The Predictive Role of Cell Cycle Marker Tumor Protein 53 (TP53) in the Pathophysiology and Biological Behavior of Urinary Bladder Cancer

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Abstract

Background: Nearly 50% of bladder cancers have mutation of the tumor suppressor gene TP53, and 76% of samples had TP53 functionally inactivated.

Objectives: The purpose of this study was to evaluate the TP53 mutational status in patients with invasive bladder cancer (BC) (T2) receiving neoadjuvant chemotherapy followed by radical cystectomy or bladder preservation protocol to predict response and recurrence.

Patients and methods: Fifty patients over the age of eighteen and had a primary diagnosis of transitional BC were included in this retrospective cross-sectional study from July, 2023, to July, 2024. The patients had to have enough tissue that was embedded in formalin-fixed paraffin for molecular analysis (DNA extraction from paraffin blocks, followed by conventional PCR and Sanger sequence method)..

Results: There was point mutation in 8 patients of various stages (T2, T3 and T4) they were found in exon 4 chromosome 17 namely Pro72Arg was caused by G-C transversion (Those were missense mutation according to NCBI). Regarding gender, age, smoking status, and comorbidities such as diabetes, hypertension, and heart disease, there was no statistically significant difference between cases with and without TP53 mutations ($p > 0.05$).

There is no statistically significant difference between the TP53 mutation and cancer grade ($p > 0.05$). There is no statistically significant difference between the TP53 mutation and metastasis or treatment response ($p>0.05$).

Conclusion: There is no significant correlation between P53 expression and bladder cancer grade. P53 mutations were discovered to be substantially linked to muscle-invasive bladder cancers in their late stages (pT2-pT4). The overall survival of patients with p53 mutations differs significantly from that of patients without p53 abnormalities, according to survival analysis.

Keywords: Bladder cancer; Cell cycle marker; Tumor protein53.

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Introduction

According to **Siegel et al. (2018)**, bladder cancer (BC) is the most common urothelial malignancy and the primary cause of morbidity and death worldwide. The diagnosis of muscle-invasive bladder cancer (MIBC) occurs in 25% of BC patients. Rapid development, metastasis, and a poor prognosis are characteristics of muscle invasive BC (Kulkarni et al., 2019). It's the more combative kind. It is linked to a low survival rate and is correlated with lymph node metastases **(Witjes et al., 2021)**.

There is growing evidence that the tumor microenvironment (TME) is associated with the malignant phenotype of tumors **(Harmon et al., 2020; Zhang et al., 2020).** It is noteworthy that MIBC is an immune-sensitive cancer that has several tumor-infiltrating lymphocytes (TILs), such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) **(Jiang et al., 2020; Cao et al., 2021).**

Therefore, advancements in the detection, therapy, and prognosis of BC necessitate the integration of various new methods, including genomic profiling, the development of biomarkers, and immunotherapy **(Witjes et al., 2021)**.

According to reports, the most frequent causes of mutations in MIBC are tumor protein 53 (TP53) genes **(Nassar et al., 2019; Sjödahl et al., 2020)**. Nearly 50% of bladder cancers have the mutant version of the tumor suppressor gene TP53 (TP53-MT), and 76% of samples had TP53 functionally inactivated **(Donehower et al., 2019)**. In order to initiate cell death upon DNA damage or cell cycle arrest, the TP53 gene binds directly to chromatin and senses cellular stress or damage**.** However, TP53 loses its ability to suppress tumors and simultaneously promotes tumorigenesis when it is mutated (mostly missense). Cells then escape from DNA damage, which leads to the unrestricted growth of tumor cells and ultimately cancer **(Powers et al., 2020).**

Many biological processes downstream of p53, including metabolism, autophagy, translational control, and epigenetic regulation, have been better understood as a result of connections between p53 and these many physiological circumstances **(Levine, 2019)**.

In order to predict response and recurrence, the purpose of this study was to evaluate the TP53 mutational status in patients with invasive BC (T2) undergoing neoadjuvant chemotherapy followed by radical cystectomy or bladder preservation protocol (CCRTH).

Patients and methods

Study Setting: Shefa El Orman Hospital and Qena University Hospitals.

