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MMP-2 and MMP-9 as prognostic markers for the early detection of urinary bladder cancer

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Abstract

The present study assessed protein and gene expression levels of tissue inhibitor of metalloproteinase-2 (TIMP-2), matrix metalloproteinase-2 (MMP-2), and MMP-9 in urine and blood samples of 50 patients with bladder carcinoma. The expression of TIMP-2, MMP-2, and MMP-9 levels with tumor stage and grade was also assessed. Results showed that the expression levels of MMP-2 and MMP-9 in both blood and urine were significantly elevated in group 1 when compared with groups 2 and 3 healthy subjects. The discriminatory ability in the diagnosis of bladder carcinoma of MMP-2 and MMP-9 expression was confirmed by receiver operating characteristic curve analysis that revealed a sensitivity and specificity of 100%. MMP-2 and MMP-9 levels were not correlated with grade or stage of the tumor. With respect to TIMP-2 blood and urine levels, results showed a significant decrease in gene expression levels in bladder carcinoma group, whereas, TIMP-2 protein showed a significant increase in bladder carcinoma.

KEYWORDS

bladder cancer, matrix metalloproteinase-2, matrix metalloproteinase-9, western blot analysis

1 | INTRODUCTION

Urinary bladder tumors are one of the most prevalent urologic malignancies worldwide.[1] In Egypt, the common risk factor is *Schistosoma haematobium* infestation, which usually leads to the development of squamous cell carcinoma (SCC) in the bladder. Other risk factors include tobacco smoking, sex, age, exposure to toxins, and carcinogens, as well as certain genetic and inherited risk factors.[2]

To date, cystoscopy is still the gold standard for the diagnosis and follow up of urinary bladder malignancies. However, cystoscopy is an uncomfortable, invasive, and costly diagnostic technique. Therefore, the development of noninvasive diagnostic biomarkers for bladder malignancies is mandatory.[3]

Tissue invasion is an essential characteristic that occurs in malignancies. The extracellular matrix (ECM) is a major barrier to tissue invasion. Matrix-degrading enzymes, the matrix metalloproteinases (MMPs), have the ability to degrade some constituents of the ECM and the basement membrane, facilitating tumor cell invasion.[4] Moreover, MMPs play major roles in the maintenance of a supportive tumor microenvironment that promotes malignant tumor cell proliferation. MMP-2 and MMP-9 have a great ability to degrade the major components of the basement membrane. These MMPs are mostly associated with the malignant genotype and phenotype of tumor tissue and their gene and protein expression levels have been found to be elevated in several types of malignant tumors.[5]

The activities of the MMPs are highly controlled at several levels, including gene transcriptional control, their secretion as inactive precursors, and functional inhibition by tissue inhibitor of metalloproteinases (TIMPs). TIMP-2 inactivates both MMP-2 and MMP-9. Dysregulation of the balance between TIMPs and MMPs expression promotes malignant tumor progression, metastasis, and recurrence.[6]

In the present study, blood and urine levels of MMP-2 and MMP-9, as well as TIMP-2 were assessed by western blot analysis and by real-time polymerase chain reaction (PCR) to measure expression levels of the proteins and genes, respectively.

2 | MATERIALS AND METHODS

2.1 | Study subjects

Subjects of the present study were selected from Kasr El-Ainy Hospital, Urology Department, Cairo University. Laboratory work was conducted in Unit of Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University. The study protocol was conducted according to the professional ethics of the Declaration of Helsinki. All subjects of the study signed written informed consents.

Subjects of the present study were divided into the following group: group I: included 50 patients with urinary bladder carcinoma diagnosed by pathological examination of cystoscopy biopsy tissue samples; group II: included 20 patients with cystitis (a benign condition); and group III: included 20 healthy normal subjects.

2.2 | Inclusion criteria include

The Inclusion criteria were as follows: bladder cancer patients, patients not receiving any medications, surgery or radiological interventions, no associated chronic diseases or their complications, or any other type of tumors.

