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The Role of Matrix Metalloproteinase-2 and -9 *in situ* Hybridization in Bladder Cancer Progression

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Abstract

Background Transitional cell carcinomas (TCC) of the bladder are a major health problem and can be a leading cause of death. There are several proteolytic enzymes which are responsible for the degradation of the extra cellular components and have an essential role in tumor invasion and metastasis. MMP-2 and MMP-9 are the most important class of these enzymes.

Objective To assess the In situ hybridization expression of MMP-2 and MMP-9 in TCC of the bladder.

Methods Fifty formalin fixed, paraffin embedded of TCC of the bladder tissue blocks from Specialized Surgical Hospital in Baghdad, were included in this study. In addition ten apparently normal bladder autopsies were collected from the Forensic Medicine Institute Archives used as control group. Tissue blocks were sectioned on charged slides to be used for In situ hybridization, for the detection of MMP-2 and MMP-9.

Results The expression of MMP-2 and MMP-9 in TCC of the bladder tissues in the present study was 64 % for both and strong relationship between expression of MMP-2 and MMP-9 and TCC of the bladder was detected.

Conclusion MMP-2 and MMP-9 play an important role in progression of transitional cell carcinoma of the bladder.

Key words Bladder cancer, Matrix Metalloproteinases, invasion, metastasis, carcinogenesis.

Introduction

Urinary bladder cancer is one of the most common cancers worlds wide; with the highest incidence in industrialized countries [(1)].It's occurrence is strongly associated with cigarette smoking and the use of certain chemicals [(2)].

More than 90% of bladder cancers begin in the lining of the bladder wall and are known as transitional cell carcinomas (TCC). About 5% of bladder cancers are squamous cell carcinomas (SCC). There are also uncommon bladder cancers, such as adenocarcinoma and small cell carcinoma, which are responsible for less than 2% of all bladder cancers [(3)].

There are many markers associating with the progression of bladder carcinoma, such as depth of invasion, stage, grade and multiplicity. Unfortunately they are inaccurate, that is why more clinical prognostic markers are needed. Matrix metalloproteinases are a family of endopeptidases that are capable of degrading most components of the extracellular matrix (ECM) [(4)]. Of them, gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are able to degrade extracellular matrix protein, including type IV collagen. Gelatinases have been linked to cell invasion and the process of metastasis [(5)].

Several studies that zymographical analysis of the levels of MMP-9 and active MMP-2 showed a significant increase with tumor grade and invasiveness, however, the correlation between the levels of both gelatinases with recurrence in superficial tumors or progression in invasive tumors was not significant [6].

To our knowledge there is no Iraqi study had focused on the possible role of MMP-2, MMP-9 during bladder cancer progression in order that this study will try to take the first step in detection of these markers and study the correlation with different parameters such as age, gender, grade and pattern of growth and presence or absence of muscle invasion in transitional cell carcinoma of the bladder.

Methods

Patients and tissue samples

Fifty patients with bladder carcinoma, 35 males and 15 female with an age ranged from 25 to 70 years, were included in this retrospective study, The patients' samples were collected from the archives of histopathology laboratories of Specialized Surgical Hospital in Baghdad From February 2009 till June 2009. The diagnosis of these tissue blocks were primarily based on the obtained histopathological records of bladder biopsy samples that had been accompanied in the hospital laboratory. Confirmatory histopathological re-evaluation of each obtained tissue blocks was done by specialist pathologist. In addition ten apparently normal bladder autopsies were collected from the Forensic Medicine Institute Archives. They were 5 males and 5 female and the range of the age was the same as patients group. Formalin-fixed, paraffin embedded blocks tissue were sectioned (4µm) thickness, from each tissue block, one section was stained with Haematoxylin and Eosin, and 2 sections were mounted on charged slides to be used for *In situ* hybridization for the detection of MMP-2 and MMP-9**.**

