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Abstract

High-grade non-muscle invasive bladder cancer (HG-NMIBC) is a clinically unpredictable disease with greater risks of recurrence and progression relative to their low-intermediate-grade counterparts. The molecular events, including those affecting the epigenome, that characterise this disease entity in the context of tumour development, recurrence and progression, are incompletely understood. We therefore interrogated genome-wide DNA methylation using HumanMethylation450 BeadChip-arrays in 21 primary HG-NMIBC tumours relative to normal bladder controls. Using strict inclusion-exclusion criteria we identified 1,057 hypermethylated CpGs within gene promoter-associated CpG islands, representing 256 genes. Bisulphite Pyrosequencing validated the array data and examined 25 array-identified candidate genes in an independent cohort of 30 HG-NMIBC and 18 low-intermediate-grade NMIBC. These analyses revealed significantly higher methylation *frequencies* in high-grade tumours relative to low-intermediate-grade tumours for the *ATP5G2*, *IRX1* and *VAX2* genes ($p<0.05$), and similarly significant increases in *mean levels* of methylation in high-grade tumours for the *ATP5G2*, *VAX2*, *INSRR*, *PRDM14*, *VSX1*, *TFAP2b*, *PRRX1*, and *HIST1H4F* genes ($p<0.05$). Although inappropriate promoter methylation was not invariantly associated with reduced transcript expression, a significant association was apparent for the *ARHGEF4*, *PON3*, *STAT5a*, and *VAX2* gene transcripts ($p<0.05$). Herein, we present the first genome-wide DNA methylation analysis in a unique HG-NMIBC cohort, showing extensive and discrete methylation changes relative to normal bladder and low-intermediate-grade tumours. The genes we identified hold significant potential as targets for novel therapeutic intervention either alone, or in combination, with more conventional therapeutic options in the treatment of this clinically unpredictable disease.

Key words: *High-grade Non-Muscle Invasive Bladder Cancer, Epigenetics, Methylation, HumanMethylation450 BeadChip Array, Gene Expression*

Introduction

Bladder cancer is the ninth most common cancer worldwide.[1] The majority of bladder cancers are transitional cell carcinomas (TCC), of which 70-80% are non-muscle invasive (NMIBC) at presentation.[2] Poorly differentiated 'high-grade' (HG)-NMIBC is a clinically important sub-type, accounting for approximately 10-15% of all NMIBCs at presentation.[3, 4], These high-grade tumours are typically more aggressive than their low- and intermediate-grade counterparts, manifest by higher rates of recurrence and progression to invasive and metastatic disease despite intensive and prolonged intravesical treatment.[5, 6]

The majority of NMIBCs are thought to be consequent to, and represent initiation and progression from, a complex interplay between sporadic, environmental, and heritable risk factors, including those that impact upon genetic and epigenetic pathways. NMIBCs and muscle invasive bladder cancers (MIBCs) have been shown to develop independently ('the two pathway model') on the basis of gain of function fibroblast growth factor receptor 3 (*FGFR3*) mutations in NMIBC, and loss of function mutations in retinoblastoma 1 (*RB1*) and tumour protein 53 (*p53*) in MIBC,[7-10] and have been shown to evolve from different cell types. [11, 12] However, the molecular pathways responsible for the evolution, outgrowth and progression of HG-NMIBC have not been subject to comprehensive study or investigation; indeed, it is currently unclear whether HG-NMIBCs arise as a discrete disease entity, whether they represent step-wise progression from low-intermediate-grade NMIBC tumours, or whether they sit at a molecular crossroads between NMIBC and MIBC.[7, 13 11] This uncertainty is illustrated by the findings that high-grade tumours harbour abnormalities in common with low-intermediate-grade NMIBC, such as mutations of *FGFR3* and/or rat sarcoma viral oncogene homolog (*RAS*) pathway genes[14, 15], but also display extensive genetic instability and compromised regulation of vital cellular processes more in keeping with MIBC.[14, 16]

Epigenetic modifications are frequently implicated in the development of human malignancies, and in these cases, are typically apparent as inappropriate gene promoter CpG island DNA methylation, histone tail modification(s), aberrant expression of micro- and long non-coding-RNAs, and less frequently, loss of gene body/intergenic methylation.[17, 18] These heritable modifications, or *epimutations*, impact upon gene expression either alone or in combination, and promote tumour evolution and/or progression by suppressing the expression of growth inhibiting and/or apoptosis promoting genes, and less frequently by leading to relaxed control of expression of growth promoting genes.[17, 19, 20]

