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Exploring urine sediments as a non-invasive method for DNA methylation detection in bladder cancer

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Abstract

Background: The main epigenetic event occurring during the bladder carcinogenesis process is DNA methylation, affecting genes involved in various metabolic pathways and cell regulation. The use of biological fluids such as urine sediments could be used as a non-invasive approach to enhance bladder cancer management. In this study, we aim to determine the promoter methylation status of a panel of genes in bladder cancer on tumor biopsies and urine sediments to evaluate the usefulness of urine samples as a non-invasive approach for methylation status assessment.

Methods: Using the methylation-specific PCR technique, we explored the promoter methylation status of *hTERT*, *TWIST1*, *VIM* and *NID2* genes in 40 tumor biopsies and their paired urine samples from Moroccan bladder cancer patients.

Results: In this study, bladder tumors showed promoter hypermethylation frequency of individual genes as 90%, 85%, 62.5% and 72.5% in *TWIST1*, *hTERT*, *NID2* and *VIM* genes, respectively.

Interestingly, the specificity of methylation detection in urine samples was 100% and the sensitivity to detect hypermethylation of *TWIST1*, *hTERT*, *NID2* and *VIM* genes reached 91.7%; 97.1%; 84% and 82.8%, respectively.

Conclusions: Our results clearly show that the assessment of promoter hypermethylation in urine samples is highly specific and has high sensitivity. Furthermore, urine sediments would be a useful approach to detect the DNA methylation status of genes and its potential association with bladder cancer development.

Keywords: DNA methylation, Non-invasive test, Bladder cancer, Urine sediments

1 Background

Worldwide bladder cancer (BC) is the tenth most commonly diagnosed cancer, with approximately 570,000 new cases per year and more than 210,000 deaths annually [1].

Currently, BC modalities for diagnosis and follow-up are cystoscopy and urine cytology; Cystoscopy is highly

sensitive for most tumors but it may fail to identify smaller and flat tumors such as in situ carcinoma. Moreover, it is an expensive and an invasive procedure that shows insufficient power to predict precisely the patient outcome. Conversely, urinary cytology is a noninvasive and highly specific modality, but it has a poor sensitivity for low-grade and well-differentiated lesions [2]. Therefore, other non-invasive, rapid and accurate alternatives with high sensitivity and specificity are needed to improve the BC management.

In the recent years, extensive efforts are made to identify a molecular signature of BC to improve patients'

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treatments and prognosis prediction. In this context, many epigenetic changes have been reported to correlate with cancer malignancy and progression. The main epigenetic event occurring during the bladder carcinogenesis process is DNA methylation; which affects genes involved in various metabolic pathways and cells regulation [3]. Furthermore, the use of biological fluids such as urine sediments has attracted much attention as a non-invasive method of bladder cancer detection and surveillance.

Accordingly, several studies have shown that urine fluid is a reliable matrix to detect molecular alterations in all stages and grades of bladder cancer [4], and could be used as a non-invasive approach to identify a molecular signature for enhancing bladder cancer management [5]. In this optic, the present study was planned to evaluate the promoter methylation status of a panel of genes known for their involvement in bladder carcinogenesis, in tumor biopsies and their paired urine sediments to assess the usefulness of urine samples as a non-invasive approach of promoters' methylation detection and bladder cancer management.

2 Methods

2.1 Specimens

A total of 40 fresh frozen tumor samples were obtained by transurethral resection of the bladder or cystoscopy at the Urology Department of University Military Hospital in Rabat, Morocco and paired urine samples were obtained before surgery from every patient. The protocol of the study was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat – Morocco (Ref 82/19) and written informed consent was obtained from each recruited patient.

2.2 Methylation specific PCR (MSP)

Tissue specimens were immediately stored at -80°C . The voided urines (50 mL fresh urines) were spun down by centrifugation at 4500 g for 15 min, and the pelleted

