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Clinical Cancer Research

# Comprehensive Genome Methylation Analysis in Bladder Cancer: Identification and Validation of Novel Methylated Genes and Application of These as Urinary Tumor Markers

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Human Cancer Biology

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# Comprehensive Genome Methylation Analysis in Bladder Cancer: Identification and Validation of Novel Methylated Genes and Application of These as Urinary Tumor Markers

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## Abstract

**Purpose:** Epigenetic alterations are common and can now be addressed in a parallel fashion. We investigated the methylation in bladder cancer with respect to location in genome, consistency, variation in metachronous tumors, impact on transcripts, chromosomal location, and usefulness as urinary markers.

**Experimental Design:** A microarray assay was utilized to analyze methylation in 56 samples. Independent validation was conducted in 63 samples by a PCR-based method and bisulfite sequencing. The methylation levels in 174 urine specimens were quantified. Transcript levels were analyzed using expression microarrays and pathways were analyzed using dedicated software.

**Results:** Global methylation patterns were established within and outside CpG islands. We validated methylation of the eight tumor markers genes *ZNF154* ($P < 0.0001$), *HOXA9* ($P < 0.0001$), *POU4F2* ($P < 0.0001$), *EOMES* ($P = 0.0005$), *ACOT11* ($P = 0.0001$), *PCDHGA12* ($P = 0.0001$), *CA3* ($P = 0.0002$), and *PTGDR* ($P = 0.0110$), the candidate marker of disease progression *TBX4* ($P < 0.04$), and other genes with stage-specific methylation. The methylation of metachronous tumors was stable and targeted to certain pathways. The correlation to expression was not stringent. Chromosome 21 showed most differential methylation ($P < 0.0001$) and specifically hypomethylation of keratins, which together with keratin-like proteins were epigenetically regulated. In DNA from voided urine, we detected differential methylation of ZNF154 ($P < 0.0001$), POU4F2 ($P < 0.0001$), HOXA9 ($P < 0.0001$), and EOMES ($P < 0.0001$), achieving 84% sensitivity and 96% specificity.

**Conclusions:** We initiated a detailed mapping of the methylome in metachronous bladder cancer. Novel genes with tumor, chromosome, as well as pathway-specific differential methylation in bladder cancer were identified. The methylated genes were promising cancer markers for early detection of bladder cancer. *Clin Cancer Res; 17(17); 5582–92. ©2011 AACR.*

## Introduction

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence (1). Several forms of epigenetic regulation exist and these include histone modifications and DNA methylation. DNA methylation occurs during critical normal processes like development, genomic imprinting, and X-chromosome inactivation (2–4). Alterations in epigenetic control have been associated with several human pathologic conditions including cancer (5). CpG sites are sparsely distributed throughout the genome except for areas named CpG islands (6, 7). CpG dinucleotides outside CpG islands are generally hypermethylated in normal cells and undergo a substantial loss of DNA methylation in cancers. CpG sites within CpG islands are usually in an unmethylated state permissive to transcription in normal cells but become hypermethylated at certain promoters in cancers. Transcriptional inactivation by CpG island promoter hypermethylation is a well-established mechanism for gene silencing in cancer including bladder cancer (8–18), and aberrant methylation is associated with stage, grade of the tumors as well as recurrence rate and progression (19–24).

Cancer of the urinary bladder is a common disease being the fifth most common neoplasm in the industrialized countries. In 75% of all cases, the primary tumor will

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## Translational Relevance

Cancer of the urinary bladder is one of the major cancers, being the fifth most common neoplasm in the industrialized countries. Superficial bladder cancer is characterized by frequent recurrences after resection and up to 25% will develop an aggressive phenotype. Patients diagnosed with superficial bladder cancer frequently attend outpatient clinics for cystoscopy controls for an extended time period. Frequent visits to the outpatient clinic cause discomfort for the patient and is costly for society. This study reports novel methylation markers of bladder cancer and identified 3 markers as highly promising urinary cancer markers. In the future, urinary cancer markers have the potential to decrease the cost for society and lessen the discomfort of patients. Some methylation events were related to stage and progression of the disease and to pathways of relevance for cancer development. These findings may help optimize cancer therapy and follow-up.

present as a non–muscle-invasive tumor stage Ta or T1, the remaining will present with invasion of the bladder muscle, stages T2–4 (25). Stage Ta bladder cancer is characterized by frequent recurrences after resection, in as many as 60% of patients (26). Often one or more tumors will appear each year over an 8- to 10-year period without any progression; however, up to 25% will eventually develop an aggressive invasive phenotype (27).

In bladder cancer, methylation of single gene has been identified and a possible function as stage marker, or as a urinary marker, has been tested (13, 19, 28–35). However, few studies have used a more global array-based approach. One study with 10 normals and 10 transitional cell carcinoma identified 84 CpG island clones with differential DNA methylation between normals and cancers (14). Another study with bacterial artificial chromosome arrays showed that methylation could discriminate between normals and cancers (36).

In this study, we used microarrays to investigate the aberrant DNA methylation at 27,000 CpG sites. We identified novel methylation markers of bladder cancer, some of which were highly promising as urinary cancer markers.

## Materials and Methods

### Patient material

A total of 119 tissue samples analyzed by Infinium array or methylation-sensitive high-resolution melting (MS-HRM; Table 1) were obtained fresh from transurethral resection, embedded in Tissue-Tek (optimum cutting temperature) Compound (Sakura Finetek), and immediately snap frozen in liquid nitrogen. Patients with bladder cancer had no other malignant disease. Normal bladder urothelium was obtained from individuals who had benign prostate hyperplasia or bladder stones. Most patients provided metachronous tumors. Informed written consent was obtained from all patients. Research protocols were approved by The Central Denmark Region Committees on Biomedical Research Ethics. Samples were macro (tumor) or laser (normal) dissected to obtain a urothelial cell percentage of at least 75%. Sample composition was confirmed by hematoxylin and eosin evaluation of sections cut before and after those used for extraction.