Epigenetic modifications and associated gene silencing have been shown in NMIBC, and specific patterns of DNA methylation, histone modifications and microRNA expression have been reported as associated with tumour growth characteristics, patient/clinical outcomes and with field defect phenomena.[21, 22] However, the majority of these reports have described epigenetic changes in heterogeneous populations of NMIBC, with an abundance of low- and intermediate-grade tumours relative to high-grade tumours. With the exception of our recent candidate-gene study[23] and a single report investigating the Myopodin A gene[24], HG-NMIBCs have not been considered as a discrete entity for the investigation of epigenetic modifications.

In this study, we interrogated DNA methylation on a genome-wide scale using methylation BeadChip-array technology, in a unique cohort of HG-NMIBCs. Through comparisons with methylation levels and gene-expression in low/intermediate-grade tumours, we extend the current understanding of bladder cancer tumourigenesis and identify potential epigenetic mechanisms implicated in the development of high-grade NMIBC, and those that might represent novel therapeutic drug-targets.

Results

Technical Validation of array by Pyrosequencing:

Subsequent to array processing, normalisation and peak-based correction (see patients and methods), a technical validation was performed by comparing array-derived β-values with Pyrosequencing-derived methylation values. Across 120 data-points (5 CpGs, 24 samples) encompassing a broad range of array β-values, a strong positive correlation was found between the methylation values (Spearman's rank correlation r=0.912, p<0.00001; **Supplemental Figure S1**).

In-house filtering criteria:

CpGs showing differential methylation in HG-NMIBC relative to normal bladder controls were identified following a series of stringent filtering criteria, as described previously and shown in **Figure 1**.[25, 26] On the basis of these criteria, a total of 1,057 CpGs, representing 256 genes, were identified as hypermethylated (≥0.4 β-value increase) in 15 or more of the 21 high-grade tumours, relative to their mean values in the normal bladder controls.

Hierarchical clustering analyses:

The filtered dataset was next subject to unsupervised hierarchical cluster analysis (**Figure 2)**: the high-grade tumours cluster independently from the normal bladder control samples. In these cases, methylation is barely detectable within the normal bladder samples, whereas 15 or more of the high-grade tumours show inappropriate methylation across all 1,057 CpG dinucleotides, spanning 256 gene-promoter-associated CpG islands (**Supplemental Table S2**).

Independent validation by Pyrosequencing:

We next selected 25 genes for independent validation by Pyrosequencing on the basis of their frequent methylation in the discovery cohort that comprised 21 high-grade tumours. These analyses revealed similar frequencies and mean levels of methylation as those apparent from the BeadChip array for 24 of the 25 genes. As further confirmation, we extended the Pyrosequence analyses to an independent investigation cohort of 30 HG-NMIBC tumours. Similar frequencies and mean levels of methylation between the discovery and investigation cohorts reinforced our confidence in the array-derived data (**Supplemental Table S3**). At this stage, and to assess for potential confounders, we assessed associations between patient demographic data and methylation patterns across these 25 genes, using separate multivariate models. No correlations were identified in these analyses, suggesting demographic factors did not significantly impact upon the methylation patterns identified (data not shown).

Differential subtype-specific promoter methylation in NMIBC:

We next determined methylation across the 25 genes described above in HG-NMIBC relative to that apparent in low-intermediate-grade tumours and in comparison to normal bladder controls (**Supplemental Table S4**). Similar to other groups[27 28], we displayed these methylation data, across the high-grade and low-intermediate-grade tumours and normal controls, by heatmap (**Figure 3**). This demonstrated heterogeneous patterns of methylation across the 51 high- and 18 low-intermediate-grade tumours relative to the normal bladder controls. Gene-specific differences in methylation were apparent between the high-grade tumours and their low-intermediate-grade counterparts on visual inspection. Closer examination of these data showed that the differences appeared to impact on either the relative *frequency* and/or the *mean levels* of methylation between these tumour subtypes. As

examples of these differences, the ten most differentially methylated genes are shown in **Table 1**.

