Original Article

Alteration in Serum Matrix Metalloproteinase-9 Gelatinase Activities in Lymphoma-Bearing Dogs with Chemotherapies

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Abstract

Matrix metalloproteinase-2 and -9 (MMP-2 and -9), gelatinases among MMPs, are associated with cell migration, invasion, metastasis and angiogenesis in human solid tumor. The prognostic value of gelatinases in canine lymphoma remains equivocal. To evaluate the relation between serum gelatinases and canine lymphoma, gelatin zymography was used to detect the levels of serum MMP-2 and MMP-9 gelatinase activities in lymphoma-bearing dogs. In this retrospective study, twenty-four canine patients with different subtypes of lymphoma were identified according to the histopathogical examination, and 10 healthy dogs were included as control. Serum samples from the lymphoma-bearing dogs were collected before and after chemotherapy. Results showed that the MMP-9 levels increased in the lymphoma-bearing dogs (median: 199.37, interquartile difference: 37.67) and decreased after a complete response was achieved (median: 140.64, interquartile difference 48.62); however, the MMP-2 levels among the controls and lymphoma-bearing dogs with treatment were similar (before: median: 138.42, interquartile difference: 35.7; complete response: median: 119.26, interquartile difference: 39.03). Both MMP-2 and MMP-9 levels were higher in T-cell lymphoma than in B-cell lymphoma patients. Compared with MMP-2, changes in the level of serum MMP-9 gelatinase activity could be a prognostic factor in canine lymphoma.

Keywords: canine lymphoma, gelatinase activity, MMP-2, MMP-9

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Introduction

Matrix metalloproteinases (MMPs) are a group of endopeptidases responsible for the degradation of the extracellular matrix (Egeblad and Werb, 2002); they play a role in altered proteolysis in cancer, which results in uncontrolled tumor growth and metastasis (Kessenbrock et al., 2010). Recently it has been shown that MMP-2 and MMP-9, two kinds of gelatinase among MMPs, are associated with the progression of human solid tumor and canine lymphoma (Zhong et al., 2008; Aricò et al., 2013). However, the role of MMP-2 and MMP-9 in haemapoietic neoplasia such as lymphoma is still unclear (Hazar et al., 2004).

Plasma or serum samples at the time of admission, and after treatment from histologically diagnosed lymphoma patients, were collected and gelatin zymography or ELISA were used to detect the levels of MMP-2, MMP-9 and other MMP-associated factors in relation to lymphoma subtypes and prognosis (Gentilini et al., 2005; Pennanen et al., 2008). The genetic and proteinous expressions, and the level of MMP-9 gelatinase activity was also verified in lymphoma-bearing dogs (Aricò et al., 2013). In canine, significantly higher levels of MMP-9 and TIMP-1 (tissue inhibitor metalloproteinases-1) mRNA expression were observed in T-cell lymphomas compared to B-cell lymphomas and healthy controls. The mRNA expression of MMP-2 and TIMP-2 was significantly higher in the healthy dogs, whereas that of TIMP-1 was significantly higher in the lymphoma dogs (Aricò et al., 2013).

In canine, the plasma levels of MMP-9 is higher in lymphoma-bearing dogs than in healthy dogs (Aresu et al., 2014). According to their chemotherapeutic strategies, MMP-9 level decreased in B-cell lymphomas during chemotherapy (Aresu et al., 2014). However, the role of MMP-2 and MMP-9 in canine lymphoma is still unclear and only insufficient data are available on chemotherapies in veterinary medicine. Herein, the objective of this study was to prove our hypothesis about the correlation of the gelatinase activities of MMP-2 and MMP-9 in the sera of lymphoma-bearing dogs with different tumor stage, tumor subtype, and tumor immunophenotype and to develop chemotherapeutic strategies/responses. Therefore, measurement of the gelatinase activity levels in canine lymphoma patients during chemotherapies was performed. Results of this study might help clinical veterinarian in predicting prognosis and survival time, and employing therapeutic strategies for canine lymphoma.

