IMMUNOHISTOCHEMICAL AND QUANTITATIVE REAL TIME PCR EXPRESSION OF MATRIX METALLOPROTEINASES (MMPs) IN CANINE MAMMARY TUMOURS

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Abstract: Expression of matrix metalloproteinases (MMP2) was evaluated by immunohistochemistry and quantitative Real time PCR. All the 19 malignant canine mammary tumours expressed MMP2 immunohistochemically as diffuse and granular reaction, which was restricted to cytoplasm of neoplastic epithelial cells, endothelial cells, fibroblasts, inflammatory cells especially macrophages and faintly in myoepithelial cells. The present study observed 16 malignant tumours that over expressed and three tumours that under expressed the MMP2. qRT PCR revealed an expression of MMP2 in all canine mammary tumours but expression was highly varied within tumour types and indicated that the tumours were in various stages in their progression towards attainment of malignancy with invasive and metastatic characteristics. Results of qRT PCR accorded well with the immunohistochemical results obtained with anti-human MMP2 monoclonal antibody (Clone K-20).

Key word: Metalloproteinases, Canine mammary tumors

INTRODUCTION

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes, which are implicated in the degradation and remodeling of extra cellular matrix and in vascularization [1]. Furthermore, the MMPs are also known to be involved in tumour progression and their increased concentrations in cancer tissues have been associated with invasion, metastasis and poor prognosis in numerous human malignancies including breast cancer [2]. Tumor derived MMP2 has been related with extracellular matrix cleavage and matrix adhesion promotion in the initial steps of tumorigenesis. In canine tumours, invasion and metastasis of tumour cells to the surrounding tissues and other organs frequently occur. Reports of expression of MMPs in the canine tumours are few in number but include mammary tumours and osteosarcomas, respectively [3,4]. The present study was undertaken to evaluate the expression of MMPs in canine mammary tumours by immunohistochemical and quantitative real time PCR methods.

MATERIALS AND METHODS

Collection of samples: Twenty surgically excised spontaneous mammary tumors were collected from Department of Veterinary Surgery and Radiology, Veterinary College, Bangalore. From the excised tumor tissue, representative samples were collected from multiple sites for histopathological examination in 10% Normal buffered formalin (NBF) were processed by routine paraffin embedding technique. Sections of 4 µm thickness were taken using microm
rotary microtome and were stained with haematoxylin and eosin [5]. Tissues were also collected in TRIzol® (Invitrogen) for RNA extraction and qRT-PCR.

**Histopathology and histologic staging of tumours:** Canine mammary gland tumours were classified based on diagnostic criteria proposed by World Health Organization [6] and accordingly histological staging was done [7].

**Immunohistochemistry:** Paraffin tissue sections were mounted on 3-aminopropyltriethoxy-silane (APES) coated slides, dried at 37°C for three hours and deparaffinized using xylene and rehydrated using descending grades of ethanol. Endogenous peroxidase was blocked by covering the whole section with 3% hydrogen peroxide in methanol (100µl), incubated at room temperature for fifteen minutes and later, washed thrice in 0.01M PBS. Heat induced epitope retrieval (HIER) was carried out by immersing tissue sections in a pressure cooker containing citrate buffer (pH 6.0), allowed to get cooked for 2 minutes after maximum pressure was attained, sections were let to cool down in room temperature for approximately 30 minutes and later, washed in three changes of PBS. MMP2 antibody (Clone-K-20, raised against an epitope sequence between amino acids 600-650 of MMP2 of human origin procured from Santa Cruz Biotechnology, Inc. USA., Cat No: sc-8835) were added to cover the sections at a dilution rate of 1:50, subsequently the sections were incubated at 37°C in humidified chamber for two hours and washed with PBS. The sections were covered with HRPO conjugate and incubated at 37°C in humidified chamber for one hour. After incubation, sections were washed with PBS as mentioned earlier. Freshly prepared DAB with 3% per cent H₂O₂ was poured to cover the whole sections, washed and counter stained with Harris haematoxylin.

