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Evaluation of matrix metalloproteinases (MMP)-2 and MMP-9 activity in serum and biochemical and hematological parameters in spontaneous canine cutaneous tumors before and after surgical treatment

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Abstract

Recently, matrix metalloproteinases (MMP), in particular the gelatinases MMP-2 and MMP-9, generally considered as tumor markers for clinical applications. A longitudinal 2-years follow-up survey was performed on dogs with cutaneous tumor. Serum samples were obtained from 22 dogs with different cutaneous tumors and 22 health dogs at the time of surgery and one month, three months and one year after surgery. Gelatin zymography, hematological and biochemical assessment were performed for all serum samples. The serum alkaline phosphatase activity in dogs with malignant tumors was significantly higher than that in dogs with benign tumors and control cases. Latent forms of MMP-2 and MMP-9 were detected in all of the tumor cases. Gelatin zymography showed active form of MMP-9 in 12 cases (three benign and nine malignant tumors) and active form of MMP-2 in one fibrosarcoma case. Serum activity of active-MMP-9 and total MMP-9 was significantly higher in dogs with cutaneous tumors than those in controls. Tumor cases had higher serum activity of active-MMP-9 rather than controls. MMPs and alkaline phosphatase activities in serum were decreased significantly after surgery. Only one case with perianal gland adenoma showed recurrence of tumor four months after surgery in which active form of MMP-9 had identified one month before recurrence. According to the findings, it will be useful to measure ALP, MMP-2 and MMP-9 activities in the serum of dogs with cutaneous tumor for determination of tumor behavior before surgical treatment.

ارزیابی فعالیت سرمی ماتریکس متالوپروتئینازهای MMP-2 و MMP-9 و پارامترهای بیوشیمیایی و هماتولوژی در تومورهای پوستی خود به خودی سگ‌ها قبل و پس از درمان جراحی

چکیده

اخیراً ماتریکس متالوپروتئینازها، بویژه ژلاتینازهای MMP-2 و MMP-9 عموماً به‌عنوان مارکرهای تومور برای کاربردهای بالینی مورد توجه قرارگرفته‌اند. یک بررسی دو ساله آینده‌نگر برای سگ‌های مبتلا به تومورهای پوستی انجام گردید. نمونه سرمی در روز جراحی، یک ماه، سه ماه و یک سال پس از جراحی از ۲۲ بیمار مبتلا به انواع تومورهای پوستی و ۲۲ سگ سالم اخذ گردید. زایموگرافی ژلاتین و ارزیابی هماتولوژی و بیوشیمی بر روی تمام نمونه های سرمی انجام گردید. فعالیت سرمی آلکالین فسفاتاز در تومورهای بدخیم به‌طور معنی‌داری بالاتر از موارد خوش‌خیم و کنترل بود. فرم غیرفعال MMP-2 و MMP-9 در تمام موارد تومور شناسایی گردید. زایموگرافی ژلاتین، فرم فعال MMP-9 را در ۱۲ مورد (سه تومور خوش‌خیم و نه تومور بدخیم) و فرم فعال MMP-2 را تنها در یک مورد فیبروسارکوما نشان داد. فعالیت سرمی MMP-9 فعال و MMP-9 تام به‌طور معنی‌داری در سگ‌های مبتلا به تومورهای پوستی نسبت به موارد کنترل بالاتر بودند. موارد مبتلا به تومور فعالیت سرمی MMP-9 فعال بالاتری را نسبت به گروه کنترل داشتند. فعالیت سرمی آلکالین فسفاتاز وMMP ها به‌طور معنی‌داری پس از جراحی کاهش داشت. تنها یک مورد تومور آدنوم غدد پره‌آنال پس از چهار ماه برگشت مجدد را پس از جراحی نشان داد که فرم فعال MMP-9 یک ماه قبل از برگشت شناسایی شده بود. با توجه به یافته‌ها، اندازه‌گیری ALP و فعالیت سرمی MMP-2 و MMP-9 برای تعیین رفتار تومور قبل از درمان جراحی می‌تواند مفید باشد.

