TP53 Mutation for Prediction of Tumor Recurrence and Metastasis in Bladder Cancer Patients

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Abstract

Background: Bladder cancer (BC) is one of the most commonly diagnosed malignancies worldwide. Its complex etiopathogenesis is dependent on numerous risk factors that can be divided into three distinct categories: genetic and molecular abnormalities, chemical or environmental exposure and previous genitourinary disorders and family history of different malignancies.

This study highlights the genetic and molecular abnormalities that considered to be part of bladder carcinogenesis such as genetic mutations of TP53.

To date, cystoscopy remains the gold standard for diagnosis, butit is invasive and uncomfortable. Hence, many efforts are focusing on the development of accuratenon-invasive diagnostic tests for BC, consequently, this study aims to develop a new non-invasive and reliable test to accurately detect TP53 mutations in urine sediments of Egyptian BC patients and to evaluate the influence of their genetic status on tumor metastasis and recurrence as they
are the main problems during the disease.

Methods: A total of 150 BC patients and 50 healthy volunteers as controls were enrolled in this study, urine samples were obtained from all participants. DNA was extracted and TP53 mutations were examinedin exons (2+3), 4 and 5 by polymerase chain reaction (PCR). All PCR products were subjected to bidirectional sequencing.

Results: Fifty four (36.0%) patients of 150 were mutated in exon (2+3) with statistically higher significant in patients when compared to controls (P = 0.001),while 96 patients(64.0%) in Exon 4, and 111 patients(74.0%) in Exon 5 with the same (P = 0.001).Moreover, a significant association was observed between TP53 status with tumor stage and grade, being alterations more common in high-stage and high-grade tumors.

Conclusion: We conclude that TP53genetic mutations are independent prognostic factors for tumor metastasis and recurrence and the genetic alternation of TP53 could be used for prediction of bladder tumorigenesis.

Introduction

Bladder cancer (BC) is one of the most common urological cancers all over the world. Urothelial (transitional cell) carcinoma is the predominant histologic type in the United States and Europe, where it accounts for 90 percent of all bladder cancers **(Joaquim, 2021).** It is classified as either non–muscle-invasive bladder cancer (NMIBC) or muscle-invasive bladder cancer (MIBC), due to distinct implications for patient management **(Kamoun, et al., 2020).** At the molecular level, MIBC is a heterogeneous disease that is characterized by genomic instability and a high mutation rate.

Bladder cancer ranks the fourth in male patients and eleventh in female patients in terms of its morbidity among all kinds of tumors. According to the statistical analysis in 2021, It was interesting that the percentage of male patients was three times more compared with the percentage of female patients, and in which smoking might be the main cancer-promoting factor for those male patients with BC. **(Siegel, et al., 2020).**

The crucial issue today is to be able to detect BC easily and early in order to treat it sooner using less invasive methods. Over the past decade, progress has been made to improve detection methods using novel urinary biomarkers **(Charpentier, et al., 2021).**

The first step to detect BC is the presence of a painless visible hematuria, the most

common symptom in BC **(Ellen, et al., 2021).** In the case of BC suspicion, several tests exist. One of them is urine cytology. It is easy assay to perform with high specificity and low cost method. But, the real issue with this test is its low sensitivity for low-grade lesions and its high rate of equivocal results. Therefore, it is always used in combination with cystoscopy to

confirm the diagnosis **(Ruan, et al., 2021).** Cystoscopy is the gold standard for the detection of BC. It gives information about the number, localization, aspect and size of the tumor. It has a sound sensitivity

(68.3 to 100%) and a good specificity (57 to 97%) but can give false negative result and it is an invasive assay and causes patient discomfort, possible urinary tract

infection, and anxiety **(Zhu, et al., 2019).**

Analysis of genomic alterations in BC has revealed complex genomic patterns underlying bladder carcinogenesis **(Lindskrog, et al., 2021).**

It has been reported that various cell cycle modulators, such as TP53 was frequently deregulated in BC and the tumor protein p53 (TP53) was recognized as a tumor suppressor factor for several common tumors **(Liao et al., 2021).**

TP53 was recognized as a tumor suppressor gene for several common tumors, it is located on the short arm of chromosome 17p13 and it was first discovered in 1979 **(**Eccles & Phipps, 2012**).** The mutation of TP53 was frequently observed in lots of BC patients, and TP53 mutation might cause the progress of BC because TP53 and TP53 related pathways regulated many important carcinogenesis-related signal pathways **(Liao et al., 2021).**

The human tumor suppressor gene p53 maps to chromosome 17p13.1, consists of 11 exons spanning over 20 kb of DNA and encodes for a 393 amino acids, 53kDa nuclear protein **(Saleh, et al., 2021)**. The p53 protein has several biological functions such as involvement in cell cycle regulation, programmed cell death, senescence, differentiation and development, transcription, DNA replication, DNA repair and maintenance of genomic stability. Genetic changes in the p53 gene are found in almost every kind of human cancer **(Feroz and Sheikh, 2020).**

The main objective of our study is to develop a new non-invasive and reliable test to accurately detect TP53 mutations in urine sediments of Egyptian BC patients and to evaluate the influence of their genetic status on tumor metastasis and recurrence.

Results

The descriptive statistics of the patients and controls:

Mutations of TP53 exons were analyzed in 150 urine samples of BC patients.

The studied cases showed, a statistically significant increase in age when compared to controls and the older ages were more susceptible to BC with odd ratio (OR) (95% Confidence interval C.I) = 1.19 (1.13–1.26), (P = 0.001). In addition, males, smokers, positive patients for bilharzia and HCV were more likely to be at risk of bladder cancer [OR(95% C.I), P value], [3.55(1.80–6.97), 0.001], [4.13(2.08–8.17), 0.001], [21.00(8.93–49.40), 0.001], and [4.13(1.81–9.38), 0.001] respectively (Table 2).

Clinical data of the patients:

Clinical data and pathological findings of the studied cases are represented in (Table 3). Histological examination allowed for identification of 84 squamous cell carcinoma and 66 were pathologically diagnosed as transitional cell carcinoma. Twenty seven tumors were classified as T1 (18%), 96 as T2 (64%), 24 as T3 (16%) and only 3 tumors were T4 (2%). A total of 18 tumors were low-grade (12%), 78 tumors were GII (52%), whereas 54 were high-grade (36%), according to the WHO classification **(**Eble J, 2004**)** (Table 3).

Clinical data and pathological findings of the studied cases were represented in (Table 3).

Table 1: Primer sequences used and their fragment sizes.

TP53 Exon	Primer sequence	Fragment size
2+3	F- GATCCCCACTTTTCCTCTTG	287 bp
	R- GTC CCA GCCC AACCCTTGT	
4	F- CTGGTCCTCTGACTGCTCTT	358 bp
	R- AGGCATTGAAGTCTCATGGA	
5	F- TGTTTGTTTCTTTGCTGCCGTGT	310 bp
	R- CAACCAGCCCTGTCGTCTCT	

Table 2
Descriptive statistics of the studied groups

		Groups			**Risk assessment**	
		Control	**Cases**	**P. value**	**OR(95% C.I)**	**P. value**
		N = 50	**N = 150**			
Age		46.3 ± 13.1	64.2 ± 7.3	0.001**	1.19(1.13–1.26)	0.001**
Sex	Female	25(50.0%)	33(22.0%)	0.001**	3.55(1.80–6.97)	0.001**
	Male	25(50.0%)	117(78.0%)			
Smoking	No	34(68.0%)	51(34.0%)	0.001**	4.13(2.08–8.17)	0.001**
	Yes	16(32.0%)	99(66.0%)			
Schistosomiasis	Negative	42(84.0%)	30(20.0%)	0.001**	21.00(8.93–49.40)	0.001**
	Positive	8(16.0%)	120(80.0%)			
HCV	Negative	42(84.0%)	84(56.0%)	0.001**	4.13(1.81–9.38)	0.001**
	Positive	8(16.0%)	66(44.0%)			

Age was represented as Mean ± SD; the data were analyzed by student t test. While Sex, Smoking, Schistosomiasis and HCV were represented as F (%) frequency and percent; the data were analyzed by X^2 test.