Voided urine was collected from 115 bladder cancer patients and 59 individuals with benign prostate hyperplasia or bladder stones (Table 1). Nineteen of the controls were stix positive for nitrite indicating bacterial infection. Urine specimens were collected immediately before urinary cytology or cystoscopy, pelleted by centrifugation, and frozen at −80°C.

### DNA extraction and bisulfite modification

Tissue DNA was extracted using the Puregene DNA Purification Kit (Gentra Systems). One microgram of DNA extracted from fresh frozen tissue was bisulfite modified using EZ-96 DNA methylation D5004 (Zymo Research) or EpiTect (Qiagen) for the Infinium array or MS-HRM, respectively. Urine DNA was extracted using the Puregene DNA Purification Kit (Gentra Systems) according to the manufacturer's recommendations. Tissue and urine DNA purity was assessed using the $OD_{260/280}$ ratio.

### Infinium array

One microgram of DNA from each sample was whole genome amplified and hybridized overnight to Infinium arrays, scanned by a BeadXpress Reader instrument (Illumina), and data analyzed by the Bead Studio Methylation Module Software (Illumina) and exported to Excel for further analysis. The CpG island status was obtained from Bead Studio. For each of the 27,578 probes, the Infinium assay returns with a β value, which approximately corresponds to the average percentage methylation in the sample analyzed. Illumina reports that the Infinium array is accurate with Δβ values above 0.2. The Δβ cutoff value for differential methylation was conservatively set to ±0.25.

### Cloning and bisulfite sequencing

Primers for bisulfite sequencing of CpG island regions were designed using MethPrimer (Supplementary Table S1; ref. 37). PCR for cloning was carried out with the Accuprime Taq DNA Polymerase System (Invitrogen) according to the manufacturer's instructions in a final volume of 25 μL using 4 μL of bisulfite-modified DNA as template. Amplification protocols can be seen in Supplementary Table S1. PCR amplicons were gel purified (Qiagen) and TOPO TA cloned for sequencing (Invitrogen) according the manufacturer's instructions. Twelve random colonies from each gene were used for colony PCR in a final volume of 25 μL using the TEMPase Kit (Ampliqon) according to the manufacturer's instructions. Primers were M13 forward and M13 reverse from the TOPO TA Cloning Kit (Invitrogen). The sequencing reactions were analyzed in a 3130x genetic analyzer (Applied Biosystems).

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**Table 1.** Demographic and clinical characteristics of bladder cancer patients and control individuals

| Characteristics | Discovery set (Infinium array) | Validation set (MS-HRM) | Urine specimens |
|---|---|---|---|
| *Controls* | 6 | 8 | 59 |
| Gender, *n* (%) | | | |
| Male | 6 (100) | 8 (100) | 53 (88) |
| Female | 0 | 0 | 7 (12) |
| Age, mean (min–max) | 72 (67–87) | 61 (52–72) | 61 (30–88) |
| Nitrite test, n (%) | | | |
| Positive | N/A | N/A | 19 (32) |
| Negative | N/A | N/A | 33 (55) |
| *Tumors* | 26[a] | 55 | 115 |
| Gender (%) | | | |
| Male | 18 (69) | 39 (70.9) | 89 (77) |
| Female | 8 (31) | 16 (29.1) | 26 (23) |
| Age, mean (min–max) | 67 (38–87) | 70 (39–89) | 68 (35–93) |
| Ta | 63 (38–80) | 68 (39–87) | 67 (35–93) |
| T1 | 72 (53–83) | 71 (63–78) | 69 (50–79) |
| T2–4 | 78 (69–87) | 72 (56–89) | 68 (45–89) |
| Pathologic stage, *n* (%) | | | |
| Ta | 17 (65) | 25 (45) | 59 (51) |
| T1 | 5 (19) | 15 (27) | 27 (23) |
| T2 | 4 (15) | 14 (25) | 28 (24) |
| T3 | 0 | 1 (2) | 1 (1) |
| T4 | 0 | 0 | 0 |
| Grade, *n* (%) | | | |
| I | 6 (23) | 6 (10.9) | 17 (15) |
| II | 10 (38) | 19 (34.5) | 37 (32) |
| III | 10 (38) | 27 (49.1) | 57 (50) |
| IV | 0 | 2 (3.6) | 4 (3) |
| N/A | 0 | 1 (1.8) | 0 |
| Nitrite test, *n* (%) | | | |
| Positive | N/A | N/A | 5 (4) |
| Negative | N/A | N/A | 108 (94) |
| Tumor cells in urine, *n* (%) | | | |
| Positive | N/A | N/A | 39 (34) |
| Negative | N/A | N/A | 15 (13) |

Abbreviation: N/A, not available.

[a]Additional metachronous tumor information used for intrapatient analyses (Supplementary Table S8).

### MS-HRM

MS-HRM was carried out in triplicate with 15 sets of primers (Supplementary Table S1) using 1.5 μL (15 ng) of bisulfite-modified DNA as template in a final volume of 10 μL LightCycler 480 High Resolution Melting Master (Roche). Each plate included a no-template control and a standard curve (100%, 75%, 50%, 25%, 5%, and 0% methylated samples): CpGenome Universal Methylated DNA (Millipore) diluted with unmethylated DNA (peripheral blood DNA). Melting curves were analyzed on a LightScanner (Idaho Technology, Inc.).

### RNA purification and gene expression microarray

RNA was purified using RNeasy (Qiagen). The RNA integrity and RIN number was assessed with the 2100 Bioanalyzer (Agilent). A total of 500 ng of RNA from each sample was loaded on a Human Exon 1.0 ST arrays (Affymetrix). Microarray analysis and data handling were conducted as described previously (38).

