

Published in final edited form as:

*Oncogene*. 2010 April 8; 29(14): 2104–2117. doi:10.1038/onc.2009.493.

## Identification of Candidate Tumour Suppressor Genes Frequently Methylated in Renal Cell Carcinoma

Mark R. Morris<sup>(1),(2)</sup>, Christopher Ricketts<sup>(1),(2)</sup>, Dean Gentle<sup>(1),(2)</sup>, Mahera Abdulrahman<sup>(2)</sup>, Noel Clarke<sup>(3)</sup>, Michael Brown<sup>(3)</sup>, Takeshi Kishida<sup>(4)</sup>, Masahiro Yao<sup>(4)</sup>, Farida Latif<sup>(1),(2)</sup>, and Eamonn R Maher<sup>(1),(2),(5)</sup>

<sup>(1)</sup> Cancer Research UK Renal Molecular Oncology Group, University of Birmingham, Birmingham B15 2TT, UK

<sup>(2)</sup> Department of Medical and Molecular Genetics, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK.

<sup>(3)</sup> Paterson Institute for Cancer Research, University of Manchester, Manchester, M20 4BX, UK

<sup>(4)</sup> Department of Urology, Yokohama City University School of Medicine, Yokohama, Japan

<sup>(5)</sup> West Midlands Region Genetics Service, Birmingham Women's Hospital, Edgbaston, Birmingham B15 2TG, UK.

### Abstract

Promoter region hypermethylation and transcriptional silencing is a frequent cause of tumour suppressor gene (TSG) inactivation in many types of human cancers. Functional epigenetic studies, in which gene expression is induced by treatment with demethylating agents, may identify novel genes with tumour-specific methylation. We used high-density gene expression microarrays in a functional epigenetic study of 11 renal cell carcinoma (RCC) cell lines. Twenty eight genes were then selected for analysis of promoter methylation status in cell lines and primary RCC. Eight genes (*BNC1*, *PDLIM4*, *RPRM*, *CST6*, *SFRP1*, *GREM1*, *COL14A1* and *COL15A1*) demonstrated frequent (>30% of RCC tested) tumour-specific promoter region methylation. Hypermethylation was associated with transcriptional silencing. Re-expression of *BNC1*, *CST6*, *RPRM*, and *SFRP1* suppressed the growth of RCC cell lines and RNAi knock-down of *BNC1*, *SFRP1* and *COL14A1* increased the growth of RCC cell lines. Methylation of *BNC1* or *COL14A1* was associated with a poorer prognosis independent of tumour size, stage or grade. The identification of these epigenetically inactivated candidate RCC tumour suppressor genes can provide insights into renal tumourigenesis and a basis for developing novel therapies and biomarkers for prognosis and detection.

### Keywords

Renal cell carcinoma; methylation; epigenetics

## INTRODUCTION

Kidney cancers account for about 2% of all cancers and more than 200,000 new cases of kidney cancer are diagnosed in the world each year (Ferlay et al., 2007). The most common form of kidney cancer in adults is renal cell carcinoma (RCC). Most RCC (~75%) are

classified as clear cell (conventional) and the next most frequent subtype is papillary RCC (~15% of all cases) (Mancini et al., 2008). The prognosis of advanced RCC is poor, although newer treatments, based on knowledge of the molecular pathology of RCC but characterisation of the molecular pathology of RCC may provide a basis for developing novel approaches to therapy. Thus the most frequent genetic event in the evolution of sporadic clear cell RCC is inactivation of the *VHL* tumour suppressor gene (TSG) (Clifford et al., 1998; Foster et al., 1994; Herman et al., 1994; Latif et al., 1993). *VHL* inactivation leads to stabilisation of HIF-1 and HIF-2 transcription factors and activation of a wide repertoire of hypoxic response genes (Maxwell et al., 1999). HIF-mediated RCC growth may be antagonised by multi tyrosine kinases inhibitors such as sunitinib and sorafenib (Chowdhury et al., 2008). Hence identification of mechanisms of tumourigenesis in RCC can provide a basis for therapeutic intervention. Although large scale mutation analysis studies of RCC are in progress (see <http://www.sanger.ac.uk/genetics/CGP/cosmic/>), with the exception of *VHL*, none of the thousands of genes tested to date are mutated in >15% of tumours. Epigenetic inactivation of TSGs by methylation promoter region of CpG dinucleotides has also been implicated in the pathogenesis of RCC and some important TSGs are frequently inactivated by promoter hypermethylation but rarely mutated (e.g. *RASSF1A*) (Dallol et al., 2002; Morris et al., 2003; Morrissey et al., 2001). Hence, strategies to identify epigenetically inactivated TSGs are an important approach to elucidating the molecular pathogenesis of RCC. Previously, in order to investigate the utility of a functional epigenomic approach to identify candidate novel epigenetically inactivated RCC TSGs, we performed a pilot study using gene expression profiling of four RCC cell lines treated with the demethylated agent 5-Aza-2'-deoxycytidine (Morris et al., 2008; Morris et al., 2005). This led us to identify *HAI-2/SPINT2* as a novel epigenetically inactivated RCC TSG (Morris et al., 2005). We now report the results of a large functional epigenetic screen of RCC in which 11 RCC cell lines were analysed using a high density gene expression microarray platform.

