Cell proliferation markers in the transplanted canine transmissible venereal tumor

[*Marcadores de proliferação celular no tumor venéreo transmissível canino transplantado*]

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ABSTRACT

Adult male mongrel dogs were subcutaneously transplanted with the canine transmissible venereal tumor (TVT) on the hypogastric region. Twelve specimens of tumors were collected, half during the proliferative phase and the other half during the regressive phase. Fragments of the tumor were fixed in 10% buffered formalin and routinely processed for light microscopy. Sections of 4μm were stained by Schorr or AgNOR or either immunostained for MIB1 (Ki67). Schorr stain, AgNOR and MIB1 showed an increased proliferative activity through mitotic index, nuclear argyrophilic protein stain and cycling tumoral cells in the growing tumors, respectively. All of the three cell proliferation markers were able to distinguish the TVT in both evolution phases. MIB1 monoclonal antibody was the best in the morphologic evaluation of growth and regression of TVT. This resulted in higher values than AgNORs counting and mitotic index. MIB1 immunostaining was the most effective parameter of the proliferative activity of TVT. However, a significant correlation has been detected only between mitosis counting and AgNORs.

Keywords: dog, venereal tumors, MIB1, AgNOR

RESUMO

Cães machos, adultos, mestiços, foram transplantados com células do tumor venéreo transmissível canino (TVTC), na região hipogástrica. Foram coletados doze espécimes do TVTC, sendo metade durante a fase proliferativa e metade durante a fase regressiva. Fragmentos do tumor foram fixados em formol a 10%, tamponado e processado rotineiramente para microscopia de luz. Secções de 4μm foram coradas pelo Shorr, ou pela AgNOR, ou ainda, imunocorado para MIB 1 (Ki67). As colorações pelo Shorr, AgNOR, ou MIB 1 mostraram um aumento do índice mitótico, coloração da proteína argirofílica nuclear e células tumorais ciclando em tumores em crescimento, respectivamente. Todos os três marcadores de proliferação celular foram capazes de distinguir o TVTC em ambas as fases de evolução. O anticorpo monoclonal MIB 1 foi o melhor na avaliação morfológica, de crescimento e regressão do TVTC. Isto resultou em um valor maior que a contagem de AgNOR e do índice mitótico. A imunomarcação com MIB1 foi o parâmetro mais efetivo da atividade proliferativa. No entanto, só foi observada uma correlação positiva entre a contagem de mitose e a AgNOR.

Palavras-chave: cão, tumor venéreo, MIB 1, AgNOR

INTRODUCTION

The canine transmissible venereal tumor (TVT) is an undifferentiated neoplasm of round cells that is sexually transmitted and installs primarily in the genital organs of both sexes. Studies using experimental transplantation have demonstrated that integral viable cells can produce TVT in dogs of all ages (Rogers, 1997). The routes of inoculation include: subcutaneous, intraperitoneal and subaracnoideal (Nielsen and Kennedy, 1990; Das and Das, 2000). Following subcutaneous inoculation, the TVT cells induce

to tumors of 3-6mm after 2-3 weeks. The development of the tumor is very quick in the beginning and slows down as time passes.

A major characteristic of the TVT cells is their high proliferative capacity (Robenhorst *et al.*, 1993; Kreipe *et al.*, 1995). The number of proliferative cells constitutes an important parameter in the prognosis of several different tumors (Quinn and Wright, 1990; Robenhorst *et al.*, 1993). Among the methods developed to evaluate the growth of a neoplasia are the determination of the mitotic index and the detection of proliferative markers through histochemical and immunohistochemical reactions. They are the most useful and commonly applied methods (Robenhorst *et al.*, 1993).

Determining the number of mitoses through the traditional morphological approach in routine stained slides is still very important for the prognosis (Kreipe *et al.*, 1995).

Histochemically, the argyrophilic nucleolar organizing regions (AgNOR) technique has been frequently used in oncology. The silver impregnation results in dark brownish dots that can be counted and their areas estimated within the nucleus. The number and area of the AgNORs represent parameters that reflect the degree of the proliferative activity of the cells. Both of them are increased in malignant neoplasias (Chung *et al.*, 1994). Therefore, AgNORs contribute to the diagnosis and prognosis of the tumor pathology (Crocker *et al.*, 1989; Ploton, 1994; Rüschoff *et al.*, 1994).

The use of the Ki-67 (MIB1) as a proliferative cell marker shows an almost accurate evaluation of the cellular growth fraction (Löhr *et al.*, 1997). MIB1 identifies the Ki-67 antigen epitopes that are formalin and paraffin resistant. MIB1 augments the practical applicability of the Ki-67 antigen as a method to evaluate the cellular proliferation in tumors (Schmitt and Ferreira, 1995).