### Data analysis

Genespring GX 10 software (Agilent) was used for exon array analysis. Data was quantile normalized using ExonRMA16 with transcript level core (17,881 transcripts) and by using antigenomic background probes. The statistical analysis was conducted with independent samples only, except for the 2 analyses of metachronous tumors. Ta(stable) and Ta(stable2) consist of the first and second tumor from patients with a stable Ta disease. Ta (prog) consists of Ta tumors from patients with subsequent progression to T1 or T2–4. When more than 1 Ta tumor exists, we used the Ta tumor closest to the stage progression.

### Gene ontology and Ingenuity pathway analysis

Gene symbols of genes showing hypo- or hypermethylation were used as input in gene ontology analysis. The undivided list was submitted to Ingenuity pathway analysis (IPA; 2000–2008 Ingenuity Systems) and the data were analyzed to identify (adjusted for multiple testing by the Benjamini–Hochberg method) top network–associated functions and canonical pathways.

### Statistical analysis

Stata 10 (StataCorp LPA) was used for analyzing methylation data from MS-HRM using the nonparametric Wilcoxon–Mann–Whitney test. The interobserver agreement coefficient (κ) was calculated for MS-HRM. The Infinium array data were analyzed using the nonparametric Wilcoxon–Mann–Whitney test or Wilcoxon signed-rank test in R (http://www.r-project.org/) to evaluate differential methylation between independent groups or related samples, respectively. As metachronous lesions were very similar in methylation, only 1 from each patient was included for statistical calculation. We did not adjust for multiple testing because of limited group sizes, the most interesting CpG sites were instead validated on an independent sample set. The $\chi^2$ test was used for evaluation of chromosomal distribution. A receiver operating characteristic curve was made for each marker by plotting sensitivity against (1−specificity) and the area under the curve (AUC) was calculated. Excel (Microsoft) was used for 2-tailed Student's *t* test to evaluate different mRNA expression between groups and Pearson correlations.

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## Results

### Genome-wide methylation in urinary bladder cancer

We profiled the genome-wide DNA methylation status of 6 normal urothelium samples and 50 urothelial carcinomas of the urinary bladder, using microarrays interrogating 27,000 CpG sites. To study the methylation over time in single individuals, we analyzed metachronous tumors (2–3 tumors from 18 patients). We subdivided patients with stage Ta into stable disease [named Ta(stable) and Ta (stable2) when same patient] and progressing disease [named Ta(prog) when going from stage Ta to T1 or higher].

The average CpG site methylation within CpG islands was increased (Student's *t* test, $P = 0.013$) in the aggressive Ta(prog), T1, and T2–4 tumors, compared with normals and Ta(stable) tumors. Sites outside CpG islands measured a decrease (Student's *t* test, $P = 0.0095$) in average CpG site methylation reaching 18.5% in the Ta(stable) group and 10.6% in the T2–4 tumor group compared with normal tissue. Using Ta(stable) as a reference group, it was evident that the majority of changes in methylation occurred in the transition from normal to cancer. These findings are in concordance with other findings in cancer tissues compared with normal tissues.

### Gene-specific methylation differences

Of the 19 most differentially methylated genes between normals and tumors, 11 showed hypomethylation and 8 hypermethylation in cancer (Table 2). Nine other genes showed a high sensitivity and specificity when comparing normal and cancer (Table 2), see flow chart for gene selection (Supplementary Fig. S1B). Eleven genes were validated by bisulfite sequencing and 8 genes also by an independent biological validation (Table 2). The methylation profiles for the tumor markers *ZNF154*, *HOXA9*, *POU4F2*, and *EOMES* are shown in Fig. 1A; the remaining *ACOT11*, *PCDHGA12*, *CA3*, *PTGDR*, *HIST1H4F*, *SLC22A12*, and *GRM4* in Supplementary Fig. S2A.

We identified the following number of genes with significantly (Mann–Whitney, $P < 0.0001$ to $P < 0.05$) altered methylation between stages: stage Ta versus T2–4, 697 genes (including *CHRNB1*, *BRF1*, and *SOCS3*; Supplementary Fig. S3A); stage Ta versus T1, 176 genes; stage T1 versus T2–4, 137 genes (including *SCARF2*; Supplementary Fig. S3A); muscle-invasive versus noninvasive tumors, 148 genes; and low- versus high-grade tumors, 375 genes.

Furthermore, we identified 149 genes as related to progression, being potential candidate methylation markers of disease progression as they were altered in progressing Ta tumors compared with stable Ta tumors (e.g., TBX4; Supplementary Fig. S3A).

### Technical validation of the MS-HRM technique

To test the robustness of the PCR-based MS-HRM technique, we conducted a technical validation prior to the independent validation. MS-HRM primers for 8 bladder cancer marker genes (selection criteria, Supplementary Fig. S1B) were tested on 12 clinical samples (2 normal and 10 tumor samples) also included on the Infinium array. The Pearson correlation coefficient between the Infinium array and the MS-HRM ranged from 0.75 to 0.99, which was acceptable.

### Validation of microarray data

To confirm the microarray findings, we used the MS-HRM technique on an independent sample set consisting of 8 normals and 55 cancers (Table 1).

We were able to successfully validate all 8 tumor markers on the independent validation set ($P < 0.011$; Fig. 1B, Supplementary Fig. S2B and Supplementary Table S2). In addition to the tumor markers, we also validated markers of stage, invasiveness, and candidate markers of tumor progression (Supplementary Fig. S3B and Supplementary Table S3). We were able to validate most but not all of these markers in the independent validation set. The interobserver agreement ($\kappa$ value) of the MS-HRM validation assay was good (0.58–1.00; Supplementary Tables S2 and 3). None of the markers identified were independent of each other (Supplementary Table S4). This indicates that one single methylation mechanism may account for the majority of the methylation alterations we discovered.

### Bisulfite sequencing of DNA surrounding Infinium probes

Eleven tumor marker genes and 1 stage marker were selected for analytic validation by bisulfite sequencing to obtain detailed information on the sequence surrounding the Infinium array probe source sequence and the sequence analyzed by MS-HRM. Bisulfite sequencing corresponded well with the array and MS-HRM–based findings (Fig. 2 and Supplementary Figs. S4–6).

