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Diagnostic Performances of Urine Cytology and TERT Promoter Mutations in Bladder Cancer

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ABSTRACT

Background: Detecting bladder cancer (BC) using urinary biomarkers may provide a valuable opportunity for screening and management. The best hope for reducing bladder cancer mortality and morbidity remains early detection. Two hotspot mutations in the promoter region of the C228T and C250T, are frequently found in several tumor types, and considered as an early event in BC tumorigenesis.
This study aims to assess the validity and diagnostic potential of these mutations to detect BC in urine tDNA-based liquid biopsy in patients and evaluate the expression of NMP-22 and MMP-9 in the urine of patients and controls, analyze the diagnostic efficacy of them and to examine their expression in relation to the TERT mutant and wild patients.
Methods & Results: 210 BC patients and 95 healthy volunteers served as controls were screened for TERT promoter mutations by PCR from urine samples, in addition to Enzyme-Linked Immunosorbent Assay (ELISA) detection for NMP-22 and MMP-9 levels, a significant increase in the expression level of NMP-22 and MMP-9 was detected indicating a significant diagnostic capability for BC, and was higher for TERT mutant variants. 141 patients (67.1%) were identified to harbor C228T TERT promoter mutations, while C250T was detected in 64 patients (30.4%). Univariate logistic regression analysis revealed that the 2 mutations were statistically associated with BC, in addition to an association with high grades, tumor recurrence and invasiveness. Conclusion: Detection of TERT promoter mutations in urine could present a reliable noninvasive diagnostic marker for BC, with patient survival time, disease recurrence and invasiveness as a unique predictor marker with individualized prognostic potential.

Keywords: Urine, Bladder cancer, TERT mutations, recurrence, survival time.

INTRODUCTION

Bladder cancer (BC) is considered the most common urinary tract malignancy, ranking 9th and 13th in terms of frequency mortality rates respectively [1]. BC occurrences and mortalities are generally associated in regions with high human development index [2]. Disease progression has been linked to environmental, lifestyle, and hereditary causes [3].

Major risk factors include tobacco smoking and occupational exposure to carcinogens, where reports have indicated incidences deceasing in regions that have lower smoking populations [4]. Bladder cancer is classified as non-muscle invasive bladder cancer (NMIBC) or muscle invasive bladder cancer (MIBC), depending on the presence or absence of invasion of the primary tumor into the muscle wall of the bladder [5].

Histologically, BC can be classified into urothelial carcinoma (UC), which is the most common sub-type (around 90%) and other rare non-urothelial tumors [6]. The International Agency for Research on Cancer (IARC) mark BC awareness month throughout May, 2022 by highlighting research on the prevention and detection of this disease. Every year, almost 600,000 people worldwide are diagnosed with bladder cancer. It is a complex and highly recurrent disease and is one of the most challenging and expensive cancer types to diagnose and treat. Therefore, IARC scientists are working on various projects in this area, including the development of a simple urine test with the potential to significantly improve early detection of bladder cancer. The test is based on the detection of mutations in the promoter of the telomerase reverse transcriptase (TERT) gene, which are the most common mutations in bladder cancer. Hotspot mutations of the TERT promoter C228T and C250T are frequently identified in primary tumors from patients with various types of cancer and tumor stages, including precancerous lesions [7].

Due to both recent advances in genomic, epigenomic and proteomic studies of tumor fluids, and the development of sensitive analytical molecular techniques, a new approach for cancer detection has emerged, namely liquid biopsy. This technique is based on the analysis of various tumor-related targets circulating in biological fluids, such as blood, urine, saliva and cerebrospinal fluid [1]. These targets include cell-free tumor DNA (tDNA), different types of tumor RNAs, peptides, metabolites, exosomes, endosomes, and even circulating tumor cells [8]. The TERT promoter 'hotspot' mutations C228T and C250T were selected as tDNA biomarkers, since they appear to be common in BC tumors and are completely absent in healthy somatic cells [9].

The strong need for the discovery of novel markers with the development of high-throughput techniques that provide highly sensitive analysis of protein content in tissues and body fluids, using proteomics, has opened new chapter in biomarker discovery [10].

The field of "proteomics" was first proposed and has been described as a characterization of the presence and activity of all proteins in a tissue, cell, or fluid [11]. Several proteomics studies in this field of early detection of BC have been described as outlined in a number of reviews [12,13].

The technical advances have allowed the field of proteomics to become extremely productive in the identification of proteins in the blood, urine, or tissue of BC patients, many of which may have the potential to be utilized in the diagnosis of bladder tumors. Currently, an increasing number of studies are being carried out with various goals aiming to improve the therapeutic treatment of BC and to better understand the potential prognostic role of proteomics in BC after follow-up [14].

Nuclear matrix protein (NMP) was first set out in 1974. It is a nonchromatin structure present in the nucleus of tumor cells. After cell death, NMP-22 is released and is available in human urine in the form of soluble complexes or fragments. Urinary NMP-22 is directly released by tumor cells, so the test results are more reliable [15]. In recent years, there have been a lot of clinical studies on NMP-22 in non-invasive bladder tumor screening and monitoring. NMP-22 is considered to be an efficient substitute, either alone or in combination with urine cytology (UC). It is utilized for the diagnosis and screening of bladder tumors, grading and staging and predicting prognosis [16].

Matrix metalloproteases (MMPs) are a multigene family of zinc-dependent endopeptidases with a comparable structure and the ability to degrade practically every extracellular matrix component collectively [17]. Matrix metalloproteinases use their endopeptidase activity to destroy proteins in the extracellular matrix.

The MMP family consists of at least 28 members and all MMPs contain two conserved domains, a catalytic domain and a prodomain that are crucial for their activity and substrate specificity [18]. MMP-9 belongs to the gelatinase subfamily and is the most complex member of the MMP family, with proteolytic activity against type IV collagen. MMPs are particularly important in tumor invasion, metastasis, and angiogenesis. Cancer cell

proliferation, tumor development, and epithelial-to-mesenchymal transition have all been linked to MMP-9 [19].

