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Original Article

Analysis of bladder cancer tumor CpG methylation and gene expression within The Cancer Genome Atlas identifies *GRIA1* as a prognostic biomarker for basal-like bladder cancer

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Abstract: Increased methylation levels at cytosines proximal to guanines (CpG) in the promoter regions of tumor suppressor genes have been reported to play an important role in the development and progression of bladder cancer. In this study, we conducted a genome-wide analysis using data from The Cancer Genome Atlas to better characterize CpG methylation and mRNA expression patterns in urothelial carcinomas and to identify new epigenetic biomarkers of survival. Across 408 tumors, we identified 223 genes that displayed significant relationships between CpG methylation and mRNA expression levels. Hypermethylation within 200 base pairs upstream of the transcription start site and hypomethylation within the 3' untranslated region and body region were associated with gene silencing. These 223 genes were functionally enriched for their role in glutamate receptor signaling and among them was a novel, tumor-stage-independent epigenetic biomarker of overall mortality, *GRIA1*. *GRIA1* hypermethylation and elevated mRNA expression levels were associated with significantly worse survival outcomes in patients with basal-like urothelial carcinomas. Furthermore, 70 genes associated with glutamate receptor signaling were differentially expressed between basal (n = 203 tumors) and luminal (n = 205 tumors) subtypes of bladder cancer, including genes involved in glutamate receptor-mediated activation of the calmodulin, PI3K/Akt, and EGFR signaling pathways. The majority of genes displayed increased expression levels in basal-like subtypes. This research highlights glutamate receptors as targets for investigation in the development and pharmacological treatment of urothelial cancer.

Keywords: Bladder cancer, CpG methylation, epigenetics, glutamate receptors, The Cancer Genome Atlas

Introduction

Urothelial bladder carcinoma is a highly prevalent cancer in the United States, and its incidence is on the rise [1]. As the public health burden from this cancer increases, better understanding of the biological mechanisms underlying its etiology is critical [2]. Mechanistic studies have identified several biological pathways that may be targeted in the development of these cancers, and genetic analyses have identified somatic mutations in multiple genes that are associated with bladder cancer tumors [2, 3]. Research has also implicated that epigenetic mechanisms play a key role in bladder carcinogenesis, and chromatin-modifying genes are frequently mutated in bladder cancer [2, 4].

Methylation of cytosines proximal to guanines (CpG) is an epigenetic mechanism that is known to be involved in carcinogenesis [5]. Large clusters of CpG sites, CpG islands, are often found in the promoter regions of genes [6]. In tumors, global hypomethylation has been implicated in a loss of cell cycle control and other cellular processes, thus leading to malignant growth [2]. In some instances, increases in CpG methylation in promoter regions of genes have been associated with gene silencing [5]. Numerous studies have identified genes that display promoter hypermethylation in bladder cancer (reviewed

in [7]), although few studies report corresponding gene expression levels, and recent research has implicated that methylation in other regions of the gene may play an important role in epigenetic regulation [5, 6, 8].

In this study, we aimed to better characterize the epigenomic profiles of CpG methylation in urothelial bladder tumors across all intragene regions and to identify functional epigenetic biomarkers of bladder cancer. Therefore, methylation and mRNA expression data sets from The Cancer Genome Atlas (TCGA) were analyzed to identify genes that are both differentially expressed and methylated in bladder cancer and that display a significant relationship between CpG methylation and mRNA expression levels.

Materials and methods

Data acquisition

All available data files from Infinium HumanMethylation 450 BeadChip arrays (n = 440) were downloaded from the TCGA and were read into SAS V9.3 [9]. Data were merged on probe identifier, and, for quality control purposes, probes where approximately 1% of the data were missing were removed. Data were log-transformed and probes corresponding to single nucleotide polymorphism (SNPs) were removed [10]. The final data set consisted of n = 412 methylation arrays of genome-wide DNA methylation for tumor tissue and n = 21 methylation arrays of genome-wide DNA methylation for non-tumor tissue, each containing 332,950 genomic sites.

All available RNASeqV2 normalized count data files (n = 427), each containing data across 20,531 genes, were downloaded from the TCGA. These data were normalized using the RSEM method by the TCGA [11]. Data were then imported into SAS V9.3 and normalized count values were summarized by gene. There were 408 data files that corresponded to tumor samples and 19 data files that corresponded to non-tumor samples. Gene expression levels across all samples for each gene were log-transformed, as in prior publications from the TCGA [12].

All available clinical data files (n = 417), each containing 54 variables from bladder cancer tumors were downloaded from the TCGA. These included demographic factors, including subject sex (male vs. female), age at initial diagnosis (continuous variable), tumor pathologic subtype (papillary vs. non-papillary), smoking status (ever vs. never), and race (white vs. non-white), and clinical data, such as the American Joint Committee on Cancer (AJCC) tumor pathologic stage and days until death. Of the 408 subjects that had both CpG methylation and RNASeq data available for tumor samples, 381 also had clinical data files.