### Association between methylation status and clinicopathologic variables in the validation set

The possible association with the clinicopathologic parameters stage and grade were investigated (Table 3). Only methylation of *ACOT11* was associated with stage (Fisher's exact test, $P = 0.049$). *ACOT11* was more frequently methylated in the T1 and T2–4 stage tumors than in the superficial Ta tumors. *CA3* was less frequently methylated in grade I tumors compared with grade II and III tumors (Fisher's exact test, $P = 0.011$). There was no significant association between methylation and age. However, higher stage was associated with increasing age (Fisher's exact test, $P = 0.041$).

### Identification of methylated biomarkers in urinary specimens from bladder cancer patients

To test the potential of the validated tumor-specific methylation of the genes *ZNF154*, *POU4F2*, *HOXA9*, and *EOMES* as urinary markers for early detection of bladder cancer, we analyzed urine from 115 patients with cancer and 59 control urine samples using MS-HRM (Table 4). The methylation difference between urine from healthy individuals and patients was highly significant for *ZNF154* ($P < 0.0001$), *POU4F2* ($P < 0.0001$), *HOXA9*

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**Table 2.** List of the 19 most highly differentially methylated genes between controls and tumors, as well as selected genes (see flow chart in Supplementary Fig. S1B) validated alone by bisulfite sequencing[a] or by bisulfite sequencing and independent validation,[b] sorted by Δβ values

| Gene | Δβ value | *P* | Sensitivity, % | Specificity, % | Pearson correlation | Distance to TSS | Chr | CpG island | Infinium targetID |
|---|---|---|---|---|---|---|---|---|---|
| Most hypermethylated | | | | | | | | | |
| *ZIC1* | 0.52 | **<0.0001** | 100 | 83 | −0.08 | 171 | 3 | + | cg14456683 |
| ***ZNF154***[b] | 0.52 | **0.0018** | 85 | 100 | **−0.68** | 68 | 19 | + | cg21790626 |
| *SPAG6* | 0.52 | **0.0001** | 96 | 83 | 0.05 | 361 | 10 | + | cg25802093 |
| *MYCL2* | 0.50 | **0.0009** | 77 | 100 | ND | 6 | X | + | cg12537796 |
| ***HOXA9***[b] | 0.50 | **0.0003** | 92 | 100 | **−0.46** | 35 | 7 | + | cg07778029 |
| *KCNA1* | 0.50 | **0.0009** | 92 | 83 | −0.16 | 148 | 12 | + | cg08832227 |
| ***ZNF154*** | 0.50 | **0.0049** | 81 | 100 | **−0.75** | 100 | 19 | + | cg08668790 |
| *HSPA2* | 0.50 | **0.0004** | 96 | 83 | 0.07 | 850 | 14 | + | cg27120999 |
| Selected | | | | | | | | | |
| ***POU4F2***[b] | 0.47 | **0.0004** | 92 | 100 | −0.13 | 38 | 4 | + | cg24199834 |
| ***HIST1H4F***[a] | 0.45 | **0.0005** | 92 | 100 | ND | 266 | 6 | + | cg08260959 |
| ***ACOT11***[b] | 0.44 | **0.0004** | 92 | 100 | 0.33 | 192 | 1 | − | cg10266490 |
| ***EOMES***[b] | 0.44 | **0.0004** | 88 | 100 | −0.01 | 1498 | 3 | + | cg15540820 |
| ***PCDHGA12***[b] | 0.43 | **0.0001** | 96 | 100 | ND | 21 | 5 | + | cg07730329 |
| ***CA3***[b] | 0.42 | **0.0001** | 88 | 100 | −0.09 | 123 | 8 | + | cg18674980 |
| ***PTGDR***[b] | 0.39 | **0.0218** | 58 | 100 | 0.08 | 98 | 14 | + | cg09516965 |
| Most hypomethylated | | | | | | | | | |
| ***GRM4***[a] | −0.43 | **<0.0001** | 96 | 100 | −0.19 | 476 | 6 | + | cg01962826 |
| ***SLC22A12***[a] | −0.46 | **<0.0001** | 88 | 100 | −0.08 | 335 | 11 | + | cg07220939 |
| *FTHL17* | −0.51 | **<0.0001** | 96 | 100 | 0.25 | 478 | X | − | cg04515986 |
| *KRTAP11-1* | −0.51 | **<0.0001** | 96 | 100 | 0.19 | 114 | 21 | − | cg07014174 |
| *MMP26* | −0.51 | **<0.0001** | 100 | 83 | 0.10 | 113 | 11 | − | cg12493906 |
| *ERAF* | −0.51 | **<0.0001** | 100 | 100 | 0.27 | 31 | 16 | − | cg02989940 |
| *REG3G* | −0.51 | **<0.0001** | 96 | 100 | ND | 384 | 2 | − | cg00918005 |
| *FFAR2* | −0.52 | **<0.0001** | 100 | 100 | −0.05 | 245 | 19 | + | cg15479752 |
| *CNTNAP4* | −0.52 | **<0.0001** | 100 | 100 | −0.10 | 119 | 16 | − | cg06793062 |
| *TNFSF11* | −0.52 | **<0.0001** | 100 | 100 | 0.19 | 326 | 13 | − | cg21094154 |
| *CNOT6* | −0.53 | **0.0001** | 96 | 100 | −0.25 | 835 | 5 | + | cg15241708 |
| *EBPL* | −0.54 | **<0.0001** | 100 | 100 | −0.33 | 616 | 13 | + | cg20399252 |
| *MAGEB6* | −0.62 | **<0.0001** | 100 | 100 | 0.26 | 34 | X | − | cg10127415 |

NOTE: Δβ values calculated as average tumor methylation β value minus average control methylation β value. Pearson correlation coefficient between methylation and expression are shown. Infinium array target id, the presence of a CpG island, chromosome number, and distance of CG dinucleotides to TSS are specified. Statistics were made using a 2-sample Wilcoxon rank-sum (Mann–Whitney) test. Bold indicates genes of special interest. *P* values below 0.05 and Pearson correlation below −0.4 are shown in bold. Abbreviations: ND, not determined; TSS, transcription start site.

