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Could dysregulation of *RASSF1* expression be a mechanism of tumorigenesis in CTVT?[1]

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ABSTRACT.- Fêo H.B., Flórez L.M.M., Yamatogi R.S., Duzanski A.P., Araújo Junior J.P., Oliveira R.A. & Rocha N.S. 2022. **Could dysregulation of *RASSF1* expression be a mechanism of tumorigenesis in CTVT?** *Pesquisa Veterinária Brasileira 42:e07082, 2022*. Departamento de Clínica Veterinária, Faculdade de Medicina e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Doutor Valter Maurício Corrêa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. E-mail: halinebf@hotmail.com

Canine transmissible venereal tumor (CTVT) is the oldest known somatic cell lineage. It is a transmissible cancer that propagates naturally in dogs and reportedly contains gene mutations. *RASSF1* participates in DNA damage repair, and its downregulation, results in tumor progression. Hence, *RASSF1* is a tumor suppressor gene. Its expression was quantified in tumors from seventeen animals and three cell cultures derived from tumors. In general, *RASSF1* was underexpressed in 65%, and absent in 35% of tumor samples. Cells from tumor tissue cultures showed decreased expression of *RASSF1* in 67% and elevated expression in 33% of samples tested. The tumor tissues showed significantly lower levels of *RASSF1* expression compared to cultured cells. Previously we reported that both the tumor microenvironment and the host immune system appear to influence the tumorigenesis and stage of CTVT. This is the first article to demonstrate the expression of *RASSF1* in CTVT. Decreased *RASSF1* possibly helps tumor progression.

INDEX TERMS: Dysregulation, RASSF1, tumorigenesis, canine transmissible venereal tumor, CTVT.

RESUMO.- [**A desregulação da expressão de *RASSF1* pode ser um mecanismo da tumorigênese em TVTC?**] O tumor venéreo transmissível canino (TVTC) é a linhagem de células somáticas mais antiga conhecida. É um câncer transmissível que se propaga naturalmente em cães e mutações genéticas já foram relatadas. O gene *RASSF1* atua no reparo de danos ao DNA e presume-se que, quando suprimido ou com expressão gênica reduzida, o TVTC tende a progredir. A expressão do gene supressor de tumor, como *RASSF1*, foi quantificada em tecidos de dezessete animais e três culturas de células de tecidos tumorais. Em geral, o gene *RASSF1* apresentou prevalência de subexpressão (65%) e ausência em 35% dos demais tecidos analisados. Células isoladas de culturas de tecidos tumorais também demonstraram 67% com expressão diminuída e 33% com expressão elevada, com diferença significativa entre os níveis de expressão gênica em amostras de tecido quando comparadas às culturas de células, com tecidos apresentando níveis mais baixos de expressão gênica em comparação com células. Anteriormente, relatamos que tanto o microambiente tumoral quanto o sistema imunológico

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do hospedeiro parecem influenciar a tumorigênese e o estágio do TVTC. Este é o primeiro artigo a demonstrar a expressão de *RASSF1* no TVTC, possivelmente alterando sua tumorigênese e auxiliando no aumento da progressão tumoral.

TERMOS DO INDEXAÇÃO: Desregulação, *RASSF1*, expressão, tumorigênese, tumor venéreo transmissível canino, TVTC.

INTRODUCTION

The canine transmissible venereal tumor (CTVT) is a transmissible cancer that is spread naturally between dogs (Fêo et al. 2018), passed by the transfer of live cancer cells during coitus. The genome of CTVT bears the imprint of the evolutionary history of this extraordinary cell lineage. Furthermore, the genome variation captured in global CTVT populations has highlighted some of the unique adaptations that have driven this lineage to become the longest-living and most prolific cancer known in nature (Strakova & Murchison 2015). In addition it provides an excellent research model for comparative oncology (Fêo et al. 2020).

The RASSF1 gene (family of the Ras 1 isoform association domain) acts in the repair of DNA damage. It participates in the activation of cell proliferation, controlling cell cycle, and inhibiting apoptosis that is induced by the Bcl-2 family of apoptotic proteins (García-Gutiérrez et al. 2020).

More and more reports are showing the occurrence of gene mutations in CTVT. Murchison et al. (2014) identified a genetic marker in the transmission cycle, with approximately 40% of all mutations caused by exposure to solar ultraviolet radiation. Additional studies have shown the presence of a specific LINE-1/*c-myc* rearrangement in CTVT samples from different countries (Fonseca et al. 2012). This alteration can be considered as a molecular diagnostic marker for CTVT.

Thus, tumor progression depends on processes regulated by *RASSF1A*, an isoform of *RASSF1*, such as cell cycle and modulation of apoptosis (García-Gutiérrez et al. 2020). Loss of expression due to hypermethylation in its promoter region is the most observed cause of deregulation of *RASSF1A*.

