

Three Epigenetic Biomarkers, *GDF15*, *TMEFF2*, and *VIM*, Accurately Predict Bladder Cancer from DNA-Based Analyses of Urine Samples

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Abstract

Purpose: To identify a panel of epigenetic biomarkers for accurate bladder cancer (BlCa) detection in urine sediments.

Experimental Design: Gene expression microarray analysis of BlCa cell lines treated with 5-aza-2'-deoxycytidine and trichostatin A as well as 26 tissue samples was used to identify a list of novel methylation candidates for BlCa. Methylation levels of candidate genes were quantified in 4 BlCa cell lines, 50 BlCa tissues, 20 normal bladder mucosas (NBM), and urine sediments from 51 BlCa patients and 20 healthy donors, 19 renal cancer patients, and 20 prostate cancer patients. Receiver operator characteristic curve analysis was used to assess the diagnostic performance of the gene panel.

Results: *GDF15*, *HSPA2*, *TMEFF2*, and *VIM* were identified as epigenetic biomarkers for BlCa. The methylation levels were significantly higher in BlCa tissues than in NBM ($P < 0.001$) and the cancer specificity was retained in urine sediments ($P < 0.001$). A methylation panel comprising *GDF15*, *TMEFF2*, and *VIM* correctly identified BlCa tissues with 100% sensitivity and specificity. In urine samples, the panel achieved a sensitivity of 94% and specificity of 100% and an area under the curve of 0.975. The gene panel could discriminate BlCa from both healthy individuals and renal or prostate cancer patients (sensitivity, 94%; specificity, 90%).

Conclusions: By using a genome-wide approach, we have identified a biomarker panel that allows for early and accurate noninvasive detection of BlCa using urine samples. *Clin Cancer Res*; 16(23); 5842–51. ©2010 AACR.

Bladder cancer (BlCa) is one of the leading causes of cancer-related morbidity and mortality. Global estimates for 2002 indicate that approximately 357,000 BlCa cases were diagnosed and that approximately 145,000 patients succumbed to the disease (1). In the United States, BlCa is

the second most common genitourinary malignancy, with about 71,000 new cases and more than 14,000 deaths in 2009 (2). The incidence of BlCa increases with age, with an average age at the time of diagnosis in the 60s, and it is 3 times more common in men than in women (3). Although several risk factors (e.g., smoking habits and exposure to carcinogens) have been identified, effective strategies for early detection are still not available (4).

The present gold standard strategy for BlCa diagnosis is noninvasive, voided urine cytology, followed by cystoscopic examination. However, both methods have low sensitivity, especially for low-grade tumors (3). Several BlCa markers were recently revised by Vrooman and Witjes (5), but they have concluded that the analysis of those markers is costly and did not improve diagnostics compared with routine urinary cytology. Additional tests have been developed during the last few years; however, their clinical usefulness has yet to be established using large clinical trials (5, 6).

Urothelial carcinoma comprises the most common form of BlCa, 70% of which present as papillary nonmuscle-invasive tumors, although as many as 50% to 70% of these tumors (pTa and pT1, classified according to AJCC/UICC; ref. 7) will recur and approximately 10% to 20% will progress to invasive disease (3). To predict which patients

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Translational Relevance

Our study identified a 3-gene panel of epigenetic biomarkers that accurately detect bladder cancer (BlCa) in urine sediments and discriminate it from prostate cancer and renal epithelial tumors. This panel might be easily applied for routine screening of BlCa in voided urine samples.

will progress from superficial to invasive disease remains a challenge. Patients with a diagnosis of early-stage BlCa undergo frequent monitoring, currently based on cystoscopy and cytology, resulting in BlCa becoming one of the most costly cancer diseases to manage (8). The development of innovative, noninvasive tests for early detection of BlCa is essential to lower the morbidity and mortality associated with BlCa, and such tests will also be economically beneficial.

The alteration of DNA methylation pattern in CpG islands is emerging as a key event in transcriptionally repressed regions of the genome. Aberrant promoter methylation at several gene loci has been reported for BlCa, and some of them have been shown to be associated with cancer development, stage, recurrence, progression, and survival (9). Because of the high prevalence of cancer-related gene promoter hypermethylation, several BlCa epigenetic biomarkers have been identified in urine for the purposes of disease detection and monitoring (10). Although most of these markers are promising from exploratory studies, many of them fail to show comparable results in independent studies and a great majority of them are reported in various cancers and thus are not specific for BlCa (11). Consequently, there is a need to identify improved epigenetic biomarkers, which might enable a more reliable and accurate detection of the disease as well as for predicting the prognosis of BlCa patients.

We have used a genome-wide strategy (12) to identify novel epigenetic biomarkers in BlCa and followed a strict pipeline to pinpoint and validate those most suitable for noninvasive, urine-based diagnostic testing.

Materials and Methods

A flow chart depicting the different steps followed in this study is provided in Supplementary Figure 1.

Cancer cell lines

Cell lines representative of bladder (5637, J82, SCaBER, and TCCSUP), renal cell (786-O, ACHN, Caki-1, and Caki-2), and prostate (22Rv1, DU 145, LNCaP, and PC-3) cancer were obtained from the American Type Culture Collection. All cell lines were cultured according to the manufacturer's specifications, with 10% fetal bovine serum (Gibco, Invitrogen) and antibiotics (100 units/mL of penicillin G and

100 µg/mL of streptomycin; Gibco), in a humidified atmosphere of 5% CO₂ at 37°C.

