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Macrophage Migration Inhibitory Factor Level and Its Receptor CD74 and CD44 Expression in Urinary Bladder Carcinoma

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A B S T R A C T

Macrophage migration inhibitory factor (MIF) is multifunction'sproinflammatory cytokine. MIF binds to CD74 which form a complex with CD44 characterized by high affinity for inducing signaling cascade. This study was designed to evaluate the role of MIF in bladder cancer progression. Seventy three biopsy and blood samples have been collected from urinary bladder carcinoma (UBC) patients after cystoscopy surgery and 10 normal bladder biopsies collected from forensic autopsy. MIF level was estimated in seventy three patients with (UBC) at different stages, 32 patients with urinary bladder disorders (UBD) and 30 healthy volunteers by sandwich ELISA. Sections were stained immunohistochemically for CD74and CD44. MIF level was significantly higher in UBC in comparison with UBD and healthy especially in stages T3 and T4 (high grade).Fifty (68.49) % out of 73 UBC gave positivity staining of CD74 and score+3 showed the highest frequency while all normal tissues expressed negative pattern . Moreover, 64(87.7) % of UBC and 9 (90) % normal urothelium gave positive staining results for CD44and score+2represented the highest frequency among scores. Serum level of MIF is significantly elevated with higher grade of advanced stage than low grade of primary stage .Over expression of MIF with higher expression of CD74 and CD44 may promote malignant cell transformation in urinary bladder carcinoma.

Introduction

Cancer is the uncontrolled proliferation of the cells and It's progression is a complex multi-step process that consists of transformation, tumor growth, invasion and metastasis(1,2).Bladder cancer is the 2nd most frequent malignancy of the genitourinary tract and the fourth most common cancer in men. The most common presenting pathology of bladder tumors is transitional cell carcinoma (90% of cases), while squamous cell carcinomas, adenocarcinomas and other rare subtypes comprise a minority of case. Tumors of the bladder rarely occur before the age of 40 years arising most commonly in the seventh decade of life. Smoking and occupational exposure to environmental carcinogens like aromatic amines, radiation and

chemotherapy are main factor strongly associated with bladder cancercases (3,4,5).

MIF is proinflammatory cytokine shown to promote tumorigenesis discovered in 1966. The *MIF* gene coded 12.5 kDa polypeptide consists of 115 amino acids which lies on chromosome 22q11.2 and regulation of the gene by two polymorphic sites in the promoter region(6,7). MIF was thought to inhibit the migration of macrophages thus hence derived it's name and helps macrophage in its functions such as phagocytosis, adherence, spreading, and metabolism. MIF is thought to be released from monocytes/macrophage in presence of glucocorticoids which acts as the inflammatory mediator to stimulate the expression of other cytokines like TNF-α, IL-1, IL-6. It's binding receptor was identified recently CD74 which is the cell-surface form of the MHC class II invariant chain that signal transduction requires the recruitment and activation of an additional protein CD44(8,9).

MIF over expression in developing malignancies may act in concert to facilitate increased tumor growthwhich present an important link between inflammation and cancer due to its pro-inflammatory role. Its molecular mechanisms involve, among others, the inhibition of p53 which promote tumor cell proliferation, cell survival and tumor-associated neoangiogenesis(10,11)

CD74 is an integral membrane protein has a molecular weight 33KD which consists of 296 amino acids. It's genewhich is located on chromosome 5q32(12,13).Cluster of differentiation 74 performs multiple roles in B cells, T cells, and antigen-presenting cells within the immune system, It has two main functions MHC II chaperon andCD74 as cell surface receptor.The cytokine MIF was found to be the natural ligand of CD74. MIF binds to the extracellular domain of CD74 with high affinity and initiates a signaling cascade.CD74 forms a complex with CD44, which is essential for the MIF-induced signaling cascade (14,15).

CD44 is a transmembrane glycoprotein that has been postulated to play important roles in a variety of biological processes in healthy and diseased tissues.CD44's encoding gene is located on chromosome 11p3and consists of at least 21 exons.CD44 is a widely expressed cell surface antigen that serves as an adhesion molecule in cell-cell and cell-matrix interactions Expression of the CD44gene becomes disorderly in the early stages of carcinogenesis, and excessive quantities of many inappropriate alternatively spliced CD44 variants accumulate in cancer cells which promotes signaling pathways that induce tumor growth, survival as well as cancer cell invasion (16,17,18).

