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Original article

Immunohistochemical detection of P-glycoprotein in various subtypes of canine lymphomas

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Abstract

Combination chemiotherapy is the current standard of care for dogs with lymphoma. Multidrug resistance is one of the most important factors contributing to the efficacy of chemiotherapy. The major protein responsible for this phenomenon is P-glycoprotein. Little is known about P-glycoprotein expression in particular subtypes of lymphomas. The aim of the study was evaluation of P-glycoprotein expression in various subtypes of canine lymphomas. Positive reaction with P-glycoprotein was found in 12/25 cases of various morphological subtypes of lymphomas, however, in 3/11 lymphomas the percentage of positively weakly stained cells was <10% and those tumors were also considered negative. Tumors with 10-50% P-glycoprotein positive cells were found in single cases of centroblastic and centroblastic-centrocytic tumors. In 5 lymphomas P-glycoprotein expression exceeded 50% of tumor cells. Those cases were found among centroblastic, centroblastic-centrocytic, lymphoblastic and Burkitt-like subtypes. Positive reaction was observed mainly in the cell cytoplasm, however, in some cases prominent perinuclear dot-like staining pattern was found. In 2 cases focal staining pattern comprised dominant type of immunolabelling. Among all lymphomas containing P-glycoprotein positive cells intensity of imunolabelling was assessed as weak (6/25), moderate (2/25) and strong (3/25). Our results indicate that P-glycoprotein expression is present in nearly one third of newly diagnosed canine lymphomas of different morphological subtypes including those most commonly occurring, such as cenroblastic lymphomas. Hence, determination of P-glycoprotein expression at the time of diagnosis could provide valuable information for the design of treatment protocols. Moreover, our results have shown that P-glycoprotein expression in canine tumors could be located in Golgi-zone.

Key words: dog, lymphoma, P-glycoprotein, immunolabelling

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Introduction

Chemiotherapy is widely used in treatment of many types of canine tumors. For many of them, including lymphomas, it is the mainstay of therapy. One of the most important factors contributing to the efficacy of chemiotherapy is tumor cell drug resistance (Nooter and Herweijer 1991, Ginn 1996) defined as the ability of tumor cells to survive exposure to toxic agents at maximum doses tolerated by normal tissues (O'Brien and Cordon-Cardo 1991). Multidrug resistance (MDR) refers to simultaneous resistance to variety of cytotoxic agents of different mechanisms of action and diverse chemical structures (Nooter and Herweijer 1991, Arceci 1993). It can be an intrinsic property of neoplastic cells or be acquired by population of tumor cells that were initially sensitive to chemotherapy, but subsequently become resistant after exposure to cytotoxic agents (O'Brien and Cordon-Cardo 1991). The major protein responsible for MDR is P-glycoprotein (P-gp) (Nooter and Herweijer 1991, Arceci 1993).

P-gp is a membrane glycoprotein belonging to the ATP-binding cassette transmembrane transporter superfamily and acts as an energy-dependent efflux pump that extrude endogenous and exogenous substrates out of the cell (Nooter and Herweijer 1991). Different P-gp isoforms have been identified and they are encoded by family of closely related genes termed *MDR* (multidrug resistance genes) in human, mice and dogs. In humans two P-gp isoforms (MDR1 and MDR3) with 80% amino acid homology have been identified, however only MDR1 is responsible for multidrug resistance phenomenon (Nooter and Herweijer 1991). In dogs the *MDR1* and *MDR3* genes were discovered. However, it has not yet been determined if the *MDR3* confers multidrug resistance (Martinez et al. 2008).

P-gp plays an important role in cellular physiology. It is implicated in the transport and regulation of endogenous molecules such as hormones or phospholipids, the transport of cytokines, the initiation of immune responses, proliferation and differentiation of hematopoietic stem cells. In physiological conditions P-gp is expressed in high concentration on epithelial cells of the liver, renal tubules, intestine and capillary endothelium of the blood-brain and blood-testis barriers (Nooter and Herweijer 1991, Arceci 1993, Ginn 1996). However, it plays also an important role in pathology. It has been suggested that certain diseases, such Crohn's disease, ulcerative colitis, Alzheimer's and Parkinson's diseases may be associated with a decrease in P-gp expression and/or activity (Martinez et al. 2008). A breed-related sensitvity to ivermectin in collies and several other dog breeds is also caused by mutation in *MDR1* gene coding P-gp (Martinez et al. 2008). Moreover, P-gp prevents the entry of chemotherapeutics into the neoplastic cells by effluxing them out of these cells decreasing the intracellular concentration of anticancer drugs, thereby limiting cytotoxicity at their target site (Campos et al. 2014). Overexpression of P-gp is associated with resistance to different types of chemotherapeutical agents including doxorubicin, mitoxantrone, etoposide, vinblastine, paclitaxel, actinomycin B, vinca alkaloids and glucocorticosteroids (Arceci 1993). Many of them are commonly used in veterinary patients.

