# ASSESSMENT OF TP53 MUTATIONS AND RAGE IMMUNOHISTOCHEMICAL EXPRESSION IN BLADDER CANCER

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**Abstract**

**Objective:** Urothelial carcinoma (UC) is one of the most common malignancies with complex pathogenesis, tumor recurrence, and metastasis. Recent multiplex expensive molecular assays can accurately identify the diagnostic genetic aberrations in urine cytology; however, they lack worldwide availability and are of high cost. To date, cystoscopy, an invasive procedure, remains the gold standard for diagnosis. Receptor for Advanced Glycation End Products (RAGE) and TP53 mutations are involved in carcinogenesis and invasiveness of many human cancers. Hence, this study aims to develop simpler reliable molecular and non-invasive UC identification and follow-up tools in Egyptian patients harboring these aberrations.

**Patients and methods:** The study enrolled 200 patients with urinary symptoms. Urines and bladder biopsies were obtained from all participants. High-Grade Urothelial Carcinoma cells (HGUC) were diagnosed in urine cytology according to the Paris system for reporting urine cytology. TP53 mutations were assessed in urine cytology using Polymerase Chain Reaction (PCR) followed by bidirectional Sanger sequencing. The expression of RAGE in UC biopsies was evaluated by immunohistochemistry (IHC). Recurrence and lymph node metastasis follow-up data were collected and Meta-analysis was performed.

**Results:** Positive HGUC in urine significantly showed mutations in TP53 exons 2+3, 4, and 5 representing 36%, 64%, and 74% respectively with significant concordance with higher tumor grade, stage, recurrence, and lymph nodes metastasis, which parallelly, exhibited higher RAGE IHC expression in biopsies compared to cystitis.

**Conclusions:** TP53 mutations assessment in urine cytology is promising as a simple molecular non-invasive tool for screening and follow-up UC patients. In the same context, RAGE IHC expression in UC biopsies could be suggested as a potential novel pathological screening for molecular-targeted UC therapies and follow-up.

**Keywords:** Urothelial carcinoma, TP53, metastasis, recurrence, RAGE, urine, cytology, non- invasive, biopsy, IHC, HGUC, urinary bladder, exons, mutations.

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## 1. INTRODUCTION

Urothelial carcinoma (UC) is one of the most common urological cancers worldwide. In Egypt, squamous cell carcinoma (SCC) is associated with endemic schistosomiasis 1. According to the WHO 2022 2, UC is classified as non–muscle-invasive bladder cancer (NMIBC) or muscle-invasive bladder cancer (MIBC), due to distinct implications on patient management 3.

Urine cytology is an easy non-invasive test of low cost for early detection of bladder cancer; however, due to low sensitivity, cystoscopy and biopsies are considered the gold standard for UC diagnosis 4; Nevertheless, being invasive, it causes patient discomfort, possible urinary tract infection and anxiety 5. Therefore, in order to overcome this problem, Paris System for reporting urinary cytology 6 has been implemented as an internationally validated and approved standardized cytological reporting system for pathological analysis of urine cytology samples with the ultimate goal of identification and diagnosis of High-Grade Urothelial Carcinoma (HGUC), whether in situ (CIS) or invasive, in urine smears, with high negative predictive value, high sensitivity and specificity ‘’6-8’’. Accordingly, HGUC in urine cytology is clinically meaningful,

significantly associated with a high prevalence of tumor recurrence, and valuable preoperatively for prediction of the patient prognosis and tumor progression''6,7''.

With the huge advancement in molecular assays, urine cytology, being noninvasive and easy to collect 9, is recently assessed using multiplex molecular assays such as Next Generation Sequencing (NGS) 10 and digital droplet PCR 11 in order to detect the tumor coding mutations and genetic aberrations. In this regard, NGS-based molecular assays can reliably and accurately can replace the biopsy''12,13''. In the same context, many other molecular tests in urine cytology such as Urovision were reported as non-accurate in advanced bladder cancer screening and detection 14. Therefore; due to the high cost of and unavailability of NGS in low-resourced countries, the need for a simpler cheaper, however accurate and reliable, predictive and prognostic molecular tool is needed for urothelial carcinoma detection, which may serve as a non-invasive surveillance tool in the follow-up of bladder cancer patients harboring such aberrations.

Over the past decade, progress has been made to improve the detection of bladder cancer using novel urinary biomarkers 9. According to Scott et al., 2017 12 and Sun et al., 2021 15, TP53 is among the most altered genes in urothelial cancer. In comprehensive NGS analysis of urine cytology, mutations of TP53 were frequently identified in HGUC and CIS with high concordance between cytology and biopsies, high sensitivity, and specificity 15. Simultaneously, using NGS in urinary bladder biopsies, TP53 was the most frequently mutated gene in CIS according to Garczyk et al., 2020 16 and it was regarded as a potential predictive biomarker for targeted urothelial carcinomas in situ therapies 16.

TP53 (17p13) is a tumor suppressor gene 17. Its mutations cause the progression of UC through various carcinogenesis-related signaling pathways 14 affecting cell cycle regulation, programmed cell death, senescence, differentiation and development, transcription, DNA replication, DNA repair, and maintenance of genomic stability 18 with the progression of NMIBC from Ta stage to higher stages as MIBC14.

Receptor for Advanced Glycation End products (RAGE) is a 45 KD multiligand transmembrane receptor that plays a role in aging, diabetes, and cancer 19. It attracted considerable attention as therapeutic applications for many human cancers since RAGE inhibin potentially inhibits cancer progression19; nevertheless, this is not yet explored in urinary bladder cancer.

RAGE is a damaged-associated molecular pattern (DAMP)19,20 that affects cell growth, apoptosis, and metastasis21,19, causing oxidative stress, leading to cell growth, stress response, and inducing changes in cellular motility; thus, promoting carcinogenesis, tumor propagation, and metastasis and angiogenesis such as in oral carcinomas 19. Nonetheless, its role in the pathogenesis of urothelial carcinoma has not been well studied.

Coming along, the relationship of RAGE and P53 is yet unclear. In non-neoplastic processes, RAGE is a receptor for Advanced Oxidation Protein Products (AOPPs), which in endothelial cells, induce cell senescence and evasion of apoptosis through inhibition of autophagy. Thus, RAGE subsequently modulates P53 to determine the fate of senescent cells whether to undergo apoptosis or escape apoptosis 22. Moreover, in adipocytes, RAGE is required for the impairment of P53 expression and P53 function in senescent preadipocytes 23. Furthermore, depletion of RAGE reduces degradation of P53, affecting cellular apoptosis 24.

Being measurable in the blood, sRAGE can easily be used as a clinical biomarker for prediction, prognosis, therapy monitoring, and staging of cancers25. In the same context, the soluble form of RAGE is detected at the protein level in tissues using immunohistochemistry (IHC) staining 26. Accordingly, increased RAGE expression was reported to be associated with increased tumor histological progression of colorectal and gastric carcinomas 19, but has not yet been explored in the bladder.

In light of the above, this study aims to develop a simple reliable, and cheap molecular and non-invasive tool for urothelial carcinoma identification and follow-up in Egyptian patients harboring TP53 and RAGE abnormalities, which potentially may show novel roles in molecular-targeted UC management and follow up.

## 2. PATIENTS AND SAMPLES

A total of 200 patients (150 urothelial carcinoma patients and 50 non-UC patients with only cystitis as controls) were enrolled in this study. They visited the outpatient clinic at Theodor Bilharz Research Institute Hospital (TBRI), Giza, Egypt seeking medical advice for their urinary symptoms. Before recruitment, signed informed consent was obtained from all participants. The research was conducted according to the Declaration of Helsinki and was approved by the TBRI institutional review board of the Ethics Committee, in accordance with the institutional guidelines. The participants were cystoscopically examined. Urines and biopsies for histopathological diagnosis were collected. The inclusion criteria for UC patients targeted those who did not receive any type of therapy. The participants have been followed up over a period of 5 years (2017-2022).