واژه های کلیدی: تومورهای پوستی سگ، زایموگرافی ژلاتین، ماتریکس متالوپروتئیناز ۲ ، ماتریکس متالوپروتئیناز ۹

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Introduction

Matrix metalloproteinases (MMPs) are enzymes secreted in a latent form, and then activated by a zinc ion at their active site. These enzymes involve in cancer invasion, metastasis and angiogenesis. The active forms of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are known for their ability for proteolytic degradation of components of the extracellular matrix.[1,2] MMP-2 and MMP-9 have an important role in angiogenesis, invasion, metastases and growth of the solid tumors like breast carcinoma, gynecological neoplasias, prostatic neoplasia and melanoma. In several studies, the prognostic value of MMP-2 and MMP-9 for different neoplastic diseases have been reported.[1-3]

Increased gelatinase in tumor tissue and serum may be correlated with tumor aggressiveness. In addition, histopathology in combination with MMPs assessment have been recommended for predicting tumor metastasis or recurrence.[4] In a study, it has been has suggested that serum MMP-9 levels may be used as a tumor marker in human patients with sarcoma.[5] The serum activity level of MMP's has been used as a diagnostic value for differentiation of breast cancer subgroups.[6] The pretreatment serum levels of MMP-9 has been proposed as a new powerful prognostic marker in non-small-cell lung cancer.[7] Circulating levels of MMP-2 and MMP-9 have prognostic value in canine spontaneous non-Hodgkin lymphoma (NHL).[8] The active form of MMP-9 has been detected in the plasma of dogs with mammary tumors.[9]

It has been shown that pro-MMP-2 and pro-MMP-9 expression is higher in malignant than those in benign tumors.[10] High levels of pro-MMP-2, pro-MMP-9 and active MMP-2 have been identified in most canine tumors. The level of MMPs in canine tumors are significantly higher than those in dogs without tumor and also the level of MMPs in dogs with malignant tumors are significantly higher compared to those in dogs with benign tumors.[11] The active and inactive forms of MMP-2 and MMP-9, measured by gelatin zymography method, increased significantly in dogs with a higher histological grade of mast cell tumors.[12] In addition, a significant correlation has been observed between high pro-and active forms of MMP-2 and MMP-9 and malignancy in canine cutaneous mast cell tumors[13] and other tumor types[11] by gelatin zymography.

Cutaneous neoplasms are the most frequent tumors in dogs.[14-19] Cutaneous tumors are often removed surgically and histopathology is essential for prognosis and determination of an appropriate treatment.[19,20] Recently, MMP activity assessment in tissue and serum samples has been used as a useful prognostic and predictive marker in a variety of skin tumors in dogs especially in cutaneous mast cell tumor.[4,11-13] In the present work, our purpose was to identify latent and active forms of MMPs in serum of dogs with spontaneous cutaneous tumor before and after surgical removal of the tumor masses. We also determined usefulness of the serum MMPs and alkaline phosphatase activities as prognostic markers after surgical treatment of canine cutaneous tumors and for predicting its recurrence and malignancy.

Materials and Methods

Animals. We performed a clinical study on dogs with spontaneous canine cutaneous tumors referred to the Veterinary Teaching and Research Hospital of Faculty of Veterinary Medicine, University of Tehran and Paytakht Pet Hospital. Dogs with palpable cutaneous and subcutaneous mass and without any clinical symptoms of other abnormalities were followed by histology. A total of 105 cutaneous biopsy specimens from dogs suspicious for cutaneous tumor were collected and submitted to the Department of Clinical Pathology, College of Veterinary Medicine, and Tehran University (Fig. 1). The mass and nearby visually normal stromal tissue (within 3 cm of the tumor) were obtained at the time of surgery. The specimens were fixed in 10.00% neutral buffered formalin, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin. Cutaneous tumors were classified into two histologic types, including benign (nine dogs) and malignant (13 dogs) tumors according to the published criteria.[16,21,22] These twenty-two dogs diagnosed with spontaneous cutaneous tumors, were undergone surgical treatment and with their owners' consent. At the time of surgery, signalment, historical data, and relevant clinical details were recorded, including the site, size, and consistency of the lesions. Chest radiograph and abdominal ultrasound were used for detection of distant metastases at the time of surgery and during the follow-up examinations. The patient cases were divided into malignant and benign groups. Serum samples were collected from 22 healthy dogs (control group) with the same age and sex as the patients group. Patient were also

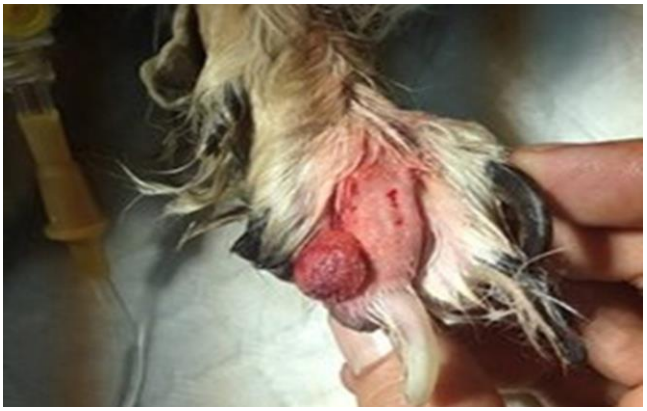

Fig. 1. Gross lesion of fibrosarcoma in a male Shih Tzu dog (interdigital of the right forelimb) at the time of surgery, Paytakht Pet Hospital (Tehran, Iran).

classified into two groups according to the size of tumors, group with the tumor diameter less than two cm and the group with tumor diameter of 2 cm or more.

