

Urinary NDRG2 and MCM8 Gene Expression as New Noninvasive Biomarkers for Diagnosis and Differentiation between Muscle and Non-Muscle Invasive Bladder Cancer

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Noninvasive diagnosis of cancer bladder remains a challenge. The study aimed to evaluate the urinary gene expression of NDRG-2 (N-Myc downstream-regulated gene2) and MCM8 (the mini chromosome maintenance proteins) genes and their importance as novel urinary biomarkers for bladder cancer. In addition, to assess their diagnostic value in comparison with voided urine cytology is the focus of this work. The study included twenty healthy controls and fifty patients with bladder cancer. Quantitative real-time polymerase chain reaction (qRT-PCR) and voided urine cytology (VUC) were performed to demonstrate the NDRG2 and MCM-8 gene expression levels in the urine of healthy controls and bladder cancer patients. There was a statistically significant decrease in NDRG-2 gene expression in bladder cancer group (4.38 ± 0.66) compared to the control group (8.29 ± 1.67). Gene expression of MCM-8 showed a statistically significant increase in bladder cancer group (5.57 ± 0.79) in comparison to control group (4.55 ± 1.39) with a significant negative correlation ($r = -0.77$) between NDRG-2 expression levels and tumor grade in cancer group ($p < 0.001$), and a positive significant correlation ($r = 0.453$) between MCM-8 expression levels and tumor grade in cancer group ($p = 0.001$). NDRG-2 had the highest ability to predict bladder carcinoma (AUC of 1.0). In addition, the most precise differentiation between non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) with AUC of 0.814. Expression of NDRG-2 and MCM-8 may be novel potential noninvasive biomarkers for diagnosis and prognosis of bladder cancer and a good tool for differentiation between NMIBC and MIBC with NDRG-2 is the most precise for diagnosis and differentiation over MCM-8, VUC and combined use of NDRG-2 and MCM-8.

Keywords: Bladder Carcinoma; Mini Chromosome Maintenance Proteins; NDRG-2; Polymerase Chain Reaction; Voided Urine Cytology.

Bladder cancer (BC) is one of the most challenging expensive cancers to diagnose and treat. Its diagnosis depends on cystoscopy, which is invasive and expensive procedure, and voided urine

cytology that lacks sensitivity in low-grade bladder cancer. Therefore, there is a challenge to identify highly sensitive noninvasive new biomarkers for BC¹.

Muscle invaded BC (MIBC) is aggressive tumor with poor prognosis, a high risk of metastasis and recurrence. While Non-muscle invaded BC, (NMIBC) has a good prognosis. MIBC is usually treated by radical cystectomy and may be followed by postoperative chemotherapy, while NMIBC is commonly treated with transurethral resection. Therefore, an accurate early diagnosis of BC is a vital factor for clinical BC management especially when it can differentiate between MIBC and NMIBC².

Interestingly, research revealed variable correlations between N-myc downstream-regulated gene 2 (NDRG2) and cancer pathogenesis. Collectively, the expression of NDRG2 is decreased within human tumors, while its overexpression suppresses the capacity of cancer cells to proliferate, invade and metabolize. Nevertheless, knowledge about the exact role of NDRG2 within bladder cancer is still restricted³.

Several studies suggested that NDRG-2 functions as cell stress-related gene and a candidate tumor suppressor. Recently, many studies concluded that NDRG-2 is down regulated in primary tumors and various human cancer cell lines through regulation of invasion and cell proliferation in bladder cancer⁴.

The mini chromosome maintenance proteins (MCMs) are ubiquitously expressed proteins. Previous studies have demonstrated that MCMs are implicated in DNA replication with eccentric expression with prognostic value in cancer. For instance, many recent studies suggested that MCM 2, 8, 10 were overexpressed in hepatocellular carcinoma⁵. However, the role of MCMs family members remains unclear in the pathogenesis and progression of bladder cancer, suggesting that increased expression of MCM8 might be associated with increased malignancy⁶.

Consequently, we conducted our study to evaluate the urinary cell expression of NDRG-2 and MCM8 genes and their importance as novel noninvasive urinary biomarkers for bladder cancer and to assess their diagnostic value compared with voided urine cytology (VUC).

MATERIALS AND METHODS

The Committee of Ethics in the Faculty of Medicine, Mutah University, Jordan approved

the study under number (882022). Signed informed written consent was obtained from the included study participants. The work has been carried out according to the Ethics of the World Medical Association (Declaration of Helsinki).

