DNA Hypermethylation of a Panel of Genes as an Urinary Biomarker for Bladder Cancer Diagnosis

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Purpose: Several studies have shown frequent changes in DNA methylation in bladder cancer (BCa), which vary among different geographical areas. The aim of this study is to examine the diagnostic accuracy of a panel of DNA methylation biomarkers in a Greek clinical setting contributing to the development of a universal panel of urine biomarkers.

Materials and Methods: Individuals with primary BCa and control individuals matching the gender, age and smoking status of the cancer patients were recruited. DNA methylation was assessed for the gene promoters of RASSF1, RARB, DAPK, TERT and APC in urine samples collected by spontaneous urination using quantitative Methylation Specific PCR (qMSP). All genes had been previously separately associated with BCa.

Results: Fifty patients and 35 healthy controls were recruited, with average age of 70.26 years and average smoking status of 44.78 pack-years. In the BCa group, DNA methylation was detected in 27 (61.4%) samples. RASSF1 was methylated in 52.2% of samples. Only 3 (13.6%) samples from the control group were methylated, all in the RASSF1 gene promoter. The specificity and sensitivity of this panel of genes to diagnose BCa was 86% and 61% respectively. The RASSF1 gene could diagnose BCa with specificity 86.4% and sensitivity 52.3%.

Conclusion: Promoter DNA methylation of this panel of five genes could be further investigated as urine biomarker for the diagnosis of BCa. The RASSF1 could be a single candidate biomarker for predicting BCa patients versus controls. Studies are required in order to develop a geographically adjusted diagnostic biomarker for BCa.

Keywords: urinary bladder neoplasms; DNA methylation; RASSF1; urine biomarker

INTRODUCTION

At present, the gold standard for bladder cancer (BCa) diagnosis is cystoscopy and urine cytology. Cystoscopy is invasive, cost-intensive, has an 85-90% sensitivity and involves a low risk of urine tract infection, hematuria and suboptimal compliance with management recommendations[(1)]. Urine cytology is non-invasive, has low sensitivity in low-grade tumors (16%) and a variable interpretation among pathologists[(2)]. Several urinary-based BCa biomarker tests have been developed to improve the detection of BCa including the UroVysion (sensitivity 72%, specificity 72%), ImmonoCyt (sensitivity 67-86%, specificity 75-79%), BTAstat (sensitivity 58-71%, specificity 73%), BTA-track (sensitivity 69-71%, specificity 66-90%), NMP22 (sensitivity 71-73%, specificity 73-78%), but are mostly lacking randomized controlled trials to establish their efficacy[(3)].

The application of epigenetics may allow for a risk-adapted approach and more cost-effective method of diagnosis of BCa. Numerous epigenetic changes such as DNA methylation, histone modifications, microRNA expression and nucleosome positioning are characteristic of the epigenome of BCa cells[(4,5,6)]. In urine samples, DNA methylation in RASSF1 (Ras association domain family member 1), DAPK (Death associated protein kinase), RARB (retinoic acid receptor, beta), TERT (telomerase reverse transcriptase) and APC (APC regulator of WNT signaling pathway) gene promoters has been strongly associated with BCa [(7,8,9)]. RASSF1 promoter methylation is significantly higher in both BCa tissue, compared to adjacent macroscopically non-cancerous bladder tissue, and in urine samples of BCa patients compared to healthy controls [(10)]. Similarly, hypermethylation of DAPK promoter is almost six times more frequent in BCa patients than in healthy individuals (OR: 5.81; 95%CI: 3.83-8.82, $P < .00001$)[(11)]. Finally, the hypermethylation of TERT leads to upregulated activity of the enzyme resulting in cancer cells' immortalization [(9)]. To date, no single diagnostic biomarker could replace cystoscopy as the primary diagnostic tool for

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Table 1. Demographic characteristics of patients and controls

	Patients	Controls	*p*-value
N	44	22	< . 05
Age, year; mean ± SD (range)	70.78 ± 9.6 (46 – 88)	69,05 ± 10,9 (50-86)	< .05
Smoking status, pack years; median/ mean ± SD (range)	45, IQR 64 (0 – 168)	61,7 ± 44,5 (0-168)	< . 05
pTaLg	22 (50%)		
pTaHg	3 (7%)		
pT1Lg	2 (4%)		
pT1Hg	10 (23%)		
CIS	2 (5%)		
pT2	5 (11%)		

BCa[12]. As, additionally, geographical and ethnic differences in methylation patterns exist[13], a more potent panel of diagnostic biomarkers may be in demand.