Hematological and biochemical analysis. Blood samples were collected in EDTA coated tubes for CBC, reticulocytes count (vital staining with new methylene blue) and ESR using automatic impedance cell analyzer (Exigo EOS Vet, Stockholm, Sweden) calibrated for canine blood. The differential leukocyte count was performed manually by counting 100 leukocytes in Giemsa-stained blood smears. Blood for biochemical analysis was collected in plain tubes, and serum was separated by centrifugation within 30 min. Biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH), creatine kinase (CK), total protein, albumin, amylase, lipase, acid phosphatase (ASP), uric acid, iron, total iron binding capacity (TIBC), magnesium, calcium, phosphorus, glucose, triglycerides, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), urea, creatinine, total and direct bilirubin were measured by commercial kits (Pars Azmoon, Tehran, Iran) using chemistry auto-analyzer (EliTech Diagnostic, Puteaux, France).

Gelatin zymography. Obtained serums to be used for zymography were immediately stored at −80 ˚C until assayed. Gelatin zymography was performed using the routine protocol described by Loukopoulos *et al*.,[11] with some modifications. Fifteen µL of each serum sample was diluted in sample buffer 4X (10 mL of 250 mM Tris-HCl, pH 6.8; 40.00% glycerol; 8.00% SDS; and 0.01% bromophenol blue) (1:1 ratio). An amount of 15 µL diluted serum was subjected to electrophoresis on a 10.00% SDS-PAGE gel copolymerized with 0.10% bovine gelatin. Recombinant human MMP-2 and MMP-9 (0.5 ng per lane; Sigma, St. Louis, USA) and standard proMMP-9 and proMMP-2 mixture solutions (1 ng per lane) (Bio-Rad, Richmond, USA) were loaded on the separated lanes. Electrophoresis was performed in 20 mA and 96 V under non-reducing condition. The gels were incubated twice, 30 min in Triton-100 X (Sigma) at room temperature and then 24 hr in 0.5 M Tris-HCl (Merck, Darmstadt, Germany) buffer, pH = 7.4 with 10 mM $CaCl_2$ (Merck) at 37 ˚C. The gel was stained by Coomassie brilliant blue and de-stained in mixture of 7.00% acetic acid (Merck) and 40.00% methanol (Merck). The clear bands against a blue background are accepted as MMPs. Gelatin zymography was repeated several times on these samples to allow the production of clear bands and the subsequent densitometric quantification of MMPs. The 62, 64, 66 and 68 kDa bands were accepted as MMP-2.[11,23] The 72, 88 and 92 kDa bands were also identified as pro-MMP-2, MMP-9 and pro-MMP-9, respectively.[11] The integrative intensity of the gelatinase bands was quantified using ImageJ (version 1.50; National Institute of Mental Health, Bethesda, USA).

Follow-up examinations. Dogs were evaluated before surgery, four weeks after surgery and every four months thereafter for a two-years period. Each evaluation included a complete physical examination, thoracic radiographs and abdominal ultrasound. Hematological and biochemical parameters and serum zymography were performed four weeks, three months and one year after surgery and compared with the initial results at the time of surgery.

Statistical analysis. All zymography, hematological and biochemical results were analyzed by statistical software SPSS (version 22.0; IBM Corp., Armonk, USA). The first day results of the patients group were compared with control group by using the Student's *t*-test and Mann-Whitney test for non-normally distributed variables. Total MMP was calculated as sum of the pro-MMP and active MMP values. One-way ANOVA, Tukey's post hoc and Kruskal–Wallis test were used for comparison of the first day results of the benign and malignant groups with the control group results. One-way repeated measures analysis of variance and Friedman test for non-normally distributed variables were used for analysis of the repeated measurements for malignant and benign cutaneous tumors after surgery. In all statistical comparisons, $p < 0.05$ was considered as significant.

