Original Research Article

The Egyptian Journal of Immunology
Volume 31 (2), 2024: 28–43.
www.Ejimmunology.org

Plasma metadherin mRNA expression in bladder cancer

Zeinab A. Abd Elhameed[1], Lubna M. Tag El Din[1], Tahra Sherif[1], Amal M. Abdel Aal[1], Ahmed M. Moeen[2], Esraa N. Abd El Hakeem[1] and Eman M. Abdelrahman[1]

[1]Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt.
[2]Department of Urology, Faculty of Medicine, Assiut University, Assiut, Egypt.

Corresponding author: Esraa N. Abd El Hakeem, Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt.
Email: esraanasr1991@gmail.com

Abstract

Urinary bladder cancer (BC) is the ninth most common cancer worldwide. At present, the clinical diagnosis of BC depends on self-reported symptoms, tissue biopsy specimens by cystoscopy and from voided urine cytology. However, cystoscopy is an invasive examination and voided urine cytology has low sensitivity, which might provoke misdiagnosis. The search for cancer biomarkers in blood is worthy of intense attention due to patients' comfort and ease of sampling. This work aimed to study expression of mRNA metadherin (MTDH) in plasma, serum BC specific antigen 1 (BLCA-1) and cystatin C as biomarkers of BC and their relation to different disease stages. This study included 59 BC patients, 11 patients with benign bladder lesion and 18 subjects as normal controls. MTDH expression was assessed by real time polymerase chain reaction, BLCA-1, and cystatin C by the enzyme linked immunosorbent assay. The three biomarkers were elevated in BC patients than patients with benign bladder diseases and controls. Patients with BC grade 3 and 4 had higher cystatin C, BLCA-1 and MTDH in comparison to patients with grade 1 and grade 2 (p=0.000). The receiver operating characteristic curve analysis showed that BLCA-1 at a cutoff point of 32.5 ng/ml and area under the curve of 1.00, had 100% accuracy, 100% sensitivity, 100% specificity, 100% positive predictive values and 100% negative predictive value. In conclusion, BLCA-1 was a better biomarker than cystatin C and MTDH. Cystatin C, BLCA-1 and MTDH levels, can differentiate between benign bladder lesion and BC and correlated with tumor grades.

Keywords: Bladder cancer, Metadherin, Bladder cancer specific antigen-1.

Date received: 12 September 2023; **accepted:** 19 February 2024

Introduction

Urinary bladder cancer (BC) is the ninth most common cancer worldwide. It is the fifth most common malignancy in males[1] with an annual incidence of around 430,000 cases.[2] The estimated incidence was 7.9% of all cancer. BC incidence increases with age with a strong male predominance of the disease with a 4:1 male-to-female ratio.[3] Approximately 75% of BC are diagnosed as superficial, confined to mucosa and sub mucosa while 25% are muscle invasive.[4] The prognosis of BC is poor as a result of highly invasive properties of tumor cells.[5] Almost 50%

of patients will experience recurrence of their disease within four years of their initial diagnosis. The optimal treatment depends on early diagnosis as well as accurate staging and grading.[6]

The clinical diagnosis of BC is via the perceived self-reported symptoms of the presenting patient, detailed analysis of tissue biopsy specimens by cystoscopy and voided urine cytology. However, cystoscopy is an invasive examination, and voided urine cytology has a low level of sensitivity, which might provoke the reported misdiagnosis.[7] The search for cancer biomarkers in blood and urine is worthy of intense attention due to patients' comfort and ease of sampling.[8]

Cystatin C is an endogenous cysteine protease inhibitor and most nucleated cells are considered to be the source of its secretion. Thus, it plays a role in inflammation and pathogenesis of cancers by acting as an inhibitor of cathepsins.[9] Cystatin C was shown to play a role in cancer development, protein catabolism, and regulation of hormone processing and bone resorption and modulating inflammation.[10]

Bladder Cancer-1 (BLCA-1) is a member of six bladder-specific nuclear matrix proteins. They are expressed in BC tissue. They belong to the nuclear matrix protein group and are nuclear transcription factors and therefore play an important role in carcinogenesis process.[11] BLCA-1 is considered a specific marker of BC. In particular, it is associated with tumor cell proliferation and survival.[11] BLCA-1 levels are related to invasion of the tumor mucosa, and tumor muscular coat, which indicated that BLCA-1 might play a significant role in tumor progression by participating in the secretory process of the tumor microenvironment and improving the invasive ability of bladder tumors.[7]

Metadherin (MTDH) is an oncogene, also called astrocyte-elevated gene-1, and known as protein Lyric. In 2002, it was initially identified in human fetal astrocytes, and reported as a novel late response gene induced in human fetal astrocytes after human immunodeficiency virus infection or treatment with viral glycoprotein 120 (gp120) or tumor necrosis factor (TNF)-α.[12] MTDH plays key roles in the activation of a group of signaling pathways, such as phosphatidylinositol 3-kinase (PI3K)/ protein kinase (Akt), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), Wnt/β-catenin, and mitogen-activated protein kinase (MAPK) signaling pathways. They are involved in cancer proliferation, invasion, angiogenesis, metastasis, turning on epithelial-mesenchymal transition and chemoresistance. MTDH inhibits cell proliferation, migration and induces apoptosis of BC cells.[13]

To date, there are no published reports on using the plasma MTDH test for the diagnosis of BC. All studies on the role of MTDH in BC have used urine samples or tissue samples. Therefore, we investigated whether MTDH could be detected in plasma and whether its high expression in plasma can be used for diagnosis of BC.

Subjects and Methods

This study included 59 BC patients, 11 patients with benign bladder mass and 18 age and sex matched normal controls. The patients were selected from the Department of Urology, Assiut University Hospitals, within the period from January 2021 till November 2021.

Group I (malignant group) included 59 newly diagnosed BC patients, they were 36 males and 23 females. The diagnosis of BC was based on upper urinary tract radiological imaging (ultrasound or computed tomography) and verified by cystoscopy and histopathology. According to histopathology all patients had transitional cell carcinoma. BC patients were further classified according to WHO classification into grades (G)[14]: G1: 25 patients, they included 15 males and 10 females. G2: 24 patients, they included 15 males and 9 females. G3, G4: 10 patients, they included 6 males and 4 females. BC patients were further classified according to TNM staging into: Stage I: 51 patients, included 31 males and 20 females, Stage II: 8 patients, 5 males and 3 females.

Group II (Benign group) included 11 patients newly diagnosed with benign bladder lesion. They were 7 males and 4 females. Group III (Control group) included 18 age and sex

matched normal controls. They were 11 males and 7 females.

All participants were subjected to full medical history including family history of BC, full clinical examination, and laboratory investigations. Body mass index (BMI) was calculated according to this formula: weight (kg)/height (m) ².