Subjects and Method

Subjects

One hundred and thirty five subjects divided into three groups. Seventy three patients with different stages of UBC, 60 male and 13 female with an average age 65.2 years and a range from (43 to 85) years, 32 patients had UBD and 30 healthy control. Subjects collected through the period from March 2014 to the November 2014. They attended to Urology Unit at Al-Yarmook hospital and Al-Jabchi privet hospital. The tumors were graded as low or high on the basis of WHO classification criteria.

Estimation of Serum MIF

Serum level of MIF was measured in sera of UBC, UBD and healthy by using ELISA kit (R&D, USA), based upon coating wells of a high protein binding ELISA plate with monoclonal antibody specific for human

MIF. All reagents, working standards, and samples were prepared. One hundred µl of assay Diluent was added to each well and 50 µl of standard then 50µl of sample were added to appropriate well followed by covering the plate with the adhesive strip and incubates for 2 hours at room temperature then washed four times with wash buffer. Two hundreds µl of cytokine conjugate was added to each well and covered with a new adhesive strip , incubated for 2 hours at room temperature on the shaker then washed four times .Two hundreds µl of substrate solution was added to each well and incubated for 30 min at room temperature in dark.Finally,50 µl of Stop Solution was added to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.The optical density of each well was measured within 30 minutes, using a microplate reader set to 450 nm.

Immunohistochemical Staining

Immunohistochemical staining was performed by using labeled streptavidin-biotin immunostaining method. Briefly, 5-mm thick sections were deparaffinized and rehydrated. Most formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining. Antigen retrieval was performed by using Tris-EDTA buffer pH=9 for CD74 and Sodium Citrate buffer pH=6 for CD44, then placed in microwave histoprocesser at 850 w for 20 min. After cooling for 10-15 min at 4°C, the slides removed and washed by wash buffer for 10 min. Hydrogen Peroxidase block was added for 10 min, Protein Block was applied and incubated for 10 min at room temperature, then washed 3 times in PBS buffer. Diluted mouse monoclonal primary antibody at a ratio (1/50 for CD74 and 1/200 for CD44) was added to tissue sections and incubated 20 min for CD44 while incubation period was 30 min for CD74 primary antibody then washed 4 times in PBS buffer. Biotinylated Goat Anti-Mouse was applied and incubated for 15 min in humidity chamber at room temperature after washed 4 times in PBS buffer; Streptavidin Peroxidase was applied and incubated for 10 min at room temperature then rinsed 4 times in PBS buffer. Two hundreds µl of DAB solution was and incubated 10 min at room temperature. Sections were rinsed 4 times in PBS buffer, and counter stain was added for 2 min at room temperature and washed in tap water. The slides were dehydrate and mounted with DPX and coverslip. Then Slides were examined by light microscope 10X, 20X and 40X. Results were compared with positive control which determined according to leaflet of the kit. Positivity was assessed semi-quantitatively by the intensity and percentage of staining. Score was determined for CD74 and CD44 according to a scale when membrane of the cell has been stained with brown color (19,20,21).

Results and Discussion

Results in table (1) showed the mean level of serum MIF in UBC patients was significantly higher than that observed in UBD patients and healthy control (55.57 and 39.08 *vs*. 18.53) pg/ml respectively.

The relationship between serum mean level of MIF and tumor stages of UBC patients showed that the highest level was recorded in sera of UBC patients in stage T3(91.48 pg/ml),then T4(86.62 pg/ml), T2(64.21 pg/ml) and T1(43.06 pg/ml) while in Tawas (37.63 pg/ml) as shown as in table (2). Result illustrated in table (3) showed that mean serum level of MIF was significantly elevated in higher grade of advanced stage (69.26 pg/ml) than low grade of primary stage (38.98 pg/ml).

