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ORIGINAL ARTICLES

Determination of MMP-2 and MMP-9 activities in urine of schistosomal bladder carcinoma: A diagnostic tumor biomarkers

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ABSTRACT

Schistosoma haematobium is the most common species in Egypt and more implicated in bladder tumor development. Matrix Metalloproteinases including MMP-2 and MMP-9 are the main enzymes involved in ECM degradation and are involved in tumor invasion, metastasis as well as in the early stages of carcinogenesis. The aim of the present study is to evaluate the activity of urinary MMP-2 and MMP-9 in schistosomal related bladder carcinoma. The activity of both MMP-2 and MMP-9 were determined using zymographic analysis and western blot techniques on urine of 50 Egyptian bladder cancer patients (bilharzial, 20 and non bilharzial, 30) with different types of tumor (TCC, 32 and SCC, 18). Urine of 50 healthy control volunteers was included in the study. High frequency of uMMP-2 and uMMP-9 was detected in bladder cancer patients (58% and 66% respectively). None of the healthy control showed any of the activities ($p< 0.001$). A none significant increase of MMP-9 activity was reported in bilharzial related TCC type (66.7%) as compared to non bilharzial TCC type (55%). Also, non significant changes of uMMP-2 and uMMP-9 were reported between TCC and SCC type. We concluded that measurement of urinary MMP-2 and MMP-9 activity might have clinical applications. High level of uMMP-9 in bilharzial TCC of bladder might have a diagnostic value but future large studies are needed to confirm this finding.

Key words: Matrix metalloproteinases, MMP-2, MMP-9, bilharzial bladder cancer.

Introduction

Bladder schistosomiasis (bilharzia) has been considered a definitive cause of urinary bladder cancer with an associated five-fold risk. Schistosomiasis is the second most common parasitic infection after malaria, with about 600 million people exposed to infection in Africa, Asia, South America and the Caribbean. Although there is a well-established relationship between squamous cell carcinoma of the bladder and schistosomiasis, the trends are changing for bladder cancer in endemic zones, such as Egypt. Data from the National Cancer Institute (NCI) Cairo, the largest tertiary cancer hospital in Egypt, showed that patients diagnosed in 2005 had a six-fold higher odds of developing transitional cell carcinoma compared with patients diagnosed in 1980(Felix *et al*. 2008). The decline in the frequency of bladder cancer is related to a decline in the detection of bilharzia eggs in urine samples, probably due to better control of the disease in rural populations (Gouda *et al*. 2007&Chambers *et al*. 2002). Matix metalloproteinases (MMPs) are the main enzymes involved in ECM degradation, and have been shown to be critically involved in a number of other physiological processes, including organ development, wound healing and angiogenesis (Haas *et al*. 2000). On the other hand, increased MMP activity has an important role in several pathological conditions, such as arthritis, cardiovascular disease and cancer (Burrage *et al*. 2006). There is clear evidence that different MMPs are causally involved in tumor invasion and metastasis. Increasing evidence suggests that MMPs are also involved in the early stages of carcinogenesis which is primarily related to their non – ECM degrading functions such as regulation of tumor growth, apoptosis, cell adhesion and angiogenesis(Egeblad and Werb, 2002).

The main goal of the present study was to evaluate the activity of urinary MMP-2 and MMP-9 as a non – invasive diagnostic biomarker to differentiate between bilharzial and non bilharzial bladder cancer.

Subjects and Methods:

One hundred samples were analyzed in this study, including 50 samples (31 males and 19 females; mean age 56± 10yr, range: 29-76) from patients of bladder carcinoma admitted to Egyptian National Cancer Institute between 2008- 2011 and diagnosed by biopsy obtained by transuretheral resection of the bladder or radical

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cystectomy. Diagnosis of Schistosomiasis was based on the presence of calcified eggs in tissue biopsy. Of those patients, 32 were diagnosed by histopathology with transitional cell carcinoma (TCC) (12 with bilharzial and 20 without bilharzial) and 18 with squamous cell carcinoma (SCC) (10with bilharzial and 8 without bilharzial). Fifty healthy control volunteers without any history of cancer, inflammatory or immunodeficiency diseases (36 males and 14 females; mean age 49± 8 yr, range: 31-57) were recruited from the hospital laboratory staff. Urine was collected according to the institutional bioethical guidelines. Specimens were obtained before surgical or other therapeutic intervention following informed consent. Samples were collected in sterile containers and immediately frozen at -20°C. Urine was tested for presence of blood and leukocytes using Urinalysis strip and samples containing blood or leukocyte were excluded from the study. Protein concentration of urine was determined by the Bradford method according to the manufacture's protocol (Bio-Rad, CA).

