Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Institute for Cancer Research

2. **Reporting Period (start and end date of grant award period):** 1/1/2011 – 6/30/2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Maria Minko Gill

4. **Grant Contact Person's Telephone Number:** 215-728-2659

5. **Grant SAP Number:** 4100054848

6. **Project Number and Title of Research Project:** 03 - A Gene Methylation Progression Model of Bladder Cancer

7. **Start and End Date of Research Project:** 1/1/2011 – 6/30/2014

8. **Name of Principal Investigator for the Research Project:** Paul Cairns, Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $455,661

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Cairns, John P.	PI	22% years 1-2; 60% year 3	$123,129.63
Ibragimova, Ilsiya	Postdoctoral Associate	25% years 1-2	$17,081.65
Almeria, Maria S.	Research Associate	15% year 1; 4% year 2; 56% year 3; 74% year 4	$80,425.46

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
Apollo 100XL System	The Apollo 100XL System is a microfluidics robot system for performing Sanger sequencing reactions in very small (1 microliter) volumes, enabling very significant cost-savings in reagent consumption, making sequencing much cheaper per run. Also, since all pipetting is controlled by a mechanical actuator, volumes are more reproducible than with manual pipetting, so results are more reliable.	$65,585

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you

able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No_____X____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

Yes, I plan to submit a R21 to the NCI on the testing of a methylation panel for prediction of behavior of superficial urothelial tumors.

12. Future of Research Project. What are the future plans for this research project?

We plan to test a panel of genes on a retrospective set of tumors that progressed cured to examine if methylation status is predictive of behavior and outcome. The gene and pathway data is being interrogated for druggable targets. Analysis of pathways and molecular themes continues as the field develops.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				1
Unknown				
Total				**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				1
Unknown				
Total				**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White				1
Black				
Asian				
Other				
Unknown				
Total				**1**

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No_____X____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

The Bioinformatics Facility gained expertise with analysis of Infinium beadchip technology

and large gene methylation and specimen data sets. The Genomics Facility gained expertise in pyrosequencing of bisulfite-modified DNA.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No_____X____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No_____X____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending

publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

The goal of this project is to have our team use our expertise and state of the art genome-wide technology to rapidly screen for genes differentially methylated between pathological stages of bladder cancer. This will address the key clinical issue that the subset of individuals with superficial bladder cancer that will progress cannot be identified in advance.

The successful completion of this project could have future impact through improved care and outcome of bladder cancer. Our work could lead to an aggressiveness index to stratify aggressive superficial tumors for potentially curative cystectomy while still allowing patients with more indolent lesions to have bladder-sparing treatment. The genes identified may also have clinical utility in other tumor types with similar management issues, e.g., indolent versus aggressive prostate tumors. Lastly, we have expertise in translational application to bladder cancer and gene methylation tests have reached the clinic.

Our Specific Aims are
1. To profile genome-wide aberrant methylation of genes in pathological stages of bladder cancer.
2. To validate aberrant methylation by direct bisulfite sequencing of the genes identified.
3. To identify functional themes, pathways and networks in bladder tumor invasion and progression.

We expect to identify genes with a statistically significant different frequency of aberrant methylation between pathological stages based on depth of invasion of bladder cancer.

We expect to verify 25 genes aberrantly methylated in bladder cancer but unmethylated in normal transitional cells.

We expect to identify functional themes, pathways or networks within the 25 genes.

The project will improve health status through:

- Further elucidation of the methylome of the human bladder cancer cell
- Candidate markers for risk stratification of superficial bladder tumors to be tested further
- Candidate markers for diagnosis, and novel targets for chemoprevention or therapy
- Insight into the biology of bladder cancer and, in particular, tumor invasion
- Insight into the biology of, and markers for, other cancers e.g. prostate cancer

Summary of Changes

No major changes were made to the research goals/objectives/aims, methods or design since the original grant application was submitted. Changes to the relative numbers of tumors in the pathologic subgroups were necessary to complete the work in a timely manner. Other changes were made because experience over the course of the study determined that they represented a superior choice. These changes were that we used a published gene expression dataset instead of the CGAP SAGE database because the latter did not include data from normal urothelium. Change in the method of verification from bisulfite sequencing to mining an independent tumor set in the TCGA database was considered more appropriate once the TCGA data became available. For statistical analysis, we examined several approaches but ultimately favored the Wilcoxon rank sum test because this test made the least distributional assumptions. The timeline was extended from an end date of 12/30/2012 to 6/30/2014.

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 1*. 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 years; the median age of UC patients at diagnosis in the United States during 2005-9 is 73 years (http://seer.cancer.gov/statfacts/html/urinb.html).