70 subjects of both genders chosen from the Urology Department, Al Karak governmental Hospital, Mutah, Jordan during the period from September 2019 to March 2022. The participants were in 2 groups. Fifty patients were diagnosed with bladder cancer with no history of chemotherapy or radiotherapy before sampling. Moreover, age sex matched 20 healthy control who attended hospital seeking medical treatment for bladder disease requiring cystoscopy with obtained renal biopsy during endoscopic removal of renal or ureteric stones to ensure that they are free from cancer.

Sampling

A naturally voided midstream single urine sample was collected in 100-mL sterile cups, put on ice immediately, and delivered to the laboratory within 1 hour.

Voided urine cytology (VUC)

Approximately 10 ml were used for the preparation of the specimen. Then centrifugation for 30 minutes at 800 rpm was done and microscope slide was prepared from the precipitate. Then the slides were carefully submerged in 96% ethanol to fix the sediments for 30 minutes. Finally, they were stained using the Papanicolaou method⁷. A pathologist examined stained cytology slides.

The remaining urine specimen was processed as follows: centrifugation of urine samples at 4000 rpm for 10 min at 4 °C. Then the supernatant was removed without disturbing pellets. After that, twice washing the cell pellet with ice-cold buffer phosphate saline (PBS) was carried out. Then adding 1 ml PBS to the pellet, and the remaining ~0.5 ml of urine. The pellet was re-suspended by pipetting and were frozed at “80 °C to detect gene expression levels of NDRG-2 and MCM8 by qRT-PCR.

RNA extraction and quantitative RT-PCR

RNA-Spin total RNA extraction kit (iNtRON Biotechnology/South Korea) was used for extraction of total RNA from the collected urine samples. RNA purity and concentration were measured by Nanodrop (Biowave II, Germany). The extracted RNA was reverse transcribed using a one-step RT PCR-PreMix kit (iNtRON Biotechnology/

South Korea). In brief, 8µl of ONE-STEP RT-PCR PreMix Kit were placed into PCR Eppendorfs. Then RNA templates and gene-specific primers were added to the PCR tubes. After that, tubes were completed to a total volume of 20µl using distilled H₂O, and the mixture was mixed thoroughly. RT-PCR reaction of samples was performed using PCR thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA) as follows: a cycle of two steps, reverse transcription reaction at 45°C for 30 minutes. Followed by denaturation of RNA: cDNA hybrid at 94°C for 5 minutes followed by 40 cycles of 94! /60sec, 68! /60sec then 72! / 1min followed by Final Extension at 72! /5min. Integrated DNA Technologies/USA supplied Primers for both NDRG2 and MCM8 genes. Primers for NDRG2 were F-(5/-GCCAGCGATCCTTACCTACC-3/) (R-5/ GGCTGCCCAATCCCAACC-3/). Primers for MCM8 were F-(5/-ATGGCTTTTCTTTGTGCTGC-3/) (R-5/ CCAGTCCATCGTAACTGTGAGA-3. We used β actin as a housekeeping control gene. Sequences of its primers were (F-5/-GGC ATC GTG ATG GAC TCC G-3/ and R -5/-GCT GGA AGG TGG ACA GCG A-3/). The relative mRNA gene expression fold changes of the studied genes were estimated by the method of 2^{- Δ ct}.

Statistical analysis

The data were analyzed using SPSS 20. Quantitative data were described as mean \pm standard deviation and qualitative data as distribution and frequency. Mann-Whitney test and Student's *t*-test were used to compare the mean of two groups of quantitative data of non-parametric and parametric data, respectively. The Fisher exact and chi square test (X^2 value) test compared categorical data. The correlation between NDRG-2 and MCM8 expression, tumor grade and size was assessed by Spearman correlation coefficient (ρ ; \tilde{r}) was used. P-values <0.05 were considered statistically significant.

RESULTS

Fifty patients with diagnosed bladder cancer (BC) were included in this cohort. Figure (1) shows photos of urine cytology findings of one of those patients highlighting malignant features of high-grade urothelial carcinoma. As regards staging of BC participants, there were 56% MIBC

(n= 28) and 44% NMIBC (n= 22), with non-significant statistical differences between MIBC and NMIBC groups regarding the voided urine cytology. (*p*-value > 0.05). Table I.