[a]Validated by bisulfite sequencing.

[b]Validated by bisulfite sequencing and independent biological validation.

($P < 0.0044$), and *EOMES* ($P < 0.0001$). The sensitivity observed for the individual markers was 62% to 74%. To ensure a high specificity of the combined markers, we selected methylation cutoff values achieving 100% specificity for *ZNF154*, *POU4F2*, and *EOMES* and 96% for *HOXA9*. The selected cutoff values represented 6%, 4%, 3%, and 0% methylation levels for *ZNF154*, *POU4F2*, *EOMES*, and *HOXA9*, respectively. Combining all 4 markers, including only samples with readings from at least 3 of the 4 markers, we obtained sensitivity of 84% and specificity of 96%; with positive predictive value (PPV) of 98% and negative predictive value (NPV) of 74%.

Given that cytology has less sensitivity in low-stage lesions, we analyzed the combined markers on urine from patients with Ta tumors. The sensitivity was 84% and specificity 96%, the AUC (95% CI) was 0.90 (0.84–0.96), the PPV was 96%, and the NPV was 85% (Supplementary Table S5). The sensitivities in urine from patients with T1 and T2–4 tumors were 85% and 83%, respectively. The performance of the combined markers on urine from patients with grade I

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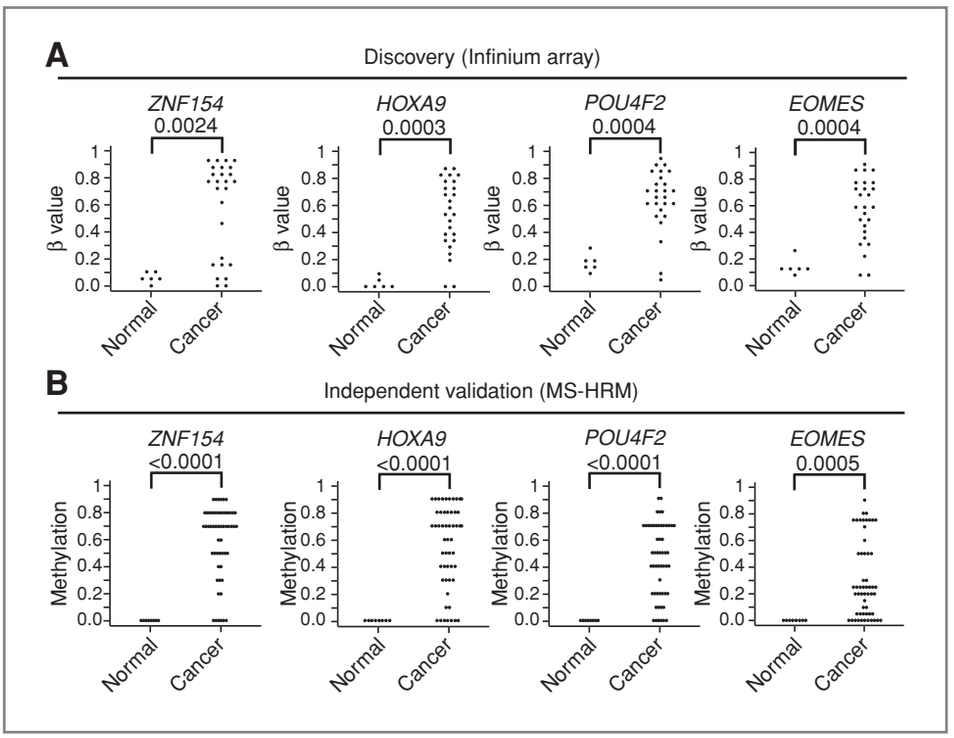


**Figure 1.** Methylation data from microarrays and MS-HRM–based validation. A, genes with differential methylation between normals and cancers. A β-value of 0 means no methylation, whereas 1 means fully methylated. Normals ($n = 6$), cancers ($n = 50$). B, MS-HRM validation of tumor markers. A methylation value of 0 means no methylation, whereas 1 means 100% methylated. Normals ($n = 8$), cancers ($n = 55$).

tumors was as follows: sensitivity, 75%; specificity, 96%; AUC (95% CI), 0.86 (0.74–0.97); PPV, 86%; and NPV, 93% (Supplementary Table S5). The sensitivity on urine specimens with tumor cells detected by the pathologist was 95%, whereas it was 93% in urines where the pathologist did not detect tumor cells. On the basis of this, the urinary methylation assay seemed much more sensitive than urine cytology for the detection of bladder tumors.

We had matched methylation data from urine specimens and tumor samples from 33 patients. The analytic sensitivity on these ranged from 81% to 97% and combination 94% (Supplementary Table S6).

### Association between methylation status and clinicopathologic variables on urine specimens

We analysed the association of the 4 urinary markers of bladder cancer with stage, grade, age, cytology, and nitrite status (Supplementary Table S7). Methylation of *ZNF154* was associated with higher stage (Fisher's exact test, $P = 0.019$) and grade (Fisher's exact test, $P = 0.002$), whereas methylation of *EOMES* was associated with high grade (Fisher's exact test, $P = 0.036$). The frequency of methylation of *HOXA9* and *EOMES* was independent of cytology being positive or negative for tumor cells (Fisher's exact test, $P > 0.05$). No association was observed between the frequency of methylation and age for any of the markers (Fisher's exact test, $P > 0.05$).