In situ hybridization procedure

Serial tissue sections were cut 4µm thick and were positioned on positive charged slides. The slides were placed in 60˚C oven over night. The tissue sections were deparaffinized, the slides were dehydrated by graded alcohol concentration (100%, 95%, 70%) and distal water. The slides were treated with proteinase K solution and dehydrated. One drop of the biotinylated long cDNA probe for human MMP-2 and MMP-9(Maxim Biotech Cat. No.: IH-60025 and IH-60028). Hybridization/ detection kit were used purchased from Maxim Biotech/USA Cat. Number IH-6001(IHD-0050) was placed on the tissue section in oven at 70˚C for 8-10 minutes. After that the slides were placed in a humid chamber and incubated over night at 37°C to allow hybridization of the probe with the target nucleic acid. The slides were soaked in 1X detergent wash at 37˚C until the cover slips fall, and then treated with RNase A solution and streptavidin-AP-conjugate. One to two drops of 5-bromo-4-chloro-3-indolyl phosphartel/nitro blue tetrazolium substrate-chromogen solution (BCIP/NBT) conjugate were placed on tissue section at room temperature for about 30 minutes; the latter was monitored by viewing the slides under the microscope. A blue colored precipitate will from at the site of the probe in positive cells. Slides were then counterstained using nuclear fast red and sections were mounted with a permanent-mounting medium (DPX). Finally the examination and scoring were done under light microscope by a pathologist at power 400 according to the scoring system of [7].

Statistical analysis was done using Chi-Square test for tables with frequencies percentages, range, mean and standard deviation. Values were considered statistically significant when $p<0.05$.

Results

Histopathpological classification

Fifty formalin-fixed, paraffin embedded blocks were collected from bladder carcinoma patients and histopathologial re-examination with hematoxylin and eosin stain was done. The specimens were graded according to world health organization classification [8]. As follow: Grade I: well differentiated transitional cell carcinoma of the bladder (n=4) (8%), Grade II: moderately differentiated transitional cell carcinoma of the bladder (n=31) (62%) and Grade III: poorly differentiated transitional cell carcinoma of the bladder (n=15) (30%).

Each carcinoma was also distributed according to the pattern of growth, as follows: papillary type (n=28) (56%) and solid type (n=22) (44%). More ever, muscle invasion was seen in 26 cases (52%) while non-invasion was seen in 24 cases (48%).

Results of in situ hybridization detection of MMP-2 and MMP-9

The results showed in table 1 and figure 1, 2 which were demonstrated that 32 cases (64%) of bladder carcinoma cases were positive for both of them, while 18 cases (36%) cases were negative for both of them. On the other hand statistical analysis were demonstrated a highly significant differences in MMP-2 and MMP-9 expression among patients with transitional cell carcinoma of the bladder when compared with healthy control group.

Table 1. The expression of MMP-2 and MMP-9 in patients with transitional cell carcinoma of the bladder.

Result of MMP expression			MMP-2 Expression	MMP-9 Expression	Comparison of Significance	
Patients	Positive	Low Intermediate High Total	10 17 5 32 (64 %)	19 10 3 32 (64 %)	p- value 0.01	Sig. Highly Sig. P<0.01
	Negative	N %	18 (36%)	18 (36%)		
	Total	N %	50 (100%)	50 (100%)		
Controls	Positive Negative	N % N %	0 10 (100%)	0 10 (100%)		

Table (2): Correlation of MMP-2 scores and related with different parameters.

Parameters		MMP-2 scores			Comparison of Significance	
		Low	Intermediate	High	p-value	Sig.
Age	25-39 40-54 55-70	1 (10%) 1 (10%) 8 (80%)	0 3 (17.6%) 14 (82.4%)	1 (20%) 1 (20%) 3 (60%)	0.48	Non Sig. (P>0.05)
Gender	Male Female	8 (80%) 2 (20%)	11 (64.7%) 6 (35.3%)	2 (40%) 3 (60%)	0.33	Non Sig. (P>0.05)
Tumor grade	I II III	0 7 (70%) 3 (30%)	1 (5.6%) 11 (64.7%) 5 (29.4%)	0 1 (20%) 4 (80%)	0.13	Non Sig. (P>0.05)
Pattern of growth	Papillary Solid Invasive	5 (50%) 5 (50%) 4 (40%)	8 (47.1%) 9 (52.9%) 10 (58.8%)	1(20%) 4 (80%) 4 (80%)	0.07	Non Sig. (P>0.05)
Muscle invasion	Non invasive	6(60%)	7 (41.2%)	1 (20%)		