Substrate gel electrophoresis:

Gelatinases (MMP-2 and MMP-9) in the urine were detected using gelatin zymography as described previously (Moses *et al*. 1998). Briefly, urine (40 µL) from controls or cancer patient and pure (2ng) MMP-2, MMP-9 were mixed with non-reducing sample buffer (4% SDS, 0.15 M Tris, pH 6.8, 20% v/v glycerol, and 0.5% w/v bromphenol blue) and were separated on a 10% polyacrylamide gelcontaining 0.1% gelatin (Bio-Rad, Hercules, CA). After electrophoresis, gels were washed twice with 2.5% Triton X-100 (15 min/each wash). Substrate digestion was carried out by incubating the gel in 50 mMTris-HCl, pH7.6, containing 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 1% Triton X-100, and 0.02% NaN3 at 37°C for 24 h. The gel was stained with 0.1% Coomassie Brilliant Blue R250 (Bio-Rad), and the location of gelatinolytic activity were detected as zones of clearance on a background of uniform blue staining and imaged using densitometer (BIOMETRA).

Western blot analysis:

Immunoblotting was used to verify the presence of MMP-2 and MMP-9 in urine. Equal amounts of proteins (20µg) were separated by SDS-PAGE under reducing conditions followed by semi-dry protein transfer into nitrocellulose membranes (Bio-Rad, CA). Monoclonal antibodies against human MMP-2 and MMP-9 (Santa Cruz Biotechnology, Inc) were used. After extensive washing, the membranes were incubated with a secondary horseradish peroxidase conjugated goat anti-mouse antibody (Sigma Chemical Co., ST. Louis, Mo), hydrogen peroxidase (BDH) and diaminobenzidine (Sigma-Aldrich). Protein bands were evaluated using Scion Image software (Scion Corporation, MD).

Statistical Method:

Data was analyzed using SPSSwin statistical package version 17 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables.

Results:

Using zymographic analysis technique in the present study, the overall activity of both MMPs (MMP-2 and MMP-9) detected in the urine of bladder cancer patients was about 74% (37 out of 50). The activity of each MMP-2 and MMP-9 recorded was 58% (29 out of 50) and 66% (33 out of 50) respectively. No activity was identified in the urine of the 50 healthy control volunteers. A representative gelatin zymogram of urine from four bladder cancer patients is shown in figure (1a). Four bands seen are corresponding with latent MMP-9 (MW 92 KDa), activated MMP-9 (MW 84 KDa), latent MMP-2 (72 KDa) and activated MMP-2 (64 KDa). Western blot analysis technique was done in the present study using antibodies specific to MMP-9 and MMP-2 to confirm the data obtained by zymographic analysis. MMP-2 and MMP-9 bands appeared in western blot analysis for all cases that showed the activities. Figure (1b) showed a representative samples.

The activity of MMP-2/MMP-9 in urine of schistosomal versus non schistosomal bladder cancer:

To correlate between the activities of both MMPs examined in the present study with the presence of bilharziasis, we classified the bladder cancer patients into two groups, bilharzial (22 patients) and non bilharzial (28 patients). MMP-2 activity didn't show any significant association between both groups where about 59.1% of activity detected were associated with the presence of bilharzial ova and about 57.1 were free of bilharziasis (p value was > 0.05). The activity of MMP-9 recorded was higher in bilharzial group (72.7%) as compared to non bilharzial (60.7%) but this increasing didn't reach the significant value (p value was > 0.05)table (1).

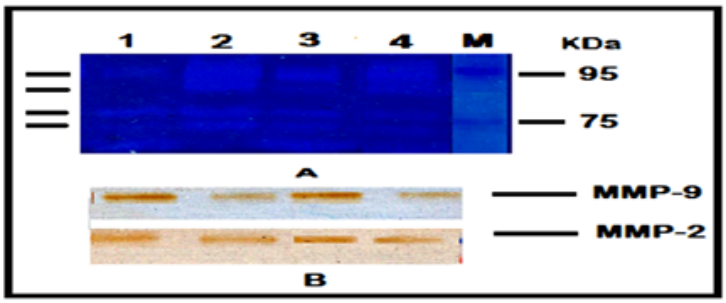

Figure (1): A. Urinesamples from bladder cancer patients analyzed by Substrate gel electrophoresis(zymography). Bands of enzyme activity were detected as zones of clearance on a background of uniform blue staining. Protein marker use to detect the molecular weight. B, **western blot used to confirm the presence of MMP2 and MMP9 in urine of bladder cancer patients**. Arrows indicate MMP9 and MMP2.