Materials and Methods

Animals and Samples: Patient information was collected by reviewing medical records at the Veterinary Medical Teaching Hospital of National Chung Hsing University (VMTH, NCHU) from 2003 to 2012. Twenty-four patients with different subtypes of lymphoma and 10 healthy control dogs (assigned according to blood and blood-biochemistry examination) were included (Table 1). Serum samples were collected from dogs at admission and follow-ups. All serum samples obtained for the measurement of MMP-2 and MMP-9 gelatinase activities were placed in pure serum from blood collected in tubes (BD Vacutainer® Plus plastic Serum Tube, Becton, Dickinson and Company, USA). After this preparation, the serum samples were centrifuged, collected in Eppendorf and frozen at -70°C within a few minutes, stored under the same conditions of the serum samples, and then separately assayed in triplicate. Three kinds of chemotherapy were applied to the lymphoma-bearing dogs: L-CHOP protocol (UW-25), including L-asparaginase (LEUNASE, Kyowa Hakko kirin CO., Ltd. Fuji Plant, Japan), cyclophosphamide (ENDOXAN, Baxter Oncology GmbH, Germany), doxorubicin (DOXOR LYO, Nang Kuang, Taiwan), vincristin (ONCOVIN, Eli Lilly& CO., USA), and prednisolone (KINGCORT, Synpac-Kingdom Pharmaceutical Company, Taiwan); single-agent prednisolone. Which one of them would be used depended on the owner's decision. The single-agent doxorubicin protocol included three courses and 4 times of drug administration in each course. Dosing intervals between the 4 times of administration in the first, second and third courses were 3 weeks, 6 weeks and 9 weeks, respectively. Evaluation of treatment response was performed. After the dog achieved a complete response or finished a course, the owner was asked to decide whether the next course should proceed. If a relapse occurred, the dog was put on the previous course again.

General disease response of target lesion was defined as follows: CR, disappearance of all evidence of disease; PR, at least a 30% decrease in the mean sum LD of target lesions compared to baseline; PD, at least a 20% increase in the mean sum LD, compared to the smallest mean sum LD at baseline or during follow-up; SD, neither sufficient decrease to qualify for PR nor sufficient increase to qualify for PD. Response of non-target lesion was defined as follows: CR, pathologic lymph nodes return to normal size; PD, unequivocal progression of existing lesions or appearance of new lesions; Non-CR/Non-PD, maintains stable (Vail et al., 2010).

Gelatin Zymography: MMP zymography was used to detect serum levels of MMP-2 and MMP-9 gelatinase activities. The protocol was slightly modified from Troeberg and Nagase (2005). Serum samples with protein quantification and 10 times dilution were loaded into gelatin gel wells. Electrophoresis was performed in a constant power supply at 90-100V, until the bands migrated over the line between the separating and stacking gels. Then, 130V was used until electrophoresis was done. The gel was twice washed with 2.5% Triton-X 100 (TRITON-X 100, **Gerbu Biotechnik GmbH, Germany**) for 30 mins, then it was transferred to incubation buffer and incubated overnight at 37ºC. Finally, the gel was stained and destained until the digested bands of MMP activity were observed. A Photo scanner was used to capture images, and the area of digestion was analysed using semi-quantification software, Image J.

Statistical Analysis: As the levels of MMP activities did not follow normal distribution, the results of different treatment groups were expressed by median

and quantile difference (QD). Variables in independent group were compared with the healthy group by Mann-Whitney U-test. Correlations between survival time and levels of gelatinase activities were performed using the Spearman Test for non-parametric data. All statistics were conducted with SPSS and SAS. *p*-values lesser than 0.01 were regarded as statistically significant.