Studies in human have shown that significant number of drug resistant tumors express P-gp. Moreover, P-gp expression within certain tumors is of prognostic value and is associated with poor prognosis, disease progression or predictable MDR (Arceci 1993).

Expression of P-gp has been investigated in canine species. Some of the studies have focused on P-gp distribution in normal tissues (Ginn 1996) whereas other have analyzed its expression in pathological conditions (Van der Heyden et al. 2011).

Studies of P-gp expression in various types of canine tumors are not numerous. Single papers focused on expression of P-gp in mast cell tumors (Teng et al. 2012), mammary tumors (Kim et al. 2012), thyroid carcinoma (Campos et al. 2014) and pulmonary carcinoma (Hifumi et al. 2010) are available. More attention is given to canine lymphomas. Several studies from this scope have been published. In one of them lymphoma cell line with MDR potential mediated with canine P-gp has been developed (Uozurmi et al. 2005). Other studies have focused on relationship between P-gp expression and response to chemotherapy or MDR development (Moore et al. 1995, Bergman et al. 1996, Lee et al. 1996, Dhaliwal et al. 2013). However, there are no studies with detailed analysis of P-gp expression in particular histological subtypes of lymphomas. Only Flood-Knapik et al. (2013) investigated expression of this protein in canine indolent lymphomas classified according to WHO scheme, but they did not found its presence in any of the examined cases. Moreover, Lee et al. (1996) also classified morphologically examined lymphomas, but they did not give any information regarding P-gp expression in particular subtypes of lymphoma. They used Working Formulation scheme, which is now considered obsolete. Thus the aim of this study was an immunohistochemical evaluation of P-gp expression in various subtypes of canine lymphomas.

Table 1. Clinical and pathological characteristics of lymphoma cases.

Case no	Breed	Age (years)	Sex	Phenotype of lymphoma	Subtype of lymphoma	Clinical stage at time of biopsy
1	Boxer	8	Female	T	Pleomorphic mixed, small and large cell	V
2	Boxer	7	Female	T	Pleomorphic mixed, small and large cell	V
3	Mix	6	Female	B	Small lymphocytic	IV
4	American Staffordshire terrier	10	Male	B	Centroblastic-centrocytic	III
5	Stafford	13	Female	B	Centroblastic-centrocytic	III
6	Rottweiler	5	Male	B	Centroblastic-centrocytic	III
7	Giant schnauzer	4	Male	B	Centroblastic-centrocytic	IV
8	Mix	11	Male	B	Centroblastic	IV
9	Standard schnauzer	4	Male	B	Centroblastic	IV
10	German shepherd	3	Female	B	Centroblastic	IV
11	Boxer	11	Female	B	Centroblastic	IV
12	Rottweiler	4	Female	B	Centroblastic	III
13	Mix	7	Male	B	Centroblastic	IV
14	Cane corso	6	Male	B	Centroblastic	IV
15	Mastino neapolitano	4	Female	B	Centroblastic	IV
16	Mix	6	Male	B	Centroblastic	IV
17	German shepherd	4	Male	B	Centroblastic	IV
18	Mix (Doberman crossbread)	12	Female	B	Lymphoblastic	III
19	Great Dane	7	Male	B	Lymphoblastic	IV
20	Great Dane	8	Female	B	Burkit-like	IV
21	Tossa-Inu	8	Female	B	Burkit-like	IV
22	Mix	7	Female	B	Burkit-like	IV
23	Boxer	6	Female	B	Burkit-like	V
24	Mix	5	Male	B	Burkit-like	III
25	Irish setter	6	Male	B	Burkit-like	IV

Materials and Methods

Histological examination

Twenty five dogs with multicentric lymphoma were included in this study. All patients were classified using modified World Health Organization (WHO) staging system (Moulton and Harvey 1990). Popliteal lymph nodes were collected during surgical biopsy from dogs with suspected lymphoma. All specimens were fixed in neutralized 10% formalin, paraffin wax-embedden and cut into 3 μm sections. Histopathological diagnosis was performed on the sections stained with haematoxylin and eosin (HE). Tumors were classified according to the updated Kiel classification adapted to canine species by Ponce et al. (2010).