Sample size calculation the study will be carried out on 50 cancer patients according to Steven K Thompson equation to calculate sample size where n= z: confidence level at 95%, d: Error proportion (0.05), p: Probability (50%), and we reach sample size (n)=50.

$$\frac{N\ X\ p(1-p)}{[N-1\ X\ (d2\div z2]+[p(1-p)]}$$

Study design and population

This was retrospective cross-sectional research involved 50 patients with a primary diagnosis of transitional bladder cancer treated with neoadjuvant CTH followed by radical cystectomy or bladder preservation protocol who were over 18 years old, had no prior history of chemotherapy or radiation therapy, and had enough formalin-fixed paraffin-embedded (FFPE) tissue available for molecular analysis were included in the study. between 2019 and 2023. Regarding the type of neoadjuvant chemotherapy received, 80% of cases received GEMZAR cisplatin, while 20% received GEMZAR carboplatin. Regarding the number of cycles, most cases (78%) received four cycles of chemotherapy. Concerning the disease outcome, 66% had a regressive course,

20% had a stationary course, and 14% had a progressive one

Any form of cancer other than BC was excluded. Finally, the study did not include individuals less than 18 years of age.

Ethical consideration: The study protocol was approved by Shefa El Orman hospital number 22.2023, also approved by the South Valley university of Medicine council, the ethical approval code: SVU-MED-PHY003-1-23-4-620. All patients signed a written informed consent before participating in the study.

Study procedures

1. **DNA extraction:** The DNA extraction from FFPE tissues was performed using the QIAamp DNA FFPE Tissue Kit (Cat. No. 56404) from Qiagen (Venlo, Netherlands), as outlined in the provided flowchart (**Fig. 1**). The initial step involved the removal of paraffin, which was achieved by dissolving the paraffin using xylene, followed by its subsequent elimination from the sample. Subsequently, the sample underwent lysis under denaturing conditions, with the aid of proteinase K, to promote the release of DNA from samples. Heat incubation at 90°C was then applied to reverse the formalin crosslinking. In (FFPE) tissues, formalin is commonly used as a fixative to preserve tissue structures for pathological analysis.