Methylation frequencies in high- and low-intermediate-grade tumours:

For ten of the genes we took forward for further analyses (*ATP5G2*, *HIST1H4F*, *INSRR*, *IRF8*, *IRX1*, *PRDM14*, *PRRX1*, *TFAP2b*, *VAX2* and *VSX1*), there was an higher frequency of methylation in high-grade tumours versus low-intermediate grade tumours (**Table 1**). Moreover, the increases were statistically significant for the *ATP5G2*, *VAX2* and *IRX1* genes ($p<0.05$), and approached significance for the *INSRR*, *IRF8*, *PRDM14* and *VSX1* genes.

Mean levels of methylation in high- and low-intermediate-grade tumours:

The mean levels of methylation in the high-grade tumours were next assessed by Pyrosequencing (right-sided panel of **Table 1**, and **Figure 4**); for eight of the ten genes, mean levels of methylation were significantly greater in high-grade tumours relative to their low-intermediate-grade counterparts. In addition, and as low-intermediate-grade tumours were not subject to array analyses relative to normal bladder, further pairwise-testing was performed. This analysis identified significant differences between mean levels of methylation in the low-intermediate-grade tumours and normal bladder in four of the ten genes assessed. The range, distribution and mean levels of methylation are shown in **Figure 4**, and show for each of the genes, a stepwise trend toward increasing methylation from normal bladder to low-intermediate and high-grade tumours.

Methylation-Associated Changes in Gene Expression:

Across the high-grade NMIBC tumours, sufficient sample was available for gene expression analyses for 17 of the 25 genes. With the exception of the *ARHGEF4* gene, promoter-associated CpG island methylation was negatively correlated with transcript expression for all genes assessed (data not shown). Furthermore, the presence of promoter methylation was significantly correlated with reduced transcript expression for the *PON3*, *STAT5a* and *VAX2* genes (Spearman's correlation coefficients -0.60, -0.50 and -0.48 respectively, all $p<0.05$). Conversely, promoter methylation was significantly positively correlated with gene transcript expression for the *ARHGEF4* gene (Spearman's correlation coefficient 0.62, $p<0.05$). **Figure 5** shows the expression levels for these four genes across the high-grade tumours.

Gene Ontology analysis of inappropriately methylated genes:

Gene Ontology analyses of the 256 differentially methylated genes identified 'over-representation' of multiple categories of biological processes, molecular functions and pathways. In particular, highly significant over-representation was identified for specific biological processes, including regulation of RNA polymerase II activity and DNA transcription, and for pathways involving cell adhesion and PI3K-Akt signalling (**Supplemental Table S5**).

Discussion

In common with most other tumour types, bladder cancers harbour epigenetic aberrations which are frequently apparent as inappropriate DNA methylation.[8, 22, 29] However, reports are limited and largely confined to heterogeneous patient cohorts of NMIBC or MIBC;[30] despite their clinical importance, high-grade NMIBC tumours are rarely investigated as a discrete entity in the context of disease and/or subtype-specific epigenetic modifications.[23] To address this, we performed genome-wide analyses of DNA methylation using BeadChip array technology in high-grade NMIBC, comprising a discrete cohort of tumours recruited at initial presentation. This analysis, the first '450K array' interrogation in bladder cancer, revealed multiple and novel frequently differentially methylated genes in these tumours relative to normal bladder. Through Pyrosequence analysis of sodium bisulphite converted DNA, we extended our analyses to include independent cohorts of high- and low-intermediate-grade tumours. These investigations confirmed the array-derived data for the high-grade tumours, and showed them as harbouring significantly increased frequencies and/or mean levels of gene-specific methylation relative to low-intermediate-grade tumours. Moreover, for some of the genes investigated, a significant inverse correlation between promoter methylation and gene expression levels was apparent and suggests their potential as targets for therapeutic intervention.[29 31 32]

Initially we performed a technical validation of the discovery cohort data by Pyrosequence analysis of converted DNA.[25 33 34] In common with previous reports and across multiple genes, these analyses confirmed and reinforced the array-derived data.[34 35 36] These analyses also showed that for the majority of regions investigated, methylation extended to include contiguous promoter-associated CpG sites. On the basis of previous reports from our own and other groups,[37 38] we employed stringent criteria (β-value differences ≥0.4) to identify differentially methylated genes across multiple CpG sites; such criteria are more

consistently associated with *bona fide* changes in methylation, and are more likely to show associations with gene expression.[37, 39 40, 41]