Table 1 Basic characteristics of lymphoma-bearing and healthy control dogs

Patients characteristics		*n*	%	Lymphoma case	Healthy control
Age (years)	Median			7	3.75
	Range			1-15	1-6
Body weight (kg)	Median			15.5	27.1
Body weight (lb)				34.17	59.75
Body weight (kg)	Range			3.5-36.6	16–29.8
Body weight (lb)				7.72-80.69	35.27-65.7
Gender	Female	11	45.8		
	Male	13	54.2		
Immunophenotype	T-cell	3	33.3		
	B-cell	6	66.7		
Stage	Early (I-III)	3	14.4		
	Advanced (IV-V)	18	85.6		
Substage	b	3	33.3		
	a	6	66.7		
Subtype	Gastric	1	4.5		
	Plasmatoid	1	4.5		
	Cutaneous	4	18.3		
	Multicentric	16	72.7		

Some dogs had incomplete medical records or diagnostic procedures (e.g. immunophenotyping); therefore, the total number of each group was not always 24.

Table 2 Levels of serum MMP-2 and MMP-9 gelatinase activities in the groups of different treatment responses

	MMP-2 (AU)	MMP-9 (AU)
	Median (QD)	Median (QD)
Healthy control (*n* = 10)	105.92 (14.78)	93.22 (10.9) †, ‡, §
All diseased (*n* = 167)	116.77 (41.56)	154.81 (79.61) †
Before treatment (*n* = 13)	138.42 (35.7)	199.37 (37.67)
Complete response (*n* = 48)	119.26 (39.03)	140.65 (48.62)
Partial response (*n* = 39)	117.58 (62.88)	230.01 (136.19) ‡
Stable disease (*n* = 11)	113.86 (43.53)	138.12 (131.93) §
Progressive disease (*n* = 20)	132.66 (31.67)	211.3 (144.89)

AU, arbitrary unit (the value from a healthy control animal is taken as standard); QD, quartile deviation. Data with the same superscript (†, ‡, §) are significantly different ($p < 0.01$).

Results

There were no significant differences in the levels of serum MMP-2 gelatinase activity between any two groups (e.g. no significant difference between before treatment and complete response groups; complete response and progressive disease groups; Table 2). In terms of MMP-9, a significant difference was observed between the healthy controls and all diseased groups. Moreover, a difference also existed between the complete response and before treatment groups. The serum MMP-9 gelatinase activity level of all lymphoma-bearing dogs were higher than that of the healthy control dogs, despite the kind of treatment response they achieved. The MMP-9 gelatinase activity levels were also higher in the before treatment and progressive disease groups compared to the healthy group, but the difference was not significant. However, there was a significant difference in MMP-9 in the partial response and stable disease groups compared to the healthy group (Table 2). The MMP-9 levels increased in the lymphoma-bearing dogs and remained in high levels even after treatment despite the kind of treatment response achieved. However, the MMP-9 gelatinase activity levels decreased when a complete response was achieved (Fig 1).

The survival time of dead cases (case numbers = 7, sample numbers = 59) did not statistically correlate with the levels of MMP-2 and MMP-9 gelatinase activities (MMP-2: Spearman correlation coefficients = -0.26, *p*-value = 0.57; MMP-9: Spearman correlation coefficients = -0.25, *p*-value = 0.58). The mean survival

time of dead cases was 4.3 months (Table 3). There were no significant differences in the levels of MMP-2 and MMP-9 gelatinase activities between male and female groups, substage a and substage b groups, cutaneous and multicentric lymphoma groups. The level of MMP-9 gelatinase activity of early stage groups was also not different from advanced stage groups. Conversely, the level of MMP-2 gelatinase activity was higher in the advanced stage group, in younger (≤ 6 years) and in heavier (> 16 kg) patients. In the immunophenotype group, both MMP-2 and MMP-9 gelatinase activity levels were higher in T-cell lymphomas than in B-cell lymphomas (Table 4).