The aim of this paper was to investigate and compare the use of some cell proliferation markers such as mitotic index, number and area of AgNORs and immunolabeling for MIB1 during the growing and regression phases of the TVT.

MATERIAL AND METHODS

The tumor cells were obtained from two mongrel adult male dogs with TVT of natural occurrence, according to Santos *et al.* (2008). Imprints of the tumors were fixed in methanol and stained with May Grünwald-Giemsa (Luna, 1968) to confirm the cytology of the TVT.

The TVT cells were transplanted in adult mongrel male clinically healthy dogs that were obtained from a Center of Zoonosis. Animals were fed with commercial food and water was given *ad libitum*. Each dog was inoculated subcutaneously with 10^7 viable cells/ml in the hypogastric region in four different places, 10cm apart from each other (Koike *et al.*, 1979). Tumors were measured daily with a caliper, their volumes were calculated through the formula [length x width x height x $\pi/4(cm^3)$] proposed by Liao *et al.* (2003).

After six months, twelve specimens of tumors were collected: (1) six with growing tumors and (2) six with regressive tumors. All procedures with the animals were carried out in accordance to the International Guiding Principles for Biomedical Research Involving Animals.

The TVT fragments were fixed in 10% buffered formalin and routinely processed. Sections of 4µm stained by Schorr trichromic (Luna, 1968) were used for the quantification of the mitotic index and for evaluation of the histological pattern of the tumor. Only the cells with unequivocal mitosis were counted, according to Schmitt and Ferreira (1995). The total number of mitosis was counted in 10 consecutive fields using the magnification of 400X. Hyperchromatic apoptotic nuclei and lymphocyte infiltrate were not included, as previously cited by Elston and Ellis (1991). The mitotic index was calculated using the following formula: Mit= Σ of mitotic cells/Σ of total cells per field.

Other sections went through AgNORs technique (Ploton *et al.*, 1986) modified by Aubele *et al.* (1994) for the quantification under light microscopy of nucleolar organizing regions. A cut-off value of the minimal representative number of microscopic fields was established by the standard deviation (SD) instability analysis described elsewhere (Moro *et al.*, 2004). The AgNORs counting was carried out with a digital image analyzer. Thirty cells were analyzed per

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slide in the magnification of 1000X. By densitometry, AgNORs numbers and areas were obtained within the nuclei (Santos *et al.*, 1998).

Another set of 4μm thick sections were used in immunohistochemistry. The sections were deparaffinized, hydrated and submitted to antigen retrieval using the retrieval solution (DAKO) in water bath (100^0C). Peroxidase was quenched with 3% hydrogen peroxide. The sections were treated with normal serum (UV block) followed by the primary monoclonal antibody MIB1 and incubated in a humid chamber. The secondary antibody (Biotiny Goat Anti-Polyvalent - UltraVision Large Volume Detection System Anti-Polyvalent, HRP - LabVision) was applied and slides were again incubated in a humid chamber. The reaction was developed by diaminobenzidine (DAB peroxidase reagent); the slides were counter stained with Harris's hematoxilyn and mounted. The immunostaining by MIB1 was measured in 1000 cells (15 to 34 fields, depending on its cellularity) with a 100X objective. Only the labeled cells were considered for the histological and morphometrical analysis. The result was expressed as the immunolabeling index for MIB1 (MIB). The calculation of the MIB was carried out using the following formula: MIB = Σ of the immunolabeled cells/Σ of the total cells inside that field.

The experimental design was entirely randomized. The proliferative indexes (PI) (i.e. Mit, AgNORn, AgNORa and MIB) of each tumor underwent ANOVA. The experimental unit was the dog. One tumor of each animal was evaluated for each treatment. The treatments were analyzed through Fisher's test (P<0.05) (Sampaio, 1998).

RESULTS

Mitotic figures were easily recognized as dense dark plaques of chromosomes with brush borders. The nuclear envelope was absent and the cytoplasm was lightly eosinophilic or colorless. The aspect of the chromatin lumps allows the identification of the mitotic phases, such as metaphases, anaphases and telophases (Figure 1).

The number of mitotic figures varied from 2-19/field in the growing TVTs and 0-7/field in the regressive ones. The average number of mitosis in 10 consecutive fields was 8.10 (SD = 3.47) for the growing and 2.80 (SD = 1.80) for the regressive TVTs. The ANOVA of the Mit showed differences (P<0.05%) between the growing and the regressive phases.

