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Original article

Immunohistochemical assessment of metalloproteinases MMP2 and MMP9 expression in canine various subtypes of lymphomas in relation with proliferative and apoptotic markers

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Abstract

Matrix metalloproteinases 2 and 9 (MMP2 and MMP9) are proteolytic enzymes involved with extracellular matrix degradation. They play a role in tumor invasion and metastases. Because of their ability to degrade signaling molecules presented in extracellular matrix, MMPs contribute to tumor proliferation and apoptosis. The aim of this study was to evaluate expression of MMP2 (latent and both active and latent forms) and MMP9 (active, latent, active and latent forms) in different subtypes of canine lymphomas and their relationship with proliferative (mitotic index and percentage of Ki67-positive cells) and apoptotic (apoptotic index) markers. Expression of MMPs was assessed immunohistochemically using an immunoreactive score system. Expression of both MMPs was found in all 20 examined lymphomas belonging to six subtypes. Most cases showed a moderate level of all analyzed forms of MMP2 and MMP9. High expression of MMPs was found in single cases. Except for a positive correlation between the active form of MMP9 and the mitotic index for all lymphoma cases, no other correlations between any remaining forms of MMPs and neither proliferative nor apoptotic markers were found, irrespective of whether the analysis encompassed all cases or the most numerous lymphoma subtypes i.e. centroblastic and Burkitt-like. Our results were not able to clearly confirm the influence of MMPs on the proliferation and apoptotic activity of canine lymphoma cells. However, further studies examining MMPs activity by zymography, expression of their inhibitors and other factors involved in activation of cell proliferation and apoptosis inhibition are needed to clarify the role of MMPs, especially the active form of MMP9, in the behavior of canine lymphoma cells.

Key words: apoptotic markers, dog, immunolabelling, lymphoma, matrix metalloproteinases, proliferative markers

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Introduction

Clinically, the most important features of malignant tumors are their invasive growth and metastasis. Infiltration of adjacent and distant tissues by neoplastic cells is a complex multistep process, in which the initial events are disruption of the extracellular matrix (ECM) and invasion of the basal membrane (BM) (Liotta 1986, Kwiatkowski et al. 2008, Aresu et al. 2011, Gialeli et al. 2011). This process involves the selective action of a group of proteases that can collectively degrade components of ECM. These proteases are known as matrix metalloproteinases (MMPs) belonging to the family of Ca^{2+} and Zn^{2+}-dependand proteolytic enzymes. Depending on their substrate specifity, MMPs are broadly divided into collagenases, stromelysins and gelatinases. The latter group, comprising Gelatinase A (72 kDa type IV collagenase, MMP2) and Gelatinase B (92 kDa type IV collagenase, MMP9), degrade denaturized collagens (gelatin), native type IV and V collagens and elastin. Because type IV collagen is the integral component of BM, uncontrolled expression of MMP2 and 9 is believed to play a critical role in the invasion of BM by tumor cells. Moreover, MMPs, including gelatinases, have the ability to degrade growth factor receptors, cytokines, chemokines, adhesion molecules, apoptotic ligands, and angiogenic factors presented in ECM, contributing to tumor proliferation, differentiation, motility, apoptosis and angiogenesis (Liotta, 1986, Vu and Werb 2000, McCawley and Matrisian 2001, Kwiatkowski et al. 2008, Gialeli et al. 2011, Pires et al. 2013). Consequently, the presence of gelatinases and other MMPs in malignant tumors is often correlated with tumor aggressiveness and unfavorable prognosis (McCawley and Matrisian 2001, Kwiatkowski et al. 2008, Pires et al. 2013).

MMPs expression has been investigated in a wide range of human tumors (*e.g.* Okada et al. 2004, Yousef et al. 2014) as well as in canine neoplasms (*e.g.* Loukopoulos et al. 2003, Pires et al. 2013). Studies in human medicine have shown that lymphomas are able to produce MMP2 and 9 and that these enzymes play a role in the pathogenesis and prognosis of these tumors (Kossakowska et al. 1993, 1998, Sakata et al. 2004, 2007). However, studies on MMP 2 and 9 in canine lymphomas are not numerous (Gentilini et al. 2005, Newman et al. 2008, Aricò et al. 2013, Aresu et al. 2014) and are focused mainly on either their role in angiogenesis (Aricò et al. 2013, Aresu et al. 2014) or prognostic values of serum MMPs levels (Gentilini et al. 2005). According to our knowledge, there are no papers analyzing MMPs expression in particular subtypes of lymphomas and their impact either on proliferation or apoptotosis of tumor cells. Thus, aim of this study was to evaluate expression of MMP2 and 9 in various subtypes of canine lymphomas and their relationship with proliferative and apoptotic markers.

