Epigenetic silencing of Na,K-ATPase β₁ subunit gene *ATP1B1* by methylation in clear cell renal cell carcinoma

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The Na,K-ATPase or sodium pump carries out the coupled extrusion of Na$^+$ and uptake of K$^+$ across the plasma membranes of cells of most higher eukaryotes. We have shown earlier that Na,K-ATPase-β₁ (NaK-β) protein levels are highly reduced in poorly differentiated kidney carcinoma cells in culture and in patients› tumor samples. The mechanism(s) regulating the expression of NaK-β in tumor tissues has yet to be explored. We hypothesized that DNA methylation plays a role in silencing the NaK-β gene (*ATP1B1*) expression in kidney cancers. In this study, to the best of our knowledge we provide the first evidence that *ATP1B1* is epigenetically silenced by promoter methylation in both renal cell carcinoma (RCC) patients' tissues and cell lines. We also show that knockdown of the von Hippel-Lindau (VHL) tumor suppressor gene in RCC cell lines results in enhanced *ATP1B1* promoter AT hypermethylation, which is accompanied by reduced expression of NaK-β. Furthermore, treatment with 5-Aza-2′-deoxycytidine rescued the expression of *ATP1B1* mRNA as well as NaK-β protein in these cells. These data demonstrate that promoter hypermethylation is associated with reduced NaK-β expression, which might contribute to RCC initiation and/or disease progression.

Introduction

Kidney cancers account for about 2% of all cancers, and more than 200 000 new cases of kidney cancer are diagnosed worldwide each year.[1] The most common form of kidney cancer in adult is renal cell carcinoma (RCC). Approximately 75% of the RCC cases are classified as clear cell RCC (ccRCC).[2] The most common genetic event in the evolution of sporadic ccRCC is inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene (TSG).[3-6] VHL inactivation leads to stabilization of the hypoxia-inducible transcription factor (HIF) and activation of a wide repertoire of hypoxia response genes.[7] The frequency of *VHL* mutations in sporadic ccRCC has been reported to be as high as 80% (although *VHL* mutations are rare in non-clear-cell forms of RCC).[8]

TSG inactivation may result from genetic or epigenetic events, and it is well recognized that epigenetic silencing of TSGs has a significant role in the pathogenesis of human cancers. Indeed, epigenetic silencing via promoter hypermethylation of *VHL* in RCC[5] was one of the first examples of this phenomenon. In fact, *VHL* mutation and methylation have been shown to be mutually exclusive, with methylation-induced silencing of *VHL* observed in 7% of RCCs.[9] From initial reports, approximately 60 genes were suggested to be epigenetically dysregulated in RCC.[10] Subsequently, work from The Cancer Genome Atlas (TCGA) that profiled over 400 tumors indicated that 289 genes display evidence of silencing by DNA methylation in at least 5% of tumors.[9] Identification of potential tumor suppressors silenced by methylation will facilitate a better understanding of the etiology of the disease and promote novel therapeutic approaches to treat ccRCC.[9,11,12]

The Na,K-ATPase is an abundantly expressed protein in epithelial cells and plays a crucial role in kidney function. Localized to the basolateral plasma membrane in epithelial cells, the
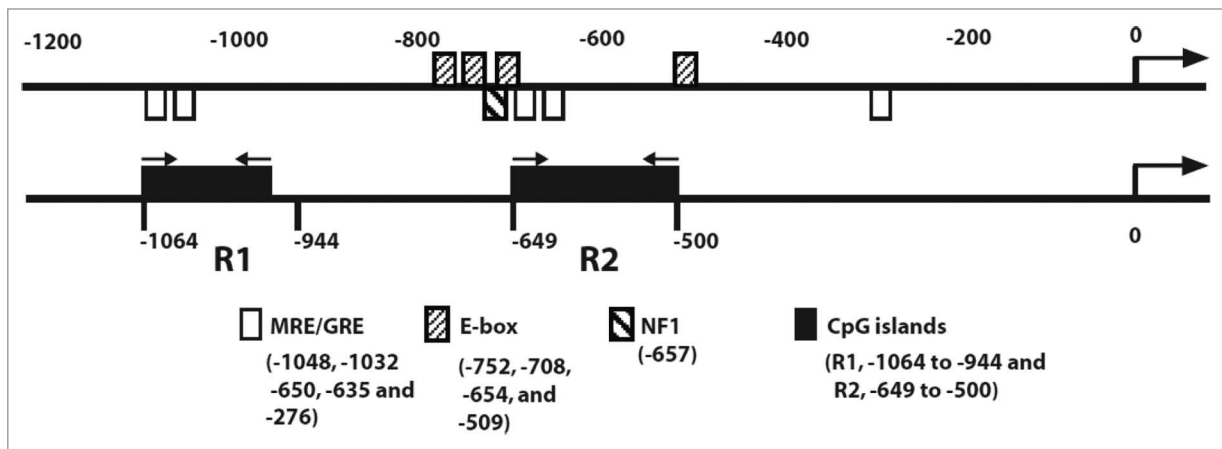
Figure 1. Promoter methylation analysis of *ATP1B1*. Schematic representation of NaK-β1 subunit promoter elements (upper panel) and CpG islands (lower panel). GRE, glucocorticoid responsive elements.

