Short Communication

The Activity of Plasma Matrix Metalloproteinase-9 as a Marker to Reflect the Tissue Level of Mammary Glands Tumor in Dogs

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Abstract

In order to understand the change of MMP-2 and MMP-9 in dogs with mammary gland tumor (MGT), the activity of MMP-2 and MMP-9 in plasma samples, MGT tissues and near by normal mammary gland tissues were evaluated by gelatin zymography. The levels of MMP-2 and MMP-9 were significantly higher in tumor tissues than those in normal gland tissues ($p<0.05$). Plasma MMP-9 levels in malignant cases were higher than those in benign ($p=0.15$) and non-tumor cases ($p<0.05$). Likewise, plasma MMP-2 levels in malignant MGT cases were higher than non-tumor ($p<0.01$) and benign cases ($p=0.54$). Furthermore, plasma MMP-9 levels were reflected and positively correlated with MMP-9 in tumor tissues ($r=0.78$, $p=0.02$), but not MMP-2. Our results indicated that the MMP-2 and MMP-9 levels are associated with tumor malignancy, and plasma MMP-9 activity can become a potential marker both in follow-up and prognosis of canine MGT patients.

Keywords: dog, mammary tumor, matrix metalloproteinase

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บทคัดย่อ

ค่าพลาสมา Matrix Metalloproteinase-9 ที่ใช้เป็นค่าบ่งชี้ในเนื้อเยื่อเนื้องอกเต้านมสุนัข

Wei-Yau Shia[1] Shih-Ming Liu[1] Chun-Sheng Lee[2] Jian-Liang Lin[2] Chih-Chung Chou[1] Yumi Yuasa[1] Shih-Chieh Chang[1,2] Wei-Ming Lee[1, 2]*

วัตถุประสงค์ของการศึกษา เพื่อเข้าใจการเปลี่ยนแปลงค่า MMP-2 และ MMP-9 ในสุนัขที่ตรวจพบเนื้องอกเต้านม โดยทำการตรวจหาค่า MMP-2 และ MMP-9 ในพลาสมา และเนื้อเยื่อเนื้องอกเต้านม และเนื้อเยื่อปกติข้างเคียง โดยวิธี gelatin zymography พบว่าระดับของค่า MMP-2 และ MMP-9 มีค่าสูงขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับเนื้อเยื่อเต้านมปกติ (p<0.05) ค่าพลาสมา MMP-9 ในกลุ่มมะเร็งเต้านม มีค่าสูงกว่ากลุ่มเนื้องอกเต้านม อย่างมีนัยสำคัญทางสถิติ (p=0.15) และกลุ่มควบคุมปกติ (p<0.05) เช่นเดียวกับค่าพลาสมา MMP-2 ในกลุ่มมะเร็งเต้านม มีค่าสูงกว่ากลุ่มเนื้องอกเต้านมอย่างมีนัยสำคัญทางสถิติ (p=0.54) และกลุ่มควบคุมปกติ (p<0.01) ค่าพลาสมา MMP-9 มีความสัมพันธ์กับค่า MMP-9 ที่ตรวจพบในก้อนเนื้องอก (r=0.78, p=0.02) แต่ค่า MMP-2 ไม่พบความสัมพันธ์ดังกล่าว สรุปผลการศึกษาแสดงถึงค่า MMP-2 และ MMP-9 มีความเกี่ยวข้องกับความรุนแรงของมะเร็งเต้านมในสุนัข และค่าพลาสมา MMP-9 สามารถนำมาใช้ในการพยากรณ์และเฝ้าติดตามโรคเนื้องอกเต้านมในสุนัขได้

คำสำคัญ: สุนัข เนื้องอกเต้านม matrix metalloproteinase

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Introduction

Mammary gland tumor (MGT) is one of the most common neoplasms in female dogs, approximately 40-50% of which are considered malignant (Lee et al., 2004). Tumor recurrence and metastasis are the most important causes of patient death after surgery. The matrix metalloproteinase (MMP) family is thought to be responsible for the accelerated breakdown of extra-cellular matrix (ECM) associated with tumor invasion and metastasis (Kawai et al., 2006).

