TERT promoter mutations in Moroccan bladder cancer patients

Introduction

Bladder urothelial carcinoma (BUC) is the most common malignancy of the urinary system and is a fatal invasive malignancy. In the world, bladder cancer ranks 12th, with about 573,278 cases and 212,536 deaths in 20201). In Morocco, as reported by the regional cancer register of Casablanca, bladder cancer is a major public health problem with an estimation of 1540 new cases yearly and is affecting more men than women2). Overall, 75–80% of bladder cancer cases are classified as non-muscle-invasive bladder cancer (NMIBC), compromising stages Tis, Ta, and T1, and most of them have a high recurrence rate within five years and up to 20% progress to muscle-invasive bladder cancer (MIBC)3).

Worldwide, NMIBC detection and monitoring are mainly based on cystoscopy and urine cytology, showing many limitations. Cystoscopy is an invasive and expensive method and cannot accurately identify small or flat in situ carcinoma (CIS) lesions. Moreover, it is more difficult to detect and distinguish between benign reactive lesions and malignancy, particularly for patients undergoing transurethral Resection (TURBT) or intravesical BCG treatment4, 5). Urine cytology is a high specific microscopic evaluation widely used to detect bladder lesions but is poorly sensitive and depends on the pathologist’s interpretation6).

Original papers

Evaluation of TERT promoter mutations in tumor biopsies and urine sediment of Moroccan bladder cancer patients

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Abstract

Background: Human telomerase reverse transcriptase (hTERT) promoter mutations are common genetic events in bladder cancer (BC) and have been recognized as potential biomarkers for BC diagnosis and prognosis. Detection of TERT promoter mutations as urine-based tests has previously been reported to detect primary and recurrent BC. This study was planned to evaluate hTERT promoter mutations in both biopsies and urines from BC patients to assess the interest and the usefulness of introducing these biomarkers for better management of BC in Morocco.

Methods: In a cohort study involving a series of BC 70 patients with different stages and grades, hTERT promoter mutations were identified in both fresh biopsies and urine sediments by PCR amplification and DNA sequencing.

Results: Overall, hTERT promoter mutations were reported in 60% of cancer biopsies (42/70), the hotspot mutations C228T and C250T were respectively identified in 80.95% (34/42) and 16.67% (7/42) of positive cases. Other mutations: A161C and G149T were also reported and were obtained in 2.38% (1/42) and 2.38% (1/42), respectively. hTERT promoter mutations were identified in 30% of matched urine sediments (21/70) and showed an overall sensitivity of 50% and specificity of 100%. In patients with non-muscle invasive bladder cancer, no statistically significant association was detected between TERT promoter mutations and recurrence-free survival (HR: 1.224, 95% CI: 0.373–4.011, p = 0.739), and overall survival (HR: 0.363, 95% CI: 0.041–3.244, p = 0.364).

Conclusion: hTERT promoter mutations are early events occurring with high frequencies and can be identified in the exfoliated cells, making them an interesting non-invasive biomarker for diagnosis and follow-up of bladder cancer.

Keywords: TERT promoter region, Bladder cancer, Mutations, Non-invasive detection, Urine sediment

(Received November 1, 2021; Accepted January 5, 2022)
Recent advances in molecular biology have highlighted the presence of various genetic and epigenetic biomarkers that could be used in molecular diagnosis and prognosis of bladder cancer and therapeutic targets. Interestingly, these molecular biomarkers are usually assessed in clinical specimens and urine samples as a simple, non-invasive and effective method for early detection, diagnosis, and follow-up of bladder cancer patients.

In cancer cells, telomerase activation plays a crucial role in cellular immortalization and cancer development. In tumor cells, telomerase activation is promoted by genetic mutations in the gene promoter region. Currently, two recurrent mutations of TERT promoter gene (C228T and C250T) are the most detected mutations associated with telomerase dysregulation and are reported in several human cancers. In bladder cancer, TERT is mutated in approximately 60–85% of cases and are detected in all clinical stages and tumor grades, suggesting that these alterations are early events in bladder carcinogenesis and are considered as promising non-invasive urinary biomarkers for the detection and surveillance of bladder cancer. Recent publications highlight the interest in detecting hTERT gene mutations as a urinary-based biomarker and report a higher sensitivity and specificity in primary and recurrent bladder cancer patients, varying between 46.4% and 80.5% and from 73% to 96%, respectively.

In this context, the present study was planned to evaluate the presence of genetic mutations of hTERT gene promoter in bladder cancer cases and their association with cancer clinicopathological parameters to assess the interest and the usefulness of these biomarkers in the global management of bladder cancer in Morocco.

