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Spontaneous canine transmissible venereal tumor: association between different phenotypes and the insertion LINE-1/c-myc¤

Tumor venéreo transmisible canino espontáneo: asociación entre diferentes fenotipos y la inserción LINE -1/c-myc

Tumor venéreo transmissível canino espontâneo: associação entre diferentes fenótipos e a inserção LINE-1/c-myc

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Summary

Objective: this study aimed to evaluate the LINE-1 transposon inserted in c-myc gene as a specific genetic alteration in cells of spontaneous canine Transmissible Venereal Tumor (TVT) with either lymphocytoid or plasmacytoid phenotypes. Methods: tumoral biopsies from 35 dogs were collected by puncture or exfoliation. Polymerase Chain Reaction (PCR) was carried out with primers myc.s and LINE. A, specific to the LINE-1 segment to detect the presence of LINE-1/c-myc molecular marker. Results: sequence alignment of DNA samples from lymphocytoid and plasmacytoid TVT cells did not show polymorphisms, and the comparison with sequences from the GenBank identified them as a LINE-1/c-myc rearrangement. Conclusions: considering the aggressive nature of the plasmacytoid phenotype, there is no apparent relation between LINE-1/c-myc and the malignancy of TVT. Further studies are needed to establish molecular differences associated with the aggressiveness of the various phenotypes of canine TVT.

Key words: *dogs, oncogenes, Polymerase Chain Reaction, transposition elements.*

Resumen

Objetivo: evaluar la expresión del trasposón LINE-1 insertado en el oncogén c-myc como una alteración genética específica en células de Tumor Venéreo Transmisible canino espontáneo con fenotipos

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linfocitoideos o plasmocitoideos. Métodos: se tomaron muestras citológicas de 35 caninos naturalmente afectados por Tumor Venéreo Transmisible (TVT), separándolas en dos grupos de acuerdo al fenotipo predominante. La identificación del marcador molecular LINE-1/c-myc fue posible mediante la técnica de Reacción en Cadena de la Polimerasa (PCR) usando los primers myc.s y LINE. A, específicos para el segmento LINE-1. Resultados: el resultado del alineamiento de las secuencias obtenidas a partir del DNA de cada uno de los fenotipos de TVT no presentó variación entre ellos y al compararlas con el alineamiento de otras secuencias depositadas en el GenBank, pudo observarse que se trata de un reordenamiento LINE-1/c-myc. Conclusiones: teniendo en cuenta la naturaleza agresiva del fenotipo plasmocitoide, se estableció que no hay polimorfismo genético entre los grupos analizados, siendo necesario realizar nuevos estudios tendientes a establecer diferencias moleculares asociadas con la agresividad de los diferentes fenotipos del TVT canino.

Palabras clave*: oncogenes, perros, Reacción en Cadena de la Polimerasa, transposición genética.*

Resumo

Objetivo: foi avaliado o elemento de transposição LINE-1 inserido no gene c-myc como alteração genética específica nas células do TVT espontâneo canino nos grupos fenotípicos previamente classificados como linfocitóide e plasmocitóide. Métodos: amostras da lesão de 35 cães foram colhidas por punção ou esfoliação. Para identificar o marcador molecular LINE-1/c-myc nas amostras foi utilizada a técnica de Reação em Cadeia da Polimerase empregando-se os primers myc.s e LINE. A, específicos para o segmento LINE-1. Resultados: o resultado do alinhamento das sequências obtidas das amostras de DNA das células de TVT linfocitóide e plasmocitóide não apresentou polimorfismos e, por meio do alinhamento com outras sequências depositadas no GeneBank, identificou-se que trata-se do rearranjo LINE-1/c-myc. Conclusões: parece não haver relação entre este aspecto molecular analisado com a agressividade do tumor.