Identifying differentially expressed genes (DEGs) between urothelial tumor and non-tumor tissue

All possible subjects were identified that had RNASeq data available for matched tumor and non-tumor tissue (n = 19). Individual fold changes (FC) were calculated using the log-transformed values in the formula FC = RNASeq value (tumor)/RNASeq value (non-tumor) for each subject across all genes available for analysis and the median FC per gene was calculated across subjects. In addition, ANCOVA of the log-transformed RNASeq values across the 20,531 genes was conducted contrasting on tumor vs. non-tumor tissue, controlling for sex, age at initial diagnosis, pathologic subtype, smoking status, and race. FDR (false discovery rate) q-values were computed. DEGs were defined by the following criteria: (i) FDR q-value < 0.05 and (ii) the median absolute FC for the gene between tumor and non-tumor tissue across all matched subjects was $\geq |2.0|$.

Identifying differentially methylated genes (DMGs) between urothelial tumor and non-tumor tissue

Subjects were identified that had DNA methylation data available for matched tumor and non-tumor tissue (n = 21). ANCOVA analysis of the DNA methylation beta-values across all CpG sites was conducted contrasting on tumor vs. non-tumor tissue controlling for sex, age at initial diagnosis, pathologic subtype, smoking status, and race. FDR q-values were computed. The Infinium HumanMethylation 450 BeadChip array annotates each probe to a gene and to one of six intragene sites- (i) from 200-1500 base pairs upstream of the gene transcription start site (TSS1500), (ii) within 200 base pairs upstream of the gene transcription start site

(TSS200), (iii) in the 5' untranslated region of the gene (5' UTR), (iv) in the first exon of the gene (1st Exon), (v) in the body of the gene (Body), and (vi) in the 3' untranslated region of the gene (3' UTR) [13]. Individual beta-differences were calculated using the formula beta-difference = beta-value (tumor) - beta-value (non-tumor) for each subject across all CpG sites available for analysis and the median beta difference per gene was calculated across subjects across all CpG sites associated with a gene and within each of the six intragene sites. DMGs were defined by the following criteria: (i) at least one probe associated with the gene had a FDR q-value < 0.05 and (ii) the median beta difference across all CpG sites associated with a gene or at least one of the intragene sites between tumor and non-tumor tissue across all matched subjects was ≥ |0.10|, representing a 10% difference in methylation, as this methylation difference threshold resulted in approximately a 95% true positive rate using the beta-value method of detecting differences in CpG methylation values [14]. Permutation testing using R was used to test if the number of genes hypermethylated versus hypomethylated in the promoter regions (TSS1500 and TSS200) in tumor versus non-tumor tissue differed from a pure 0.5 probability of methylation directionality.

Identifying genes demonstrating a significant relationship between CpG methylation and mRNA expression in urothelial tumor tissue

In order to identify genes whose expression was associated with methylation levels in tumors, Spearman rank correlations were run on all genes that were both differentially methylated and expressed between tumor and non-tumor tissue. Individual median methylation values for all CpG sites associated with a gene and for the six intragene regions were calculated for all individuals who had both methylation and RNASeq tumor data available (n = 408). In separate analyses, these methylation values were log-transformed and then tested via Spearman rank analysis with the individuals' log-transformed RNASeq values. A significant relationship between CpG methylation and mRNA expression was defined as a Spearman rank correlation *p*-value < 0.05. In order to integrate these three analyses, we determined that genes that met the following criteria could serve as potential epigenetic biomarkers of bladder carcinogenesis: (i) differentially expressed between matched tumor and non-tumor tissue, (ii) differentially methylated between matched tumor and non-tumor tissue, and (iii) displayed a significant association between CpG methylation and mRNA expression in tumor tissue.

Network analysis of potential epigenetic biomarkers of urothelial cancer

In order to examine the higher-level biological processes related to the genes identified as both differentially methylated and expressed between bladder cancer tumor and non-tumor tissue and with a significant relationship between CpG methylation and gene expression in tumor tissue, we analyzed these genes in Ingenuity Network Analysis (IPA) (Ingenuity Systems®, Redwood City, CA, USA). Canonical pathways were identified as enriched using the right-tailed Fisher's Exact test, where significance was set at *p*-value < 0.001 [15].

Assessment of prognostic potential of epigenetic biomarkers in urothelial tumors

ANCOVA was used as an initial screen to identify specific genes among the 223 that were differentially methylated and expressed among survivors and non-survivors of bladder cancer. ANCOVA of the log-transformed RNASeq values across the 223 genes and of the log-transformed median CpG methylation beta-values of all differentially methylated regions (DMRs) associated with gene expression was performed, contrasting on survival status in the 408 bladder cancer tumors, controlling for sex, age at initial diagnosis, pathologic subtype, smoking status, and race. Genes that were differentially expressed and methylated between survivors and non-survivors at an alpha level of 0.10 were further analyzed using Kaplan-Meier analysis with log-rank statistics. For genes significant in Kaplan-Meier analysis, a Cox regression model was run, with AJCC Pathologic Tumor Stage incorporated as a co-predictor of survival. For both analyses, subjects were stratified into two groups of (i) mean - SD and (ii) mean + SD based on CpG methylation or gene expression level and significance was defined as *p*-value < 0.05.