The aim of this study was to explore the diagnostic potential of a panel of five hypermethylated gene promoters, whose sensitivity and specificity have been proven when studied in a separate fashion. Ideally, this would lead to the development of a DNA methylation-based diagnostic protocol in urine samples and optimization of its sensitivity and specificity.

MATERIALS AND METHODS

Study design – participants

This was a prospective, case-control study conducted in the Urology Department of a public teaching Hospital. The Hospital's Review Board and the local University Bioethics Committee approved the study protocol. The study has been registered in the Australian New Zealand Clinical Trials Registry (registration reference: ACTRN12620000258954). Urine samples were collected following written informed consent from patients who consecutively attended the cystoscopy clinics, from June 2014 till December 2016. Recruits were subjects with non-muscle invasive or muscle invasive BCa who were able to provide a urine sample and undergo a cystoscopy before any treatment for BCa. Control subjects matching the gender, age and smoking status of the BCa patients were recruited among healthy volunteers with no known urological disease or malignancy (**Table 1**). Individuals with metastasis in urinary bladder or other malignancies were excluded.

Eighty-five subjects were recruited, 50 patients and 35 healthy controls, with an average age of 70.26 years and average smoking status 44.78 pack-years. All individuals were Caucasian. Eighty-four percent of patients were diagnosed with non-muscle invasive BCa (57% Ta and 27% T1 grade), 11% with muscle invasive cancer and 5% with carcinoma in situ (CIS)(**Table 1**).

Sample collection

Urine samples (approximately 50 mL) were prospectively collected before scheduled cystoscopy or any specific treatment in sterile container with urine preservative (NORGEN BIOTEK CORP., Thorold, Canada), in a blinded fashion and were stored according to manufactures' instructions for maximum two years at room temperature until DNA extraction. No sample was first morning urine. Patients with positive urine culture were excluded from the study as well as all otherwise healthy individuals but with urinary tract symptoms.

DNA extraction and treatment

DNA was extracted from urine sediments using the Cells and Tissue DNA Isolation Kit (NORGEN BIOTEK CORP., Thorold, Canada). Both integrity and purity were confirmed via spectrophotometry and agarose gel electrophoresis. Extracted DNA was stored at -40°C until the modification with sodium bisulfite using the EZ DNA Methylation-GoldKit (Zymo Research, Orange, CA). Modified DNA was then stored at -20°C until further analysis.

Gene promoter methylation assay

Quantification of the percentage of methylation of DNA in the gene promoter of DAPK[14], APC[15], RAR-B2 [15], RASSF1[14] and TERT[14] was performed with Luna Universal Probe qPCR Master Mix (New England Biolabs, Massachusetts, USA), according to the manufacturer's instructions and performed on Applied Biosystems

Table 2. The sequences of primers and probes for the quantitative methylation specific real-time PCR

Gene	Primer/ Probe	Sequence
APC[13]	Forward	5'-GAACCAAAACGCTCCCCAT-3'
	Reverse	5'-TTATATGTCGGTTACGTGCGTTTATAT-3'
	Probe	5'-/56-FAM/CCCGTCGAA/ZEN/AACCCGCCGATTA/31ABkFQ/3'
DAPK[12]	Forward	5'-TCGTCGTCGTTTCGGTTAGTT-3'
	Reverse	5'-TCCCTCCGAAACGCTATCG-3'
	Probe	5'-/56-FAM/CGACCATAA/ZEN/ACGCCAACGCCG/31ABkFQ/3'
RARB[13]	Forward	5'-GGGATTAGAATTTTTTATGCGAGTTGT-3'
	Reverse	5'-TACCCCGACGATACCCAAAC-3'
	Probe	5'-/56-FAM/TGTCGAGAA/ZEN/CGCGAGCGATTCG/31ABkFQ/3'
RASSF1[12]	Forward	5'-ATTGAGTTGCGGGAGTTGGT-3'
	Reverse	5'-ACACGCTCCAACCGAATACG-3'
	Probe	5'-/56-FAM/CCCTTCCA/ZEN/ACGCGCCA/31ABkFQ/3'
TERT[12]	Forward	5'-GGATTCGCGGGTATAGACGTT-3'
	Reverse	5'-CGAAATCCGCGCGAAA-3'
	Probe	5'-/56-FAM/CCCAATCCC/ZEN/TCCGCCACGTAAAA/31ABkFQ/3'
ACTB [12]	Forward	5'-TGGTGATGGAGGAGGTTTAGTAAGT-3'
	Reverse	5'-AACCAATAAAACCTACTCCTCCCTTAA-3'
	Probe	5'-/56-FAM/ACCACCACC/ZEN/CAACACACAATAACAAACACA/31ABkFQ/3'