## METHODS

### Patients and samples

DNA from up to 61 primary RCCs (~80% clear cell and 20% non-clear cell) and matched adjacent macroscopically normal renal tissue and normal renal tissue (not required for surgical pathology) from 6 patients undergoing non-renal cancer surgery (mean age 57 years, range from 23-79 years) were analysed. Local research ethics committees approved the collection of samples and informed consent was obtained from each patient. This study was conducted according to the principles expressed in the Declaration of Helsinki.

### Cell lines, 5-Aza-2'-deoxycytidine treatment and microarray analysis

RCC cell lines KTCL 26, RCC4, UMRC2, UMRC3, SKRC18, SKRC39, SKRC45, SKRC47, SKRC54, 786-0 and Caki-1 were routinely maintained in DMEM (Invitrogen, San Diego, CA) supplemented with 10% FCS at 37°C, 5% CO<sub>2</sub>. The demethylating agent 5-Aza-2'-deoxycytidine (Sigma) was freshly prepared in dd H<sub>2</sub>O and filter sterilized. Cell lines were plated in 75-cm<sup>2</sup> flasks in DMEM supplemented with 10% FCS at differing densities, depending upon their replication factor, to ensure that both control and 5-Aza-2'-deoxycytidine treated lines reached approximately 75% confluency at the point of RNA extraction. Twenty-four hrs later, cells were treated with 5 μM 5-Aza-2'-deoxycytidine. The medium was changed 24 hrs after treatment and then changed again after 72hrs. RNA was prepared 5 days after treatment using RNABee (AMS Biotechnology). Total RNA from all 11 cell lines +/-5-Aza-2'-deoxycytidine was isolated using RNA-Bee reagent following manufacturer's instructions (AMS Bio) followed by purification using RNeasy Mini-columns (Qiagen). cRNA probes were prepared using the Affymetrix protocol and

hybridized to HG-U133 plus2 GeneChip oligonucleotide arrays (Affymetrix). Array hybridisation and data production was done by the CRUK Paterson Institute Microarray Service (<http://bioinformatics.picr.man.ac.uk/mbcf/>).

### RT PCR conditions

PCR cycling conditions consisted of 5 min at 95°C followed by 30 cycles of 45 sec of denaturation at 95°C, 45sec of annealing at 55-60°C and 45sec of extension at 72°C. Semi-quantitative analysis of expression was done using LabWorks software (Ultraviolet products, California). (RT-PCR primers and conditions upon request).

### Bisulfite Modification and Methylation Analysis

Bisulfite DNA sequencing was performed as described previously (Morris et al., 2008; Morris et al., 2005). Briefly, 0.5–1.0 µg of genomic DNA was denatured in 0.3 M NaOH for 15 min at 37°C, and then unmethylated cytosine residues were sulfonated by incubation in 3.12 M sodium bisulfite (pH 5.0; Sigma)/5 mM hydroquinone (Sigma) in a thermocycler (Hyaid) for 20 cycles of 30 s at 99°C and 15 min at 50°C. The sulfonated DNA was recovered using the Wizard DNA cleanup system (Promega) in accordance with the manufacturer's instructions. The conversion reaction was completed by desulfonating in 0.3 M NaOH for 10 min at room temperature. The DNA was ethanol-precipitated and resuspended in water.