For the laboratory investigations, a venous blood sample (10 ml) was collected from each study participant under complete aseptic conditions and divided into four aliquots. The first aliquot (4 ml) was collected in two EDTA coated tubes. Of these, a portion of 2 ml was used for CBC. The second portion of 2 ml was centrifuged at 400 xg for 10-min and plasma collected, and carefully transferred into an RNAase free tube, stored in aliquot at -80°C till time of RNA extraction for MTDH PCR analysis. An aliquot of 2 ml was collected into a tube containing sodium citrate for prothrombin time and concentration. The tube was centrifuged at 400 xg for 20-min and used immediately for determination of prothrombin time and concentration. The fourth aliquot (4 ml) was collected into tubes containing clot activator for serum separation. The tubes were centrifuged at 400 xg for 20-min and serum was collected and divided into three portions: the first was used for routine chemical investigations. The second portion was stored at -20°C till time of assay of cystatin C. The third portion was stored at -80°C till time of assay of bladder cancer specific antigen-1 (BLCA-1).

Laboratory investigations

Routine investigations as hematological investigations including complete blood count (CBC) was done by a hematology analyzer (ADVIA 2120i, Siemens Healthineers, Germany), according to the manufacturer's instructions. Prothrombin time, concentration, and international normalization ratio (INR) was done using fully automated coagulation system (Sysmex CS-5100, Siemens Healthineers, Germany), according to the manufacturer's instructions. Routine biochemical tests including kidney function tests, liver function tests, glucose and uric acid were done using a Clinical Chemistry System (ADVIA 1800, Siemens Healthineers, Germany), according to the manufacturer's instructions.

Special investigations

Serum Cystatin C was measured by an enzyme linked immunosorbent assay (ELISA) technique using Human Cystatin C commercial ELISA kits (Catalog No: SG-10555, provided by Sinogene Clone Biotech company, China), according to the manufacturer's instructions. Serum Human Bladder Cancer specific Antigen-1(BLCA-1): was measured by ELISA technique using commercial BLCA-1 ELISA Kits (Catalog No: SG-15733H2297, provided by Sinogene Clone Biotech Company, China), according to the manufacturer's instructions.

Expression of plasma MTDH: Relative expression of plasma metadherin (MTDH) mRNA was performed by quantitative real-time reverse transcription polymerase chain reaction (qRT- PCR) using a 7500 fast real time PCR machine (Applied Biosystems, USA), according to the manufacturer's instructions. The first step was extraction of RNA from stored plasma samples, using the commercial miRNeasy Mini Kits (Cat.No. 217004; Qiagen, Germany), according to the manufacturer's instructions. Then RNA was reversed **transcribed** into complementary DNA (cDNA) using the Thermo Scientific RT kits (Cat. No. K1622; Qiagen, USA), according to the manufacturer's instructions. Then cDNA was amplified and detected using the Thermo Scientific SYBR Green PCR Kits (Catalog Number: K0251, provided by QIAGEN, Germany), according to the manufacturer's instructions. The qRT-PCR used the following primer set: (forward primer for MTDH 5'-CACGCCATGATGGAAAGGA-3') and (reverse primer for MTDH 5'-GCGGTTGTAAGTTGCT CGGT-3'). The glyceraldehyde3phosphate dehydrogenase (GAPDH) was used as an internal control to normalize RT-Q PCR readout.

The 7500 fast real time PCR machine was programmed according to the following conditions: incubation period at 95°C for 15 minas a preliminary activation step for DNA polymerase, followed by 40 amplification cycles, each cycle included three consecutive steps of

DNA denaturation at 94°C for 15 sec., annealing at 55°C for 30 sec., and extension at 70°C for 30 sec. Fluorescence measurement was made at every cycle.

The expression levels of the investigated mRNA were calculated using the ΔCt method. The results were expressed as fold changes compared to the control sample, considered the normal value, and assumed to equal 1.

Delta-Delta method for comparing Relative Quantitation results in Real-time PCR
ΔCt Sample = Ct MTDH - Ct GAPDH
ΔCt Control = Ct MTDH - Ct GAPDH
ΔΔCt Sample = ΔCt Sample - ΔCt Control
Relative quantitation (Fold Change, FC) of sample = 2-ΔΔCt
Relative quantitation (Fold Change, FC) of control Mean = 1.

Statistical Analysis

Data entry and data analysis were done using the statistical package for social science (SPSS) version 22. The normal distribution of the quantitative variables was tested with Shapiro-Wilk test. The Chi-square test was used to compare qualitative variables. Fisher Exact test was used if more than 20% of cells have expected frequency less than 5. ANOVA/ post-hoc (least significant difference/LSD) test was used to compare quantitative variables among groups in case of parametric data. The Mann-Whitney test was used to compare quantitative variables between two groups and Kruskal Wallis Test for more than two groups in case of non-parametric data. Pearson correlation was used to measure correlation between quantitative variables in case of parametric data, and Spearman correlation for non-parametric data. Data were statistically described in terms of mean +/- standard deviation (SD), range and median as appropriate. The Medcalc statistical software version 11.3 was used to perform the receiver operating characteristic (ROC) curve analysis and to calculate sensitivity, specificity, positive and negative predictive values, and. A p-value was considered statistically significant at $p< 0.05$.

Results

The personal, demographic data and clinical history of the studied groups

Age was significantly higher in the malignant group compared to patients in the benign group. BMI was significantly higher in the malignant group compared to the control group and higher in benign group compared to the control group (Table 1). Smoking, schistosomiasis, and family history of BC were not different between the three study groups. Recurrent of urinary tract infection (UTI) showed statically significant elevation in the malignant group than the control group and in the benign group than the control group. However, recurrent UTI showed no differences between the malignant group and benign group. The frequency of patients who had history of food consumption was significantly elevated in the malignant group than the control group and elevated in the benign group than the control group. However, there was no difference between the malignant and the benign groups (Table 1).

Table 1. Demographic and clinical history data of the studied groups.

Personal data	Malignant group (n= 59)	Benign group (n= 11)	Control group (n= 18)	*p*-value1	*p*-value2	*p*-value3	*p*-value4
Age (years)							
Mean ± SD	61.18 ± 6.02	55.55 ± 5.89	57.50 ± 8.31	0.013*	0.012*	NS	0.453
Range	42.0-73.0	44.0-62.0	39.0-71.0				
BMI							
Mean ± SD	30.04 ± 4.47	30.32 ± 4.10	25.65 ± 1.99	<0.0001*	NS	<0.0001*	0.003*
Range	23.0-38.0	24.5-37.0	23.0-32.0				

Table 1. Continued.