Table.1 Serum Level of MIF in UBC, UBD and Control

Group	Mean ± S.E of MIF pg/ml	Sig. between Groups	p value
UBC	**55.91 ±2.97 a**	(LSD = 6.721)	0.001
UBD	**39.08±2.37 b**		
Healthy	**18.53±2.26 c**		

Different letters represent significant differences between means (Duncan test)

Table.2 Serum Level of MIF in UBC Patient at Different Stages

Stage	No.	Mean ±S.E of MIF pg/ml	Significant between Groups	P Value
Ta	**12**	**37.63 ± 5.06 c**	(LSD = 8.963)	0.0012
T1	**28**	**43.06 ± 4.04 c**		
T2	**22**	**64.21±3.66 b**		
T3	**7**	**91.48 ±5.19 a**		
T4	**4**	**86.62±6.84 a**		

Different letters represent significant differences between means(Duncan test)

Table.3 Serum Level of MIF in UBC Patient at Different Grades

Grade	No.	Mean ± MIF pg/m	Sig .between Groups	P value
Low	**33**	38.98±3.66 b	(LSD = 9.205)	0.0001
High	**40**	69.26 ±3.17 a		

Different letters represent significant differences between means(Duncan test

Table.4 CD74 Expression in UBC and Control

Group	CD 74 Expression				Total		P-Value
	Positive		Negative				
	No.	%	No.	%	No.	%	
UBC	50	68.49	23	31.51	73	88	0.0027 *
Control	0	0.00	10	100	10	12	
Total	50	60.2	33	39.8	83	100	

* (significant)

Table.5 Frequency of CD74 Scores in UBC and Control

CD 74 Score	UBC		Control		Total	
	No.	%	No.	%	No.	%
Scorer 0	23	31.5	10	100	33	39.8
Score +1	12	16.4	0	0.0	12	14.5
Score +2	13	17.8	0	0.0	13	15.6
Score +3	19	26.1	0	0.0	19	22.9
Score +4	6	8.2	0	0.0	6	7.2
Total	73	88	10	12	60	100
p-value	0.0001 *					

* (significant)

Table.6 Association Between CD74 Expression and UBC Stages

Stage	CD74 expression Score										Total positive	
	Negative		+1		+2		+3		+4		No.	%
	No.	%	No.	%	No.	%	No.	%	No.	%		
Ta	8	34.8	1	8.3	3	23.1	0.0	0.0	0.0	0.0	4	8
T1	12	52.2	8	66.7	4	30.8	4	21.1	0.0	0.0	16	32
T2	2	8.7	1	8.3	5	38.5	8	42.1	6	100	20	40
T3	0	0.0	2	16.7	0.0	0.0	5	26.3	0.0	0.0	7	14
T4	1	4.3	0.0	0.0	1	7.6	2	10.5	0.0	0.0	3	6
Total	23	31.5	12	16.4	13	17.8	19	26.1	6	8.2	50	100
P value	0.001*											

* (significant)

Table.7 Association Between CD74 Expression and UBC Grade

Grade	CD74 expression Score										Total positive	
	Negative		+1		+2		+3		+4		No.	%
	No.	%	No.	%	No.	%	No	%	No.	%		
Low	19	82.6	10	83.3	4	30.8	0	0.0	0	0.0	14	28
High	4	17.4	2	16.7	9	69.2	19	100	6	100	36	72
Total	23	31.5	12	16.4	13	17.8	19	26.1	6	8.2	50	100
P value	0.001**											

*significant

Table.8 CD44 Expression in UBC Patients and Control

Group	CD 44 Expression				Total		P-Value
	Positive		Negative				
	No.	%	No.	%	No.	%	
UBC	64	87.7	9	12.3	73	88	0.0001 *
Control	9	90	1	10	10	12	
Total	73	88	10	12	83	100	

*significant

Table.9 Frequency of CD44 Score in UBC and Control

CD 44 Score	UBC		Control		Total	
	No.	**%**	**No.**	**%**	**No.**	**%**
Score 0	9	12.3	1	10	10	12
Score +1	10	13.7	1	10	11	13.3
Score +2	24	32.9	5	50	29	35
Score +3	23	31.5	3	30	26	31.3
Score +4	7	9.6	0	0.0	7	8.4
Total	73	100	10	100	83	100
p-value	0.00361 **					

*significant

Table.10 Association Between CD44 Expression and UBC Stages

Stage	CD44 expression Score										Total of positive	
	Negative		+1		+2		+3		+4		No.	%
	No.	%	No.	%	No.	%	No.	%	No.	%		
Ta	2	22.2	0	0.0	3	12.5	6	26.1	1	14.3	10	15.6
T1	0	0.0	2	20	9	37.5	13	56.6	4	57.1	28	43.8
T2	4	44.5	6	60	9	37.5	1	4.3	2	28.6	18	28.1
T3	1	11.1	2	20	2	8.3	2	8.7	0	0.0	6	9.4
T4	2	22.2	0	0.0	1	4.2	1	4.3	0	0.0	2	3.1
Total	9	12.3	10	13.7	24	32.9	23	31.5	7	9.6	64	100
P value	0.001*											