This work is part of a study that has already been published in the form of two articles that quantified: the expression of genes related to the immune system such as IL-6, IFN-γ, and TGF-β, as well as angiogenic factors (VEGF, CXCR4) (Fêo et al. 2018), and the expression of tumor suppressor genes, such as TP53, P21, and apoptosis-related genes, such as BAX, BCL-2, and BCL-xL (Fêo et al. 2020).

This study however has a new approach, that is to study the differences in the expression of the tumor suppressor gene RASSF1 in vivo and in vitro (primary cell culture) in CTVT. This article is the first to report the expression of RASSF1 in CTVT, and that decreased expression of *RASSF1* possibly results in increased tumor progression.

MATERIALS AND METHODS

Ethics Committee. All experiments and methods involving animals in this study were approved by the Ethics Committee on Animal Use (CEUA) of the "Faculdade de Medicina Veterinária e Zootecnia" (Faculty of Veterinary Medicine and Animal Science - FMVZ), protocol no. 81/2014.

Tumor collection. Seventeen animals with CTVT diagnosis confirmed through cytological and histological analyses (Amaral et al. 2007) were anaesthetized for total cleansing of the tumor site. The samples were collected by incisional biopsy, obtaining fragments of approximately 1cm^3 (Table 1). All samples were taken from the patients prior to chemotherapy. The samples were stored at room temperature in saline and phosphate solution (PBS) pH 7.4 and in RNA

Table 1. CTVT patients at the Veterinary Hospital included in the study and the numerical (QR) values related to the RT-qPCR analysis for the *RASSF1* gene in CTVT tissue and cell culture samples

Sample	Sex	Age (years)	Breed	Location	*RASSF1 (QR)*
T1	M	9	Mixed	Penis	0.008
T2	F	9	Dachshund	Vagina	0.001
T3*	M	7	Mixed	Penis	0.002
T4	M	9	Mixed	Penis	0
T5	M	–	Mixed	Skin - penis	0
T6	M	10	Mixed	Penis	0
T7	M	–	Mixed	Penis	0
T8*	F	10	Mixed	Vagina	0.002
T9*	M	5	Mixed	Penis	0.005
T10	M	6	Mixed	Penis	0.001
T11	M	–	Mixed	Penis	0
T12	M	8	Poodle	Oral cavity	0.029
T13	M	10	Mixed	Penis	0.011
T14	M	10	Mixed	Penis	0.002
T15	M	16	Mixed	Skin - penis	0.001
T16	M	–	Bull Terrier	Penis	0
T17	M	–	Mixed	Penis	0.027
C1	N/A	N/A	N/A	N/A	0.098
C2	N/A	N/A	N/A	N/A	1.360
C3	N/A	N/A	N/A	N/A	0.038

* Tumor samples that were used to perform isolations for cell cultures; M = male, F = female, N/A = not applicable, T = tissue, C = cell; (–) Not specified by owner.

Later (Qiagen, Venlo, Limburg, the Netherlands) in the Department of Pathology/FMVZ, until the material was processed. To verify that tissues and cultured cells pertained to CTVT, samples were subjected to analysis of chromosome numbers in the Animal Genetics Laboratory of the "Instituto de Biociências" (Institute of Biosciences).

CTVT primary culture. Insulations of CTVT cultures from the tumor collections (Table 1) were performed according to the protocol described by Bassani-Silva et al. (2007), Hsiao et al. (2008), Flórez et al. (2017) and Fêo et al. (2018). Aseptic fragments of three samples from tumor tissues, placed in saline PBS pH 7.4, were transported to the Laboratory for *in vitro* Fertilization and Cellular Cultures in the "Departamento de Cirurgia Veterinária e Reprodução Animal" (Department of Animal Reproduction and Veterinary Radiology - DCVRA/FMVZ). Subsequently, the fragments were transferred to a trypsin solution (TrypLE Select–Invitrogen 12563-029) at 37.5°C, and kept for 40 minutes in a magnetic homogenizer. Next, the solution was centrifuged (820g, 4°C, 25 minutes) discarding the supernatant. The pellet was resuspended and conditioned in two 25cm flasks (Sarstedt -Germany) with 5mL of DMEM high glucose culture (Dulbecco's modified essential medium - Gibco). This material was supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts), with a combination of 100U/mL penicillin and 100mg/mL streptomycin (Life Technologies - Gibco) and 3µg/mL amphotericin B (Life Technologies - Gibco). The samples were then incubated in a 5% CO_2 culture incubator, at 95% humidity and 37.5°C. Cell viability and concentration were assessed by an exclusion test using trypan blue, and cells were resuspended in DMEM high glucose culture (DMEM - Gibco, Life Technologies).

