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Original article

Survivin expression in correlation with apoptotic activity in canine lymphomas

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Abstract

Survivin regulates cell cycle and mitosis and has antiapoptotic properties. Because of its dual function survivin has been the subject of much research focusing on its role in tumorigenesis and the relationship between survivin expression and apoptotic and/or proliferative activity in many types of human tumor including non-Hodgkin's Lymphomas. Such studies have not been conducted in canine lymphomas. The aim of this study was to evaluate the expression of survivin in canine lymphomas of low (5/25) and high (20/25) grades in relation to apoptotic markers (apoptotic index and index of caspase-3). Survivin was found in all examined lymphomas. Most tumors (18/25) showed survivin expression in 10%-25% of positive cells. Only in single cases was lower (0-10% positive cells, 1/25) or higher (25%-50% and >50% positive cells, 5/25 and 1/25, respectively) survivin expression. No significant differences between mean values of either index of survivin or apoptotic index was found between low and high grade lymphomas. However, such a difference among lymphoma grades was shown regarding the caspase-3 index. No correlation between the survivin index and either the apoptotic index or caspase-3 index was found, irrespective of the method of quantification: in whole specimens or in areas of low and high survivin expression. Positive correlation was consistently noted only between both apoptotic markers. The results indicate that survivin is commonly expressed in canine lymphomas. It seems that survivin does not exhibit anti-apoptotic activity in canine lymphomas. Lack of correlation between survivin expression and apoptotic markers could indicate its potential role in cell cycle activation in lymphoma cells.

Key words: apoptotic markers, dog, lymphoma, survivin

Introduction

Survivin is a protein belonging to the family of inhibitor of apoptosis proteins (IAPs) and was first described by Ambrosini et al. (1997) in human B-cell lymphoma. It is transiently expressed during embryological development, but barely detectable in normal differentiated adult tissues (Garg et al. 2016). However, its overexpression has been found in many human and canine neoplasms of different origin (Fu et al. 2014, Soleimanpour and Babaei 2015, Garg et al. 2016, Shamsabadi et al. 2016).

Survivin is essential for the proper completion of various stages of cell division (Andersen et al. 2007).

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Its expression is regulated in a cell-cycle dependent manner, with the maximum level during the G2/M phase. It plays a role in the mitotic checkpoint control and proper kinetochore attachment to spindle formation, and acts as a cytoprotective factor at cell division (Garg et al. 2016, Shamsabadi et al. 2016).

Survivin, via interaction with multiple regulators (e.g. Bcl-2 family members, Fas, TRIAL, FLIP, caspase-8 and -9), counteracts both intrinstic and extrinsic apoptosis pathways (Soleimanpour and Babaei 2015, Shamsabadi et al. 2016). Survivin is unable to directly attach to caspases. Hence, it interacts with several adaptor or cofactor molecules, such as XIAP. This complex activates the NFκB signaling pathway, resulting in the inhibition of caspase-9 and -3 (Shamsabadi et al. 2016). It can also act by binding to SMAC/DIABLO mitochondrial proapoptotic protein preventing caspase-9 activation (Garg et al. 2016, Shamsabadi et al. 2016). It also inhibits apoptosis via a caspase-independent mechanism via AIF with no change in caspase-3 activation. Moreover, upregulation of survivin inhibits, while downregulation promotes, cell autophagy (Soleimanpour and Babaei 2015). Interestingly, in tumor cells, survivin inhibits Fas-mediated apoptosis induced by immune cells (Shamsabadi et al. 2016).

The C-terminus of survivin is required for cell division and the N-terminus is dispensable for apoptosis. It may therefore be possible that survivin expression acts as a vital checkpoint for induction of apoptosis in cells undergoing aberrant divisions (Garg et al. 2016).

One of the propounded causes of tumor progression is disturbance of the balance between cell proliferation and apoptosis. Because of its dual function survivin has been the subject of many studies focusing on its role in tumorigenesis and the relationship between its expression and proliferative and/or apoptotic activity in many types of human tumors including non-Hodgkin's Lymphomas (NHLs) (Ito et al. 2000, Kutler et al. 2002, Ansell et al. 2004, Martinez et al. 2004, Li and Wu 2006, Zuo et al. 2006, Zuo et al. 2007, Mellai et al. 2008). However, their results are not fully consistent.

Recently, we investigated the expression of survivin in canine NHLs. Our results showed the relationship between its expression and proliferative activity in tumor cells, suggesting a potential role of survivin in cell cycle activation in this type of canine tumor (Sokołowska et al. 2015). However, to more completely characterize the role of survivn in tumorigenesis in canine NHLs the role of survivin in apoptosis inhibition should also be analyzed. Thus the aim of this study was to evaluate the expression of survivin in canine NHLs in relation to apoptotic markers on the same set of cases as in our previous study.

Materials and Methods

Histological examination: Popliteal lymph nodes collected during surgical biopsy from 25 dogs with confirmed multicentric lymphoma were included in this study. Tumor specimens were fixed in 10% neutral buffered formalin, processed by common paraffin technique and cut on 3 µm slides. Histopathological diagnosis was performed on sections stained with haematoxylin and eosin (HE) and by immunophenotyping. Tumors were classified according to the updated Kiel classification adapted to the dog by Ponce et al. (2010).

Immunohistochemistry: Lymphoma phenotype was determined by immunohistochemistry 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 survivin was determined by using anti-survivin rabbit polyclonal antibody (Novus Biologicals Inc., Littleton, Colorado, USA).

