Iraqi Journal of Cancer and Medical Genetics

Urinary marker (MMP-9) and bladder cancer

Nahi Yousif Yaseen[1], Rana S. Aziz[2], Refif Sabih AL-Shawk[3], Maysoon Ali Saleem[3], Issam Salman Al-Azzawi[4] and Huda Sadoon AL-biaty[3]

1 Iraqi Center for Cancer and Medical Genetics Research, Al-Mustanseria University, Baghdad-Iraq
2 Department of histopathology- Teaching Laboratories / Al- Yarmouk Teaching hospital, Baghdad-Iraq.
3 Department of microbiology ,College of Medicine, Al-Mustansiriy University,Baghdad-Iraq
4 Department of urosergery ,College of Medicine, Al-Mustansiriy University, Baghdad-Iraq

Abstract:

Bladder cancer is a complex disease and this study of bladder cancer was conducted to assess the significance of level difference of MMP-9 in urine and tissue biopsy which has a role in tumor progression in bladder cancer patients. MMP-9 evaluated in urine of 48 bladder cancer patients and 40 healthy controls by ELISA test, and in 57 biopsies of bladder cancer and 40 normal urothelium (autopsies) by immunohistochemistry (IHC). Result indicated that urine MMP-9 showed high specificity in diagnosis bladder cancer patients, and has a role in discrimination between (newly diagnosed vs recurrence), but urine level and tissue expression of MMP-9 were unrelated to grade, muscle invasion. Some risk factors like Schistosomiasis and family history of cancer showed a significant association with MMP-9 tissue expression.

Key words: *MMP-9, Bladder cancer, Schistosomiasis, Elisa, muscle invasion, Immunohistochemistry*

Introduction:

Bladder cancer is the ninth most commonly diagnosed cancer worldwide, with more than 150,000 deaths per year, and an estimated male-female ratio of 3.8:1.0 [1]. Incidence of bladder cancer in Iraq on constant rise, with (80%) were males and (20%) were female[2], with this cancer is associated of an infection of Schistosoma [3]. Several years ago, data from the Baghdad Tumor Registry, showed that the urinary bladder cancer is the dominant tumor, squamous-cell carcinomas being the majority and an evidence of infection with Schistosoma haematobium is associated with the occurrence of this histological type [4]. Although the major histological cell type of such cancer in Iraq was SCC, there is a trend for increasing frequency of TCC among patients infected with schistosomiasis [5]. The age of highest incidence of bladder carcinoma over (50) years, majority of them male, they have low level education, and most of them smoking [6]. risk factor like positive family history, seems to play now more important roles in the development of this disease [7], Bladder cancer risk is 1.8 times higher in people with family members of the disease, of which in some cases, these members of family may be all exposed to the same cancer-causing chemical, and may also share changes in some genes like Glutathione S-transferases M1(GSTM1),[8].

The most system of classification for grading urothelial cancer has been World Health Organization (WHO) classification of the 1973, for papilloma and grades 1, 2, and 3 carcinomas, then in 2004, WHO and International Society of Urological pathology (ISUP) described new category called papillary urothelial neoplasm of low malignant potential (PUNLMP[9], more recently, WHO has recommended changing bladder cancer grading to only two categories: low and high grade, which is used in the latest version of the American Joint Committee on Cancer (AJCC) 7thedition Staging System(2010).

Ghafouri-Fard etal.,2014 noted that at the time of diagnosis about one fifth of primary bladder cancers have reached the muscle layer of the bladder wall and have a poor prognosis[10], as well [11]noted that, 80% of bladder tumors or more are in the mucosa or lamina propria non-muscle invasive (NMIBC). On regard to recurrence, the NMIBC recurrence phenomenon makes bladder cancer one of the most prevalent cancers worldwide [12], with papillary and superficial tumors recur in 70% of patients after surgical excision of tumor[13].

In Iraq, staging showed that most cases were in Ta group, both new or receive treatment cases and the type of tumor

Corresponding Address:
Huda Sadoon AL-biaty
Department of microbiology ,College of Medicine, Al-Mustansiriy University,Baghdad-Iraq
Email: huda.sadoon@yahoo.com

mostly resected was urothelial cell carcinoma[2]. As well, [14] reported that, more than half of the cases with papillary growth pattern had a superficial non-invasive disease while, the remaining cases was a sessile growth pattern which had a deeper muscle invasive disease.

Diagnosis, treatment with follow-up of Bladder cancer require laborious, expensive, invasive in addition to time-consuming investigations that make this type of cancer un easy clinical condition [15], therefore there is an increasing need for exploring non invasive markers for it, so urine tumor markers used as an indicators of disease in patients presented with hematuria, also, aid in diagnosis of recurrence and survival[16], and these markers were used as an adjunct to urine cytology, result in reducing the cystoscopy frequency in the follow-up patients with bladder cancer[17]. Like Matrix metallopeptidase 9 (MMP-9) which is in particular the gelatinases have been associated with degradation of extracellular matrix, metastasis with invasion of tumor cell in many human cancers and aid in the diagnosis of Bladder cancer(BCa) through urinalysis[18]. The aim of this study is to evaluate the significances of MMP-9 biomarkers in urine of bladder cancer patients (as non-invasive procedure), and in bladder tissue and compare this biomarker in patients at different cancer grade, invasion and relation with risk factors, then evaluate their effect on the prognosis of the illness.

Materials and Methods:

Subjects

This prospective study conducted on 137 individuals (111 males and 26 females) over the period from June 2013 to April 2014 in Al- Yarmouk and Baghdad Teaching Hospital in Baghdad, of which urine sample and tissue biopsy were collected from patients with suspected carcinoma of bladder who were undergoing cystoscope, as well, urine samples were collected from apparently healthy volunteers (who were free of diabetes mellitus, hypertension, infections especially UTI and Schistosomiasis and with no previous history of cancer especially bladder cancer, cigarette smoking and alcohol drinking). In addition, apparently normal bladder autopsies were collected from the Forensic Medicine Institute, Baghdad, Iraq. These studied individuals were classified into three groups:

Group 1: 57 bladder cancer patients with average age (63± 9.3), this were sub divided into 28 newly diagnosed bladder tumor patients (25 of them were men and 3 were women), and 29 recurrent tumor patients (relapse), of which they previously received intravesical chemotherapy and /or radiotherapy (25 men and 4 women).