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

Table 3
Clinicopathological characteristics of 150 bladder cancers

		Cases N = 150
Pathological diagnosis	Negative	0(0.0%)
	SqCC	84(56.0%)
	TCC	66(44.0%)
Papillary	Negative	117(78.0%)
	Positive	33(22.0%)
Number	Negative	0(0.0%)
	Single	90(60.0%)
	Multi	60(40.0%)
Size		10.2(2.0–22.0)
Lymph Node (LN)	Negative	72(48.0%)
	Positive	78(52.0%)
Grade	Negative	0(0.0%)
	GI	18(12.0%)
	GII	78(52.0%)
	GIII	54(36.0%)
Stage	Negative	0(0.0%)
	T1	27(18.0%)
	T2	96(64.0%)
	T3	24(16.0%)
	T4	3(2.0%)
Cytology	Negative	105(70.0%)
	Positive	45(30.0%)
Carcinoma In Situ (CIS)	Negative	120(80.0%)
	Positive	30(20.0%)
Follow up	No	87(58.0%)
	RE	63(42.0%)

Size was represented as Median with Interquartile range (25% -75%), while the remaining parameters were represented as F (%) frequency and percent.

The mutation frequencies among bladder cancer patients:

Mutation frequency of TP53 varies according to the exon. Fifty four (36.0%) patients of 150 were mutated in exon (2 + 3) with statistically higher significant in patients when compared to controls (P = 0.001), while 96 patients (64.0%) in Exon 4

(P = 0.001), and 111 patients (74.0%) in Exon 5 (P = 0.001) (Table 4) (Fig. 1).

Also, It was observed that 48 patients (32%) had mutations in exon (2 + 3) and exon 4 together, 51 patients (34%) had mutations in exon (2 + 3) in combination with exon 5,

78 patients (52%) were mutated in exon 4 and 5, while 48 (32%) were mutated in the 3 studied exons.

Univariate logistic regression analysis:

Logistic regression analysis of the 3 targeted exons mutation was carried out to evaluate their efficiency to use as prognostic biomarkers, the analysis revealed that all of the studied exons were statistically associated with BC, the mutation of exons (2 + 3), 4 and 5 of TP53 gene may be used as predictor and/or prognostic parameters for BC prospection, an increase in 1 degree of exon (2 + 3) increased the odds of being BC by a factor of OR (95% C.I) = 0.66 (0.58–0.74) with (P = 0.001), while OR (95% C.I) = 0.52 (0.43–0.62) in exon 4 (P = 0.001), and OR (95% C.I) = 0.44 (0.35–0.55) in exon 5 (P = 0.001) (Table 4).

Table 4
Univariate logistic regression analysis

TP53 Exons	Groups			Prognostic viability		
	Control	**Cases**	**P. value**	**OR(95% C.I)**	**P. value**	
	N = 50	**N = 150**				
Exon (2 + 3)	Wild	50(100.0%)	96(64.0%)	0.001**	0.66(0.58–0.74)	0.001**
	Mutant	0(0.0%)	54(36.0%)			
Exon 4	Wild	50(100.0%)	54(36.0%)	0.001**	0.52(0.43–0.62)	0.001**
	Mutant	0(0.0%)	96(64.0%)			
Exon 5	Wild	50(100.0%)	39(26.0%)	0.001**	0.44(0.35–0.55)	0.001**
	Mutant	0(0.0%)	111(74.0%)			

The distribution of the studied exons was represented as F (%) frequency and percent; the data were analyzed by X2 test.

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

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

Statistical evaluation of diagnostic performance of TP53 mutation using ROC Analysis:

Receiver Operating characteristic (ROC) analysis is a useful and fundamental tool for evaluating the performance of diagnostic tests and more generally for evaluating the accuracy of a statistical model. The Area Under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal). It measures the entire two-dimensional area underneath the entire ROC curve.

In our statistical analysis, ROC Curve was established to assess the diagnostic performance of Exons (2 + 3), 4 and 5 of TP53 gene in BC patients and to evaluate the specificity and sensitivity of BC prediction and also to evaluate their discriminatory properties of the patients and healthy individuals. Analysis of the prognostic value of TP53 mutations showed promising results, indicating that these mutations are associated with convenient disease characteristics.

For discrimination, It was found that, the mutations of exon (2 + 3) with sensitivity of 36.0% and specificity of 100.0% with an area under curve (AUC) of 0.680 (P < 0.0001, 95% C.I: 0.611–0.744) and accuracy 36.0%. The mutants of exon 4 were with sensitivity of 64.0% and specificity of 100.0% with an (AUC) of 0. 820 (P < 0.0001, 95% C.I: 0.760–0.871) and accuracy 64.0%, whilst the mutants of exon 5 were with sensitivity of 74.0% and specificity of 100.0% with an (AUC) of 0.870 (P < 0.0001, 95% C.I: 0.815–0.913) and accuracy 74.0% (Table 5) (Fig. 2).

Table 5
Diagnostic performance of Exons (2 + 3), 4 and 5.

TP53	Sn.	Sp.	PPV	NPV	Accuracy	AUC	95%C.I	P. value
Exon(2 + 3)	36.00	100.00	100.0	34.2	36.00	0.680	0.611–0.744	< 0.0001**
Exon4	64.00	100.00	100.0	48.1	64.00	0.820	0.760–0.871	< 0.0001**
Exon5	74.00	100.00	100.0	56.2	74.00	0.870	0.815–0.913	< 0.0001**
Sn: Sensitivity, Sp: Specificity, PPV: Positive predictive value, NPV: negative predictive value, AUC Area under curve and C.I: 95% Confidence Interval.								
* P value < 0.05 is significant, ** P value < 0.01 is highly significant.								

Association analysis between mutation frequency and the studied parameters among BCa patients:

Regarding the association analysis between the mutation frequency and the studied parameters among BC patients.

For exon (2 + 3), an association was observed between the occurrence of mutation with smoking OR (95% C.I) = 2.39 (1.12–5.12) (P = 0.02), TCC cases regarding the pathological diagnosis OR (95% C.I) = 6.64 (3.16–13.97) (P = 0.001), GIII cases OR (95% C.I) = 17.00 (5.31–54.47) (P = 0.001), T3 stage OR (95% C.I) = 3.00 (1.19–7.56) (P = 0.02), and positive cytology OR (95% C.I) = 6.75 (3.13–14.57) (P = 0.001) (Table 6).

Regarding exon 4, the clinicopathological factors which were significantly associated with the overall occurance of mutation included, TCC cases regarding the pathological diagnosis OR (95% C.I) = 2.95 (1.44–6.04) (P = 0.001), GIII cases OR (95% C.I) = 17.00 (5.31–54.47) (P = 0.001), T2 stage OR (95% C.I) = 3.00 (1.89–4.76) (P = 0.001), T3 OR (95% C.I) = 3.00 (1.19–7.56) (P = 0.02), positive cytology OR (95% C.I) = 3.00 (1.31–6.86) (P = 0.02), and patients with positive carcinoma in situ (CIS) OR (95% C.I) = 2.67 (1.01–7.01) (P = 0.04) (Table 7).

While regarding exon 5, the results showed an association between mutation occurrence with old age patients OR (95% C.I) = 1.08 (1.03–1.14) (P = 0.001), positive patients for schistosoma OR (95% C.I) = 2.30 (0.98–5.36) (P = 0.05), TCC cases regarding the pathological diagnosis OR (95% C.I) = 2.13 (0.98–4.63) (P = 0.05), GII cases OR (95% C.I) = 2.71(1.65–4.48) (P = 0.001), T2 OR (95% C.I) = 4.33 (2.60–7.23) (P = 0.001), T3 OR (95% C.I) = 7.00 (2.09–23.47) (P = 0.001), positive cytology OR (95% C.I) = 2.98 (1.15–7.73) (P = 0.02), and patients with positive CIS OR (95% C.I) = 3.86 (1.10- 13.53) (P = 0.03) (Table 8).

Association between TP53 mutations and tumor metastasis:

In addition, the previous data analysis regarding the association between the studied exons mutation frequency and lymph node metastasis, the results indicated that (45/54, 83%) of the mutant patients of exon (2 + 3) had positive lymph node with risk to metastasis, OR (95% C.I) = 9.55 (4.16–21.90) and P = 0.001 (Table 6), in exon 4, (69/96, 71.9%) of the mutant patients had positive lymph node with risk to metastasis, OR (95% C.I) = 12.78 (5.50- 29.68) and P = 0.001 (Table 7).