Materials and Methods

Histological examination

Twenty canine popliteal lymph nodes with multicentric lymphoma collected during surgical biopsy were included in this study. All 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 immunochemistry with anti-CD3 and anti-CD79α antibodies, detecting neoplastic cells of T-cell and B-cell origin, respectively. Antibody against Ki67 and TUNEL methods were used to estimate proliferation activity and apoptosis of tumor cells, respectively. Expression of MMPs was determined by using antibodies against pro- and both pro- and active forms of MMP2 (proMMP2 and a/proMMP2, respectively) as well as pro-, active and pro- and active forms of MMP9 (proMMP9, aMMP9 and a/proMMP9, respectively).

All immunohistochemical procedures were performed according to the manufacturer's protocols. Antigen unmasking was performed using a microwave (two cycles: 7 and 5 min, 700 W). A block of unspecific staining with 5% goat serum (1 hour at room temperature) was than carried out and slides were incubated with primary antibody for 1 hour at room temperature or overnight at 4°C. 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 hematoxylin. Details of the primary antibodies and antigen retrieval methods used in immunohistochemical evaluation are presented in Table 1.

The TUNEL method was used with an 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 Ki67 antibodies as well as for the TUNEL method. Sections of canine osteosarcoma and canine skin with inflammatory infil-

Table 1. Primary antibodies and antigen retrieval methods used in immunohistochemical evaluation of canine lymphomas.

Primary antibody	Clonality	Dilution	Antigen unmasking method	Manufacturer
CD79α	Monoclonal, clone HM57	1:25[a]	Microwave, citrate buffer (pH 6.0)	Dako, Glostrup, Denmark
CD3	Polyclonal	1:50[a]	Microwave, citrate buffer (pH 6.0)	Dako, Glostrup, Denmark
Ki67	Monoclonal, clone MIB-1	1:50[a]	Microwave, citrate buffer (pH 6.0)	Dako, Glostrup, Denmark
proMMP2	Monoclonal, clone CA-4001/ CA719E3C	1:50[b]	Microwave, citrate buffer (pH 6.0)	Abcam, Cambridge, UK
a/proMMP2	Monoclonal, clone A-Gel VC2	1:25[b]	Microwave, Dako Target Retrieval Solution (pH 9.0)	Merck Millipore, Darmstadt, Germany
proMMP9	Monoclonal, clone IIA5	1:50[b]	Microwave, citrate buffer (pH 6.0)	ThermoFisher Scientific, Waltham, MA USA
aMMP9	Monoclonal, clone 4A3	1:50[b]	Microwave, citrate buffer (pH 6.0)	Merck Millipore, Darmstadt, Germany
a/proMMP9	Monoclonal, clone EP1255Y	1:50[b]	Microwave, citrate buffer (pH 6.0)	Abcam, Cambridge, UK

[a] 1 hour incubation at room temperature; [b]Overnight incubation at 4°C

tration containing macrophages and neutrophiles were used as positive controls for MMPs antibodies. Substitution of primary antibody by TBST (Dako, Glostrup, Denmark) was used for negative controls for all immunohistohemical methods.

Assessment of cell proliferation

Tumor cell proliferation was estimated in the sections stained with HE and with anti-Ki67 antibody. The proliferative activity was estimated on the basis of the mitotic index (MI) and proliferative index (PI) in each specimen. The MI was assessed as the mean number of metaphase and anaphase nuclei in 10 visual fields (HE, ×400 magnification). The PI was defined as the number of Ki67-positive lymphoma cells in 1000 tumors cells (×1000 magnification). Both indices were estimated in triple counting.

Assessment of cell apoptosis

Estimation of lymphoma cell apoptosis was made in sections stained using the TUNEL method. Tumor cell apoptosis was estimated on the basis of apoptotic index (AI) in each specimen, defined as the number of positive lymphoma cells and apoptotic bodies in 1000 tumor cells in triple counting (×1000 magnification).

Analysis of MMPs immunolabelling

Expression of the MMPs was assessed semiquantitatively in 20 randomly selected visual fields (×400 magnification) through all specimens using the immunoreactive score system. This system defines two parameters: quantity score (QS) corresponding to the percentage of malignant cells expressing each MMPs and staining intensity score (SIS) assessing labeling intensity. QS was estimated as: 0 = negative; 1 = <25%; 2 = 25-50%; 3 = 51-75%; 4 = >75% of positive cells. SIS was defined as: 0 = negative; 1 = weak; 2 = moderate; 3 = strong. In each case the mean values of QS and SIS were assigned. Mean values of QS and SIS were than multiplied to provide the total immunohistochemical score (TIS) ranging from 0 to 12 (Beltran et al. 2013). TIS scores of 1 to 4 were considered to represent low levels of MMPs expression, scores of >4 to 9 were classified as moderate, while scores of >9 to 12 were considered as high levels of MMPs expression (Chu et al. 2011, Yousef et al. 2014).

