

Expression of MMP-10 in Human Transitional-Cell Carcinoma of the Bladder

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Introduction

In order that malignant tumors can grow, spread and metastasise they must possess the potential to degrade and remodel both the extracellular matrix (ECM) and basement membranes, promote an angiogenic response, evade immune surveillance and avoid elimination¹. Multiple proteolytic enzymes have been demonstrated to be involved in these processes, most prominent among which are the matrix metalloproteinases (MMPs)^{1,2}. The MMPs are a family of at least 24 structurally related zinc-dependent endopeptidases central to tumor invasion, metastasis, angiogenesis and malignant cell proliferation^{1,3,4}. Most MMPs, with the exception of the membrane type MMPs (MT-MMPs), are secreted as inactive zymogens (proMMPs) which must be subsequently activated extracellularly to generate catalytically active MMP^{1,5,6}.

Compared to other MMPs, MMP-10 has a relatively broad substrate specificity which, in addition to activation of proMMPs -1, -7, -8, -9 and -13^{5,6}, includes several ECM proteins^{1,7,8}. Overexpression of MMP-10 has been demonstrated in several human tumors of epithelial origin including lung, head and neck, oesophageal and oral squamous cell carcinoma and squamous and basal cell carcinomas of the skin⁹⁻¹³. Unlike several other MMPs which are localised predominantly in tumor stroma^{13,14}, tumor cells themselves appear to express MMP-10^{6,9,13,15}. The expression profile of MMP-10 in several tumor types and localization of MMP-10 predominantly to tumor cells coupled with a broad substrate specificity strongly supports MMP-10 as a valid target for therapeutic intervention^{7-9,11-13}.

Bladder cancer is the fourth most common cancer in men, with an incidence of 7,880 new diagnoses in the UK in 2000 and a mortality of 3,230 in 2002^{16,17}. Transitional cell carcinoma (TCC) accounts for approximately 90% of all bladder

cancers and is classified as either a superficial (T_a and T₁) or muscle invasive (\geq T₂) stage. Although superficial TCC generally has a good prognosis compared to muscle invasive disease, approximately 70% of superficial tumors recur, with about 20% of these recurring as aggressive invasive disease¹⁶. A better understanding of the molecular pathology of TCC and prediction of tumor behaviour are central to improving diagnosis, clinical surveillance and treatment.

Several studies have addressed the expression of MMPs in TCC with the aim of ascertaining their role in tumor progression, value as prognostic markers and potential as therapeutic targets. Elevated levels of MMP-2, -9, -11 and -13 have all been reported in invasive compared to superficial TCC¹⁸⁻²⁰. MMP-2 and -9 are detectable in urine^{21,22} and are associated with tumor stage and a poor prognosis^{23,24}. On the basis of findings so far, the involvement and roles of individual MMPs in TCC of the bladder seem to be complex and unclear. In the present study, we investigated the expression of MMP-10 by immunohistochemistry using tissue microarrays (TMAs) to determine any correlation between MMP-10 and clinicopathological factors and assess the potential of MMP-10 as a therapeutic target for human bladder cancer.

MATERIALS AND METHODS

Human tissues

A total of 60 formalin-fixed, paraffin-embedded specimens of human bladder transitional cell carcinoma (TCC) were used for this study. The study also includes 10 histologically normal human bladder tissue specimens, excised from patients with early stage TCC but distant to the tumor mass. Tumors were representative of all grades (11 Grade 1; 32 Grade 2; 17 Grade 3) of both superficial (20 pT_a; 18 pT₁) and

muscle-invasive ($22 \geq pT_2$) stages of human bladder TCC. 52 of the tumor blocks were used as tissue microarrays (TMAs). All normal human bladder blocks ($n = 10$) and 20 tumor blocks, inclusive of 12 blocks also used for TMA construction, were used for conventional immunohistochemistry. All experiments were performed after first obtaining consent from the local research and ethics committee according to Medical Research Council regulations. Patient details were anonymised to ensure confidentiality.

Cell culture and protein isolation

The human bladder tumor cell lines EJ138 and RT112 (National Cancer Institute, Frederick, MD) were cultured in RPMI supplemented with 10% foetal bovine serum and L-glutamine (2mM). Before confluence, cells were lysed in RIPA buffer, sonicated (3 x 15 s, 4°C) and centrifuged at 10,000 x g. Protein concentrations of the resultant supernatants were determined using the BioRad protein assay (BioRad, UK).