2.3 | Exclusion criteria include

Exclusion criteria were as follows: patients with chronic kidney, liver, cardiac diseases, hypertension, diabetes mellitus, autoimmune disease, pregnant women, severe obesity, or patients receiving any medications.

2.4 | Sample collection

Whole blood samples on ethylenediaminetetraacetic acid were collected and divided into two parts; 3 mL for separation of the mononuclear cell layer by Ficoll Paque (Munich, Germany) and 3 mL for serum separation. Blood and serum samples were stored at −80°C till the time of laboratory assays.

Voided morning urine samples (50-100 mL) were collected from all patient groups before they received any medications. Urine samples were collected before cystoscopy, surgery, or any radiological interventions. Samples were centrifuged at 4000 *g* for 20 minutes. Urine supernatant was stored at −80°C until used for TIMP-2, MMP-2, and MMP-9 protein assay. Urine sediments were stored at −80°C until used for TIMP-2, MMP-2, and MMP-9 gene assays.

2.5 | Biochemical tests

2.5.1 | Quantitative real-time PCR gene expression of TIMP-2, MMP-2, and MMP-9 in blood, urine sediments and bladder carcinoma tissue

Total RNA was isolated from mononuclear cells and urine sediments by the Maxwell 16 Total RNA Purification Kit (catalogue number AS1050; Promega, Madison, WI) according to the manufacturer's protocol. The extracted RNA was quantified by spectrophotometer (Jenway, Staffordshire, UK) at 260 nm.

The extracted RNA was reverse-transcribed into complementary DNA (cDNA) by a Reverse Transcription System Kit (catalogue number A3500; Promega, Madison, WI). The cDNA was generated from 5 μg of total RNA extracted with 1 μL (20 pmol) of antisense primer and 0.8 μL of superscript AMV reverse transcriptase for 60 minutes at 37°C. The relative abundances of the mRNA species were assessed by the SYBR Green method and Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR primers used were designed with Gene Runner Software (Hastings Software Inc, Hastings, NY) from RNA sequences in GenBank (Table 1). Primer sets had a calculated annealing temperature of 60°C. Quantitative RT-PCR analyses were performed in a 25-μL reaction volume comprising about 2× SYBR Green PCR Master Mix, 900 nM of each primer, and 3 μL from cDNA. The amplification was performed for 40 cycles, cycle conditions were 2 minutes at 50°C, 10 minutes at 95°C, and denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 10 minutes. The relative expression levels of TIMP-2, MMP-2, and MMP-9 were computed by the comparative C_t method (Tables 2–4).

2.5.2 | Estimation of TIMP-2, MMP-2, and MMP-9 protein levels by Western blot analysis (using V3 Western Workflow Complete System; Bio-Rad Laboratories Inc, Hercules, CA)

In brief, 5 mg of bladder tissue or urine samples containing 5 mg protein or mononuclear cells from whole blood containing 5 mg proteins were

TABLE 1 PCR primers of TIMP-2, MMP-2, and MMP-9

Metallopeptidase inhibitor 2 (TIMP-2), NM_003255.4	Forward: 5′-TCTCGACATCGAGGACCCAT-3′
	Reverse: 5′-TGGACCAGTCGAAACCCTTG-3′
Matrix metallopeptidase 2 (MMP-2), NM_001302510.1	Forward: 5′-CATCTGGGCAGTTGCTAAGGG-3′
	Reverse: 5′-CTGGGGCAGTCCAAAGAACT-3′
Matrix metallopeptidase 9 (MMPP), NG_011468.1	Forward: 5′-GAGCCAGTTTGCCGGATACA-3′
	Reverse: 5′-AGAACACTTTCTGGCACGTAGA-3′
Glyceraldehyde-3 phosphate dehydrogenase (GAPDH), NG_007073.2	Forward: 5′-CCTGAGGGTTCTTTGTGCTGA-3′
	Reverse: 5′-AAAGGCTCAACCTTCCCCAT-3′

TABLE 2 Blood and urine protein levels of TIMP-2, MMP-2/TIMP-2, and MMP-9/TIMP-9 ratios by Western Blot analysis