Group 2: 40 healthy volunteers (28 men and 12 women with average age 51±13.3).

Group 3: 40 autopsies (apparently normal urothelium), of which 33 were men and 7 were women with average age (51± 13.7).

Previous laboratory finding was obtained from each patient and a full history for diseases, besides a cystoscopic examination by which transurethral resection (TUR) biopsies were taken from the apparent lesion, processed by standard oncological procedures, tumor grade and muscle invasion was defined by a specialist pathologist according to (WHO/ISUP) and American Joint Committee on Cancer (AJCC, 7th ed.,2010).

• Urine collection and ELISA.

After collection of freshly voided urine samples in clean sterile Container in cool box. Sample (50) ml were centrifuged at 3000 rpm for 10 minutes at 4°C and the supernatant was aliquoted, stored at −80°C in deep freez until analyzed. Before analysis, samples were slowly thawed and Centrifuge again before assaying to remove any additional precipitates that may appear after storage. Urinary level for MMP-9(abcam,UK) quantitatively determined and done for collected samples accordingly . Enzyme-Linked Immunosorbent Assay (ELISA) for mmp-9performed for patients and healthy urine samples by commercially available ELISA kits. The assay were read in a microplate reader (Memmert, Germany) and method is a solid phase sandwich ELISA .

• Immunohistochemical detection of MMP-9 proteins in paraffin embedded sections

Biopsies were processed by fixation in(neutral buffered formalin10%, dehydrated through a graded series of ethanol, cleared in xylenes, after that, paraffin embedding step took place then stained with routine stains of(H&E), as well as immunohistochemically, following the procedure described in the texts [19]. Hematoxylin and eosin section were examined for histological grading and muscle invasion examination.

Immunohistochemistry procedures

• Slide baking in tissue-drying oven.
• Deparaffinization by washing in xylene.
• Rehydration with alcohol.
• Then Peroxidase Block (enough drops of H_2O_2), followed by Antigen Retrieval, Buffer and protein block was added .
• Optimally, (100µl) of diluted primary antibody applied on the sections
• The secondary antibody of complement (rabbit anti mouse secondary antibody) were applied on the sections
• HRP-conjugate of secondary antibody (Goat anti –rabbit HRP Conjugate) were applied covering the specimen
• The DAB –chromogen were applied
• Counter- stain: Mayer's heamatoxylin stain was applied to the sections
• Dehydration: in ethanol and xylene
• Between each of the previous steps there was washing step with buffer
• Mounting media: of DPX was applied
• Slides were examined and the stained cells were counted with assistance of an experienced histopathologist by light microscope. Using a two tier scoring system was carried out, for scoring of MMP-9expression, (cytoplasmic and membranous staining)

• Scoring For MMP-9 as (Yousefetal.,2014):

The quantity score (QS) of stained tumor cells was estimated

as follows:

Score 1: 0, 1-10%, Score 2: >10-50%, Score 3: >50-70%, Score 4: >70-100%.

Staining intensity score was estimated as follows:

Score 0: no staining, Score 1: weak staining, Score 2: moderate staining, Score 3: strong staining.

The total IHC score result from multiply the percentage and the staining intensity scores that ranges from 0 to 12. Low levels of expression represented by scores of 0 to 4 and high levels of expression score was from >4 to 12.

Results:

Assessment of matrix metalloproteinase (MMP-9)

Urine MMP-9 level assessment

The mean±SD (pg/ml) of urine MMP-9 level was elevated in patients (6747.74±2150.31), compared to that of healthy controls (1131.53±1554.81), with statistical significant difference (P<0.001) as seen in (Table1).

Table 1: Mean ±SD, SE and mode of MMP-9 in bladder cancer patients and controls.

MMP-9 (pg/ml)	Bladder cancer patients	Healthy volunteers
.No	48	40
Mean	6747.7	1131.53
Standard Deviation (SD)	2150.3	1554.81
Standard error of Mean (SE)	310.3	245.83
Mode	9336.8	381.05
Minimum	2447.5	210.88
Maximum	9336.8	6400.19
Median	7064.3	542.41
T test .Sig	18.6 0.001>	

MMP-9 level in bladder cancer groups

On regard to patients groups, as shown in (Table 2) the mean ± SD(pg/ml)of urine MMP-9 level for newly diagnosed was (6176.8±2366.9), recurrent cases was (7368.3± 1730.19) and healthy controls was (1131.53 ±1554.80). The statistical analysis showed that there was a significant difference between and within groups with P value <0.001, with a significant higher level in recurrence groups (7368.3pg/ml) to be followed by newly diagnosed (6176.8pg/ml) then it was significantly getting down in healthy groups (1131.53pg/ml). The distribution of bladder cancer cases and healthy control according to the median MMP-9 level (pg/ml) is shown in the (Figure 1).

Table 2: Mean ±SD, SE and mode of MMP-9 in healthy, newly diagnosed andrecurrent cases of bladder cancer.

MMP-9 pg/ml	Healthy controls	Newly diagnosed cases	Recurrent cases
Mean	1131.53	6176.8	7368.3
Standard Deviation (SD)	1554.80	2366.9	1730.19
Standard error of Mean (SE)	245.83	473.3	360.7
Mode	381.05	4244.2	9336.8
Minimum	210.88	2447.5	2785.5
Maximum	6400.19	9336.8	9336.8
Percentile 05th	256.94	2900.354	4312.7
25th	301.59	4244.2	6544.4
50th (Median)	542.41	6821.3	7902.6
75th	917.04	8110.4	8719
95th	5100.31	8923.791	9316.664
99th	5893.24	9332.7	9336.8
F test .Sig	73.117 0.001>		

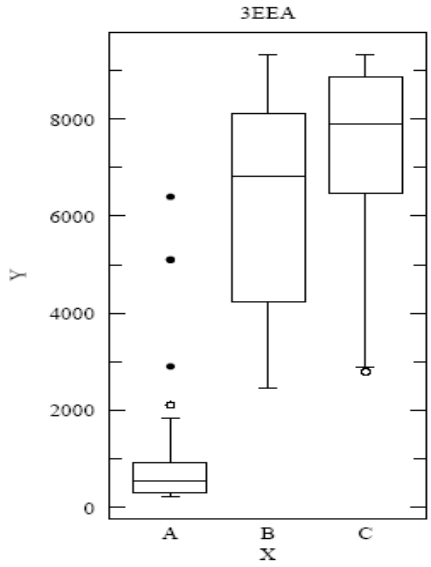

Figure 1: Distribution of median MMP-9 level in healthy, newly diagnosed and recurrent cases of bladder cancer.

