Serum DNA Hypermethylation in Patients with Bladder Cancer: Results of a Prospective Multicenter Study

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Abstract. *Background: Cell-free serum DNA levels are increased in patients with cancer, and at least partially, these DNA fragments are derived from cancer cells. A few reports indicated that methylated serum DNA in patients with bladder cancer (BCA) is a useful non-invasive biomarker. The purpose of this prospective multicenter study was to validate earlier studies. Materials and Methods: In total, 227 consecutive participants (non-muscle invasive BCA, n=75; muscle-invasive BCA, n=20; transurethral bladder resection (TURB) without BCA, n=48; benign disease, n=31; healthy individuals, n=53), were recruited for this study. Cell-free serum DNA was isolated and digested with methylation-sensitive restriction-enzymes (Bsh1236I, HpaII and HinP1I) to quantify the amount of methylated (TIMP3, APC, RARB, TIG1, GSTP1, p14, p16, PTGS2 and RASSF1A) DNA fragments. Results: The amount of methylated DNA was usually small (<10%), and the methylation frequencies varied for different genes (e.g. frequent: TIMP3; moderate: APC, RARB, TIG1; infrequent: p16, PTGS2, p14, RASSF1A, GSTP1). Methylation levels at each gene site and the number of methylated genes were increased in BCA compared to healthy individuals, but were similar in BCA and patients with non-malignant disease. The number of methylated genes allowed for discrimination (62% sensitivity, 89% specificity) of BCA patients from healthy individuals. DNA hypermethylation was not correlated with advanced stage or grade in patients with BCA. Conclusion: The detection of hypermethylated DNA in serum allows for discrimination of patients with BCA and healthy individuals, but there is no difference between patients with BCA and those with non-malignant disease, thereby limiting its value as a non-invasive biomarker.*

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Key Words: Bladder cancer, hypermethylation, cell-free DNA, serum.

Epigenetic alterations (*e.g.* DNA hypermethylation and histone modifications) are common during bladder carcinogenesis (1, 2). It was shown that DNA CpG island hypermethylation occurs at various genes [*e.g.* adenomatous-polyposis-coli (*APC*), 21-100% (3-6), glutathione S-transferase pi gene (*GSTP1*), 1-11% (3, 4), alternative protein product of the CDKN2A locus (p14), 10-31% (7, 8), cyclin-dependent kinase inhibitor-2A (p16), 1-40% (4, 7-9), retinoic acid receptor beta gene (*RARB*), 10-75% (3, 4, 6, 8) and 32-73% (3-5, 8, 10), tissue inhibitor of metalloproteinase-3 (*TIMP3*) (20% (11)] in urothelial bladder cancer (BCA), and allows distinguishing of malignant from normal bladder tissues. Furthermore, DNA methylation correlated with recurrence in non-muscle invasive BCA [*e.g. p14* (12), apoptotic peptidase activating factor-1 (*APAF-1*), insulin-like growth factor binding protein-3 (*IGFBP3*) (13), *TIMP3* (11)] and overall survival in muscle-invasive BCA [*e.g.* runt-related transcription factor-3 (*RUNX3*) (14), secreted frizzled-related protein (*SFRP*) (15)]. Thus, DNA hypermethylation may be used as a diagnostic and prognostic biomarker. An increase of cell-free circulating serum DNA was described for patients with malignancy, including patients with BCA (16, 17). In 2002, Valenzuela *et al.* reported the highly specific detection of methylated *p16* DNA in the serum of patients with BCA (18), while only few studies have investigated serum DNA hypermethylation in patients with BCA. Ellinger *et al.* reported a high frequency of methylation at *APC*, *GSTP1* and retinoic acid receptor responder (tazarotene-induced)-1 (*TIG1*) (combined sensitivity 80%) and an increased BCA-specific mortality in patients with APC methylation (17, 19). Lin *et al.* reported cadherin-13 (*CDH13*) methylation in 30% of patients with BCA, with higher methylation frequencies in patients with advanced disease (19). Given the potential diagnostic and

prognostic role of serum DNA hypermethylation, we analyzed DNA hypermethylation patterns at *APC*, *GSTP1*, *p14*, *p16*, *RARB*, *RASSF1A* and *TIMP3* in a prospective, multicenter cohort of patients with BCA.