Validation of a prognostic epigenetic biomarker in an independent cohort

In order to validate prognostic indicators of overall mortality in urothelial bladder tumors,

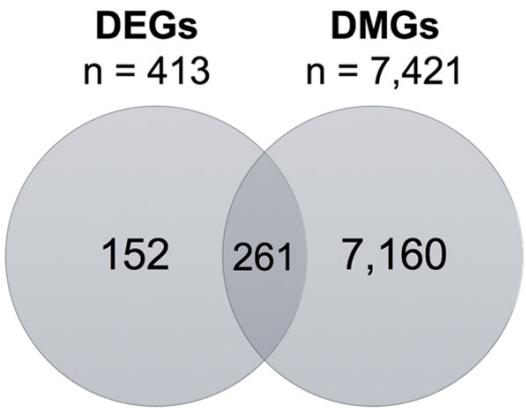

Figure 1. Venn Diagram of the overlapping DEGs and DMGs between matched tumor and non-tumor tissues (n = 38 and n = 42, respectively) in the TCGA.

expression levels originally reported in Choi et al. 2014 were obtained from the Gene Expression Omnibus (GSE48277) (n = 146) [16]. As the sample size of this cohort was approximately one-third the size of the TCGA cohort, individuals were stratified into two groups of mean ± [SD]/2, in order to have a sufficient number of individuals within each stratification group. Kaplan-Meier analysis with log-rank statistics was used to test for differences in survival. A Cox regression model was used to test if observed differences were independent of AJCC Pathologic Tumor Stage. For both analyses, significance was defined as a *p*-value < 0.05.

Analysis of the prognostic potential of an epigenetic biomarker in basal and luminal subtypes of urothelial tumors

In order to elucidate potential biological roles of the identified epigenetic prognostic biomarker, we tested if this gene had significantly different expression and methylation levels in basal- versus luminal subtypes of bladder cancer. Methodology of tumor subtyping is described in detail elsewhere [17]. ANCOVA of the log-transformed RNASeq values and of the log-transformed median CpG methylation beta-values of the significant prognostic intragene region was performed, contrasting on basal (n = 203) versus luminal (n = 205) subtype, controlling for sex, age at initial diagnosis, smoking status, and race. Tumor pathologic subtype and tumor stage were not controlled for in this analysis as it is likely correlated with basal and luminal subtype classification. The median FC and median beta difference of the log-transformed RNASeq values of the gene and of the log-transformed median CpG methylation beta-values of the significant prognostic intragene region were also calculated between basal and luminal subtypes of urothelial cancers. Then, the prognostic power of mRNA expression and CpG methylation levels of the identified gene was assessed separately in basal and luminal subtypes using Kaplan-Meier analysis with log-rank statistics independently among the basal and luminal subtypes. Cox regression models were used to test if observed differences were independent of AJCC Pathologic Tumor Stage. For all analyses, subjects were stratified into two groups of (i) mean - SD and (ii) mean + SD based on CpG methylation or gene expression level, and significance was defined as *p*-value < 0.05.

Assessment of DEGs between basal and luminal subtypes of urothelial cancer

To assess whether there is a difference in glutamate receptor signaling in basal-like versus luminal-like bladder cancers, we tested for differential expression between basal and luminal subtypes of bladder cancer in a total of n = 1,776 genes associated with glutamate receptor signaling. ANCOVA of log-transformed RNASeq values was performed, contrasting on basal versus luminal subtype in the 408 bladder cancer tumors, controlling for sex, age at initial diagnosis, smoking status, and race. Tumor pathologic subtype was not controlled for in this analysis as it is likely correlated with basal and luminal subtype classification. FDR q-values were generated to control for multiple tests. Significance for DEGs was defined as a FDR q-value < 0.05 and a median FC (basal/luminal) ≥ |2.00|.

Results

Gene expression and CpG methylation differences between urothelial tumor and non-tumor tissue

A total of 413 DEGs were identified between matched tumor and non-tumor tissue samples (n = 38) (**Figure 1**). The majority of genes (261/413 = 63.2%) displayed decreased expression in tumor tissue versus non-tumor tissue, while 152/413 (36.8%) displayed increased expression in tumor versus non-tumor tissue.

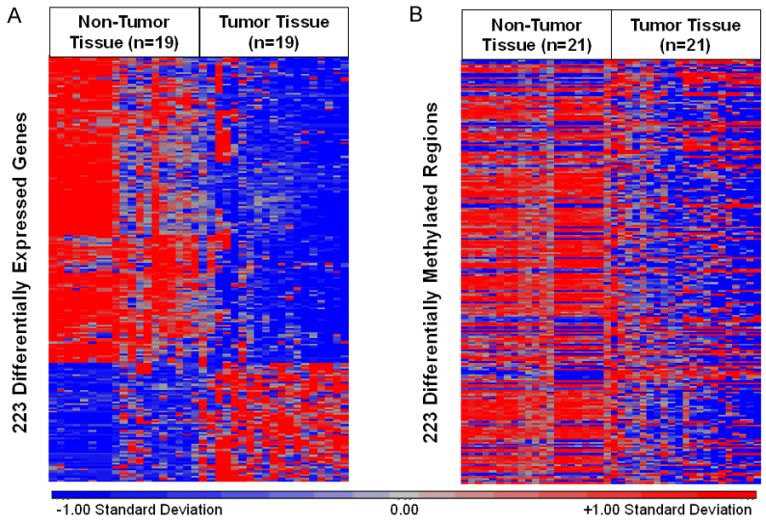

Figure 2. A. A total of 223 DEGs in n = 19 matched controls and cases. Red indicates relatively higher expression. Blue indicates relatively lower expression. B. A total of 223 DMRs with strongest correlation to gene expression in n = 21 matched non-tumor and tumor tissues. Red indicates relatively higher levels of methylation. Blue indicates relatively lower levels of methylation.

