



Detection of matrix metalloproteinase -2 and -9 in various types of canine tumours

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ABSTRACT

A study was carried out to detect the matrix metalloproteinases (MMP-2 and MMP-9) in tissues by gelatin zymography in various types of canine tumours. Fresh canine tissue samples (24) up to 0.5 cm were collected at the time of biopsy, surgery and necropsy. Tissue samples were homogenated, filtered and subjected to gelatin zymography. All the tumours samples were proteolytically active as they fully degraded the gelatin. Except with minor variation, in all tumours, the major bands were observed at 220, 135 and 92 kDa of MMP-9 and 72 kDa of MMP-2 band. Among all the groups, histiocytoma and malignancy tumours showed 3–4 times higher intensity bands than the other groups. This clearly showed that the expression of the active form of MMP-2 and intensity of other bands depends upon the degree and stage of tumour. The latent form of 72 kDa MMP-2 was thicker than that of 92 kDa MMP-9 as in mammary gland tumour and fibroadenoma. In histiocytoma, the expression of MMP-2 and -9 was almost similar. It was concluded that MMP-2 and MMP-9 could be used as a diagnostic tool/marker to ascertain the degree and stage of particular tumour, whether it has reached to the stage of metastasis or not. Further study on the difference between serum MMP and tissue MMP could be carried out to assess the degree of metastasis.

Key words: Canine tumours, Gelatin zymography, Matrix metalloproteinases

The extracellular matrix (ECM), a complex meshwork of protein and carbohydrate polymers, is secreted by the cells of connective tissues. The connections between the ECM, cell-surface integrins and cytoskeletal actin fibers support the conformational changes and migration exploited by normal, reactive and malignant cells (Giancotti *et al.* 1999). Management of the ECM is a specialized function of mesenchymal cells. Modulation of the ECM is important in the development and progression of malignancy, during neo angiogenesis and through the complex processes involved in metastasis (Stetler-Stevenson *et al.* 1990, Nguyen *et al.* 2001).

High-grade soft tissue sarcomas including malignant fibrous histiocytoma (MFH), leiomyosarcoma and undifferentiated sarcoma are aggressive, rapidly growing tumours accounting for 2% of deaths from malignancy. Early metastasis to the lungs is common and 10% of cases have advanced disease at the initial examination. Matrix metalloproteinases are zinc-dependent endopeptidases that are capable of degrading almost all ECM components at collectively. Substrate specificity is determined by the C-terminal domain; however, there is considerable overlap which cautions against attributing specific degradative

functions in *in vivo*. Biological activity is regulated at the level of gene transcription, protein activation and by the presence of specific inhibitors.

Most MMPs are secreted as inactive zymogens activated through the enzymatic cleavage of a propeptide from the zinc-active site by active MMPs and cysteine or serine proteases activated through the enzymatic cleavage of a propeptide from the zinc-active site (Nagase *et al.* 1997, Creemers *et al.* 1998). The main groups, defined according to substrate specificity, are collagenases (MMP-1, MMP-8, MMP-13, and MMP-18) that degrade interstitial collagens, gelatinases (MMP-2 and MMP-9) that degrade basement membrane type-IV collagen, stromelysins (MMP-3, MMP-10 and MMP-11) that primarily degrade fibronectin and laminin, and membrane-type MMPs (MMP-14 through MMP-17) that have a wide range of substrates and localize matrix degradation to the cell surface.

The expression of MMPs in carcinomas varies with the histologic type and stage and as a function of the MMP-9. Increased levels of one or more MMPs particularly MMP-2 have been demonstrated in breast, prostate, ovary, lung, colorectal, gastric, thyroid and liver carcinomas. Levels of activated MMP-2 and MMP-9 correlated with progression or metastasis in many carcinomas. Zymography proved to be a sensitive and quantitative technique for the assessment of MMP presence but has the limitation of requiring fresh tissue (Loukopoulos *et al.* 2003). Hence, the present study was carried out to find out the existence of MMPs in various types of canine tumours through gelatin zymography.

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MATERIALS AND METHODS

Collection of tissue samples: Fresh canine tissue samples (24), up to 0.5 cm in diameter were collected at the time of biopsy, surgery or during necropsy examination. Tissue samples included tumours, tissues immediately surrounding the tumours and normal tissues from a range of organs. Samples were taken from the marginal and central areas of the tumours wherever feasible. Excised tumour lesions were immediately divided into aliquots.