Table 1. Clinicopathological data of 101 UC patients.
The age of 2 patients was not available.
All tumors were urothelial (transitional cell) carcinoma
Twelve tumors were annotated as Grade II and histology slides were unavailable.
Pathologic stage is given in the far left column.
T2-T4 UC are grouped together as MI.

Gender	Male n=68	Female n=33	
Age	median 71	range 41-89	
Stage	Low Grade	High Grade	Grade II
Ta or T1	1		
Ta	25	6	6
T1	9	12	5
MI		36	1

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.[1] 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 27,578) probes with missing β-values (N-bead value = 0 in at least 1 beadchip) were removed. In addition, up to 7,510 probes where β= <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 1,080 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 ingenuity pathway analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) 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 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.

RESULTS

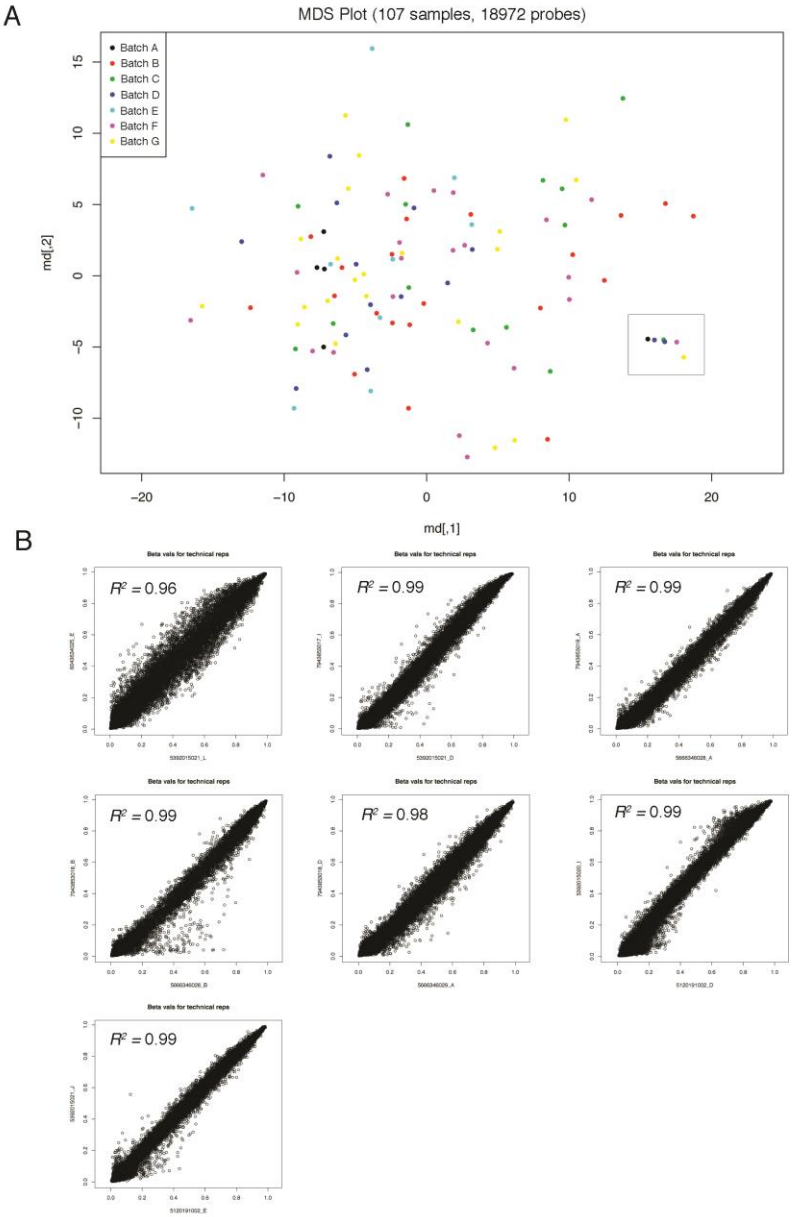
Aim 1: To profile genome-wide aberrant methylation of genes in pathological stages of bladder cancer.

We will examine DNA from 25 non-invasive pTa, 25 low grade minimally invasive pT1, 25 high grade pT1, 25 muscle invasive ≥pT2-T4 and 5 normal transitional cells from age-matched individuals with no bladder cancer for genome-wide methylation by the Infinium BeadChip.

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 above in Methods. Multi-dimensional 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 (*Figure 1A*). 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 (*Figure 1B*) 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. The 6 individuals from whom NU was sampled varied in age (range 28-78 years), which may account for some of the difference in methylation.

Figure 1. A) MDS analysis of Infinium HM27 data from different beadchips and dates of the 101 UC and 6 NU specimens. The batches (by color) appear intermingled. The box highlights that the 6 NU group together. B) Correlation plots and R^2 of the 7 pairs of technical replicates.