Palavras chave: *cão, elementos de transposição, oncogene, Reação em Cadeia da Polimerase.*

Introduction

Transmissible Venereal Tumor (TVT) is a spontaneous naturally occurring neoplasia of round cells with lymphocytoid and plasmacytoid phenotypes, frequently observed in dogs of the Botucatu region in the state of São Paulo, Brazil (Amaral *et al.*, 2007). As a rule, it shows no predilection for breed, sex, or age and can be transmitted by transplant of viable tumor cells and between animals through copulation, licking, biting, and scratching, whenever there is a loss of mucosa or skin integrity (Cohen, 1985; Das and Das, 2000; Brandão *et al*., 2002; Amaral *et al*., 2004; Nak *et al*., 2005; Amaral *et al*., 2007; Lefebvre *et al*., 2007).

TVT cells are genetically complex, exhibiting great differences between different areas across the world. The normal diploid assembly of the domestic dog cell has 78 chromosomes. All autosomes show acrocentric morphology, with the X chromosome being the largest submetacentric and the Y chromosome the smallest. On the other hand, cytogenetic studies on TVT cells showed wide deviation in the karyotype chromosome number, which exhibits between 57 and 59 chromosomes including several with submetacentric morphology, suggesting the occurrence of rearrangements. These rearrangements may have resulted from balanced fusions and not from the gain or loss of genetic material (Cohen, 1985; Vonholdt and Ostrander, 2006; Murgia *et al*., 2006; Vázquez-Mota *et al*., 2008).

Prominent among the known TVT molecular alterations is the rearrangement of the protoncogene c-*myc*, resulting from the insertion of a repetitive DNA segment of 1.5 Kb belonging to the family of gene transposition elements known as LINE (*Long Interspersed Nuclear Element*), which do not occur in normal dog cells (Katzir *et al*., 1985; Katzir *et al.*, 1987; Amariglio *et al*., 1991; Choi *et al*., 1999; Chu *et al.*, 2001; Choi *et al.*, 2002; Vonholdt and Ostrander, 2006; Murgia *et al*., 2006).

Although rearrangement of the LINE-1/c-*myc* sequence supports the hypothesis that all the cells of this tumor originate from a single tumoral cell

(Vonholdt and Ostrander, 2006; Murgia *et al*., 2006), it has recently been demonstrated that gene mutations can be acquired at a late stage resulting in genetic variants of TVT Vázquez-Mota *et al*. (2008). These events can cause genomic instability and lead to progressive modifications that may contribute to its malignant phenotype.

Amaral *et al.* (2007) demonstrated different cytomorphological types in cytological samples of Transmissible Venereal Tumor. There is a predominance of round cells, scarce cytoplasm, and high nucleus to cytoplasm ratio in the lymphocytic pattern. There is predominance of ovoid cells, ample cytoplasm, and eccentric nucleus in the plasmacytic pattern. The presence of both morphological types does not yield predominance of either in the mixed pattern. According to Bassani-Silva *et al.*, 2007 when TVT cells of lymphocytoid and plasmacytoid morphologies where exposed to extracts of propolis *in vitro*, the plasmacytoid phenotype was more resistant. This could be an indication that the plasmacytoid TVT is more aggressive than the lymphocytoid.

Considering the absence of data in the literature relating to the molecular characteristics of the different phenotypes of this neoplasia, more research is needed in this area. Thus, the present work aimed to compare the element of LINE-1 transposition inserted into the gene c-*myc* as a specific genetic alteration of this tumor in cells of canine spontaneous TVT by Polymerase Chain Reaction (PCR) in the groups previously classified as lymphocytoid and plasmacytoid.

Materials and methods

Sample origin and phenotypic classification

The study used 35 dogs of different breeds received at the Veterinary Hospital, FMVZ, UNESP, Botucatu, São Paulo, Brazil. The lesion samples were collected by puncture or exfoliation with consideration to the anatomical localization of TVT. Cellular morphology was evaluated by placing half of the collected material on a microscope slide where it was smeared. Slides were subsequently fixed in methanol (Merck, Germany) and stained using the Giemsa method (Merck, Germany). One thousand neoplastic cells per slide were quantified utilizing a polarized light microscope (Axio Imager A1; Carl Zeiss, Germany) connected to a computer program (*Axionvision Software Rel. version 4.3;* Zeiss Vision, Germany). The TVT was classified as lymphocytoid or plasmacytoid according to the description by Amaral *et al.* (2007).