The AgNORs were identified as dark brownish dots that varied in shape, size and distribution. Additionally, they were more frequent and intense in the growing phase than in regressive TVTs. The dots were found isolated or aggregated, round or irregularly shaped, diffusely distributed inside the nucleus or around the nucleolus, resembling satellites (Figure 2A and 2B). The morphometry showed that TVTs in both evolution phases had the same shape, size and distribution AgNORs patterns.

ANOVA showed that growing tumors had a higher average number and area of the AgNORs within the nucleus than the regressive TVTs (Table 1).

Table 1. Statistical analysis of the AgNORs number and area in TVT cells nuclei of the growing and regressive phases

Variables *	Growing	Regressive	P < 0.05
AgNORn	15.35± 4.00b	9.96± 3.71a	0.01
AgNORa	6.04± 1.54b	2.57± 1.34a	4.24E-06

*AgNORn: densitometric quantification of AgNORs within the nuclei; AgNORa: densitometric measurement of the total AgNORs area within the nuclei.

Growing tumors did not present correlation between the number and the area of the AgNORs within the nucleus (P>0.05%). However, the same correlation was shown to be positive within the regressive tumors.

MIB1 labeling was confined to the nucleus and its intensity varied between the cases and the phases. The growing tumors presented more intense, numerous and diffuse labeled cells than the regressive ones (Figure 3A). The regressive tumors showed few labeled cells that were localized near the capsule (Figure 3B).
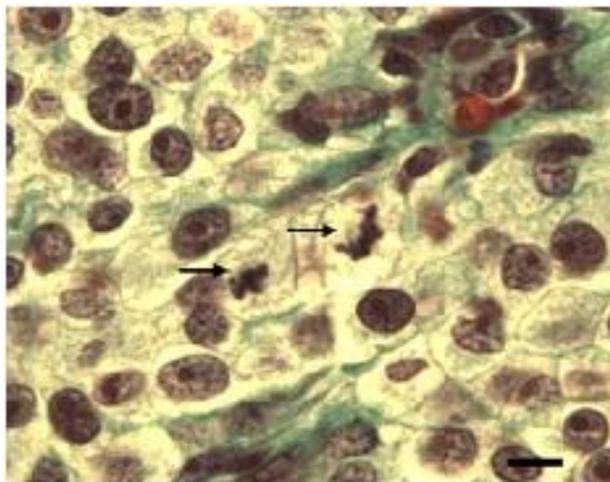
Figure 1. Transplanted canine venereal tumor during the growing phase. Observe the mitosis figures (arrows) (Schorr, bar = 10μm).
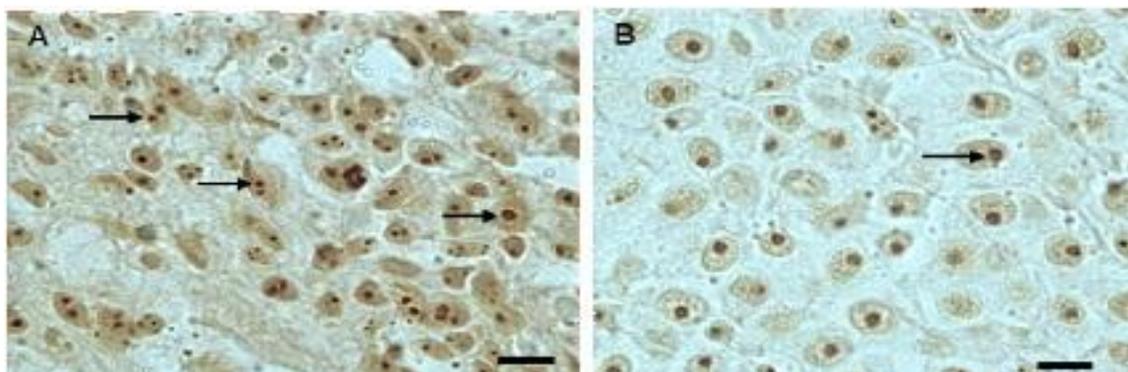