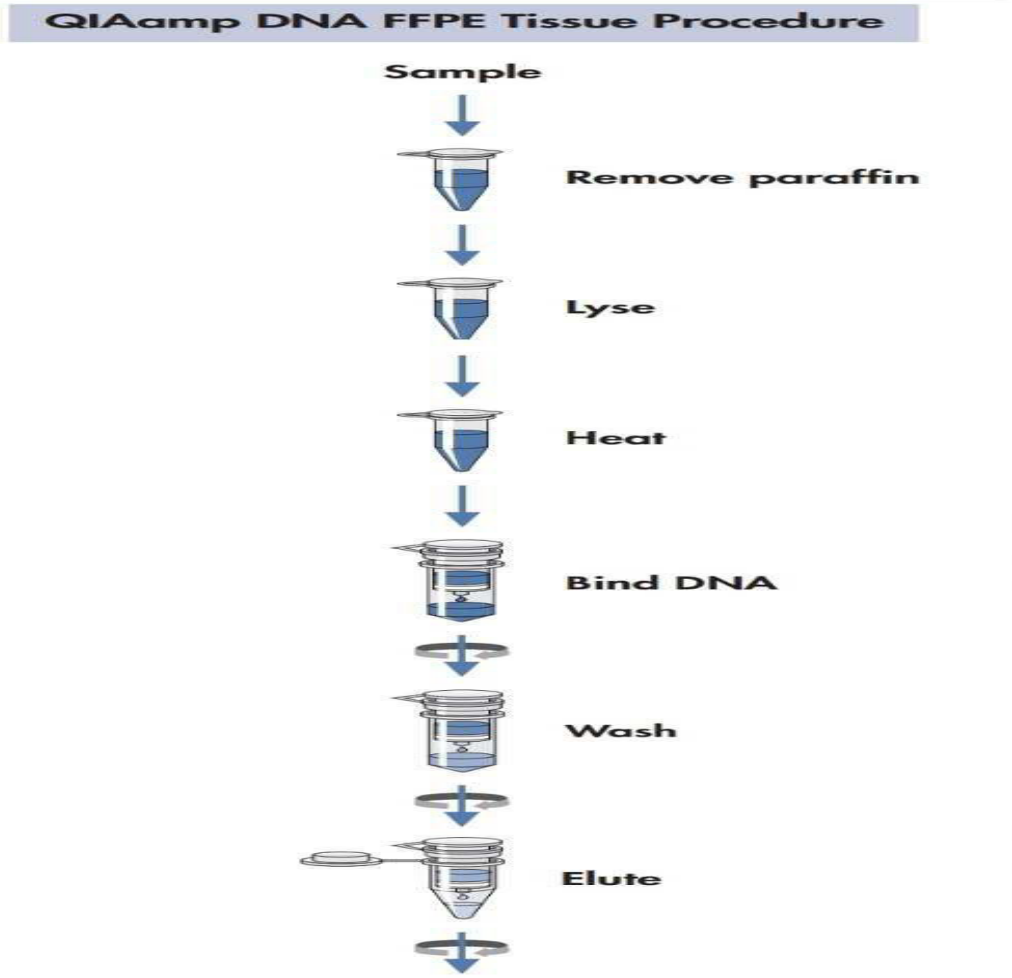

Fig.1. QIAamp DNA formalin-fixed-paraffin-embedded (FFPE) Tissue

To ensure the purity of the DNA, a series of washing steps were executed to eliminate any residual contaminants effectively. Finally, the DNA, now in a pure and concentrated form, was eluted from the membrane, resulting in the successful extraction of high-quality DNA. This extracted DNA was well- suited for

subsequent downstream analysis. The assessment of DNA sample purity was carried out using a microplate reader, specifically the Thermo Scientific™ Multiskan Sky Microplate Spectrophotometer. This evaluation involved measuring the absorbance at two specific wavelengths: 260 and 280 nanometers. This analytical method allows for a comprehensive examination of the DNA samples purity, which is of paramount importance in ensuring the quality and integrity of the genetic material under scrutiny. Following the purity assessment, the purified DNA samples were portioned into aliquots and then storage was done at a temperature of -80°C. Following the DNA extraction and purity assessment, gel electrophoresis was performed to evaluate integrity and the quality of the extracted DNA. The DNA samples were loaded into a 1% agarose gel and subjected to electrophoresis at a constant voltage. The gel was subsequently stained with ethidium bromide, allowing for the visualization of the DNA bands under UV light.

The storage of the purified DNA was done at -80°C until used for polymerase chain reaction (PCR) and Sanger sequencing.

2. **PCR :** PCR amplification was carried out using the FIREPol® Master Mix Ready to Load (5x) with a 12.5 mM $MgCl_2$ concentration. The reactions were performed on a Veriti™ 96-Well Thermal Cycler, utilizing forward and reverse primers designed to target Exon-4. The forward primer (Exon-4_F) had the sequence 5′-CTGGTCCTCTGACTGCTCTT-3′, with a GC content of 55% and a melting temperature of 61.7°C. The reverse primer (Exon-4_R) had the sequence 5′-AGGCATTGAAGTCTCATGGA-3′, with a GC content of 45% and a melting temperature of 58.3°C. For each PCR reaction, the final volume was 20 µL, which contained 4 µL of FIREPol® Master Mix (5x), 0.6 µL of each primer, 10 ng/µL of gDNA template, and nuclease-free water to adjust the volume. The thermal cycling protocol included an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 60 seconds, and extension at 72°C for 4 minutes. The amplification was completed with a final extension step at 72°C for 7 minutes. The PCR product was analyzed using gel electrophoresis, with the resulting band observed at 510 base pairs, as shown in (**Fig.2**).