oligomeric Na,K-ATPase catalyzes the ATP-dependent transport of three Na+ out and two K+ into the cell per pump cycle to maintain Na+ and K+ gradients across the plasma membrane. This Na+ and K+ homeostasis in epithelia is necessary regulate the functions of various ion and solute transporters which is essential for the directional transport of solutes across the epithelial cell layer (vectorial transport).[13] The Na,K,ATPase is composed of two essential polypeptide subunits, the α-subunit (112 kDa) and the β-subunit (55 kDa),[14,15] and an optional regulatory γ-subunit with tissue-specific expression (7 kDa).[16] Of the four α- and three β-subunit isoforms known, α_1 (NaK-α) and β_1 (NaK-β) are predominantly expressed in kidney.[14,15]

Our laboratory previously demonstrated that NaK-β protein expression is reduced in ccRCC patients' tumor samples.[17] Subsequently, we showed that oncogenic transformation of kidney epithelial cells resulted in the reduced expression of NaK-β and promoted invasive and metastatic behaviors of these cells.[18,19] NaK-β levels were also reduced in a wide variety of carcinoma cells that have undergone epithelial to mesenchymal transition (EMT), which is one of the events associated with cancer progression into metastatic disease.[20] Ectopic expression of NaK-β in these transformed kidney epithelial cells delayed EMT,[21] abolished anchorage independent growth (the ability of tumor cells to grow in soft agar), and suppressed the growth of tumor xenografts in vivo.[19] Anchorage-independent growth and the ability to form tumors in immunocompromised mice (tumorigenicity) are primary features of malignant transformation, and TSGs inhibit both of these characteristics.[22-25] Since NaK-β repletion suppressed these characteristics, we proposed that NaK-β is a potential tumor suppressor.[19]

Inactivating mutations of the NaK-β gene (*ATP1B1*) have not been reported in cancer, yet many tumor suppressors can be silenced by promoter hypermethylation instead of mutation.[26] Given the putative tumor suppressor role of NaK-β and its general reduction in ccRCC, we hypothesized that NaK-β is epigenetically repressed in ccRCC. In this study, by analyzing a large number of patients' data from TCGA in conjunction with our own in vitro experiments we show a strong correlation

between *ATP1B1* expression and methylation. Using methylation specific PCR (MSP) in ccRCC patients' tumor samples, the *ATP1B1* promoter displays a stage-dependent increase in hypermethylation. Furthermore, we demonstrate that the *ATP1B1* promoter is preferentially hypermethylated in RCC cell lines deficient in VHL expression, which correlates with an increase in the expression of DNA methyltransferase (DNMT) 1 and 3A in these cells. Importantly, inhibition of DNMT activity using 5-Aza-2′-deoxycytidine (5-Aza-dC) rescued NaK-β expression in VHL knockdown cell lines.

Results

ATP1B1 promoter is hypermethylated in ccRCC patient tumor samples

Analysis of the *ATP1B1* promoter sequences using MethPrimer[27] showed two CpG islands located at bases -944 to -1064 and –500 to -649 (termed as CpG regions R1 and R2, respectively) (**Fig. 1**). This finding suggests that methylation of 5′ regulatory CpG sites might be one of the mechanisms involved in the transcriptional repression of *ATP1B1* in ccRCC.

We then analyzed *ATP1B1* promoter methylation in tumor samples obtained from RCC patients. To determine DNA methylation, methylation-specific PCR (MSP) primers were designed using the online tool Methprimer to the R1 and R2 regions (**Fig. 1**; **Table 1**). Analyses of the methylation pattern of ccRCC tumor samples with matched morphologically normal tissues from six patients are shown in **Figure 2**. Compared with matched normal tissues, tumor tissues showed more intense bands corresponding to methylated promoter regions (compare lanes 8 vs 4). Interestingly, the intensity of methylated promoter regions positively correlated with the stage of the tumor (lane 8). In the R1 region the promoter is largely unmethylated in normal compared with tumor tissues (compare lanes 1 vs 5). In the R2 region of stage 4, adjacent normal tissues showed higher methylation compared with stages 1, 2, and 3 (lane 4). The intensity of the methylated band was maximal in stage 4 tumors (lane 8).