The analysis of the discovery cohort of high-grade NMIBC identified 1,057 CpGs, across 256 gene-promoter-associated CpG islands. Cluster analysis and heat map display of these regions revealed extensive and frequent differential methylation in the tumours relative to normal bladder controls. As our study represents the first 450K analysis of high-grade bladder cancer a direct 'like-for-like' comparisons of our findings with those of other groups was not possible; however, the number of differentially methylated sites we identified appeared to be lower than those previously reported in other tumour types.[42 43] Potential explanations for these findings are the tumour type *per se* and/or the stringency of our inclusion-exclusion criteria and definition of differential methylation.[44]

For the genes identified, we performed gene ontology and KEGG pathway analyses. In these cases we identified significant over-representation of genes in processes and pathways previously reported by other groups as subject to epigenetically-mediated dysregulation in tumour development. For examples, these included transcription and cell signalling and adhesion[45-47], suggesting possible similar roles in high-grade bladder tumours, and their validity as targets for further investigation.

We next extended our investigation of multiple novel genes to an independent cohort of high-grade tumours, and a cohort of low-intermediate-grade tumours for comparison. Similar frequencies and mean levels of methylation, as determined by Pyrosequence analysis, were apparent within the discovery and investigation cohorts of high-grade tumours, suggesting our approach for the identification of candidates by array analysis was robust. Interestingly, many of the genes identified as novel and differentially methylated were also inappropriately methylated in low-intermediate-grade tumours. However, and despite the absence of genes as being exclusively associated with either high- or low-intermediate-grade tumours, the frequency and mean levels of gene-promoter methylation in the high-grade tumours were

242 significantly higher than in the low-intermediate-grade tumours. Indeed, similar observations
243 with respect to differences in the frequencies of methylation between high- and low-grade
244 bladder tumours were first suggested by Ibragimova *et al*.[47] Similar subtype and/or grade-
245 associated differences have been reported in other tumour types including, pituitary, breast,
246 and colon cancer subtypes.[37, 48, 49] In our analysis of NMIBC it remains unclear whether the
247 increase in frequency and/or mean levels of methylation in the more aggressive tumours
248 represents a more rapid accumulation of epigenetic changes during tumour progression, or
249 reflects distinct epigenetic pathways of tumour development and outgrowth.[50, 51] Our findings
250 may therefore reflect either of the described scenarios in the more aggressive (high-grade)
251 tumours and suggests that these tumours are either consequent to progression from low-
252 intermediate-grade tumours, or are the progeny of aberrations in distinct epigenetic
253 pathways within these NMIBC subtypes. Moreover, the identification of different patterns of
254 methylation between tumours represents an important area for future investigation. In this
255 case, methylation may hold promise as an 'at diagnosis' biomarker of long-term tumour
256 outcome, similar to that described in colorectal, breast and lung cancers. [52-54]

257 Although many of the novel genes we identified have not been previously reported in
258 bladder cancer, their inappropriate methylation, accompanied with gene-silencing, has been
259 reported in the context of other tumour types and suggests potential roles as tumour
260 suppressor genes.[55, 56 57] To determine associations between methylation and gene
261 expression, we confined our studies to genes showing frequent and/or high mean levels of
262 methylation. For the majority of gene-transcripts we investigated, promoter methylation was
263 negatively correlated with reduced transcript expression, although not significantly so (data
264 not shown). However, as described by our own and other groups, this may reflect a
265 passenger-driver phenomenon where, in the 'passenger' context, gene expression is not
266 directly influenced by the observed epigenetic modification(s).[58 59] However, for four of
267 seventeen transcripts we examined, significant correlations between methylation and
268 transcript expression were apparent. In these cases, and for the *PON3*, *STAT5a* and *VAX2*

genes, promoter methylation was significantly associated with reduced gene expression, whilst the converse was true for the *ARHGEF4* gene. Such associations are similar to those described previously in multiple other cancers and in NMIBC. [20, 21 43] Indeed, for two of these genes, *PON3* and *STAT5a*, previous studies in mice and cell-line models have described potential tumour suppressor roles.[60 61] If this is the case, then these genes may represent important targets for further studies of functional the significance of methylation and reduced expression in a bladder tumour context, including *in-vitro* investigations of de-methylating agents designed to restore gene expression.

In summary, we have presented the first comprehensive genome-wide DNA methylation analysis of NMIBC in a unique cohort of high-grade tumours. The study has reported an increase in the frequency and/or mean levels of methylation at gene promoter-associated CpG islands in high-grade tumours relative to their low-intermediate-grade tumour counterparts, that in some cases is associated with reduced gene expression. These findings suggest that epigenetic modifications, alone or in combination with other aberrations, are causal in the development and/or progression of this tumour type. Further studies are required to assess the functional significance of epigenetic changes in HG-NMIBC; however, we suggest that the genes identified hold significant potential as targets for novel therapeutic interventions alone, or in combination, with conventional therapeutic options in the treatment of this clinically unpredictable disease.