Additionally, (69/111, 62.2%) of the mutant patients in exon 5 were significantly associated to lymph node metastasis with risk, OR (95% C.I) = 5.48 (2.37–12.66) and P = 0.001 (Table 8).

Table 6

The associations between Exon (2 + 3) of TP53 gene with the studied parameters

		TP53 Exon(2 + 3)			Risk assessment	
		Wild N = 96	Mutants N = 54	P. value	OR(95% C.I)	P. value
Age		64.1 ± 7.5	64.4 ± 7.0	0.8	1.01(0.96–1.05)	0.8
Sex	Female	21(21.9%)	12(22.2%)	0.6	0.98(0.44–2.19)	0.9
	Male	75(78.1%)	42(77.8%)			
Smoking	No	39(40.6%)	12(22.2%)	0.02*	2.39(1.12–5.12)	0.02*
	Yes	57(59.4%)	42(77.8%)			
SCHISTO	Negative	21(21.9%)	9(16.7%)	0.3	1.40(0.59–3.32)	0.4
	Positive	75(78.1%)	45(83.3%)			
HCV	Negative	57(59.4%)	27(50.0%)	0.2	1.46(0.75–2.86)	0.3
	Positive	39(40.6%)	27(50.0%)			
Pathological diagnosis	SqCC	69(71.9%)	15(27.8%)	0.001**	6.64(3.16–13.97)	0.001**
	TCC	27(28.1%)	39(72.2%)			
Papillary	Negative	72(75.0%)	45(83.3%)	0.2	0.60(0.26–1.41)	0.2
	Positive	24(25.0%)	9(16.7%)			
Number	Single	42(43.8%)	48(88.9%)	0.001**	0.10(0.04–0.25)	0.001**
	Multi	54(56.3%)	6(11.1%)			
Size		12.6(5.0- 21.9)	2.0(1.5–22.0)	0.01*	0.96(0.94–0.99)	0.02*
LN	Negative	63(65.6%)	9(16.7%)	0.001**	9.55(4.16–21.90)	0.001**
	Positive	33(34.4%)	45(83.3%)			
Grade	GI	15(15.6%)	3(5.6%)	0.01*	0.20(0.06–0.69)	0.01*
	GII	78(81.3%)	0(0.0%)	0.01*	-	-
	GIII	3(3.1%)	51(94.4%)	0.001**	17.00(5.31–54.47)	0.001**
Stage	T1	24(25.0%)	3(5.6%)	0.001**	0.13(0.04–0.42)	0.001**
	T2	66(68.8%)	30(55.6%)	0.001**	0.45(0.30–0.70)	0.001**
	T3	6(6.3%)	18(33.3%)	0.03*	3.00(1.19–7.56)	0.02*

Age was represented as Mean ± SD; the data were analyzed by student t test. While Sex, Smoking, Schistosomiasis, HCV, Pathological diagnosis, Papillary, Number, LN, Grade, Stage, Cytology, CIS, and Follow up were represented as F (%) frequency and percent; the data were analyzed by X^2 test. But Size of tumor was represented as Median with Interquartile range (25% -75%), the data were analyzed by Mann-Whitney U test.

OR; Odd Ratio, C.I; Confidence Interval and 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.

		TP53 Exon(2 + 3)			Risk assessment	
		Wild N = 96	Mutants N = 54	P. value	OR(95% C.I)	P. value
	T4	0(0.0%)	3(5.6%)	0.1	-	-
Cytology	Negative	81(84.4%)	24(44.4%)	0.001**	6.75(3.13–14.57)	0.001**
	Positive	15(15.6%)	30(55.6%)			
CIS	Negative	72(75.0%)	48(88.9%)	0.03*	0.38(0.14–0.99)	0.04*
	Positive	24(25.0%)	6(11.1%)			
Follow up	No	39(40.6%)	48(88.9%)	0.001**	0.09(0.03–0.22)	0.001**
	RE	57(59.4%)	6(11.1%)			

Age was represented as Mean ± SD; the data were analyzed by student t test. While Sex, Smoking, Schistosomiasis, HCV, Pathological diagnosis, Papillary, Number, LN, Grade, Stage, Cytology, CIS, and Follow up were represented as F (%) frequency and percent; the data were analyzed by X^2 test. But Size of tumor was represented as Median with Interquartile range (25% -75%), the data were analyzed by Mann-Whitney U test.

OR; Odd Ratio, C.I; Confidence Interval and 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.

Table 7
The associations between Exon 4 of TP53 gene with the studied parameters

		TP53 Exon 4			Risk assessment	
		Wild N = 54	Mutant N = 96	P. value	OR(95% C.I)	P. value
Age		63.0 ± 7.6	64.8 ± 7.0	0.1	1.04(0.99–1.08)	0.1
Sex	Female	6(11.1%)	27(28.1%)	0.01*	0.32(0.12–0.83)	0.02*
	Male	48(88.9%)	69(71.9%)			
Smoking	No	15(27.8%)	36(37.5%)	0.2	0.64(0.31–1.32)	0.3
	Yes	39(72.2%)	60(62.5%)			
SCHISTO	Negative	15(27.8%)	15(15.6%)	0.06	2.08(0.92–4.67)	0.07
	Positive	39(72.2%)	81(84.4%)			
HCV	Negative	30(55.6%)	54(56.3%)	0.5	0.97(0.50–1.90)	0.6
	Positive	24(44.4%)	42(43.8%)			
Pathological diagnosis	SqCC	39(72.2%)	45(46.9%)	0.001**	2.95(1.44–6.04)	0.001**
	TCC	15(27.8%)	51(53.1%)			
Papillary	Negative	36(66.7%)	81(84.4%)	0.01*	0.37(0.17–0.82)	0.01*
	Positive	18(33.3%)	15(15.6%)			
Number	Single	30(55.6%)	60(62.5%)	0.3	0.75(0.38–1.48)	0.4
	Multi	24(44.4%)	36(37.5%)			
Size		11.3(1.5–20.0)	6.8(2.0- 26.3)	0.7	1.00(0.98–1.03)	0.8
LN	Negative	45(83.3%)	27(28.1%)	0.001**	12.78(5.50- 29.68)	0.001**
	Positive	9(16.7%)	69(71.9%)			
Grade	GI	18(33.3%)	0(0.0%)	0.01*	-	-
	GII	33(61.1%)	45(46.9%)	0.1	1.36(0.87–2.14)	0.2
	GIII	3(5.6%)	51(53.1%)	0.001**	17.00(5.31–54.47)	0.001**
Stage	T1	24(44.4%)	3(3.1%)	0.001**	0.13(0.04–0.42)	0.001**

Age was represented as Mean ± SD; the data were analyzed by student t test. While Sex, Smoking, Schistosomiasis, HCV, Pathological diagnosis, Papillary, Number, LN, Grade, Stage, Cytology, CIS, and Follow up were represented as F (%) frequency and percent; the data were analyzed by X^2 test. But Size of tumor was represented as Median with Interquartile range (25% -75%), the data were analyzed by Mann-Whitney U 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.

		TP53 Exon 4			Risk assessment	
		Wild N = 54	Mutant N = 96	P. value	OR(95% C.I)	P. value
	T2	24(44.4%)	72(75.0%)	0.001**	3.00(1.89–4.76)	0.001**
	T3	6(11.1%)	18(18.8%)	0.01*	3.00(1.19–7.56)	0.02*
	T4	0(0.0%)	3(3.1%)	0.8	-	-
Cytology	Negative	45(83.3%)	60(62.5%)	0.01*	3.00(1.31–6.86)	0.02*
	Positive	9(16.7%)	36(37.5%)			
CIS	Negative	48(88.9%)	72(75.0%)	0.03*	2.67(1.01–7.01)	0.04*
	Positive	6(11.1%)	24(25.0%)			
Follow up	No	24(44.4%)	63(65.6%)	0.01*	0.42(0.21–0.83)	0.01*
	RE	30(55.6%)	33(34.4%)			

Age was represented as Mean ± SD; the data were analyzed by student t test. While Sex, Smoking, Schistosomiasis, HCV, Pathological diagnosis, Papillary, Number, LN, Grade, Stage, Cytology, CIS, and Follow up were represented as F (%) frequency and percent; the data were analyzed by X^2 test. But Size of tumor was represented as Median with Interquartile range (25% -75%), the data were analyzed by Mann-Whitney U 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.