Table 1. PCR primers

Na,K,ATPase β1:
5'-CATCAGAGAGCAACTGATCC-3' and
5'-ATGCCATTCCGAATCTCAGC-3'
R1 region
Unmethylation
5'-GAA GGG TTG GAA TTG TAA TTG T-3'
5'-AAA AAA AAT CAC TCC AAA ACA C-3'
Methylation
5'-GAA GGG TCG GAA TTG TAA TC-3'
5'-AAA AAA ATC ACT CCG AAA CG-3'
R2 region:
Unmethylation
5'-TTT TTT TGT GTT GGT TTT GA -3'
5'-AAA AAA TTA ACT ACA CCC ACC-3'
Methylation
5'-GGT TTT TTT GTG TCG GTT TC-3'
5'-GAA AAA TTA ACT ACG CCC GC-3'

Figure 2. *ATP1B1* promoter methylation, tumor stage, and gene expression in patient RCC tumor samples. Analysis of RCC patient tumor samples. U and M indicate amplicons generated using primers specific for unmethylated and methylated NaK-β1 promoter alleles.

To validate our observation of *ATP1B1* promoter methylation and explore the relation of *ATP1B1* methylation with gene expression in RCC, we accessed data from The Cancer Genome Atlas (TCGA) of renal cell carcinoma (tumor n = 272, normal n = 21).[9] There are 15 CpG sites associated with *ATP1B1* on the Illumina 450K methylation array platform; six CpGs 5' of the TSS, three CpGs in the first exon, and six CpGs in the gene body with nine of the CpGs in a CpG island. Interestingly, the nine CpG island CpGs were relatively unmethylated in RCC, with none of the nine sites having a maximum average β greater than 0.17 (17% methylated). Among the nine CpG island CpG sites, one CpG was mapped to each of the regions measured with MSP (R1 and R2), though not within the primer sequences. The maximum methylation among TCGA RCCs was 0.14 (14% methylated) for the CpG in R1, and 0.09 (9% methylated) for the CpG in R2. The range of median methylation values among the nine CpG island CpG sites was from 0.009 to 0.054 (less than 1% methylated to 5% methylated). However, five of the remaining six CpG sites each demonstrated a wide range of methylation values among RCC tumors, with median methylation ranging from 0.29 to 0.60 (29% methylated to 60% methylated). Methylation of each of these five *ATP1B1* CpG sites was significantly negatively associated with *ATP1B1* gene expression. For example, methylation of cg13104274 in the South CpG island shore region of *ATP1B1* was significantly associated with reduced *ATP1B1* gene expression (*P* = 5.3E-09, **Fig. 3A**). In addition, methylation of cg13104274 was significantly lower in normal kidney samples (n = 21, mean methylation 0.203) compared with RCC (n = 272, mean methylation 0.46; *P* = 3.0E-06, **Fig. 3B**). Concomitantly, *ATP1B1* gene expression was significantly lower in RCC compared with normal kidney (*P* = 3.0E-12, **Fig. 3C**). Altogether, these results indicate that the *ATP1B1* promoter is hypermethylated in ccRCC tumors relative to matched morphologically normal tissues.
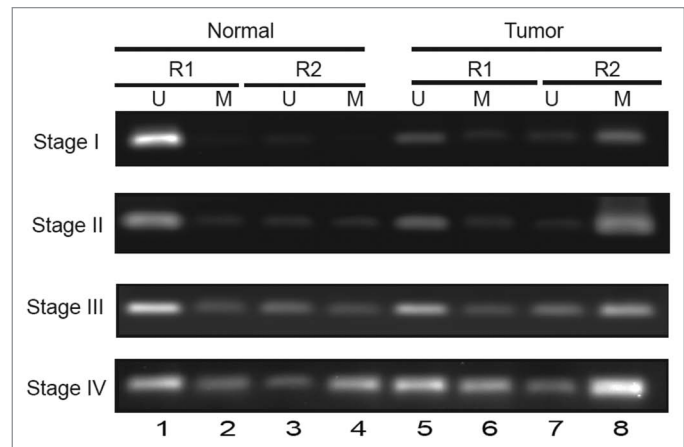
Knockdown of VHL enhances ATP1B1 promoter methylation

To further understand the molecular mechanism of *ATP1B1* repression by promoter hypermethylation, we used two well characterized isogenic pairs of human ccRCC cell lines that differ only in VHL expression levels due to lentiviral transfer of VHL-specific shRNA (SN12C and SN12C-VHL; ACHN and ACHN-VHL).[28] Methylation-specific PCR of the genomic DNA isolated from these cell lines revealed enhanced methylation of *ATP1B1* promoter in the VHL knockdown cells compared with the parental cells (**Fig. 4A**). In the R1 region, methylation of the promoter was not detected in the parental cell lines (lane 1), whereas it was considerably methylated in VHL-knockdown cell lines (lane 5). In the R2 region moderate methylation was observed in VHL-positive cell lines (lane 3) while the promoter was heavily methylated in VHL-knockdown cell lines (lane 7). Taken together, these data suggest that *ATP1B1* promoter is hypermethylated in ccRCC cell lines and that VHL deficiency associated with *ATP1B1* promoter methylation.

