# Prognostic Value of Circulating Matrix Metalloproteinase-2 (MMP-2) and Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) in Human Bladder Cancer

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**Abstract:** Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play an important role in the metastasis and invasiveness of bladder cancer. The balance of secreted MMPs and their specific inhibitors is crucial in maintaining connective tissue homeostasis in physiological conditions. In neoplastic diseases, an unbalance of MMPs and TIMPs is supposed to be linked to the invasive character of tumor cells. This study was designed to evaluate the role of serum MMP-2 and TIMP-2 as non-invasive prognostic parameters in bladder carcinoma. Sixty seven patients were included in the study; 28 with chronic cystitis (11 schistosomal and 17 non-schistosomal) and 39 with malignant bladder lesions [12 schistosomal squamous cell carcinoma (Sqcc) and 27 transitional cell carcinoma (TCC) of whom 15 were schistosomal and 12 were non-schistosomal]. TCC cases were stratified according to their histopathological stage into: superficial TCC (13 cases) and invasive TCC (14 cases). Thirteen healthy individuals served as controls. Serum levels of both MMP-2 and TIMP-2 were measured by enzyme immunoassay (sandwich ELISA). All the malignant cases of the study showed a highly significant increase in MMP-2 serum levels with a corresponding highly significant decrease in their TIMP-2 serum levels when compared to both control and chronic cystitis cases ($p<0.001$). On stratifying TCC cases according to their histopathological grade and stage; serum levels of MMP-2 were significantly increased while TIMP-2 levels were significantly decreased in association with the increase in grade ($p<0.01$ & $p<0.001$ respectively) and stage ($p<0.001$, $p<0.05$ respectively). Meanwhile, the ratio of MMP-2/TIMPs was progressively increasing with a high significance with the progress in grade and stage in TCC cases. In conclusion, High serum levels of MMP-2 and low levels of TIMP-2 exist in malignant bladder carcinoma and these levels are proportional to TCC grading and staging which may have a prognostic value. Moreover, the MMP-2/TIMP-2 ratio may play a more significant role in the determination of aggressiveness and clinical out come of bladder cancer.

**Key words:** Human bladder cancer, circulating matrix metalloproteinase-2 (MMP-2), tissue inhibitor.

## INTRODUCTION

In Egypt, Carcinoma of the urinary bladder is the most common cancer in men constituting about 30% of all cancers (Helal *et al.,* 2006). It is characterized by high frequency of squamous cell carcinoma due to schistosomiasis which induces squamous metaplasia of the urothelium (Mostafa *et al.,* 1999). However, the frequency of transitional cell type in schistosomiasis–associated bladder cancer has been increased in the last decade (Gad EL – Mawala *et al*., 2000). Bladder cancer is usually divided into two categories; superficial tumor (Ta & T1), which is in 75-80% of cases confined to the mucosa, and muscle invasive tumors (T2 & T3). Seventy percent of superficial bladder carcinomas show recurrence after treatment, from which, unfortunately, 30% progress to muscle-invasive tumors (Stein *et al*., 1998). The outcome for patients with invasive disease at presentation remains poor, with distant metastasis occurring in over 50% within 2 years and an average 5-year survival of only 50% (Busch and Algaba, 2002).

One of the essential alterations that occur in malignancy is tissue invasion and metastasis (Hanahan and Weinberg, 2000). Tumor growth involves alteration in stromal extracellular matrix, and malignant tumors often induce a fibroproliferative response in adjacent stroma characterized by increased expression of type I and type III collagens. The formation of tumor stroma is often viewed as a non specific host attempt to wall off the tumor and it is thought to have a negative effect on tumor progression (Stetter-stevenson *et al*., 1993).

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Degradation of the basement membrane and the extracellular matrix (ECM) is a prerequisite for tumor invasion. Matrix metalloproteinases (MMPs) belong to the group of ECM degradation enzymes. The balance of secreted MMPs and their specific inhibitors (TIMPs) plays an important role in maintaining connective tissue homeostasis in normal tissue (polette *et al.*, 2004). In neoplastic diseases an imbalance of MMPs and TIMPs, leading to an excess of degradative activity, is supposed to be linked to the invasive character of tumor cells (Liotta *et al.*, 1991 and polette *et al.*, 2004). In urothelial carcinoma, several of the well characterized MMPs including MMP-2, MMP-9 and MMP-14, demonstrate increased expression and activity (Kanda *et al.*, 2000; Hara *et al.*, 2001).