Statistical analysis

Data, presented as mean values ± SD, were analyzed using Statistica 13.3 for Windows (Tibco Software Inc.). Statistical comparisons were made using the Mann-Whitney U-test. Correlations between expression of MMPs and MI, PI and AI were established by the significance of Spearman's rank correlation coefficient. $p \leq 0.05$ was considered significant.

Results

Histological examination

Among 20 canine lymphomas 2 were of T-cell phenotype ($CD3^+CD79\alpha^-$) and belonged to a pleomorphic mixed, small and large cell lymphoma (PMCL) subtype, whereas other tumors were of B-cell origin and were classified into 5 subtypes: centroblastic (CBL) – 7 cases, Burkitt-like (BLL) – 6 cases, centrocytic-centroblastic (CC/CBL) – 3 cases, lymphoblastic (LBL) - 1 case and small lymphocytic (SLL) – 1 case.

Table 2. Proliferation and apoptotic markers and expression of matrix metalloproteinases 2 and 9 in particular subtypes of lymphoma.

Lymphoma subtype	MI		PI [%]		AI [%]		proMMP2		a/proMMP2		aMMP9		proMMP9		a/proMMP9	
	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	Range of values	Mean value±SEM	Range of values	Mean value±SEM
PMCL	6.56-8.2	7.38±1.16	21.87-25.47	23.67±2.55	3.86-4.2	4.03±0.24	7.48-9.49	8.48±1.42	5.5-7	6.25±1.06	4.17-4.93	4.55±0.53	5.11-7.67	6.39±1.81	8-10.95	9.48±2.09
SLL	3.2	-	21.57	-	2.17	-	1.7	-	5.04	-	3.53	-	7.43	-	7.75	-
CC/CBL	1.33-6.27	4.44±2.71	36.13-38.3	37.22±1.53	1.84-3.87	2.7±1.05	2.69-6.83	4.33±2.2	3.68-4.78	4.08±0.61	0.04-5.36	3.23±2.82	1.11-5.43	3.08±2.18	4.9-10.2	7.6±2.65
LBL	12.73	-	19.1	-	2.1	-	6.38	-	4.96	-	9.06	-	4.88	-	8.14	-
CBL	2.75-11.2	5.52±3.07	20.66-35.47	26.26±5.59	2.65-8.17	5.78±2.29	1.28-9.8	5.95±2.82	4.32-5.93	4.98±0.54	0.13-10.07	3.69±4.32	0.26-9.72	3.94±4.19	4.06-9.59	6.82±1.98
BLL	4.96-13.2	9.73±3.14	34.82-68.47	46.61±13.93	1.17-7.2	4.21±2.21	3.24-7.69	5.93±1.71	4.59-6.57	5.3±0.69	0.15-6.99	4.36±2.98	0.46-6.02	3.81±2.01	5.58-8.23	7.36±1.05
Total	**1.33-13.2**	**7.05±3.62**	**19.1-68.47**	**32.94±13.18**	**1.17-8.17**	**4.31±2.18**	**1.28-9.8**	**5.76±2.43**	**3.68-7**	**5.07±0.8**	**0.04-10.07**	**4.17±3.26**	**0.26-9.72**	**4.24±2.94**	**4.06-10.95**	**7.48±1.76**

Abbreviations: **PMCL** – pleomorphic mixed, small and large cell lymphoma, **CC/CBL** – centrocytic-centroblastic lymphoma, **LBL** – lymphoblastic lymphoma, **CBL** – centroblastic lymphoma, **BLL -** Burkitt-like lymphoma, **SLL** – lymphocytic lymphoma, **MI** – mitotic index, **PI** – proliferative index, **AI** – apoptotic index, **proMMP** – latent form of matrix metalloproteinase, **aMMP** – active form of matrix metalloproteinase, **a/proMMP** – latent and active forms of matrix metalloproteinase.

Table 3. Proliferation and apoptotic markers and expression of matrix metalloproteinases 2 and 9 estimated according to immunoreactive score system in particular cases of canine lymphomas.