Personal data	Malignant group (n= 59)		Benign group (n= 11)		Control group (n= 18)		*p*-value1	*p*-value2	*p*-value3	*p*-value4
Sex:	No.	%	No.	%	No.	%				
Male	36	61.0	7	63.6	11	61.1	NS	NS	NS	NS
Females	23	39.0	4	36.4	7	38.9				
Job:										
Farmer	38	64.4	9	81.8	9	50.0				
Employee	12	20.3	2	18.2	4	22.2				
Skilled worker	1	1.7	0	0.0	3	16.7	NS	NS	NS	NS
Housewife	6	10.2	0	0.0	2	11.1				
Worker	2	3.4	0	0.0	0	0.0				
Residence:										
Rural	43	72.9	7	63.6	9	50.0	NS	NS	NS	NS
Urban	16	27.1	4	36.4	9	50.0				
Risk factor										
Smoking:	No.	%	No.	%	No.	%				
Yes	15	25.4	5	45.5	5	27.8	NS	NS	NS	NS
No	44	74.6	6	54.5	13	72.2				
UTI										
Yes	22	37.3	6	54.5	1	5.6	0.011*	NS	0.010*	0.006*
No	37	62.7	5	45.5	17	94.4				
Schisto.										
Yes	13	22.0	3	27.3	1	5.6	NS	NS	NS	NS
No	46	78.0	8	72.7	17	94.4				
Food consumption										
Yes	32	54.2	5	45.5	1	5.6	0.001*	NS	0.0001*	0.018*
No	27	45.8	6	54.5	17	94.4				
Family history of BC										
Yes	4	6.8	0	0.0	0	0.0	NS	NS	NS	NS
No	55	93.2	11	100	18	100				

BMI: Body mass index, UTI: Urinary tract infection, Schisto: Schistosomiasis. Quantitative data are presented as mean ± SD, qualitative data are presented as number (percentage), $p > 0.05$ is not significant (NS). *ANOVA test was used to compare mean difference between groups. **Post-hoc (LSD). *p*-value1 Comparison among all groups, *p*-value2 Comparison between Malignant and Benign, *p*-value3 Comparison between Malignant and Control, *p*-value4 Comparison between Benign and Control.

Blood cystatin C, BLCA-1 and MTDH levels among the studied groups

Patients in the malignant group had significantly higher cystatin C, BLCA-1 and MTDH in comparison to patients in the benign group (p= 0.00) and the control group (p< 0.001). Also, patients in the benign group had significantly higher cystatin C, BLCA-1 and MTDH in comparison to the control (p<0.001) (Table 3, Figure 1, 2, 3).

Table 2. Results of Cystatin C, BLCA-1 and MTDH among the studied groups.

Markers	Malignant group (group I) (n= 59)	Benign group (group II) (n= 11)	Control group (group III) (n= 18)	*P**-value1	*P***-value2	*P***-value3	*P***-value4
Cystatin C (mg/l):							
Mean ± SD	1.50 ± 0.51	0.80 ± 0.15	0.66 ± 0.11	<0.001*	<0.001*	<0.001*	NS
Range	0.92-3.10	0.60-0.98	0.49-0.89				
BLCA-1(ng/ml):							
Mean ± SD	77.18 ± 49.08	27.23 ± 4.92	16.77 ± 5.16				
Median	64.5 ()	28.5 ()	16.0 ()	<0.001*	<0.001*	<0.001*	<0.001*
range	35.0-275.5	15.0-32.5	5.5-24.0				
MTDH(FC):							
Mean ± SD	2.16 ± 2.66	0.17 ± 0.07	0.08 ± 0.03)	<0.001*	<0.001*	<0.001*	<0.001*
Median	1.10	0.15	0.09				
Range	0.20-15.45	0.10-0.35	0.01-0.13				

Quantitative data are presented as mean ± SD, $p > 0.05$ is not significant (NS). *ANOVA test was used to compare mean difference between groups. **Post-hoc (LSD). *P*-value1 Comparison among all groups. *p*-value2 Comparison between Malignant and Benign, *p*-value3 Comparison between Malignant and Control, *P*-value4 Comparison between Benign and Control.

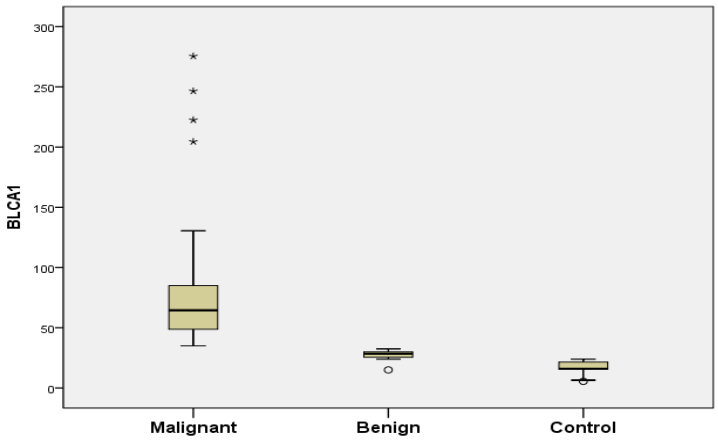

Figure 1. Mean level of serum BLCA-1 among the studied groups.

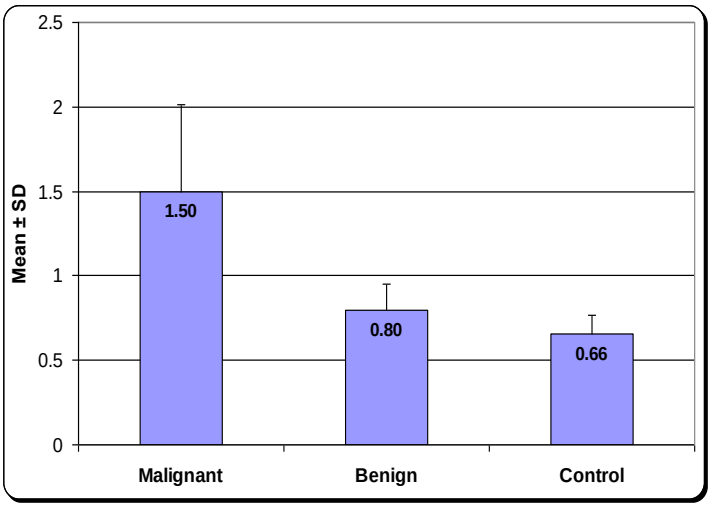

Figure 2. Mean level of serum cystatin C among the studied groups.

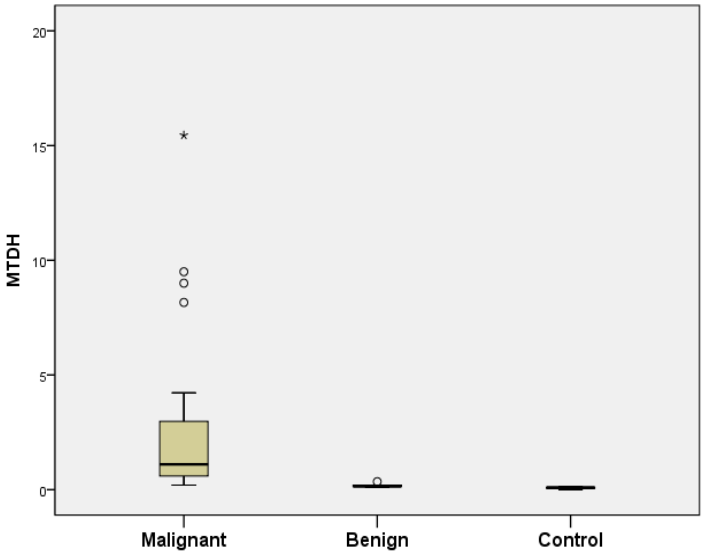

Figure 3. Mean level of plasma MTDH expression among the studied groups.