Parameter	Malignant group	Benign group	Normal group	*P* value
TIMP-2 in urine	40.7	17.13	0.0	<0.001
TIMP-2 in blood	18.84	7.6	4.5	<0.001
MMP-2/TIMP-2 ratio in urine	6.55 ± 0.34	0.15 ± 0.008	0	<0.0001
MMP-2/TIMP-2 ratio in blood	5.9	0.15	0.2	<0.0001
MMP-9/TIMP-2 ratio in urine	5.69 ± 6.16	0.148 ± 0.008	0	<0.0001
MMP-9/TIMP-2 ratio in blood	7.03	0.14	0.23	<0.0001

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

TABLE 3 Blood and urine gene expression levels of TIMP-2, MMP-2/TIMP-2, and MMP-9/TIMP-9 ratios by real-time PCR

Parameter	Malignant group	Benign group	Normal group	*P* value
TIMP-2 in urine	0.027	0.31	0.55	<0.001
TIMP-2 in blood	0.015	0.19	0.35	<0.001
MMP-2/TIMP-2 urine ratio	5.0	0.19	0.09	<0.0001
MMP-2/TIMP-2 blood ratio	6.0	0.17	0.09	<0.0001
MMP-9/TIMP-2 urine ratio	2.0	0.11	0.06	<0.0001
MMP-9/TIMP-2 blood ratio	3.8	0.19	0.09	<0.0001

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

homogenized in radioimmunoprecipitation assay buffer, then centrifugation at 12 000 rpm for 20 minutes. The protein concentration for each homogenized sample was determined using the Bradford assay. Equal amounts of protein (20-30 μg of total protein) were separated by SDS-PAGE and then transferred to a poly vinylidene di fluoride (PVDF) membrane. The membrane was blocked in Tris-buffered saline buffer containing 5% skim milk and 0.1% Tween 20 at room temperature for 1 hour and incubated with TIMP-2, MMP-2, and MMP-9 primary antibodies supplied by Thermo Fisher Scientific (Rockford, Illinois) overnight at pH 7.6 at 4°C with gentle shaking. After washing, peroxidase-labeled secondary antibodies were added, and the membranes were incubated at 37°C for 1 hour. Band intensity was analyzed by ChemiDoc Imaging System with Image Lab software (version 5.1; Bio-Rad Laboratories Inc). β-Actin was used to normalize each gene protein levels. TIMP-2 monoclonal antibody 3A4 was obtained from Thermo Fisher Scientific (catalogue number MA5-12207). MMP-2 monoclonal antibody 101 was obtained from Thermo Fisher Scientific (catalogue number 436000). MMP-9 monoclonal antibody 5G3 was obtained from Thermo Fisher Scientific catalogue number MA5-15886. Dilution of TIPM2 and TIMP-2 antibodies used is 1: 200, dilution of MMP-9 is 1:500, final concentration is 2 μg/mL for all antibodies. Rat anti-mouse secondary antibody horse radish peroxidase conjugate was obtained from Thermo Fisher Scientific (catalogue number 18-4015-82). Detection of proteins blots in PVDF membranes was

TABLE 4 Urine protein expression levels of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2, and MMP-9/TIMP-9 ratios in different clinicopathological parameters in bladder cancer group