Table 8

The associations between Exon 5 of TP53 gene with the studied parameters

		TP53 Exon 5			Risk assessment	
		Wild N = 39	Mutant N = 111	P. value	OR(95% C.I)	P. value
Age		61.0 ± 8.0	65.3 ± 6.7	0.001**	1.08(1.03–1.14)	0.001**
Sex	Female	3(7.7%)	30(27.0%)	0.01*	0.23(0.06–0.79)	0.02*
	Male	36(92.3%)	81(73.0%)			
Smoking	No	9(23.1%)	42(37.8%)	0.07	0.49(0.21–1.14)	0.08
	Yes	30(76.9%)	69(62.2%)			
SCHISTO	Negative	12(30.8%)	18(16.2%)	0.04*	2.30(0.98–5.36)	0.05*
	Positive	27(69.2%)	93(83.8%)			
HCV	Negative	21(53.8%)	63(56.8%)	0.4	0.89(0.43–1.85)	0.5
	Positive	18(46.2%)	48(43.2%)			
Pathological diagnosis	SqCC	27(69.2%)	57(51.4%)	0.04*	2.13(0.98–4.63)	0.05*
	TCC	12(30.8%)	54(48.6%)			
Papillary	Negative	27(69.2%)	90(81.1%)	0.1	0.53(0.23–1.20)	0.2
	Positive	12(30.8%)	21(18.9%)			
Number	Single	15(38.5%)	75(67.6%)	0.001**	0.30(0.14–0.64)	0.001**
	Multi	24(61.5%)	36(32.4%)			
Size		13.5(2.0–20.0)	6.0(2.0–22.0)	0.8	0.99(0.96–1.01)	0.3
LN	Negative	30(76.9%)	42(37.8%)	0.001**	5.48(2.37–12.66)	0.001**
	Positive	9(23.1%)	69(62.2%)			
Grade	GI	18(46.2%)	0(0.0%)	0.01*	-	-
	GII	21(53.8%)	57(51.4%)	0.001**	2.71(1.65–4.48)	0.001**
	GIII	0(0.0%)	54(48.6%)	0.01*	-	-
Stage	T1	18(46.2%)	9(8.1%)	0.08	0.50(0.22–1.11)	0.09
	T2	18(46.2%)	78(70.3%)	0.001**	4.33(2.60–7.23)	0.001**
	T3	3(7.7%)	21(18.9%)	0.001**	7.00(2.09–23.47)	0.001**

Age was represented as Mean ± SD; the data were analyzed by student t test. While Sex, Smoking, Schistosomiasis, HCV, Pathological diagnosis, Papillary, Number, LN, Grade, Stage, Cytology, CIS, and Follow up were represented as F (%) frequency and percent; the data were analyzed by X^2 test. But Size of tumor was represented as Median with Interquartile range (25% -75%), the data were analyzed by Mann-Whitney U 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.

		TP53 Exon 5			Risk assessment	
		Wild N = 39	Mutant N = 111	P. value	OR(95% C.I)	P. value
	T4	0(0.0%)	3(2.7%)	0.1	-	-
Cytology	Negative	33(84.6%)	72(64.9%)	0.01*	2.98(1.15–7.73)	0.02*
	Positive	6(15.4%)	39(35.1%)			
CIS	Negative	36(92.3%)	84(75.7%)	0.02*	3.86(1.10- 13.53)	0.03*
	Positive	3(7.7%)	27(24.3%)			
Follow up	No	15(38.5%)	72(64.9%)	0.001**	0.34(0.16–0.72)	0.001**
	RE	24(61.5%)	39(35.1%)			

Age was represented as Mean ± SD; the data were analyzed by student t test. While Sex, Smoking, Schistosomiasis, HCV, Pathological diagnosis, Papillary, Number, LN, Grade, Stage, Cytology, CIS, and Follow up were represented as F (%) frequency and percent; the data were analyzed by X^2 test. But Size of tumor was represented as Median with Interquartile range (25% -75%), the data were analyzed by Mann-Whitney U 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.

Association between TP53 mutations with tumor Grade and Stage:

Regarding the association between mutation frequency and tumor grade, It was observed that TP53 mutations were most frequent in high grade, with less occurrence in low grade. In GI, only 5.6% were mutants in exon (2 + 3), and no GI mutations were detected in exons 4 and 5. For GII, no mutations were detected in exon (2 + 3), 46.9% mutants in exon 4 and 51.4% in exon 5. Finally, for GIII, 94.4% were mutants in exon (2 + 3), 46.9% in exon 4 and 48.6% in exon 5 (Fig. 3).

Regarding the association between mutation frequency and tumor stage, It was observed that TP53 mutations were most frequent in high stage, with less occurrence in low stage. In T1, only 5.6% were mutants in exon (2 + 3), 3.1% exon 4, and 8.1% in exon 5.

In T2, 55.6% were mutant in exon (2 + 3), 75% in exon 4, and 70.3% in exon 5. In T3, 33.3% were mutant in exon (2 + 3), 18.8% in exon 4 and 18.9% in exon 5. In T4, 5.6% were mutant in exon (2 + 3), 3.1% in exon 4 and 2.7% in exon 5 (Fig. 4).

A significant association was observed in exon (2 + 3) between mutation occurrence and patients pathologically classified as GIII and T3 with P (0.001, 0.02) respectively. Regarding exon 4, significant association was also observed in cases with GIII and T3 with P (0.001, 0.02) respectively, while in exon 5, the association was with patients with GII and T3 with P (0.001, 0.001) respectively.

Association between TP53 mutations with tumor recurrence:

The overall tumor recurrence in our patient population was (63/150, 42.0%).

Regarding the recurrence function by Kaplan-Meier analysis, we found that the recurrence time was much closer in the cases with mutation in exon (2 + 3), the mean time to recurrence in mutants was 23.00 ± 0.45 months (range 22.12–23.88) with (P = 0.001). For mutants of exon 4, the mean time to recurrence in mutants was 30.5 ± 1.8 months (range 26.9–34.0)

with (P = 0.008), while Kaplan-Meier analysis for tumor recurrence showed no statistically significant difference between mutants of exon 5 with the wild cases (Table 9) (Fig. 5).

Table 9
Recurrence analysis

		Total No.	No. of RE	Recurrent Time		Log Rank (Mantel-Cox)	P. value
				Mean ± SE	**95% C.I**		
TP53 Exon(2 + 3)	**Wild**	96	57	36.47 ± 1.31	33.90–39.05	27.253	0.001**
	Mutant	54	6	23.00 ± 0.45	22.12–23.88		
TP53 Exon4	**Wild**	54	30	40.4 ± 1.3	37.9–42.9	7.062	0.008**
	Mutant	96	33	30.5 ± 1.8	26.9–34.0		
TP53 Exon5	**Wild**	39	24	38.1 ± 1.8	34.6–41.7	0.736	0.4
	Mutant	111	39	33.4 ± 1.7	30.0 -36.7		

C.I: Confidence Interval, the data were analyzed by Kaplan-Meier test,

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

Regarding the recurrence association with the clinicopathological features, we found that the tumor recurrence was significantly associated with multiple number of tumor mass with risk to recurrence OR (95% C.I) = 81.0 (27.3- 240.6) (P = 0.001), associated also with tumor size with OR (95% C.I) = 1.15 (1.10–1.20) (P = 0.001), patients with GII OR (95% C.I) = 1.60 (1.01–2.52) (P = 0.04), likwise in positive CIS, with risk to recurrence OR (95% C.I) = 8.3 (3.1–22.0), (P = 0.001) (Table 10).

