Genome-Wide DNA Methylation Profiling in Bladder cancer and Identification of Diagnostic and Prognostic Markers

Raju Kandimalla

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Genoomwijde profilering van DNA methylatie in blaaskanker en identificatie van diagnostische en prognostische merkers

Thesis

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By

Raju Kandimalla
Born in India

Doctoral Committee

Promotor: Prof.dr. E.C. Zwarthoff

Other members: Prof.dr. C.H. Bangma
Dr. P.M.J.J. Berns
Dr. G.J.L.H. Van Leenders

Copromotor: Dr. A.G. Van Tilborg

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Abbreviations

AUC	Area under the curve
BC	Bladder cancer
BS-SNaPshot	Bisulfite specific single nucleotide primer extension
CGI	CpG island
CIMP	CpG island methylator phenotype
CIS	Carcinoma *in situ*
CpG	Cytosine phosphate Guanine
DAC	Decitabine
DMH	Differential methylation hybridization
DNMT	DNA methyltransferase
EAU	European association of urology
EORTC	European Organisation for Research and Treatment of Cancer
GGMA	Golden gate methylation assay
GWS	Genome-wide study
HDAC	Histone deacetylase
LOOCV	Leave one out cross-validation
MIBC	Muscle-invasive bladder cancer
NMIBC	Non-muscle invasive bladder cancer
NPV	Negative predictive value
PCA	Principal component analysis
PcG	Polycomb group
PPV	Positive predictive value
PreTUR	Before *trans*-urethral resection
ROC	Receiver operating characteristic curve
TSA	Trichostatin
TUR	*Trans*-urethral resection

Chapter 1

General introduction and outline of the thesis

Introduction and scope of the thesis

1.1 Bladder cancer: clinical characteristics

Bladder cancer (BC) is the fifth most common cancer in the western world after prostate, breast, lung and colorectal cancer with an estimated 386,300 new cases and 150,200 deaths in the year 2008 worldwide. The majority of bladder cancers occur in men, with a male: female ratio of 3:1 [1]. The incidence is about 20 new cases per year per 100,000 people in the U.S [1]. BC has been associated with exposure to a number of environmental factors. Smoking is the most important risk factor associated with bladder cancer [2]. Other risk factors are contact with heterocyclic amines in several industrial settings such as rubber manufacturing, petrochemical industry, dyestuffs and textile printing [3]. The most common symptom of bladder cancer is the development of haematuria (blood in the urine). This may be either macroscopic (visible to the eye) or microscopic (only detected by laboratory testing). Other symptoms include more frequent urination, dysuria (pain or burning sensation) and urgency. These symptoms do not always indicate bladder cancer. More often they are caused by infections, benign (non-cancerous) tumours, stones in the kidney or bladder, or other benign kidney diseases. If a bladder tumour is suspected cystoscopy (Figure 1) is performed, which is sometimes combined with cytology. More than 90% of BCs are urothelial cell carcinomas (UCCs). The other histological types encompass squamous cell carcinoma (6-8%) and adenocarcinoma (1-2%). In Egypt and other countries where endemic spread of schistosomiasis is more common, squamous cell carcinoma is the predominant subtype. The majority of UCC is found in the bladder. UCC of the renal pelvis, ureter and urethra accounts for less than 10% of the carcinomas.

Most bladder tumours (70-80%) are non-muscle invasive BC (NMIBC) at the time of initial diagnosis and have a good prognosis [4]. NMIBC comprises stages pTa, pT1 and pTis (Figure 2). Unfortunately, 70% of the NMIBC cases will recur after transurethral resection (TUR), and 10-20% will eventually progress to muscle-invasive BC (MIBC) [5-7]. MIBC patients and those who progress to MIBC have a poor prognosis with a 5 years survival of 10 to 50% depending on stage [8], in spite of radical cystectomy and chemotherapy. Somatic mutations in the *FGFR3* gene accompanied by losses of chromosome 9 are more frequent in NMIBC, while *TP53* mutations are associated with MIBC [9-11]. Based on the known genetic alterations a two pathway model for BC pathogenesis was described, which is depicted in Figure 3 [12, 13]. Besides these genetic aberrations, alterations in the epigenetic landscape like DNA methylation, histone modifications and nucleosome remodeling are associated with bladder cancer [14, 15]. At this moment, BC treatment strategies and follow-up mostly depend on the Tumor, Node, Metastasis classification (TNM, Table 1) and grading proposed by the World Health Organization (WHO) in 1973 and 2004 (Table 2) [16].

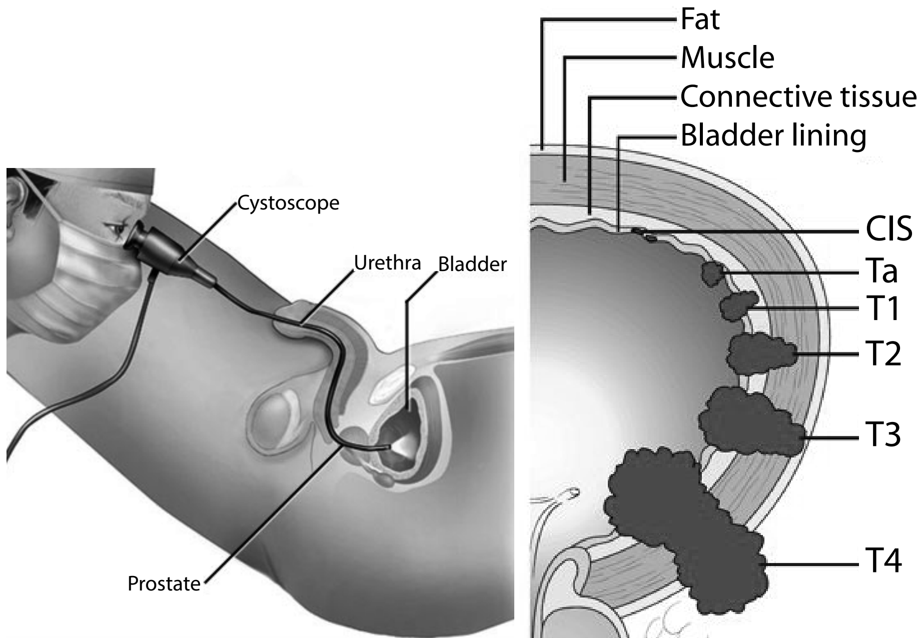

Figure 1: Diagram of a flexible cystoscopy performed on a male patient. The lubricated cystoscope is passed through the urethra and fills the bladder with water. The lighted tip allows the surgeon to view the inner wall of the bladder. *Illustration by Mayo Foundation for Medical Education and Research.*

Figure 2: Stage classification of bladder cancer. CIS: carcinoma in situ; Ta: Non-invasive papillary carcinoma; T1: Tumor invades connective tissue. T2: Tumor invades muscle. T3: Tumor invades fat layer. T4: Tumor invades other organs like prostate, uterus or vagina. CIS , Ta and T1 are NMIBC and T2, T3 and T4 are MIBC. *Illustration by CancerHelp UK, the patient information website of Cancer Research UK: www.cancerhelp.ork.uk*

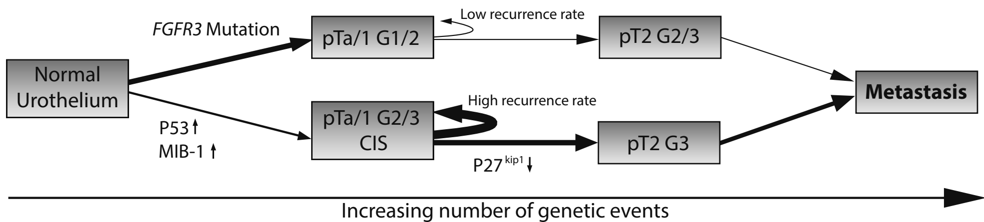

Figure 3: Two-pathway model for disease pathogenesis of bladder cancer. Arrow thickness indicates the percentage of tumours. Chromosomal alterations are represented by the bottom arrow.11,13,14,21,38–43 *FGFR3*, fibroblast growth factor receptor 3 gene; ↑, increased expression (MIB-1 and P53); ↓, reduced expression (P27kip1); CIS, carcinoma-in-situ. *This figure is adapted from van Rhijn B W et al. JCO 003;21:1912-1921*

Table 1: 2009 TNM classification of urinary bladder cancer (from EAU guidelines)

T	Primary tumour
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma in situ: 'flat tumour'
T1	Tumour invades subepithelial connective tissue
T2	Tumour invades muscle
	T2a Tumour invades superficial muscle (inner half)
	T2b Tumour invades deep muscle (outer half)
T3	Tumour invades perivesical tissue:
	T3a Microscopically
	T3b Macroscopically (extravesical mass)
T4	Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall
	T4a Tumour invades prostate, uterus, or vagina
	T4b Tumour invades pelvic wall or abdominal wall
N	**Lymph nodes**
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N2	Metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N3	Metastasis in a common iliac lymph node(s)
M	**Distant metastasis**
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 2: World Health Organisation grading in 1973 and in 2004; CIS = carcinoma in situ. (from EAU guidelines)

1973 WHO grading
Urothelial papilloma:
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated
2004 WHO grading
Flat lesions:
Hyperplasia (flat lesion without atypia or papillary)
Reactive atypia (flat lesion with atypia)
Atypia of unknown significance
Urothelial dysplasia
Urothelial CIS
Papillary lesions:
Urothelial papilloma (which is a completely benign lesion)
Papillary urothelial neoplasm of low malignant potential
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma

NMIBC is treated by transurethral resection (TUR), followed by one cycle of chemotherapy. High grade (G3) and T1 tumours are further treated with bacillus Calmette Guérin (BCG) instillations to delay recurrence and to prevent progression. MIBC is treated with cystectomy, radiation or chemotherapy to reduce the disease-specific mortality.
In principle, the three major risks for patients with NMIBC after initial treatment include tumor recurrence, progression to a higher grade or stage, and for MIBC death due to metastatic disease. Conventional clinical and pathologic parameters are widely used to grade and stage tumors in order to predict clinical outcome of BC. For NMIBC risk scores for recurrence and progression have been developed by Sylvester [17] and these have been taken up in the EAU guidelines [18]. (Table 3 and 4). However, these risk scores were based on a cohort of patient from the nineteen eighties, a time when TUR was not followed by an initial installation of a chemotherapeutic drug such as mitomycin C and when treatment of patients with high risk NMIBC with BCG was not carried out yet. In addition, especially grading has been shown to be rather subjective with considerable interobserver variation [19]. Molecular markers such as the specific point mutations in the *FGFR3* gene that occur in over 75% of stage Ta tumours have been associated with a relatively benign disease course [10]. Moreover, reproducibility of the *FGFR3* mutation assay was shown to be 100%. Besides the *FGFR3* mutations other molecular markers with similar performance would be very welcome to better predict disease course in NMI as well as in MIBC.
Cystoscopy is the gold standard for surveillance of patients with a previous bladder tumour, however, it is an invasive and uncomfortable procedure [20, 21]. Moreover, even with cystoscopy, there is 20 to 30% chance to miss a tumour [22, 23]. Due to the high recurrence rate, depending on the risk group of the patient, NMIBC patients are monitored by cystoscopy every 3-12 months after TUR in order to spot potential recurrences. This sums to an average of about 20 cystoscopies per patient in the first decade following removal of the primary tumour, thus making BC one of the most expensive cancers to treat [24]. Cytological examination of voided urine can identify tumour cells with a high sensitivity if a high-grade tumour is present [25]. However, for low stage and grade tumours the sensitivity is low. This low sensitivity induced the development of urine-based assays in the past decade [8]. In summary, these assays are based on immunological assays to detect tumour cells, differentially expressed genes, tumour-associated proteins and tumour-specific DNA alterations [26-29]. Three of these tests have been approved by the FDA (Federal Drug Administration, USA), namely NMP22, UroVysion, and ImmunoCyt [25, 30]. Although most tests have better sensitivity than urinary cytology, their specificity is lower and their sensitivity for low grade recurrent tumours is also insufficient. Hence, none of them have been accepted as a standard diagnostic procedure in routine urology to date. The *FGFR3* mutations test is an excellent diagnostic test for recurrent cancer in patients presenting with NMIBC if their primary tumour harbors a mutation. Evidently, other tests are required for those patients with *FGFR3* wild-type tumours.

Table 3: Weighting used to calculate recurrence and progression scores. (from EAU guidelines)

Factor	Recurrence	Progression
No. of tumours		
Single	0	0
2–7	3	3
≥8	6	3
Tumour diameter		
<3 cm	0	0
≥3 cm	3	3
Prior recurrence rate		
Primary	0	0
≤1 recurrence per year	2	2
>1 recurrence per year	4	2
Category		
Ta	0	0
T1	1	4
Concomitant CIS		
No	0	0
Yes	1	6
Grade (1973 WHO)		
G1	0	0
G2	1	0
G3	2	5
Total score	0–17	0–23

Table 4: Probability of recurrence and progression according to total score. CI = confidence interval. (from EAU guidelines)

Recurrence score	Probability of recurrence at 1 yr		Probability of recurrence at 5 yr		Recurrence risk group
	%	(95% CI)	%	(95% CI)	
0	15	(10–19)	31	(24–37)	Low risk
1–4	24	(21–26)	46	(42–39)	Intermediate risk
5–9	38	(35–41)	62	(58–65)	Intermediate risk
10–17	61	(55–67)	78	(73–84)	High risk

Progression score	Probability of progression at 1 yr		Probability of progression at 5 yr		Progression risk group
	%	(95% CI)	%	(95% CI)	
0	0.2	(0–0.7)	0.8	(0–1.7)	Low risk
2–6	1	(0.4–1.6)	6	(5–8)	Intermediate risk
7–13	5	(4–7)	17	(14–20)	High risk
14–23	17	(10–24)	45	(35–55)	High risk

1.2 Epigenetics

Conrad Waddington introduced the term epigenetics in the early 1940s [31]. Epigenetics refers to ''the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence'' [32, 33]. At present, there are two major components of the epigenome, which are DNA methylation and histone modifications. A great variety of post-translational covalent histone modifications (acetylation, methylation, phosphorylation etc.) are associated with actively transcribed or inactive chromatin (Figure 4) [33]. DNA methylation is mediated by a family of enzymes termed DNA methyltransferases (DNMTs). The components of the epigenome together function in highly dynamic processes, responsible for activation or deactivation of gene expression throughout the life span of a cell. Alterations of the epigenetic landscape in a normal cell can lead to malignant transformation and cause cancer. The epigenetic progenitor model of cancer described by Feinberg [34] claims that cancer develops in three steps. The first being an epigenetic alteration of a stem or progenitor cell within a given tissue and the second a gate-keeper mutation followed by genetic and epigenetic instability leading to further tumour evolution. This hypothesis is supported by studies that showed that epigenetic alterations precede the initial mutations in cancer [35-37].

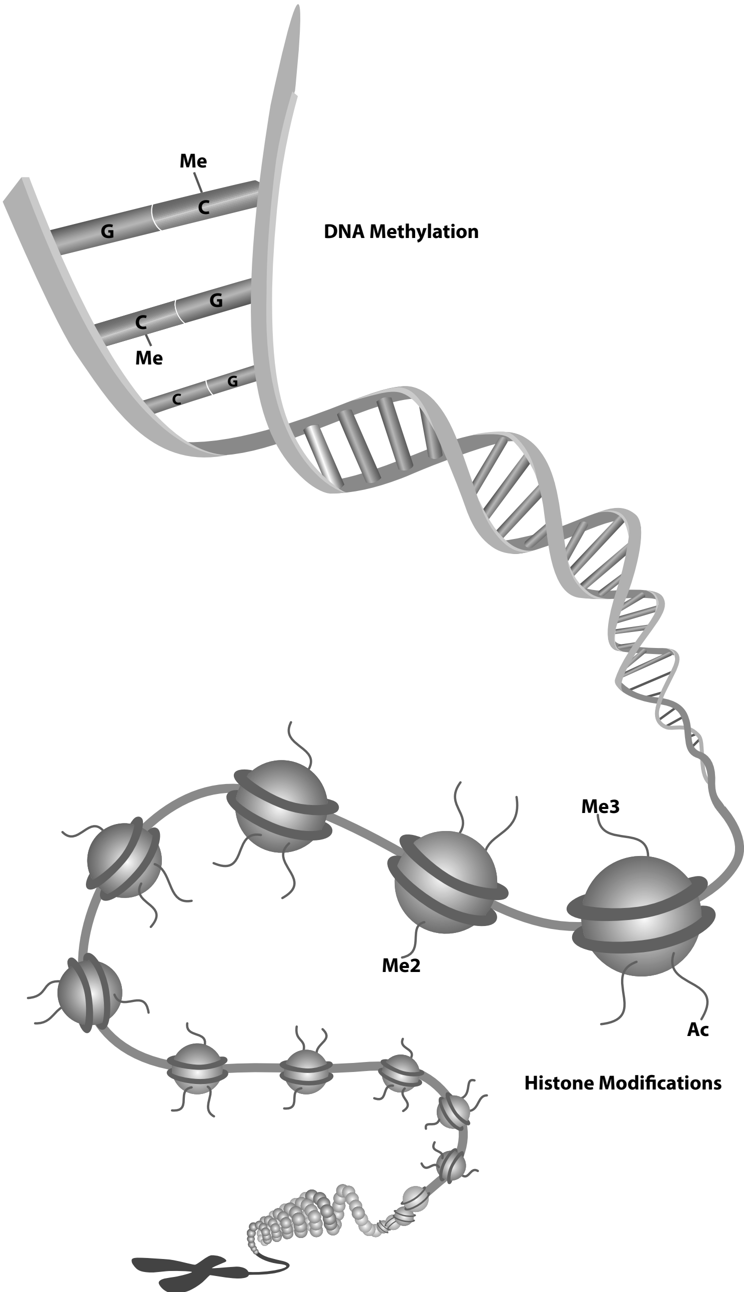

Figure 4: The two main components of the epigenetic code. DNA methylation and histone modifications. *This figure is adapted from Applied Biosystems.*

1.3 DNA methylation

DNA methylation is currently the most widely studied epigenetic aberration. DNA methylation is mediated by the cytosine DNA methyltransferase (DNMT) family of enzymes that catalyze the transfer of a methyl group from S-adenosyl methionine to cytosines, thereby creating a 5-methylcytosine (5 mC). This occurs only in the background of a cytosine (C) followed by a guanine (G) in the DNA. The CG dinucleotide is commonly known as CpG. DNMT1 is primarily responsible for maintaining the methylation signature by copying the pre-existing methylation pattern onto the daughter strand after DNA replication. DNMT3A and DNMT3B are *de novo* enzymes that target previously unmethylated CpGs [38, 39]. DNMT1 is the most abundant DNMT in the cell [40, 41].

DNA methylation is essential for normal development [42] and is associated with a number of key functions such as genomic imprinting [43], X-chromosome inactivation in females [44] and suppression of repetitive elements. CpG dinucleotides are not distributed uniformly throughout the human genome. Rather, they are most frequently found within dense CpG sequence stretches termed CpG islands, defined as "regions of more than 200 bases with a G + C content of at least 50% and a ratio of observed to statistically expected CpG frequencies of at least 0.6" [45]. Although CpG islands (CGIs) constitute only 1% of the total genome, they are present in >50% of human gene promoters indicating their role in regulation of gene expression.

DNA methylation of the promoter regions is generally associated with transcriptional repression through different mechanisms, including the inhibition of transcription factor binding and the recruitment of methyl-binding domain proteins (MBD1, MBD2 and MeCP2) and their associated complexes [46]. In normal cells, promoter CpG islands are usually unmethylated while the genome is globally methylated thereby preventing genome instability (Figure 5) [46].

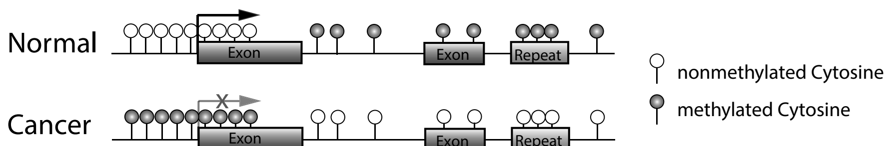

Figure 5: DNA methylation in normal and cancer cells. In normal cells, nearly all of the CpG dinucleotides are methylated whereas CpG islands, mostly residing in 5′ regulatory regions of genes, are unmethylated. In cancer cells, many CpG islands become hypermethylated, in combination with silencing of their associated genes, while global hypomethylation, mostly at repetitive elements, occurs.

Recently it is discovered that 5-mC can be converted into 5-hydroxymethylcytosine (5-hmC) by the 2-oxoglutarate– and Fe(II)-dependent oxygenases TET1, TET2 and TET3 [47]. These modifications have recently been detected in embryonic stem (ES) cells and Purkinje neurons and are shown to be involved in ES cell self-renewal and embryonic inner cell

mass specification. Conversion of 5-mC to 5-hmC results in reactivation of gene expression [48].
Cancers can be classified according to their degree of methylation, and those cancers with high degrees of methylation (CpG island methylator phenotype, or CIMP) represent a clinically and etiologically distinct group that is associated with 'epigenetic instability'. This mechanism was first described in colorectal cancer, but later on confirmed in many different cancers namely glioblastomas, gastric cancer, liver cancer, pancreatic cancer, esophageal cancer, ovarian cancer, acute lymphocytic leukaemia and acute myelogenous leukaemia [39, 49]. PcG complexes repress gene transcription by altering histone modifications around the transcription start site of genes involved in lineage specification during development. The altered histones can attract DNMTs and this can result in aberrant DNA methylation and irreversible shut down of the genes in question. It has been established that PcG target genes are frequently aberrantly hypermethylated by DNA methylation in cancer [50-52]. These studies revealed that PcG target genes are as much as 12 times more likely to be aberrantly silenced by DNA methylation in cancer than non-PcG target genes. It is therefore thought that hypermethylation in cancer occurs early in the tumorigenic process and may already be present in some cells during development.

1.4 Histone modifications

Histone modifications influence chromatin structure which plays an important role in gene regulation and carcinogenesis [53]. Chromatin is a highly ordered structure consisting of repeats of nucleosomes connected by linker DNA. Each nucleosome encompasses 146 bp of DNA wrapped around an octamer of histone proteins. These octamers consist of two subunits of each of the following core histone proteins: H2A, H2B, H3 and H4 [54]. Chromatin consists of DNA, histones, and non-histone proteins condensed into nucleoprotein complexes and functions as the physiological template of all eukaryotic genetic information [55]. Histones are small basic proteins containing a globular domain and a flexible charged NH2 terminus known as the histone tail, which protrudes from the nucleosome. Regulation of gene expression occurs through posttranslational modifications of the histone tails provided by covalent modifications including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization, and ADP ribosylation [56-58]. Posttranslational modifications regulate the structural status of chromatin and thereby transcriptional status of genes within a particular locus. These modifications are reversible and are controlled by a group of enzymes. The enzymes that add and remove such modifications are, respectively, histone acetyltransferases (HATs) and deacetylases (HDACs and sirtuins), methyltransferases (HMTs) and demethylases (HDMs), kinases and phosphatases, ubiquitin ligases and deubiquitinases, SUMO ligases and proteases [57, 58]. Chromatin is divided into two distinct conformation states: heterochromatin, which is densely compacted and transcriptionally inert and euchromatin, which is open and transcriptionally active. Euchromatin is characterized by high levels of

acetylation and trimethylated H3K4, H3K36 and H3K79. On the other hand, heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation [59-61]. Genome-wide studies have revealed that various combinations of histone modifications in a specific genomic region can lead to a more 'open' or 'closed' chromatin structure resulting in the activation or repression of gene expression. For example, trimethylation of lysines (K) 4, 36 or 79 on H3 (H3K4me3, H3K36me3 and H3K79me3, respectively), monomethylation of H4K20 and H2BK5 (H4K20me and H2BK5me), and acetylation of H3K9 and H3K14 (H3K9ac and H3K14ac) result in gene activation, whereas di or trimethylation of H3K9 (H3K9me2 and H3K9me3) and trimethylation of H3K27 (H3K27me3) lead to gene repression [60, 62, 63]. In embryonic stem cells, key developmental genes remain poised for lineage-specific activation or repression as a result of their bivalent domains, a combination of two modifications in their promoter regions, H3K4me3 for active and H3K27me3 for repressive marks [63].

1.5 DNA methylation as a biomarker and therapeutic target in cancer

DNA methylation shows much promise as a potential biomarker for clinical application in cancer. DNA is the most stable biological macromolecule and DNA methylation being a covalent modification is a very stable bond. Unlike genetic mutations, which could occur anywhere in a gene, cancer-specific DNA hypermethylation occurs in defined regions, usually in or near the promoter of genes. DNA methylation affects long sequences of DNA rather than single nucleotides, allowing more efficient and robust assay designs. Promoter hypermethylation occurs frequently in human cancer and is an early event in cancer development making it an ideal candidate for early diagnosis, prognosis, predictive and therapeutic target [64, 65]. To be clinically useful, a tumour biomarker should be able to be detectable in clinical specimens through non-invasive procedures and DNA hypermethylation seems to fulfill this requirement. Because of the fact that these aberrations can also be detected in the exfoliated cells from voided urine, this gives a possibility for non-invasive detection of BC.

Unlike genetic abnormalities, epigenetic modifications are to some extent reversible. Currently two major classes of epigenetic drugs have been developed and these are being used in clinical trials. The first class of epigenetic drugs involves histone deacetylase (HDAC) inhibitors, which targets histone acetylation. Histone acetylation leads to open chromatin helping gene expression, inhibition of deacetylation by HDACs will consequently induce gene expression. Presently, there are two FDA approved HDAC inhibitors: suberoylanilide hydroxamic acid (SAHA, Zolinza) and romidepsin (Istodax). The HDAC inhibitors SAHA and romidepsin are approved for the treatment of cutaneous T-cell lymphoma [66, 67]. Many other HDAC inhibitors are currently undergoing clinical testing for several cancers. The second class of epigenetic drugs are DNMT inhibitors, which are typically cytidine analogues: 5-azacytidine (Azacytidine) [68] and 5-azadeoxycytidine

(Decitabine) [69]. Cell division is required for the action of DNMT inhibitors as they compete with normal cytidine for incorporation into the DNA to function. Cytidine analogues are recognized by DNA methyltransferases as natural substrate and the enzymes will initiate the methylation reaction by binding to the DNA covalently, which blocks the DNA methyltransferase function. In addition, the covalent binding also compromises the functionality of DNA and triggers DNA damage signaling, resulting in the degradation of trapped DNA methyltransferases. As a consequence, methylation marks become lost during DNA replication [70]. 5-Azacytidine and decitabine have shown significant clinical benefit in the treatment of myelodysplastic syndrome [71, 72] and are also used for the treatment of myeloid leukemias. The use of these epigenetic drugs is still under investigation to treat solid tumours. Though these drugs have a tremendous potential in cancer treatment, their non-specificity and toxicity are the major limitations.

1.6 Scope and outline of this thesis

The identification of epigenetic aberrations in BC could clarify the pathogenesis of this disease, provide insight into the possible different epigenetic patterns, and eventually result in useful clinical tools. **Chapter 1** gives a general background of BC and DNA methylation. The review in **chapter 2** summarizes the DNA methylation biomarkers discovered in BC so far. The general scope of the thesis is to understand the pathogenesis of BC, detect molecular epigenetic biomarkers for diagnosis and surveillance in urine and prognosis of BC in various clinical settings.

In **chapter 3** we describe a genome-wide DNA methylation analysis on 44 bladder tumours using the Agilent 244K Human CpG Island Microarray. Validation of identified methylated CGIs was done using a custom Illumina 384-plex assay (Illumina, San Diego, CA, USA) in 77 independent tumours. This genome-wide investigation revealed distinct DNA methylation patterns in BC subtypes. Furthermore, extensive methylation indicative of a CpG island methylator phenotype was observed in *FGFR3* wild-type NMI tumours. Most *de novo* methylated genes in bladder cancer were found to be known targets of repression by polycomb group proteins in embryonic stem cells. The genome-wide screening and subsequent validation allowed us to confirm 110 CGIs that significantly differed in methylation in tumours when compared to urine-derived DNA from age-matched non-BC controls. These CGIs represent potential biomarkers for urine tests (see below). Furthermore, four markers (*TBX2*, *TBX3*, *GATA2* and *ZIC4*) for predicting progression were identified in pTa (n = 24) tumours and they were validated in an independent series of 41 pTa tumours by the SNaPshot method. We further found that the accuracy of predicting progression using the EAU risk scores as developed by Sylvester [73] is improved by 23% by adding methylation of *TBX2*, *TBX3*, and *GATA2* to the model.

We next decided to develop a diagnostic assay for the detection of recurrent bladder cancer in voided urine by using specific CGIs from the genome-wide study. This is described in **chapter 4**. To this end, we selected eight candidate CGIs methylated in

bladder cancer but not in urinary cells from healthy individuals. This was performed on a test and validation set of recurrence associated urines from 196 BC patients and 70 urines from age-matched non-bladder cancer controls. We found a panel of three methylation markers in combination with the *FGFR3* mutation assay detecting recurrent BC in voided urine with a sensitivity of 79% at a specificity of 90%. This is the first study specifically used recurrence associated urines from primary Ta G1/G2 tumours as it is important to note that the recurrent tumours are smaller than the primary tumours. As the earlier studies used a mixture of primary and recurrence associated urines, the sensitivity is high.
Our genome-wide methylation study in bladder cancer revealed that DNA methylation in bladder tumours was equally distributed over both promoter and gene body CpG islands. Many previous studies had shown that promoter methylation leads to repression of gene expression, however, the effect of methylation of intragenenic CGIs was not known and is still being investigated. We decided to study the effect of gene body methylation in more detail and this is described in **chapter 5**. To identify the effect of intragenic methylation on gene silencing, we selected the 21 most hypermethylated genes in bladder cancer and studied the gene reactivation pattern by treating bladder cancer cell lines with the DNA demethylating agent decitabine and with decitabine in combination with the deacetylase inhibitor trichostatin A. In general we found that intragenic methylation could also lead to gene silencing, but further experiments are necessary to unravel the mechanism. Finally in **chapter 6** we discuss the results described in this thesis along with suggestions for the future**.**

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Chapter 2

DNA methylation based biomarkers in bladder cancer: A systematic review

Raju Kandimalla and Ellen C. Zwarthoff
Department of Pathology, Erasmus MC, Rotterdam, The Netherlands

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Abstract

Context

Urinary bladder cancer (BC) is the fifth most common cancer in the Western world. DNA methylation contributes to the pathogenesis of various cancers including BC, and may serve as a useful biomarker for diagnostic, prognostic, and predictive purposes.

Objective

In this systematic review, we investigated DNA methylation in BC, its relation to pathogenesis and its use as a diagnostic and/or prognostic marker.

Evidence Acquisition

A PubMed search was performed using MESH terms (Urinary Bladder Neoplasms and DNA Methylation) and general search terms (DNA methylation and Bladder cancer) until April 2012. This yielded a total of 259 articles. The following articles were excluded: reviews, non-English articles and non-bladder cancer related articles. This reduced the number of articles to 86, which are discussed in this review.

Evidence Synthesis

Genome-wide methylation studies implicated several aspects of BC pathogenesis. Methylation of normal urothelium next to the tumour was observed, defined as epigenetic field defect. This suggests that methylation precedes tumorigenesis. Most methylated genes are found to be PcG targets. This again implies that methylation could be an early event. Furthermore, distinct methylation patterns were identified between non-muscle invasive (NMI) and muscle-invasive (MI) BC, as well as between *FGFR3* mutant and wild type tumours. The profound methylation observed in *FGFR3* wild type tumours could indicate the CpG island methylator phenotype (CIMP). Thus like the genetic alterations, epigenetic alterations also differ between subgroups of BC. These genome-wide studies together with small scale cancer specific gene studies have led to the identification of several tumour and urine biomarkers for the diagnosis, prognosis and prediction of BC.

Conclusions

Methylation of Myopodin, *TBX2*, *TBX3*, *GATA2* and *ZIC4* genes were shown to be associated with progression in NMIBC on two independent patient cohorts. Methylation of *RASSF1A*, CDH1, *RUNX3* and *TIMP3* were shown to be associated with progression in different studies. Methylation of *CDH1, FHIT, LAMC2, RASSF1A, TIMP3, SFRP1, SOX9, PMF1,* and *RUNX3* were shown to be associated with poor survival in MIBC in different studies. *PMF1* gene methylation was identified to predict bacillus Calmette-Gue´rin (BCG) response in T1G3 BC. Several studies have identified sensitive urine biomarkers, but most used a mixture of primary and recurrent tumour associated urines. *TWIST1* and *NID2* genes have shown a good sensitivity in detecting primary tumours, while *OTX1*, *ONECUT2* and *OSR1* genes have

displayed a good sensitivity in detecting recurrent bladder tumours in voided urine. However, most of these studies were retrospective and therefore there is a need to perform large randomized prospective multicenter validation studies to prove which markers are valuable to bring into the clinic. Further testing of urine biomarkers in a randomized control trial together with cystoscopy will reveal whether urine testing can replace or add to the cystoscopy. The markers which are identified to predict progression, survival and BCG response will help in clinical decision making and eventually for individualized treatment.