Knockdown of VHL decreases NaK-β expression but increases DNMT1 and 3A expression

Since we observed enhanced *ATP1B1* promoter methylation in the VHL knockdown cells, we assessed whether NaK-β expression is reduced in these cells. We found that knockdown of VHL produced a dramatic reduction in both NaK-β transcript (**Fig. 4B**) and protein (**Fig. 4C**) expression. There was a 60% reduction in the NaK-β protein levels. These data confirm that enhanced *ATP1B1* promoter methylation leads to reduced NaK-β expression in VHL-knockdown cells.

DNA methylation is performed by methyltransferase enzymes such as DNMT1 and DNMT3A.[29] We investigated whether expression of DNMT1 and DNMT3A were affected in the isogenic ccRCC cells. Strikingly, the VHL-knockdown cells exhibited an increase in both DNMT1 and DNMT3A protein expression levels compared with the parental cell lines (**Fig. 4D**). The increase was more pronounced for both DNMT1
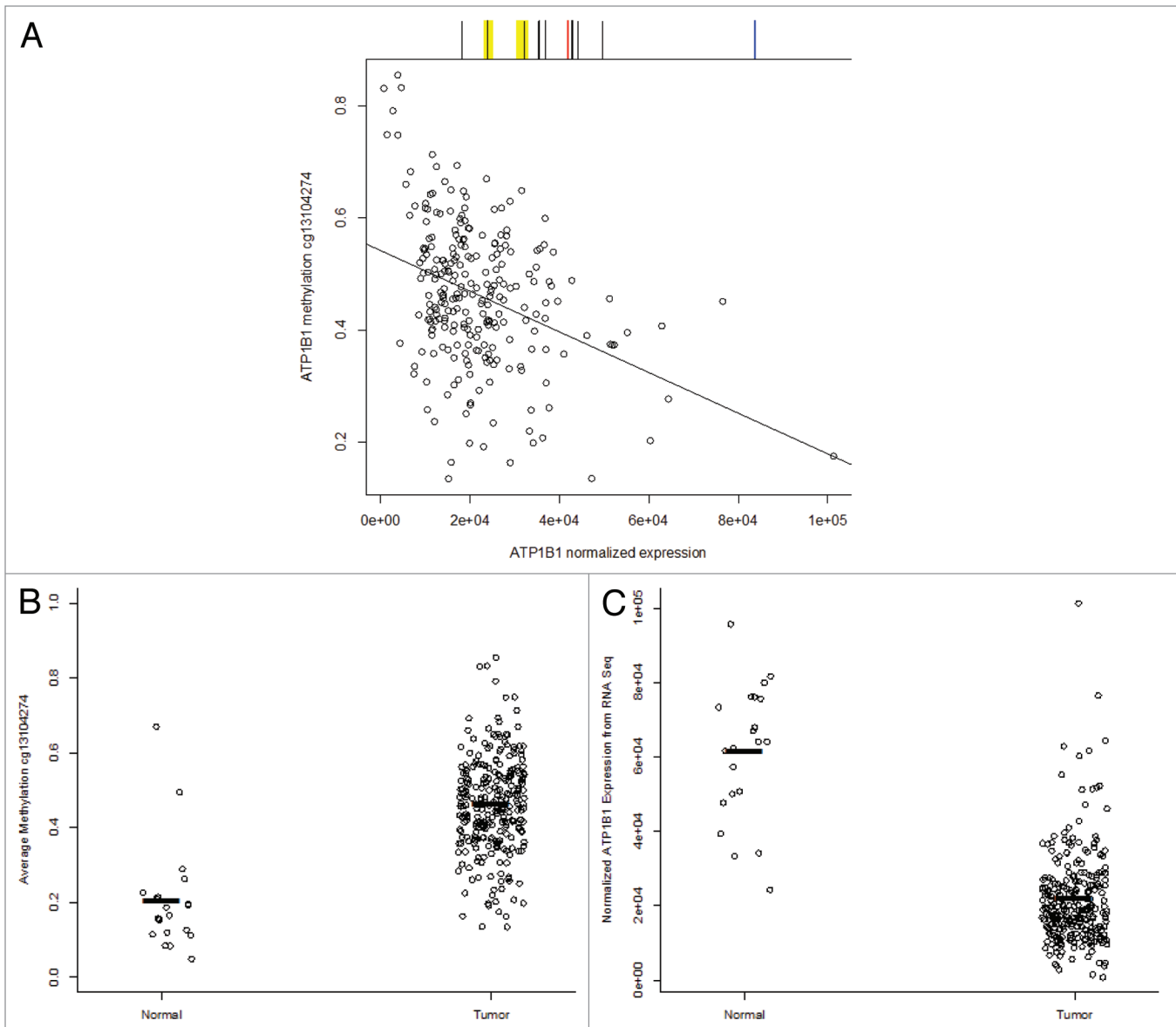
Figure 3. TCGA data analysis of *ATP1B1* methylation and gene expression in normal kidney and RCC. (**A**) Normalized gene expression of *ATP1B1* is plotted vs. *ATP1B1* methylation at cg13104274 in RCC (n = 272) and demonstrates a significant association between DNA methylation and reduced gene expression (*P* = 5.3E-09). Above the plot is a scheme of CpG sites measured on the Illumina 450K platform in black, the yellow regions represent R1 (left) and R2 regions measured with MSP, the red line is the TSS, and the blue line is the plotted CpG site. (**B**) Methylation of cg13104274 is significantly higher in RCC (n = 272) compared with normal kidney (n = 21, *P* = 3.0E-06). Black bars represent mean methylation values: normal tissue mean methylation = 0.20, tumor mean methylation = 0.46 (C) *ATP1B1* gene expression is significantly higher in normal kidney (n = 21) compared with RCC (n = 272, *P* = 3.0E-12). Black bars represent mean expression values: normal tissue mean expression = 6.1E+04, tumor mean methylation = 2.2E+04.