Table 10
Recurrence association with the clinicopathological factors for BC patients

		Follow up			Risk assessment	
		No-RE N = 87	RE N = 63	P. value	OR(95%C.I)	P. value
Number	Single	81(93.1%)	9(14.3%)	0.001**	81.0(27.3- 240.6)	0.001**
	Multi	6(6.9%)	54(85.7%)			
Size		2.0(1.5- 5.0)	20.0(13.5–32.5)	0.001**	1.15(1.10–1.20)	0.001**
Grade	GI	9(10.3%)	9(14.3%)	0.8	1.00(0.40–2.52)	0.9
	GII	30(34.5%)	48(76.2%)	0.03*	1.60(1.01–2.52)	0.04*
	GIII	48(55.2%)	6(9.5%)	0.001**	0.13(0.05–0.29)	0.001**
Stage	T1	15(17.2%)	12(19.0%)	0.6	0.80 (0.37–1.71)	0.6
	T2	54(62.1%)	42(66.7%)	0.3	0.78(0.52–1.16)	0.2
	T3	18(20.7%)	6(9.5%)	0.01*	0.33(0.13–0.84)	0.02*
	T4	0(0.0%)	3(4.8%)	0.05*	-	-
CIS	Negative	81(93.1%)	39(61.9%)	0.001**	8.3(3.1–22.0)	0.001**
	Positive	6(6.9%)	24(38.1%)			

Number, Grade, Stage, and CIS were represented as F (%) frequency and percent; the data were analyzed by X^2 test. But Size of tumor was represented as Median with Interquartile range (25% -75%), the data were analyzed by Mann-Whitney U 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.

Association between tumor metastasis and recurrence with double and triple mutation patients:

Most patients carried only one mutation, while others had double and triple mutations,

the association analysis between mutation frequency and lymph node positiviety expressing tumor metastasis showed that, for exon (2 + 3) & 4, an association was observed between double mutation occurrenc and lymph node positivity with OR (95% C.I) = 54.1(15.8- 185.2) (P = 0.001), likewise in exon (2 + 3) & 5, exon 4 & 5 and for patients with triple mutation in all the studied exons, OR (95% C.I) = 26.4(10.3–67.2) (P = 0.001), OR (95% C.I) = 19.3(9.3–39.8) (P = 0.001), and OR (95% C.I) = 54.1(15.8- 185.2) (P = 0.001) respectively. While the association study revealed no links between mutation frequency and tumor recurrence in double and triple mutation patients (Table 11).

Table 11
The association between positive LN and tumor recurrence with double and triple mutation patients.

			TP53			Risk assessment	
			Wild	**Mutant**	**P. value**	**OR(95%C.I)**	**P. value**
LN	**Exon 2 + 3 & 4**	**Negative**	69(67.6%)	3(6.3%)	0.001**	54.1(15.8- 185.2)	0.001**
	Wild = 102, Mutant = 48	**Positive**	33(32.4%)	45(93.8%)			
	Exon 2 + 3 & 5	**Negative**	66(66.7%)	6(11.8%)	0.001**	26.4(10.3–67.2)	0.001**
	Wild = 99, Mutant = 51	**Positive**	33(33.3%)	45(88.2%)			
	Exon 4 & 5	**Negative**	54(75.0%)	18(23.1%)	0.001**	19.3(9.3–39.8)	0.001**
	Wild = 72, Mutant = 78	**Positive**	18(25.0%)	60(76.9%)			
	Exon 2 + 3 & 4 & 5	**Negative**	69(67.6%)	3(6.3%)	0.001**	54.1(15.8- 185.2)	0.001**
	Wild = 102, Mutant = 48	**Positive**	33(32.4%)	45(93.8%)			
Follow up	**Exon 2 + 3 & 4**	**No**	45(44.1%)	42(87.5%)	0.001**	0.24(0.1–0.6)	0.001**
	Wild = 102, Mutant = 48	**RE**	57(55.9%)	6(12.5%)			
	Exon 2 + 3 & 5	**No**	42(42.4%)	45(88.2%)	0.001**	0.22(0.1–0.54)	0.001**
	Wild = 99, Mutant = 51	**RE**	57(57.6%)	6(11.8%)			
	Exon 4 & 5	**No**	30(41.7%)	57(73.1%)	0.2	0.7(0.4–1.3)	0.3
	Wild = 72, Mutant = 78	**RE**	42(58.3%)	21(26.9%)			
	Exon 2 + 3 & 4 & 5	**No**	45(44.1%)	42(87.5%)	0.001**	0.24(0.1–0.6)	0.001**
	Wild = 102, Mutant = 48	**RE**	57(55.9%)	6(12.5%)			

LN and Follow up were represented as F (%) frequency and percent; 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.

Mutational landscape of TP53:

All the studied exons of TP53 gene were examined by sequencing. In general, (54/150, 36%) patients had mutations in exon (2 + 3). Missense mutation was the most frequently detected mutation (33/54, 61.1%) as the alignment results showed a nucleotide substitution at codon 15 where Histidine (His) was substituted by Aspartic (Asp) amino acid, Likewise at codon 112, Leucine (Leu) was substituted by Valine (Val) amino acid in (21/54, 38.8%) leading to incorrect amino acid sequence, which may produce a malfunction protein. (Fig. 6) (Table 12).

Ninety six patients (96/150, 64%) were mutated in exon 4, the alignment results showed a nucleotide substitution at codon 100 leading to “missense mutation”, where Proline (Pro) was substituted by Serine (Ser) amino acid in (45/96, 46.8%), while there was a nucleotide substitution at codon 150 “Silent mutation” was detected in (27/96) where the codon of amino acid “Leu” unaltered than reference. It was interesting to detect an insertion of a single nucleotide occurred at codon 151in (9/96, 9.3%) followed by a missense mutation at codon 152 which lead to frameshift of DNA nucleotide resulting in an abnormal amino acid sequence in (15/96, 15.6%) (Fig. 7) (Table 12).

For exon 5, mutations were dominant among patients (111/150, 74%). The alignment results showed a nucleotide substitution at codon 77 leading to “missense mutation”, where Aspartic acid was substituted by Alanine (Ala) amino acid in (22/111, 19.8%), Likewise in codon 107, Valine (Val) was substituted by Ala amino acid in (19/111, 17.1%), a missense mutation was detected at codon 175 where Arginine (Arg) was substituted by Histidine (His) in (57/111, 51.3%). A nucleotide substitution was observed at codon 180 “Silent mutation”, where the codon of amino acid Tyrosine (Tyr) unaltered than reference in (13/111, 11.7%) (Fig. 8) (Table 12).

Table 12
Mutational analysis of TP53 in bladder tumors

Cases number	Exon	Codon	Change	Triplete	Amino acid	Type
33	2 + 3	15	C → G	CAC	His →Asp	Missense
21	2 + 3	112	T → G	TTA	Leu →Val	Missense
45	4	100	C → T	CCG	Pro →Ser	Missense
27	4	150	G → G	CTG	Leu → Leu	Silent
9	4	151	C			Insertion
15	4	152	G → A	GCA	Ala → His	Frame shift
22	5	77	A → C	GAT	Asp → Ala	M issense
19	5	107	T → C	GTT	Val → Ala	Missense
57	5	175	G → A	CGC	Arg → His	Missense
13	5	180	T → C	TAT	Tyr → Tyr	Silent

Discussion

Bladder cancer is the fourth and the 11th most common cancer in men and women, respectively, representing 6.6% of all cancer types **(Rubio, et al., 2021).**

The need for non-invasive tools for diagnosis and follow up led to the identification of biomarkers in the urine **(Georgantzoglou, et al., 2021).**

Liquid biopsies are increasingly used for the diagnosis and follow-up of cancer patients. Urine is a body fluid that can be used to detect cancers and others diseases. It is noninvasive and easy to collect **(Charpentier, et al., 2021).**

In the present study, we have analysed the TP53 mutational spectra in urine sediments of urinary bladder tumors by studying the mutation frequency in exons (2 + 3), 4 and 5 in a material consisting of TCCs and SqCCs of various stage and grade. We used PCR technique for mutation screening followed by sequencing for confirmation and identification of mutations.

Our results indicated that 54 (36%) patients of 150 enrolled in this study were mutated in exon (2 + 3) with statistically higher significant in patients when compared to controls (P = 0.001), while 96 patients (64.0%) in Exon 4 (P = 0.001), and 111 patients (74.0%) in Exon 5 (P = 0.001).

For double mutation, it was indicated that 48 patients (32%) had mutations in exon (2 + 3) and exon 4 together, 51 patients (34%) had mutations in exon (2 + 3) in combination with exon 5, 78 patients (52%) were mutated in exon 4 and 5, while 48

(32%) were mutated in the 3 studied exons.