All BlCa cell lines were subjected to treatment with a combination of the demethylating drug 5-aza-2'-deoxycytidine (1 µmol/L for 72 hours) and the histone deacetylase inhibitor trichostatin A (0.5 µmol/L added in the last 12 hours). In parallel, the same cell lines were cultured without treatment for 72 hours and were harvested before confluency.

Patients and tumor sample collection

The 50 BlCa samples that were included in this study were obtained from a consecutive series of patients diagnosed and treated between 2005 and 2006 at the Portuguese Oncology Institute – Porto, Porto, Portugal. Tumor tissues were collected after transurethral resection or radical cystectomy. A small tumor sample was immediately snap-frozen, stored at –80°C, and subsequently cut in cryostat for DNA and RNA extraction. The bulk material was routinely processed for routine pathologic examination allowing for tumor classification and WHO/ISUP grading (13, 14). An independent set of 20 NBMs from BlCa-free individuals (prostate cancer patients submitted to radical prostatectomy) was used as controls. Relevant clinical data was collected from patient's clinical records (see Supplementary Table S1). Patients were enrolled following informed consent. These studies were approved by the institutional review board of the Portuguese Oncology Institute – Porto.

Urine sample collection and processing

Morning voided urine samples (1 sample per patient) were collected from 51 patients with a diagnosis of BlCa and treated between 2004 and 2006 at the Portuguese Oncology Institute – Porto, from 19 patients with renal cell tumor and from 20 patients with prostate cancer. Controls were randomly chosen among healthy donors with no personal or family history of cancer. Relevant demographic data are provided in Supplementary Table S2. Patients and controls were enrolled after informed consent. Urine storage and processing conditions were standardized: each sample was immediately centrifuged at 4,000 rpm for 10 minutes; the pelleted urine sediment was then washed twice with phosphate-buffered saline, and stored at –80°C.

Isolation of nucleic acids

DNA was extracted from the frozen urine cell pellets and treated and untreated cancer cell lines, using a standard phenol–chloroform procedure. Total RNA from cancer cell lines was isolated using Trizol (Invitrogen). From tissue samples, DNA and total RNA were extracted using the AllPrep DNA/RNA Mini kit (Qiagen Inc.). DNA and RNA concentrations were determined using an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies), and the RNA quality was measured in a 2100 bioanalyzer (Agilent Technologies).