Table 3 Survival time and levels of serum MMP-2 and MMP-9 gelatinase activities in the dead cases

	n	Survival time (months)	Mean MMP-9 (AU)	Mean MMP-2 (AU)
case 1	6	1.75	243.72	154.97
case 2	5	2	223.98	152.42
case 3	8	2	266.29	188.5
case 4	10	5	99.93	80.92
case 5	6	5	49.47	40.2
case 6	4	6	443.68	239.37
case 7	20	8.5	94.39	104.99

AU, arbitrary unit (the value from a healthy control animal is taken as standard); *n* = total sample number of the case. Survival analysis could not be performed in this study due to the lack of sample number. However, the correlation between survival time and gelatinase levels was analyzed. Although a statistic correlation was not found, there was a tendency for MMP-9 levels to decrease as survival time increased.

Table 4 Statistical analysis of levels of serum MMP-2 and MMP-9 gelatinase activities grouped by patient characteristics

			MMP-2 (AU)		MMP-9 (AU)	
Patient characteristics	***n***	**%**	**Median (QD)**	***p*-value**	**Median (QD)**	***p*-value**
Age (years)						
> 6	12	50	101.26 (33.12)	0.003*	148.08 (108.62)	0.415
≤ 6	12	50	126.38 (45.49)		154.8 (71.87)	
Body weight (kg) [lb]						
> 16 [35.27]	11	45.8	119.52 (43.98)	0.008*	155.74 (72.58)	0.481
≤ 16 [35.27]	13	54.2	102.05 (42.24)		138.73 (104.05)	
Gender						
Female	11	45.8	114.82 (46)	0.253	206.84 (117.28)	0.108
Male	13	54.2	120.62 (42.34)		153.53 (62.16)	
Immunophenotype						
T-cell	3	33.3	154.1 (44.79)	0.001*	235.88 (50.42)	0.001*
B-cell	6	66.7	97.34 (36.94)		124.13 (54.42)	
Stage						
Early (I-III)	3	14.4	61.91 (35.47)	< 0.001*	190.39 (154.12)	0.779
Advanced (IV-V)	18	85.6	120.62 (41.17)		154.71 (70.58)	
Substage						
b	3	33.3	86.68 (21.59)	0.146	98.58 (51.05)	0.25
a	6	66.7	117.94 (41.56)		155.28 (77.4)	
Subtype						
Cutaneous	4	20	106.68 (31.52)	0.964	115.12 (83.35)	0.211
Multicentric	16	80	119 (43.82)		160.71 (76)	

AU, arbitrary unit (the value from a healthy control animal is taken as standard); QD, quartile deviation (Q_3-Q_1/2). *: Statistically significant.

Discussion

In this study, increased serum levels of MMP-9 gelatinase activity and unaltered levels of MMP-2 were observed in the lymphoma-bearing dogs. Compared with the dogs in the healthy group, the MMP-9 level was increased in all the patients with lymphoma. It may be associated with the increased number of circulating leukocytes. In fact, a significantly positive correlation was observed between the serum levels of MMP-9/MMP-2 and the leukocytes counts, especially the neutrophil counts. MMPs secreted from leucocytes are thought to be involved in the migration of these cells through the basement membrane by ensuring the destruction of connective tissue (Birkedal-Hansen et al., 1993; Guedez et al., 1996). MMP-2 and MMP-9 are also reported to play a pivotal role in facilitating the extravasation and migration of neutrophils by breaking down the basement membrane (Delclaux et al., 1996). Therefore, circulating leucocytes may injure endothelial cells and also migrate into the vascular wall, by producing an excessive amount of inflammatory mediators such as MMP-9 and MMP-2 and are thus suggested to contribute to the pathogenesis of canine lymphoma. After treatment, the MMP-9 level was still significantly increased in the dogs only in the partial response group and in the stable disease group, while the MMP-9 level was decreased in the dogs in the complete response group but showed no significance. However, although there was no significant difference in the MMP-9 level in the dogs among the before treatment group, the progressive group and the healthy group, the MMP-9 level was higher than that in the healthy group. The MMP-9 level in the progressive group was also higher than that in the before treatment group. A possible explanation for these results is that the sample size was insufficient, as well as the variation among individuals. Taken together, MMP-9 can still be an indicator to evaluate the treatment of lymphoma.