Materials and Methods

Study design and specimens collection

A total of 70 patients’ specimens from the Urology Department of the Mohammed V Military Hospital in Rabat-Morocco were collected between 2017 and 2019 with a retrospective and prospective follow-up between January 2015 and May 2021. For these patients, urine samples were obtained at the transurethral resection of bladder tumor (TUR-BT). The bladder tumor staging and grading were performed according to the TNM (tumor node metastasis) classification and World Health Organization (WHO)/International Society of Urologic Pathology criteria, respectively. hTERT mutational status was assessed in both bladder cancer biopsies and voided urine samples for patients with NMIBC (≤ PT1) and MIBC (> PT1).

The study protocol was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat – Morocco (Ref 82/19), and written informed consents were obtained from all recruited patients.

Genomic DNA extraction

Genomic DNA was extracted from fresh-frozen samples and urine cell sediments using the phenol/chloroform method. All extracted DNA was quantified with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). DNA samples were used immediately or stored at -20°C until use.

Mutation Detection

The mutational status was assessed by PCR – sequencing. A 193 bp fragment of TERT promoter was amplified using TERT-F (5’-CACCCGTCTGCCCCCTTCACCTT-3’) and TERT-R (5’-GGCCTTCACGTGCGCAGCAGGA-3’) primers. The amplification reaction was performed in a total volume of 25 µL, containing 10 µM of each consensus primer, 10 µM of each dNTP (dATP, dCTP, dGTP, and dUTP), 1.5 mM MgCl₂, 5 units Taq DNA polymerase per µL, and 2 µL of DNA sample in 1x Taq polymerase buffer. The mixture was first denatured at 95°C for 5 min. Then, thirty-five cycles of PCR were performed with denaturation at 94°C for 30 sec, primer annealing for 40 sec at 60°C, and primer extension for 40 sec at 72°C. At the end of the last cycle, the mixture was incubated at 72°C for 7 min. Negative control in which DNA template was omitted from the amplification mixture is included for every reaction.

Purification of PCR products was done using the ExS-Pure enzymatic PCR cleanup kit (Nimagen, The Netherlands) and was sequenced on an ABI 3130XL DNA analyzer using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequencing reactions were performed in a final volume of 10 µL, containing 1 µL of 2.5X Big Dye ready reaction mix v.3.1, 10 pmol of forward primer, and 100 ng of purified PCR product. The mixtures were incubated at 96°C for 1min, and 25 cycles were performed: denaturation at 96°C for 10 sec, primer annealing at 50°C for 5 sec and extension at 60°C for 4 min. Sequencing reaction products were finally purified using Sephadex G-50 gel-exclusion chromatography (GE Healthcare Life Sciences). Obtained sequences were then matched with the gene reference sequence collected from the GenBank database (NCBI Reference Sequence: NG_009265.1). The sequence alignments were performed using the Clustal W program in BioEdit Software.

Statistical analysis

All statistical analyses were carried out using SPSS for Windows, version 23 (SPSS Inc, Chicago). The correlation between mutation status of hTERT gene promoter and clinical-histopathological parameters (tumor’s stage, grade, age of patients, smoking status, and recurrence/
progression) was evaluated by \( \chi^2 \), and \( p \) values < 0.05 were interpreted as statistically significant.

Univariate and multivariate Cox regression models were performed to compare variables and outcomes. Evaluation of the association between TERT promoter mutations / clinicopathological factors, Overall Survival (OS), and Recurrence-Free Survival (RFS) was performed by univariate cox regression to estimate the hazard ratio and \( p \)-values of each covariate. Recurrence-free survival was calculated from the first treatment assignment to the date of first recurrence or the last follow-up visit. Progression-free survival was calculated from the first treatment assignment to the date of the first progression detected, the date of death, or the last follow-up visit. Overall survival was defined as the interval from the date of diagnostic to the date of death from any cause or last follow-up.

A multivariate analysis was performed if the \( p \)-value of one or more variables was less than <0.05. Survival curves were performed by the Kaplan-Meier method, and the follow-up period was defined from the date of first TUR-BT treatment to the moment of death for deceased cases or the date of the last follow-up for survivors.

### Results

#### Characteristics of the study population

Clinico-pathological characteristics of the 70 recruited patients are reported in Table 1. Overall, 68 patients were male with a sex ratio of 34. The mean age of the patients was 67, with extreme ages at 47 and 85 years. Moreover, 40% of patients were smokers. Clinico-pathological characteristics of patients showed that 74.29% of cases were staged \( \leq \) PT1 (52/70), and 61.43% had high tumor grades (43/70). Among NMIBC cases, 23.08% have recurred (12/52), and 9.62% have progressed upon relapse (5/52).