Tumor cell apoptosis was estimated using the TUNEL method and immunohistochemically with anti-caspase-3 rabbit polyclonal antibody (ab4051, Abcam, Cambridge, UK) reacting with the active form of caspase-3. Serial sections were used for all staining methods. All immunohistochemical procedures were performed according to the manufacturer's protocols. Antigen unmasking was performed by treating the slides with high temperature. CD3, CD79α and caspase-3 were unmasked by microwaving twice (7 and 5 min, 700 W in citrate buffer pH 6.0). Survivin was unmasked using a pressure cooker (10 min in citrate buffer pH 6.0). The slides were than incubated with primary antibody (diluted 1:50, 1:25, 1:100 and 1:500 for CD3, CD79α, caspase-3 and survivin, respectively) for 1 hour at room temperature or overnight at 4°C (caspase-3 and survivin). The REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark) visualization system was used for antigen detection. The sections were counterstained wit Erlich's hematoxylin.

The TUNEL method was used with ApopTag® Peroxidase In Situ Apoptosis Detection Kit (catalog number S7100, Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol. The sections were counterstained with methyl green.

Reactive canine lymph nodes were used as a positive control for CD3, CD79α and caspase-3 antibodies, as well as for the TUNEL method, and canine cutaneous squamous cell carcinoma for survivin antibody. Substitution of primary antibody by TBST (Dako, Glostrup, Denmark) was used for negative controls for all immunohistochemical methods.

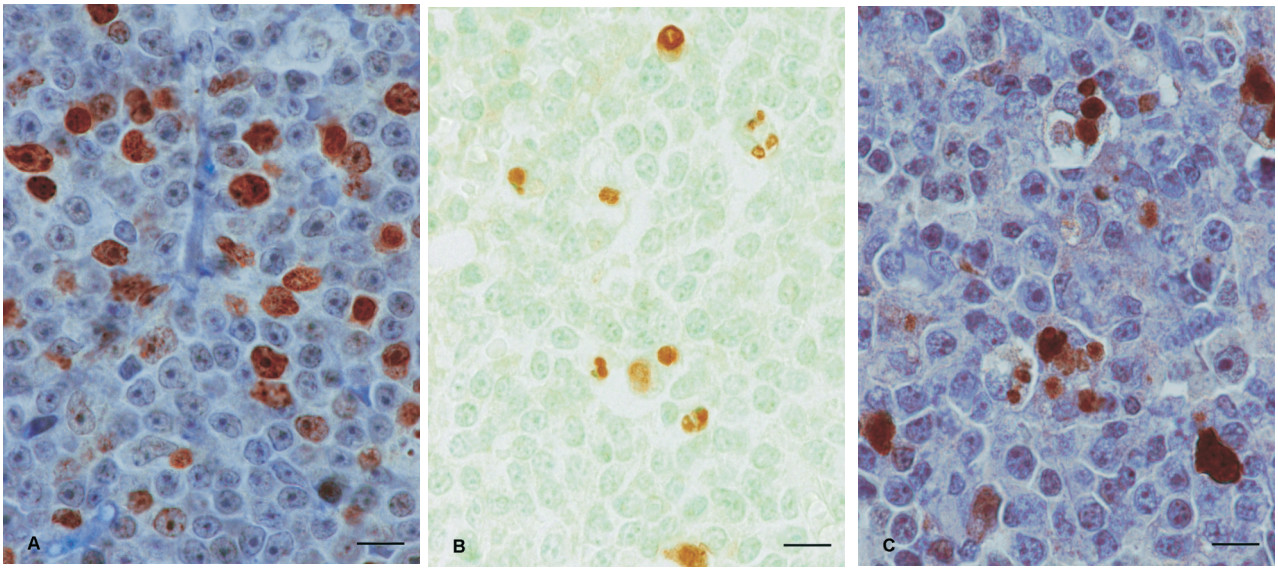

Fig. 1. Immunohistochemical staining of survivin and caspase-3 as well as TUNEL-positive cells in canine lymphomas: A) Survivin-positive nuclei. B) TUNEL-positive apoptotic bodies and single nuclei. C) Expression of caspase-3 in lymphoma cells, and apoptotic bodies, some of them are phagocytosed by macrophages; Bar = 10 μm.

Apoptosis marker scoring: Estimation of lymphoma cell apoptosis was made in the sections stained using the TUNEL method and immunohistochemically with anti-caspase-3 antibody. Tumor cell apoptosis was estimated on the basis of apoptotic index (AI) and the index of caspase-3 (Casp3I) in each specimen. Both indices were defined as the number of positive lymphoma cells and apoptotic bodies in a 1000 tumor cell population in triple counting. Calculation of AI and Casp3I was made, firstly in fields of view (1000x) randomly selected along the long axis of each specimen, then separately in the areas of high and low survivin expression.

Survivin scoring: Survivin expression was classified based on its subcellular localization (cytoplasmic/nuclear) and the index of survivin (SI) defined as the percentage of positive cells in a 1000 tumor cell population in triple counting. Calculation of cells with survivin expression was made, firstly in fields of view (1000x) randomly selected along the long axis of each specimen, then separately in the areas of high and low expression. 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) (Sokołowska et al. 2015).

Association between survivin expression and apoptotic markers: In order to evaluate the relationship between survivin expression and the number of cells undergoing apoptosis, two areas were selected in each specimen: one with low and the second with high number of survivin positive cells. In each area the SI was calculated in triple counting. In the same localization the AI and Casp3I were than scored in triple counting.

Statistical analysis: Data, presented as mean values ± SEM, were analyzed using Statistica 8.0 for Windows. Statistical comparisons were made using the Mann-Whitney U-test. Correlations between expression of survivin, and apoptotic markers (AI, Casp3I) were established by the significance of Spearman's rank correlation coefficient. $p \leq 0.05$ was considered significant.