	MMP-2	MMP-9	TIMP-2	MMP-2/TIMP-2	MMP-9/TIMP-2
Bilharziasis					
Bilharzial	233.2 ± 19.6	226.5 ± 16.8	40.2 ± 11.9	6.2 ± 0.61	5.2 ± 0.43
Non-bilharzial	263.2 ± 16.1	221.8 ± 19.8	46.1 ± 15.3	6.5 ± 0.12	6.1 ± 0.25
P	<0.001	–	–	<0.05	<0.05
Type					
TCC	252.06 ± 22.5	226.6 ± 16.1	40.1 ± 3.7	6.2 ± 0.73	5.4 ± 0.31
SCC	253.06 ± 24.6	220 ± 22.1	41.3 ± 2.9	6.4 ± 0.94	6.2 ± 0.96
Grade					
Low (1 and 2)	300.4 ± 17.2	225 ± 184.	45.9 ± 12.3	6.1 ± 0.38	5.4 ± 0.47
High (3)	297.8 ± 21.6	224.6 ± 17.7	40.2 ± 10.1	6.9 ± 0.11	6.7 ± 0.15
P	–	–	–	<0.05	<0.05
Stage					
Early (a, 1 and 2)	254.9 ± 21.4	230.4 ± 4.0	41.7 ± 12.3	6.2 ± 0.26	5.2 ± 0.35
Late (3 and 4)	248.6 ± 24.8	216.6 ± 20.6	46.3 ± 13.9	6.9 ± 0.42	6.8 ± 0.38
P	–	–	–	<0.05	<0.05

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

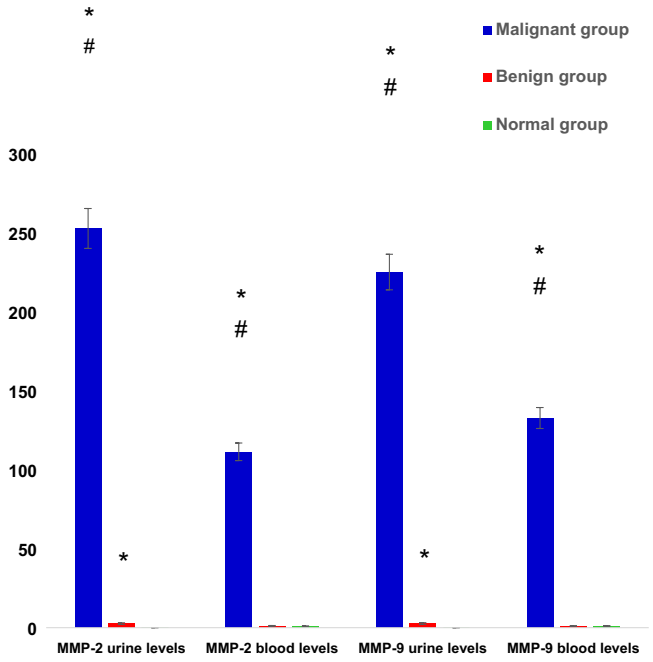

FIGURE 1 Blood and urinary protein levels of MMP-2 and MMP-9 in groups of the study by Western blot. *Significant *P* versus control group, #significant *P* versus benign bladder disorders group. *Y* axis represents a ratio between each target gene expression and housekeeping gene expression. MMP, matrix metalloproteinase

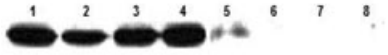

FIGURE 2 Levels of MMP-2 expression by protein normalization ratio in urine samples of the studied groups. Lanes 1-4: bladder cancer samples and lanes 5-6: cystitis as benign bladder disorder, and lanes 7-8: healthy control samples. MMP, matrix metalloproteinase

FIGURE 3 Levels of MMP-9 expression by protein normalization ratio in urine samples of the studied groups. Lanes 1-5: bladder cancer samples and lanes 6-7: cystitis as benign bladder disorder, and lanes 8-9: healthy control samples. MMP, matrix metalloproteinase

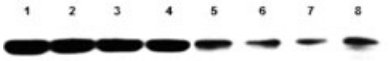

FIGURE 4 Levels of MMP-2 expression by protein normalization ratio in serum samples of the studied groups. Lanes 1-4: bladder cancer samples and Lanes 5-6: cystitis as benign bladder disorder, and Lanes 7-8: healthy control samples. MMP, matrix metalloproteinase

conducted by Chemiluminescent Detection (Immun-Star WesternC Chemiluminescence Kit; catalogue number 1705061; Bio-Rad Laboratories Inc). Protein standards for Western blot analysis were obtained from Bio-Rad Precision plus westernC protein standards (catalogue No. 161–0385; http://www.bio-rad.com/en-eg/applications-technologies/detection-methods#3). Chemiluminescence detection was conducted using ChemiDocMP Imaging System with Image Lab software (version 5.1; Bio-Rad Laboratories Inc)