Materials and Methods

Patients and sample collection. The cohort of this prospective multicenter study was previously described (16). In brief, serum samples from 227 consecutive patients at four urological departments (University Hospital Bonn, Sankt-Josef Hospital Troisdorf, Evangelische Kliniken Bonn, Katholische Kliniken Oberberg) were collected between November 2006 and July 2007. Among these patients, 132 underwent transurethral resection of the bladder (TURB; presence of urothelial BCA in 84 patients) and 11 patients underwent radical cystectomy; 31 patients with non-malignant urological disorders and cystoscopically excluded BCA (*e.g.* surgery for incontinence or benign prostate hyperplasia) and 53 healthy individuals served as controls. Patients with history of cancer (except for BCA) were excluded. All patients gave written informed consent; the study was approved by the Ethics Committee of the University of Bonn (vote number 019/03). See Table I for clinical information of the study patients. Blood withdrawal was performed before surgery in a Serum-S Monovette with clotting activator (Sarstedt, Nürnbrecht, Germany). Centrifugation (1800 x*g*, 10 min) was performed after clotting (30-240 minutes), and serum was then separated and stored at –20˚C. The samples were shipped to the University Hospital Bonn on dry ice one week following collection, and stored thereafter at –80˚C.

DNA isolation and methylation-sensitive (PCR). Cell-free DNA was isolated from 1 ml serum using the ChargeSwitch gDNA Kit (Invitrogen, Paisley, Scotland, UK), as described in detail before (20); the final elution volume was 50 μl. Twenty microliters of DNA eluate was digested with methylation-sensitive restriction enzymes (*Bsh*1236I, recognition site: CGCG; *Hpa*II, CCGG; *HinP*1I, GCGC); the enzymes cut the unmethylated DNA sequence, whereas methylation prevents cutting. Thus, a target sequence may only be amplified in a subsequent PCR if all restriction sites are methylated. DNA was digested with 20 U of Bsh1236I, HpaII and HinP1I (Fermentas, St. Leon-Rot, Germany) in a total volume of 45 μl 1X Tango Buffer at 37˚C (4 h). To ensure complete digestion, an additional 5 U of each enzyme were added and incubated for 14 h followed by inactivation at 65˚C for 20 min. The detection of DNA methylation was essentially performed using a PCR as described earlier (20). The PCR primers covered at least one restriction site of *Bsh*1236I, *Hpa*II or *HinP*1I in the promoter CpG island of *APC*, *GSTP1*, *p14*, *p16*, *PTGS2* and *RASSF1A*. An *ACTB* primer without any restriction sites was used to determine the amount of input DNA. Relative DNA methylation levels were calculated as the methylation ratio (MR; ratio of methylated target templates to *ACTB* templates in a sample). Quantitative real-time PCR was carried out in triplicate on an ABIPrism 7900HT (Applied Biosystems, Foster City, CA, USA). Each 10-μl reaction consisted of 1× SYBRGreenER (Invitrogen), 200 nmol forward/reverse primer and 1 μl of digested DNA. PCR was carried out by the following procedure: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. Melting curve analysis was performed to confirm specificity of the PCR products. The copy number was determined using a standard curve. Each run included a positive control (*SssI*-treated DNA) and water blanks.

Statistical analysis. Methylation levels and clinicopathological parameters were correlated using the Mann-Whitney test or the Chi-square test, were appropriate. The area under the curve (AUC), sensitivity and specificity were determined by receiver-operating characteristic (ROC) analysis. Statistical tests were performed using the SPSS Statistics v20 (IBM Cooperation, Armonk, NY, USA). Significance was concluded at $p<0.05$.