Matrix metalloproteinases (MMP) are a family of zinc-containing, proteolytic enzymes implicated in the degradation of extracellular matrix (Blavier et al., 2006). These proteolytic enzymes have been implicated in a variety of physiologic and pathological conditions. Under physiologic condition, for example, they are expressed by a variety of cell and tissue types and are involved in processes such as trophoblast invasion (Cross et al., 1994), development (Matrisian and Hogan, 1990), tissue remodeling (Salamonsen et al., 1999; Blavier et al., 2006), ovulation (Russell et al., 1995), angiogenesis (Birkedal-Hansen, 1995), wound healing (Madlener et al., 1998), etc. Meanwhile, these proteolytic enzymes are implicated in the pathologic processes in animals including tumor invasion and metastasis in experimental animals (Sawaya et al., 1998) and humans (Moses et al., 1998; Kleiner and Stetler-Stevenson, 1999). It has been documented that increased MMPs activity are associated with invasion, metastasis and prognosis in human and animal malignancies (Moses et al., 1998; Lana et al., 2000; Hirayama et al., 2002; Loukopoulos et al., 2003; Kawai et al., 2006) such as breast cancer (Talvensaari-Mattila et al., 1998; Dalberg et al., 2000). Recently tissue MMP-2 and -9 were documented in canine tumors and a high level of pro-MMP-9, pro-MMP-2 and active MMP-2 were detected in most canine tumors. In addition, high levels of MMP-9 activity were found in the sera of canines with mammary adenocarcinoma indicating that MMP-9 plays an important role in the progression of a canine mammary tumor and that serum MMP-9 analysis provides as early diagnosis of adenocarcinoma (Yokota et al., 2001). In humans, plasma MMP-9 is a useful marker in the follow-up and in the assessment of prognosis in breast cancer patients (Farias et al., 2000; Ranuncolo et al., 2003). In this study the zymographic band patterns of MMPs from plasma and mammary gland tissues including tumor and nearby normal tissue were measured by Image J software (National Institutes of Health, USA).

Therefore, the aim of this study was to find a potential marker from MMPs to reflect the tissue level of mammary glands tumor in dogs.

Materials and Methods

Animals: Surgically resected tissues from twelve cases of canine mammary gland tumor were obtained at the Veterinary Medical Teaching Hospital, National Chung Hsing University. Six healthy female beagles were used in this study as a control group for the determination of plasma MMP-2 and MMP-9 activity. The animals were used with the owner's consent.

Histopathologic examination: For histological examination, part of the tumor and nearby normal tissues were fixed in 10% formalin and embedded in paraffin. Thin sections were then prepared and stained with hematoxylin & eosin. The biopsy sections were histopathological diagnosed according to the guideline of World Health Organization (Misdrop et al., 1999) as nine malignant tumors including five malignant mixed type mammary tumors, one fibrosarcoma, three adenocarcinomas and three benign mammary tumors.

Tissue specimens: Twelve tumor tissues were clinically classified by TNM system, including two TNM stage II, eight TNM stage III, and two TNM stage IV (Rutteman and Kirpensteijn, 2003). Among the 12 MGT cases, five cases (three TNM stage III and two TNM stage IV) had bilateral enlarged lymph nodes (retropharyngeal and/or inguinal lymph node) and four cases (TNM stage III) had ipsilateral enlarged lymph nodes. Tumor tissues were collected from the inter-zone between the central and margin of the tumor and cut into several 0.3x0.3x0.3 cm size pieces and store in cryogenic freezing tube (Nalgene®, Thermo Fisher Scientific, USA) at -70°C refrigerator. Two hundred mg of each tumor mass were collected and ground in a tissue grinder with 10 ml phosphate buffer saline (PBS, buffer solution. The ground tumor tissues were homogenized at 1,000 revolution for 8 min in a homogenizer（Macro ES Digital Programmable Homogenizer, OMNI, USA）and then ground by hand (Kimbo Kontes, USA) for 5 min. The homogenate was placed into micro-centrifuge tube and centrifuged at 10,500xg for 30 min. The supernatant was collected and kept at -20°C until MMPs determination.

All plasma samples were collected by jugular vein puncture from the 12 cases of canine mammary gland tumor and 6 normal spayed beagle bitches for MMPs determination. Additionally, blood samples from adenocarcinoma and one case of malignant mixed mammary tumor before and after mastectomy were collected to observe the change of plasma MMP-2 and MMP-9.