The Promoter Methylome of Urothelial Cancer

The difficulty in obtaining treatment-naïve tumors led to the inclusion of 31 low grade pTa, 6 high grade pTa, 15 low grade pT1, 12 high grade pT1, and 37 muscle-invasive (≥pT2-T4) tumor specimens. Some of the statistical comparisons were performed with slightly smaller cohorts of tumors since we excluded tumors with equivocal histopathology. In total, we analyzed 101 urothelial tumors broadly representative of the disease at presentation, with no prior immunotherapy, chemotherapy or radiotherapy and 6 normal urothelium. Seven technical replicates were run.

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.[2] In addition, we have found probes with $\beta<0.15$ to typically read as unmethylated (0%) by pyrosequencing of the identical CpG loci.[3] 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 than or equal to 0.2. This $\Delta\beta\geq0.2$ cut-off was again based on the Illumina report that a Δβ sensitivity of 0.2 could be detected with 95% confidence across more than 90% of probes.[2] A $\Delta\beta\geq0.2$ cut-off [4] or a $\Delta\beta\geq0.1$-0.3 (relaxed-stringent) cut-off range has also been used by TCGA.[5,6] An initial 980 probes had a significant Wilcoxon *p* value for hypermethylation after the 101 UC were compared to 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. 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.[7]

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.[8] Based on the cluster analysis in Figure 1 of Sanchez-Carbayo et al.,[8] 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 dataset.[8]

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 to 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 to the 6 NU. 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. 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 2*). *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[9] 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[10] and in endometrial cancer.[11] Consistent with this, *CIDEA* mRNA expression was

downregulated in UC compared to NU from the Sanchez-Carbayo et al. dataset.[8] 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.

Two genes were significantly more frequently hypermethylated in S UC compared with MI UC (*Table 2*). 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.[12] 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. [13]). *TET2* promoter methylation has been reported in low grade glioma without *IDH1* or *IDH2* mutation.[14] *TRPA1* mRNA expression was downregulated in UC compared to NU while *TET2* was not present in the expression array dataset used.[8]

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.[15] We used Fisher's exact test as before to compare 35 low grade (LG) vs. 54 high grade (HG) UC. 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* (*Table 2*). 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.[16] 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,[17] we examined non-invasive UC (37 pTa) vs. invasive UC (63 pT1-T4). 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 analyses (*Table 2*). 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, alpha 1*) involved in cell adhesion and extracellular matrix (ECM) remodeling, and the *ITPKB* gene described above.

Table 2. List of genes with significant differential methylation between MI and S, HG and LG, Invasive and non-invasive UC. 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.

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				+	
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					+

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 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). 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.[18,19] AJAP1 is known to interact with CDH1 and CTNNB1 in adherens junctions in epithelial cells[20] and with BSG/CD147 to regulate cellular invasion.[19] Aberrant hypermethylation of *AJAP1* associated with downregulation of mRNA expression has been reported in glioma.[21] *COL12A1* (*collagen, type XII, alpha 1*) as a structural constituent of the ECM is also implicated in cell adhesion.

Differential Methylation in pTa vs. Muscle-Invasive UC

This comparison identified 14 genes with significantly different methylation in MI UC. The 14 genes were all included in the list of the 47 genes that differed between superficial UC and MI UC from our earlier analysis. No genes were differentially methylated with statistical significance when pT1 UC were compared to MI UC.

Aim 2: To validate aberrant methylation by direct bisulfite sequencing of the genes identified. We will validate a prioritized set of genes. Genes that show the greatest difference in frequency of methylation between pathological stages will be prioritized, as will genes with a true CpG island in the promoter region as methylation of such genes is more likely to be of functional relevance. Genes that show down-regulation of expression in bladder tumor compared to normal cells in the Cancer Genome Anatomy Project (CGAP) Serial Analysis Gene Expression (SAGE) database (http//cgap.nci.nih.gov) will be prioritized. Known imprinted genes, X-chromosome genes, or genes with tissue specific methylation in normal cells should be largely absent from the prioritized set.

Verification of Infinium Methylation Score by Pyrosequencing

We selected genes for verification on the basis of greatest difference in frequency of methylation between pathological stages, presence of a true CpG island in the promoter region, down-regulation of expression in UC compared to NU and genes of known importance in cancer. Known imprinted genes, X-chromosome genes, or genes with tissue specific methylation in normal cells were largely absent from the prioritized set. We selected 7 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 bisulfite DNA pyrosequencing of *CIDEA*, *TRPA1*, *ITPKB*, *TET2*, *RASSF1A*, *ADHFE1* and *AJAP1*. Constraints of the sequence context on assay performance meant we could not analyse 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. Between 35-51 specimen DNAs (of the 101 UC and 6 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 (*Figure 3*). Some of the other genes e.g. *CIDEA* 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 (*Figure 4*). These particular specimen DNAs may have a single nucleotide polymorphism within the sequence homologous to the Infinium probe.[22]

Figure 3. 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.