Level of Cystatin C, BLCA-1and MTDH according to grades of bladder cancer

Cystatin C, BLCA-1 and MTDH had statistically significantly higher levels in patients with grade 3 and 4 in comparison to patients with grade 1 and grade 2 ($p< 0.001$), (Table 3, Figure 4, 5, 6).

Table 3. Cystatin C, BLCA-1 and MTDH levels according to grades of bladder cancer.

Markers	Grade 1 (n= 25)	Grade 2 (n= 24)	Grade 3, 4 (n= 10)	*p*-value1	*p*-value2	*p*-value3	*p*-value4
Cystatin C (mg/l):							
Mean ± SD	1.16 ± 0.16	1.51 ± 0.30	2.36 ± 0.52	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Range	0.92-1.50	0.99-1.98	1.70-3.10				
BLCA-1 (ng/ml):							
Mean ± SD	46.14 ± 7.64	75.02 ± 9.52	159.95 ± 69.55	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Median	45.5	74.0	124.3				
Range	35.0-58.5	61.5-91.0	95.5-275.5				
MTDH (FC):							
Mean ± SD	0.51 ± 0.21	2.09 ± 0.79	6.43 ± 4.03	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Median	0.48	2.16	4.15				
Range	0.20-0.89	0.90-3.20	3.30-15.45				

Quantitative data are presented as mean ± SD, significance defined at $p< 0.05$. * ANOVA test was used to compare mean difference between groups. ** Post-hoc (LSD). *p*-value1 Comparison among all grades, *p*-value2 Comparison between grade 1 and grade 2. *p*-value3 Comparison between grade 1 and grade 3 & 4. *p*-value4 Comparison between grade 2 and grade 3 & 4.

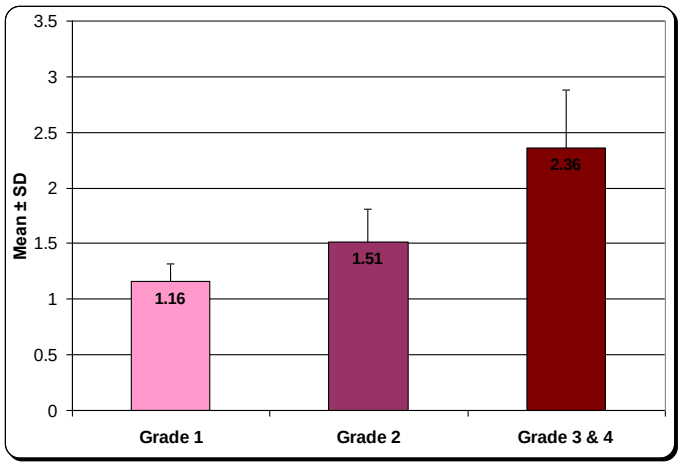

Figure 4. Mean level of serum Cystatin C according to grades of bladder cancer.

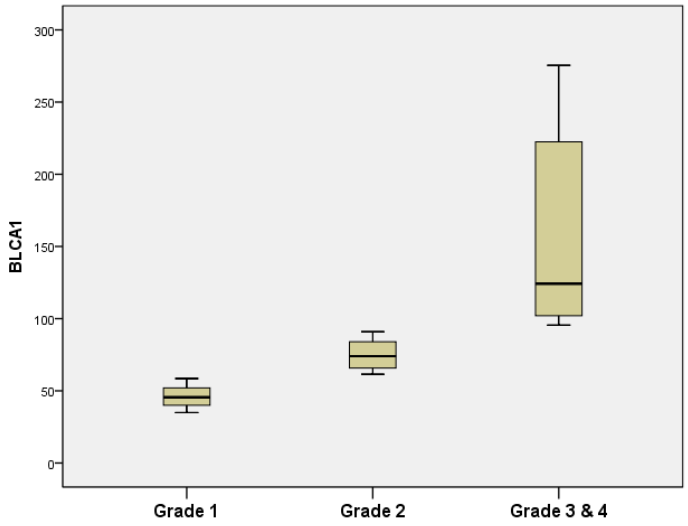

Figure 5. level on BLCA-1 according to grades of bladder cancer.

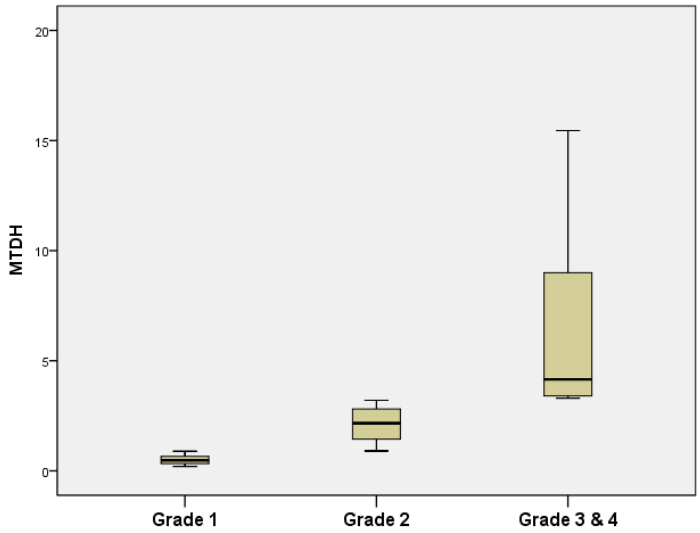

Figure 6. Level of MTDH according to grades of bladder cancer.

Levels of Cystatin C, BLCA-1and MTDH according to stages of bladder cancer

Levels of Cystatin C, BLCA-1 and MTDH were statistically significantly higher in patients with stage II in comparison to patients with stage I ($p< 0.001$), (Table 4).

Table 4. Levels of Cystatin C, BLCA-1and MTDH according to stages of bladder cancer.

Markers	Stage I (n= 51)	Stage II (n= 8)	*p*-value
Cystatin C(mg/l):			
Mean ± SD	1.36 ± 0.32	2.44 ± 0.56	< 0.001*
Range	0.9-2.1	1.7-3.1	
BLCA-1(ng/ml):			
Mean ± SD	65.88 ± 35.59	149.19 ± 63.69	< 0.001*
Median (Range)	61.5 (35.0-275.5)	110.8 (95.5-246.5)	
MTDH(FC):			
Mean ± SD	1.61 ± 2.22	5.69 ± 2.69	< 0.001*
Median (Range)	0.9 (0.2-15.5)	4.2 (3.3-9.5)	

Quantitative data are presented as mean ± SD, significance defined at $p< 0.05$. * ANOVA test was used to compare mean difference between groups. ** Post-hoc (LSD). *p*-value1 Comparison among all groups, *p*-value2 Comparison between Malignant and Benign, *p*-value3 Comparison between Malignant and Control. *p*-value4 Comparison between Benign and Control.