More than five decades ago, Dr. George Papanicolaou hypothesized that microscopic evaluation of exfoliated cells in the urine was a potentially useful method to detect bladder malignancies [20]. Urine cytology has been a standard diagnostic test to aid in the diagnosis of bladder cancer and is increasingly accepted as a diagnostic tool in the detection and follow-up of patients with BC.

The aim of the present study is to examine the profile of TERT promoter mutations including TERT C228T and C250T**,** in urine samples of BC patients and to assess its diagnostic potential as non-invasive early biomarkers, and analyze the association of these mutations with recurrence, metastasis and surveillance of BC patients. In addition, to evaluate the expression of NMP-22 and MMP-9 in the urine of patients and controls**,** analyze the diagnostic efficacy of them and to examine their expression in relation to the TERT mutant and wild patients.

SUBJECTS AND METHODS

Patients. Two hundred and ten patients from the urology department of Theodor Bilharz Research Institute (TBRI, Egypt) presenting BC irrespective of histological grade and stage, were initially enrolled in this study. To enhance validity of the study, 95 healthy individuals without BC were also included as controls.

Informed consent was obtained from all participants to use their specimens for research purposes, as required by the Ethics Committee of TBRI, in accordance with the institutional guidelines. This study complies with the latest version of the Declaration of Helsinki and general guidelines for good clinical practice. Fifty milliliters of morning voided urine samples were collected from patients and controls, samples were centrifuged at 3000 rpm for 20 min, the supernatant was decanted and the pellet was re-suspended in 1x pbs (PH7.2), centrifuged again and the pellet was used for urine cytology and DNA extraction.

Cytology. Mid-stream urine samples, preferably the morning ones were collected in sterile cups with caps. Samples were centrifuged using the cytospin. Smears were stained with Hematoxylin and eosin as well as Papanicolaou stain, cover-slipped and then screened and evaluated by a cyto-pathologist. After cytological evaluation, the specimens were classified according to Paris classification [21].

Diagnostic categories for The Paris System for Reporting Urinary Cytology

1 Nondiagnostic/unsatisfactory
2 Negative for high-grade urothelial carcinoma (NHGUC)
3 Atypical urothelial cells (AUC)
4 Suspicious for high-grade urothelial carcinoma (SHGUC)
5 High-grade urothelial carcinoma (HGUC)
6 Low-grade urothelial neoplasm (LGUN)
7 Other: primary and secondary malignancies and miscellaneous lesions

Histopathology. Tumor stage and histological grade were assessed according to the International Union Against Cancer tumor, node, metastases system and the 2004 World Health Organization classification [22]. Histopathology served as the gold standard for cancer diagnosis (True Positive).

Molecular testing. Genomic DNA was extracted from urine sediments using Qiagen DNeasy kit (Hilden, Germany) according to the manufacturer's recommendations. The purified DNA was dissolved in 50 µl water, measured on a Nanodrop ND-2000c (Thermo Scientific, Waltham, MA, USA) and stored at −20°C for further analysis.

Mutations of TERT promoter were analyzed in C228T and C250T by PCR in a final volume of 25 µl containing 100 ng of urine sediment DNA. The PCR reactions were assembled on ice and initially denatured at 95°C for 5 minutes, 35 cycles at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. Positive controls (DNA of healthy volunteers) were included in each PCR reaction.

PCR products were resolved on 3% agarose gel, electrophoresed on a Bio-RAD electrophoresis chamber, with 5 µl of 100-1000 bp DNA ladder RTU used as a marker and visualized by ethidium bromide staining. The gel image was analyzed using Cleaver micro DOC gel documentation system.

Evaluation of the urine levels of NMP-22 and MMP-9. All urine samples were tested for NMP-22 and MMP-9 levels by ELISA method according to manufacturer's instructions using Human assay kits (Sunlong Biotech Co., Ltd). Briefly, 100 µl of prepared standards and samples were added to appropriate wells of ELISA plate and then assayed according to the manufacturer's instructions. The absorbance was measured at 450 nm in a microtest plate spectrophotometer, (Abcamm CA, USA), NMP-22 and MMP-9 levels were quantified. Each standard or sample was assayed in duplicate. Diagnostic yields of these markers were evaluated by analysis of the ROC curves.

Statistical Analysis

The data were analyzed using Microsoft Excel 2016 and statistical package for social science 'IBM SPSS Statistics for Windows, version 28 (IBM Corp., Armonk, N.Y., USA)'. Continuous normally distributed variables were represented as mean±SD. with 95% confidence interval, and using the frequencies and percentage for categorical variables; a P value < 0.05 was considered statistically significant. To compare the means of normally distributed variables between groups, the student's t test was performed. $\chi 2$ test or Fisher's exact test was used to determine the distribution of categorical variables between groups. The diagnostic performance of the studied markers was assessed by receiver operating characteristic (ROC) curves. The area under the ROC (AUC) was calculated as an accuracy index for prognostic performance of selected tests. The risk assessment OR (95% C.I) was done by using the logistic regression analysis. Survival analysis was performed using Log Rank (Mantel-Cox).

RESULTS

Patient demographics and pathological characteristics

TERT mutation status was analyzed in 210 specimens including radical cystectomy and transurethral resection samples. At time of diagnosis, the patients aged 45-78 years with mean age of 64.3±7.2. They including 164 (78.1%) males and 46 (21.9%) females. Ninety-five healthy individuals with no history of bladder disease were included as a control group aged 23-65 years with mean of (41.9±12.9) including 63 male (66.3%) and 32 female (33.7%). Both age and sex had statistically highly significant risk assessment to BC with 95% confidence interval (95% C.I) of 1.233(1.173 - 1.296) and 1.811(1.059 - 3.097) respectively with P value <0.001 for age and P=0.03 for sex.

Of the 210 cases of BC analyzed, 139(66.2%) patients were smokers, 168(80%) were Schistosoma infected and 93(44.3%) were infected with hepatitis C virus (HCV).