Results

The frequency and types of canine cutaneous tumors. During the 2-year study period, 22 (20.90%) cases were diagnosed as canine cutaneous tumors among a total of 105 examined biopsies (Fig. 2). Cutaneous tumors had the highest frequency in the Shih Tzu and Terrier breeds (Table 1). Eleven types of canine cutaneous tumors were identified in 16 males and six female dogs. There were no statistically significant differences between male and female dogs regarding cutaneous tumors and other clinical and laboratory findings. Of these 22 tumor cases, nine tumors were diagnosed to be benign and 13 tumors were malignant. Perianal gland adenoma (four cases) and sebaceous adenoma (three cases) in benign tumors and fibro-sarcoma (three cases) and canine malignant cutaneous histiocytoma (three cases) in malignant tumors were the most frequent tumors (Table 2).

No evidence of tumor metastasis was detected in the dogs using thoracic radiography and abdominal ultra-sound after surgical treatment. Although, one of the perianal gland adenomas showed evidence of recurrence four months after surgery. Nine tumors had more than 2 cm diameter and 13 tumors had 2 cm diameter and less. (Table 1). All the dogs were alive until the end of study. Tumor malignancy was not associated with age, breed, sex and tumors size.

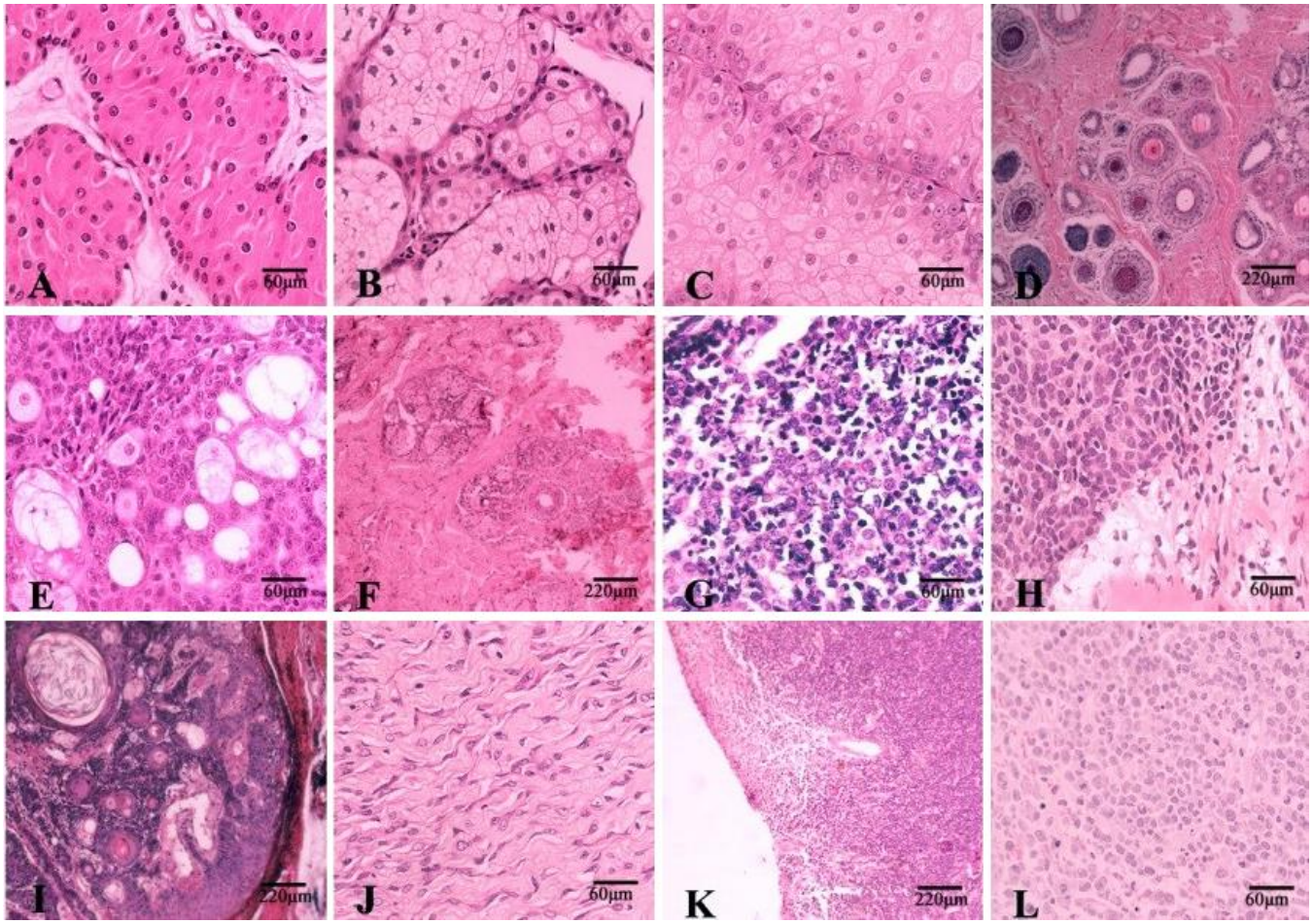

Fig. 2. A) Perianal gland adenoma, B) Sebaceous adenoma, C) Sebaceous epithelioma, D) Trichoblastoma, E) Trichoepithelioma, F) Apocrine gland adenoma, G) Cutaneous plasmacytoma, H) Apocrine gland adenocarcinoma, I) Squamous cell carcinoma, J) Fibrosarcoma, K and L) Canine malignant cutaneous histiocytoma (Hematoxylin and Eosin staining).

Table 1. Characteristics of twenty-two dogs with spontaneous cutaneous tumor.

No.	Tumor type	Age (Year)	Tumor Size (cm)	Sex	Breed	Involved area	Recurrence
1	Perianal Gland Adenoma	6.7	2.5×2.0×1.0	Female	Spitz	Perianal area	-
2	Perianal Gland Adenoma	10.2	2.0×1.6×1.5	Male	Terrier	Perianal area	After 4 month
3	Perianal Gland Adenoma	7.3	1.8×1.5×1.0	Female	Shih Tzu	Perianal area	-
4	Perianal Gland Adenoma	9.5	2.2×1.1×1.7	Male	Chihuahua	Perianal area	-
5	sebaceous Adenoma	5.1	1.0×0.7×1.2	Male	Boxer	Forehead	-
6	sebaceous Adenoma	4.0	2.0×1.1×0.5	Male	Terrier	Tail	-
