Polish Journal of Veterinary Sciences Vol. 18, No. 1 (2015), 113–122

DOI 10.1515/pjvs-2015-0015

Original article

Survivin expression in canine lymphomas in relation with proliferative markers

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Abstract

Survivin is a member of apoptosis inhibiting proteins family. Apart from its antiapoptotic activity it plays a critical role in regulating the cell cycle and mitosis. It is overexpressed in most human malignancies. While the prognostic significance of survivin expression is widely investigated in human non-Hodgkin's lymphomas, little is known about its expression in canine lymphomas. The aim of the study was to evaluate the expression of survivin in canine lymphomas in relation to proliferation markers (mitotic index and percentage of Ki67-positive cells). Survivin was found in all examined lymphomas belonging to 6 different morphological subtypes with nuclear immunoreactivity. In most of lymphomas (18/25) survivin expression ranged 10%-25% of positive cells. Only single cases had lower (0-10% positive cells, 1/25) or higher (25-50% and >50% positive cells, 5/25 and 1/25, respectively) index of survivin. Neither mitotic index nor proliferative index correlated with survivin expression when the values quantified randomly in whole specimens were compared. However, when survivin expression were quantified in selected tumor areas of low and high proliferation activity the high correlations between survivin expression and proliferation index were found. The results indicated that survivin is commonly expressed in canine lymphomas. Nuclear labelling together with the relation of its expression and proliferative activity in highly proliferative areas of neoplastic tissue suggest a potential role of survivin in cell cycle activation in canine lymphoma cells. However, further studies of the relation between expression of survivin and other proteins involved in cell cycle regulation are needed. Moreover, the results suggest that survivin may pose the therapeutic target in canine lymphomas.

Key words: dog, lymphoma, proliferative markers, survivin

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Introduction

Non-Hodgkin's Lymphomas (NHLs) are the most frequently diagnosed tumors arising from hematopoietic tissue in dogs. Because of their incidence and seriousness as well as very high sensitivity to chemotherapy they are a major concern in veterinary medicine. NHLs constitute a heterogeneous group of lymphoproliferative disorders with respect to presentation, clinical course, response to treatment and prognosis. They have been classified into subtypes on the basis of histological criteria. However, histology alone is not always predictive for clinical behavior, especially as lymphomas of the same histological subtype but different clinical courses have been reported (Schwartz et al. 1989). Thus, other prognostic factors are investigated to better predict the tumor's behavior. This factors include cell proliferation markers such as argyrophilic nucleolar organizer regions (AgNORs), expression of Ki67 or proliferating cell nuclear antigen (PCNA) and mitotic index (MI).

Recently a special attention has been given to survivn. Survivin is a member of apoptosis inhibiting proteins (IAPs) family. It inhibits both caspase-dependent and caspase-independent apoptosis and plays a critical role in regulating the cell cycle and mitosis (Altieri 2003, Li and Ling 2006). Survivin exhibits peak expression during mitosis and is critical for normal cell division (Li et al. 1998). It is located in various components of mitotic apparatus, including centrosomes, microtubules of the mitotic spindle (Altieri 2003) and is involved in the maintenance of chromosomal stability during mitosis (Kallio et al. 2001). Moreover, more recent evidence suggests that survivin also enhances telomerase activity which indicates that survivin participates not only in inhibition of apoptosis, but also in prolonging cellular lifespan (Endoh et al. 2005). Survivin is strongly and broadly expressed in embryonic and fetal organs, but becomes undetectable in most terminally differentiated normal adult tissues (Altieri 2003). Weak expression of survivin is observed in tissues with very high proliferative potential and cell turnover including placenta, endometrium, hematopoietic stem cells, thymocytes and intestinal epithelium (Urbaniak 2004). However, in malignant cells survivin is overexpressed and has been shown to be an almost universal tumor antigen being expressed in most human neoplasms (Andersen and thor Straten 2002, Altieri 2003). In tumor cells survivin is critical for cell division and inhibition of apoptosis (Altieri 2003). It also seems to have a roles in tumorigenesis, drug resistance and metastases (Dohi et al. 2004, Li and Ling 2006). Moreover, in many tumors including hematological neoplasms it has been shown as prognostic marker of aggressive and unfavorable form of disease (Adida et al. 2000, Altieri 2003, Martinez et al. 2004, Urbaniak 2004, Mitrović et al. 2011). Survivin is also considered as target for cancer therapy (Altieri 2003, Urbaniak 2004, Li and Ling 2006).