Results

Histological examination: The study included 25 cases of canine lymphoma. Two of these were of T-cell phenotype ($CD3^+CD79\alpha^-$) and belonged to the pleomorphic mixed, small and large cell lymphoma (PMCL) subtype, whereas others were B-cell tumors and were classified morphologically into 5 subtypes: centroblastic (CBL) – 10 cases, Burkitt-like (BLL) – 6 cases, centroblastic-centrocytic (CB/CCL) – 4 cases with only minimally follicular pattern, lymphoblastic (LBL) – 2 cases and small lymphocytic (SLL) – 1 case. The SLL and CB/CCL belong to low grade tumors, while all other subtypes are classified as high grade malignancies.

Survivin expression in whole sections: All examined cases were positive for survivin. This reaction had a nuclear staining pattern in the vast majority of lymphoma cells (Fig. 1A). The cytoplasmic perinuclear reaction was found only in single cells. Intensity of the immunostaining was moderate to strong. Expression of survivin was observed in both non-mitotic nuclei and mitotic figures. In all examined lymphomas survivin-positive cells were not distributed uniformly

Table 1. Apoptotic markers and survivin expression in particular subtypes of lymphoma quantified in randomly selected tumor areas.

Subtype of lymphoma	AI [%]		Casp3I [%]		SI [%]							
					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
T-cell lymphomas: Pleomorphic mixed, small and large cell	3.86-4.2	4.03± 0.24	1.95-2.11	2.03± 0.11			16.78-18.48 (2)	17.63± 1.20				
B-cell lymphomas:												
Small lymphocytic	2.17	–	0.93	–	8.4 (1)	–						
Centroblastic-centrocytic	2.4-3.87	3.04± 0.61	0.8-1.1	0.95± 0.13			12.89-13.97 (2)	13.43± 0.76	26.78-27.42 (2)	27.1± 0.45		
Lymphoblastic	1.6-2.1	1.85± 0.35	0.87-0.93	0.9± 0.04			13.03-14.23 (2)	13.63± 0.85				
Centroblastic	2.65-8.17	5.51± 2.02	1-8.6	4.71± 2.79			14.4-17.57 (10)	16.33± 1.69				
Burkitt-like	1.17-7.2	4.62± 1.96	0.93-10.53	4.9± 3.11			13.98-14.56 (2)	14.27± 0.41	25.51-26.43 (3)	25.97± 0.65	55.13 (1)	–
Total	1.17-8.17	4.36± 1.99	0.8-10.53	3.48± 2.85	8.4	–	12.89-18.48	15.1± 1.86	25.51-27.42	26.53± 0.69	55.13	–

The number of cases is given in parenthesis
Abbreviations: AI – apoptotic index, Casp3I – index of caspase-3, SI – index of survivin

throughout the tumor; they formed areas of high survivin expression surrounded by areas of lower expression of this protein.

Our results indicate, that most lymphomas (18/25) had survivin expression at the level of 10-25% positive cells. Only in single cases was survivin expression either lower than 10% (1/25) or higher than 50% of positive cells (1/25). In the remaining lymphomas (5/25) survivin expression ranged from 25% to 50% of positive cells. Detailed data on survivin expression in particular subtypes of lymphomas are given in Table 1.

Apoptotic index in whole sections: Positive TUNEL reaction was observed in the nuclei of lymphoma cells and in apoptotic bodies either dispersed between tumor cells or phagocytosed by macrophages (Fig. 1B). They were distributed randomly and relatively uniformly throughout the whole specimen, with a tendency towards accumulation in the central part of neoplastic infiltration in cases of the highest apoptotic activity.

The range of AI values for all examined lymphomas was 1.17-8.07 with a mean AI value of 4.36±1.99. In most cases (12/25) AI ranged from 3% to 7% positive cells with a mean AI value of 4.77±0.84. These tumors belonged to the following subtypes: CBL (5 cases), BLL (4 cases), PMCL (2 cases) and CB/CCL (1 case). In 9/25 tumors AI was lower than 3% with a mean value of 2.33±0.63 and these lymphomas were classified as CB/CCL (3 cases), LBL (2 cases), CBL (2 cases), BLL (1 case) and SLL (1 case). The remaining 4/25 cases had apoptotic activity higher than 7%. The mean value of AI for this group was 7.7±0.45. These lymphomas belonged to the CBL (3 cases) and BLL (1 case) categories. Detailed data on AI in particular subtypes of lymphomas are given in Table 1.

Caspase-3 index in whole sections: Active caspase-3 displayed mainly cytoplasmic positivity and less frequently a nuclear pattern of immunostaining. Positive reaction was observed in lymphoma cells and less frequently in apoptotic bodies (Fig. 1C). Distribution of caspase-3-positive cells within lymphoma specimens was similar to apoptotic cell staining using the TUNEL method.