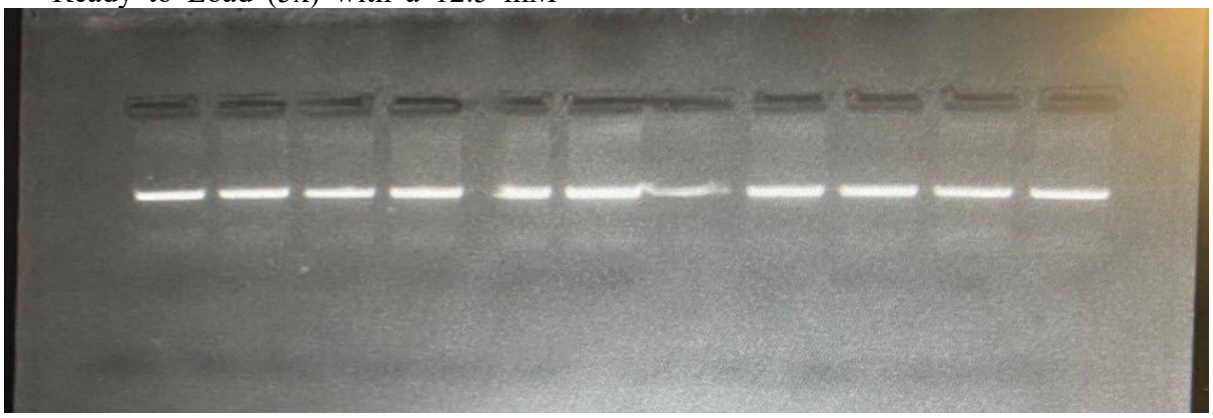

Fig.2. gel electrophoresis of PCR products (510 base pair)

3. **PCR Product Purification:** Purification: Purify the PCR products using a column-based purification method to remove unincorporated primers, nucleotides, and enzymes.

Quantification: Measure the concentration of the purified PCR products using a

spectrophotometer to determine the amount of DNA for sequencing.

4. **Sequencing Reaction:** Following the manufacturer's instructions, the BigDye Terminator v1.1 Cycle Sequencing Kit was used to prepare the sequencing reaction. Every sequencing response contained: One microliter of the purified PCR product, one microliter of the BigDye Terminator v1.1 reagent , one microliter of sequencing buffer, 3.2 pmol of sequencing primer , nuclease free water to reach a final volume of 10 µL.

   The cycle sequencing was performed with the following thermal conditions: Initial denaturation: 96°C for 1 minute, 25-30 cycles of denaturation: 96°C for 10 seconds, annealing: 50-60°C for 5-10 seconds, extension: 60°C for 4 minutes.
5. **Dye Termination Cleanup:** After sequencing, the reaction products were cleaned to remove excess dye terminators using either an ethanol precipitation method or the BigDye X Terminator purification kit. In the precipitation method, then adding ethanol to the reaction, which then incubated at -20°C, followed by centrifugation and washing with 70% ethanol.
6. **Capillary Electrophoresis:** Loading: After being cleaned, the sequencing reactions were re-suspended in 10 µL of formamide and allowed to denature for five minutes at 95°C. Sequencing: Following the manufacturer's instructions, the produced sample was loaded onto an ABI 3730xl System capillary electrophoresis device. The fragments were separated according to size throughout the sequencing process, and the fluorescence released by the dye terminators was observed. The Sanger sequencing was performed by Macrogen sequencing labs in Seoul, South Korea (Macrogen) (**Fig. 3 & Fig. 4**) illustrate the sequencing results. Figure 3 shows a point mutation on chromosome 17, specifically Pro72Arg, while Figure 4 displays the normal gene sequence without mutation.

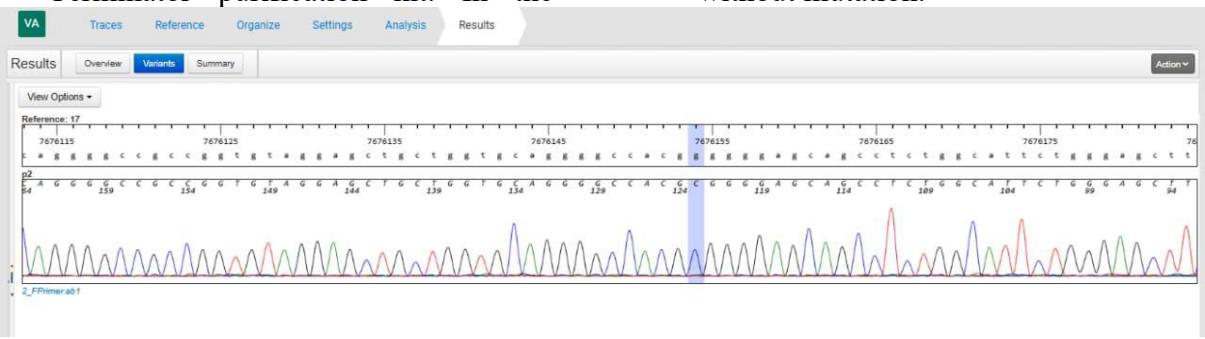

Fig.3. Point mutation in chrosome17 namely Pro72Arg

Fig.4. Normal gene sequence without mutation

7. **Data analysis:** The ab1 files of the 25 patients were firstly quality checked and then aligned against the TP53 reference gene using Applied Biosystems™ Analysis Module Variant Analysis software, version 2.1.3. The alignment stringency was chosen to be medium, and the variant score threshold is set to be 40.