In veterinary medicine, in contrast to human oncology, much less studies have been focused on survivin expression in malignant cells. Immunohistochemical studies have revealed expression of this protein in some canine tumors including transitional cell carcinomas of urinary bladder (Rankin et al. 2008), cutaneous tumors (Bongiovanni et al. 2009, 2012b) and osteosarcomas (Bongiovanni et al. 2012a, Davies et al. 2012, Shoeneman et al. 2012).

While expression of survivin and its prognostic significance is widely investigated in human NHLs, to the author's knowledge only two studies focusing on survivin expression in canine lymphomas has been published. One of them has evaluated prognostic relevance of its expression in relapsed canine lymphomas (Rebhun et al. 2008). The second one is the methodological paper focused on establishing of a reliable method for immunohistochemical detection of survivin in canine tissues including samples of different normal tissues and lymphomas (Wimmershoff et al. 2010). Thus the aim of this study was to evaluate the expression of survivin in canine lymphomas in relation to proliferation markers.

Materials and Methods

Animals

Forty seven dogs, both males and females, of different breeds, aged from 3 to 13 years were examined because of clinical signs of lymphoadenopathy. The tentative diagnosis of multicentric lymphoma was made on the basis of clinical and laboratory examinations. From all dogs the fine needle biopsy samples were collected. On the basis of microscopic evaluation fine needle biopsy samples diagnosis of lymphoma was made. Finally, 25 dogs were included in the study. All patients were classified using modified World Health Organization (WHO) staging system (Moulton and Harvey 1990).

Histological examination

Popliteal lymph nodes were collected during surgical biopsy from all 25 dogs. All specimens were fixed in 10% neutral buffered formalin and processed by common paraffin technique. Histopathological diagnosis was performed on the sections stained with hae-

matoxylin and eosin (HE) and by immunohistochemical phenotyping. Tumors were classified according to the updated Kiel classification adapted to the dog 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 Ki67 antigen and survivin were determined by using anti-MIB-1 mouse monoclonal antibody (Dako, Glostrup, Denmark) and anti-survivin rabbit polyclonal antibody (Novus Biologicals Inc., Littleton, Colorado, USA), respectively. All immunohistochemical procedures were performed on serial sections according to the manufacturer's protocols. Antigen unmasking was performed by treating the slides with high temperature. CD3, CD79α and Ki67 were unmasked by microwaving twice (7 and 5 min, 700 W in citrate buffer pH 6.0). Survivin was unmasked using the pressure cooker (10 min in citrate buffer, pH 6.0). Then, they were incubated with primary antibody (diluted 1:50, 1:25, 1:100 and 1:500 for CD3, CD79α, Ki67 and survivin, respectively) for 1 hour at room temperature or overnight at 4°C (survivin). 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, CD79α and Ki67 antibodies and canine cutaneous squamous cell carcinoma for survivin antibody. Substitution of primary antibody by TBST (Dako, Glostrup, Denmark) was employed for negative controls.

Proliferation markers scoring

Estimation of proliferation activity was made in the sections stained with HE and immunohistochemically with anti-Ki67 monoclonal antibody. The proliferation activity was estimated on the basis of mitotic index and proliferative index in each specimen. Mitotic index (MI) was calculated as the mean number of metaphase and anaphase nuclei in 10 visual fields in triple counting (HE, 400x). Proliferative index (PI) was defined as the number of Ki67-positive lymphoma cells in 1000 tumors cell population in triple counting. Calculation of cells with Ki67 expression was made, first in fields of view (1000x) randomly selected along long axis of each specimen, then separately in the areas of intense and low proliferation activity.