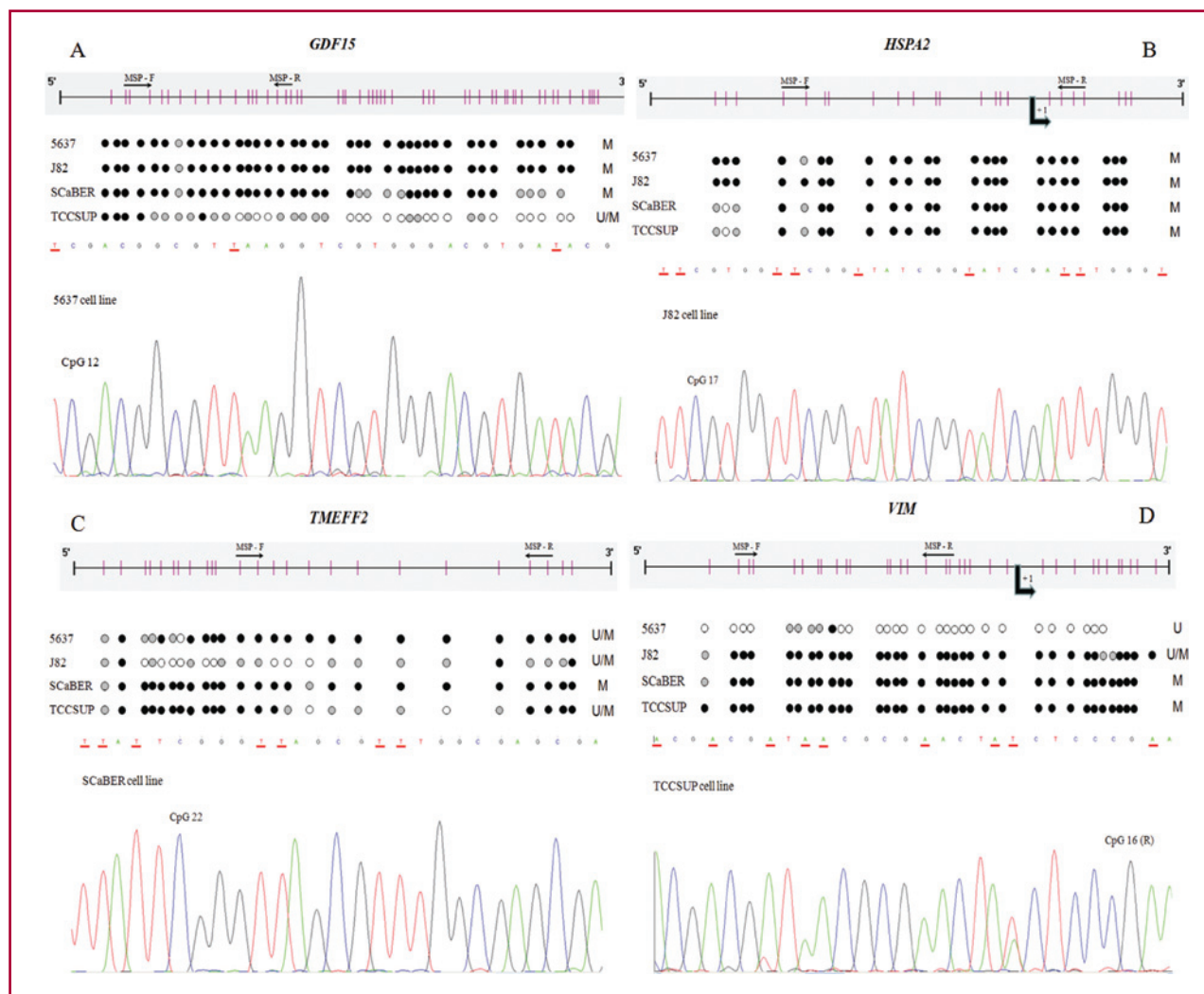


Fig. 1. Characterization of the methylation status of individual CpG dinucleotides by bisulfite sequencing of the *GDF15* (A), *HSPA2* (B), *TMEFF2* (C), and *VIM* (D). Top, schematic representation of the CpG island in the area of the transcription start (+1). Vertical bars indicate the location of individual CpG sites and the 2 arrows indicate a location of MSP primers. Middle, unfilled circles represent unmethylated CpGs, black filled circles represent methylated CpGs, and gray filled circles represent partially methylated sites. The column of U, M, and U/M, at the right side, lists the methylation status of the cell line from MSP analysis of the corresponding gene. Bottom, section of the bisulfite sequence electropherogram, where cytosines in CpG sites are in blue and cytosines that have been converted to thymines are underlined in red.

Gene expression microarrays

Treated and untreated BlCa cell lines were analyzed with the Applied Biosystems Human Genome Survey Microarray (P/N 4337467), which contains 31,700 60-mer oligonucleotide probes representing 27,868 individual human genes. Digoxigenin-UTP-labeled cRNA was generated and amplified from 1.5 µg of total RNA from each sample, using Applied Biosystems chemiluminescent RT-IVT labeling kit (P/N 4365716) according to the manufacturer's protocol. Array hybridization was carried out for 16 hours at 55°C, using 10 µg of the labeled cRNA. Chemiluminescence detection, image acquisition, and analysis were carried out using an Applied Biosystems Chemiluminescence Detection kit (P/N 4342142) and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (P/N 4338036)

following the manufacturer's protocol. Images were auto-gridded and the chemiluminescent signals were quantified, background subtracted, and spot- and spatially normalized using the above-mentioned Microarray Analyzer software. All samples were postprocessed and normalized with the R-script "ABarray" and Bioconductor. Normalized postprocessed data had a selected cutoff of 25% relatively to the array signal.

Microarray analysis of bladder carcinomas (*n* = 21) and NBM samples (*n* = 5) was carried out in parallel. The relative gene expression in tumor samples was calculated using the median value of expression of the normal tissues.

Arrays elements, upregulated more than 4-fold after 5-aza-2'-deoxycytidine and trichostatin A treatment in at least 2 of 4 BlCa cell lines and simultaneously downregulated in

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tumor samples compared with normal tissue, were considered to be potential targets for DNA methylation.

CpG island search, bisulfite treatment, and methylation-specific PCR

The resulting top 100 target genes from the microarray approach were analyzed for the presence of promoter CpG islands. We retrieved the RefSeqs, from the UCSC Genome browser database,¹ including 1000-bp upstream and 500-bp downstream of the transcription start point. Presence of promoter CpG islands was determined using default settings in the CpG Island Searcher software (15). For CpG island-containing genes, MSP primers specific to methylated and unmethylated sequences were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems). Primer sequences are summarized in Supplementary Table S3 (16), along with amplicon lengths, positions, and PCR conditions. Prior to methylation-specific PCR (MSP) analyses, DNA samples were bisulfite modified. Tissue samples were treated with the EpiTect bisulfite kit (Qiagen Inc.), and bladder and urine samples were treated with the EZ DNA methylation Gold kit (Zymo Research). The modified DNA was eluted in 60 μ L of water and then stored at -80°C .

The promoter methylation status of the first 20 to 25 CpG island-containing target genes was analyzed in BICa cell lines. Genes methylated in all 4 BICa cell lines were also analyzed in renal and prostate cancer cell lines to determine their tumor specificity. All results were confirmed with a second independent round of MSP. Bisulfite-treated DNA from normal lymphocytes and *in vitro* methylated human DNA (Chemicon International) represented the unmethylated and the methylated positive controls, respectively. Water, replacing bisulfite-treated template, was the negative control in both reactions. PCR amplifications were done as follows: a 10-minute 94°C incubation step followed by 35 cycles of 94°C for 30 seconds, annealing temperature for 30 seconds, and 72°C for 30 seconds. A 7-minute elongation step at 72°C completed the PCR amplification program. PCR products were loaded onto nondenaturing 2% agarose gels, stained with ethidium bromide, and visualized under an ultraviolet transilluminator.

