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A global profile of gene promoter methylation in treatment-naïve urothelial cancer

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The epigenetic alteration of aberrant hypermethylation in the promoter CpG island of a gene is associated with repression of transcription. In neoplastic cells, aberrant hypermethylation is well described as a mechanism of allele inactivation of particular genes with a tumor suppressor function. To investigate the role of aberrant hypermethylation in the biology and progression of urothelial cancer, we examined 101 urothelial (transitional cell) carcinomas (UC), broadly representative of the disease at presentation, with no prior immunotherapy, chemotherapy or radiotherapy, by Infinium HM27 containing 14 495 genes. The genome-wide signature of aberrant promoter hypermethylation in UC consisted of 729 genes significant by a Wilcoxon test, hypermethylated in a CpG island within 1 kb of the transcriptional start site and unmethylated in normal urothelium from aged individuals. We examined differences in gene methylation between the two main groups of UC: the 75% that are superficial, which often recur but rarely progress, and the 25% with muscle invasion and poor prognosis. We further examined pairwise comparisons of the pathologic subgroups of high or low grade, invasive or non-invasive (pTa), and high grade superficial or low grade superficial UC. Pathways analysis indicated overrepresentation of genes involved in cell adhesion or metabolism in muscle-invasive UC. Notably, the *TET2* epigenetic regulator was one of only two genes more frequently methylated in superficial tumors and the sole gene in low grade UC. Other chromatin remodeling genes, *MLL3* and *ACTL6B*, also showed aberrant hypermethylation. The Infinium methylation value for representative genes was verified by pyrosequencing. An available mRNA expression data set indicated many of the hypermethylated genes of interest to be downregulated in UC. Unsupervised clustering of the most differentially methylated genes distinguished muscle invasive from superficial UC. After filtering, cluster analysis showed a CpG Island Methylator Phenotype (CIMP)-like pattern of widespread methylation in 11 (11%) tumors. Nine of these 11 tumors had hypermethylation of *TET2*. Our analysis provides a basis for further studies of hypermethylation in the development and progression of UC.

Introduction

More than 72 000 new cases of, and over 15 000 deaths from, urothelial cancer (UC) of the urinary bladder are estimated in the US in 2014.[1] UC is the fourth most common cancer in males. There is a male to female incidence ratio of approximately 3:1 and the average age at diagnosis is 73 y in the US. Pathological stage is the most important factor for prognosis and is based on the depth of invasion into the bladder wall. Tumor grade is significant within superficial UC as higher grade is associated with increased rate of recurrence and progression to higher pathologic stage. At presentation, 20–25% of tumors present as muscle invasive or more advanced stage disease (pT2–4) and have a poor prognosis. The other 75–80% of cases present as non-invasive (pTa), lamina propria invasive (pT1) or carcinoma in situ (pTis) disease and are collectively classified as superficial cancers. However, 60–70% of superficial tumors recur at least once within 5 y and 10–20% progress to pT2–4 muscle invasive cancer. pTis or high grade T1 are at a greater risk of progression than are other superficial tumors. It is unclear whether muscle-invasive tumors at diagnosis have progressed from a superficial tumor or develop by means of a different pathway. There are established associations between tobacco exposure or occupational exposure to aromatic amines and UC, although UC can present in individuals with no prior exposures. In the Western world, more than 90% of UC is transitional cell carcinoma (TCC) by histology. In Africa and the Middle East, the majority of UC is squamous cell carcinoma (SCC) and is related to bilharzia infection.[2,3]

The biological relevance of aberrant methylation at promoter CpG islands has been clearly demonstrated in the transcriptional silencing of classical tumor suppressor genes such as *CDKN2A/p16, BRCA1,* and *MLH1* found in tumor cells having hypermethylation compared with an unmethylated state in the normal cell of origin.[4,5] Candidate gene studies have identified several genes as aberrantly hypermethylated in bladder cancer such as *RASSF1A*, *APC*, and *SFRP2*.[2] Recently, there have been more

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global beadchip or array-based studies of methylated genes in UC by Infinium Goldengate,[6,7] HM27,[8] and CpG island arrays[9,10] but all were limited in either the number of genes or the number and type of samples assayed. Beyond elucidation of the biology underlying the development of UC, there is considerable interest in the study of DNA methylation in UC for diagnosis, prognosis, prediction of response to therapy and reactivation by epigenetic therapy.[2,11]

To gain insight into the biology and progression of UC, we used the Infinium HM27 beadchip with 27 578 probes with an average of 2 CpG sites derived from 14 495 genes to survey the promoter methylome of 101 urothelial (transitional cell) carcinomas (64 superficial, 37 muscle-invasive) with no prior chemotherapy, immunotherapy or radiotherapy for UC and 6 normal urothelium specimens from patients in the seventh decade of life with no history or evidence of UC.

Results and Discussion

Assay performance

We first examined the Infinium HM27 beadchip data from the 101 treatment-naïve urothelial (transitional cell) carcinomas and 6 normal urothelium (NU) specimens for consistency of assay performance. Superficial (S) urothelial cancers (UC) of different stage and grade, muscle-invasive (MI) UC, and NU specimens were hybridized across different beadchips on different dates in order to lessen any batch effects. Probes with poor performance were removed as described in Materials and Methods. Multidimensional scaling (MDS) analysis by each beadchip and date revealed no apparent batch effects. The 6 NU specimens were in close proximity to each other and formed a distinct group from the 101 UC specimens (**Fig. S1A**). Seven technical replicates (7% of total specimens) were run on different beadchips and dates and the R^2 correlation coefficient of the replicate pairs ranged from 0.9606 to 0.9907 with a median of 0.9852 (**Fig. S1B**) indicating little variation. Probes that map to the X or Y chromosome were removed before further analysis. We next measured the variation in overall DNA methylation between the NU specimens plotted against a common reference (a synthetic array formed by taking the median β-value for each probe among the 6 normal samples). The R^2 correlation coefficient ranged from 0.9581 to 0.9821 (**Fig. S1C**). The 6 individuals from whom NU was sampled varied in age (range 28–78 y), which may account for some of the difference in methylation.

Unsupervised clustering by differential methylation

Unsupervised two-dimensional hierarchical clustering of the 200 most differentially methylated probes (SD 0.2583) within the 101 UC showed that of the two highest level clusters (**Fig. 1**), the cluster on the left contained a majority of S UC (45 superficial of 58 total) whereas the right cluster had a majority of MI UC (24 MI of 43 total). The difference in proportions was significant (Fisher's exact test $P = 0.0008$, two-sided). We noted that within the top 200 differentially methylated probes there was a tendency for multiple probes for the same gene locus to cluster together. This suggests that methylation was uniform across distances of several hundred base pairs of the CpG island in such genes. Co-methylation of CpG sites across distances of ≤ 1kb has been reported in human genome bisulfite sequencing studies.[12-14]

The promoter methylome of urothelial cancer

To identify genes hypermethylated in UC but unmethylated in NU cells, we first applied a stringent condition that each of the 6 NU must have a $\beta < 0.15$ for a probe to be considered unmethylated. We chose this cut-off because Illumina has reported that $\beta < 0.15$ can be due to the background noise of the assay chemistry at the unmethylated state.[15] In addition, we have found probes with $\beta < 0.15$ to typically read as unmethylated (0%) by pyrosequencing of the identical CpG loci.[16] We used the Wilcoxon Rank sum test in a two-group comparison with a P value < 0.05 as significant. A probe was considered hypermethylated in a UC specimen when the difference between the β-value of the tumor and the mean β-value of the NU samples was greater or equal to 0.2. This $\Delta\beta \geq 0.2$ cut-off was again based on the Illumina report that a $\Delta\beta$ sensitivity of 0.2 could be detected with 95% confidence across more than 90% of probes.[15] A $\Delta\beta \geq 0.2$ cut-off[17] or a $\Delta\beta \geq 0.1$–0.3 (relaxed-stringent) cut-off range has also been used by TCGA.[18,19]