Figure 2. Micrography of a transplanted canine transmissible venereal tumor (TVT): (A) TVT in the growing phase. Observe that AgNORs are distributed diffusely and in higher quantity inside the nuclei and nucleoli (arrows). (B) TVT in the regressive phase with AgNORs that are more frequent inside the nucleoli (arrows) (Silver nitrate, bar = 10μm).
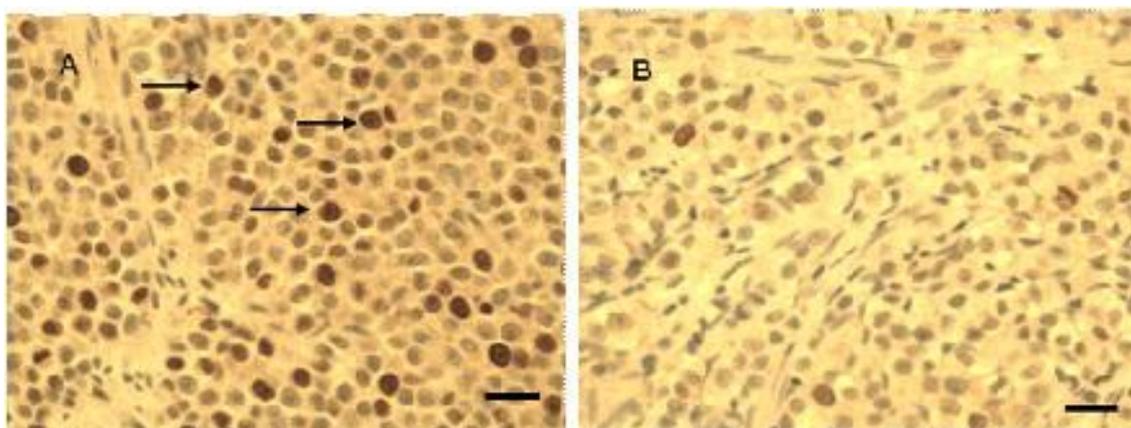

Figure 3. Micrography of a transplanted transmissible venereal tumor. Immunohistochemistry (streptavidine-biotine-peroxidase): (A) Growing tumor with an intense MIB1 expression (white arrows) and mitosis figures (black arrows). (B) Regressive tumor with a mild MIB1 expression (counter stained with Harris hematoxylin, bar = 10 μm).

The fraction of the MIB1 immunolabeling in growing and regressive tumors is presented in table 2. The variance analysis in MIB1 percentage of immunolabeled cells showed difference between both TVT evolution phases ($P < 0.05$).

The correlation between MIB1 immunolabeling and other proliferation markers are summarized in table 3.

A correlation between MIB and Mit patterns was not observed ($r = - 0.1370$). The result was confirmed by the Pearson's correlation test ($P = 0.79$ %). The same occurred considering MIB and AgNORn ($r = - 0.47$; $P = 0.33$%). On the other hand, a positive correlation was evident between Mit and AgNORn ($r = 0.8990$; $P < 0.05$%).

Table 2. Variance analysis and descriptive statistic of the percentage of MIB1 immunolabeled cells in growing and regressive TVTs

Phases	Variables			
	Minimum	Maximum	Average	P
Growing	40.20	87.16	59.32±16.59	0.002
Regressive	8.38	41.86	25.90±11.58	

Table 3. Correlation between MIB1 immunolabeling, mitotic index and AgNORn

Variables	N	Pearson's coefficient correlation (R)	Significance (P)
MIB X Mit	12	-0.13	0.79
MIB X AgNORn	12	-0.47	0.33
Mit X AgNORn	12	-0.89	0.01

Mit – mitotic index

DISCUSSION

Traditionally, the counting of mitotic figures has been utilized to evaluate the cellular proliferation in tumor sections stained by HE (Kreipe *et al.*, 1995). Here, the mitotic index was higher in growing phases than in regressive phases of the TVT. This result differs from that presented by Cohen and Steel (1972), who did not succeed in distinguishing both TVT evolution phases through mitotic index. A major stimulus for the evaluation of proliferation indexes is the conviction that it could provide data to support a more accurate procedure with the patient. Until recently, the knowledge of the biological, biochemical and molecular events that precede or regulate mitosis was scarce. Consequently, the cellular proliferation was estimated by counting the mitotic figures. However counting mitotic cells is only possible during the cellular cycle phase of mitosis. Cells in interphase cannot be considered in this analysis, although this phase corresponds to a very long period of the cellular cycle (Robenhorst *et al.*, 1993). According to van Diest *et al.* (1998), the counting of mitotic cells can be a tool for diagnosis and prognosis in tumor pathology, mainly when it is correlated to other proliferation markers.