urine sediments were washed twice with phosphate buffered saline (PBS) and stored at -80°C . Genomic DNA was extracted from fresh tumor biopsies and urine cell sediments using phenol/chloroform method [6]. Then, 500 ng of genomic DNA extracted from each sample was used for bisulfite modification as previously described [7]. The Sodium bisulfite modification and DNA purification were carried out by the EZ DNA Methylation Kit (Zymo Research, USA). The modified DNA was subject to methylation-specific PCR (MSP) using methylation-specific and unmethylation-specific primers of a panel of genes "TWIST1, hTERT, VIM and NID2" [8–11]. The amplification reaction was carried out in a final volume of 25 μl containing 2 μl of modified DNA, 1X PCR buffer, 1.5 mM MgCl_2 , 10 μM of each dNTP, 10 μM of each primer and 1U Platinum Taq Polymerase (Invitrogen, USA). Mixtures were first denatured at 95°C for 10 min. Then, 40 cycles of PCR were performed with denaturation at 95°C for 30 s, primer annealing for 45 s at the corresponding temperature (Table 1) and primer extension for 45 s at 72°C . At the end of the cycles, mixtures incubation was carried out at 72°C for 10 min. A negative control, in which DNA was omitted from the amplification mix, was included in each reaction. PCR products were analyzed by electrophoresis on 2% agarose gel followed by staining with ethidium bromide (10 mg/ml). The sensitivity reflects the fraction of patients in which the urine DNA was methylated among cases with methylation in matched tumor DNA of the same gene promoter. The specificity reflects the number of methylated tumor biopsies among cases showing methylation in matched urine DNA of the same gene promoter.

2.3 Statistical analysis

The hypermethylation status of genes promoters in tumor biopsies and urine sediments was statistically evaluated using percentages and confidence intervals (CI) of 95% by IBM SPSS software version 23.

Table 1 Genes primers and corresponding annealing temperatures for MSP

Gene		Forward (5' → 3')	Reverse (5' → 3')	Product size (pb)	Tm (°C)
TWIST1	M	CCGTCGCCTTCTCCGACGA	TGCGTTAGGGTTCGGGGCGT	100	64
	U	TTGGATGGGGTTGTTATTGT	CCTAACCCAAACAACCAACC	79	63
hTERT	M	GAGGTATTTCCGGAGGTTTCGC	ACTCCGAACACCACGAATACCG	121	58
	U	GGGAGGTATTTGGGAGGTTTTGT'	CAAACCTCCAAACACCACAATACCA	126	58
NID2	M	TAGTATTGGTAACGACGATAGTATC	AAATTCGAAACTAACGCGACACG	141	58
	U	TAGTATTGGTAATGATGATAGTATT	AAATTCAAAATAACACAACACA	141	55
VIM	M	TTATAAAAATAGCGTTTTCCGGC	ATAACGCGAACTAACTCCCG	143	59
	U	GGGTTATAAAAATAGTGTTTTTGGT	ACAATAACACAACTAACTCCCA	149	57

Table 2 Tumor characteristics of recruited cases

Parameter	Total cases	Percentage (%)
<i>Gender</i>		
Male	39	97.5
Female	1	2.5
<i>Age</i>		
≤ 50	1	2.5
50–70	24	60
> 70	15	37.5
<i>Smoking</i>		
Yes	22	55
No	18	45
<i>Tumor stage</i>		
≤ PT1	30	75
> PT1	10	25
<i>Tumor grade</i>		
Low grade	14	35
High grade	26	65

Table 3 Comparison between methylation status in bladder biopsies and voided urine sediments

Genes	Hypermethylation detection in fresh tumor biopsies		Hypermethylation detection in urine sediments		Sensitivity % (95% CI)
	N	% [95% CI]	N	% [95% CI]	
<i>TWIST1</i>	36	90 [76.3–97.2]	33	82.5 [67.2–92.7]	91.7 [77.5–98.2]
<i>hTERT</i>	34	85 [70.2–94.3]	33	82.5 [67.2–92.7]	97.1 [84.7–99.9]
<i>NID2</i>	25	62.5 [45.8–77.3]	21	52.5 [36.1–68.5]	84 [63.9–95.5]
<i>VIM</i>	29	72.5 [56.1–85.4]	24	60 [43.3–75.1]	82.8 [64.2–94.2]

CI = confidence interval

3 Results

Clinico-pathological characteristics of tumor specimens are summarized in Table 2. The mean age of patients was 67 years, ranging from 47 to 84 years. The most recruited cases were staged ≤ PT1 and have high tumor grade (Table 2).

Promoter's methylation was assessed in tumor biopsies and matched urine sediments from all patients and results are summarized in Table 3. Globally, DNA hypermethylation was found in all bladder tumor biopsies in at least one of the studied genes. Comparison between hypermethylation of the studied genes in bladder biopsies and paired urine sediments, showed that all cases with hypermethylation in DNA from urine sediments have an aberrant methylation in the corresponding DNA from bladder biopsies; suggesting a specificity of methylation detection in urine sediments of 100%.

In this study, the sensitivity of hypermethylation detection in urine sediments was different according to the studied gene. It was higher for *hTERT* 97.1% [95% CI = 77.5–98.2] and lower for *VIM* gene 82.8% [95% CI = 64.2–94.2] (Table 3).