The expression of NDRG2 and MCM8 in urinary samples by RT- PCR

A statistically significant decline in NDRG-2 gene expression in the bladder cancer group (4.38 \pm 0.66) compared to the control group (8.29 \pm 1.67) (*p*<0.001). While gene expression of MCM-8 was significantly increased in the bladder cancer group (5.57 \pm 0.79) as compared to control group (4.55 \pm 1.39) (*p*<0.001) as shown in figure (2A).

Although VUC showed a non-significant statistical difference between MIBC and NMIBC groups (*p*> 0.05), gene expression of NDRG-2 and MCM-8 showed a significant decrease (*p*<0.001) and increase (*p*=0.001) between MIBC and NMIBC groups, respectively. Figure (2B).

Relationship between clinicopathologic parameters and urinary NDRG-2, MCM-8 gene expression of patients with bladder cancer

There was a statistically significant difference between NDRG-2 gene expression in the cancer group as regards the tumor stage (*p*<0.001) and grade (*p*<0.001). As regards MCM-8, there was also a statistically significant difference between MCM-8 gene expression in the cancer group regarding the tumor stage (*p*=0.001) and grade (*p*=0.004). However, for both genes, there was no statistically significant difference between cancer groups regarding sex, smoking, Bilharziasis, tumor type, tumor onset, tumor size, or voided urine cytology (*p*>0.05). Table II

A significant negative correlation (\tilde{r} = -0.77) between NDRG-2 expression level and tumor grade in the cancer group (*p*<0.001).

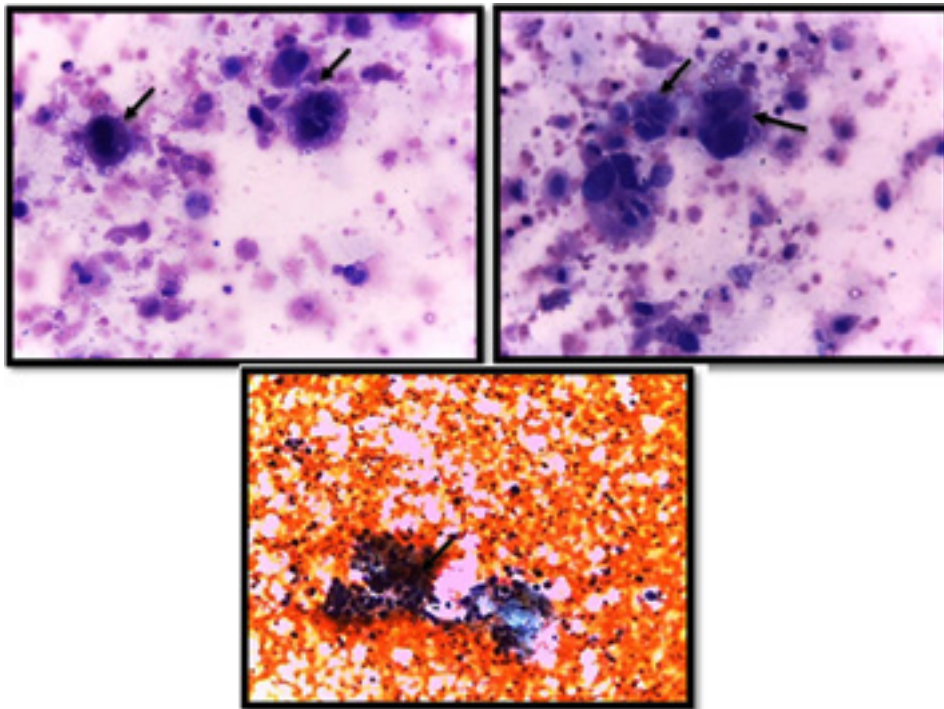
Table 1. Voided urine cytology in bladder cancer group

Voided urine cytology	MIBC		NMIBC		p
	No.	%	No.	%	
Positive	17	60.7	14	63.6	0.37
Negative	10	35.7	5	22.7	
Missed	1	3.6	3	13.6	

p=0.05 significant *p*>0.05 non-significant

Table 2. Relationship between urinary NDRG-2, MCM-8 gene expression and clinicopathologic parameters of patients with bladder cancer