It has been reported that TIMP-2 mainly forms a complex with Pro MMP-2 and could inhibit the enzymatic activity of this enzyme (lokeshwar *et al.,* 1999).Therefore, the secretion ratio of MMP to TIMP play an essential role in the determination of the aggressiveness and prognosis of bladder cancer (Kexin *et al.*, 2002).

On the basis of the concept that MMPs are synthesized in tissue and released into the blood stream, this study was designed to evaluate the possibility of using MMP-2 and TIMP-2 serum levels and ratio as non-invasive prognostic markers for the clinical behavior and aggressiveness of human bladder carcinoma.

## MATERIALS AND METHODS

This study included 67 urological patients (53 males and 14 females) with age range (17-45 years). All patients were referred from the urology department in TBRI. Thirteen healthy individuals (8 males and 5 females) with age range 20-48 years served as controls. Patients were subjected to detailed history taking, complete clinical examination, full routine laboratory investigations; abdomino-pelvic ultrasonography, intravenous urography, (computed tomography in selected patients), cystoscopy, transuretheral resection of any bladder lesion and routine histopathological examination of the bladder biopsy specimens. Diagnosis of schistosomal infestation was based on detection of schistosomal eggs in urine or tissue with detection of circulating anti-schistosomal antibodies in sera of patients by enzyme immunoassay.

*Accordingly Patients Were Classified Into:*
*Chronic Cystitis (Ch Cyst) Group:*                                                              (28 cases)

*According to Schistosomal Infestation this Group Was Subdivided Into:*
- Chronic schistosomal cystitis (Ch sch cyst)                                                    (11 cases)
- Chronic non-schistosomal cystitis (Ch nonsch cyst)                                             (17 cases)

*Malignant Group:*                                                                               (39 cases)
*According to Histopathological Diagnosis They Were Divided Into:*
-Squamous cell carcinoma (SqCC).                                                                 (12 cases)
All cases were infested with schistosomiasis
- Transitional cell carcinoma (TCC)                                                              (27 cases)

*TCC Group Was Subdivided According to Schistosomal Infestation Into:*
- Schistosomal TCC  (sch TCC)                                                                    (12 cases)
-Non schistosamal TCC (nonsch TCC)                                                               (15  cases)

*Histopathological Examination:*
Tissue sections were fixed in 10% buffered formalin, paraffin embedded and processed routinely. Serial sections 5 µm thick were taken on poly L- lysine coated slides. Hematoxylin and Eosin  stained  slides were used to evaluate the pathological diagnosis of all bladder lesions, and to assess TCC cases for pathological grades as outlined by Hanham (1991) and Rosai and Ordonez (1996).

*Enzyme Immunoassay for Serum MMP-2 and TIMP-2:*
MMP-2 and TIMP-2 serum levels were measured by sandwich  ELISA technique using commercially available kits (Quantiken MMP-2 and TIMP-2 immunoassay, R&D system, Inc. USA).  Polyclonal antibodies specific for MMP-2 and TIMP-2 had been pre-coated onto a microplate. Standars and samples were pipetted into the wells, and MMP-2 or TIMP-2 was bound by the immobilized antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for MMP-2 or TIMP-2 was added to the wells.

Following washing to remove unbound antibody enzyme reagent, a substrale solution was added to the wells and color development was stopped and color intensity was measured at 450 nm within 30min using a spectrophotometric plate reader (Bio-Rad). MMP-2 and TIMP-2 concentrations in the samples were determined by comparing the optical density of the samples to standard curves.

### Statistical Analysis:

Statistical analysis was performed using SPSS.9 software program. ANOVA test was used to compare mean serum levels of MMP-2 and TIMP-2 in the different groups. The observed difference between MMP-2 / TIMP-2 ratio in the different groups was evaluated using the test of proportion. $P < 0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

### Results:

Both MMP-2 and its inhibitor TIMP-2 could be detected in sera of healthy controls with a mean of (9.6 ± 0.8, 4.2 ± 0.5 ng/ml respectively).

In ch cyst gp the mean serum concentration of MMP-2 was found to be significantly higher in comparison to the healthy control gp ($P < 0.001$). As regards the malignant group; MMP-2 serum concentration in both TCC and SqCC was significantly elevated compared to both healthy controls and ch cyst gps ($p < 0.001$) (Table 1). On the other hand, mean serum concentration of TIMP-2 was significantly reduced in ch cyst gp compared to healthy control gp ($p < 0.001$). In the malignant group, significant reduction in the mean serum concentration of TIMP-2 in both TCC and SqCC gps compared to their corresponding concentrations in both control and ch cyst gps ($p < 0.001$ and $p < 0.01$ respectively) (Table 1).