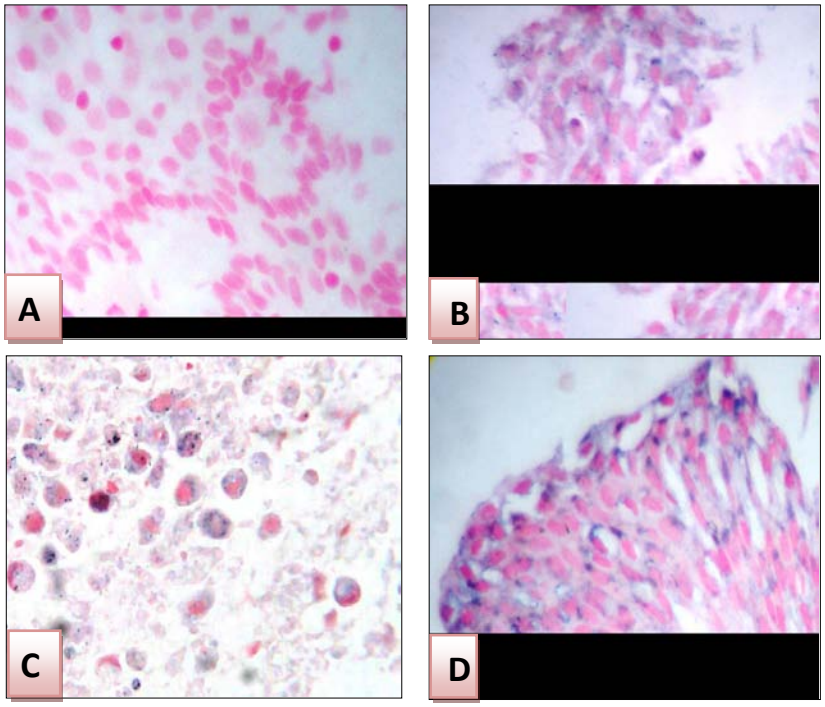

Figure 1. *In situ* hybridization for MMP-2 of patient with TCC of the bladder, stained by BCIP/NBT-Chromogen and counter stained with nuclear fast red (NFR), magnification power, 400. A-Negative expression, B-low MMP-2 positive expression, C-intermediate MMP-2 positive expression, D-High MMP-2 positive expression.