Survivin scoring

Immunolabelling of survivin was classified based on the subcellular localization (cytoplasmic/nuclear) and the index of survivin defined as the percentage of positive cells in 1000 tumors cell population in triple counting. Calculation of cells with survivin expression was made, firstly in fields of view (1000x) randomly selected along long axis of each specimen, then separately in the areas of intense and low proliferation activity. According to the survivin expression assessed in randomly selected areas, all lymphomas were grouped into 5 classes (0%, 0-10%, 10-25%, 25-50% and >50% positive cells) (Adida et al. 2000, Rankin et al. 2008, Davies et al. 2012).

Association between survivin expression and proliferative index

In order to evaluate relationship between the number of proliferating cells and survivin expression in each specimen two areas were selected: one with low and the second with high number of Ki67-positive cells. In each area the PI was calculated in triple counting. Then, in the same localization the index of survivin was scored in triple counting.

Statistical analysis

Data, presented as mean values ±SEM, were analyzed using the Statistica 8.0 for Windows. Statistical comparisons were made with the Mann-Whitney U-test. Correlations between expression of survivin, and proliferative indices (MI, PI) were established by the significance of Spearman's rank correlation coefficient. $P \leq 0.05$ was considered significant.

Results

Histological examination

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

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	7	Female	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 napolitano	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	Irisch setter	6	Male	B	Burkit-like	IV

B-cell lymphomas represented the following subtypes: centroblastic-centrocytic (CB/CCL) – 4 cases, centroblastic (CBL) – 10 cases, Burkitt-like (BLL) – 6 cases, lymphoblastic (LBL) – 2 cases and small lympocytic (SLL) – 1 case. The SLL and CB/CCL are low grade tumors while all others subtypes belong to high grade lymphomas. Clinical and pathological characteristics of all cases of lymphomas are presented in Table 1.

Proliferation markers in whole sections

The range of MI for all lymphomas examined was 3.2-13.2 with the mean IM value of 8.84. In most cases (15/25) MI ranged from 5 to 10 mitoses per field at 400x. Those tumors belonged to PMCL (2 cases), CB/CCL (4 cases), CBL (7 cases) and BLL (2 cases) subtypes. Nine cases had MI higher than 10 (4 BLL, 2 LBL and 3 CBL tumors). Only SLL had MI value less then 5.

Expression of Ki67 was observed in whole specimens, however highly proliferating areas were frequently surrounded by the areas with lower Ki67 expression. When the PI was counted in randomly selected areas most of lymphomas (19/25) had proliferation activity at the level of 20%-40% Ki67-positive cells (2 PMCL, 4 CB/CCL, 1 LBL, 9 CBL, 3 BLL). Only in single cases expression of Ki67 was lower than 20% of positive cells (SLL) or reached almost 70% of positive cells (1 LBL, 1 CBL, 3 BLL). Detailed data on MI and Ki67 expression in particular subtypes of lymphomas are given in Table 2.

Survivin expression in whole sections

Survivin expression was observed in all examined cases. Neoplastic cells exhibited nuclear survivin expression of moderate to strong staining reaction (Fig. 1A). Positive reaction was found in both interphase nuclei and mitotic phases (metaphase, anaphase) (Fig. 1B). In small percentage of cells the cytoplasmic perinuclear reaction was observed. In all cases survivin-positive cells were distributed irregularly throughout the tumor. They form clusters of positive cells surrounded with areas of lower expression of this protein. In most of examined lymphomas (18/25) survivin expression ranged from 10% to 25% of positive cells Those tumors belonged to the following

Table 2. Proliferative indices and survivin expression in particular subtypes of lymphoma quantified in randomly selected tumor areas.