Fifty milliliters of morning voided urine samples were collected from cancer patients and controls, and 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. After checking cytological adequacy, urine cytology smears were assessed according to the Paris system for reporting urine cytology 6,7 categories. Positive cytology is defined when at least 5-10 (HGUC) cells must essentially exhibit high N/C ratio >0.7 (where the nucleus occupies >70% of cytoplasm), together with

either hyperchromasia, nuclear pleomorphism, irregular nuclear membranes, and irregular chromatin pattern. Their biopsies were histopathologically diagnosed and confirmed by two independent pathologists.

**DNA extraction from urine samples**

DNA extraction was carried out using Qiagen DN easy 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 analysis.

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

Mutations of TP53 exons were analyzed in 150 urine samples of UC patients. TP53 mutations in exons 2+3, 4, and 5 were screened in a volume of 25 µl containing 100 ng of urine sediment DNA, using (PCR). Exons (2+3), 4, and 5 of the TP53 gene were amplified using the primers shown in Table 1 according to Bakkar et al., 2003 27. After that, 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 images were analyzed using Cleaver Scientific's micro-DOC gel documentation system.

**DNA Sanger Sequencing analysis**

All PCR exon products were subjected to Exonuclease I- Shrimp Alkaline Phosphatase PCR product treatment (Thermo Fisher, catalog no.78200) in order to hydrolyze the excess primers and nucleotides in a single step. Then, purified samples were subjected to bidirectional sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The abnormal sequencing results were re-confirmed 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 28. After that, all sequences were aligned by using the Bio Edit software which depended on the Clustal W multiple alignment conditions**. Cytology preparations of urine smears slides:**

For each urine sample, a PAP-stained slide was prepared. Urine was centrifuged, then the sediment was smeared on a glass slide and fixed immediately in 95% alcohol for 10 mins followed by 6 dips in each for the following solution of ethanol 80%, 70%, and 50% respectively, then rinsed gently in distilled water, stained in diluted Harris's Hematoxylin for 3 minutes, rinsed again in distilled water, dipped in 0.25% hydrochloric acid 6 times, placed under running tap water for 5 minutes. Then, smears were dehydrated by dipping them in distilled water, alcohol solution of ascending concentrations 50%, 70%, and 95% respectively, 6 dips in each solution, then stained in Orange G-6 for 2 minutes, rinsed in 95% alcohol, 3 changes, 6 dips each, followed by staining in Eosin A-50 for 2 minutes, rinsed in 95% alcohol, 3 changes, followed by dehydration of the smears in absolute alcohol for 2 changes. Finally, the slides were dipped in Xylene for 6 dips, followed by 3 changes in xylene for 6 dips, then mounted using DPX and examined under the light microscope.

**Immunohistochemistry staining method (IHC)**

Anti-RAGE antibody mouse monoclonal antibody (Santa Cruz Biotechnology) was used for immunohistochemical detection of RAGE protein expression in UC biopsies. Paraffin sections (4 µm thick) were treated in PT link (DAKO apparatus) for antigen retrieval, incubated at 90°C for 30 min, then in 0.03% hydrogen peroxide for 10 min at room temperature to remove endogenous peroxidase activity, then in blocking serum (0.04% bovine serum albumin, A2153, Sigma-Aldrich, Shanghai, China, and 0.5% normal goat serum X0907, Dako Corporation, Carpinteria, CA, USA, in PBS) for 30 min at room temperature. After that, anti-RAGE antibody (A11): sc- 80652 RAGE Antibody was added at a dilution of 1:100 and incubated with the sections overnight at 4°C. Sections were then washed three times for 5 min in Phosphate-Buffered Saline (PBS). Non-specific staining was blocked using 5% normal serum for 30 min at room temperature. Finally, sections were counter-stained with hematoxylin. In negative controls, RAGE antibody was replaced with PBS.

**Evaluation of RAGE protein immunohistochemistry staining in biopsies:**

The expression of RAGE in tissue sections was semi-quantitatively estimated as the total membrano-cytoplasmic immunostaining scores in the tumor cells, as a sum of proportion score and intensity score. The proportion and intensity of staining were evaluated independently. The proportion score reflected the percentage of positively stained cells (score 0: <5%, score 1:5%-10%, score 2:10%-50%, score 3:50%-75%, score 4:>75%). The intensity of the staining was scored

as (0: absent staining, 1: weak positive, 2: moderate positive, 3: strong positive). Finally, a total expression score was given ranging from 0 to 12 as an h-score.

**Statistical analysis methods:**

Microsoft Excel 2016 and its statistical package 'IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA) was used. Continuous normally distributed variables were represented as mean±SD with a 95% confidence interval, while nonnormal variables were summarized as median with 25 and 75 percentile, and using the frequencies and percentage for categorical variables; in which $P< 0.05$ is statistically significant. Student's t-test was performed for comparison of normally distributed variables among the groups. Mann-Whitney U test assessed the non-normal variables. Chi-square ($\chi 2$) test and/or Fisher's exact test were used to determine the distribution of categorical variables between groups. Logistic regression analysis was performed to identify predictors associated with the risk of BC occurrence. The Kaplan-Meier method was used to estimate the time to survive. Receiver Operating Characteristic (ROC) curves evaluated the diagnostic performance of the studied exons mutations. The area under the ROC (AUC) illustrated the accuracy index for the prognostic performance of selected tests.

## 3. RESULTS

A total of 200 urine cytology samples for 150 UC and 50 non-UC (with cystitis as control) were assessed. The mutational frequency of TP53 varied according to exons among the 150 UCs. Fifty-four patients (36.0%) were significantly mutated in exon (2+3), 96
(64.0%) in exon 4, and 111 (74.0%) in Exon 5, compared to controls with no detected mutations ($P = 0.001$) (Table 2) (Figure 1a). Moreover, double exons mutations such as exons (2+3 & 4), (2+3 & 5), and (4&5), representing 48 (32%), 51 (34%), 78 (52%) respectively as well as triple exons mutations in 48 (32%) cases were identified (Table 3).

Mutational landscape of TP53:

All the studied exons of TP53 gene were sequenced. Regarding exons 2+3, missense mutations were the most frequently detected (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. Leucine (Leu), likewise, at codon 112, was substituted by Valine (Val) amino acid in (21/54, 38.8%) leading to an incorrect amino acid sequence, which may produce a malfunctioning protein (Figure 2a) (Table 3).

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 nucleotide substitution at codon 150 "Silent mutation" was detected in (27/96) where the codon of amino acid "Leu" unaltered than reference. Interestingly, an insertion of a single nucleotide was detected at codon 151in (9/96, 9.3%) followed by a missense mutation at codon 152 which led to frameshift of DNA nucleotide resulting in an abnormal amino acid sequence in (15/96, 15.6%) (Figure 2b) (Table 3).

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) was unaltered than reference in (13/111, 11.7%) (Figure 2c) (Table 3).

TP53 exons mutations diagnostic performance evaluation:

ROC Curve was established to assess the diagnostic performance of TP53 Exons (2+3), 4, and 5 mutations in UC patients, to evaluate the specificity and sensitivity of UC prediction in cytology samples, and to assess their discriminatory properties among patients and healthy individuals. TP53 mutations prognostic value analysis showed promising results; thus, indicating that these mutations are associated with convenient disease characteristics.