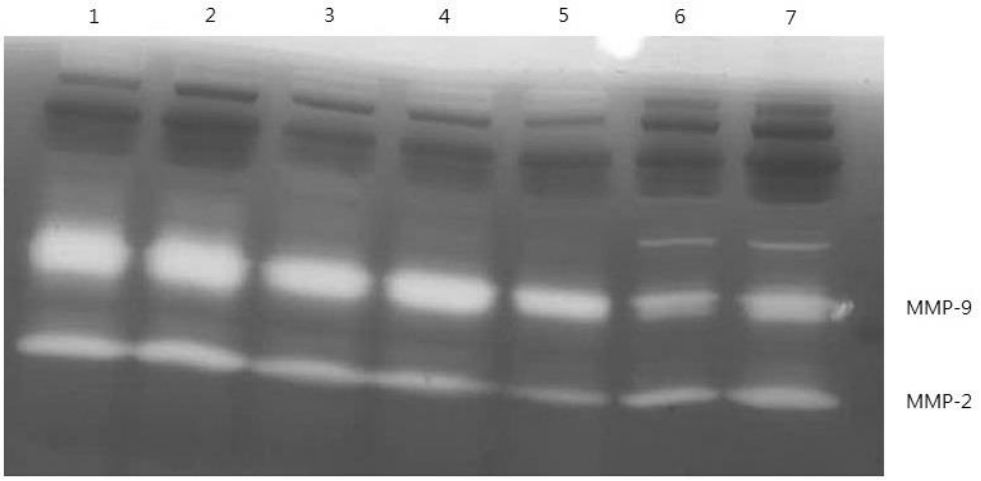

Figure 1 The dog received the A 6-month modified protocol of the University of Wisconsin (UW)-Madison chemotherapy protocol (UW-25). A complete response was achieved after 4 months. When the complete response was achieved, the level of MMP-9 significantly decreased. The bands on the upper and the lower row (arrows) are MMP-9 (92 kDa) and MMP-2 (72 kDa), respectively.

MMP-2 and MMP-9 are believed to play an important role in invasion and metastasis of malignant neoplasms. A previous study showed that MMP-9 mRNA expression increased before therapy and decreased after therapy (Negaard et al., 2009). The overexpression of MMP-9 was related to tumor aggressiveness (Lalancette et al., 2000) and poor prognosis (Kuittinen et al., 2002; Kuittinen et al., 2003). In this study, the increased MMP-9 gelatinase activity level was observed before therapy similar to the results of other studies, but the decreased MMP-9 gelatinase activity only occurred in the complete response group after chemotherapy. The duration of after chemotherapy was variable and indefinite between studies as mentioned above; an absolute therapy response would help the comparison. As a result, appropriate groupings of different treatment response were performed in this study in order to explore the exact serum levels of gelatinases.

Unchanged MMP-2 expression was reported in canine lymphoma (Newmana et al., 2008). Increased MMP-2 in canine lymphoma was also observed in a study (Gentilini et al., 2005). Aricò et al. (2013) pointed out that MMP-2 mRNA level in healthy dogs was significantly higher than in lymphoma-bearing dogs. It seemed that MMP-2 only increased in some subtypes of lymphoma such as human Hodgkin's lymphoma (Kuittinen et al., 2002). The MMP-2 gelatinase activity level was not increased in this study, but they were analysed without the subgroup classification of Hodgkin's or non-Hodgkin's lymphoma owing to the seldom use of this classification in dogs. The relationship between treatment responses and MMP-2 levels have also been studied. The MMP-2-TIMP-2 complex, proMMP-2/TIMP-2 and proMMP-2 were higher in remission and in active disease group than in healthy controls (Pennanen et al., 2008). Although there have been many studies discussing the relation between gelatinase and human lymphomas, there are