A total of 7,421 genes were significantly differentially methylated between tumor and non-tumor tissue between matched tumor and non-tumor tissue samples (n = 42) (**Figure 1**). This high number of DMGs observed between tumor and non-tumor tissue in this analysis has also been described in other types of cancer [18, 19]. Of these 7,421 genes, 2,666 (35.9%) had a median beta difference across all CpG probes ≥ |0.10|, representing a 10% increase or decrease in methylation in the entire gene.

In further analyses of these data by intragene region, the TSS1500 and TSS200 regions, which are predicted to contain gene promoters, comprised 3,081 (41.5%) and 2,054 (27.7%) DMGs, respectively. Furthermore, 631 (20.5%) genes in the TSS1500 region and 696 (33.9%) genes in the TSS200 regions were hypermethylated. Permutation testing revealed that this was significantly fewer hypermethylated genes than would be expected to result from chance alone in both the TSS1500 and TSS200 regions (p-values < 0.0001), a surprising result as promoter CpG hypermethylation is often discussed in the context of bladder carcinogenesis [7, 20, 21]. Some known tumor suppressor genes were hypermethylated in their promoter regions in tumor versus non-tumor tissue, including *DBC1*, *PAX6*, *RUNX3*, and *WT1*, although these methylation changes were not associated with decreases in gene expression. Others, such as *BRCA1*, *PTEN*, *TP53*, and *RB1* were not present among the DMGs. There were 261 genes that overlapped between the lists of DMGs and DEGs (**Figure 1**).

CpG methylation is associated with mRNA expression in urothelial tumor tissue

An association measure was calculated between CpG methylation and mRNA expression for the 261 DMGs and DEGs to assess whether CpG methylation in tumor tissue had a functional effect on mRNA expression. Of the 261 overlapping DMGs and DEGs, 223 displayed a significant relationship between DNA methylation and gene expression. The majority of these genes (n = 161, 72%) were decreased in expression in tumor tissue versus non-tumor tissue (**Figure 2A**). Interestingly, only 69 genes (31%) displayed significant promoter-associated hypermethylation. In addition, 160 (72%) DMRs that displayed the strongest correlation with gene expression for each gene in tumor tissue demonstrated a loss of methylation in tumor versus non-tumor tissue. An inverse relationship between mRNA expression levels and CpG methylation levels was not observed among these samples (**Figure 2B**).

Further analysis of these patterns by intragene locality revealed several interesting findings. First, a consistent trend in gene suppression via promoter hypermethylation was observed in the TSS200 region, but not in the TSS1500 region. These results suggest that proximal promoter hypermethylation (e.g. TSS200) may have a greater role in cancer-associated gene silencing than hypermethylation at more distal nucleotides (e.g. TSS1500). Second, the majority of hypomethylated DMRs in the TSS1500, 5' UTR, gene body, and 3' UTR regions displayed gene activation (**Figure 3**). These results support that intragene location of methylation is a critical determinant of gene expression.

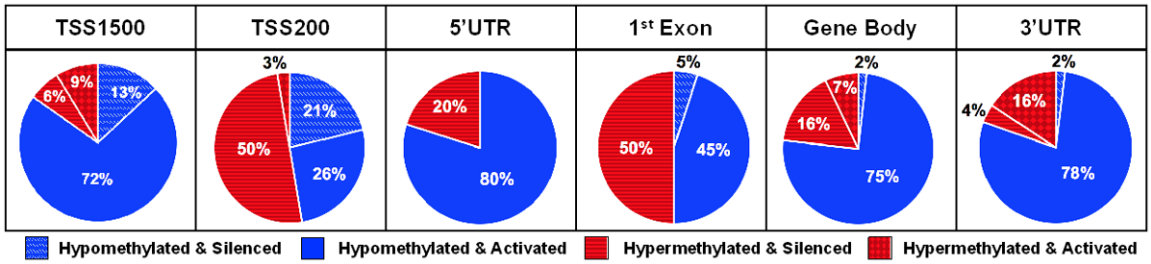

Figure 3. Intragene regional distribution of hypo- and hypermethylation of DMRs and DEGs and directionality of CpG methylation and mRNA expression correlation among TCGA urothelial tumors (n = 408).

Table 1. Canonical pathways enriched among N = 223 DMGs and DEGs

Canonical Pathways	*P*-Value	Associated Genes
Glutamate Receptor Signaling	1.29e-6	*CALML5, GRIA1, GRIK3, GRIN2A, GRM4, GRM7, SLC1A6*
Transcriptional Regulatory Network in Embryonic Stem Cells	2.29e-6	*CDX2, FOXD3, ISL1, LHX5, OTX1, SIX3*
cAMP-Mediated Signaling	1.12e-5	*ADRB3, CALML5, CHRM2, CNGA3, GPR17, GRM4, GRM7, HTR1B, PDE1C, SLC1A6, TULP2, VIPR2*
G-Protein Coupled Receptor Signaling	3.24e-4	*ADRA1D, ADRB3, CHRM2, GPR17, GRM4, GRM7, HTR1B, PDE1C, TULP2, VIPR2*

Significance was defined as a right-tailed Fisher's Exact test *p*-value < 0.001.