### Promoter Methylation Analysis

CpG islands were identified on the human genome browser and *putative* promoter regions were predicted by Promoter Inspector software (Genomatix). Details of bisulphite sequencing primers and *COL15A1* MSP primers are provided in supplementary table 1. Examples of direct tumour-DNA sequencing traces are shown in supplementary figure 1. Methylation specific PCR analysis for the following genes was carried out using previously described MSP primers: *BNC1* (Shames et al., 2006), *SFRP1* (Nomoto et al., 2007), *REPRIMO* (Sato et al., 2006).

### Plasmid Constructs and Colony Formation Assay

The *CST6*, *SFRP1* and *REPRIMO* expression constructs were made by cloning the full-length human coding regions amplified from kidney cell lines into the EcoRI-BamHII sites of pCDNA3.1 (Invitrogen) or pFLAG-CMV4 (Sigma-Aldrich) vectors. *BNC1* was amplified from the I.M.A.G.E clone 40080551. Plasmid constructs were verified by sequencing. Six micrograms of empty vector and an equal Molar amount of expression vector were transfected, using Fugene (Roch), following the manufacturer's instructions, into  $5 \times 10^5$  target cells (SKRC39, RCC4, 786-0, SKRC47 or HEK-293). Forty-eight hours after transfection, cells were seeded in a serial dilution and maintained in DMEM and 10% foetal bovine serum supplemented with 1 mg/mL G418 (Life Technologies). Surviving colonies were stained with 0.4% crystal violet (Sigma) in 50% methanol, 21 days after initial seeding, and counted. Each transfection was carried out in triplicate. Additionally, replicate experiments were carried out to obtain further clones for expression analysis. Expression was confirmed by RT-PCR and Western blot analysis.

### Anchorage Independent Growth Assay

RCC clones stably expressing *BNC1*, *CST6*, *REPRIMO* or the empty vector control were suspended in 2ml DMEM 10% FCS, 3% agar. Cells were maintained by addition of 200 µl of DMEM 10% FCS, supplemented with 1mg/ml G418, weekly. After 5 weeks of growth a final count of colonies was performed. RNAi “silencer select” oligos against *BNC1* (s2012), *COL14A1* (s14677) and *SFRP1* (s12713) or “Silencer select” control oligo no.1 (Ambion)

were transfected into HEK-293 or KTCL26 cells using Interferin reagent (Polyplus) following the manufacture's instructions. After 24h incubation cells were seeded into 2ml DMEM 10% FCS, 3% agar. Cells were maintained by addition of 200  $\mu$ l of DMEM 10% FCS weekly. After 3 weeks of growth a final count of colonies was performed. Cells not seeded into agar were incubated for a further 24h before efficiency of knock-down was assessed by RT-PCR.

**Statistical analysis** was performed as indicated with a significance level of 5%.

## RESULTS

### Identification of candidate silenced genes involved in RCC

Eleven RCC-derived cell lines were treated with the demethylating agent 5-Aza-2'-deoxycytidine (5  $\mu$ M) for 5 days to re-activate epigenetically silenced/down-regulated genes and changes in gene expression were measured by Affymetrix U133 plus-2 microarrays that contain probes for >47000 unique transcripts. In order to prioritise genes for methylation analysis, we initially focussed on genes which showed a ten-fold increase in expression following de-methylation in three or more cell lines (RT-PCR analyses demonstrated a good correlation with microarray based estimates of changes in gene expression (see Supplementary Fig 1, Fig 1A and Fig 2A)), Transcripts that represented hypothetical proteins were also removed. 405 unique genes were left following this filter. Next we excluded genes that did not have a predicted promoter specific CpG island (as predicted by the human genome browser ([www.genome.ucsc.edu](http://www.genome.ucsc.edu)) and Genomatix promoter inspector ([www.genomatix.de](http://www.genomatix.de))), 201 genes were left following this filter step. Genes known to be imprinted and genes that are not expressed in renal tissue (Array express ([www.ebi.ac.uk/arrayexpress/](http://www.ebi.ac.uk/arrayexpress/))) were also removed, leaving 155 genes. Of these, 19 genes have previously been assessed for promoter methylation in RCC including *KRT19*, *COL1A1* and *IGFBP1* that are frequently methylated in RCC (banez de Caceres et al., 2006; Morris et al., 2008) (Supplementary table 2 lists the genes identified by this filtering process in full).