Table 3. The two multiple logistic models with the same predictors but with different estimation methods:

Table 3a. Model 1, using ML logistic regression

Odds Ratio Estimates using ML				
Effect	**Point Estimate**	**95% Wald Confidence Limits**		**Two-sided *P*-value**
RAR_B2	> 999.999	< .001	> 999.999	.9577
RASSF1	4.098	.976	17.202	.0540
APC	> 999.999	< .001	> 999.999	.9496

Table 3b. Model 2, using exact logistic regression

Exact Odds Ratios					
Parameter	**Estimate**		**95% Confidence Limits**		**Two-sided *P*-Value**
RAR_B2	1.056	*	.056	Infinity	.9730
RASSF1	3.987			.855	26.001 .0874
APC	2.937	*	.450	Infinity	.3655

* indicates a median unbiased estimate.

StepOnePlus Real Time PCR System (Thermo Fisher Scientific, Inc.). 30 ng of modified DNA were used in each reaction. The cycling conditions were as follows: 95˚C for 1 min, then 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec. The primers and probe were designed to specifically amplify the bisulphite-converted promoter of the gene of interest and their sequences are listed in Table 2. Positive and negative controls were used and the methylation status of the genes was calculated by the StepOne™ and StepOnePlus™ Software v2.0 software. No further sequencing of the samples was conducted since the method was specific enough. All primers were synthesized by IDT (Integrated DNA Technologies, Iowa, United States). In order to quantify and compare the amplification products, Cq data corresponding to the target genes were normalized relative to those of the internal housekeeping gene, actin beta (ACTB)[14]. Furthermore, a standard 100% methylated control human DNA and a 100% non-methylated control human DNA were used (EpiTect PCR Control DNA set, Qiagen, Germany. Methylation specific quantitative PCR (MSP-qPCR) was run in duplicate.

All laboratory methods and analyses were performed at the Laboratory of Biological Chemistry of the Medical School, Aristotle University of Thessaloniki.

Statistical Analysis

Sample size calculation. Assuming that the percentage of DNA methylation among controls is 20%[10], and the desired OR will be 4, then the total sample size needed would be 78 subjects (39 patients and 39 controls) in order to achieve 80% power with alpha set at 5% (for each of the 5 biomarkers) based on Pearson Chi-Square test for two proportions. Since this was a pilot study, no correction for multiple testing was made.

Statistical tests. Descriptive statistics, univariate analysis, Shapiro Wilk normality test, Mann-Whitney for comparison of two independent non-parametric samples as well as multivariate and exact logistic regression were used for all variables with meaningful number of data points between patients and controls. ROC analysis was performed.