Diagnostic accuracy of cystatin C, BLCA-1 and MTDH in detection of bladder cancer

Cystatin C at a cutoff point of 0.98 mg/l and AUC of 0.996, had accuracy of 95.45%, sensitivity of 93.22%, specificity of 100%, PPV 100 % and NPV 87.9%. BLCA-1 at a cutoff point of 32.5 ng/ml and AUC of 1.00, had accuracy of 100%, 100% sensitivity, 100% specificity, 100% PPV and 100% NPV. MTDH at a cutoff point of 0.2 ng/ml, and AUC of 0.993, had accuracy of 95.45%, sensitivity of 94.92%, specificity of 96.55%, PPV of 98.2 % and NPV of 90.3%, (Table 5, Figure 7).

Table 5. Diagnostic accuracy of cystatin C, BLCA-1 and MTDH in detection of bladder cancer.

	Cut-off	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy	AUC
Cystatin C	0.98	93.22	100.00	100.0	87.9	95.45	0.996
BLCA-1	32.5	100.00	100.00	100.0	100.0	100.0	1.00
MTDH	0.2	94.92	96.55	98.2	90.3	95.45	0.993

AUC: Area under the curve

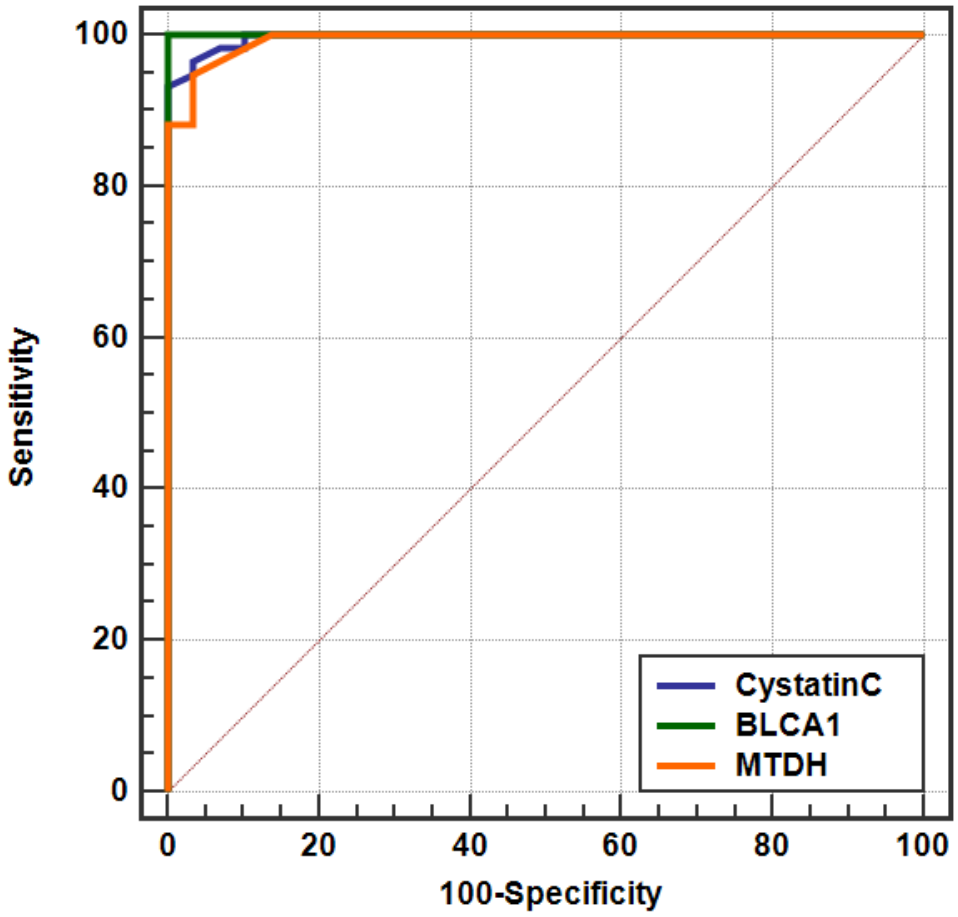

Figure 7. Receiver operating characteristic (ROC) curve analysis for Cystatin C, BLCA-1 and MTDH as markers for diagnosis of bladder cancer.

Diagnostic accuracy of Cystatin C in detection of early bladder cancer (grade 1 versus benign cases)

Cystatin C at a cutoff point of 0.98 mg/l and AUC of 0.978, had accuracy of 88.89%, sensitivity of 84.0%, specificity 100%, PPV 100 % and NPV 73.3%. BLCA-1 at a cutoff point of 32.5 ng/ml and AUC of 1.00, had 100% accuracy, 100% sensitivity, 100% specificity, 100% PPV and 100% NPV. MTDH at a cutoff point of 0.2 ng/ml and AUC of 0.958, had accuracy of 88.89%, sensitivity of 88.0%, specificity of 90.91%, PPV of 95.7% and NPV of 76.9%, (Table 6, Figure 8).

Table 6. Diagnostic accuracy of Cystatin C, BLCA-1 and MTDH in detection of Bladder Cancer grade 1 versus benign cases.

	Cut-off	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy	AUC
Cystatin C	0.98	84.00	100.00	100.0	73.3	88.89	0.978
BLCA-1	32.5	100.00	100.00	100.0	100.0	100.0	1.000
MTDH	0.2	88.00	90.91	95.7	76.9	88.89	0.958