To evaluate the microarray fold-change filtering selection criteria we analysed the methylation status of 3 genes (*LRCH1*, *KLF2* *FZD8*) that had expression fold-changes of <10 (range 2 to 7-fold). All three genes were unmethylated in the cell lines (supplementary table 1).

24 genes from our shortlist were analysed for promoter methylation in RCC cell lines and primary tumours. In addition we analysed promoter methylation status of *RPRM* which, although not meeting the selection criteria had previously been shown to be inactivated by methylation in a number of other tumour types (Bernal et al., 2008; Sato et al., 2006; Takahashi et al., 2005). One of the 25 genes (*NOG*) did not demonstrate detectable promoter region hypermethylation. Six genes (*DMRTB1*, *SCYA20*, *CLDN6*, *TPM2L1*, *LOXL* and *ZFP42*) were methylated in RCC cell lines and tumours but also in non-malignant renal tissue and so were not investigated further. A further 10 genes demonstrated both promoter region methylation in RCC cell lines but no (*FOXP1B*, *RARRES1*, *DKK1* and *UQCRH*) or infrequent (*RGPD5* (7%), *ITGBL* (9%), *KLF4* (10%), *TLL1* (10%), *PTGIS* (13%) and *EMX2* (18%)) promoter methylation in primary RCC tumours.

Eight of 28 (29%) genes (*BNC1*, *COL14A1*, *COL15A1* *CST6*, *SFRP1*, *GREM1*, *RPRM* and *PDLIM4*) demonstrated frequent (methylation frequency >30%, range 35-53%) methylation in primary RCC, no methylation in normal kidney from non-RCC patients and upregulation of gene expression following demethylation of methylated RCC cell lines (Table 1, Fig 1A and 2A). Five of 8 genes were methylated in renal tumours but not (or rarely) in matched normal renal tissue (*BNC1*, *COL14A1*, *CST6*, *PDLIM4* and *SFRP1*).

The promoter region of the 5' CpG island of *BNC1* was methylated in 46% (27/59) of primary RCC (Fig 2B). One of 20 (5%) adjacent normal samples (from a patient with a methylated tumour) also demonstrated promoter methylation. Methylation of the *COL14A1* promoter was detected in 44% (18/41) primary RCC tumours analysed (1/20 adjacent normal kidney samples (from a patient with a methylated tumour) and 0/6 samples from patients without kidney cancer) (Fig 1B). Methylation of *PDLIM4* promoter was detected in 43% (13/30) primary RCC but not in any normal renal tissue samples (n=22) (Fig 1C). *SFRP1* promoter methylation was present in 34% (20/58) sporadic primary RCC but not in adjacent normal tissue samples (Fig 2B). The *CST6* predicted promoter was methylated in 46% (28/61) primary RCC. A small number of normal kidney samples adjacent to RCC (11%, 4/35) demonstrated a low level of promoter methylation (all of which showed methylation in the matched tumour tissue) (Fig 1D).

Three genes, *COL15A1*, *RPRM* and *GREM1* demonstrated frequent promoter methylation in primary RCC (53% (34/65), 44% (23/52) and 41% (11/27) respectively) (Fig 2B), but methylation was also detected relatively frequently in macroscopically normal renal tissue from patients with RCC (30% (9/30), 18% (8/44), 24% (7/29) respectively (P=0.034, P=0.009 and P=0.025 respectively compared to primary RCC). However there was a strong correlation between the presence of methylation in normal renal tissue and in tumour material. Thus 8/9 normal renal tissue with *COL15A1* methylation in normal tissue also displayed methylation in the corresponding tumour and the corresponding figures for *RPRM* and *GREM1* were 7/8 and 5/7.