For discrimination, exons (2+3) mutations revealed 36.0% sensitivity and 100.0% specificity with an area under the curve (AUC) of 0.680 ($P< 0.0001$, 95% C.I: 0.611 - 0.744) and accuracy 36.0%. Exon 4 mutations exhibited 64.0% sensitivity and 100.0% specificity with
an (AUC) of 0. 820 ($P< 0.0001$, 95% C.I: 0.760 - 0.871) and accuracy of 64.0%, compared to exon 5 where sensitivity and specificity reached 74.0% and 100.0% respectively with an (AUC) of 0.870 ($P< 0.0001$, 95% C.I: 0.815 - 0.913) and accuracy 74.0% (Table 4) (Figure 1b).

Association analysis between TP53 mutations frequency and the studied parameters among UC patients:

a- Association between TP53 mutations and tumor grade:

Overall, TP53 mutations were most frequent in high grade tumors compared to low grades. In GI, only 5.6% were mutants in exon (2+3) with no

exons 4 or 5 mutations. On the other hand, GII tumors exhibited exon 4 mutations in 46.9% and exon 5 mutations in 51.4% of UCs, with no exons (2+3) mutation. Similarly, regarding GIII, exon 4 significantly showed mutations in 53.1% and 48.6% for mutations in exon 4 and 48.6% for mutations in exon 5 (Table 5), (Figure 1c).

b- Association between TP53 mutations and tumor stage:

Overall, TP53 mutations were most frequent in high tumor stages compared to low stages. For T1, only 5.6% showed mutations in exons (2+3), 3.1% in exon 4, and 8.1% in exon 5.

On the other hand, 55.6% of T2 tumors significantly exhibited mutations in exons (2+3), 75% in exon 4, and 70.3% in exon 5. Regarding T3 tumors, 33.3% were significantly mutant in exons (2+3), 18.8% in exon 4, and 18.9% in exon 5 (Table 5), (Figure 1d). The mutations were not significant in T4 due to the low number of cases.

c- Association between TP53 mutations and Lymph nodes metastasis:

Results elucidated that (45/54, 83%) of TP53 mutant patients at exons (2+3) showed positive lymph nodes with risk OR (95% C.I) = 9.55 (4.16- 21.90) and P=0.001 (Table 5). Similarly, regarding exon 4 mutations, (69/96, 71.9%) of the mutant patients had lymph node metastasis with risk OR (95% C.I) = 12.78 (5.50- 29.68) and P=0.001 (Table 5). In the same context, (69/111, 62.2%) of the mutant patients in exon 5 were significantly associated with lymph node metastasis with risk OR (95% C.I) = 5.48 (2.37- 12.66) and P=0.001 (Table 5).

d- Association between TP53 mutations with tumor recurrence:

Sixty-three patients showed urothelial carcinoma recurrence (63/150, 42.0%). Overall recurrence significantly correlated with tumor multifocality, with risk of recurrence OR (95% C.I) =81.0(27.3- 240.6) (P=0.001), bigger tumor size OR (95% C.I) = 1.15(1.10- 1.20)

(P=0.001), GII tumors OR (95% C.I) =1.60 (1.01- 2.52) (P=0.04) as well as presence of CIS with risk to recurrence OR (95% C.I) = 8.3(3.1- 22.0), (P= 0.001) (Table 6).

Using Kaplan-Meier analysis, significantly closer recurrences in the cases with mutations in exons (2+3) were found with a mean time of 23.00±0.45 months (range 22.12 - 23.88) (P= 0.001) compared to the wild type. Similarly, mutants in exon 4, showed significant meantime recurrence 30.5±1.8 months (range 26.9 - 34.0) (P= 0.008), contrasting the absence of statistically significant difference between exon 5 mutants and wild type (Table 7) (Figure 3, a-c).

e- Association between lymph node metastasis, double/triple TP53 exons mutations, and risk of recurrence in urothelial carcinoma follow-up:

Most patients carried only one mutation, while others had double and triple mutations (Table 3). Association analysis revealed a significant correlation between double and/or triple TP53 mutations and lymph node metastasis as illustrated in Table 8, with mutants in exons (2+3) & 4 OR (95% C.I) =54.1(15.8- 185.2) (P = 0.001). Likewise in exons (2+3) & 5, exons

4&5, and for patients with triple mutations 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 correlation between mutation frequency and tumor recurrence in double and triple mutations patients (Table 8).

Histopathological assessment of the urinary biopsies:

Urinary bladder biopsies (N=200) as 150 urothelial carcinomas and 50 chronic cystitis (including bilharzia cystitis) were examined. The UC cases included 84 Squamous cell carcinomas (SCC) and 66 transitional cell carcinomas (TCC), 96 tumors were classified as low grade (64.0%) and 54 tumors as high grade (36.0%), 27 cases were NMIBC (18.0%) and 123 cases were MIBC (82.0%), according to the WHO 2022 2 (Table 9).

RAGE Immunohistochemical evaluation in biopsies and correlations:

Overall, urothelial carcinomas significantly exhibited stronger cytoplasmic expression (Figure 4 a-e) with higher h-scores of RAGE in contrast to cystitis, which showed either negative or weak cytoplasmic staining and low h-score of RAGE expression (P<0.01) (Table 9). SCC showed the highest scores of RAGE expression compared to TCC.

The mean score of RAGE expression was significantly correlated with the higher tumor grades (GII & GIII) and to higher tumor stages in MIBC compared to low grade tumors (GI) and NMIBC respectively) (P<0.01). Moreover, positive lymph node metastasis showed a higher score of RAGE expression than negative lymph node metastasis, with high statistical significance (P<0.001). Furthermore, patients with recurrent UC exhibited significantly higher RAGE expression scores contrasting those without recurrence (P<0.001) (Table 9).

On the other hand, schistosomiasis-associated and schistosomiasis-non-associated UC did not show significant statistical differences (P>0.05) (Table 9). In addition, there was no significant correlation between RAGE immunohistochemical expression score with the prevalent TP53 exons mutations among the studied UC cases.