At the time of diagnosis, there were 113 (53.8%) SqCC, 97 (46.2%) TCC, Among the 210 of the urinary bladder patients, 127 (60.5%) were with single tumor, 83 (39.5%) with muti-tumor, 131(62.4%) were low grade and 79 (37.6%) were high tumor grade, 37 (17.6%) were pathologically diagnosed as T1, 132(62.9%) were T2, 36(17.1%) were T3 and only 5(2.4%) were T4, Table 1 summarizes the other patient characteristics and clinical findings (Table 1).

Table 1. Clinico-Pathological Characteristics of BC Patients:

		Groups	
		Number	%
Smoking	No	71	33.8%
	Yes	139	66.2%
Schistosomiasis	Negative	42	20.0%
	Positive	168	80.0%
HCV	Negative	117	55.7%
	Positive	93	44.3%
Pathological diagnosis	SqCC	113	53.8%
	TCC	97	46.2%
Papillary	Negative	165	78.6%
	Positive	45	21.4%
Number of tumors	Single	127	60.5%
	Multi	83	39.5%

Maximum tumor diameter			3.6±1.9
Lymph node	Negative	99	47.1%
	Positive	111	52.9%
Grade of tumor	Low	131	62.4%
	High	79	37.6%
Stage	T1	37	17.6%
	T2	132	62.9%
	T3	36	17.1%
	T4	5	2.4%
Urine cytology	Negative	144	68.6%
	Positive	66	31.4%
Carcinoma In Situ	Negative	169	80.5%
	Positive	41	19.5%
Tumor recurrence	No	123	58.6%
	Yes	87	41.4%
Time for recurrence			35.1±10.1
Tumor invasiveness	No	37	17.6%
	Yes	173	82.4%
5y Survival	No	180	85.7%
	Yes	30	14.3%
Tumor progression	No	29	13.8%
	Yes	181	86.2%
C228T	Wild	69	32.9%
	Mutant	141	67.1%
C250T	Wild	146	69.5%
	Mutant	64	30.5%

Levels of NMP-22 and MMP-9

Independent t test was used to analyze the concentrations of NMP-22 and MMP-9 in urine samples of BC patients and healthy volunteers, the data showed highly significant increase in the expression level of NMP-22 and MMP-9 in the urine of BC patients than healthy volunteers with 95% C.I of 2.211(1.126 - 3.435) and 2.209(1.118 - 2.986) respectively and with the same P value <0.001 (Table 2, Fig. 1).

Table 2. Urine levels of NMP22 and MMP-9 with risk assessment in the studied groups.

	Control N=95	Cases N=210	P. vale	Risk assessment	
				OR (95% C.I)	P. vale
NMP22 ng/ul	1.1±0.8	15.3±4.2	<0.001**	2.211(1.126 - 3.435)	<0.001**
MMP-9 ng/ul	1.3±1.05	13.7±5.3	<0.001**	2.209(1.118 - 2.986)	<0.001**

NMP-22 and MMP-9 are represented as Mean ±SD; the data were analyzed by independent t test.
OR; Odd Ratio, C.I; Confidence Interval, P value of risk assessment was calculated depending on logistic regression analysis.
* P. value <0.05 is significant, ** P. value <0.01 is highly significant.

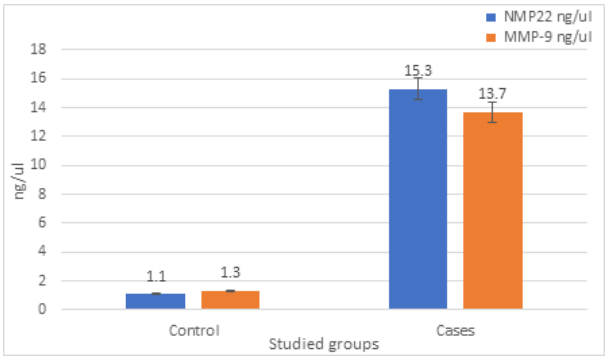


Fig. 1: NMP-22 and MMP-9 concentration levels in BC patients and controls.

Diagnostic performances of NMP-22 and MMP-9 in urine samples

Receiver Operating Characteristic (ROC) Curves were established to show the diagnostic performances of the 2 biomarkers regarding the studied groups. Statistical analysis showed that NMP-22 and NMP-9 were significantly able to diagnose BC with P value <0.0001 and to differentiate between the urine sample of BC patient and that of a healthy volunteer. ROC analysis showed that NMP-22 was at cut-off value of ≥4.2, with sensitivity and specificity of 100.0%, the area under curve (AUC) was 1, with 0 standard error, while MMP-9 was at cut-off value of ≥4.13, with sensitivity and specificity of 100.0%, with an AUC was 1, with 0 standard error (Table 3, Fig. 2).

Table 3. Diagnostic performances of NMP-22 and MMP-9.

Test Result Variable(s)	Cut-off	Sn. %	Sp. %	AUC	S. E	95% C. I		P. value
						Lower Bound	Upper Bound	
NMP-22 ng/ul	≥4.2	100.0	100.0	1.000	0.000	1.000	1.000	<0.0001**
MMP-9 ng/ul	≥4.13	100.0	100.0	1.000	0.000	1.000	1.000	<0.0001**

Sn: Sensitivity, Sp: Specificity, AUC Area under curve, S.E; Standard Error and C.I: 95% Confidence Interval.

* P value <0.05 is significant, ** P value <0.01 is highly significant.

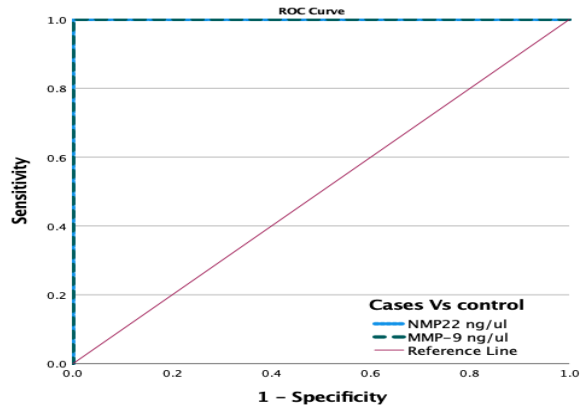


Fig. 2: ROC Curve of NMP-22 and MMP-9 for BC patients and controls.