*significant

Table.11 Association Between CD44 Expression and UBC Grade

Grade	CD44 expression Score										Total of positive	
	Negative		+1		+2		+3		+4		No.	%
	No.	%	No.	%	No`.	%	N0.	%	No	%		
Low	2	14.3	2	14.3	10	37.5	16	85.7	3	42.9	31	48.4
High	7	85.7	8	85.6	14	62.5	7	14.3	4	57.1	33	66
Total	9	12.3	10	13.7	24	32.9	23	31.5	7	9.6	50	100
P value	0.461*											

* Not Significant ; (P<0.01)

Figure.1 Negative Immunohistochemical Staining of CD74.A: Non Invasive TCC (Stage T0, Low Grade). B: Invasive TCC (Stage T2, High Grade) under 10 X

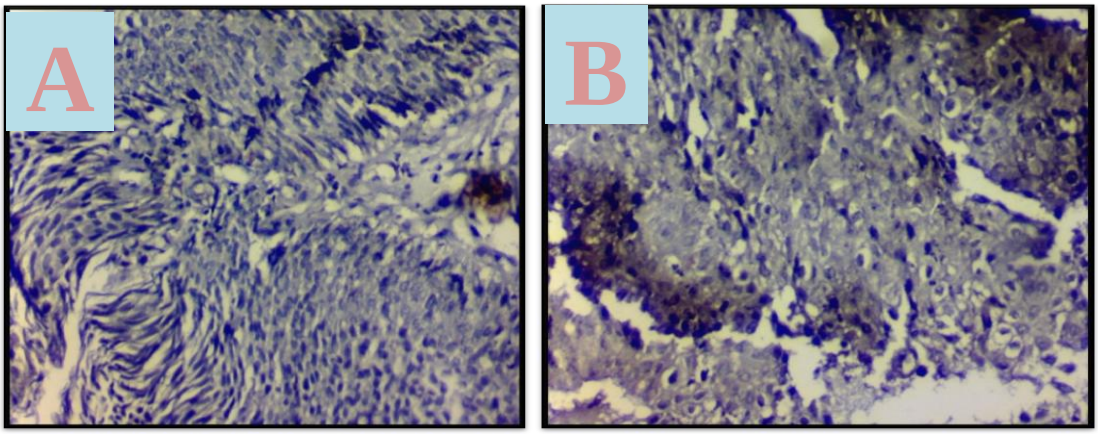


Figure.2 Immunohistochemicalstaining of CD74 (Invasive TCC Stage: T2, High Grade) Expressed Positive Staining (Score +3). A: Section under 10 X B: Section under 40 X

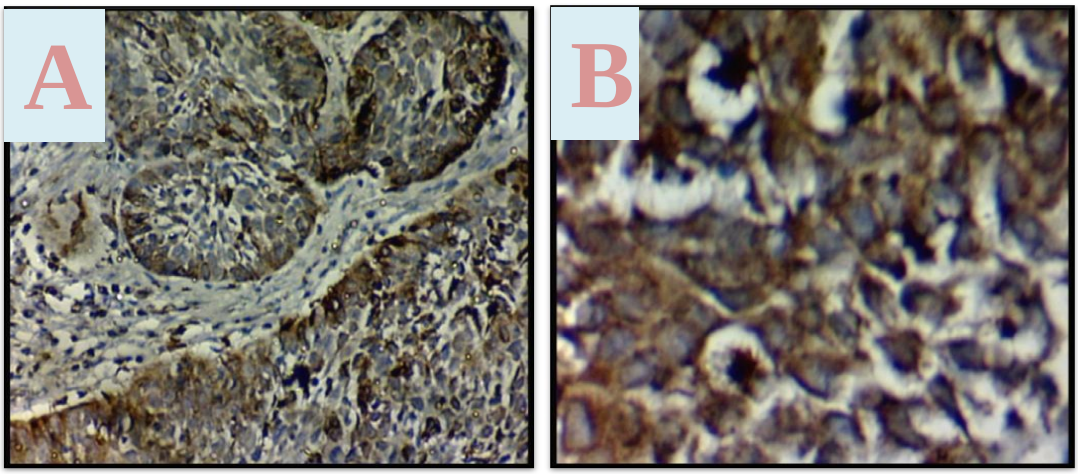


Figure.3 Positive Immunohistochemical staining of CD44 (Normal Urothelium):A: Section under 10X B: Section under 40X