Preparation of tissue: Tissue (0.5 g) was weighed in a clean aluminum foil and added with 2 ml of Lamelli buffer. It was triturated aseptically in mortar and pestle and centrifuged at 6,000 rpm for 10 min at 4°C. The supernatant was collected and it was stored for further analysis.

Preparation of sample: Gelatin zymography was used to detect precursor and active forms of MMP-2 and -9 in fresh and frozen tissues. Tissues were weighed and homogenized and MMPs were extracted from the tissues (Woessner *et al.* 1995, Gilbert *et al.* 1997).

Gelatin zymography: The samples were subjected to modified SDS-PAGE (modification of Laemmli's method 1970) as per Heussen and Dowdle (1980) by the addition of co-polymerizing substrate of gelatin (0.3%) (final concentration 0.15%) to the resolving gel 0.8%. The samples were electrophoresed at 100V for 20 min. Renaturation was carried out with 2.5% Triton X-100 for 3 h on a mechanical shaker with a mild agitation. Then developing was done by incubating the gel in 10 mM CaCl₂, 0.15 M NaCl and 50 mM Tris pH 7.5 for 18 h at 37°C. The gel was stained with 0.25% coomassie brilliant blue for 2 h, followed by destaining with destaining solution for 1 h and finally the gel was washed with distilled water.

Analyzing the results of gelatin zymogram: Human capillary blood gelatinase was used as the standard marker for comparing the zymogram bands as per Makowski and Ramsby (1996). Samples were added with 20× volume of Laemmli buffer and mixed thoroughly. The aliquots were stable for 3 months at -20°C.

RESULTS AND DISCUSSION

All the tissue samples collected from various canine tumours were subjected to gelatin zymography (Fig.1). In

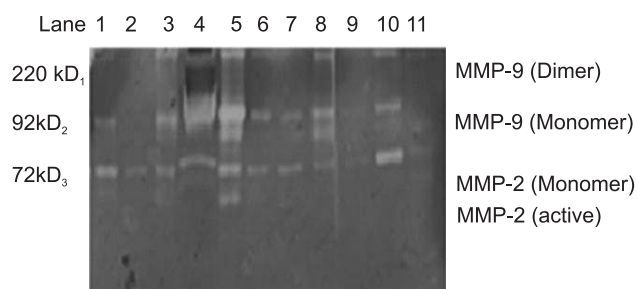


Fig. 1. Gelatin zymography of canine tumours. Lane 5, 7, 10, histiocytoma; lane 1, 6, mixed mammary tumour; lane 2, 9, perianal gland adenoma; lane 3, fibroadenoma; lane 8, malignancy; lane 11, mastocytoma; lane 4, marker.

the mixed mammary tumour (lane 1 and 6), the major bands, 92 kDa of MMP-9 and 72 kDa of MMP-2 were observed. The 220 kDa of MMP-9 was fainter than the other groups and histiocytoma (lane 5). The results were in accordance with results of Loukopoulos *et al.* (2003) and Aresu *et al.* (2011). MMP-2, MMP-9 and MT1-MMP are synthesized by epithelial cancer cell and cancer associated fibroblast plays an important role in malignant canine mammary tumours (Aresu *et al.* 2011).

Loukopoulos *et al.* (2003) observed that MMP-2 (gelatinase) was detected in its precursor (72 kDa) and active forms (66 and 62 kDa) and MMP-9 (gelatinase) was detected in its precursor form (92 kDa) and active form (82 kDa) in tumours collected from canine tumour. Production of each MMP with the exception of Pro-MMP-9 was significantly greater in cartilageneous tumour even when compared with osteosarcomas and fibrosarcomas. Margaret *et al.* (2015) suggested that modulation of MMP activity, particularly endothelial MMP-2 activity is an essential process that supports the requirement for increased substrate supply for an expanding cell population. Inherent in these modifications are the reduction in attachments between lesional cells and the matrix and degradation of interstitial collagen in the peritumoral tissue facilitating the local invasion and metastasis.