Introduction

BC is the fifth most common cancer in the western world with an estimated 386,300 new cases and 150,200 deaths in the year 2008 worldwide [1]. Most bladder tumours (70-80%) present as non-muscle invasive (NMIBC) and have a good prognosis. Muscle-invasive BC (MIBC) and patients who progress to MIBC have a poor prognosis with a 5 year survival rate of 10-50% depending on stage and despite treatment [2]. NMIBC comprises stages pTa, pT1 and pTis. Unfortunately, 70% of the NMIBC cases will recur after transurethral resection (TUR) and 10-20% will eventually progress to muscle-invasive BC [3-5]. Genomic alterations like somatic mutations in the *FGFR3* gene accompanied with losses of chromosome 9 are more frequent in NMIBC, while *TP53* mutations are associated with MIBC [6-8]. Besides these genetic aberrations, alterations in the epigenetic landscape like DNA methylation, histone modifications, miRNAs and nucleosome remodeling are associated with bladder cancer pathogenesis [9, 10].

Conrad Waddington introduced the term epigenetics in the early 1940s [11]. Epigenetics refers to ''the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence'' [12, 13]. DNA methylation has been shown to contribute to the pathogenesis of various cancers including bladder cancer [14-17]. DNA hypermethylation is often found in CpG islands (CGIs) in the 5' regions of genes overlapping promoter regions. DNA methylation is mediated by the cytosine DNA methyltransferase (DNMT) family of enzymes that catalyze the transfer of a methyl group from S-adenosyl methionine to DNA. DNMT1 is primarily responsible for maintaining the methylation signature by copying the pre-existing methylation pattern onto the daughter strand after DNA replication. DNMT3A and DNMT3B are *de novo* enzymes that target previously unmethylated CpGs [18, 19]. DNA methylation negatively effects gene expression and may contribute to cancer pathogenesis, and consequently DNA modifications may serve as useful biomarkers, both for diagnostic, prognostic and predictive purposes.

At this moment, BC treatment strategies and follow-up mostly depend on the Tumor, Node, Metastasis classification (TNM) and grading proposed by the World Health Organization (WHO) in 1973 and 2004 [2]. NMIBC is treated by transurethral resection (TUR), followed by one cycle of chemotherapy. High grade (G3) and T1 tumours are further treated with bacillus Calmette Guérin (BCG) instillations to delay recurrence and to prevent progression. MIBC is treated with cystectomy, radiation or chemotherapy to reduce the disease-specific mortality.

In principle, the major risks for patients with NMIBC after initial treatment include tumor recurrence, progression to a higher grade or stage, and for MIBC death due to metastatic disease. Conventional clinical and pathologic parameters are widely in use to grade and stage tumors in order to predict clinical outcome of BC. For NMIBC, risk scores for recurrence and progression have been developed by Sylvester [20] and these have been taken up in the EAU (European Association of Urology) guidelines [21]. However, these risk scores were based on a cohort of patient from the nineteen eighties, a time when TUR was

not followed by an initial installation of a chemotherapeutic drug such as mitomycin C and when treatment of patients with high risk NMIBC with BCG was not carried out yet. In addition, especially grading has been shown to be rather subjective with considerable interobserver variation [22]. Molecular markers such as the specific point mutations in the *FGFR3* gene that occur in over 75% of stage Ta tumours have been associated with a relatively benign disease course [7]. Moreover, reproducibility of the *FGFR3* mutation assay was shown to be 100%. Besides the *FGFR3* mutations other molecular markers with similar performance are necessary to better predict disease course in NMI as well as in MIBC.

Cystoscopy is the gold standard for surveillance of patients with a previous bladder tumour, however, it is an invasive and uncomfortable procedure [23, 24]. Moreover, even with cystoscopy, there is 20 to 30% chance to miss a tumour [25, 26]. Due to the high recurrence rate, depending on the risk group of the patient, NMIBC patients are monitored by cystoscopy every 3-12 months after TUR. This sums to an average of about 20 cystoscopies per patient in the first decade, thus making BC one of the most expensive cancers to treat [27]. Cytological examination of voided urine can identify tumour cells with a high sensitivity if a high-grade tumour is present [28]. However, for low stage and grade tumours the sensitivity is low. The *FGFR3* mutations test is an excellent diagnostic test for recurrent cancer in patients presenting with NMIBC if their primary tumour harbors a mutation. Evidently, other tests are required for those patients with *FGFR3* wild-type tumours.

In the past decade several studies addressed DNA methylation patterns in BC using genome-wide studies or studies genes that were shown to be methylated in other tumours in order to understand BC pathogensis and to develop biomarkers for the above mentioned problems. The aim of the present review is to summarize the literature from 2001 to April 2012 on DNA methylation based biomarkers for the diagnosis, surveillance, prognosis and response to BCG treatment of BC comprehensively.

Evidence acquisition

The purpose of this review is to summarize the DNA methylation based biomarkers studied so far in BC. A PubMed search was performed using MESH terms (Urinary Bladder Neoplasms and DNA Methylation) and general search terms (DNA methylation and Bladder cancer) until April 2012. This yielded a total of 259 articles. We excluded reviews, non-English articles, and studies where these terms were mentioned but not studied, reducing the selection to 86 articles.

Evidence synthesis

DNA methylation studies in BC

In the past decade several studies were published on DNA methylation based biomarkers in BC. From 2001 to 2008, most studies focused on the genes commonly methylated in other cancers. These studies are summarized in the first part of Table 1. Besides the

methylation frequency in tumour and normal DNA, Table 1 also indicates if a marker was significantly correlated with stage, grade, and occurrence of recurrence, progression or survival. From 2009 to 2012 several genome-wide studies (GWS) were performed using different approaches. Below we discuss those studies that identified markers based on a genome-wide discovery phase. In the last part of this section we address studies dealing with methylation in relation to age and carcinogenic exposure. All studies are taken up in Table 1 in a chronological order.

The first GWS by **Nishiyama 2009** [29] used a Bacterial Artificial Chromosome (BAC) array based methylated CpG island amplification (BAMCA) to map methylation patterns in 18 normal urothelia, 17 non-cancerous urothelia from BC patients and 40 BCs. Increased methylation levels were reported in these categories of samples, respectively. In addition, several of the BAC clones were reported to methylated in BC and few specific for predicting recurrence but none of these were validated independently. As there was no information of the genes involved, this study was not taken up in Table 1. **Wolff 2010** [30] used the Illumina GoldenGate methylation assay comprising of 1370 CpG sites selected for their frequent methylation in different cancers and studied methylation patterns in 49 NMIBC, 38 MIBC with matched normal-appearing urothelium, and urothelium from 12 age-matched BC-free patients. Distinct patterns of hypomethylation in non-invasive tumours and widespread hypermethylation in invasive tumours was reported. Methylation of *IPF1*, *GALR1*, *TAL1*, *PENK* and *TJP2* was shown to be significantly higher in MIBC, which was confirmed by validation using pyrosequencing on independent set of tumours. Methylation of *ZO2*, *MYOD* and *CDH13* were shown to be higher in tumour associated normal urothelium but was significantly increased in the corresponding tumour. The authors concluded that this indicated the presence of an epigenetic field defect, i.e. methylation was already present in normal looking cells before onset of tumorigenesis. **Chung 2011** [31] used Methylated CpG Island Amplification and Microarray (MCAM) and pyrosequencing on 85 primary NMI bladder tumours and 26 MI cystectomy specimens to report a panel of 10 genes highly methylated in BC with methylation frequency ranging from 62 to 92%. **Augustin 2011** [32] studied methylation patterns in different cancer types including 44 BC and 8 normal urothelial samples using the same Illumina GoldenGate methylation assay as Wolff. The main outcome of the study was the finding of DNA methylation fingerprints which are able to identify the origin of cancers of unknown primary origin (CUPs). We were not able to deduce whether the same genes were identified by both Wolff and Augustin. **Reinert 2011** [33] used the Illumina Infinium 27K microarray to map the methylation patterns in 56 bladder tumours and 14 normal urothelial samples followed by validation in 63 samples using methylation specific high resolution melting (MS-HRM) and bisulfite sequencing. *POU4F2* and *HOXA9* were reported to have the highest frequency of methylation (92%) in BC tissues, while normal urothelium was unmethylated. **Kandimalla 2012** [34] performed a GWS using the Agilent 244K microarray on 44 fresh-frozen BC and subsequently validated the findings using a custom

Illumina GoldenGate methylation assay in 77 BC tissues. This resulted in the identification of 96 highly methylated CpG islands with a highest methylation frequency of 94% for the *MEIS1* gene. This study also indicated that, NMIBC *FGFR3* wild type tumours were more methylated than the *FGFR3* mutant tumours. Vallot [35] and Costa [36] took a different approach and reported genes methylated and reactivated in bladder cancer cell lines using epigenetic reactivating drugs. **Vallot 2011** [35] reported that multiple chromosomal regions are epigenetically silenced in BC, defined as multiple regional epigenetic silencing (MRES) phenotype. However, this was not due to DNA methylation, instead it was because of histone methylation and hypoacetylation. **Costa 2010** [36] reported a panel of three genes *GDF15*, *TMEFF2* and *VIM* studied in 50 BC and 20 normal urothelial samples using methylation specific PCR (MSP) and showed a methylation frequency of 100% in tumour DNA, while no methylation was observed in normal urothelial tissue. **Lin-Hui 2010** [37] studied methylation of *e-cadherin (CDH1)*, *p16*, *p14* and *RASSF1A* in 50 BC tissues and associated normal urothelium. In general methylation was observed in both tissue types but increased from normal urothelium to BC significantly. *p14* hypermethylation was found to be associated with shorter recurrence-free survival (p=0.019). **Serizawa 2011** [38] were the first to combine mutation analysis with methylation. They studied mutation of six genes (*FGFR3*, *PIK3CA*, *TP53*, *HRAS*, *NRAS* and *KRAS*) together with promoter methylation of 11 genes in 101 BC tumours and corresponding urines and 33 controls. Combining *FGFR3* mutations with methylation status of 3 markers (*APC*, *RASSF1A* and *SFRP2*) resulted in methylation frequency of 90% and 93% in two independent tumour sets with no methylation in normal tissue.

DNA methylation is associated with age and carcinogenic exposure. **Marsit 2006** [39] studied methylation of *p16*, *RASSF1A* and *PRSS3* in a large set of 351 BC and found that methylation of *p16* and methylation of *RASSF1A* and *PRSS3* were significantly associated with smoking and arsenic exposure respectively. **Owen 2010** [40] studied methylation of 8 tumour suppressor genes (TSG) in younger patients. The methylation index (MI) of these TSG increased from younger age group patients to older (MI-37.5 in age group <20, MI-62.5 in age group 20-45). **Sobti 2010** [41] and **Wolff 2008** [42] showed that methylation of *DAPK* and *RUNX3* was significantly associated with age and smoking.

Table 1: Overview of all DNA methylation markers in BC until April 2012.

Study	Genes	% methylated in tumours	% methylated in normal	Stage	Grade	Prog[1]	Sur[2]	Rec[3]	Method[4]
Bornman 2001[43]	e-cadherin	43 (20/47)	66 (6/9)						MSP
Habuchi 2001[44]	DBCCR1	52 (36/69)	NA (NA/21)						MSP
Lee 2001[45]	RASSF1A	57 (31/55)	100 (15/15)						MSP
Maruyama 2001[46]	CDH1	36 (35/98)	NA				0.02		MSP
	RASSF1A	35 (34/98)	NA	0.05	0.02				MSP
	APC	35 (34/98)	NA	0.02	0.05				MSP
	CDH13	29 (28/98)	NA		0.04				MSP
	FHIT	16 (16/98)	NA				0.04		MSP
	RARB	15 (15/98)	NA						MSP
Chan 2002[47]	DAPK	58 (57/98)	100 (7/7)						MSP
	RARB	88 (86/98)	43 (3/7)						MSP
	e-cadherin	63 (62/98)	100 (7/7)						MSP
	P16	27 (26/98)	100 (7/7)						MSP
	DAPK, RARB, e-cadherin, P16	100 (98/98)	43 (3/7)						MSP
Ribeiro 2002[48]	e-cadherin	69 (35/51)	12 (6/51)						MSP
Tada 2002[49]	MLH1	13 (7/55)	NA (NA/5)						MSP
	MGMT	17 (9/55)	NA (NA/5)						MSP
	P16	11 (6/55)	NA (NA/5)						MSP
	VHL	4 (2/55)	NA (NA/5)						MSP
	DAPK	29 (16/55)	NA (NA/5)					0.001	MSP
	GSTP1	13 (7/55)	NA (NA/5)						MSP
	e-cadherin	48 (26/55)	NA (NA/5)						MSP
	MLH1, MGMT, P16, VHL, DAPK, GSTP1, e-cadherin	73 (40/55)	NA (NA/5)						MSP
Horikawa 2003[50]	CDH1	47 (23/49)	NA						MSP
Dulaimi 2004[51]	APC, RASSF1A, P14	100 (45/45)	100 (5/5)						MSP
Satyanarayana 2004[52]	LAMA3, LAMB3, LAMC2	55 (70/128)	100 (10/10)	0.0001	0.0001				MSP
	LAMB3	NA (NA/128)	NA (NA/10)	0.01	0.017				MSP

	LAMC2	NA (NA/128)	NA (NA/10)				0.002		MSP
Catto 2005[53]	RARB	25 (23/94)	NA						MSP
	e-cadherin	60 (61/101)	NA						MSP
Catto 2005[53]	RASSF1A	54 (57/106)	NA	0.04	0.04	0.04	0.05		MSP
Friedrich 2005[54]	TIMP3	NA (NA/105)	NA				0.05	0.036	Q-MSP
Kim 2005[55]	RUNX3	73 (91/124)	100 (20/20)	0.01		0.01		0.02	MSP
Marsit 2005[56]	SFRP1	18 (64/355)	NA						Q-MSP
	SFRP2	52 (185/355)	NA						Q-MSP
	SFRP4	9 (32/355)	NA						Q-MSP
	SFRP5	37 (131/355)	NA						Q-MSP
	SFRP1, SFRP2, SFRP4, SFRP5	62 (220/355)	NA	0.05			0.0003		Q-MSP
Christoph 2006[57]	DAPK1	77 (62/80)	8 (2/20)		0.001				MSP
	APAF1	100 (80/80)	11 (2/20)	0.04	0.002				MSP
Christoph 2006[58]	DAPK1	74 (81/110)	20 (4/20)					0.04	Q-MSP
	APAF1	100 (110/110)	15 (3/20)	0.01	0.04			0.05	Q-MSP
	CASP-8	3.6 (4/110)	100 (20/20)						Q-MSP
	IGFBP3	66 (73/110)	10 (2/20)					0.002	Q-MSP
Marsit 2006[59]	p16	31 (109/351)	NA						MSP
	RASSF1A	32 (112/351)	NA	0.0001					MSP
	PRSS3	33 (116/351)	NA	0.04					MSP
Neuhausen 2006[60]	SFRP1	55 (53/96)	NA (NA/19)						MSP
	APC	45 (43/96)	NA (NA/19)						MSP
	RASSF1A	35 (34/96)	NA (NA/19)						MSP
	DAPK1	29 (28/96)	NA (NA/19)						MSP
	RARB2	19 (18/96)	NA (NA/19)						MSP
	CDKN2A	2 (2/96)	NA (NA/19)						MSP
Urakami 2006[61]	SFRP1,SFRP2, SFRP4, SFRP5, WIF1, DKK3	77 (42/54)	67 (36/54)	0.05					MSP, BS-SEQ
Boireau 2007[62]	CLDN4	NA (NA/39)	NA (NA/39)						MSP
Wu 2007[63]	LOXL1	70 (45/64)	67 (8/12)						BS-SEQ
	LOXL4	40 (26/64)	100 (12/12)						BS-SEQ
	LOXL1, LOXL4	92 (59/64)	67 (8/12)						BS-SEQ
Yates 2007[64]	RASSF1A, e-cadherin, TNFSR25, EDNRB, APC	97 (93/96)	75 (23/30)			0.05			Q-MSP
Aleman 2008[65]	SOX9	56.4 (57/101)	90 (9/10)		0.032		0.025		MSP

Aleman 2008[66]	PMF1	83 (421/507)	NA (NA/10)	0.025			0.001		MSP
Brait 2008[67]	CCNA1	57 (53/93)	100 (26/26)	0.02					Q-MSP
	MINT1	31 (29/93)	100 (26/26)						Q-MSP
	CRBP	39 (36/93)	96 (25/26)						Q-MSP
	CCND2	33 (31/93)	100 (26/26)	0.02					Q-MSP
	PGP9.5	41 (38/93)	100 (26/26)	0.04					Q-MSP
	CALCA	28 (26/93)	88 (23/26)						Q-MSP
	AIM1	54 (50/93)	100 (26/26)						Q-MSP
Jarmalaite 2008[68]	p16, RARB, RASSF1A, DAPK,MGMT	62 (36/58)	NA (NA/2)	0.05	0.02				MSP
Kim 2008[69]	RUNX3	72 (85/118)	NA	0.007		0.013	0.05		MSP
Shi 2008[70]	DBC2	59 (44/75)	86 (49/57)						MSP
Cebrian 2008[71]	Myopodin	69 (322/466)	NA (NA/20)	0.0005	0.037	0.05	0.008		MSP
Wolff 2008[42]	RUNX3	39 (133/342)	NA (NA/10)						Q-MSP, MS-SNP
	RASSF1A	37 (127/342)	NA (NA/10)						Q-MSP, MS-SNP
	EDNRB	30 (103/342)	NA (NA/10)						Q-MSP, MS-SNP
	BCL2	28 (96/342)	NA (NA/10)						Q-MSP, MS-SNP
Mori 2009[72]	COL1A2	NA (NA/67)	NA (NA/10)						Q-MSP
Ali Hosseini 2010[73]	P14ARF, P16INK4a	51.25 (41/80)	NA						MSP
Costa 2010[36]	GDF15, TMEFF2, VIM	100 (50/50)	100 (20/20)						MSP
	TMEFF2	NA (NA/50)	NA (NA/20)	0.026	0.005				MSP
Hui Lin 2010[37]	e-cadherin, P16, P14, RASSF1A	90 (51/57)	NA						MSP
Lin Hui 2010[74]	e-cadherin	48 (24/50)	NA						MSP
	P16	64 (32/50)	NA						MSP
	P14	36 (18/50)	NA					0.05	MSP
	RASSF1A	62 (31/50)	NA						MSP
Marsit 2010[75]	HOXB2	NA (NA/582)	NA	0.0001	0.01				Microarray, Pyroseq
	KRT13	NA (NA/582)	NA		0.0001				Microarray, Pyroseq
	FRZB	NA (NA/582)	NA		0.01				Microarray, Pyroseq
Matsumoto 2010[76]	FHL1	NA (NA/70)	NA (NA/10)						Q-MSP
Alvarez 2010[77]	Myopodin	NA (NA/170)	NA			0.03	0.028	0.01	MSP
Owen 2010[40]	8 TSG	NA (NA/76)	NA						Q-MSP
Renard 2010[78]	NID2	73 (66/91)	100 (39/39)						MSP
	TWIST1	68 (62/91)	100 (NA/NA)						MSP

Serizawa 2011[38]	FGFR3, APC, RASSF1A, SFRP2	90 (91/101)	NA						Q-MSP
	RASSF1A	NA (NA/101)	NA	0.01	0.01				Q-MSP
Sobti 2010[41]	DAPK	38 (39/103)	92 (44/48)						MSP
Toki 2010[79]	CRBP1	43 (28/65)	100 (16/16)						COBRA
Cabello 2011[80]	BRCA1	NA (NA/31)	NA		0.017				MS-MLPA
	RARB	NA (NA/31)	NA						MS-MLPA
	WT1	NA (NA/31)	NA	0.025					MS-MLPA
	THSB1	NA (NA/31)	NA						MS-MLPA
	VHL	NA (NA/31)	NA		0.022				MS-MLPA
	MGMT	NA (NA/31)	NA		0.03				MS-MLPA
Chung 2011[31]	A2BP1	62 (16/26)	NA						Pyroseq
	NPTX2	88 (23/26)	NA						Pyroseq
	SOX11	77 (20/26)	NA						Pyroseq
	PENK	92 (24/26)	NA						Pyroseq
	NKX6-3	69 (18/26)	NA						Pyroseq
	DBC1	69 (18/26)	NA						Pyroseq
	MYO3A	65 (17/26)	NA						Pyroseq
	CA10	85 (22/26)	NA						Pyroseq
Durat Periera 2011[81]	OPCML	60 (55/91)	96 (24/25)				0.022		Q-MSP
Reinert 2011[33]	ZNF154	85 (89/105)	100 (14/14)						MS-HRM
	POU4F2	92 (97/105)	100 (14/14)						MS-HRM
	HOXA9	92 (97/105)	100 (14/14)						MS-HRM
	EMOES	88 (92/105)	100 (14/14)						MS-HRM
	TBX4	NA (NA/NA)	NA			0.05			MS-HRM
Vinci 2011[82]	BCL2, TERT, DAPK	91 (98/108)	60 (63/105)	0.05	0.05				Q-MSP
Xuan 2011[83]	CD99	32 (26/82)	100 (10/10)	0.01	0.05				MSP
Kandimalla 2012[34]	TBX2, TBX3, GATA2, ZIC4	NA (NA/65)	NA			0.003			BS-SNP
Kim Sang 2012[84]	RASSF1A	34 (102/301)	NA	0.026	0.001	0.004			MSP
Lin 2012[74]	H-Cadherin	35 (47/133)	100 (43/43)	0.0006	0.01	0.00	0.007	0.01	MSP
Yan 2012[85]	RUNX3	59 (110/186)	NA	0.05	0.009	0.006		0.04	MSP

[1]Prog-Progression [2]Sur-Survival [3]Rec-Recurrence [4]MSP - Methylation Specific PCR; Q-MSP - Quantitative MSP; BS-SEQ - Bisulfite Sequencing; MS-SNP - Methylation Specific Single Nucleotide Primer Extension; COBRA - Combined Bisulfite Restriction Analysis; MS-MLPA - Methylation Specific Multiplex Ligation-dependent Probe Amplification; MS-HRM - Methylation Specific High Resolution Melting; BS-SNP - Bisulfite Specific SNP. NA - Not Available

Prediction of disease course using DNA methylation

MIBC and patients who progress to MIBC have a poor survival in spite of radical cystectomy and therefore markers predicting progression in this group of patients are urgently needed. Progression to MI disease in pTa tumours is much lower than for T1 tumours. Several studies have reported genes methylated in BC with a potential of predicting progression. Below, we discuss these studies, the details are included in Table 1 in chronological order.

Maruyama 2001 [46] were the first to report methylation based prognostic markers in BC. They studied methylation of 10 tumour suppressor genes in BC using MSP in 98 BC patients comprising of 15 stage 0, 6 with stage I, 8 with stage II, 18 with stage III, 16 with stage IV and 35 cases of unknown stage with a maximum follow-up time of 120 months. Methylation of *CDH1* or *FHIT* was shown to be associated significantly with poor survival (p=0.003 and 0.04 respectively). *CDH1* methylation was shown to be an independent predictor of survival in a multivariate analysis (p=0.02). **Satyanarayana 2004** [52] showed that methylation of the *LAMC2* gene studied in 91 BC using MSP was significantly associated with survival (p=0.002). These samples comprised MI and NMI tumours, but the exact stage information of the 91 tumours was not mentioned. **Catto 2005** [53] studied methylation of 11 CpG islands using MSP in 116 bladder and 164 upper urinary tract tumours with a median follow-up time of 56 months. Methylation was found in 86% of all tumours, was noticeably more profound in upper tract tumours than in BC and was generally associated with advanced grade, stage, progression and mortality. Methylation of *RASSF1A*, *DAPK* and *MINT31* was associated with tumour progression (p=0.008, 0.014 and 0.008 respectively). Furthermore *RASSF1A* and *MINT31* methylation were shown to be associated with mortality (p=0.018 and 0.007). Multivariate analysis showed that in addition to tumour stage (p=0.002) and grade (p=0.025), methylation of *RASSF1A* (p=0.028) and *DAPK* (p=0.039) were associated with tumour progression. **Marsit 2005** [56] studied the methylation of *WNT* antagonist *SFRP* genes using MSP in 355 BC with a median follow-up of 60 months. Overall patient survival was shown to be significantly poorer with any *SFRP* gene methylation (p=0.0003). With a proportional hazard modeling, patients with methylation of any *SFRP* gene revealed a significantly poorer overall survival (p=0.02), when controlled for *TP53* staining and other survival associated factors. **Kim 2005** [55] showed methylation of *RUNX3* studied in 124 BC using MSP to be associated with tumour recurrence (p=0.02) and progression (p=0.01) in NMIBC. **Freidrich 2005** [54] studied methylation of 20 cancer related genes in 105 BC using methyl light. KM analysis revealed that methylation of *TIMP3* was significantly associated with recurrence free survival (p=0.036). Contrary, patients in which *TIMP3* was methylated had a better disease free survival than patients without (p<0.05). **Yates 2007** [64] performed q-MSP at 17 gene promoters suspected to be associated with tumour progression in 96 malignant and 30 normal urothelial samples. KM analysis revealed 5 loci (*RASSF1A, E-Cadherin, TNFSR25, EDNRB* and *APC*) to be associated with progression to more advanced stage (p<0.05). Multivariate analysis revealed that the overall degree of methylation was more

significantly associated with subsequent progression and death (p=0.002) than tumour stage (p=0.008). Moreover likelihood and timing of tumour progression was identified with a sensitivity of 97% and specificity of 75% using a predictive model. **Aleman 2008** [65] described the methylation of *SOX9* gene in 101 BC using MSP and showed that it is significantly associated with shorter overall survival (p=0.025). **Hoque 2006** [86] studied methylation of *TIMP3* in 175 BC urines (12 months follow up information was available for 85 urines) using MSP. Univariate analysis showed risk of death was significantly higher in patients with high *TIMP3* methylation (p=0.01). Multivariate analysis revealed that *TIMP3* methylation was an independent prognostic factor for bladder cancer survival with stage and metastasis (p=0.001 and 0.02 respectively). Multivariate Cox regression models revealed that *RUNX3* methylation status was a strong predictor of tumour progression and cancer specific survival. **Cebrian 2008** [71] studied methylation of the Myopodin gene in 466 BC using MSP and showed that its methylation was significantly associated with tumour stage, grade, progression and survival (p<0.05). **Aleman 2008** [66] studied *PMF1* methylation in 507 BC using MSP and found it was significantly associated with increasing stage (p=0.025), Immunohistochemical analyses revealed that *PMF1* methylation was associated with cytoplasmic *PMF1* expression loss (p=0.032) and *PMF1* protein expression patterns were significantly associated with stage (p=0.001), grade (p=0.001), and poor overall survival using univariate and multivariate (p=0.001 and 0.011) analyses. In a large series of BC, **Marsit 2010** [75] showed that methylation of *HOXB2* was associated with muscle invasive BC. **Alvarez 2010** [77] studied Myopodin methylation in 170 T1G3 BC using MSP. Univariate and multivariate analysis revealed that Myopodin methylation was associated with an increased recurrence rate (p=0.004), progression (p= 0.002) and shorter disease specific overall survival (p =0.020). Myopodin methylation was also shown to be associated with BCG response. In a GWS performed by by **Reinert 2011** [33] it was found that methylation of the *TBX4* gene was associated with progression in NMIBC (p<0.04). **Yan 2012** [85] studied methylation of *RUNX3* in 186 BC using MSP and showed its significant association with progression in univariate and multivariate analysis (p=0.016 and 0.043 respectively). GWS by **Kandimalla 2012** [34] showed that methylation of *TBX2*, *TBX3*, *GATA2* and *ZIC4* genes in a test and a validation set of pTa tumours (n=65) was associated with progression (each p<0.003). Multivariate analysis indicated that methylation of *TBX3* and *GATA2* are independent predictors of progression when compared to clinicopathological variables (p = 0.04 and 0.03, respectively). The predictive accuracy improved by 23% by adding methylation of these genes to the European Organization for Research and Treatment of Cancer risk scores. **Kim Sang 2012** [84] showed that among patients with recurrent NMIBC (n=111), *RASSF1A* methylation was associated with shorter time to progression by KM analysis (p=0.004) and identified methylation of this gene as an independent predictor of cancer progression by multivariate Cox regression analysis (p=0.014). **Agundez 2011** [87] studied the methylation status of 25 tumour suppressor genes for predicting Bacillus Calmette-Guerin (BCG) response in 91 patients with T1G3

high risk BC using MS-MLPA. The genes most frequently methylated in these tumours were *STK11* (95%), *MSH6* (81%), *BRCA1* (73%), *PAX5A* (68%), *MGMT* (67%), *CDH13* (63%), and *IGSF4* (62%). In a multivariate analysis the combination of *MSH6* and *THBS1* predicted progression most significantly (p=0.004). *PAX6* and *GATA5* predicted recurrence and disease specific survival respectively (p=0.025 and 0.037 respectively). Recently **Alvarez 2012** [88] reported methylation *PMF1* gene predicting BCG response by studying 108 T1G3 bladder tumours.

Urine methylation markers for the diagnosis of BC

Urine methylation markers are promising tools for the surveillance of NMIBC patients to spot potential recurrences, and this can reduce the burden of invasive cystoscopy. In the past decade several studies described urine methylation markers. However, in all studies, except those by Zuiverloon and Kandimalla (see below), markers were tested on urine samples that were obtained from patients with both primary and recurrent tumours. As primary tumours are often larger and of higher stage and grade, the sensitivity of the markers is not realistic for the purpose of surveillance [89]. The markers are discussed below and detailed information can be found in Table 2 again in chronological order.

For the analysis of gene methylation in voided urine different techniques have been used like methylation specific PCR (MSP), quantitative methylation specific PCR (Q MSP), methylation specific multiplex ligation probe amplification (MS-MLPA), methylation specific high resolution melting (MS-HRM) and bisulfite specific single nucleotide primer extension (BS-SNaPshot). Using MSP the following studies have determined the sensitivity and specificity of markers in urine DNA. **Yu 2007** [90] studied methylation of 11 genes in urines from 99 patients with a primary and 33 with a recurrent tumour along with 23 normal urines and reported 92% sensitivity and 87% specificity. **Renard 2010** [78] identified and validated methylation of *TWIST* and *NID2* in 157 patient urines from primary tumours and 339 normal controls with a sensitivity of 84% and specificity of 96%. This was higher than the sensitivity of cytology, which was 48%. **Dulaimi 2004** [51] studied methylation of *APC*, *RASSF1A* and *p14* in 45 patient urines and 21 normal urines revealing a combined sensitivity of 87% and specificity of 100%. **Eissa 2012** [91] combined methylation of *RARB2* with hyaluronidase levels and cytology in 100 patient urines and 111 control urines, which resulted in a sensitivity of 95% and a specificity of 82%. **Yang-Xing 2012** [92] studied methylation of 5 genes *VAX1*, *KCNV1*, *TAL1*, *PPOX1* and *CFTR* in 212 urines comprising of 157 primary and 55 recurrence associated and 190 normal urines to report a sensitivity of 89 and specificity of 88%. Q-MSP was used in the following studies. **Hoque 2006** [86] studied methylation of four genes in 175 patient urines comprising of 128 primary, 29 recurrent and 18 unknown along with 94 control urines to report a sensitivity of 69 and a specificity of 100%. **Roupret 2008** [93] combined methylation with microsatellite analysis on 40 primary tumour urines and reported a sensitivity of 86 and specificity of 84%. **Costa 2010** [36] studied methylation of three genes *GDF15, TMEFF2* and

VIM in 51 patient and 20 control urines to report a sensitivity of 94 and a specificity of 100%. **Serizawa 2011** [38] combined methylation of three genes with *FGFR3* mutations in 101 primary tumour urines and 30 normal urines and reported a sensitivity of 62 and a specificity of 100%. **Chung 2011** [31] studied methylation of a four gene panel in 128 patient urines comprising of 88 primary and 40 recurrent along with 110 control urines and reported a sensitivity of 81 and a specificity of 97%. **Vinci 2011** [82] studied methylation of a three gene panel in 108 patient and 105 control urines and reported a sensitivity of 79 and a specificity of 90%. **Reinert 2011** [33] used MS-HRM to study methylation of a four gene panel in 115 patient and 59 control urines and reported a sensitivity of 84 and a specificity of 96%.

