P21 expression may also be related to some other signaling mechanism independent of TP53, such as the unregulated presence of c-myc or ultraviolet radiation, which leads to negative regulation of p21 and whose alteration has already been reported in the human cancer cell (WANG et al., 1999), as well as in CTVTs (MURCHISON et al. 2014).

According to KLUMB and JÚNIOR (2002), the mutation in TP53 results in failure to induce P21 gene expression. MÖLLER et al. (1999) demonstrated that the expression of p53 associated with the absence of p21 has 100% specificity and sensitivity as predictive of mutation in large non-Hodgkin’s lymphomas. VILLUENDAS et al. (1997) observed that these same lymphomas, in the presence of mutations of the TP53 gene, had absence or underexpression of p21 and MDM2, suggesting that these mutations are related to the inability to transactivate p21 and MDM2. These researchers proposed that there is a likely alteration in the expression of this gene, which would result in loss of protein function.

Some tissues also presented normalized expression of P21, related to both absence (18%) and overexpression (53%) of TP53. According to SHEIKH et al. (1994) mechanisms that regulate the expression of P21 involve both dependent and independent signaling pathways of TP53. In human ovarian cancer cells, it has been shown that induction of P21 expression can occur through activation of the protein kinase C in cells lacking p53 (AKASHI et al., 1999), occurring at both transcriptional and post-transcriptional levels (ABBAS & DUTTA, 2009).

In prostate cells, the expression of P21 can be increased by either the direct action of TP53 (HERNANDEZ et al., 2003) as well as by the action of growth factors (ROBSON et al., 1999). Thus, it is questionable whether this normalized expression of P21 in CTFT is in fact associated with TP53, or is related to some other mechanism of independent signaling of this gene, as an immediate-early response to inducers of cell differentiation, or by the deregulated presence of c-myc, which leads to the decoupling of p21 in G1 (STEINMAN et al., 1994), and whose alteration has already been demonstrated in this tumor (COI et al., 2002; LIAO et al., 2003).

The BAX, BCL-2 and BCL-XL genes belong to the same family, which comprises approximately 25 genes. In the CTFT, there are no reports on the
expression of BCL-xL; however, the expression of the BCL-2 protein has been identified. According to STOCKMANN et al. (2011), the overexpression of BCL-2 occurs independently of the stage of tumor development. STOCKMANN et al. (2011) still suggested that its overexpression would promote the acquisition of characteristics in the tumor, associated with progression and survival. Likewise, AMARAL et al. (2011) highlighted that CTVTs with a lower degree of aggressiveness has a high apoptotic index, and may even have a better prognosis.

The decrease in BCL-2 expression was observed in three samples, as well as the decrease in BCL-xL in thirteen samples. Thus, the results differ from STOCKMANN et al. (2011), who reported an increase in this protein in CTVT. These authors argued that the increase of BCL-2 may be associated with the survival of tumor cells, which causes cell selection to acquire functions associated with tumor progression. WOLF & ROTTER, (1984) mentioned that there may still be interactions between the family of proteins Bcl-2 and p53.

In breast cancers, TP53 has the ability to sub-regulate BCL-2. Likewise, in ovarian cancers, the expression of BCL-2 is inversely related to the expression of TP53 (BASU & HALDAR, 1998). KUMARAGURUPARAN et al. (2006), on the other hand, observed an increase in BCL-2 related to an increase in TP53 in breast tumors. In the present study, there was an increase in TP53, but with no significant alterations for BCL-2. Divergences in the expression of BCL-2 and TP53 have been described in other studies (BASU & HALDAR, 1998).

With regard to BCL-xL, increased expression of it has already been reported in some types of cancers (GOBÉ et al., 2002; FESIK, 2005). LEITER et al. (2000) observed increased expression of this gene in melanoma metastases, indicating that the expression of BCL-xL, as well as BCL-2, increases with the progression of the neoplasia. Thus, BCL-xL could reflect an increase in the potential for malignancy, caused by the inhibition of apoptosis, resulting in growth advantages for melanoma cells. Likewise, in pancreatic cancers, their increased expression was associated with shorter patient survival (FRIESS et al., 1998).

However, the same situation was not observed in the present study. In the present study the decrease in BCL-2 expression, as well as BCL-xL, was observed in 18% and 76% of the samples, respectively, suggesting that intrinsic apoptosis is not occurring in the tumor microenvironment (Figure 2). This fact may be occurring due to a possible non-functional increase in p53 as, despite its high expression, this does not necessarily indicate that this protein is being translated. Conversely, the expression levels of BCL-xL in tissue samples presented, for the most part, lower values than in samples from cell cultures, so that the tumor microenvironment and its components present in the tissue samples are possibly influencing the levels of pro and antiapoptotic regulation in these cases.

Another apoptotic pathway stimulated by TP53 is that of BAX (CZABOTAR et al., 2014; LACROIX et al., 2006). In the tissues, 41% presented high expression, and the others 29% presented normal expression, while 41% did not express the gene. The expressions of the cells in culture were all normal.

Reports on gene mutations in CTVT have already been described. MURGIA et al. (2006) and FONSECA et al. (2012) described the presence of the specific LINE-1/c-myc rearrangement in CTVTs from different countries, while MURCHISON et al. (2014) identified a genetic marker in the
transmission cycle, with approximately 40% of all mutations being caused by exposure to solar ultraviolet light.

Thus, the understanding and characterization of different carcinogenic types has the potential to open new areas of research to increase knowledge about the biology of these cells, as well as studies on gene expression, mutations, and epigenetic alterations.

CONCLUSION

The overexpression of TP53 is evident in the majority of tissues and cells, as well as the variability of the expression of genes P21, BCL-2, BCL-xL, and BAX, possibly occurring due to the presence of genetic and/or epigenetic alterations in CTVT, leading to loss of function in these genes and contributing to the development of tumors through the inactivation of their inhibitory function.

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BIOETHICS COMMITTEE APPROVAL

This study was approved by the Ethics Committee on the Use of Animals (CEUA) of the Faculty of Veterinary Medicine and Animal Science: Protocol nº 81/2014.

DECLARATION OF CONFLICTS OF INTERESTS

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.
AUTHORS’ CONTRIBUTIONS

JP AJ and NSR conceived and designed experiments. APD supervised and coordinated the animal experiments and provided clinical data. HBF and LMMF performed the experiments and prepared the draft of the manuscript. RSY carried out the lab analyses. RAO performed statistical analyses of experimental data. All authors critically revised the manuscript and approved of the final version.

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