RESULTS

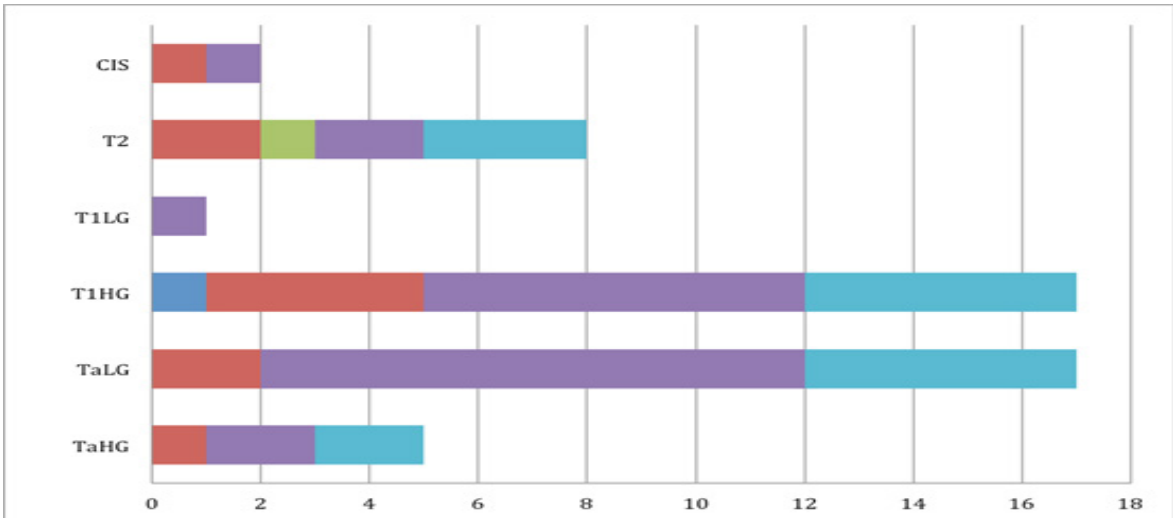
DNA was successfully extracted from 66 urine samples; 44 patients and 22 healthy controls. DNA was found to be methylated in 27 (61.4%) patient samples as opposed to only 3 of 22 (13.6%) control-samples ($P < .001$). RASSF1 was hypermethylated in 52.2% of patients followed by APC (34%), RARB (22.7%), DAPK (2.2%) and TERT (2.2%). The only gene promoter that was methylated in controls was RASSF1.

The gene promoters were hypermethylated in 57% of individuals with non-muscle invasive BCa; in particular, 24% of them had one, 16% two, 14% three, 3% four and none five hypermethylated gene promoters. By comparison, 80% of subjects with muscle invasive tumor had hypermethylated gene promoters: 20% one, 40% two and 20% three genes respectively. According to the grade classification of tumors, the gene promoters were hypermethylated only in 50% of the patients with low-grade urothelial cancer (one in 29%, two in 17% and three in 4%) while up to 72% of patients with high grade or CIS had hypermethylated gene promoters (one in 17%, two in 22%, three in 28% and four in 5%)(Figure 1). The hypermethylation was not significantly different between patients with muscle and non-muscle invasive BCa as well as between patients with high-grade and low-grade ($p = .369$ and $p = .148$, respectively). The gene promoters of DAPK and TERT were hypermethylated in one patient each.

According to our statistical analysis the specificity and sensitivity of this diagnostic panel of biomarkers were 86.4% and 61.4%, respectively, while the positive and negative predictive values were estimated at 90% and 53%, respectively. The area under the curve (AUC = .76) derived from a multivariate logistic regression model (**Table 3**). The diagnostic panel was considered positive when it had at least one methylated promoter. A multivariate logistic regression model with RASSF1, APC and RAR-B2 as predictors estimated an area under

Table 4. The cut-off, sensitivity, specificity and AUC (95% CI), p-value for all markers and logistic models

	Specificity	**Sensitivity**	**AUC Area**	**95% CI**	***P*-value**	**Cut-off**
Diagnostic panel	86.4	61.4	0.76	.67-.85	< .0001	at least one methylated
DAPK	100	2.3	0.51	.49-.53	.3173	methylated
RAR-B2	100	22.7	0.61	.55-.67	.0004	methylated
TERT	100	2.3	0.51	.49-.53	.3173	methylated
RASSF1	86.4	52.3	0.69	.59-.79	.0003	methylated
APC	100	34.1	.67	.59-.74	< .0001	methylated
Multiple logistic regression model of APC, RAR-B2, RASSF1	86.4	61.4	.76	.67-.85	< .0001	predicted probability = .78