An initial 980 probes had a significant Wilcoxon P value for hypermethylation after the 101 UC were compared with the 6 NU. We then excluded 127 probes: of these 64 were not located in a true CpG island, a further 25 were not located within 1kb of the transcriptional start site (TSS), 58 were not annotated by Infinium for a TSS while 20 of the 58 probes were rescued after manual examination of the TSS by Ensembl (http://useast.ensembl.org/Homo_sapiens/Info/Index). In total, 853 probes from 713 genes were located in a CpG island and within 1kb of the TSS. Ninety-three of 853 probes and 91 of 713 genes were hypermethylated in a single tumor only (**Table S2**). We included genes hypermethylated in only a small number of UC for several reasons: hypermethylation of the *VHL* gene occurs in only 7–8% of clear cell RCC by Infinium HM27 analysis[16,20] but has clear biological relevance; dysregulation of a pathway in cancer may be common through the cumulative sum of a relatively low frequency of mutation of each of several different genes within, or that act upon, the pathway in different individuals, e.g., Wnt signaling,[21] BRCA1/2 and other genes in homologous recombination[22,23] and the PI3K/AKT pathway[20]; a recent report of a dramatic response to a targeted therapy in a UC patient with a rare mutation of *NF2* (as well as *TSC1*) present in <1% of UC.[24] The mean number of probes/genes hypermethylated in the 101 UC was 297/247 with a median of 290/244 and range of 44–606/42–488 probes/genes. The total number of 713 genes aberrantly hypermethylated is similar to the number of 465 predicted somatic mutations reported by whole-exome sequencing of 9 MI UC.[25] Because of inter-tumor heterogeneity, sequencing of a larger number of UC would likely have resulted in a number greater than 465.

Unsupervised clustering of the 200 most differentially methylated probes (SD 0.2242) after filtering within the 101 UC showed that of the two highest level clusters (**Fig. 2**), the left cluster had a preponderance of S UC (9 MI, 26 S) whereas the right cluster was more even (28 MI, 38 S). The difference in

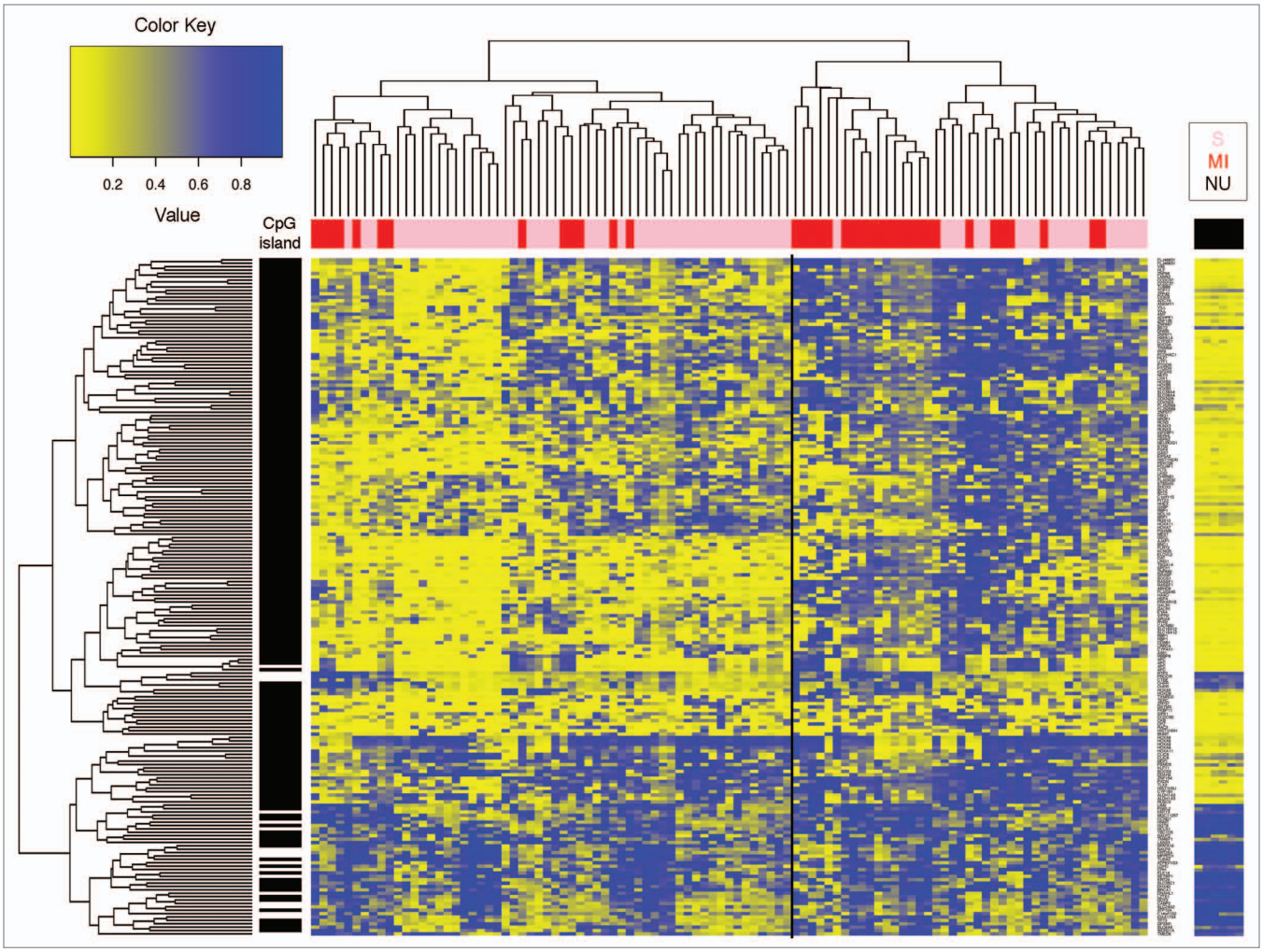

Figure 1. Unsupervised two-dimensional hierarchical clustering analysis of the 200 most differentially methylated probes in UC. 101 UC are identified as S or MI by color across the top of the heatmap. Top left is color scale for methylation status: unmethylated is yellow (β = 0), methylated is blue (β = 1). Probes located in a CpG island are shown in black (left). Gene name is given (right). Methylation status of the 6 NU for each probe is shown at far right.

proportions was not significant (Fisher's exact test P = 0.1293, two-sided). That the unfiltered differential methylation better discriminated between S and MI UC suggests that alterations in methylation in addition to promoter CpG island hypermethylation vary between S and MI tumors for example hypomethylation,[26] and hypermethylation outside the promoter CpG island.[27] More comprehensive coverage of the UC methylome by whole genome bisulfite sequencing (WGBS) or Infinium 450K will determine the importance of aberrant methylation outside CpG islands.

The hypermethylated genes included genes not previously reported in UC such as *WNK2* (*lysine deficient protein kinase 2*) a serine/threonine kinase with a key role in the regulation of cell signaling, survival, and proliferation known to be hypermethylated in glioblastoma[28] and colorectal cancer,[27] and *ITPKB* (*inositol-trisphosphate 3-kinase B*) which regulates inositol phosphate metabolism and is involved in cellular signaling through regulation of intracellular calcium levels. ITPKB was also recently implicated in wound healing in an animal model.[29] The *ACTL6B* (*actin-like 6B*) gene belongs to a family of actin-related proteins (ARPs) involved in different cellular processes, including vesicular transport, spindle orientation, nuclear migration and chromatin remodeling (http://www.ncbi.nlm.nih.gov/gene). This gene encodes a subunit (BAF53B) of the BAF (BRG1/brm-associated factor) complex in mammals[30] that is functionally related to the SWI/SNF complex. ACTL6B was first reported as selectively expressed in the brain,[30] however there is evidence of expression in other organs including low expression in normal urinary bladder and moderate nuclear staining in parts of some UC specimens by the Human Protein Atlas (http://www.proteinatlas.org/ENSG00000077080). Another gene *PTPRN* (*protein tyrosine phosphatase, receptor type, N*) is a member of the protein tyrosine phosphatase (PTP) family of signaling molecules that regulate cell growth, differentiation, mitotic cycle, and oncogenic transformation among other cellular processes. PTPRN has a role in the epithelial adherens junctions pathway (http://www.ncbi.nlm.nih.gov/gene).