### Functional Analysis of the tumour suppressor activity of epigenetically inactivated genes

The effect of re-expressing four of the epigenetically silenced RCC genes (*SFRP1*, *RPRM*, *CST6*, and *BNC1*) on *in vitro* growth characteristics of RCC cell lines was assessed using colony formation and anchorage independent growth assays.

**Colony formation**—The tumour suppressor activity of *SFRP1*, *RPRM*, *CST6*, and *BNC1* was assessed by *in vitro* colony formation assays. Following transfection of wild-type *BNC1*, *CST6*, *RPRM* and *SFRP1* expression plasmids into non-expressing RCC cell lines (RCC4, 786-0, SKRC39 and SKRC47 respectively) there was a significantly reduced number of G418 resistant colonies compared to cell lines transfected with an empty vector control for 3 independent experiments (Fig 3A).

The number of *BNC1* expressing RCC4 clones was reduced by 77% compared to those transfected with an empty plasmid (SD=10%, t=12.43, P<0.0001). Re-expression of *CST6* in 786-0 cells reduced their colony forming ability by 47% (SD=6%, t=6.629, P=0.003). *RPRM* expressing SKRC39 clone numbers were reduced by 40% (SD=5%, t=8.328, P=0.001). The number of *SFRP1* expressing SKRC47 clones was reduced by 76% compared to SKRC47 clones containing the empty vector (SD=5%, t=16.644, P<0.0001) (Fig. 3A).

**Anchorage independent growth**—The effect of re-expression of, *RPRM* and *CST6* on anchorage-independent growth in a soft agar colony formation assay was assessed in stably transfected RCC cell line clones. Colonies were counted after initial seeding and incubation in soft agar for 5 weeks. Each experiment was done in triplicate with 3 independent clones. Cells transfected with empty vector showed robust colony growth, whereas, colony growth was greatly reduced when *CST6* was re-expressed in 786-0 cells, both the number and size of colonies was statistically significantly reduced. The number of colonies  $100\mu\text{m}$  was reduced by 53% (SD=4%, t=16.15, P<0.0001) in clones expressing *CST6* when compared to the control clones (Fig. 3B).



Re-expression of *RPRM* in SKRC39 cells did not significantly alter the anchorage independent growth capabilities of SKRC39 cells. Both clones re-expressing *RPRM* and those with *RPRM* silenced grew robustly when suspended in soft agar.

As the *BNC1* silenced RCC4 cell line did not grow well in an anchorage independent manner, to investigate the effect of alterations of *BNC1* expression on cell growth we produced *BNC1* over-expressing clones of HEK293s. Whereas HEK293 cells transfected with an empty vector grew robustly, *BNC1* overexpressing clones produced statistically significantly fewer colonies 100 $\mu$ m (mean ( $\pm$ SD) reduction 54% ( $\pm$ 5%),  $t=5.92$   $P=0.001$ ) (Fig 3B).

To investigate the possible tumourigenic advantage of loss of expression of *BNC1*, *SFRP1* and *COL14A* we utilised RNAi methodology to knock down *BNC1* and *SFRP1* expression in HEK293s and *COL14A* expression in the KTCL26 RCC cell line (sufficient knockdown of *COL14A* expression was obtained in HEK293 cells). Twenty-four hours after RNAi transfection cells were seeded into 3% agar, colonies >100 $\mu$ m were counted 21 days later. 55% more colonies >100 $\mu$ m were produced by HEK293 cells with reduced *BNC1* (SD=11%,  $t=-6.9$ ,  $p<0.0001$ ) expression compared to cells transfected with a control RNAi oligo (Fig 4A). Reduced expression of *SFRP1* resulted in 74% more colonies >100 $\mu$ m (SD=6%,  $t=-14.3$ ,  $p<0.0001$ ) than control cells (Fig 4A). The growth suppressing activity of re-expression of *SFRP1* in RCC cell lines has been previously demonstrated (Gumz et al., 2007). However, to our knowledge, this is the first time that an increase in growth potential has been associated with reduced *SFRP1* expression in RCC. The colony forming capability of the RCC cell line KTCL26 was less than that of HEK293s. However, this was increased by 93% in those cells where *COL14A* expression was reduced by RNAi (SD=9%,  $t=-10.44$ ,  $p<0.0001$ ) (Fig 4B).