Variable		NDRG-2		MCM-8	
		Mean± SD	p	Mean± SD	p
Sex	Females	4.18±0.58	0.40	5.63±0.92	0.84
	Males	4.41±0.67	5.56±0.77		
Smoking	smokers	4.35±0.66	0.60	5.54±0.80	0.60
	Non-Smokers	4.46±0.66	5.67±0.76		
Bilharzias	Positive	4.26±0.71	0.77	5.69±0.50	0.79
	Negative	4.38±0.66	5.57±0.80		
Type	TCC	4.42±0.65	0.068	5.56±0.76	0.63
	SCC	3.71±0.17	5.79±1.38		
Onset	Denovo	4.40±0.66	0.54	5.52±0.82	0.28
	Recurrent	4.24±0.68	5.85±0.51		
Stage	MIBC	4.06±0.46	<0.001**	5.89±0.61	0.001**
	NMIBC	4.78±0.66	5.17±0.81		
Grade	I	5.11±0.66	<0.001**	4.99±0.95	0.004**
	II	4.73±0.42	5.40±0.74		
	III	3.88±0.27	5.90±0.60		
Tumor size	<3cm	4.58±0.59	0.18	5.59±0.60	0.93
	>3cm	4.30±0.67	5.57±0.85		
Voided urine cytology	Positive	4.36±0.65	0.97	5.49±0.82	0.46
	Negative	4.41±0.73	5.79±0.64		
	Missed	4.33±0.52	5.42±1.09		

**Fig. 1.** Urine cytology findings of high-grade urothelial carcinoma according to The Paris System for reporting urine cytology. Large pleomorphic hyperchromatic cells with high nuclear cytoplasmic ratios (black arrows)

While there was a significant positive correlation ($\tilde{r}=0.453$) between MCM-8 expression levels and tumor grade in the cancer group ($p= 0.001$) Figure 3

To assess and compare the validity of VUC, NDRG-2, and MCM-8 as biomarkers for cancer bladder prediction and each biomarker’s ability to differentiate MIBC, a receiver operating characteristic (ROC) analysis for each area under the curve (AUC) was calculated. Our results revealed that NDRG-2 had the highest AUC of 1.0 (1.0-1.0), followed by the combined NDRG-2+MCM-8 with an AUC of 0.88 (0.802-958), followed by VUC with an AUC of 0.837 (0.743-0.930) while MCM-8 had the lowest AUC 0.744 (0.615-0.873). Moreover, NDRG-2 had the best to differentiate MIBC with an AUC of 0.814 (0.692-936), followed by MCM-8, which had an AUC equal to that of the combined markers (NDRG-

2+MCM-8) 0.774 (0.637-0.912) and the least was VUC [AUC=0.554 (0.384-0.723)]. Figure 4, 5

DISCUSSION

Previous studies found that NDRG2 regulates invasion and cell proliferation in bladder cancer⁸. In addition to its roles in DNA replication, MCM-8 reportedly indicated that it is related to several human tumors’ metastasis and invasion. The current study reported that MCM8 overexpression underlined prostate cancer aggressiveness⁹. More importantly, the increased expression of MCM8 was considered as a valuable independent prognostic marker in pancreatic cancer¹⁰. However, the impact of MCM8 and NDRG2 on bladder cancer remains away from being fully demonstrated.

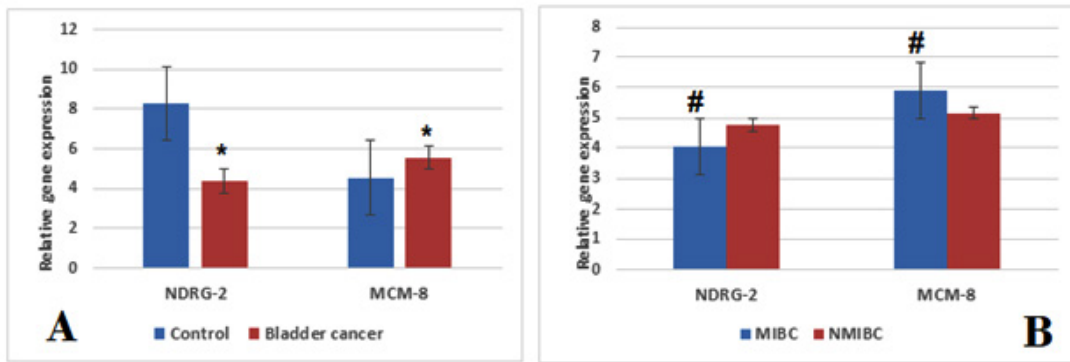


Fig. 2. A: Comparison between urinary gene expression level of NDRG-2, MCM-8 showing significant decrease, increase in bladder cancer and control groups respectively (A). Fig. 2B showing significant decrease, increase in MIBC and NMIBC groups respectively. Data are presented as mean± SD