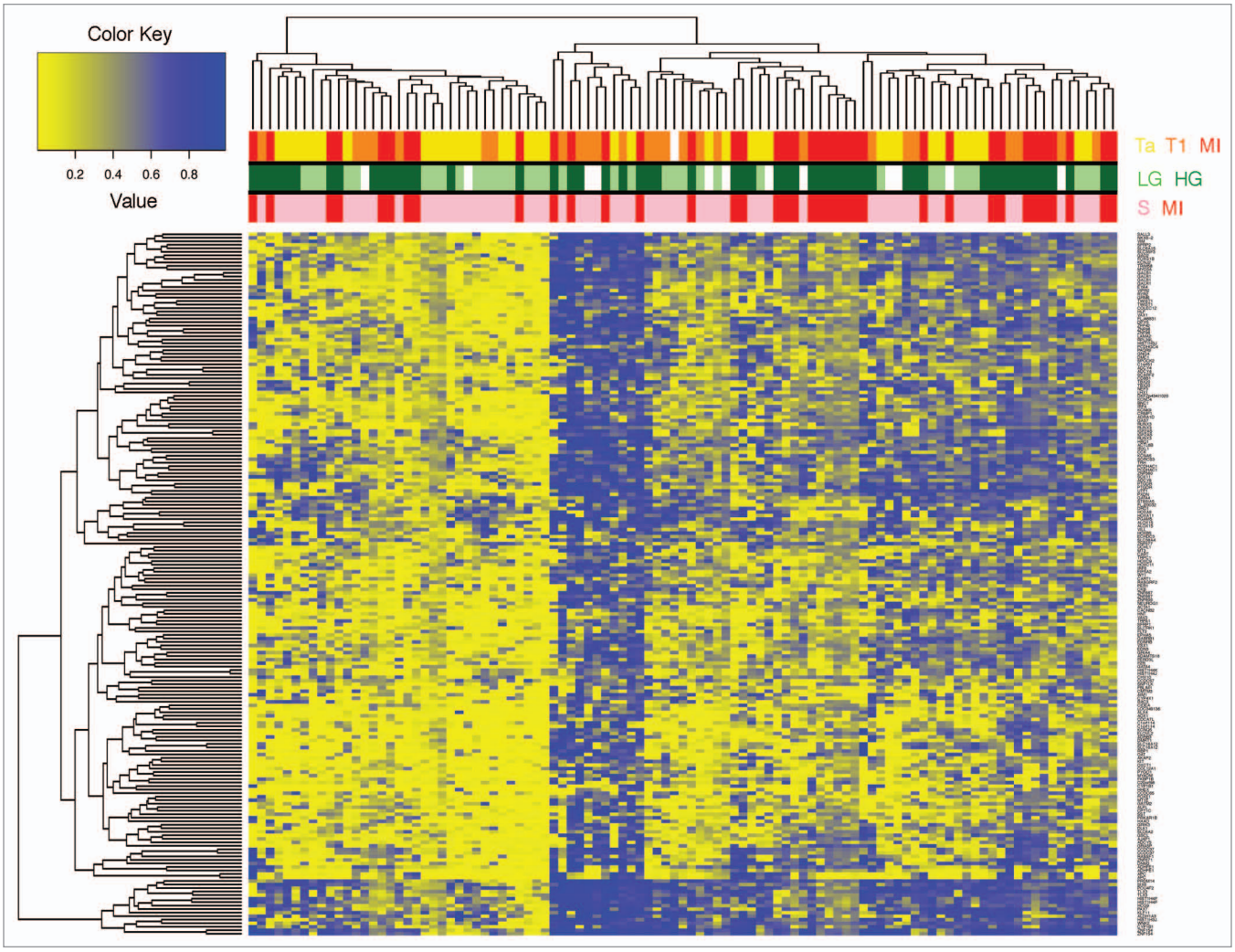

Figure 2. Unsupervised two-dimensional hierarchical clustering analysis of the 200 most differentially methylated promoter CpG island probes in UC unmethylated in NU. 101 UC are identified as S or MI, LG or HG, and pTa, T1 or MI by color top right, white = excluded because of insufficient histopathology. Top left is color scale for methylation status: unmethylated is yellow (β = 0), methylated is blue (β = 1). Gene name is given (right).

For a preliminary assessment of an association between aberrant promoter hypermethylation and mRNA downregulation of a gene, we used the normalized mRNA expression data from Sanchez-Carbayo et al.[31] Based on the cluster analysis in Figure 1 of Sanchez-Carbayo et al.,[31] we excluded the normal specimens present in the tumor cluster and the tumor specimens in the normal cluster. We then used Limma for a pairwise comparison of the level of expression of a gene in 43 NU specimens from 35 patients to 98 UC from 80 patients. *ITPKB*, *ACTL6B,* and *PTPRN* were downregulated in the UC while *WNK2* was not present in the expression array data set.[31]

Genes known to have aberrant hypermethylation in UC, e.g., *GREM1*, *RUNX3*, *RASSF1A* and *NID2* including classical tumor suppressor genes, i.e., *APC*, (reviewed in ref. 2) were also present among the genes hypermethylated in our study. Polycomb group (PcG) target genes were overrepresented in the total number of hypermethylated genes per urothelial tumor as there were 38% with 1, and 19% with 3, of the polycomb occupancy marks of the PRC2 subunits SUZ12 and EED associated with H3K27 methylation, among the 713 genes. This can be compared with 9.5% of genes with 1 mark and 4% with 3 marks in the human ES cells by Lee et al.[32] Ingenuity Pathways Analysis (IPA) of the 713 hypermethylated genes indicated many canonical pathways to be significantly over-represented ($P < 0.05$). These pathways may be broadly categorized as G-protein coupled receptors (GPCRs) and signal transduction, inflammation response, bladder cancer signaling, various epithelial cancer types, neuronal regulation, and stem cells (**Table S3**).

A separate analysis of 3 NU and 68 UC from males in the 101 patient set for probes mapped to the X or Y-chromosome identified 23 probes/20 genes before, and 19 probes/16 genes after, filtering to be hypermethylated by the criteria used for autosomal genes above (**Table S2**).

Differential methylation in muscle-invasive and superficial UC

We next examined separately the two main clinical types of bladder cancer. The 64 S UC (pTa and T1) were compared with the 6 NU using the Wilcoxon test and the same conditions

as before. Only T1 UC with muscularis propria present in the biopsy were included in our study. The 37 MI UC were similarly compared with the 6 NU (**Table S2**). The S tumors had a mean 258/221, median 251.5/220.5 and range of 41–472/39–406 probes/genes hypermethylated. The MI tumors had a mean 382/316, median 388/320 and range of 74–740/65–581 probes/genes hypermethylated. It should be noted that the degree of hypermethylation (β-value) might be underestimated more in MI compared with S UC. This is because of the typically higher level of normal cell contamination (from the muscularis propria, supporting mesenchymal tissue, as well as reactive lymphocyte and other inflammatory cells) in the often flat, infiltrating MI tumors compared with the more exophytic S tumors.

We then used Fisher's Exact Test with a false discovery rate (FDR) significance cut-off of $P < 0.05$ to identify genes differentially methylated in the MI tumor set compared with the S tumor set. Forty-seven probes from 40 genes were significantly more frequently hypermethylated in MI UC (FDR $P < 0.002$). The most significant were two probes for each of *CIDEA*, *ADHFE1*, and *GLOX1* (**Table 1**; **Table S4**). *CIDEA* (*cell death-inducing DFFA-like effector a*) maps to 18p11.21, is the human homolog of the mouse protein Cidea reported to activate apoptosis in the mouse[33] and thought also to be involved in transcriptional regulation and lipid metabolism. The methylation status of the *CIDEA* promoter is inversely correlated with transcription in different human tissues[34] and in endometrial cancer.[35] Consistent with this, *CIDEA* mRNA expression was downregulated in UC compared with NU from the Sanchez-Carbayo et al. data set.[31] ADHFE1 (alcohol dehydrogenase, iron containing, 1) has a function in retinol metabolism and also the TCA cycle. *GLOXD1* (*glyoxalase domain containing 1*) is an alias for *HPDL* (*4-hydroxyphenylpyruvate dioxygenase-like*) and is involved in aromatic amino acid family metabolism.