Immunohistochemistry

Lymphoma phenotype was determined by immunochemistry with anti-CD3 rabbit polyclonal antibody (Dako, Glostrup, Denmark) and anti-CD79α mouse monoclonal antibody (clone HM57, Dako, Glostrup, Denmark), detecting neoplastic cells of T-cell and B-cell origin, respectively. Expression of P-gp was determined by immunohistochemistry using mouse monoclonal antibody against human P-gp (clone C494, Covance, Dedham, USA).

All immunohistochemical procedures were performed according to the manufacturer's protocols. All antigens were unmasked by microwaving twice (7 and 5 min, 700 W) in citrate buffer (pH 6.0). Then, they were incubated with primary antibody (diluted 1:50, 1:25 and 1:100 for CD3, CD79α and P-gp, respectively) for 1 hour at room temperature. Prior to incubation with primary antibody, sections stained with P-gp were blocked with goat serum for 1 hour at room temperature. The REAL™ EnVision™ Detection System, Peroxidase/DAB$^+$, Rabbit/Mouse (Dako, Glostrup, Denmark) visualization system was used for antigen detection. The sections were counterstained with Erlich's haematoxylin.

Reactive canine lymph nodes were used as a positive control for CD3 and CD79α antibodies and normal canine liver for P-gp antibody. Substitution of primary antibody by TBST (Dako, Glostrup, Denmark) was employed for negative controls.

Analysis of P-gp immunolabelling

Quantification of P-gp immunolabelling was performed by evaluation of entire section at 200x magnification and estimating the percentage of lymphoma cells with positive reaction using the immunoreactive score (IRS) system. This system defines IRS = staining intensity (SI) x percentage of positive cells (PP). SI was determined as 0 = negative; 1 = weak; 2 = moderate; 3 = strong. PP was defined as 0 = no signals; 1 = ≤10% of positive cells; 2 = 10-50% of positive cells; and 3 = ≥50% of positive cells. Cases with IRS scores of 2 or 3 were considered positive (Lee et al. 1996, Kim et al. 2012, Teng et al. 2012).

Results

From the 25 examined lymphomas 2 cases were of T-cell origin ($CD3^+CD79\alpha^-$) and 23 cases were of B-cell phenotype ($CD3^-CD79\alpha^+$). The T-cell tumors were classified morphologically as pleomorphic mixed, small and large cell lymphoma (PMCL) and B-cell lymphomas represented the following subtypes: centroblastic-centrocytic (CB/CCL) with diffuse architecture – 4 cases, centroblastic (CBL) – 10 cases, Burkitt-like (BLL) – 6 cases, lymphoblastic (LBL) – 2 cases and small lympocytic (SLL) – 1 case. Clinical and pathological characteristics of all cases of lymphomas are presented in Table 1.

Positive reaction with P-gp antibody was found in 12/25 (48%) of canine lymphomas, however, in 3/11 of them the percentage of positively weakly stained cells did not exceed 10% and those tumors were also considered negative (totally 68% tumors were negative). Those lymphomas belonged to CBL, BLL, SLL subtypes. Tumors with 10-50% P-gp positive cells were found in 2 cases (8%) and classified as CBL and CB/CCL. In 5 lymphomas (20%) P-gp expression exceeded 50% of tumor cells. Those cases were found among CB/CCL, CBL, LBL and BLL subtypes. The detailed data on PP in particular subtypes of lymphomas are given in Table 2.

Positive reaction was observed mainly in the cell cytoplasm, however, in some cases prominent perinuclear intensely stained dot-like foci were observed (Fig. 1A and 1B). Dot-like staining pattern was observed either in all specimen (2/11, CB/CCL and CBL) or only in some percentage of tumor cells (4/11, PMCL, LBL, CBL and BLL). In the latter case cells with such staining pattern did not form the dominant cell population. Among lymphomas with P-gp expression, intensity of imunolabelling was assessed as weak (6/25, 24%), moderate (2/25, 8%) and strong (3/25, 12%) (Table 2). P-gp staining intensity in CB/CLL and CBL tumors with dot-like staining pattern were considered strong.