4 Discussion

Recent advances in oncology research report that molecular signature of tumors is a key factor for cancer stratification, personalized therapy and a reliable prognosis [12]. Molecular characterization of tumors is mainly based on the assessment of genetic alterations leading to genetic activation/inactivation and cellular pathways deregulation.

During last decade, a great interest was given to DNA methylation changes as the main epigenetic event associated with cancer development and progression [13]. In bladder cancer, DNA hypermethylation have been used as targets for detection and diagnosis of tumor cells in clinical specimens such as tissue biopsies and urine sediments [4].

In this study, we have used MSP assay to assess the promoter methylation status of a panel of genes in both tumor biopsies and urine sediments DNA from Moroccan patients with bladder cancer. The interest was focused on, *TWIST1*, *hTERT*, *NID2* and *VIM* genes for their implication in many cellular pathways and their widely reported data as highly methylated genes in bladder cancer [14–17].

In this study, bladder tumors showed promoter hypermethylation frequency of individual genes as 90%, 85%, 62.5% and 72.5% in *TWIST1*, *hTERT*, *NID2* and *VIM* genes, respectively.

These findings are in good agreement with already reported data on Moroccan patients [17]. Of interest, medium frequency of *TERT* (53%) and high frequency of *TWIST1* (98%) promoters' methylation in BC were reported by Leão et al. [14] and Yegin et al. [16], respectively.

The methylation study of each gene promoter was extended to the matched urine sediment DNA for all studied patients. Overall, the promoter hypermethylation for at least one gene was detected in 39 analyzed samples; thereby the detection coverage of methylation in urine reached 97.5% [95% CI = 86.8–99.9]. Interestingly, the sensitivity of *TWIST1* and *TERT* promoters' hypermethylation detection in urine samples was higher; reaching 91.7% and 97.1%, respectively. These results are supported by Yegin et al. (2013) and Vinci et al. (2011), who showed that *TWIST1* and *TERT* hypermethylation have high sensitivities (87.5% and 76%, respectively) and specificities (93.3% and 100%, respectively) for bladder cancer detection in urine sediments [16, 18]. Similar

results were reported by Renard et al. (2010) that have identified high sensitivity and specificity (90% and 93%, respectively) of *TWIST1* methylation detection in urine sediments [19].

In this study, sensitivity of detecting *NID2* and *VIM* hypermethylation in urine sediments was 84% and 82.8%, respectively. These results are slightly different from those reported by Yegin et al. (2013) and Costa et al. (2010), showing higher sensitivity frequencies (95.8% and 96%, respectively) in urinary bladder cancer patients [16, 20]. This difference could be due to the smaller cohort in our study. Scientific evidences have shown that the urine DNA is presumably shed from the original primary tumor or neoplastic lesions in the bladder wall, and consequently genetic and epigenetic alterations in urine sediments will reflect the whole situation in the bladder tumors' tissues. Moreover, DNA methylation assessment in urine sediments has much significant potential added value for clinicians and patients: (1) a non-invasive approach; (2) reducing the number of cystoscopies required for the diagnosis and surveillance of bladder cancer and (3) saving time and budget.

In this study, of particular interest, a specificity of 100% was obtained for the all studied genes. Indeed, the methylation was only detected in those patients whose tumor tissue also showed gene promoter methylation, and consequently no false-positive result was detected, with 100% specificity for all the studied genes. These results are of a great interest and highlighted the interest of using urine sediments for biomarkers' assessment.

This study is very informative and clearly highlights the usefulness of urine sediment as a valuable and non-invasive matrix for epigenetic studies. However, there were several limitations due to the use of the MSP technique which identifies only the qualitative level of DNA methylation and to the small scale of our study cohort. Further studies on a large population are necessary to confirm the obtained results.

5 Conclusions

Our investigation clearly showed that promoters' hypermethylation assessment in non-invasive urine samples is highly specific, shows a great sensitivity, and would be a useful approach for studying epigenetic alterations and their potential association with bladder cancer development.

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Author contributions

ME performed the experiments, analyzed the data and wrote the paper. HE analyzed the data and contributed to experiments and paper writing. CHA, IC and LB contributed to experiments and paper writing. MT, IH and MB provided patient samples and follow up. MO, AA and AA provided intellectual contributions and revised the manuscript. ME provided intellectual contribution critically revised the manuscript. MA supervised all the experiments, critically revised the manuscript and gave final approval to the publication. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

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 consent was obtained from each recruited patient.

Consent for publication

I declare that all participants have given their consent for publication.

Competing interests

The authors declare that they have no competing interests.

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