Statistical analysis

SPSS v27 (IBM SPSS) was used for the statistical analysis. The mean and standard deviation (SD) were used to display quantitative variables. Frequencies and percentages (%) were used to display the qualitative factors. The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to determine normality of the data. The chi-square test was used to compare groups based on qualitative factors, The Fisher exact test was applied when the chi-square assumptions were not satisfied. When comparing numerical variables with parametric distributions, the independent t-test is utilized. The disease-free survival of several chemotherapy regimens was compared using the log-rank test. A two-tailed P value of less than 0.05 was deemed significant, and the 95% confidence interval was established.

Results

The expression of p53 mutation in axon 4 was evaluated in 50 patients with invasive transitional cell carcinoma all of them received neoadjuvant CTH. There was a point mutation in 8 patients of various stages (HMF@misr64HMFT2, T3, and T4); they were found in exon 4 of chrosome 17 namely Pro72Arg. Pro72Arg was caused by G-C transversion. Those were missense mutations according to National Center for Biotechnology Information (NCBI) (annotated variant rs1042522)

(https://www.ncbi.nlm.nih.gov/snp/rs1042522#clinical_significance)

Patients' demographic and clinical parameters

Ages range from 47 to 86 years old with the mean age being 59.14 ± 8.264 years old and 86% were males. Twenty-one patients (42%) had comorbidity, 46% were smokers, and 22% had a history of bilharziasis. mutation occurred in 8% of cases (**Table.1**).

Table 1. Patients' demographic and clinical parameters (n=50)

Parameters		Number	Percentage (%)
Gender	**Male**	**43**	**86%**
	Female	**7**	**14%**
Age (years)	**47-60**	**32**	**64%**
	61-86	**18**	**36%**
	Mean ± SD	**59.14 ± 8.264**	
	Median (range)	**57 (47-86)**	
Comorbidity	**Diabetes**	**8**	**16%**
	Hypertension	**7**	**14%**
	Cardiac disease	**6**	**12%**
Smoking status	**Smoker**	**23**	**46%**
	Non-smoker	**27**	**54%**
Bilharziasis		**11**	**22%**
Tumor protein 53 Mutation		**4**	**8%**

Patients' tumor parameters

Most cancers were poorly differentiated (96%), 58% of cases had T2-stage, and 64% had N0-stage. The most common site of cancer metastasis was to the liver and lung (8%) for each followed by bone and brain (4%) for each (**Table.2**).

Table 2. Tumor pathological parameters (n=50)

Parameters		Number	Percentage (%)
Grade	**1**	**1**	**2%**
	2	**1**	**2%**
	3	**48**	**96%**
T-stage	**T2**	**29**	**58%**
	T3	**18**	**36%**
	T4a	**2**	**4%**
N-stage	**N0**	**32**	**64%**
	N1	**6**	**12%**
	N2	**9**	**18%**
Metastasis sites	**Liver**	**4**	**8%**
	Lung	**4**	**8%**
	Bone	**2**	**4%**
	Brain	**2**	**4%**
	Peritoneal nodules	**1**	**2%**
	Skin nodules	**1**	**2%**
	Pancreas	**1**	**2%**
	Pleural effusion	**1**	**2%**

T refers to size and extent of the main tumor, N refers to the number of nearby lymph nodes have cancer, M refers to whether the cancer has metastasized.

Neoadjuvant chemotherapy and other treatment modalities

The time from diagnosis to neoadjuvant chemotherapy administration was 1 to 3 weeks in 40%, 4 to 8 weeks in 56%, and 16 to 44 weeks in 4% of cases. Nine cases (18%) had neoadjuvant chemotherapy toxicity or interrupted it. Regarding the type of neoadjuvant chemotherapy received, 80% of cases received GEMZAR cisplatin, while 20% received GEMZAR carboplatin. Regarding the number of cycles, most cases (78%) received four cycles of chemotherapy. Concerning the disease outcome, 66% had a regressive course, 20% had a stationary course, and 14% had a progressive one **(Table.3**).