The final IRS score ranged from 0 to 9 with mean 4.09±3.45. The detailed data on IRS score in particular cases of lymphoma expressing P-gp are presented in Table 3.

Discussion

This study evaluated P-gp expression in various histological subtypes of lymphomas collected from dogs before initiation of chemiotherapy. Large discrepancies in P-gp expression level in untreated canine lymphomas exist in available literature. It has been reported to occur from 3.3% (Moore et al. 1995) to 80% (Dhaliwal et al. 2013) of cases. We found P-gp expression in 28% of examined tumors. Our results are consistent with studies of Ginn (1996) and Lee et al. (1996) who observed P-gp expression in 27.3% and 32.9% of examined lymphomas, respectively. Similarly, large discrepancies in the percentage of P-gp positive tumors exist in human lymphomas. Among untreated lymphomas the percentage of tumors positive for P-gp range from about 10% or less (Liang et al. 2002) to about 70% or even more positive cases (Jillella et al. 2000, Jerkeman et al. 2004). In one study none of examined cases of diffuse large B-cell lymphomas (DLBCL) was considered positive for P-gp (Rujirojindakul et al. 2011).

Our results indicate that P-gp expression occurs in lymphomas of different histological subtype including CBL which is the most often diagnosed type of canine lymphomas (Ponce et al. 2010). In human medicine P-gp expression has been observed mainly in cutaneous T-cell lymphomas (Jillella et al. 2000), nasal NK/T lymphomas (Saglam et al. 2008), acute lymphoblastic leukemia (ALL) (Plasschaert et al. 2003), chronic lymphocytic leukemia (Jamroziak et al. 2004) and DLBCL (Ohsawa et al. 2005). In our study positive P-gp labeling was observed in LBL, CBL, BLL and CB/CCL. According to WHO classification CBL is included into DLBCL whereas CB/CCL fall into follicular lymphoma (FL) category (Ponce et al. 2010).

Little information is available on P-gp expression in CB/CCL in both man and dogs. One study reported the experimental group of lymphomas that included single cases of FL, but all were negative (Liang et al. 2002). Similarly, two cases of canine FL analyzed by Flood-Knapik et al. (2013) were also negative. On the contrary, Schiozawa et al. (2003) observed P-gp expression in the majority of examined cases of FL. Similarly, in our study 2/4 cases of CB/CCL were P-gp positive. One of them was characterized by weak P-gp

Table 2. Expression of P-glycoprotein in lymphoma subtypes.

Subtype of lymphoma	Number of cases	Phenotype	Immunoreactive score system (IRS)							
			PP				SI			
			0%	≤10%	10-50%	≥50%	negative	weak	moderate	strong
Pleomorphic mixed, small and large cell	2	T	1/2	1/2			1/2		1/2	
Small lymphocytic	1	B		1/1				1/1		
Centroblastic-centrocytic	4	B	2/4		1/4	1/4	2/4	1/4		1/4
Lymphoblastic	2	B	1/2			1/2	1/2		1/2	
Centroblastic	10	B	6/10	1/10	1/10	2/10	6/10	3/10		1/10
Burkit-like	6	B	4/6	1/6		1/6	4/6	1/6		1/6
TOTAL [%]	25		14/25 56%	4/25 16%	2/25 8%	5/25 20%	14/25 56%	6/25 24%	2/25 8%	3/25 12%