There are only two studies so far; where solely recurrence associated urines were used to investigate the sensitivity of methylation markers. **Zuiverloon 2012** [94] used MS-MLPA to study methylation of *APC*, *TERT* and *EDNRB* for the detection of recurrent BC in a test (68 positive and 91 negative urines) and validation set (49 positive and 60 negative urines). Sensitivities of 63, 72% were reported for the test and validation set with a specificity of 58 and 55% respectively. **Kandimalla 2012** used a BS-SNaPshot assay to study methylation of the *OTX1*, *OSR1* and *ONECUT2* for the detection of recurrent bladder tumours in a test and validation set consisting of 198 recurrence associated urines and 70 control urines together. Combination of methylation assay with the *FGFR3* mutation assay resulted in a sensitivity of 68%, 79% at a specificity of 90% for the detection of recurrent BC in the test and validation set respectively.

Out of the few serum based methylation markers, **Ellinger 2008** [95] studied methylation of *APC* and *GSTP1* in 45 normal and 45 patient serum DNA using MSP and reported a sensitivity of 80 and specificity of 93%. **Valenzuela 2002** [96] studied methylation of *p16* in 86 BC patients and 49 normal serum DNA using MSP, which resulted in a sensitivity of 23% and a specificity of 95%.

Table 2: Urine methylation markers in BC.

Study	Genes	Sensitivity	Specificity	Urines studied[1]	Method
Chan 2002[47]	DAPK	45 (10/22)	100 (17/17)	NA	MSP
	RARB	68 (15/22)	23.5 (4/17)		MSP
	e-cadherin	59 (13/22)	100 (17/17)		MSP
	P16	14 (3/22)	100 (17/17)		MSP
	DAPK, RARB, e-cadherin, P16	91 (20/22)	24 (4/17)		MSP
Tada 2002[49]	DAPK1	29 (16/55)	NA	P/R	MSP
Dulaimi 2004[51]	APC, RASSF1A, P14	87 (39/45)	100 (21/21)	NA	MSP
Friedrich 2004[97]	DAPK, BCL2, TERT	78 (29/37)	100 (20/20)	P/R	Q-MSP
Satyanarayana 2004[52]	LAMA3, LAMB3, LAMC2	49 (35/71)	100 (10/10)	P/R	MSP
Friedrich 2005[54]	TIMP3	NA (NA/26)	NA (NA/69)	P	MSP
Hoque 2006[86]	CDKN2A, ARF, MGMT, GSTP1	69 (121/175)	100 (94/94)	P/R	Q-MSP
	ARF	NA (NA/175)	NA (NA/94)		Q-MSP
	MGMT	NA (NA/175)	NA (NA/94)		Q-MSP
	GSTP1	NA (NA/175)	NA (NA/94)		Q-MSP
Urakami 2006[61]	SFRP1, SFRP2, SFRP4, SFRP5, WIF1, DKK3	61 (15/24)	94 (19/20)	P/R	MSP
Yates 2006[98]	RASSF1A, e-cadherin, APC	69 (24/35)	60 (41/69)	P/R	MSP
Yu 2007[90]	SALL3, CFTR, ABCC6, HPP1, RASSF1A, MT1A, ALX4,CDH13, RPRM, MINT1, BRAC1	92 (121/132)	87 (20/23)	P/R	MSP
Aleman 2008[66]	PMF1	65 (22/34)	95 (80/84)	NA	MSP
Hoque 2008[99]	TIMP3	NA (NA/85)	NA	P/R	MSP
Roupret 2008[93]	IFNA, MBP, ACTBP2, D9S162, RASSF1A, WIF1	86 (34/40)	84 (NA/25)	P	Q-MSP
V Cebrian 2008[71]	Myopodin	65 (26/40)	78 (97/124)	P/R	MSP
Costa 2010[36]	GDF15, TMEFF2, VIM	94 (48/51)	100 (20/20)	P/R	Q-MSP
Hui Lin 2010[37]	e-cadherin,P16, P14, RASSF1A	83 (47/57)	100 (20/20)	P	MSP
Renard 2010[78]	NID2, TWIST1	90 (141/157)	93 (315/339)	P	MSP
Serizawa 2010[38]	FGFR3, APC, RASSF1A, SFRP2	62 (63/101)	100 (30/30)	P	Q-MSP
Cabello 2011[80]	BRCA1	65 (62/96)	64 (32/50)	NA	MS-MLPA
	RARB	39 (37/96)	92 (46/50)		MS-MLPA

	WT1	37 (36/96)	96 (48/50)		MS-MLPA
Chung 2011[31]	MYO3A, CA10, NKX6.2, DBC1	81 (104/128)	97 (107/110)	P/R	Q-MSP
Dudziec 2011[100]	mir 152, mir 328, mir 1224-3p	81 (55/68)	75 (40/53)	P/R	MSP
PiChen Chen 2011[101]	DAPK	27 (8/30)	90 (17/19)	P/R	Q-MSP
	IRF8	57 (17/30)	95 (18/19)		Q-MSP
	P14	28 (8/30)	100 (19/19)		Q-MSP
	RASSF1A	30 (9/30)	90 (17/19)		Q-MSP
	SFRP1	41 (12/30)	100 (19/19)		Q-MSP
	IRF8, P14, SFRP1	87 (26/30)	95 (18/19)		Q-MSP
Reinert 2011[33]	ZNF154	62 (71/115)	100 (59/59)	P/R	MS-HRM
	POU4F2	66 (76/115)	100 (59/59)		MS-HRM
	HOXA9	74 (85/115)	96 (57/59)		MS-HRM
	EOMES	68 (78/115)	100 (59/59)		MS-HRM
	ZNF154, POU4F2, HOXA9, EOMES	84 (97/115)	96 (57/59)	P/R	MS-HRM
Vinci 2011[82]	BCL2, TERT, DAPK	79 (85/108)	90 (95/105)	P/R	Q-MSP
Eissa 2012[91]	RARB2	65 (65/100)	90 (100/111)	P/R	MSP
	Hyaluronidase	89 (89/100)	91 (101/111)		MSP
	Cytology	53 (53/100)	91 (101/111)		MSP
	COMBO THREE	95 (95/100)	82 (91/111)		MSP
Kandimalla 2012	OTX1, OSR1, ONECUT2, FGFR3	79 (156/198)	90 (63/70)	R	BS-SNaPshot
Yan 2012[85]	RUNX3	NA (NA/186)	NA	P/R	MSP
Yangxing 2012[92]	VAX1, KCNV1, TAL1, PPOX1, CFTR	89 (189/212)	88 (167/190)	P/R	MSP
Zuiverloon 2012[94]	APC, TERT, EDNRB	72 (84/117)	55 (83/151)	R	MS-MLPA

[1]P-Primary, R-Recurrence, P/R-Primary and recurrent, NA-Not available

Conclusions

DNA methylation is a common feature of BC and plays an important role in bladder carcinogenesis as well as in disease progression. Wolff [30] and Reinert [102] have shown that normal urothelium next to the tumour is methylated, and defined this as an epigenetic field defect. This suggests that methylation precedes tumorigenesis. This could have implications for urine testing as methylation will persist in the normal urothelium after the resection of the tumour. However, this will depend on the extent of epigenetic field defect and on the analytical sensitivity of the urine assay. From the studies by Wolff and Kandimalla [103] it appeared that a high proportion of genes that are *de novo* methylated in BC are repressed by PcG complexes in embryonic stem cells. These complexes attract DNMTs and aberrant DNA methylation in some cells early in development may be the outcome [104]. This again implies that methylation could be an early event in tumorigenesis as was first proposed by Feinberg [105]. Serizawa and Kandimalla [38, 103] identified that DNA methylation is more profound in NMI *FGFR3* wild type BC compared to the NMI *FGFR3* mutant BC indicating a CpG island methylator phenotype (CIMP). Wolff et al also showed that MIBC and NMIBC have different methylation patterns. Together these studies show that like the genetic alterations, epigenetic alterations also differ between subgroups of BC. Vallot [35] identified that multiple chromosomal regions are epigenetically silenced in BC, defined as multiple regional epigenetic silencing (MRES) phenotype. However, this was not due to DNA methylation, but because of histone methylation and hypoacetylation.

Urine methylation markers are important for the surveillance of BC to spot potential recurrences, which can ultimately decrease the burden of invasive cystoscopy. In most studies, however, the sensitivity of the urine markers was assessed on a convenience set of urines that comprises urines from primary and recurrent tumours and urines from patients with high stage and grade tumours. Before these markers can be used for surveillance, they first need to be validated on a representative cohort of patients. So far only two studies identified recurrence specific urine methylation markers [94] [103]. The 3-plex methylation assay in the study by Kandimalla [103] in combination with the *FGFR3* mutation assay achieved a sensititvity of 79% for recurrent tumour detection. The sensitivity of this assay combination is similar to the sensitivity of the current gold standard white light cystoscopy with sensitivity in the range of 68 to 83% when compared to the more sensitive blue light cystoscopy [106, 107]. Given the comparable sensitivities of urine testing and cystoscopy, patients under surveillance for recurrent BC in the low/intermediate risk groups could benefit from urine testing followed by cystoscopy only when the urine test is positive. This should be validated further in a prospective manner.

Prediction of progression for patients with NMIBC has been a major clinical challenge. Currently prediction of progression in NMIBC is based on the progression scores developed by Sylvester [20], that have been taken up in the EAU guidelines. These risk scores were based on a cohort of patient from the nineteen eighties, a time when TUR was

not followed by an initial installation of a chemotherapeutic drug such as mitomycin C and when treatment of patients with high risk NMIBC with BCG was not carried out yet. In addition, especially grading has been shown to be rather subjective with considerable interobserver variation [22]. Several of methylation markers have been associated with disease course in multiple studies. *RASSF1A* was shown to be associated with progression in NMIBC by three different studies [53, 64, 108]. *CDH1* (e-cadherin), *TIMP3*, Myopodin and *RUNX3* were also shown to predict progression in NMIBC in two different studies [46, 55, 64, 71, 77, 85, 86]. All these studies used a mixture of pTa and pT1 tumours except two [77][102]. Alvarez [77] who showed that methylation of the Myopodin gene promoter was able to predict progression in pT1 tumours. The same group had earlier demonstrated that Myopodin was also significantly associated with progression in a mixture of pTa and pT1 tumours [71]. Kandimalla [103] identified and validated a set of four genes *TBX2*, *TBX3*, *GATA2* and *ZIC4* predicting progression in pTa tumours exclusively and these markers improved the accuracy of the EAU scores. Taking survival as endpoint, different studies have shown that methylation of *CDH1, FHIT, LAMC2, RASSF1A, TIMP3, SFRP1, SOX9, PMF1, RUNX3* and Myopodin was associated with poor survival in MIBC [46, 52, 54-56, 65, 66, 71, 77].

Intravesical bacillus Calmette-Guerin (BCG) immunotherapy is a successful therapy for patients with high grade NMI tumours [109]. A significant portion of these patients fail to respond to BCG therapy and their tumours not only persist or recur, but they may become invasive or metastatic [110]. To predicted BCG response in T1G3 bladder tumours, methylation of a set of tumour suppressor genes and methylation of PMF1 were identified and validated [87, 88].

To conclude, several very promising biomarkers based on DNA methylation have been identified for diagnosis, prediction of progression and survival and for therapy response. Several of these markers were validated in retrospective studies. To bring these promising markers to the clinic, it is necessary to perform large prospective multicenter validation studies. It is recommended that future studies should adhere to STARD [111] and REMARK [112] guidelines when reporting diagnostic and prognostic markers as this was not the case with many studies presented in this review.

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Chapter 3

Genome-wide Analysis of CpG Island Methylation in Bladder Cancer Identified TBX2, TBX3, GATA2, and ZIC4 as pTa-Specific Prognostic Markers

Raju Kandimalla[1], Angela A. G. van Tilborg[1], Lucie C. Kompier[1], Dominique J.P.M. Stumpel[2], Ronald W. Stam[2], Chris H. Bangma[3], Ellen C. Zwarthoff[1]
[1]Department of Pathology, Erasmus MC, Rotterdam, The Netherlands
[2]Department of Paediatric Oncology, Erasmus MC, Rotterdam, The Netherlands
[3]Department of Urology, Erasmus MC, Rotterdam, The Netherlands

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Abstract

Background

DNA methylation markers could serve as useful biomarkers, both as markers for progression and for urine-based diagnostic assays.

Objective

To identify bladder cancer specific methylated DNA sequences for predicting pTa specific progression and detecting bladder cancer in voided urine.

Design, setting and participants

Genome-wide methylation analysis was performed on 44 bladder tumors using Agilent 244k human CpG island microarrays. Validation was done using a custom Illumina 384-plex assay in a retrospective group of 77 independent tumors. Markers for progression were identified in pTa (n=24) tumors and validated retrospectively in an independent series of 41 pTa tumors by the SNaPshot method.

Measurements

The percentage methylation in tumor and urine samples was used to identify markers for detection and related to the endpoint progression to muscle-invasive disease with Kaplan-Meier models and multivariate analysis.

Results and limitations

In the validation set, methylation of the *TBX2*, *TBX3*, *GATA2* and *ZIC4* genes was associated with progression to muscle-invasive disease in pTa tumors ($p=0.003$). Methylation of *TBX2* alone showed a sensitivity of 100%, a specificity of 80%, PPV of 78% and NPV of 100% with an AUC of 0.96 ($p<0.0001$) for predicting progression. Multivariate analysis showed that methylation of *TBX3* and *GATA2* are independent predictors of progression when compared to clinicopathological variables ($p=0.04$ and 0.03 respectively). The predictive accuracy improved by 23% by adding methylation of *TBX2, TBX3* and *GATA2* to the EORTC risk scores. We further identified and validated 110 CpG islands that are differentially methylated between tumor cells and control urine. The limitation of this study is the small number of patients analyzed for testing and validating the prognostic markers.

Conclusions

We have identified 4 methylation markers that predict progression in pTa tumors thereby allowing stratification of patients for personalized follow-up. In addition, we identified CGIs that will enable detection of bladder tumors in voided urine.

Key words

Bladder cancer, DNA methylation, Biomarkers, Epigenetics, Progression, Prognostic markers

Introduction

Urinary bladder cancer is the fifth most common cancer in the western world [1]. Bladder tumors present either as non-muscle (stages pTa, pT1 and CIS) or as muscle-invasive carcinomas (stage >pT1), and are associated with different genetic changes. Somatic mutations in the *FGFR3* gene accompanied with losses of chromosome 9 are more frequent in NMIBC (Non-muscle invasive bladder cancer), while *TP53* mutations are associated with MIBC (Muscle-invasive bladder cancer) [2-4]. Patients diagnosed with MIBC and those who progress to MIBC have an unfavourable prognosis, despite radical cystectomy and chemotherapy.

pTa tumors represent about 60% of all bladder tumors upon presentation. Progression to MI disease in pTa tumors is much lower than for T1 tumors, however, the risk is still 10% and up to 60% of patients will have on average 3 recurrences, warranting long-term surveillance [5-7]. Until now, no good biomarkers are available that predict progression in this large subgroup of BC. The long survival of NMIBC patients and the need to monitor them makes BC the most costly cancer when calculated per patient. In recent years, it became clear that recurrent BC can be detected using urine-based assays [8]. In this context, we have previously shown that loss-of-heterozygosity (LOH) by microsatellite analysis or detection of *FGFR3* mutations in DNA isolated from urine is able to predict the presence of recurrent bladder tumors [9, 10]. As these assays do not cover all bladder tumors, additional biomarkers are required.

Epigenetic modifications, such as DNA methylation, contribute to the pathogenesis of various cancers, including BC. Cancer-associated hypermethylation of CGIs may serve as a useful biomarkers for diagnostic, prognostic and predictive purposes. For instance, methylation of the *MGMT* gene is associated with sensitivity of patients with glioblastoma for temozolomide therapy [11]. To identify the best bladder-specific epigenetic biomarkers, we performed a genome-wide screen for DNA methylation in BC.

Materials and methods

Patient samples, ethics statement

For the genome-wide study using Agilent arrays, we collected 44 freshly frozen bladder cancer (BCa) tissues, representing 29 non-muscle invasive (NMI) tumours (19 with *FGFR3* mutation (NMI-MT) and 10 without (NMI-WT)), and 15 muscle-invasive (MI) tumours via the department of Urology at Erasmus MC, Rotterdam. For the Golden Gate Methylation (GGMA) validation assay, we used a retrospective group of 77 formalin-fixed, paraffin-embedded (FFPE) BCa samples (23 Ta NMI-MT, 10 Ta NMI-WT, 7 T1-MT, 13 T1-WT, 24 MI) collected from the department of Pathology at our institute. This set of tumours consist of 5 Ta progressors and 19 Ta non-progressors from individual patients with a median follow up time of 60 mo, which we considered as a test set to find prognostic markers. For the validation of progression markers in an independent set of tumours, we used DNA from a retrospective group of fresh frozen tissue of 22 non-progressors and 19 progressors with a

median follow up time of 88 mo collected via the department of Urology at Erasmus MC. The median age of these patients was 68 yr. Ten patients were female. Generally, all patients were followed and treated according to the guidelines of the European Association of Urology [12]. Progression in both groups was defined as progression from Ta NMI-BCa (NMI-BC) to MI-BCa (MI-BC) as determined by pathology. We have used the REMARK criteria to address the prognostic markers [13]. The medical-ethical committee at Erasmus MC approved the project. All patients have given written informed consent. Patient data for the tumours included can be found in Supplementary Table S1-3 (http://www.europeanurology.com/article-asset/S0302-2838(12)00013-9/mmc1/pdf/).
FFPE samples were used according to the standards presented in "The Code for Proper Secondary Use of Human Tissues in the Netherlands" (http://www.federa.org/). Tumour samples were included only if at least 80% of the sample consisted of cancer cells, as verified by H&E staining. Tumour samples were extracted from FFPE tumor tissue by de-waxing with xylene and ethanol. Tumour DNA from FFPE and fresh frozen tissue was isolated using the DNeasy Tissue kit (Qiagen, Hilden, Germany), according to the protocol. Commercially available normal human genomic blood DNA (Promega, Madison, WI, USA) was used as a reference in the genome-wide study. As reference DNA in the GGMA validation assay we used DNA isolated from cells present in the normal urine of four healthy men aged >50 yr. Urine was collected and assessed for leukocytes, erythrocytes and nitrite using Multistix® 8 SG. Each sample was centrifuged for 10 minutes at 3,000 rpm. Cell pellets were washed once with 10 ml phosphate buffered saline, resuspended in 1 ml phosphate buffered saline, transferred to an Eppendorf vial and centrifuged for 5 minutes at 6,000 rpm. Supernatant was discarded and each cell pellet was stored at −20°C until DNA isolation. DNA was extracted from the cell pellet using the QIAamp DNA mini-kit according to the manufacturer protocol. All analyses were done with blinded sample information.

Differential Methylation Hybridization (DMH) and Data Analysis
DNA amplicons were prepared for hybridization according to the differential methylation hybridization (DMH) protocol, originally described by Yan *et al* [14] and modified according to Stumpel *et al* [15]. Briefly, 0.5 µg of genomic DNA was digested with the four base (T^TAA) restriction enzyme *MseI*, which restricted genomic DNA into fragments <250 bp in length, while leaving the GC-rich CpG islands (CGIs) relatively intact. Subsequently, the sticky ends of the fragments were ligated to linkers. The samples were then digested with the methylation-sensitive restriction enzymes *HpaII* and *BstUI*. Reference samples were treated in the same way to generate amplicons. Polymerase chain reaction (PCR; 20 cycles) was performed using the purified, digested, linker-ligated DNA as template to generate final target amplicons, followed by coupling to fluorescent dyes (Cy3 in the case of human genomic reference DNA and Cy5 in the case of tumor samples) before hybridizing to the Agilent 244K CpG island microarray. The pooled amplicons were co-hybridized on the array. Using this approach, genomic DNA fragments containing unmethylated CpG sites in

one sample (e.g. the human genomic reference DNA) were degraded by restriction digestion, and not amplified. However, corresponding DNA fragments in the other sample (the bladder tumor) that contain methylated restriction sites were protected from digestion and subsequently amplified by PCR. Differentially methylated sequences were identified by comparing hybridization signals between fluorescently labeled tumor (Cy5) and reference (Cy3) amplicons. Labeling and hybridization of the arrays were performed at Service XS (Leiden, the Netherlands), which also provided the quality control reports about the hybridization and performance of the arrays according to the manufacturers recommendation. All the arrays passed the quality parameters described by the Agilent technologies (Agilent feature extraction software (v9.5) reference guide).
All microarray data generated is compliant with current Minimum Information About a Microarray Experiment (MIAME) standards according to Brazma et al [16, 17]. Normalization, preprocessing of raw data and statistical analysis were done using Bioconductor packages in an R programming environment (http://www.r-project.org/). Raw hybridization signals were normalized by applying the within-array global "lowess" normalization method [18], which assumes that the bulk of the probes on the array are not differentially expressed. Because the normalization also includes a pre-processing step, the normalized intensities were log2-transformed. The log2-transformed intensities were then subjected to further statistical testing to determine which loci were differentially methylated. For this, we applied the linear model "limma" [19], to fine differentially methylated genes in bladder tumours vs normal and between all different subgroups. These comparisons are analogous to a classical two-sample *t*-test analysis.
The *p*-value threshold was calculated using the Benjamini and Hochberg method [20]. Loci corresponding to a p-value of <0.05 and a log fold change of either greater than 0.5 or less than -0.5 and with more than 2 probes from the same CGI were classified as *differentially methylated*. We choose to use Log fold change of plus or minus 0.5 as an arbitrary cutoff to end up with a reasonable number (not too much or too low) of probes for further validation.

Illumina custom Golden Gate Methylation assay and statistical analysis
We designed GGMA interrogating 384 CpG-containing probes representing 238 CGIs. We selected the probes based on the identified differentially methylated loci in the genome-wide analysis. The methylation assay is an adaptation of the Illumina high-throughput SNP genotyping assay described by Fan et al [21]. Briefly, nonmethylated cytosines were converted to uracil by treatment with bisulfite. Sodium bisulfite modification of genomic DNA was carried out using the EZ DNA Methylation Gold Kit (Zymo Research Corp, Orange, CA, USA) according to the manufacturer's protocol, using 0.8 µg of DNA. The bisulfite conversion efficiency was monitored by PCR with BS-specific primers. Arrays were run at Service XS according to the manufacturer's protocol and as described by Bibikova et al [22]. Data were analyzed with Illumina's BeadStudio Methylation module software. All array-data points were represented by fluorescent signals from both methylated (M) and

unmethylated (U) alleles. The average methylation value β was derived from approximately 30 replicate methylation measurements for each locus. The methylation level was given by $\beta = (\max(M, 0))/(|U| + |M| + 100)$. The β-value ($0 \leq \beta \leq 1$) reflects the methylation level of each CpG site, where 1 represents fully methylated and 0 represents unmethylated. At each locus for each sample, the detection *p*-value was defined as 1 minus *p*-value computed from the background model, characterizing the chance that the signal was distinguishable from negative controls. Using this as a metric of quality control for sample performance, we dropped 13 FFPE samples from the analysis, because they either had very low overall signal intensities or > 25% of loci failed. Differences in methylation between loci were considered when the average β-value ratio was larger than 1.2 and/or the difference between the average β-value (Δβ) was > 0.1.

Validation of prognostic markers and statistical evaluations
We used the Bisulfite Specific-Snapshot method described by van Oers et al [9] to validate our prognostic markers. Leave-one-out crossvalidation (LOOCV) method was used to re-check the prognostic markers found as significant by student *t*-test and log-rank test, and the best probes were validated in an independent set of tumours by BS-Snapshot (A list of primers and probes used is given in Supplementary Table S4). The method involves bisulfite conversion of genomic DNA (EZ DNA methylation gold kit, Zymo Research Corp, Orange, CA, USA), followed by subsequent amplification of interesting CGIs. The PCR products were treated with two units of Exonuclease I (ExoI) and three units of Shrimp Alkaline Phosphatase (SAP; USB, Cleveland, Ohio USA). This was followed by a single-nucleotide probe- extension assay using a SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA) and probes designed to anneal to either the forward strand or the reverse strand of a PCR product adjacent to the mutation site of interest. These probes were fitted with T-tails of different length at their 5′ ends to allow separation of the extension products by size [23]. The mutation detection reactions were performed in a total volume of 10 µl containing 2 µl SAP/ExoI treated PCR product, 2.5 µl SNaPshot Multiplex Ready Reaction mix, 1 x Big Dye sequencing buffer, and 1 µl probe mix. Thermal cycler conditions were: 25 cycles of 10 seconds at 95˚C, 5 seconds at 50˚C, and 30 seconds at 60˚C. The products were treated with 1 unit SAP at 37°C for 60 min, and at 75°C for 15 min, and were analyzed on an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems) with the fluorescent label on the incorporated ddNTP indicating the presence or absence of a mutation. For analysis of the data, we used GeneMarker Software version 1.7 (SoftGenetics, State College, PA, USA). Methylation percentages were calculated by using the formula: Height of the C/G-peak / (Height of the C/G-peak + Height of the T/A peak) x 100.

Statistical evaluations
Statistical analyses were completed using SPSS v. 15.0 (IBM Corp. Arnmonk, NY, USA). Differences were considered significant when P-values were below 0.05. Survival curves were calculated using the Kaplan-Meier method, with significance evaluated by two-sided

log-rank statistics. Progression free survival was measured from the day of surgery until progression to MI disease. A multivariate Cox regression model was used to test the independent prognostic relevance of clinical/investigational factors and methylation markers. Only patients for whom the status of all variables was known were included in the proportional hazard models. The clinical variables age at diagnosis, multiplicity, histological grade, tumor size, previous recurrence and adjacent CIS were dichotomized (<65 vs >65 years, G1-2 vs G3, <3 cm vs >3 cm, yes vs no). [24]

Results

A genome-wide methylation profile for bladder cancer

The study design is described in Figure 1. Patient characteristics are given in Table 1. To investigate global DNA methylation in BC, we analyzed 44 bladder tumors with microarrays containing 244,000 CpGs. We found 731 probes representing 392 unique CpG islands to be methylated in BC. We clustered the different bladder tumor subgroups on the basis of all probes differentially methylated between the respective subgroups (Figure 2). These analyses showed that subgroups can be identified on the basis of their methylation patterns. NMI-WT tumors showed a 3.9 to 5.5 times higher number of hypermethylated genes than NMI-MT and MI. Polycomb group (PcG) target genes have been shown to be particularly prone to DNA methylation in cancer [25, 26]. Notably, we found that 56.5% of the hypermethylated genes in BC were PcG target genes (results not shown).

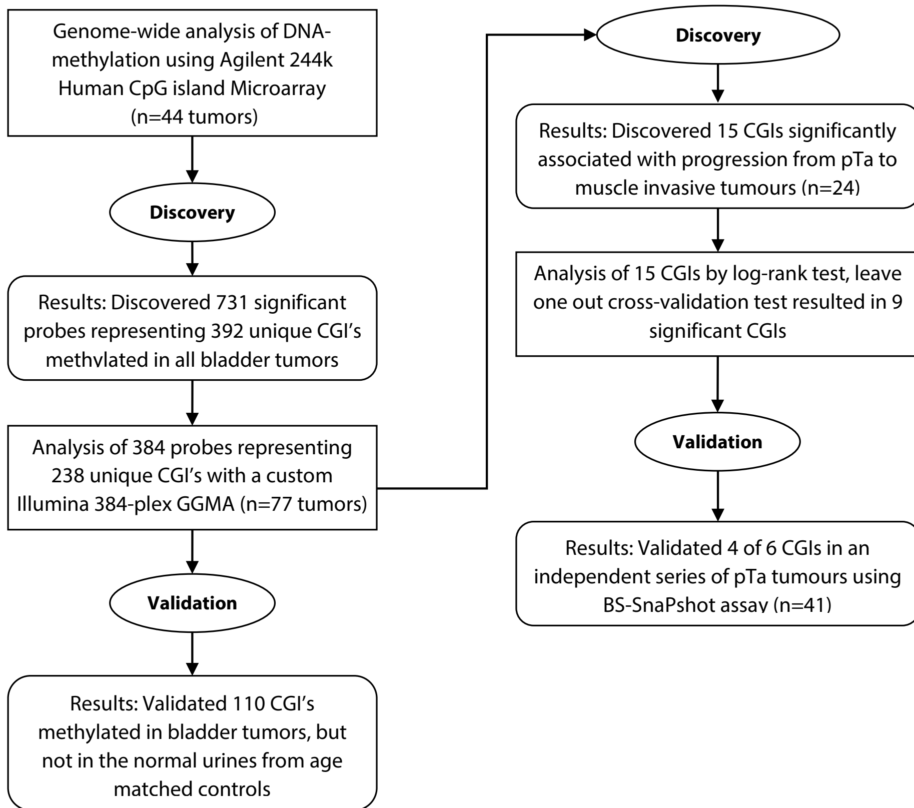

Figure 1: Study design CGI= CpG Island; GGMA= Golden Gate Methylation Assay.

Table 1: Patient characteristics

Clinical Parameter	*Discovery set (Agilent, n=44)*	*Prognostic marker test set (n=24)*	*Prognostic marker validation set (n=41)*
Gender			
Male	38	16	29
Female	6	8	12
Unknown	0	0	0
Age			
<65	15	14	16
>65	29	10	25
Unknown	0	0	0
Post operative treatment			
Yes	15	3	22
No	6	8	12
Unknown	23	13	7
Primary/Recurrent			
Primary	24	11	32
Recurrent	19	13	9
Unknown	1	0	0
Previous recurrence			
Yes	15	6	9
No	28	16	32
Unknown	1	2	0
Multiplicity			
Solitary	23	9	20
Multiple	20	12	21
Unknown	1	3	0
Adjacent CIS			
Yes	4	1	4
No	39	20	37
Unknown	1	3	0
Tumor Size			
<3 cm	17	8	23
>3 cm	4	5	13
Unknown	23	11	5
T category			
Ta	26	24	41
T1	3	0	0
T2	12	0	0
T3	3	0	0
Grade			
G1	8	6	15
G2	23	15	24
G3	13	3	2
Progression			
Yes	4	5	19
No	25	19	22
Unknown	0	0	0
Survival			
Yes	31	20	17
No	13	4	20
Unknown	0	0	4
Cause of Death			
Malignant disease	5	2	12
Other	8	2	4
Unknown	0	0	4

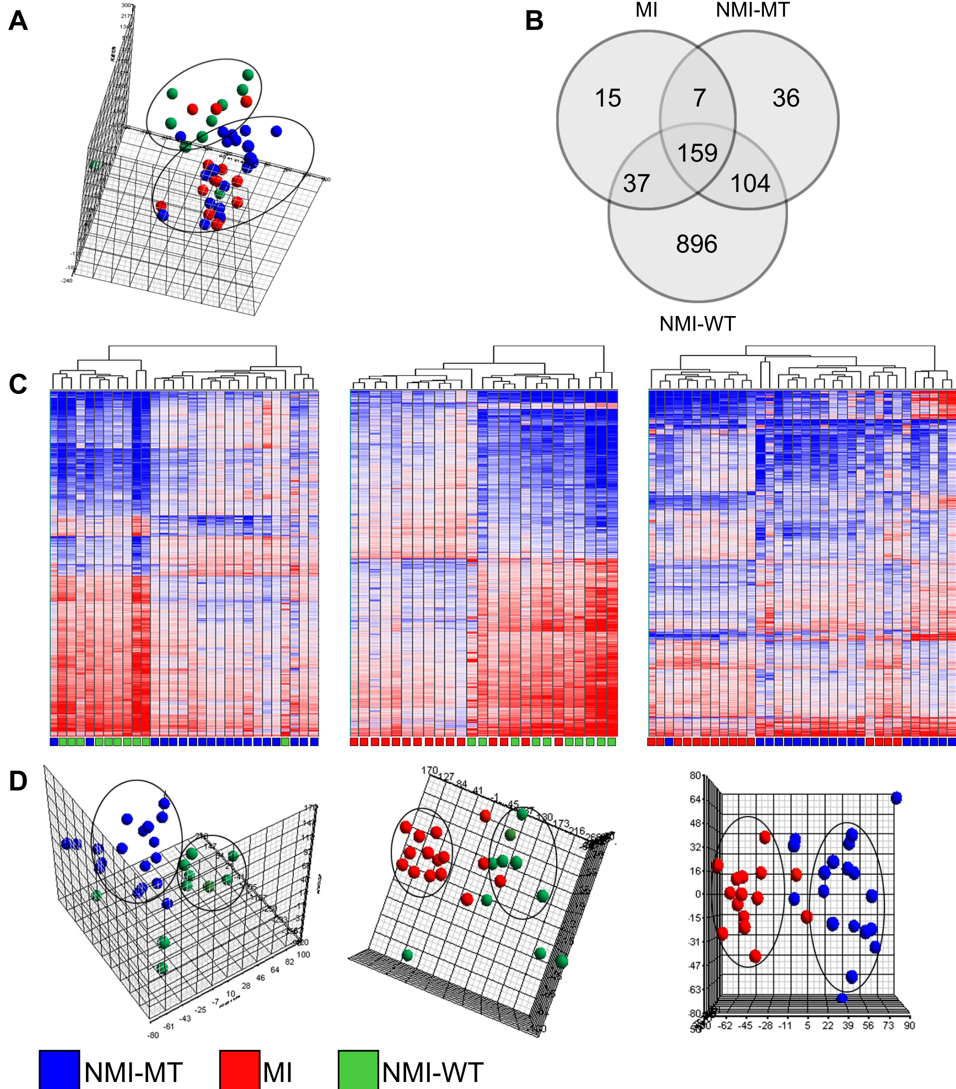

Figure 2: Comparison of methylation patterns between BC subgroups. (A) Unsupervised principal component analysis with all probes present on the 244K Agilent array: the NMI-WT (Non-muscle invasive FGFR3 wild type) bladder tumor group clusters separately from the NMI-MT (Non-muscle invasive FGFR3 mutant) and MI tumors (Red: MI, Blue: NMI-MT and Green: NMI-WT), (B) Venn diagram showing the number of genes that are hypermethylated in a specific group of bladder tumors, (C) Semi-supervised analyses for bladder tumor subgroups by hierarchical clustering of NMI-MT vs NMI-WT tumors, MI vs NMI-WT tumors and NMI-MT vs MI tumors, (D) Principal component analyses for bladder tumor subgroups as in (C).