Subtype of lymphoma	MI		PI [%]		Index of survivin [%]							
					0-10%		10-25%		25-50%		>50%	
	range of values	mean value ±SEM	range of values	mean value ±SEM	range of values	mean value ±SEM	range of values	mean value ±SEM	range of values	mean value ±SEM	range of values	mean value ±SEM
Pleomorphic mixed, small and large cell	7.6-8.8	8.2±0.85	21.67-22.07	21.87±0.28			16.78-18.48(2)	17.63±1.20				
Small lymphocytic	3.2		21.57		8.4(1)							
Centroblastic-centrocytic	5.65-6.35	6.0±0.49	35.79-38.63	37.21±2.01			12.89-13.97(2)	13.43±0.76	26.78-27.42(2)	27.1±0.45		
Lymphoblastic	12.65-12.81	12.73±0.11	18.77-19.43	19.1±0.47			13.03-14.23(2)	13.63±0.85				
Centroblastic	5.63-11.2	8.12±2.83	21.6-35.47	27.06±7.39			14.4-17.57(10)	16.33±1.69				
Burkitt-like	9.4-13.2	11.16±1.92	37.2-68.47	48.18±17.59			13.98-14.56(2)	14.27±0.41	25.51-26.43(3)	25.97±0.65	55.13(1)	
Total	3.2-13.2	8.84±3.17	18.77-68.47	31.04±15.57	8.4		12.89-18.48	15.1±1.86	25.51-27.42	26.53±0.69	55.13	

The number of cases are given in parenthesis

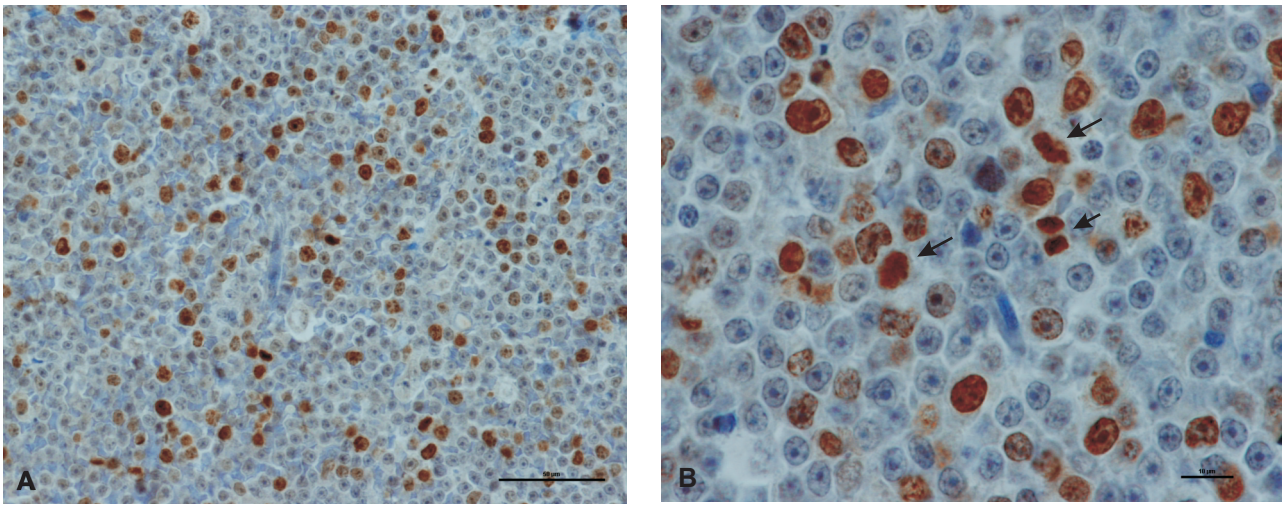

Fig. 1. Survivin expression in canine lymphomas: A) Representative area of canine lymphoma demonstrating survivin staining pattern; Bar=50 μm. B) Positive staining of mitotic figures – metaphases (long arrows) and anaphase (short arrow); Bar=10 μm.