Karyotyping of CTVT cell cultures. CTVT cell cultures at 50%, confluence were submitted to the chromosomal analysis in the Animal Genetics Laboratory of the "Instituto de Biociências". For this, the supernatant in cell culture was discarded, the cells were detached by adding 5mL of hypotonic KCl solution (0.075M), incubated for 40 minutes at 38°C and centrifuged at 1500rpm for 5 minutes. The cells were then washed in 3:1 methanol:acetic acid solution, until the supernatant was clear and resuspended in 0.5mL of the solution to prepare slides. 3-4 drops of suspension obtained were placed on a clean histological slide and maintained in ice-cold distilled water. The prepared slides were dried at room temperature and kept in a refrigerator until chromosome analysis. The slides were analyzed individually in each sample and mitoses were counted.

Primer design for *RASSF1*. The primers for *RASSF1* gene (Ras association – RalGDS/AF-6 – domain family member 1), were designed with the aid of the primer blast program[8], sequence, accession number: NC_006602.3; range 39014609-39023393. To confirm the gene, the amplified product of this primer (*RASSF1*) was sequenced and analyzed with the help of bioinformatics software.

For primer design, *RASSF1* gene sequences were searched in the database of the National Center for Biotechnology Information (NCBI)[9]. For the *RASSF1* gene, two sequences were found and to select the final sequence of the gene, the complete bibliographic information (*RASSF1*, NC_006602.3; range 39014609-39023393) was considered. The primers were designed in the online program Primer 3[10]. Gene sequences were inserted separately in the FASTA format. Once designed, the specificity of the primers was confirmed using the BLAST algorithm[11]. The primers were synthesized by Molecular Brasil.

Real time PCR (RT-qPCR). RNA extraction was performed according to the protocol described by Flórez et al. (2017) and Fêo et al. (2018) and the relative concentration of the *RASSF1* gene was normalized according to Larionov et al. (2005), through the most stable endogenous control among the three tested detailed in Table 2 (RPS5, RPS19, and ACTB) (Brinkhof et al. 2006).

Statistical analysis. All reactions were performed in duplicate and a decrease in expression was considered when QR <0.5 and an increase when QR >2.0. The results of the gene expression measurements obtained from tumor tissues were compared to the expression of the same genes obtained from *in vitro* assays using Wilcoxon non-parametric tests and statistically significant differences were indicated by the acronym QR, considered when the test *P* value was below the significance level of 0.05 ($P<0.05$).

RESULTS

The *RASSF1* gene was under expressed in 65% (11/17; QR=0.001 to 0.029) and absent in 35% (6/17) of the analyzed tumor samples. Cells from cultures derived from tumor tissues also showed decreased expression of *RASSF1* in about 67% (2/3) (QR=0.038 and 0.098), and 33% with normal expression (QR=1.360). There was a significant difference between the levels of gene expression in tumor samples and cell cultures, with tumors showing lower levels of gene expression compared to cells ($P<0.01$) (Fig.1).

Table 2. Primer sequences of the genes analysed in the RT-qPCR

Gene	Sense (5' → 3')	Antisense (3' → 5')
*RPS5**	GAGGCGTCAGGCTGTCGAT	AGCCAAATGGCCTGATTCAC
*RPS1**	GGGTCCTCCAAGCCCTAGAG	CGGCCCCCATCTTGGT
*ACTB**	GGCATCCTGACCCTCAAGTA	CTTCTCCATGTCGTCCCAGT
RASSF1	TGTCGGATGATGAACAGCCC	CCTGCCCAATGACAACCAAG

* Brinkhof et al. (2006).

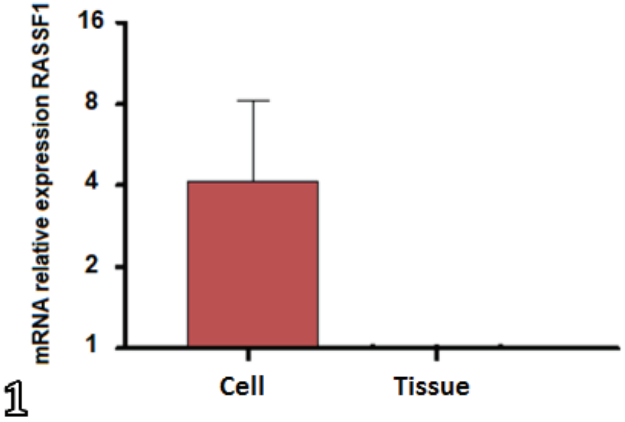

Fig.1. Graphic representation of the relative mRNA expression of *RASSF1*, showing a statistically significant difference between the levels of expression in tumor tissue samples and cell cultures derived from tumors ($P<0.01$).