#### Evaluation of TERT promoter mutational status

All bladder cancer specimens have shown successful amplification and direct sequencing of the hTERT promoter fragment. An example of a nucleotide sequence of hTERT promoter, showing C228T and C250T mutations, is reported in Fig. 1. Overall, mutations in the hTERT promoter were reported in 60% of the bladder cancer specimens (42/70), C228T mutation prevailed and was obtained in 80.95% of positive cases (34/42). Other mutations: C250T, A161C, and G149T were also reported and were obtained in 16.67% (7/42), 2.38% (1/42), and 2.38% (1/42), respectively. Of particular interest, 1 case haro-

| Parameter          | Total | Percentage | Parameter          | Total | Percentage |
|--------------------|-------|------------|--------------------|-------|------------|
| Sex                |       |            | Tumour stage       |       |            |
| Male               | 68    | 97.14      | \( \leq \) PT1     | 52    | 74.29      |
| Female             | 2     | 2.86       | \( >\)PT1          | 18    | 25.71      |
| Age                |       |            | Tumour grade       |       |            |
| < 50               | 1     | 1.43       | Low grade          | 27    | 38.57      |
| 50–70              | 44    | 62.86      | High grade         | 43    | 61.43      |
| > 70               | 25    | 35.71      | Tumour recurrence  |       |            |
| Yes                | 12    | 23.08      | Yes                | 12    | 23.08      |
| No                 | 40    | 76.92      | No                 | 40    | 76.92      |
| Smoker             |       |            | Tumour progression |       |            |
| Yes                | 28    | 40         | Yes                | 5     | 9.62       |
| No                 | 42    | 60         | No                 | 47    | 90.38      |

Fig. 1 Representative sequencing electropherograms of the human TERT promoter with mutations C228T (A) and C250T (B).
boured both C228T and C250T mutations.

The correlation between TERT promoter mutations and patients’ characteristics is summarized in Table 2. No significant association was obtained between the mutational profile and patients’ age, gender, and smoking status ($p > 0.05$). The mutations frequencies were similar in early and advanced stages and high and low grades. The distribution of TERT mutations according to the cancer evolution was assessed in the 52 NMIBC cases and showed that TERT mutations were found in both cases with and without recurrence ($p = 0.647$). Among the 5 patients showing progressive tumors, TERT C228T mutation was reported in 1 case (20%), whereas TERT mutations were reported in 63.83% of cases with no progressive tumors. However, the difference was not statistically significant due to the very low number of cases with progressive tumors ($p = 0.083$).

**Patients outcomes**

The results of univariate and multivariate Cox regression analysis are reported in Table 3. In the univariate analysis, TERT mutations and clinicopathological features do not significantly influence the Recurrence-Free Surivals ($p > 0.05$).

The univariate analysis also indicated that TERT mutations, age, tumors’ grade, and stage do not significantly influence overall survival ($p > 0.05$).

These results are confirmed by the Kaplan-Meier sur-

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**Table 2** Distribution of TERT gene promoter mutations according to clinicopathological parameters

| Parameter          | N   | Overall Mutations in hTERT |
|--------------------|-----|---------------------------|
|                    |     | +  | %  | -  | %  | $p$  |
| Gender             |     |    |    |    |    |      |
| Male               | 68  | 41 | 60.29 | 27 | 39.71 | 0.751 |
| Female             | 2   | 1  | 50.00 | 1  | 50.00 |      |
| Age                |     |    |    |    |    |      |
| < 50               | 1   | 1  | 100.00 | 0 | 0.00 | 0.00  |
| 50–70              | 44  | 28 | 63.64 | 16 | 36.36 | 0.154 |
| > 70               | 25  | 13 | 52.00 | 12 | 48.00 |      |
| Smoking            |     |    |    |    |    |      |
| Yes                | 28  | 18 | 64.29 | 10 | 35.71 | 0.941 |
| No                 | 42  | 24 | 57.14 | 18 | 42.86 |      |
| Stages             |     |    |    |    |    |      |
| ≤ PT1 (NMIBC)     | 52  | 31 | 59.62 | 21 | 40.38 | 0.860 |
| > PT1 (MIBC)      | 18  | 11 | 61.11 | 7  | 38.89 |      |
| Grades             |     |    |    |    |    |      |
| LG                 | 27  | 15 | 55.56 | 12 | 44.44 | 0.538 |
| HG                 | 43  | 27 | 62.79 | 16 | 37.21 |      |
| Tumour recurrence  |     |    |    |    |    |      |
| Yes                | 12  | 7  | 58.33 | 5  | 41.67 | 0.647 |
| No                 | 40  | 24 | 60.00 | 16 | 40.00 |      |
| Tumour progression |     |    |    |    |    |      |
| Yes                | 5   | 1  | 20.00 | 4  | 80.00 | 0.083 |
| No                 | 47  | 30 | 63.83 | 17 | 36.17 |      |