The range of Casp3I for all cases was 0.8-10.53 with a mean Casp3I value of 3.48±2.85. In most cases (13/25) expression of caspase-3 was found in <3% of lymphoma cells, with a mean Casp3I value of 1.23±0.53. These tumors were classified as: CB/CCL (4 cases), CBL (3 cases), PMCL (2 cases), LBL (2 cases), BLL (1 case) and SLL (1 case). In 8/25 cases Casp3I was at the level of 3%-7% of positive cells with

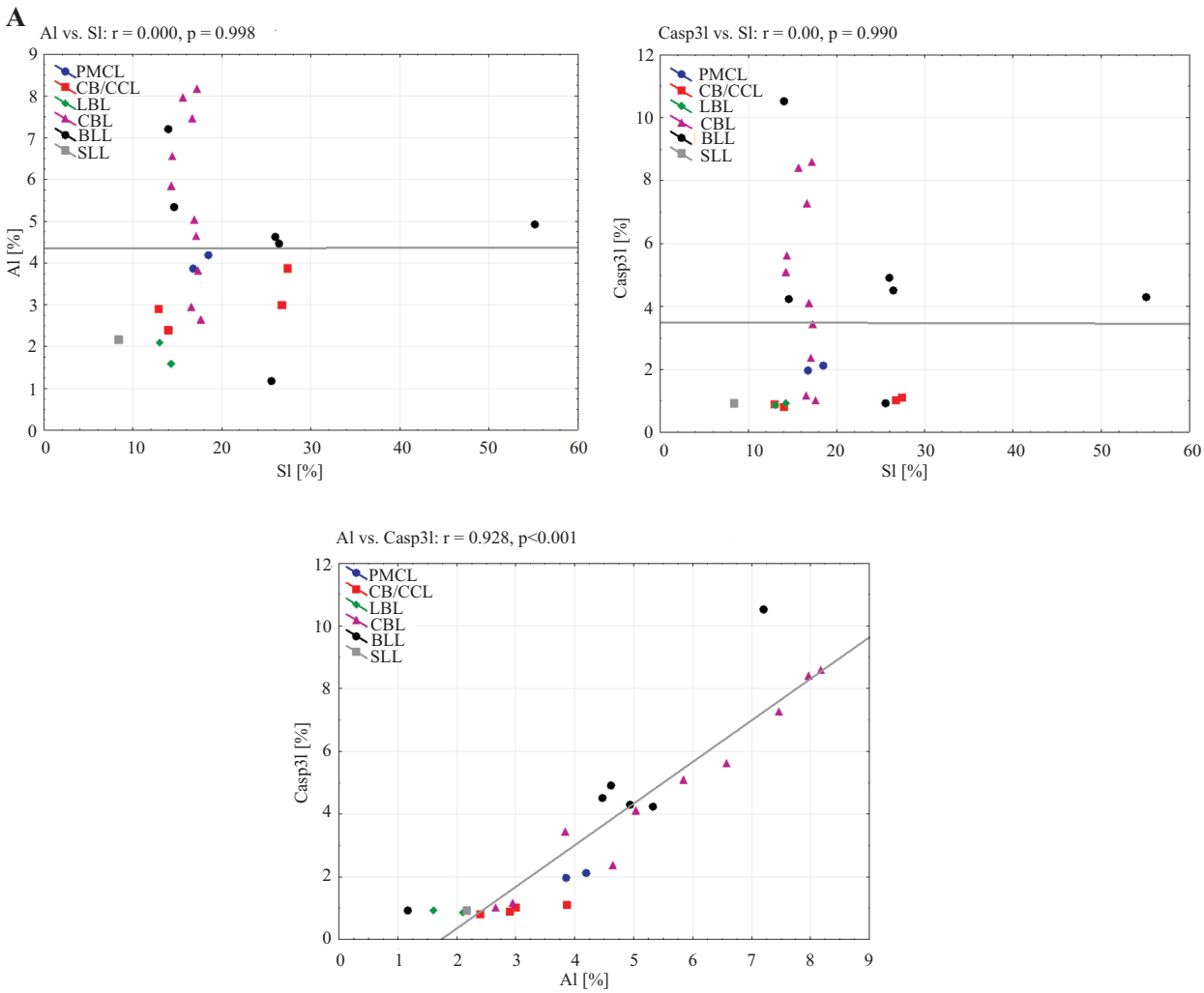

Fig. 2A

a mean Casp3I value of 4.52±0.68. They were classified as CBL (4 cases) and BLL (4 cases) subtypes. In the remaining CBL (3 cases) and BLL (1 case) Casp3I was higher than 7% with a mean Casp3I value of 8.7±1.35. Moreover, those lymphomas with the highest Casp3I were characterized also by the highest AI values. Detailed data on Casp3I in particular subtypes of lymphomas are given in Table 1.

Correlation between expression of survivin and apoptotic markers in whole sections: A very high positive correlation was found between AI and Casp3I ($r=0.94$; $p\leq0.05$). However, the expression of survivin did not correlate with either AI or Casp3I (Fig. 2A).

We also compared the mean values of examined indices between groups of different TUNEL and caspase-3 expression i.e. <3%, 3%-7% and >7% of positive cells. Where all examined cases were grouped according to AI values, the mean values of SI were: 16.54±6.02 (range of values: 8.4-26.78), 22.05±11.47 (range of values: 14.4-55.13) and 15.85±1.41 (range of values: 13.98-17.2), for groups with <3%, 3%-7% and >7% of TUNEL-positive cells, respectively. The mean values of Casp3I for these 3 groups were: 0.95±0.1 (range of values: 0.8-1.17), 3.64±1.44 (range of values: 1.1-5.63) and 8.7±1.35 (range of values: 7.27-10.53), respectively. Only mean values of Casp3I differed significantly among the groups with various AI ($p\leq0.01$, except 0-3% *vs.* 3%-7% groups with $p\leq0.001$). When we grouped examined lymphomas according to Casp3I values, the mean values of SI were: 17.59±5.72 (range of values: 8.4-27.42), 23.11±13.86 (range of values: 14.27-55.13) and 15.85±1.41 (range of values: 13.98-17.2), for groups with >3%, 3%-7% and <7% of caspase-3-positive cells, respectively. The mean values of AI for these 3 groups were: 2.89±1.03 (range of values: 1.17-4.65), 5.08±0.85 (range of values: 3.83-6.57) and 7.7±0.44 (range of values: 7.2-8.17), respectively. Only mean values of AI differed significantly among the groups with various Casp3I ($p\leq0.001$, except 0-3% *vs.* >7% groups with $p\leq0.01$).