Gelatin zymography: Gelatin zymography of MMP-2 and MMP-9 from the plasma and tissue was performed as previously described (Gerlach et al., 2005; Souza-Tarla et al., 2005; Gerlach et al., 2007). Briefly, Dual Mini Slab Kit (Model: AE-6450, ATTO, Japan) was used in this study for the electrophoresis. The tissue and plasma samples were subjected to electrophoresis on 8% SDS-PAGE co-polymerized with gelatin (0.2%) as the substrate. 15 µl of homogenized supernatant were loaded in each well. Besides, 2 µl human MMP-2 marker (Chemicon®, CA) and 10 µl human MMP-9 markerZ (R&D®, USA) were loaded in the same gel. After the electrophoresis was complete, the gel was washed by 200 ml 2.5% Triton X-100 for 1 hour, and then washed by DW several times. The gel was incubated in 10 times diluted digestion buffer (15.6 g Tris-HCl and 1.47 g $CaCl_2$ were dissolved in DW, pH=7.8) at 37°C for 19 hours. The gel was then stained with 2% Coomassie Blue R-250 in 50% methanol and 10% acetic acid for 30 min, and destained in 40% methanol and 10% acetic acid, until the active bands became clear.

Measurement of zymographic band patterns: The digestive areas of the gels were photographed. The digested area was measured by Image J (Image Processing and Analysis in Java) software (National Institute of Health, USA). The value depended on the size and brightness of the digestive area.

Statistics: Differences in parameters between tumor and normal tissue were evaluated using the Mann-Whitney U test. Correlation coefficient (r) was calculated between different factors using the Spearman correlation. Values are expressed as mean±SEM. $P<0.05$ was considered statistically significant.

Results and Discussion

The zymographic band pattern of MMP-2 and MMP-9 were presented on the gel. The latent form and active form of MMP-2 were 92 kDa and 86 kDa and of MMP-9 were 68 kDa and 62 kDa, respectively (Fig. 1). There was an increase in both the latent and active form of MMP-9, but only an increase in the potential activity of MMP-2 was found in our study. The difference of the latent form of MMP-2 in

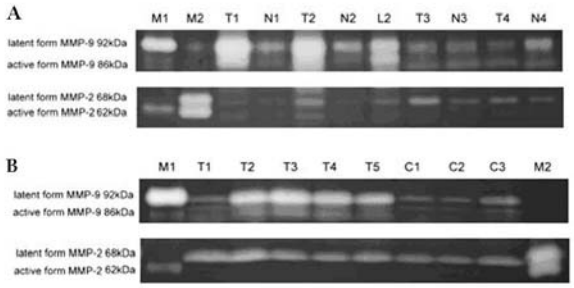

Figure 1 The activity of MMP-2 and MMP-9 in different tissues (A) and plasma (B) in dogs with MGT. Latent form of MMP-2 and MMP-9 were 68 kDa and 92 kDa, respectively. Active form of MMP-2 and MMP-9 were 62 kDa and 86 kDa, respectively. (A) M1: human MMP-9 marker, M2: human MMP-2 marker, T: tumor tissue, N: normal tissue, L: lymph node.

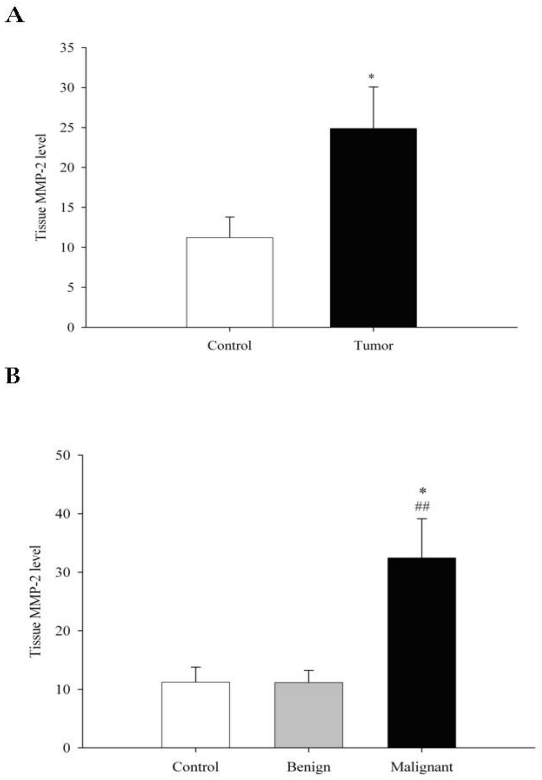

Figure 2 The activity of MMP-2 in tumor tissue and nearby normal mammary gland tissue. (A; tumor tissue MMP-2 was significantly higher than that in normal tissue, B: MMP-2 in the malignant tumor tissue was significantly higher than that in the benign tumor tissue.) * indicate significant difference compared to the control (p<0.05); ## indicated significant difference compared to benign tumor tissue (p<0.01)