subtypes: PMCL (2 cases), CB/CCL (2 cases), LBL (2 cases), CBL (10 cases) and BLL (2 cases). Only single cases were characterized by lower (0-10% positive cells, 1/25, SLL) or higher (25-50% and >50% positive cells, 5/25 and 1/25, respectively) index of survivin. Tumors with 25-50% positive cells belonged to CB/CCL (2 cases) and BLL (3 cases) subtypes. Lymphoma with the highest index of survivin was BLL. Detailed data on survivin expression in particular subtypes of lymphomas are given in Table 2. No correlations were found between survivin expression and either MI or PI when the values quantified randomly in whole specimens were compared.

Survivin expression and PI in tumor areas of different proliferation activity

In the areas of intense proliferation activity the range of PI values of all examined lymphomas were 22.8-72.75 with the mean PI value of 40.71±13.04. In most cases PI ranged from 30% to 40% of Ki67-positive cells (17/25 including the following subtypes: PMCL – 2 cases, CB/CCL – 4 cases, LBL – 1 case, CBL – 7 cases and BLL – 3 cases). Only in single cases PI reached lower or higher values. In SLL tumor the percentage of Ki67-positive cells was about 20% and in other 4 cases was higher than 45% (1 LBL, 1 BLL and 2 CBL), exceeding even 70% of Ki67-positive cells in 3 cases (1 CBL and 2 BLL). In the areas of low proliferation activity the range of PI values of all cases was 8.75-40.7 with the mean PI value of 18.66±8.66. In most tumors (14/25) PI ranged from 15% to 25%. Those lymphomas were classified as: PMCL (2 cases), CB/CCL (1 case), LBL (2 cases), CBL (6 cases) and BLL (3 cases) In 5/25 cases PI was lower than 15% (3 CB/CCL and 2 CBL) and in one of them the percentage of Ki67-positive cells did not reach 10% (SLL). In 4/25 cases PI was higher than 25% (2 BLL and 2 CBL) and in another 1 case PI exceeded 40% of Ki67-positive cells (BLL). The mean values of PI in areas of high and low prolifative activity differed significantly ($P \leq 0.001$).

Expression of survivin in corresponding areas of each specimen behave similarly to intensity of proliferation activity of tumor cells. In areas with high PI, higher percentage of survivin positive cells compared to region of tumor with low PI was noted. Those differences were not high and the percentage of survivin-positive cells usually ranged from 10% to 25% in both localizations. In some cases differences in number of cells expressing survivin between areas of low and high PI were higher (10-25% in areas of low proliferation activity *vs.* 25-50% or even >50% of positive cells in areas of intense proliferation). The range of survivin expression in areas with high PI was 15.65-57.65 with the mean value of 27.16±12.31. In the areas of low proliferation, the range of survivin-positive cells was 6.45-29.1 with the mean value of 15.59±6.02. The mean values of index of survivin in areas of high and low prolifative activity differed significantly ($P \leq 0.01$). The high correlation between survivin expression and PI was found in areas of lymphoma either of low or high Ki67 expression ($r = 0.75$; $P \leq 0.05$; $r = 0.84$; $P \leq 0.05$, respectively) (Fig. 2).