7	sebaceous Adenoma	4.2	1.2×0.8×0.6	Female	Poodle	Face	-
8	Sebaceous epithelioma	5.3	0.6×0.9×0.7	Male	German shepherd	Tail	-
9	Trichoblastoma	6.8	1.6×1.5×0.6	Male	Terrier	Submandibular	-
10	Trichoepithelioma	10.6	2.0×1.7×0.9	Male	Terrier	Left hindlimb	-
11	Trichoepithelioma	5.8	2.3×1.5×1.1	Female	Doberman	Upper eyelid	-
12	Apocrine gland adenoma	7.6	3×2.5×01.8	Male	Terrier	Lumbosacral	-
13	Cutaneous plasmacytoma	7.8	1.7×1.2×0.7	Male	Bulldog	Right tarsus	-
14	Cutaneous hemangioma	6.3	3.0×1.5×0.1	Male	Shih Tzu	Abdominal	-
15	Apocrine gland adenocarcinoma	7.2	1.7×1.1×1.0	Male	Cocker Spaniel	On the paw	-
16	Squamous cell carcinoma	14.0	1.3×1.4×0.5	Male	Terrier	Head	-
17	Fibrosarcoma	13.3	2.3×1.6×0.8	Male	Shih Tzu	Interdigital of right forelimb	-
18	Fibrosarcoma	3.2	2.5×1.7×1.4	Female	Dalmatian	Face	-
19	Fibrosarcoma	7.2	2.1×1.7×1.1	Female	Shih Tzu	Face	-
20	Cutaneous histiocytoma	2.7	1.8×1.2×0.6	Male	Terrier	Left hindlimb	-
21	Cutaneous histiocytoma	2.4	2.0×1.5×1.6	Male	Boxer	Right hindlimb	-
22	Cutaneous histiocytoma	2.5	2.1×0.8×0.7	Male	Shih Tzu	Left hindlimb	-

Table 2. Histogenetic classification, prevalence and sex distribution of cutaneous tumors in the examined dogs from October 2013 to November 2015.

Tumor type	Number of cases (%)	Male: Female ratio
Epithelial tumors	14 (63.66)	10:4
Perianal gland adenoma	4 (18.18)	2:2
Sebaceous adenoma	3 (13.64)	2:1
Trichoepithelioma	2 (9.09)	1:1
Sebaceous epithelioma	1 (4.55)	1:0
Trichoblastoma	1 (4.55)	1:0
Apocrine gland adenoma	1 (4.55)	1:0
Apocrine gland adenocarcinoma	1 (4.55)	1:0
Squamous cell carcinoma	1 (4.55)	1:0
Lymphohistiocytic tumors	4 (18.19)	4:0
Cutaneous plasmacytoma	1 (4.55)	1:0
Malignant cutaneous histiocytoma	3 (13.64)	3:0
Mesenchymal tumors	4 (18.19)	2:2
Fibrosarcoma	3 (13.64)	1:2
Cutaneous hemangioma	1 (4.55)	1:0
Other non-neoplastic masses	83 (79.00)	43:40
Total	105 (100)	16:6

Hematology. The first day assessment of hematological parameters demonstrated no significant differences between the patients and control groups. In addition, there was no significant difference in hematological parameters in the first day between the dogs with benign and malignant tumors and the normal (control) dogs. There wasn't any specific alteration in the hematological parameters measured at four weeks, three months and one year after surgery.