and DNMT3A in the SN12C cells (2.5- and 3.0-fold) than the ACHN cells (1.8 and 1.6-fold) indicating that there may be additional cell-type specific effects that affect DNMT enzyme expression.

DNMT inhibitor 5-AZA-dC rescues NaK-β expression in VHL knockdown cell lines

5-Aza-dC has been effectively used to re-express genes that have been silenced by promoter hypermethylation.[30] We treated the isogenic ccRCC cell lines with 5-Aza-dC and found that treatment enhanced NaK-β protein expression in the VHL-knockdown cells but not the parental cells (**Fig. 5A**). In support

of this, transcript analysis revealed a 50% increase in the NaK-β mRNA levels in 5-Aza-dC-treated VHL-knockdown cells (**Fig. 5B**). These results indicate that inhibition of methyltransferase enzymes rescues NaK-β expression in VHL-knockdown cells.

To further probe this effect, we performed bisulfite pyrosequencing to quantitatively analyze the *ATP1B1* promoter methylation and its loss of methylation following 5-Aza-dC treatment in the isogenic ccRCC cell lines (**Fig. S1**). SN12C cells showed 51% methylation, and ACHN cells showed 16% promoter methylation (16%). 5-Aza-dC treatment had no significant effect on
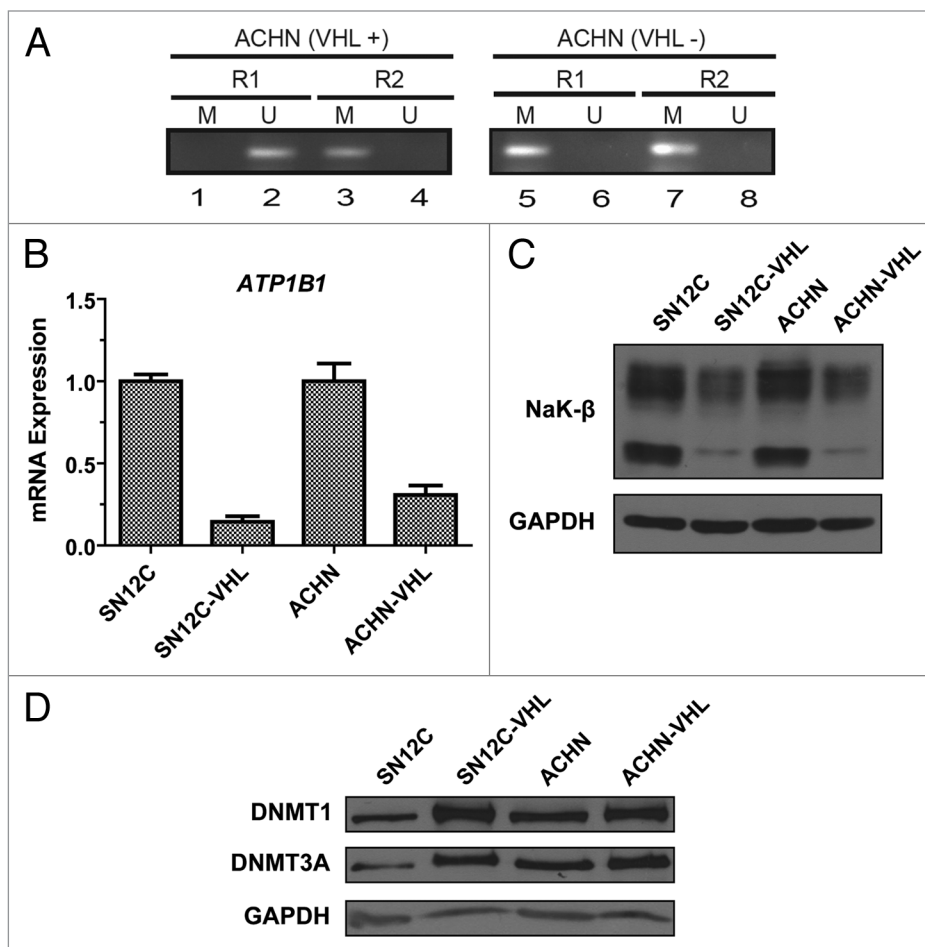
Figure 4. *ATP1B1* promoter methylation, NaK-β expression, and DNMT expression in isogenic ccRCC cell lines. (**A**) Methylation-specific PCR analysis of *ATP1B1* promoter methylation in the isogenic ccRCC cells lines. U and M indicate amplicons generated using primers specific for unmethylated and methylated *ATP1B1* promoter alleles. (**B**) Analysis of *ATP1B1* transcript expression. Graph depicts mean ± SD of one representative experiment. (**C**) Analysis of NaK-β1 protein expression. (**D**) Analysis of DNMT1 and DNMT3A protein expression.