Moreover, the case of CBL with the highest total PI counted in randomly selected tumor areas that reached about 70% of Ki67-positive cells was characterized by the highest survivin expression exceeding 50% of positive cells. However, similar situation

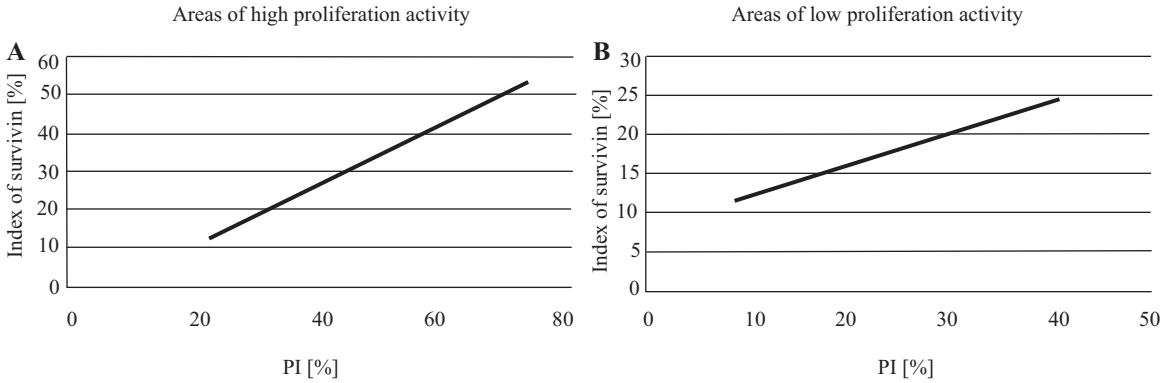

Fig. 2. Correlation of survivin expression and PI quantified in areas of different proliferative activity: A) In areas of high proliferation activity. B) In areas of low proliferation activity.

was not observed in case of tumor with the lowest total PI (LBL) in which the expression of survivin was found in 13.63% positive cells.

Discussion

In the present study we examined immunohistochemically the expression of survivin in the samples of canine NHLs and the relation between survivin expression and proliferative activity of lymphoma cells. We used the polyclonal antibody against full-length human survivin, as canine survivin has the same length and is 91.5% homologous to its human counterpart at the amino acid level (Uchide et al. 2005). This antibody has been used in previous studies conducted in human medicine (Martinez et al. 2004, Sohn et al. 2006, Margulis et al. 2007) as well as on canine tissues (Rankin et al. 2008, Rhebun et al. 2008, Bongiovanni et al. 2009, Wimmershoff et al. 2010, Bongiovanni et al. 2012a,b). Recently, Wimmershoff et al. (2010) confirmed that this polyclonal antibody is a reliable and sensitive tool for the detection of survivin in canine tissues.

In immunohistochemical studies investigating survivin expression, 5% of positive cells is considered as the cutoff value for this protein (Adida et al. 2000, Rankin et al. 2008, Davies et al. 2012). Because in all our cases belonging to different histological subtypes the expression of survivin significantly exceeding 5%, we considered them positive. It indicates that expression of survivin is frequent phenomenon in canine lymphomas. The results of two studies on survivin expression in canine NHLs also indicate that this protein is often present in this type of tumor (Rebhun et al. 2008, Wimmershoff et al. 2010). Wimmershoff et al. (2010) using polyclonal anti-survivin antibody showed expression of this protein in all examined cases. Similarly, Rebhun et al. (2008) detected survivin protein in majority of examined cases (in 94% and 88% of primary and relapsed lymphomas, respectively). However, there are some discrepancies regarding exact percentage of cells expressing survivin in canine NHLs. In the study of Wimmershoff et al. (2010) using polyclonal anti-survivin antibody in about 4% of cases the presence of survivin was demonstrated in 40-70% of cells and in 96% of cases the percentage of positive cells exceeds 70%. In contrast, the range of survivin expression for majority of lymphomas examined in our study was 10%-25%. Only single cases with higher percentage of survivin expression were noted. It seems that Rebhun et al. (2008) obtained similar results although those authors did not present detailed data but only median survivin score of 2.25. However, taking into account that they applied the same numeric scoring system to asses the percentage of survivin-positive cells, their results are comparable to ours. Our results confirmed previous observations (Rebhun et al. 2008, Wimmershoff et al. 2010) that survivin is expressed in canine NHLs of both B and T origin belonging to different morphological subtypes, especially high grade. Similarly, survivin expression was observed in most human NHLs of various morphological subtypes and often exceeded 70%-80% of examined cases. In the study of Gu and Lin (2004) the differences of survivin expression between high and low grade lymphomas were found. In the latter group survivin expression was observed in 20%-40% of cases depending of its histological subtype. Similar results were obtained in other studies (Li and Wu 2006, Zuo et al. 2007). In contrast, Mazur et al. (2004) observed the expression of survivin with equal frequency in low and high grade lymphoma cases. However, the group of indolent lymphomas was characterized by lower number of positive cells. In human NHLs the percentages of cells expressing survivin were also high (Kuttler et al. 2002). In most cases it exceeded 50% and often