	TaHG	TaLG	T1HG	T1LG	T2	CIS
DAPK	0	0	1	0	0	0
RAR-B2	1	2	4	0	2	1
TERT	0	0	0	0	1	0
RASS-1	2	10	7	1	2	1
APC	2	5	5	0	3	0

Figure 1. The frequency and the type of the hypermethylated genes among the different histological types of BCa

the curve (AUC) of .76. (**Table 4 and Figure 2**).
Due to the high correlation observed, only RASSF1, APC and RAR-B2 promoters remained in the model; however, APC and RAR-B2 had very unstable estimates (Quasi-complete separation of data points was detected). Thus, further analysis using exact logistic regression was performed in order to explore the multicollinearity effects (**Tables 3a, b**). As a result, RASSF1 gene promoter could be a single candidate for predicting patients versus controls with specificity 86.4% and sensitivity 52.3%. The odds ratio (OR) and AUC estimates for the diagnostic biomarker RASSF1 are shown in **Tables 3 and 4**.
Further analysis was performed for the diagnostic accuracy of the current panel for MIBC or high-grade tumors. Based on the 5-year recorded prevalence of BCa in the Greek population[16] and the known prevalence of 20-30% for MIBC or high-grade tumors among firstly diagnosed BCa patients, we calculated that the current panel had a positive predictive value of .21 and a negative predictive value of .86, with .86 specificity and .72 sensitivity.

DISCUSSION

In this pilot, controlled study the gene promoters of individuals with BCa were more commonly hypermethylated compared to healthy controls. The panel of genes tested was found to have 86.4% specificity and 61.4% sensitivity in the diagnosis of BCa, quite similar to the specificity and sensitivity of the RASSF1 promoter gene alone (86.4% and 52.3% respectively with OR 6.9).
Despite the relatively small sample size, the study sample reflects the disease's demographics. In general, non-muscle invasive cancer (NMIBC) can be found in 70-80% of all BCa and only 10-30% constitute muscle invasive BCa (MIBC)[17]. In our sample 84% of the patients had NMIBC and 11% MIBC.
Regarding the diagnostic potential of the hypermethylated gene promoters' panel of our study, the sensitivity (61%) is lower compared to the cystoscopy's sensitivity for all kinds of BCa (68-83%) but higher compared to cytology, particularly for low-grade tumors (50%)[18]. Furthermore, the specificity is considerably higher than the cytology's specificity for patients with low-grade cancer and comparable to the specificity of the invasive cystoscopy[4]. However, the diagnostic accuracy of the methylation of the panel of Twist Family BHLH Transcription Factor 1 (TWIST1) and Nidogen 2 (NID2) genes as urine biomarker was higher than the diagnostic accuracy of our panel of genes, with 90% sensitivity and 93% specificity[19]. Similarly, a meta-analysis and systematic review of 24 articles revealed that the overall sensitivity and specificity of DNA methylation urine biomarkers was 84% and 92% respectively, higher than our results[20]. Studies by Zhang et al. and van der Heijden et al. achieved higher AUC (.894 and .874 respectively) compared to our study's AUC (.7634) [21,22]. However, Zhang et al. investigated the diagnostic potential of a panel of seven gene promoters in a non-Caucasian (Chinese) population, while van der Heijden et al. were focused on monitoring BCa and not on diagnosis.
But since DNA methylation varies among different human groups regarding macro- and micro-geographical scales, numerous studies from different areas are required in order to investigate the methylation profile of the patients with BCa across human populations[13,23]. This may lead to a common panel of gene promoters that could be used worldwide to differentiate the BCa from healthy subjects but, in addition, a more individual approach may be necessary depending on subjects'