Both TVT evolution phases presented similar distribution patterns, organization and shape of the AgNORs inside the nuclei and around the nucleoli. The AgNORs were found isolated or grouped forming agglomerates under a cordon shape, or under circular shape inside the nucleus. They were also found in satellite shape around the nucleolus and as necklaces in the nucleolus, as described by others (Crocker *et al.*, 1989; Rüschoff *et al.*, 1989; Toikkanen and Joensuu, 1993). The number and the total area of AgNORs in the nucleus were higher in growing TVTs than in regressive ones. Both parameters were utilized by Santos *et al.* (1998) to show the difference between genital and extra genital TVTs of natural occurrence. Harmelin *et al.* (1995) compared the number and the area of AgNORs in the nucleus between genital TVTs and their metastasis. They found that metastasis had significantly higher averages than the genital TVTs.

The evaluation of the proliferation represented by the AgNOR index was positively correlated to the mitotic index. According to van Diest *et al.* (1998), the AgNORs are positively correlated to Ki-67 (MIB1) immunolabeling and mitotic

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indexes. AgNORs technique may be also used to estimate the cellular cycle length. The shorter the cellular cycle the more ribosomes and proteins can be produced and more AgNORs per unit of time are presented (Derenzini and Ploton, 1991; Trerè, 1993; Rüschoff *et al.*, 1994).

Again, there was a positive correlation between the number and the area of AgNORs in the regressive TVTs. Growing TVTs did not show the same correlation. It may suggest that growing TVTs present a morphological and distribution pattern of AgNORs different from that showed by regressive TVTs. Additionally, growing TVTs have an AgNOR pattern similar to malignant tumors (Underwood and Giri, 1988; Derenzini and Ploton, 1991; Trerè, 1993; Rüschoff *et al.*, 1994; Löhr *et al.*, 1997; Santos *et al.*, 1998).

MIB1 immunolabeled cells were found scattered in the growing TVTs and occurred as isolated groups in regressive tumors. Besides, growing TVTs presented more immunolabeled cells than the regressive ones, suggesting that in the regressive TVTs the cellular proliferation activity was decreased.

Regressive TVTs had less MIB1 immunolabeled cells and mitotic figures than the growing ones. Similar results were observed during TVT spontaneous (Pérez *et al.*, 1998) and chemotherapy (Gonzalez *et al.*, 2000) induced regression of natural occurrence. This result summed up to the highest apoptotic index observed and this phase (Santos *et al.*, 2008) plays an important role in the natural involution of the TVT.

A correlation between MIB1 immunolabeling and mitotic index was not found, although both methods differentiated well the two TVT evolution phases. Conversely, according to Schmitt and Ferreira (1995), the correlation between both markers has been used and described in women breast cancer. In addition, Van Diest *et al.* (1998) reported a partial correlation between MIB1 immunolabeling and mitotic index. Griffey *et al.* (1999) also found a positive correlation between both cellular proliferation markers. They also observed that the correlation decreased when the counting of mitotic cells was made out of the MIB1 immunolabeling area. In spite of that, the

correlation still remained significant. Alves *et al.* (1999) proposed that the AgNORs evaluation could give an estimation of the cell cycling velocity. The association of methods used to calculate the growth fraction (MIB1) and the generation time (AgNOR) could help the estimation of the proliferate activity of a neoplasia. It could also be used to select faster proliferating tumors more accurately (Alves *et al.*, 1999).

However, MIB1 immunolabeling did not correlate with the proliferation index obtained with AgNORs counting. Similar results were showed by Chung *et al.* (1994) with squamous cell carcinoma of the cervix. They have also found a correlation between both proliferation markers and the histological grade of the tumor. Löhr *et al.* (1997) verified that the MIB1 immunolabeling had better prognostic importance when associated with histopathologic, clinic, macroscopic and anamnestic patterns than the AgNOR technique.

In this study, MIB1 presented higher values than number and area of AgNORs and Mit. Chung *et al.* (1994) concluded that a higher MIB1 average compared to the number of AgNORs was correlated to the tumor recurrence. Recurrence was not evaluated in the present study. Dervan *et al.* (1989) found a high correlation (P < 0.001) between AgNORs and MIB1 in malignant lesions. However, the correlation was significant between groups with benign lesions (P = 0.02).

The usefulness and the reliability of cellular cycle markers are linked to the number of studies that associate marker detection with cellular proliferation and compare them with other methods already standardized (Robenhorst *et al.*, 1993). The present paper characterized the use of different cellular proliferation markers during different evolution phases of the TVT.

On the whole, the three cellular proliferation markers (Mit, AgNOR and MIB1) are able to distinguish growing and regressive TVTs. Besides, the monoclonal antibody MIB1 is the most effective cellular proliferation marker in growing or regressive phases of TVT. However, AgNOR can be used instead of MIB1 considering its easy and quick performance and low cost.

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