Urine cytology remains to be the test of choice in the detection of BC due to its favorable sensitivity. ROC analysis was used to evaluate the diagnostic performances of urine cytology, C228T and C250T TERT mutations, it was noted that the urine cytology test and the 2 mutations were significantly able to detect BC in urine samples indicating a significant diagnostic power of them. Regarding urine cytology test, the sensitivity was 70.42%, specificity 88.24%, with an AUC of 0.74, 95% C.I (lower bound-upper bound) was (0.67-0.81) and P=0.001. Our results of the diagnostic performance concerning C228T mutation showed that it is more efficient than urine cytology test in the detection of BC with sensitivity was 74.63%, specificity 100%, with an AUC of 0.79, 95% C.I (0.72-0.86) and P=0.001, while the sensitivity of C250T was 58.82%, specificity 100%, with an AUC of 0.65, 95% C.I (0.59-0.72) and P=0.01. The combination of the 2 mutations showed significant diagnosis for BC with sensitivity 75.76%, specificity 100%, with an AUC of 0.8, 95% C.I (0.74-0.89) and P=0.001. The highest sensitivity was observed when combing urine cytology and the TERT C228T mutation of

87.72%, specificity 100%, with an AUC of 0.93, 95% C.I (0.38-0.79) and P<0.001, this means that urine cytology in combination with the TERT C228T mutation can significantly detect BC with highly sensitive test (Table 4).

Table 4. Diagnostic performance of urine cytology and the studied mutations.

Test Result Variable(s)	Sn. %	Sp. %	AUC	S. E	95% C. I		P. value
					Lower Bound	Upper Bound	
Urine cytology	70.42	88.24	0.74	0.023	0.67	0.81	0.001**
C228T	74.63	100.0	0.79	0.042	0.72	0.86	0.001**
C250T	58.82	100.0	0.65	0.012	0.59	0.72	0.01*
C228T&C250T	75.76	100.0	0.8	0.02	0.74	0.89	0.001**
Urine cytology+C228T	87.72	100.0	0.93	0.02	0.38	0.97	<0.001**

Sn: Sensitivity, Sp: Specificity, AUC Area under curve, S.E; standard Error and C.I: 95% Confidence Interval.
* P value <0.05 is significant, ** P value <0.01 is highly significant.

TERT mutation analysis and associations with patient's outcome

Tumor DNA derived from 210 patients with BC and 95 healthy volunteers was analyzed for the TERT promoter status using PCR. A total of 141 patients (67.1%) were identified to harbor C228T TERT promoter mutations in their tumors, and were absent among matched controls who did not develop any bladder disease. Mutation frequency increased with age in patients with OR (95% CI) = 1.10(1.05- 1.15) (P <0.001). An association was found between TERT mutations and sex, among the mutant patients, 119 (84.4%) were male with OR (95% CI) =2.885(1.472- 5.652) (P=0.002) indicating that sex is significantly associated with mutation prevalence while only 22(15.6%) were mutant females.

A significant association was observed between TERT C228T promoter mutation and multicentric number of tumors with OR (95% C.I) = 2.457(1.378- 5.552) P<0.001, maximum diameter tumor size with OR (95% C.I) = 2.3(1.8- 2.9) P<0.001, positive lymph node with OR (95% CI) = 1.917(1.07- 3.436) P= 0.028, TERT promoter mutation prevalence was associated with high tumor grade compared to wild but this association did not reach BC risk assessment. There was a higher frequency of TERT mutations among T3 tumor stage than other stages with OR (95% CI) = 8.0(2.8- 22.6) P<0.001, positive urine cytology and positive carcinoma in situ (CIS) were associated with TERT promoter mutation prevalence with OR (95% CI) = 2.948(1.451- 5.991) P= 0.002, 5.781(1.969- 16.975) P<0.001 respectively.

During the follow-up period, 79(56.0%) among the mutant patients experienced BC recurrence with OR (95% CI) = 9.716(4.328- 21.808) P<0.001, this finding indicates that the patients with the TERT C228T mutation had significantly greater chance of bladder recurrence compared to wild patients. It was observed that the time consumed for tumor recurrence was shorter in mutants with mean of 34.5±10.3 months than wild patients with mean time of 41.0±5.3 months. Chi-squared test was used to analyze the tumor invasiveness that was closely associated with mutation prevalence with OR (95% CI) = 2.977(1.44- 6.155) P= 0.003. A significant association of TERT promoter mutation was observed with increased death frequency with OR (95% CI) = 1.617(0.55- 3.692) P<0.001. Regarding NMP-22 and MMP-9, higher concentrations were detected in the urine of TERT C228T mutants than wild patients with OR (95%CI) = 1.5(1.3- 1.7), 1.3(1.2- 1.4) respectively and P<0.001 (Table 5, Fig. 3).

Table 5. The association between C228T mutation status and clinicopathological data in BC patients.