SDS-PAGE and western blotting

Cell lysates (30 μ g protein per lane), recombinant MMP-3 (100 ng; R&D systems, UK) and recombinant MMP-10 (100 ng; R&D systems) were separated on SDS-polyacrylamide gels (10%) and proteins transferred to polyvinylidenedifluoride (PVDF) membrane (Hybond-P; Amersham, UK). Non-specific protein binding was blocked (2% ECL advanced blocking reagent; Amersham), blots were incubated with a monoclonal antibody, raised against human MMP-10 (5E4, Novocastra, UK; diluted 1:1000) at 4°C overnight. Immunoreactive bands were localised using an HRP-conjugated secondary antibody and visualised using an enhanced chemiluminescence (ECL) detection kit (Amersham). For antigenic adsorption, the primary antibody was

incubated with a two-time excess of recombinant MMP-10 protein (R&D systems, UK) overnight at 4°C prior to being incubated with the blot.

Tissue microarray construction

TMAAs were constructed from paraffin embedded blocks to represent the various grades (G1-G3) and the various stages (pTa, pT₁, pT₂) of human bladder TCC. TMA construction was achieved using a Beecher Instruments microarrayer (Silver Spring, MD, USA) using a modified method of that by Bubendorf et al.²⁵. Briefly, cylindrical cores (600 µM) were punch-biopsied from representative areas of each donor block and transferred into a recipient block. Tissue sampling used four cores from each tumor block to provide representative data on each parent block²⁶. A total of 108 core samples representing 26 patients were included per TMA block, of which two were constructed (total of 52 patients). Sections, 5 µM thick, were cut from the TMA blocks and mounted using a tape transfer system (Instrumedics, USA). H&E staining for verification of histology and sample integrity was performed on the first and every subsequent tenth TMA section. TMA slides were then subject to immunohistochemical analyses.

Immunohistochemistry

Localization of MMP-10 was assessed by immunohistochemistry of human bladder TCC and histologically normal bladder tissue, as previously described in our laboratory⁹. Following inhibition of non-specific antibody binding, sections were incubated for 90 min at room temperature with primary antibody (5E4; diluted 1:50). Controls were performed using normal mouse IgG instead of primary antibody. Immunoreactivity was detected using an anti-mouse biotinylated secondary antibody

(Vector Laboratories, USA; diluted 1:200), followed by amplification and detection using a Vectastain ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (DAB). Sections were then counterstained with Harris' hematoxylin and mounted in DPX mountant (Sigma, UK).

Semi-quantitative analysis of immunohistochemistry

Positive immunostaining was scored semi-quantitatively by two independent observers. Each TMA core was scored for intensity and extent of MMP-10 in the epithelial compartment on a scale from 0 (no staining) to 4 (maximal staining). An average score was then calculated for each tumor of the TMA. Whole sections were analysed in the same manner and assigned a score using the same scale. In cases where both TMA and whole sections were available (n=12), average expression in the TMA was compared to that of whole sections. In all cases, MMP-10 expression was compared for any relationships to clinicopathological parameters.

Statistical analysis

Statistical analyses were performed using SPSS statistical software. Because expression of MMP-10 is not normally distributed, the average values for each group of patients were reported as medians with interquartile ranges. Statistical analyses were performed using non-parametric tests (Mann-Whitney *U*-test and Kruskal-Wallis). Values of P less than 0.05 in two-tailed analyses were considered significant.

RESULTS

Specificity of antibody against MMP-10

Using western blotting, the antibody detected bands at approximately 52 kD (pro-MMP-10) and 44 kD (active MMP-10) with recombinant MMP-10. No bands were detected with recombinant MMP-3, the closest structural homologue to MMP-10 (Figure 1A). Antibody specificity and suitability for use in immunohistochemistry was provided by the detection of both pro- and active-MMP-10 in the EJ138 and RT112 human bladder cell lines (Figure 1A). Antigenic adsorption of this antibody using recombinant MMP-10 protein considerably diminished detection of MMP-10 by western blotting (data not shown), further proving the MMP-10 specificity of this antibody.