Table 3. Neoadjuvant chemotherapy and other treatment modalities among study participants (n=50)

Parameters		Number	Percentage (%)
Time from diagnosis to neoadjuvant chemotherapy (weeks)	**1-3**	**20**	**40%**
	4-8	**28**	**56%**
	16-44	**2**	**4%**
Neoadjuvant Chemotherapy toxicity or interruption		**9**	**18%**
Type of NACTH	**GEMZAR cisplatin**	**40**	**80%**
	GEMZAR carboplatin	**10**	**20%**
Number of cycles	**1-3**	**9**	**18%**
	4	**39**	**78%**
	5-6	**2**	**4%**
Response	**Regressive**	**33**	**66%**
	Stationary	**10**	**20%**
	Progressive	**7**	**14%**
Radiotherapy		**40**	**80%**
Radical cystectomy		**20**	**40%**

Relation between mutation and demographic characteristics

Mutation was found in 8 patients in axon 4 chromosome 17 namely Pro72Arg, According to NCBI, Pro72Arg was caused by G-C transversion. Those were missense mutations, so there was no statistically significant difference between cases with TP53 mutation and those without mutation in terms of gender, age, smoking status, comorbidity such as diabetes, hypertension, and heart disease (P>0.05), and there is no statistically significant difference between TP53 mutation and cancer grade, T-stage, and N-stage (**Table.4**). Eight patients (16%) had a point mutation in chromosome 17, PRO72ARG.

Table 4. Relation between mutation and demographic characteristics

Parameters		Tumor protein 53		P value
		Mutation (n=8)	No mutation (n=42)	
		Number (%)	Number (%)	
Gender	Male	3 (75%)	40 (87%)	0.509(1)
	Female	1 (25%)	6 (13%)	
Age (years)	47-60	2 (50%)	30 (65.5%)	0.543(1)
	61-85	2 (50%)	16 (34.8%)	
	Mean ± SD	57.75 ± 11.843	59.26 ± 8.051	0.730(2)
Smokers		2 (50%)	21 (45.7%)	0.867(1)
Diabetes		1 (25%)	7 (15.2%)	0.609(1)
Hypertension		1 (25%)	6 (13%)	0.509(1)
Cardiac disease		0 (0%)	6 (13%)	0.441(1)
Bilharziasis		1 (25%)	10 (21.7%)	0.880(1)
Grade	1	0 (0%)	1 (2.2%)	0.155
	2	1 (25%)	0 (0%)	
	3	3 (75%)	45 (97.8%)	
T-stage	T2	4 (100%)	25 (54.3%)	0.342
	T3	0 (0%)	18 (39.1%)	
	T4a	0 (0%)	2 (4.3%)	
N-stage	N0	3 (75%)	29 (63%)	0.805
	N1	0 (0%)	6 (13%)	
	N2	1 (25%)	8 (17.4%)	

(1) Chi-square test; (2) Student t-test.

Association between mutation and cancer recurrence and treatment response

No statistically significant difference exists between mutation and chemotherapy response and metastasis as p≥0.05, (**Table.5**).

Table 5. Association between mutation and cancer recurrence and treatment response and other pathological features

Parameters		Tumor protein 53		P-value
		Mutation (n=8)	No mutation (n=42)	
		Number (%)	Number (%)	
Local Recurrence		3 (37.50%)	3 (7.1%)	0.015*
Response	Regressive	4 (50%)	29 (69%)	0.137
	Stationary	1 (12.5%)	9 (21.4%)	
	Progressive	3 (37.5%)	4 (9.5%)	
Metastasis		3 (37.5%)	8 (20.5%)	0.301

*: significant; Chi-square test

Mean survival time concerning chemotherapy

Mean disease-free survival time, as shown in (**Fig.5**) was 16.571 months concerning carboplatin and 45.698 months concerning cisplatin chemotherapy (**Table.6**).