Other genes significantly more frequently hypermethylated in MI UC included *RASSF1* (*Ras Association (RalGDS/AF-6) Domain Family Member 1*) known to be hypermethylated in many types of cancer and with putative tumor suppressor function through inhibition of proliferation, mediation in apoptosis, and involvement in response to DNA damage (http://www.ncbi.nlm.nih.gov/gene). *ABO* promoter methylation in UC has been described[36] although the biological rationale for loss of expression of the ABO blood group gene in cancer is unclear.[37] *GRASP* (*GRP1 [general receptor for phosphoinositides 1]-associated scaffold protein*), by similarity to other members of this family, functions as a molecular scaffold, linking receptors, including metabotropic glutamate receptors, to neuronal proteins and has a role in intracellular trafficking. *GRASP* was recently reported as the most differentially methylated gene between colorectal tumors and normal tissue or adenomas[38] and to be methylated in breast cancer.[39] *FOXE3* (*forkhead box E3*) is a member of the forkhead family of transcription factors. *PRKAR1B* (*protein kinase, cAMP-dependent, regulatory, type I,* β) is involved in cAMP signaling, signaling in FGFR, G-protein signaling, and metabolism. *BTG4* (*B-cell translocation gene 4*) is a negative regulator of proliferation, previously described as methylated with downregulation in colorectal and other types of cancer.[40]

Of these genes, *CIDEA*, *RASSF1*, *ABO*, *FOXE3*, *PRKAR1B,* and *BTG4* were downregulated in UC compared with NU while *ADHFE1*, *GLOXD1* and *GRASP* were not present in the expression array data set.[31] IPA analysis of the 40 genes found 24 canonical pathways to be over-represented. The pathways may be broadly categorized as cell adhesion, metabolism, inflammatory response, neuronal regulation, stem cells, G-protein coupled receptors and signal transduction including the PI3K/AKT/mTOR pathway (**Fig. 3**).

Two genes were significantly more frequently hypermethylated in S UC compared with MI UC. The first gene was *TRPA1* (*Transient Receptor Potential Cation Channel, Subfamily A, Member 1*) a protein of calcium channel activity. Calcium signaling is essential to cell growth control and cellular differentiation and Stokes et al. have reported that an increased TRPA1 protein level was associated with a transformed phenotype in tumors.[41] The second gene was *FLJ20032*, better known as *TET2* (*tet methylcytosine dioxygenase 2*), a catalyst for the conversion of methylcytosine to 5-hydroxymethylcytosine (hmC). TET2 is thought to affect chromatin structure and recruit specific factors and to have an intermediary role in cytosine demethylation. Inactivating point mutations of *TET2* are well-described in myeloid malignancies (reviewed in ref. 42). *TET2* promoter methylation has been reported in low grade glioma without *IDH1* or *IDH2* mutation.[43] *TRPA1* mRNA expression was downregulated in UC compared with NU while *TET2* was not present in the expression array data set used.[31]

Differential methylation in high grade and low grade UC

We next examined methylation by tumor grade independent of stage. We excluded 12 tumors, originally annotated as grade II, from this analysis as histology slides could not be retrieved for the pathologist to reassess as low or high-grade according to current recommendations.[44] We used Fisher's Exact test as before to compare 35 low grade (LG) vs. 54 high grade (HG) UC (**Table S4**). Forty-six probes from 40 genes were significantly more frequently hypermethylated in HG UC. Some of the most frequently hypermethylated genes in the HG UC were similar to MI UC i.e., *CIDEA*, *ADHFE1*, *RASSF1,* and *GRASP*. This was expected since virtually all MI UC are of high grade. Sixteen of the 40 genes were different to the MI analysis. The 16 genes included *HOXD9* (*homeobox D9*) a transcription factor that provides cells with specific positional identities during developmental morphogenesis and *KL* (*Klotho*) an inhibitor of insulin and IGF1 signaling as well as the FGF pathway. *KL* is downregulated and hypermethylated in cancer.[45] One probe, for *FLJ20032*/*TET2*, was more frequently hypermethylated in LG UC and is discussed above.

Differential methylation in invasive UC

Since the acquisition of the ability to invade is of major importance in tumor progression,[46] we examined non-invasive UC (37 pTa) vs. invasive UC (63 pT1-T4) (**Table S4**). One UC specimen was excluded as it was unclear from the pathology annotation whether this tumor was pTa or T1. Fifty-seven probes from 47 genes had a significantly higher frequency of hypermethylation in the invasive UC. The most significant by *P* value *CIDEA*, *ADHFE1*, *RASSF1,* and *GRASP* were similar to the MI and HG

Table 1. List of genes with significant differential methylation between MI and S, HG and LG, invasive and non-invasive UC

TargetID	SYMBOL	Description	Chr. Location	S	MI	LG	HG	Inv
cg20950011	CIDEA	Cell Death-Inducing DFFA-Like Effector A1	18p11.21		+		+	+
cg08090772	ADHFE1	Alcohol Dehydrogenase, Iron Containing, 1	8q13.1		+		+	+
cg10164640	GLOXD1	4-Hydroxyphenylpyruvate Dioxygenase-Like	1p34.1		+		+	
cg21554552	RASSF1	Ras Association (RalGDS/AF-6) Domain Family Member 1	3p21.31		+		+	+
cg07241568	ABO	ABO system transferase	9q34.2		+			
cg04034767	GRASP	GRP1 (General Receptor For Phosphoinositides 1)-Associated Scaffold Protein	12q13.13		+		+	+
cg18815943	FOXE3	Forkhead Box E3	1p33		+			
cg13577076	PRKAR1B	Protein Kinase, CAMP-Dependent, Regulatory, Type I, Beta	7p22.3		+		+	
cg22879515	BTG4	B-Cell Translocation Gene 4	11q23.1		+		+	+
cg04970117	SLC6A20	Solute Carrier Family 6 (Proline IMINO Transporter), Member 20	3p21.31		+			+
cg04922810	CRHR2	Corticotropin Releasing Hormone Receptor 2	7p14.3		+		+	+
cg11846236	COL7A1	Collagen, Type VII, Alpha 1	3p21.31		+		+	+
cg17547792	GPR30	G Protein-Coupled Estrogen Receptor 1	7p22.3		+		+	
cg16098981	SYNDIG1	Synapse Differentiation Inducing 1	20p11.21		+			
cg21250296	HIST1H2BB	Histone Cluster 1, H2bb	6p22.2		+			
cg26131019	LRIG1	Leucine-Rich Repeats And Immunoglobulin-Like Domains 1	3p14.1		+			
cg22598028	ZNF660	Zinc Finger Protein 660	3p21.31		+			
cg20835282	C3orf62	Chromosome 3 Open Reading Frame 62	3p21.31		+			+
cg19776201	ZNF132	Zinc Finger Protein 132	19q13.43		+			+
cg19018097	FLJ30934	Sorting Nexin 32	11q13.1		+		+	
cg00888479	SLC24A3	Solute Carrier Family 24 (Sodium/Potassium/Calcium Exchanger), Member 3	20p11.23		+		+	
cg17525406	AJAP1	Adherens Junctions Associated Protein 1	1p36.32		+		+	+
cg02599464	HIST1H4I	Histone Cluster 1, H4i	6p22.1		+		+	
cg15134649	MT1E	Metallothionein 1E	16q12.2		+		+	+
cg02622316	ZNF96	Zinc Finger And SCAN Domain Containing 12	6p22.1		+		+	+
cg21516478	GPX3	Glutathione Peroxidase 3 (Plasma)	5q33.1		+		+	
cg02144933	AOX1	Aldehyde Oxidase 1	2q33.1		+		+	+
cg21604803	CPT1C	Carnitine Palmitoyltransferase 1C	19q13.33		+		+	+
cg01561916	HAAO	3-Hydroxyanthranilate 3,4-Dioxygenase	2p21		+			+
cg20312228	CCDC37	Coiled-Coil Domain Containing 37	3q21.3		+		+	+
cg03070194	GSTM2	Glutathione S-Transferase Mu 2 (Muscle)	1p13.3		+			+
cg20449692	CLDN11	Claudin 11	3q26.2		+			
cg23290344	NEF3	Neurofilament, Medium Polypeptide	8p21.2		+		+	+
cg19697981	NR2E1	Nuclear Receptor Subfamily 2, Group E, Member 1	6q21		+			
cg07237939	SLC22A3	Solute Carrier Family 22 (Organic Cation Transporter), Member 3	6q25.3		+			
cg00112517	PPP1R1B	Protein Phosphatase 1, Regulatory (Inhibitor) Subunit 1B	17q12		+			
cg26416466	MEGF11	Multiple EGF-Like-Domains 11	15q22.31		+		+	+
cg19620294	TNFRSF11B	Tumor Necrosis Factor Receptor Superfamily, Member 11b	8q24.12		+		+	+
cg06165395	GRIK3	Glutamate Receptor, Ionotropic, Kainate 3	1p34.3		+		+	+
cg05472874	SULT4A1	Sulfotransferase Family 4A, Member 1	22q13.31		+			+
cg06493386	TRPA1	Transient Receptor Potential Cation Channel, Subfamily A, Member 1	8q13.3	+				
cg08924430	TET2	Tet Methylcytosine Dioxygenase 2	4q24	+		+		
cg14991487	HOXD9	Homeobox D9	2q31.1				+	