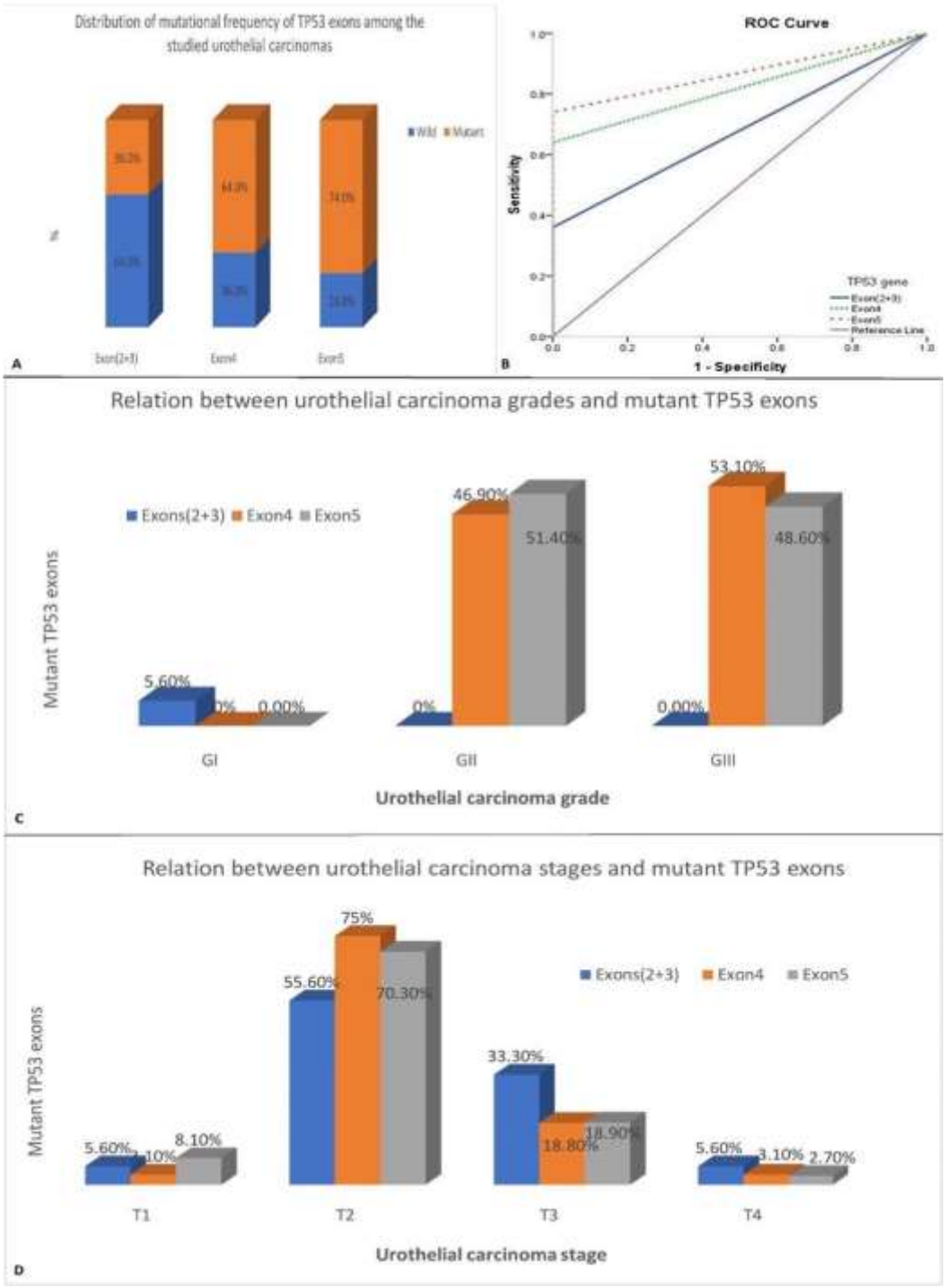


**Figure (1):** Statistical analysis. **A**- Distribution of TP53 exons mutational frequency among the studied urothelial carcinoma patients. **B**- ROC Curve of the studied TB53 exons mutations for diagnostic performance assessment. **C**- The significant correlation between the urothelial carcinoma grades and mutant TP53 exons. **D**- The significant correlation between the urothelial carcinoma stages and mutant TP53 exons.

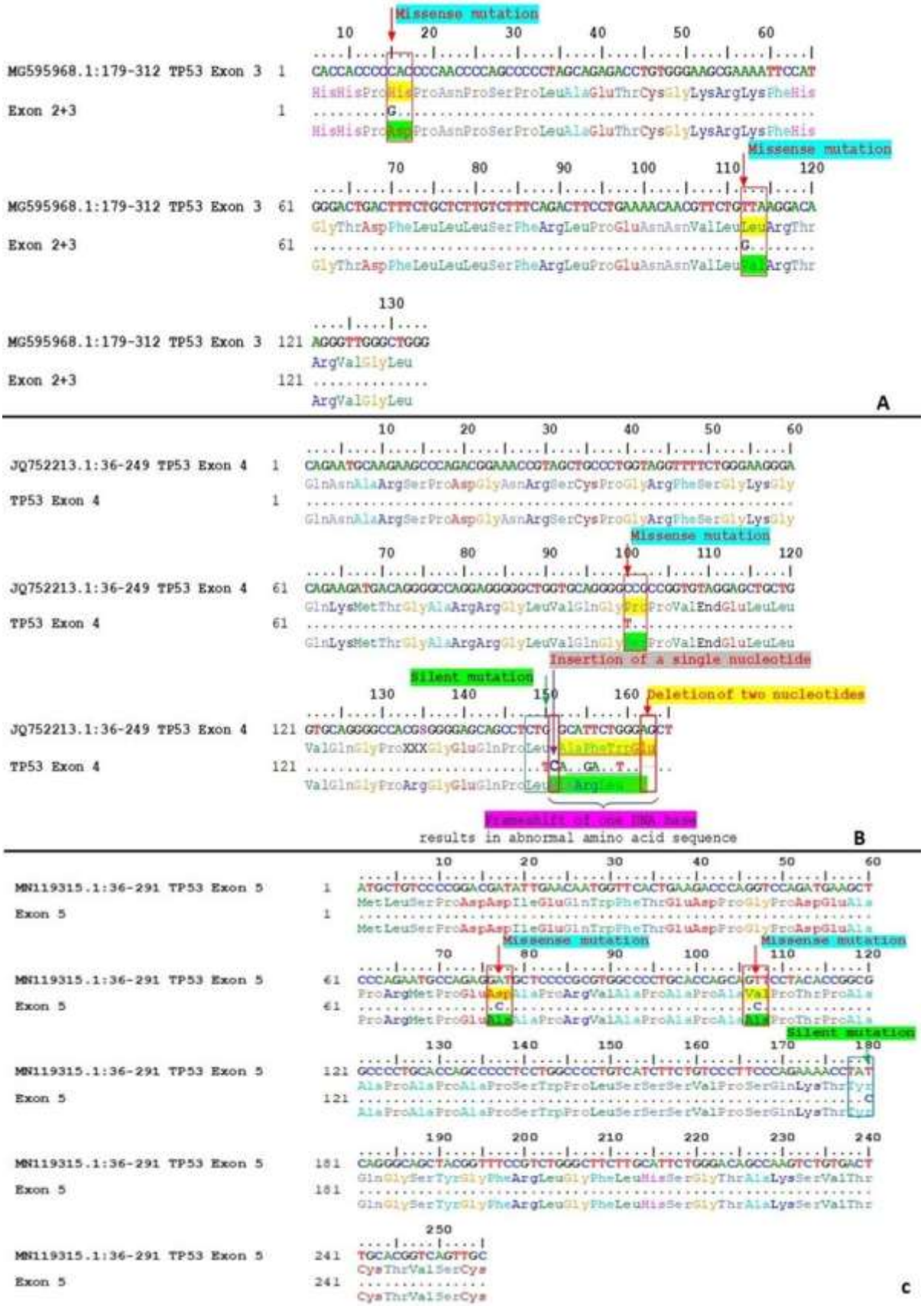


**Figure (2):** TP53 gene mutations identification according to NCBI blast reference alignment results. **A**- Exons (2+3) mutations with reference alignment "MG595968.1:179-312 TP53 Exon 3". **B**- Exon 4 mutations with reference alignment "JQ752213.1:36-249 TP53 Exon 4". **C**- Exon 5 mutations with reference alignment "MN119315.1:36-291 TP53 Exon 5".

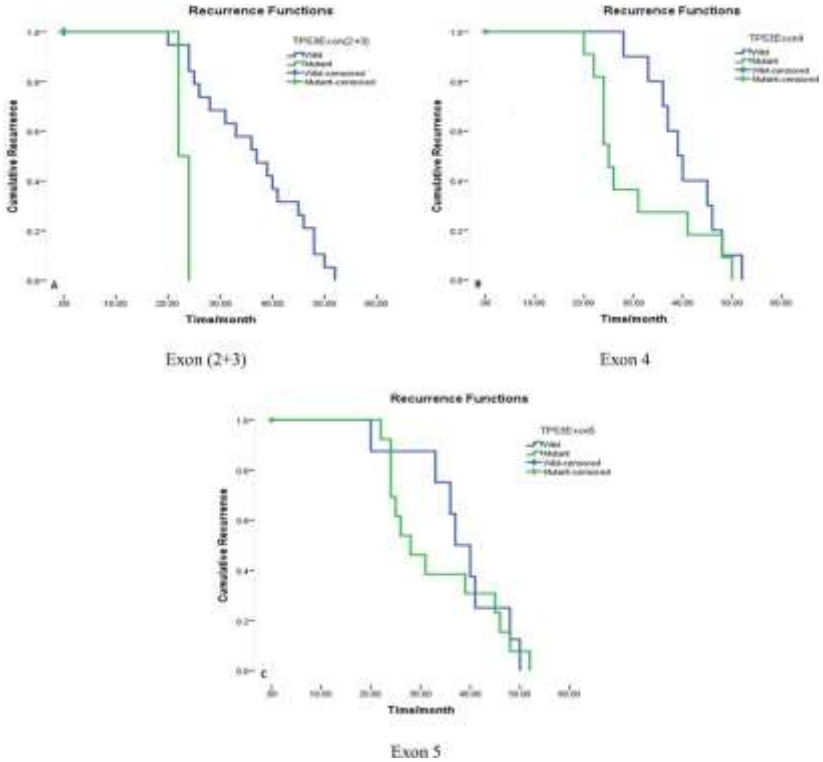


**Figure (3):** Kaplan-Meier analysis of TP53 mutation regarding tumor recurrence. Significant closer recurrences in mutant TP53 exons compared to wild type. **A-** Exons (2+3). **B-** Exon 4. C-Exon 5.