Wallerand et al., 2005 indicated that of the 110 tumors studied, 22 harbored

TP53 mutations (20%). These mutations were located in exons 4–9; one mutation was located at a splice acceptor site of intron 6. **Wallerand** also reported that no mutations were found in exons 2, 3 which is on the contrary to our results as the mutation frequency in exon (2 + 3) was 36%, his study detected only 4 patients had double mutation (3.6%) while our results indicated that the highest percentage of double mutation was 52% in exons 4 and 5.

Al-Kashwan et al., 2012 reported that the sequencing results confirmed 10 cases of the 29 harbored one or more TP53 mutations (37.9%) and among them 7 patients (63.6%) showed single mutation and 3 (27.3%) had double mutations. On the other hand, **Noel et al., 2015** revealed that the functional TP53 mutations were 56 out of the 103 analyzed tumors (54% of cases).

The study of **Ecke et al., 2008** has reported that the mutations were detected in 26 of 75 patients (34.7%) and only 6 mutations (8%) were detected in exon 5 and these results contradict our results which showed a large percentage of mutations (74%) in exon 5.

The univariate logistic regression analysis of the present study revealed that all the studied exons were statistically associated with BC and the prevelence of mutations of exons (2 + 3), 4 and 5 of TP53 gene may be used as predictor and/or prognostic parameters for BC prospection.

Regarding sensitivity, the probabilities of detecting a TP53 mutation in the urinary

sediment for exons (2 + 3), 4 and 5 were 36%, 64% and 74% respectively for the urine test. Moreover, the specificity and the positive predictive value of the 3 studied exons were 100% and the negative predictive value of (2 + 3), 4 and 5 exons were 34.2%, 48.1% and 56.2% respectively while the results of **Noel et al., 2015** indicated that the sensitivity was 34% and the specificity was 87%, with a positive predictive value of 76% and a negative predictive value of 53%.

Our results demonstrated that in exon (2 + 3), smoking, TCC cases were significantly associated with mutation frequency. Whilst, in exon 4, significant association was observed with TCC cases, positive patients for CIS. Furthermore to the previous factors, age and bilharzial patients were significantly associated with mutation frequency in exon 5. Futhermore, the present study revealed a significant association between the 3 studied exons and lymph node metastasis and as a consequence, it may be considered as a useful prognostic indicator for tumor metastasis.

Notably, our results support the notion that a higher proportion of mutations was found in high tumor grade than in low grade and in advanced stage. In addition, a significant association was observed in exon (2 + 3) between mutation occurrence and patients pathologically classified as GIII and T3 with P (0.001, 0.02) respectively. Regarding exon 4, significant association was also observed in cases with GIII and T3 with P (0.001, 0.02) respectively, while in exon 5, the association was with patients with GII and T3 with P (0.001, 0.001) respectively.

The above findings matched those of **Liao et al., 2021** who stated that high stage of BC has obviously higher level of TP53 mutation than the lower stage and also consistent with those of **Shao et al., 2021** who reported that TP53 mutations were most frequent among BC patients with high tumor grade. The overall analysis provides a strong support to the initial findings, which further confirmed the potential diagnosis role of TP53 mutation in advanced BC.

The occurrence or recurrence of BC is a molecular biological change or process that is effected by occupational factors, non-occupational factors, genomics and proteomics factors **(Fan, et al., 2021).**

The clinical value of TP53 in bladder cancer as a predictive marker of tumor recurrence and treatment selection is still in debate **(Sobhani et al., 2021).** But the results of our study settled this matter and statistically proved the the clinical value

of TP53 in bladder cancer as a predictive marker of both tumor metastasis and recurrence.

Analysis of the data using Log Rank Mantel-Cox for tumor recurrence showed a significant association between mutation frequency in exons (2 + 3), 4 and tumor recurrence with P = 0.001, 0.008 respectively while no significant association was observed in exon 5. Hence, TP53 mutation is an independent predictor of tumor recurrence in mutant BC patients in exons (2 + 3) and 4. However, other studies have shown a cosiderable discrepancy regarding tumor recurrence as **Ecke et al., 2008** who stated that Kaplan-Meier analysis for tumor recurrence showed that the tumor recurrence frequency was 69.4% in patients with TP53 wild-type, and 88.5% in patients with TP53 mutation.

Previous studies reported the vital role of TP53 in urothelial carcinogenesis, however, other factors may contribute to it and to tumor recurrence because not all the mutant patients who were followed up have recurrence. Accordingly, the present study clarified the association between tumor recurrence and clinicopathological parameters in BC patients according to TP53 mutation frequency. It was noted that tumor recurrence was significantly associated with multiple number of tumor mass, tumor size, patients with carcinoma in situ and patients with GII while no association was observed among patients with GIII and T3. These results can be explained by the little chance of recurrence to occur in stage 3 BC because the line of treatment is usually radical cystectomy, which is not the case in treating lower stages of BC.

Saoud et al., 2021 reported in his case study that he presented this case to highlight how even patients with NMIBC disease may rapidly progress towards metastatic MIBC and death and hypothesized that in addition to histological analysis of the tumor, early molecular and cytogenetic characterization of resected tissue is essential in predicting prognosis of the disease based on identifiable gene mutations. Interestingly, our results revealed a significant assosiation between TP53 mutation frequency and tumor metastasis in all the studied exons with the same P value of 0.001.

Generally, our results demonstrated a higher abundance of double mutation comparing to the data published in previous studies, the findings revealed 48 patients (32%) had mutations in exon (2 + 3) and exon 4 together, 51 patients (34%) had mutations in exon (2 + 3) in combination with exon 5, 78 patients (52%) were mutated in exon 4 and 5, while 48 (32%) were mutated in the 3 studied exons. These results do not coincide with **Erill et al., 2004** 's results who detected only 2 cases displayed double mutation of 76 BC patients while **Ecke et al., 2008** detected five patients had mutations in two TP53 exons of 75 BC patients.

Furthermore, the double mutation frequency with all the probabilities and triple mutations were significantly associated with lymph node positivity expressing tumor metastasis, but no links were observed between mutation frequency and tumor recurrence in double and triple mutation patients.

Notably, our results unveils the evidence that the most common mutations observed in TP53 DNA were missense mutations while frameshift and silent types were found to be less frequent.

Our results suggest a larger number of missense mutation, C→G in 33 mutants and T→G in 21 mutant patients in exon (2 + 3), this genomic instability may reflect the role of other factors in carcinogenesis as smoking (77.8%) and mutant patients with bilharziasis (83.3%),

it was also found a high frequency of missense mutation C→T (45/96, 46.8%) in mutant patients in exon 4, silent mutation G→T (27/96, 28.1%), an insertion of C nucleotide at codon 151 (9/96, 9.3%) and a frameshift mutation G→A at codon 152 (15/96, 15.6%). Surprisingly, **Schroeder et al, 2003** reported a nucleotide substitution at codon 110 G→A that leads to amino acid change (Arginine→Histidine), while at the same codon and exon, **Wallerand et al., 2005** reported a nucleotide substitution G→T, leads the Arginie to be changed to Leucine in mutation of exon 4.

It has been indicated that missense mutations were the most prevalent type of TP53 mutation in exon 5, A→C in 22 mutants, T→C in 19 and G→A in 57 mutants (98/111, 88.2%) and finally 13 silent mutations T→C were observed at codon 180 13/111, 11.7%) where Tyrosine unaltered than reference, the detected silent mutation at codon 180 in exon 5 catches

our attention because **Schlichtholz, 2004** and his team mates detected a missense mutation at which G→A leads to amino acid change (Glutamic acid→Lysine).

In summary, our results show, that the mutation of exons (2 + 3), 4 and 5 of TP53 gene may be used as predictor and/or prognostic parameters for BCa prospection. Furthermore, our study provides a foundation could help clinicians to predict tumor recurrence and metastasis, this finding is even more valuable, because TP53 mutations can be analyzed in sediments of urine cells by non-invasive methods. Since TP53 mutation frequency is significantly associated to clinicopathological features of

BC patients, consequently, the inclusion of both TP53 mutation status and genetic analysis into the predictive panel of tumor markers for bladder cancer is recommended.

Methods

Patients and samples

A total of 150 patients and 50 healthy volunteers as controls were enrolled in this study, it included a patient criterion of diagnosed BC patients who did not receive any type of therapy, and the diagnosis was confirmed by histo-pathological examination of the removed tumor tissues by 2 independent pathologists. Before recruitment, a signed informed consent was obtained from all participants. The research protocol was conducted according to the guidelines of the ethical principles outlined in the declaration of Helsinki and was approved by the institutional review board of the Ethics Committee of Theodor Bilharz Research Institute, in accordance with the institutional guidelines. 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 stored at − 80°C until the DNA extraction.