Genes are listed in order of Wilcoxon *P* value and FDR *P* value. Gene symbol and name per HUGO Gene Nomenclature Committee (HGNC), chromosomal location per Ensembl.

Table 1. List of genes with significant differential methylation between MI and S, HG and LG, invasive and non-invasive UC (continued)

TargetID	SYMBOL	Description	Chr. Location	S	MI	LG	HG	Inv
cg25763788	HTR1B	5-Hydroxytryptamine (Serotonin) Receptor 1B, G Protein-Coupled	6q14.1				+	
cg16097079	HLA-C	Major Histocompatibility Complex, class I, C	6p21.33				+	
cg19246110	ZNF671	Zinc Finger Protein 671	19q13.43				+	
cg23282559	KL	Klotho	13q13.1				+	+
cg08958015	CCDC65	Coiled-Coil Domain Containing 65	12q13.12				+	
cg09872233	ALOX15	Arachidonate 15-Lipoxygenase	17p13.2				+	
cg17872757	FLI1	Fli-1 Proto-Oncogene, ETS Transcription Factor	11q24.3				+	+
cg18349835	VIPR2	Vasoactive Intestinal Peptide Receptor 2	7q36.3				+	
cg02026235	RHBDL1	Rhomboid, Veinlet-Like 1 (*Drosophila*)	16p13.3				+	
cg04448487	GDAP1L1	Ganglioside Induced Differentiation Associated Protein 1-Like 1	20q13.12				+	
cg22325703	GPR83	G Protein-Coupled Receptor 83	11q21				+	
cg20645065	ALPL	Alkaline Phosphatase, Liver/Bone/Kidney	1p36.12				+	
cg01283289	ACSS3	Acyl-CoA Synthetase Short-Chain Family Member 3	12q21.31				+	
cg08009622	COL12A1	Collagen, Type XII, Alpha 1	6q14.1				+	+
cg25228126	FZD2	Frizzled Family Receptor 2	17q21.31				+	+
cg18952647	BNC1	Basonuclin 1	15q25.2					+
cg18338311	TMEM132E	Transmembrane Protein 132E	17q12					+
cg21790626	ZNF154	Zinc Finger Protein 154	19q13.43					+
cg14950072	LAMA1	Laminin, Alpha 1	18p11.23					+
cg18592174	CHAT	Choline O-Acetyltransferase	10q11.23					+
cg15984661	CCDC8	Coiled-Coil Domain Containing 8	19q13.32					+
cg01259619	ITPKB	Inositol-Trisphosphate 3-Kinase B	1q42.12					+
cg05382123	CSMD2	CUB And Sushi Multiple Domains 2	1p34.3					+
cg20276750	PPM1M	Protein Phosphatase, Mg2+/Mn2+ Dependent, 1M	3p21.2					+
cg19450025	SULT1A3	Sulfotransferase Family, Cytosolic, 1A, Phenol-Preferring, Member 3	16p11.2					+
cg04660410	VILL	Villin-Like Protein	3p22.2					+
cg18453621	LMX1B	LIM Homeobox Transcription Factor 1, Beta	9q33.3					+
cg19751300	ST8SIA5	ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 5	18q21.1					+
cg06274159	ZFP42	ZFP42 Zinc Finger Protein	4q35.2					+
cg00250430	DMRT2	Doublesex And Mab-3 Related Transcription Factor 2	9p24.3					+
cg06621126	HSF4	Heat Shock Transcription Factor 4	16q22.1					+
cg02244695	HCA112	Transmembrane Protein 176A	7q36.1					+
cg23472215	GSTM3	Glutathione S-Transferase Mu 3	1p13.3					+
cg08918749	LPL	Lipoprotein Lipase	8p21.3					+
cg07671603	C7orf13	Chromosome 7 Open Reading Frame 13	7q36.3					+

Genes are listed in order of Wilcoxon *P* value and FDR *P* value. Gene symbol and name per HUGO Gene Nomenclature Committee (HGNC), chromosomal location per Ensembl.

analyses. Twenty genes were not in the previous two analyses. The most statistically significant were *BNC1* (*basonuclin 1*) a transcription factor in squamous epithelium, *LAMA1* (*laminin, α 1*) involved in cell adhesion and extracellular matrix (ECM) remodeling, and the *ITPKB* gene described above.

Differential methylation in high grade superficial UC

Because high grade superficial UC are considered to be at greater risk of progression we examined hypermethylated probes in 18 HG pTa or T1 vs. 35 LG Ta or T1 UC. Fisher's exact test did not identify any probe differentially methylated between these two groups at a significant FDR. Probes for two genes were significant for the Fisher's *P* value but not for the FDR ($P > 0.05$). The two genes were *CCDC65* (*coiled-coil domain containing 65*) a sperm tail protein of unknown function in the epithelial cell and *FLJ21963/ACSS3* (*acyl-CoA synthetase short-chain family member 3*) that, by similarity, activates acetate used for lipid synthesis