the tissue and plasma between the tumor and normal sample was not as much as MMP-9. This could be because MMP-9 synthesized in cell cytosol, the enzyme can be stored in either a latent or an active form, which is contrast to MMP-2, which can be stored only in a latent form (Nguyen et al., 2001). The activity of MMP-2 in the tumor tissue (24.86±5.22) was significantly higher (p<0.05) than that in normal tissue (11.22±2.56) (Fig. 2A). In addition, the activity of MMP-2 in the malignant tumor tissue (32.45±6.67) was significantly higher (p<0.05) than that in the benign tumor tissue (11.14±2.09) (Fig. 2B). The proteolytic activity of the malignant tumor tissues balance shifted to a more enzymatic activity, very likely by increased activation or decreased inhibitor level. The result suggested that malignant tissues had higher proteolytic activities which was in agree with the result of Lee et al. (1996). Similar to MMP-2, the activity of MMP-9 in the tumor tissue (86.17±17.27) was significantly higher (p<0.05) than that in the normal tissue (30.14±5.81) (Fig. 3A). The activity of MMP-9 in the malignant tumor tissue (83.34±16.36) was higher than the benign tumor tissue (63.94±13.14) but without significant difference (Fig. 3B). This might be because of the smaller sample sizes and higher standard deviation between individuals. Referring to the result of the level of MMP-2 and MMP-9 in

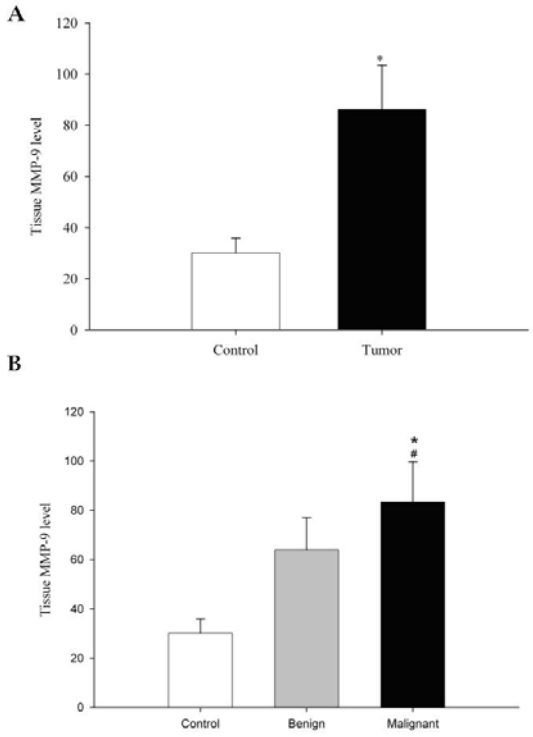

Figure 3 The activity of MMP-9 in tumor tissue and nearby normal mammary gland tissue. (A: MMP-9 in the tumor tissue was significantly higher than that in the normal tissue, B; there was no significant difference of the activity of MMP-9 between the malignant tumor tissue and the benign tumor tissue) * indicate significant difference compared to the control (p<0.05); ## indicated significant difference compared to benign tumor tissue (p<0.05)

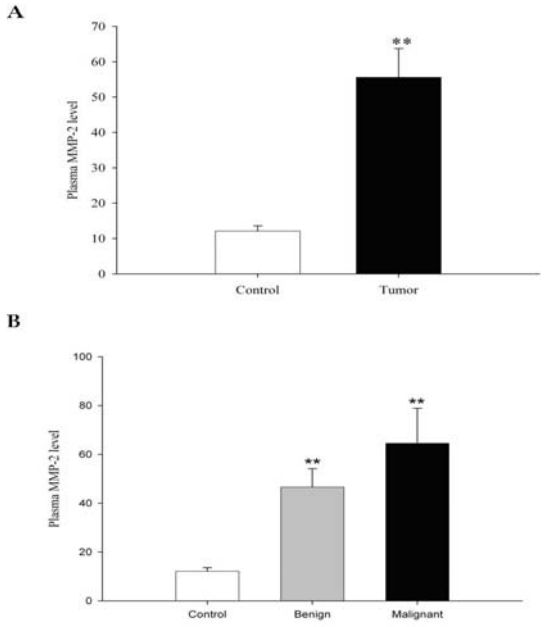

Figure 4 The activity of plasma MMP-2 in dogs with and without MGT. A: plasma MMP-2 was significantly higher than that in control dogs, B: plasma MMP-2 in cases with malignant tumor tissue and with benign MGT were significantly higher than that in control dogs. ** indicated significant difference compared to the control (p<0.01)