Clinical biochemistry. The first day assessment of biochemical parameters showed a significant increase in serum ALP in the tumor group (182.70 ± 27.20 U L^{-1}) in comparison with that in control group (84.70 ± 5.80 U L^{-1}), ($p < 0.01$). In addition, serum ALP activity was higher in dogs with malignant tumors (237.40 ± 38.60 U L^{-1}) than that dogs with benign tumor (128.10 ± 15.90 U L^{-1}) and the control (84.70 ± 5.80 U L^{-1}) group ($p < 0.01$). In addition, serum ALP activity in the dogs with benign tumors was mildly higher than dogs in the control group, although the difference was not statistically significant (Fig. 3A). Repeated measure ANOVA showed a significant decrease in the serum ALP activity after the surgical removal of the tumors in the dogs with malignant tumors ($p < 0.05$). However, there was no significant change in serum ALP activity in the dogs with benign tumors before and after the surgery (Fig. 3B).

Zymography. Active and inactive forms of MMP-9 and MMP-2 were detected in serum of the dogs with cutaneous tumors. The pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) bands were detected in all samples (first sampling) examined in all of the dogs with tumor and control dogs. Only one of the dogs with fibrosarcoma exhibited band for the active form of MMP-2 (66 kDa) in its first day blood sample (Fig. 4A). The active form of MMP-9 (82 kDa) was detected in 12 cases in their first day blood samples including perianal gland adenomas (two cases) in benign tumors and cutaneous plasmacytoma, cutaneous hemangioma, apocrine gland adenocarcinoma, squamous cell carcinoma, fibrosarcomas (three cases), and malignant cutaneous histiocytomas (three cases) in malignant tumors.

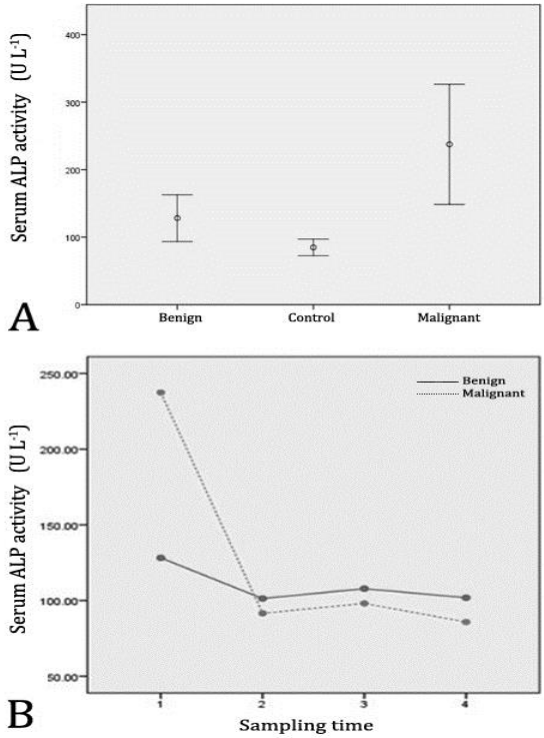

Fig. 3. A) The differences between serum activity of alkaline phosphatase (ALP) of the control dogs and the dogs with benign and malignant tumors. Data (mean ± SE) are expressed in U L^{-1}. B) Serum ALP activity at the first day (before surgery) (1), 4 weeks (2), 3 months (3) and 1 year after surgery (4). Error bars = 95% CI.