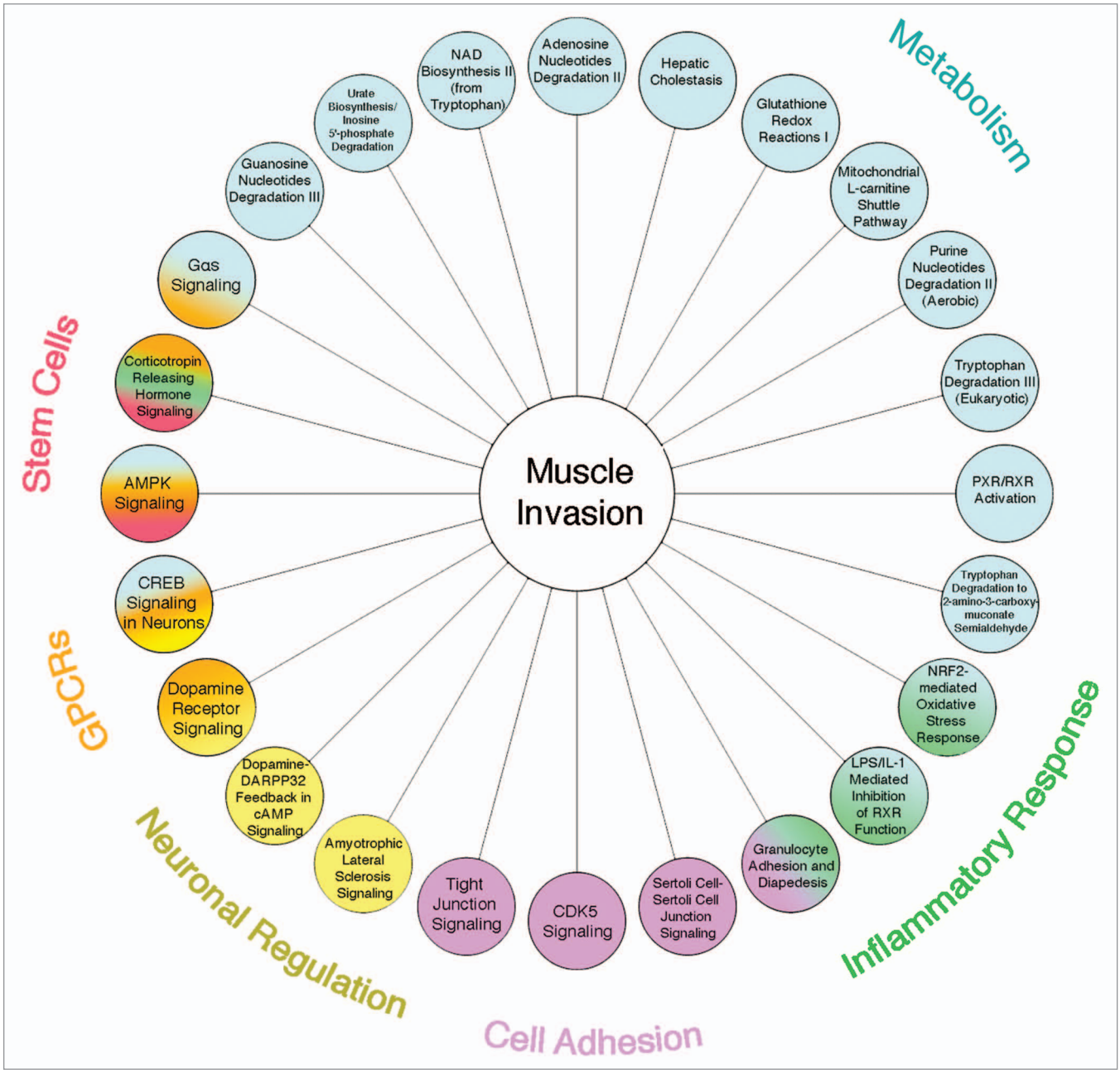

Figure 3. Pathways significantly overrepresented by aberrant gene methylation in MI compared with S UC. IPA analysis of genes significantly more frequently hypermethylated in MI UC compared with S UC identified 24 canonical pathways that may be broadly grouped as shown.

or for energy generation (http://www.ncbi.nlm.nih.gov/gene). We then examined hypermethylation in the subset of S UC with the highest risk of progression i.e., 12 HG pT1 vs. the 41 other superficial tumors (any grade pTa and LG T1) (**Table S4**). Probes for the *AJAP1* and *COL12A1* genes were significantly more frequently hypermethylated in the HG T1 tumors. *AJAP1* (*adherens junctions associated protein 1*) is involved in cell adhesion and cell migration.[47,48] AJAP1 is known to interact with CDH1 and CTNNB1 in adherens junctions in epithelial cells[49] and with BSG/CD147 to regulate cellular invasion.[48] Aberrant hypermethylation of *AJAP1* associated with downregulation of mRNA expression has been reported in glioma.[50] *COL12A1* (*collagen, type XII,* α *1*) as a structural constituent of the ECM is also implicated in cell adhesion.

Hypomethylation in UC

We examined hypomethylation in UC by the Wilcoxon Rank sum test with inversion of the cut-offs used for hypermethylation: that is a probe showed $\beta > 0.85$ in all 6 NU and hypomethylation was defined as $\Delta\beta \geq 0.2$ below the mean β-value of the 6 NU. In total 506 probes were hypomethylated in UC. After filtering 163 probes/156 genes were hypomethylated (cf. 853 probes/713 genes hypermethylated) (**Table S5**). As expected, the majority of hypomethylated probes (68%) were located outside a CpG island and/or >1 vkb from the TSS. Two members of the GP40 family of G

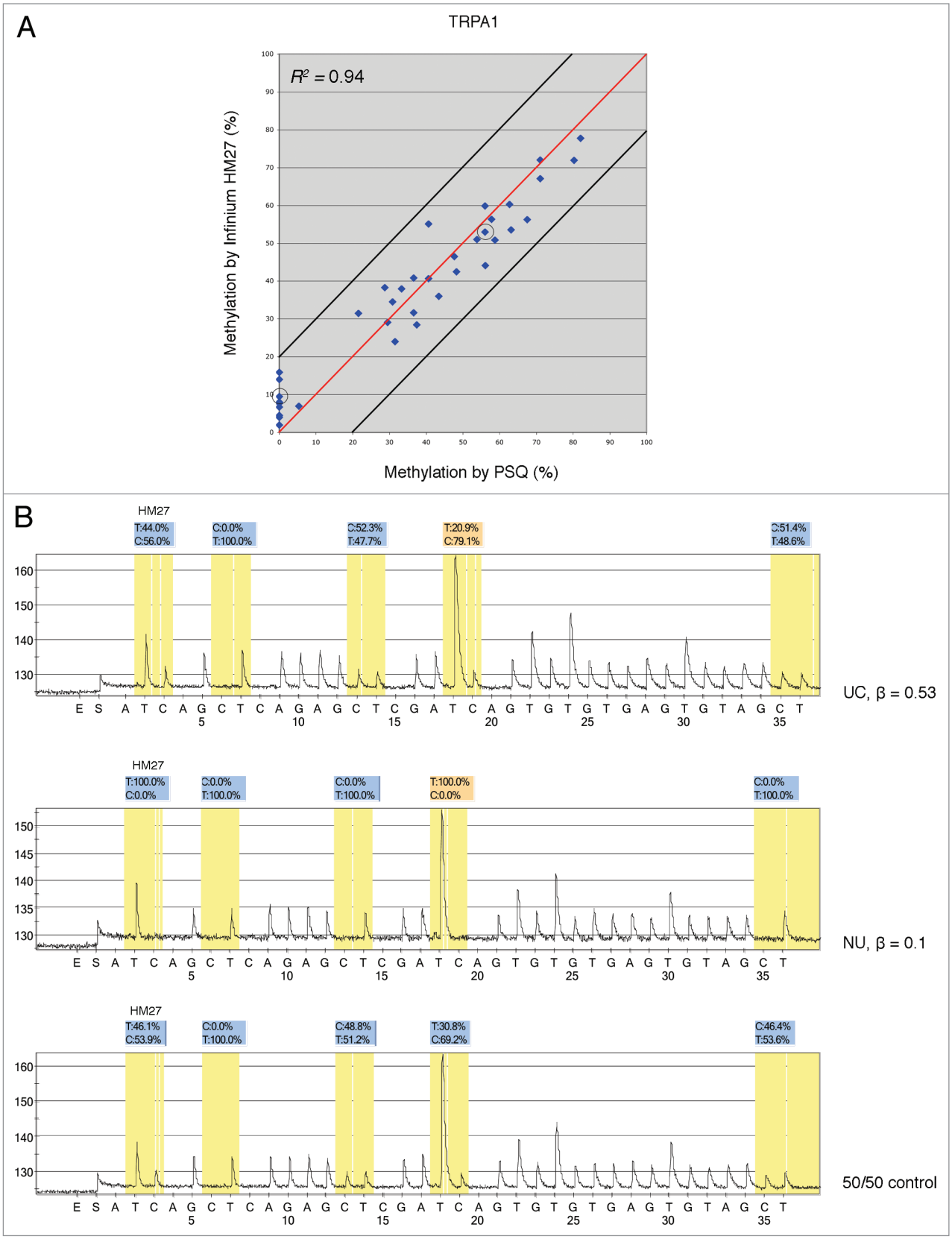

Figure 4. Verification of *TRPA1* Infinium HM27 β-value by pyrosequencing. (**A**) Correlation between Infinium methylation score and pyrosequencing of *TRPA1*. The R^2 is the Pearson coefficient. The two circled points correspond to the hypermethylated UC specimen and unmethylated NU specimen shown in the pyrogram below. (**B**) Pyrograms of *TRPA1* CpG loci hypermethylated in a UC but unmethylated in NU and in vitro methylated 50:50 unmethylated DNA control are shown. The CG loci from the HM27 probe are indicated.

protein-coupled receptors that are clustered together on chromosome 19q13.1, the free fatty acid receptors *FFAR2* and *FFAR1* were among the most frequently hypomethylated genes. These proteins act as receptors for short chain free fatty acids through a G(i)-protein-mediated inhibition of adenylyl cyclase and elevation of intracellular calcium and may be involved in the inflammatory response (http://www.ncbi.nlm.nih.gov/gene).