We also compared the mean values of all indices between low and high lymphoma grades. Both groups were characterized by similar values of mean SI:

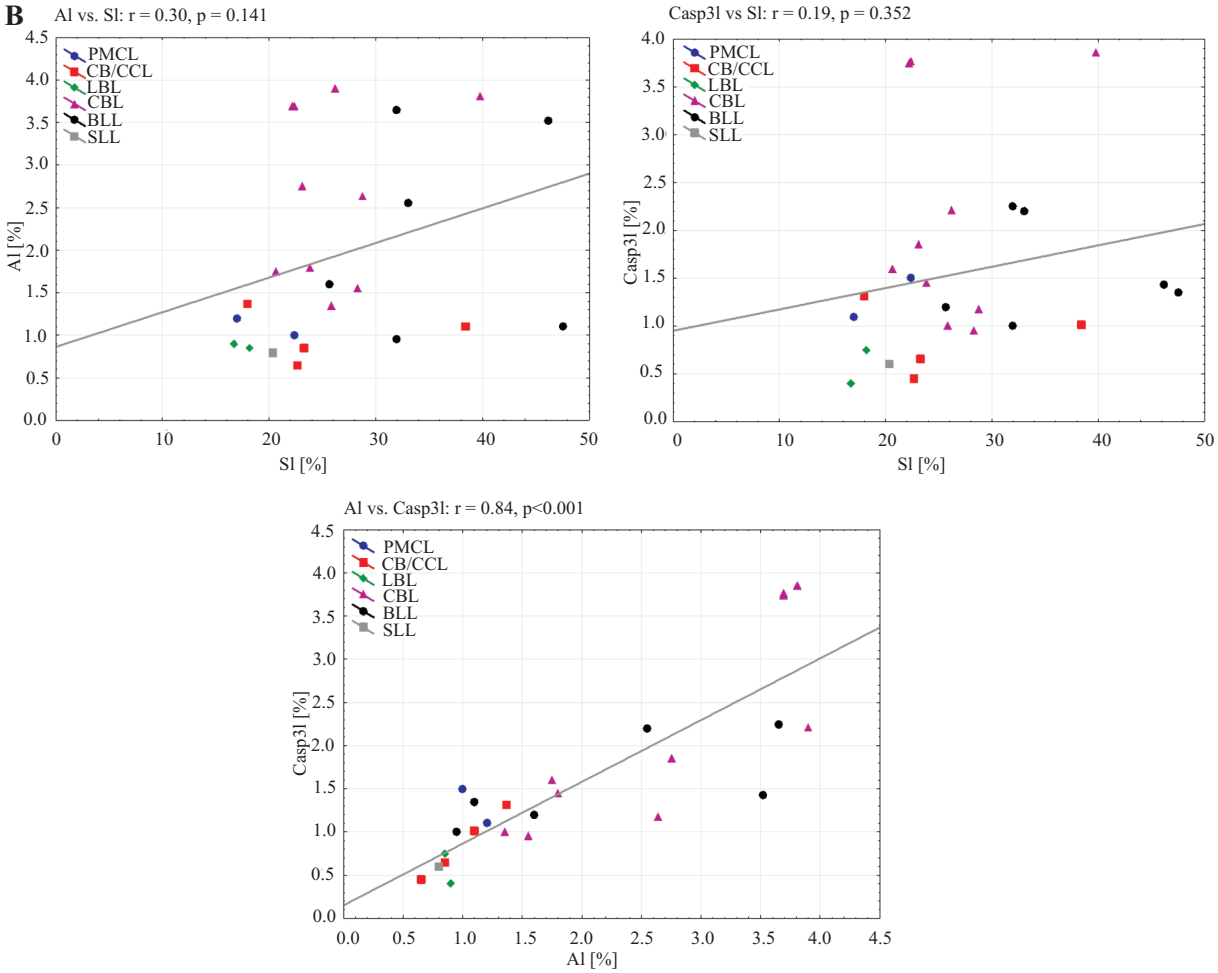

Fig. 2B

17.89±8.66 (range of values: 8.4-27.42) and 19.37±9.29 (range of values: 13.03-55.13) for low and high grade malignancy, respectively. In the group of indolent lymphomas, the mean AI value was 2.87±0.66 (range of values: 2.17-3.87) and the mean Casp3I value was 0.95±0,11 (range of values: 0.8-1.01), whereas the high grade group was characterized by higher values of these indices: 4.73±2.05 (range of values: 1.17-7.97) and 4.17±2.86 (range of values: 0.87-10.53), for AI and Casp3I, respectively. However, only mean values of Casp3I differed significantly among lymphoma grades ($p \leq 0.01$).

Survivin index and apoptotic markers in tumor areas of different survivin expression: In the areas of intense survivin expression the range of SI values of all examined lymphomas was 16.7-47.5 with a mean SI value of 26.95±8.54. In 13/25 cases SI ranged from 10% to 25% of positive cells. In most tumors of this group survivin expression exceeded 20% of positive cells (9/13). The remaining 12/25 cases had survivn expression ranging from 25% to 50% of positive cells. Among these 8/12 cases were characterized by survivin expression at the level of 25%-35% of positive cells and in 4 cases SI was higher than 35% of positive cells.