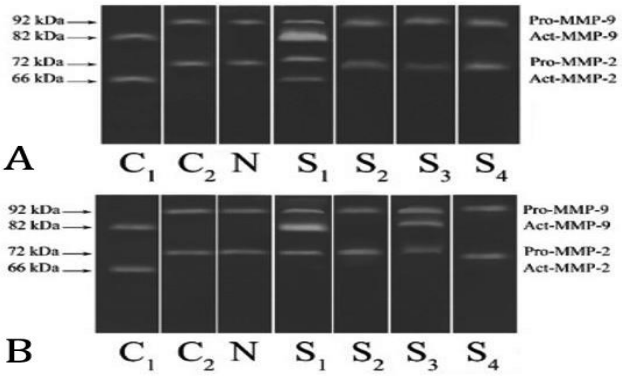

Fig. 4. A) Zymography of cutaneous fibrosarcoma, and **B)** Zymography of canine perianal gland adenoma. The bands of canine pro-MMP-2 (72 kDa), active MMP-2 (66 kDa), pro-MMP-9 (92 kDa), and active MMP-9 (82 kDa) are seen. The 82 kDa band (active MMP-9) can be observed at third sampling in canine perianal gland adenoma. Lane C_1, recombinant human MMP-2 and MMP-9; lane C_2, pro-MMP-9 and pro-MMP-2 mixture solutions; lane N, control dog; S_1, sampling at the first day; S_2; sampling after four weeks; S_3; sampling after three months; S_4, sampling after 1 year.

The MMPs activities have been increased in the serum of the fibrosarcomas cases as more intense bands of the pro- and active types of MMP-2 and -9 than those of the other tumors were observed. In all of the repeated measurements of MMPs (4 weeks, 3 months and 1 year after surgery) only pro-MMP-2 and pro-MMP9 bands were seen. Interestingly, an additional 82 kDa band, representing active MMP-9 was detected before the recurrence of the perianal gland adenoma three month after surgery (Fig. 4B). The serum activity of the latent and active forms of matrix MMP were increased in dogs with cutaneous tumors in comparison with the control dogs at their first blood samples. However, this increase was statistically significant only for active-MMP-9 and total MMP-9 ($p < 0.01$, Table 3). Furthermore, serum activity of the MMP-9 was significantly higher in the dogs with malignant tumors than that in the dogs with benign tumors and the control group in their first blood sample ($p < 0.01$). Total MMP-2 was significantly higher in dogs with malignant tumors than that in control group ($p < 0.01$).

Table 3. Mean values of the first day matrix metalloproteinase (MMPs) serum activity (ng per lane) in healthy (control) dogs (n = 22) and dogs with cutaneous neoplasm (n = 22). Data are presented as mean ± SE.

Parameters	Healthy	Neoplastic	Significant level
Pro-MMP-2	1.02 ± 0.14	1.12 ± 0.45	$p = 0.338$
Active MMP-2	0.00	0.06 ± 0.01	$p = 0.323$
Pro-MMP-9	0.83 ± 0.17	0.92 ± 0.35	$p = 0.222$
Active MMP-9	0.00	0.47 ± 0.43	$p = 0.001$
Total MMP-2	1.02 ± 0.14	1.13 ± 0.46	$p = 0.287$
Total MMP-9	0.93 ± 0.17	1.25 ± 0.47	$p = 0.001$

The increased MMPs serum activity did not differed significantly between dogs with benign tumors and the control dogs. All forms of the MMP were decreased after surgery in the repeated measurements and the active forms of MMP were disappeared, although these changes were not statistically significant (Table 4). The MMPs serum activity was mildly higher (but not significant) in the tumors with more than 2 cm dimeter than that in the tumors with 2 cm or less in diameter.

Discussion

Many researchers have reported a relation between serum levels of MMP-2 and MMP-9 with tumors in dogs and human.[3-13] In this study, we identified both latent and active forms of MMP-2 and MMP-9 in serum of dogs with a variety of canine cutaneous tumors at the time of surgery. The presence of pro-MMP-2 and pro-MMP9 was demonstrated in all serums of control group. Both pro and active MMP-2 and MMP-9 serum levels were higher in dogs with tumor than those in the control group, but only active-MMP-9 and total MMP-9 showed statically significant differences. Moreover, the serum levels of MMP-9 were significantly higher in the dogs with malignant tumors than that in dogs with benign tumors. The total MMP-2 serum levels were significantly higher in the dogs with malignant tumors than that in the control group. Active forms of MMP-2 and MMP-9 were not detected after surgical removal of the tumors. These findings could represent that the serum level of active MMP-9 is consistent with the gelatinase activity that is due to the presence of an ongoing process of tumor. Furthermore, MMP-2 and MMP-9 serum levels could indicate malignant progression of tumors.