DNA extraction from urine samples:

DNA extraction was carried out using Qiagen DNeasy kit (Hilden, Germany) as per manufacturer instructions, the purified DNA was dissolved in 50 µl of water, measured on a Nanodrop ND-2000c (Thermo Scientific, Waltham, MA, USA), and stored at − 20°C for further analyses.

TP53 mutation analysis for exons 2 + 3, 4 and 5 by PCR

For TP53, we screened for mutations in exons 2 + 3, 4 and 5 by PCR in a final volume of 25 µl containing 100 ng of urine sediment DNA, exons (2 + 3), 4 and 5 of the TP53 gene were amplified using the primers shown in (Table 1) **(Bakkar, et al., 2003).** Cycling conditions were as follows: initial denaturation 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 30 seconds, The mixture was then heated at 72°C for 10 min as a final extension step.

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 Scientific's micro DOC gel documentation system.

DNA Sequence analysis

The PCR products for all exons were subjected to Exonuclease I-Shrimp Alkaline Phosphatase PCR product treatment (Thermo Fisher, catalogue no. 78200). This enzymatic treatment hydrolyzes excess primers and nucleotides in a single step. The Exonuclease I-Shrimp Alkaline Phosphatase-purified samples were subjected to bidirectional sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The abnormal sequencing results were reconfirmed by at least 2

repeats right from PCR amplification. Furthermore, a wild-type sequencing control was run for comparison of abnormal sequencing results.

Nucleotide sequencing and analysis

Exons sequences of TP53 gene were matched with reference sequences registered in the GenBank database through BLAST-NCBI (https://blast.ncbi.nlm.nih.gov), after that, all sequences were aligned by using the BioEdit software which depending on the ClustalW multiple alignment conditions.

Statistical analysis methods

The data were analysed using Microsoft Excel 2016 and statistical package for social science 'IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA)'. Continuous normally distributed variables were represented as mean ± SD. with 95% confidence interval, while nonnormal variables were summarized as median with 25 and 75 percentile, 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, and Mann-Whitney U test was used in non-normal variables. Chi-square ($\chi 2$) test or Fisher's exact test were used to determine the distribution of categorical variables between groups. Logistic regression analysis was performed to identify predictor associated with the risk of BC occurrence. The diagnostic performance of the studied exons 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.

Declarations

- **Ethics approval and consent to participate:**

This research work was approved by the ethical committee of Theodor Bilharz Research Institute, Cairo, Egypt according to the regulations adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964.

Trial Registration Number (TRN): FWA 00010609

Date of registration: 21/4/2016

- **Consent for publication:**

Not applicable

- **Availability of data and materials:**

All data and source of used materials are available up-on request.

- **Competing interests:**

No competing interests from any author of the current work.

- **Funding:**

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- **Authors' contributions:**

1. M.: Suggest the idea of the current work and was responsible for the major part of molecular biology techniques and writing the manuscript.

2. K.: Share in doing the molecular biology technique.
3. A.: Was responsible for the histopathological study of the research.
4. S.: Was responsible for coordination of different aspects of study, collection of refferences and sharing in writining the manuscript.
5. E.: Was responsible for clinical diagnosis of patients, doing the cystoscopic examination and collecting the biopsy samples of the study.

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Figures

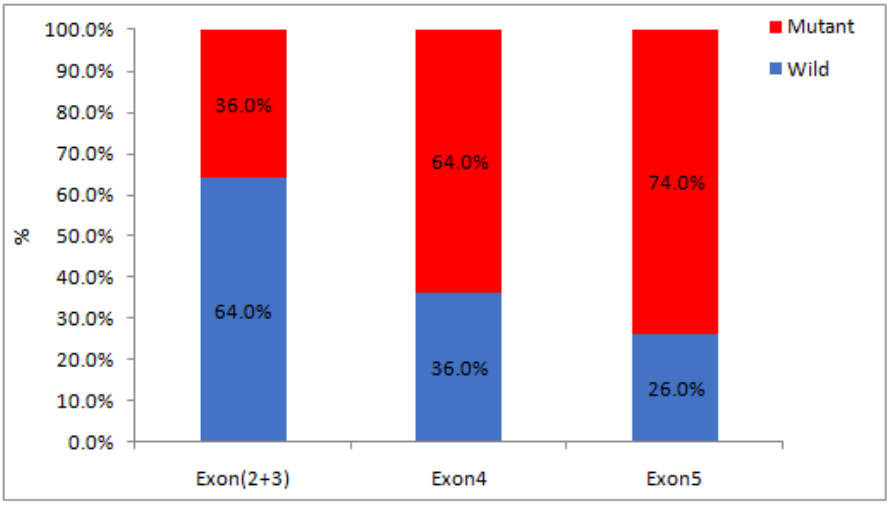

Figure 1

Distribution of the studied exons regarding BC patients.

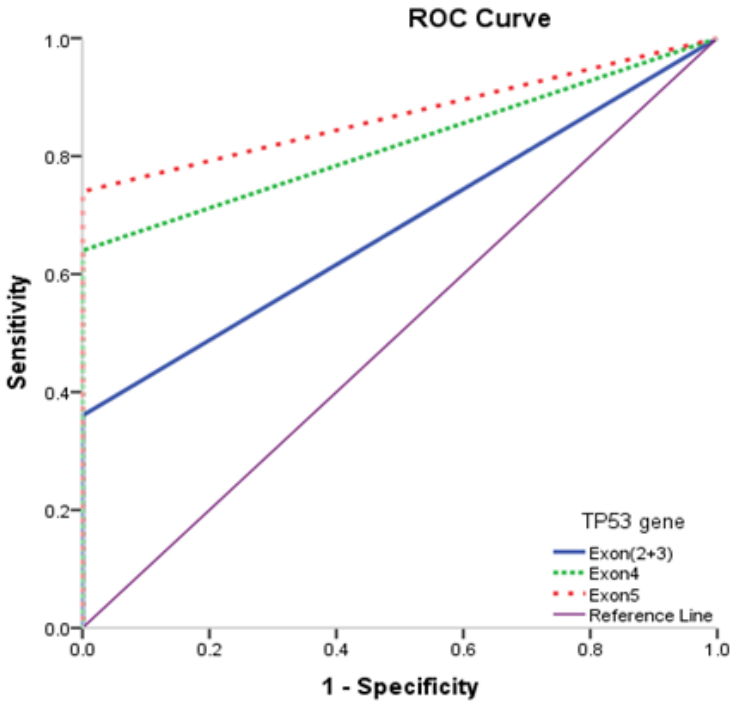

Figure 2

ROC Curve of the studied exons in TP53 gene.

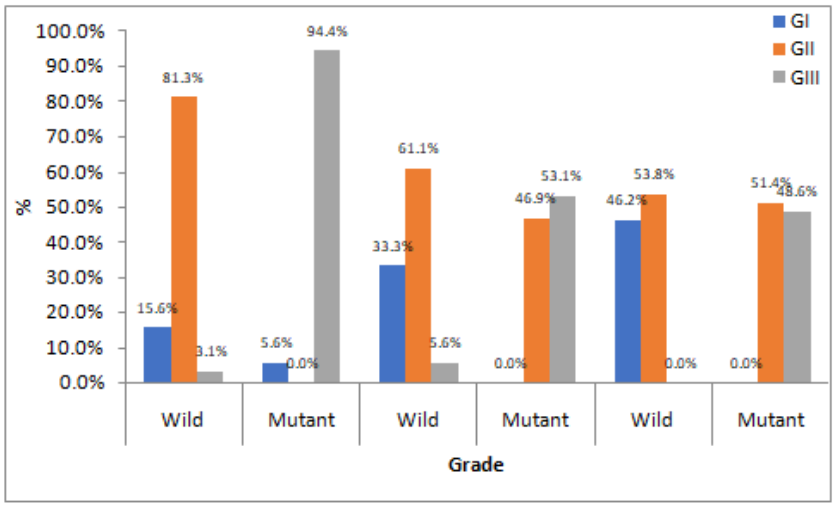

Figure 3

The association between the mutation frequency of the studied Exons and tumor grade.