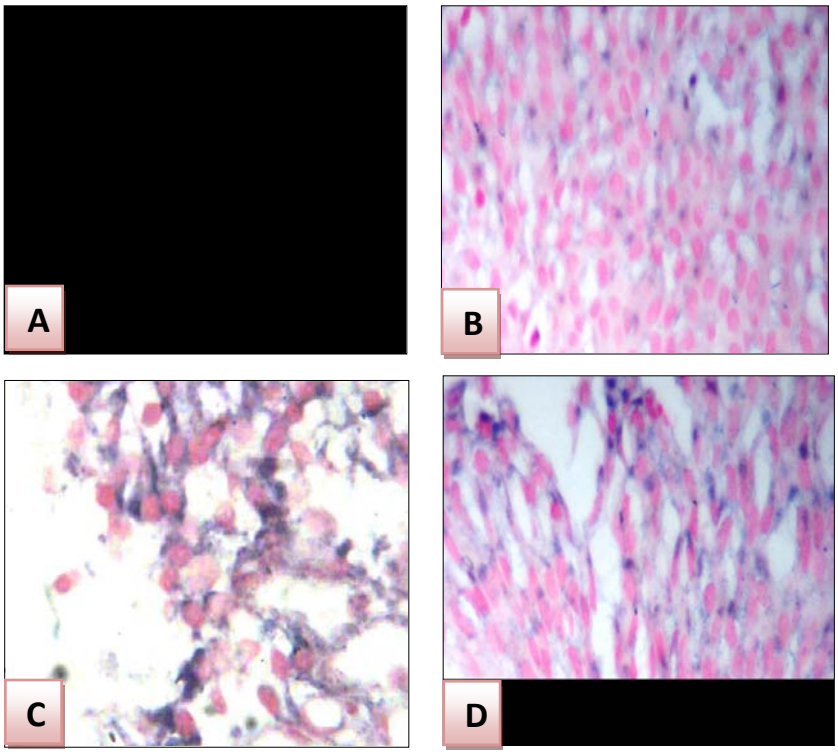

Figure 2. *In situ* hybridization for MMP-9 of patient with TCC of the bladder, stained by BCIP/NBT-Chromogen and counter stained with nuclear fast red (NFR), (Magnification power, 400). A-Negative expression, B-low MMP-9 positive expression, C-intermediate MMP-9 positive expression, D-High MMP-9 positive expression.

muscle invasive. The results of frequency distribution of positive and negative MMP-2 and -9 showed significant correlations between MMP-2 expression and pattern of growth while no correlation occur with MMP-9 based on Chi-square test of analysis (Table 4 and 5).

Table 2 and 3 demonstrated the correlation between expression of MMP-2 score and MMP-9score, with different variables. The results showed that there were no significant differences between *in situ* hybridization expression of both MMP-2 and MMP-9 with age, gender, grade, pattern of growth, and

Table 3. Correlation of MMP-9 scores and related with different parameters.

Parameters		MMP-2 scores			Comparison of Significance	
		Low	Intermediate	High	p-value	Sig.
Age	25-39 40-54 55-70	0 4 (21.1%) 15 (78.9%)	1 (10%) 1 (10%) 8 (80%)	1 (33.3%) 1 (33.3%) 1 (33.3%)	0.37	Non Sig. (P>0.05)
Gender	Male Female	14 (73.7%) 5 (26.3%)	9 (90%) 1 (10%)	2 (66.7%) 1 (33.3%)	0.28	Non Sig. (P>0.05)
Tumor grade	I II III	3 (15.8%) 12 (63.3%) 4 (21.1%)	0 6 (60%) 4 (40%)	0 1 (33.3%) 2 (66.7%)	0.50	Non Sig. (P>0.05)
Pattern of growth	Papillary Solid Invasive	11 (57.9%) 8 (42.1%) 10 (52.6%)	5 (50%) 5 (50%) 3 (30%)	1 (33.3%) 2 (66.7%) 2 (66.7%)	0.80	Non Sig. (P>0.05)
Muscle invasion	Non invasive	9 (47.4%)	7 (70%)	1 (33.3%)		

Table 4. *In situ* hybridization expression of positive and negative MMP-2 and related with clinicpathological profile of patients with TCC.

Variables		MMP-2		Comparison of Significance	
		positive	negative	p-value	Sig.
Age	25-39 40-54 55-70	2 (6.3%) 5 (15.6%) 25 (78.1%)	2 (11.1%) 6 (33.3%) 10 (55.6%)	0.24	Non Sig. (P>0.05)
Gender	Male Female	21 (65.6%) 11 (34.4%)	14 (77.8) 4 (22.2%)	0.36	Non Sig. (P>0.05)
Tumor grade	I II III	1 (3.1%) 19 (59.4%) 12 (37.55%)	3 (16.7%) 12 (66.7%) 3 (16.7%)	0.10	Non Sig. (P>0.05)
Pattern of growth	Papillary Solid	14 (43.8%) 18 (56.2%)	14 (77.8%) 4 (22.2%)	0.02	Sig. (P<0.05)
Muscle invasion	Invasive Non invasive	18 (56.2%) 14 (43.8%)	6 (33.3%) 12 (66.7%)		

Table 5. *In situ* hybridization expression of positive and negative MMP-9 and related with clinicpathological profile of patients with TCC.