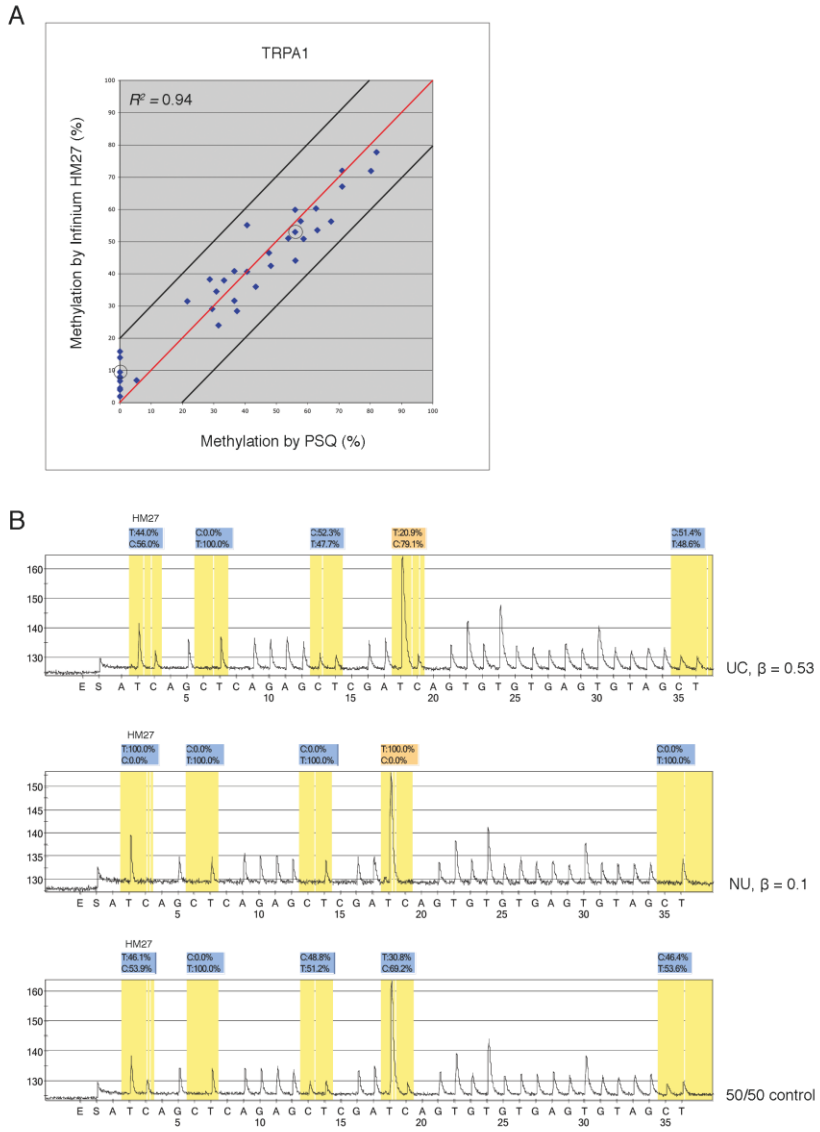
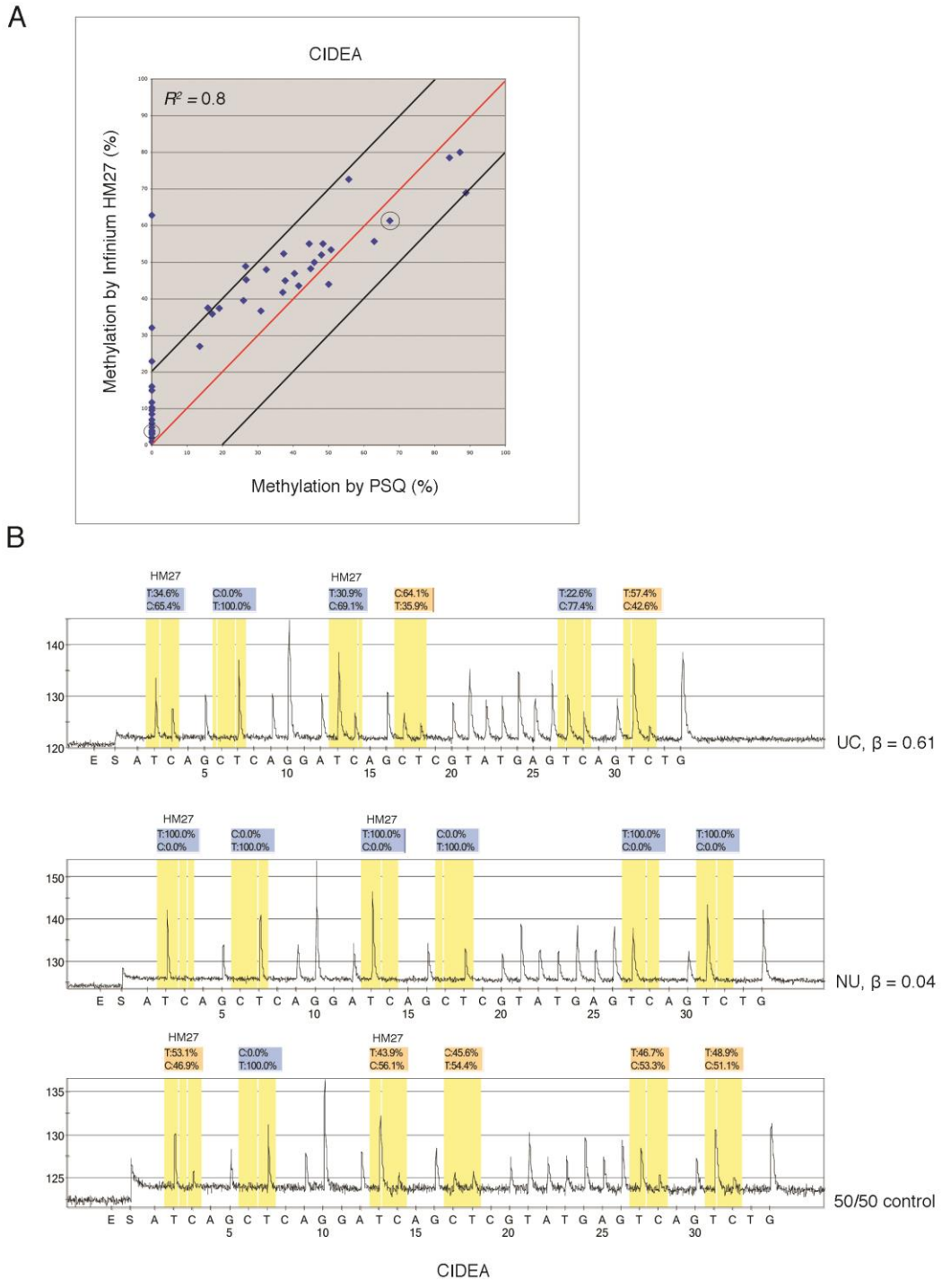