Results

The amount of methylated DNA was determined using methylation-sensitive PCR; in patients with detectable methylated DNA, the amount was usually low, with a methylation ratio <10% (irrespective of the gene of interest). The methylation profile of the studied patients is displayed in Figure 1. The frequency of methylation varied among the target genes: *e.g. TIMP3* methylation was frequent (38-88%), *GSTP1* methylation was uncommon (2-20%); see Table II for detailed methylation frequencies. The methylation frequency of all target genes was significantly increased in patients with BCA compared to healthy individuals ($p=0.022$ to $p<0.001$); however, the methylation frequencies were similar in patients with BCA compared to those undergoing TURB without BCA ($p=0.357$-0.936) or with benign urological disease ($p=0.074$-0.960; except of *RASSF1A* $p=0.042$). The mean number of methylated genes was higher in BCA patients (mean=3.8 genes) compared to healthy individuals (mean=0.9 genes; $p<0.001$), but was similar to patients undergoing TURB without BCA (mean=4.0 genes; $p=0.203$) and patients with benign disease (mean=3.2 genes; $p=0.561$). The number of methylated genes allowed for discrimination with moderate sensitivity (62.1%) and high specificity (88.7%) of patients with BCA from healthy controls (AUC=0.825, 95% confidence interval=0.761-0.890). The ROC analysis for single-methylated genes was less informative, *i.e.* the AUC was between 0.738 (*TIMP3*) and 0.585 (*GSTP1*). Methylation frequencies were similar in patients with non-muscle invasive and muscle-invasive BCA ($p=0.145$-0.987) for all genes. Moreover, there was no correlation of serum DNA hypermethylation and any clinicopathological parameter (pT stage, grade, lymph node metastasis, smoking, age, sex; all $p>0.05$).

Discussion

The existence of cell-free tumor-specific DNA fragments in serum/plasma of patients with cancer is well-established (21). In patients with BCA, the presence of tumor-associated alterations [DNA hypermethylation, mutations, loss of heterozygosity (22)] in circulation was confirmed; DNA methylation in plasma and tissue correlated well (18).

Table I. *Clinicopathological parameters*.

	NMIBC n=75 (%)	MIBC n=20 (%)	TURB w/o BCA n=48 (%)	Benign disease n=31 (%)	Healthy control n=53 (%)
Age, years					
Mean	72.3	73.5	67.5	62.0	31.1
Median	74.0	77.0	69.0	65.0	28.0
Range	38-91	44-94	36-86	27-83	18-56
Gender					
Male	51 (68.0)	18 (90.0)	37 (77.1)	26 (83.9)	36 (67.9)
Female	24 (32.0)	2 (10.0)	11 (22.9)	5 (16.1)	18 (34.0)
Center					
UKB	17 (22.7)	5 (25.0)	11 (22.9)	28 (90.3)	53 (100)
EKB	3 (4.0)	3 (15.0)	6 (12.5)	2 (6.4)	0 (0)
SJH	33 (44.0)	9 (45.0)	18 (37.5)	1 (3.3)	0 (0)
KKO	22 (29.3)	3 (15.0)	13 (27.1)	0 (0)	0 (0)
History of BCA	45 (60.0)	10 (50.0)	13 (27.1)	0 (0)	0 (0)
Smoker status					
Non-smoker	24 (32.0)	2 (10.0)	12 (25.0)	4 (12.9)	0 (0)
Former smoker	32 (42.7)	10 (50.0)	21 (43.8)	0 (0)	2 (3.8)
Smoker	15 (20.0)	3 (15.0)	11 (22.9)	0 (0)	6 (11.3)
Unknown	4 (5.3)	5 (25.0)	4 (8.3)	27 (87.1)	45 (84.9)
Stage					
pT0	1 (1.3)	0 (0)	n.a.	n.a.	n.a.
pTis	3 (4.0)	0 (0)	n.a.	n.a.	n.a.
pTa	48 (64.0)	0 (0)	n.a.	n.a.	n.a.
pT1	22 (0)	0 (0)	n.a.	n.a.	n.a.
pT2	0 (0)	15 (75.0)	n.a.	n.a.	n.a.
pT3	0 (0)	4 (20.0)	n.a.	n.a.	n.a.
pT4	0 (0)	1 (5.0)	n.a.	n.a.	n.a.
LNM	0 (0)	6 (30.0)	n.a.	n.a.	n.a.
Grade					
G1	17 (22.7)	0 (0)	n.a.	n.a.	n.a.
G2	40 (53.3)	2 (10.0)	n.a.	n.a.	n.a.
G3	18 (24.0)	18 (90.0)	n.a.	n.a.	n.a.