these cell lines (-4% and +1%, respectively). In contrast, the SN12C and ACHN VHL-knockdown cell lines showed a methylation rate of 72 and 74%, respectively, and 5-Aza-dC treatment reduced promoter methylation in these cell lines (-20% and -9%, respectively). These results further confirm that in VHL-knockdown cells the *ATP1B1* promoter is methylated and that 5-Aza-dC treatment results in loss of methylation.

Discussion

Aberrant DNA methylation patterns have been linked to altered gene expression in certain genetic diseases and tumors.[31,32] In this study, to our knowledge, we provide the first evidence that the *ATP1B1* promoter is hypermethylated in ccRCC compared with paired adjacent non-malignant tissues, a finding that we validated using TCGA tumor methylation data. TCGA RCC tumor data indicated a significant association between *ATP1B1* methylation and decreased *ATP1B1* gene expression. Furthermore, in the TCGA data, normal kidney tissue samples had both significantly lower methylation and significantly higher gene expression compared with with RCCs. In our tumor samples,

we observed that *ATP1B1* promoter methylation increased with increasing tumor stage, although we did not observe a consistent trend for increasing methylation with tumor stage in the TCGA data. Using isogenic ccRCC cell lines that differ in only VHL expression, we further showed that *ATP1B1* hypermethylation is enhanced in VHL-deficient cell lines and that inhibition of DNMTs by 5-Aza-dC relieves promoter hypermethylation which leads to renewed expression of Na,K-β transcript and protein. We reported earlier that ectopic expression of Na,K-β in kidney carcinoma cells reduces in vivo tumor growth and abolishes anchorage-independent growth which are hallmark functions of TSGs.[19] Along with previous observations, the present study further confirms that Na,K-β has tumor suppressor functions in kidney epithelial cells.

Patients' tumor samples obtained from stage IV tumors showed heavy methylation by methylation-specific PCR (MSP) compared with stage I, II, and III tumors. Interestingly, the matched morphologically normal adjacent tissue from stage IV patients showed higher methylation than the stage II or III patients. This result indicates that *ATP1B1* promoter is also hypermethylated in adjacent normal tissues, and suggests that *ATP1B1* promoter

methylation may be an early event that occurs prior to changes in the morphology of kidney cells. Based upon our MSP results, the R2 region appears to be methylated to a greater degree than the R1 region in tumors, although this relationship was not confirmed in the TCGA data. While these results may seem inconsistent, there are key differences in the methods used. Provided that there is correlation among the methylated CpG sites in the primers, MSP assays can detect relatively low levels of methylated DNA (< 5%).[33] Interestingly, despite the canonical relation of CpG island hypermethylation with gene silencing for tumor suppressor genes in cancer, TCGA methylation and expression data indicated a strong relation of methylation at island-adjacent CpG sites with gene expression. The importance of so-called CpG island shore methylation in human cancer has become well recognized in recent years.[34,35] We proposed earlier that Na,K-β expression could be used as a biomarker whose expression is reduced in ccRCC.[17] Since the morphologically normal tissues adjacent to advanced tumors showed *ATP1B1* promoter hypermethylation by MSP, and significant differences between normal and tumor tissue were found from the TCGA data, the methylation pattern of *ATP1B1* promoter may have potential as an early biomarker for the diagnosis of ccRCC.