Verification of infinium methylation score by pyrosequencing

We selected 4 hypermethylated genes of interest in which to verify the promoter methylation status of identical, and adjacent, CG loci to the Infinium probe by an independent technology. We therefore designed assays for pyrosequencing of *CIDEA*, *TRPA1*, *ITPKB,* and *TET2*. Constraints of the sequence context on assay performance meant we could not analyze all CG dinucleotides in the relevant Infinium probe, however at least one of the CG dinucleotides pyrosequenced was identical and the others were by definition adjacent. The number and position of CG dinucleotides are shown in **Table S1**. Between 35–51 specimen DNAs (of the 107 UC and NU specimen DNAs) representative of the range of Infinium β-values were pyrosequenced for each gene. The pyrosequencing data for *TRPA1* showed excellent concordance as all 39 specimen DNAs had a pyrosequence score within 0.20 of the Infinium β-value (**Fig. 4**). The other genes showed a similar concordance for the majority (85–91%) of specimen DNAs examined, however the pyrosequence scores of several specimen DNAs were outside the 0.20 range (**Fig. S2**). These particular specimen DNAs may have a single nucleotide polymorphism within the sequence homologous to the Infinium probe.[51]

A subset of UC have widespread CpG island methylation

To investigate for the presence of a CpG island methylator phenotype (CIMP) in UC, we performed unsupervised clustering of the most differentially methylated (SD 0.224) gene probes after filtering across the 101 UC. The use of the filtered data first excluded any probes that did not meet the criteria of being located in a true CpG island thus adhering to the original definition of CIMP and second, included only probes that were unmethylated (β < 0.15) in all 6 NU thereby removing age-related methylation.[52] Within one of the two highest clusters, a sub-cluster of 11 tumors showed more widespread methylation and, to some degree, concordant methylation of true CpG island genes unmethylated in NU (**Fig. 2**). Of the Weisenberger et al. panel of 5 genes diagnostic for CIMP in colorectal tumors[53] *RUNX3* and *NEUROG1* were present in the top 200 probes in UC. Neither *CACNA1G* nor *IGF2* were present but the related genes *CACNAB2* and *IGF2AS* were in the top 200 probes. The fifth gene *SOCS1* was absent. Of the genes diagnostic for CIMP in Toyota et al.[52] *MLH1* and *THBS* were absent in the top 200 probes in UC while HM27 has no probe in the promoter CpG island of *CDKN2A/p16*. The 11 putative CIMP-positive tumors contained 7 S and 4 MI UC from 9 male and 2 female patients of 71.8 y average age. Seven patients had a history of smoking, 1 was a never smoker and information was unavailable for the remaining 3 patients. In a recent study of CIMP in colorectal cancer, all CRC with *MLH1* hypermethylation were CIMP-positive.[54] *MLH1* was unmethylated in NU and hypermethylated (but not significant by Wilcoxon) in 4/101 UC: none of which were among the group of 11 UC. Since inactivation of TET2 could result in aberrant hypermethylation,[42] we also examined if *TET2* hypermethylation was associated with CIMP. Nine of 11 (82%) UC in the putative CIMP subset had *TET2* hypermethylation compared with 50 (56%) of the remaining 90 UC. The difference in proportions was not significant (Fisher's exact test $P = 0.1159$, two-tailed).

Methylation as an alternative to point mutation for inactivation of TSGs

Several classical tumor suppressor genes (TSGs) are known to be hypermethylated, albeit some relatively infrequently, in UC (reviewed in ref. 2). We examined the location and the β-value of the relevant probes on HM27. In agreement, we found *APC* was hypermethylated in 39% of UC. Four UC showed hypermethylation of *MLH1* (cg02279071) although the Wilcoxon p-value was not significant. Only one UC showed hypermethylation of *CDKN2A/p14ARF* (cg26673943) but it should be noted that methylation may be more frequent at other areas of the promoter CpG island. Infinium HM27 does not contain a probe for the promoter CpG island of *CDKN2A/p16INK4A*. The promoter methylation status of other classical TSG has either not yet been investigated or examined only in limited numbers of UC. A probe for *RB1* (cg17055959) was unmethylated (β < 0.15) in NU and UC confirming a previous small study by ourselves.[55] A probe for *NF2* (cg16293088) was similarly unmethylated in all UC. A probe for *TSC1* (cg04648087) is located just outside the 3′ end of the promoter CpG island and was unmethylated in all UC (< 0.15), A second probe for *TSC1* (cg19393006) is located within the promoter CpG island but the mean β-value was 0.45 in NU and 0.39 in UC. However, 4 UC did have a β-value of 0.2 higher than NU. The two Infinium HM27 probes for *TSC2* are both located outside the promoter CpG island and showed high β-values in NU as well as UC.

A next generation sequencing study of 9 MI UC[25] found inactivating point mutations of several TSG known from other types of cancer. Because these TSG might also show allelic inactivation by aberrant promoter methylation in UC, we examined the location and the β-value of the relevant Infinium HM27 probes. Both probes for *ARID1A* were β < 0.1 in all 101 UC and were therefore excluded from further analysis. The probe for *KDM6A*/*UTX* is located outside, and relatively distant to, the promoter CpG island and so the methylation status is unlikely to be associated with transcription of this gene. A probe for *MLL3* (cg03634234) was unmethylated in NU and hypermethylated in 18/101 (18%) UC and had a significant Wilcoxon *P* value. The promoter region of *MLL3* is known to have high sequence homology to a pseudogene.[56] This Infinium probe, located 871bp downstream of the TSS, has very high homology to the pseudogene as only 3 base pairs of the probe differ. The other Infinium *MLL3* probe (cg20919133) is specific for *MLL3* but is located 1044bp upstream of the TSS and showed a β-value of 0.18 in one NU, and 0.16 in another NU although the mean of the 6 NU was β = 0.11. Three UC showed hypermethylation for this probe ranging from β = 0.32–0.57. In a verification set of 97 UC, Gui et al. reported 5% to have a non-silent point mutation of *MLL3*.[25]

Our data suggests that aberrant hypermethylation is a candidate mechanism of allelic inactivation of *MLL3*. Lastly, a single bladder tumor in our series had hypermethylation of the probe for *NF1* (cg22289810).

Other global studies of gene methylation in UC

Aleman et al. profiled 10 invasive UC and matched NU using differential methylation hybridization on custom-made CpG arrays. The study identified promoter hypermethylation of 84 clones as simultaneously present in 7 or more of the 10 tumors. One of the clones, *SOX9*, was verified as hypermethylated by independent technology and associated with downregulation of *SOX9* expression.[9] In our study, *SOX9* was unmethylated in NU and hypermethylated in 14/101 UC but the Wilcoxon *P* value was not significant. Wolff et al. used Infinium Goldengate containing 1370 loci/784 genes in 49 S and 38 MI UC as well as NU specimens. More genes were methylated in MI than S UC although most genes overlapped. The main focus was on a field defect in the urothelium of patients with MI tumors indicated by a clear increase of methylation in the corresponding normal-appearing tissues. Three individual genes were highlighted: a tight junction binding protein *TJP2*, *MYOD1,* and *CDH13*.[7] All 3 genes were hypermethylated in UC in our study however *TJP2* showed a $\beta > 0.15$ in NU and the Wilcoxon *P* value for *CDH13* only approached significance ($P = 0.0504$). Marsit et al.[6] also used Goldengate on a large series of invasive vs. non-invasive UC. They identified increased methylation of *HOXB2*, *KRT13* and *FRZB*/*SFRP3* in high grade non-invasive UC, and of *HOXB2* in invasive UC. *HOXB2* and *KRT13* were methylated in NU although invasive UC had a higher level of methylation. In our study, these two genes had a $\beta > 0.15$ in NU while *SFRP3* was significantly hypermethylated in UC vs. NU. Reinert et al.[8] examined 17 pTa, 5 T1 and 4 MI UC as well as NU with HM27. In their list of 403 probes/328 genes hypermethylated in UC, 129 probes overlap with the 854 probes from 713 genes in our study. Kandimalla et al. used an Agilent CpG island array in 44 UC vs. blood for discovery and a custom Goldengate 384-probe chip for validation in an independent set of tumors vs. normal urine. The transcription factors *TBX2*, *TBX3*, *GATA2,* and *ZIC4* were highlighted as highly methylated in pTa tumors that later showed progression to MI UC.[10] There is no probe for the *ZIC4* gene on HM27 and the other 3 genes did not fulfill our criteria.