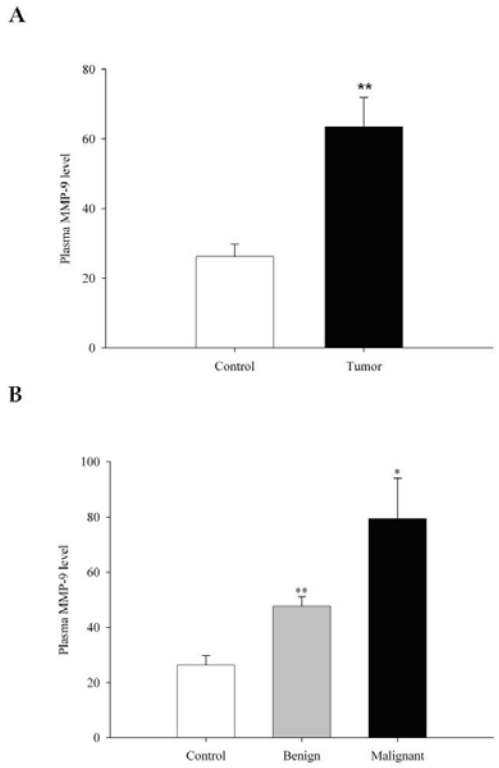

Figure 5 The activity of plasma MMP-9 in dogs with and without MGT. A: plasma MMP-9 in tumor tissue was significantly higher than that in control dogs, B: there was no significant difference in the activity of plasma MMP-9 between cases with malignant tumor and cases with benign tumor. * indicate significant difference compared to the control (p<0.05); ** indicated significant difference compared to the control (p<0.01)

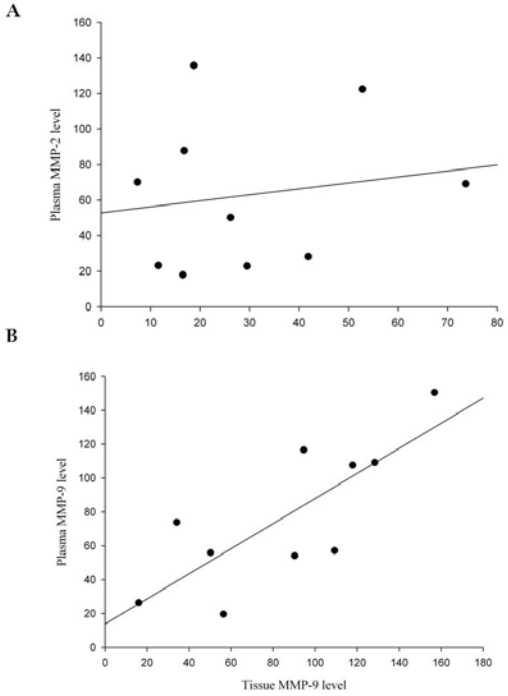

Figure 6 Correlation of MMP-9 between tumor tissues and plasma samples (B, r=0.78, p=0.02), but not in MMP-2 (A, r=0.15, p=0.68).

