

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

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1 **Quantitative genome-wide methylation analysis of high-grade non-muscle**  
2 **invasive bladder cancer.**

3

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23 **Abstract**

24 High-grade non-muscle invasive bladder cancer (HG-NMIBC) is a clinically unpredictable  
25 disease with greater risks of recurrence and progression relative to their low-intermediate-  
26 grade counterparts. The molecular events, including those affecting the epigenome, that  
27 characterise this disease entity in the context of tumour development, recurrence and  
28 progression, are incompletely understood. We therefore interrogated genome-wide DNA  
29 methylation using HumanMethylation450 BeadChip-arrays in 21 primary HG-NMIBC  
30 tumours relative to normal bladder controls. Using strict inclusion-exclusion criteria we  
31 identified 1,057 hypermethylated CpGs within gene promoter-associated CpG islands,  
32 representing 256 genes. Bisulphite Pyrosequencing validated the array data and examined  
33 25 array-identified candidate genes in an independent cohort of 30 HG-NMIBC and 18 low-  
34 intermediate-grade NMIBC. These analyses revealed significantly higher methylation  
35 *frequencies* in high-grade tumours relative to low-intermediate-grade tumours for the  
36 *ATP5G2*, *IRX1* and *VAX2* genes ( $p<0.05$ ), and similarly significant increases in *mean levels*  
37 of methylation in high-grade tumours for the *ATP5G2*, *VAX2*, *INSRR*, *PRDM14*, *VSX1*,  
38 *TFAP2b*, *PRRX1*, and *HIST1H4F* genes ( $p<0.05$ ). Although inappropriate promoter  
39 methylation was not invariantly associated with reduced transcript expression, a significant  
40 association was apparent for the *ARHGEF4*, *PON3*, *STAT5a*, and *VAX2* gene transcripts  
41 ( $p<0.05$ ). Herein, we present the first genome-wide DNA methylation analysis in a unique  
42 HG-NMIBC cohort, showing extensive and discrete methylation changes relative to normal  
43 bladder and low-intermediate-grade tumours. The genes we identified hold significant  
44 potential as targets for novel therapeutic intervention either alone, or in combination, with  
45 more conventional therapeutic options in the treatment of this clinically unpredictable  
46 disease.

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

## 49 Introduction

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

57

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

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

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

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

98

99 **Results**

100 *Technical Validation of array by Pyrosequencing:*

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

107

108 *In-house filtering criteria:*

109 CpGs showing differential methylation in HG-NMIBC relative to normal bladder controls were  
110 identified following a series of stringent filtering criteria, as described previously and shown  
111 in **Figure 1**.<sup>25, 26</sup> On the basis of these criteria, a total of 1,057 CpGs, representing 256  
112 genes, were identified as hypermethylated ( $\geq 0.4$   $\beta$ -value increase) in 15 or more of the 21  
113 high-grade tumours, relative to their mean values in the normal bladder controls.

114

115 *Hierarchical clustering analyses:*

116 The filtered dataset was next subject to unsupervised hierarchical cluster analysis (**Figure**  
117 **2**): the high-grade tumours cluster independently from the normal bladder control samples.  
118 In these cases, methylation is barely detectable within the normal bladder samples, whereas  
119 15 or more of the high-grade tumours show inappropriate methylation across all 1,057 CpG  
120 dinucleotides, spanning 256 gene-promoter-associated CpG islands (**Supplemental Table**  
121 **S2**).

122

123 *Independent validation by Pyrosequencing:*

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

136

137 *Differential subtype-specific promoter methylation in NMIBC:*

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

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

150

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

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

157

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

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

169

170 *Methylation-Associated Changes in Gene Expression:*



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

181

182 *Gene Ontology analysis of inappropriately methylated genes:*

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

189

190 **Discussion**

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

208

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

216 consistently associated with *bona fide* changes in methylation, and are more likely to show  
217 associations with gene expression.<sup>37, 39 40, 41</sup>

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

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

233 We next extended our investigation of multiple novel genes to an independent cohort of  
234 high-grade tumours, and a cohort of low-intermediate-grade tumours for comparison. Similar  
235 frequencies and mean levels of methylation, as determined by Pyrosequence analysis, were  
236 apparent within the discovery and investigation cohorts of high-grade tumours, suggesting  
237 our approach for the identification of candidates by array analysis was robust. Interestingly,  
238 many of the genes identified as novel and differentially methylated were also inappropriately  
239 methylated in low-intermediate-grade tumours. However, and despite the absence of genes  
240 as being exclusively associated with either high- or low-intermediate-grade tumours, the  
241 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.*<sup>47</sup> Similar subtype and/or grade-  
245 associated differences have been reported in other tumour types including, pituitary, breast,  
246 and colon cancer subtypes.<sup>37, 48, 49</sup> 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.<sup>50, 51</sup> 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.<sup>52-54</sup>

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.<sup>55, 56 57</sup> 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).<sup>58 59</sup> 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*

