

Spontaneous canine transmissible venereal tumor: cell morphology and influence on P-glycoprotein expression

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Abstract: The present study aimed to determine P-glycoprotein expression according to TVT cell morphology in 42 dogs with confirmed TVT, classified into lymphocytoid, plasmocytoid, and mixed. The chemotherapy efficiency was investigated along with its relation to P-glycoprotein expression in tumoral cells, evaluated by immunocytochemistry, considering positive tumors more than 10% stained. Among the samples collected, 50.00% possessed plasmocytoid morphology, 18.63% lymphocytoid, and 31.37% mixed. The plasmocytoid presented greater immunoreactivity to the anti-P-glycoprotein antibody in relation to the lymphocytoid. It was observed that the plasmocytoid was less sensitive to chemotherapy. The cases that had partial clinical response in the lymphocytoid were less compared with those in the plasmocytoid. Therefore, we can infer that the plasmocytoid presents a potential drug resistance. Based on this study, it can be concluded that the plasmocytoid presents a higher frequency of partial clinical response than the lymphocytoid and mixed, possibly through its higher percentage of anti-P-glycoprotein antibody expression.

Key words: Dog, TVT, P-glycoprotein, cell morphology

Introduction

Spontaneous canine transmissible venereal tumor (TVT) is a neoplasia of round cells that can be transmitted by transplant of viable tumor cells from one animal to another during copulation or through licking, biting, or scratching, especially in the presence of

abrasions or loss of surface integrity. Although extragenital localization is also possible, it occurs most frequently in the mucous membrane of the external genitalia of stray, sexually active medium-sized dogs, of both sexes, and presents global distribution, though is most common in tropical and subtropical regions (1-3).

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The TVT metastases may reach virtually all organs including the superficial and deep lymph nodes in the subcutaneous abdominal and thoracic cavities, liver, spleen, kidneys, lungs, and mediastinum (3-6).

Cytology is the method of choice for diagnosis since it is rapid, simple, and presents little invasiveness (7,8). Cytological samples of TVT are generally described as multicellular with round or oval cells whose cytoplasmic borders are well defined. The nucleus, round or oval, is frequently eccentric and of variable size with thick granular chromatin and 1 or 2 prominent nucleoli (9). However, considering the absence or presence of cytoplasmic vacuoles, the cellular size and form, and the nucleus-to-cytoplasm ratio, TVT can be classified into 3 groups denominated plasmocytoid, lymphocytoid, and mixed (8,10).

Among the various treatment modalities such as cryosurgery, radiotherapy, surgical resection, and chemotherapy, the last of these is regarded as the most effective and least invasive (11-15).

Although chemotherapy treatment may utilize vincristine, vimblastine, doxorubicin, and cyclophosphamide as single agents or in combination, administered in up to 8 cycles at weekly intervals (16-18), resistance to drugs has been frequently observed in TVT patients (10).

Resistance to chemotherapy is a multifactorial phenomenon that can be conferred by various cellular mechanisms related to defects in apoptosis regulation, increase in intracellular detoxification, alterations in DNA repair systems, and the activation or overexpression of molecules such as P-glycoprotein.

P-glycoprotein constitutes part of a family of cell transporters that function as an efflux pump dependent on the energy generated by hydrolysis of ATP, capable of translocating a series of drugs to the cell exterior, thus reducing their intracellular concentrations to levels of low lethality (19,21-23).

This protein is normally expressed in the adrenals, kidneys, liver, colon, brain, lungs, peripheral blood, and bone marrow (20,23). However, in both veterinary and human oncology, it was demonstrated that elevated P-glycoprotein expression levels correlated positively with lack of response or

remission of neoplasias after adequate forms of chemotherapy (24,25). Nevertheless, there are data in the literature that report the expression of P-glycoprotein among other cell groups different from TVT.

The present study aimed to verify whether the different cytomorphological groups of transmissible venereal tumor (lymphocytoid, plasmocytoid, and mixed) express P-glycoprotein at the same intensity, enabling, in the future, adequate anticipatory treatments given the observation of growing resistance to chemotherapy in patients with this neoplasia, especially in those with the plasmocytoid pattern.