Table 2: Hypermethylated genes and CGIs in bladder cancer

CGI	Symbol	Fold Change
chr2:6652593	*MEIS1*	4.8
chr15:946887	*NR2F2*	2.9
chr13:941521	*SOX21*	2.8
chr13:349505	*MAB21L1*	2.7
chr2:6313453	*OTX1*	2.7
chr19:637845	*MGC2752*	2.6
chr16:528731	*IRX3*	2.6
chr14:362052	*PAX9*	2.6
chr6:1549606	*FOXC1*	2.5
chr7:9648815	*DLX5*	2.5
chr7:9648990	*DLX5*	2.5
chr2:1568926	*NR4A2*	2.5
chr2:1568938	*NR4A2*	2.5
chr17:380892	*CNTNAP1*	2.4
chr6:1338049	*FOXF2*	2.4
chr10:944413	*HHEX*	2.3
chr1:2072175	*PRKCZ*	2.3
chr1:2106293	*PRKCZ*	2.3
chr7:2716470	*HOXA7*	2.3
chr5:1740912	*MSX2*	2.3
chr8:1422883	*SLC45A4*	2.2
chr2:1115916	*FLJ44006-*	2.2
chr19:405992	*MAP2K2*	2.2
chr7:1552884	*SHH*	2.2
chr13:496055	*chr13*	2.2
chr7:2082894	*MAD1L1*	2.2
chr17:440743	*hsa-mir-196a-*	2.2
chr7:8449658	*NXPH1*	2.2
chr18:518624	*chr18*	2.1
chr10:218286	*C10orf114*	2.1
chr2:4501493	*SIX3*	2.1
chr2:4501339	*SIX3*	2.1
chr6:1053030	*TFAP2A*	2.1
chr6:1049802	*TFAP2A*	2.1
chr15:744145	*ISL2*	2.1
chr15:744193	*ISL2*	2.1
chr2:7098470	*VAX2*	2.0
chr7:2724531	*AMZ1*	2.0
chr17:588653	*CYB561*	2.0
chr2:1767376	*HOXD3*	2.0
chr6:1010038	*SIM1*	2.0
chr6:1010024	*SIM1*	2.0
chr13:105943	*EFNB2*	1.9
chr2:1713847	*GAD1*	1.9
chr4:4915358	*MSX1*	1.9
chr4:4910534	*MSX1*	1.9
chr14:371371	*FOXA1*	1.9
chr6:2267756	*HDGFL1*	1.9

CGI	Symbol	Fold Change
chr13:495959	*chr13*	1.8
chr16:873724	*chr16*	1.8
chr7:2725116	*EVX1*	1.8
chr5:5455481	*UNG2 (CCNO)*	1.8
chr18:532541	*ONECUT2*	1.8
chr21:439021	*HSF2BP*	1.8
chr1:1193506	*chr1*	1.8
chr1:6355498	*FOXD3*	1.8
chr20:225148	*FOXA2*	1.8
chr20:225107	*FOXA2*	1.8
chr20:225055	*FOXA2*	1.8
chr6:1019534	*GRIK2*	1.8
chr2:4524937	*chr2*	1.8
chr14:601787	*SIX1*	1.8
chr7:2717044	*HOXA9*	1.8
chr1:4767171	*FOXD2*	1.7
chr1:4768230	*FOXD2*	1.7
chr1:4767224	*FOXD2*	1.7
chr14:361228	*NKX2-8*	1.7
chr19:634200	*ZNF274*	1.7
chr20:600616	*TAF4*	1.7
chr2:4508528	*SIX2*	1.7
chr8:1459093	*chr8*	1.7
chr4:3033030	*PCDH7*	1.7
chr4:1117744	*PITX2*	1.7
chr2:8521346	*TCF7L1*	1.7
chr6:1049295	*chr6*	1.7
chr8:1062402	*SOX7*	1.7
chr12:527269	*HOXC4*	1.7
chr5:1343909	*PITX1*	1.7
chr5:1407906	*PCDHGA12*	1.6
chr6:1704228	*chr6*	1.6
chr19:609070	*EPN1*	1.6
chr7:9646932	*DLX6*	1.6
chr2:1767231	*hsa-mir-10b*	1.6
chr2:1767206	*hsa-mir-10b*	1.6
chr21:369900	*SIM2*	1.6
chr12:184400	*CACNA2D4*	1.6
chr7:2714913	*HOXA5*	1.6
chr2:1058642	*NCK2*	1.6
chr2:1942444	*OSR1*	1.6
chr17:759360	*C17orf27*	1.6
chr6:1690517	*chr6*	1.6
chr2:1766523	*EVX2*	1.5
chr10:768251	*ZNF503*	1.5
chr5:9293199	*chr5*	1.5
chr17:764041	*KIAA1303*	1.5
chr17:763901	*KIAA1303*	1.5

Validation of hypermethylated CGIs

We validated the selected CGIs on a custom 384-plex Illumina platform (GGMA). We included CGIs methylated in all bladder tumors (Table 2) and CGIs differentially methylated between bladder tumour subgroups. Hierarchical clustering clearly separated the bladder tumors from blood and normal urine (Figure 3A). We found 217 of 384 probes methylated in BC. Of these 203 probes (93.5%) from 133 CGIs were also methylated on the Agilent array. We also included DNA isolated from urine of healthy individuals on the GGMA array. These cells are derived from normal urothelium and serve as a control in the selection of cancer-associated methylated CGIs that can be used to detect primary and recurrent tumors in urine DNA. In total, we discovered 110 CGIs that are methylated in bladder tumors but not in control urine-derived DNA (Figure 3B and Table 3). A urine test based on the best performing CGIs is in development and will be the subject of a separate paper.

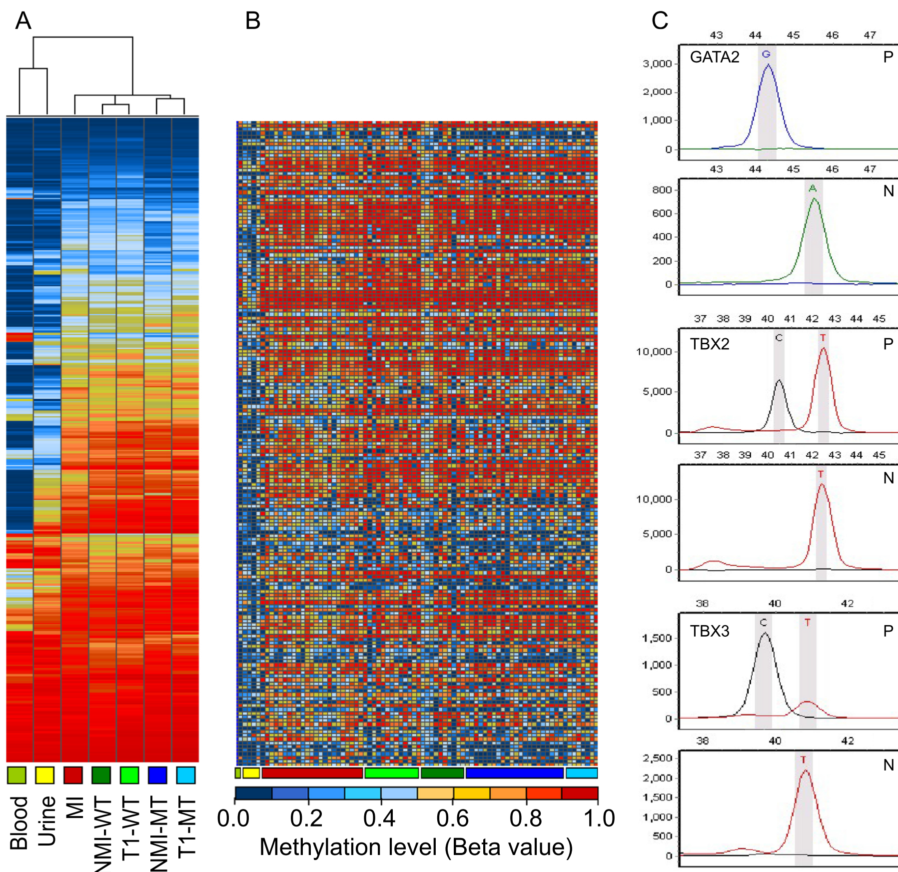

Figure 3: Identification and validation of probes useful for urine diagnosis (A) Hierarchical clustering of GGMA data of all the investigated samples. The BC samples cluster separately from normal blood and urine samples. (B) Heat map showing the probes which are methylated in all tumor subgroups vs blood and urine. (C) Methylation status of CpGs in the GATA2, TBX2 and TBX3 CGIs as detected with BS-Snapshot. Depending on whether the probe used is hybridizing to the sense or the antisense strand, black/blue peaks represent methylated CpGs (C or G), red/green peaks represent umethylated CpGs (T or A) after bisulfite conversion. P: progressor, N: non-progressor.

Table 3: Overview of the CGIs differentially methylated between tumor and control urine

CpG island location	*Gene Name*	*Average Beta Ratio Cancer vs Urine*	*Delta beta Cancer vs Urine*
chr18:53254153-53259851	*ONECUT2*	27.2	0.5
chr7:27170441-27172987	*HOXA9*	15.9	0.5
chr21:36990064-36995761	*SIM2*	13.6	0.6
chr2:119329502-119332035	*EN1*	12.8	0.4
chr4:147778656-147781351	*POU4F2*	12.2	0.3
chr1:178464743-178471598	*LHX4*	12.0	0.3
chr4:30330303-30333940	*PCDH7*	11.8	0.6
chr2:176652334-176656692	*EVX2*	11.1	0.5
chr11:115955870-115957122	*chr11*	10.8	0.2
chr10:102986025-102986636	*LBX1*	10.8	0.2
chr1:219134071-219134808	*HLX1*	10.7	0.2
chr7:121727243-121727884	*FEZF1*	10.5	0.6
chr1:47682300-47683607	*FOXD2*	10.2	0.2
chr8:65662303-65662699	*BHLHB5*	10.0	0.4
chr4:134291113-134293078	*PCDH10*	9.8	0.2
chr1:163590111-163590435	*LMX1A*	9.2	0.4
chr3:148619591-148620015	*ZIC1*	8.9	0.2
chr2:19424445-19425131	*OSR1*	8.8	0.5
chr3:148591199-148594390	*ZIC4*	8.6	0.3
chr18:26875488-26877115	*DSC3*	8.5	0.1
chr10:26544390-26547440	*GAD2*	8.3	0.2
chr2:156893804-156894601	*NR4A2*	7.8	0.7
chr2:66525936-66527140	*MEIS1*	7.4	0.6
chr14:37137198-37138958	*FOXA1*	7.3	0.4
chr2:63134539-63134851	*OTX1*	7.2	0.5
chr10:21828640-21829594	*C10orf114*	7.2	0.4
chr2:111591678-111597436	*FLJ44006-BCL2L11*	7.0	0.4
chr3:62331811-62332352	*FEZF2*	6.0	0.1
chr1:232106922-232108080	*SLC35F3*	5.7	0.5
chr10:76825135-76839606	*ZNF503*	5.5	0.5
chr10:124891898-124892607	*HMX2*	5.4	0.5
chr16:47868915-47869809	*CBLN1*	4.5	0.4
chr15:58083428-58085812	*FOXB1*	4.5	0.2
chr4:122521018-122521740	*GPR103*	4.5	0.2
chr2:85213468-85216104	*TCF7L1*	4.4	0.6
chr2:70984709-70985764	*VAX2*	4.2	0.6
chr5:92931996-92934631	*chr5*	4.2	0.4
chr1:47672249-47672972	*FOXD2*	4.0	0.3
chr7:32768010-32768497	*chr7*	4.0	0.6
chr18:68359955-68362770	*CBLN2*	4.0	0.2
chr1:63554983-63563059	*FOXD3*	3.9	0.3
chr19:57648469-57649057	*chr19*	3.9	0.3
chr2:115635208-115637235	*DPP10*	3.9	0.2

CpG island location	*Gene Name*	*Average Beta Ratio Cancer vs Urine*	*Delta beta Cancer vs Urine*
chr5:140790679-140792801	*PCDHGA12*	3.7	0.4
chr18:5186244-5187389	*chr18*	3.7	0.3
chr3:129688183-129694961	*GATA2*	3.5	0.4
chr13:94152191-94153185	*SOX21*	3.5	0.5
chr14:36122886-36123441	*NKX2*	3.4	0.3
chr7:27164708-27165039	*HOXA7*	3.4	0.6
chr5:134390992-134393045	*PITX1*	3.4	0.4
chr19:63420090-63420541	*ZNF274*	3.4	0.4
chr6:101953488-101953856	*GRIK2*	3.2	0.3
chr17:67627870-67631593	*SOX9*	3.2	0.2
chr6:101003802-101004342	*SIM1*	3.2	0.4
chr5:122461778-122463450	*chr5*	3.0	0.2
chr8:57520681-57521969	*PENK*	3.0	0.5
chr15:58079172-58079459	*FOXB1*	2.8	0.1
chr12:94776152-94776377	*SNRPF*	2.8	0.4
chr8:10624024-10624296	*SOX7*	2.7	0.2
chr7:8449658-8450236	*NXPH1*	2.7	0.4
chr1:119333515-119333719	*TBX15*	2.7	0.5
chr2:42182944-42183157	*chr2*	2.6	0.1
chr2:45085286-45086054	*SIX2*	2.5	0.4
chr20:54012011-54014085	*CBLN4*	2.5	0.3
chr10:102497473-102499636	*PAX2*	2.4	0.2
chr2:176737660-176738187	*HOXD3*	2.2	0.4
chr21:33316999-33322115	*OLIG2*	2.2	0.1
chr13:34950554-34951119	*MAB21L1*	2.2	0.4
chr4:111774415-111774953	*PITX2*	2.2	0.4
chr8:65653638-65653873	*BHLHB5*	2.1	0.2
chr20:22505518-22507240	*FOXA2*	2.1	0.2
chr12:52726910-52727810	*HOXC4*	2.1	0.4
chr2:171384799-171385226	*GAD1*	2.1	0.4
chr15:35174679-35174906	*MEIS2*	2.0	0.4
chr2:72224630-72228512	*CYP26B1*	2.0	0.1
chr7:155288454-155292175	*SHH*	1.9	0.4
chr7:96488158-96489487	*DLX5*	1.9	0.2
chr6:101002495-101002783	*SIM1*	1.8	0.4
chr7:96469320-96469736	*DLX6*	1.8	0.3
chr15:94705727-94706054	*chr15*	1.8	0.4
chr10:94441310-94441717	*HHEX*	1.8	0.3
chr12:113589232-113589931	*TBX3*	1.7	0.1
chr6:1338049-1339169	*FOXF2*	1.7	0.3
chr8:72916429-72917309	*MSC*	1.7	0.3
chr8:65661156-65661382	*BHLHB5*	1.7	0.2
chr1:71284813-71286392	*PTGER3*	1.7	0.2
chr7:96489900-96490182	*DLX5*	1.6	0.3

CpG island location	*Gene Name*	*Average Beta Ratio Cancer vs Urine*	*Delta beta Cancer vs Urine*
chr6:1549606-1560865	*FOXC1*	1.6	0.1
chr19:4059920-4060207	*MAP2K2*	1.6	0.2
chr5:174091287-174092335	*MSX2*	1.5	0.3
chr14:60178708-60179539	*SIX1*	1.5	0.2
chr14:98781593-98783184	*BCL11B*	1.5	0.3
chr16:52873104-52882105	*IRX3*	1.5	0.2
chr13:49595986-49600287	*chr13*	1.4	0.3
chr5:54554812-54555385	*UNG2*	1.4	0.2
chr15:94688798-94689034	*NR2F2*	1.4	0.2
chr14:36205265-36206099	*PAX9*	1.4	0.3
chr2:43251545-43251780	*chr2*	1.4	0.3
chr7:27219207-27220360	*chr7*	1.4	0.2
chr12:113657998-113659205	*chr12*	1.3	0.2
chr7:2082894-2083307	*MAD1L1*	1.3	0.2
chr10:22804715-22807056	*chr10*	1.3	0.2
chr19:63784504-63785085	*MGC2752*	1.3	0.2
chr17:75531280-75531511	*TBC1D16*	1.3	0.2
chr2:176723195-176723460	*hsa-mir-10b*	1.3	0.2
chr7:27100623-27100828	*HOXA1*	1.3	0.1
chr2:45249374-45251690	*chr2*	1.2	0.2
chr12:1844001-1845219	*CACNA2D4*	1.2	0.1
chr5:140724146-140724826	*PCDHGA5*	1.2	0.1
chr17:44074360-44075233	*hsa-mir-196a-1*	1.2	0.1

Identification and validation of CGIs predicting progression

The GGMA validation assay contained 5 pTa progressors and 19 pTa non-progressors from individual patients with a median follow up time of 60 months. This we considered as a test set to find prognostic markers. Performing a student t-test identified 15 CGIs (18 probes) associated with progression to MIBC (Supplementary Table S5). A log-rank test and leave-one-out cross-validation test reduced the number to 9 CGIs. We successfully designed a bisulfite PCR-SNaPshot assay (Figure 3C) for 6 of these CGIs and tested them in an independent validation set of pTa tumors comprising 19 progressors and 22 non-progressors with a median follow up time of 88 months. This allowed us to confirm that methylation of 4 CGIs in the *TBX2*, *TBX3*, *GATA2* and *ZIC4* genes was associated with progression. For *TBX2*, *TBX3* and *GATA2* any methylation was associated with progression, whereas *ZIC4* was methylated in most tumors, however, very high methylation (over 45%) was associated with progression. Univariate analysis showed that all clinical factors except tumor size and adjacent CIS along with *TBX2*, *TBX3*, *GATA2* and *ZIC4* methylation were associated with progression. Multivariate analysis showed that *TBX3* and *GATA2* are independent predictors of progression (Table 4). Kaplan-Meier curves for these 4 CGIs for both the test set (GGMA) and the validation set are shown in Figure 4A and 4B. Sensitivity, specificity, NPV and PPV of the markers are given in Table 5. The EORTC risk scores were

significant in predicting progression in univariate analysis, P=0.012, log rank test (Figure 5A). The predictive accuracy of the EORTC model for progression information was 72.7%. The improvement in predictive accuracy by adding methylation percentages (*TBX2*, *TBX3* and *GATA2*) was 23.3 %, resulting in a predictive accuracy of 96% for the model with methylation markers as determined by AUC (Area under the curve). The major part of this effect was caused by the influence of methylation on the intermediate EORTC score. Combining the intermediate EORTC risk score and methylation grade (Figure 5B) led to more accurate prediction of progression (P=0.007).

Table 4: Progression-free survival analysis of clinicopathological and molecular factors. HR= Hazard Ratio; CI= Confidence Interval; * *p*<0.05.

Variable	Categorization	Univariate			Multivariate		
		Patients	Events	*p*	HR	95% CI	*p*
Clinicopathological factors							
Age at diagnosis	<65 years	16	5	0.028*	103.1	3.1-3401	0.009*
	≥ 65 years	25	14				
Multiplicity	Solitary	20	5	0.002*	12.3	0.91-165	0.058
	Multiple	21	14				
Histological grade	G1-G2	39	17	0.002*	0.08	0.001-5.6	0.244
	G3	2	2				
Tumor size	< 3 cm	23	11	0.672			
	≥ 3 cm	13	5				
Previous recurrence	No	32	11	0.003*	0.12	0.001-2.17	0.046*
	Yes	9	8				
Adjacent CIS	No	37	17	0.983			
	Yes	4	2				
Molecular factors							
TBX2	unmethylated	16	0	0.000*	13.44	0.69-264	0.08
	methylated	18	14				
TBX3	unmethylated	31	10	0.000*	39.4	1.18-1412	0.044*
	methylated	10	9				
GATA2	unmethylated	13	1	0.001*	34.6	1.41-852	0.03*
	methylated	19	14				
ZIC4	unmethylated	24	8	0.003*	0.38	0.03-3.84	0.41
	methylated	17	11				

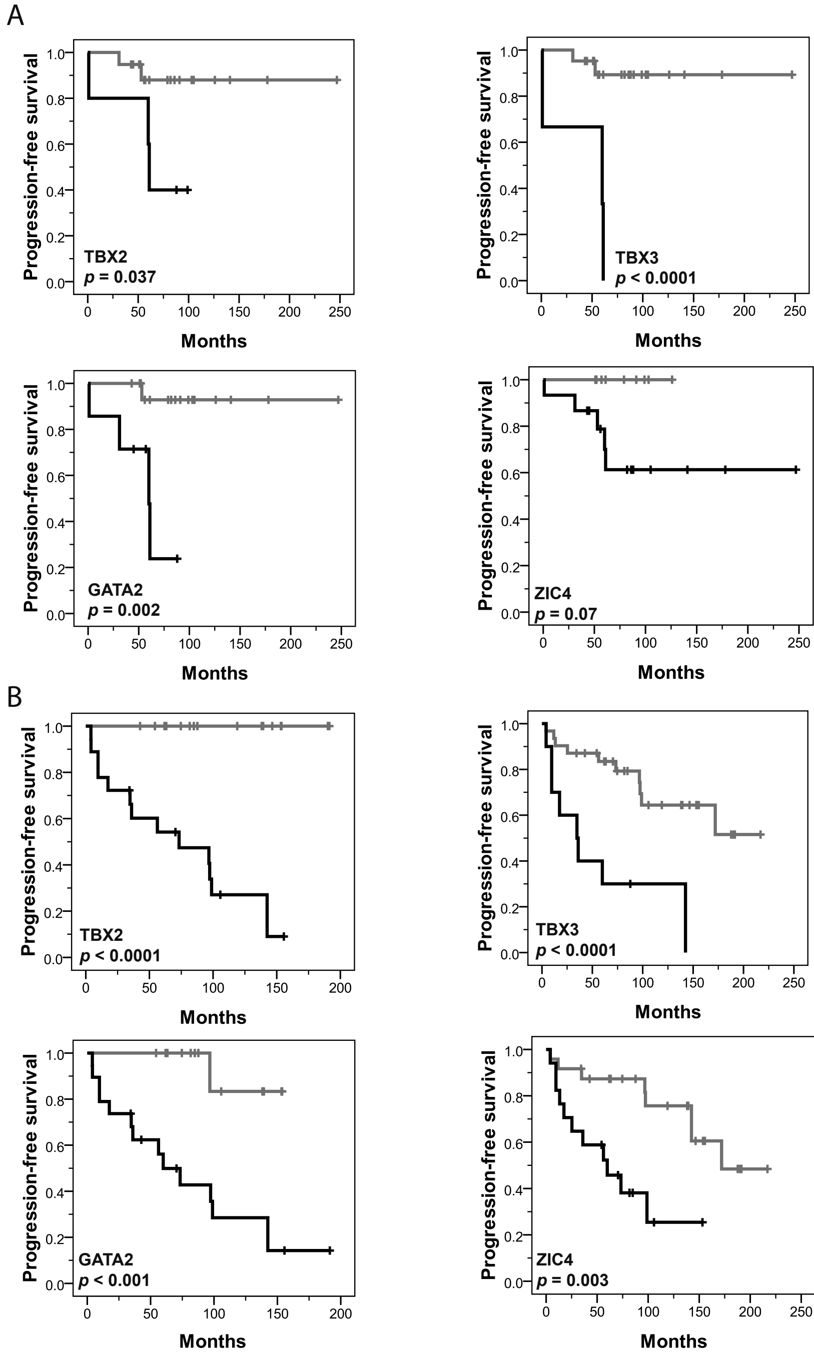

Figure 4: Methylation of specific CGIs can predict progression to muscle-invasive disease in NMI pTa tumors. Patients without methylation of TBX2, GATA2, ZIC4, and TBX3, have a significantly better progression-free survival (A) Test set, GGMA assay. (B) Validation set. The differences between the groups were significant as indicated (log-rank test). Green line indicates methylation, Blue line indicates no methylation and (+) indicates censored data.

Table 5: Sensitivity, Specificity, Negative Predictive value and Positive Predictive value of prognostic markers for predicting progression.

	Sensitivity	Specificity	Negative Predictive value	Positive Predictive value	AUC (*p* value)
TBX2	100	80	100	78	0.96 (0.0001)
TBX3	48	96	68	90	0.72 (0.019)
GATA2	94	71	93	74	0.88 (0.0001)
ZIC4	58	73	67	65	0.71 (0.021)

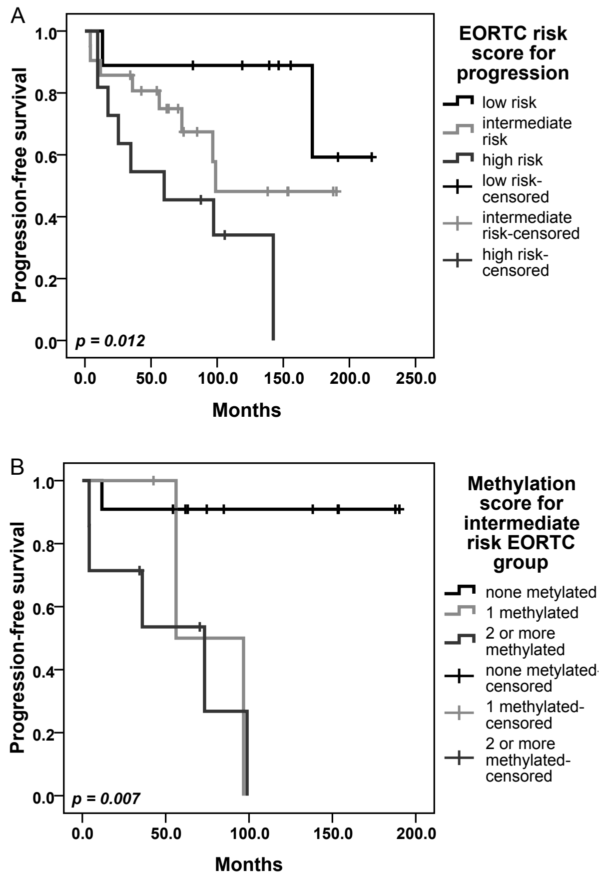

Figure 5: Comparison of EORTC risk scores and methylation scores for predicting progression in the validation set (n=41) (A) Kaplan-Meier plot for progression-free survival according to the EORTC risk score. Based on these scores, patients score low, intermediate or high risk for progression (p=0.012) (B) Methylation grade improved prediction of progression in intermediate-risk EORTC group (p=0.007). Methylation grade was determined as (0-No gene methylated, 1-One gene methylated, 2- Two or more genes methylated).

Discussion

The epigenetic progenitor model of cancer described by Feinberg [27] claims that cancer develops in three steps. The first being an epigenetic alteration of a stem or progenitor cell within a given tissue and the second a gate-keeper mutation followed by genetic and epigenetic instability leading to further tumor evolution. This hypothesis is supported by studies that showed that epigenetic alterations precede the initial mutations in cancer [28-30]. Here, we report an investigation of BC associated aberrant DNA methylation. The genome-wide screening and subsequent GGMA validation assay allowed us to confirm 110 CGIs that significantly differed in methylation in tumors when compared to urine-derived DNA from age-matched non-bladder cancer controls. These CGIs represent potential biomarkers for urine tests.

We identified and validated four genes that are highly methylated in pTa tumors that later showed progression to MIBC. pTa tumors represent about 60% of all primary bladder tumors. Progression to MIBC in pTa tumors is much lower than for pT1 tumors, however, the risk is still 10% and 60-70% of patients will have on average 3 recurrences, warranting long-term surveillance [5-7]. We found that methylation of the *TBX2*, *TBX3*, *GATA*2 and *ZIC4* genes was significantly associated with progression. These genes encode transcription factors that are important in lineage decisions during development [31]. Gene expression profiling studies showed that all these genes are more expressed in NMIBC than in MIBC [32], which fits with our findings as we observed that these genes are more methylated in MIBC. *TBX2* and *TBX3* are transcriptional repressors that inhibit expression of the ARF gene a.o., thereby finally inhibiting P53 activation [33]. This may present an alternative for mutation of P53, which is frequent in MIBC. Mutations that reduce DNA binding of *GATA2* were discovered in familial myelodysplastic syndrome [34]. Currently prediction of progression in NMIBC is based on the progression scores developed by Sylvester [7], that have been taken up in the EAU guidelines. The markers described in this work enhanced the predictive accuracy of these risk scores and could thus contribute to a further objective stratification of patients. We previously showed that mutations in the *FGFR3* gene are associated with a low chance of progression and combining the here identified markers with *FGFR3* may also help to stratify patients presenting with pTa tumors for further clinical management, for instance, by increasing the number of follow-up visits for patients with methylation of the identified genes and lacking an *FGFR3* mutation. An additional advantage is that both methylation and *FGFR3* mutation analysis can be performed on DNA isolated from FFPE tissue, which is easier to obtain than fresh tissue. Other studies also reported prognostic markers based on genetic and epigenetic alterations that can be analysed on DNA isolated from FFPE tissue [24, 35]. Reinert et al showed methylation of *TBX4* gene associated with progression in pTa tumors, while our study found methylation in *TBX2* and *TBX3* association with progression, this suggests the important role of T-box genes in BC progression [36]. Yates et al. identified a group of 5 genes that could significantly discriminate progressors from non-progressors in pTa and pT1 tumors combined. No multivariate analysis to compare the markers with clinical variables was performed. In the paper by Friedrich et al. methylation of *TIMP3* also predicted progression in a set of patients with pTa and pT1 tumors, however, the marker was not compared to

other parameters. In conclusion, we present here validated easy-to-assay progression markers specific for pTa bladder tumors.

Most genes discovered in the present study have never been shown to be methylated in BC or in other types of cancer. Some of the previously described methylated genes in bladder cancer are *SOX9, CDKN2A, TERT, DAPK1, EDNRB, DBC1, NID2, TWIST1, LAMB3, GDF15, TMEFF2, VIM, MYO3A, CA10, SOX11*, and *NKX6-2*. Most of these were also found to be methylated in our study, although only *SOX9* and *CDKN2A* made it to the list of our 96 highly methylated CGIs [37-40]. A recent study by Reinert et al showed that a panel of 8 markers are methylated in BC, our study also found 3 of these markers representing *HOXA9*, *POU4F2*, *PCDHGA12* to be higly methylated in BC [36]. We observed that NMIBCs with a wild-type *FGFR3* gene have more methylated CGIs and the intensity of methylation is more profound than in the *FGFR3* mutant NMIBC and MIBC groups. Interestingly similar results were recently described by Serizawa et al [41]. The difference in methylation presents additional evidence that the two subgroups of NMIBC develop along different pathogenesis pathways. The extensive methylation in WT NMIBC is reminiscent of the CpG island methylator phenotype (CIMP) in colorectal cancers (CRC) and gliomas [42-45]. When comparing the genes methylated in this study with those found by us, there was hardly any overlap. This confirms that CGI methylation is tissue and tumour type specific [46].