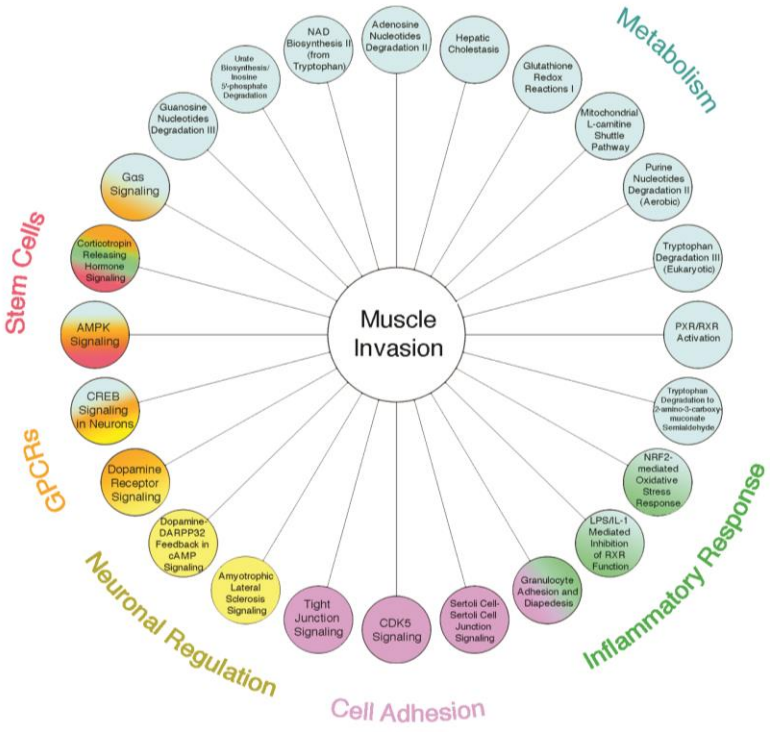
Figure 4. Verification of *CIDEA* Infinium HM27 β-values by pyrosequencing.
A) Correlation between Infinium methylation score and pyrosequencing of *CIDEA*. The R^2 is the Pearson coefficient. The two circled points correspond to the hypermethylated UC specimen and unmethylated NU specimen shown in the pyrogram below. B). Pyrograms of *CIDEA* 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.