Expression of TUNEL-positive cells in areas of high survivin expression ranged from 0.65 to 3.9 with a mean AI value of 1.96±1.15. Only in 6/25 cases was AI >3% of positive cells; however, in all these tumors AI did not exceed 4% of positive cells. In the majority of examined lymphomas (19/25) AI was lower than 3%. Among these, 10/25 cases were characterized by AI at a level of 1%-2% of positive cells, in 6 tumors AI was <1% and in 3 lymphomas it was within 2%-3% of positive cells.

Expression of caspase-3 in corresponding areas of each specimen was similar to AI and ranged from 0.4 to 3.86, with a mean Casp3I value of 1.55±0.98. Except for 3 cases with Casp3I at the level of 3%-4% of positive cells, all other lymphomas were characterized by caspase-3 expression lower than 3% of positive cells. In most of these (13/22) Casp3I ranged from 1% to 2% of positive cells, in 6 tumors expression of caspase-3 was <1% and in the remaining 3 lymphomas it was within 2%-3% of positive cells.

In the areas of low survivin expression, the range of SI values of all cases was 2.4-19.5, with a mean

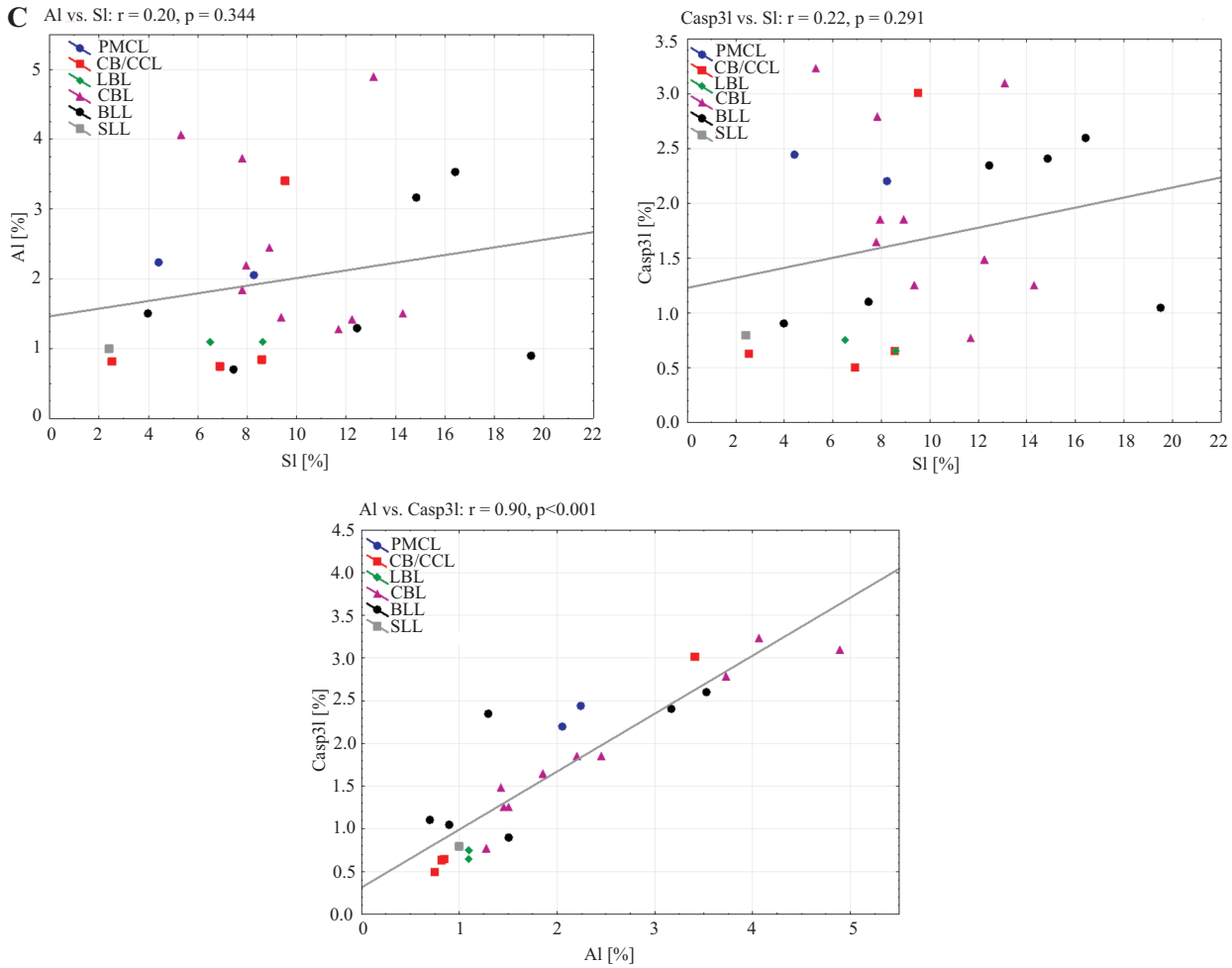

Fig. 2. Correlations of survivin expression and apoptotic markers: A) In whole section. B) In areas of high survivin expression. C) In areas of low survivin expression. Significant correlations were found only between AI and Casp3I.
Abbreviations: **PMCL** – pleomorphic mixed, small and large cell lymphoma, **CB/CCL** – centroblastic-centrocytic lymphoma, **LBL** – lymphoblastic lymphoma, **CBL** – centroblastic lymphoma, **BLL** – Burkitt-like lymphoma, **SLL** – lymphocytic lymphoma, **AI** – apoptotic index, **Casp3I** – index of caspase-3, **SI** – index of survivin

SI value of 9.23±4.26. Only 8/25 cases were characterized by survivin expression within 10%-25% of positive cells; however, in all of these cases SI was <20%. In the remaining 17/25 lymphomas SI was at the level of 0-10% of positive cells. In 13 of these the expression of survivin was >5% and in 4 lymphomas it was <5% of positive cells.