**Table 1:** Mean serum levels of MMP-2 and TIMP-2 in the studies groups.

| Groups | | Serum MMP-2 (ng/ml), X ± SD | Serum TIMP-2 (ng/ml), X ± SD | Ratio of Serum MMP-2/TIMP-2, X ± SD |
|---|---|---|---|---|
| Control | (13) | 9.6 ± 0.8 | 4.2 ± 0.5 | 2.3 ± 0.3 |
| Ch cyst | (28) | 14.3 ± 2.9[a] | 2.3 ± 0.4[a] | 6.4 ± 1.9[a] |
| Ch non sch cyst | (17) | 16.1 ± 2.8 | 2.2 ± 0.4 | 7.4 ± 1.6 |
| Ch sch cyst | (11) | 11.9 ± 0.5 | 2.6 ± 0.5 | 4.7 ± 0.9 |
| Malig lesions | (39) | 37.5 ± 4.7[ab] | 1.3 ± 0.4[ac] | 32.1 ± 12.3[ab] |
| SqCC | (12) | 33.3 ± 2.8 | 1.8 ± 0.3 | 19.5 ± 3.2 |
| TCC | (27) | 39.4 ± 4.2 | 1.1 ± 0.3 | 37.7 ± 10.5 |
| - Non sch TCC | (12) | 39.7 ± 4.04 | 1.3 ± 0.5 | 35.6 ± 7.4 |
| -Sch TCC | (15) | 39.39 ± 5.3 | 1.2 ± 0.3 | 35.7 ± 12.5 |

Data are expressed as Mean ± SD. Ch cyst = chronic cystitis. Malig = Malignant. Sqcc = squamaus cell carcinoma. Ch non sch cyst = chronic non-schistosmal cystitis. Ch sch cyst = chronic schistosmol cystitis.
Sch TCC = schistosomol transitional cell carcinoma. Nonsch TCC = non schistosomol transitional cell carcinoma. a= $P < 0.001$ relative to control. b= $P < 0.001$ relative to ch cyst. c= $P < 0.01$ relative to ch cyst

When the ratio between MMP-2 and TIMP-2 was calculated in the different gps, a high significant increase in the ch cyst gp (3 folds) and the malignant gp (16 folds) was found when both gps were compared to normal controls ($p < 0.001$). Moreover, the increase in the ratio among the malignant gp was highly significant compared to the corresponding increase in the ch cyst gp ($p < 0.001$). (Table 1).

On classifying the studied cases according to their schistosomal infestation, no significant difference was detected in the mean serum levels of both MMP-2 and TIMP-2 between schistosomal and non-schistosomal ch cyst and TCC gps.

In TCC cases mean serum level of MMPs was significantly increased with the progress in both grade and stage ($p < 0.01$ and $P < 0.001$ respectively), while the mean serum level of TIMP-2 was found significantly reduced with the progress in both grade and stage ($p < 0.001$ and $P < 0.05$ respectively) (Table 2).

**Table 2:** Mean serum levels of MMP-2 and TIMP-2 in malignant cases stratified according to their histopathological grades and stages.

| Histopathological grade and stage | | MMP-2 (ng/ml)X ± SD | TIMP-2 (ng/ml)X ± SD | Ratio of MMP-2/TIMP-2 |
|---|---|---|---|---|
| G II | (16) | 37.1 ± 10.47 | 1.77 ± 0.45 | 35.6 ± 11.8 |
| G III | (11) | 46.8 ± 5.5[b] | 0.94 ± 0.2[a] | 50.6 ± 11.6[a] |
| Sup (Ta+T1) | (13) | 36.1 ± 2.5 | 1.2 ± 0.4 | 30.8 ± 7.1 |
| Inv (T2+T3) | (14) | 42.5 ± 2.9[c] | 1.0 ± 0.2[d] | 44.1 ± 9.0[c] |

Data were expressed as Mean ± SD, G = Grade, T = Stage, Sup = superficial, Inv= Inivasive
a = $P < 0.001$ relative to G II          c = $P < 0.001$ relative to sup gp
b = $p = 0.01$ relative to G II          d = $P < 0.05$ relative to sup gp

The ratio of MMP-2 to TIMP-2 in invasive TCC was found to be significantly higher than that in superficial TCC (p<0.001). Also, this ratio was observed to be significantly increased with the progress in tumor grade (p<0.01) (Table 2).