few studies in canine (Gentilini et al., 2005; Newmana et al., 2008; Aricò et al., 2013; Aresu et al., 2014). One study demonstrated that both MMP-2 and MMP-9 levels were increased in canine lymphomas (Pennanen et al., 2008). Others showed only MMP-9 increase (Newmana et al., 2008; Aricò et al., 2013; Aresu et al., 2014). Our results were similar to those of the latter. A possible cause of discrepancy in the MMP-2 and MMP-9 gelatinase activity levels is the different matrix examined from plasma and serum. Additionally, other possible reasons as sample size and the classification of tumor subgroup should be considered.

In this study, we found a tendency for younger (≤ 6 years) and heavier (> 16 kg) canine lymphoma patients, and patients with an early stage (I-III) of lymphoma or T-cell lymphoma, to have higher level of serum MMP-2 gelatinase activity according to the characteristics of age, body weight, disease stage and immunophenotype. In contrast to MMP-2, a discrepancy of MMP-9 was only observed in the immunophenotype group, which was higher in the T-cell lymphoma- than in B-cell lymphoma-bearing dogs (Table 4). Because age, body weight, tumor stage and tumor immunophenotype have been reported as prognostic factors for lymphoma (Troeberg and Nagase, 2005), compared with MMP-9 in this study, MMP-2 showed a good correlation to these factors. Histologic grade, b symptoms and extranodal involvement were also found for evaluation of prognosis for lymphoma, but no significant changes in gelatinase levels associated with these factors have been reported[7]. MMP-2 and MMP-9 were increased in WHO substage b (with b symptoms) compared to substage a (without b symptoms) (Gentilini et al., 2005). MMP-9 was higher in both T-cell lymphoma and advanced stage (stage V). In this study, the MMP-9 levels were decreased during chemotherapy for lymphoma, which is similar to a previous study of canine B-cell lymphoma (Aresu et al., 2014). The other factors did show a correlation to gelatinase, but due to the small sample size or the shallow classification of tumor subgroup, the power of this study was not so strong. It is suggested that a larger group of canine lymphoma patients is needed to accurately confirm the results of our study. Recently, a larger group of canine lymphoma patients (n = 37) and healthy control (n = 10) was used to evaluate the correlation of MMP-9 gelatinase activity at admission and disease status, clinical stage and tumor immunophenotype. Higher MMP-9 gelatinase activity was shown in the lymphoma-bearing dogs. MMP-9 gelatinase activity was significantly higher in T-cell lymphomas, and in stage V compared with stages III–IV disease, regardless of immunophenotype. During chemotherapy, MMP-9 gelatinase activity decreased in B-cell lymphomas (Aricò et al., 2013). Although some results were different from our results, these evidences confirm that MMP-9 gelatinase activity correlates with tumor immunophenotype and chemotherapeutic strategies, suggesting a possible predictive role in the lymphoma-bearing dogs and providing a novel target therapeutic strategy to substitute for the traditional chemotherapy.

In this work, among all the cases (24), there were seven dead dogs that had complete treatment and follow-up data that can be applied to a correlation test. The others were excluded because they were in the end stage when diagnosed with lymphoma and received no or only a few treatments. The effects of survival time and treatment responses to gelatinase levels have been studied (Pennanen et al., 2008). Survival analysis could not be performed in this study due to the lack of sample numbers. However, the correlation between survival time and gelatinase activity levels was analysed. Although a statistical correlation was not found, there was a tendency for the MMP-9 gelatinase activity level to decrease as the survival time increased.