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

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

288

289

290

291 **Patients and methods**

292 *Human tissue samples*

293 Primary tumour and normal bladder tissues used were provided by the Bladder Cancer  
294 Prognosis Programme (BCPP, National Research Ethics Service East Midlands - Derby  
295 06/MRE04/65.)<sup>62</sup>, the University of Birmingham Human Biomaterials Resource Centre  
296 (National Research Ethics Service (North West 5): 09/H1010/75), and the University  
297 Hospitals of North Midlands NHS Trust (National Research Ethics Service (South Central –  
298 Oxford C): 12/SC/0725). All samples were confirmed histologically as normal bladder  
299 urothelium (control,  $n=4$ ), G3pT1 TCC (high-grade: discovery cohort  $n=21$ , investigation  
300 cohort  $n=30$ ), and G1/2 pTa/1 TCC (low/intermediate-grade:  $n=18$ ). As previously  
301 described<sup>23</sup>, patients received repeat bladder tumour resection (TURBT), cystectomy and/or  
302 intra-vesical therapy as recommended by European Association of Urology guidelines.<sup>63</sup> All  
303 samples (details are provided in **Supplemental Table S1**) were stored at  $-80^{\circ}\text{C}$  prior to  
304 nucleic acid extraction, as described below.

305

306 *DNA extraction and bisulphite modification*

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

314

315

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<sup>35, 47, 65</sup>, 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.<sup>66</sup>

325 Raw array data were processed using GenomeStudio software and the bioinformatical  
326 platform 'NIMBL', as we<sup>67, 68</sup> and others<sup>69</sup> 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.<sup>40</sup> 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<sup>33</sup>; 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.<sup>68</sup>

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<sup>68</sup>,  
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

341 inappropriate methylation, defined as a  $\beta$ -value difference  $\geq 0.4$ , in tumour samples relative  
342 to the mean of the normal bladder controls.

343

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

349

#### 350 *Technical validation of Methylation Bead-Chip Array Data*

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

357

#### 358 *Pyrosequencing<sup>TM</sup> of sodium bisulphite-converted DNA*

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



367 the mean of the normal controls.<sup>37</sup> The number of tumours methylated for any given gene  
368 describes the *frequency* of methylation, whereas the mean percentage methylation *per se* of  
369 all of the CpGs surveyed within a gene describes the *mean level* of methylation.

370

### 371 *Quantitative RT-PCR*

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

381

### 382 *Non-Array Informatics and statistics.*

383 STATA (version 8, Stata Corporation, College Station, TX) was used to analyse methylation  
384 and gene expression data in tumour and normal cohorts using Fisher's exact tests  
385 (frequency of methylation), Student's t-tests (mean level of methylation), **and Spearman**  
386 **correlation coefficients (associations between methylation and gene expression)**. *p*-values  
387 <0.05 were considered statistically significant.

388

389 **Ethics Committee Approvals**

390 East Midlands - Derby: 06/MRE04/65.

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

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

395

396 **Reagents**

397 EZ DNA Methylation Gold kit, Zymo Research, D5005

398 HumanMethylation450 BeadChip arrays, Illumina, WG-314-1003

399 PyroMark Gold Q24 Reagents, Qiagen, 970802

400 SYBR III brilliant green, Agilent, 600882

401

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408

409

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634

635

636 **Figure Legends**

637

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

643

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

652

653 **Figure 3. Heatmap for 25 hypermethylated gene promoter-associated CpG islands.**  
654 Pyrosequencing validation of 25 gene promoter-associated CpG islands, identified as  
655 frequently differentially methylated in high-grade tumours by 450k BeadChip-array analysis.  
656 As indicated above the heatmap, the four normal bladder controls are presented to the left-  
657 side of the heatmap, followed by 18 low-intermediate-grade tumours, and 51 high-grade  
658 tumours (the combined discovery and investigation cohorts). Each row represents the  
659 promoter-associated CpG island of the indicated gene, and each colour block the *mean level*  
660 *of methylation* across the island. The colour scale beneath the heatmap represents  
661 methylation status: unmethylated is green (0.0% methylation), and fully methylated is red  
662 (100.0% methylated).

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

671

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

681

682

683

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

687 **Table 1. Genes showing the greatest methylation increase in high-grade relative to**

688 **low-intermediate-grade NMIBC tumours.** Top ten genes showing an increase in frequency

689 of methylation (left side of table), and/or an increase in mean level of methylation (right side

690 of table) in high-grade tumours relative to low-intermediate-grade tumours. For the left side

691 of the table, the number and proportion of tumours methylated are displayed for the low-

692 intermediate- and high-grade cohorts, with *p*-value (Fishers exact, *p*<0.05 significant). For

693 the right side of the table, the mean level of methylation across the low-intermediate- and

694 high-grade tumour cohorts are displayed with *p*-value (Student's T-Test, *p*<0.05 significant).

695 Statistically significant *p*-values are displayed in bold.

696 **Supplemental Data**

697

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

702

703 **Table S1. Sample characteristics.**

704

705 **Table S2. List of 256 differentially methylated genes.**

706

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

708

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

711

712 **Table S5. Gene Ontology and KEGG pathway annotation lists.**

713

714 **Table S6. Primer sequences.**

715

716

717