Summary

We have examined the profile of DNA methylation, with a focus on promoter CpG islands, in a large series of UC broadly representative of the disease although with over-sampling of muscle invasive UC since such tumors are the most lethal. Only pT1 UC with muscularis propria present in the biopsy were included and specimens of equivocal grade were excluded from specific analyses. A limitation was that in our specimen set there were no de novo Tis cases which accounts for less than 1–3% of UC.[57] Importantly, the UC set had received no prior chemotherapy (e.g., mitomycin C) or immunotherapy (i.e., Bacillus Calmette-Guerin) for superficial UC and no prior chemotherapy or radiotherapy for MI UC. While, to our knowledge, direct experimental supporting evidence is lacking, it seems likely that, for example, an inflammatory response induced by BCG could potentially alter the epigenome of UC. Indeed, studies have demonstrated a hypermutation phenotype after treatment with temozolomide,[58] or temozolomide and radiation,[59] in glioblastoma. Another study found heavily treated castration-resistant prostate tumors to have more point mutation and copy number alterations than treatment-naïve high grade prostate tumors.[60] A further example is that ovarian tumors can develop secondary mutations in *BRCA1* or *BRCA2* after cisplatin[61,62] or PARP inhibition therapy.[63] Another important point is that UC specimens from both smokers and non-smokers were included in our study. Therefore, the novel alterations in DNA methylation we have identified should be more pertinent to the biology that underlies the development and progression of this disease.

Table 2. Clinicopathological data of 101 UC patients

Gender	Male n = 68	Female n = 33	
Age	Median 71	Range 41–89	
	Low Grade	High Grade	Grade II
Ta or T1	1		
Ta	25	6	6
T1	9	12	5
MI		36	1

The age of 2 patients was not available. All tumors were urothelial (transitional cell) carcinoma. 12 tumors were annotated as Grade II and histology slides were unavailable.

Through MDS analysis, technical replicates, and verification by pyrosequencing we found the Infinium HM27 technology to perform well, consistent with results of our prior study[16] as well as studies by others.[15,18] HM27 has extensive but not full coverage of genes with promoter CpG islands. The present study and the work of others with HM27[8] comprise a first pass of the UC methylome that will be extended by future studies.

We found a number of genes not previously described as hypermethylated in UC including the chromatin modifying genes *TET2*, *MLL3,* and *ACTL6B*. Since a source of expression data in UC is unavailable for some of the genes of interest, it will be important to demonstrate an inverse relationship between hypermethylation and expression of a gene before further investigation of function in UC. The more frequent hypermethylation of TET2 found in S or LG UC is intriguing as too is the association of TET2 hypermethylation with 9 of the 11 UC showing widespread methylation. Evidence for more widespread methylation in a subset of UC (independent of higher grade or stage) is suggestive of a CIMP phenotype and it will be important to further characterize a putative CIMP in UC. The predominant molecular themes of the set of genes more frequently hypermethylated in MI UC included cell adhesion and metabolism. Further analysis of the particular genes and pathways may provide novel targets for therapy as well as for establishing a differential prognosis for more aggressive subsets of superficial UC. DNA methylation is a promising target for early detection of UC in urine[64] and particular genes or sets of genes, identified in our study as hypermethylated, may have utility for molecular diagnosis.

Materials and Methods

Specimens

Snap-frozen urothelial (transitional cell) carcinoma tissues were embedded in OCT, cut and stained with H&E before examination by the pathologist, Dr. E. Dulaimi, for an area of ≥ 70% tumor cell content to be used for DNA extraction. Clinicopathological data for the 101 tumors are given in **Table 2**. Specimens were collected from 1993 to 2012. Normal urothelium (NU) specimens were obtained by dissection of a cross-sectioned ureter from six patients (3 male, 3 female) with no history or evidence of urothelial cancer (UC) who underwent radical nephrectomy for renal cell carcinoma. Four were never smokers and two had a history of smoking. The six NU patients had a mean age of 61 y; the median age of UC patients at diagnosis in the United States during 2005–9 is 73 y (http://seer.cancer.gov/statfacts/html/urinb.html). The Fox Chase Cancer Center Institutional Review Board approved the study and all patients provided consent.

DNA isolation and bisulfite modification

DNA was extracted from fresh-frozen tissue using a standard technique of digestion with proteinase K followed by phenol-chloroform extraction and ethanol precipitation.[65] One microgram of genomic DNA from each sample was bisulfite modified using the EZ-DNA Methylation kit (Zymo Research Corporation D5002) according to the manufacturer's protocol with the alternative incubation conditions as stated for use with the Infinium beadchip.

Bead chip based DNA methylation analysis

Bisulfite treated DNA was isothermally amplified, enzymatically fragmented and hybridized to the Infinium HM27 BeadChip (Illumina WG-311-2201). We took care to distribute specimens of each histological type across different beadchips on different dates. We also ran 7 technical replicates on different beadchips on different dates. During hybridization, single-stranded DNA anneals to locus-specific DNA oligomers linked to individual bead types. Each bead type corresponds to each CpG locus: one to the methylated and the other to the unmethylated state. Allele-specific primer annealing is followed by single-base extension using dinitrophenyl (DNP)- and Biotin-labeled ddNTPs. After extension the BeadChip was fluorescently stained. The fluorescent intensity of the beads is detected by the Illumina BeadArray Reader and analyzed using Illumina BeadStudio software. DNA methylation values, described as β-values, vary between 0 (unmethylated) and 1 (fully methylated), representing the ratio of the intensity of the methylated bead type to the combined locus intensity.

Data analysis

Methylation data were analyzed using the R/Bioconductor platform. The N-bead value averaged 18 bead replicates for each probe across all 107 beadchips. β-values were used to exclude poor performance probes prior to comparison of the tumor groups. Up to 119 (of 27578) probes with missing β-values (N-bead value = 0 in at least 1 beadchip) were removed. In addition, up to 7510 probes where $\beta < 0.1$ in all 107 specimens were excluded. The exact number of probes removed depended upon the particular specimen groups compared. We also initially removed 1080 probes mapping to chromosome X and Y as otherwise gender specific methylation could skew clustering analysis. We imposed cut-offs and ranked probes by Wilcoxon ranked sum test in a two-group comparison with $P < 0.05$ considered significant. Based on this approach the set of genes that are differentially methylated in urothelial tumors and NU cells were ranked and thus prioritized for further analysis. We used IPA (Ingenuity Systems,) to identify significantly over-represented canonical pathways in the lists of differentially methylated genes between UC and NU or subsets of UC. We considered IPA pathways with enrichment scores ≥ 1.3 equivalent to a non-log scale P value < 0.05 as significant.

Pyrosequencing

Primers for PCR amplification and pyrosequencing (**Table S1**) were designed using Biotage software (Qiagen). For pyrosequencing analysis, the PyroMark Gold Reagent Kit (Qiagen 972812) was used. An internal control, a C not in a CG dinucleotide, for the efficiency of modification was included in the assay for promoter methylation for all genes with the exception of *ITPKB*. A 50:50 unmethylated:fully methylated DNA control was examined to identify amplification or sequencing bias for each assay.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/28078

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