Genes epigenetically dysregulated in urothelial tumors are associated with glutamate receptor signaling

In order to examine the function of these 223 genes, they were analyzed for enriched canonical pathways (**Table 1**). The most significantly enriched canonical pathway was glutamate receptor signaling. The seven genes identified in this pathway included *CALML5*, *GRIA1*, *GRIK3*, *GRIN2A*, *GRM4*, *GRM7*, and *SLC1A6*. Three genes, namely *CALML5*, *GRM4,* and *SLC1A6*, displayed increased expression levels in tumor tissue. *GRIA1, GRIK3, GRIN2A,* and *GRM7* displayed decreased expression levels in tumor tissue.

GRIA1 is prognostic indicator of overall survival independent of tumor stage

To further explore the functional significance of the 223 DMGs and DEGs, all genes were analyzed for prognostic significance of overall patient survival and potential targets were validated in an independent cohort of urothelial tumors (n = 146) [16]. Analysis of all 223 genes revealed one gene whose CpG methylation and mRNA expression levels displayed significant relationships to overall survival independent of tumor stage. Specifically, it was found that increased mRNA expression levels of *GRIA1* and increased CpG methylation in the TSS1500 region were significantly associated with overall mortality in bladder cancer tumors (**Figure 4A** and **4B**). These findings remained significant when tumor stage was included as a co-predictor of mortality (Wald chi-squared *p*-values = 1.34e-3; 1.05e-2, respectively). Tumor grade was not assessed as a co-predictor as all tumors were high-grade urothelial bladder tumors. These findings are supported by the significant positive correlations observed among tumor tissues between *GRIA1* TSS1500 CpG methylation and mRNA expression values among tumors. However, *GRIA1* was found to be both hypomethylated in the TSS1500 region and decreased in expression in tumor versus non-tumor tissue (TSS1500 Median Beta Difference = -0.24; RNASeq FC = -2.51). *GRIA1* TSS1500 methylation levels were significantly correlated with *GRIA1* expression levels, suggesting that hypermethylation of the TSS1500 region may activate *GRIA1* mRNA expression (**Figure 5**). To note, a fraction of the samples displayed low-level expression of *GRIA1.* These findings underscore the complex relationship between CpG methylation and mRNA expression patterns in tumor tissue. Both *GRIA1* TSS1500 methylation levels and *GRIA1* mRNA levels are novel biological endpoints associated with mortality in bladder cancer patients.