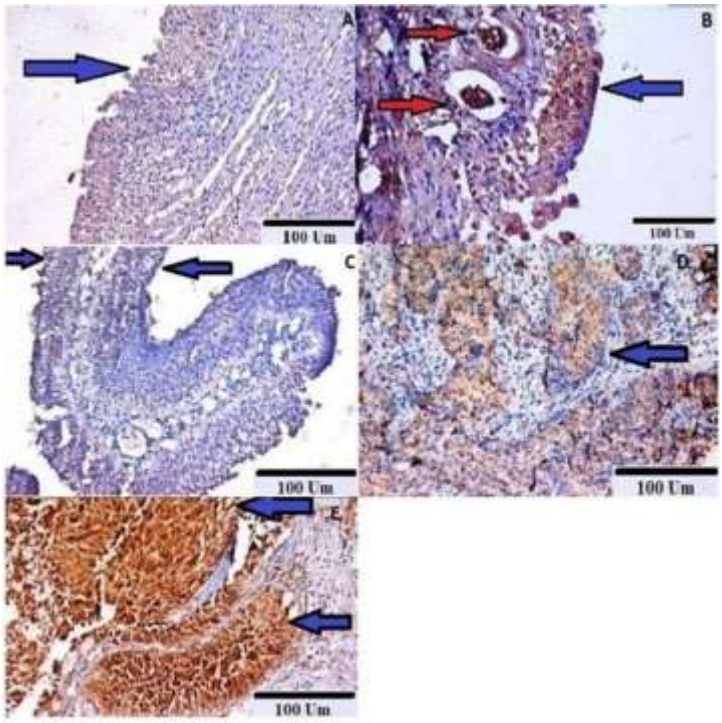


**Figure (4):** RAGE immunohistochemical (IHC) expression among the studied urinary bladder biopsies. **A-** Chronic cystitis as control with focal weak RAGE cytoplasmic staining in the urothelium (blue arrow) (IHC for RAGE, using DAB, X200). **B-** Schistosomal cystitis with moderate RAGE expression in the urothelium (blue arrow) and two bilharzia ova (red arrows) (IHC for RAGE, using DAB, X200). **C-** Low grade non-invasive papillary urothelial carcinoma, GI, Ta, with weak RAGE urothelial staining (IHC for RAGE, using DAB, X200). **D-** High grade muscle invasive transitional cell carcinoma, GII, T2 with diffuse moderate RAGE expression in malignant urothelial cells (IHC for RAGE, using DAB, X400). **E-**: High grade squamous cell carcinoma with strong diffuse RAGE expression by the malignant cells (IHC for RAGE, using DAB, and X400).

**Table (1):** Primer sequences used and their fragment sizes

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

**Table (2):** Univariate logistic regression analysis of TP53 mutations

| TP53 Exons | | Groups | | | Prognostic viability | |
|---|---|---|---|---|---|---|
| | | Control N=50 | Cases N=150 | P.value | OR (95% C.I) | P. value |
| **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 $X^2$ 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.

**Table (3):** TP53 Mutational frequencies and landscape among the studied urothelial carcinoma cases

| Number of mutant cases | Exon | Codon | Change | Triplet | 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 | Missense |
| 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 |
| 48 | 2+3 &4 | **Double exons mutations** | | | | |
| 51 | 2+3 &5 | | | | | |
| 78 | 4&5 | | | | | |
| 48 | 2+3 &4 & 5 | **Tripple exons mutations** | | | | |

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

| TP53 | Sn. | Sp. | PPV | NPV | Accuracy | AUC | 95%C. I | P. value |
|---|---|---|---|---|---|---|---|---|
| **Exons (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.

**Table (6):** Overall UC recurrence association with the clinicopathological parameters

| | | Follow up | | | Risk assessment | |
|---|---|---|---|---|---|---|
| | | **No-RE N=87** | **RE N=63** | **P. value** | **OR (95%C. I)** | **P. value** |
| **Tumor Number** | **Single** | 81(93.1%) | 9(14.3%) | 0.001** | 81.0(27.3- 240.6) | 0.001** |
| | **Multifocal** | 6(6.9%) | 54(85.7%) | | | |
| **Tumor Size (mm)** | | 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 (urothelial carcinoma in situ presence) 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 (7):** Urothelial carcinoma recurrence correlation with TP53 exons mutations frequency

| | | **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 | | |

RE: Recurrence rate. 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.

**Table (8):** Lymph nodes metastasis association with double / triple TP53 exons mutations and risk of urothelial carcinoma recurrence

| | | | TP53 | | | Risk assessment | |
|---|---|---|---|---|---|---|---|
| | | | **Wild** | **Mutant** | **P. value** | **OR (95%C. I)** | **P. value** |
| **LN** | **Exons 2+3 & 4** Wild=102, Mutant=48 | **Negative** | 69(67.6%) | 3(6.3%) | 0.001** | 54.1(15.8- 185.2) | 0.001** |
| | | **Positive** | 33(32.4%) | 45(93.8%) | | | |
| | **Exons 2+3 & 5** Wild=99, Mutant=51 | **Negative** | 66(66.7%) | 6(11.8%) | 0.001** | 26.4(10.3- 67.2) | 0.001** |
| | | **Positive** | 33(33.3%) | 45(88.2%) | | | |
| | **Exons 4 & 5** Wild=72, Mutant=78 | **Negative** | 54(75.0%) | 18(23.1%) | 0.001** | 19.3(9.3- 39.8) | 0.001** |
| | | **Positive** | 18(25.0%) | 60(76.9%) | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Exons 2+3 & 4 & 5** Wild=102, Mutant=48 | **Negative** | 69(67.6%) | 3(6.3%) | 0.001** | 54.1(15.8- 185.2) | 0.001** |
| | | **Positive** | 33(32.4%) | 45(93.8%) | | | |
| **Follow up** | **Exons 2+3 & 4** Wild=102, Mutant=48 | **No** | 45(44.1%) | 42(87.5%) | 0.001** | 0.24(0.1- 0.6) | 0.001** |
| | | **RE** | 57(55.9%) | 6(12.5%) | | | |
| | **Exons 2+3 & 5** Wild=99, Mutant=51 | **No** | 42(42.4%) | 45(88.2%) | 0.001** | 0.22(0.1- 0.54) | 0.001** |
| | | **RE** | 57(57.6%) | 6(11.8%) | | | |
| | **Exons 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%) | | | |
| | **Exons 2+3 & 4 & 5** Wild=102, Mutant=48 | **No** | 45(44.1%) | 42(87.5%) | 0.001** | 0.24(0.1- 0.6) | 0.001** |
| | | **RE** | 57(55.9%) | 6(12.5%) | | | |

LN: Lymph nodes. RE: Recurrence rate. Lymph nodes metastasis and recurrence follow up data were represented as F (%) frequency and percent; 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.