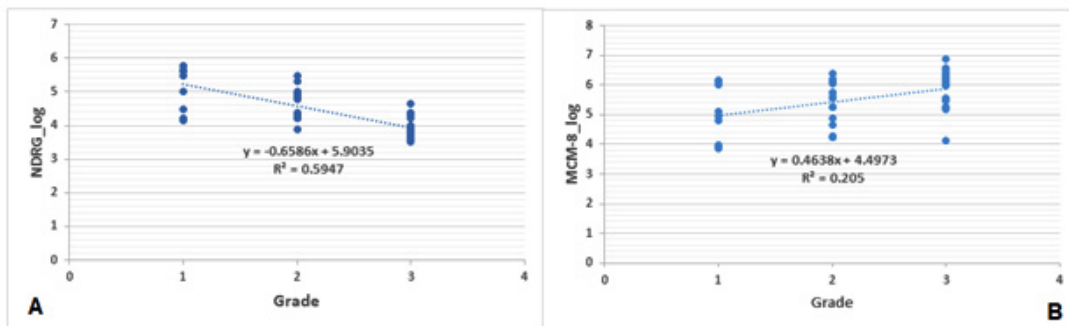


Fig. 3. Spearman’s correlation of NDRG-2 (A), MCM-8 (B) expression levels with the tumor grade of bladder cancer patients showing a significant negative correlation ($\tilde{r}= -0.77$) between NDRG-2 expression levels and tumor grade in cancer group ($p<0.001$). While there was a significant positive correlation ($\tilde{r}=0.453$) between MCM-8 expression levels and tumor grade in cancer group ($p<0.001$)

Consequently, we recruited 50 bladder cancer patients and 20 healthy controls. QRT-PCR and Voided urine cytology (VUC) were performed to measure the NDRG2 and MCM-8 expression in urine of healthy controls and patients with bladder cancer.

Our results demonstrated that MCM-8 was up-regulated in bladder cancer while NDRG-2

gene expression was statistically downregulated in bladder cancer group compared to control group, as shown in figure (2A). ROC curve analysis showed NDRG2 might be a good diagnostic marker, with an AUC of 0.888, with specificity and sensitivity were 81.4% and 85.5% respectively while in our study, the ROC curve showed AUC of 0.1 with the specificity and sensitivity were 100% and 98%,