Expression of MMP-10 in bladder cancer

Immunohistochemical analyses were used to evaluate the expression of MMP-10 protein in human bladder TCC. Studies using TMAs had been shown previously to be highly representative of results obtained from whole histological sections²⁷. This observation was also mirrored in our study, with a very close agreement of expression in the 12 specimens analysed by both TMA and as whole sections (shown in figure 1 B-G). MMP-10 was present in all superficial (pTa, pT₁; n=38) tumors and 19/22 muscle invasive (\geq pT₂) tumors. In all cases, MMP-10 was predominantly localised in the tumor epithelia rather than the stroma (Figure 1). Occasionally weak MMP-10 expression was also detected within the stroma of a small subset of tumors, in general, MMP-10 was expressed by tumor cells.

Although expression of MMP-10 was observed in histologically normal bladder tissue (Table 1), significantly higher levels were observed in TCC (Table 1). No significant difference in expression was observed between stages pTa and pT₁ of superficial TCC (Table 2; $P = 0.964$). Conversely, a significant difference was

observed between superficial non-muscle invasive (pTa, pT₁) and muscle invasive (\geq pT₂) TCC (Table 2; $P = 0.014$), MMP-10 expression being lower in the muscle invasive tumors (Table 1). Interestingly, unlike many MMPs, no correlation is observed between MMP-10 expression and the invasive potential of bladder TCC. Expression of MMP-10 in histologically normal bladder tissue is not significantly different from that observed in tumors demonstrating invasion into the muscle layer (\geq pT₂ stage) (Table 2; $P = 0.50$).

High levels of MMP-10 were observed in all tumor grades (degree of cellular differentiation) in comparison to normal bladder (Table 1). Although expression appeared to show a negative correlation to tumor grade (Table 1), this was not statistically significant (Table 2, $P = 0.379$). Interestingly, expression in histologically normal bladder tissue was significantly different from grade 1 (Table 2; $P = 0.02$) but not grade 2 or 3 tumors (Table 2), supporting MMP-10 as an early event in bladder tumorigenesis. The lack of difference in expression between tumor grades suggest that MMP-10 does not strongly associate with the differentiation status of the tumor (Table 2).

Comment

Breakdown and remodelling of the ECM is essential for tumor growth, survival and invasion. Although involving many enzymes, the MMPs are believed to be the central mediators of these processes. In this study, MMP-10 was expressed at much higher levels in human bladder TCCs than normal bladder. In contrast to other MMPs, MMP-10 localises predominantly to the tumor epithelial mass rather than the tumor stroma²⁸. This epithelial localization is substantiated by similar observations in human skin carcinomas¹³ and our studies in lung carcinomas⁹. With reference to

bladder carcinoma, MMP-13 expression is also predominantly in the tumor cells rather than tumor stroma²⁰. In contrast to MMP-13 which is restricted to cells at the invading edge²⁰, MMP-10 was expressed strongly throughout the tumor, suggesting it has a role in bladder tumorigenesis but may not be directly related to muscular invasion, unlike MMP-13.

Converse to the majority of MMPs, which show tight affiliation with tumor invasion, we have demonstrated an inverse relationship between MMP-10 expression and muscle invasive potential. Although MMP-10 levels were significantly higher in superficial TCC compared to normal tissue, levels were significantly lower in muscle-invasive disease. In addition, a weak negative trend was observed between MMP-10 and tumor grade, suggesting MMP-10 overexpression as an early event in bladder tumorigenesis. These data strongly suggest that MMP-10 is involved in the expansion of the neoplastic cell mass rather than invasion or progression to metastatic disease. The recent crystallization of the catalytic site of MMP-10²⁹ hopefully will lead to the development of a specific inhibitor to address and resolve this theory.

Recently, a close relationship has been suggested between expression of MMP-10 and laminin-5, an ECM protein central to cell migration and cell adhesion^{30,31}. Interestingly, enhanced loss from the basement membrane of laminin-5 occurs early in urothelial cell carcinoma, is associated with development of an invasive tumor phenotype and reorganization of the ECM at the invasive tumor front³¹. Although co-expression of these proteins are often observed, the interplay between MMP-10 and laminin-5 has not yet been shown *in vivo*³¹. Therefore it is conceivable to suggest that MMP-10 may be involved in laminin-5 reorganization and thus cell motility and tumor expansion.