[8] Available at <http://www.ncbi.nlm.nih.gov/nuccore> Accessed on Jan. 22, 2016.

[9] Available at <http://www.ncbi.nlm.nih.gov/> Accessed on Jan. 22, 2016.

[10] Available at <http://biotools.umassmed.edu/bioapps/primer3_www.cgi> Accessed on Jan. 22, 2016.

[11] Available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> Accessed on Jan. 22, 2016.

DISCUSSION

The prevalence of *RASSF1* gene under expression in most tumor tissues and cells corroborates with the results described by Lagiou et al. (2016) who made a similar observation in 50.6% of samples from breast cancer. According to Fernandes et al. (2013) the *RASSF1* gene can be downregulated and/or inactivated by both genetic and epigenetic mechanisms, with *RASSF1A* being the most studied isoform that is commonly inactivated epigenetically in cancer (Blandino & Sardo 2019). The inactivation of *RASSF1A* has mainly been attributed to an increase in specific methylation of the gene of clusters of CpG sites (hypermethylation of the CpG island) of its promoter region, thus silencing the expression of the gene (Lung et al. 2019). Statistical analyses also showed a significant difference between the levels of the gene expression in tissue samples when compared to those from cell cultures of CTVT, which may be due to the presence of molecules such as growth factors, in the tumor microenvironment, that are possibly absent in cell cultures (Fêo et al. 2018). Thus, the tumor progression in the analyzed CTVT samples could have presumably occurred due to the dysregulation of processes that are regulated by *RASSF1A*, such as cell cycle control, microtubule stabilization, cell migration, and inefficient regulation of apoptosis modulation (García-Gutiérrez et al. 2020).

In this study, the tumor sample was a primary tumor. Hence the risk of contamination and cell death is high. Therefore, of the total samples collected for the work, only three met the criteria to be used for the study (cell proliferation index, karyotype, viability, time and confluence capacity, freezing resistance, among others). Additionally, as the objective was to compare the cultured cells with the original tumors, even though few samples were analyzed, the results showed a significant difference in the expression of *RASSF1* between tumors and cell cultures. This result is very important for further studies on molecular pathways of CTVT.

Strunnikova et al. (2005) suggested that histone H3 deacetylation and histone H3K9 trimethylation precede DNA methylation during the progression of *RASSF1A* inactivation in human mammary epithelial cells. Similarly, Kawamoto et al. (2007) observed that prostate tumor cell lines, LNCaP and PC3, presented silencing of *RASSF1A* mediated by hypermethylation and enrichment of the histone H3K9 me2 in its promoter. Other studies have also shown that transcriptional repression of the *RASSF1A* gene in mammary glands during the early stages of carcinogenesis was associated with increased trimethylation of histones H3K9 and H3K27 and methylation of the *RASSF1A* promoter (Starlard-Davenport et al. 2010). Thus, gene silencing caused by DNA methylation is closely linked to repressive modifications of histones in the gene promoter of *RASSF1A* in tumor cells, and abnormal epigenetic gene silencing appears to be as important as mutations in DNA sequences in the development of cancers (Alberts et al. 2010).

However, in addition to epigenetic alterations, several genetic changes related to *RASSF1A* have also been described in cancer cells (Ahmad et al. 2020). According to Kashuba et al. (2009), of the 144 *RASSF1A* clones sequenced, 129 mutations were detected in exons 1-2. In exons 3-5, where 98 clones were analyzed, 146 mutations were found, showing that simple nucleotide polymorphisms occur in this gene in different epithelial malignancies (Kashuba et al. 2009). In nasopharyngeal carcinomas, Pan et al. (2005) observed 35 mutations identified in 74% of cases, including 30 substitutions, three nonsense mutations (stop codon), and two base pair deletions (frameshift), with the majority of these mutations resulting in amino acid alterations.

Studies on gene expression, mutations, and epigenetic alterations are revolutionizing the way in which biology and medicine are explored. Such studies in CTVT, can provide a study model for other cancers, as the dog species is very close to humans. Thus, our results demonstrate that the under expression of the *RASSF1* gene, which possibly occurs as a result of a genetic alteration, influences the tumorigenesis of CTVT. The presence of molecules, such as growth factors, in the tumor microenvironment, which are possibly absent in cell culture, seem to support this inference.

CONCLUSIONS

The present study demonstrates that the under expression of the *RASSF1* gene possibly occurs as a result of an epigenomic alteration, and influences the tumorigenesis of canine transmissible venereal tumor (CTVT).

The presence of molecules, such as certain growth factors, in the tumor microenvironment, which are possibly absent in cell culture, could be responsible for the reduced expression of *RASSF1* in CTVT tumors compared to cell cultures derived from such tumors. However, future studies should be conducted to confirm this observation.

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Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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