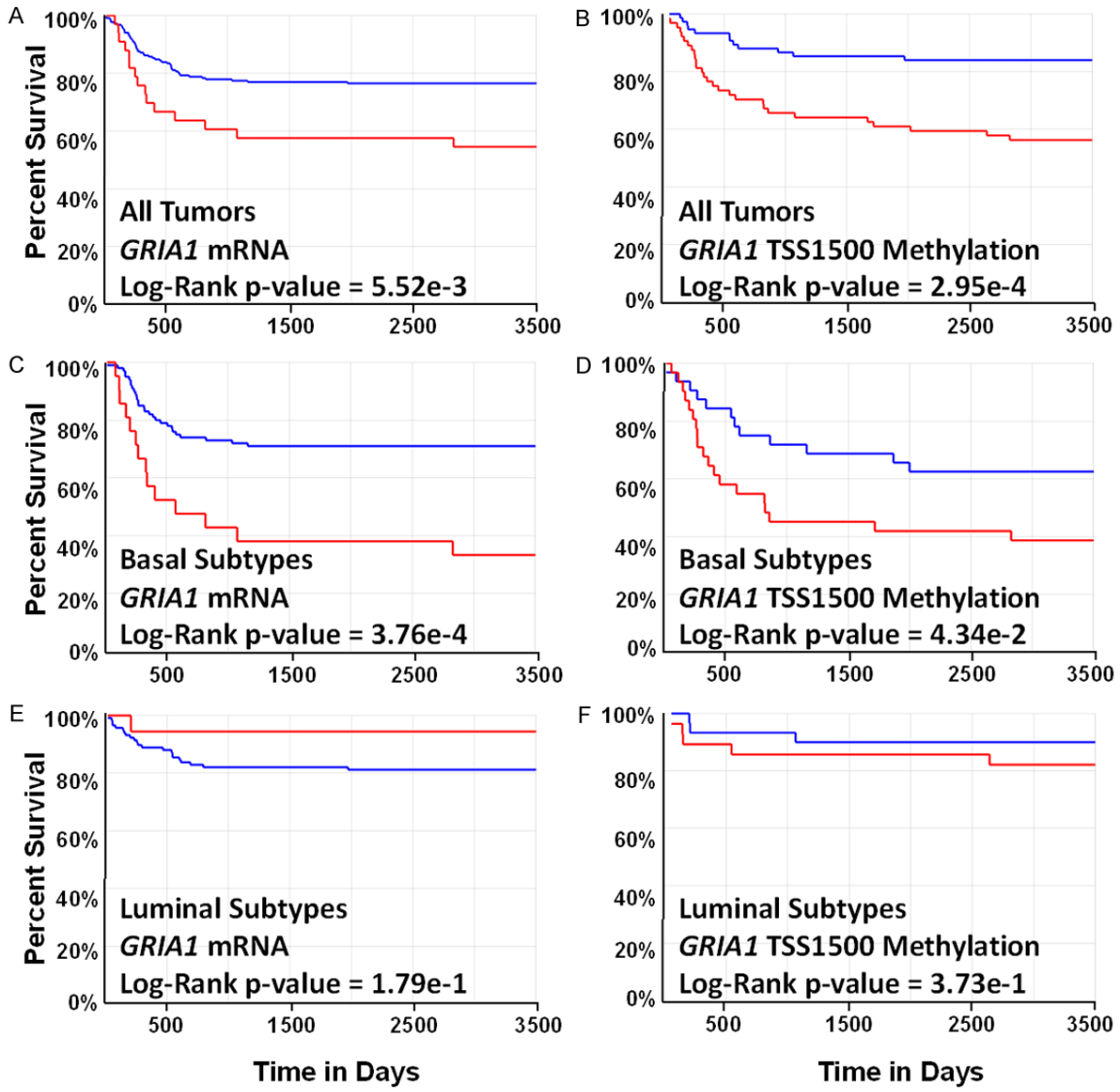

Figure 4. Differences in TCGA patient survival associated with *GRIA1* mRNA expression and TSS1500 CpG methylation levels. In all plots, blue represents low levels of expression or methylation and red represents high levels of expression or methylation. A. Kaplan-Meier plot of overall survival in subjects with low versus high *GRIA1* mRNA expression. B. Kaplan-Meier plot of overall survival in subjects with low versus high *GRIA1* TSS1500 methylation levels. C. Kaplan-Meier plot of overall survival in subjects with basal-like bladder cancer with low versus high *GRIA1* mRNA expression. D. Kaplan-Meier plot of overall survival in subjects with basal-like bladder cancer with low versus high *GRIA1* TSS1500 methylation levels. E. Kaplan-Meier plot of overall survival in subjects with luminal-like bladder cancer with low versus high *GRIA1* mRNA expression. F. Kaplan-Meier plot of overall survival in subjects with luminal-like bladder cancer with low versus high *GRIA1* TSS1500 methylation levels.

Validation of GRIA1 mRNA levels as a prognostic indicator of overall survival independent of tumor stage

The prognostic significance of increased *GRIA1* mRNA expression levels in predicting overall survival of patients with bladder cancer tumors was confirmed using data from an independent cohort of 146 tumors published in a previous study by Choi et al. 2014 (**Figure 6**) [16]. In a Cox regression analysis, the association was also confirmed to be independent of tumor stage (*p*-value = 2.98e-2).

GRIA1 prognostic power is specific to basal-like urothelial cancers

As two distinct molecular subtypes of urothelial bladder cancers have been classified, we sought to examine whether *GRIA1* was a sub-

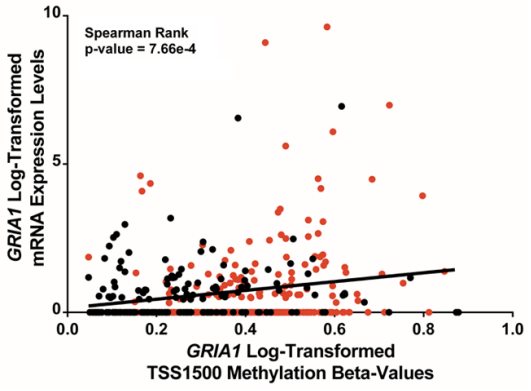

Figure 5. *GRIA1* mRNA expression versus *GRIA1* TSS1500 methylation. Log-transformed *GRIA1* RNA-Seq mRNA expression values are plotted against log-transformed *GRIA1* CpG methylation beta-values. Red points indicate values from basal-like subtypes and black points indicate values from luminal-like subtypes.

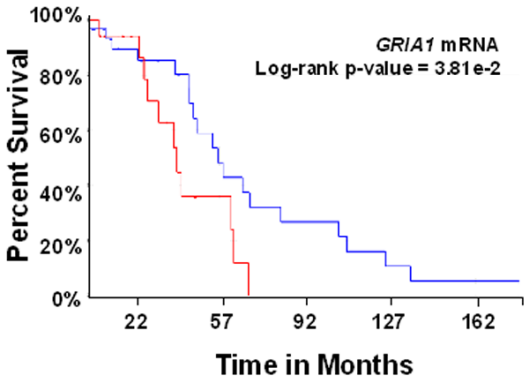

Figure 6. Kaplan-Meier plot of overall survival in subjects with low (blue) versus red (high) *GRIA1* mRNA expression in the MD Anderson Cancer Center discovery and validation cohorts.

type-specific prognostic biomarker [16]. *GRIA1* mRNA expression levels were found to be higher in basal versus luminal subtype bladder cancers (FDR q-value = 2.66e-3, FC = 1.80). CpG methylation levels in the TSS1500 region of *GRIA1* were also significantly greater in basal-like subtypes (FDR q-value = 2.61e-14, median beta difference (basal-luminal) = 0.17). Stratified Kaplan-Meier analyses between basal and luminal subtypes of urothelial tumors revealed that the prognostic power of *GRIA1* is significant in basal subtypes of bladder cancer, but not in luminal subtypes. Specifically, increased mRNA expression levels of *GRIA1* and increased CpG methylation in the TSS1500 region remained significantly associated with overall mortality in basal-like bladder cancer tumors (**Figure 4C** and **4D**), while neither expression levels or TSS1500 methylation levels were significant among luminal-like bladder cancers (**Figure 4E** and **4F**). In addition, higher transcript levels of *GRIA1* and increased CpG methylation in the TSS1500 region remained significantly associated with overall mortality in basal-like bladder independent of tumor stage (Wald chi-squared *p*-values = 6.30e-5; 1.86e-2, respectively). These results potentially suggest a unique role for dysregulation of glutamate receptor signaling in basal-like bladder cancers.

