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RESEARCH PAPER

Quantitative genome-wide methylation analysis of high-grade non-muscle invasive bladder cancer

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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 characterize this disease entity in the context of tumor development, recurrence, and progression, are incompletely understood. We therefore interrogated genome-wide DNA methylation using HumanMethylation450 BeadChip arrays in 21 primary HG-NMIBC tumors 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. We validated the array data by bisulphite pyrosequencing 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 tumors relative to low-intermediate-grade tumors for the *ATP5G2, IRX1* and *VAX2* genes ($P<0.05$), and similarly significant increases in *mean levels* of methylation in high-grade tumors 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 tumors. 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.

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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 tumors 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 tumor 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 tumors, or whether they sit at a molecular crossroads between NMIBC and MIBC.[7,11,13] This uncertainty is illustrated by the findings that high-grade tumors harbor 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

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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 tumor 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 tumor 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 tumors relative to high-grade tumors. 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 tumors, we extend the current understanding of bladder cancer tumorigenesis and identify potential epigenetic mechanisms implicated in the development of high-grade NMIBC, and those that might represent novel therapeutic drug-targets.

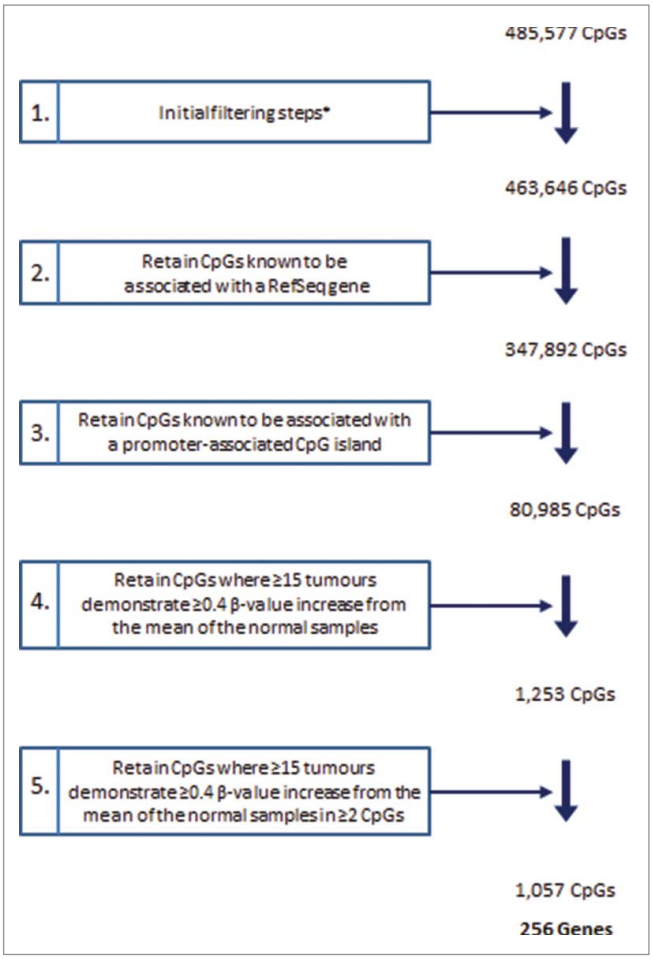

Figure 1. Array filtering steps. Summary of the steps implemented for the identification of CpGs hypermethylated in HG-NMIBC. The initial filtering steps (*) included exclusion of non-significant probe data, probes with missing data and probes located on allosomes. RefSeq (National Center for Biotechnology Information Reference Sequence Database). CpG island based upon the UCSC genome browser definition from Gardiner-Garden and Frommer.[78]

Results

Technical validation of array by pyrosequencing

Subsequent to array processing, normalization, 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 Fig. 1.[25,26] On the basis of these criteria, a total of 1,057 CpGs, representing 256 genes, were identified as hypermethylated (β-value increase ≥ 0.4) in 15 or more of the 21 high-grade tumors, relative to their mean values in the normal bladder controls.