		C228T			Risk assessment	
		Wild N=69	Mutant N=141	P. value	OR (95% C.I)	P. value

Age		61.1±5.5	65.9±7.5	<0.001**	1.10(1.05- 1.15)	<0.001**
Sex	Female	24(34.80%)	22(15.6%)	0.002**	2.885(1.472- 5.652)	0.002**
	Male	45(65.20%)	119(84.4%)			
Smoking	No	28(40.60%)	43(30.5%)	0.098	1.556(0.855- 2.835)	0.147
	Yes	41(59.40%)	98(69.5%)			
Schistosomiasis	Negative	4(5.80%)	38(27.0%)	<0.001**	0.167(0.057- 0.489)	<0.001**
	Positive	65(94.20%)	103(73.0%)			
HCV	Negative	32(46.40%)	85(60.3%)	0.04*	0.57(0.319- 1.019)	0.057
	Positive	37(53.60%)	56(39.7%)			
Pathological diagnosis	SqCC	41(59.40%)	72(51.1%)	0.16	1.403(0.783- 2.514)	0.254
	TCC	28(40.60%)	69(48.9%)			
Papillary	Negative	45(65.20%)	120(85.1%)	0.001**	0.328(0.166- 0.647)	<0.001**
	Positive	24(34.80%)	21(14.9%)			
Number of tumors	Single	69(100.00%)	58(41.1%)	<0.001**	2.457(1.378- 5.552)	<0.001**
	Multi	0(0.00%)	83(58.9%)			
Maximum tumor diameter		2.0±1.4	4.3±1.7	<0.001**	2.3(1.8- 2.9)	<0.001**
Lymph node	Negative	40(58.00%)	59(41.8%)	0.02*	1.917(1.07- 3.436)	0.028*
	Positive	29(42.00%)	82(58.2%)			
Grade of tumor	Low	49(71.00%)	82(58.2%)	0.048*	1.763(0.95- 3.272)	0.071
	High	20(29.00%)	59(41.8%)			
Stage	T1	20(29.00%)	17(12.1%)	0.564	0.9(0.4- 1.6)	0.622
	T2	45(65.20%)	87(61.7%)	0.1	1.1(0.3- 2.8)	0.2
	T3	4(5.80%)	32(22.7%)	<0.001**	8.0(2.8- 22.6)	<0.001**
	T4	0(0.00%)	5(3.5%)	-	-	-
Urine cytology	Negative	57(82.60%)	87(61.7%)	0.001**	2.948(1.451- 5.991)	0.002**
	Positive	12(17.40%)	54(38.3%)			
CIS	Negative	65(94.20%)	104(73.8%)	<0.001**	5.781(1.969- 16.975)	<0.001**
	Positive	4(5.80%)	37(26.2%)			
Tumor recurrence	No	61(88.40%)	62 (44.0%)	<0.001**	9.716(4.328- 21.808)	<0.001**
	Yes	8(11.60%)	79(56.0%)			
Time for recurrence		41.0±5.3	34.5±10.3	0.006*	0.9(0.9- 1.0)	0.093
Tumor invasiveness	No	20(29.00%)	17(12.1%)	0.002**	2.977(1.44- 6.155)	0.003**
	Yes	49(71.00%)	124(87.9%)			
5y survival	Alive	69(100.00%)	111(78.7%)	<0.001**	1.617(0.55- 3.692)	<0.001**
	Died	0(0.00%)	30(21.3%)			
Tumor progression	No	12(17.40%)	17(12.1%)	0.199	1.536(0.688- 3.427)	0.293
	Yes	57(82.60%)	124(87.9%)			
NMP22 ng/ul		12.0±3.8	17.0±3.3	<0.001**	1.5(1.3- 1.7)	<0.001**
MMP-9 ng/ul		10.2±3.5	15.5±5.2	<0.001**	1.3(1.2- 1.4)	<0.001**

Age, Max tumor diameter, Time for recurrence, NMP22, and MMP-9 are represented as Mean± SD; the data were analyzed by independent t test. While Sex, Smoking, Schistosomiasis, HCV, Pathological diagnosis, Papillary, Number of tumors, Lymph node, Grade, Stage, Cytology, Carcinoma In-situ (CIS), Recurrence, Invasiveness, 5y survival, and Tumor Progression are represented in (%); the data were analyzed by x2 test.

OR; Odd Ratio, C.I; Confidence Interval, P value of risk assessment were calculated depending on logistic regression analysis.

* P. value <0.05 is significant, ** P. value <0.01 is highly significant.

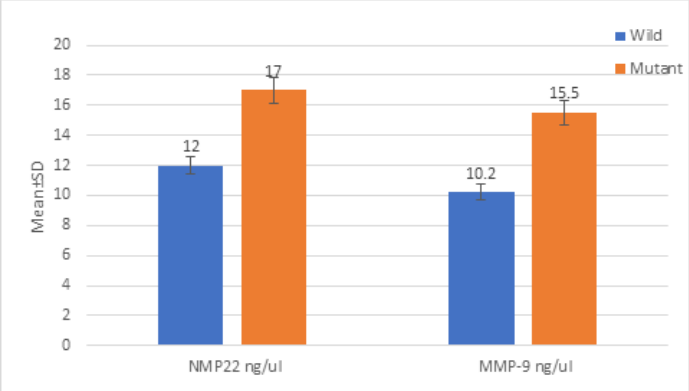


Fig. 3: The association between C228T TERT mutation with the NMP-22 and MMP-9 levels in urine.

Among TERT promoter hotspot mutations, C250T was less prevalent in BC patients and was detected in 64 patients (30.4%), while all the 95 controls do not have any mutation. We then determined a potential association between TERT promoter mutations and clinical variables in patients with BC, the median age of C250T mutant patients was 65.9±6.1 years, the frequency of TERT promoter mutations in older patients was obviously higher than younger aged patients with OR (95% CI) = 1.05(1.00- 1.10) P=0.03. There was no difference in sex between patients with and without TERT C250T mutation (P = 0.431). A highly significant association was observed between C250T mutation prevalence and mutant patients pathologically diagnosed with TCC with OR (95% CI) = 2.367(1.297- 4.32) P=0.005, multicentric number of tumors with OR (95% C.I) = 2.044(1.124- 3.715) P=0.018, maximum diameter tumor size with OR (95% C.I) = 1.9(1.6- 2.4) P<0.001, positive lymph node with OR (95% CI) = 3.952(2.056- 7.599) P<0.001. There was a higher frequency of TERT C250T mutations among high grade tumors than among low grade ones with OR (95% CI) = 4.573(2.448- 8.542) P<0.001.

Patients presenting with BC are known to be at risk of recurrence with more invasive tumors. We therefore evaluated whether TERT in urine could detect such recurrence, by analyzing the association of TERT status with tumor recurrence, it was observed that presence of TERT C250T promoter mutation in urine was strongly associated with tumor recurrence with OR (95% CI) =1.814(1.001- 3.286) P= 0.048. Similarly, as mentioned in C228T mutation, it was observed that the time consumed for tumor recurrence was shorter in mutants with mean of 30.4±10.3 months than wild patients with mean time of 37.9±9.0 months. A highly significant association of C250T TERT promoter mutation prevalence was observed with tumor invasiveness with OR (95% CI) = 1.587(1.416- 1.779) P<0.001. Our data showed tendency of poor survival in the patients that carried the mutations in tumors with OR (95% CI) = 2.673(1.217- 5.875) P= 0.013. Regarding NMP-22 and MMP-9, higher concentrations were detected in the urine of TERT mutants than wild patients with OR (95%CI) = 1.8(1.5- 2.2), 1.3(1.2- 1.4) respectively and P<0.001 (Table 6, Fig. 4).