Aim 3: To identify functional themes, pathways and networks in bladder tumor invasion and progression.
We will initially use Ingenuity Pathways Analysis (IPA) and the NIH Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/content.jsp?file=functional_annotation.html) for the identification of biological processes over networks of genes that can be masked at the level of the individual gene.

IPA analysis of the 40 genes significantly more frequently hypermethylated in MI UC found 24 canonical pathways to be significantly over-represented (IPA pathways with enrichment scores ≥1.3 equivalent to a non-log scale *p* value <0.05). The pathways are shown in *Figure 5* and may be broadly categorized as functional themes of cell adhesion, metabolism, inflammatory response, neuronal regulation, stem cells, G-protein coupled receptors and signal transduction including of the PI3K/AKT/mTOR pathway (*Figure 5*).

Figure 5. Pathways significantly overrepresented by aberrant gene methylation in MI compared to S UC.
IPA analysis of genes significantly more frequently hypermethylated in MI UC compared to S UC identified 24 canonical pathways that may be broadly grouped as shown.



IPA analysis of the genes significantly more frequently hypermethylated in HG UC found 22,

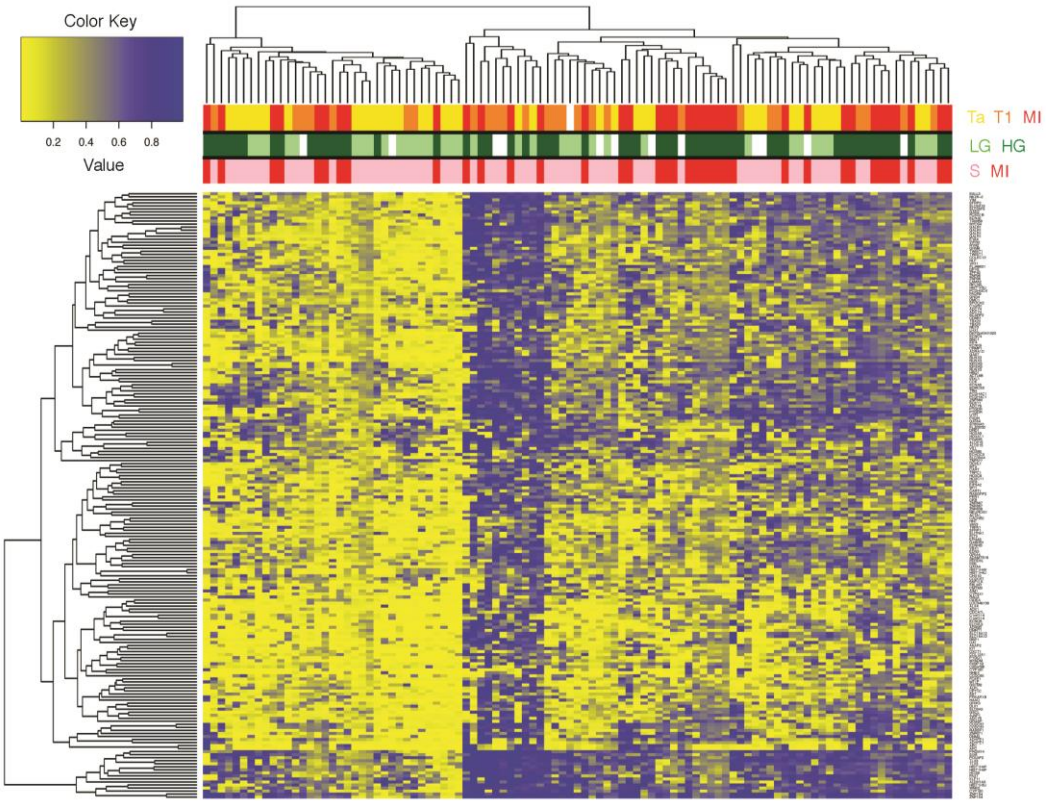
and in invasive UC 27 canonical pathways to be significantly over-represented. There was substantial overlap of the pathways between each analysis suggesting a signature of aggressiveness. Analysis by DAVID highlighted G-protein signaling coupled to cyclic nucleotide second messenger, cyclic-nucleotide-mediate signaling, intracellular signaling cascade and response to endogenous stimulus and also cellular homeostasis, response to wounding, and transcription.

In addition to the aims and objectives in the original research proposal, we also examined whether a CIMP-like subgroup exists in UC, methylation as an alternative to point mutation, and hypomethylation in UC.

A Subset of UC Have Widespread CpG Island Methylation

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*. *Figure 6* shows this set of tumors as a distinct cluster just left of center.

Figure 6. Unsupervised two-dimensional hierarchical clustering analysis of the 200 most differentially methylated promoter CpG island probes in UC unmethylated in NU.
One hundred and one 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).