### Analysis of patient survival and gene methylation

We initially investigated whether the presence of tumour methylation for *BNC1*, *PDLIM4*, *RPRM*, *CST6*, *SFRP1*, *GREM1*, *COL14A1* and *COL15A1* was associated with changes in patient survival (time to cancer death) or recurrence/cancer death. No significant association was detected for *CST6* ( $P=0.38$  and  $P=0.76$  respectively by Kaplan-Meier analysis), *COL15A1* ( $P=0.25$  and  $P=0.16$ ), *GREM1* (0.07 and 0.08), *RPRM* ( $P=0.83$  and 0.94), or *PDLIM4* ( $P=0.38$  and 0.32). However methylation of *BNC1* ( $P=0.017$  (Hazard ratio (HR)= 3.69 (95% CI= 1.27-10.93) and  $P=0.018$  (HR=3.07 (95% CI= 1.22-8.16)), *COL14A1* ( $P=0.005$  (HR= 4.07 (95% CI= 1.73-20.3) and  $P=0.007$  (HR=3.49 (95% CI= 1.55-16.1)) or *SFRP1* ( $P=0.002$  (HR=4.88 (95% CI= 2.76-87.95)) and  $P=0.003$  (HR=4.44 (95% CI= 2.75-86.2)) was associated with a significantly poorer prognosis (see Figure 4A-C). We then investigated the relationship between survival, tumour grade, size and stage and methylation status of *BNC1*, *COL14A1* and *SFRP1* using Cox proportional hazard analysis. Analysis for *SFRP1* methylation, grade, size and stage gave a strong association with survival ( $P=0.0002$ ) but the effect of *SFRP1* methylation status was not statistically significant ( $P=0.4$ ). However for a similar analysis for *BNC1*, tumour stage ( $P=0.019$ ; HR=2.4536 (95% confidence interval (95% CI)= 1.16-5.19)), *BNC1* methylation ( $P=0.033$ ; HR=4.87 (95% CI 1.14-20.888) and tumour size ( $p=0.04$ ; HR=1.23 (95% CI= 1.009-1.49) were each significantly associated with prognosis, and under a similar analysis for *COL14A1*, methylation of *COL14A1* ( $P=0.0067$ ) (Hazard ratio (HR)= 6.56 (95% CI 1.69 to 25.38) and tumour stage ( $p=0.0009$ ) (HR=3.42 (95% CI 1.67 to 7.02) were identified as significant prognostic factors. A combined analysis was then performed for survival and *BNC1* and *COL14A1* methylation status, tumour grade, size and stage. Methylation of *COL14A1* ( $P=0.0067$ ; HR=18.37 (95% CI=2.27-148.8)) was the most significant predictor of survival, followed by stage ( $P=0.015$ ; HR=3.5 (95% CI=1.28-9.57) and grade ( $P=0.08$ ; HR=2.03 (95% CI=0.92-4.46) (*BNC1* methylation status was not a significant prognostic factor ( $P=0.38$ ) (Figure 4D).

There were no significant differences in *BNC1*, *PDLIM4*, *RPRM*, *CST6*, *SFRP1*, *GREMI*, *COL14A1* and *COL15A1* promoter methylation frequencies between clear cell RCC and non-clear cell RCC) and no significant differences between methylation status of *VHL* mutated and *VHL* wild-type clear cell RCC.

## DISCUSSION

Cancer specific genetic and epigenetic alterations resulting in tumour suppressor gene (TSG) inactivation are frequent events in RCC. However, whereas the mutation spectrum causing TSG inactivation is usually diverse (so limiting the utility for tumour screening programmes of detecting an individual TSG mutation), TSG inactivation by promoter hypermethylation provides a more homogeneous target for molecular screening strategies. Furthermore, large scale gene sequencing studies of RCC and other cancers have revealed that relatively few genes are mutated frequently. Thus, apart from *VHL*, there are no reports of a TSG that is frequently mutated in RCC (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>). In contrast, at least 10 genes have been reported to be hypermethylated >20% of RCC (13 and references within) and we have now reported a further 8 genes that demonstrate promoter methylation in >30% of RCC.