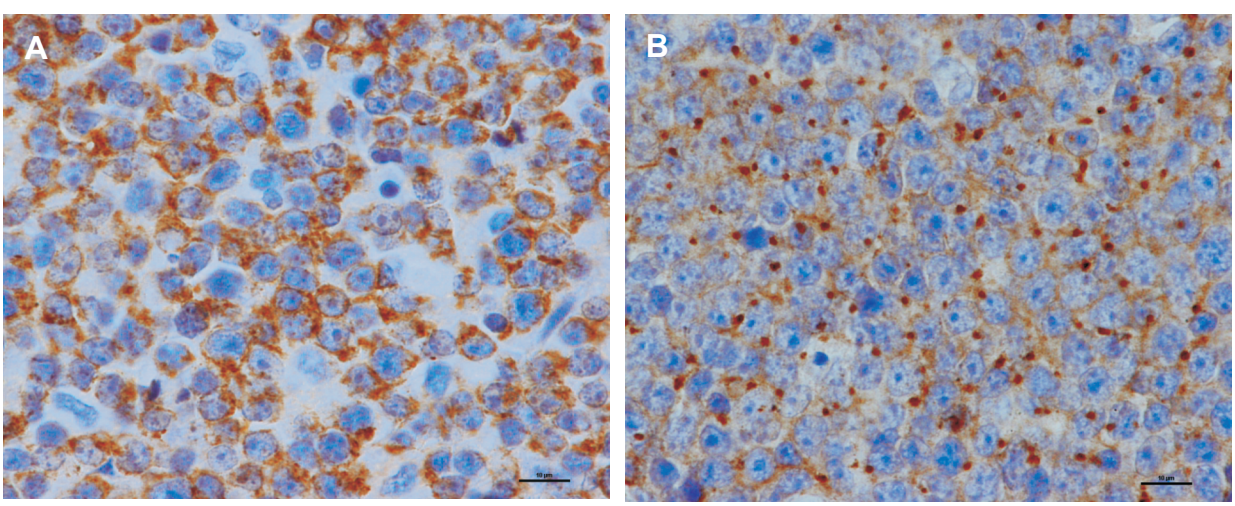

Fig. 1. Immunohistochemical expression of P-glycoprotein in canine lymphomas: A) Strong diffuse cytoplasmic immunoreactivity; B) Dot-like staining pattern; Bar = 10 μm.

Table 3. Expression of P-glycoprotein in lymphoma positive cases including those with P-glycoprotein weakly positive cells less than 10%.

Case no	Subtype of lymphoma	Immunoreactive score system (IRS)		
		PP	SI	Final score
1	Pleomorphic mixed, small and large cell^	1	2	2
2	Small lymphocytic	1	1	1
3	Centroblastic-centrocytic*	3	3	9
4	Centroblastic-centrocytic	2	1	2
5	Lymphoblastic^	3	2	6
6	Centroblastic	1	1	1
7	Centroblastic*	3	3	9
8	Centroblastic^	3	1	3
9	Centroblastic	2	1	2
10	Burkit-like	3	3	9
11	Burkit-like^	1	1	1
	TOTAL (mean ± SEM)	2.09 ± 0.94	1.73 ± 0.90	4.09 ± 3.45

* – dot-like staining pattern

^ – cases with diffuse cytoplasmic staining pattern containing cells with dot-like perinuclear P-gp expression

expression observed in less than 50% of tumor cells. However, in the second case the P-gp expression was strong and found in more than 50% of lymphoma cells. Our results are consistent with observations of Schiozawa et al. (2003) who also found variations in P-gp expression in FL. Moreover, these authors did not observe increase in P-gp expression after transformation FL to DLBCL. CB/CCL cases included in our study showed complete disappearance of neoplastic follicles existing in the tumor at the first diagnosis and corresponded to definition of transformed FL (Schiozawa et al. 2003). It can indicate that P-gp is not involved in lymphoma progression though such role of this protein has been suggested (Burger et al. 2003). Our results have confirmed observations of Lee et al. (1996) that P-gp did not differ significantly within any morphological subtype. Moreover, they suggest that in dogs, similar to human lymphomas, expression of P-gp does not differ significantly among low and high grade tumors (Marie 1999).

Except CB/CCL, P-gp expression was found also in LBL and BLL subtypes. Presence of P-gp in LBL is in the agreement with results of human studies, since expression of P-gp in ALL is frequently detected (Plasschaert et al. 2003). One of the 2 examined T lymphomas was positive for P-gp. Expression of this protein is frequently observed in human T-cell lymphomas of different subtypes (Jillella et al. 2000, Saglam et al. 2008). In contrast, P-gp expression was observed in BLL. In the available literature no information regarding P-gp expression in human Burkitt lymphoma or Burkitt-like lymphoma was found. However, it should be underlined that definition of canine BLL is based only on the histological similarities with human Burkitt lymphoma, including cell morphology and very high proliferative potential but have nothing to do with its ethiopathogenesis (Ponce et al. 2010).