Materials and methods

Forty-two dogs of both sexes and of different breeds were utilized, between the ages of 2 and 14 years, from the routine of the FMVZ, UNESP, Veterinary Hospital, Botucatu, SP, Brazil, with cytomorphological diagnosis of lymphocytoid, plasmocytoid, or mixed TVT (8). For each animal, the clinical genital and extragenital expression of the lesion and the presence or absence of metastasis were taken into consideration.

Samples of 102 neoplastic masses were collected by puncture or exfoliation. Half of each sample was fixed and stained with Giemsa, and 100 neoplastic cells were read and quantified per slide in a mechanical counter, for inclusion in the experimental groups: lymphocytoid, plasmocytoid, and mixed. The other half of each sample was stored in Eppendorf microtubes containing 1.5 mL of buffered saline solution (PBS).

The samples suspended in PBS were divided into aliquots of 50 μ L and cytocentrifuged at 500 rpm for 3 min, for accomplishment of the immunocytochemistry technique. Immunostaining utilized the monoclonal antibodies anti-human anti-P-glycoprotein clone 5B12 (Lab Vision, Fremont, CA, USA) and anti-P-glycoprotein clone C494 (Signet Laboratories, Dedham, MA, USA).

The slides were dehydrated by sequential passage through alcohol. This was followed by the blocking of endogenous peroxidase with 10 volumes of hydrogen peroxide and then by washing with distilled running

water. Afterwards, antigen recuperation was performed in microwaves at maximum power while maintaining the slides in citrate buffer, pH 6.0, for the antibody anti-P-glycoprotein clone 5B12. For the antibody anti-P-glycoprotein clone C494, the slides were incubated with BSA 2%, for 1 h, at room temperature.

Next, the slides were incubated with primary antibody in a humid chamber at 4 °C for 18 h. Table 1 displays the antibody data like type of staining, clone, brand, and standardized dilutions.

This was followed by incubation of the secondary complex, utilizing the LSAB Kit (Dako Corporation, Carpinteria, CA, USA) according to the manufacturer's instructions. For reaction revelation the chromogen 3'-3' diaminobenzidine was utilized (Dako Carpinteria, CA, USA) for 5 min. Between all steps the slides were washed with tris buffer.

The slides were counterstained with methyl green for 5 min and washed with isopropyl alcohol for 2 min (2 washings), and then passed through a battery of dehydration and mounting.

The P-glycoprotein positive control utilized histological cuts and an impression of a normal canine liver while the negative control employed cytological TVT samples incubated only with diluent of primary antibody.

The slides were observed in an optical microscope, at 40× magnification, and the results were expressed as the percentage of positive cells among the 100 cells per sample. Cells were considered positive if the cytoplasmic membrane and cytoplasm were brown, independent of the staining intensity, as described by Ginn (22) and Miyoshi et al. (25).

To evaluate the efficacy of the response to chemotherapy and its relation to expression of membrane antigens, the 42 dogs received a therapeutic protocol identical to vincristine sulfate (0.75 mg/ m², IV, q 7 d). The treatment duration

varied from 4 to 8 weeks. The response was considered complete when tumor regression did not surpass 4 weeks and partial when the regression was from 5 to 8 weeks.

The relation of the clinical response by cytomorphological group and P-glycoprotein expression was investigated. Furthermore, the relation of P-glycoprotein immunostaining to the clinical response to chemotherapy was studied in 42 neoplastic masses.

The samples were compared according to cytomorphological type and biological behavior and analyzed at a 5% significance level by the chi-square test with the Goodman test applied for comparison of multinomial proportions.

Results

The tested antibody anti-P-glycoprotein clone 5B12 did not present positive staining in tissues that physiologically express P-glycoprotein such as rigid kidney and liver. Furthermore, antigen expression was not demonstrated in TVT cells, even following the recommendations of the literature and of the manufacturer. After the attempts at antigen recuperation by heating with EDTA and citrate and the use of secondary systems and different amplification without success, it was not incorporated into the panel.

When the antibody anti-P-glycoprotein clone C494 was utilized, positive staining was achieved at 1:100 dilution of primary antibody, both in positive controls and in TVT cells. In the initial processing it was not necessary to employ antigen recuperation by heat, but for the diminution of non-specific stainings and depth reduction, before incubation with primary antibody, the cytological preparations were incubated with BSA 2% for 1 h at room temperature. For the P-glycoprotein positive control histological cuts or the impression of a normal canine liver was utilized and

Table 1. Primary antibody, staining type, brand, and standardized dilutions utilized.