reached at least 75% of positive cells, regardless of their histological subtype (Vassallo et al. 2010).

All examined lymphomas exhibited nuclear survivin expression of moderate to strong labelling. It seems that Rebhun et al. (2008) also observed only the nuclear staining pattern of tumor cells however, they did not define exact pattern of survivin labelling, showing only figures with representative areas of lymphoma samples. In contrast, in canine NHLs examined by Wimmershoff et al. (2010) cytoplasmic pattern of staining predominated. In human NHLs variable staining pattern of survivin is also observed. In some studies conducted for example on diffuse large B cell lymphomas (DLBCL) the nuclear staining pattern predominated (Martinez et al. 2004, Vassallo et al. 2010) while in other cytoplasmic reaction (Ambrosini et al. 1997, Adida et al. 2000) or both patterns (Mitrović et al. 2011) have been observed. Similarly, in anaplastic large cell lymphoma different patterns of staining reaction have been found (Vassallo et al. 2010). Such discrepancy in survivin localization in neoplastic cells have also been described in canine tumors. For example, in osteosarcoma cytoplasmic or nuclear staining reaction was predominant, as observed by Bongiovanni et al. (2012a) and Shoeneman et al. (2012), respectively.

Mechanisms controlling survivin nuclear and/or cytoplasmic localization in tumor cells are often a debated question, since the item could acquire a different prognostic significance depending on the tumor type. Five different splice variants of survivin with different subcellular localization have been identified in humans. Survivin and survivin-2B are localized in the cytoplasm while survivin-ΔEx3 is localized in the nucleus (Urbaniak 2004). Survivin isoforms differ in their functions. Survivin-2A may attenuate the anti-apoptotic activity of full length survivin, while survivin-ΔEx3 has been shown to have apoptotic functions (Urbaniak 2004, Li and Ling 2006). However, existence of survivin splice variants have not been confirmed in canine species. According to other studies it has been proposed that subcellular distribution of survivin is regulated by an active import into the nucleus and a chromosomal region maintenance 1 (CRM1) mediated export to the cytoplasm, suggesting that survivin may be considered a nuclear shuttling protein. Therefore, the almost exclusively cytoplasmic localization in a high number of tumor cells may be the result of a higher rate of nuclear export (Rodriguez et al. 2002). Generally, it is considered that nuclear survivin is likely to participate in promoting cell proliferation, whereas cytoplasmic survivin may be involved in cell survival by regulation of apoptosis (Li et al. 2005). However, the exact molecular mechanisms underlying nuclear survivin expression in tumors are not completely understood.

Results of microarray-based studies of human NHLs indicated that survivin expression in aggressive B-cell lymphomas, such DLBCL or Burkitt lymphoma was associated with overexpression of many cell proliferation-related genes, including cell cycle control, DNA repair and polymerase as well as apoptosis-inhibition genes which may contribute to the aggressive phenotype and poor prognosis (Kuttler et al. 2002). Increased survivin expression in DLBCL was found to be significantly associated with a higher expression of genes encoding cell cycle regulators (cyclin A, B) and anti-apoptotic factors and downregulation of genes encoding cell cycle inhibitors (Kuttler et al. 2002). Results of Kuttler et al. (2002) indicated the preferential relation between survivin and cyclin B expression, suggesting that cycline B overexpression, when linked to survivin overexpression, might demonstrate a specific G2/M transition promotion and enhancement of cell proliferation.