***Discussion:***

Proteolytic degradation of ECM is a fundamental aspect of cancer development and a key event in the regulation of tumor proliferation and metastasis. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are collectively capable of degrading most components of the basement membrane and ECM and disrupting local tissue architecture to facilitate cell migration, allow tumor growth and break down basement membrane barriers for cancer invasion and metastasis (Gontero *et al.,* 2004). MMPs are secreted as inactive proenzymes and are transformed into active forms after cleavage of a propeptide domain of the molecule. They are tightly regulated at various levels, including expression level, latent form activation, and the balance between enzyme levels and their inhibitors (TIMPs). The balance between MMPs and TIMPs is critical in maintaining the integrity of ECM and its regulatory role in organ development, cell growth and differentiation (El-Badry *et al.*, 2007).

In the present study, serum levels of MMP-2 were significantly increased while TIMP-2 levels were significantly decreased in the ch cyst gp compared to control gp. Many authors had mentioned the upregulation of MMPs expression during chronic inflammation (Gomez *et al,* 1999 and Shen *et al.,* 2001). Blood levels of MMPs and TIMPs are considered as a reflection to the presence of these enzymes in tissues as found by Sier *et al*, 2000 and Staak *et al.*,2006.

Sanders *et al.*, 2004 reported that MMPs have been firmly linked to inflammation and the essential step in their activation is the cleavage by proteases derived from activated inflammatory cells, especially neutrophils and monocyts. Gene expression of MMP-2 was shown to correlate in different stages with inflammation (Gomez *et al.*, 1999), also pro-inflammatory cytokines have been shown to induce transcription and expression of several MMPs (Horstrup *et al.*, 2002).

Interaction between tumor cells and matrix components are important for the growth and invasion of malignant tumors (Sier *et al.*, 2000).

The data of this study have revealed a significant increase in MMP-2 serum levels together with significant reduction in TIMP-2 levels in the malignant gp compared to both ch cyst and control gps. Comparable results were obtained by Wallard *et al.* (2006) they demonstrated increased levels of MMP-2 and MMP-9 in bladder cancer tissue as they regulate tumor development, metastasis and promote the invasiveness of malignant cells. Expression of MMPs was found by Daveis *et al.* (1993), Okada *et al.* (1994) and Kexin *et al.* (2002) in both normal bladder and Bladder cancer tissues with significant difference in the quantity of MMPs expression between the two kinds of tissues.

The balance between production and activation of MMPs and their inhibitors TIMPs is a critical aspect of cancer invasion and metastasis (Zucker *et al.,* 1999). In this work, in TCC cases, mean serum level of MMP-2 were significantly increased while, mean serum levels of TIMP-2 were significantly reduced with progress in both grades & stages of malignancy. Similar results were obtained by Vasala *et al.* (2003) who concluded that MMP-2 was revealed to serve as tissue indicator of aggressiveness and poor clinical outcome. Significant high expression of MMP-2 in bladder tumor line was found by Kallakury *et al,* 2001 and an elevated trend of expression was observed with increased staging and grading of bladder cancer. Kexin *et al.* (2002) reported that increased level of MMP-2 was correlated with the prognosis of bladder cancer. TIMP-2 is involved in regulation of apoptosis and is associated with an adverse prognosis in pateints with TCC of the bladder as reported by Gukiopoulou *et al.* (2003). Gohji *et al.* (1998) found elevated serum concentrations of MMP-2 and MMP-3 in patients with advanced TCC of the bladder in comparison to their levels in patients with superficial tumors. Over expression of MMP-2,3,13,14 promotes, while over expression of TIMPs inhibits the invasion of cancer cell lines (Kanayama, 2001).

In the present study, the ratio of MMP-2/TIMP-2 was found to be significantly increasing with the progress in tumor grade and stage. Xu *et al.* (2002) reported that when MMP-2 / TIMP-2 levels are unequal, either due to increased MMP-2 with less TIMP-2 or without changes of TIMP-2, they reliably predict aggressiveness and this ratio is highly correlated with the aggressiveness of bladder cancer .

In summary, the results of our study suggest that, serum levels of both MMP-2 and TIMP-2 may be used as non-invasive markers for the prediction of tumor behavior and its clinical outcome in patients with bladder cancer. The MMP-2 to TIMP-2 ratio may play a more significant role in the determination of the aggressiveness and metastatic potentials of the tumor.

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