Table 6. Mean survival time among patients concerning chemotherapy

Variables	Chemotherapy		P value
	Carboplatin	Cisplatin	
Mean disease-free survival time (months)	**16.571**	**45.698**	**0.654**

Log-rank test

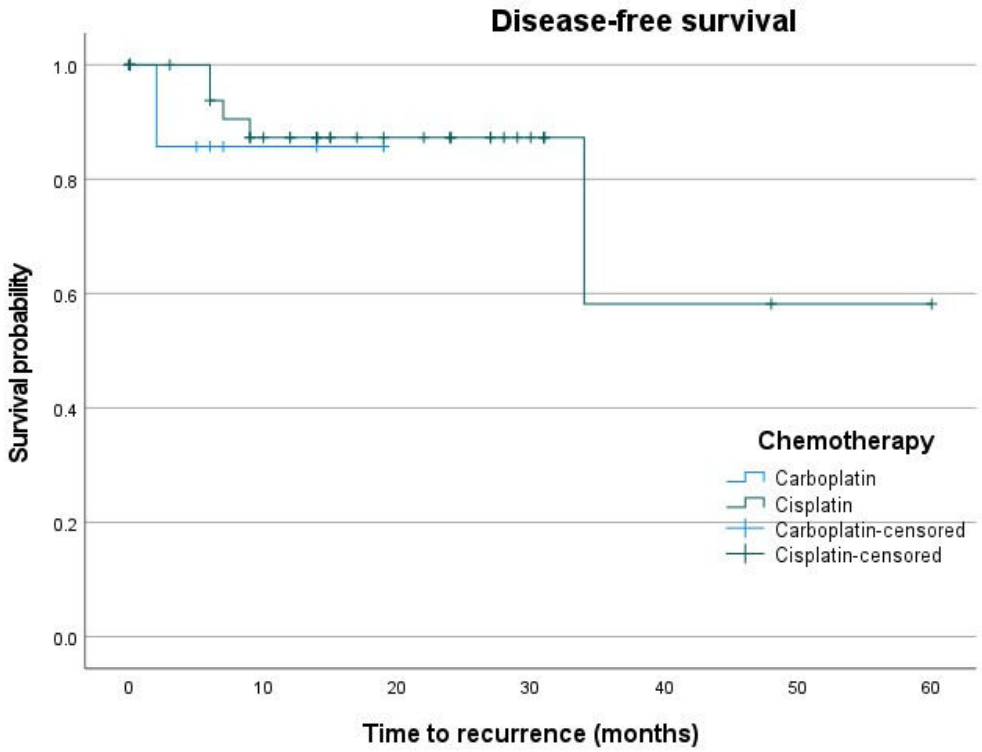

Fig. 5. Kaplan-Meier curve of DFS in different types of chemotherapy.

Discussion

According to new data, TP53 also modifies the Tumor micro environment(TME) by reprogramming its constituent parts. By boosting the release of chemokines and cytokines, P53 (encoded by TP53) malfunctioning in cancer-associated fibroblasts can encourage tumor aggressiveness **(Tao et al., 2022)**. The connection between TP53 and BLCA, however, has not been well studied, and it is unknown how it affects tumor growth and patient outcomes **(Lyu et al., 2020; Wu et al., 2020; Chatterjee et al., 2024)**.

In the present study, most cancers were poorly differentiated (96%), 58% of cases had T2-stage, and 64% had N0-stage. Eleven cases (22%) had metastasis. The most common sites of cancer metastasis the liver and lung (8%) for each followed by bone and brain (4%) for each. In line with our findings. **Aljabery et al. (2023)** claimed that non-organ-confined tumors (pT3-4) were present in 80% of cases. Forty-two percent (42%) of the patients had lymph node metastases.

Richters et al. (2022) reported that 44% had metastasis. The most common site of cancer metastasis was to the viscera (49%) for each, followed by bone (29%) and then liver (14%), which is slightly higher than our findings. This

difference may be attribute to different study settings and a larger sample size.

We noted that the time from diagnosis to neoadjuvant chemotherapy administration was 1 to 3 weeks in 40%, 4 to 8 weeks in 56%, and 16 to 44 weeks in 4% of cases. Nine cases (18%) had neoadjuvant chemotherapy toxicity or interrupted it. Regarding the type of neoadjuvant chemotherapy received, 80% of cases received GEMZAR cisplatin, while 20% received GEMZAR carboplatin. Regarding the number of cycles, most cases (78%) received four cycles of chemotherapy. Concerning the disease outcome, 66% had a regressive course, 20% had a stationary course, and 14% had a progressive one.