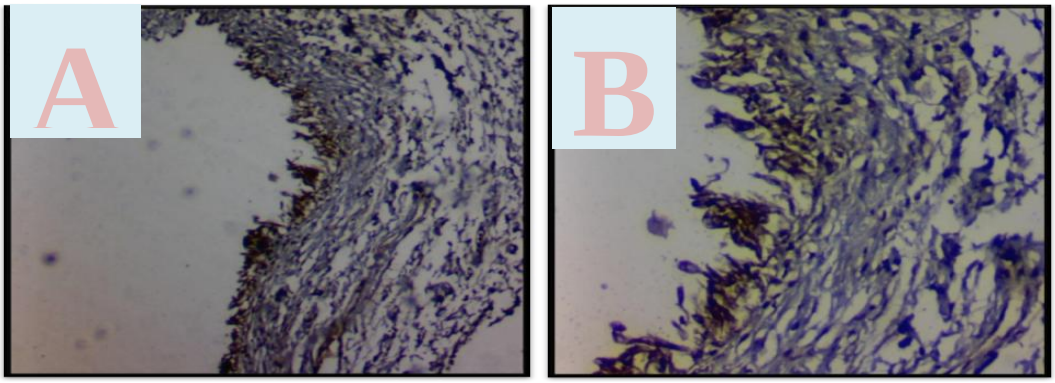


Figure.4 Positive Immunohistochemical Staining of CD44 (Invasive TCC, StageT2, High Grade, Score 4+).A: Section under 10X B:Section under 40X

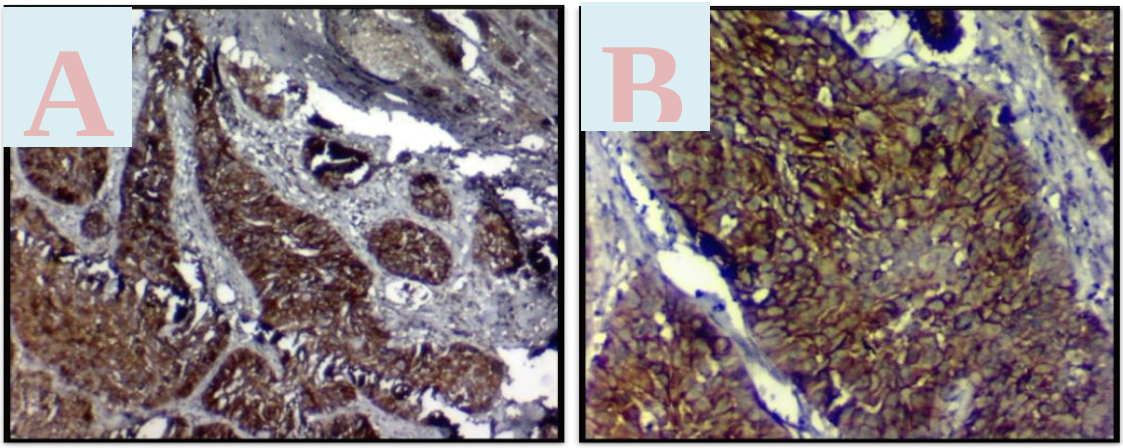
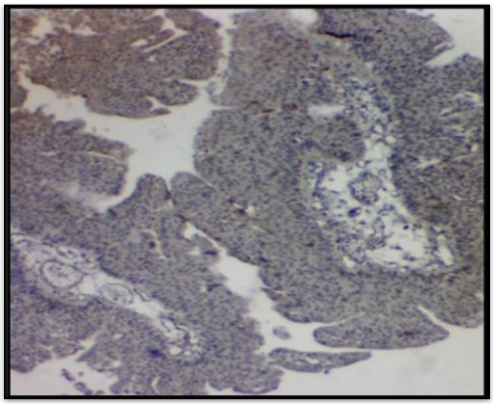


Figure.5 Negative Immunohistochemical Staining of CD44(Stage T1, Low Grade)



In order to study the expression of CD74 molecules, staining was done by using anti-CD74 clone then compared between CD74 expressions. Results have shown in table (4) exhibited positive staining 50(68.49) % with different scores and 23(31.51%) gave negative staining with highly significant differences, while all the bladder tumors free tissue expressed negative staining.