The AI and Casp3I in areas of low survivin expression were similar to each other and to their distribution in parts of the specimens with high survivin expression. The AI ranged from 0.7 to 4.89 with a mean value of 1.97±1.18. TUNEL-positive cells ranging from 3%-4% were found only in 6/25 cases. In the majority of examined tumors (19/25) AI was <3% of positive cells. In 10 of them AI was within the range of 1%-2% of positive cells and in the remaining 5 and 4 cases AI was <1% and >2%, respectively.

Expression of caspase-3 in corresponding areas ranged from 0.5 to 3.23 with a mean Casp3I value of 1.65±0.89. All but 3 tumors were characterized by Casp3I at the level of 0-3% of positive cells with an equal number of cases with caspase-3 expression lower than 1% and within the range of 1%-2% of positive cells (8 cases per group). In the remaining 6 cases Casp3I was >2%. Only in 3 tumors was AI >3% of positive cells; however, in all these tumors it did not exceed 4% of positive cells.

The mean values of SI in areas of high and low expression of this protein differed very highly significantly ($p \leq 0.001$) however, such statistical differences were not found between the mean values of AI and Casp3I. A very high correlation between AI and Casp3I was found in areas of lymphoma either of low or high survivin expression ($r = 0.9$; $p \leq 0.05$; $r = 0.87$; $p \leq 0.05$, respectively). No correlation between SI and either AI or Casp3I was found in both areas of different survivin expression (Fig. 2B and 2C).

Discussion

Defects in regulatory pathways resulting in inhibition of apoptosis together with increased proliferation are important mechanisms involved in cancer formation. By extending the lifespan of cells and increasing the frequency of cell division, they favor the accumulation of transforming mutations. Studies in NHLs have suggested that these mechanisms are important in lymphoma cells and that these cells are commonly resistant to apoptosis and have an increased proliferative index (Ansell et al. 2004). In our previous study we examined the impact of survivin expression on proliferation activity in canine lymphomas. We have shown the relationship between expression of this IAP and proliferative markers (Sokołowska et al. 2015). In the present study we investigated the impact of survivin on apoptosis inhibition using the same series of slides, to more completely characterize the role of survivin in canine NHLs. According to our knowledge this is the first study investigating the relationship between apoptotic markers and survivin expression in canine lymphomas and one of very few papers focusing on this topic in canine oncology (Fu et al. 2014).

In human medicine papers investigating the relationship between the presence of survivin and degree of apoptosis in various types of tumors are considerably more numerous; however, their results are not consistent. Some of them have shown a negative correlation between survivin expression and AI. In the study of Sarela et al. (2002) examining pancreatic adenocarcinoma a positive correlation between the above-mentioned indices was found. Other studies have not confirmed any correlation between survivin expression and degree of apoptosis (Ito et al. 2000, Mellai et al. 2008). Studies analyzing this relationship in human NHLs are not numerous and their results are also inconsistent. In the papers of Zuo et al. (2006, 2007) apoptotic index correlated positively with expression of survivin, whereas Li et al. (2006) found a negative correlation between survivin expression and either AI or expression of caspase-3. No correlation between survivin expression and caspase-3 expression was found in Markovic et al. (2011) and Martinez et al. (2004).

Any correlation study of survivin with apoptosis and proliferation activities must take into consideration two characteristics: the regional heterogeneity of these markers and the existence of diverse pathways of apoptosis. Most studies dedicated to the above-mentioned correlations had different aims and did not focus on such characteristics. Because of the regional heterogeneity, the above-mentioned markers calculated in histological sections may or not be prognostic (Mellai et al. 2008). Taking into account this bias of regional heterogeneity of apoptotic cell distribution, we analyzed all examined indices in serial sections, firstly in randomly chosen areas of each lymphoma, then in selected tumor areas of high and low survivin expression. However, irrespective of the mode of assessment, no correlation was found between survivin expression and any examined apoptotic markers with simultaneous presence of very high positive correlations between both apoptotic indices. Moreover, no statistically significant differences in the levels of survivin expression were found when values of SI were compared between the three groups of lymphomas with various apoptotic activity. Taken together, our results suggest that survivin expression does not exhibit direct anti-apoptotic activity in canine lymphoma cells. However, it is possible that the intervention of survivin in apoptosis inhibition can be indirect and mitosis-dependent expression of survivin can elevate its mitochondrial stores and raise the antiapoptotic threshold during cell division (Mellai et al. 2008).

Initial observations suggested that survivin could directly suppress activation of caspase-3; however, more recent studies have demonstrated that survivin lacks such ability (Banks et al. 2000, Garg et al. 2016). Despite these doubts we chose active caspase-3 as the one of the apoptotic indices because it belongs to executive caspases, and its level should reflect the anti-apoptotic properties of survivin regardless of whether the impact of survivin on apoptotic activity is direct or indirect. However, we have found no correlation between caspase-3 expression and expression of survivin. This is in the agreement with results of other studies conducted on human lymphomas (Martinez et al. 2004, Markovic et al. 2011). Moreover, our findings show that expression of caspase-3 was very low in all examined lymphomas. Martinez et al. (2004) investigating the expression of cleaved caspase-3 and survivin in mantle cell lymphoma had similar results. Interestingly, using double immunostaining they showed that survivin and cleaved caspase-3 were never expressed in the same lymphoma cell. Unfortunately, the authors did not comment this finding.