**Quantitative real time polymerase chain reaction (qRT PCR):** Complementary DNA was prepared for RNA segments encoding MMP2 canines using gene specific primers (Forward - TTCAGGCT and reverse - GCCCTCTTTGAGAC-AGTTTCC, GeneBank No: AF177217.1). Real-time PCR amplification reaction was carried out as per the protocol [8]. It was per-formed in a 25µl reaction mixture containing 12.5 µl SYBR green mastermix (Fermentas®), primers - 5 pmol each, ROX solution (0.25µl), cDNAs (1µl) and nuclease free water (9.25µl) and each sample was measured in duplicate. Amplification was followed by denaturation (95°C/15 secs), annealing and extension (60°C for and 1 min) for 40 cycles each. The standard curve was constructed using duplicate dilutions of RT-PCR products. The design of the primers and the thermocycling conditions ensured that no genomic DNA was amplified and detected during this reaction. The data were normalized with respect to β-actin (Forward- TCC-ATAATGAAGTGTGAT- and reverse- GGACCTGACTCGTCATACTC) mRNA levels. The relative quantity of each mRNA was calibrated with the amounts in control. The relative quantity was calculated by the 2-ΔΔCt method [9].

**Statistical analysis:** Statistical analysis was performed using the statistical software GraphPad Prism®, Version 5. Correlation was considered significant for 2 tailed p-value d” 0.05.

**RESULTS AND DISCUSSIONS**

**Histopathology:** Histopathological classification was undertaken (Table 1) according to the WHO classification of canine mammary tumours [6] and observed that 18 cases were malignant epithelial tumours and they were (a). Papillary adenocarcinoma simple type, (b). Papillary adenocarcinoma complex type, (c). Tubulopapillary adenocarcinoma, (d). Adeno squamous carcinoma and (e). Myoepithelioma. Further, one each of fibrosarcoma and non neoplastic hyperplasia were also observed.

**Immunohistochemistry:** In the current study, MMP2 expression was observed in all the 19 malignant mammary tumours (Table 2). The MMP2

**Explanation of figures:**

**Fig. 1:** Picture of papillary adenocarcinoma of canine mammary gland showing MMP2 positive reaction (3+) in epithelial cells of papillary structure involving 50-75 % of neoplastic cells IHC X 200. **Fig. 2:** Picture of papillary adenocarcinoma of canine mammary gland showing MMP2 positive reaction (3+) in epithelial cells of papillary structure involving 50-75 % of neoplastic cells IHC X 200. **Fig. 3:** Picture showing MMP2 positive reaction in macrophages. IHC X 1000. **Fig. 4:** Picture showing intense reaction to MMP2 involving squamous component of adenosquamous carcinoma of canine mammary gland. IHC X1000.
expression was observed as diffuse and granular brownish reaction restricted to the cytoplasm of neoplastic epithelial cells and also in endothelial cells, fibroblasts, inflammatory cells especially macrophages and faintly in myoepithelial cells. Intense staining of MMP2 in fibroblast was observed according to Livak and Schmittgen, [10] who reported that the staining intensity in the fibroblast was almost equivalent to that in tumour epithelial cells and the expression was significantly higher in fibroblasts of carcinoma than benign neoplasms.

In the present study, the expression of MMP2 was graded as overexpression with a score of 2+ and 3+ and as under expression with a score of 1+. A total of 16 cases revealed an overexpression and three underexpression. A total of 16 cases revealed an overexpression and three underexpression. In papillary adenocarcinoma complex type, MMP2 expression was observed in tumour cells lining papillary structures, cystic ducts and more prominently in neoplastic cells invading the stroma. Fraga et al. [9] also reported a prominent expression at the edge of the tumours where invasion of stroma was observed. In the current study, other tumour types such as papillary adenocarcinoma simple type and tubulopapillary adenocarcinoma also revealed MMP2 expression, which did not much variations. Similar observations have also been made by Loukopoulos et al.[2], who reported an absence of correlation between MMPs expression and tumour types or tumour grades. In one case of adenosquamous carcinoma, in the current study, only a mild reaction was observed with intense staining of keratin material. Keratinocytes have been shown to be highly immunoreactive for MMPs not only in tumours but also in normal tissues [2]. In fibrosarcoma in the present study, the MMP2 expression was intense and was over expressed in neoplastic fibroblasts. It was observed that MMP2 and MMP9 expression were greater in malignant neoplasms than benign and in sarcomas than carcinomas with intense expression in fibroblasts [2,10].