Case no.	Lyphoma subtype	MI	PI	AI	proMMP2			a/proMMP2			aMMP9			proMMP9			a/proMMP9		
					QS	SIS	TIS	QS	SIS	TIS	QS	SIS	TIS	QS	SIS	TIS	QS	SIS	TIS
1	PMCL	6.56	25.47	3.86	3.37	2.21	7.48	3.55	1.55	5.5	3.14	1.57	4.93	2.81	1.8	5.11	3.5	2.3	8
2	PMCL	8.2	21.87	4.2	3.45	2.75	9.49	4	1.75	7	3.15	1.33	4.17	3.65	2.1	7.67	3.65	3	10.95
3	SLL	3.2	21.57	2.17	1.7	1	1.7	3.15	1.6	5.04	2.48	1.43	3.53	2.7	2.75	7.43	3.1	2.5	7.75
4	CC/CBL	1.33	29.92	1.84	2.15	1.25	2.69	2.8	1.35	3.78	0.35	0.1	0.04	2.25	1.2	2.7	3.4	3	10.2
5	CC/CBL	5.73	36.13	3.87	3.5	1.95	6.83	3.68	1.3	4.78	3.25	1.65	5.36	3.5	1.55	5.43	3.85	2	7.7
6	CC/CBL	6.27	38.3	2.4	2.35	1.48	3.47	2.68	1.38	3.68	3.13	1.38	4.3	1.7	0.65	1.11	2.65	1.85	4.9
7	LBL	12.73	19.1	2.1	3.75	1.7	6.38	3.68	1.35	4.96	3.63	2.5	9.06	3.75	1.3	4.88	3.7	2.2	8.14
8	CBL	2.75	28.78	7.47	0.8	1.6	1.28	2.85	1.65	4.7	0.55	1	0.55	0.7	1.3	0.91	2.1	2.4	5.04
9	CBL	3.03	30.97	7.97	3.25	1.55	5.04	3.9	1.2	4.68	0.25	0.5	0.13	0.8	1.1	0.88	2.8	1.45	4.06
10	CBL	3.03	20.66	8.17	3.4	1.45	4.93	3.75	1.4	5.25	0.55	0.4	0.22	0.35	0.75	0.26	3.85	1.5	5.78
11	CBL	5.47	22.23	3.83	3.5	1.9	6.65	3.2	1.35	4.32	0.9	0.4	0.36	0.6	1.25	0.75	3.55	2.7	9.59
12	CBL	7.53	21.6	2.65	4	2.2	8.8	3.95	1.5	5.93	3.8	2.65	10.07	3.35	2.65	8.88	3.35	2.6	8.71
13	CBL	5.63	24.1	3.8	3.5	2.8	9.8	3.65	1.45	5.29	3.35	2.3	7.71	3.6	2.7	9.72	3.2	2.3	7.36
14	CBL	11.2	35.47	6.57	3.05	1.7	5.19	3.6	1.3	4.68	3.73	1.83	6.8	3.55	1.75	6.21	3.35	2.15	7.2
15	BLL	4.96	34.82	7.2	2.38	1.36	3.24	3.1	1.55	4.8	1.25	0.8	1	1.67	1.53	2.55	3.32	2.4	7.97
16	BLL	10.87	37.2	5.33	3.25	2.35	7.64	3.68	1.45	5.33	3.58	1.73	6.17	3.35	1.4	4.69	3.5	2.35	8.23
17	BLL	9.4	38.87	1.17	3.75	2.05	7.69	3.75	1.23	4.59	3.58	1.78	6.35	3.65	1.65	6.02	3.35	2.45	8.21
18	BLL	13.2	68.47	4.93	3.48	1.38	4.78	3.93	1.68	6.57	3.55	1.55	5.5	3.25	1.25	4.06	3.75	2	7.5
19	BLL	7.43	40.63	4.47	3.6	1.7	6.12	3.65	1.45	5.29	0.75	0.2	0.15	0.4	1.15	0.46	3.85	1.45	5.58
20	BLL	12.5	59.64	2.13	3.23	1.89	6.1	3.39	1.54	5.22	3.48	2.01	6.99	3.29	1.54	5.07	2.93	2.28	6.68

Abbreviations: **PMCL** – pleomorphic mixed, small and large cell lymphoma, **CC/CBL** – centrocytic-centroblastic lymphoma, **LBL** – lymphoblastic lymphoma, **CBL** – centroblastic lymphoma, **BLL** – Burkitt-like lymphoma, **SLL** – lymphocytic lymphoma, **MI** – mitotic index, **PI** – proliferative index, **AI** – apoptotic index, **proMMP** – latent form of matrix metalloproteinase, **aMMP** – active form of matrix metalloproteinase, **a/proMMP** – latent and active forms of matrix metalloproteinase, **QS** – quantity score, **SIS** – staining intensity score, **TIS** – total immunohistochemical score

Proliferative markers examination

The MI in all examined lymphomas ranged from 1.33-13.2 with a mean MI value of 7.05±3.62. In most cases (9/20) MI ranged from 5-10 mitoses per high-power field with the mean MI value of 6.91±1.33. In 6/20 cases the MI was <5 and in the remaining 5/20 cases was >10 with a mean MI value of 3.05±1.16 and 12.1±1.01, respectively.