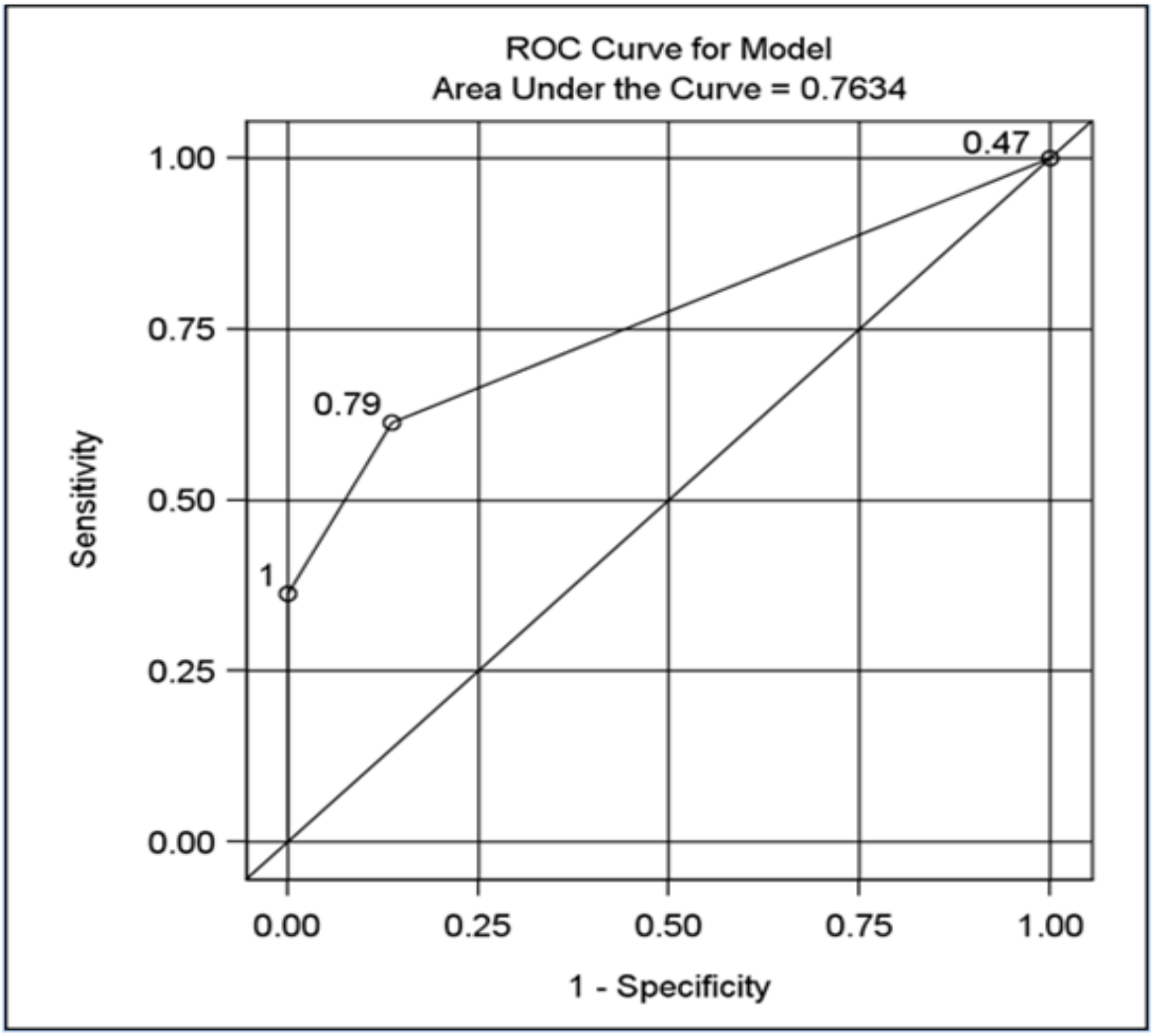


Figure 2. ROC curve and AUC for the gene panel.

residency.

Our results also indicate that the hypermethylation of suppressor gene promoter of RASSF1 might be a potential single urine biomarker in BCa with specificity 86.4%, sensitivity 52.3% and OR 6.9. By contrast, a previous study which investigated the diagnostic accuracy of RASSF1 in BCa, showed lower specificity and sensitivity of RASSF1 than in our study (17% and 58% respectively), whereas a recent meta-analysis revealed that the risk for BCa in those individuals who have hypermethylated RASSF1 promoter in urine samples was 95% CI:9.25-42.45, OR = 19.82; [10,24]. This risk was found to be higher among Mixed-race individuals (95% CI: 8.39 - 65.05, OR = 23.36;) and Asians (95% CI: 15.01 - 38.69, OR = 24.10) and lower for Caucasians (95% CI:6.47 - 30.25, OR = 13.99) (all $P < .0001$). RASSF1 can constitute a fairly unique diagnostic biomarker for BCa since methylation of RASSF1 is rarely detected in normal bladder tissue[25]. Functional analysis of RASSF1 shows a potential involvement in inhibition of cell proliferation, promoting cell apoptosis and aging and the maintenance of microtubule stability. It is also known that the expression of RASSF1 is absent in many tumor cells as a consequence of methylation of gene promoter[25]. However, Chen et al. found that the use of a panel of genes had higher diagnostic accuracy compared to the use of a single gene promoter[19].