Table 6. The association between C250T mutation status and clinicopathological data in BC patients.

		C250T			Risk assessment	
		Wild N=146	Mutant N=64	P. value	OR (95% C.I)	P. value
Age		63.6±7.6	65.9±6.1	0.02*	1.05(1.00- 1.10)	0.03*
Sex	Female	33(22.60%)	13(20.30%)	0.431	1.146(0.557- 2.358)	0.712
	Male	113(77.40%)	51(79.70%)			
Smoking	No	50(34.20%)	21(32.80%)	0.485	1.066(0.572-1.99)	0.84
	Yes	96(65.80%)	43(67.20%)			
Schistosomiasis	Negative	30(20.50%)	12(18.80%)	0.461	1.121(0.532- 2.361)	0.764
	Positive	116(79.50%)	52(81.30%)			

HCV	Negative	78(53.40%)	39(60.90%)	0.196	0.735(0.404-1.337)	0.313
	Positive	68(46.60%)	25(39.10%)			
Pathological diagnosis	SqCC	88(60.30%)	25(39.10%)	0.004**	2.367(1.297- 4.32)	0.005**
	TCC	58(39.70%)	39(60.90%)			
Papillary	Negative	118(80.80%)	47(73.40%)	0.154	1.524(0.764- 3.042)	0.23
	Positive	28(19.20%)	17(26.60%)			
Number of tumors	Single	96(65.80%)	31(48.40%)	0.014*	2.044(1.124- 3.715)	0.018*
	Multi	50(34.20%)	33(51.60%)			
Max tumor diameter		2.9±1.5	4.9±2.0	<0.001**	1.9(1.6- 2.4)	<0.001**
Lymph node	Negative	83(56.80%)	16(25.00%)	<0.001**	3.952(2.056- 7.599)	<0.001**
	Positive	63(43.20%)	48(75.00%)			
Grade	LOW	107(73.30%)	24(37.50%)	<0.001**	4.573(2.448- 8.542)	<0.001**
	HIGH	39(26.70%)	40(62.50%)			
Stage	T1	37(25.30%)	0(0.00%)	-	-	-
	T2	95(65.10%)	37(57.80%)	<0.001**	0.4(0.3- 0.6)	<0.001**
	T3	14(9.60%)	22(34.40%)	0.156	1.6(0.8- 3.1)	0.186
	T4	0(0.00%)	5(7.80%)	-	-	-
Cytology	Negative	103(70.50%)	41(64.10%)	0.22	1.344(0.721- 2.504)	0.351
	Positive	43(29.50%)	23(35.90%)			
CIS	Negative	122(83.60%)	47(73.40%)	0.067	1.839(0.907- 3.727)	0.088
	Positive	24(16.40%)	17(26.60%)			
Recurrence	No	92(63.00%)	31(48.40%)	0.035*	1.814(1.001- 3.286)	0.048*
	Yes	54(37.00%)	33(51.60%)			
Time for recurrence		37.9±9.0	30.4±10.3	<0.001**	0.9(0.9- 1.0)	0.001**
Invasiveness	No	37(25.30%)	0(0.00%)	<0.001**	1.587(1.416- 1.779)	<0.001**
	Yes	109(74.70%)	64(100.00%)			
5y survival	Alive	131(89.70%)	49(76.60%)	0.012*	2.673(1.217- 5.875)	0.013*
	Died	15(10.30%)	15(23.40%)			
Tumor Progression	No	29(19.90%)	0(0.00%)	<0.001**	1.547(1.389- 1.723)	<0.001**
	Yes	117(80.10%)	64(100.00%)			
NMP22 ng/ul		13.6±3.4	19.2±3.0	<0.001**	1.8(1.5- 2.2)	<0.001**
MMP-9 ng/ul		11.9±4.4	17.9±4.8	<0.001**	1.3(1.2- 1.4)	<0.001**

Age, Max tumor diameter, Time for recurrence, NMP22, and MMP-9 are represented as Mean± SD; the data were analyzed by independent t test. While Sex, Smoking, Schistosomiasis, HCV, Pathological diagnosis, Papillary, Number of tumors, Lymph node (LN), Grade, Stage, Cytology, carcinoma In-situ (CIS), Recurrence, Invasiveness, 5y survival, and Tumor Progression are represented in (%); the data were analyzed by X^2 test. OR; Odd Ratio, C.I; Confidence Interval, P value of risk assessment were calculated depending on logistic regression analysis. * P. value <0.05 is significant, ** P. value <0.01 is highly significant.

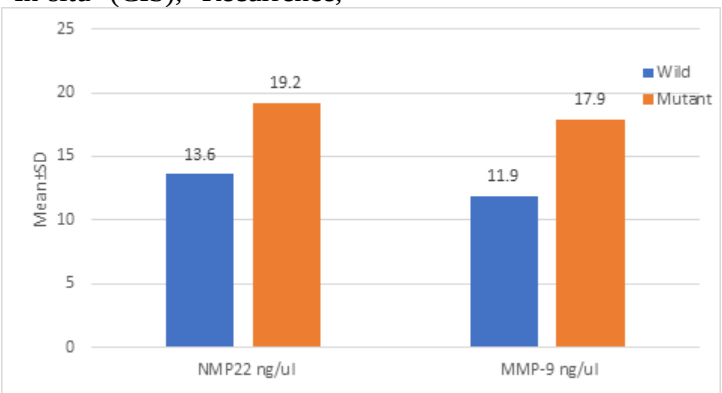


Fig. 4: The association between C250T TERT mutation with the NMP22 and MMP-9 levels in urine.

Kaplan-Meier analysis of TERT mutation regarding survival time.