Tumors arising from tissues without intrinsic P-gp expression have a membranous and/or diffuse cytoplasmic pattern of labelling (Cordon-Cardo et al. 1990, Ginn 1996). This staining pattern have been described in canine tumors of different origin (Hifumi et al. 2010, Kim et al. 2012, Teng et al. 2012, Campos et al. 2014) including lymphomas (Bergman et al. 1996, Ginn 1996, Lee et al. 1996). However, in our study, except typical cytoplasmic reaction, a dot-like staining pattern was found. It was observed either is some percentage of neoplastic cells or it consisted of dominant type of immunolabelling in two cases. This staining pattern has not been reported to date in canine lymphomas. Its presence could be explained by unspecific cross-reaction with other than P-gp intracellular proteins. However, the P-gp is highly conserved across species and the monoclonal antibody clone C494 used in our study is known to cross-react with canine tissues (Ginn 1996). This antibody has been frequently used during the last two decades in studies on P-gp expression in dogs in normal tissues (Ginn 1996), pathological conditions (Van der Heyden et al. 2011) and in various types of tumors (Ginn 1996, Lee et al. 1996, Hifumi et al. 2010, Campos et al. 2014). Clone C219 is another most widely used monoclonal antibody for P-gp detection in canine tissues (Bergman et al 1996, Lee et al. 1996, Uozurmi et al. 2005, Van der Heyden et al. 2011, Kim et al. 2012, Teng et al. 2012, Flood-Knapik et al. 2013). However, we chose clone C494 of antibody because, according to Ginn (1996) results, C494 is the antibody of choice for P-gp detection in canine tissues. Moreover, whereas C219 binds to both MDR1 and MDR3, C494 detects only MDR1 (Georges et al. 1990). Despite described in literature specificity of C494 binding to P-gp (Georges et al. 1990), cross-reactivity of C494 with an antigen other than P-gp cannot be excluded, especially that this antibody is believed to cross-react with pyruvate carboxylase (Rao et al. 1994).

On the other hand, dot-like perinuclear P-gp immunoreactivity has been described in canine adrenal cortex cells (Gin 1996). Moreover, studies conducted in man have shown that P-gp expression can take a form of single dot-like signal in perinuclear area where the Golgi apparatus is located. Such staining pattern termed Golgi-zone pattern has been described in normal colon and urether epithelium cells (Weinstein et al. 1990) as well as in human cancers including colon carcinoma and endometrial adenocarcinoma (Axiotis et al. 1999). Staining of the Golgi apparatus could have resulted from the presence of newly synthesized P-gp (Sai et al. 1999) and it was suggested that P-gp is responsible for the accumulation of doxorubicin in the Golgi apparatus (Molinari et al. 1998).

The Golgi apparatus staining is not limited only to the tumors arising from epithelium cells with intrinsic P-gp expression since the same staining pattern has been observed in some cases of human leukemia, including ALL and acute myeloid leukemia (Kaczorowski et al. 1996). Unfortunately, the cited authors did not specified in how many cases and of what particular type of leukemia this staining pattern was found. It should be stressed that Golgi-zone pattern of P-gp expression was observed using clones C219 and JSB1 of P-gp antibodies (Weinstein et al. 1990). However, Axiotis et al. (1991) also used clone C494 of P-gp antibody in their study. Results of the above mentioned papers suggest that focal P-gp staining pattern observed in our study could be specific and the Golgi-zone immunolabelling could also exist in canine tumors. According to our knowledge this staining pattern has not been described previously in

canine neoplastic lesions. However, to additionally identify this protein assessment of P-gp expression with the second clone, for example C219, recognizing spatially distinct epitopes of P-gp should be performed as it was suggested by Beck et al. (1996). Moreover, molecular confirmation of *MDR1* gene expression and Western blots analyses on parallel specimens should be conducted to exclude immunohistochemical false-positive results.

Our results indicate that P-gp expression is present in nearly one third of newly diagnosed canine lymphomas of different morphological subtypes including those most commonly occurring, such as CBL. Hence, determination of P-gp expression at the time of diagnosis could provide valuable information for the design of treatment protocols. Moreover, our results have shown that P-gp expression in canine tumors may have not only membranous/cytoplasmic pattern but it also could be located in Golgi-zone.

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