During EMT, the expression of cell-cell adhesion molecules is reduced which facilitates invasion and metastatic dissemination of carcinoma cells. NaK-β is a key cell-cell adhesion molecule in kidney epithelial cells, and its expression is downregulated during EMT[21] by the transcriptional suppressor Snail which binds to E-boxes within the *ATP1B1* promoter and downregulates its expression.[20] Although we cannot rule out that Snail is induced and involved in the suppression of NaK-β in VHL-knockdown cells, the enhanced NaK-β transcript and protein levels following 5-Aza-dC treatment suggests that hypermethylation is one of the mechanisms involved in reducing NaK-β expression in ccRCC. Furthermore, loss of VHL function leads to constitutive expression of hypoxia-inducible factor (HIF) which promotes EMT.[36] Therefore, it is possible that HIF might be involved in the downregulation of NaK-β levels in VHL-knockdown cells.

Enhanced expression of both DNMT1 and DNMT3A in VHL-knockdown cells suggest that loss of VHL might be associated with hypermethylation of multiple genes associated with the EMT process. In fact, it has been shown that DNMTs are induced during EMT in several cancers, including pancreatic cancer, non-small cell lung cancer,[37] and breast cancer.[38] There are no reports available on the influence of HIF-1α on DNMT activity; however multiple reports are available on the interaction between HIF-1α and histone demethylases JMJD1A, (demethylates dimethylated histone H3 lysine 9 (H3K9Me2) and JMJD2B (demethylates trimethylated H3K9).[39,40] A recent report suggests that VHL depletion results in the HIF-dependent regulation of H3K4 trimethylation by decreasing histone demethylase
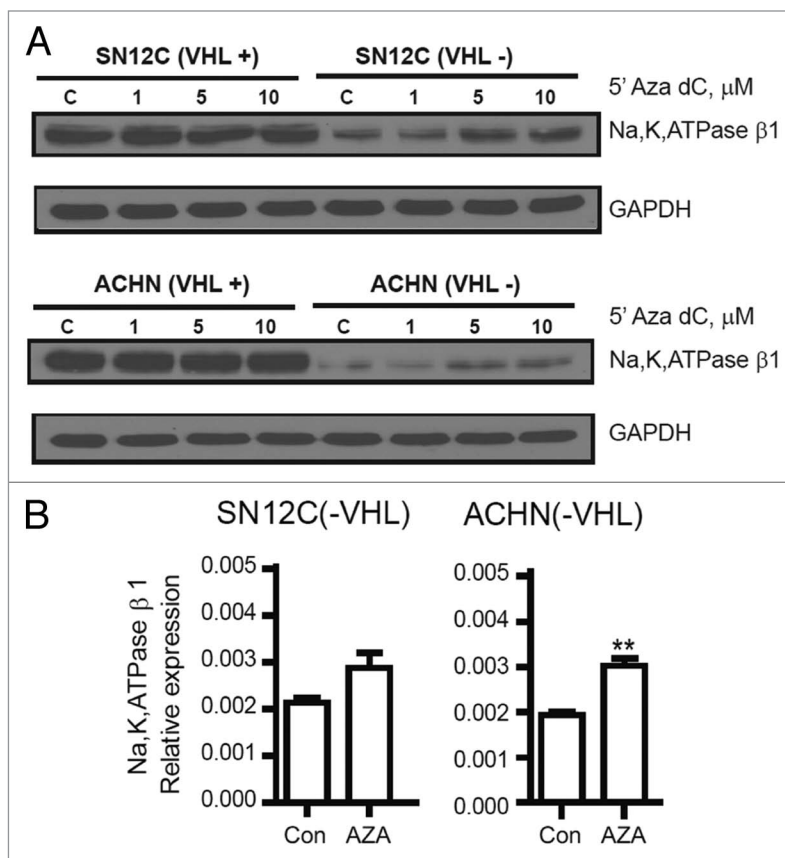
Figure 5. Effect of 5-Aza-dC on the expression of NaK-β in ccRCC cell lines. (**A**) Analysis of NaK-β protein expression in isogenic SN12C and ACHN cells treated with increasing concentrations of 5-Aza-dC (0, 1, 5 and 10 μM) for 48hr. (**B**) Analysis of NaK-β transcript expression in VHL-knockdown cells treated with 5-Aza-dC.

JAR1D1C.[41] Future investigations are necessary to understand the mechanisms of DNMT induction by loss VHL in ccRCC. We reported earlier that ectopic expression of NaK-β in kidney carcinoma cells reduces in vivo tumor growth and abolishes anchorage-independent growth, which are hallmark functions of TSGs.[19] Along with our previous observations, the present study further confirms that NaK-β has tumor suppressor functions in kidney epithelial cells.