Real-time quantitative MSP

Primers and probes for real-time quantitative MSP (qMSP) were specifically designed to bind to bisulfite-converted DNA (17), spanning 11 to 13 CpG dinucleotides. Sequences and annealing temperatures are provided in Supplementary Table S3. *GDF15*, *HSPA2*, *TMEFF2*, and *VIM* were amplified and normalized for DNA input by using ALU as a reference gene. Amplification reactions were carried out in triplicates consisting of 10 μ L of TaqMan Universal PCR Master Mix No AmpErase UNG (Applied

Biosystems); 900 nmol/L concentration of forward and reverse primers; 200 nmol/L of probe; and 3 μ L of bisulfite-modified DNA as a template, and were carried out at 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute, in 384-well plates in a 7900HT Fast Real-Time PCR System (Applied Biosystems), and analyzed by a sequence detector system (SDS 2.3; Applied Biosystems). Each plate included patient DNA samples, positive (*in vitro* methylated human DNA; Chemicon) and negative (normal leukocyte human DNA) controls, and multiple water blanks. Leukocyte DNA from a healthy individual was methylated *in vitro* with excess SssI methyltransferase (New England Biolabs) to generate completely methylated DNA, and serial dilutions (32.5–0.052 ng) of this DNA were used to construct a calibration curve for each plate to quantify the amount of fully methylated alleles in each reaction.

A run was considered valid when the following 6 criteria were met: 1) slopes of each standard curve above -3.60 corresponding to a PCR efficiency of greater than 90%; 2) R^2 of at least 4 relevant data points 0.99 or greater; 3) no amplification in no-template controls; 4) the positive methylation control had to provide a methylated signal; 5) the negative control had no signal; and 6) threshold cycle value for each gene 40 or less.

The relative level of methylated DNA for each gene in each sample was determined using the following formula: $[(\text{gene}/\text{ALU})^{\text{sample}}/(\text{gene}/\text{ALU})^{\text{in vitro methylated DNA}}] \times 1,000$. To categorize samples as methylated or unmethylated, a cutoff value was chosen on the basis of the highest methylation ratio value of the respective normal samples, ensuring the specificity of the assay.

Bisulfite sequencing

GDF15, *HSPA2*, *TMEFF2*, and *VIM* were subjected to direct bisulfite sequencing in BICa cell lines. Primer sequences, overlapping with the MSP products, amplicons, and annealing temperatures are listed in Supplementary Table S3. PCR reactions included a 10-minute 94°C denaturation step followed by 40 cycles of 94°C for 30 seconds, annealing temperature for 30 seconds, and 72°C for 30 seconds. PCR products were loaded onto a nondenaturing 2% agarose gels, stained with ethidium bromide, and visualized under an ultraviolet transilluminator. Excess primer and nucleotides were removed by Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, USB Corporation) following the protocol of the manufacturer. The purified products were sequenced using the dGTP BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The approximate amount of methyl cytosine of each CpG site was calculated by comparing the peak height of the cytosine signal with the sum of the cytosine and thymine peak height signals (18). CpG sites with ratio ranges 0–0.20, 0.21–0.80, and 0.81–1.0 were considered unmethylated, partially methylated, and fully methylated, respectively.

¹<http://genome.ucsc.edu>.

Table 1. Frequency and distribution of promoter methylation levels in NBM and BlCa tissue samples measured by qMSP

Gene	NBM, <i>n</i>	NBM med (IQR)	BlCa, <i>n</i>	BlCa med (IQR)	<i>P</i> ^a
GDF15	0/20	4.35 (1.22–16.82)	33/50	219.39 (13.82–480.80)	<0.001
HSPA2	0/20	1.14 (0.76–1.91)	32/50	31.79 (3.02–184.58)	<0.001
TMEFF2	0/20	0.38 (0.08–1.41)	24/50	10.89 (1.32–143.74)	<0.001
VIM	0/20	0.0 (0.0–0.0)	48/50	237.15 (94.22–466.59)	<0.001

Abbreviations: *n*, number of positive cases; med, median; IQR, interquartile range.

^aMann–Whitney test.

Quantitative gene expression analyses

RNA from 4 BlCa cell lines untreated, treated either with 1 or 5 μmol/L of 5-aza-2'-deoxycytidine for 72 hours, and treated with the combination of 1 μmol/L of 5-aza-deoxycytidine (72 hours) and 0.5 μmol/L of trichostatin (added in the last 12 hours) was analyzed. For each sample, 0.5 μg of total RNA was reverse transcribed into cDNA, using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas Inc.), including random hexamer primers. cDNA was used as the template for real-time PCR reaction. *GDF15* (Hs00171132_m1), *HSPA2* (Hs00356607_g1), *TMEFF2* (Hs01086901_m1), *VIM* (Hs00185584_m1), and endogenous controls assays *GUSB* (Hs99999908_m1) and *HPRT1* (Hs99999909_m1) were amplified separately in 96-well plates following the recommended protocol (Applied Biosystems), and the real-time quantitative gene expression was measured by the 7500 Real-Time PCR System (Applied Biosystems). All samples were analyzed in triplicate, and the mean value was used for data analysis. The human universal reference RNA (Stratagene) was used to generate a standard curve on each plate, and the resulting quantitative expression levels of the tested genes were normalized against the mean value of the 2 endogenous controls to obtain a ratio that was then multiplied by 1,000 for easier tabulation.