In line with previous reports in solid tumors [25, 26, 47], a high proportion of the genes we find *de novo* methylated in BC are repressed by polycomb complexes in embryonic stem cells. PcG target genes are as much as 12 times as likely to be silenced by DNA methylation in cancer as non-PcG target genes. Our findings confirm those of Wolff et al, where they showed 40% methylated genes in BC as PcG targets [48]. These findings strongly suggest that methylation occurs early in bladder cancer development.

Conclusions

We have identified four methylation markers that predict progression in pTa tumors thereby allowing stratification of patients for personalized follow-up. In addition, we identified 110 CGIs as putative biomarkers for early detection of BC in voided urine.

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Chapter 4

A 3-plex methylation assay for sensitive detection of recurrent bladder cancer in voided urine

Raju Kandimalla[1], Roy Masius[1], Willemien Beukers[1], Chris H. Bangma[2], Torben F. Orntoft[3], Lars Dyrskjot[3], Angela A. G. van Tilborg[1] , Ellen C. Zwarthoff[1]
[1]Department of Pathology, Erasmus MC, Rotterdam, The Netherlands
[2]Department of Urology, Erasmus MC, Rotterdam, The Netherlands
[3]Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark

Submitted for publication

Abstract

Background
Aberrant DNA methylation is associated with the pathogenesis of bladder cancer (BC) and can serve as a diagnostic biomarker.

Objective
To develop a sensitive urine assay for the diagnosis of recurrent bladder tumours in patients with a previous primary non-muscle invasive BC (NMIBC) G1/G2.

Design, setting, and participants
We selected eight CpG islands (CGIs) methylated in BC. Sensitivity of the CGIs for recurrences detection was investigated on a test set of 101 preTUR (before Trans Urethral Resection) urines using a bisulfite specific single nucleotide primer extension assay (BS-SNaPshot). Specificity was determined on 70 urines from healthy males >50 years. A 3-plex assay for the best combination was developed and validated on an independent set of 95 preTUR urines, 39 urines associated with a primary G1/G2 tumour and 40 urines from patients who were recurrence free for at least 6 months.

Outcome measurements and statistical analysis
ROC curves were used to predict the best single marker and binary logistic regression was performed to select the best combination.

Results and limitations
The 3-plex assay identified recurrent BC in voided urine with a sensitivity of 74% in the validation set (specificity of 90%). Sensitivity for the detection of primary G1/G2 tumours was 80%. Combining the methylation assay with the *FGFR3* (*fibroblast growth factor receptor 3*) mutation assay resulted in a sensitivity of 79%. The 3-plex methylation assay was more sensitive than cytology and the *FGFR3* assay. There were 22% false positives in patients that were recurrence free for >6 months.

Conclusion
The combination of the methylation and FGFR3 assays efficiently detects recurrent BC without the need for stratification of patients regarding methylation/mutation status of the primary tumour. We conclude that sensitivity of this combination is in the same range as white light cystoscopy and we suggest that, a subsequent study should be performed investigating a modified surveillance protocol consisting of the urine test followed by cystoscopy only when the urine test is positive, for patients in the low/intermediate risk group.

Introduction

BC is the fifth most common cancer in the western world with an estimated 386,300 new cases and 150,200 deaths in the year 2008 worldwide [1]. Most bladder tumours (70-80%) are non-muscle-invasive (NMIBC) at presentation and patients have a good prognosis [2]. NMIBC comprises stages pTa, pT1 and pTis. Unfortunately, 70% of patients with NMIBC will have recurrence after transurethral resection (TUR) and 10-20% of patients will eventually have progression to muscle-invasive BC (MIBC) [3-5]. As a consequence, patients are monitored by cystoscopy every 3-12 months after TUR, making BC one of the most expensive cancers to treat [6]. Currently cystoscopy is the gold standard for surveillance, however, it is an invasive and uncomfortable procedure [7, 8]. Moreover with cystoscopy, there is 20 to 30% chance to miss a tumour [9, 10].

Cytological examination of voided urine can identify tumour cells with a high sensitivity if a high-grade tumour is present [11]. However, for low stage and grade tumours the sensitivity is low. This low sensitivity induced the development of urine-based assays in the past decade. Although some assays have been approved by the FDA, they have so far not been taken up in routine clinical practice [12-16]. In most studies the sensitivity of the markers was assessed on a convenience set of urines that comprises urines from primary and recurrent tumours and urines from patients with high stage and grade tumours. Hence when tested on urines from patients under surveillance the sensitivity of these markers is disappointing [14, 17]. Because of these problems our group has specifically focused on markers that are better in detecting recurrent BC in patients who presented with a primary NMIBC [18-20]. Up to 80% of the pTa tumours have a mutation in the *fibroblast growth factor receptor 3* (*FGFR3)* oncogene [21-23]. We showed that a multiplex assay for the most common mutations was able to detect about 75% of tumours smaller than 1.5 cm and 100% if the tumours were larger than 3 cm [24, 25]. A great advantage of the *FGFR3* assay is that since these mutations are extremely rare in normal cells, an assay to detect *FGFR3* mutations has a specificity of 100%. A disadvantage is that the patients need to be stratified up front for the presence of an *FGFR3* mutation and therefore other assays are required for patients with wild-type tumours.

It is evident from many studies that methylated CpG islands (CGIs) may present useful biomarkers. In order to find the most promising CGIs for BC prognosis and diagnosis we have previously performed a genome-wide study [26]. In the current study, we investigated selected CGIs for the detection of recurrent BC in voided urine.

Materials and methods

Bladder cancer patient samples and other control samples

Forty-eight FFPE BC DNAs from a mixture of primary and recurrent tumours, 101 pre-TUR urines (test set) from patients with a previous primary NMIBC G1/G2, 70 urines from males (> 50 years of age) without any history of BC as controls containing 20 urines with a high leucocyte count, 39 preTUR urines from primary tumours (pTaG1 and pTaG2), and 40

urines were collected during surveillance in a period in which no recurrences occurred within 6 months in the Departments of Pathology and Urology, Erasmus MC. Out of these 40 patients, 24 are under and 16 are over 65 years. There were 27 males and 13 females. The median follow-up was 24 months. These patient samples were used according to “The Code for Proper Secondary Use of Human Tissues in the Netherlands” (http://www.federa.org/). An additional set of 95 pre-TUR urines (validation set) from patients with a previous primary NMIBC G1/G2 was obtained from Aarhus University Hospital, Denmark . The test and validation set urines were collected before transurethral resection of the corresponding recurrent tumour. With 70 controls and 95 validation samples we have 90% power to detect 20% difference in methylation. Patient characteristics are presented in Table 1. There is no overlap of the samples used. Urine samples (25-100 ml) were collected one day before TUR and stored at 4 °C. Within 6 hrs after voiding, samples were centrifuged at 4000 rpm for 10 minutes, followed by washing the pellet twice with cold PBS, then stored at -20 °C for DNA isolation. DNA was isolated using DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany). Recurrences were registered only when proven by histology. The study adhered to the STARD guidelines for the reporting of studies of diagnostic accuracy [27].

Table 1: Patient characteristics

Characteristics	**Test set Urines (n=101)**	**Validation set Urines (n=95)**	**Primary Tumour Urines (n=39)**	**FFPE tumours (n=48)**
Gender				
Male	78	52	29	39
Female	23	43	10	9
Age				
<65	38	36	17	15
>65	63	59	22	33
Stage				
Ta	73	93	39	39
T1	13	0	0	8
>=T2	14	1	0	1
NA	1	1	0	0
Grade				
G1	23	33	16	15
G2	46	52	23	25
G3	27	9	0	2
NA	5	1	0	0
FGFR3				
Mutant	10	49	15	39
Wild type	91	46	24	9

Bisulfite conversion and BS-SNaPshot assay

Bisulfite conversion and quantitative assessment of methylation was done as described previously [26]. In short: DNA was converted with sodium bisulfite (EZ DNA methylation gold kit, Zymo Research Corp, Orange, CA, USA). PCR of selected CGIs was performed using 20 ng of converted DNA, 20 pmols of primers and 10 µL of KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, USA) in a total volume of 20 µl. The PCR products were treated with 2 units of Exonuclease I (ExoI) and 3 units of Shrimp Alkaline Phosphatase (SAP) (USB, Cleveland, Ohio USA). This was followed by a single-nucleotide probe extension assay using a SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA) and probes designed to anneal to either the forward or reverse strand adjacent to the investigating CpG site. These probes were fitted with T-tails of different length at their 5′ ends to allow separation of the extension products by size [22, 25]. The single nucleotide primer extension reactions were performed in a total volume of 10 µl containing 2 µl SAP/ExoI treated PCR product, 2.5 µl SNaPshot Multiplex Ready Reaction mix, 1 x Big Dye sequencing buffer, and 1 µl probe mix. Thermal cycler conditions were: 25 cycles of 10 seconds at 95˚C, 5 seconds at 50˚C, and 30 seconds at 60˚C. The products were treated with 1 unit SAP at 37°C for 60 min, and at 75°C for 15 min, and were analyzed on an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems) with the fluorescent label on the incorporated ddNTP indicating incorporation of a C or T or an A or G depending on the strand investigated. The primers and probes of 8 CGI markers are given in Supplementary Table S1. We further developed a multiplex BS-SNaPshot assay aimed at detecting the methylation status of the three most interesting CGIs (*OTX1*, *ONECUT2* and *OSR1*) in a single PCR and a single nucleotide primer extension reaction. Concentrations of primers and probes for this assay are given in Supplementary Table S1. A typical multiplex assay is shown in the Figure 4.

FGFR3 mutation analysis

FGFR3 mutation analysis was performed as described previously [25]. Briefly, we performed a multiplex polymerase chain reaction (PCR) of the 3 exons containing the most common *FGFR3* mutations (exons 7, 10 and 15). Subsequently we performed single nucleotide polymorphism analysis using primers that anneal to the PCR product adjacent to the mutation site. Mutation probes were extended with a labeled dideoxynucleotide and products were analyzed on an ABI PRISM® 3100 Genetic Analyzer with the label indicating the presence or absence of a mutation. GeneScan® Analysis Software, version 3.7 was used for data analysis. Primers and probes are given in Supplementary Table S1.

Statistical analysis

For the analysis of BS-SNaPshot data, we used GeneMarker Software version 1.7 (SoftGenetics, State College, PA, USA). The methylation percentage for each gene and each sample was calculated as the ratio of the height of the C/G peak divided by the height of the C/G peak plus T/A peak multiplied by 100. We calculated the sensitivity and specificity

of the different combinations of the 8 markers by plotting the receiver operating characteristic curve (ROC) and calculating the area under the curve (AUC) using SPSS statistical software V17.0. To predict the best combination of markers, we performed a binary logistic regression on all possible combinations of the markers. Binary logistic regression will assign a beta (Coefficient) value for each marker based on the contribution in predicting an outcome. From these beta values we calculated the predictive probability value for that particular combination in all individual samples investigated using a formula $\frac{1}{1+e^{-z}}$, where z = constant + % methylation marker A * β value marker A + % methylation marker B * β value marker B etc. We chose a cut-off value allowing 10% false positives in the control group (specificity=90%). From this analysis we picked the best 3-marker combination that showed the highest sensitivity.

Results

Identification and validation of methylated CGIs in BC

Study design is described in Figure 1. Patient characteristics for all cohorts are given in Table 1. Previously we identified 110 CGIs methylated in BC but not in control urine [26] (PCT(NL2010)/050213). For this study the 21 CGIs with the largest methylation difference were selected. First we tested the performance of 42 CpGs from these 21 CGIs in BS-SNaPshot assays on DNA obtained from BC cell lines (results not shown). We selected 8 candidate CpGs that performed best regarding PCR efficiency and probe signal. We then validated the methylation status of these eight CpGs in an independent set of 48 bladder tumours and 70 normal urines. Figure 2 shows the methylation % of the 8 markers.

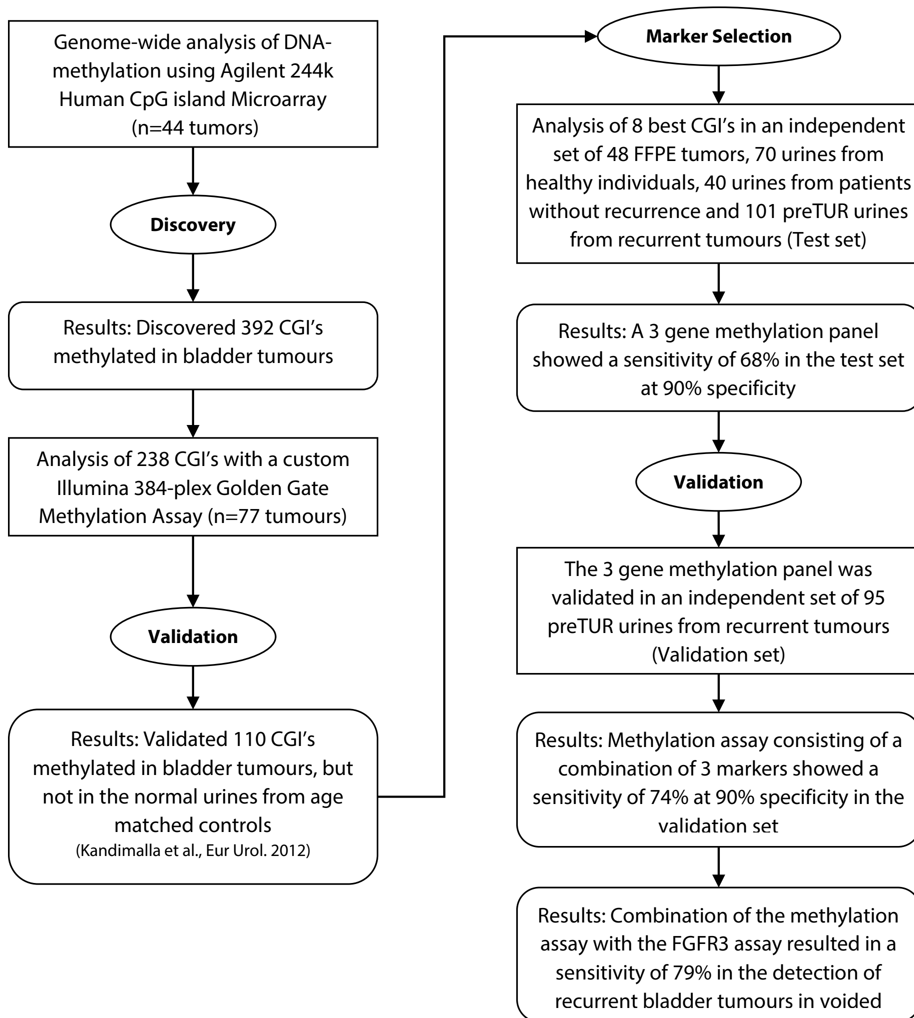

Figure 1: Study design.

Selection of the optimal combination of markers for the detection of recurrent BC in voided urine

The eight markers were then analyzed on a test set of 101 voided urines collected from different patients before resection of a recurrent tumour. We calculated the power of each methylation marker by calculating the AUC using the 101 recurrent urines against the 70 control samples. Specificity was set at 90%. The best single marker in the test set was *OTX1* with a sensitivity of 65%. Performance of the 8 markers on the test set is shown in Table 2 and Figure 2. The p-values in Table 2 indicate that the markers are significantly different between urines from patient and healthy individuals. In addition we analyzed sensitivity of each marker regarding detection of different stage and grade recurrences. Sensitivity increased with stage and grade as shown in Table 3.

Table 2: Sensitivity, PPV, NPV and AUC of the individual markers in the test set (n=101). Specificity is 90%).The combination of OTX1, ONECUT2 and OSR1 was found to be the most sensitive combination of markers

Gene	Sensitivity	PPV	NPV	AUC	95% CI-Lower	95% CI-Upper	P-value
OTX1	65 (65/101)	90	65	.805	.740	.871	.000
MEIS1	46 (46/101)	87	54	.749	.678	.821	.000
ONECUT2	52 (52/101)	88	57	.737	.664	.810	.000
SIM2	49 (49/101)	88	56	.753	.683	.824	.000
FOXA1	38 (38/101)	84	50	.659	.579	.739	.000
ZNF503	52 (52/101)	88	57	.784	.717	.852	.000
HOXA9	62 (62/101)	90	63	.829	.770	.888	.000
OSR1	44 (44/101)	86	53	.705	.630	.781	.000
OTX1+ONECUT2+OSR1	68 (68/101)	91	67	.801	.734	.867	.000

Table 3: Sensitivity of the methylation markers for detection of different stage and grade recurrences in the test set (n=101). Specificity of the markers is 90%.

Gene/Combination	Ta (n=73)	T1 (n=13)	T2 (n=14)	G1 (n=23)	G2 (n=46)	G3 (n=27)
OTX1	60 (44/73)	77 (10/13)	86 (12/14)	48 (11/23)	63 (29/46)	81 (22/27)
MEIS1	44 (32/73)	62 (8/13)	50 (7/14)	30 (7/23)	48 (22/46)	59 (16/27)
ONECUT2	49 (36/73)	77 (10/13)	50 (7/14)	43 (10/23)	46 (21/46)	70 (19/27)
SIM2	40 (29/73)	85 (11/13)	71 (10/14)	22 (5/23)	46 (21/46)	78 (21/27)
FOXA1	33 (24/73)	62 (8/13)	43 (6/14)	4 (1/23)	43 (20/46)	59 (16/27)
ZNF503	47 (34/73)	77 (10/13)	64 (9/14)	35 (8/23)	50 (23/46)	70 (19/27)
HOXA9	60 (44/73)	77 (10/13)	64 (9/14)	52 (12/23)	61 (28/46)	78 (21/27)
OSR1	37 (27/73)	69 (9/13)	57 (8/14)	17 (4/23)	37 (17/46)	74 (20/27)
OTX1_ONECUT2_OSR1	64 (47/73)	77 (10/13)	86 (12/14)	57 (13/23)	65 (30/46)	81 (22/27)

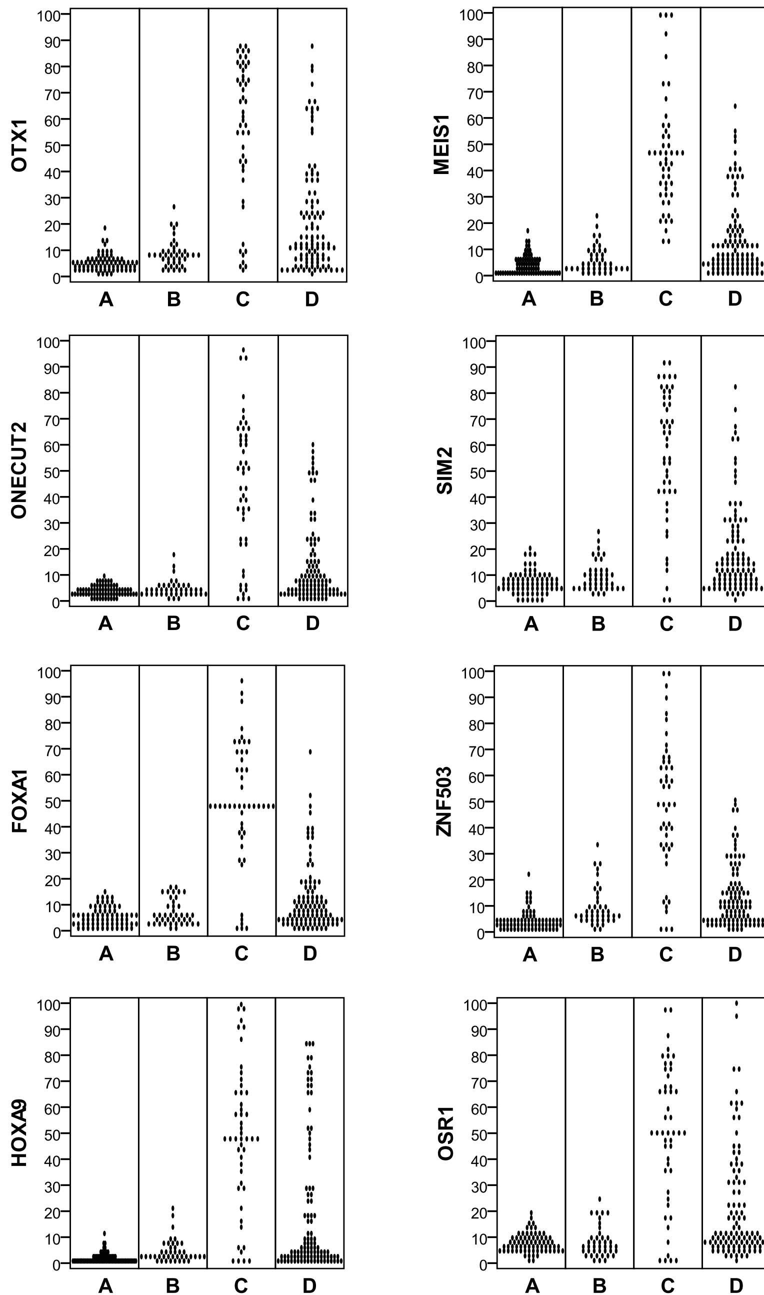

Figure 2: Scatter plots of methylation percentage of each marker in the samples investigated. A) Urines from healthy individuals (n=70) B) Urines from patients without recurrence (n=40) C) Tumour tissue (n=48) D) Test set urines (n=101).

A 3-plex sensitive and reproducible methylation assay

Next we performed a binary logistic regression with all possible combinations and picked the combination of three CGIs (in the *OTX1*, *ONECUT2* and *OSR1* genes) with the highest sensitivity. The predictive probability value was calculated using the following formula: z = -1.618 + % methylation *OTX1* * 0.168 + % methylation *ONECUT2* * 0.050 + % methylation *OSR1* * 0.016. A predictive probability cut-off value of 0.580 was chosen, allowing 10% false positives in the normal urines, which is shown in Figure 3. This resulted in a sensitivity of 68% in the test set. Sensitivity, AUC, PPV, NPV of this combination are presented in Table 2. The 3 markers were subsequently combined in a 3-plex BS-SNaPshot assay as depicted in Figure 4. The 3-plex methylation assay showed sensitivities of 64, 77 and 86% for Ta, T1 and T2 recurrences, respectively and 57, 65 and 81% for detecting G1, G2 and G3 tumours (Table 3). Reproducibility of the assay was investigated independently by two investigators on 16 urine samples. Percentages of methylation were highly correlated (Supplementary Figure 1). In order to get an impression of methylation in the case of multiple metachronous tumours, we selected patients with multiple tumours (30 tumours from 11 patients) from the original validation array [26]). Methylation of the 3 genes was highly consistent within a patient (Supplementary Table 2).

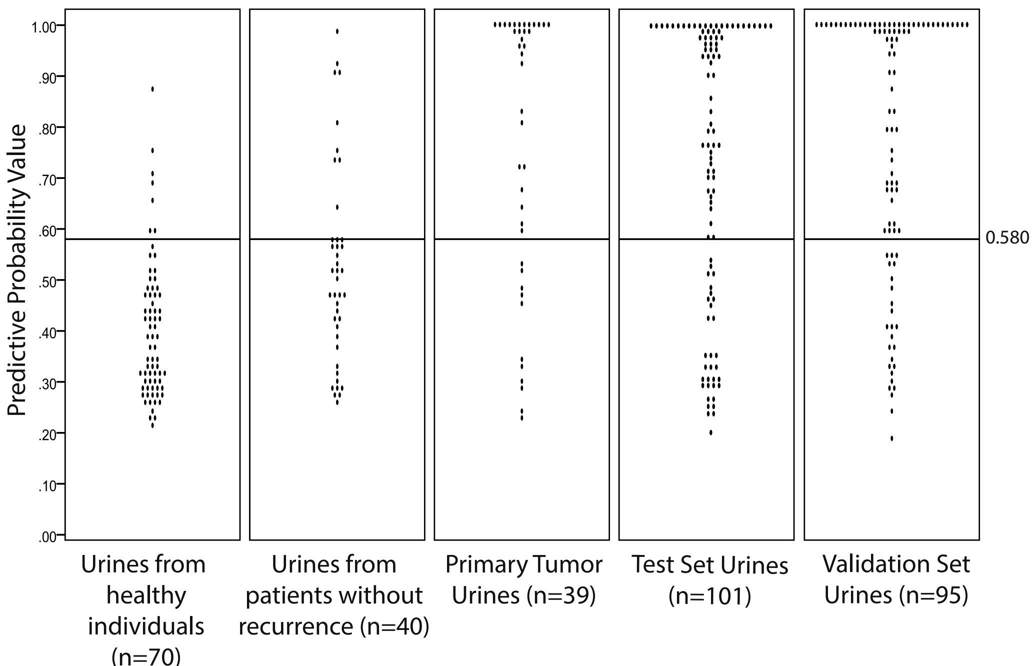

Figure 3: Predictive Probability Value plot of the 3-plex methylation assay. The chosen cut-off was 0.58.

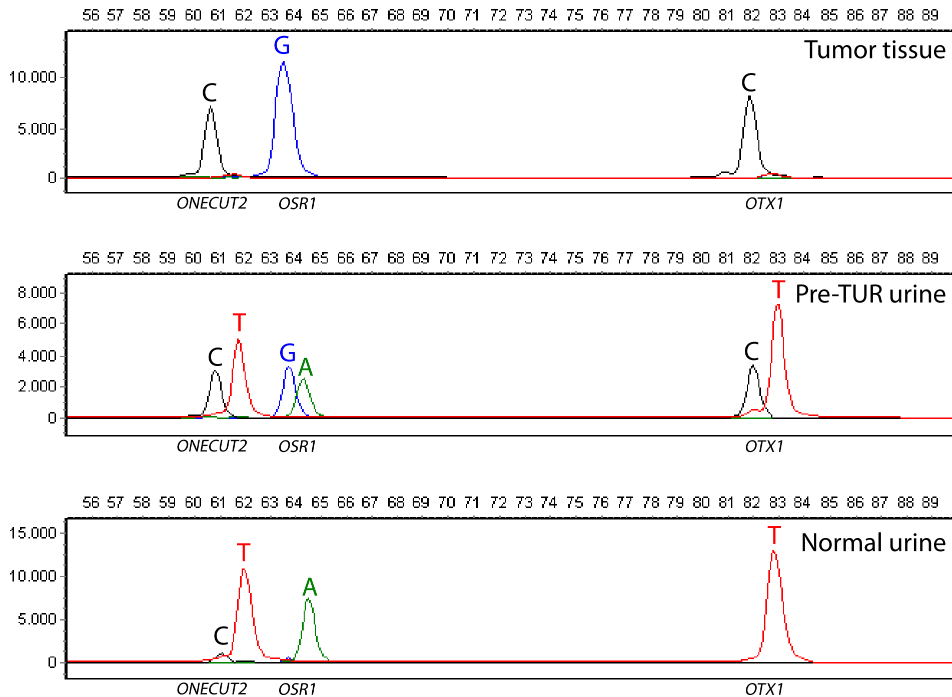

Figure 4: Multiplex Methylation Assay. The figure shows a typical ABI Sequencer output file analyzed by the GeneMarker Software. An analysis of tumour tissue, preTUR urine and normal urine is shown in top, middle and bottom panels, respectively. Peaks labeled with. C or G represent the methylated nucleotide and T or A the not methylated nucleotide.

Validation of the best combination of markers in an independent set of urines

Subsequently, the 3-plex assay was tested on an independent validation set of 95 preTUR urines from patients with a previous NMIBC G1/G2. The best single marker in the test set *OTX1* also showed the highest sensitivity (72%) in the validation set. The 3-plex assay had a sensitivity of 74% with an AUC of 0.86 (Table 4, Figure 5). We achieved a sensitivity of 80% for the detection of primary NMIBC G1/G2 tumours. We further observed 22% false positives in 40 urines investigated from BC patients who did not have a recurrence 6 months following urine collection (Figure 2 and 3).

Next we combined the 3-gene methylation panel with *FGFR3* status of the preTUR urine. This lead to an increase of 5% sensitivity in the validation set achieving 79% sensitivity with an AUC of 0.89 for the detection of recurrent BC in voided urine (Table 4). The p-values in Table 4 indicate that the markers/assays are significantly different between urines from patient and healthy individuals. The AUC curve for the combination of the methylation and the *FGFR3* assay is shown in Figure 5. The data of cytology, FGFR3 and methylation was available for 72 urines. For this set of urines we calculated the sensitivity, PPV, NPV and AUC of cytology alone, methylation+cytology and methylation+cytology+FGFR3. Results are shown in Table 4.

Table 4: Validation of 3-plex methylation assay in an independent set of 95 preTUR urines (validation set, n=95) alone and in combination with the FGFR3 assay and cytology. Specificity of 3-plex assay, 3-plex assay plus FGFR3 assay, 3-plex assay plus cytology and 3-plex assay together with cytology and FGFR3 is 90%, while it is 100% for the FGFR3 assay and cytology.

Assay	Sensitivity	PPV	NPV	AUC	95% CI-Lower	95% CI-Upper	P-value
Methylation (OTX1+ONECUT2+OSR1)	74	91	72	.864	.808	.919	.000
FGFR3	52	100	58	.762	.692	.832	.000
Methylation + FGFR3	79	92	76	.886	.835	.938	.000
Cytology	57	100	70	.785	.707	.863	.000
Methylation + Cytology	77	89	79	.890	.833	.947	.000
Methylation + Cytology + FGFR3	82	89	83	.904	.850	.959	.000

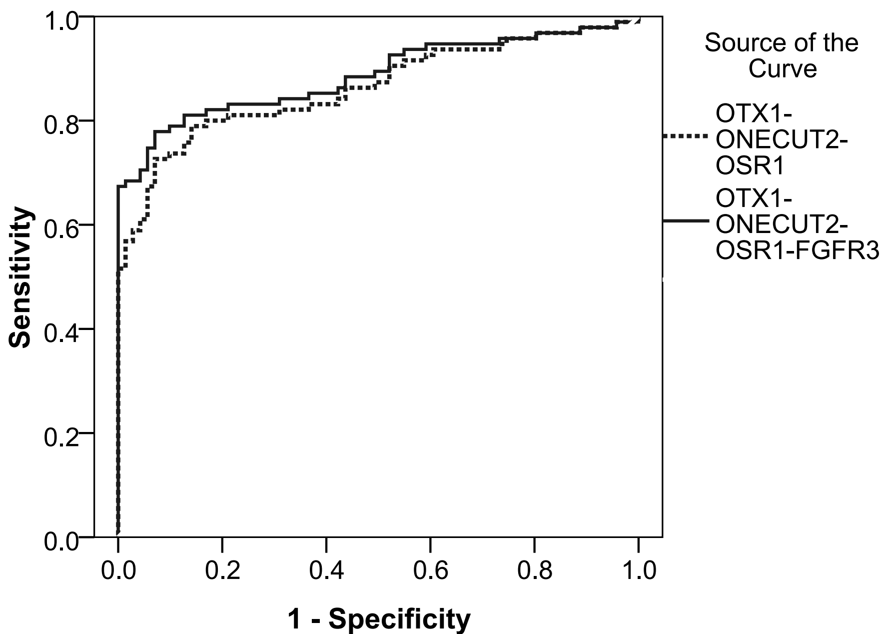

Figure 5: ROC curve of methylation assay (dotted line) and methylation + FGFR3 assay (thick line) for the validation set.

The 3-plex methylation assay is more sensitive than the FGFR3 and cytology

Next, we compared the 3-plex methylation assay with the *FGFR3* assay and cytology in the validation set. Methylation, *FGFR3* mutation status and cytology information was available for 72 urine samples. The methylation assay showed a sensitivity of 74%, while it was 57% for cytology and 52% for *FGFR3,* respectively. When we split this group according to grade, the sensitivity increased from low to high grade as shown in Table 5. The sensitivities of the combinations Methylation + *FGFR3*, Methylation + Cytology and Methylation + *FGFR3* +

Cytology were 79, 77 and 82%, respectively. These results show that the methylation assay is more sensitive than cytology and the *FGFR3* assay (when patients were not stratified based on the *FGFR3* or methylation status of their primary tumour).

Table 5: Comparison of the sensitivities of the methylation assay, FGFR3 and cytology and sensitivities of the various combinations (validation set, n=95).