Functional epigenomic screens provide a useful strategy to identify novel TSGs and have been employed to identify epigenetically inactivated TSGs in a number of different tumour types (Lodygin et al., 2005; Sato et al., 2003; Shames et al., 2006; Yamashita et al., 2002), including two studies of RCC (Ibanez de Caceres et al., 2006; Morris et al., 2008; Morris et al., 2005). Previously we identified *SPINT2* as a novel RCC TSG (Morris et al., 2005), but many candidate TSGs upregulated after treatment of RCC cell lines with 5-Aza-2'-deoxycytidine do not show tumour-specific promoter methylation (Morris et al., 2008). Therefore in order to maximise the potential for identifying additional genes epigenetically silenced in RCC we extended our previous study (Morris et al., 2008; Morris et al., 2005) to include more cell lines (11 versus 4) and higher density microarrays (47000 transcripts versus 11000 genes). Seven of the 8 genes we report in this study (*BNC1*, *RPRM*, *CST6*, *PDLIM4*, *GREMI*, *COL14A1* and *COL15A1*) have not previously been reported to be methylated in RCC, and, to our knowledge, *COL14A1* and *COL15A1* have not previously been reported to be methylated in neoplasia. While this study was in progress *SFRP1* was reported to be methylated in RCC (Awakura et al., 2008; Dahl et al., 2007; Gumz et al., 2007)

*SFRP1* encodes a negative regulator the Wnt/beta-catenin pathway. Thus SFRP1 molecules bind to and sequester Wnt molecules away from their cognate receptors, the Frizzled family. Activation of the Wnt/beta-catenin pathway is associated with upregulation of a range of oncogenic targets (e.g. Cyclin D1, VEGF, cMYC and cMET) implicated in the pathogenesis of cancer. We detected *SFRP1* promoter methylation in 34% of RCC compared to 68% in the study of Dahl et al (Dahl et al., 2007). Re-expression of *SFRP1* in a null RCC-cell line has previously been shown to associate with reduced tumour cell growth (Gumz et al., 2007). We confirmed this and also demonstrated that reduced expression of endogenous *SFRP1* increases the tumorigenic potential of an RCC-cell line.

*RPRM* (Reprimo, TP53 dependent G2 arrest mediator candidate) is a downstream mediator of p53-induced G2 cell cycle arrest (Ohki et al., 2000). Reprimo induced cell cycle arrest is thought to be mediated by an indirect inhibition of Cdc2-CyclinB1 complex translocation to the nucleus (Ohki et al., 2000). Epigenetic silencing of *RPRM* has previously been reported in a variety of cancers including gastric, gall bladder, colorectal, oesophageal, breast and prostate (Bernal et al., 2008; Sato et al., 2006; Takahashi et al., 2005). We have now

demonstrated that it is also frequently methylated in RCC and that re-expression *RPRM* reduces the tumour forming properties of RCC-derived cell lines.

*CST6* is a type 2 secreted cystatin. It inhibits Cathepsin B, L, H and V, members of a family of lysosomal proteases. Cathepsins are involved in multiple biological processes such as apoptosis, intracellular protein catabolism and pericellular matrix re-modelling (Bromme & Kaleta, 2002). It has been suggested that an imbalance of cathepsins and their respective inhibitors may promote tumour cell invasion (Bellail et al., 2004) and so loss of *CST6* expression by epigenetic silencing might be predicted to contribute to the malignant phenotype. Consistent with this we found that re-expression of *CST6* reduced the ability of RCC cells to form colonies in an anchorage-independent manner. *CST6* has also been shown to be epigenetically silenced and reduced *in vitro* tumourigenicity in breast cancer (Ai et al., 2006; Shridhar et al., 2004) and gliomas (Qiu et al., 2008).

*PDLIM4* encodes a LIM domain candidate tumour gene mapping to 5q31.1. Although methylation of *PDLIM4* has not been reported previously in RCC tumours, Bumber et al (Bumber et al., 2007) reported frequent *PDLIM4* promoter methylation in acute myelogenous leukaemia and colon cancer. Gremlin (*GREM1*) is an inhibitor of TGFbeta signalling that has previously been reported to be frequently methylated in lung, breast and bladder cancers (Suzuki et al., 2005). We found frequent methylation of *GREM1* suggesting that this may contribute to the perturbed TGFbeta signalling that is detected in many human cancers, including RCC. *GREM1* methylation was detected in some samples of adjacent normal renal tissue from RCC patients with *GREM1* tumour methylation. Similar findings were obtained for *RPRM* and *COL15A1*, these results would be consistent with the hypothesis that *GREM1*, *RPRM* and *COL15A1* methylation might occur as part of a premalignant field effect, as has been described in bronchial epithelium (Wistuba, 2007).