In conclusion, our data provide some new information on the gelatinase activities of MMP-2 and MMP-9 in canine lymphoma with or without the traditional chemotherapies. A significant correlation between serum levels of gelatinases (MMP-2 and MMP-9) and lymphoma in dogs is proposed in this study. The serum MMP-9 levels increased before chemotherapy and decreased after a complete response was achieved. Higher MMP-2 and MMP-9 levels in T-cell lymphomas than in B-cell lymphomas were also observed. Most of these results reveal a tendency for gelatinases to be used as prognostic factors of canine lymphoma.

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บทคัดย่อ

การเปลี่ยนแปลงการทำงานของ Matrix Metalloproteinase-9 Gelatinase ในซีรั่มของสุนัขที่มีมะเร็งต่อมน้ำเหลืองและได้รับเคมีบำบัด

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Matrix metalloproteinase-2 และ -9 (MMP-2 และ -9) เป็นเอนไซม์ gelatinase ในกลุ่ม MMP ซึ่งเกี่ยวข้องกับการเคลื่อนที่ของเซลล์ การบุกรุกเซลล์ การแพร่กระจายของเซลล์มะเร็ง และการสร้างหลอดเลือดใหม่ของมะเร็งชนิดเป็นก้อนในคน แต่การพยากรณ์โรคมะเร็งต่อมน้ำเหลืองในสุนัขด้วยเอนไซม์ gelatinase ยังคงคลุมเครือ เพื่อประเมินความสัมพันธ์ระหว่างเอนไซม์ gelatinase ในซีรั่มและมะเร็งต่อมน้ำเหลืองในสุนัข จึงทำ gelatin zymography เพื่อตรวจวัดระดับการทำงานของ MMP-2 และ MMP-9 gelatinase ในซีรั่มของสุนัขที่มีมะเร็งต่อมน้ำเหลือง ในการศึกษาย้อนหลังครั้งนี้ประกอบด้วยสุนัขจำนวน 24 ตัวที่มีมะเร็งต่อมน้ำเหลืองชนิดย่อยแตกต่างกันซึ่งระบุได้ด้วยการตรวจสอบทางจุลพยาธิวิทยา และสุนัขปกติในกลุ่มควบคุมอีกจำนวน 10 ตัว ทำการเก็บตัวอย่างซีรั่มของสุนัขก่อนและหลังการทำเคมีบำบัด ผลการทดลองพบว่าระดับ MMP-9 เพิ่มสูงขึ้นในสุนัขที่มีมะเร็งต่อมน้ำเหลือง (ค่ามัธยฐาน 199.37, ค่าพิสัยควอไทล์ 37.67) และมีระดับลดลงหลังจากการตอบสนองอย่างสมบูรณ์ (ค่ามัธยฐาน 140.64, ค่าพิสัยควอไทล์ 48.62) อย่างไรก็ดีระดับ MMP-2 มีค่าใกล้เคียงกันระหว่างสุนัขกลุ่มควบคุมและสุนัขที่มีมะเร็งต่อมน้ำเหลืองและได้รับการรักษาแล้ว (ก่อน: ค่ามัธยฐาน 138.42, ค่าพิสัยควอไทล์ 35.7: การตอบสนองอย่างสมบูรณ์: ค่ามัธยฐาน 119.26, ค่าพิสัยควอไทล์ 39.03) สุนัขที่มีมะเร็งต่อมน้ำเหลืองชนิด T-cell มีระดับ MMP-2 และ MMP-9 สูงกว่าสุนัขที่มีมะเร็งต่อมน้ำเหลืองชนิด B-cell เมื่อเปรียบเทียบกับ MMP-2 การเปลี่ยนแปลงของระดับ MMP-9 ในซีรั่มสามารถใช้เป็นค่าพยากรณ์โรคสำหรับสุนัขที่มีมะเร็งต่อมน้ำเหลือง

คำสำคัญ: มะเร็งต่อมน้ำเหลืองในสุนัข, gelatinase activity, MMP-2, MMP-9

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