NMIBC, Non-muscle-invasive bladder cancer; MIBC, muscle-invasive bladder cancer; TURB w/o BCA, patients undergoing transurethral resection with histological exclusion of bladder cancer; LNM, lymph node metastasis; n.a., not applicable; UKB, University Hopital Bonn; KKO, Katholische Kliniken Oberberg; EKB, Evangelische Kliniken Bonn; SJH, Sankt-Josef-Hospital, Troisdorf, all Germany.

Examination of DNA alterations in serum allows for conclusions to be drawn on the characteristics of the primary tumor and, thus, they may be regarded as useful biomarkers. To date, only few, small-scaled and single-center studies reported findings on cell-free circulating DNA hypermethylation in patients with bladder cancer: Domínguez *et al*. reported hypermethylation frequencies at *p14* (56%) and *p16* (18%) in plasma samples; in addition, hypermethylation at *p14* was associated with tumor size, multifocality and BCA recurrence (22). Valenzuela *et al*. reported a similar frequency of *p16* methylation (22%) in serum from patients with BCA (18), while only one patient with benign disease exhibited methylated *p16*. Ellinger *et al*. investigated methylation in the serum of patients with muscle-invasive BCA; a multigene pattern [*APC*, *GSTP1*, *TIG1*, death-associated protein kinase-1 (*DAPK1*), *PTGS2*] allowed sensitive (80%) and specific (93%) discrimination from controls, and methylation of *APC* was prognostic for poor BCA-specific survival (17). Recently, Lin *et al*. reported that serum cadherin-13 (*CDH13*) was specifically methylated in 31% of patients with BCA, and correlated with clinicopathological parameters (tumor size, stage and grade) and BCA recurrence (19). Jablonowski *et al*. studied patients with non-muscle-invasive BCA, and reported methylation frequencies of 64% for DAPK and 41% for p16 (9). We, thus, investigated this hopeful approach in a prospective, multicenter study, and studied-methylation profile instead of the single the gene to increase the sensitivity of our assay. Indeed it was possible to increase the sensitivity of the assay if multiple genes were used: A threshold of two genes allowed for patients with BCA to be distinguished from healthy individuals, with a sensitivity of 61% and a specificity of 89%. However, methylation of the