AUC: Area under the curve

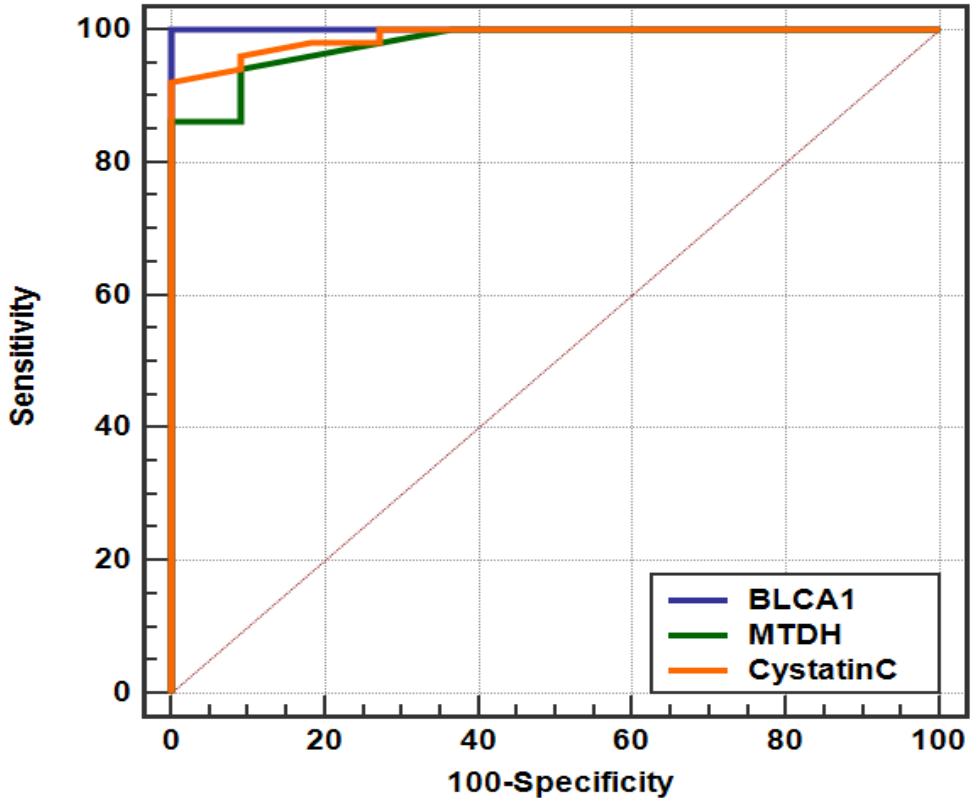

Figure 8. Receiver operating characteristic (ROC) curve analysis for Cystatin C, BLCA-1 and MTDH as markers for detection of early bladder cancer.

Discussion

The present work aimed to evaluate the diagnostic value of plasma mRNA MTDH expression as a noninvasive biomarker in BC patients and its relation to BC grading and staging. In addition, the study aimed to compare the diagnostic performance of the previously used serum markers Cystatin C and BLCA-1 with MTDH expression.

In our study, patients in the malignant group had older age compared to the benign group (p=0.012). Age is a risk factor for the incidence of BC, as BC develops more in older age than young age. This is because patients with BC have experienced long periods of exposure to other established risk factors for the development of BC, such as cigarette smoke and various chemical carcinogens.[15] A previous study reported that patients in the malignant group had older age compared to the benign group.[16]

Regarding sex, male predominance was observed in our study patients. This may be attributable to increased incidence of BC among males.[17] Male predominance in BC patients was also reported in previous studies.[18,19] However, other studies did not find significant sex differences in BC patients.[16,20] BC appears to be influenced by sex-hormones.[21] Androgen and estrogen receptors (AR & ER) are expressed in bladder urothelium and hence they and their respective hormones may have a role in the development and outcome of BC.[22] AR expression was noted to decrease with increasing the BC pathological stage. It is believed that androgens could enhance the susceptibility of the urothelium to carcinogens, downregulate carcinogen breakdown or directly stimulate cancer cell growth.[23] Estrogens may inhibit BC development.[22]

In our study, BMI was significantly higher in the malignant group than in the control group and significantly higher in the benign group than the control group.[24] Previous studies confirmed

that obesity and BMI are positively correlated with the increased risk of BC.[25] Obesity increases the production of insulin and insulin-like growth factor-I which modify cell proliferation, angiogenesis, and apoptosis.[26] Obesity also promotes chronic inflammation by altering the levels of cytokines, thereby initiating an immune cascade that ultimately promotes carcinogenesis.[27]

Smoking was positively correlated with the increased risk of BC.[28] Tobacco smoke contains known carcinogens such as beta-naphthylamine and polycyclic aromatic hydrocarbons. These particles promote inflammation, and their metabolism, in the bladder and throughout the body, culminates in DNA-adduct formation and permanent genetic mutation. Such mutations can activate oncogenes or suppress tumor suppressor genes, promoting carcinogenesis. Certain inherited genotypes associated with abnormal detoxification enzymes have been shown to increase the susceptibility to cancer among those who smoke.[29] However, in our study smoking was not significantly different between the studied groups. This may be due to the female/male ratio of the study groups, as smoking behavior is lower in Egyptian females.

In our study, the frequency of patients with history of urinary tract infection (UTI) was significantly higher in BC patients than in the control group and in the benign group than in the control group. Also, a previous study has confirmed that UTI was positively correlated with increased risk of BC than those with benign bladder lesions and the control group.[30] UTI is related to the virulence of the infecting organism. The virulence of an organism is related to its ability to adhere to epithelial cells. *Escherichia coli* has hair-like structures called pili or fimbriae that interact with glycoprotein and glycolipid receptors on uroepithelium.[31]

In our study, we found that the frequency of schistosomiasis showed no significant difference between the studied groups. However, a previous study found that schistosomiasis was positively correlated with the increased risk of BC.[32] In our study, we found that the frequency of patients with history of food consumption (coffee and meat consumption) was significantly higher in the malignant group than the control group and significantly higher in the benign group than the control group. Previous studies confirmed that food consumption was positively correlated with the increased risk of BC.[33,34] In addition, a previous study indicated an association between the inflammatory potential of diet and the risk of bladder cancer.[35] Chronic inflammation was implicated in BC and diet can modulate inflammation which has a role in BC development.[36]

Genome-wide association studies (GWAS) identified multiple single nucleotide polymorphisms (SNPs) associated with the inherited risk of BC. These SNPs have an effect on BC risk. In our study we found that family history showed no significant difference between the studied groups. According to histopathological types of bladder cancer, we found that all cases were transitional cell carcinoma. It was reported that TCC is the most common histopathological type of BC.[37]

In the present study, Cystatin C had statistically significantly higher level in patients with BC than in patients with benign bladder lesion and in the studied controls. Also, patients in the benign group had significantly higher Cystatin C in comparison to the control group. However, in a previous study, Cystatin C did not show significant difference between patients with BC compared to patients with benign lesions and normal controls.[18]

In the present study, classification of the BC group according to grades and TNM staging, revealed significant elevation of serum Cystatin C levels in BC patients with grade 3 compared to grade 1 and grade 2 patients.

Also, BC patients with grade 2 had significantly higher levels of serum Cystatin C than patients in grade 1. There was a significant elevation in serum Cystatin C levels in BC patients with stage II compared to stage I patients. However, a previous study did not find difference in Cysteine C between BC patients with different grades or stages.[7] Cysteine cathepsins are involved in the degradation of extracellular matrix, facilitating the growth, invasion, and metastasis of tumor cells, and also in tumor angiogenesis.[38] It plays a role in inflammation and pathogenesis of cancers.[39]

Using the ROC curve analysis, we found that serum Cystatin C level can discriminate between BC and both benign and control groups at a cutoff point of 0.98 mg/l, and an area under curve (AUC) of 0.996, had % accuracy of 95.45, sensitivity of 93.22%, 100% specificity (p<0.0001).