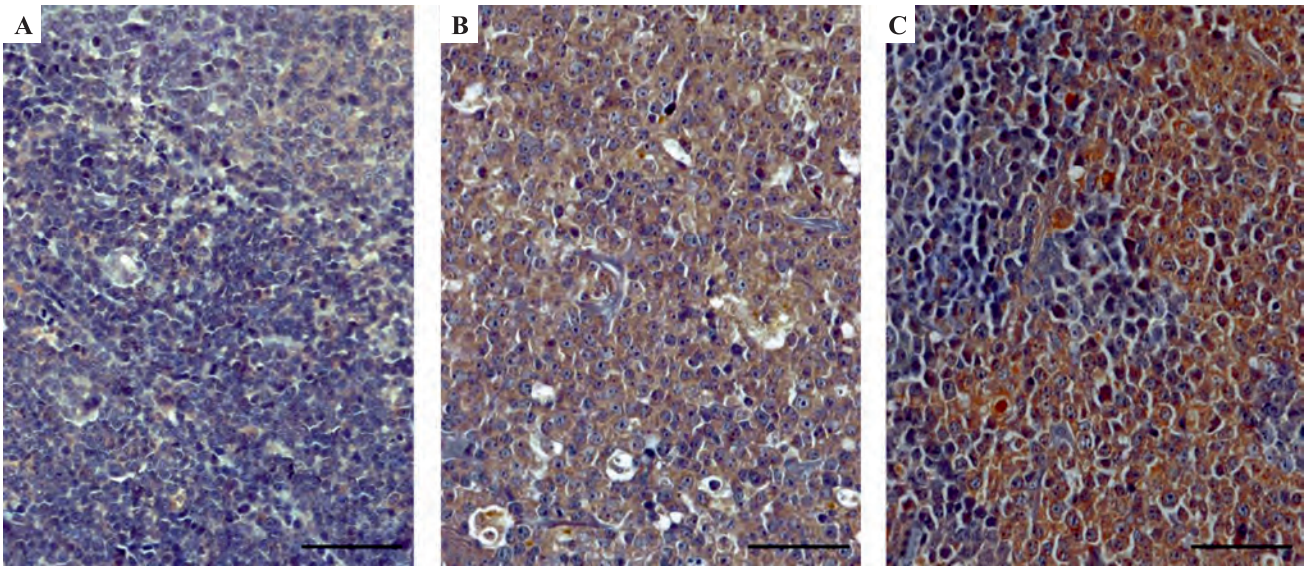

Fig. 1. Immunohistochemical staining of metalloproteinases (MMPs) in canine lymphomas: A) Expression of latent form of MMP2 in <25% of cells with a low labeling intensity. B) Expression of latent form of MMP9 in >75% of cells with a moderate labeling intensity. C) Expression of active form of MMP9 in 50-75% of cells with a strong labeling intensity; bar = 50 µm.

All examined lymphomas showed comparable expression of Ki67 with the mean PI value of 32.94±13.18 (range of values: 19.1-68.47). In 12/20 cases the PI was <35% Ki67-positive cells with the mean PI of 24.65±4.9 (range of values: 19.1-34.82). In 8/20 lymphomas the PI was >35%; however, only in 3 cases did it exceed 40%. The mean PI for this group was 44.34±12.5 (range of values: 35.47-68.47). Detailed data on the MI and the PI in particular subtypes and cases of lymphomas are presented in Tables 2 and 3.

Apoptotic index examination

The range of AI values for all examined lymphomas was 1.17-8.17 with the mean AI value of 4.31±2.18. In most cases (8/20) the AI ranged from 3-7% of positive cells with the mean AI value of 4.54±0.93. In 7/20 cases the AI was <3% positive cells with a mean AI value of 2.07±0.47. In the remaining 4/20 lymphomas the AI was >7% of positive cells with the mean AI value of 7.7±0.45. Detailed data on AI in particular subtypes and cases of lymphomas are presented in Tables 2 and 3.

MMP2 and MMP9 expression

Positive reactions with all MMPs antibodies were observed in lymphoma cells and in macrophages presented within tumor tissue. MMPs were diffusely located in the cytoplasm of lymphoma cells. All examined tumors showed expression of all analyzed forms of MMP2 and 9. Positive correlation was found between expression of aMMP9 and proMMP9 ($r = 0.79$; $p \leq 0.01$).