Measurement of MMP-2 and MMP-9 in the serum can be used in decision making for surgical intervention in cutaneous masses in dogs and can help to understand the risk extent of these masses. Aresu *et al*. have demonstrated that the active MMP-9 is present in the plasma of dogs with malignant and benign mammary gland tumors, and pro-MMP-2 and pro-MMP9 were detected in the plasma of all of the dogs with tumor and the normal dogs.[9]

There are several reports on the serum activity of MMPs in human and dogs that demonstrate their usefulness as tumor marker,[5] differentiation of breast cancer subgroups, [6] and as prognostic marker in patients with non-small cell lung cancer[7] and canine NHL.[8] Miya *et al*. showed that gelatinase activity in serum was positively correlated with that in tumor extracts and increased gelatinase in tumor tissue and

Table 4. Mean value of the repeated measurements of matrix metalloproteinase (MMP; ng per lane) in the dogs with malignant (n = 9) and benign (n = 13) cutaneous tumors in different times. Data are presented as mean ± SE.

Parameters		S_1	S_2	S_3	S_4
	ProMMP-2	1.12 ± 0.14	1.05 ± 0.07	0.94 ± 0.10	0.84 ± 0.11
	Active MMP-2	0.03 ± 0.03	0.00	0.00	0.00
Malignant	**ProMMP-9**	1.09 ± 0.12	0.11 ± 0.06	0.11 ± 0.10	0.65 ± 0.05
	Active MMP-9	0.79 ± 0.13	0.00	0.00	0.00
	Total MMP-2	1.15 ± 0.09	1.05 ± 0.07	0.94 ± 0.10	0.84 ± 0.11
	Total MMP-9	1.88 ± 0.15	0.11 ± 0.06	0.11 ± 0.10	0.65 ± 0.05
	ProMMP-2	1.20 ± 0.15	0.93 ± 0.05	0.88 ± 0.07	0.80 ± 0.71
	Active MMP-2	0.00	0.00	0.00	0.00
Benign	**ProMMP-9**	0.94 ± 0.11	0.82 ± 0.06	0.84 ± 0.06	0.77 ± 0.05
	Active MMP-9	0.18 ± 0.01	0.00	0.06 ± 0.05	0.00
	Total MMP-2	1.20 ± 0.15	0.93 ± 0.05	0.88 ± 0.07	0.80 ± 0.71
	Total MMP-9	1.12 ± 0.12	0.82 ± 0.06	0.90 ± 0.09	0.77 ± 0.05

S_1: sampling at the first day, S_2: sampling after four weeks, S_3: sampling after three months, S_4: sampling after 1 year.

serum may be correlated with tumor aggressiveness.[4] Latent and active forms of gelatinolytic activity, MMP-2 and MMP-9, in the serum were almost higher in masses with malignancy than those in benign tumors and it may be used as a facile method for detecting malignant cutaneous tumors.

In our study, ALP activity in serum were significantly higher in dogs with cutaneous tumors than that in healthy dogs. Significant increase of total ALP has been reported in dogs with breast tumors[24,25] and other neoplasms,[26] but it has never been associated with malignancy and histological types of tumors. Increasing level of total ALP in serum may also indicate localized production of isoenzyme by tumor cells similar to the human's Regan isoenzymes.[24] The measurement of serum ALP is not a specific index in the canine cutaneous tumors, and therefore, measurement of ALP isoenzymes will have a higher diagnostic and prognosis value than ALP. Serum ALP activity was significantly higher in malignant tumors than that in dogs with benign tumors and healthy dogs and the ALP activity was decreased significantly after surgical removal of the masses.

One of the most interesting findings of the present study was that the serum of dog with fibrosarcoma exhibited an active MMP-2 band (66 kDa). Fibrosarcomas showed the most intense bands of MMP-2 and MMP-9 activity in comparison with other diagnosed tumors. This finding may be associated with fibroblasts proliferation and increased production of the active form of MMP-2. Moreover, it has been suggested that serum MMP-9 levels may be used as a tumor marker in patients with sarcoma and may be helpful in the follow-up of patients.[5]

An additional 82 kDa band representing active MMP-9 was detected a month before the recurrence of the perianal gland adenoma three month after surgical removal of the tumor. The higher gelatinase activity in malignant tumors with recurrence has been reported.[4] Thus, gelatinase activity could be a useful marker for predicting tumor metastasis and recurrence. Based on the findings of this study, it is recommended to determine the levels of MMP-2 and MMP-9 proteolytic activity using a modified gelatin zymography at least one month and three months after surgery.

In conclusion, canine spontaneous cutaneous tumors must be completely removed by surgical intervention. In many cases, there are many concerns about the unknown skin masses and whether they will become metastatic or re-emerge in future. Consequently, assessment of gelatinase and ALP activity in serum may be useful for predicting of malignancy and behavior of canine cutaneous tumors. However, more studies are needed about serum activity of MMPs in canine cutaneous tumors.

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Conflict of Interest

The authors state that they have no conflict of interest.

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