BLCA-1 is a member of six bladder-specific nuclear matrix proteins (BLCA-1 to BLCA-6). They were identified as being specifically expressed in bladder cancer tissues. They belong to the nuclear matrix protein group and are nuclear transcription factors, and therefore play an important role in carcinogenesis process.[11] In the present study, serum BLCA-1 had statistically significantly higher level in patients with BC than in patients with benign bladder disease and the studied controls. Also, patients with benign bladder disease had significantly higher BLCA-1 in comparison to the control group. These results are consistent with findings of previous studies, which reported that serum BLCA-1 was significantly higher in patients with bladder cancer compared to patients with benign lesions and normal controls.[7,20,40]

In our study, there was a significant difference in the mean value of BLCA-1 levels according to the grade of BC among the studied cases. There was a significant elevation of serum BLCA-1 levels in BC patients with grade 3 compared to grade 1 and grade 2 patients. Also, BC patients with grade 2 had significantly higher levels of serum BLCA-1 than patients with grade 1. According to TNM staging, there was significant elevation of serum BLCA-1 levels in BC patients with stage II compared to stage I patients. This agreed with previous studies who found that BLCA-1 expression differed significantly according to TNM staging with higher expression in muscle invasive bladder cancer than non-muscle invasive bladder cancer.[20,40] They also found gradual increase in expression of BLCA-1 with progression of the tumor grade.

Using the ROC curve analysis, we found that at a cutoff point of 32.5 ng/ml and an AUC of 1.00, serum BLCA-1 level can discriminate between BC and both benign and control groups, with 100 % accuracy, 100% sensitivity, and 100 % specificity (p<0.0001). In a similar manner, a previous study reported that using serum BLCA-1 in bladder cancer, in ROC curve analysis at AUC of 0.743, the sensitivity was 74% and specificity 69% for detecting malignant cases (p = 0.042).[20]

A previous study reported that MTDH enhances the metastatic potential of cancer cells by regulating multiple signaling pathways. miRNAs and various tumor-related proteins have been shown to interact with MTDH, making it a potential therapeutic target as well as a biomarker in human malignancies.[41] In our study, the plasma expression of the MTDH biomarker was significantly higher in the malignant group than patients in the benign group and the studied controls. Our result agreed with findings of a previous study, reported higher expression of MTDH in bladder cancer tissue in BC patients than in patients with benign bladder disease.[42] Other studies reported that the expression level of mRNA MTDH was notably higher in the bladder cancer tissues than in the normal tissues.[43,44]

In this study, there was significant elevation of plasma mRNA MTDH expression in BC patients with grade 3 compared to grade 1 and 2 patients. Also, BC patients with stage II had significantly higher expression levels of plasma mRNA MTDH than those with grade 1. Moreover, in the present study, according to TNM staging, there was significant elevation of plasma mRNA MTDH expression levels in BC patients with stage II compared to stage I patients. These results agreed with those reported in a previous study which found that plasma mRNA MTDH expression was correlated with grades of BC disease.[42]

To evaluate the role of plasma mRNA MTDH expression in diagnosis of BC, the ROC analysis was performed. At a cutoff value of 0.2, and an AUC of 0.993, the sensitivity was 94.92% and specificity 96.55%, and accuracy 95.45 (p<0.0001). In conclusion, our study data indicated that assessment of blood Cystatin C, BLCA-1and MTDH levels, can be used to differentiate between benign bladder lesion and bladder cancer. Also, they correlated with bladder cancer tumor grades. BLCA-1 was a better marker than Cystatin C and MTDH

Acknowledgements

The authors would like to thank all study subjects for their cooperation and participation in the study.

Author Contributions

ZAA, LMT, TS, AMA, AMM, ENA, EMA; contributed to the study conception and design, contributed to material preparation, data collection and analysis. AMM, provided clinical support. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) denies receipt of any financial support for the research, authorship, and/or publication of this article.

Ethical approval

The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Assiut University (approval dated, June 2020).

Informed consent

A written informed consent was obtained from each participant before being enrolled in the study.

References

1. Asker, N. & Gabal, R. A. (2022). Theoretical and Experimental Approach for the Sensation of Urine Ubiquitin Conjugating Enzyme E2 C (UBE2C) mRNA Related to Bladder Cancer by Polyacrylamide Sensor Doped with Zinc Oxide. *Trends in Sciences,* 20(2):3566.

2. Mamdouh, S., Khorshed, F., Hammad, G., et al. (2022). Molecular Detection of Genetic Susceptibility to Bladder Cancer in Egyptian Patients. *Asian Pacific Journal of Cancer Prevention: APJCP*, 23(1):221.

3. Essa, H., Abeer, A., El-Morshedy, S.A.M.A.R. et al. (2022). A Clinico-Epidemiological Study and Clinical Outcome in Patients with Urinary Bladder Cancer at Assiut University Hospital from 2015-2019 (Hospital Based Study). *The Medical Journal of Cairo University*, 90 (12):2673-2681.

4. Yu, C., Wang, S., Lai, W. F., et al. (2023).The Progress of Chitosan-Based Nanoparticles for *Intravesical Bladder Cancer Treatment. Pharmaceutics,* 15(1): 211.

5. Guo Wang, S. S., Zhai, G. Q., Chen, G., et al. (2022). Metadherin Promotes the Development of Bladder Cancer by Enhancing Cell Division. *Cancer Biotherapy & Radiopharmaceuticals*

6. Atag, E., Coban, E., Sari, M., et al. (2023). Treatment Approach for Muscle-Invasive Bladder Cancer: *Real-Life Data from a Single Center.*

7. Wang, Z., Li, H., Chi, Q., et al. (2018). Clinical significance of serological and urological levels of bladder cancer-specific antigen-1 (BLCA-1) in bladder cancer. Medical Science Monitor: *International Medical Journal of Experimental and Clinical Research,* 24:3882.

8. Guszcz, T., Szymańska, B., Kozlowski, R., et al. (2020). Plasma aromatase as a sensitive and selective potential biomarker of bladder cancer and its role in tumorigenesis. *Oncology Letters*, 19(1): 562-568.

9. Onopiuk, A., Tokarzewicz, A., &Gorodkiewicz, E. (2015). Cystatin C: a kidney function biomarker. *Advances in clinical chemistry*, *68*:57-69.

10. Dikovskaya, M. A., Trunov, A. N., Chernykh, V. V., et al. (2013). Cystatin C and lactoferrin concentrations in biological fluids as possible prognostic factors in eye tumor development. *International Journal of Circumpolar Health,* 72(1):21087.

11. Santoni, M., Catanzariti, F., Minardi, D., B et al. (2012). Pathogenic and diagnostic potential of BLCA-1 and BLCA-4 nuclear proteins in urothelial cell carcinoma of human bladder. *Advances in Urology*.

12. Sarkar, D., & Fisher, P. B. (2013). AEG-1/MTDH/LYRIC: Clinical Significance. *Advances in cancer research,* 120:39-74.