Similarly, **Richters et al. (2022)** indicated that of the 1041 patients with mUCB, 3359 received either cisplatin (n = 170; 47%) or carboplatin (n = 189; 53%) as first line. However, most cases (36%) underwent six cycles of treatment.

No statistically significant difference between cases with TP53 mutation and those without mutation concerning gender, age, smoking status, and comorbidity as diabetes, hypertension, and heart disease ($P > 0.05$).

A statistically significant difference between TP53 mutation and recurrence; 37.5% of cases with TP53 mutation had recurrence compared to 7.1% of cases without mutation ($p = 0.015$). No statistically significant difference exists between TP53 mutation and chemotherapy response, cancer grade, T-stage, N-stage, and metastasis.

No statistically significant difference exists between TP53 mutation and chemotherapy response, cancer grade, T-stage, N-stage, and metastasis.

The relationship between TP53 mutations and demographic characteristics in BC is a complex interplay that influences the prognosis and progression of the disease. TP53, a tumor suppressor gene, is frequently mutated in BC, and these mutations are associated with various clinical outcomes **(Strandgren and Wiman, 2024)**.

Similarly, **Kelsey et al. (2005)** claimed that older cases and men had lower rates of TP53 inactivation (as determined by persistent IHC staining) and TP53 mutation prevalence, although these differences were not statistically significant. A bigger sample size could be the cause of this discrepancy. Also, **Chen et al. (2016)** who observed that the TP53 mutation pattern was a significant predictive factor for bladder recurrence in the Cox proportional hazard model's univariate analysis. The relationship between the TP53 mutation and metastasis, T-stage, N- stage, and cancer grade is not statistically significant. Similarly, **Kim et al. (2016)** found that when TP53 status and baseline variables were analyzed, TP53 mutation or expression was unaffected by baseline parameters such as age, stage, and grade.

The disease-free survival time for carboplatin and cisplatin treatment, respectively, was 16.571 and 45.698 months in the current study. A number of variables pertaining to the pharmacological characteristics and varying performance of carboplatin-based chemotherapy and cisplatin-based regimens in invasive BC may be responsible for the reported shorter mean disease-free survival (DFS) time in these patients. According to studies, tumors in patients on carboplatin may show either acquired or intrinsic resistance to platinum drugs. While carboplatin may be less effective in cases of aggressive or advanced disease, cisplatin's higher DNA-damaging action is more successful at overcoming **(Huang et al., 2021; Mendoza-Valderrey et al., 2024; Stefàno et al., 2024)**.

Similar to our findings, **Richters et al. (2022)** found that the median OS (mOS) periods for the cisplatin and carboplatin groups were 13.1 (95% CI: 12.2–16.8) and 11.5 (10.3– 13.5) mo, respectively. After IPW adjustment, the

mOS periods were 16.7 (12.5–not estimated) and 12.3 (10.7– 14.5) mo, respectively.

Also, **Bamias et al. (2019)** revealed that the 2-year COS for patients who survived 3 years after starting cisplatin-based treatment was 83% (95% CI: 59.7–93.5), and those eligible patients treated with cisplatin had 31.6% probabilities of 3-year survival (95% CI: 25.1–38.3). Patients who were either ineligible for cisplatin or who were not treated with cisplatin while being eligible had respective probability of 14% (95% CI: 10.8–17.6) and 49.3% (95% CI: 28.2–67.4). Up to three years after the start of chemotherapy, there was still a substantial difference in the two-year COS between these two groups.

Limitations: The sample size was relatively small. The study was in a single center. The study did not explore additional molecular markers or pathways that might influence treatment outcomes, potentially missing interactions between TP53 and other genetic alterations.

Conclusion

TP53 mutations significantly predict disease recurrence in invasive BC (T2) patients after radical cystectomy and neoadjuvant chemotherapy. Patients with TP53 mutations had higher recurrence rates (50%). The choice of platinum drug influenced outcomes, with cisplatin-based therapy showing better disease-free survival than carboplatin.

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