Kaplan-Meier estimate is the simplest way of computing the survival over time in spite of all these difficulties associated with subjects or situations. It is also called as "product limit estimate". It involves computing of probabilities of occurrence of event at a certain point of time.

In the present study, Log Rank (Mantel-Cox) was used for survival analysis which showed that TERT promoter mutations were significantly associated with poor survival with OR (95% CI) = 1.617(0.55- 3.692) P<0.001 for C228T mutation and OR (95% CI) = 2.673(1.217- 5.875) P= 0.013 for C250T.

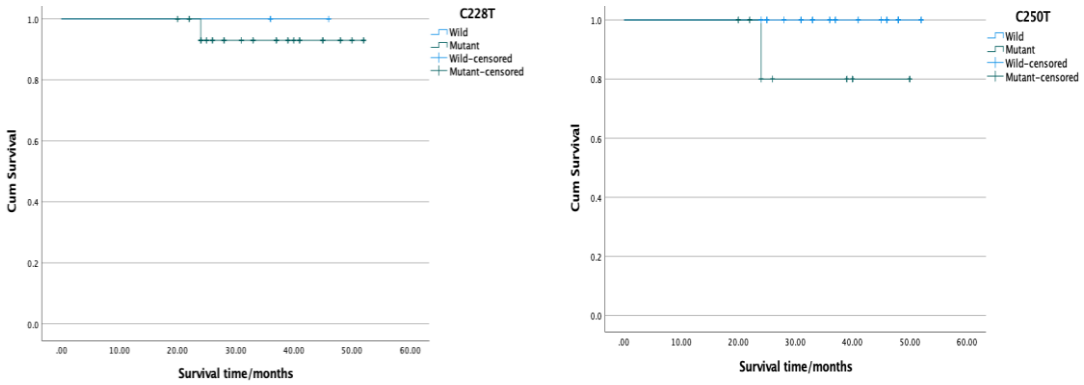
We also used Kaplan–Meier survival curves to further determine the effect of these two mutations on the survival of BC patients. TERT promoter mutations significantly affect the survival of BC patients. The patients with TERT promoter mutations had significantly shorter survival times than wild patients. Surprisingly, the analysis revealed that C228T did not reach statistically significant with number of events was 5, mean estimate of 0.930 months, Log Rank (Mantel-Cox)= 0.594 and P=0.441 while, TERT C250T mutation was significantly associated with worse survival of BC patients as compared with C228T with number of events was 5, mean estimate of 0.8 months, Log Rank (Mantel-Cox) = 11.384 and P <0.001 (Table 7, Fig. 5).

Table 7: Survival analysis

		No of Events	Mean Estimate	Std. Error	Log Rank (Mantel-Cox)	P. value
C228T	Wild	0	-	-	0.594	0.441
	Mutant	5	0.930	0.030		
C250T	Wild	0	-	-	11.384	<0.001**
	Mutant	5	0.8	0.08		

P. value depending on the Kaplan-Meier test.

* P value <0.05 is significant, ** P value <0.01 is highly significant.



a) C228T b) C250T

Fig. 5: Survival Curve of the studied TERT mutations.

DISCUSSION

More than 300 thousand new cases of bladder cancer are diagnosed in the world annually [23]. Identifying accurate non-invasive biomarkers that can facilitate early detection and improve post-treatment monitoring in BC patients might significantly contribute to reducing the mortality, morbidity, and economic burdens of BC worldwide [24].

Telomeres preserve genomic stability by preventing chromosomal fusions, the recent discovery that human tumors harbor mutations in the promoter region of the telomerase gene (TERT) produced a flurry of research aimed at

elucidating the role of these mutations in cancer development [25]

Urine-based tests are a non-invasive diagnostic tool for urological malignancies including BC. Since normal human cells lack TERT promoter mutations, they are likely ideal urinary markers for diagnosis and disease monitoring of these urological cancers [26,27].

In the present study, we highlighted the potential of transcription-activating mutations in the promoter of the TERT gene detected in the urine as promising simple non-invasive biomarker for the detection of BC and surveillance and investigated the possible relationships between TERT mutant patients and the level of NMP-22 and MMP-9 that may contribute to the development of urogenital cancers.

Tumor DNA derived from 210 patients with BC was analyzed for the TERT promoter status using PCR, a total of 141 patients (67.1%) were identified to harbor C228T TERT promoter mutations in their tumors, 64 (30.4%) were carried C250T TERT promoter mutations, while no mutation was detected in the DNA of the 95 healthy individuals. Our result regarding mutations prevalence refers to that TERT promoter C228T mutation appeared to be the more frequent than C250T. Our result is slightly high and different from those reported by Wang et al. 2014 as they detected only 23 of 122 (19%) BC tumors had the mutation (15 cases with C228T and 8 with C250T) and partly similar to those of Jian et al. 2021 who reported that TERT promoter C228T mutation appeared to be the most frequent, as it was detected in 14/27 (51.8%) patients, while C250T mutation was detected in 4/27 (14.8%) patients [1,27]. Pakmanesh et al. 2022 reported that the most frequent TERT mutations among BC cases was C228T (18/31, 58%), C250T (4/31, 12.9%), these results come a little closer to our results, especially with regard to C228T [28].

These discrepancies may reflect differences in patient population, tumor location, sample size, methodology of mutation detection and environmental factors affecting the state of living. However, obstacles remain before urine telomerase activity-based assays can be translated into clinical practice [29]. Accordingly, it was of clinical interest to search for novel non-telomerase activity-based urinary biomarkers for the detection of BC, for this reason, we evaluate the concentrations of NMP-22 and MMP-9 in urine of BC patients and healthy individuals as recent advances in proteomics have facilitated the high-throughput profiling of data generated from bladder cancer–related proteins or peptides in parallel with high sensitivity and specificity, providing a wealth of information for biomarker discovery and validation [29].