Hierarchical clustering analyses

The filtered dataset was next subject to unsupervised hierarchical cluster analysis (Fig. 2): the high-grade tumors 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 tumors 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 tumors. 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 pyrosequencing analyses to an independent investigation cohort of 30 HG-NMIBC tumors. Similar frequencies and mean levels of

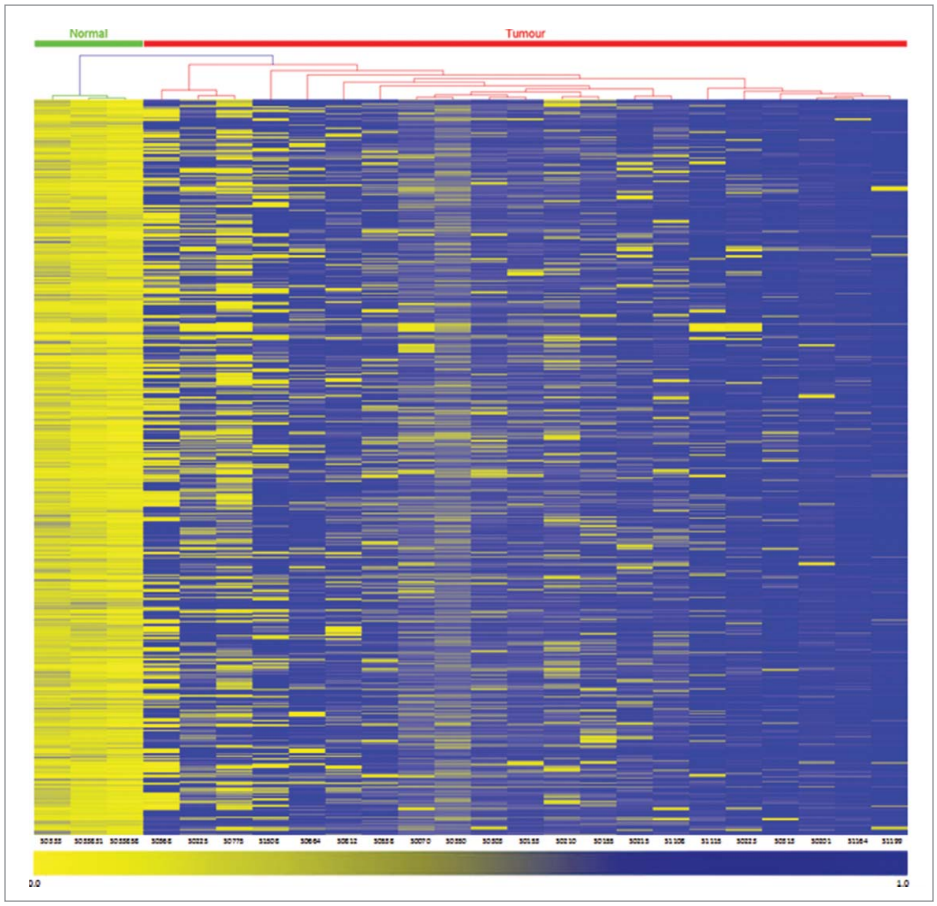

Figure 2. Unsupervised hierarchical clustering analysis of the 1,057 gene promoter-associated hypermethylated CpGs in HG-NMIBC. Heatmap and dendrogram of differentially methylated gene promoter-associated CpG sites identified by array analysis. The dendrogram above the heatmap separates normal bladder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

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 tumors 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 tumors and normal controls, by heatmap (Fig. 3). This demonstrated heterogeneous patterns of methylation across the 51 high- and 18 low-intermediate-grade tumors relative to the normal bladder controls. Gene-specific differences in methylation were apparent between the high-grade tumors 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 tumor 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 tumors

For ten of the genes we took forward for further analyses (*ATP5G2, HIST1H4F, INSRR, IRF8, IRX1, PRDM14, PRRX1, TFAP2b, VAX2* and *VSX1*), there was a higher frequency of methylation in high-grade tumors vs. low-intermediate grade tumors (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 tumors

The mean levels of methylation in the high-grade tumors were next assessed by pyrosequencing (right-sided panel of, Table 1 and Fig. 4); for eight of the ten genes, mean levels of methylation were significantly greater in high-grade tumors relative to their low-intermediate-grade counterparts. In addition, and as low-intermediate-grade tumors were not subject to array