Statistics

Differences in quantitative methylation values were assessed by the Kruskal–Wallis test, followed by pairwise comparisons using the Mann–Whitney *U* test. The relationship between methylation ratios and other standard clinicopathologic variables (gender, tumor stage, grade) were evaluated using the Mann–Whitney or Kruskal–Wallis tests. A Spearman nonparametric correlation test was additionally carried out compare age and methylation levels. Disease-specific survival curves (Kaplan–Meier with log-rank test) were computed for standard variables such as tumor stage and grade, and also for methylation status. A receiver operator characteristics (ROC) curve was created by plotting the true positive rate (sensitivity) against the false-positive rate (1 – specificity), and the area under the curve (AUC) was calculated. All 2-tailed *P*-values were derived from statistical tests, using a computer-assisted program (SPSS Version 15.0), and considered statistically significant at *P* < 0.05.

Results

Identification of a list of novel DNA methylation candidate targets in bladder cancer

Five hundred eighty microarray elements were found to be upregulated at least 4-fold after the epigenetic-modulating treatment in at least 2 of 4 BlCa cell lines analyzed. Among these elements, 409 were present in the postprocessed microarray data set from 21 bladder carcinomas and 5 NBM samples. The median expression levels of 226 of these genes were downregulated across the panel of tumor samples relative to NBM. Among the first 130 array elements, nearly 100 contained a CpG island located around their transcription start sites (Supplementary Table S4).

Methylation status of novel candidate genes *in vitro* and *in vivo*

Twenty-one of the DNA methylation candidate genes were analyzed by MSP in BlCa cell lines. The 4 best performing markers, hypermethylated in at least 3 cell lines, were selected for further validation: *GDF15*, *HSPA2*, *TMEFF2*, and *VIM* (summarized in Supplementary Table S5). With the exception of *TMEFF2*, the markers were more often methylated in BlCa than in kidney and prostate cancer cell lines. Illustrative examples of MSP results are shown in Supplementary Figure 2. Using qMSP, *GDF15*, *HSPA2*, *TMEFF2*, and *VIM* were found to be methylated in 66%, 64%, 48%, and 96% of the bladder tumors, respectively, and in none of the normal urothelium (Table 1). Differences in quantitative methylation levels between cancer patients and BlCa-free individuals were statistically significant for all genes (Mann–Whitney, *P* < 0.001). Finally, a statistically significant correlation between methylation levels of *HSPA2*, on the one hand, and *TMEFF2* (Spearman test, *r* = 0.592, *P* < 0.001), and *VIM* (*r* = 0.506, *P* < 0.001), on the other, was also found.

Bisulfite sequencing verification of promoter methylation status

To verify the promoter methylation status assessed by MSP, *GDF15*, *HSPA2*, *TMEFF2*, and *VIM* were subjected to bisulfite sequencing in BlCa cell lines (Fig. 1). In general, CpG island methylation patterns observed by bisulfite sequencing correlated well with the MSP scoring data.

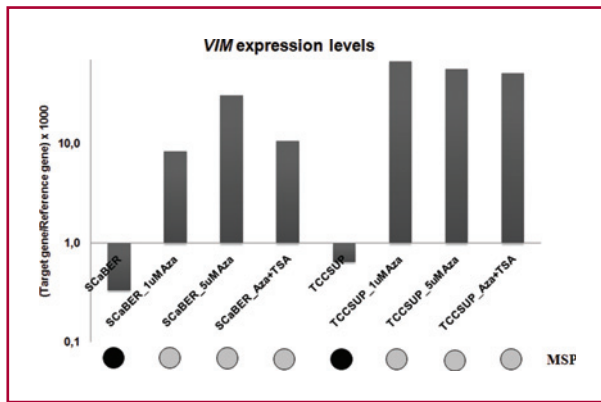


Fig. 2. *VIM* promoter methylation and mRNA expression levels in BICa cell lines after pharmacologic treatment with the demethylating 5-aza-2'-deoxycytidine alone (1 and 5 $\mu\text{mol/L}$) and in combination with the deacetylase inhibitor trichostatin A (0.5 $\mu\text{mol/L}$). Below each sample the respective methylation status is shown, as assessed by MSP. Black circles represent promoter methylation of the genes and gray circles represent the presence of both unmethylated and methylated alleles.

Association between CpG island hypermethylation and transcriptional gene silencing in bladder cancer cell lines

BICa cell lines hypermethylated for *GDF15*, *HSPA2*, *TMEFF2*, and *VIM* showed low transcript expression, which restored or increased after promoter demethylation induced either by 1 or 5 $\mu\text{mol/L}$ of 5-aza-deoxycytidine alone or by the combined treatment with 5-aza-deoxycytidine and trichostatin A (illustrated for *VIM* in Fig. 2). Overall, these

results indicate a correlation between promoter methylation and decreased gene expression levels.

Association between quantitative promoter methylation levels and clinicopathologic variables in primary tumors

Relationship between methylation status and clinicopathologic variables of BICa patients is summarized in Supplementary Table S6. With the exception of *GDF15*, high-grade, muscle-invasive tumors displayed higher methylation levels than superficial low-grade tumors. A significant association of methylation levels with increasing tumor grade and stage was found for *TMEFF2* (Kruskal-Wallis, $P = 0.005$ and $P = 0.026$, respectively) and with tumor grade for *HSPA2* ($P = 0.032$). No statistically significant association was found between gene promoter methylation and patient's age, even after age matching between BICa patients and controls (data not shown).

As expected, high tumor grade and stage were significantly associated with shorter overall survival (log-rank, $P < 0.001$). However, methylation levels were not predictive of outcome.