In perianal gland adenoma (lane 2 and 9), there was only 1 major band as the latent form of 72 kDa of MMP-2 and there was no band noticed in 200 kDa and a fainter band observed in 92 kDa of MMP-9. The present study results accorded with those of Loukopoulos *et al.* (2003) and Hussaini *et al.* (2007). The intensity of 92 kDa and 72 kDa MMP bands was comparatively lesser than the other groups, especially the groups of histiocytoma and malignancy. Hussaini *et al.* (2007) suggested an increase in the expression level and activity of MMP-9 in invasive adenoma.

Among all the groups, in histiocytoma (lane 5, 7 and 10), the major bands were observed at 220, 92 kDa of MMP-9 and 72, 62 kDa of MMP-2. Lane 5 had a major band at 62 kDa indicating the active form of MMP-2 indicating degree and metastatic stage of tumour. The intensity of active form of MMP-9 is 3-4 times higher than the other tumour group. MMP-2 is explicitly observed in certain stage, degree and severity of tumour (Loukopoulos *et al.* 2003). The 62 kDa form of active MMP-2 was detected only in high grade, p53 positive metastatic malignancies.

In this study, except lane 5, 62 kDa of MMP-2 was not observed, it might be due to severity of metastasis and other tumour might not have reached the stage of metastasis. The results were in accordance with the results of Benassi *et al.* (2003), Loukopoulos *et al.* (2003), Hussaini *et al.* (2007). Yang *et al.* (2014) also suggested that MMP-2, MMP-9, TIMP-1 and TIMP-2 may have important roles in the development and progression of MFH and that the degree of expression of these metalloproteinases and their inhibitors, especially MMP-2 could be useful as a prognostic factor related to metastasis in animals.

Benassi *et al.* (2003) observed that pro-MMP2 and pro-MMP9 were strongly expressed in high-risk cases with histiocytoma. The latent pro-enzyme levels were statistically associated with prognosis. In fibroadenoma (lane 3), the major bands were observed at 220, 92 kDa of MMP-9 and 72 kDa of MMP-2. The intensity of active form of MMP-9 (92 kDa) band was 3–4 times higher than mammary tumour and perianal gland tumour and lesser than histiocytoma and malignant tumour. The results of this study were in accordance with those of Maiti *et al.* (2014), who suggested that matrilysins is the one of the matrix metalloproteinases, which can promote cancer invasion by proteolytic cleavage of the ECM substrates. Matrilysin activates other MMPs such as proMMP-2 and proMMP-9 to facilitate tumour invasion. Matrilysin influences the early stage of tumour genesis.

In mastocytoma (lane 11), only latent form of MMP-2 was expressed and there was no band in active and latent form of MMP-9. This could be due to the influence of different tumours and their stage. The expression of MMP could be attributed to define the degree and stage of tumour. The results were in accordance with those of Fang *et al.* (2015), who suggested that mastocytoma gelatinase is a member of 92 kDa gelatinase/gelatin B/MMP-9 family. These features include electrophoretic mobility similar to that of 92 kDa gelatinase. Mastocytoma cells secrete a 92-kD gelatinase, which is activated outside of the cell by chymase. Mastocytoma 92-kD gelatinase is the second MMP found to be directly cleaved and activatable by chymase. The novel demonstration of extracellular activation of 92-kDa progelatinase by chymase suggested that cells can use secondary proteases to activate MMP proenzymes not only in the cytosol and on the cell membrane surface, but also in the immediate extracellular milieu. In malignant case, active and latent form of MMP-9 (200, 92 kDa) and latent form of 72 kDa MMP-2 was observed.

Loukopoulos *et al.* (2003) suggested that metastatic malignancies (3 of 7) produced the 62-kDa form of active MMP-2. All metastatic osteosarcomas produced active MMP-2 (66 or 62 kDa). Metastatic malignancies produced on average approximately 5 times higher levels of proMMP-9 and 1.6 times higher levels of proMMP-2 compared with non-metastatic malignancies. On the basis of this study, it was clear that MMP-2 and MMP-9 concentrations increased in a wide range of canine tumours and it appears that MMPs are particularly involved in one or more stages of the pathogenesis or clinical advancement. MMPs are responsible for degradation of extracellular matrix proteins thought to be important for metastasis of tumour cells. It was concluded that MMP-2 and MMP-9 could be used as a diagnostic tool/marker to ascertain the degree and stage of tumour particular whether it has reached to the stage of metastasis or not. Further, studies should be carried out to find out the difference between serum MMP and tissue MMP and also to find out which method is more reliable to find out the degree of metastasis.

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