The range of TIS values for proMMP2 for all lymphomas was 1.28-9.80 with a mean TIS value of 5.76±2.43. Most cases of lymphomas (13/20) had a moderate level of proMMP2 expression. In 5/20 cases expression of proMMP2 was weak and in the remaining 2/20 cases was high. The range of TIS values for a/proMMP2 for all cases was 3.68-7 with a mean TIS value of 5.07±0.8. All but two cases had moderate a/proMMP2 expression. Non of the examined lymphomas was characterized by a high level of a/proMMP2 expression.

The mean TIS values for MMP9 were: 4.17±3.26 (range of values: 0.04-10.07), 4.24±2.94 (range of values: 0.26-9.72) and 7.48±1.76 (range of values: 4.06-10.95) for aMMP9, proMMP9 and a/proMMP9, respectively. In most cases expression of all forms of MMP9 was moderate (in 10/20, 11/20 and 17/20 cases for aMMP9, proMMP9 and a/proMMP9, respectively). Low levels of aMMP9 and proMMP9 were observed in 8 cases in each group, whereas non of the examined lymphomas showed low expression of a/proMMP9. High levels of aMMP9, proMMP9 and a/proMMP9 expression were observed in 2/20, 1/20 and 3/20 cases, respectively. Detailed data on MMP2 and MMP9 expression in particular subtypes and cases of lymphomas are given in Tables 2 and 3, whereas representative images of MMPs immunolabelling in lymphoma cells are shown in Fig. 1.

Association of MMPs expression with prolirerative and apoptotic markers

Expression of all forms of MMPs did not correlate with either proliferative or apoptotic markers, irrespec-

Table 4. Expression of matrix metalloproteinases 2 and 9 depending on the values of mitotic, proliferative and apoptotic indices.

Parameter	proMMP2		a/proMMP2		aMMP9		proMMP9		a/proMMP9	
	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
MI										
<5	1.28-5.04	3.15±1.58 [a]	3.78-5.25	4.71±0.5	0.04-3.53	0.91±1.33 [b,B]	0.26-7.43	2.45±2.62	4.06-10.2	6.58±2.18
5-10	3.47-9.8	7.37±1.94 [a]	3.68-7	5.12±0.96	0.15-10.07	4.82±3.18 [b]	0.46-9.72	5.02±3.53	4.9-10.95	7.89±1.86
>10	4.78-7.64	6.02±1.12	4.68-6.57	5.41±0.73	5.5-9.06	6.9±1.34 [B]	4.06-6.21	4.98±0.78	7.2-8.2	7.8±0.4
PI										
<35% Ki67$^+$cells	1.28-9.8	5.62±2.97	3.78-7	5.1±0.82	0.04-10.07	3.48±3.73	0.26-9.72	4.31±3.44	4.06-10.95	7.8±2.04
>35% Ki67$^+$cells	3.47-7.69	5.97±1.45	3.68-6.57	5.02±0.83	0.15-6.99	5.2±2.22	0.46-6.21	4.13±2.18	4.9-8.23	7±1.21
AI										
<3% positive cells	1.7-8.8	5.26±2.67	3.68-5.93	4.68±0.77	0.04-10.07	5.76±3.45	1.11-8.88	5.16±2.65 [b]	4.9-10.2	7.99±1.58
3-7% positive cells	4.78-9.8	7.11±1.72 [b]	4.32-7	5.42±0.87	0.15-7.71	4.57±2.65	0.46-9.72	4.9±2.97	5.58-10.95	8.01±1.52 [b]
>7% positive cells	1.28-5.04	3.62±1.76 [b]	4.68-5.25	4.96±0.31	0.13-1	0.47±0.4	0.26-2.55	1.15±0.98 [b]	4.06-6.68	5.39±1.11 [b]

Identical letters in the same column indicate statistically significant differences: a- the difference very highly significant (P≤0.001); b, B – the difference significant (p≤0.05)
Abbreviations: **MI** – mitotic index, **PI** – proliferative index, **AI** – apoptotic index, **proMMP** – latent form of matrix metalloproteinase, **aMMP** – active form of matrix metalloproteinase, **a/proMMP** – latent and active forms of matrix metalloproteinase

tively of whether we analyzed all cases or the most numerous lymphoma subtypes *i.e*. CBL and BLL, with the exception of a high positive correlation between MI and expression of aMMP9 established for all examined cases (r = 0.69; p≤0.01).