**Real time PCR:** In the current study, the MMP2 expression in canine mammary gland tumour by real time PCR revealed, expression of MMP2 in all the malignant tumours, which ranged from 0.2 to 5,24,197.2 folds higher in comparison with that of control mammary tissue. In the present study, the MMP2 expression highly varied between as well as within tumour types. In papillary adenocarcinoma complex type, the MMP2 expression ranged from 5.4 to 8077.8 folds. The variation in the expression indicated that the tumours were in various stages in their progression towards attainment of malignancy with invasive and metastatic characteristics as MMPs (MMP2 and MMP9) are involved in accelerating extra cellular matrix (ECM) breakdown that facilitate tumour invasion and metastasis [2,4,12,13,14].

In the current study, though histologically all the tumours were identified as malignant types with local invasion and some with metastasis, the MMP2 expression varied highly with some tumours expressing lesser mRNA fold difference values. As reported by several workers, the tumour progression is controlled by several factors including tissue inhibitors of metallo proteinases (TIMPs), which regulate the activity of MMPs. These TIMPs (TIMP-1 & TIMP-2) preferentially bind to MMPs (MMP9 and MMP2 respectively) to exert their inhibitory function and any unbalanced activity of MMPs and TIMPs with more of MMPs contributed to tumour progression [10,15]. In addition, each one of these pro- and inhibitory factors is reported to be involved in different stages and processes during tumour progression [15]. Involvement of MMP2 in tumour invasion and metastasis was rightly proved in the current study, where in the only two mammary

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**Table 1: Histological classification of induced rat mammary tumour (n=20)**

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Histological type</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Papillary adenocarcinoma (PAC)</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Papillary adenocarcinoma predominantly cribriform</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Papillary adenocarcinoma predominantly comedo</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Tubular adenocarcinoma</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Fibrosarcoma</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Fibroadenoma</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2: Immunohistochemical expression score of MMP2 in induced rat mammary tumours**

<table>
<thead>
<tr>
<th>Scoring</th>
<th>MMP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3+</td>
<td>3</td>
</tr>
<tr>
<td>2+</td>
<td>13</td>
</tr>
<tr>
<td>1+</td>
<td>4</td>
</tr>
</tbody>
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**Table 3: Details of C\_t values & 2^{-\delta\delta C\_t} (Fold Difference) values of MMP2 gene mRNA expression in induced rat mammary tumours by real time PCR**

<table>
<thead>
<tr>
<th>Tumours</th>
<th>MMP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.06</td>
</tr>
<tr>
<td>House</td>
<td>22.54</td>
</tr>
<tr>
<td>CT</td>
<td>2-6\delta C_t</td>
</tr>
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By Livak and Schmittgen, [10] who reported that the staining intensity in the fibroblast was almost equivalent to that in tumour epithelial cells and the expression was significantly higher in fibroblasts of carcinoma than benign neoplasms.
tumours, adenosquamous carcinoma and tubulopapillary adenocarcinoma, which showed metastasis revealed a very high expression of MMP2 mRNA by 2,75,702 to 5,24,197.2 fold difference.

In the present study, one case of fibrosarcoma, which was encountered, revealed MMP2 mRNA expression by 123.7 fold difference than the control tissue. The expression was comparatively less in comparison with other tumour types such as adenocarcinoma, though immunohistochemically fibroblasts

CONCLUSION

Tumour metastasis and progression are essential for tumour growth and is regulated by matrix metalloproteinases (MMPs) which play important roles in paving way for angiogenesis. Techniques like IHC and qRT PCR are reliable tools to measure expression of MMP2. Higher expression of MMP2 is recorded in malignant tumours compared to benign and non-neoplastic condition.

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REFERENCES