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Immunohistochemical evaluation of MMP-2 and TIMP-2 in canine mammary tumours: A survival study

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ABSTRACT

Canine mammary tumours (CMTs) are a very heterogeneous group of neoplasms with variable prognosis. Their aggressiveness is mainly due to their ability to invade locally and to metastasize. The degradation of extracellular matrix components is an important determinant of the invasive phenotype. The aims of this study were to analyse by immunohistochemistry and double immunofluorescence the expression of metalloproteinase 2 (MMP-2) and tissue inhibitor of metalloproteinase 2 (TIMP-2) in eight normal canine mammary glands and 118 CMTs (24 benign, 94 malignant) and to investigate relationships with metastatic disease and survival.

MMP-2 and TIMP-2 expression was higher in malignant tumours than in normal canine mammary tissue and benign tumours. The main difference between benign and malignant CMTs was the pattern of expression of both molecules: benign tumours presented TIMP-2 and MMP-2 immunoreactivity in the myoepithelial cells lining the basement membrane of tubuloalveolar structures, while malignant tumours showed mainly diffuse expression in neoplastic cells. In malignant tumours, increased TIMP-2 expression was significantly associated with the development of distant metastases, lower overall survival and lower disease-free survival. MMP-2 expression was not significantly associated to any of these parameters. These results suggest that the immunohistochemical expression of TIMP-2 is a useful prognostic factor in CMTs.

Introduction

Tumour metastasis involves a multi-step process that includes disruption of the interstitial connective tissue and basement membranes (BM) allowing tumour cells to escape from the primary tumour and to invade surrounding tissues (Hojilla et al., 2008). Matrix metalloproteinases (MMPs) are among the most important proteolytic enzymes implicated in the degradation of various extracellular matrix (ECM) components (Pupa et al., 2002).

MMPs are a family of zinc-dependent endopeptidases (Köhrmann et al., 2009), that are secreted as inactive zymogens (pro-MMPs) and that require extracellular activation by other MMPs (such as membrane-type MMPs) and serine proteases (Toth et al., 2000). According to their substrate specificity, MMPs are divided into several subfamilies, namely, collagenases, gelatinases, stromalysins, matrilysins, membrane-type MMPs, elastases and others (Somerville et al., 2003). Many human breast cancer investigators have focused on the gelatinases (MMP-2 and MMP-9), since they are able to degrade type IV collagen, an important component of BM (Köhrmann et al., 2009).

Evidence has recently emerged showing that the role of MMPs goes beyond digesting ECM components (Somerville et al., 2003). It is believed that MMPs influence tumour behaviour through their ability to cleave (and activate) growth factors, cell surface receptors, cell adhesion molecules and pro-apoptotic factors, thereby promoting tumour cell growth, migration and dissemination (Köhrmann et al., 2009).

The expression and activity of MMPs are tightly regulated at multiple levels, including gene transcription, pro-enzyme activation and the action of specific physiological inhibitors, known as the tissue inhibitors of metalloproteinases (TIMPs) (Somerville et al., 2003; Peterson et al., 2009). The TIMP family comprises four distinct members: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Lambert et al., 2004). TIMP-2 plays a dual paradoxical function in regulating MMP-2 activity (Duffy et al., 2000; Lambert et al., 2004), and whereas TIMP-2 is an endogenous inhibitor of MMP-2, it is also involved in pro-MMP-2 activation (Toth et al., 2000).

CMTs have a very heterogeneous behaviour and histological evidence of malignancy does not invariably imply a malignant

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clinical course (Lana et al., 2007). Therefore, it is important to search for prognostic factors other than histopathology to predict individual tumour behaviour. Currently, there are no studies regarding the possible associations of MMP-2 and TIMP-2 expression and clinical outcome. Hence, the aims of this study were to evaluate the expression of MMP-2 and TIMP-2 by immunohistochemistry in benign and malignant CMTs and to investigate association between their expression and clinical outcome.