Evaluation of the biomarkers diagnostic potential by using tissue and urine samples

Among the possible gene combinations tested, 3 gene methylation markers—*GDF15*, *TMEFF2*, and *VIM*—demonstrated the best performance in terms of sensitivity and specificity for discriminating BICa patients from controls (Table 2). Remarkably, a sensitivity of 100% (17/17) was apparent for early-stage Ta and low-grade BICa. The ROC

Table 2. Frequency and distribution of promoter methylation levels in urine sediments

	GDF15	HSPA2	TMEFF2	VIM
BICa patients				
Freq methylation	24/51	30/51	32/51	40/51
med (IQR)	9.34 (2.50–33.12)	8.15 (2.52–71.84)	4.63 (1.10–40.37)	45.47 (7.50–254.48)
HD				
Freq methylation	0/20	0/20	0/20	0/20
med (IQR)	1.64 (0.68–3.53)	1.54 (0.25–3.02)	0.0 (0.0–0.51)	0.25 (0.0–1.40)
P^a	<0.001	<0.001	<0.001	<0.001
RCT patients				
Freq methylation	1/19	2/19	2/19	1/19
med (IQR)	0.32 (0.0–1.07)	1.78 (0.64–2.72)	0.0 (0.0–0.22)	0.24 (0.0–0.45)
P^a	<0.001	<0.001	<0.001	<0.001
PCa patients				
Freq methylation	4/20	3/20	0/20	1/20
med (IQR)	1.08 (0.36–9.72)	1.46 (0.37–2.71)	0.0 (0.0–0.14)	0.0 (0.0–0.38)
P^a	0.012	<0.001	<0.001	<0.001

NOTE: Samples were scored as methylation positive when their value was higher than the highest value across the respective normal healthy donors. P value refers to the statistical comparison with BICa.

Abbreviations: HD, healthy donors; BICa, bladder cancer; RCT, renal cell tumor; PCa, prostate carcinoma; Freq, frequency; med, median; IQR, interquartile range.

^aMann-Whitney test.

Table 3. Performance of epigenetic biomarkers for BlCa in tissue and urine sediments

	Sensitivity % (n positive/n total)	Specificity % (n negative/n total)	PPV %	NPV %
Tissue samples				
<i>VIM</i>	96 (48/50)	100 (20/20)	100	91
<i>VIM/TMEFF2</i>	98 (49/50)	100 (20/20)	100	95
<i>VIM/TMEFF2/GDF15</i>	100 (50/50)	100 (20/20)	100	100
<i>VIM/TMEFF2/GDF15/HSPA2</i>	100 (50/50)	100 (20/20)	100	100
Urine samples (BlCa patients vs. HD)				
<i>VIM</i>	78 (40/51)	100 (20/20)	100	64
<i>VIM/TMEFF2</i>	82 (42/51)	100 (20/20)	100	69
<i>VIM/TMEFF2/GDF15</i>	94 (48/51)	100 (20/20)	100	87
<i>VIM/TMEFF2/GDF15/HSPA2</i>	94 (48/51)	100 (20/20)	100	87
Urine samples (BlCa patients vs. HD, RCT patients, and PCa patients)				
<i>VIM</i>	78 (40/51)	97 (57/59)	95	84
<i>VIM/TMEFF2</i>	82 (42/51)	95 (56/59)	93	86
<i>VIM/TMEFF2/GDF15</i>	94 (48/51)	90 (53/59)	89	95
<i>VIM/TMEFF2/GDF15/HSPA2</i>	94 (48/51)	86 (51/59)	86	94

Abbreviations: HD, healthy donors; RCT, renal cell tumor; PCa, prostate carcinoma; PPV, positive predictive value; NPV, negative predictive value.

curve based on these epigenetic biomarkers (Supplementary Fig. 3) resulted in an AUC of 0.995, with a 95% confidence interval (CI) of 0.985 to 1.000, at a significance of $P < 0.001$. Equal sensitivity and specificity rates were obtained with the 4-gene panel.

In an independent training set of urine sediments from noncancerous donors, methylation levels of BlCa, renal cell carcinoma, and prostate cancer patients were found to be significantly higher in urine from BlCa patients than in normal samples from noncancerous donors for all genes (Table 2; Mann-Whitney, $P < 0.001$). The differences in methylation levels in urine samples from BlCa patients and from other urological tumors was also statistically significant ($P < 0.001$). Moreover, a statistically significant correlation was found between methylation levels of *HSPA2* and *TMEFF2* (Spearman test, $r = 0.638$, $P < 0.001$) and *VIM* ($r = 0.546$, $P < 0.001$), as well as between methylation levels of *VIM* and *GDF15* ($r = 0.289$, $P = 0.040$) and *TMEFF2* ($r = 0.598$, $P < 0.001$).

Interestingly, the relative methylation levels of the genes tested were not significantly different among noncancerous donors, renal cell tumor, and prostate cancer patients, except for *GDF15* levels that differed between donors and renal cell tumor ($P = 0.009$).

The same 3-gene panel displaying the best performance for detecting BlCa in tissue samples demonstrated a sensitivity of 94% (48/51) and a specificity of 100% (20/20) for BlCa detection in urine samples (Table 3). ROC curve analysis showed an AUC of 0.975 (95% CI = 0.948–1.000, $P < 0.001$; Fig. 3). Moreover, when the sets of healthy donors and other urological cancer patients were combined to constitute the control group, the specificity rate

decreased slightly (90%), displaying an AUC of 0.948 (95% CI = 0.912–0.985, $P < 0.001$; Supplementary Fig. 4).