Assay	G1 (n=33)	G2 (n=52)	G3 (n=9)	Overall (n=94)
Methylation	67 (22/33)	77 (40/52)	78 (7/9)	74 (70/94)
FGFR3	60 (20/33)	48 (25/52)	56 (5/9)	52 (49/94)
Cytology	36 (9/25)	67 (26/39)	75 (6/8)	57 (41/72)
Methylation + FGFR3	73 (24/33)	81 (42/52)	78 (7/9)	79 (74/94)
Methylation+ Cytology	68 (17/25)	82 (32/39)	87 (7/8)	77 (55/72)
Methylation+FGFR3+Cytology	72 (18/25)	87 (34/39)	88 (7/8)	82 (59/72)

Discussion

A major problem in the management of patients presenting with NMIBC is that 70% will develop one or more recurrences and that recurrences can keep on developing for up to 25 years [6]. Surveillance of these patients by cystoscopy is warranted [28]. However, cystoscopy is an invasive diagnostic procedure that is not well tolerated by many patients. Cytology has a high sensitivity for high-grade lesions, but lacks sensitivity for low-grade tumours [14]. To provide an alternative for cystoscopy and cytology, the development of molecular non-invasive tests using voided urine has been a major undertaking in the last decade. However, what is still lacking are tests that address the patient population in question, i.e. patients under surveillance for potential recurrences after a primary G1/G2 NMIBC. One-third of these patients do not develop recurrences at all and low/intermediate risk patients may develop only few recurrences over a long period of time. Hence, surveillance by too frequent cystoscopies can be considered as overtreatment of many of these patients. An ideal test for surveillance of BC that can replace cystoscopy should be urine based, sensitive, cost-effective, easy to perform with limited material, and with no intra observer variability.

To achieve this, we developed a 3-plex assay for the diagnosis of recurrent BC. To our knowledge this is the first study, where the methylation markers were assessed during follow-up of patients with a primary Ta/T1G1/G2 tumour. Our three gene methylation panel consisting of *OTX1*, *ONECUT2* and *OSR1* had a sensitivity of 68 and 74% in the test and validation set respectively with a specificity of 90% for the detection of recurrent bladder tumours in voided urine. Previously we and Serizawa et al., [29] showed an inverse correlation between *FGFR3* mutation and methylation, therefore a combination of these assays could increase sensitivity for the detection of recurrent BC. We therefore combined the 3-plex methylation assay with the *FGFR3* mutation assay. The combination of both assays increased sensitivity to 79% in the validation set.

It is our experience that many urine samples contain low amounts of cells and a yield of 50 nanogram DNA from 50 ml of urine is no exception. The *FGFR3* and 3-plex assays require 5 and 30 nanogram DNA each. Hence, there will be sufficient DNA in most urine samples to perform these assays. Both assays are easy to perform in a standard molecular diagnostic laboratory. Moreover, we have shown that they are highly reproducible between different operators. Combined material costs of the two assays, including DNA isolation, amount to about 30€. Personnel costs depend on the number of assayed samples, being cheaper when many samples are analyzed simultaneously.
The fact that not all recurrences are detected with the urine assays is most probably due to the absence or low concentration of tumour cells in the urine sample. This could probably be improved by analyzing more than one urine sample as we showed previously for the *FGFR3* mutation assay [24]. Another possibility would be to increase the analytical sensitivity of the assays. For the *FGFR3* assay we obtained an analytical sensitivity of about 5% (the mutation is detected when more than 5% of the cells harbor the mutation [25]). For the similar 3-plex methylation assay this would be the same. Higher analytical sensitivity can possibly be obtained by using next-generation sequencing (NGS). However, at the moment the costs of methylation+*FGFR3* as performed in this work is still much cheaper. We further observed that multiple tumours from a patient have highly concordant methylation of the 3 markers, which underlines the usefulness of the markers for surveillance. The sensitivity of our assay combination is similar to the sensitivity of the current gold standard white light cystoscopy with sensitivity in the range of 68 to 83% when compared to the more sensitive blue light cystoscopy [30, 31]. Given the comparable sensitivities of urine testing and cystoscopy, we suggest that, a subsequent study should be performed investigating a modified surveillance protocol consisting of the urine test followed by cystoscopy, only when the urine test is positive, for patients in the low/intermediate risk BC group.
A number of recent studies have reported high sensitivities with DNA methylation biomarkers for the detection of BC in voided urine [32-43]. Three of these studies used methylation specific PCR (MSP) and reported sensitivities in the range of 85 to 94% at a specificity in the range of 93 to 100%. Renard *et al* showed a high sensitivity for the combination of the *TWIST1* and *NID2* genes for the diagnosis of primary BC. Reinert and colleagues used a methylation sensitive high resolution melting analysis and reported 84% sensitivity at 96% specificity. The study by Zuiverloon [44] et al., reported a four gene methylation panel to detect recurrent bladder tumours with a sensitivity of 72% at a specificity of 55%. However, all these studies, with the exception of Zuiverloon *et al*, used urines from patients with primary and recurrent tumours, including high-grade and MIBC. Hence the detection rate of recurrent tumours developing after a primary NMIBC G1/G2 using these markers is probably much lower. The CGIs found by others to be methylated in BC were largely also found to be methylated in our genome-wide study, but to a lesser extent than the ones we chose for our diagnostic assay.

The *OTX1* (orthodenticle homeobox 1) gene is methylated in lung cancer [45] and its expression is regulated by p53 in breast cancer [46]. The *OSR1* (odd-skipped related 1) gene is methylated in lung and breast cancer [45, 47]. The *ONECUT2* (one cut homeobox 2) gene participates in the network of transcription factors regulating liver differentiation and metabolism [48]. This gene is methylated in lymphomas and lung cancer [49, 50]. The methylation of these genes in BC was not reported before.

Conclusion

Here we report a genome-wide methylation investigation in BC, followed by a selection and validation strategy to develop a 3-plex methylation assay specific for the detection of recurrent BC. The combination of the 3-plex methylation assay and the *FGFR3* assay efficiently detects recurrent BC without the need for up-front stratification of patients. Given the comparable sensitivities of urine testing and cystoscopy, we suggest that, a subsequent study should be performed investigating a modified surveillance protocol consisting of the urine test followed by cystoscopy, only when the urine test is positive, for patients in the low/intermediate risk BC group.

Further validation of the presented markers in a prospective longitudinal study is underway.

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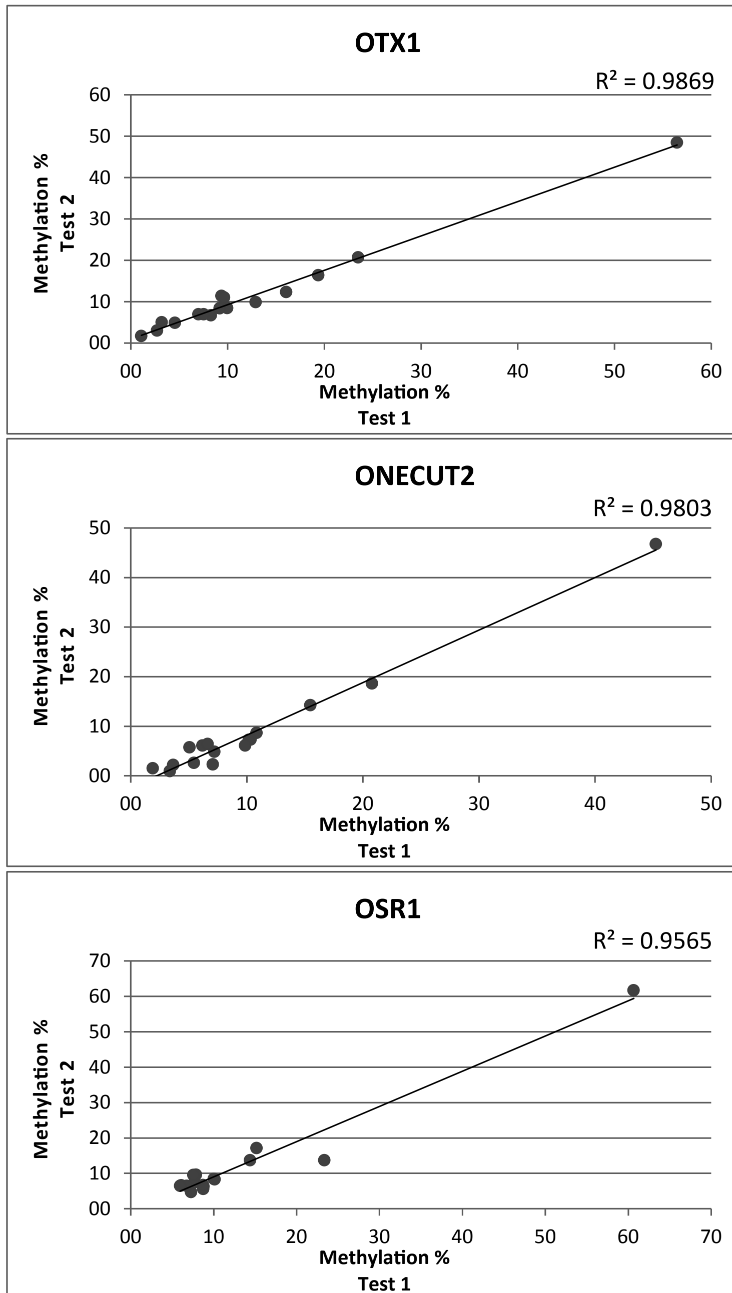

Supplementary Figure 1: Consistency of methylation assay

Supplementary Table 1: Primers and probes used for the BS-SNaPshot assay.

Marker	Primer/Probe	Sequence	Concentration pmol
Single-plex Methylation Assays			
HOXA9	Forward primer	5'-TTGGGGAAAAAATTATAAGTGG-3'	10
	Reverse primer	5'-AATTTCCAACCCTAACCTTAAAC-3'	10
	Amplicon size	270 bp	
	SNaPshot probe	5'-TTTYGTTTTGGTTATTTA-3'	2
ONECUT2	Forward primer	5'-GGGGTTTTTTGTTTTTTGTATTTTT-3'	10
	Reverse primer	5'-TCATTTTCAAACTTAAACTTAATCACC-3'	10
	Amplicon size	206 bp	
	SNaPshot probe	5'-GTTTGGGYGGTTGGGTT-3'	2
FOXA1	Forward primer	5'-TTAGGTAGGTAGAAGTAGAGGGAGA-3'	10
	Reverse primer	5'-AAAAAAAACCCTAAAAAAACTC-3'	10
	Amplicon size	204 bp	
	SNaPshot probe	5'-TGGTAGATAGTAGGGTTTGGGT-3'	2
SIM2	Forward primer	5'-GGTTTTTGTTGATTTTTAGGTTA-3'	10
	Reverse primer	5'-ACCTCTTCCAAAAATACAACTTTC-3'	10
	Amplicon size	171 bp	
	SNaPshot probe	5'-GTTAAGGTAAGGAATTGTTTTG-3'	2
MEIS1	Forward primer	5'-GGGTTTTTAGAGGTTAGGGGAA-3'	10
	Reverse primer	5'-CAACTAAATAACCAAACCTCTCCTC-3'	10
	Amplicon size	256 bp	
	SNaPshot probe	5'-GGAGAGGGGGGTTATGATGTTAGG-3'	2
OSR1	Forward primer	5'-GTTTTTTTAGTAGAGATTAGGTTTTTAGTT-3'	10
	Reverse primer	5'-TAAAAACTACTCAATTTTCACTCC-3'	10
	Amplicon size	297 bp	
	SNaPshot probe	5'-TTAAATTTTAGTTTTTTTTT-3'	2
ZNF503	Forward primer	5'-GTTTGTGTTTGGAGAATTTTTAGAG-3'	10
	Reverse primer	5'-AAACTACCTCCTCCCCAATTTAAT-3'	10
	Amplicon size	263 bp	
	SNaPshot probe	5'-TTGGGTGATTTAGTTTGGTT-3'	2
OTX1	Forward primer	5'-TTTTGAGAGGTATAGAGAGGGGTAGT-3'	10
	Reverse primer	5'-CCCCTAACAAACCCAAATCTC-3'	10
	Amplicon size	172 bp	
	SNaPshot probe	5'-TTTATTTGTGGTTTTTTAGGTT-3'	2
Multiplex Methylation Assay			
OTX1	Forward primer	5'-TTTTGAGAGGTATAGAGAGGGGTAGT-3'	10
	Reverse primer	5'-CCCCTAACAAACCCAAATCTC-3'	10
	Amplicon size	172 bp	
	SNaPshot probe	5'-TTTATTTGTGGTTTTTTAGGTT-3'	5
ONECUT2	Forward primer	5'-GGGGTTTTTTGTTTTTTGTATTTTT-3'	10
	Reverse primer	5'-TCATTTTCAAACTTAAACTTAATCACC-3'	10
	Amplicon size	206 bp	
	SNaPshot probe	5'-GTTTGGGYGGTTGGGTT-3'	5
OSR1	Forward primer	5'-GTTTTTTTAGTAGAGATTAGGTTTTTAGTT-3'	10
	Reverse primer	5'-TAAAAACTACTCAATTTTCACTCC-3'	10
	Amplicon size	297 bp	
	SNaPshot probe	5'-TTAAATTTTAGTTTTTTTTT-3'	10

FGFR3 Assay			
Exon 7	Forward primer	5′- AGTGGCGGTGGTGGTGAGGGAG 3′	18
	Reverse primer	5′- GCACCGCCGTCTGGTTGG 3′	18
Exon 10	Forward primer	5′- CAACGCCCATGTCTTTGCAG 3′	7.5
	Reverse primer	5′- AGGCGGCAGAGCGTCACAG 3′	7.5
Exon 15	Forward primer	5′- GACCGAGGACAACGTGATG 3′	10
	Reverse primer	5′- GTGTGGGAAGGCGGTGTTG 3′	10
R248C	SNaPshot probe	5'-T46CGTCATCTGCCCCCACAGAG	2
S249C	SNaPshot probe	5'-T36TCTGCCCCCACAGAGCGCT	2.4
G372C	SNaPshot probe	5'-T29GGTGGAGGCTGACGAGGCG	0.4
S373C	SNaPshot probe	5'-T19GAGGATGCCTGCATACACAC	1
Y375C	SNaPshot probe	5'-T43ACGAGGCGGGCAGTGTGT	0.6
G382R	SNaPshot probe	5'-T56GAACAGGAAGAAGCCCACCC	0.6
A393E	SNaPshot probe	5'-T34CCTGTTCATCCTGGTGGTGG	2.4
K652M/T	SNaPshot probe	5'-T20CACAACCTCGACTACTACAAGA	0.8
K652E/Q	SNaPshot probe	5'-T50GCACAACCTCGACTACTACAAG	1.2

Supplementary Table 2: Methylation in multiple metachronous tumours. Predictive probability value was calculated for all metachronous tumours using the formula specified in the methods and results section. The cut-off chosen was 0.58. The table shows that all metachronous tumors are detected and moreover the PPVs are highly correlated. Methylation Beta value range is 0 to 1; 0 represents no methylation and 1 represents 100% methylation.

		Methylation Beta value			
Patient	**Tumour ID**	**ONECUT2**	**OSR1**	**OTX1**	**PPV**
Patient 1	Ta:70	0.77	0.11	0.67	1.00
Patient 1	Ta:77	0.44	0.11	0.71	1.00
Patient 1	Ta:73	0.46	0.07	0.54	1.00
Patient 1	Ta:17	0.68	0.22	0.68	1.00
Patient 2	Ta:71	0.43	0.31	0.46	1.00
Patient 2	Ta:19	0.60	0.21	0.59	1.00
Patient 3	Ta:8	0.43	0.78	0.33	1.00
Patient 3	Ta:79	0.49	0.73	0.61	1.00
Patient 4	Ta:3	0.53	0.85	0.46	1.00
Patient 4	Ta:1	0.18	0.57	0.32	1.00
Patient 4	Ta:42	0.69	0.44	0.52	1.00
Patient 4	Ta:2	0.33	0.49	0.35	1.00
Patient 5	T1:20	0.97	0.68	0.02	0.99
Patient 5	T2:16	0.83	0.54	0.81	1.00
Patient 6	Ta:4	0.69	0.78	0.75	1.00
Patient 6	T1:15	0.84	0.91	0.86	1.00
Patient 6	T1:32	0.92	0.87	0.34	1.00
Patient 7	Ta:72	0.33	0.19	0.07	0.82
Patient 7	Ta:74	0.35	0.35	0.11	0.93
Patient 7	T1:24	0.55	0.08	0.15	0.98
Patient 8	T2:38	0.24	0.82	0.80	1.00
Patient 8	T2:48	0.32	0.79	0.69	1.00
Patient 9	T1:11	0.82	0.84	0.77	1.00
Patient 9	T1:10	0.84	0.75	0.84	1.00
Patient 10	T2:43	0.81	0.66	0.80	1.00
Patient 10	T2:45	0.75	0.80	0.75	1.00
Patient 10	T2:66	0.48	0.65	0.16	0.99
Patient 10	T2:69	0.68	0.73	0.74	1.00
Patient 11	T2:83	0.54	0.74	0.76	1.00
Patient 11	T2:88	0.34	0.48	0.77	1.00

Chapter 5

Decitabine and TSA reactivate genes in bladder cancer cell lines irrespective of the position of the CpG island

Raju Kandimalla[1], Roy Masius[1], Merel Willekes[1], Angela A. G. van Tilborg[1], Ellen C. Zwarthoff[1]
[1]Department of Pathology, Erasmus MC, Rotterdam, The Netherlands

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Abstract

Aberrant DNA methylation leads to gene silencing and is associated with the pathogenesis of bladder cancer. These modifications serve as biomarkers for the detection, prognosis, prediction and therapy response of bladder cancer. Epigenetic modulating drugs have shown promise in treating hematological malignancies like myelodysplastic syndrome and leukemia. Currently many of these drugs are in clinical trials also for the treatment of solid tumors. We have previously performed a genome-wide DNA methylation profiling in bladder cancer and found many genes methylated in promoter as well as in intragenic CpG islands (CGI). It is well documented that promoter methylation can lead to gene silencing, but intragenic methylation is still under investigation. To identify the effect of intragenic methylation on gene silencing, we selected the most hypermethylated and lowly expressed genes in bladder cancers and studied their reactivation pattern by treating bladder cancer cell lines with decitabine (DAC) and trichostatin A (TSA). For this we selected a set of 21 most methylated genes, nine of which were methylated in the promoter region, 10 in intragenic regions and in two the methylation CGI was downstream of the gene. First we confirmed the methylation of these genes in bladder cancer cell lines T24 and RT112. We treated both cell lines either with DAC or with DAC and TSA and investigated gene expression with q-RT-PCR. Among eight genes which are exclusively methylated at intronic CGIs in the tumours, four genes *NR4A2, PCDH7*, *TCF7L1* and *GATA2* showed similar methylation in the cell lines and were also silenced. These four genes were re-expressed after the treatment with DAC and TSA. This shows that gene body CpG island methylation can also lead to gene silencing.

Introduction

DNA methylation is currently the most widely studied epigenetic aberration. DNA methylation is mediated by the cytosine DNA methyltransferase (DNMT) family of enzymes that catalyze the transfer of a methyl group from S-adenosyl methionine to DNA. DNMT1 is primarily responsible for maintaining the methylation signature by copying the pre-existing methylation pattern onto the daughter strand after DNA replication. DNMT3A and DNMT3B are *de novo* enzymes that target previously unmethylated CpGs [1, 2]. Many studies have shown that promoter CGI methylation leads to the repression of gene expression [3, 4], while the effect of intragenic CGI methylation was less clear. There is currently more data to support the hypothesis that intragenic DNA methylation is a consequence of other mechanisms of transcriptional regulation, including histone modifications, nucleosome positioning and replication timing [5]. Interestingly, recent evidence suggested that variable methylation levels of individual CpG sites affect the binding affinity of transcription factors to nearby binding sites [6, 7]. This may offer a further mechanism by which specific intragenic CpG sites can affect transcription.

Drugs that inhibit DNA methyltransferases (DNMTs) were shown to reactivate silenced genes and induce differentiation or apoptosis of malignant cells [8]. Currently, patients with myelodysplastic syndrome and leukemia are treated with DNA methylation inhibitors such as azacitidine and decitabine [9, 10]. Histone acetylation is associated with transcriptional activation, whereas conversely, deacetylation of histones is associated with gene silencing and transcriptional repression. HDAC inhibitors like trichostatin inhibit deacetylation and hence increase gene transcription.

Bladder cancer (BCa) is the fifth most common solid neoplasm in humans [11]. About 80% of bladder cancers present as non-muscle invasive (NMIBC), 70% of which will recur and 10-20% of which will eventually progress to muscle-invasive disease (MIBC) [12, 13]. A high number of low grade NMIBC has a mutation in the *fibroblast growth factor receptor 3* (*FGFR3)* oncogene [14, 15]. MIBC is associated with the inactivation of tumor suppressor genes like *TP53* and *RB1* [16-18]. Besides the genetic aberrations, alteration in the epigenetic landscape like DNA methylation, histone modifications and nucleosome remodeling are associated with neoplasms including bladder cancer [19, 20].

In this regard we previously performed a genome-wide methylation study and found many genes methylated in bladder cancer [21]. Most of these genes were targets of polycomb repressor complexes (PcGs), which play a crucial role in the early development [22]. This genome-wide methylation study in bladder cancer has shown that methylation is equally distributed over both promoter CpG islands and gene body CpG islands. However, what the consequence of this intragenic methylation is regarding expression of the gene is unknown. Given these interesting results, we aimed to identify the effect of intragenic methylation on transcription in bladder cancer cell lines. Therefore we studied the expression of genes methylated in BCa using BCa cell lines before and after the treatment with epigenetic reactivating drugs DAC and Trichostatin A (TSA).

Materials and methods

Bladder cancer cell lines and drug treatment

Two bladder cancer cell lines T24 and RT112 were obtained from the American Type Culture Collection and cultured with standard Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 25 units/ml penicillin and 25 µg/ml of streptomycin in a humid atmosphere of 5% CO_2 at 37°C. Decitabine (Sigma-Aldrich, St Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) as a 0.1 M stock solution and stored at -80˚C. Cells were seeded ($3*10^5$ cells/100 mm dish) and treated 24 h later with 3 µM decitabine for 72 hrs. For decitabine treatment, the medium with the drug was refreshed every 24 h. For the combination treatment, 0.5 µM trichostatin A (TSA, Calbiochem) was added in the last 12 hrs. The control group comprised cells grown under the same condition but treated with DMSO. After 72 hrs the cells were harvested, subsequently DNA and RNA were isolated for methylation detection and gene expression analysis by RT-PCR. We performed two independent treatments with the cell lines using both DAC alone and together with TSA and harvested the RNA separately for gene expression analysis.

Nucleic acid isolation and bisulfite conversion

DNA was extracted with the QIAamp DNA mini kit (Qiagen) according to the manufacturer's recommended protocol. RNA was extracted with the RNeasy Mini-kit (Qiagen, Valencia, California, USA) according to the manufacturer's recommended protocol. The concentration and purity of RNA and DNA was measured using the NanoDrop ND-1000 Spectrophotometer (Thermo scientific, Wilmington, USA). Bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Gold Kit (Zymo, CA, USA) according to the manufacturer recommendations.

cDNA synthesis and real time RT-PCR

For cDNA synthesis we used the enzyme reverse transcriptase (RT), an RNA-dependent DNA polymerase which is isolated from a retrovirus; Moloney Murine Leukemia Virus (M-MLV RT). The reverse transcription reaction (M-MLV Reverse Transcriptase from Invitrogen, Paisley, UK) was carried out as follows: a mixture of 1 µg of the isolated RNA, 1.5 µL random primer, 1 µL 10mM dNTP mixture (10 mM of dATP, dGTP, dCTP and dTTP each at neutral pH) and water to a final volume of 12 µL was incubated at 65˚C for 5 min. Then, the mixture containing 4 µL 5x First-Strand buffer (250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM MgCl2), 1 µl RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/µl) and 2 µL 0.1M DTT was added. This reaction mixture was incubated at 37˚C for 2 min. Finally, 1 µl (200 units) of M-MLV RT was added and incubated at 25˚C for 10 min followed by 37˚C for 50 min. The reaction was inactivated by heating at 70˚C for 15 min. Real time PCR was performed using 12.5 ng cDNA, 7.5 pmol of primers and 12.5 µl of SYBR Green Master Mix (SensiMix SYBR Hi-ROX Kit from BIOLINE). The primers used are listed in

Supplementary Table 1. *RPLP0* gene was used as housekeeping gene to which all tested genes were normalized. *H19* and *SMPD3* genes were used as positive controls for the treatment with decitabine and TSA respectively. H19 is an imprinted gene and which was shown to be methylated in BCa cell lines and which can be re-expressed with the DAC treatment [23]. *SMPD3* is a gene that was proven to be silenced in BCa cell lines by histone methylation and hypoacetylation, but not by DNA methylation and which can be re-expressed with the TSA treatment [24]. All the reactions were performed in triplicates. The PCR conditions were as follows: 95°-10 min (activation of the enzyme), 95°-15 sec (denaturation) and 60°-1 min (annealing). Forty cycles were performed. Real time PCR was performed on the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, CA, USA). The ΔΔCt is (Normalized Ct value of the treatment and control with the house keeping gene, followed by subtracting the Ct value of the treatment with the control) calculated for all the genes investigated in the cell lines by treating with DAC and TSA. We calculated the fold change of re-expression using 2^n, where n represents the ΔΔCt.

Quantitative methylation specific PCR (Q-MSP)
The primers selected for real-time quantitative MSP (Q-MSP) assays were designed for CpG dinucleotides located in the promoter and intronic regions of the selected genes using Methyl Primer software [25]. The primers used are listed in Supplementary Table 1. Q-MSP reactions were performed in the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, CA, USA) as follows: each reaction included 5 µl of bisulfite converted DNA (12.5 ng), 7.5 µl of primers (7.5 pmol), 12.5 µl of SYBR Green Master Mix. In each 96-well plate, multiple negative controls (DEPC treated water) were included. Bisulfite converted Human Universal Methylated DNA Standard (Zymo, CA, USA) was used as control.

BS-SNaPshot
We used the Bisulfite Specific Snapshot method described in [21] to investigate the methylation of selected genes in BCa cell lines T24 and RT112. A list of primers and probes used is given in Supplementary Table 1. The method involves bisulfite conversion of genomic DNA (EZ DNA methylation gold kit, Zymo Research Corp, Orange, CA, USA), followed by subsequent amplification of interesting CGIs. The PCR products were treated with 2 units of Exonuclease I (ExoI) and 3 units of Shrimp Alkaline Phosphatase (SAP) (USB, Cleveland, Ohio USA). This was followed by a single-nucleotide probe- extension assay using a SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA) and probes designed to anneal to either the forward strand or the reverse strand of a PCR product adjacent to the CpG site of interest. These probes were fitted with T-tails of different length at their 5′ ends to allow separation of the extension products by size [26]. The mutation detection reactions were performed in a total volume of 10 µl containing 2 µl SAP/ExoI treated PCR product, 2.5 µl SNaPshot Multiplex Ready Reaction mix, 1 x Big Dye sequencing buffer, and 1 µl probe mix. Thermal cycler conditions were: 25 cycles of 10 seconds at 95˚C, 5 seconds at 50˚C, and 30 seconds at 60˚C. The products were treated with 1 unit SAP at 37°C for 60

min, and at 75°C for 15 min, and were analyzed on an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems) with the fluorescent label on the incorporated ddNTP indicating the presence or absence of a mutation. For analysis of the data, we used GeneMarker Software version 1.7 (SoftGenetics, State College, PA, USA). Methylation percentages were calculated by using the formula: Height of the C/G-peak / (Height of the C/G-peak + Height of the T/A peak) multiplied by 100.

Results

Methylation of intronic CGIs is associated with reduced gene expression

Promoter methylation leads to gene silencing, while the effect of the intragenic methylation is still under investigation. Our earlier genome-wide study [21] in bladder tumours revealed no specific preference for methylation of promoter regions. Instead, methylation was distributed proportionally over CGIs in promoter regions, within genes and in intergenic regions in accordance with the number of probes present on the array in the respective regions (Figure 1). Twenty-six percent of the probes represented on the array were located in promoter regions and a similar percentage of probes were methylated. Likewise, 53% of the probes were located in gene bodies, and the same percent of probes was methylated. To investigate whether methylation outside the promoter regions was also associated with low or absent gene expression, we next used the Oncomine database to compare genes with methylated CGIs with the available genome-wide expression profiles of bladder cancer [27-32]. We selected 71 highly methylated genes to look for the expression profile. This information could be obtained for 54 out of 71 genes. Of these 54 genes, 22 showed promoter CGI methylation, 17 (77.3%) of which were positively correlated with low expression. The remaining 32 genes showed intragenic methylation. Interestingly, 23 (72%) of these genes were also lowly expressed in bladder cancers (data not shown). These data suggest that not only promoter CGI methylation but also methylation of intronic CGIs can lead to reduced gene expression.

Selection of genes to study re-expression with DAC and TSA

We selected a set of 21 methylated CGIs from different genes, these comprised nine genes with promoter methylation only, 2 genes with CGIs methylated in promoter and an internal CGI, 8 with intragenic methylation and two that had methylation of a downstream CGI. Positions of the CGIs with respect to the gene i.e. promoter, inside etc. were as indicated by Agilent. The methylation profile of these 21 CGIs in tumors was determined previously [21] and is shown in Figure 2 for 8 genes (see below) with internal methylation and in Supplementary Figure 1 for the remaining 13. First we investigated the methylation of these CGIs in cell lines T24 and RT112 using the BS-SNaPshot assay. This clearly showed that the CGIs were also methylated in the cell lines (Table 1).

Subsequently, we then focused on the eight genes *NR4A2, MEIS1, PCDH7, VAX2, TCF7L1, OTX1, TBX15* and *GATA2* that were exclusively methylated in the gene body in tumours (n=44). We analyzed the methylation of upstream and internal CGIs in these genes in more

detail using Q-MSP. The position of the Q-MSP primers relative to the transcriptional start site is indicated in Figure 2. As the Q-MSP primers cover at least 3 to 4 CpGs, this analysis provides a more thorough picture of methylation than the array. Results of the Q-MSP are depicted in Figure 3. For instance, the *OTX1* gene as depicted in Figure 2 was methylated in tumours on the green CGI overlapping exon 4 and only low levels of methylation were detected in the blue CGI that covers the promoter and first exon. Figure 3 shows that in the two bladder cancer cell lines two regions upstream of position -1586 relative to the transcription start site (TSS) are methylated as is the internal CGI (position 3258-3392), but not the region (-316)-(-214) that is closes to the TSS. Together these results show that methylation of *NR4A2, PCDH7, TCF7L1* and *GATA2* in the two cell lines is similar to the methylation profile of the tumours. Among these four genes *NR4A2*, *PCDH7* and *TCF7L1* displayed exclusive intragenic CGI methylation, while the promoter CGIs are unmethylated. *MEIS1* was found to be methylated to a large extent in the gene body, while the promoter region closes to the gene body was found methylated in the cell lines unlike tumours. *OTX1*, *TBX15* and *VAX2* genes were found to be methylated in the promoter and gene body as opposed to the methylation pattern we observed in the tumours. This is most probably because of the difference in methylation pattern between the tumours and cell lines.

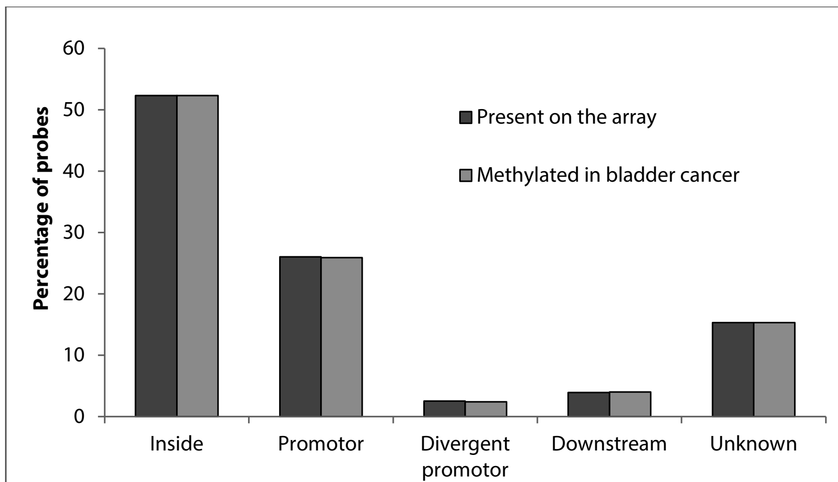
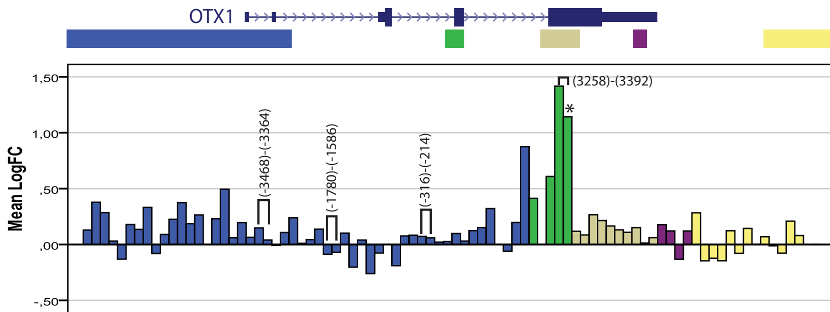


Figure 1: DNA methylation in bladder cancer is not promoter specific, instead it is evenly distributed over the genome according to the probes present on the array. Positions of the probes are according to the annotation of Agilent.