Materials and methods

Specimens

Tumours ($n = 118$) were surgically removed from 118 female dogs, aged 5–16 years. Animals with malignant mammary tumours (MMTs) were enrolled in a 2-year post-operative follow-up study with no adjuvant therapy. Samples of normal mammary tissue were obtained from eight bitches that were humanely euthanased as part of the national stray dog control programme.

All specimens were fixed in 10% neutral buffered formalin for 48 h. Tumours $\leqslant$1 cm were paraffin-embedded in one block, while larger tumours were cut sequentially at 5 mm intervals to provide a series of tissue blocks representative of the entire lesion. After dehydration and embedding in paraffin, 3 μm sections were cut from each block. One section was stained with haematoxylin and eosin (HE) for diagnostic purposes and two representative blocks from tumours >1 cm were selected for immunohistochemical studies. Tumours were evaluated independently by two observers (F.G. and I.A.) according to the criteria of the World Health Organization (WHO) for the histological classification of mammary tumours of domestic animals (Misdorp et al., 1999).

MMP-2 and TIMP-2 immunohistochemistry

Selected tumour sections adjacent to those used for HE staining were analysed by immunohistochemistry (IHC) using a polymer based system (Novocastra Novo-Link Max Polymer Detection System) according to the manufacturer's instructions. Sections (3 μm) were de-waxed in xylene and rehydrated in graded alcohols. Antigen retrieval was performed by immersion in citrate buffer (10 mM, pH 6.0) for 30 min at 100 °C in a water bath. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Slides were incubated overnight at 4 °C in a wet chamber with the anti-MMP-2 mouse monoclonal (clone A-Gel VC2, NeoMarkers) and anti-TIMP-2 rabbit polyclonal (H-140, Santa Cruz Biotechnology) antibodies, diluted 1:75 and 1:40, respectively, in Tris-buffered saline (TBS) solution with 5% bovine serum albumin (BSA). Colour was developed with a solution of 3,3′-diaminobenzidine for 2 min at room temperature and the sections were counterstained with haematoxylin.

For the negative controls, the anti-MMP-2 antibody was replaced with mouse IgG and the anti-TIMP-2 antibody with non-immune rabbit immunoglobulin. Negative controls did not show any staining. Positive controls consisted of sections of human breast cancer known to express MMP-2 and TIMP-2 and of normal canine mammary tissue (Papparella et al., 1997). The epidermis was used as internal positive control. Positive controls showed diffuse granular cytoplasmic labelling with strong intensity. Consulting GenBank indicated that canine MMP-2 and TIMP-2 proteins are conserved among several species including human and dog. A BLAST analysis revealed strong homologies (>95%) between human and dog proteins as previously reported (Lana et al., 2000; Hirayama et al., 2002). Therefore, the antibodies were considered specifically recognizing as canine MMP-2 and TIMP-2.

The evaluation of MMP-2 and TIMP-2 expression was semi-quantitative and based on the percentage of positively stained cancer cells (<10%; 10–25%; 25–50%; >50%) and staining intensity (weak; moderate; strong). The level of expression was considered low when tumours showed <25% of positive tumour cells with strong intensity, <50% with moderate intensity, or any percentage of cells with weak intensity. Tumours were classified as demonstrating high level of TIMP-2 and MMP-2 expression when they had >25% of strongly labelled cancer cells or >50% of cells with moderate labelling intensity. Tumours were also described according to the pattern of expression of both enzymes: basal expression (when the immunoreactivity was observed in normal myoepithelial cells lining BM) vs. diffuse expression in neoplastic cells. Slides were examined independently by two observers (A.S. and A.M.) and when there was disagreement (<5% of the slides), a consensus was obtained using a multi-head microscope.