13. Wang, S.S., Zhai, G.Q., Chen, G., et al. (2022). Metadherin Promotes the Development of Bladder Cancer by Enhancing Cell Division. *Cancer Biotherapy & Radiopharmaceuticals.*

14. Cancer.Net Editorial Board*.,* 2020, Robins, D J., (2020). "MP86-17 the 2020 American Joint Committee on Cancer eighth edition cancer staging manual: changes in staging guidelines for cancers of the kidney, renal pelvis and ureter, bladder, and urethra.The Journal of Urology 197.4S (2020): e1163-e1163.

15. Saginala, K., Barsouk, A., Aluru, J.S., et al. (2020). Epidemiology of bladder cancer. *Medical sciences*, 8(1):15.

16. Zhang, L., Xu, C., Chen, X et al. (2018). SOCS-1 suppresses inflammation through inhibition of NALP3 inflammasome formation in smoke inhalation-

17. Witjes, J. A., Babjuk, M., Bellmunt, J., et al. (2020). EAU-ESMO Consensus statements on the management of advanced and variant bladder cancer-an international collaborative multistakeholder effort†: under the auspices of the *EAU-ESMO Guidelines Committees. European Urology*, 77(2): 223-250.

18. Tokarzewicz, A., Guszcz, T., Onopiuk, A., et al. (2018). Utility of cystatin C as a potential bladder tumor biomarker confirmed by surface plasmon resonance technique. *The Indian journal of medical research,* 147(1): 46.

19. Okbah, A.A., Al-Shamahy, H.A., Al-Shamahi, E.H. et al. (2022). Renal lesions: Differentiation of malignant and benign tumors, sex and age distribution and variables associated with renal cell carcinoma in Sana'a City, Yemen. *Universal Journal of Pharmaceutical Research,* 7, pp.34-39.

20. Wang, R., Ke, Z. F., Wang, F., et al. (2015). GOLPH3 overexpression is closely correlated with poor prognosis in human non-small cell lung cancer and mediates its metastasis through upregulating MMP-2 and MMP-9. *Cellular Physiology and Biochemistry*, 35(3): 969-982.

21. Daugherty, S. E., Lacey Jr, J. V., Pfeiffer, R. M., et al. (2013). Reproductive factors and menopausal hormone therapy and bladder cancer risk in the NIH-AARP Diet and Health Study. *International journal of cancer,* 133(2): 462-472.

22. Mancini M, Righetto M, Baggio G. (2020). Spotlight on gender-specific disparities in bladder cancer. *Urologia.;* 87(3):103–114.

23. Marks, P., Soave, A., Shariat, S. F., et al. (2016). Female with bladder cancer: what and why is there a difference? *Translational andrology and urology*, *5*(5):668.

24. Zhao, L., Tian, X., Duan, X., et al. *(2017).* Association of body mass index with bladder cancer risk: a dose-response meta-analysis of prospective cohort studies. *Oncotarget,* 8(20): 33990.

25. Karra, P., Winn, M., Pauleck, S., et al. (2022). Metabolic dysfunction and obesity-related cancer: Beyond obesity and metabolic syndrome. *Obesity*, 30(7):1323-1334.

26. Pollak, M. (2012). The insulin and insulin-like growth factor receptor family in neoplasia: an update. *Nature Reviews Cancer*, 12(3): 159-169.

27. Arthuso, F. Z., Fairey, A. S., Boulé, N. G., et al. (2022). Associations between body mass index and bladder cancer survival: Is the obesity paradox short-lived. *Canadian Urological Association Journal,* 16(5):E261.

28. Zhao, X., & Liang, C. (2022). Cigarette smoking and risk of bladder cancer: a dose–response meta-analysis. *International Urology and Nephrology*, 1-17.

29. Weiss, K.G., Matulewicz, R.S., Moreton, E., et al. (2023). History of the Relationship Between Smoking and Bladder Cancer: A Public Health Perspective. Urology, 171:.6-10.

30. Pottegård, A., Kristensen, K. B., Friis, S., et al. (2020). Urinary tract infections and risk of squamous cell carcinoma bladder cancer: a Danish nationwide case–control study. *International Journal of Cancer*, *146*(7): 1930-1936.

31. Wurpel, D. J., Beatson, S. A., Totsika, M., et al. (2013). Chaperone-usher fimbriae of *Escherichia coli. PloS one,* 8(1): e52835.

32. Jalloh, M., Cassell, A., Diallo, T., et al. (2020). Is schistosomiasis a risk factor for bladder cancer? Evidence-based facts. *Journal of tropical medicine*, 2020.

33. Di Maso, M., Turati, F., Bosetti, C., et al. (2019). Food consumption, meat cooking methods and diet diversity and the risk of bladder cancer. *Cancer Epidemiology*, 63:101595.

34. Wigner, P., Bijak, M., & Saluk-Bijak, J. (2022). Clinical Potential of Fruit in Bladder Cancer Prevention and Treatment. *Nutrients*, 14(6): 1132.

35. Abufaraj M, Tabung FK, Shariat SF, et al. (2019). Association between inflammatory potential of diet and bladder cancer risk: results of three United States prospective cohort studies. *J Urol.* 2019; 202:484–9

36. Ricordi C, Garcia-Contreras M, Farnetti S. (2015). Diet and inflammation: possible effects on immunity, chronic diseases, and life span. *J Am Coll Nutr.* 34:10–3.

37. Garalla, H. M., & Darraz, K. M. (2022). Clinicopathological Study of Urinary Bladder Cancer.

38. Gocheva, V., & Joyce, J. A. (2007). Cysteine cathepsins and the cutting edge of cancer invasion. Cell cycle, 6(1):60-64.

39. Onopiuk, A., Tokarzewicz, A., &Gorodkiewicz, E. (2015). Cystatin C: a kidney function biomarker. *Advances in clinical chemistry*, *68*: 57-69.

40. Feng, C., Wang, L., Ding, G., et al. (2015). BLCA-1 expression is associated with angiogenesis of bladder cancer and is correlated with common pro-angiogenic factors. *International Journal of Clinical and Experimental Medicine*, *8*(9): 16259.

41. Chen, Y., Huang, S., Guo, R., et al. (2021). Metadherin-mediated mechanisms in human malignancies. *Biomarkers in Medicine*, 15(18): 1769-1783.

42. Zhou, J., Li, J., Wang, Z., et al. (2012). Metadherin is a novel prognostic marker for bladder cancer

progression and overall patient survival. *Asia-Pacific Journal of Clinical Oncology*, 8(3): e42-e48.

43. Nikpour, M., Emadi-Baygi, M., Fischer, U., et al. (2014). MTDH/AEG-1 contributes to central features of the neoplastic phenotype in bladder cancer. In Urologic Oncology: *Seminars and Original Investigations.* 32(5): 670-677.

44. Zhang, L., Yang, G., Chen, H., et al. (2017). Depletion of astrocyte elevated gene-1 suppresses tumorigenesis through inhibition of Akt activity in bladder cancer cells. *American Journal of Translational Research*, *9*(12).