The positive score +3 represented the highest frequency (26.1 %) then score +2 represent (17.8%) followed by score +1 (16.4%). Score +4 represent the lowest frequency (8.2%) and negative score represent (31.51%) with highly significant differencesas shown in table (5) but also noticed some tumors had necrosis thus membrane of the cancerous cells not expressed the CD74 clearly.

In addition, result in table (6) showed higher positive expression of CD74 was observed in stage T2 and T1 (40 and 32) % respectively. Lower positive expression was recorded in stage T3, Ta and T4 (14,8 and 6) % respectively.

Results in table (7) revealed higher positive expression was recorded in high grade (72) % and (28) % in low grade. The highest negative expression in low grade was (56.6) % followed by (11.1) % in high grade as shown in Figure (1) and (2).

CD44 IHC Score

Results showed highly positive immunohistochemical expression of CD44 in bladder tumor tissues, 64 (87.7%) gave positive result of staining with different score as shown as in table (8) and 9(12.3%) (one with low grade tumor and eight with high grade tumor) gave negative result while 9(90%) out of ten normal urothelium gave positive expression with highly significant differences.

Results in table (9) revealed that and score +2 represented the highest frequency 24 (32.9%) followed by score +3(31.5%) ,score +1 (13.7%)and score +4(9.6%)while negative score represented 9(12.3%).

Results explained by table (10) demonstrated that the highest positive expression of CD44 was 28 (38.4) % out of 64 in T1 and 19 (26) % in T2 respectively followed by 10(13.7) % in Ta, 5(6.8) % in T3 and 2 (2.7) % in T4 respectively with highly significant differences.

Results exhibited that positive expression intensity of CD44 was 31 (48) % out of 64 lower in low grade than high grade 33 (52) % with no significant differences and negative expression was lower 2 (14.3)% out of 9 in low grade and higher 7 (85.7)% in high grade as shown in table (11) Figure (3),(4), and (5).

The present study demonstrated overexpression of MIF in advanced tumor stage and grade**.** MIF is the initial inflammatory mediator to stimulate the expression of other cytokines such as TNF-α and IL-1 via suppression of the anti-inflammatory actions of glucocorticoids. MIF has the potential to inhibin action of the tumor suppressor gene p_{53} and suppress transcriptional activity ofp_{21}. Macrophages lacking MIF are sensitized to p53-dependent activation-induced apoptosis while cells containing MIF are significantly more resistant. In the tumor microenvironment, bypass of p53 by high concentrations of MIF expressed intrinsically by transformed cells or provided by surrounding inflammatory cells would enhance cell proliferation, extend lifespan, create a deficient response to genotoxic damage and allow for the accumulation of oncogenic mutations (22,23).

MIF play a central role in uncontrolled tumor cell division, angiogenic stimulation or suppression of tumor cell immune surveillance. The MIF it's receptor CD74 when they bound,initiate survival pathways and cell proliferation thuswere highly expressed in invasive stages than non invasive tumors. Overexpressed in most tumor types has been shown to promote malignant cell transformation, inhibit tumor cell-specific immune cytolytic responses and strongly enhance neovascularization(24, 25).

Result disagreed with (26)who reported that the expression of MIF protein was found predominantly in tumor cell and inversely correlated with tumor stage and grade. The expression of MIF in non muscle invasive bladder cancer was more frequently than in the muscle invasive disease**.**

CD74 expression is increased in high-grade, invasive carcinoma of the bladder. Its expression was significantly associated with older age at diagnosis. Also, generally accepted with results reported by (27)who found that CD74 expression primarily on the malignant cells in the tumor would suggest that MIF might be working through antagonism of apoptotic pathways, or by autocrine regulation of angiogenic factor expression. In many tumors the malignant cells themselves formed advanced invasive tumor strongly expressed CD74 and a larger proportion of CD74-negative tumors were stage I-II.

Current findings on CD44 expression are in agreement with previously reported studies by (28)reported that there was high association between the expression level of CD44 and tumor type, grade and lymph node metastasis and may play important roles in cancer progression and metastasis. Also, agreed with (29) higher CD44 expression was revealed in low grade and noninvasive tumors and all tumors diagnosed as T1 stage showed 100% positive expression for CD 44.

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