Over the last few years, the identification of MMPs as being tumor specific and necessary for metastasis has resulted in the development of MMP inhibitors as anticancer therapeutics. Until recently, clinical trials using these inhibitors have been disappointing with very little success³. The failure of these drugs was attributed to their broad spectrum of MMP inhibition, activity against normal cells and use in late-stage cancer clinical trials, which bypasses MMPs involved earlier in tumorigenesis, such as MMP-10^{3,32}. A greater understanding of MMP expression, their activity profiles and interrelationships is required in order to design better MMP mediated anticancer therapeutics^{3,32}.

Conclusions

This is the first study showing expression of MMP-10 in human TCC of the bladder. Unlike the majority of MMPs, MMP-10 appears to be an early event in TCC growth and development and does not relate to specifically to tumor invasion. Further evaluation of MMP-10 in the bladder is required to address levels of active enzyme, relationship to prognosis and its viability as a target for therapeutic intervention in human TCC of the bladder.

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Figure 1: (A) Immunoblot of human MMP-10 demonstrating antibody specificity. The antibody detects both pro- and active MMP-10. No cross reactivity was observed against MMP-3. (B-G) Expression of MMP-10 in human transitional cell carcinoma (TCC) of human bladder by immunohistochemical analyses. (B, D) demonstrate MMP-10 expression in full histological sections and (C, E) demonstrate MMP-10 expression in the same tissue within the TMA, respectively. (B, C) pTa grade 1 TCC, (section expression score = 4, TMA score = 4.0); (D, E) pT₁ grade 3 TCC, (section expression score = 4, TMA score range = 3.0-4.0). (F) Expression of MMP-10 in histologically normal bladder tissue (expression score = 2). (G) Expression of MMP-10 in muscle invasive pT₂ grade 2 TCC (expression score = 1). Scale bars are included in each image.

Table 1

Expression of MMP-10 protein expression in human TCC of the bladder.

| Characteristic | Number of Patients | Median immunohistochemical score (interquartile range) |
|---------------------------|---------------------------|---|
| <i>Tumor Stage</i> | | |
| Normal Bladder | 10 | 2.00 (2.00-3.00) |
| pTa | 20 | 3.11 (2.94-3.54) |
| pT ₁ | 18 | 3.14 (2.67-3.90) |
| ≥ pT ₂ | 22 | 2.50 (1.00-3.33) |
| <i>Tumor Grade</i> | | |
| Normal Bladder | 10 | 2.00 (2.00-3.00) |
| G1 | 11 | 3.11 (3.00-3.90) |
| G2 | 32 | 3.00 (2.50-3.70) |
| G3 | 17 | 2.88 (2.60-3.60) |

Table 2

Comparison of MMP-10 expression with clinicopathological features in human bladder TCC

| Clinicopathological feature | | Median MMP-10 scores | Difference in MMP-10 expression (P value) |
|-----------------------------|-------------------|----------------------|--|
| <i>Tumor stage</i> | | | 0.003 ^a |
| pTa | pT ₁ | 3.11 / 3.14 | 0.964 ^b |
| pTa | ≥ pT ₂ | 3.11 / 2.50 | 0.008 |
| pT ₁ | ≥ pT ₂ | 3.11 / 2.50 | 0.005 |
| Normal | Superficial | 2.00 / 3.11 | 0.010 |
| Superficial | Invasive | 3.11 / 2.50 | 0.014 |
| Normal | Invasive | 2.00 / 2.50 | 0.500 ^b |
| <i>Tumor Grade</i> | | | 0.379 ^{ab} |
| Normal | Grade 1 | 2.00 / 3.11 | 0.020 |
| Normal | Grade 2 | 2.00 / 3.00 | 0.100 ^b |
| Normal | Grade 3 | 2.00 / 2.88 | 0.130 ^b |
| Grade 1 | Grade 2 | 3.11 / 3.00 | 0.174 ^b |
| Grade 1 | Grade 3 | 3.11 / 2.88 | 0.329 ^b |
| Grade 2 | Grade 3 | 3.00 / 2.88 | 0.747 ^b |

^a Determined by Kruskal-Wallis one-way analysis

^b Not Significant

Superficial: Non-muscle invasive human bladder carcinoma, combined pTa and pT₁;

Invasive: Muscle invasive human bladder carcinoma, ≥ pT₂.

