

DNA DAMAGE IN CANINE TRANSMISSIBLE VENEREAL TUMOR CELLS.

DANOS NO ADN DAS CÉLULAS DO TUMOR VENÉREO TRANSMISSÍVEL CANINO

Anne Santos do Amaral¹; Isabelle Ferreira²; Marcia Moleta Colodel²; Dayse Maria Fávero Salvadore³; Noeme Sousa Rocha²

¹ Department of Small Animal Clinic, UFSM, Santa Maria, RS, Brazil

² Department of Clinical Veterinary Medicine, FMVZ/UNESP, Botucatu, SP, Brazil

³ Department of Pathology, FMB/UNESP, Botucatu, SP, Brazil

Abstract: *The Transmissible Venereal Tumor (TVT) has been classified according to the predominant cell type as follows: lymphocytoid, plasmocytoid, and mixed. Various degrees of aggressiveness with large array of biological behaviour have been described according to the TVT cell lineages, the present study was designed to investigate the level of DNA damage in the three TVT cell types aiming a better understanding of mechanisms related to the aggressiveness of this neoplasia. A total of 35 dogs were evaluated, with no restriction regarding sex, age or breed, and with clinical and cytological diagnoses for TVT. Cell samples from the 35 tumoral neoplasmas were obtained by fine needle aspiration and taken to cytology and DNA damage analysis by the comet assay. From the 35 TVT cases, 12 (34.3%) were plasmocytoid, 11 (31.4%) lymphocytoid, and 12 (34.3%) mixed. Statistically significant ($p < 0.05$) higher level of DNA damage was detected in lymphocytoid TVT cells when compared to the other two types.*

Resumo: *O Tumor Venéreo Transmissível (TVT) tem sido classificado de acordo com o tipo celular predominante da seguinte forma: linfocitóide, plasmocitóide e misto. Vários graus de agressividade com grande variedade de comportamento biológico, têm sido descritos de acordo com as morfologias das células do TVT. O presente estudo teve como objectivo investigar o nível de danos no DNA nos três tipos de células do TVT, visando uma melhor compreensão dos mecanismos relacionados com a agressividade dessa neoplasia. Um total de 35 cães, sem restrição quanto à idade, sexo ou raça, e com diagnóstico clínico e citológico de TVT foram avaliados. Amostras de células foram obtidas a partir de 35 tumores, por aspiração com agulha fina, e realizada citologia e análise dos danos no DNA, pelo Ensaio Cometa. Dos 35 casos de TVT, 12 (34,3%) foram plasmocitóide, 11 (31,4%) linfocitóide, e 12 (34,3%) misto. Estatisticamente ($p < 0,05$) o TVT linfocitóide apresentou maior nível de danos no DNA quando comparado com os outros dois tipos.*

SHORT COMMUNICATION

Transmissible venereal tumor (TVT) canine is an allogeneic transplantable tumor that is usually transmitted during coitus. It might be presented as a single or multiple mass, almost always located in the genital, oral, nasal and/or conjunctival mucosae. Although uncommon, metastasis can be found in superficial inguinal, iliac, and mesenteric lymph nodes, kidney, spleen, brain, pituitary, skin, subcutaneous tissue, lungs and peritoneum (Amaral et al., 2007; Gaspar et al., 2010).

Studies have shown that TVT origin is related to an ancestral neoplastic cell, originated from a single host, through successive selections and clonal expansion

(Murgia et al., 2006, Vásquez-Mota et al., 2008). Samples from different countries share common histopathological features and genetic alterations, such as aneuploid karyotype with the presence of 57 to 59 chromosomes (Murgia et al., 2006), insertion of LINE-1 (Long Interspersed Element) at the end c-MYC gene or rearrangement of LINE-1 c-MYC (Choi & Kim, 2002).

Despite the common origin, peculiarities have been observed. For example, the DQA1 locus might be diploid or haploid in some TVT samples. In addition, two genetic subtypes have been characterized based on mitochondrial DNA analysis (Murgia et al., 2006). Other authors found a mutation in TVT cells of asian dogs at position 963 of

the TP53 gene (Choi & Kim, 2002), which was not observed in tumor samples from dogs in Mexico (Vázquez-Mota *et al.*, 2008). Together, these data suggest the existence of several TVT subtypes, probably due to acquisition of subsequent genetic alterations, after developing the original clone of TVT (Vázquez-Mota *et al.*, 2008). The recent evidences that TVT exhibit morphological differences between strains lead us to use several diagnostic criteria to its classification. The system adopted by the School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (UNESP), Botucatu, São Paulo, Brazil is based upon cytological characteristics such as the predominant cell type and degree of aggressiveness. On this basis, TVT cells might be classified as lymphocytoid, plasmocytoid and mixed cell type (lymphocytoid plus plasmocytoid cells) (Amaral *et al.*, 2007; Bassani-Silva *et al.* 2007; Gaspar *et al.*, 2010) (table 1).