**Table 3** Univariable Cox regression results for the association of clinical-pathological parameter with overall survival (OS) and recurrence-free survival (RFS) among NMIBC patients

| Overall Survival | Recurrence-free survival |
|------------------|--------------------------|
| HR (95% CI)      | $p$ value                |
| HR (95% CI)      | $p$ value                |

| TERT Mutation     | Overall Survival | p value | Recurrence-free survival | p value |
|-------------------|------------------|---------|--------------------------|---------|
| Wild type         | 1.00             | 0.364   | 1.00                     | 1.224   |
| Mutant            | 0.363 (0.041–3.244) | 0.0041–3.244 | 1.00 | 0.363 (0.041–3.244) | 1.00 |
| Gender            | Overall Survival | p value | Recurrence-free survival | p value |
| Male              | 8.919 (0.978–81.369) | 0.052 | 0.047 (0.000–135528.454) | 0.687 |
| Female            | 1.00             | 1.00    | 1.00                     | 1.00    |
| Age               | Overall Survival | p value | Recurrence-free survival | p value |
| ≥ 70              | 1.00             | 0.119   | 2.040 (0.552–7.543)      | 0.285   |
| < 70              | 0.175 (0.20–1.565) | 1.00    | 2.040 (0.552–7.543)      | 0.285   |
| Stages            | Overall Survival | p value | Recurrence-free survival | p value |
| ≤ PT1 (NMIBC)    | 1.00             | 0.035   | 1.00                     | 1.00    |
| > PT1 (MIBC)     | 0.318 (0.35–2.842) | 0.035   | 1.00                     | 0.035   |
| Grades            | Overall Survival | p value | Recurrence-free survival | p value |
| LG                | 1.00             | 0.18    | 1.00                     | 0.474   |
| HG                | 0.223 (0.025–1.998) | 1.00    | 1.00                     | 0.474   |
vival curves estimating survival rates for all cases with TERT promoter wild-type (WT) and mutant sequences and with respect to clinicopathological features (Fig. 2). Indeed, a borderline significance was obtained with age ($p = 0.078$), and no significant association was reported between TERT promoter mutations and the remaining clinicopathological features, including tumors stage, tumors grade, and Overall Survival ($p > 0.05$).

### Table 4 Frequency of mutations detected in biopsies and urine for different stages and grades types

| Parameter | N   | Overall Mutations in hTERT | Overall Mutations in hTERT |
|-----------|-----|----------------------------|----------------------------|
|           |     | +  | %  | p   | +  | %  | p   |
| **Stages**|     |    |    |     |    |    |     |
| ≤ PT1 (NMIBC) | 52  | 31 | 59.62 | 0.860 | 16 | 30.77 |
| > PT1 (MIBC)   | 18  | 11 | 61.11 |        | 15 | 27.78 |
| **Grades**     |     |    |    |     |    |    |     |
| LG             | 27  | 15 | 55.56 | 0.538 | 7  | 25.93 |
| HG             | 43  | 27 | 62.79 |        | 14 | 32.56 |

Sensitivity of Urinary test [95% CI] 50% [34.2%–65.8%]
Specificity of Urinary test [95% CI] 100%

### Evaluation of TERT promoter mutations in matched urine sediments

In this study, TERT Promoter mutational status was also assessed in matched urine sediments from all the 70 recruited patients, and the results are summarised in Table 4. TERT mutations were obtained in 30% of specimens (21/70). TERT mutations were detected in urines from both high and low grades cases and both cases with early and advanced stages, with no statistically signifi-
significant difference ($p > 0.05$).

Comparison between TERT promoter mutations in bladder biopsies and paired urine sediments was also assessed and is reported in Table 4. All cases showing TERT promoter mutations in urine sediments have mutations of TERT promoter in the corresponding DNA from bladder biopsies. Meanwhile, among the 42 TERT mutations harboring cases, only 21 were detected in urine samples. Accordingly, the specificity and sensitivity of detecting TERT promoter mutations in urine sediments was 100% and 50%, respectively.