Patients and methods

Human tissue samples

Primary tumour and normal bladder tissues used were provided by the Bladder Cancer Prognosis Programme (BCPP, National Research Ethics Service East Midlands - Derby 06/MRE04/65.)[62], the University of Birmingham Human Biomaterials Resource Centre (National Research Ethics Service (North West 5): 09/H1010/75), and the University Hospitals of North Midlands NHS Trust (National Research Ethics Service (South Central – Oxford C): 12/SC/0725). All samples were confirmed histologically as normal bladder urothelium (control, *n*=4), G3pT1 TCC (high-grade: discovery cohort *n*=21, investigation cohort *n*=30), and G1/2 pTa/1 TCC (low/intermediate-grade: *n*=18). As previously described[23], patients received repeat bladder tumour resection (TURBT), cystectomy and/or intra-vesical therapy as recommended by European Association of Urology guidelines.[63] All samples (details are provided in **Supplemental Table S1**) were stored at -80^{o}C prior to nucleic acid extraction, as described below.

DNA extraction and bisulphite modification

Genomic DNA was extracted from tumour and control tissues using a standard phenol-chloroform procedure [64], then bisulphite-converted using the EZ DNA Methylation Gold kit (Zymo Research) as we have previously described.[37] Bisulphite-conversion of DNA was confirmed in all cases by successful PCR using primers specific to bisulphite-converted DNA (primer sequences in **Supplemental Table S6**). To increase the relative amount and stability of bisulphite-converted DNA, whole-genome amplification (WGA) was performed as previously described.[37]

316 *Illumina 450K Methylation Bead-Array Analyses*

317 Bisulphite-converted DNA from 21 bladder tumours and three normal controls was
318 hybridised to Infinium-based HumanMethylation450 BeadChip arrays (Illumina, San Diego,
319 CA, USA) to quantify DNA methylation at approximately 480,000 CpG positions across the
320 genome, representing more than 21,000 RefSeq genes. In this case, normal bladder was
321 used as control for consistency with previous array analyses [35, 47, 65], and also to permit
322 comparisons with earlier reports of non-muscle invasive bladder cancer. Arrays were
323 processed according to the manufacturer's instructions (performed by Barts and the London
324 Genome Centre, UK), as described by us previously.[66]

325 Raw array data were processed using GenomeStudio software and the bioinformatical
326 platform 'NIMBL', as we [67, 68] and others[69] have described. For each probe, the methylation
327 status was reported as a methylation 'β-value', where 'β' is defined as the ratio of the
328 methylated signal intensity over the summed intensity of the methylated and unmethylated
329 signals + 100.[40] β-values range from 0 (unmethylated) to 1 (fully methylated). NIMBL was
330 used to perform 'peak-based' correction, to adjust for potential differences in array probe-
331 type sensitivity previously reported[33]; all comparative analyses of high-grade tumours to
332 normal bladder controls, were performed on peak-based corrected β-values, as described by
333 us previously.[68]

334 Each array passed quality control assessment based upon the performance of internal
335 controls and the distribution of β-values across all array CpGs. As previously described[68],
336 and represented by **step 1** of **Figure 1**, we excluded all CpGs for which any of the 24
337 samples displayed: (i) probe detection *p*-values >0.05 (unreliable probe data), or (ii) missing
338 β-values (preventing analyses of all samples). We also excluded all CpG loci on allosomes
339 (reducing confounding gender-based methylation differences). We used a series of stringent
340 filtering criteria, shown in **Figure 1** and described in the Results section, to identify

inappropriate methylation, defined as a β-value difference ≥0.4, in tumour samples relative to the mean of the normal bladder controls.

Unsupervised hierarchical clustering using average linkage criteria was performed using Genesis software (v1.7.6).[70] Gene Ontology (GO) analyses were performed using http://geneontology.org/ and http://gather.genome.duke.edu/, and Kyoto Encyclopaedia of Genes and Genomes (KEGG) analyses with http://www.genome.jp/kegg/ online platforms, respectively. Bonferroni correction[71] was employed in all GO and KEGG pathway analyses.