Although urine cytology has been fraught with its lower diagnostic accuracy in the diagnosis of low-grade urothelial neoplasms, it continued to be the test of choice in many institutions in the detection of high-grade urothelial carcinomas (HGUC) due to its acceptable sensitivity [30]. Urine cytology is a valuable and practical tool for BC screening, our results revealed that the diagnostic performance of C228T TERT mutation is more sensitive and specific than urine cytology and C250T mutation. This result renders TERT mutation more important and sheds light on it because its sensitivity and specificity are superior to that of urine cytology, where is the latter was not able to detect the disease in all BC cases and negative urine cytology cases were reported. Our results indicated that the combination of the 2 mutations showed significant diagnosis for BC with a higher sensitivity of 75.76% than urine cytology and higher than each mutation alone. And what attracts attention is that the highest sensitivity was observed when combing the urine cytology and the TERT C228T mutation of 87.72%.

Enzyme-linked immunosorbent assay was performed to measure NMP-22 and MMP-9 protein

levels in urine samples of patients and control subjects, the results form ELISA clearly showed that the expression levels of NMP-22 and MMP-9 protein in BC group were significantly higher than the control group, and the difference between the two groups was statistically significant, the results also showed that the sensitivity and specificity of these two markers were 100.0%. This overexpression of NMP-22 and MMP-9 in urine of BC patients suggested that these proteins could cause tumorigenesis. Our results are identical to those of who published that urine levels of NMP-22 were significantly higher in the BC group

compared to controls ($p < 0.001$) [31]. According to Yang et al. 2022 who supported our results regarding MMP-9 levels, as he demonstrated that MMP9 expression was increased in bladder cancer tissues and BC cell lines [32].

This is the first study showing that TERT promoter mutations prevalence is closely related to the concentrations of NMP-22 and MMP-9 in the urine samples of BC patients, where mutant patients have higher concentrations, while wild patients have lower concentrations of NMP-22 and MMP-9.

Our findings confirm that both TERT mutations are significantly associated with high grade tumors although they are widespread through different stages and grades of the disease, suggesting their role as an early tumorigenic event and also focusing on the diagnostic efficiency of these mutations to detect BC at early stage and low grade.

Some studies agree with this finding and others do not, Vinagre et al. 2013 reported that TERT mutations were frequently detected in BC tumors with higher percentage in low-grade (67%) than high-grade tumors (56%) while Siraj et al. 2020 reported that TERT mutations were identified in 68.6% (140/204) of bladder cancer cases, with 68.8% being present in high grade tumors and 68.4% in low grade tumors [9,33].

Our results showed that the presence of TERT mutations in urine is a dynamic marker of recurrence as the mutation prevalence was significantly associated with tumor recurrence, it also showed that the time taken for tumor recurrence was shorter in mutants than wild patients, these results are matched with those reported by Hayashi et al. 2020, they published that patients with the TERT C228T mutation had a significantly greater chance of bladder recurrence compared

to patients without mutation ($p < 0.005$) [34].Moreover, Hayashi et al. 2021 found that C228T mutation detected in urinary cell-free DNA (cfDNA) was associated with bladder tumor recurrence in patients after transurethral surgery for NMIBC [35]. Batista et al. 2020 ’s results are similar to ours as they found an association between TERT mutations and tumor recurrence [33].

The present study clarified a highly significant association between TERT mutation and tumor invasiveness, this finding is in agreement with the study by Wu et al. 2014 as they found that the TERT promoter mutations were more prevalent in muscle-invasive tumors than in non–muscle-invasive tumors, and were more prevalent in bladder cancer patients with advanced tumor stages (T2–4) than those with low stage tumors (Ta or T1) [36]. Furthermore, chi-squared test identified CIS as significant predictors for BC especially for mutants in C228T and not for C250T mutants, this is in agreement with Descotes et al. 2017 who reported that TERT remaining positive after initial surgery was associated with residual carcinoma in situ [37].

The striking correlation between TERT promoter mutations and distant metastasis in BC suggests a role of TERT in the tumor dissemination, our result supports this suggestion through the association found between TERT mutation and positive lymph node which elucidates tumor metastasis, this finding goes parallel with that of Wang et al. 2014 who reported that distant metastasis was closely associated with the presence of TERT promoter mutations ($P = 0.001$)

[36]. On the contrary, a study by Siraj et al. 2020 reported that the TERT mutations were inversely associated with distant metastasis [9].

Although many studies of bladder cancer failed to detect any significant association between TERT promoter mutations and clinical outcomes, others have suggested an increased risk of recurrence, distant metastasis and decreased survival in patients with TERT promoter mutations.

The Kaplan–Meier estimator, also known as the product limit estimator, is a non-parametric statistic used to estimate the survival function from lifetime data.

Our results showed tendency of poor survival in the patients that carried mutations in tumors, this finding is identical with that of Rachakonda et al. 2013 who reported a poor survival in TERT mutant patients than wilds [38]. Wu and his colleagues, 2014 published results similar to ours as they reported that the Kaplan-Meier survival analysis revealed that the survival rate of patients with TERT mutations was significantly lower than that of patients without TERT mutations ($P < 0.001$)

[36]. Our result regarding survival time is in accordance with what Batista and others described in 2020, they reported that patients with the TERT mutations presented a poorer survival than wild BC patients [33]. On the contrary, Siraj et al. 2020 observed a trend towards good overall 5 years survival in patients with TERT mutations ($P = 0.0585$) [9]. de Kouchkovsky et al. 2021 supports the results of Siraj as they observed a significant association between TERT mutation and improved survival [39].

This study concluded that detection of TERT promoter mutations in urine is a reliable noninvasive early diagnostic marker for BC. The association of the mutations with patient survival time, disease recurrence and invasiveness can be a unique predictor marker with individualized prognostic potential.

DECLARATIONS

Ethical approval and Consent to participate *This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by* the Research Institute office for IRB (TBRI-IRB). Informed consent was obtained from all individual participants included in the study.

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Competing Interests, the authors have no relevant financial or non-financial interests to disclose.

Availability of Supporting data the datasets generated during and/or analysed during the current study are available from the corresponding author or Samah Mamdouh on reasonable request.

Author Contributions All authors contributed to equally to the research project. S.M. Conception and writing of manuscript, GH, practical work, and manuscript preparation, TA, Practical for IHC, data analysis, GS, study design, editing and reviewing the manuscript, KE samples, clinical data, and approval acquisition, and review of the manuscript.

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