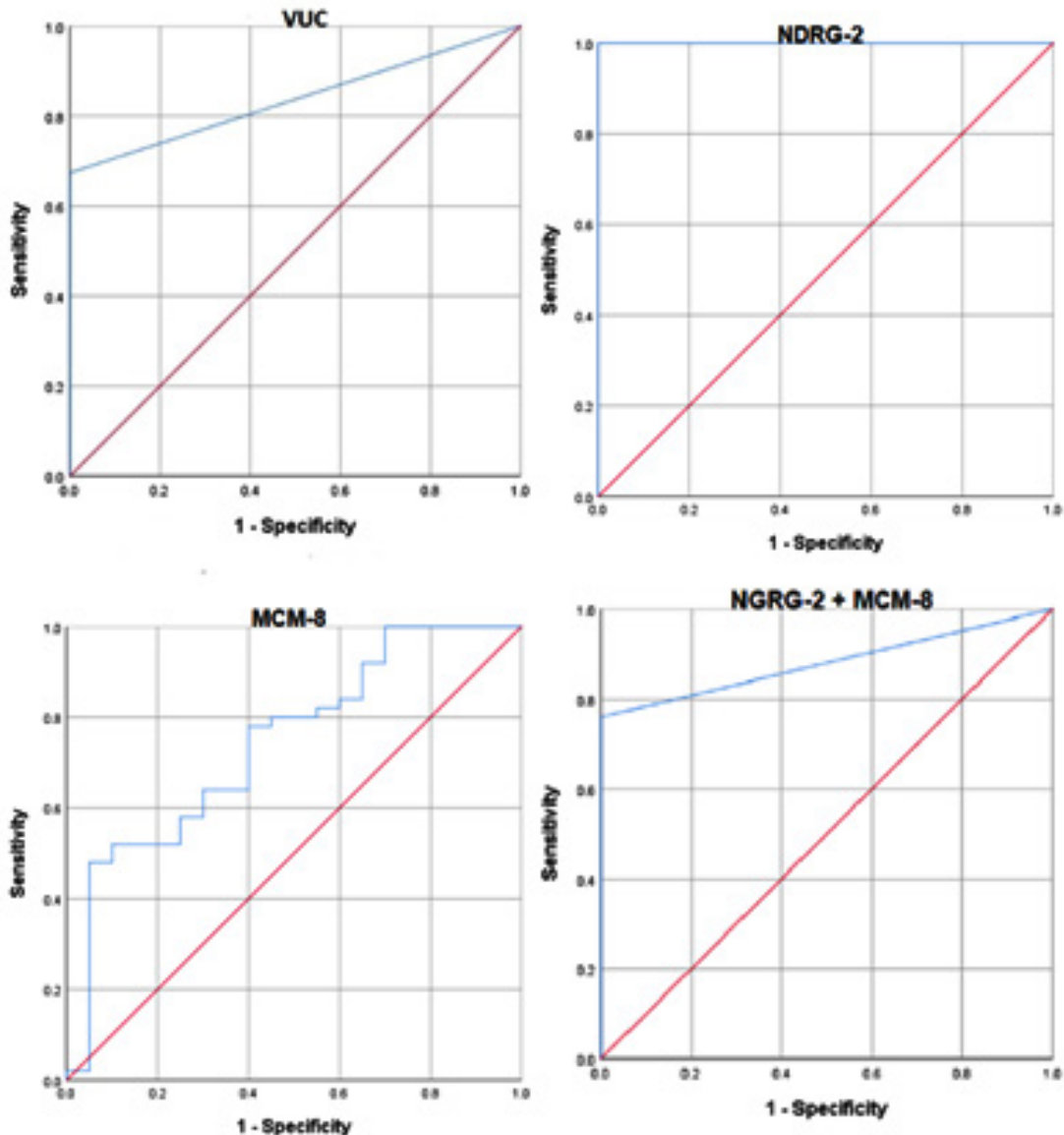


Fig. 4. Show the validity of VUC, NDRG-2 and MCM-8 as biomarkers for prediction of cancer bladder. AUC of NDRG-2 gene expression in prediction of cases was the highest (1), with cut-off point of 5.71, VUC with an AUC of 0.837 (0.743-0.930) while MCM-8 had the lowest AUC 0.744 (0.615-0.873). AUC of combined use of NDRG-2 & MCM-8 gene expression in prediction of cases was 0.88

respectively indicating it could be a potential gold standard biomarker for non-invasive diagnosis of bladder cancer. Our results were in agreement with most of the published studies, such as the study of Zhang and his colleagues in 2017¹¹ and they reported that the relative NDRG2 expression was significantly decreased both at protein and

mRNA levels in the urine of BC patients, and its low expression was dramatically correlated with tumor staging and grading.

Compared to other reported urinary biomarkers, Eissa *et al.*¹² reported that the Tissue inhibitor of metalloproteinase 2 (TIMP-2) and survivin might be considered urine biomarkers