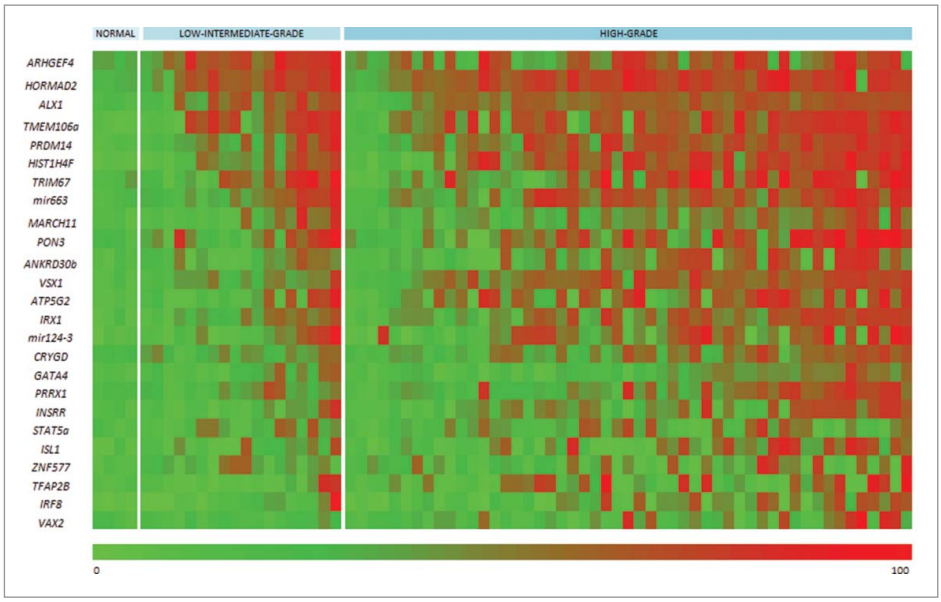

Figure 3. Heatmap for 25 hypermethylated gene promoter-associated CpG islands. Pyrosequencing validation of 25 gene promoter-associated CpG islands, identified as frequently differentially methylated in high-grade tumors by 450 K BeadChip-array analysis. As indicated above the heatmap, the four normal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

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 tumors and normal bladder in four of the ten genes assessed. The range, distribution and mean levels of methylation are shown in Fig. 4, and show for each of the genes, a stepwise trend toward increasing methylation from normal bladder to low-intermediate and high-grade tumors.

Methylation-associated changes in gene expression

Across the high-grade NMIBC tumors, 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, $P<0.05$). Fig. 5 shows the expression levels for these four genes across the high-grade tumors.

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

Table 1. Genes showing the greatest methylation increase in high-grade relative to low-intermediate-grade NMIBC tumors. 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 tumors relative to low-intermediate-grade tumors. For the left side of the table, the number and proportion of tumors 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 tumor cohorts are displayed with *P*-value (Student's T-Test, $P<0.05$ significant). Statistically significant *P*-values are displayed in bold.

	METHYLATION FREQUENCY			MEAN LEVEL OF METHYLATION		
Gene Symbol	High-grade *Number (%)*	Low-intermediate-grade *Number (%)*	*P* value	High-grade *(%)*	Low-intermediate-grade *(%)*	*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