### Correlation between DNA methylation and transcription

Considering the genes in Table 2, only *HOXA9* and *ZNF154* had an absolute Pearson correlation between methylation and expression equal to or larger than 0.4, and only *HOXA9* was differentially expressed between normal and tumor samples (Student's $t$ test, $P = 0.0022$). As expected, the level of HOXA9 transcript was lower in tumor than normal samples. The bisulfite sequencing did not provide additional information, as the array probes seemed to reflect the methylation event well in the sequenced areas (Supplementary Figs. S4 and 5).

### Intrapatient variation in methylation

The intrapatient stability of methylation was high for both Ta(stable) and Ta(prog) tumors, as 92% and 89% of changes, respectively, found in early tumors were present later on.

The number of changes was independent of time between tumors ($R^2 = 0.0029$) and mRNA transcript level of DNA methyltransferases. However, to study if this was based on a systematic change in methylation of certain genes over time, we made a group comparison across the metachronous samples (Supplementary Table S8). This analysis revealed that no single gene was differentially methylated between the first and second tumor within the stable or progressing groups ($P > 0.05$; Wilcoxon signed-rank test).

### Pathway analysis of differentially methylated genes

Using gene ontology, the 149 differentially methylated genes between Ta stable and Ta progressing tumors belonged to mainly 22 overrepresented pathways, having up to 7 methylation changes. Hypermethylated pathways were related to cellular development in particular epidermal development ($P < 0.037$). Hypomethylated pathways

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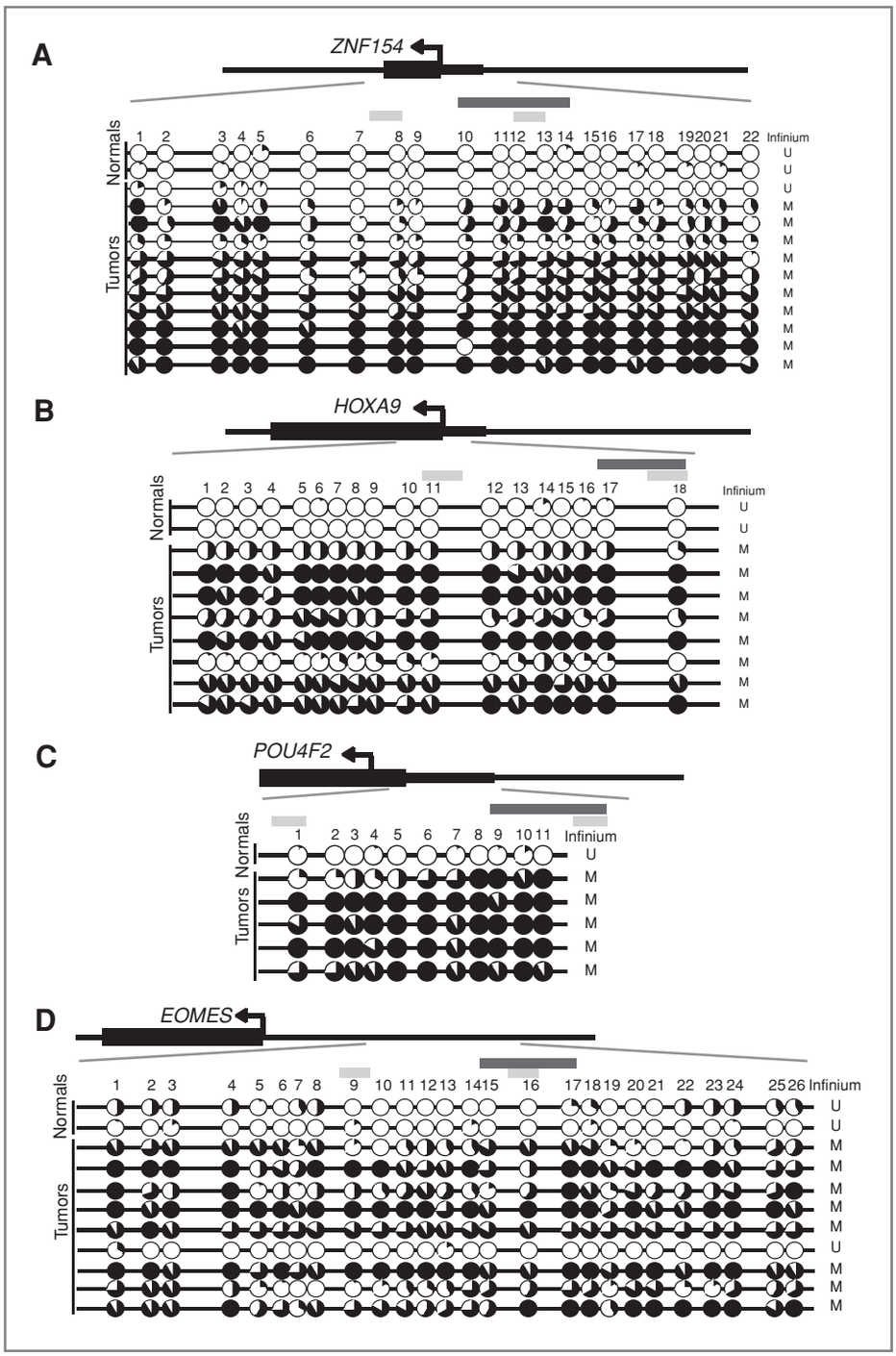


**Figure 2.** Analytic validation by bisulfite sequencing of the bladder tumor markers *ZNF154* (A), *HOXA9* (B), *POU4F2* (C), and *EOMES* (D). The top part of each panel provides a schematic representation of the transcription start site. The dark gray bar indicates Infinium probe annealing site and the light gray bars represent MS-HRM primer–binding sites. The numbers shows the CpG sites in the sequence. The column on the right side lists the methylation status of the gene (above or below cutoff point) reported by the Infinium array (U, unmethylated; M, methylated). On the left side, the sample type is given as normal or tumor. Each circle represents the average methylation of 10 to 12 clones. A hollow circle means no methylation, whereas a filled circle means 100% methylated.