The remaining material from each sample was stored in cryotubes (TPP Techno Plastic Products, Switzerland) containing 1.5 mL of buffered saline solution (PBS) (Sigma-Aldrich, EUA), and stored at -20 ºC for subsequent extraction of genomic DNA.

Three mL samples of whole blood from animals with and without TVT were collected in EDTA vacutainer tubes (Becton, Dickinson and Company, USA).

Extraction of genomic DNA

Illustra Blood GenomicPrep Mini Spin Kit (Amersham Biosciences, Switzerland) was used to extract the genomic DNA according to the manufacturer's protocol. The DNA was quantified in a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, EUA) and then diluted to 10 ng/µL concentration.

LINE-1 insertion

Identification of the molecular marker LINE-1/c-*myc* was accomplished by PCR, as described by Chu *et al*. (2001), using *myc*.s and LINE.A primers designed by Amariglio *et al*. (1991) and specific for the LINE-1 segment. The human hemoglobin gene primer (HGBA; Venta *et al*.,1996), which amplifies a segment of 480 bp, was used as internal reaction control.

The PCR reaction was carried out in 35 one-minute cycles at 94 °C, 1 minute at 64 °C and 1 minute at 72 °C. Each reaction contained 10 ng of genomic DNA, 10 µM of each primer, 4 µL of PCR Master Mix (Promega), and 6 µL of Mili-Q autoclaved water.

DNA sequencing

From all the amplifications, samples of lymphocytoid and plasmacytoid phenotypes were chosen and submitted for sequencing. Direct sequencing reaction followed *Big Dye Terminator Cycle Sequencing Ready Reaction* kit manufacturer's protocol (Applied Biosystems, USA). Electrophoresis was completed using an *ABI Prism™ 377 DNA Sequencer (*Applied Biosystems, USA*)*. The sequences generated were aligned and compared with those deposited at GenBank using the *BioEditW version 7.0.4.1* program and the *Clustal W Multiple Sequence Alignment Program, v1.7*.

Clinical manifestations

The lymphocytoid and plasmacytoid phenotypic groups were classified by age, breed, sex, and localization of genital and/or extragenital neoplasia.

Results

Phenotypic classification

Tumor cells of the 35 dogs were phenotypically classified into lymphocytoid (n=6) or plasmacytoid (n=29).

LINE-1 insertion

The amplified fragments of TVT samples presented approximately 480 bp and 340 bp in reference to the HGBA gene and the target region LINE-1/c-*myc*, respectively. Conversely, there was no amplification of rearrangement in the PCR negative control (DNA from canine TVT carriers) (Figure 1).

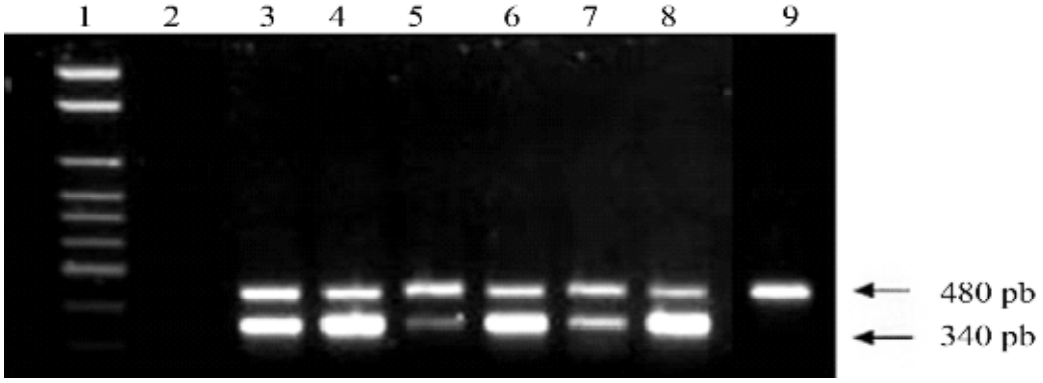

Figure 1. Genomic DNA from TVT cells. Column 1: molecular weight marker 100 pb; Column 2: NTC (no template control); Columns 3 to 8: samples of amplified TVT; Column 9: positive control.