Table 1. DNA damage (tail intensity) in canine Transmissible Venereal Tumor (TVT) cells of different morphology subtypes.

TVT/ morphologies subtype	Animals (n)	Tail intensity
Plasmocytoid	12	7.48 ± 3.65 ^a
Lymphocytoid	11	11.65 ± 3.88 ^b
Mixed cell type	12	7.89 ± 3.69 ^a

Different letters, in the same column, mean with significant statistical difference ($p < 0.05$).

In addition to the morphological differences, there are also biological differences between the morphological subtypes (Bassani-Silva *et al.*, 2007; Gaspar *et al.*, 2010). Almost all cases related to resistance to chemotherapy or metastases are associated to plasmocytoid cytotype (Amaral *et al.*, 2007; Bassani-Silva *et al.* 2007; Gaspar *et al.*, 2010).

Considering the hypothesis that different morphological subtypes of TVT might be

related to different DNA damage and the Single Cell Gel Electrophoresis (SCGE) or Comet Assay has been widely used to assess this damage (Tice *et al.*, 2006; McKenna *et al.*, 2008), the aim of this study was to investigate DNA lesions in canine TVT cells, and the relationship between the level of DNA damage and cell morphology.

A total of 35 dogs, regardless of their gender, age or breed, but with positive clinical diagnosis and cytomorphology for TVT, were assisted at the Veterinary Hospital, FMVZ, UNESP, Botucatu, São Paulo, Brazil. Cell samples from 35 tumoral nodule were obtained by fine needle (24³/₄G caliber) aspiration. Cell suspension was divided into two aliquots: one for cytology and other for the comet assay.

For cytomorphology, two slides with cells obtained from each tumoral nodule were left to dry at room temperature, fixed with methanol P.A. (Merck®, Darmstadt, Germany) and stained with Giemsa for the cytomorphological analysis using a standard light microscope. The analyses were initially done under 10x magnification in order to evaluate cellularity, color pattern, and cell distribution. Then, the observations were done at 250x and 400x to better characterize and count the cells. Carefully detailed cytomorphological analysis that delimited the cell-count field was also performed. At least 100 cells were counted from each sample.

Tumors were classified according to Amaral *et al.* (2007) in Lymphocytoid - 60% or more cells with round morphology, scarce and finely granular cytoplasm, presence of vacuoles tracking the cell periphery, and round nucleus with rough chromatin and one or two protruding nucleoli (Fig. 1A); Plasmocytoid - 60% or more cells with ovoid morphology, more abundant cytoplasm (lower nucleus:cytoplasm ratio), and eccentrically located nucleus (Fig. 1B); Mixed-cellularity between lymphocytoid and plasmocytoid cell types, but none surpassed 59% of the total.

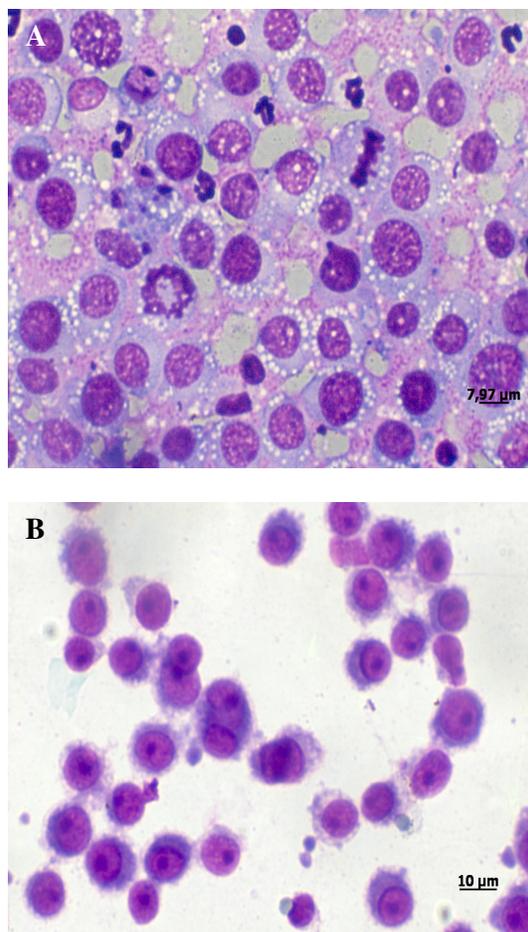


Figure 1 – Photomicrography of the cytological preparation from canine Transmissible Venereal Tumor: (A) Lymphocytoid; (B) Plasmocytoid. Giemsa, 400x magnification.