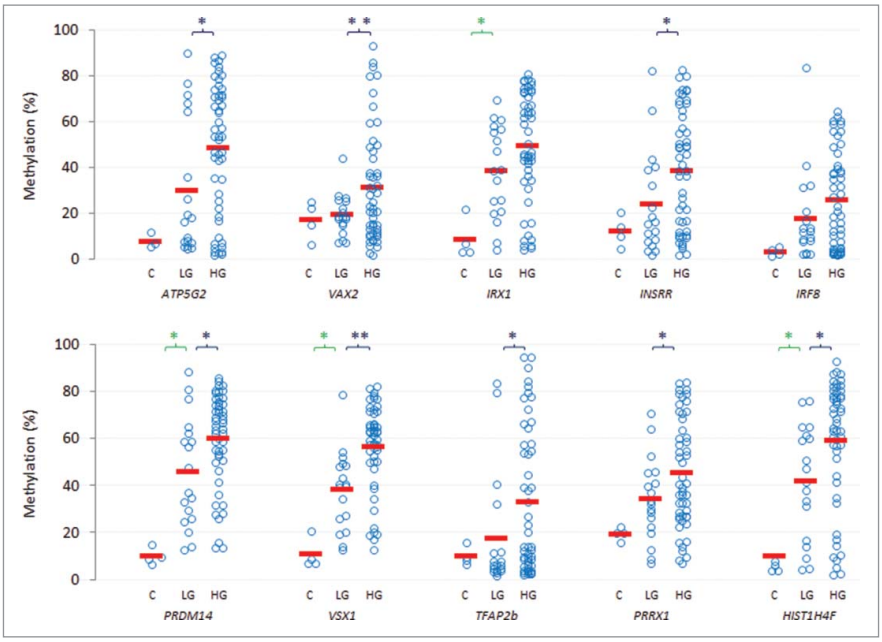

Figure 4. Mean levels of methylation in high-grade tumors relative to low-intermediate-grade tumors and normal bladder. Top ten genes showing an increase in mean level of methylation (solid red bar) in high-grade tumors (HG, n = 51) relative to low-intermediate-grade tumors (LG, n = 18) and in comparison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

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 signaling (Supplemental Table S5).

Discussion

In common with most other tumor types, bladder cancers harbor 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 tumors 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 tumors recruited at initial presentation. This analysis, the first '450 K array' interrogation in bladder cancer, revealed multiple and

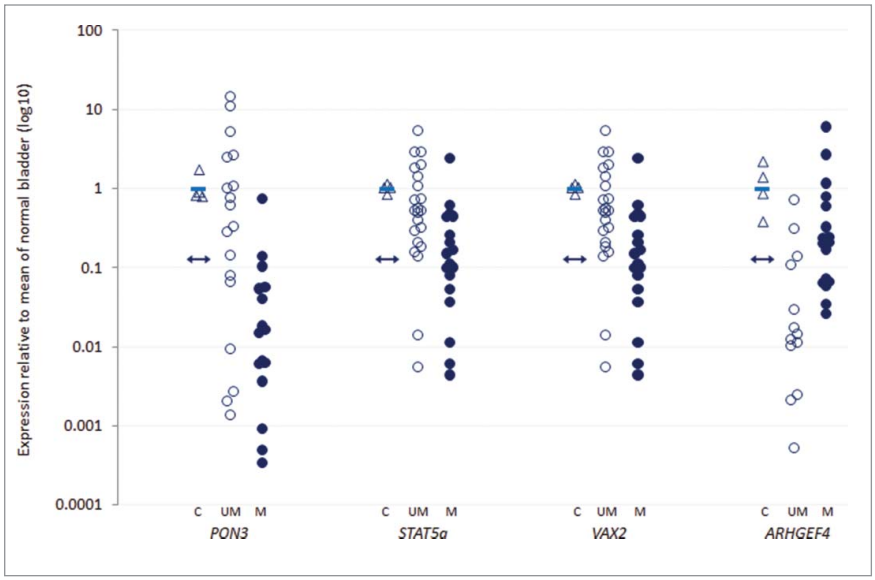

Figure 5. Association of methylation with gene transcript expression in HG-NMIBC. Tumor transcript expression in unmethylated (UM, unfilled circles) and methylated (M, filled circles) high-grade tumors, relative to normal bladder control (C, unfilled triangles) for the 4 genes showing significant Spearman's 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 tumor samples.

novel frequently differentially methylated genes in these tumors 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 tumors. These investigations confirmed the array-derived data for the high-grade tumors, and showed them as harboring significantly increased frequencies and/or mean levels of gene-specific methylation relative to low-intermediate-grade tumors. 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 $\geq$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 tumors relative to normal bladder controls. As our study represents the first 450 K 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 tumor types.[42,43] Potential explanations for these findings are the tumor 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 tumor development. For example, these included transcription and cell signaling and adhesion,[45-47] suggesting possible similar roles in high-grade bladder tumors, and their validity as targets for further investigation.