2.6 | Statistical analysis

Data was analyzed by Sigma Plot version 12.5. Data were summarized as mean ± SD. Differences between groups were analyzed by the Kruskal-Wallis test, Shapiro-Wilk test, and the Student *t* test. Post hoc testing was performed by the Tukey test to compare the difference between the groups. Simple linear correlation (Pearson's correlation coefficient test) (*r*) was also done to test for linear relations between MMP-2 and MMP-9 and other variables. *P* value is considered significant if < 0.05. Receiver operating characteristics (ROC) curves were used to assess the diagnostic performance of all studied parameters.

3 | RESULTS

3.1 | Biochemical characteristics of the studied subject

Demographic and clinicopathological variables of all groups of the study are group I, composed of 12 females (24%) and 38 males (76%). The history of the smoking variable was significantly higher in malignant group patients ($P = 0.002$). The frequencies of bilharzia in patients in the malignant group were 36%. Histopathologic examination of bladder carcinoma tissues revealed: transitional cell carcinoma in 36 cases (72%) and squamous cell carcinoma in 14 cases (28%). Regarding the tumor stage, the frequencies of non-muscle–invasive (Ta-T1 and T2) and muscle-invasive (T3-T4) tumors were 58% and 42%, respectively. Regarding tumor grade, those frequencies of G-1 and G-2 were 50%, also of G-3 were 50%. Group II was composed of five females (20%) and 20 males (80%). The history of the smoking variable was 60% in benign group patients. The frequencies of bilharzia in benign group patients were 20%. Group III was composed of three females (12%) and 22 males (88%). There is no history of smoking or bilharzia infection.

3.2 | MMP-2 and MMP-9 blood and urine protein levels assessed by Western blot analysis

Blood and urine protein levels of MMP-2 and MMP-9 assessed by protein normalization ratio in all the studied groups are shown in Figures 1–5.

3.3 | MMP-2 and MMP-9 blood and urine levels by PCR

Blood and urine gene levels of MMP-2 and MMP-9 assessed by real-time PCR in all the studied groups (Figure 6).

FIGURE 5 Levels of MMP-9 expression by protein normalization method in serum samples of the studied groups. Lanes 1-4: bladder cancer samples, lanes 5-6: cystitis as benign bladder disorder, and lanes 7-8: healthy control samples. MMP, matrix metalloproteinase

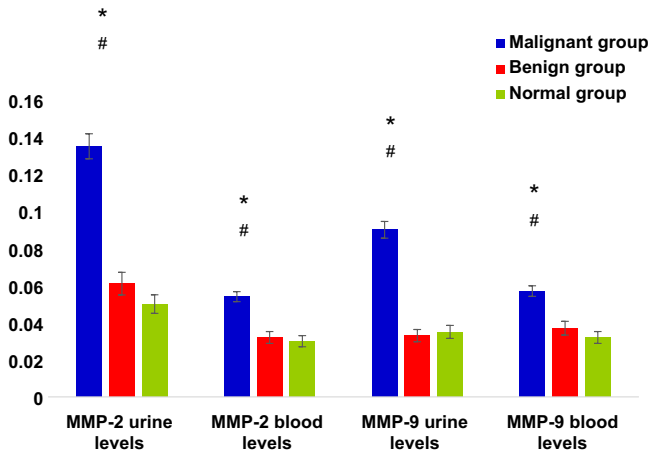

FIGURE 6 Blood and urinary gene expression levels of MMP-2 and MMP-9 in the studied groups. *Significant *P* versus control group, #significant *P* versus benign bladder disorders group. *Y* axis represents a ratio between each target gene expression and housekeeping gene expression. MMP, matrix metalloproteinase

3.4 | ROC curve

ROC curves were carried out to assess sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of MMP-2, and MMP-9 protein levels in urine and blood. Results are summarized in Table 5.