According to the age and gender, (Table 3, 4, 5, 6) demonstrate the mean ± SD (pg/ml) of urine MMP-9, showed to be non -significant for patients and healthy subject.

Table 3: Mean ± SD of MMP-9 in different age groups of bladder cancer.

MMP-9(pg/ml)	Age in years				F test Pvalue
	<50	50-59	60-69	>70	
No.	2(4.16%)	10(20.83%)	26(54.16%)	10(20.83%)	Ftest 1.554 Pvalue 0.22
Mean	5435.1	5903.9	6871.9	7527.6	
Standard Deviation	3772.5	2355.1	1984	2049.4	

Table4: Mean, SD and SE of MMP-9 in bladder cancer patients according to the gender.

MMP-9 (pg/ml)	**Gender**	
	Male	**Female**
No.	42	6
Mean	6662.4	7344.6
Standard Deviation	2240.2	1349.1
Standard error of Mean (SE)	345.6	550.7
Median	7024.1	7261
T test	0.723	
Pvalue	0.47	

Table 5: Mean ± SD of MMP-9 in different age groups of healthy.

Age in years	Mean ± SD
50>	1153.37±1574.93
50-59	377.78 ± 661.95
60-69	1629.35 1105.17±
70<	±2603.72 1940.32
Ftest Pvalue	0.7104 0.55

Table 6: Mean ± SD of MMP-9 in healthy according to gender.

MMP-9(pg/ml)	Gender	
	Male(28)	Female(12)
Mean ± SD	1223.10 ± 1780.92	917.86± 845.38
Pvalue	0.45 (P>0.05)	

3-3-1-B. Urine MMP-9 in low and high grade bladder cancer patients

For MMP-9 in patients with low and high grade bladder cancer, it showed that the mean were (6419.5 vs 7169.7) respectively, with no significant difference between them (p > 0.05), as shown in (Table7 and Figure2)

Table 7: Mean urine MMP-9 level in low and high grade bladder cancer patients.

MMP9 level	Low grade tumor	High grade tumor
No.	27	21
Mean	6419.5	7169.7
Standard Deviation (SD)	2429.8	1690.6
Standard error of Mean (SE)	467.6	368.9
Mode	4244.2	7064.3
Median	6983.9	7457.6
T –test	1.20	
P-value	0.23	

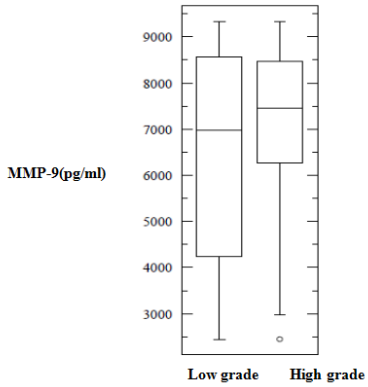

Figure2: Median urine MMP-9 level in bladder cancer patients in relation to tumor grade

Urine MMP-9 level in bladder cancer patients in relation to tumor muscle invasion

There was no statistically significant difference in MMP-9 between muscle invasion and non-muscle invasion with mean level of (7385.7 vs6251.5) as noted in (Table 8).

Table 8: Mean urine MMP-9 level in bladder cancer patients in relation to tumor muscle invasion.

MMP-9 level	Muscle invasion tumor(27)	Non- muscle invasion tumor(21)
Mean	7385.7	6251.5
Standard Deviation (SD)	1302.2	2543.5
Standard error of Mean(SE)	284.18	489.49
Mode	7064.34	4244.24
Minimum	4929.69	2447.5
Maximum	9336.8	9336.8
Median	7458 6983	
T –test **P value**	1.86 0.069	

Urine levelof MMP-9 in relation to selected risk factors in bladder cancer patients

For the relation of some factors with bladder cancer, in regard to urine MMP-9 level, Table 9 Showed non-significant relation to smoking (P= 0.63), Schistsoma infection (P= 0.25), UTI (P= 0.62), stone (P= 0.41) as well as family history of cancer (P =0.62).

Table 9: Urine level of MMP-9 in relation to selected risk factors in bladder cancer patients.

MMP	Smoking		*Schistosomiasis*		UTI		Stones		FamilyHistory	
(Pg/ml)	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
.No	29	19	8	40	29	19	6	42	7	41
(%)	(60.41)	(39.58)	(16.66)	(83.3)	(60.4)	(39.5)	(12.5)	(87.5)	(14.5)	(85.4)
Mean	5846.4	5372.1	5942.6	6908	6621.1	6940.9	7434.44	6649.6	6400.5	6817.1
SD	3408.6	3062.5	2294.4	2113.7	2163.2	2174.5	1529.7	2221.7	2230.8	2156.2
T-test	0.490		1.16		0.500		0.834		0.496	
Pvalue	0.63		0.25		0.62		0.41		0.62	

Receiver Operating Characteristic (ROC) analysis

The area under the ROC curve (AUC) gives an idea about the usefulness of a tested parameter in differentiating between groups, the closer the area to one, the more useful it is in discrimination. So this ROC was used to determine the cut-off value for different parameters for diagnosis of bladder cancer as well as differentiating them from healthy controls by the MMP-9 a highly significant (P= 0.000), with ROC area 0.971with high sensitivity and specificity, as shown in (Table 10, Figure 3) .

Table 10: The ROC analysis for urine IL-8, MMP-9 and BLCA-4 level in bladder cancer patients.