We next extended our investigation of multiple novel genes to an independent cohort of high-grade tumors, and a cohort of low-intermediate-grade tumors 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 tumors, 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 tumors. However, and despite the absence of genes as being exclusively associated with either high- or low-intermediate-grade tumors, the frequency and mean levels of gene-promoter methylation in the high-grade tumors were significantly higher than in the low-intermediate-grade tumors. Indeed, similar observations with respect to differences in the frequencies of methylation between high- and low-grade bladder tumors were first suggested by Ibragimova et al.[47] Similar subtype and/or grade-associated differences have been reported in other tumor types including, pituitary, breast, and colon cancer subtypes.[37,48,49] In our analysis of NMIBC it remains unclear whether the increase in frequency and/or mean levels of methylation in the more aggressive tumors represents a more rapid accumulation of epigenetic changes during tumor progression, or reflects distinct epigenetic pathways of tumor development and outgrowth.[50,51] Our findings may therefore reflect either of the described scenarios in the more aggressive (high-grade) tumors and suggests that these tumors are either consequent to progression from low-intermediate-grade tumors, or are the progeny of aberrations in distinct epigenetic pathways within these NMIBC subtypes. Moreover, the identification of different patterns of methylation between tumors represents an important area for future investigation. In this case, methylation may hold promise as an 'at diagnosis' biomarker of long-term tumor outcome, similar to that described in colorectal, breast and lung cancers.[52-54]

Although many of the novel genes we identified have not been previously reported in bladder cancer, their inappropriate methylation, accompanied with gene-silencing, has been reported in the context of other tumor types and suggests potential roles as tumor suppressor genes.[55,56,57] To determine associations between methylation and gene expression, we confined our studies to genes showing frequent and/or high mean levels of methylation. For the majority of gene-transcripts we investigated, promoter methylation was negatively correlated with reduced transcript expression, although not significantly so (data not shown). However, as described by our own and other groups, this may reflect a passenger-driver phenomenon where, in the 'passenger' context, gene expression is not directly influenced by the observed epigenetic modification(s).[58,59] However, for four of 17 transcripts we examined, significant correlations between methylation and transcript expression were apparent. In these cases, and for the *PON3, STAT5a* and *VAX2* genes, promoter methylation was significantly associated with reduced gene expression, while 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 tumor 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 tumor context, including *in vitro* investigations of demethylating 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 tumors. The study has reported an increase in the frequency and/or mean levels of methylation at

gene promoter-associated CpG islands in high-grade tumors relative to their low-intermediate-grade tumor counterparts, which 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 tumor 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 tumor and normal bladder tissues used were provided by the Bladder Cancer Prognosis Program (BCPP, National Research Ethics Service East Midlands - Derby 06/MRE04/65.),[62] the University of Birmingham Human Biomaterials Resource Center (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 tumor 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°C prior to nucleic acid extraction, as described below.

DNA extraction and bisulphite modification

Genomic DNA was extracted from tumor 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]

Illumina HumanMethylation450 BeadChip array analyses

Bisulphite-converted DNA from 21 bladder tumors and three normal controls was hybridized to Infinium-based HumanMethylation450 (450K) BeadChip arrays (Illumina, San Diego, CA, USA) to quantify DNA methylation at approximately 480,000 CpG positions across the genome, representing more than 21,000 RefSeq genes. In this case, normal bladder was used as control for consistency with previous array analyses,[35,47,65] and also to permit comparisons with earlier reports of non-muscle invasive bladder cancer. Arrays were processed according to the manufacturer's instructions (performed by Barts and the London Genome Center, UK), as described by us previously.[66]

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

Each array passed quality control assessment based upon the performance of internal controls and the distribution of β-values across all array CpGs. As previously described,[68] and represented by step 1 of Fig. 1, we excluded all CpGs for which any of the 24 samples displayed: (i) probe detection P-values >0.05 (unreliable probe data), or (ii) missing β-values (preventing analyses of all samples). We also excluded all CpG loci on allosomes (reducing confounding gender-based methylation differences). We used a series of stringent filtering criteria, shown in Fig. 1 and described in the Results section, to identify inappropriate methylation, defined as a β-value difference ≥0.4, in tumor 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 Beadchip array data

Five CpG loci encompassing a broad range of β-values derived from 450 K 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^{TM} of sodium bisulphite-converted DNA

Validation of array data (discovery cohort) and further quantitative assessment of methylation in the independent (investigation) tumor 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 tumors 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 tumors 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 tumor samples using a standard guanidinium thiocyanate-phenol-chloroform protocol.[72] cDNA (cDNA) was synthesized as described previously.[73] Thermal cycling using SYBR Green was as previously described,[74] with target genes normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the endogenous control gene (Supplemental Table S6). Relative quantification of transcript expression was performed using the $2^{-\Delta\Delta CT}$ method,[75] as previously described.[76] Reduced transcript expression in a tumor 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 analyze methylation and gene expression data in tumor 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 Center (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

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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