Technical validation of Methylation Bead-Chip Array Data

Five CpG loci encompassing a broad range of β-values derived from 450k array analyses, were assessed by Pyrosequencing (described below), using identical samples, to independently validate the array data (β-values *vs.* methylation %). Correlation between the methods was assessed across a total of 120 CpGs using Spearman's rank correlation, as shown in **Supplemental Figure S1**. Primer sequences are provided in **Supplemental Table S6**.

Pyrosequencing™ of sodium bisulphite-converted DNA

Validation of array data (discovery cohort) and further quantitative assessment of methylation in the independent (investigation) tumour cohort were performed by Pyrosequencing of sodium bisulfite-converted DNA, as previously described by us[66], using a PyroMark Q24 Pyrosequencer, PyroMark Q24 Software 2.0 and PyroMark Gold Q24 Reagents. Dependent on the specific gene, and the density of CpGs within their promoter-associated CpG island, between five and nine consecutive CpG sites were assessed. Promoter methylation was defined in tumours if the mean level of methylation across the assessed CpG island was greater either than four standard deviations (4SD), or 20% above,

the mean of the normal controls.[37] The number of tumours methylated for any given gene describes the *frequency* of methylation, whereas the mean percentage methylation *per se* of all of the CpGs surveyed within a gene describes the *mean level* of methylation.

Quantitative RT-PCR

Total RNA was extracted from control and tumour samples using a standard guanidinium thiocyanate-phenol-chloroform protocol [72]. Complementary DNA (cDNA) was synthesised as described previously[73]. Thermal cycling using SYBR Green was as previously described[74], with target genes normalised to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the endogenous control gene (**Supplemental Table S6**). Relative quantification of transcript expression was performed using the $2^{-\Delta\Delta}$ cycle threshold (CT) method[75], and as previously described[76]. Reduced transcript expression in a tumour was defined where expression was at least 3-fold lower than the mean level of expression observed in control samples; the converse was true for increased transcript expression.[37 38 77]

Non-Array Informatics and statistics.

STATA (version 8, Stata Corporation, College Station, TX) was used to analyse methylation and gene expression data in tumour and normal cohorts using Fisher's exact tests (frequency of methylation), Student's t-tests (mean level of methylation), and Spearman correlation coefficients (associations between methylation and gene expression). *p*-values <0.05 were considered statistically significant.

Ethics Committee Approvals

East Midlands - Derby: 06/MRE04/65.

The University of Birmingham Human Biomaterials Resource Centre (National Research Ethics Service (North West 5): 09/H1010/75.

The University Hospitals of North Midlands NHS Trust (National Research Ethics Service (South Central – Oxford C): 12/SC/0725.

Reagents

EZ DNA Methylation Gold kit, Zymo Research, D5005

HumanMethylation450 BeadChip arrays, Illumina, WG-314-1003

PyroMark Gold Q24 Reagents, Qiagen, 970802

SYBR III brilliant green, Agilent, 600882

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Figure Legends

Figure 1. Array filtering steps. Summary of the steps implemented for the identification of CpGs hypermethylated in HG-NMIBC. The initial filtering steps (*) included exclusion of non-significant probe data, probes with missing data and probes located on allsomes.

RefSeq (National Center for Biotechnology Information Reference Sequence Database).

CpG island based upon the UCSC genome browser definition from Gardiner-Garden and Frommer[78].

Figure 2. Unsupervised hierarchical clustering analysis of the 1,057 gene promter-associated hypermethylated CpGs in HG-NMIBC. Heatmap and dendrogram of differentially methylated gene promoter-associated CpG sites identified by array analysis. The dendrogram above the heatmap separates normal bladder (green bar, *n*=3) and high-grade-NMIBC bladder tumours (red bar, *n*=21). Each row represents an individual CpG locus, and each column represents a normal control or tumour sample (listed beneath the heatmap). The colour scale beneath the heatmap represents methylation status: unmethylated is yellow (β-value=0.0), and fully methylated is blue (β-value=1.0).

Figure 3. Heatmap for 25 hypermethylated gene promoter-associated CpG islands. Pyrosequencing validation of 25 gene promoter-associated CpG islands, identified as frequently differentially methylated in high-grade tumours by 450k BeadChip-array analysis. As indicated above the heatmap, the four normal bladder controls are presented to the left-side of the heatmap, followed by 18 low-intermediate-grade tumours, and 51 high-grade tumours (the combined discovery and investigation cohorts). Each row represents the promoter-associated CpG island of the indicated gene, and each colour block the *mean level of methylation* across the island. The colour scale beneath the heatmap represents methylation status: unmethylated is green (0.0% methylation), and fully methylated is red (100.0% methylated).