4 | DISCUSSION

Detection of MMPs and their TIMPs in urine or serum of patients could provide essential information in the diagnosis and prognosis of malignant tumors.[7] The present study was done to evaluate if MMP-2, MMP-9, MMP-2/TIMP-2 ratio, and MMP-9/TIMP-2 ratio have significant diagnostic value in urinary bladder cancer, and also if these markers have significant utilization in discrimination between different stages and grades of the tumor.[8]

Results of the current study showed a significant elevation in gene and protein expression levels of MMP-2 and MMP-9 in both urine and blood of patients with bladder cancer as compared with nonmalignant bladder disorders and healthy control subjects. Several lines of evidence stated overexpression of MMPs in malignant tumors including urinary bladder cancer.[9,10] A similar study reported that MMP-2 and MMP-9 were elevated in voided urine samples of bladder cancer and exhibited sensitivity and specificity of 67.3% and 90%, respectively.[11]

There are several evidence that the mitogen-activated protein kinases family is also involved in malignant tumor progression and metastasis and this effect could be mediated via upregulation of MMPs expression.[12]

A recent study proved that MMP-2 and MMP-9 are involved in adhesion, migration, and invasion of human bladder cancer cells and this effect is inhibited by the use of resveratrol, a polyphenolic compound of plant origin. Resveratrol inhibited ERK1/2 and JNK signaling pathways with subsequent downregulation of MMP-2 and MMP-9.[10,13]

The disruption of the balance between MMPs and their inhibitors, TIMPs results in tumor cell proliferation, invasion, and metastasis.[14,15] MMP-2 will be compulsory for the activation of MMP-9.[16]

In the present study, TIMP-2 gene expression was significantly decreased in malignant bladder group when compared with the other nonmalignant groups. In contrast, protein levels of TIMP-2 were significantly elevated in malignant bladder group as compared with the other nonmalignant groups and this could be explained by amplification during gene expression and translation into proteins. Moreover, the ratio between MMPS/TIMPs is more important and has a significant impact on the imbalance between MMPs and their inhibitors. Results of the current study demonstrated significant elevation of the ratio of MMP-2/TIMP-2 and the ratio of MMP-9/TIMP-2 in bladder cancer group in both urine and in blood samples. The present study agrees with previous studies that proved higher MMP-2 to TIMP-2 ratio (MMP-2/TIMP-2) in cancer patients than in controls.[17]

Moreover, results of the present study demonstrated that MMP-2/TIMP-2 and MMP-9/TIMP-2 could significantly discriminate between high-grade and advanced-stage tumors from low-grade and early-stage tumors, respectively. A higher ratio indicates an

TABLE 5 ROC curves of MMP-2 and MMP-9 protein levels in urine and blood

ROC	Cut off value	Sensitivity, %	Specificity, %	PVP, %	NPV	AUC[a], %
MMP-2 in urine	100%	100	100	100	100	100
MMP-2 in blood	45.1	100	100	100	100	100
MMP-9 in urine	98	100	100	100	100	100
MMP-9 in blood	50.7	100	100	100	100	100

Abbreviations: AUC, area under the curve; MMP, matrix metalloproteinase; NPV, net present value.
[a]AUC represents the accuracy.

imbalance between MMPs and their inhibitors and this promote metastasis through remodeling of ECM.[18,19] Furthermore, MMPs are able to produce highly aggressive tumor by degradation of proapoptotic factors with subsequent generation of apoptosis-resistant cells.[20] MMPs could have a significant impact on angiogenesis in malignant tumors via activation of proangiogenic factors, [20] as well as the generation of angiogenesis inhibitors as endostatin and angiostatin.[21,22] In conclusion, urinary bladder cancer exhibited significant upregulation of blood and urine levels of MMP-2 and MMP-9, ratio of MMP-2/TIMP-2 and ratio of MMP-9/TIMP-2. The ratio between MMP-2, MMP-9, and TIMP-2 is significantly elevated in the advanced stage and high-grade tumors.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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