**Table (9):** Immunohistochemical expression of RAGE and association with the studied parameters

| | | **All Cases N=200** | **RAGE H-Score (Mean±S.D.)** | **P value** |
|---|---|---|---|---|
| **Pathological diagnosis** | Control (Cystitis) | 50 (100.0%) | 1.31±0.36 | <0.01* |
| | SCC | 84(56.0%) | 10.22±2.59 | |
| | TCC | 66(44.0%) | 8.30±2.51 | |
| **TCC (Papillary pattern)** | Non-Papillary | 24(36.4%) | 10.20±2.71 | >0.05 |
| | Papillary | 42(63.6%) | 6.55±1.53 | |
| **Number of tumors in the Bladder** | Single | 90(60.0%) | | |
| | Multifocal | 60(40.0%) | | |
| **Tumor size (mm) Median (Range)** | | 10.2(2.0- 22.0) | | |
| **Lymph Node Metastasis (150)** | Negative | 72(48.0%) | 6.07±1.29 | <0.001** |
| | Positive | 78(52.0%) | 10.87±2.62 | |
| **Grade** | Low Grade GI | 96(64.0%) | 6.25±1.53 | <0.001** |
| | High Grade (GII&GIII) | 54(36.0%) | 11.69±3.44 | |
| **Stage** | NMIBC (T1) | 27(18.0%) | 6.13±1.40 | <0.001** |
| | MIBC (T2, T3&T4) | 123(82.0%) | 10.05±3.37 | |
| **Follow up (80)** | No | 47(58.7%) | 7.51±2.33 | <0.001** |
| | RE | 33(41.3%) | 10.27±3.74 | |
| **Schistosomiasis association** | Negative | 30 (20.0%) | 8.08±2.82 | >0.05 |
| | Positive | 120 (80.0%) | 8.81±3.77 | |

SCC: Squamous cell carcinoma. TCC: Transitional cell carcinoma. LN: Lymph node. NMIBC: Non muscle invasive bladder cancer. MIBC: Muscle invasive bladder cancer RE: Recurrence rate. Size was represented as Median with Interquartile range (25% -75%), while the remaining parameters were represented as F (%) frequency and percent. * P. value <0.05 is significant, ** P. value <0.01 is highly significant.

**Table 5**
**Association between TP53 exons mutations and the studied parameters**

| Studied parameters | | Exons (2+3) mutations | | | | | Exon 4 mutations | | | | | Exon 5 mutations | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Wild N=96 | Mutant N=54 | P value | OR (95% C.I) | P. value | Wild N=54 | Mutan tN=96 | P value | OR(95% C.I) | P. value | Wild N=39 | Mutant N=111 | 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 | 63.0±7.6 | 64.8±7.0 | 0.1 | 1.04(0.99- 1.08) | 0.1 | 61.0±8.0 | 65.3±6.7 | 0.001** | 1.08(1.03- 1.14) | 0.001** |
| Sex | Female | 21(21.9%) | 12(22.2%) | 0.6 | 0.98(0.44- 2.19) | 0.9 | 6(11.1%) | 27(28.1%) | 0.01* | 0.32(0.12- 0.83) | 0.02* | 3(7.7%) | 30(27.0%) | 0.01* | 0.23(0.06- 0.79) | 0.02* |
| | Male | 75(78.1%) | 42(77.8%) | | | | 48(88.9%) | 69(71.9%) | | | | 36(92.3%) | 81(73.0%) | | | |
| Smoking | No | 39(40.6%) | 12(22.2%) | 0.02* | 2.39(1.12- 5.12) | 0.02* | 15(27.8%) | 36(37.5%) | 0.2 | 0.64(0.31- 1.32) | 0.3 | 9(23.1%) | 42(37.8%) | 0.07 | 0.49(0.21- 1.14) | 0.08 |
| | Yes | 57(59.4%) | 42(77.8%) | | | | 39(72.2%) | 60(62.5%) | | | | 30(76.9%) | 69(62.2%) | | | |
| Schistosomiasis association | Negative | 21(21.9%) | 9(16.7%) | 0.3 | 1.40(0.59- 3.32) | 0.4 | 15(27.8%) | 15(15.6%) | 0.06 | 2.08(0.92- 4.67) | 0.07 | 12(30.8%) | 18(16.2%) | 0.04* | 2.30(0.98- 5.36) | 0.05* |
| | Positive | 75(78.1%) | 45(83.3%) | | | | 39(72.2%) | 81(84.4%) | | | | 27(69.2%) | 93(83.8%) | | | |
| HCV | Negative | 57(59.4%) | 27(50.0%) | 0.2 | 1.46(0.75- 2.86) | 0.3 | 30(55.6%) | 54(56.3%) | 0.5 | 0.97(0.50- 1.90) | 0.6 | 21(53.8%) | 63(56.8%) | 0.4 | 0.89(0.43- 1.85) | 0.5 |
| | Positive | 39(40.6%) | 27(50.0%) | | | | 24(44.4%) | 42(43.8%) | | | | 18(46.2%) | 48(43.2%) | | | |
| Pathological diagnosis | SCC | 69(71.9%) | 15(27.8%) | 0.001** | 6.64(3.16- 13.97) | 0.001** | 39(72.2%) | 45(46.9%) | 0.001** | 2.95(1.44- 6.04) | 0.001** | 27(69.2%) | 57(51.4%) | 0.04* | 2.13(0.98- 4.63) | 0.05* |
| | TCC | 27(28.1%) | 39(72.2%) | | | | 15(27.8%) | 51(53.1%) | | | | 12(30.8%) | 54(48.6%) | | | |
| Papillary morphology (66) | Negative | 32(76.2%) | 20(83.3%) | 0.2 | 0.60(0.26- 1.41) | 0.2 | 16(66.7%) | 36(85.7%) | 0.01* | 0.37(0.17- 0.82) | 0.01* | 12(70.6%) | 40(81.6%) | 0.1 | 0.53(0.23- 1.20) | 0.2 |
| | Positive | 10(23.8%) | 4(16.7%) | | | | 8(33.3%) | 6(14.3%) | | | | 5(29.4%) | 9(18.4%) | | | |
| Tumour Number | Single | 42(43.8%) | 48(88.9%) | 0.001** | 0.10(0.04- 0.25) | 0.001** | 30(55.6%) | 60(62.5%) | 0.3 | 0.75(0.38- 1.48) | 0.4 | 15(38.5%) | 75(67.6%) | 0.001** | 0.30(0.14- 0.64) | 0.001** |
| | Multifocal | 54(56.3%) | 6(11.1%) | | | | 24(44.4%) | 36(37.5%) | | | | 24(61.5%) | 36(32.4%) | | | |
| Tumour Size (mm) | | 12.6(5.0- 21.9) | 2.0(1.5-22.0) | 0.01* | 0.96(0.94- 0.99) | 0.02* | 11.3(1.5 20.0) | 6.8(2.0- 26.3) | 0.7 | 1.00(0.98- 1.03) | 0.8 | 13.5(2.0- 20.0) | 6.0(2.0- 22.0) | 0.8 | 0.99(0.96- 1.01) | 0.3 |
| LN metastasis | Negative | 63(65.6%) | 9(16.7%) | 0.001** | 9.55(4.16- 21.90) | 0.001** | 45(83.3%) | 27(28.1%) | 0.001** | 12.78(5.50- 29.68) | 0.001** | 30(76.9%) | 42(37.8%) | 0.001** | 5.48(2.37- 12.66) | 0.001** |
| | Positive | 33(34.4%) | 45(83.3%) | | | | 9(16.7%) | 69(71.9%) | | | | 9(23.1%) | 69(62.2%) | | | |
| | GI | 15(15.6%) | 3(5.6%) | 0.01* | 0.20(0.06- 0.69) | 0.01* | 18(33.3%) | 0(0.0%) | 0.01* | - | - | 18(46.2%) | 0(0.0%) | 0.01* | - | - |
| Grade | GII | 78(81.3%) | 0(0.0%) | 0.01* | - | - | 33(61.1%) | 45(46.9%) | 0.1 | 1.36(0.87- 2.14) | 0.2 | 21(53.8%) | 57(51.4%) | 0.001** | 2.71(1.65- 4.48) | 0.001** |
| | GIII | 3(3.1%) | 51(94.4%) | 0.001** | 17.00(5.31- 54.47) | 0.001** | 3(5.6%) | 51(53.1%) | 0.001** | 17.00(5.31- 54.47) | 0.001** | 0(0.0%) | 54(48.6%) | 0.01* | - | - |
| Stage | T1 | 24(25.0%) | 3(5.6%) | 0.001** | 0.13(0.04- 0.42) | 0.001** | 24(44.4%) | 3(3.1%) | 0.001** | 0.13(0.04- 0.42) | 0.001** | 18(46.2%) | 9(8.1%) | 0.08 | 0.50(0.22- 1.11) | 0.09 |
| | T2 | 66(68.8%) | 30(55.6%) | 0.001** | 0.45(0.30- 0.70) | 0.001** | 24(44.4%) | 72(75.0%) | 0.001** | 3.00(1.89- 4.76) | 0.001** | 18(46.2%) | 78(70.3%) | 0.001** | 4.33(2.60- 7.23) | 0.001** |
| | T3 | 6(6.3%) | 18(33.3%) | 0.03* | 3.00(1.19- 7.56) | 0.02* | 6(11.1%) | 18(18.8%) | 0.01* | 3.00(1.19- 7.56) | 0.02* | 3(7.7%) | 21(18.9%) | 0.001** | 7.00(2.09- 23.47) | 0.001** |
| | T4 | 0(0.0%) | 3(5.6%) | 0.1 | - | - | 0(0.0%) | 3(3.1%) | 0.8 | - | - | 0(0.0%) | 3(2.7%) | 0.1 | - | - |
| Cytology | Negative | 81(84.4%) | 24(44.4%) | | | | 45(83.3%) | 60(62.5%) | | | | 33(84.6%) | 72(64.9%) | | | |
| | Positive HGUC | 15(15.6%) | 30(55.6%) | 0.001** | 6.75(3.13- 14.57) | 0.001** | 9(16.7%) | 36(37.5%) | 0.01* | 3.00(1.31- 6.86) | 0.02* | 6(15.4%) | 39(35.1%) | 0.01* | 2.98(1.15- 7.73) | 0.02* |
| CIS | Negative | 72(75.0%) | 48(88.9%) | 0.03* | 0.38(0.14- 0.99) | 0.04* | 48(88.9%) | 72(75.0%) | 0.03* | 2.67(1.01- 7.01) | 0.04* | 36(92.3%) | 84(75.7%) | 0.02* | 3.86(1.10- 13.53) | 0.03* |
| | Positive | 24(25.0%) | 6(11.1%) | | | | 6(11.1%) | 24(25.0%) | | | | 3(7.7%) | 27(24.3%) | | | |
| Follow up | No | 39(40.6%) | 48(88.9%) | 0.001** | 0.09(0.03- 0.22) | 0.001** | 24(44.4%) | 63(65.6%) | 0.01* | 0.42(0.21- 0.83) | 0.01* | 15(38.5%) | 72(64.9%) | 0.001** | 0.34(0.16- 0.72) | 0.001** |
| | RE | 57(59.4%) | 6(11.1%) | | | | 30(55.6%) | 33(34.4%) | | | | 24(61.5%) | 39(35.1%) | | | |