were related to cell–cell signaling, in particular negative regulators of cell death ($P < 0.038$). Using IPA, the main network–associated functions altered by methylation were cell movement of eukaryotic cells ($P = 1.65E-010$), tumorigenesis ($P = 3.37E-08$), and growth of cancer cells ($P = 4.46E-07$; Supplementary Table S9) as well as apoptosis ($P < 1.24E-06$) and proliferation of cells ($P < 3.91E-06$). The top canonical pathway was G-protein–coupled receptor signaling ($P = 9.96E-06$ to $P = 1.56E-02$; Supplementary Table S9). Pathway analysis on superficial papillomas of low histologic grade versus high-grade superficial and invasive tumors showed that many of the top networks identified between Ta stable and Ta progressing tumors were also present in this analysis (Supplementary

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**Table 3.** Association between methylation markers and stage and grade in the validation set

| | *ZNF154* | *HOXA9* | *POU4F2* | *EOMES* | *CA3* | *PCDHGA12* | *ACOT11* | *PTGDR* |
|---|---|---|---|---|---|---|---|---|
| Stage | | | | | | | | |
| pTa | 84% (21/24) | 83% (19/23) | 92% (23/25) | 68% (17/25) | 92% (22/24) | 92% (23/25) | 79% (19/24) | 44% (11/25) |
| pT1 | 100% (15/15) | 100% (15/15) | 100% (15/15) | 93% (14/15) | 100% (15/15) | 93% (14/15) | 100% (15/15) | 80% (12/15) |
| pT2–4 | 100% (15/15) | 87% (13/15) | 100% (15/15) | 87% (13/15) | 100% (15/15) | 100% (15/15) | 100% (15/15) | 67% (10/15) |
| $P^a$ | 0.184 | 0.303 | 0.495 | 0.153 | 0.497 | 0.786 | 0.049 | 0.079 |
| Grade | | | | | | | | |
| I | 67% (4/6) | 100% (6/6) | 100% (6/6) | 50% (3/6) | 67% (4/6) | 83% (5/6) | 100% 6/6) | 33% (2/6) |
| II | 95% (18/19) | 88% (15/17) | 95% (18/19) | 79% (15/19) | 100% (18/18) | 95% (18/19) | 78% (14/18) | 58% (11/19) |
| III–IV | 97% (28/29) | 90% (26/29) | 97% (28/29) | 86% (25/29) | 100% (29/29) | 97% (28/29) | 97% (28/29) | 69% (20/29) |
| *P* | 0.087 | 1.000 | 1.000 | 0.165 | 0.011 | 0.342 | 0.095 | 0.243 |

NOTE: Methylation values were dichotomized as positive or negative. The frequency of methylation is shown as well as the number of methylation-positive tumors and the total number of tumors.
[a]Fisher's exact test.

Table S9). These results suggest that methylation may hit selected networks and pathways at multiple levels, thereby impacting the malignant process.

**Epigenetic regulation of keratin, keratin-associated proteins, and small proline-rich proteins**

We found that chromosome 21 encompasses more differentially methylated genes outside CpG islands, than any other chromosome after correction for number of CpG sites ($P < 0.0001$; Supplementary Fig. S7). Chromosome 21 furthermore contains many genes encoding keratin-associated proteins (KRTAP). In 16 of these, hypomethylation was detected ($\Delta\beta < -0.25$ and $P < 0.0001$ to $P = 0.019$), and 3 of the genes (*KRTAP13-1*, *KRTAP19-2*, and *KRTAP20-2*) had significantly ($P < 0.05$) increased transcript expression. We have previously shown a set of keratin-related genes to be upregulated in bladder cancer and associated with squamous cell metaplasia (38). Analysis of this set showed the small proline-rich proteins (SPRR) 1A/2D/3 on chromosome 1 to be hypomethylated in cancer and SPRR3 expression to be upregulated ($P < 0.0001$). Of the neutral keratins located on chromosome 12, 5 showed hypomethylation KRT2A/6B/6C/7/8 ($\Delta\beta < -0.25$ and $P = 0.0001$ to $P = 0.0022$) and KRT6B/7/8 showed increased expression ($P < 0.05$). The acidic keratins on chromosome 17 showed hypomethylation of KRT10/19/20 and upregulated expression of KRT20 ($P = 0.0027$). The Pearson correlations between methylation and expression were $-0.84$, $-0.50$, $-0.66$, and $-0.91$ for KRT7/8/19/20, respectively. Thus, the keratins and keratin-related proteins seem to be epigenetically regulated in bladder cancer.

## Discussion

This study mapped details of the methylome in bladder cancer. We used microarrays to investigate aberrant DNA methylation at 27,000 CpG sites. We were able to identify (i) stability of methylation over time in metachronous tumors, (ii) distinct stage-related events inside and outside CpG islands, (iii) chromosome 21 as major epigenetic target in bladder cancer, (iv) novel methylation markers for bladder cancer, stage markers, and candidate markers of

**Table 4.** Performance of the methylation assays for *ZNF154*, *HOXA9*, *POU4F2*, and *EOMES* on DNA from urine specimens from tumor patients

| Gene | Sensitivity, % (pos./total[a]) | Specificity, % (neg./total[a]) | AUC (95% CI) | PPV, % | NPV, % | $P^b$ | κ |
|---|---|---|---|---|---|---|---|
| *ZNF154* | 62 (68/110) | 100 (57/57) | 0.84 (0.79–0.89) | 100 | 58 | <0.0001 | 0.94 |
| *POU4F2* | 66 (75/113) | 100 (54/54) | 0.88 (0.84–0.93) | 100 | 59 | <0.0001 | 0.89 |
| *HOXA9* | 74 (79/107) | 96 (46/48) | 0.84 (0.78–0.90) | 98 | 63 | <0.0001 | 0.95 |
| *EOMES* | 68 (69/101) | 100 (40/40) | 0.89 (0.85–0.93) | 100 | 56 | <0.0001 | 0.89 |
| Combined | 84 (94/112) | 96 (50/52) | 0.90 (0.86–0.94) | 98 | 74 | <0.0001 | N/A |

NOTE: Samples with readings from at least 3 of 4 markers were included in the combined panel of markers.
Abbreviation: N/A, not applicable.
[a]Some urines provided small amount of DNA, not sufficient for all analysis.
[b]Mann–Whitney *U* test.