DNA sequencing

The sequence alignment from DNA samples of TVT cells in carriers of lymphocytoid and plasmscytoid phenotypes did not present polymorphisms and, by comparison with sequences from the GenBank, LINE-1/c-*myc* rearrangement was present with homology ranging from 94 to 96%.

Clinical manifestations

Occurrence of spontaneous TVT in animals (Table 1) varied from three to nine years of age in the lymphocytoid group, and between two to fourteen years in the plasmacytoid group. Both males and females were equally affected by plasmacytoid tumors with no definitive breed predisposition. The external genitalia was primarily affected except in four cases that involved the extra-genital region where the tumor was the plasmacytoid type.

Table 1. Canine spontaneous transmissible veneral tumor: phenotypical and clinical manifestation.

Case	Breed *	Age (years)	Sex **	Localization	Morphology
1	Cocker Spaniel	2	M	Genital	plasmacytoid
2	WDB	4	F	Genital	plasmacytoid
3	WDB	3	M	Genital	plasmacytoid
4	WDB	3	M	Genital and extragenital	plasmacytoid
5	WDB	2	M	Genital	plasmacytoid
6	WDB	4	F	Genital	lymphocytoid
7	Poodle	3	F	Genital	plasmacytoid
8	Border Collie	4	F	Genital	plasmacytoid
9	WDB	9	M	Genital	lymphocytoid
10	WDB	6	M	Genital	plasmacytoid
11	WDB	7	M	Genital	plasmacytoid
12	Brazilian Terrier	4	F	Genital	lymphocytoid
13	Brazilian Fila	3	F	Genital	lymphocytoid
14	WDB	6	F	Extragenital	plasmacytoid
15	WDB	4	M	Genital	plasmacytoid
16	WDB	3	M	Extragenital	plasmacytoid
17	Poodle	6	M	Genital	plasmacytoid
18	WDB	5	M	Genital	plasmacytoid
19	WDB	14	M	Genital	plasmacytoid
20	WDB	5	M	Genital	plasmacytoid
21	WDB	5	M	Genital	plasmacytoid
22	WDB	8	F	Genital	plasmacytoid
23	WDB	5	M	Genital	plasmacytoid
24	Poodle	4	M	Genital	plasmacytoid
25	WDB	8	F	Genital and extragenital	plasmacytoid
26	WDB	4	M	Genital	plasmacytoid
27	WDB	3	F	Genital	lymphocytoid
28	WDB	1	F	Genital	plasmacytoid
29	WDB	9	M	Genital	plasmacytoid
30	WDB	9	F	Genital	plasmacytoid
31	WDB	1	F	Genital	plasmacytoid
32	WDB	14	M	Genital	plasmacytoid
33	WDB	6	M	Genital	plasmacytoid
34	Belgian Shepherd	4	F	Genital	plasmacytoid
35	WDB	5	M	Genital	lymphocytoid

*WDB: without defined breed. **M: male, F: female.

Discussion

The mechanisms involved in the development and biological behavior of neoplasias are still mostly unexplained. Nevertheless, results from genetic, biochemical, mutagenetic, and phenotypic studies have supplied extremely important data on the manner by which normal cells are transformed into malignant ones. Genomic instability leads to progressive modifications of the biological profile of these cells, altering their proliferation, differentiation and interaction with the microenvironment, all contributing factors to cell malignancy (Murphy and Jirtle, 2003; Chamary and Hurst, 2009).