Primary antibody	Type of staining	Clone	Brand	Standardized dilution
Anti-P-glycoprotein	cytoplasmic	C494	Dako	1:100

for the negative control, cytological TVT samples incubated only with diluent of the primary antibody were employed.

The replacement of streptavidine-biotin complex by a secondary antibody system and polymer associated with peroxidase, incubated at room temperature for 1 h, resulted in a reduction of non-specific stainings (background).

The cytoplasmic membrane staining of TVT cells varied from discrete to intensely colored, generally accompanied by diffuse cytoplasmic staining.

Of the 102 samples tested, 50.00% possessed plasmocytoid morphology, 18.63% lymphocytoid, and 31.37% mixed. Of the total, 46 (45%) presented P-glycoprotein expression and 56 (55%) were negative. The plasmocytoid group displayed a significantly greater immunoreactivity to the anti-P-glycoprotein antibody ($P < 0.05$) than did the lymphocytoid group (Table 2).

When the cytological preparations were regrouped into primary and non-primary masses (Table 3), it

was observed that the non-primary group produced superior immunoreactivity (67.65%), compared to the primary group (32.35%) without a significant difference.

The 42 clinical cases monitored were submitted to the same therapeutic protocol (drug, administration route, interval, and number of applications). In analyzing the complete clinical response to chemotherapy among the groups (Table 4), it was observed that the plasmocytoid group was significantly less sensitive to chemotherapy ($P < 0.05$) in relation to lymphocytoid. Still, it was noted that the percentage of cases that presented a partial clinical response to chemotherapy in the lymphocytoid group (36.37%) was lower than that in the plasmocytoid group (80.95%).

By comparing the clinical response to chemotherapy with the P-glycoprotein expression due to tumoral resistance to some chemotherapies (Table 5), it was observed that the group with partial response presented a higher percentage of P-glycoprotein

Table 2. Number of cases (n) and percentage (%) of P-glycoprotein staining in samples from different cytomorphological groups of transmissible venereal tumor.

GROUP	Positive		Negative		Total
	n	%	n	%	
Lymphocytoid	3 ^a	15.79	16	84.21	19
Plasmocytoid	29 ^b	56.86	22	43.14	51
Mixed	14 ^{ab}	43.75	18	56.25	32

^{ab}Different letters represent significant differences for $P < 0.05$ (Goodman test for contrast between multinomial proportions)

Table 3. Percentage of P-glycoprotein staining in samples of primary and non-primary neoplasias in transmissible venereal tumor.

P-glycoprotein	Primary		Non-primary	
	n	%	n	%
Staining positive	26	37.68	19	57.57
Staining negative	43	62.32	14	42.43
Total	69	100	33	100

$P = 0.11$ by the chi-square test

Table 4. Clinical response to chemotherapy among the different cytomorphological groups of transmissible venereal tumor.

GROUP	Complete response		Partial response		Total
	n	%	n	%	
Lymphocytoid	7 ^a	63.63	4	36.37	11
Plasmocytoid	4 ^b	19.05	17	80.95	21
Mixed	7 ^a	70.00	3	30.00	10

^{ab}Different letters represent significant differences at $P < 0.05$ (Goodman test for contrast between multinomial proportions)

Table 5. Distribution of P-glycoprotein staining percentage in relation to clinical response to chemotherapy in animals with transmissible venereal tumor.

GROUP	Positive		Negative	
	n	%	n	%
Complete response	6	31.57	13	56.52
Partial response	13	68.43	10	43.48
Total	19	100	23	100

$P = 0.19$ by the chi-square test

staining (68.43%). Alternatively, the group with complete response had a lower percentage of P-glycoprotein staining (31.57%). Despite the lack of a significant difference, the biological effect was apparent.

Discussion

The hypothesis that TVT presents different strains of variable aggressiveness has already motivated the use of this tripartite classification since 1994 by the Veterinary Pathology Service of FMVZ, UNESP at Botucatu, Sao Paulo, Brazil (7,26). The cells of the lymphocytoid group are small, have a regular contour with a round nucleus, and are generally concentric. Those of the plasmocytoid group are voluminous, and have an irregular contour with an eccentric nucleus and abundant cytoplasm. By analyzing the frequency of TVT cytomorphological types in the present study, it can be observed that the plasmocytoid and mixed classifications predominated in relation to the lymphocytoid group.