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disease progression all validated using an independent technique on an independent sample set, (v) detection of methylation in DNA from voided urine using novel tumor marker candidates, and (vi) a general relation between methylation and keratin transcript levels.

Many genes have been reported to be hypermethylated in bladder cancer, but it is just recently that studies with new screening approaches have identified methylation markers with high sensitivity and specificity (14, 34, 35, 39, 40). Using the Infinium array, we identified genes being hypo- or hypermethylated in bladder cancer (Supplementary Table S10). From a list of 108 genes previously reported hypermethylated in bladder cancer, 89 genes were present on the Infinium array and 32 showed methylation (Supplementary Table S11). There was a high degree of agreement (70%) between the genes reported by Renard and colleagues and our findings (32). Similar concordance was observed between the study by Wolff and colleagues using Illuminas Golden Gate technology and the Infinium array (40). Discrepancies between our results and previous results may have several reasons; the most likely explanations are position of CpG site investigated and a more conservative threshold in this study. To obtain information on the exact positions of the DNA methylation will require other methods, for example, bisulfite sequencing or next-generation sequencers that provide data at single-nucleotide resolution. Such data may have a better correlation to gene expression, especially if combined with data on nucleosome positioning.

We identified several markers well suited for urine-based detection of bladder cancer. The combination of *TWIST* and *NID2* was reported to have a sensitivity and specificity of 90% and 93%, respectively (32). Recently, 3 other novel markers (*GDF15*, *TMEFF2*, and *VIM*) have been reported to have a sensitivity of 94% and a specificity of 90% to 100%. Other marker studies include a 5-gene panel (sensitivity 75%, specificity 97%) and recently a 3-gene panel (sensitivity 75%–85%; refs. 31, 33). In our opinion, the urinary markers of early bladder cancer detection reported in this article contribute significant novel data toward developing a noninvasive test for bladder cancer. All studies mentioned above have a higher sensitivity than cytology and specificity equal or slightly lower than cytology. One way of improving the already sensitive bladder cancer detection assay is to combine methylation and mutational analysis as done by Serizawa and colleagues (35). In their study, they discovered an inverse correlation between methylation and *FGFR3* mutations. Because the 4 markers reported in this study are all associated, it is not unlikely that utilizing methylation or genetic markers without such association could improve the sensitivity. This requires that the tumors we failed to detect did not fail because of lack of tumor cells in the urine but was caused by a tumor with no methylation on those 4 genes. Furthermore, urine tests are less stressful to the patients and may provide important information for the urologist before a cystoscopy.

Several groups have reported methylation markers of progression (14, 20, 23, 24, 41, 42). We identified and validated *TBX4* as a promising candidate of disease progression, but in addition to *TBX4*, we also found markers reported previously. A comparison of previously reported markers and our findings is in Supplementary Table S12.

Some of the stage markers and the candidate markers of disease progression that we identified with the Infinium array failed the validation process (Supplementary Table S3). The most likely explanation for this is the difference in CpG sites analyzed using different techniques. Another explanation may be the small groups in the discovery phase of the experiment.

We identified a panel of 4 novel urinary methylation markers. The *HOXA9* gene located at chromosome 7p15.2 has been reported methylated in early lung cancer, where it was methylated in 8 of 10 (80%) stage 1 tumors (43). In bladder cancer, we were able to detect HOXA9 methylation in 83% of Ta tumors and in 75% of the urine specimens from patients with Ta tumors. The *ZNF154* gene is located at chromosome 19q13.43 and encodes a transcription factor belonging to the human zinc finger Krüppel family. No reports have been made about aberrant methylation of the gene, but the gene has been suggested to be deleted in thyroid adenomas (44). The *POU4F2* protein is a transcription factor encoded by a gene located at chromosome 4q31.23. *POU4F2* has been reported to be a multifunctional protein that interacts with cancer-related genes such as *BRCA1* and *TP53*. Depletion of *POU4F2* has been reported to confer cell resistance to apoptosis (45, 46). *EOMES* located at chromosome 3p24.1 is a novel methylation tumor marker in bladder cancer. The gene encodes a transcription factor involved in development processes and is silenced by methylation in diffuse large B-cell lymphomas (47). Several of the other genes that showed stage-specific methylation may also impact on the tumor progression, and future research should be devoted to the study of their exact biological function in bladder cancer progression.

The observed hypomethylation of *KRTAP*, *SPRR*, and keratins, which correlated very well with increased expression, indicated that transcription of these genes may be regulated by methylation. These genes seem to be involved in the squamous metaplasia often seen in bladder cancer and point to an epigenetic regulation of this relatively common phenomenon. It may be related to increased malignancy, as pure squamous cell carcinomas have a very poor prognosis. The coregulation of this set of genes, located at different chromosomes, is interesting and points to a common mechanism that drives their hypomethylation.

Chromosome 21 was the main target for methylation changes. This chromosome confers protection against cancer in trisomy 21 patients (48) but also houses a leukemia-related area (49). The massive alteration of keratin methylation we discovered is not located in any of the Down's syndrome or leukemia regions but indicates that chromosome 21 may play a hitherto underestimated role in bladder cancer.

Certain pathways of obvious relevance to cancer were differentially methylated to a larger extend than other pathways. This underscores the importance of the systems

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biology concept where several changes at different levels may lead to the same biological effect.

In conclusion, we have documented a number of methylation changes in bladder cancer among which some seem to form clinically useful urinary bladder cancer markers with a much better sensitivity than urinary cytology.

## Disclosure of Potential Conflict of Interest

T.F. Ørntoft is a molecular consultant for Aarhus University Hospital.

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