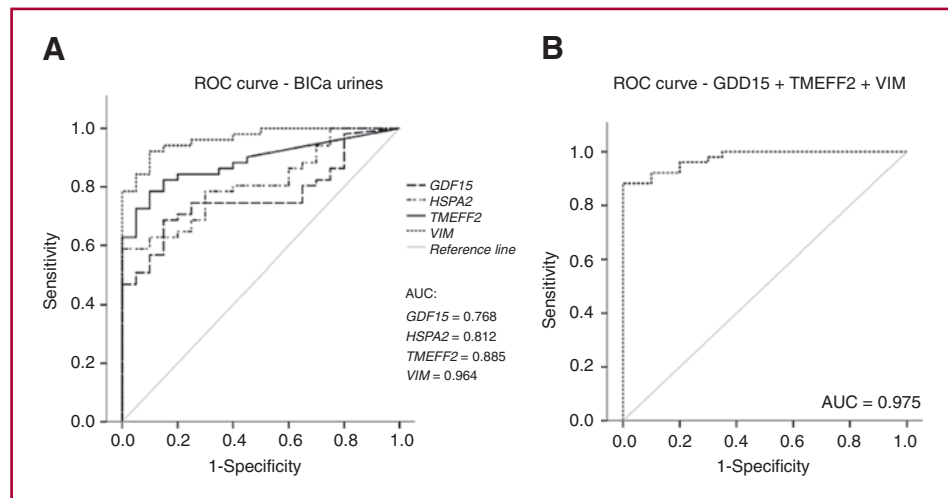
Because urine cytology is carried out only in selected cases at our institution, only 31 of the 51 (61%) BlCa urines were examined by an experienced cytopathologist. Thirteen cases were cytologically diagnosed as malignant (3 were low grade), 8 as negative for malignancy, and 10 cases were “inconclusive/suspicious for malignancy.” Remarkably, the 3-gene panel correctly identified the 13 malignant cases as BlCa and the 8 negative cases diagnosed by cytology, as well as 9 of the 10 “inconclusive/suspicious” cases. Moreover, of the 17 low-grade BlCa, only 1 was missed by the 3-gene panel in urine sediments.

Of the 51 BlCa urine samples tested, 12 were from cases in which the corresponding tissue sample was analyzed in the preceding experiments. Although not all individual gene promoters methylated in tissue samples were correspondingly detected in urine, full concordance was seen for the 3-gene epigenetic biomarker panel (*GDF15*, *TMEFF2*, and *VIM*; Supplementary Table S7).

Discussion

BlCa is a heterogeneous group of tumors that follow diverse pathways of development and progression. Because tumor behavior is difficult to predict, novel diagnostic and prognostic markers for BlCa, especially nonmuscle invasive tumors, is required to provide risk-adjusted treatment and surveillance. Although urine cytology is very sensitive for high-grade tumor detection, low-grade tumors are difficult to identify and diagnostic performance relies heavily on the

Fig. 3. ROC curve in BICa urines for each individual gene (*GDF15*, *HSPA2*, *TMEFF2*, and *VIM*) (A) and the best combination of 3 genes (*GDF15*, *TMEFF2*, and *VIM*) (B).



operator's proficiency (3). A suitable diagnostic test should provide high sensitivity and specificity, no interobserver variability, cost-effectiveness, and easy-to-perform testing, precluding the need for cystoscopic evaluation.

Epigenetic alterations, and DNA methylation in particular, are cancer hallmarks that show promise as sensitive and specific molecular markers for urological cancers (19). The main goal of this study was to discover novel epigenetic biomarkers for sensitive and specific detection of BICa in voided urine samples, enabling their use for early detection and patient monitoring as an alternative strategy to cystoscopy and urine cytology. We found that a 3-gene panel (*GDF15*, *TMEFF2*, and *VIM*), which was selected on the basis of stringent criteria, could accurately identify BICa in both tissue and urine samples with sensitivity and specificity of 94% and 100%, respectively. The performance of this gene panel clearly exceed that of conventional cytopathology, as it correctly identified BICa in 30 of 31 (97%) cases, whereas cytology only clearly diagnosed as malignant 10 of the 31 (32%) of cases. Moreover, the panel could discriminate urine samples of BICa-positive patients from patients with other urological tumors. Thus far, there have been several reports on the feasibility of detecting BICa through methylation profiling of tumor tissues (20–24). However, most of these studies were based on qualitative rather than quantitative methods and the methylated gene promoters were typically identified in several types of urological tumors, thus hampering a differential detection of BICa. The same approach has also been attempted in urine samples from BICa patients but with essentially the same caveats and lower sensitivity (25–30). Although marker specificity has been higher in most of these studies, the performance of the gene panels was not tested against other urological tumors and thus it is likely that specificity and positive predictive value would decrease. The present marker panel has the ability to discriminate BICa from prostate and renal cancer retaining both high specificity and sensitivity, offering the possibility of a future integration of this gene panel with some additional genes specific for prostate and/or renal cancers.