Some studies have postulated that detecting the specificity of LINE-1/c-*myc* rearrangement in cells of spontaneous TVT may explain the appearance, maintenance, and propagation of genetic material in this neoplasia among hosts in different continents for about 200 to 2500 years due to the fact that this

rearrangement is identical in all of the evaluated cells (Vonholdt and Ostrander, 2006; Murgia *et al*., 2006; Vázquez-Mota *et al*., 2008). This specificity was also observed in the samples of the present study, which, in addition to corroborating the data available in the literature, supports the notion that the LINE-1 insertion in the gene c-*myc* is being conserved and can be utilized as a diagnostic marker of this neoplasia. Furthermore, this abnormality did not differ between the lymphocytoid and plasmacytoid phenotypes.

After sequencing the PCR product, it was verified that part of the LINE-1/c-*myc* rearrangement was the region actually amplified and similar to sequences available at GenBank (Katzir *et al*., 1985; Amariglio *et al*., 1991; Choi *et al*., 1999; Choi and Kim, 2002; Murgia *et al*., 2006). The fact that TVT presents phenotypic differences prompted the practice of classifying this tumor as either lymphocytoid or plasmacytoid from 1994 by the Veterinary Pathology Service of FMVZ-UNESP at Botucatu (Amaral *et al*., 2004; Amaral *et al*., 2007; Bassani-Silva *et al*., 2007). As described in table 1, prevalence of the plasmacytoid phenotype was observed compared to the lymphocytoid type.

Considering that some TVTs are resistant to chemotherapy (Brandão *et al*., 2002) and almost all metastases are associated with plasmacytoid TVT cases (Amaral *et al*., 2007), it is of clinical relevance to further classify the tumor phenotype.

Changes in the c-*myc* proto-oncogene can alter cellular metabolism, growth and proliferation, and in turn, be associated with tumoral malignity. The differences in LINE-1/c-*myc* rearrangement could be involved not only in the phenotypic variation of TVT but may also be implicated in the aggressive activity of plasmacytoid TVT. Although qualitative differences were not observed in this study, comparative quantitative studies are needed between phenotypic groups. It is also necessary to conduct more studies to ascertain the true role of this genetic alteration in TVT, the transposition mechanism, and its role in this neoplasia (Katzir *et al*., 1985; Amariglio *et al*., 1991; Choi *et al.,* 1999; Choi *et al*., 2002).

A clear understanding of TVT pathogenesis differentiated by subtype will provide better methods to advise patients on the best treatment by taking into account the malignancy degree of the tumor. It is important to emphasize that there are no literature reports comparing the two studied phenotypic groups to detect the LINE-1/c-*myc* rearrangement, so that the current data on this alteration, together with new molecular studies, would help to comprehend the biological behavior of this tumor. Of the 35 dogs evaluated 21 were male and 14 female in spite of the literature, which shows a clear gender predisposition (Cohen, 1985; Das and Das, 2000). The age varied between two and fourteen years, with the highest frequency of affected dogs among those at four years of age. This is due to the fact that these animals have high sexual activity between three and five years of age (Das and Das, 2000; Amaral *et al*., 2004).

As to breed, 27 (77%) of the 35 animals studied were mixed-breed dogs (WDB), in agreement with descriptions by Brandão *et al*. (2002), Amaral *et al*. (2004), and Lefebvre *et al.* (2007). These authors grouped the WDB dogs into a population characterized as homeless and therefore more exposed to the transmission of this tumor. In both lymphocytoid and plasmacytoid groups the predominant tumor localization was genital in comparison with extragenital sites, corroborating the results by Das and Das (2000) and Nak *et al*. (2005).

In conclusion, when comparing both phenotype polymorphisms there was no relation to LINE-1 rearrangement in the c-*myc* gene after sequence alignment obtained in tumors previously classified into the lymphocytoid and plasmacytoid phenotypic groups and, therefore, there appears to be no relation between this analyzed molecular aspect and tumor aggressiveness.

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