	AUC	SE	95%CI	P-value	Cut-off	(%)Sensitivity	(%)Specificity
MMP-9 pg/ml	0.971	0.015	0.943-1.000	0.000	2616.5	93.8	87.5

AUC : Area Under ROC Curve , SE : Standard Error , CI: Confidence Interval

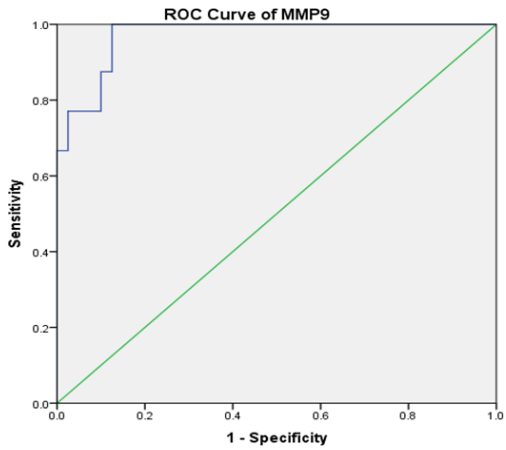

Figure3: Receiver operating characteristic curve for detectionof bladder cancer patients by reference to the level of urineMMP-9.

Immunohistochemistry of MMP-9 in bladder cancer tissue
Scoring of MMP-9 in patients and controls

The percentage of positively (PR) stained cells and Staining Intensity of positively stained cells (SI) in patients and controls were summarized in (Table 11). The product of the positively (PR) stained cells and the intensity of staining cells scores represented the final IHC score that ranges from 0 to 12. Scores of 0 to 4 were considered as low levels of expression while high levels of expression were with score from >4 to12. MMP-9 was highly expressed in 3(5.2%) out of 57 TCC tissue while tissue while all of 40 normal autopsies were with low expression, fisher's exact test showed no significant difference between MMP9 over expression and these two group (Table 12,Figure 4)

Table11: The percentage of positively (PR) stained cells andStaining Intensity of positively stained cells (SI) of MMP-9 in bladder tissue of bladder cancer patients and normal autopsies.

percentage of positively stained cells of MMP-9	Staining Intensity score						Total No.
	NO (Score0)	weak (Score1)	Moderate (Score 2)		(Strong) (Score3)		
	PatientsNo.	Controls No.	Patients No.	Controls No.	Patients No.	Controls No.	
(Score 1) 0 , 1-10%	24 , 1	30 , 0	0	0	0	0	55
(Score 2) 10-50%	13	0	0	0	0	0	13
(Score 3) >50-70%	10	0	3	0	0	0	13
(Score 4) >70-100%	6	10	0	0	0	0	16
Total No.	54	40	3	0	0	0	97

Table 12: Final score of MMP-9 in bladder cancer patients and normal urothelium.

MMP-9 expression	Patients No. (%)	Normal urothelium No. (%)	Total No. (%)
High expression (>4- 12)	3(5.2)	0	3(3)
Low expression (<4)	54 (94.7)	40(100)	94(96.9)
Total No. (%)	57(58.76)	40(41.23)	97(100)
Fisher's exact	0.265		

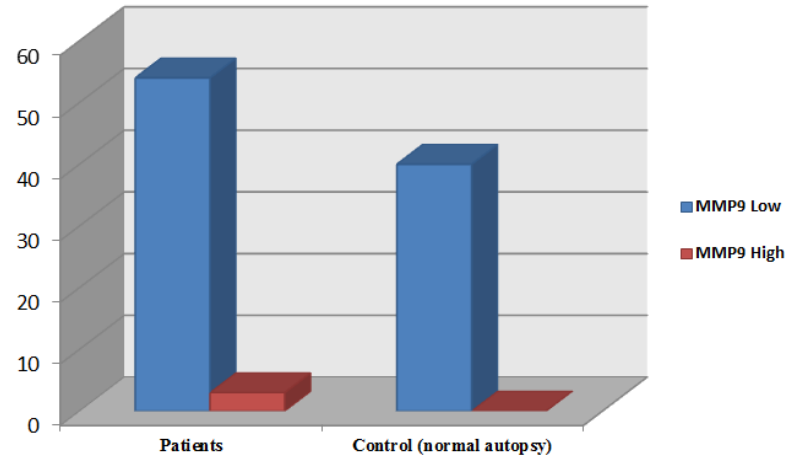

Figure4: Score of MMP-9 in bladder cancer patients and control.

Scoring of MMP-9in newly diagnosed and recurrent cases of bladder cancer

The percentage of positively(PR) stained cells, Staining intensity of positively stained cells (SI) in newly and recurrence cases shown in (Table 13). In 28 newly diagnosed bladder carcinoma, high expression of MMP-9 was detected in 1(3.5%), and low expression was found in 27 (96.4%), while in the recurrent cancer patients, 2 of them (6.8%) showed high expression and 27 (93.2%) revealed low expression, with Chi –square showed no significant difference between these two groups(Table14).

Table13: The MMP-9 percentage of positively (PR) stained cells, Staining Intensity of positively stained cells (SI) in newly diagnosed and recurrent bladder cancer cases.

percentage of positively stained cells of MMP-9	Staining Intensity score						Total No.
	NO weak (Score 0) score1		Moderate (Score 2)		Strong (Score 3)		
	Newly No.	Recurrence No.	Newly No.	Recurrence No.	Newly No.	Recurrence No.	
(Score 1) 0 , 1-10%	12 , 0	12 , 1	0	0	0	0	25
(Score 2) 10-50%	8	5	0	0	0	0	13
(Score 3) >50-70%	4	6	1	2	0	0	13
(Score 4) >70-100%	3	3	0	0	0	0	6
Total No.	27	27	1	2	0	0	57

Table 14: Final score of MMP-9 in newly diagnosed and recurrent cases of bladder cancer patients.

MMP-9 expression	Group		Total (%) .No
	Newly diagnosed (%) .No	Recurrence (%) .No	
High expression to 12 4<	(3.5)1	(6.8) 2	(5.26)3
Low expression to 4 0	(96.4) 27	(93.2) 27	(94.7)54
(%) .Total No	(49.12)28	(50.87)29	(100)57
Chi-square	0.500		
P value	0.480		

Scoring of MMP-9 in relation to bladder tumor grade and muscle Invasion

Regarding the score of MMP-9 with tumor grade, as shown in (Table15, Table16), it was with high expression in 3.3% and 7.4% of low grade and high grade TCC respectively, so no statistically significant difference between them. In addition to that, it was noted that the cytoplasmic tumor cells stained positive for MMP-9(Figure5and 6).