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[23]. We examined the location and the β-value of the relevant probes on HM27. We confirmed hypermethylation of APC and *CDKN2A/p14ARF* in UC. We also found occasional (<5%) hypermethylation of *MLH1*, *MLL3* and *NF1* among the 101 UC that to our knowledge was not previously reported.

Hypomethylation in UC
We examined hypomethylation in UC by the Wilcoxon Rank sum test with inversion of the cut-offs used for hypermethylation. After filtering 163 probes/156 genes were hypomethylated (cf. 853 probes/713 genes hypermethylated). As expected, the majority of hypomethylated probes (68%) were located outside a CpG island and/or >1kb from the TSS. Two members of the GP40 family of G 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).

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. 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,[24] or temozolomide and radiation,[25] 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.[26] A further example is that ovarian tumors can develop secondary mutations in *BRCA1* or *BRCA2* after cisplatin[27,28] or PARP inhibition therapy.[29] 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.

Through MDS analysis, technical replicates, and verification by pyrosequencing as well as database analysis of an independent set of UC, we found the Infinium HM27 technology to perform well, consistent with results of our prior study[3] as well as studies by others.[2,5] HM27 has extensive but not full coverage of genes with promoter CpG islands. The present study comprises 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[30] and particular genes or sets of genes, identified in our study as hypermethylated, may have utility for molecular diagnosis. Extensive data from the epublication in February 2014 is freely available in Supplementary Materials to be mined by the community.[31] Shortly after, in March 2014, the Cancer Genome Atlas (TCGA) published a comprehensive analysis of urothelial cancer. However, TCGA examined muscle-invasive UC only and included UC treated with BcG. TCGA did not verify any genes identified as aberrantly hypermethylated.[32] Our data generated through support from the PA Department of Health included treatment-naïve superficial UC as well as verification of a subset of genes. Therefore, we believe that we have completed all aims and objectives and provided data that advances the field and is of use to the community.

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18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. A Global Profile of Gene Promoter Methylation in Treatment-Naïve Urothelial Cancer	Ilsiya Ibragimova, Essel Dulaimi, Michael J. Slifker, David Y. Chen, Robert G. Uzzo, and Paul Cairns	Epigenetics	January 2014	☐Submitted ☐Accepted ☒Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

After further pathway and network analysis and verification studies we plan to prepare additional publications.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of

work under this health research grant? Yes_____ No___X

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Cairns, Paul, Ph.D.	Associate Professor
eRA COMMONS USER NAME pcairns	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Aberdeen (United Kingdom)	B.Sc. Honors	07/86	Genetics
University of Birmingham (United Kingdom)	Ph.D.	08/93	Clinical Genetics

A. Personal Statement

The primary focus of my research program is the translation of basic knowledge of the epigenetics of cancer to improve the early detection, prognosis, and treatment of cancer through novel molecular tests. As a postdoctoral fellow at Johns Hopkins, I identified novel mutation in cancer genes and developed new methodology to do so. Translation of this expertise resulted in one of the first studies of cancer gene hypermethylation as a target for non-invasive detection of cancer in body fluids. I began an independent research career at FCCC in November 1998 that has resulted in a track record of research publication of pioneering studies of methylation-based detection of cancer and global epigenetic signatures of cancer cells. I have obtained peer-reviewed funding, trained graduate students and postdoctoral fellows for future success. I also have both shared and gained expertise within the community as an active member of the NIH Early Detection Research Network (EDRN), NIH SPORE program and NIH Process to Advance Translational Science (PATS), as well as through invited talks at AACR, ASCO and GRC and publication of position papers.

I therefore have a demonstrated record of productivity in the important field of cancer epigenetics with high relevance for translation to the clinic. It is my long term goal to make a substantial contribution to elucidating the basic science of urological cancer, ovarian and breast cancer and to apply the knowledge gained from my research, in collaboration with surgical and medical oncologists, for successful translation in ways that will be directly relevant to individuals at risk of, and patients with cancer.

B. Positions

Ph.D. Student, Department of Clinical Genetics, University of Birmingham, U.K.
Ph.D., "Cytogenetic and Molecular Genetic Analysis of Bladder Carcinoma" 1987-1990
Postdoctoral Fellow, Molecular Genetics Laboratory,
Marie Curie Research Institute, U.K. 1990-1993
Postdoctoral Fellow, Head & Neck Cancer Research, Johns Hopkins Univ.,
Baltimore, MD 1993-1998
Assistant Professor (Associate Member), Fox Chase Cancer Center,
Philadelphia, PA 1998-2005
Associate Professor (Member), Fox Chase Cancer Center, Philadelphia, PA 2005-present

C. Selected Peer-reviewed Publications (from more than 70 articles)

1. Cairns, P., Mao, L., Merlo, A., Lee, D., Schwab, D., Eby, Y., Tokino, K., van der Riet, P., Blaugrund, J., Sidransky, D. Rates of p16 (MTS1) mutations in primary tumors with 9p loss. Science 265:415-6, 1994.