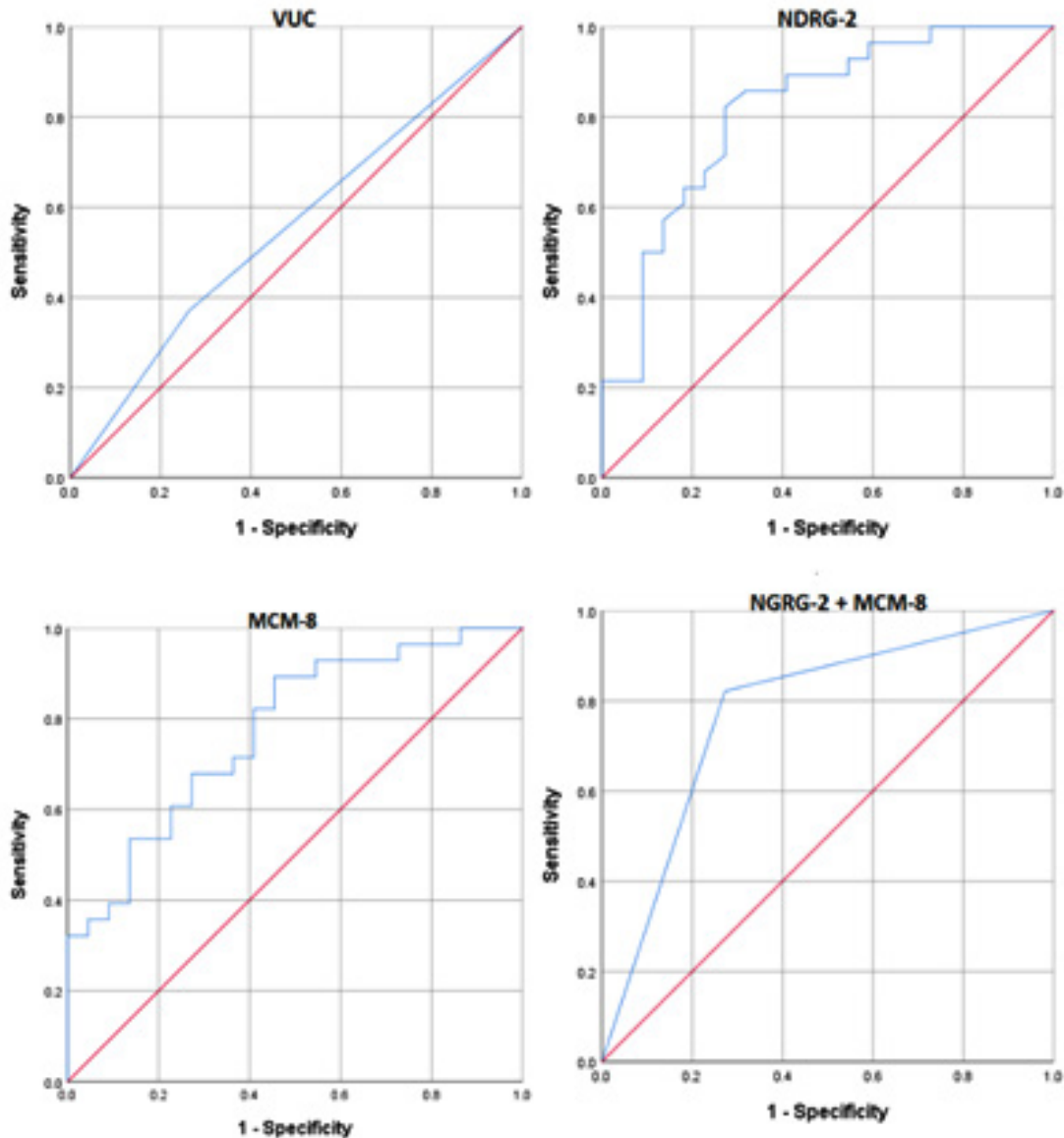


Fig. 5. Show the ability of VUC, NDRG-2 and MCM-8 biomarker to differentiate MIBC. NDRG-2 was the best to differentiate MIBC with AUC of 0.814 (0.692-936) followed by MCM-8 which had AUC of 0.774 (0.646-0.903) and that of the combined markers (NDRG-2+MCM-8) 0.774 (0.637-0.912) and the least was VUC [AUC=0.554 (0.384-0.723)]

in early detection of BC. Survivin showed 95.3% specificity and 78.6% sensitivity in diagnosis of bladder cancer, while TIMP had 83.7% specificity and 93% sensitivity. Another study concluded that Vascular endothelial growth factor (VEGF) was an accurate urinary biomarker for bladder cancer, with 87% specificity and 83% sensitivity with an AUC of 0.886¹³.

In addition, and by using Spearman's correlation, we further investigated whether NDRG2 and MCM-8 have a role in BC development, and we found a significant negative correlation ($r = -0.77$) between NDRG-2 expression levels and tumor grade in cancer group ($p = 0.001$). At the same time, a positive significant correlation ($r = 0.453$) between MCM-8 expression levels and tumor grade in the cancer group ($p = 0.001$) was found. Our findings were supported by the results of Zhang and his colleagues in 2017¹¹. Their study reported that decreased expression of NDRG2 was associated with stage and grade. However, no statistically significant relationship between cancer groups regarding other clinicopathological features such as sex, smoking, Bilharziasis, tumor type, tumor onset, tumor size, or voided urine cytology ($p > 0.05$), concluding that NDRG2 expression levels are closely associated with progression and pathogenesis of bladder cancer.