Regarding groups of different MI (Table 4), only expression of proMMP2 for groups with MI<5 and MI 5-10 (p≤0.001) as well as expression of aMMP9 for the following groups: MI<5 *vs*. MI 5-10 (p≤0.05) and MI<5 *vs*. MI>10 (p≤0.05) differed significantly. No statistical differences in expression of all forms of MMPs were found between groups of different PI (<35% Ki67-positive cells *vs*. >35% Ki67-positive cells; Table 4).

Regarding groups of different AI (Table 4), significant differences in MMPs expression were found only in the case of proMMP2 for groups with AI 3-7% and >7% of positive cells (p≤0.05), as well as for proMMP9 and a/proMMP9 for groups of AI 0-3% *vs*. >7% (p≤0.05) and AI 3-7% *vs*. >7% of positive cells (p≤0.05), respectively.

Discussion

We investigated MMP2 and 9 expression in various subtypes of canine lymphomas and according to our knowledge this is the first such study, as other authors (Newman et al. 2008, Aricò et al. 2013, Aresu et al. 2014) focused on differences in MMPs levels between lymphomas of different grade, phenotype or stage and they did not specify lymphoma subtypes. MMP2 and 9 were found in all examined cases with a moderate level of expression in the majority of them, followed by low and high expression levels. Our results are comparable with these sparse papers which analyzed MMPs in canine lymphomas. Newman et al. (2008) showed the expression of MMP2 at mRNA level in 91% of B-cell and 57% of T-cell tumors. In the study of Aricò et al. (2013) immunohistochemical expression of MMP9 was found in all examined lymphomas of both phenotypes, whereas expression of MMP2 was found in all T-cell lymphomas and 73% of B-cell tumors. It seems that the levels of expression of both MMPs in the study of Aricò et al. (2013), especially in tumors of B-cell origin, were lower than in our study; however, these authors used a distinct score system for assessing the percentage of positive cells and they did not estimate staining intensity. Such a large percentage of lymphoma cases positive for MMP2 and 9 could be partially explained by the physiological role of lymphocytes. Normal lymphocytes, especially T cells, naturally produce gelatinases which act to facilitate the movement of these cells out of the vasculature into tissues giving them an "invasive" phenotype (Goetzl et al. 1996). The expression of MMP2 and 9 in normal canine lymphocytes was confirmed immunohistochemically (Loukopoulos et al. 2003) and at the mRNA level (Aricò et al. 2013). However, sparse data on the expression of MMP2 and 9 in human non-Hodgkin lymphomas (NHLs) have not confirmed such a large frequency of MMPs expression in human NHLs. In the study of Kossakowska et al. (1993) mRNA transcripts of MMP9 were found in 83% of tumors, but transcripts of MMP2 were detected in only a few cases. In another study, immunohistochemical expression of MMP9 and 2 were found in 16% and

10% of cases, respectively (Sakata et al. 2004). Expression of these MMPs was observed in various subtypes of NHLs (Kossakowska et al. 1998, Sakata et al. 2004); however, except for diffuse large B-cell and nasal NK/T-cell lymphomas regarding both MMPs expression as well as anaplastic large cell and peripheral T cell lymphomas regarding MMP9 expression, the presence of either MMP2 or MMP9 is limited to small percentage of cases within other subtypes of NHLs (Sakata et al. 2004, 2007).

Apart from their role in disruption of the ECM and BM, MMPs play a role in the modification of the cellular behavior and modulations of biologically active ECM molecules (Manello et al. 2005, Vu and Werb 2018). These functions of MMPs that are essential in normal conditions in a changing environment may contribute to deregulation of cell behavior in a pathological state when the production and activity of MMPs are compromised (Pires et al. 2013). MMPs, including gelatinases, can regulate bioavailability and/or the activity of growth factors as well as the function of cell-surface receptors (McCawley et al. 2001, Gialeli et al. 2011). Moreover, in a tumor environment MMPs may deregulate the balance between growth and antigrowth signals (Pires et al. 2013) leading to increased proliferative activity. However, studies examining the impact of gelatinases on the proliferative activity of tumor cells are not numerous and their results are conflicting. Such relationships were found in some human (da Silva et al. 2016) and canine tumors (Nowak et al. 2008), but they were not confirmed by other studies (Okada et al. 2004, Mandara et al. 2009). Our research did not confirm the impact of MMPs on proliferation activity in canine lymphomas. There were no correlations between MMP2 and either MI or PI. We found only a correlation between aMMP9 and MI but it was not confirmed with PI. However, when we compared the mean values of TIS between groups of different MI, a trend towards the lowest expression of all examined forms of MMPs in the group of lymphomas with the lowest MI was observed, but this was confirmed statistically only in case of proMMP2 and aMMP9. The groups with medium and high MI were characterized with comparable values of TIS for all forms of MMPs, except aMMP9 where the TIS value for group with medium MI was lower than for group with the highest MI; however, this difference was not statistically significant. This tendency to increase expression of MMPs with increasing proliferation activity was not confirmed by analysis of MMPs expression between groups of different PI, as for all examined MMPs except aMMP9 the mean TIS values were similar. However, even in the case of aMMP9 these differences were not confirmed statistically.