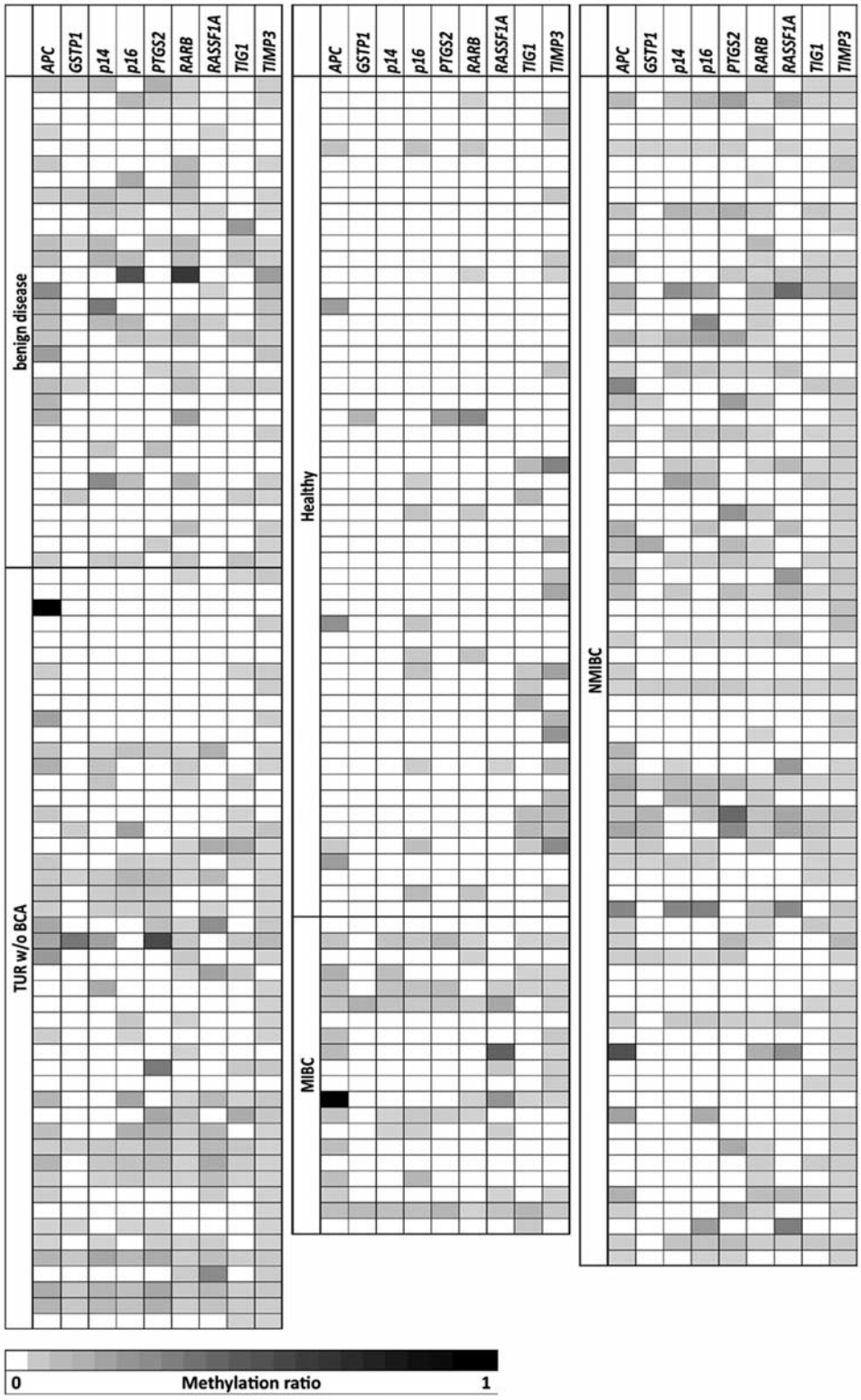

Figure 1. *Quantitative analysis of cell-free serum DNA hypermethylation at the promoter region of APC, adenomatous-polyposis-coli; GSTP1, glutathione S-transferase pi gene; p14 (ARF), alternative protein product of the CDKN2A locus; p16 (INK4a), cyclin-dependent kinase inhibitor 2A; PTGS2, prostaglandin-endoperoxide synthase-2; RARB, retinoic acid receptor beta gene; RASSF1A, Ras association (RalGDS/AF-6) domain family member 1; TIG1, retinoic acid receptor responder (tazarotene induced) 1 TIMP3, tissue inhibitor of metalloproteinase-3; The methylation ratio was scaled as no methylated DNA (white boxes) to fully methylated DNA (black boxes).*

Table II. *Methylation frequencies*.