To generate the gene panel tested in this study, we used microarray gene expression profiling in combination with 5-aza-deoxycytidine and trichostatin A treatment of BICa cell lines to identify potential epigenetically inactivated gene targets. To the best of our knowledge, only a few studies using high-throughput strategies to uncover epigenetically deregulated genes in BICa have been reported. Using CpG microarrays, Aleman and coworkers (31) associated *SOX9* promoter methylation with BICa progression, but the methylation frequency of 56.4% is much lower than for the marker panel presented here. Both *FGF18* and *MMP11* were found to be downregulated in response to 5-aza-2'-cytidine and zebularine treatment and have been proposed as potential epigenetic biomarkers for BICa, but they were not tested either in tumor tissues or in urine samples (32). More recently, Renard et al. (33) were able to detect BICa in urine samples with 90% sensitivity and 93% specificity with a 2-gene panel (*TWIST1* and *NID2*), which was superior to cytology. Although the sensitivity of our 3-gene panel was slightly lower, specificity was 100% in urine samples. Moreover, cases of prostate or renal cell cancer were not included as controls in the aforementioned studies, preventing differential detection. The added value of this approach is clearly illustrated by a case in our series that was initially allocated in the renal cell carcinoma set of urines. Because the gene panel tested positive in this case, the clinical files were reanalyzed, revealing that this renal cell cancer patient was diagnosed with BICa 3 years after the collection of the urine sample. Although this is an anecdotal case, it suggests that epigenetic alterations do, indeed, precede clinical manifestations of disease and are thereby represent promising biomarkers for early cancer detection.

Interestingly, the candidate target gene list found in our study is different from those that used similar methodologic approaches (31–33). In our strategy, we have included several strict criteria to limit the probability of selecting false positive rates (12). Only array elements upregulated more than 4-fold in at least 2 of the 4 cell lines analyzed were chosen. Then, the expression level of these genes was subsequently examined in primary bladder

carcinomas and NBM, and only genes found to be down-regulated were further selected.

The putative biological relevance of *GDF15*, *HSPA2*, *TMEFF2*, and *VIM* gene promoter methylation in carcinogenesis may provide additional support to our methodology.

Both *TMEFF2* (located at chromosome band 2q32.3, encoding a transmembrane protein with EGF-like domains and 2 follistatin-like domains 2, involved in cell proliferation control) and *VIM* (located at chromosome band 10p13, encoding the intermediate filament vimentin) have been previously found to be silenced through aberrant promoter methylation in esophageal, gastric, and colon cancer (34–40). Interestingly, a higher frequency of *TMEFF2* promoter methylation in tumor tissue than in morphologically normal tumor-adjacent tissue has been reported for BICa (41) in line with our results, although no association with grade or stage was apparent. Nevertheless, the presence of *TMEFF2* promoter methylation in apparently NBM indicates that this epigenetic alteration arises early in bladder carcinogenesis and supports its use as an epigenetic biomarker for early cancer detection. The *VIM* promoter methylation has been proposed as a colorectal tumor marker (35–37) and is currently included in a noninvasive test for colorectal cancer (ColoSure). *VIM* methylation is a “true” early diagnostic marker in colorectal cancer without biological function, as it is not expressed as a protein in the mucosa epithelium of the large bowel (35). The present study demonstrates for the first time the value of the *VIM* marker for BICa prediction in urine samples.

To the best of our knowledge, this is the first report of an association between *GDF15* (located at chromosome band 19p13.11) and *HSPA2* (located at chromosome band 14q24.1) promoter methylation and BICa. *GDF15* encodes a divergent member of the transforming growth factor (TGF)- β superfamily, a large family of secreted molecules required for normal development, differentiation, and tissue homeostasis. Its antitumorigenic activity has been suggested because of the association between *GDF15* overexpression and tumor growth arrest and increased apoptosis (42). However, other reports suggest a protumorigenic role, as high expression of *GDF15* is frequently observed in several tumors (43, 44). Thus, it has been postulated that, like other members of the TGF- β superfamily, *GDF15* might act as a tumor suppressor in early cancer stages and as a protumorigenic at later stages of tumor progression (44). Interestingly, our results fit well with this hypothesis, as *GDF15* promoter methylation was

cancer specific, but a decrease in methylation levels was apparent from low- to high-grade tumors and from superficial to muscle-invasive tumors. Interestingly, the *GDF15* gene promoter has been previously reported to be more densely methylated in renal cancer cell lines (including 2 derived from primary tumors) than in normal kidney cells, although no information is provided concerning primary renal tumors (45). Indeed, we found *GDF15* promoter methylation in urine sediments from renal cancer patients, but it was mostly vestigial and only 1 of 19 cases was above the cutoff value. Thus, although BICa and RCT might share *GDF15* promoter methylation, a quantitative assay can accurately discriminate these cancers. Finally, the role of *HSPA2* in BICa has to be further clarified, as concomitant depletion of *HSP70* and *HSPA2* has a synergistic antiproliferative effect on cancer cells (46) and elevated expression of *HSPA2* was found in primary and metastatic breast cancer samples compared with adjacent normal breast tissue (47).

In conclusion, using a highly sensitive automated and quantitative screening methodology for detecting cancer-related promoter methylation, we discovered a novel set of 4 genes frequently and specifically methylated in BICa. Moreover, a 3-gene panel derived from that set could discriminate between tumorous and nontumorous bladder tissue with high sensitivity and specificity. Finally, the same panel showed promise for early and accurate detection of BICa in urine samples, even when patients with kidney or prostate cancer were used as controls.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing interests.

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