Table 1: Activity of urinary MMP2 and MMP9 in bladder cancer with and without bilharzia in comparison with control

Marker	Bladder n=50 bilharzial n=22 (%)	non-bilharzial n= 28(%)	Control n=50 Positive n= 0 (%)	Negative n= 50(%)
MMP2				
Positive n=29(%)	13/22 (59.1%)	16/28 (57.1%)		50(100)
Negative n= 21(%)	9/22(40.9%)	12/28 (43.9%)		
MMP9				
Positive n= 33(%)	16/22(72.7%)	17/28(60.7%)		50(100)
Negative n=17 (%)	6/22(27.3%)	11/28(39.3%)		

Activity of MMPs in TCC versus SCC of bladder carcinoma

The type of tumor (TCC versus SCC) was correlated with the activity of both MMPs examined in the present study. A non significant difference was reported between different types of bladder carcinoma with the activity of both MMP-2 and MMP-9 identified in urine. The activities recorded in TCC were 56.3% and 68.8% respectively. The activities in SCC were 61.1% in both MMPs examined.

To clarify the role of bilharziasis on the activity of MMPs examined, we sub-classified the types of bladder cancer into TCC with and without bilharsiasis and SCC with and without bilharsiasis. Our data indicated that MMP-9 activity was higher in TCC with bilharzial (66.7%) as compared to non bilharzial patients (55%) but this correlation was still not significant (p value >0.05). No observed difference in the MMP-2 activity with both subgroups as shown in table (2) and figure (2).

Table 2: The activity of MMP-2 and MMP-9 in TCC and SCC of bladder carcinoma

types	TCC (32)		SCC (18)	
	bilharzial	non-bilharzial	bilharzial	non-bilharzial
MMP-2				
Positive	7/12 (58.3%)	11/20 (55%)	6/10 (60%)	5/8 (62.5%)
Negative	5/12 (41.7%)	9/12 (45%)	4/10 (40%)	3/8 (37.5%)
MMP-9				
Positive	8/12 (66.7%)	11/20 (55%)	5/10 (50%)	4/8 (50%)
Negative	4/12 (33.3%)	9/20 (45%)	5/10 (50%)	4/8 (50%)

TCC, transitional cell carcinoma SCC, squamous cell carcinoma

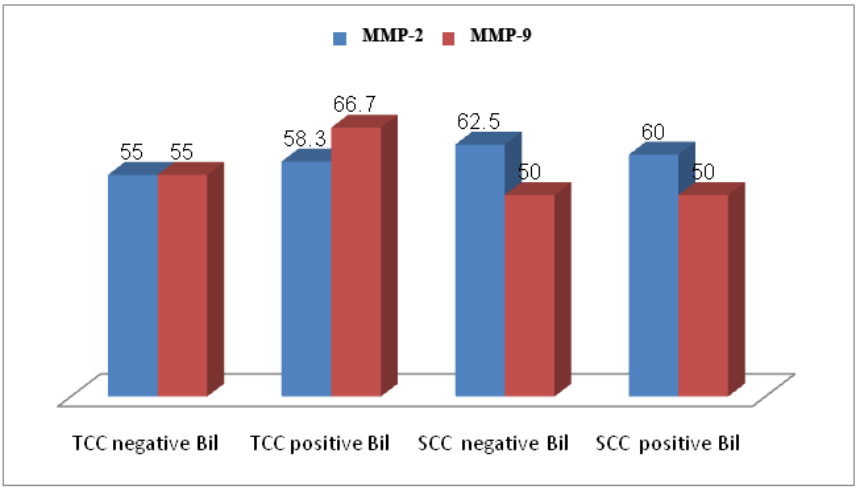

Fig. 2: Percentage of urinary MMP2 and MMP9 in TCC and SCC bladder cancer with and without bilharzia.