The antibody anti-P-glycoprotein clone 5B12 did not present positive staining in the tissues that physiologically express P-glycoprotein. In the same manner, it did not demonstrate antigen expression in TVT cells. Although, as exemplified by other antibodies such as clone C494, a cross-reaction exists, in this case it was not observed. The use of different monoclonal antibodies for the same molecule may be recommended, given that not all epitopes of the molecule have the same constitution of amino acids (27). Probably, the amino-acid constitution of the TVT cell epitope would not be compatible with the clone 5B12, since it does not present immunostaining.

When the antibody anti-P-glycoprotein clone C494 was utilized, satisfactory positive staining was obtained with a 1:100 dilution of primary antibody, both in positive controls and in TVT cells, as recommended by Erünal-Maral et al. (14). Considering that in oncology the increase in P-glycoprotein expression is related to an unfavorable prognosis (22), the standardization of this antibody

in cytological and histological preparations constitutes an innovative parameter for definition of prognosis in canine oncology and represents a pioneering step in veterinary medicine.

Starting from the expression of P-glycoprotein patterns, 4 categories were created. The first consists of those tumors that always express P-glycoprotein. The second comprise those that sometimes present P-glycoprotein expression. Tumors of the third category rarely express P-glycoprotein. The fourth category includes tumors that come to express P-glycoprotein after chemotherapy. In the context of this classification, it may be supposed that TVT would be, imprecisely, within the first and second categories (22).

The expression of P-glycoprotein by TVT suggests that it can perform an important role in resistance to chemotherapy against this neoplasia, similarly to that cited by Miyoshi et al. (25), who, working with P-glycoprotein expression canines with mastocytoma, observed that at least 26% of them expressed P-glycoprotein and, for this reason, could be resistant to several different drugs.

The findings described above still corroborate the results obtained by Moore et al. (19), Moral et al. (28), and Lee et al. (29), in P-glycoprotein staining in canine lymphoma; they observed that the most malignant forms displayed strong P-glycoprotein staining, which produced a direct relation between P-glycoprotein and multi-drug resistance in tumor cells. In an analogous manner, human oncology studies on the clinical importance of multi-drug resistance (MDR) found that elevated levels of P-glycoprotein expression correlate positively with lack of response or remission after adequate forms of chemotherapy (20).

Comparing the results of response to chemotherapy among the 3 morphological groups, it can be noted that the plasmocytoid group was significantly less sensitive to chemotherapy in relation to the lymphocytoid and mixed groups. When this response was compared with P-glycoprotein expression, a direct relation was observed between partial response to chemotherapy and strong expression of P-glycoprotein in the plasmocytoid

group. Based on this result, it can be inferred that clinical plasmocytoid TVT cases tend to present partial clinical response to chemotherapy, possibly by means of strong P-glycoprotein expression. In the same manner, it can be established that by the results obtained the lymphocytoid tumors responded completely to chemotherapy, probably through low expression of P-glycoprotein. This result agrees with that of Bassani-Silva et al. (10), who found augmented resistance to antitumoral action of propolis among plasmocytoid cases.

When cytological preparations were regrouped into primary and non-primary masses, it was observed that P-glycoprotein presented higher immunoreactivity in non-primary masses compared with the primary mass group. Even though the difference is not statistically significant, this result suggests that the non-primary neoplasias can present a potential to express resistance to chemotherapy. This fact was also observed by Lee et al. (29) and by Bergman et al. (30), who verified higher levels of P-glycoprotein expression in canine lymphomas in relapse than in their initial expression. Thus, according to Lee et al. (29), the P-glycoprotein expression before the initiation of treatment is a predictive factor independent of survival.

In conclusion, the expression of P-glycoprotein by TVT may be involved in the resistance to chemotherapy. Greater expression by plasmocytoid tumors points to the greater malignancy of this lineage. Determination of reactivity can constitute a prognostic tool for this neoplasia.

The present results suggest the need for future works that would identify oncogenes and anti-oncogenes expressed in the plasmocytoid and lymphocytoid cytomorphological groups, in order to evaluate whether the temporal sequence of mutations in fact determines the malignancy of spontaneous transmissible venereal tumors in dogs.

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