Figure 4. Mean levels of methylation in high-grade tumours relative to low-intermediate-grade tumours and normal bladder. Top ten genes showing an increase in mean level of methylation (solid red bar) in high-grade tumours (HG, *n*=51) relative to low-intermediate-grade tumours (LG, *n*=18) and in comparison to normal bladder controls (C, *n*=4). Each individual control or tumour sample is shown as an unfilled blue circle. Significant differences in the mean levels of methylation between the low-intermediate- and high-grade tumours, or between control and low-intermediate-grade tumours, are indicated by *, $p<0.05$, or **, $p<0.005$ (Student's T-test).

Figure 5. Association of methylation with gene transcript expression in HG-NMIBC. Tumour transcript expression in unmethylated (UM, unfilled circles) and methylated (M, filled circles) high-grade tumours, relative to normal bladder control (C, unfilled triangles) for the four genes showing significant Spearman's correlation coefficients between promoter methylation and gene expression (*PON3*, *STAT5a*, *VAX2* and *ARHGEF4*; $p=0.0006$, $p=0.005$, $p=0.013$ and $p=0.0007$, respectively). The double-headed arrow represents the threshold for 3-fold reduced expression relative to the mean of the normal controls (solid blue bar); expression at or below this threshold signifies reduced expression in tumour samples.

Tables

Gene Symbol	METHYLATION FREQUENCY			MEAN LEVEL OF METHYLATION		
	High-grade	Low-intermediate-grade		High-grade	Low-intermediate-grade	
	Number (%)	*Number (%)*	*P* value	*(%)*	*(%)*	*P* value
ATP5G2	37/51 (72.5)	6/18 (33.3)	**0.005**	51.04	30.20	**0.029**
VAX2	13/51 (25.5)	0/18 (0.0)	**0.015**	32.31	19.56	**0.004**
IRX1	37/51 (72.5)	8/18 (44.4)	**0.045**	49.47	38.70	0.067
INSRR	29/51 (56.9)	5/18 (27.8)	0.054	24.06	24.06	**0.028**
IRF8	25/51 (49.0)	4/18 (22.2)	0.057	26.13	17.99	0.157
PRDM14	45/51 (88.2)	12/18 (66.7)	0.066	60.14	46.06	**0.029**
VSX1	44/51 (86.3)	12/18 (66.7)	0.086	56.37	38.26	**0.0004**
TFAP2b	22/51 (43.1)	4/18 (22.2)	0.160	32.25	17.68	**0.047**
PRRX1	27/51 (52.9)	7/18 (38.9)	0.413	47.03	34.36	**0.041**
HIST1H4F	42/51 (82.4)	13/18 (72.2)	0.496	59.46	41.91	**0.017**

Table 1. Genes showing the greatest methylation increase in high-grade relative to low-intermediate-grade NMIBC tumours. Top ten genes showing an increase in frequency of methylation (left side of table), and/or an increase in mean level of methylation (right side of table) in high-grade tumours relative to low-intermediate-grade tumours. For the left side of the table, the number and proportion of tumours methylated are displayed for the low-intermediate- and high-grade cohorts, with *p*-value (Fishers exact, $p<0.05$ significant). For the right side of the table, the mean level of methylation across the low-intermediate- and high-grade tumour cohorts are displayed with p-value (Student's T-Test, $p<0.05$ significant). Statistically significant p-values are displayed in bold.

Supplemental Data

Figure S1. Technical validation of 450k BeadChip-array data. Correlation between array-derived β-values (*x*-axis) and methylation percentage as determined by Pyrosequencing (*y*-axis) for 5 CpGs (cg07778029, cg14456683, cg01227537, cg05661282 and cg26465391) across 24 samples is shown. Spearman-rank correlation coefficient r=0.912; $p<0.00001$.

Table S1. Sample characteristics.

Table S2. List of 256 differentially methylated genes.

Table S3. Methylation in discovery and investigation high-grade tumour cohorts.

Table S4. Frequency and mean levels of methylation in 25 genes for high- and low-intermediate-grade tumours.

Table S5. Gene Ontology and KEGG pathway annotation lists.

Table S6. Primer sequences.