SCC: Squamous cell carcinoma. TCC: Transitional cell carcinoma. HGUC: High grade Urothelial Carcinoma. LN: Lymph nodes. CIS: Carcinoma in situ. RE: Recurrence rate. 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 X2 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.

## 4. DISCUSSION

High grade urothelial carcinoma (HGUC) is the ultimate target of the Paris system for reporting urine cytology (using PAP-stained cytology slides only) with high sensitivity, specificity, and negative predictive value"6-8"; thus, overcoming the problem of low urine cytology sensitivity and is prognostically useful in the prediction of tumor recurrence and progression 6,7, which also led to the identification of many biomarkers in the urine 29.

Regarding TP53 mutations, being the most frequently identified mutations in HGUC (including CIS) by NGS according to Sun et al., 202115, our results revealed that positive cytology for HGUC significantly exhibited mutations in TP53 exons 2+3, 4, 5 representing 36%, 64%, 74% respectively. Moreover, these mutations significantly correlated with high tumor grades (G II & III), high stages (T2 & T3), showed recurrence over shorter follow-up duration, and lymph node metastasis. To illustrate, the majority of our patients (64.0% and 74%) were highly significantly mutated in Exon 4 and Exon 5 respectively as compared to controls (P = 0.001), while less (36%) were mutated in exons (2+3) (P = 0.001). Moreover, among mutated patients, double exons mutations were identified in 32% of exons (2+3) and exon 4 together. In the same context, double exon mutations affecting exons (2+3) in combination with exon 5 were present in 34% of UC, while 78 patients (52%) were mutated in exon 4 and 5 together. In addition, triple exons mutations were also revealed in 48 patients (32%) affecting exons (2+3) &4 &5. Our results were similar to the NGS sequencing data of Sun et al., 202115 where most of TP53 mutations in HGUC were predominantly affecting exons 4 and 5 (with fewer mutations on exons 6 & 7 compared to exons 2+3 in our cases). Our findings also came in agreement with Al-Kashwan et al., 2012 30 as they reported TP53 mutations in 37.9%, among them, 7 patients (63.6%) showed single exon mutations and 3 (27.3%) had double exon mutations. In the same context, Noel et al., 2015 31 revealed functional TP53 mutations in 54% of their cases (56 analyzed tumors out of the 103).

On the other hand, Ecke et al., 2008 32 reported that the majority of TP53 mutations were found in exons 6-8 with only a few mutations (8%) in exon 5, in comparison to 74% in our study. Our data also contrasted Wallerand et al., 2005 33, as most of their cases harbored TP53 mutations in exons 4-9 with no mutations in exons 2, 3. Simultaneously, their study reported only 4 patients with double exons mutation (3.6%) in contrast to our results which indicated a higher number of patients (78 patients, 52%) had double exons mutations in exons 4 and 5 together. Not only that, we reported triple exons mutations affecting exons (2+3) &4 &5 combined in 48 patients (32%).

The univariate logistic regression analysis of the present study revealed that all the studied exons were statistically associated with UC and the prevalence of TP53 mutations in exons (2+3), 4, and 5 may be used as predictor and/or prognostic parameters for UC prospection, with sensitivity 36%, 64% and 74% for exons (2+3), 4 and 5 respectively and with 100% specificity, 100% positive predictive value for the 3 studied TP53 exons mutations, and negative predictive value of 34.2%, 48.1%, and 56.2% for exons (2+3), 4, and 5 respectively (Table 4). Coming along, our data illustrated a significant correlation between all the studied exons mutations and lymph node metastasis (P=0.001), which can give a prognostic insight into the metastatic perspective in patients with urothelial carcinomas. On the contrary, Noel et al., 2015 31 indicated much lower sensitivity (34%), and lower specificity (87%), with a lower positive predictive value (76%) and negative predictive value of 53% for TP53 mutations in urine cytology in comparison to our results.