Disscusion:

Bladder carcinoma is one of the most common urologic malignancies occurring worldwide(Jemal *et al*. 2005). In Egypt it accounts for 30% of all cancers(El-Mawla *et al*. 2001) and has been associated with many pathologic factors, most commonly bilharzial infestation(El-Sebaie *et al*. 2005). Therefore new noninvasive methods for bladder cancer detection would open new possibilities in diagnosis and monitoring(Hughes *et al*. 2000), as well as in screening of groups at high risk for the development of this malignancy, such as in bilharzial infested patients. Soluble molecular markers secreted in urine could serve as urinary markers for bladder cancer detection, depending on their efficiency to provide early detection capabilities and insight into appropriate treatment decision as well as to monitor treatment response and tumor recurrence(Curran and Murray, 2000). Urinary MMPs levels in cancer patients could in turn reflect enhanced presence of these markers in the circulation which might be originated directly originated from tumors in the case of bladder cancer(Rémy and Trespeuch , 2005). In the present study, we used zymographic analysis to identify the activity of both MMP-2 and MMP-9 in voided urine from bladder cancer patients. Our results revealed high frequency of MMP-2 and MMP-9 activities in bladder cancer cases as compared to control healthy volunteers ($p<0.001$) 58% and 66% respectively. MMP-2 and MMP-9 degrade type IV collagen, the major component of the basement membrane. Accordingly, these MMPs received much attention during the early stage of MMP research. Previous studies demonstrated that MMP-2 concentration and activity were found to be consistently elevated in urine of patients with bladder cancer, which confirm our present finding and raising the hope that it may be used as an early detection marker for this type of cancer. However, the sensitivity proved to be similar to or even lower than that of urine cytology(Gerhards *et al*. 2001). A marked increase of MMP-2 and MMP-9 levels was detected in urine of bladder cancer group as compared to normal once(Sier *et al*. 2000&Eissa *et al*. 2007). It was noticed that this marked increasing of MMPs in urine of bladder carcinoma due to that the synthesis of MMP-2 occurs by tumor cells or by host response tumor as fibroblasts, macrophages, and vascular endothelial cells whereas MMP-9 is strongly expressed in intravascular and tissue-infiltrating leucocytes(Ozdemir *et al*. 1999). On the other hand, urinary MMP-2 levels strongly correlate with tumor stage which makes it seems to be more useful for detecting early tumor progression rather than early-stage bladder carcinoma (Szarvas *et al*. 2011). Several independent studies as well as our present study have consistently reported elevated MMP-9 activity in urine samples from patients with bladder cancer which provided the rationale to test MMP-9 as a screening of diagnostic marker(Szarvas *et al*. 2011 ;Gerhards *et al*. 2001 ; Bianco *et al*. 1998). Like MMP-2, its low sensitivity and specificity as compared to urine cytology decrease its diagnostic value for bladder cancer. The activity level of MMP-2 in urine of bladder cancer patients in the present study and previous one(Sier *et al*. 2000) were lower than for MMP-9. This is due to that MMP-2 is constitutionally regulated by many cell types and is most likely regulated at the level of proenzyme activation, whereas MMP-9 is transcriptionally regulated by inflammatory cytokines such as tumor necrosis factor and interleukins α(Leber and Balkwill, 1998).

In the Present study the percentage of TCC was (64%) while SCC was (36%) the ratio was 1.8:1. It was postulated that there is a changing of histopathological patterns of bladder cancer seen at NCI-Cairo where it was reported that 2:1 predominance of TCC over SCC with the declining of schistosmal infection. Previous study in Egypt indicated the emergence of TCC in hospital-based case control study from Alexandria Egypt, TCC comprised 67% of histologically confirmed bladder cancer while SCC accounted for 18% in 1997(Felix *et*

al. 2008). In the present data , A non significant increasing of the MMP-9 activity in both bilharzial bladder cancer group (72.7%) as compared to non bilharzial one (60.7) as well as in TCC associated with bilharziasis (66.7%) as compared to non bilharziasis (55%). No significant difference was observed in MMP-2 activity either in presence or absence of bilharziasis. It was reported that the urinary MMP-9/TIMP-2 ratio was 1.72-fold higher in bilharzial bladder cancer thannon bilharzial type suggesting its usefulness for diagnosing bilharzial bladder carcinoma either as TCC or SCC (Eissa *et al*. 2007). The same study demonstrated that urinary MMP-9 level and MMP-2/TIMP-2 ration were higher in SCC(1.31 fold and 1.27-fold respectively) than in TCC which reflected a more aggressive phenotype in this bladder cancer subtype. In another study, high level of serum MMP-2 was detected in TCC of bladder carcinoma and this level is proportional to the grade and stage of the tumor which may indicate its prognostic value(Yang *et al*. 2006).

In conclusion, measurement of urinary MMP-2 and MMP-9 activity might have clinical applications. High level of uMMP-9 in bilharzial TCC of bladder might have a diagnostic value but future large studies are needed to confirm this finding.

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