Table 15: The MMP-9 percentage of positively (PR) stained cells, Staining Intensityof positively stained cells (SI) in low and high grade bladder tumor.

Percentage of positively stained cells of MMP-9	Intensity score						Total No.
	NO (Score0)	Weak (scorre1)	Moderate (Score 2)		Strong (Score 3)		
	Low grade No.	High grade No.	Low grade No.	High grade No.	Low grade No.	High grade No.	
(Score1) 0 , 1-10%	14, 1	10 , 0	0	0	0	0	25
(Score2) 10-50%	10	3	0	0	0	0	13
(Score 3) >50-70%	4	6	1	2	0	0	13
(Score4) >70-100%	0	6	0	0	0	0	6
Total No.	29	25	1	2	0	0	57

Table16: Final score of MMP-9 in bladder cancer patients according to tumor grade.

MMP-9 expression	Grade		Total No.(%)
	Low grade No.(%)	High grade No.(%)	
High expression>4 to 12	1(3.3)	2 (7.4)	3(5)
Low expression0 to 4	29(96.6)	25(92.6)	54(94.7)
Total No.	30(52.6)	27(47.4)	57(100)
Chi-square	1.55		
P value	0.213 (not significant)		

As well, there was no significant difference between muscle invasion of tumor and MMP-9 expression (P > 0.286), with high expression in only 7.4% and 3.3% of invasive and non-invasive bladder cancer respectively, as shown in (Table17, Figure6).

Table 17: Scoring of MMP-9 in bladder cancer patients according to invasion of tumor.

MMP-9 expression	Tu.invasion		Total (%) .No
	Invasion (%) .No	Non -invasion (%) .No	
High expression >4 – 12	(7.4) 2	(3.3)1	(5.26)3
Lowexpression0 – 4	(92.6) 25	(96.6) 29	(94.73)54
(%) .Total No	(47.36) 27	(52.63)30	(100)57
Chi-square P value	1.14 (not significant) 0.286		

Scoring of MMP-9 in bladder cancer patients according to gender

Scoring of MMP-9 shows highly significant difference (P =0.000) with gender of patients, on which high expression were noted in (4.0%) of male and (14.2%) of female patients, as seen in (Table18).

Table18: Expression of MMP-9 in bladder cancer patients according to gender

MMP-9 expression	Male No. (%)	Female No. (%)	Total No. (%)
High expression (>4 – 12)	2 (4.0)	1(14.2)	3(5)
Low expression (0 –4)	48 (96.0)	6 (85.8)	54(94.73)
Total No. (%)	50(87.71)	7(12.28)	57(100)
Chi-square P-value	37.3 0.000 (highly significant)		

The relation of risk factors for TCC with MMP-9 expression in tumor tissue

Thirty four cases out of 57 cancer patients were smokers and high expression of MMP-9 was seen in 1 (2.9%) of them, while high expression of MMP-9 in non-smoker cancer patients was 2 (8.6%) of 23, with Chi –square test showed significant difference between MMP-9 over expression and smoking(P= 0.021). Nine cases of bladder cancer patients had a history of schistosomiasis, they showed high expressionin 1 (11.1%), while in 48 non- schistosomal bladder cancer cases, overexpression was detected in 2 (4.2%), statistically there was a significant association (P= 0.021). As well, a significant increase was noted in expression of this factor in patients with family history of malignancy (P= 0.000). On other hand, UTI as well as history of stone revealed no significant difference between groups (Table 19).

Table19: Scoring of MMP-9 in bladder cancer patients in relation to risk Factors.

Risk factors	MMP-9 expression			Statistical analysis
	Low/negative Expression 0-4	High Expression >4 – 12	Total No. (%)	
Smoking				Chi-square 5.36
Yes No. (%)	33(97.1)	1 (2.9)	34(59.64)	P= 0.021
NO No. (%)	21 (91.2)	2 (8.6)	23(40.35)	(significant)
S. heamatobium				Chi-square 5.36
Yes No.(%)	8 (88.9)	1 (11.1)	9 (15.78)	P= 0.021
NO No. (%)	46 (95.8)	2 (4.2)	48(84.21)	(significant)
UTI				Chi-square 0.500
Yes No.(%)	27(93.1)	2 (6.8)	29(50.87)	P= 0.480
NO No.(%)	27 (96.4)	1 (3.5)	28(49.12)	(not significant)
Stones				Fishers' exact
Yes No.(%)	6(100)	0	6(10.52)	p= 1.000
NO No. (%)	48 (94.23)	3 (5.8)	51(89.47)	(not significant)
FamilyHistory				Chi-square 295.
Yes No.(%)	6 (85.8)	1 (14.2)	7(12.28)	P= 0.000
NO N o. (%)	48 (96.0)	2 (4.0)	50(87.71)	(Significant)
Total No.(%)	54(94.73)	3(5.26)	57(100)	

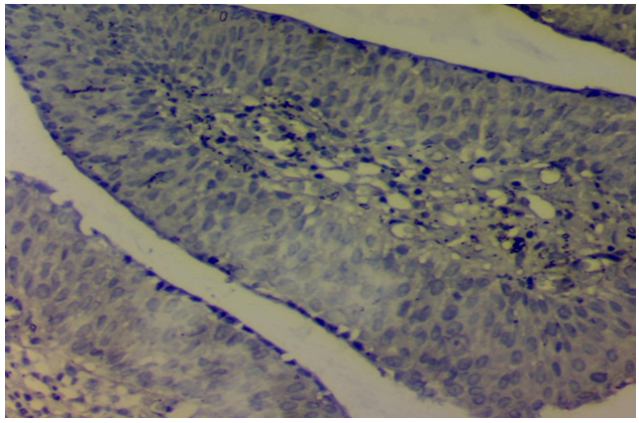

Fig5: Transitional cell carcinoma (TCC), low grade, showing no staining, (Immunohistochemical stainingfor MMP-9,X100)