For more proof, in 2013, the Immunohistochemistry results of Li and his colleagues¹⁴ showed that the increased expression of NDRG2 in bladder carcinoma tissues was significantly decreased than in normal tissues. The expression of NDRG2 decreased as the degree of bladder carcinoma malignancy increased, and they conclusively reported that silencing of NDRG2 highly attenuates p53-mediated apoptosis. In addition, there was a report that NDRG2 could increase the expression of p53 in cells of breast cancer³. This information highly suggested that NDRG2 was a critical agent in regulating tumor apoptosis. While in 2015 the results of the study of Huang and his colleagues¹⁵ indicated that NDRG2 expression in mitochondria may arrest bladder cancer cells in S phase as well as decrease cell proliferation through inducing oncosis. It was therefore proposed that NDRG2 was not only a biomarker, but also a tumor suppressor for bladder cancer.

Recently, in 2021, Zhu *et al.*¹⁶ demonstrated that MCM8 expression in bladder cancer was up-regulated in association with advanced histologic stage with poor prognosis of BC patients. Furthermore, they found that MCM8 down regulation in bladder cancer cell lines decreased cell proliferation and induced apoptosis, concluding the substantial role of MCM8 in creating a rationale for the therapeutic potency of MCM8 inhibition in human bladder cancer therapy.

Recently in 2022 and in accordance to our results, Yu and his colleagues confirmed that mRNA and protein of MCM8 were significantly overexpressed in the tissues of hepatocellular carcinoma concluding that High MCM8 protein expression was an independent risk factor in HCC patients as MCM8 expression is altered in 60% of queried HCC patients and was associated with poor prognosis.¹⁷ previously in 2020 Kang *et al* revealed that NDRG2 mRNA expression and protein levels were downregulated within both ovarian cancer tissues and cell lines. The overexpression of NDRG2 dramatically inhibited the cell viability and colony formation and tumor growth, whereas promoted the cell apoptosis, cell cycle arrest in G1 phase within ovarian cancer cells. More importantly, NDRG2 overexpression significantly enhanced the suppressive roles of cisplatin (DDP) in ovarian cancer cell viability¹⁸.

NDRG2 was repeatedly reported to be downregulated in a variety of cerebral tumors, including glioma and meningioma¹⁹. The transcription levels of human *NDRG2* are significantly reduced in human glioblastoma tissues and human glioblastoma cell lines, and exogenous overexpression of NDRG2 repressed glioblastoma cell proliferation in vitro. Moreover, the expression level of *NDRG2* was negatively correlated with the pathological grade of the brain tumors and positively correlated with survival in astrocytoma patients²⁰. The above results suggest that NDRG2 may be a potential biomarker for predicting the prognosis of human brain tumors.

Analysis of differentially expressed genes in NMIBC versus MIBC phenotype is pivotal in understanding the molecular basis of bladder cancer progression²¹. Because pathogenesis and treatment of MIBC and NMIBC differ, and pathological evaluation is inadequate to precisely

predict the prognosis of high-risk NMIBC. We found that NDRG-2 was the best to differentiate MIBC from NMIBC with an AUC of 0.814 followed by MCM-8 which had AUC equal to that of the combined markers (NDRG-2+MCM-8) 0.774 and the least was VUC AUC=0.554. Our study has a few limitations; the sample size was comparatively small. Research with a larger sample size is recommended, that remains a primary priority of our research team targets.

CONCLUSION

In summary, Expression of MCM-8 was increased in bladder cancer patients. NDRG-2 gene expression was decreased in bladder cancer patients and might be involved in its progression. NDRG2 might be a potential diagnostic and prognostic biomarker for bladder cancer. In addition to its pivotal role as a marker to differentiate MIBC from NMIBC. Research in this field would eventually provide novel methods for early diagnosis and new therapy for bladder cancer.

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Author's contribution:

Heba M. Abd el kareem: Conceptualization, methodology, writing, validation, data curation, reviewing, editing. Aiman I. Al-Qtaitat, Fardous S. Karawya: Methodology, data curation, supervision, writing, reviewing, editing. Fadi S. Sawaqed: Sample collection, original draft preparation, reviewing.

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