We were unable to find a relationship between survivin expression and apoptotic markers but we cannot exclude the possibility that our results are influenced by status of other pro- and antiapoptotic factors (e.g. p53, Bcl-2, MDM2) which all together decide whether a cell lives or dies (Zhou et al. 2002, Soleimanpour and Babaei 2015, Shamsabadi et al. 2016). The status of p53 seems to be especially important as there is evidence of the existence of interaction between p53 and survivin in the regulation of the cell cycle and apoptosis (Shamsabadi et al. 2016). Zhou et al. (2002) defined a p53-survivin signaling pathway activated by DNA damage that results in downregulation of survivin, cell cycle arrest, and apoptosis. Moreover, they found that

the presence of mutant p53 in tumor cells may contribute to upregulation of survivin and induction of G2/M arrest without inducing apoptosis.

Growing evidence from gene expression profiling conducted on human NHLs indicate that survivin upregulation is associated with overexpression of genes involved in cell cycle control rather than apoptosis inhibition. Tracey et al. (2005) showed that, in human NHLs, *BIRC5*/*SURVIVIN* expression was associated with the signature of cell proliferation i.e. overexpression of cell cycle control genes including cyclins (*CDKs, CDKIs, CDC*), DNA repair genes (*XRCC3/5, MHL1)*, polymerase genes and oncogenes (*HRAS*). Similarly, Kutler et al. (2002), investigating diffuse large B cell lymphomas, found that a high expression of survivin was associated with a significant increase in protein involved in cell cycle activation, *cyclin A* and *B*, as well as anti-apoptotic factors belonging to the Bcl-2 family (*Bfl-1, Bcl-w, Bcl-xL*) and *XIAP*. All these factors may be involved in a shared enhancement of cell proliferation. They also found a preferential survivin-cyclin B relationship suggesting that cyclin B overexpression, when linked to survivin overexpression in aggressive forms of lymphoma, might demonstrate a specific G2/M transition promotion (Kutler et al. 2002).

The possibility cannot be excluded that in canine NHLs survivin overexpression is also associated mainly with the expression of cell cycle activators, as the results of our current work may indicate, as well as our previous study (Sokołowska et al. 2015) regarding the relationship between survivin expression and proliferative activity. Moreover, nuclear localization of survivin in the examined lymphoma cases, together with the results of other research analyzing the expression of this IAP protein in canine NHLs (Rebhun et al. 2008), confirms this hypothesis. It is generally considered that nuclear expression of survivin is strongly associated with proliferative activity and regulation of cell division, whereas its location in the cytoplasm is involved in suppression of apoptosis (Li et al. 2005, Garg et al. 2016).

Our results have shown a very low level of apoptosis in canine NHLs. Only in 16% of tumors (4/25) did both examined apoptotic markers exceed 7%. Our findings are in agreement with Philips et al. (2000) which, according to our knowledge, is the only paper examining the level of apoptosis in canine lymphomas based on the TUNEL method. They also observed a very low apoptotic index ranging from 0.8 to 9 in all examined cases, with no significant differences in median values between low, intermediate and high grade tumors. The AI in low and intermediate lymphomas was 2.1 (with the range of values 0.8-5.9 and 1.2-9, respectively) and is comparable with our results regarding the group of indolent lymphomas. However, in high grade tumors there is some discrepancy between the values of AI obtained in our study and that by Philips et al. (2000), where the mean AI for high grade tumors was 2.05 (range of values 1-3.6) whereas in our study AI was 4.73 and in 55% of cases (11/20) it exceeded 4% of positively stained cells. Similarly to Philips et al. (2000), the values of AI obtained in our study were not statistically significant among lymphoma grades. However, data from human medicine indicates that such differences exist between low and high NHLs irrespective of the method of AI assessment: counting of apoptotic bodies (Symmans et al. 2000) or TUNEL positive cells (Zuo et al. 2006, 2007). A possible explanation for this could be the small number of indolent lymphoma cases or the specificity of the TUNEL assay. It is known that this method is not absolutely objective and many factors can lead to false positive or false negative staining (Tamura et al. 2000). However, using the anti-caspase-3 antibody a significant difference in the level of apoptosis between canine lymphoma of low and high grades was found. Unfortunately, we were unable to find such a difference regarding survivin expression. The results of a few studies focusing on this subject indicate that such differences exist in human NHLs (Zuo et al. 2006, 2007). Among our low grade lymphomas the SSL case had the lowest SI irrespective of the mode of assessment (in whole section and in areas of different survivin expression). This finding is in agreement with the results of de Graf et al. (2005) which shows, using quantitative PCR assay, an extremely low expression of survivin in this lymphoma subtype. However, in CB/CCL cases the values of SI were comparable with those obtained for the high grade lymphomas group. Taking into account that all analyzed cases were characterized by only a minimally follicular pattern, we cannot exclude that these tumors have undergone genetic alterations associated with their transformation into aggressive diffuse large B-cell lymphomas, especially since such transformation is associated with mutations of *p53* and some other genes involved in apoptosis, proliferation and cell cycle control (Lossos and Levy 2003) and a relationship between the mutation of *p53* and upregulation of survivin was found (Zhou et al. 2002).

The results of our current and previous studies, as well as papers of other authors, confirm that in lymphomas survivin is involved in the stimulation of proliferation rather than in apoptosis inhibition. Accelerated proliferation may play a central role in canine lymphoma tumorigenesis and survivin may be involved in this process. However, to fully confirm this hypothesis, as well as to clarify the role of survivin in the progression of canine lymphomas, further studies of the relationship between expression of survivin and other proteins involved in cell cycle regulation are needed.

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