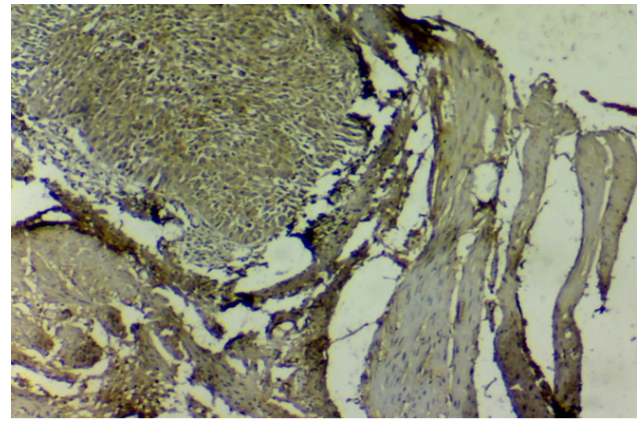

Fig 6: Transitional cell carcinoma (TCC), High grade, muscle invasion, showing elevated MMP-9 expression in the cytoplasm of cancer cells, (Immunohistochemical staining for MMP-9, X100)

Discussion:

Result of MMP-9 in urine and IHC test:

The common malignancy involving the urinary system is Bladder cancer [20]. Less accuracy in bladder cancer diagnosis with current urine-based assays, so there is a continue in search for improvement biomarkers, through proteomic and genomic profiling of urine, with many biomarkers associated with the presence of BCa, like MMP-9[21]. Many studies demonstrate that, in the progression and development of many neoplasias, such as bladder cancer the expression of MMP-9 has been implicated[22], breast cancer [23], nasopharyngeal carcinoma[24], head and neck squamous cell carcinoma[25],and lung cancer [26].

In this study the (mean+ SD)pg/ml of urine MMP-9 results for recurrence cases and newly diagnosed were(7368.3±1730.19 and 6176.8±2366.9 respectively) which was significantly higher in recurrent cases, but the level was significantly getting down in healthy group(1131.53 ±1554.80) when compared with newly and recurrence group ($P < 0.001$), these finding were comparable to other studies which consider MMP-9 urinary level was able to discriminate between bladder cancer patients and healthy control [21,18,27,28],as it was known that MMP-9 mediate growth factors activation, release of angiogenic factors or destroy the chemokine gradient development by host immune response and suppress apoptosis of tumor cell[29, 30], but in contrast to the finding of this study[31] revealed that patients with newly diagnosed bladder cancer as well as, with recurrent disease, had equivalent levels of urinary MMP-9.

In high grade TCC, urine level of MMP-9 (7169.7 pg/ml)was increased (but non-significantly)in comparison to that of low grade (6419.5pg/ml), as well a similar finding were noted in muscle invasion in comparison with non- muscle invasion(7385.7vs6251.5) this was similar [32], however[18,28] found that ELISA levels of urine MMP-9 were correlated to stage and grade of the disease. In the urine of bladder cancer patients, the detection of MMP-9 bounded to neutrophil gelatinase-associated lipocalin (NGAL) forming a complex that has the the ability to protect MMP-9 from auto degradation, and enhance its activity that is associated with metastasis [33,34] in addition another confirmation that MMP-9 has a role in tumor progression was the detection of non- significant difference in its level in both high grade and muscle invasion bladder cancer cases [32]. The urine level of MMP-9 at cut-off value (2616pg/ml) was highly significant ($P=0.000$) in bladder cancer patients with high sensitivity and specificity (93.8%, 87.5%) respectively,when compared to healthy group which indicate that significant increase in cytokines level concentration as an early sign for diagnosis of bladder cancer[35], however,[21] reported uMMP-9 sensitivity of 56% and specificity of 92% but uMMP9 data generated an AUROC of 0.533, sensitivity of 45%, specificity of 76% according to[28], and[27]reported sensitivity and specificity of (64.29% and 100%)respectively.

On regard to expression of MMP-9 in bladder tissues of patients, it was noted that high expression were present in only1(3.5%)of newly diagnosed cases, 2(6.8%) of recurrence and was low/ negative in all normal autopsies with non–significant difference among groups, the other non-significant correlation were noted between its expression with histological grade and muscle invasion as seen in (Table16, Table17), that is to say, most of tumor cells were in low expression of MMP-9 and that was in agreement with other study reported that MMP-9 staining IHC was unrelated to tumor stage , grade , multiplicity , or recurrence rate but associated with tumor size [31,32], as well urine concentrations of MMP-9 detected in samples of bladder cancer patients was in no correlation with immunehistochemical status of bladder tumor tissue sections, of which the frequency and staining intensity is graduated from absent, low then moderate to intense while ELISA measures the total of MMP-9 protein concentrations present in urine[31]. Opposite to this finding [22] reported a positive association of MMP-9 expression with higher stage of cancer, of which it is associated with cell surface and facilitate the invasion of cancer cells through epithelial cell layers into nearby stroma and blood vessels, as well other study demonstrated overexpression of this protein in human tumors [23,36], as it usually participate in angiogenesis and tumor growth [37]. In correlation with studied risk factors a significant associations between high expression of MMP-9 in IHC of tissue taken from cancer patients with Schistosoma and family history of cancer ($P= 0.02$ and 0.000 respectively), the higher expression of MMP-9 in schistosomiasis may be explained as the effect of transcriptional regulation of MMP-9by inflammatory cytokines such as interleukins and TNFα [38,39,31], while recent study of[27] on this marker demonstrated a much higher level in non-schistosomal bladder cancer, however,[32] showed no correlation between uMMPs and bilharziasis. The conclusion of this study is that MMP-9 in urine measured by ELISA showed high specificity in bladder cancer patients than normal, but low potential in diagnosis of high grade, muscle invasion and low grade, non-muscle invasion however, yet it is a good marker in detection of newly diagnosed cancer patients and recurrent cases. Immunohistochemistry of MMP-9 showed low expression in both tumor tissue and normal urothelium biopsy. Gender, age, smoking, schistosomiasis, family history and UTI were associated with bladder cancer and correlated with our markers in different manner.