Gene	NMIBC n=75 (%)	MIBC n=20 (%)	TURB w/o BCA n=48 (%)	Benign disease n=31 (%)	Healthy control n=53 (%)
APC	39 (52.0)	13 (65.0)	25 (52.1)	16 (51.6)	5 (9.4)
GSTP1	14 (18.7)	4 (20.0)	13 (27.1)	6 (19.4)	1 (1.9)
p14	22 (29.3)	7 (35.0)	15 (31.2)	10 (32.3)	0 (0)
p16	26 (34.7)	7 (35.0)	17 (35.4)	10 (32.3)	9 (17.0)
PTGS2	24 (32.0)	5 (25.0)	18 (37.5)	9 (29.0)	1 (1.9)
RARB	46 (61.3)	9 (45.0)	27 (56.2)	17 (54.8)	7 (13.2)
RASSF1A	22 (29.3)	8 (40.0)	17 (35.4)	4 (12.9)	1 (1.9)
TIG1	33 (44.0)	8 (40.0)	23 (47.9)	8 (25.8)	8 (15.1)
TIMP3	66 (88.0)	15 (75.0)	38 (79.2)	22 (71.0)	20 (37.7)

NMIBC, Non-muscle-invasive bladder cancer; MIBC, muscle-invasive bladder cancer; TURB w/o BCA, patients undergoing transurethral resection with histological exclusion of bladder cancer. *APC*, adenomatous-polyposis-coli; *GSTP1*, glutathione S-transferase pi gene; *p14* (*ARF*), alternative protein product of the CDKN2A locus; p16 (*INK4a*), cyclin-dependent kinase inhibitor-2A; PTGS2, prostaglandin-endoperoxide synthase-2; *RARB*, retinoic acid receptor beta gene; *RASSF1A*, Ras association (RalGDS/AF-6) domain family member-1; *TIG1*, retinoic acid receptor responder (tazarotene induced) 1 *TIMP3*, tissue inhibitor of metalloproteinase-3.

examined genes was also frequently observed in patients undergoing TURB without detection of BCA and patients with non-malignant disease. Unfortunately, follow-up information was not available and we cannot exclude that BCA was subsequently diagnosed in the patients. However, the value of DNA methylation is distinctly lowered. It should be noted that DNA methylation also of typical tumor suppressor genes, occurs in non-malignant diseases, and furthermore aging, nutrition and lifestyle factors contribute to altered DNA methylation. For example, methylated cell-free serum DNA fragments (*e.g. RASSF1A* and *p16*) were also found in patients with hepatitis C (23, 24). Most studies employed a bisulfite-based assay for the detection of methylated DNA. It was shown that up to 90% of DNA is lost during sample during bisulfite-treatment (25). Thus, the sensitivity may be limited due to loss of target sequences. In contrast, we treated the DNA with methylation-sensitive restriction enzymes, which cut un-methylated target sequences; this technique is less prone to DNA degradation and therefore more sensitive. However, specificity may be a cause of concern in this detection method. We argue for the specificity of our approach because the detection of methylated DNA sequences was not correlated with the amount of recovered DNA. Furthermore, un-methylated DNA samples had negative signals in our former study (20). In contrast to former studies, we also could not observe a correlation of DNA hypermethylation and prognostically-relevant parameters.

Conclusion

The detection of hypermethylated DNA in serum allows for the discrimination of patients with BCA from healthy individuals, but there is no difference between patients with BCA and those with non-malignant disease. Furthermore, serum DNA hypermethylation was not associated with advanced stage/grade. Thus, the value of DNA methylation as non-invasive biomarker for patients with BCA is limited.

Acknowledgements

The Authors would like to thank Ms. Doris Schmidt for her excellent technical assistance.

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Received January 12, 2013
Revised February 18, 2013
Accepted February 18, 2013