As survivin expression, especially in the nucleus, is related to cell proliferation promotion, we examined if the relation between survivin expression and proliferative activity can be defined in canine NHLs included in this study. We considered the most popular proliferation indices ie. MI and PI assessed as the number of mitotic figures and the percentage of Ki67-positive cells, respectively. According to our knowledge only a few such studies have been previously conducted on canine osteosarcomas (Bongiovanni et al. 2012a, Davies et al. 2012, Shoeneman et al. 2012) and sebaceous lesions (Bongiovanni et al. 2012b) and has not been previously reported in canine NHLs.

Survivin role in mitosis and cell cycle is well documented (Altieri 2003, Li and Ling 2006), however, we showed no correlation between MI and survivin expression. We found no data regarding relation between MI and survivin expression in NHLs and only a few studies conducted on other tumors. However, their results are conflicting. Some of them confirm such relation (Mellai et al. 2008) while other have not found it (Bongiovanni et al. 2009). One of the explanation of lack of such correlation could be the possibility that survivin gene may be globally deregulated in neoplasm, driving overexpression of the protein at all cell cycle phases, not just mitosis (Altieri 2003). This hypothesis can be confirmed by the fact that correlation between survivin expression and PI defined by Ki67 or PCNA is frequently found in various neoplasms including human NHLs (Martinez et al. 2004, Li and Wu 2006, Zhang et al. 2006, Zuo et al. 2007). This relation has been observed in NHLs without specifying of particular subtypes (Li and Wu 2006), in lymphomas of B-cell origin (Zuo et al. 2007) and in some histological subtypes as acute leukemia (Zhang

et al. 2006) or mantle cell lymphoma (Martinez et al. 2004).

In the present work, however, we did not found correlation between PI and percentage of survivin positive cells when the values of those parameters were quantified randomly in whole specimens. Similarly, Kuttler et al. (2002) did not found differences in Ki67 expression between groups of DLBCL of the highest and the lowest survivin gene expression. In both groups cases of similar Ki67 expression were observed. This phenomenon may be explained by the fact that when evaluating Ki67 antigen expression, proliferation rate often varies from field to field and highly proliferating areas are frequently surrounded with areas of lower Ki67 expression (Schwarz et al. 1989). Similar phenomenon was found in specimens of canine lymphomas in previous studies (Sokołowska et al. 2012) as well as in the present work. Similarly to Ki67 expression, survivin-positive cells were also distributed irregularly throughout the tumors. However, when survivin expression was quantified in selected tumor areas of high and low Ki67 expression the high correlation between survivin expression and PI was found. Similar results were obtained by Martinez et al. (2004) in human mantle cells lymphomas with double immunolabeling for Ki67 and survivin. In all cases described in cited paper a nuclear co-localization of both proteins was observed in representative areas of tumor, although the number of Ki67-positive cells was higher than survivin labelling. Off note, virtually all cells positive for survivin also expressed the Ki67 antigen.

Our results indicate that survivin is commonly expressed in canine NHLs of various morphological subtypes with a mostly nuclear staining pattern. Nuclear staining pattern together with the relation of its expression level with the proliferative activity in highly proliferative areas of neoplastic tissue suggest a potential role of survivin in cell cycle activation in canine lymphoma cells as in human counterpart. However, further studies looking for the relation between expression of survivin and other proteins involved in cell cycle regulation are needed. Moreover, the role of survivin in apoptosis inhibition should be also investigated to fully characterize its role in tumorigenesis and tumor progression in canine NHLs. Our results suggest that survivin may pose the therapeutic target in canine NHLs.

Acknowledgments

This work was supported by Committee of Scientific Research Grant No. N N308 578339 for R. Lechowski.

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