Notably, our findings supported the overall significant correlation between HGUC in urine with TP53 mutations and high grade high stage tumors and the presence of CIS, particularly for exons 4 and 5 mutations. These findings matched Liao et al., 2021 14 results which stated that high stage UC obviously exhibited a higher level of TP53 mutations than the lower stage UCs. Similarly, our data were consistent with those of Shao et al., 202134 who reported that TP53 mutations were most frequent among UC patients with high tumor grade. Preferentially according to our results, mutations in exon 4 correlated more with G3 urothelial carcinomas, while exon 5 mutations correlated more with T3. In contrast, exons (2+3) mutations correlated more with lower tumor grades (Gi). The overall analysis provided strong support to the initial findings, which further confirmed the potential diagnostic role of TP53 mutations in advanced UC prognostication.

The overall UC recurrence in our study was (63/150, 42.0%). The clinical value of TP53 as a predictive marker for UC tumor recurrence and treatment selection is debatable 35. Ecke et al., 2008 32 reported a tumor recurrence frequency of 69.4% in patients with TP53 wild- type, and 88.5% in patients with TP53 mutation. Nevertheless, we settled this issue and statistically proved the clinical value of TP53 in UC as a predictive marker of both metastasis and recurrence. To elucidate, our data analysis using Log Rank Mantel-Coxfor tumor recurrence showed a significant correlation between TP53 mutational frequency in exons (2+3), 4 and tumor recurrence (P= 0.001, 0.008 respectively); however, no significant association was observed for exon 5 mutations (Table 7). Hence, TP53 exons (2+3) and 4 mutations can serve as independent predictors of tumor recurrence.

Moreover, our results demonstrated an abundance of TP53 double exons mutations, compared to the data published in previous studies. In the current study, 48

patients (32%) had mutations in exons (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. However, these results did not coincide with Erill et al., 2004 36 results in which only 2 cases displayed double exons mutations (out of 76 UC patients), similar to Ecke et al., 2008 32 who reported the presence of double exons mutations in five patients only. On the contrary, we also elucidated triple exons mutations which, together with the double exons mutations), were significantly correlated with lymph nodes metastasis. Nonetheless, our data did not reveal a significant association between TP53 mutational frequency and tumor recurrence in double and triple exons mutated patients. This can be understood since not all the mutant patients who were followed up have recurrences. It may be due to the contribution of other factors such as clinicopathological parameters. In this regard, it was noted that tumor recurrence was significantly associated with tumor multifocality, increased tumor size, presence of CIS, as well as Schistosoma infection (only in exon 5 mutations).

Notably, our results unveiled 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. To elaborate, our results revealed a larger number of missense mutations as C→G in 33 patients and T→G in 21 mutant UC in exons (2+3). This genomic instability may reflect the role of other factors in UC carcinogenesis such as smoking (77.8%) and bilharziasis (83.3%). Interestingly, a high frequency of missense mutation C→T (45/96, 46.8%) was found in mutant patients in exon 4, in addition to 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 37 reported a nucleotide substitution at
codon 110 G→A that led to amino acid change (Arginine→Histidine), while at the same codon and exon, Wallerand et al., 2005 33 reported a nucleotide substitution G→T, leading the Arginine to be changed to Leucine in mutations of exon 4. Furthermore, our data indicated that missense mutations were also the most prevalent type of TP53 mutations in exon 5 as A→C in 22 mutants, T→C in 19, and G→A in 57 mutants (98/111, 88.2%) as well as 13 silent mutations (T→C) at codon 180 (13/111, 11.7%), where Tyrosine is unaltered than reference. Interestingly, these silent mutations at codon 180 of exon 5 cached our attention due to Schlichtholz, 2004 38 detection of a missense mutation in which G→A led to amino acid change (Glutamic acid→Lysine).

Regarding RAGE protein expression in urinary bladder biopsies, the current immunohistochemical study showed an overall significant difference between groups of various urinary bladder lesions in the staining intensity and percentage of cellular expression (represented as RAGE H-score). To clarify, TCC and SCC exhibited statistically significant higher scores of RAGE expression contrasting cases of cystitis, including bilharzial cystitis cases, that mostly showed negative/weak focal cytoplasmic RAGE IHC staining with low h-scores of RAGE expression ($P<0.001$). These results came in agreement with Aboushousha et al, 2018 39 and Khorramdelazad et al, 2015 40 as the latter demonstrated high RAGE mRNA levels, increased encoding gene expression, and high RAGE proteins levels in tumor tissues in comparison to the normal bladder.

Interestingly, SCC showed a higher score of RAGE expression compared to both chronic cystitis and TCC with a statistically significant difference ($P<0.001$), which came similar to the findings of Pilzweger and Holdenrieder, 2015 25. Surprisingly however, despite SCC being commonly associated with endemic schistosomiasis in Egypt, there was no significant correlation between RAGE IHC expression and Schistosomal infection, thus simulating Aboushousha et al., 2018 findings 39. In addition, there was no significant correlation between the RAGE immunohistochemical expression score with the prevalent TP53 exons mutations among the studied UC cases. In the same context, despite higher RAGE expression scores in UC of non-papillary morphology, they were not statistically different from those with papillary morphology as reported by Aboushousha et al., 2018 39. Also consistent with the research of Hao Q et al., 2008 41 on cervical carcinomas, our study on urothelial carcinomas did not reveal a significant correlation between RAGE IHC expression with either tumor size or focality.

Importantly, our study showed that high grade and muscle invasive UCs significantly exhibited higher H-scores of RAGE expression compared to their lower grade lower stage counterparts ($P<0.001$), thus consistent with Aboushousha et al., 2018 39 and Kuniyasu et al., 2002 42 (however in gastric cancer). In this regard, Khormaldezad et al., 2015 40 suggested that the RAGE ligand-receptor axis plays a significant role in urinary bladder carcinoma's pathogenesis and progression.

Moreover, our study confirmed the significant correlation between RAGE IHC expression and urothelial carcinomas recurrence and lymph nodes metastasis, which is in keeping with Khormaldezad et al., 2015 40 test on the urinary bladder and Hao et al., 2008 41, however on cervical carcinomas.

Based on the above and to summarize, positive HGUC in urine cytology significantly correlated with TP53 exons 2+3, 4, and 5 mutations with the significant concordance with higher tumor grade, stage, recurrence, and lymph nodes metastasis in biopsies, particularly regarding TP53 exons 4 and 5 mutations. Parallelly, high grade high stage urothelial

carcinomas exhibited higher RAGE IHC expression scores in urinary bladder biopsies compared to cystitis, with a significant correlation with UC recurrence and lymph node metastasis.

To conclude, undoubtedly, recent advanced comprehensive multiplex molecular assays can accurately identify the distinguishing genetic aberrations in urine cytology (whether for diagnosis or follow-up); nevertheless, due to the lack of worldwide availability and their high cost, there is a necessary need to develop simpler cheaper, and more importantly reliable molecular, non-invasive tool for identification and follow up of urothelial carcinomas in our Egyptian population harboring TP53 mutations and RAGE aberrations. With special interest, TP53 mutations assessment in urine cytology is a promising tool for screening, follow-up, prognostication, and prediction of recurrence and metastasis in UC patients. Parallelly, RAGE IHC expression in UC biopsies could be suggested as a potential novel pathological screening for molecular-targeted urothelial carcinoma therapies and follow-up.

**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.

**Availability of data and materials:**

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

**Conflict of interests:**

No conflict of interests to declare by authors of the current work.

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## AUTHORS' CONTRIBUTIONS:

**S. M.:** Suggested the idea of the current work, was responsible for the major part of molecular biology techniques and writing of the manuscript.

**F. K.:** Shared in doing the molecular biology techniques.

**T. A.:** Responsible for the histopathological study of the research.

G. S.: Collection of data, tabulation of results

**K. E.:** Was responsible for clinical diagnosis of patients, doing the cystoscopic examination and collecting the biopsy samples of the study.

**M.M.:** Shared in idea suggestion, writing and the immunohistochemical study of the research.

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