Genes involved in glutamate receptor signaling are differentially expressed between basal and luminal subtypes of urothelial cancer

We identified 70 DEGs associated with glutamate receptor signaling between basal and luminal molecular subtypes of bladder cancer (**Table 2**). Of these, 42 (60%) displayed increased levels and 28 (40%) genes displayed decreased levels of mRNA in basal-like subtypes. Among these 70 genes was one gene encoding for another glutamate ionotropic receptor, *GRIA2*, and two genes encoding for metabotropic glutamate receptors, namely *GRM3* and *GRM5*. *GRIA2* and *GRM5* displayed increased levels of mRNA expression in basal-like subtypes (FDR q-values = 2.17e-3; 2.36e-11, FC = 35.40; 2.11, respectively), while *GRM3* displayed decreased levels of mRNA expression in basal-like subtypes (FDR q-value = 8.00e-19, FC = -2.50). Additionally, the DEGs that displayed increased expression levels in basal-like subtypes included two genes encoding downstream effector proteins of glutamate receptors. Specifically, these were *CAMK2A* and *PIK3C2G* (FDR q-values = 9.86e-20; 4.46E-6, FC = 2.49; 66.02, respectively) [22]. Finally, two regulators of metabotropic glutamate receptor signaling were also among the 70 DEGs. *GDNF* displayed increased expression levels in basal-like subtypes (FDR q-value = 4.17e-7, FC = 36.14), and *F2* displayed decreased expression levels in basal-like subtypes (FDR q-value = 4.45e-5, FC = -60.69) [23, 24]. These results provide further evidence of differential activity of glutamate receptors in basal and luminal subtypes of urothelial cancer.

Table 2. Genes associated with glutamate receptor signaling differentially expressed in basal-like and luminal-like subtypes of bladder cancer

Gene	ANCOVA FDR q-value	Median FC (Basal/Luminal)
ASTL	2.65E-10	-2.07
CACNA2D1	3.18E-11	2.06
CALB2	3.75E-17	2.09
CAMK2A	9.86E-20	2.49
CASP14	1.31E-08	2.43
CASP5	1.18E-21	3.64
CBLN4	6.88E-04	49.23
CEACAM3	2.14E-14	2.21
CHGA	3.17E-04	2.17
CR2	1.45E-04	2.16
CRH	1.58E-21	-358.55
CSF3	8.47E-12	2.05
CYP1A1	1.77E-11	-2.45
DAB1	2.65E-21	-6.50
DLGAP2	7.75E-04	44.44
DRD1	2.88E-09	-2.04
ERBB4	1.41E-15	-2.86
F2	4.45E-05	-60.69
FMN1	5.06E-22	2.17
FOLR3	6.13E-13	76.61
GABBR2	1.13E-18	-2.46
GABRP	2.33E-13	2.15
GAL	4.87E-10	3.51
GAP43	4.82E-11	2.07
GDNF	4.17E-07	36.14
GJB1	3.40E-10	-3.64
GNRH2	3.69E-06	-33.66
GRIA2	2.17E-03	35.40
GRM3	8.00E-19	-2.50
GRM5	2.36E-11	2.11
GRP	1.89E-07	3.67
HBE1	6.12E-06	29.59
HNF1B	3.63E-27	-3.18
HRH3	1.07E-17	-125.54
HTR2A	6.52E-07	3.13
HTR3A	1.35E-06	66.51
IFNG	3.37E-14	3.06
IL13	1.23E-05	39.43
IL5RA	9.57E-03	39.70
KCNA2	6.49E-05	30.02
KCNJ6	2.39E-05	49.11
KLK11	1.28E-06	2.03
L1CAM	3.48E-20	2.01
LHX1	1.71E-12	106.28
LPA	1.10E-06	-3.06
MUC2	1.10E-13	-3.12
NR1H4	3.94E-12	-5.06
NR2E1	2.44E-06	-2.03
NRXN1	3.98E-04	2.25
PENK	9.12E-06	71.70
PIK3C2G	4.46E-06	66.02
PRKCG	5.03E-07	44.46
PTPRN	3.47E-17	2.37
PVALB	3.43E-14	-2.77
RETN	4.66E-09	2.73
RNASE3	2.02E-05	2.21
SGK2	8.93E-29	-2.43
SHH	1.47E-14	-5.66
SLC26A5	9.52E-12	-88.02
SLC5A1	2.43E-07	2.31
SLC6A4	8.57E-21	-2.50
SLCO1B3	8.89E-16	188.44
SST	2.83E-03	-28.11
TAC1	4.19E-07	-52.37
TFF2	4.53E-12	-4.13
TH	7.99E-15	-2.31
TPO	7.51E-04	42.51
TTR	1.73E-15	-187.68
TUBA4B	6.44E-04	36.59
UGT1A1	1.56E-23	-2.25

Discussion

Bladder cancer is highly prevalent throughout the world and the incidence is increasing [1]. Furthermore, bladder carcinomas are the most expensive cancer to treat over the course of a patient's lifetime [25]. While CpG methylation has been associated with bladder carcinogenesis, the role of CpG methylation in the development and progression of bladder tumors is largely unknown [2, 5, 7]. We identified a set of 223 DMGs and DEGs where CpG methylation levels were associated with gene expression levels. The identified genes are enriched for their role in glutamate receptor signaling. One of these genes, *GRIA1*, is a significant biomarker of overall mortality in patients with basal-like urothelial bladder tumors that is independent of tumor stage.