Table 1: Methylation percentages of the selected genes in two bladder cancer cell lines T24 and RT112 investigated by BS-SNaPshot.

	Methylation %	
Gene	**T24**	**RT112**
GRIK2	81	69
ZNF503	13	77
HOXA7	100	100
OSR1	93	100
FOXA1	100	100
SIM2	100	100
SNRPF	72	63
HOXA9	51	100
NR4A2	88	100
EVX2	53	96
VAX2	100	100
OTX1	100	100
GATA2	90	90
MEIS1	100	100
TBX15	89	95
ONECUT2	27	94
TCF7L1	100	100
PCDH7	36	96
SIM1	91	82
FEZF1	100	100
BHLHB5	88	97

Figure 2: Methylation pattern of multiple CGIs per gene as obtained from the Agilent 244K array. The gene and the position of its CGIs are shown above the bar graph and are based on the UCSC Genome browser NCBI36/hg18 assembly. Each colored bar represents an individual CpG probe of a similarly colored CGI. Data is shown as the average log fold change per probe for 44 tumours. Positions of the probes for the BS-SnapShot assay (results is Table 1) are indicated with an asterisk. The positions of Q-MSP primers used in Figure 3 are indicated relative to the transcription start site. All 8 genes shown in this figure were exclusively methylated at intragenic CGIs in 44 bladder tumours.


OTX1
Mean LogFC
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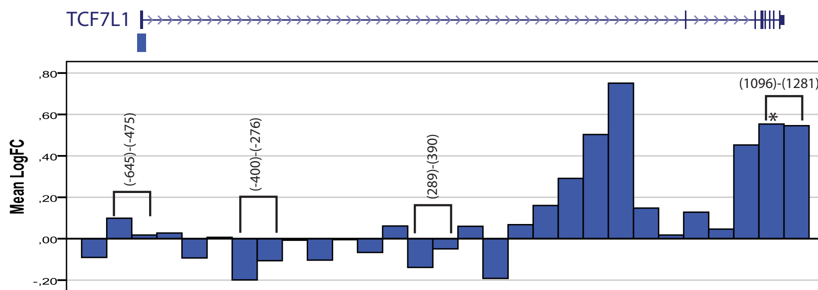
(-1780)-(-1586)
(-316)-(-214)
(3258)-(3392)
*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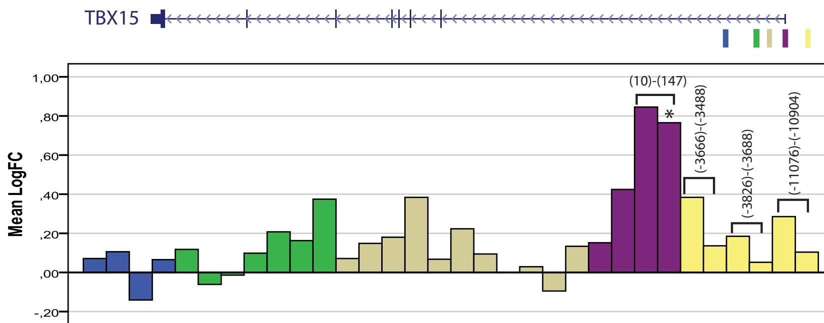
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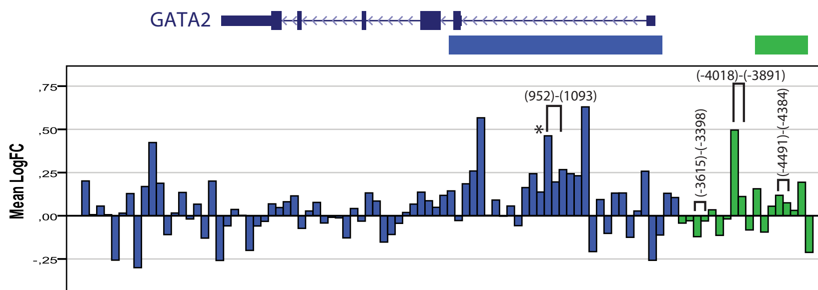
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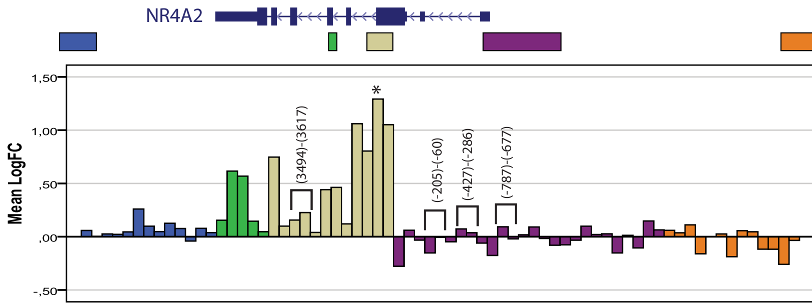
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,00
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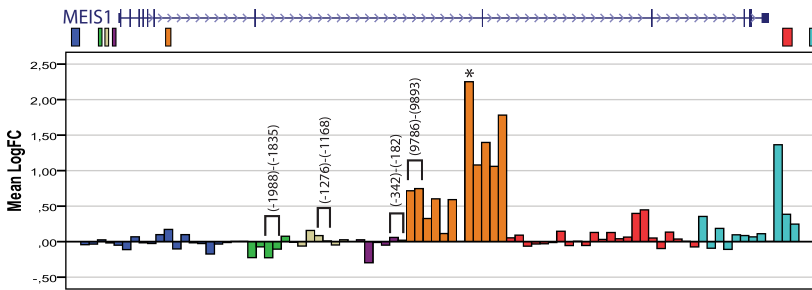
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,00
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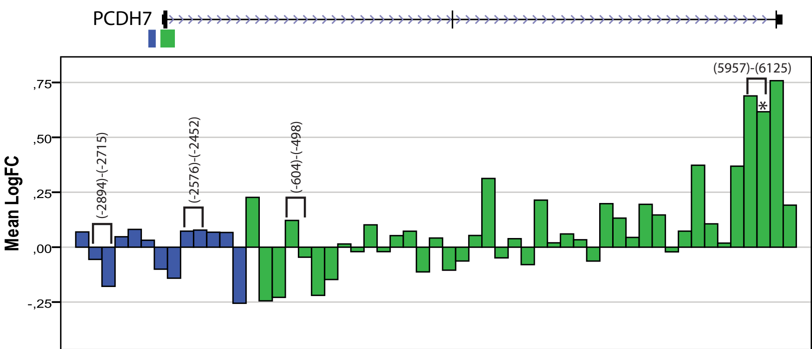
*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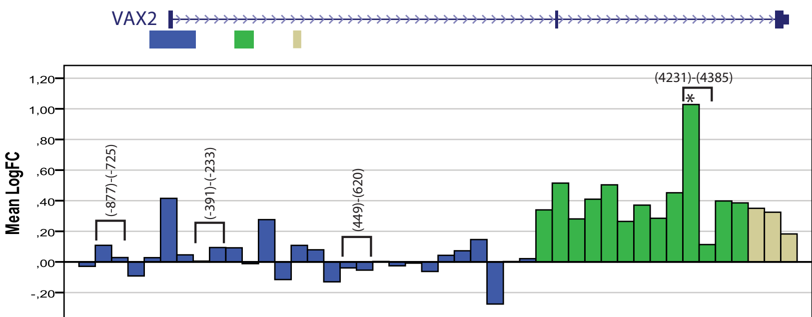
*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,20
,00
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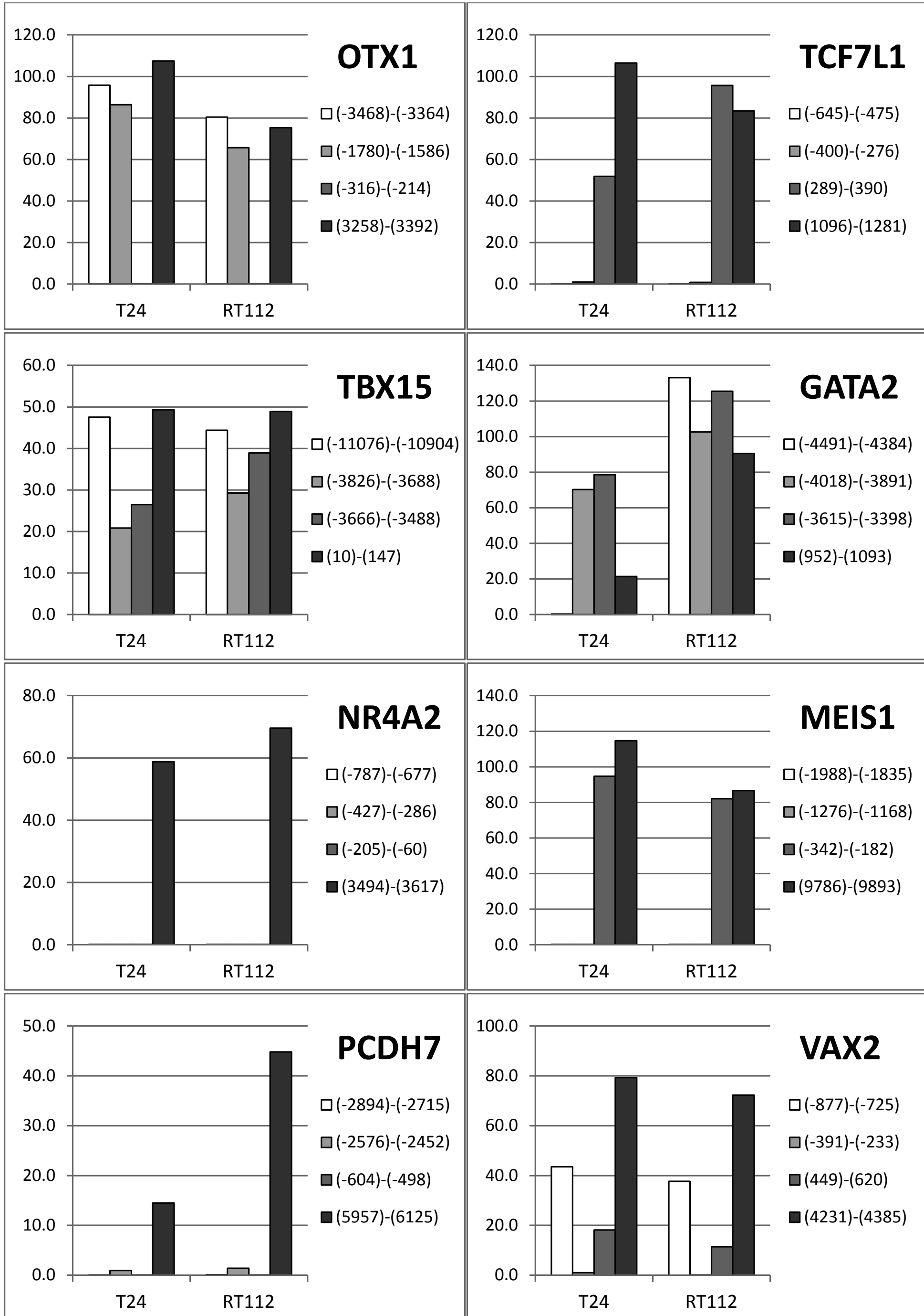
(449)-(620)
(4231)-(4385)
*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(-1780)-(-1586)
(-316)-(-214)
(3258)-(3392)
TCF7L1
(-645)-(-475)
(-400)-(-276)
(289)-(390)
(1096)-(1281)
TBX15
60.0
50.0
40.0
30.0
20.0
10.0
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GATA2
140.0
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(-3615)-(-3398)
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NR4A2
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(3494)-(3617)
MEIS1
(-1988)-(-1835)
(-1276)-(-1168)
(-342)-(-182)
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PCDH7
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(-2576)-(-2452)
(-604)-(-498)
(5957)-(6125)
VAX2
(-877)-(-725)
(-391)-(-233)
(449)-(620)
(4231)-(4385)

Figure 3: In depth methylation analysis of CGIs of the 8 selected genes. Methylation percentage of individual CpG islands investigated by Q-MSP is shown for two bladder cancer cell lines T24 and RT112. Legends represent primer position with respect to the transcription start site (TSS, see also Figure 2), Y-axis represents relative methylation percentage to in-vitro methylated DNA.

Re-expression of the genes with DAC and TSA

To assess whether the 21 genes selected are epigenetically regulated, the two bladder cancer cell lines were treated with epigenetic-modulating drugs DAC and TSA. We performed two independent treatments with both the drugs. From each treatment, we isolated RNA and performed the q-RT-PCR with all the genes in triplicates. The ΔΔCt values after treatment with DAC and DAC plus TSA are given in Table 2 (see materials and methods for a detailed explanation). Also included are positive controls *H19* and *SMPD3*. The RT112 cell line showed re-expression of all genes except *FOXA1*, *SNRPF, ONECUT2, PCDH7* and *BHLHB5*. The T24 cell line showed re-expression of all genes except *ZNF503, FOXA1*, *SNRPF*, *EVX2* and *ONECUT2*. The genes *NR4A2*, *PCDH7* and *TCF7L1*, with gene body CGI methylation were re-expressed after treatment with DAC and with DAC plus TSA. However, *PCDH7* was re-expressed only in the T24 cell line. In general we observed a higher re-expression when both the drugs DAC and TSA were used in combination. A difference in the ability to restore expression was evident among the two cell lines as RT112 cells re-expressed the studied genes to a higher extent compared to the T24 cell line. To exclude that re-expression could be due to transcription starting in the body of the gene we checked expression of *NR4A2* and *MEIS1* using multiple primer sets. There was no difference in expression (see Supplementary Figure 2).

Table 2: Re-expression of the genes in fold change for two BCa cell lines after treatment with DAC alone or DAC+TSA. Re-expression of the each experiment (Exp) as well as average re-expression of the two experiments is given. ND: not determined.

	RT112 - DAC			RT112 - DAC+TSA			T24 - DAC			T24 - DAC+TSA		
Gene	**Average**	**Exp 1**	**Exp 2**	**Average**	**Exp 1**	**Exp 2**	**Average**	**Exp 1**	**Exp 2**	**Average**	**Exp 1**	**Exp 2**
GRIK2	11.0	13.9	8.7	71.1	73.5	68.7	19.4	18.4	20.4	15.9	18.4	13.7
ZNF503	2.3	2.5	2.1	20.5	20.6	20.4	0.7	0.7	0.7	1.2	1.1	1.2
HOXA7	5.3	3.7	7.5	4.8	5.7	4.1	1.7	1.4	2.1	0.3	0.3	0.3
OSR1	3.1	2.6	3.8	3.0	3.2	2.8	1.3	1.2	1.4	1.2	1.3	1.1
FOXA1	1.0	1.0	0.9	0.8	0.7	0.9	0.5	0.5	0.5	0.1	0.1	0.1
SIM2	2.0	2.1	1.9	0.6	0.7	0.6	1.3	1.7	1.0	0.2	0.2	0.2
SNRPF	0.6	0.6	0.7	0.5	0.5	0.5	0.9	0.8	0.9	0.4	0.4	0.5
HOXA9	4371.8	14263.1	1340.0	26696.3	35119.9	20293.1	3.9	4.9	3.0	1.3	1.4	1.3
NR4A2	11.3	13.1	9.8	121.1	111.9	131.0	2.2	1.6	3.2	2.0	1.9	2.1
EVX2	15.6	21.1	11.5	111.4	477.7	26.0	0.7	1.1	0.4	6.1	14.9	2.5
VAX2	5.3	6.1	4.5	59.7	59.7	59.7	2.2	2.5	1.9	5.0	5.7	4.5
OTX1	3.6	4.0	3.3	21.5	17.6	26.2	1.4	2.0	1.0	2.1	2.3	1.8
GATA2	3.6	3.7	3.6	12.3	15.0	10.1	2.8	3.0	2.7	6.3	5.7	6.9
MEIS1	1.4	1.5	1.4	7.1	7.2	7.0	1.3	1.5	1.3	2.3	2.3	2.3
TBX15	3.2	3.2	3.1	4.9	13.0	1.8	3.6	3.2	3.9	4.4	4.6	4.2
ONECUT2	0.6	0.8	0.5	3.8	3.2	4.4	0.5	0.7	0.4	1.4	1.8	1.1
TCF7L1	1.7	1.6	1.8	3.6	3.2	3.9	1.9	1.9	1.9	1.0	1.0	1.0
PCDH7	0.7	0.9	0.6	1.0	0.9	1.1	1.7	2.2	1.4	2.1	2.2	2.0
SIM1	1.8	2.1	1.4	12.1	18.4	8.0	2.7	11.3	0.7	11.8	73.5	1.9
FEZF1	58.4	84.4	40.3	1355.3	2195.0	836.8	1.1	1.1	1.1	373.1	495.2	281.1
BHLHB5	1.0	0.9	1.1	6.1	6.5	5.8	1.1	2.5	0.5	1.8	2.8	1.1
H19	0	0	0	ND	ND	ND	19.1	21.3	17.0	ND	ND	ND
SMPD3	ND	ND	ND	268.2	222.0	315.1	ND	ND	ND	671.1	512.2	831.0

Discussion

We performed a genome wide study of CGI methylation in bladder cancer using a 244K array. Of the probes on the array about half were from intragenic CGIs and to our surprise we observed that the intragenic CGIs were as frequently methylated as promoter related CGIs. We therefore decided to investigate the effect of methylation of promoter and gene body CpG islands on gene expression. Comparing the methylated genes with gene expression profiles obtained from the public domain suggested that about 70% of the genes methylated at the intragenic or promoter CGIs had low transcriptional activity. After extensive rechecking, we identified three genes, *NR4A2*, *PCDH7* and *TCF7L1* that were methylated exclusively in CGIs in the gene body in bladder tumours as well as in two bladder cancer cell lines. We observed re-expression of these genes using RT-PCR following treatment of the bladder cancer cell lines with decitabine with or without trichostatin. By using multiple primer pairs we could exclude that this was due to activation of an internal transcription start site for *NR4A2*.

It is generally known that gene regulation is affected by promoter methylation [3, 4]. Data on the effect of methylation of internal CGIs on gene expression up till now is scarce. However, the data so far rather suggest that methylation of internal CGIs does not affect gene transcription in a negative way. Some of these papers were recently reviewed by Shenker [5] and Jones [33]. For instance, Hellman and colleagues [34] studied X-linked gene silencing using an array with 49% of the probes in the intragenic coding region. They observed that the active X-allele displayed a higher overall as well and intragenic methylation than the inactive X-allele [34]. In addition, Rauch and Maunakea [35, 36] found that intragenic methylation was even associated with higher level of gene transcription and they proposed that intragenic methylation is a mechanism which regulates the use of alternative promoters. Aran and colleagues [37] found a link between intragenic methylation and replication timing and claimed that early replicating genes inclined to be active and show high intragenic methylation levels. Salem et al., [38] showed that the *PAX6* gene was methylated in exon 5 in bladder and colon cancer cell lines, and that this was not associated with the inhibition of gene expression. Smith et al., [39] found that methylation of non-promoter CGIs were associated with up-regulation of gene expression in colorectal cancer.

In the light of these contrary results we propose two alternative explanations for our findings. Firstly, reactivation of genes with internal methylated CGIs could occur *in trans* through reactivation of a transcription factor gene by DAC treatment. Secondly, the methylated internal CGI could associate *in CIS* with the promoter region and negatively affect gene expression.

In conclusion we show that intragenic methylation can also lead to gene silencing, which can be rescued using the epigenetic modulating drugs. Further studies are necessary to unravel the mechanism behind this. Hence it is important to study the methylation all over the genome, instead of focusing on promoters only.

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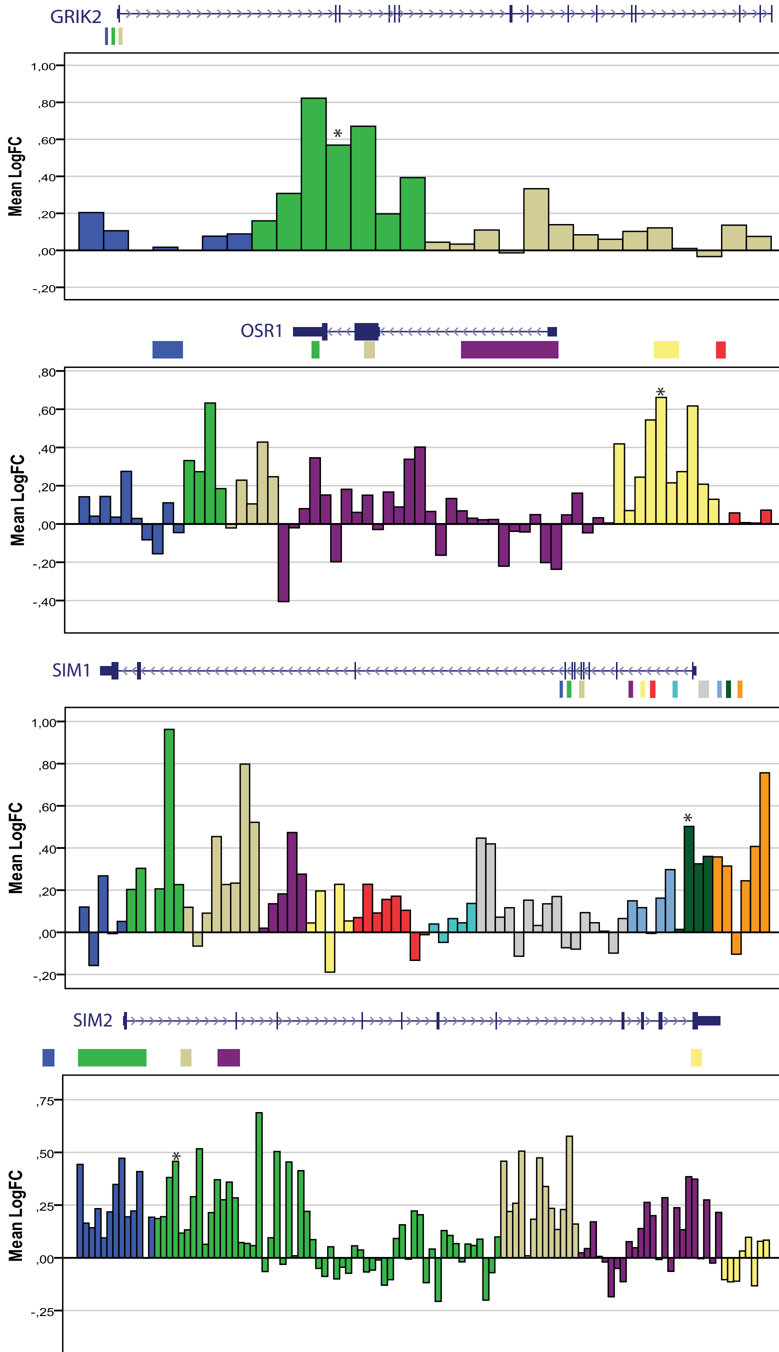
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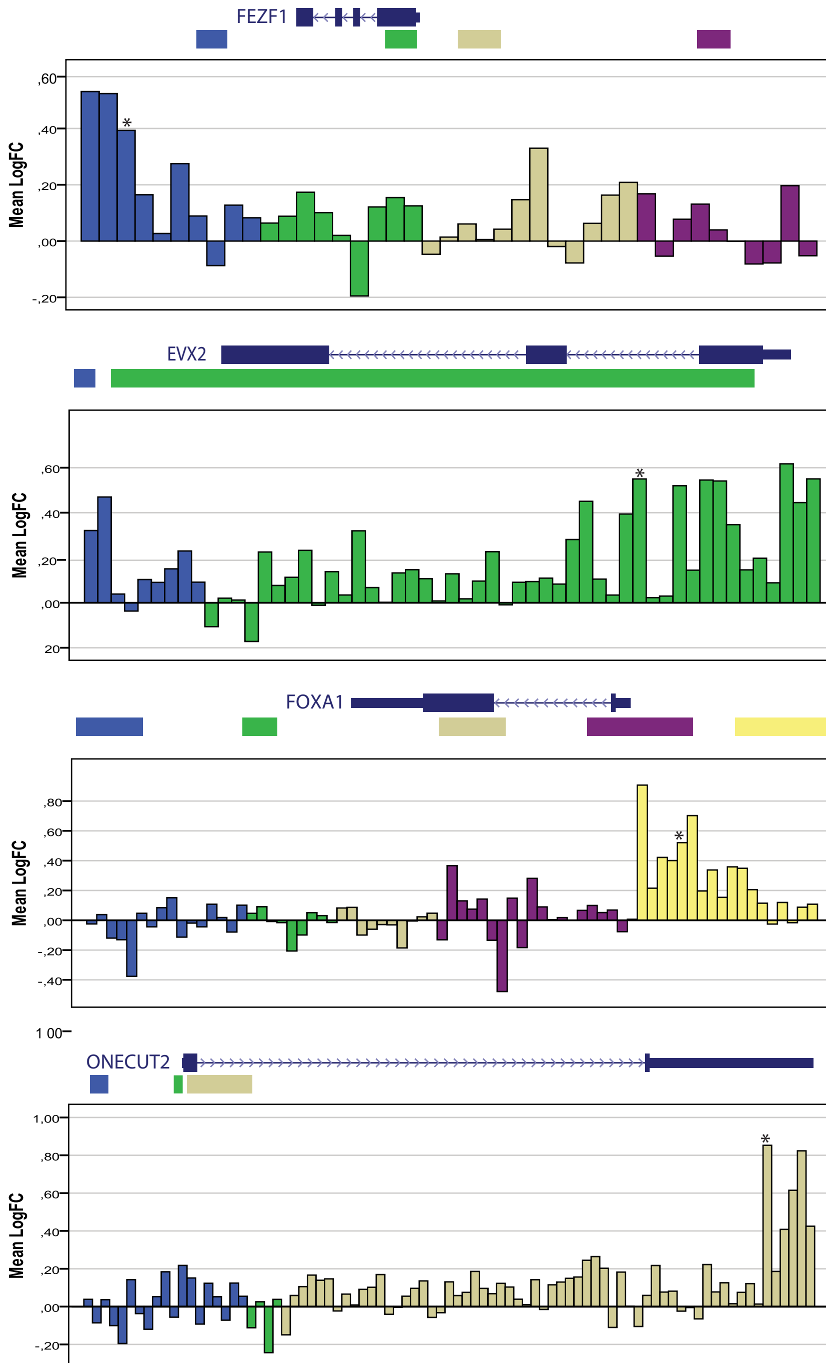
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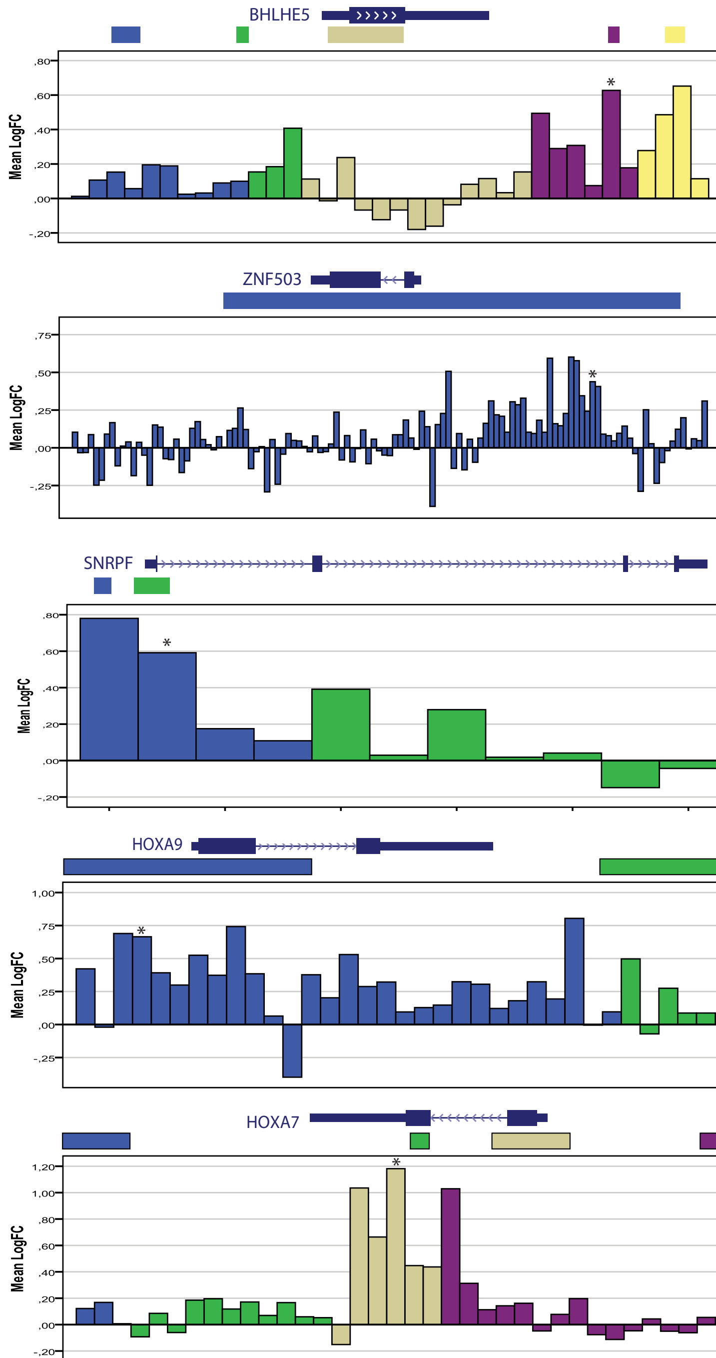
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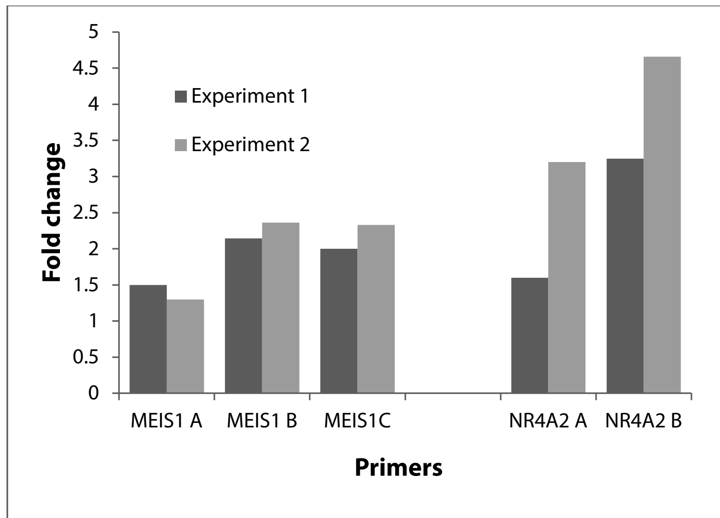
Supplementary Figure 1: (previous 3 pages) Methylation pattern of multiple CGIs per gene. The gene of interest and the position of relevant CGIs shown above the graph were based on the UCSC Genome browser NCBI36/hg18 assembly. Each colored bar represents an individual CpG probe of a specific CGI. Data is shown as the average log fold change per probe.



Supplementary Figure 2: Expression of *NR4A2* and *MEIS1* genes using multiple primer sets in T24 cell lines after treatment with DAC. *MEIS1* A primers spanned intron 12. *MEIS1* B primers spanned intron 5 and *MEIS1* C primers spanned intron 7. *NR4A2* A primers spanned intron 1 and *NR4A2* B primers spanned intron 2.

Chapter 6

General discussion

Discussion

6.1 Aim of the thesis

Bladder cancer is the fifth most common cancer in the western world and comprises distinct histopathological forms. Studies based on genomic alterations proposed a two pathway model for the pathogenesis of bladder cancer. Besides genomic changes epigenomic alterations also play a role in bladder cancer pathogenesis. Epigenetics covers several changes to the genome that do not alter the DNA sequence but can be inherited during cell division [1]. Major examples of epigenetic modifications are methylation of CG dinucleotides (CpGs) in the DNA and post-translational histone modifications. The work described in this thesis was aimed at unraveling aberrant DNA methylation in bladder tumours in order to shed light on the pathogenesis of bladder tumours and to identify and validate biomarkers that can be used in the clinic to diagnose recurrences and predict progression. To this end, we performed a genome-wide methylation profiling study and investigated the identified changes further regarding their diagnostic and prognostic performance. The results will be discussed in a wider perspective, and also implications for future research will be made.