Materials and Methods

Reagents

5-Aza-2′-deoxycytidine and β-actin mouse antibody were purchased from Sigma-Aldrich. Antibody against NaK-β (M17-P5-F11) was kindly provided by Dr William Ball, Jr (University of Cincinnati). Primary antibodies specific for GAPDH, DNMT1, and DNMT3, as well as horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling.

Patient tissues

Fresh-frozen clinical tissue samples were obtained with informed consent from the Helen F Graham Cancer Center Tumor Bank, Christiana Care Hospital with appropriate ethical

approval from the Institutional Review Boards of the Christiana Care Hospital and the Nemours/Alfred I. duPont Hospital for Children. Primary RCCs and matched adjacent macroscopically normal renal tissues from six patients undergoing renal surgery were analyzed. Tissues were evaluated by pathologists, and DNA was isolated from fresh frozen samples using the DNeasy Blood and Tissue Kit (Qiagen Inc.) and used for methylation specific PCR as described below.

The Cancer Genome Atlas data and methods

Using The Cancer Genome Atlas (TCGA) data portal, we downloaded level 3 data from 272 RCC tumors and 21 normal tissues with available matched Illumina 450K DNA methylation array and Illumina HiSeq RNASeqV2 data. DNA methylation and RNASeq files were loaded into and parsed using the R software environment (v.3.0.1), matching samples based on their TCGA ID.

Tissue culture and drug treatments

Human clear cell renal cell carcinoma cell lines (SN12C, ACHN) and isogenic VHL-knockdown cell lines (SN12C-VHL, ACHN-VHL)[28] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 25 U/mL penicillin, and 25 µg/mL streptomycin at 37 °C in 5% CO_2. Cell lines were treated with 5-aza-2′-deoxycytidine (5-Aza-dC) (Sigma) for 48 h.

Methylation-specific PCR

DNA methylation within the CpG region of the NaK-β promoter was analyzed using methylation-specific PCR after sodium bisulfite treatment of genomic DNA as described previously.[22] CpGenome universally methylated DNA (Chemicon), after bisulfite modification, served as the methylated NaK-β. PCR was performed in 50 µl reaction mixtures containing 1.0 µl of modified DNA, 1× Qiagen buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer and 1.5 U of HotStar Taq (Qiagen Inc.).

Quantitative real-time PCR

Total RNA was harvested using RNAase isolation kit (Qiagen Inc.). CDNA (cDNA) was synthesized from 2 µg of total RNA using iScript cDNA synthesis kit (Bio-Rad laboratories). CDNA was amplified in a 25 µL reaction containing 12.5 µL SybrGreen PCR Master Mix (Bio-Rad), 100 pmol primers and 0.25 µg cDNA using a Mini-Opticon Q-RTPCR machine (Bio-Rad) with the following program: 1 cycle at 95 °C for 5 min; 45 cycles at 95 °C for 45 s, 55 °C 45 s, 72 °C for 45 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used to normalize mRNA expression.

Pyrosequencing

Pyrosequencing analysis was performed using PCR product generated from bisulfite-treated DNA. PCR was performed in 50 µl reaction mixtures containing 1.0 µL of bisulfite-modified DNA, 1 × Qiagen PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer and 1.5 U of Hotstar Taq DNA polymerase. The amplification program consisted of 1 cycle at 95 °C for 15 min, 45 cycles at 95 °C for 20s, 52 °C for 20s and 72 °C for 20s. Pyrosequencing analysis was performed by EpigenDx Inc.

Immunoblot analysis

At the end of treatments with 5-Aza-2′-deoxycytidine and untreated control were analyzed by immunoblot analysis as described before (23). Briefly, cells were washed with cold PBS and lysed in buffer (20 mmol/L TRIS-HCl pH 7.4, 100 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium glycerolphosphate, 1 mmol/L sodium orthovanadate) supplemented with protease inhibitors. Cell lysates were centrifuged at 14 000 g for 15 min at 4 °C and protein concentration was determined in the supernatants using the Biorad protein assay reagent (Bio-Rad) using bovine serum albumin as standard. Equal amounts of protein were then resolved by SDS-PAGE and transferred to nitrocellulose. The membranes were blocked for one hour at room temperature in TBST (50 mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.2% Tween-20) containing 5% nonfat milk. Blots were sequentially incubated with the primary and secondary antibodies, washed in TBS-T. Membranes were developed using Amersham ECL Prime (GE Healthcare) and images were acquired using Photoshop (Adobe Systems Inc.).

Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Dunnett's Test. Statistical comparisons were considered significant at $P < 0.05$. Linear regression analysis was used to test the relation of *ATP1B1* gene expression with promoter DNA methylation. The Wilcoxon rank sum test was used to test the relation of DNA methylation or *ATP1B1* gene expression with tumor status (tumor vs. normal).

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