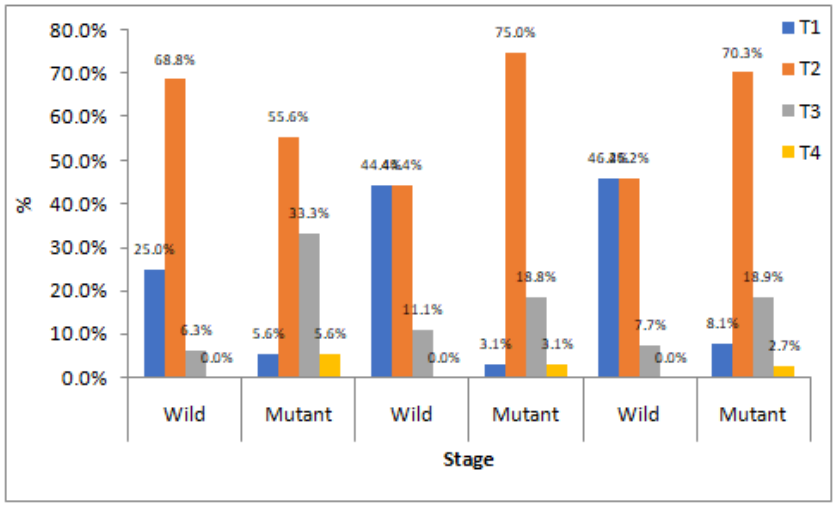

Figure 4

The association between the mutation frequency of the studied Exons and tumor stage.

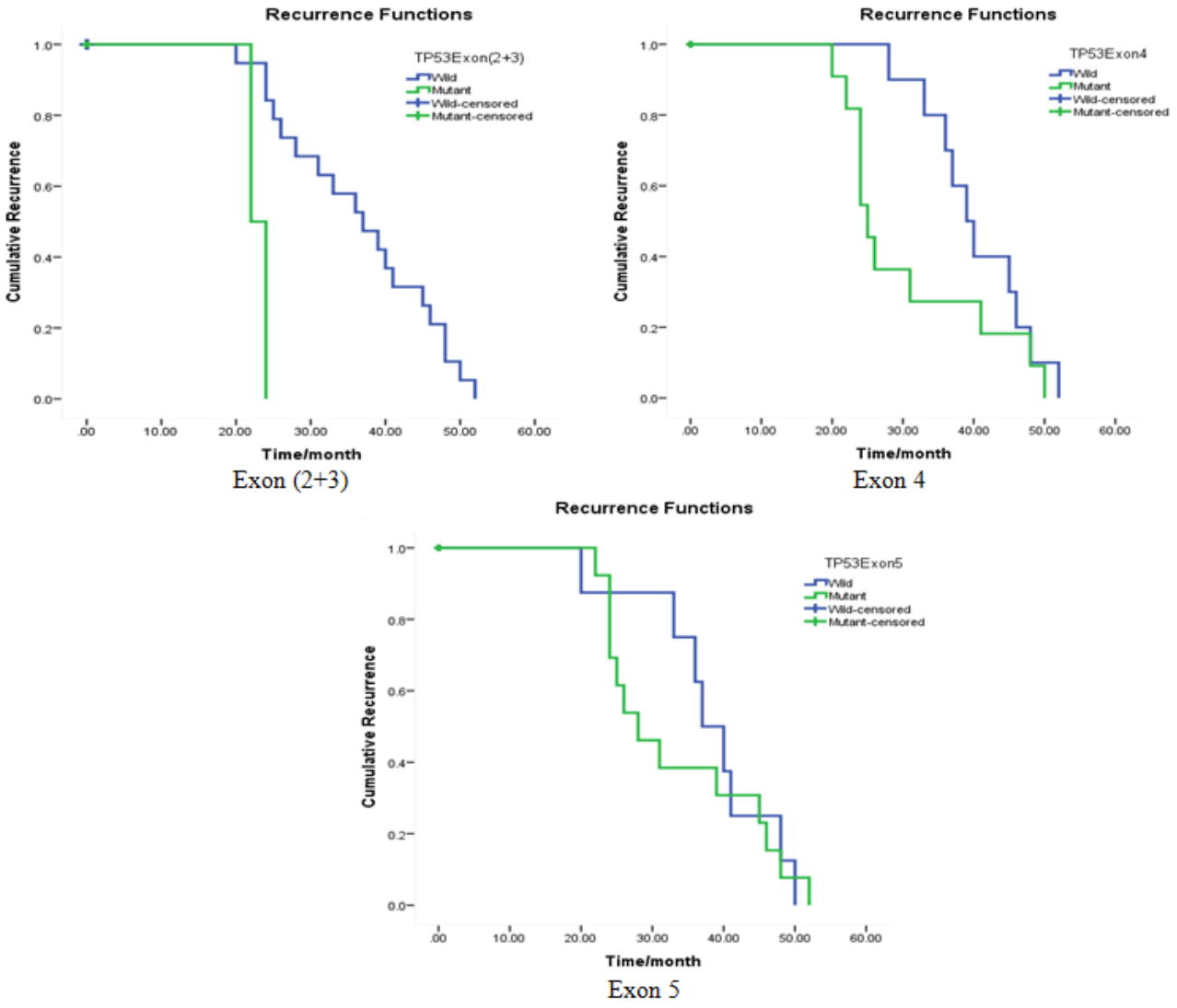

Figure 5

Kaplan-Meier analysis of TP53 mutation regarding tumor recurrence.

Figure 6

Exon (2+3) of TP53 gene with reference alignment “MG595968.1:179-312 TP53 Exon 3” as a result of NCBI blast.

Figure 7

Exon 4 of TP53 gene with reference alignment “JQ752213.1:36-249 TP53 Exon 4” as a result of NCBI blast.

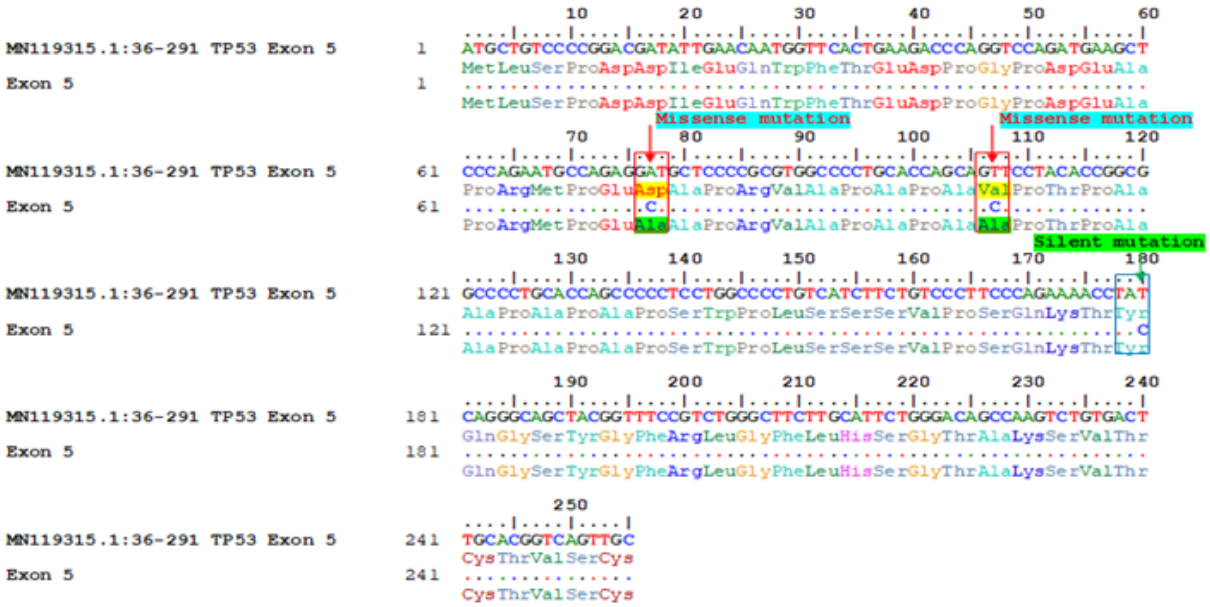

Figure 8

Exon 5 of TP53 gene with reference alignment “MN119315.1:36-291 TP53 Exon 5” as a result of NCBI blast.