The growing awareness that MMPs cleave a wide range of bioactive substrates presented in ECM including death-inducing signaling components, indicate that MMPs can up- or down-regulate cell apoptosis in a context-depend manner (McCawley et al. 2001, Manello et al. 2005, Gialeli et al. 2011). Studies conducted *in vitro* indicate that gelatinases have a proapoptotic effect in both physiological and pathological conditions, including tumors; however, opposing functions of these MMPs have also been reported (Manello et al. 2005). The majority of these studies have been conducted on various cell lines or mouse models and we were unable to find any studies conducted on tumors collected from clinical patients in both humans and dogs. We did not find any correlations between all examined forms of MMPs and AI. However, when we compared mean values of TIS between groups of different AI, we observed similar values of TIS in groups with the lowest and medium AI, and a lower level of MMPs expression in the group with the highest AI for all forms of MMPs except a/proMMP2. However, the lowest value of TIS in the group with the highest AI compared to groups with medium AI and the lowest AI was confirmed only for proMMP2, a/proMMP9 and proMMP9, respectively. Our results appear to be difficult to interpret without knowledge of the status of other pro- and antiapoptotic factors of known role in tumor biology *e.g.* P53 and BCL-2. Moreover, although TUNEL is a well-established apoptosis detection method, this assay is not objective and many factors can lead to false positive or negative staining (Tamura et al. 2000), thus use of other apoptotic markers e.g. caspase-3 is advisable to confirm our results.

In the present study we were unable to show a clear relationship between MMP2 and 9 expression and either proliferation intensity or apoptotic activity in canine lymphomas. However, our results do not exclude the impact of both gelatinases on the biological behavior of canine lymphomas. aMMP9 in particular seems to be a candidate for future studies. Moreover, our inconclusive results can mirror some limitations of this study. Firstly, we analyzed MMPs expression with immunohistochemistry, which is a relatively insensitive and qualitative method, whereas gelatin zymography proved to be both sensitive and quantitative, but has the limitation of requiring fresh tissue (Loukopoulos et al. 2003). Because we worked on archival samples we were not able to use this method. Therefore, our results remain to be verified by enzymological assays. In addition, we analyzed MMPs expression solely, without examination of the expression of their inhibitors, called tissue inhibitors of MMPs (TIMPs), which regulate MMPs activity. Among them, TIMP1 preferentially binds to MMP9 and TIMP2 to MMP2 (Aresu et al.

2011, Pires et al. 2013). TIMPs act either by suppressing proenzyme activation or inactivation of active enzymes *via* TIMP-MMP complex formation (Kwiatkowski et al. 2008). Thus the level of TIMPs can influence the activity of MMPs. In particular it has been shown that MMP9 is frequently expressed with TIMP1 by canine neoplastic cells (Aricò et al. 2013) and TIMP2 expression was found in 70% of canine B cell lymphomas at the RNA level (Newman et al. 2008). It has also been shown that TIMP1 has antiapoptotic activity (Guedez et al. 1998, Li et al. 1999). It should also be stressed that expression of MMPs and TIMPs, as well as the amount of synthesized active and latent forms of MMPs, are likely linked to specific physiologic and immunologic conditions and vary over time (Leibman et al. 2000, Newman et al. 2008). Finally, proliferation activity and apoptosis are influenced by many other factors *e.g*. growth factors, P53, BCL-2 or survivin and they could be more potent stimulators of proliferation or inhibitors of apoptosis in canine lymphoma cells than MMPs.

In summary, we performed immunohistochemical analysis of MMP2 and 9 expression in various subtypes of canine lymphomas and made an attempt to find a correlation between the expression of MMP2 and 9 and proliferation and apoptotic activities. We were not able to clearly confirm the role of MMPs in these processes in canine lymphomas; therefore, further studies examining MMPs activity by zymography, expression of TIMPs and other factors involved in the activation of cell proliferation and apoptosis inhibition are needed to clarify the role of MMPs, especially aMMP9, in the behavior of canine lymphomas. The results of our study and limited papers available in the literature indicate that canine lymphomas frequently express MMPs, and thus they could be a good candidate for treatment with MMP inhibitors.

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