malignancy of the tumor, the observation was similar to previous studies (Lana et al., 2000; Yokota et al., 2001; Loukopoulos et al., 2003). The mean activity of plasma MMP-2 and MMP-9 in the dogs with MGT (55.58±8.14, 63.55±8.30) were significantly higher (p<0.05) than those in the normal dogs (12.09±1.48, 26.30±3.54), respectively (Fig. 4A and 5A). Likewise, plasma MMP-2 in the dogs with malignant (64.55±14.33) or benign (46.62±7.53) MGT was significantly higher (p<0.05) than that in the control dogs (12.09±1.48). There were no significant differences in activity of plasma MMP-9 between the dogs with malignant MGT (79.34±14.81) and the dogs with benign MGT (47.77±3.37), although the level of plasma MMP-9 in the dogs with benign MGT increased in comparison to the normal dogs. A positive correlation was noticed in MMP-9 between the tumor tissues and the plasma samples (r=0.78, p=0.02), but not in MMP-2 (r=0.15, p=0.68) (Fig. 6). No significant correlation between the tissue and the plasma in MMP-2 was likely to occur because MMP-2 in the benign tumor tissue was did not increase parallel to plasma level. The result might be because MMP-2 antigen levels were not significantly altered in mammary tumor tissue, but active MMP-2 at higher levels in malignant tissue as compared with benign tissue (Hanemaaijer et al., 2000). The result is in accordance with our finding that the malignant tumor tissue MMP-2 was significantly higher than the benign one. The metastasis of tumor is assumed that the primary mechanism by which MMPs promote tumor metastasis is by the degradation of the ECM. Collagen IV, the main component of basement membrane, is thought to be degraded mostly by MMP-2 and MMP-9. These MMPs may therefore play a critical role in the conversion of in situ breast cancers to invasive lesions (Duffy et al., 2000). Additionally, increased MMP activity in tumor local environment results in proteolytic cleavage of membrane-associated extracellular matrix metalloproteinase inducer (EMMPRIN, CD147) releasing soluble EMMPRIN. Soluble EMMPRIN in turn acts in a paracrine fashion on stroma cells that are both adjacent and distant to tumor sites to further stimulate the production of MMPs and additional EMMPRIN, which consequently contributes to tumor angiogenesis, tumor growth, and metastasis (Tang et al., 2004). Besides the regulation of EMMPRIN on MMP-2 and MMP-9, MMP-9 is secreted by stromal cell and inflammatory cells including neutrophils, macrophage and mast cell (Deryugina and Quigley, 2006). In our study, lymphocyte was the prominent inflammatory cell, especially in malignant MGT. Neutrophil was also found in tumor tissue but not correlated with the malignancy of MGT. In addition, inflammatory cytokines were proved to recruit different inflammatory cells, for example, neutrophils recruited by IL-2, eosinophils recruited by IL-4 and IL-5, natural killer cells recruited by IL-12, and macrophages recruited by IFN-γ (Cavallo et al., 1992; Cavallo et al., 1997; Musiani et al., 1997; Di Carlo et al., 1998).

The high levels of MMP-2 and MMP-9 presented in this study indicated the level of tumor

malignancies which may be associated with the angiogenesis and inhibition of immune cell proliferation. In the micro-environment of a tumor, high levels of MMP-2 and MMP-9 are associated with the angiogenesis (Deryugina and Quigley, 2006), an important process in tumor metastasis, growth and tumor cells entering general circulation (Woodhouse et al., 1997). In addition, the expression of vascular endothelial growth factor (VEGF) can be affected by the MMP-9 during angiogenesis (Folgueras et al., 2004). Besides the angiogenesis, MMP-2 and MMP-9 can inhibit the proliferation of T-cells allowing tumor cells to escape immune surveillance (Sheu et al., 2001).

Based on our knowledge there are few publications concerning the relationship between plasma MMP-2 and MMP-9 level and malignancy of tumor. In humans, it has been documented that high plasma MMP-9 levels are present in patients with lung cancer, but no association has been reported with the tumor stage and malignancy (Farias et al., 2000). Nevertheless, the plasma level of MMP-9 has a positive correlation with the status of patients with breast cancer (Ranuncolo et al., 2003). Thus, the implementation of plasma MMP-2 and MMP-9 levels as a useful parameter in correlation with tumor malignancy is warranted. Though high plasma level of MMP-2 and MMP-9 were observed in dogs with malignant MGT in this study, only plasma MMP-9 levels reflected the activity of MMP-9 from MGT tissues. Taken together, plasma MMP-9 analysis is a potential early diagnosis method in the determination of canine MGT progression.

In conclusion, the activity of tissue MMP-2 and MMP-9 was positively correlated with the malignancy of MGT in dogs. The activity of tumor tissue MMP-9 was positively correlated with the activity of plasma MMP-9 in dogs with MGT, but not MMP-2. Although the sample size was small in this preliminary study, plasma MMP-9 level might be suggested as a useful marker in the follow-up and in the assessment of prognosis in dogs with MGT. However, it still needs further investigation.

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