Our data corroborate several other studies in reporting that the intragene region of methylation is tied to functional changes in gene expression [5, 6, 8]. Specifically, we found that

hypermethylation within the TSS200 region was most often associated with gene silencing, a finding that has been reported in both normal and cancerous tissues [5, 26, 27]. In addition, several genes previously reported to display promoter hypermethylation in bladder cancer, including *DBC1, PAX6, RUNX3,* and *WT1* also displayed promoter hypermethylation in the present study, although none were found to have altered expression [7]. This apparent incongruity may be explained by unmeasured effects of CpG methylation at distal-acting enhancer and silencer regions and demonstrates that our knowledge of the role of CpG methylation in tumor tissues remains incomplete [28-31]. Finally, our observation of a large number of hypomethylated genes in the gene body and 3' UTR regions is consistent with data from methylation profiling of colorectal cancers [32].

The data also demonstrate the surprising finding that many genes previously identified to be hypermethylated in their promoter regions in bladder cancer tumors are not hypermethylated in the TCGA samples. Our result that *BRCA1* showed promoter hypomethylation in tumor vs. non-tumor samples contrasts with previous publications that the *BRCA1* promoter is hypermethylated in bladder cancer tumors [20, 21]. In addition, other tumor suppressor genes that are commonly reported to display promoter hypermethylation in bladder cancer, including *PTEN*, *TP53*, and *RB1*, displayed minimal changes in methylation (< 1%) between tumor and non-tumor tissue in their promoter regions in the present analysis [20, 21]. A possible explanation for this discrepancy is that not all studies compare methylation status between tumor and non-tumor tissue to determine the change in methylation levels at the promoter regions. Similarly, not all studies used matched non-tumor control tissues, thus possibly failing to control for the interindividual differences in methylation [33, 34]. Our analysis indicates that promoter hypermethylation may only constitute part of an epigenetic-mediated bladder carcinogenesis signature and further contributes to the emerging picture of the complex relationship between CpG methylation and gene expression.

In the present study, we found that the DEGs and DMGs were enriched for their role in the glutamate receptor signaling pathway. Numerous genes related to glutamate receptor signaling were found to be differentially expressed between basal and luminal subtypes of bladder cancer. In addition, *GRIA1*, which encodes for glutamate ionotropic receptor AMPA type subunit 1, was found to significantly predict prognosis among basal-like urothelial bladder cancers. Glutamate is a neurotransmitter that also functions as a growth factor to stimulate proliferation in both normal and cancerous cells, and glutamate signaling has been found to be dysregulated in numerous cancers via changes in expression of glutamate receptors [22, 35-38]. Genes involved in glutamate receptor signaling have been reported to be aberrantly methylated in other malignant neoplasms [22, 35-37]. This is the first study to provide evidence of dysregulation of CpG methylation and gene expression of glutamate receptors in bladder cancer. Interestingly, glutamate receptors are reported to contribute to carcinogenesis through activation of the calmodulin, PI3K/Akt, and EGFR signaling pathways [39, 40]. These results highlight altered CpG methylation and/or mRNA expression of several genes involved in glutamate receptor signaling and these three downstream pathways, including *CAMK2A*, *PIK3C2G*, *GDNF*, and *F2* [22-24]. Activation of these pathways is known to contribute to cell growth and proliferation through various mechanisms, including activation of p63, a hallmark feature of basal-like bladder cancers [16, 41].

Several factors should be considered when interpreting the data from this study. First, intratumor sample location was unknown, and thus cellular heterogeneity within the tumor samples may be a confounding variable in our analysis. Second, there was not an available dataset from an independent cohort in which we could validate our finding that elevated levels of *GRIA1* TSS1500 methylation predict worse overall survival outcomes in urothelial bladder patients. Finally, the literature does not currently have an explanation for the apparent paradox of methylation and expression directionality from non-tumor to tumor states to tumor progression and more severe clinical outcomes (e.g. *GRIA1* mRNA expression was decreased in tumor tissue, but patients with increased mRNA expression had a worse survival outcome). This inconsistency has been observed in previous studies of associations between hypermethylation of another iono-

tropic glutamate receptor and mutations of critical enzymes and prognostic outcomes in non-small cell lung cancer and glioblastomas, respectively [42, 43].

In summary, we conclude that epigenetic profiling of urothelial bladder carcinomas increases the understanding of the development and progression of this highly prevalent neoplasm. Although bladder cancer is primarily a disease of somatic mutations, our study supports the growing body of evidence that implicates epigenetic mechanisms in urothelial bladder carcinogenesis [2, 3]. Importantly, epigenetic modifications may be reversible, and thus represent potential targets to halt tumor progression by restoring normal tissue function through epigenetic-directed pharmaceuticals [44]. As demonstrated in the present study, identification of these targets is an important first step in the development of novel cancer therapies.

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Disclosure of conflict of interest

None.

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