Variables		MMP-2		Comparison of Significance	
		positive	negative	p-value	Sig.
Age	25-39 40-54 55-70	2 (6.2%) 6 (18.8%) 24 (75%)	2 (11.1%) 5 (27.8%) 11 (61.1%)	0.58	Non Sig. (P>0.05)
Gender	Male Female	25 (78.1%) 7 (71.4%)	10 (55.6%) 8 (44.4%)	0.95	Non Sig. (P>0.05)
Tumor grade	I II III	3 (9.4%) 19 (59.4%) 10 (31.3%)	1 (5.6%) 12 (66.7%) 5 (27.8%)	0.83	Non Sig. (P>0.05)
Pattern of growth	Papillary Solid	17 (53.1%) 15 (46.9%)	11 (61.1%) 7 (38.9%)	0.58	Non Sig. (P>0.05)
Muscle invasion	Invasive Non invasive	15 (56.9%) 17 (53.1%)	9 (50%) 9 (50%)	0.83	Non Sig. (P>0.05)

Discussion

Matrix metalloproteinases (MMPs) are involved in cellular proliferation, migration, invasion and metastasis [(9)]. MMP-2 and MMP-9 are thought to play a central role in these processes, in view of their ability to degrade many Extracellular Matrix (ECM) components and other substrates [(10)].

In general, the role of MMPs in carcinogenesis seems to be very complex, sometimes even controversial, according to some preclinical findings [(11)].

In vitro studies had indicated that MMP-2 and MMP-9 activity may determine the invasion capacity of the bladder carcinoma cell line [(12)].

The current study had demonstrated that MMP-2 and MMP-9 were over expressed in transitional cell carcinoma of the bladder. These results might possibly reflect the association between cellular expression of MMP-2 and MMP-9 and bladder tumor genesis. This was in agreement with the findings of other authors [(6,13,14)] since they found over expression of this enzyme in transitional cell carcinoma of the bladder. In comparison with other studies both enzyme are increased in malignant tissues compared to their benign counterparts [(15).] This raises the question why MMPs expression was rare in benign tumor, we know that benign tumor have no metastasis and no invasion, so that there is no need for additional degradation of ECM, and finally no need for exaggerated MMPs expression. In fact, analysis of both primary and metastatic tumors demonstrated increased MMPs at the metastatic site had pointed out their role in tumor migration and spread [(16)].

In this study, the results showed no correlation between MMP-2 expression and age, gender, also any correlation with tumor grade and muscle invasion not observed. In the present study the results were in agreement with Grignon et al [(13)] who did not found any association between the expression of MMP-2 immunoreaction protein in bladder cancer tissue or the grade or stage in TCC of the bladder. However, this result was also in agreement with the results of Kanayama et al [(17)] who reported that MMP-2 contributes to the invasive properties of bladder carcinoma. Moreover, in some study where gelatine zymography was used, the expression of activated MMP-2 was higher in invasive tumor tissue [(18)]. Other study has also shown a correlation to grade and stage [(19)].

Concerning with MMP-9 expression, positivity did not correlate to the age, gender of the patients, pattern of growth, grade of the tumor. This result was in agreement with the findings of Ozdemir et al [20] who pointed out that no correlation between MMP-9 over expression and tumor grade was recorded. Mohammed et al [21] measured the level of MMP-9 in serum by western blot technique and revealed that serum level of MMP-9 showed highly significant elevation compared to healthy normal subjects but this elevation did not correlate with age, gender or even grade of the disease. Durkan and co workers [22] found no correlation to grade but instead, the MMP-9 levels measured by enzyme-linked immunoassay (ELISA) correlated to stage when measuring MMP-9 protein in urine samples of bladder cancer patients. It had been found that patients with no relapse had a higher urine MMP-9 protein level than patients with relapses, the difference being statistically significant [23].

MMP-9 is quite well examined in bladder cancer whether using tissue samples, serum or urine detection, it seems that high or elevated expression of MMP-9 enzyme correlates to clinical stage or histological grade of the tumor [24,25].

The results are consistent with result of previous studies suggesting that MMP-2 and MMP-9 may play an important role in TCC of the bladder or could facilitate its progression.

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