References:

1. Siegel, R.; Naishadham, D. and Jemal, A. (2013). Cancer statistics. CA Cancer J Clin, 63(1):11-30.
2. Al-Shwani, M.AU.R.(2013).Transurethral management of bladder cancer (2003-2008) at Department of urology, Azadi teaching hospital in Kirkukcity, Iraq. Medical Journal of Tikrit, 19(1):159-163.
3. Botelho, M.C.; Veiga, I.; Oliveira, P.A.; Lopes, C.; Teixeira, M.; daCosta, J.M.C. and Machado, J.C.(2013). Carcinogenic ability of Schistosoma haematobium possibly through oncogenic mutation of KRAS gene. Adv Cancer ResTreat, 2013: 876585.
4. Al-Fouadi, A. and Parkin, D.M.(1984). Cancer in Iraq: Seven years' data from the Baghdad tumour registry.International Journal of Cancer, 34(2): 207–213
5. Abdul–muhsin, A.S. (2008). Schistosoma –Associated Bladder Cancer in Iraq Single Center Pathologic Review. Iraqi postgraduate Medical Journal, 7(2):174-177.
6. Khalida, K.M.(2005).Assessment of Factors That Contribute to Bladder Cancer. nursing national Iraqi specility, 18 (2): 1-10.
7. Zarzour, A.H.; Selim, M.; Abd-Elsayed, A.A.; Hameed, D.A.and Abdelaziz M.A.(2008). Muscle invasive bladder cancer in Upper Egypt: the shift in risk factors and tumor characteristics BMC Cancer, 8:250.Doi:10.1186/1471-2407-8-250.
8. Ovsiannikov, D.; Selinski, S.; Lehmann, M.; Blaszkewicz, M.; Moormann, O.; Haenel, M.W.; Hengstler, J.G.and Golka, K.(2012).Polymorphic enzymes, urinary bladder cancer risk, and structural change in the local industry. J Toxicol Environ HealthA, 75(8-10):557–65.
9. Montironi, R. and Lopez-Beltran, A. (2005). The 2004 WHO classification of bladder tumors: a summary and commentary. Int J Surg Pathol, 13:143–153.
10. Ghafouri-Fard, S.; Nekoohesh, L.and Motevaseli, E.(2014). Bladder Cancer Biomarkers: Review and Update. Asian Pac J Cancer Prev, 15(6):2395-2403.
11. Babjuk, M.; Osterlinck, W.; Sylvester, R.; Kaasinen, E.; Bo¨hle,A. and Palou Redorta ,J.(2008).EAUGuidelines on Non-Muscle-Invasive UrothelialCarcinoma of the Bladder.European Urolog ,54:303–314.
12. Goodison, S.; Chang, M.; Dai, Y.; Urquidi, V. and Rosser, Ch.J.(2012). A Multi-Analyte Assay for the Non-Invasive Detection of Bladde Cancer. PLoS One,7(10):e4746. Doi: pone.004746910.1371/journal.
13. Goodison, S.; Rosser, C.J. and Urquidi, V. (2013). Bladder cancer detection and monitoring: assessment of urine- and blood based marker tests. Mol Diagn Ther, 17 (2):71-84.
14. Attarbashii, A.M. (2013). Urethrocystoscopic Features in Cases with Urinary Bladder Cancer in Mosul: "a Case Series Study"Medical Journal of Tikrit, 19(1): 134-141.
15. Mohammed, S.M.; Ali, S.H.and Kamal, M.S.(2014).Serum lysis oxidase ,nitrate oxidase and IL-8 as possible biological markers of TCC of the bladder. I J S N, 5(1):82-86.
16. Nasterlack, M.; Feil, G.; Leng, G.; Pesch, B.andHuber, S.(2011). Bladder cancer screening with urine-based tumour markers-occupational medical experience.Aktuelle Urol, 42(2): 128-34.
17. Shariat, A.; Karam, J.A.; Lotan, Y. and Karakiewizc, P.I.(2008). "Critical Evaluation of Urinary Markers for Bladder Cancer Detection and Monitoring". Rev Urol, 10(2): 120–135.
18. Chen, L.M.; Chang, M.; Dai, Y.; Chai, K.X.; Dyrskjøt, L.; Sanchez-Caebayo, M.; Szarvas, T.Z., ;warthoff, E.C.; Lokeshwar, V.; Jeronimo, C.; Parker, S.; Ross, S.; Borre, M. Orntoft, T.F.; Jaeger, T.; Beukers, W.; Lopez, L.E.; Henrique, R.;Young, P.R.; Urquidi, V.; Goodison, S.and Rosser, C.J.(2014).External validation of a multiplex urinary protein panel for the detection of bladder cancer in a multicenter cohort. Cancer Epidemiol Biomarkers Prev, 23(9):1804-12. Doi:10.1158/1055-9965.EPI-14-0029.
19. Suvarna, S.K.; Layton, C. and Bancroft, J.D.(2013). Bancroft's theory and Practice of histological techniques, 7thed. Churchil Livingstone Elsevier, England.
20. Anastasiadis, A.and deReijke, T.M.(2012). Best practice in the treatment of non-muscle invasive bladder cancer therapy. Adv Urol, 4(1): 13–32. Doi: 10.1177/1756287211431976.
21. Urquidi, V.; Chang, M.; Dai, Y.; Kim, J.; Wolfson, E.D.; Goodison, S.and Rosser, C.J.(2012).IL-8 as a urinary biomarker for the detection of bladder cancer. BMCUrology,12:12. Doi: 10.1186/1471-2490-12-12.
22. Zhang, G.; Gomes-Giacoiae, E.; Dai, Y.; Lawton, A.; Miyake, M.; Furuya, H.; Goodison, S.and Rosser, C.J.(2014).Validation and clinicopathologic associations of a urine-based bladder cancer biomarker signature. Diagnosticpathology, 9:200. Doi:10.1186/s13000-014-0200-1.
23. Yousef, E.M.; Tahir, M.R.; St-Pierre, Y.and Gaboury, L.A.(2014). MMP-9 expression varies according to molecular subtypes of breast cancer . BMC Cance, 14:609. Doi:10.1186/1471-2407-14-609.
24. Liu, Z.; Li, L.; Yang, Z.; Luo, W.; Li, X.; Yang, H.; Yao, K.; Wu, B. and Fang, W.(2010). Increased expression of MMP9 is correlated with poor prognosis of nasopharyngeal carcinoma. BMC Cancer, 10:270.
25. Ruokolainen, H.; Paakko, P. and Turpeenniemi-Hujanen, T.(2005). Serum matrix metalloproteinase-9 in head and neck squamous cell carcinoma is a prognostic marker. Int J Cancer, 116(3):422–427.
26. Hoikkala, S.; Paakko, P.; Soini, Y.; Makitaro, R.; Kinnula, V.and Turpeenniemi-Hujanen,T.(2005).Tissue MMP-2/TIMP-2-complexare betterprognostic factors than serum MMP-2, MMP-9 or TIMP-1 in stage I-III lung carcinoma. Cancer Lett, 236 (1):125–
27. El-Sharkawi, F.; El Sabah, M.; Hassan, Z. and Khaled, H. (2014). The biochemical value of urinary metalloproteinases 3 and 9 in diagnosis and prognosis of bladder cancer in Egypt. Journal of Biomedical Science, 21:72. Doi: 10.1186/s12929-014-0072-4
28. Rosser, C.J.; Dai, Y.; Miyake, M. Zhang, G.and Goodison, S.(2014). Simultaneous multi-analyte urinary protein assay for bladder cancer detection. BMCBiotechnology, 14:24.Doi: 10.1186/1472-6750-14-24.
29. Egeblad, M.and Werb, Z. (2002).New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer, 2(3):161–174.
30. Hojilla, C.V.; Mohammed, F. F. and Khokha, R.(2003). Matrix metalloproteinases and their tissue inhibitors direct cell fate during cancer development. Br. J. Cancer, 89(10): 1817-21.
31. Durkan, G.C.; Nutt, J.E.; Marsh, C.; Rajjayabun, P.H.; Robinson, M.C.; Neal, D.E.; Lunec, J. and Mellon, J.K. (2003). Alteration in Urinary Matrix Metalloproteinase-9 to Tissue Inhibitor of Metalloproteinase-1 Ratio Predicts Recurrence in Nonmuscle-invasive Bladder Cancer.Clin Cancer Res, 9(7): 2576.
32. Mohammed, M.A.; Seleim, M.F.; Abdalla, M.S.; Sharada, H.M. and Abdel Wahab,A.A.(2013).Urinary high molecular weight matrix metalloproteinases as non-invasive biomarker for detection of bladder cancer.BMCUrology,13:25.Doi:10.1186/1471-2490-13-25.
33. Yan, L.; Borregaard, N.; Kjeldsen, L. and Moses, M.A.(2001). The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neu-