In our study population, the gene promoter of DAPK was hypermethylated only in one patient contrary to a meta-analysis concluding that DAPK promoter methylation was associated with BCa risk (95% CI = 3.83-8.82, OR:5.81, $P < .00001$)[11]. Similarly, the gene promoter of TERT was methylated in only one subject with BCa; a recent study by a research group suggests that THOR (TERT Hypermethylated Oncological Region) hypermethylation is associated with disease progression and increased TERT expression, which leads to carcinogenesis[9]. To date, there are no published studies to confirm whether our results reflect a low prevalence of TERT and DAPK promoter in the Greek population.

An attempt was made to explore the diagnostic accuracy of our panel of genes for MIBC or high-grade tumors. We found a high negative predictive value of .86, with .86 specificity and .72 sensitivity Previous literature has sparsely investigated the value of methylation biomarkers in the diagnosis of MIBC or high-grade tumors. In a recent study, molecular analysis of the methylation profile of the promoters of p14ARF, p16INK4A, RASS-

F1A, DAPK and APC from urine sediments demonstrated correlations with BCa grade and stage[7], while other researchers found that the progression to MIBC in patients with primary pTaG1/2 BCa could be predicted with the methylation analysis of the gene promoters TBX2 and TBX3[26].

Finally, 13.6% of our study controls had methylated the RASSF1 gene promoter, in accordance with a previous survey, which showed that 12% of the loci in apparently normal urothelium from bladders with cancer were hypermethylated, indicating an epigenetic field defect [27].

In the control group, we detected DNA in 63% of the urine samples, which can be explained by the decreased cell exfoliation of normal urothelium[28]. Furthermore it has been shown that the procedure for collection of urine sediments can be influenced by the co-sedimentation of normal cells and the presence of crystals and substances that may inhibit downstream PCR analyses [29].

Finally, our study was adequately powered and achieved an OR[6.9] higher than the initially desired OR = 4. The attained sample size of the control group was almost half of the initially planned. This might have had an effect on the genes with nonsignificant results especially for APC where methylation was 34% as opposed to 13.6% of the control samples.

Cost-effectiveness is always an issue with novel technologies. The detection of hypermethylation of specific genes from urine samples has been previously shown to be cost-effective in the diagnosis of BCa[20,30]. When using our panel of genes, the real costs were significantly lower than cystoscopy costs in the Greek National Healthcare System (89 Euro vs. 230 Euro). However, the cost-effectiveness needs to be examined in light of the diagnostic accuracy of our panel of genes which remains to be proven in larger longitudinal case-control studies. Future studies could explore the diagnostic potential of our panel of genes in different geographical areas at a national level, additionally testing the role of this gene promoter panel in blood samples of BCa patients, in order to obtain a circulating liquid biopsy setting.

The limitations of our study are the small sample size and a lower diagnostic accuracy compared to some previous studies but as it is already mentioned, DNA methylation varies among different human populations and therefore external validation of these findings with larger prospective studies is mandatory. Additionally, clinical comorbidities or concurrent use of medications potentially able to alter urine composition were not investigated for associations with DNA methylation status of our gene panel. However, none of our study patients was treated with chemotherapeutic drugs targeting epigenetic modifications before urine sample collection and all individuals with previous or other current malignancies were excluded. Patients with urolithiasis were also excluded as well as all otherwise healthy individuals but with urinary tract symptoms.

CONCLUSIONS

Results of this study suggest that methylation of the proposed panel of genes could be a promising urine biomarker for the diagnosis of BCa, but this needs to be confirmed with validation studies within different human populations in order to develop a “universal” or “generic” test that can detect, in principle, any BCa. The methylation of RASSF1 gene promoter itself could be a potential single urine biomarker. It would be intriguing to verify in the future whether DNA hypermethylation of this five-promoter gene panel can correlate with the pharmacological response to drugs conventionally used in BCa treatment.

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CONFLICT OF INTEREST

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