MMP-2 and TIMP-2 double-labelling immunofluorescence (DIF)

Six benign and 13 malignant CMTs were also analysed by DIF. Sections (3 μm) were de-waxed in xylene and rehydrated in graded alcohols. Antigen retrieval was performed for both antibodies by immersion in boiling citrate buffer (30 min in water bath at 100 °C). Slides were then incubated for 20 min at room temperature with monkey non-immune serum (Invitrogen) at a 1:5 dilution in phosphate buffered saline (PBS) containing 1% BSA. Slides were then incubated overnight at 4 °C in a wet chamber with the first primary antibody, mouse anti-MMP-2 (diluted 1:15 in PBS with 1% BSA). After washing in PBS, slides were incubated with Alexa Fluor 568 donkey anti-mouse IgG (diluted 1:100 in PBS with 1% BSA; Invitrogen) for 60 min. Sections were washed three times in PBS and blocked with non-immune goat serum (diluted 1:5 in PBS with 1% BSA) for 20 min at room temperature. After incubation with the anti-TIMP-2 antibody (diluted 1:10 in PBS with 1% BSA; overnight at 4 °C in a wet chamber), slides were washed in PBS and then incubated for 60 min at room temperature with FITC-conjugated goat anti-rabbit IgG (diluted 1:100 in PBS with 1% BSA; Southern Biotech). Finally, slides were rinsed in PBS and mounted in Vectashield with DAPI (Vector).

Immunostained tissue sections were examined under a Laser Scanning Confocal Microscope (Olympus FV1000/IX81) with appropriate filters. After sequential scanning to assure the absence of cross-talk between channels, separate images for Alexa Fluor 568 and FITC were captured at a 400× magnification and then were merged to allow the visualization of MMP-2–TIMP-2 double immunostaining.

Follow-up study

All animals were examined prior to surgery, 3 weeks after surgery and every 3 months subsequently for a 2-year period. Each examination consisted of a complete physical examination, thoracic radiographs (three views) and complete abdominal ultrasound. Whenever necessary, additional examinations (e.g. aspiration cytology, excisional biopsy, skeletal radiography, etc.) were performed with the owner's consent. Complete necropsies were performed on all dogs that died spontaneously or were euthanased. Suspected metastases were confirmed histologically.

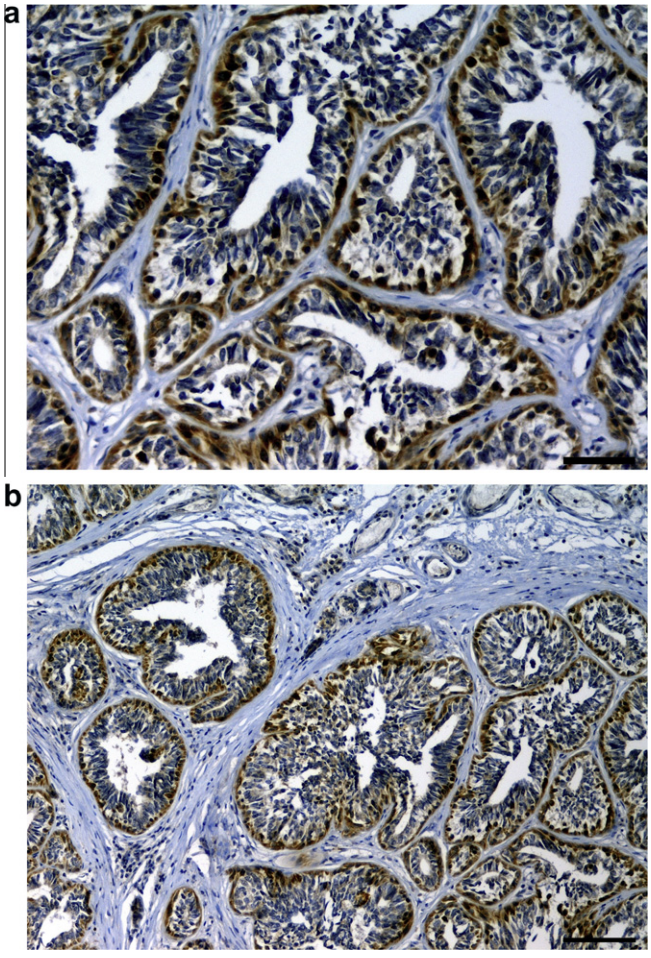

Fig. 1. Canine benign mixed mammary tumour. Strong MMP-2 (a) and TIMP-2 (b) immunoreactivity in myoepithelial cells lining the BM of tubuloalveolar structures. IHC, bar 50 μm (a) and 100 μm (b).