trophil gelatinase-associated lipocalin (NGAL).Modulation of MMP-9 activity by NGAL. J Biol Chem, 276(40):37258-37265.
34. Provatopoulou, X.; Gounaris, A.; Kalogera, E.; Zagouri, F.; Flessas, I.; Goussetis, E.; Nonni, A.; Papassotiriou, I. and Zografos G.(2009): Circulating levels of matrix metalloproteinase-9 (MMP-9), neutrophil gelatinase-associated lipocalin (NGAL) and their complex MMP-9/NGAL in reast cancer disease. BMC Cancer, 9:390. Doi:10.1186/1471-2407-9-390.
35. Fata, R.D.; Ferruelo, A.; Andrés, G.; Gimbernat, H.; Sánchez-Chapado, M. and Angulo, J.C.(2013).The role of matrix metalloproteinase MMP9 and TIMP-2 tissue inhibitor of metalloproteinases as serum markers of bladder cancer.ActasUrolEsp, 37(8):480-8.
36. Olsson, H.; Hultman, P.; Monsef, N.; Rosell, J. and Jahnson,S. (2012). Immunohistochemical Evaluation of Cell Cycle Regulators: Impact onPredicting Prognosis in Stage T1 Urinary Bladder Cancer ISRN Urology, 2012 (2012), Article ID 379081, 7 pages.
37. Vasala, K.(2008).Matrix metalloproteinase MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 in bladder carcinoma. Academic Dissertation Acta. Univ.Oul., Finland.
38. Leber, T.M.and Balkwill, F.R.(1998).Regulation of monocyte MMP-9 production by TNF-alpha and a tumour-derived soluble factor (MMPSF). Br J Cancer, 78(6):724-732.
39. Gerhards, S., Jung, K., Koenig, F., Daniltchenko, D., Hauptmann, S., Schnorr, D. and Loening ,S.A.(2001). Excretion of matrix metalloproteinases2 and 9 in urine is associated with a high stage and grade of bladder carcinoma. Urology, 57(4):675-679.

المؤشر البولي (MMP-9) وسرطان المثانة

ناهي يوسف ياسين[1]، رنا سامي عزيز[2]، رفيف صبيح الشوك[3]، ،ميسون علي سليم[3] ، عصام سلمان العزاوي[4] ،هدى سعدون البيلتي[3]

1 المركز العراقي لبحوث السرطان والوراثة الطبية بغدادالعراق
2 فرع النسيج المرضي المختبرات التعليمية مستشفى اليرموك التعليمي بغداد العراق
3 فرع الاحياء المجهرية كلية الطب الجامعة المستنصرية بغداد العراق
4 فرع الجراحة البولية كلية الطب الجامعة المستنصرية بغداد العراق

الخلاصه:

سرطان المثانة هو مرض معقد وقدأجريت هذه الدراسة لتقييم أهمية مستوى الفرق في المؤشر الحيوي MMP-9 والذي له دور في تطور الورم في مرضى سرطان المثانة في بول 48 مريضا بسرطان المثانة و 40 من الاصحاء بواسطة اختبار الاليزا وفي 57 خزعة من سرطان المثانة و 40 من الظهارة البولية الطبيعية من المتوفين في الطب العدلي من خلال فحص التعبير المناعي النسيجي Immunohistochemistry. وأشارت النتائج أن التركيز البولي لل-MMP 9 أظهر خصوصية عالية في تمييز مرضى سرطان المثانة، و في التمييز بين (المشخصين حديثا مقابل متكررين الاصابة)، إلا أن التركيز البولي والتعبير المناعي النسيجي لل MMP-9 ليس له صلة بدرجة تصنيف ومرحلة الورم. وأظهرت بعض عوامل الخطر المصاحبة لسرطان المثانة مثل البلهارسيا والتاريخ العائلي للسرطان وجود علاقة وثيقة مع MMP-9