The single cell gel comet assay was performed according to Tice et al. (2006). Cell viability was determined by trypan blue dye exclusion and was always $\geq 65\%$. Briefly, a volume of 10 μL of the cell suspension was added to 120 μL of 0.5% low-melting point agarose at 37°C, layered onto a slide that was precoated with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification at 4°C, the coverslip was removed and the slides immersed into a lysis solution (2.5M NaCl, 100mM EDTA, 10 mM Tris-HCL buffer, pH 10, 2% sodium sarcosinate containing 1% Triton X-100 and 10% DMSO added just before use) for about 1 to 7 days, at 4°C. Subsequently, the slides were

placed into a horizontal electrophoresis unit filled with fresh alkaline buffer (1mM EDTA and 300 mM NaOH, pH>13) and left for 20 minutes. Then, electrophoresis was carried out for 20 minutes, at 25V and 300mA. At the end, the slides were neutralized (0.4 M Tris, pH 7.5), fixed with absolute ethanol and stained with 50 μL ethidium bromide (20 $\mu\text{g}/\text{ml}$). Fifty cells per slide were randomly selected and examined at 400x magnification in a fluorescence microscope, using an automated image analysis system (Comet Assay II, Perceptive Instruments, UK). The parameter selected as indicator of DNA damage was the tail intensity (% tail DNA, in % pixels).

Data were statistically analyzed by the Kruskal-Wallis and Dunn tests. Statistical significance was set as $p < 0.05$.

After cytological analyses, the 35 TVT cases were classified according to the predominant cell morphology as follows: 12 (34.3%) plasmocytoid, 11 (31.4%) lymphocytoid, and 12 (34.3%) mixed-cell type. Data presented in Table 1 show the level of DNA damage (tail intensity) observed in the three types of TVT category. Increased DNA lesions ($p < 0.05$) were detected in lymphocytoid TVT cells when compared to the other two subtypes.

The Comet Assay has been widely accepted as a standard method for the assessment of DNA damage in individual cells, especially applied for environmental biomonitoring, and for clinical management of diseases (Tice et al., 2006; McKenna et al., 2008).

In the present study, the levels of DNA damage were evaluated through the Comet Assay, in TVT cells, in order to better understand the mechanisms concerning the aggressiveness of this neoplasia, and to provide information for its diagnosis, prognosis and treatment. Our present data showed higher level of DNA damage, in lymphocytoid cells. This result suggests that the different morphological subtypes of TVT have different biological behavior, as

suggested by Amaral *et al.* (2007), Bassani-Silva *et al.* (2007) and Gaspar *et al.* (2010). Recently, it was described that plasmocytoid TVTs present higher resistance to the antitumoral action of propolis (Bassani-Silva *et al.*, 2007) and vincristine sulfate (Gaspar *et al.*, 2010), higher immunoreactivity of Ki-67 (MBI-1) (Gaspar, 2005), most expression of glycoprotein-P (Gaspar *et al.*, 2010), and higher rates of metastases (Amaral *et al.*, 2007), suggested that plasmocytoid TVT cells present higher aggressiveness and higher proliferation than the lymphocytoid TVT cells.

Whereas the cellular proliferation that occurs in the progression stages of carcinogenesis due to inactivation of apoptosis can be observed from the cleavage of DNA by endonucleases, changes such as pyknosis, nuclear fragmentation and proteolysis of cytoskeleton (Hoeijmakers, 2009), significant differences related to DNA damage between the lymphocytoid, plasmocytoid and mixed cell hypothesize that the plasmocytoid subtype has a higher proliferative activity and rate of mitosis and that this would be a progression of lymphocytoid. Thus, the lymphocytoid subtype would have a better prognosis due to a higher apoptotic index. However, this possibility remains to be investigated.

In cancer, the morphological diagnostic indicates the type of cell that is proliferating, its cytological grade, and other features of the tumor. The Comet Assay results can be an important tool to investigate cancer progression. Together, these procedures can be useful to evaluate the prognosis of the patient and establish therapeutic strategies.

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