6.2 Implications of genome-wide methylation profiling in bladder cancer

The general implications of the genome-wide investigation will be discussed here. The genome-wide DNA methylation analysis revealed distinct methylation patterns in bladder cancer subtypes. We observed that NMIBCs with a wild-type *FGFR3* gene have more methylated CGIs and that the intensity of methylation is more profound than in the *FGFR3* mutant NMIBC and MIBC groups. Similar results were recently described by Serizawa et al [2]. The difference in methylation presents additional evidence that the two subgroups of NMIBC develop along different pathogenesis pathways. WT NMIBCs also have more wide spread chromosomal aberrations than *FGFR3* MT NMIBCs [3-5]. The extensive methylation in WT NMIBC is reminiscent of the CpG island methylator phenotype (CIMP) in colorectal cancers (CRC) and gliomas [6-10]. When we compared the genes representing the CIMP genotype in BC with the genes representing CIMP genotype in CRC and glioma, there was no overlap. This confirms that CGI methylation is tissue and tumour type specific [11].

Supporting the previous reports in other solid tumors [12-17], a high proportion of the genes we found *de novo* methylated in bladder cancer are repressed by polycomb complexes (PcG) in embryonic stem cells. PcG target genes are as much as 12 times as likely to be silenced by DNA methylation in cancer as non-PcG target genes. PcG complexes silence genes by methylation of histone H3 at lysine 27 (H3K27). The association between DNA methylation and PcG has further been substantiated by the finding of interactions between DNMT1, DNMT3A, DNMT3B and several PcG complex subunits like EZH2 and BMI1 [18]. Another recent study showed that 49% of the genes methylated in colon cancer are polycomb targets in embryonic stem cells [19], agreeing with Schlesinger and colleagues who showed that genes prone to tumor specific

hypermethylation in colon cancer were more likely to be marked by H3K27 methylation in normal tissues than genes lacking H3K27 methylation [15]. In this perspective, we found that 56% of the hypermethylated genes in bladder cancer are PcG targets in embryonic stem cells. Our findings confirm those of Wolff et al, who found that 40% of the methylated genes in BC are PcG targets [20]. These epigenetic alterations thus may occur in early development specifically in stem or progenitor cells and might be bladder cancer specific and this strengthens the further understanding of bladder cancer tumorigenesis. This implies that methylation is an early event in bladder cancer in accordance with Feinberg [21].

We have observed that methylation is equally distributed over both promoter CpG islands and gene body CpG islands. It is generally known that gene regulation is affected by promoter methylation [22, 23]. Data on the effect of methylation of internal CGIs on gene expression up till now is scarce. However, the data so far rather suggest that methylation of internal CGIs does not affect gene transcription in a negative way. Some of these papers were recently reviewed by Shenker [24] and Jones [25]. For instance, Hellman and colleagues [26] studied X-linked gene silencing using an array with 49% of the probes in intragenic coding regions. They observed that the active X-allele displayed a higher overall as well and intragenic methylation than the inactive X-allele [26]. In addition, Rauch and Maunakea [27, 28] found that intragenic methylation was even associated with higher level of gene transcription and they proposed that intragenic methylation is a mechanism which regulates the use of alternative promoters. Aran and colleagues [29] found a link between intragenic methylation and replication timing and claimed that early replicating genes inclined to be active and show high intragenic methylation levels. Salem et al., [30] showed that the *PAX6* gene was methylated in exon 5 in bladder and colon cancer cell lines, and that this was not associated with the inhibition of gene expression. Smith et al., [31] found that methylation of non-promoter CGIs were associated with up-regulation of gene expression in colorectal cancer.

In our study, besides promoter methylation, we found more than 50% of all methylated CpG dinucleotides to be located in CGIs within gene bodies. Given these intriguing results, we investigated the association of promoter and intragenic methylation on gene expression by treating BC cell lines with epigenetic reactivating drugs like DAC and TSA. We observed that intragenic CGI methylation was associated with low gene expression to a similar extent as was observed for promoter methylation, suggesting that gene silencing may be the result of both promoter and internal CGI methylation. We observed that it was possible to restore the gene expression with the epigenetic modulating drugs in BC cell lines, also for the genes that harbored internal methylated CGIs. In the light of these results we propose two alternative explanations for our findings. Firstly, reactivation of genes with internal methylated CGIs could occur *in trans* through reactivation of a transcription factor gene by DAC treatment. Secondly, the methylated internal CGI could associate *in CIS* with the promoter region and negatively affect gene expression. This suggests that intragenic

methylation also contributes to gene silencing. However, additional experiments are necessary to unravel the underlying mechanisms.

6.3 Distinct DNA methylation patterns exist in bladder cancer and are valuable prognostic indicators

One of the major goals of this work was to identify and validate methylated CGIs that could serve as biomarkers for the prediction of disease course. In the genome wide study we discovered several markers that were associated with disease course. The subsequent validation on a custom array resulted in the identification of four genes whose CGIs were associated with progression to MIBC in pTa tumors when the CGIs were methylated.
pTa tumours represent about 60% of all primary bladder tumours. Progression to MIBC in pTa tumours is much lower than for pT1 tumours, however, the risk is still 10% and 60-70% of patients will have on average 3 recurrences, warranting long-term surveillance [32-34]. We found that methylation of the *TBX2*, *TBX3*, *GATA*2 and *ZIC4* genes was significantly associated with progression. These genes encode transcription factors that are important in lineage decisions during development [35]. Gene expression profiling studies showed that all these genes are more expressed in NMIBC than in MIBC [36], which fits with our findings as we observed that these genes are more methylated in MIBC. *TBX2* and *TBX3* are transcriptional repressors that inhibit expression of the ARF gene a.o., thereby finally inhibiting P53 activation [37]. This may present an alternative for mutation of P53, which is frequent in MIBC. Recently, Reinert et al., showed methylation of the *TBX4* gene associated with progression in pTa tumours, this suggests the important role of T-box genes in BC progression [38]. Mutations that reduce DNA binding of *GATA2* were discovered in familial myelodysplastic syndrome [39]. Currently, prediction of progression in NMIBC is based on the progression scores developed by Sylvester [34], that have been taken up in the EAU guidelines. These scores are based on the clinicopathological factors. The markers described in this work enhanced the predictive accuracy of these risk scores by 23% and could thus contribute to a further objective stratification of patients.

6.4 Surveillance of NMIBC using markers based on tumour-specific DNA methylation

One of the major issues in the treatment of NMIBC patients is the high recurrence rate; therefore the surveillance of the patients is a major clinical challenge. Can we find molecular biomarkers based on DNA methylation for the surveillance of these patients, which can provide an alternative for the current invasive cystoscopy? The development of sensitive, non-invasive tests for diagnosis and surveillance has been performed in the last decade with not much success. Current FDA approved urine tests BTA stat, BTA TRAK, UroVysion, and Quanticyt have shown good sensitivity and specificity for low grade NMIBC, but failed to present similar results in the following studies [40-42]. Therefore these markers are not used yet in routine clinical practice. Currently, the cystoscopy and cytology on voided urine are very important predictors of recurrence at the first follow-up, three months after trans-urethral resection of the bladder tumour (TURBT) [43]. While cystoscopy

is an invasive procedure, cytology has a low median sensitivity of 35% in detecting low grade NMIBC [42].

Epigenetic modifications, especially DNA methylation showed promise in developing urine based biomarkers in BC. However, previous studies were mainly performed on primary tumors and were not aimed at the detection of recurrent bladder tumors and therefore the sensitivities reported might be too high. This is very important as most of the recurrences are small with low stage and grade. An ideal test for the surveillance of BC that can replace the cystoscopy should be urine based, sensitive, cost-effective, easy to perform with limited material, and with no interobserver variability.

The genome-wide screening and subsequent independent validation allowed us to confirm 110 CGIs that significantly differed in methylation in tumours when compared to urine-derived DNA from age-matched non-bladder cancer controls. These CGIs represented potential biomarkers for urine tests. Urine-based tests are especially important for surveillance of patients with NMIBC after transurethral resection in order to provide an alternative for cystoscopy, which is an invasive diagnostic method.

To identify recurrence specific DNA methylation markers especially for low and intermediate risk group patients, we selected 8 candidate CGIs from our genome-wide study and tested their performance in two independent sets of recurrence-associated urines along with urines from non-bladder cancer controls. This enabled us to identify a three marker combination (*OTX1*, *ONECUT2* and *OSR1*) together with *FGFR3* mutation assay detecting recurrent BC in voided urine with a sensitivity of 79% at a specificity of 90%. At least 21% of the recurrent tumours were undetected with our test. This could be because of the absence of tumour cells in the corresponding urine, which can be improved by collecting and testing multiple urines from the patient. So far this is only the second study which investigated methylation biomarkers for the exclusive detection of recurrent BC in voided urine. The study by Zuiverloon [44] et al., reported a four gene methylation panel to detect recurrent bladder tumours with a sensitivity of 72% at a low specificity of 55%.

We also observed that multiple tumours from a patient are highly concordant in their methylation percentage, which emphasize the value of the markers. This is very crucial for a recurrent diagnostic assay. Unlike the *FGFR3* mutation, methylation seems to be consistent in the primary and recurrent tumours.

Most of the times urine DNA is very limited, therefore it is challenging to perform many individual molecular tests on the limited amount of the DNA. To overcome this, we developed a sensitive and quantitative multiplex methylation assay, which require as low as 30 to 40 ng DNA unlike other assays. Moreover, we have shown that they are highly reproducible between different operators. Combined material costs of the two assays, including DNA isolation, amount to about 30€. Personnel costs depend on the number of assayed samples, being cheaper when many samples are analyzed simultaneously. The sensitivity of our assay combination is similar to the sensitivity of the current gold standard white light cystoscopy with sensitivity in the range of 68 to 83% when compared to the more sensitive blue light cystoscopy [45, 46]. Given the comparable sensitivities of urine

testing and cystoscopy, we postulate that patients under surveillance for recurrent BC in the low/intermediate risk groups could benefit from urine testing and only followed by cystoscopy when the urine test is positive.

6.5 Future perspectives

Our genome-wide study provided an insight into the methylation profiles of different subtypes of BC and identified novel biomarkers for the detection, surveillance and prognosis of BC. The possible CIMP phenotype we observed in NMI-*FGFR3* WT tumours should be further validated to find the CIMP signature. This could help in finding methylation markers specific for the NMI *FGFR3* wild type tumours. It is also interesting to study the polycomb target genes methylated in BC further. These epigenetic alterations occurring in early development specifically in stem or progenitor cells might be bladder cancer specific and this strengthens the further understanding of bladder cancer tumorigenesis in future.

The diagnostic and prognostic markers developed in this study showed very good sensitivities and specificities. Therefore, more multicenter validation studies should be initiated to take these markers a step closer to the clinical utilization. We are currently undertaking the validation of the urine biomarkers in a longitudinal prospective study. The prospective studies should also be performed to compare the markers with current gold standard methods. For example, the urine diagnostic markers should be compared with cystoscopy in a randomized trial to see if the urine markers can replace the cystoscopy and when. As we discussed in chapter 4, especially for the low and intermediate risk NMIBC surveillance, urine markers could be tested initially and a cystoscopy can be performed if the urine test is positive. This will help reducing the unnecessary cystoscopies for this group of low risk patients. Furthermore, other work has shown that recurrent tumours are often detected earlier than with cystoscopy and this is called the anticipatory effect. In addition, more recurrences are detected when the urologist who performs the cystoscopy is aware of a positive urine test result [47]. The other problem with the urine methylation markers is the low sensitivity, which mostly occurs because of the absence of tumour cells in that particular urine. As with the *FGFR3* assay, we should investigate if testing of multiple urines can improve the detection rate [48, 49]. An increase in analytical sensitivity of the *FGFR3* and the methylation assay can be further expected with the use of next generation sequencing in the future.

The prognostic markers for pTa tumours that we discovered performed better that the clinicopathological parameters combined in the EAU guidelines. The current TNM grading system is not perfect as it suffers from interobserver variability. Currently, the prognostic markers are being further validated in an independent large set of tumours. Previously, we developed a molecular grading system (mG 1.0) based on the presence/absence of an FGFR3 mutation and expression of the proliferation marker Ki-67. This molecular grading system was superior to the EAU scores [32, 50]. The disadvantage of this mG is the fact that immunohistochemistry (IHC) is required to score the Ki-67 marker. IHC is cumbersome and

not entirely reproducible. We therefore aim to combine the methylation profile with the *FGFR3* mutation status to come to an mG 2.0 system that is better than mG 1.0 and more reproducible.
It is also important to study the intragenic methylation and its effect on gene transcription further.

6.6 References

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Chapter 7

Appendices

Summary

Bladder cancer is the 5th most common cancer and the 9th leading cause of cancer death with an estimated 386,300 new cases and 150,200 deaths in the year 2008 worldwide. The majority of bladder cancers occur in men, with a male: female ratio of 3:1. The incidence is about 20 new cases per year per 100,000 people in the U.S. Smoking is the most consistent and common 3 times risk factor associated with bladder cancer. Most bladder tumours (75%) are non-muscle invasive (i.e. pTa-pT1) at first presentation. There are two major problems in the management of these patients. First, NMIBC characterized by a high risk of recurrence (55-85%), with most recurrences in the first year after trans-urethral resection (TUR). These recurrences are detected by cystoscopy, considered as the gold standard, which is performed every 3-4 months depending on the clinicopathological features. This is often in combination with urine cytology. After a two-year recurrence free period, the intensity of follow up may be lowered. Cystoscopy and cytology are routinely used in clinical practice. However, cystoscopy is invasive, inconvenient for the patients and costly, while cytology has low sensitivity for low grade tumours. Hence, it is necessary to develop urine based non-invasive and sensitive diagnostic tools for the detection of recurrent bladder cancer.

The second major concern is to prevent progression (10-15%) to muscle invasive bladder cancer. The ability to distinguish NMI tumors with invasive capacity from those not likely to become invasive would be of great clinical benefit. At this moment this prediction is based on clinicopathological factors. However, individual prediction of prognosis by clinical and pathological features is still very tricky and can suffer from interobserver variability. NMIBC patients with high chance of progression might be better treated with radical cystectomy in an early stage of the disease. Therefore it is necessary to develop more accurate predictors for progression, which helps in stratification of patients for follow-up and treatment.

Over the past decade it has become clear that cancer is an epigenetic as well as a genetic disease. Epigenetic modifications can act as surrogate markers for diagnosis and prognosis of cancer. Methylation of cytosine bases in DNA is an important epigenetic mechanism and the analysis of aberrant DNA methylation in bodily fluids has the potential to substantially improve cancer screening, detection and prognosis. Therefore, epigenetic markers may aid in solving the two major clinical problems summarized above. If these markers prove to have added value over the established clinico-pathological variables, they could become part of the clinical practice. This thesis deals with the development of molecular epigenetic biomarkers as tools for the management of bladder cancer patients. To achieve this we performed genome-wide methylation screening, followed by identification and validation of urine markers for recurrence detection as well as tissue biomarkers for predicting progression in NMIBC.

In **chapter 2**, a systematic review on DNA methylation based biomarkers identified so far for the diagnosis and prognosis of bladder cancer is presented. **Chapter 3** describes the results of genome-wide methylation study. The genome-wide screening and subsequent validation allowed us to identify and validate 110 CpG islands that significantly differed in methylation in tumours when compared to urine-derived DNA from age-matched non-BC controls. These CGIs represent potential biomarkers for urine tests. Furthermore, four markers (*TBX2*, *TBX3*, *GATA2* and *ZIC4*) for predicting progression were identified and validated in pTa tumours. The predictive accuracy of EORTC risk scores for progression, which are based on clinicopathological factors are improved by 23% by adding methylation of *TBX2*, *TBX3*, and *GATA2* to the model. Therefore these molecular markers will add to the prediction scores and will aid in improving the predictive accuracy, thereby helping in patient stratification.

An ideal test for surveillance of BC that can replace cystoscopy should be urine based, sensitive, cost-effective, easy to perform with limited material, and with no inter observer variability. To achieve this, we developed a 3-plex assay for the diagnosis of recurrent BC. Our three gene methylation panel consisting of *OTX1*, *ONECUT2* and *OSR1* achieved a sensitivity of 74% with a specificity of 90% for the detection of recurrent bladder tumors in voided urine. Previously, we showed an inverse correlation between *FGFR3* mutation and methylation, therefore we combined the 3-plex methylation assay with the *FGFR3* mutation assay. The combination of both assays increased sensitivity of detection to 79%. This assay needs as low as 35 ng of DNA, hence there will be sufficient DNA in most urine samples to perform these assays. Both assays are easy to perform in a standard molecular diagnostic laboratory. Moreover, we have shown that they are highly reproducible between different operators. Combined material costs of the two assays, including DNA isolation, amount to about 30€. Personnel costs depend on the number of assayed samples, being cheaper when many samples are analyzed simultaneously. We further observed that multiple tumours from a patient have highly concordant methylation of the 3 markers, which underlines the usefulness of the markers for surveillance. The sensitivity of our assay combination is similar to the sensitivity of the current gold standard white light cystoscopy with sensitivity in the range of 68 to 83% when compared to the more sensitive blue light cystoscopy. Given the comparable sensitivities of urine testing and cystoscopy, we postulate that patients under surveillance for recurrent BC in the low/intermediate risk groups could benefit from urine testing and only followed by cystoscopy when the urine test is positive. This is described in **chapter 4.** The genome-wide study in bladder cancer revealed that methylation is distributed equally in the promoter and intragenic regions. Promoter methylation is proved to be inversely correlated with gene expression, while intragenic methylation is still under investigation. In **chapter 5**, we addressed the effect of intragenic methylation on transcription using bladder cancer cell lines. The results indicated that, intragenic methylation can also lead to gene silencing and the expression was able to be restored by treating the cell lines with the epigenetic modulating drugs. We propose two alternative explanations for our

findings. Firstly, reactivation of genes with internal methylated CGIs could occur *in trans* through reactivation of a transcription factor gene by DAC treatment. Secondly, the methylated internal CGI could associate *in cis* with the promoter region and negatively affect gene expression. Further studies are necessary to unravel the mechanism behind this. Hence it is important to study the methylation all over the genome, instead of focusing on promoters only.

Samenvatting

Blaaskanker is één van de meest voorkomende vormen van kanker. Per jaar wordt deze ziekte bij ongeveer 386.300 mensen geconstateerd. Zo'n 150.200 mensen overlijden jaarlijks aan deze ziekte. De ziekte komt vaker voor bij mannen dan bij vrouwen, in een verhouding van ongeveer 3:1. De incidentie in de Verenigde Staten is ongeveer 20 nieuwe patiënten per jaar per 100.000 mensen. Roken is de meest duidelijke en bekende risicofactor geassocieerd met blaaskanker en verhoogt het risico ongeveer drie maal.
De meeste blaastumoren (75%) zijn bij presentatie niet-spierinvasief (NMIBC, pTa-pT1). De behandeling van deze tumoren gaat gepaard met twee lastige problemen. Ten eerste wordt NMIBC gekarakteriseerd door een hoog risico op recidieven (55-85%), vooral in het eerste jaar na een transurethrale resectie (TUR). Deze recidieven worden opgespoord met behulp van cystoscopie, een methode die, afhankelijk van de klinische en pathologische gegevens, elke 3 tot 4 maanden wordt herhaald. Cystoscopie wordt op dit moment beschouwd als de Gouden Standaard. Naast cystoscopie wordt ook vaak cytologie uitgevoerd op urine. Als na een periode van twee jaar geen recidieven zijn opgetreden, wordt de frequentie van de onderzoeken verminderd. Alhoewel cystoscopie en cytologie routinematig worden toegepast, zitten aan beide methoden toch wat nadelen: cystoscopie is een invasieve techniek die flink wat ongemak met zich meebrengt, en cytologie is niet zo'n gevoelige techniek om NMIBC tumoren op te sporen. Daarom is het noodzakelijk om onderzoek te verrichten naar de mogelijkheid voor niet-invasieve testen die met een grote gevoeligheid recidief tumoren opsporen.
Het tweede lastige probleem is het feit dat zo'n 10-15% van de NMIBC tumoren uiteindelijk toch spierinvasief wordt. Een test die kan aantonen welke patiënten het meeste risico lopen op zo'n spierinvasieve tumor zou zeer welkom zijn. Patiënten met een hoog risico zouden misschien een betere prognose hebben na radicale cystectomie. Op dit moment wordt deze voorspelling gebaseerd op klinisch-pathologische parameters. Dit kan echter nog onderhevig zijn aan interobserver variatie. Daarom is het van belang om meer accurate voorspellers te ontwikkelen, die kunnen helpen om de patiënten te stratificeren voor behandeling.
De laatste jaren is duidelijk geworden dat epigenetische veranderingen, naast genetische ook een belangrijke rol spelen in het ontstaan van kanker. Epigenetische veranderingen kunnen worden gebruikt als surrogaatmerkers voor diagnose en prognose van kanker. Methylering van cytosinebasen in DNA is een belangrijk epigenetisch mechanisme en het aantonen van afwijkende DNA-methylering in lichaamsvloeistoffen kan in hoge mate bijdragen aan het verbeteren van het (vroeg) opsporen van kanker, de diagnose en de prognose. Als deze merkers toegevoegde waarde blijken te hebben bovenop de al bestaande methoden, zouden ze kunnen worden ingepast in de standaard klinische praktijk. Dit proefschrift beschrijft de ontwikkeling van moleculaire epigenetische merkers als instrumenten om de behandeling van blaaskankerpatiënten te sturen. Om dit te

bereiken hebben we een genoombrede methylatiescreening uitgevoerd om merkers te identificeren. Deze merkers zijn gevalideerd voor het gebruik als urinegebaseerde test voor detectie van recidieven en het voorspellen van progressie naar spierinvasieve ziekte.
In **hoofdstuk 2** wordt een systematisch overzicht gegeven van op DNA-methylatie gebaseerde biomerkers die tot nu toe zijn geïdentificeerd voor de diagnose en prognose van blaaskanker. **Hoofdstuk 3** beschrijft de resultaten van een genoombrede methylatiestudie. Deze genoombrede screening en de daarop volgende validatie stelde ons in staat om 110 CpG eilanden te identificeren en te valideren waarvan de methylatie in tumoren aanzienlijk verschilde van die in DNA geïsoleerd uit urine van controlepersonen zonder blaaskanker van dezelfde leeftijd. Deze CGI's vertegenwoordigen potentiële biomerkers voor urinetesten. Verder werden vier merkers geïdentificeerd (*TBX2*, *TBX3*, *GATA2* en *ZIC4*) met voorspellende waarde voor progressie en deze zijn gevalideerd in pTa tumoren. Door toevoeging van methylering van *TBX2*, *TBX3* en *GATA2* aan het model voor de EORTC risicoscores voor progressie, die gebaseerd zijn op klinische en pathologische factoren, nam de voorspellende waarde toe met 23%. Daarom zal toevoeging van deze moleculaire merkers aan de risicoscores helpen bij het verbeteren van de predictieve nauwkeurigheid en aldus bijdragen aan de patiëntenzorg.
Een ideale test voor het volgen van patiënten met blaaskanker, die cystoscopie kan vervangen, moet niet-invasief zijn, gevoelig, goedkoop, eenvoudig uit te voeren met een beperkte hoeveelheid patiëntenmateriaal, en zonder inter-observer variabiliteit. Hiervoor hebben we een 3-plex assay ontwikkeld voor de diagnose van recidief blaastumoren. Het genmethylatiepanel van de drie genen *OTX1*, *ONECUT2* en *OSR1* bereikt een gevoeligheid van 74% met een specificiteit van 90% voor de detectie van recidieftumoren in urine. Eerder toonden we een omgekeerde correlatie aan tussen *FGFR3* mutatie en methylering, daarom hebben we de 3-plex methylatie assay gecombineerd met de *FGFR3* mutatie assay. De combinatie van beide assays verhoogde de detectiegevoeligheid tot 79%. Deze test is uit te voeren met een minimale hoeveelheid DNA van 35 ng, waardoor er voldoende DNA in de meeste urinemonsters aanwezig was om deze assays uit te voeren. Beide testen zijn gemakkelijk uit te voeren in een standaard moleculair diagnostisch laboratorium. Bovendien hebben we aangetoond dat de test zeer reproduceerbaar is tussen verschillende uitvoerders. Gecombineerde materiaalkosten van de twee assays, inclusief DNA-isolatie, bedraagt ongeveer €30. De personeelskosten zijn afhankelijk van het aantal geteste monsters en worden lager wanneer veel monsters gelijktijdig geanalyseerd worden. We hebben verder vastgesteld dat meerdere tumoren van een patiënt een zeer vergelijkbaar methylatieprofiel van de 3 merkers hebben, wat de bruikbaarheid van de test voor recidieftumoren onderstreept. De gevoeligheid van de methylatietest gecombineerd met *FGFR3* mutatie-analyse is vergelijkbaar met de gevoeligheid van de huidige gouden standaard, cystoscopie met wit licht, welke een gevoeligheid heeft tussen de 68 tot 83% vergeleken met de meer gevoelige cystoscopie met blauw licht. Gezien de vergelijkbare gevoeligheden van urinetesten en cystoscopie, nemen we aan dat patiënten onder controle voor recidieftumoren in de lage/intermediaire risicogroepen zouden kunnen

profiteren van urinetesten en alleen een cystoscopie hoeven te ondergaan als de urinetest positief is. Dit wordt beschreven in **hoofdstuk 4**. Tijdens de genoombrede studie bij blaaskanker bleek dat methylatie gelijkmatig verdeeld is in de promotor en intragene regio's. Promotormethylering is omgekeerd evenredig met genexpressie, terwijl het effect van intragene methylatie nog onduidelijk is. In **hoofdstuk 5** onderzochten we het effect van intragene methylatie op transcriptie met behulp van blaaskankercellijnen. De resultaten gaven aan dat intragene methylering ook kan leiden tot gene silencing en dat de expressie kon worden hersteld door behandeling van de cellijnen met drugs die methylering beïnvloeden. Er zijn twee mogelijke verklaringen voor onze bevindingen. Ten eerste kan reactivering van genen met intern gemethyleerde CGI plaatsvinden *in trans* door reactivering van een transcriptiefactorgen door DAC behandeling. Anderzijds zou de gemethyleerde interne CGI *in cis* met de promotor regio kunnen associëren en zo een negatieve invloed hebben op genexpressie. Verdere studies zijn nodig om het mechanisme hierachter te ontrafelen. Daarom is het belangrijk om de methylering van het hele genoom te bestuderen, in plaats van het onderzoek te beperken tot genpromoters.

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Well, where to start?! It’s been an exciting journey towards my PhD since I came to Germany in October 2005 to pursue my master’s degree. I joined Erasmus MC in 2008 and so many people have helped me throughout this very challenging period of my life. I would like to take this opportunity to express my deep and sincere gratitude to all of you.

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there to comfort me. I had the longest association with you during my PhD and sitting beside you was the best thing. Thank you for your extensive support in professional and personal life. You were always there to share my good and bad times. More than a supervisor, you are a friend and a great colleague to work with. I have learnt so much from you and I couldn't have done all this alone without your guidance. I wish you all the very best for your future career. What a coincidence, we both are now working on gynaecological cancers and I hope we will continue to work together in the future. I had a great time with you during so many meetings, especially during the AACR in Orlando. Thank you so much for reading my thesis and giving your comments to make it better. Thanks for reading all those Dutch letters and yeah Roy took over in the end. Angela, thank you so much for your help with the Dutch summary.

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Finally, special thanks to my parents, wife and sisters for your unconditional love and I am sure I couldn't have achieved this without your support. I lovingly dedicate this thesis to you all. Big thanks to my wife for all the love, support and affection in the most difficult phases of my PhD.

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Curriculum Vitae

Raju Kandimalla was born on April 21st 1980 in Warangal, AP, India. He studied Bachelor and Master of Science in Biochemistry at Kakatiya University, Warangal, India from 1998-2003. After getting his Master's degree, he worked for two years from 2003-2005 in Aristo Pharmaceuticals ltd, Hyderabad, India. In 2006 he moved to Germany and earned his International Master's degree in Biochemistry. He did his Master's thesis at Helmholtz Centre for Infection Research, Braunschweig, Germany under Prof.dr. Juergen Bode, which was funded by Cellca GmbH. In 2008 he moved to the Netherlands to start his PhD project under the guidance of Prof.dr. E.C. Zwarthoff in the Department of Pathology, Erasmus MC, Rotterdam. This project is funded by Erasmus MC, MRACE grant 2007, European Community Seventh Framework program FP7/2007-2012, grant agreement no 201663, and Dutch Cancer Society grant no. EMCR 2007-3863. The aims of his PhD project were to perform genome-wide DNA methylation profiling in bladder cancer to find novel biomarkers for the diagnosis and prognosis of bladder cancer patients. The results obtained during these four and half years effected in four publications, which are presented in this thesis. He also guided two master and two bachelor students for their internships. He also presented the work described in this thesis in the form of posters and presentations at several national and international meetings. These include the annual meeting of American Association of Cancer Research (2011-2012), EMBL Conference on Omics and Personalized Health held in Heidelberg, Germany (2012), European Association of Urology meeting held in Paris, France (2012), European Meeting on Molecular Diagnostics held in the Hague (2011), European Workshop on Cytogenetics and Molecular Genetics of Solid Tumors, Radboud University Nijmegen Medical Centre, Nijmegen (2010), KWF Tumor Cell Biology Meeting, Lunteren (2009), MGC PhD student symposium and workshop held in Leiden and Brugge (2011 and 2009), GROW PhD Course Epigenetics, Maastricht University Medical Center, Maastricht (2008) and the Molecular Medicine Day (2009-2012). In 2012 he received the prize for best poster at the European Association of Urology (EAU) meeting held in Paris. He is presently working as a post doctoral fellow at the Institute for Women's Health, University College London under Prof.dr. Martin Widschwendter.

PhD Portfolio

Name PhD student: Raju Kandimalla
Erasmus MC Department: Pathology
Research School: Molecular Medicine

PhD period: Jan 2008 - Sep 2012
Promotor: Prof. dr. E.C. Zwarthoff
Co-Promotor: Dr. A.G. van Tilborg

1. PhD training

	Year	ECTS
Courses and workshops		
Workshop on Bioinformatic Analysis, Tools and Services	2008	0.2
Course Molecular Diagnostics III	2008	0.3
Course Analysis of microarray gene expression data	2008	1.0
Course Molecular Medicine	2008	1.9
Course Classical Methods for Data analysis	2008	5.7
2nd GROW PhD Course Epigenetics, Maastricht UMC	2008	5.0
Basic Course on 'R´	2010	1.0
Biomedical English writing and communication	2010	4.0
Course on Photoshop and Illustrator CS5	2011	0.3
Next Generation Sequencing Training	2011	0.3
Workshop on InDesign CS5	2012	0.2
Oral presentations		
2nd GROW PhD Course Epigenetics, Maastricht UMC	2008	0.3
Annual presentations scientific meetings at the JNI	2009-2011	0.9
16th MGC PhD student workshop, Bruges, Belgium	2009	0.3
KWF Tumor Cell Biology Meeting, Lunteren, the Netherlands	2009	0.3
12th European Workshop on Cytogenetics and Molecular Genetics of Solid Tumors, UMC St. Radboud, Nijmegen	2010	0.3
21st MGC PhD Symposium, LUMC, Leiden	2011	0.3
7th European Meeting on Molecular Diagnostics, The Hague	2011	0.3
EMBL Conference Omics and Personalized Health, Heidelberg, Germany	2012	0.3
16th Molecular Medicine Day, Erasmus MC, Rotterdam	2012	0.3
Course Basic and Translational Oncology of the Erasmus MC Post Graduate School	2012	0.3

Poster presentations	Year	ECTS
Molecular Medicine Day, Erasmus MC, Rotterdam	2009-2011	0.9
AACR Annual Meeting, Orlando, FL, USA	2011	0.3
27th Annual EAU Congress, Paris, France	2012	0.3
AACR Annual Meeting, Chicago, IL, USA	2012	0.3
International conferences		
AACR Annual Meeting, Orlando, FL, USA	2011	1.0
27th Annual EAU Congress, Paris, France	2012	1.0
AACR Annual Meeting, Chicago, IL, USA	2012	1.0
EMBL Conference Omics and Personalized Health, Heidelberg, Germany	2012	1.0
Department meetings		
Weekly JNI scientific meetings	2008-2012	2.5
Monthly JNI oncology lectures	2009-2012	1.0
Work group discussions	2008-2012	2.5
2. Teaching activities		
Supervision of master student internship (January-September)	2010	8.0
Supervision of bachelor student internship (March-September)	2010	8.0
Supervision of master student internship (March-August)	2011	8.0
Supervision of bachelor student internship (Nov 2010-August 2011)	2011	8.0

Lovingly dedicated to my parents