**Discussion**

The main problem clinicians face in bladder cancer diagnosis is the low sensitivity of cytology and high invasiveness of cystoscopy, the two most widely used tools for cancer diagnosis and follow-up. Recent advances in molecular oncology highlighted the presence of biomarkers of particular interest in diagnosis, prognosis, and follow-up. In this field, genetic events, and DNA mutations, in particular, are cancer hallmarks that show promise as sensitive and specific molecular markers for urological cancers. In bladder cancer, the TERT gene, particularly the promoter region, is widely reported to harbor many point mutations associated with cancer development. The main goal of this study was to assess the prevalence of mutations in the promoter region of the TERT gene in Moroccan cases with bladder cancer in tumor biopsies and voided urine samples, enabling their potential use as biomarkers as an alternative strategy to cystoscopy and urine cytology for early detection, patient monitoring, and disease prognosis.

Overall, 60% of patients with bladder cancer had a mutation in the TERT promoter region. These results are in agreement with widely reported data showing that TERT is the most frequently mutated gene in bladder cancer. The two widely reported mutations considered to be associated with bladder cancer development, C228T and C250T, were reported as hotspot mutations in our study. C228T was found in 80.95% and C250T in 16.67% of bladder cancer cases. Other 2 point mutations, A161C and G149T, considered rare events, were obtained in 1 case each. Similar results were reported by Huang et al., that have identified C228T and C250T mutations in 39.7% (116/292) and 14.4% (42/292) of bladder cancer cases, respectively. Interestingly, they have also identified 2 patients with A161C and G149T mutations.

No significant difference was found between TERT promoter mutations and clinicopathological features, grade, and stage in our study. This result is shared by many authors suggesting that point mutations in TERT promoter region are early events in the cancer development and suggesting their interesting use as biomarkers for the detection and monitoring of bladder cancer.

In this study, the presence of mutations was not related to overall and recurrence-free survivals of bladder cancer. These results are supported by the findings of Allory et al., showing that overall survival was not associated with TERT promoter mutations. However, contrary results were reported elsewhere, showing that TERT promoter mutations significantly decreased overall survival and recurrence-free survival. Our results were also confirmed by the Kaplan Meier curve, and cox regression model performed on the 52 cases with NMIBC.

The Cox regression model revealed an independent association of recurrence-free survival with TERT promoter mutations, age at diagnosis, grade, and stage. In this study, TERT promoter mutational status was also evaluated in voided urine samples. Mutations in exfoliated cells were detected with a specificity of 100% but a sensitivity of 50%. Higher sensitivity ranging from 80.5% to 81.3% was already reported. The difference could be due to the cell sampling, preparation, DNA/RNA extraction, and protocol used for point mutations identification. Indeed, several analytical approaches are used for detecting mutations in TERT promoters. The difference could also be due to the rate of cancer exfoliated cells that could be recovered from the urine. The small urine volume may affect the number of exfoliated cells, and consequently, the quantity of DNA extracted from the malignant cells, causing the lack of detecting mutations in the urine sample. In line with this, Zuiverloon and colleagues have reported that the sensitivity of this test based on detection of mutations in the FGFR3 gene in urine increases with the volume of the urine sample. Of particular interest, the number of exfoliated cells depends on the tumor grade and stage. In this study, only 62.79% of recruited cases were diagnosed with high grades. Kinde et al. have reported a sensitivity of 79% on exfoliated cells recovered from 86.7% of HG cases.

It is currently widely accepted that TERT promoter mutations are interesting biomarkers for bladder cancer diagnosis and follow-up, but recent investigations showed that telomerase inhibition is a promising therapeutic target. The discovery of some inhibitors, antisense oligonucleotides, and some immunotherapeutics are under clinical trials for many cancer, including bladder cancer, to increase the therapeutic arsenal for better cancer management.

This study is very informative and clearly showed that mutations in TERT promoter region are an interesting biomarker that could be used for early diagnosis for better management of bladder cancer. However, the main limitations of the study are: (1) the small sampling size, especially the low number of NMIBC patients not allowing to accurately estimate the association between TERT promoter mutations and cancer recurrence; (2) The very
low number of women patients; (3) The follow-up period is short, causing a missing recurrence and progression data for newly collected cases.

**Conclusion**

hTERT promoter mutations are early events occurring with high frequencies and can be identified in the exfoliated cells, making them an interesting non-invasive biomarker for diagnosis and follow-up of bladder cancer. A large study on a high number of cases will be of great interest to accurately assess the usefulness of this biomarker in bladder cancer management.

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