2. Cairns, P., Polascik, T.J., Eby, Y., Tokino, K., Califano, J., Merlo, A., Mao, L., Herath, J., Jenkins, R., Westra, W., Rutter, J.L., Buckler, A., Gabrielson, E., Tockman, M., Cho, K.R., Hedrick, L., Bova, G.S., Issacs, W., Schwab, D., Sidransky, D. Frequency of homozygous deletion at p16/CDKN2 in primary human tumors. Nature Genetics 11:210-212, 1995.
3. Cairns, P., Esteller, M., Herman, J.G., Schoenberg, M., Jeronimo, C., Sanchez-Cespedes, M., chow, N-H., Grasso, M., Wu, L., Westra, W.B., Sidransky, D. Molecular detection of prostate cancer in urine by GSTP1 hypermethylation. Clin. Cancer Res. 7:2727-2730, 2001.
4. Battagli, C., Uzzo, R.G., Dulaimi, E., Ibáñez de Cáceres, I., Krassenstein, R., Al-Saleem, T., Greenberg, R.E., Cairns, P. Promoter hypermethylation of tumor suppressor genes in urine from kidney cancer patients. Cancer Res. 63:8695-8699, 2003.
5. Dulaimi, E., Uzzo, R.G., Greenberg, R.E., Al-Saleem, T., Cairns, P. Detection of bladder cancer in urine by a tumor suppressor gene hypermethylation panel. Clin. Cancer Res. 10:1887-1893, 2004.
6. Dulaimi, E., Ibáñez de Cáceres, I., Uzzo, R.G., Al-Saleem, T., Greenberg, R.E., Polascik, T., Babb, J.S., Grizzle, W.E., Cairns, P. Promoter hypermethylation profile of kidney cancer. Clin. Cancer Res. 10:3972-79, 2004.
7. Ibanez de Caceres, I., Dulaimi, E., Hoffman, A.M., Al-Saleem, T., Uzzo, R.G., Cairns, P. Identification of novel target genes by an epigenetic reactivation screen of renal cancer. Cancer Res. 66:5021-28, 2006.
8. Kagan, J., Srivastava, S., Belinsky, S.A., Cairns, P. Towards clinical application of methylated DNA sequences as cancer biomarkers: a joint NCI's EDRN & NIST workshop on standards, methods, assays, reagents and tools (SMART). Cancer Res. 67:4545-4549, 2007.
9. Cairns P. Gene methylation and early detection of genitourinary cancer: the road ahead. Nat. Rev. Cancer 7:531-543, 2007.
10. Potapova, A., Hoffman, A.M., Godwin, A.K., Al-Saleem, T., Cairns, P. Promoter hypermethylation of the PALB2 susceptibility gene in inherited and sporadic breast and ovarian cancer. Cancer Res. 68:998-1002, 2008.
11. Ibragimova, I., Ibáñez de Cáceres, I., Hoffman, A.M., Potapova, A., Dulaimi, E., Al-Saleem, T., Hudes, G.R., Ochs, M.F., Cairns, P. Global reactivation of epigenetically silenced genes in prostate cancer. Cancer Prev. Res. 3:1084-1092, 2010.
12. Hoffman, A.M., Cairns, P. Epigenetics of kidney cancer and bladder cancer. Epigenomics 3:19-34, 2011.
13. Ibragimova, I., Maradeo, M.E., Dulaimi, E., Cairns, P. Aberrant promoter hypermethylation of PBRM1, BAP1, SETD2, KDM6A and other chromatin-modifying genes is absent or rare in clear cell RCC. Epigenetics 8:486-493, 2013.
14. Ibragimova, I., Slifker, M.J., Maradeo, M.E., Banumathy, G., Dulaimi, E., Uzzo, R.G., Cairns, P. Genome-wide promoter methylome of small renal masses. PLoS One 8:e77309, 2013.
15. Gowrishankar, B., Ibragimova, I., Zhou, Y., Slifker, M.J., Devarajan, K., Al-Saleem, T., Uzzo, R.G., Cairns, P. MicroRNA expression signatures of stage, grade, and progression in clear cell RCC. Cancer Biol. Ther. 15:329-341, 2014.
16. Ibragimova, I., Dulaimi, E., Slifker, M.J., Chen, D.Y., Uzzo, R.G., Cairns, P. A global profile of gene promoter methylation in treatment-naïve urothelial cancer. Epigenetics 9:760-73, 2014.