Basonuclin (*BNC1*) is a zinc finger transcription factor that interacts with the promoters of both RNA polymerases I and II. *In silico* analysis suggests that basonuclin target genes may be implicated in chromatin structure, transcription/DNA-binding, adhesion/cell-cell junction, signal transduction, and intracellular transport (Wang et al., 2006). Shames et al (Shames et al., 2006) reported that *BNC1* was methylated in breast, lung, prostate and colon cancers, suggesting that *BNC1* methylation might be of relevant to the diagnosis and management of a range of human cancers.

Ibanez et al. have reported epigenetic silencing of *COL1A1* in RCC (Ibanez de Caceres et al., 2006). In this study we identified frequent methylation of *COL15A1* and *COL14A1* in RCC. *COL15A1* encodes a nonfibrillary proteoglycan which is found in many tissue types forming an integral unit of the collagenous network subjacent to the basement membrane (Amenta et al., 2005). The COOH-terminal end of COL15A1 which has homology to endostatin has been shown to have anti-angiogenic properties and is capable of inhibiting tumour growth in a xenograph renal model (Ramchandran et al., 1999). Moreover, recently COL15A1 was identified in a fibroblast-tumour cell hybrid screen as a potent suppressor of tumour growth, and re-expression of COL15A1 in a HeLa derivative cell line completely suppressed tumour formation in nude mice (Harris et al., 2007). We have identified frequent *COL15A1* methylation in RCC (53%). Collagen XIVA1 (*COL14A1*) is a large extracellular matrix glycoprotein that associate with mature collagen fibrils (Schuppan et al., 1990). Although methylation of *COL14A1* has not previously been reported in human cancer, Schuppan et al (1990) noted frequent absence of COL14A1 in the vicinity of invading tumours such as Kaposi sarcoma and oral squamous cell carcinoma. In addition we note data from the Sanger cancer genome re-sequencing project (<http://www.sanger.ac.uk/genetics/CGP/Studies/>) that identifies *COL14A1* mutations in ~1% (n=4) of RCC sequenced (n=412). COL14A1 interacts with Decorin (Ehnis et al., 1997) which regulates



fibrogenesis and downregulates activity of a number of receptors (EGFR, IGF-IR, LRP) implicated in cell growth and survival (Brandan et al., 2006; Santra et al., 2002; Schaefer et al., 2007).

Loss of expression of epigenetically silenced TSGs can affect a wide variety of cellular processes, including those which directly affect cell growth and proliferation. We investigated whether re-expressing *SFRP1*, *RPRM*, *CST6* and *BNC1* in RCC cell lines would influence *in vitro* analyses of tumorigenesis. *BNC1* has not previously been demonstrated to affect tumour cell growth, but we found that re-expression of *BNC1* suppressed tumour cell growth. Similar results were found for *RPRM*, *CST6* and *SFRP1*. Although *RPRM* and *CST6* have not previously been studied in RCC these findings are consistent with those reported in cervical and breast cancer cell lines (Ohki et al., 2000; Shridhar et al., 2004). We have also shown that reducing the expression of *BNC1*, *SFRP1* and *COL14A1* in an embryonic kidney cell line or an RCC-cell line increased tumorigenic potential as assessed by soft agar colony formation assays.

The methylated genes identified in this and previous studies of RCC (Ibanez de Caceres et al., 2006; Morris et al., 2008; Morris et al., 2005; Morris et al., 2003), represent a variety of functions. However we note that many of these genes can be linked to pathways associated with the *VHL* TSG that has a gatekeeper function for RCC (analogous to that of *APC* in colorectal cancer). The *VHL* gene product has a key role in regulating the hypoxia inducible transcription factors HIF-1 and HIF-2 and, in turn, these influence a wide repertoire of target genes. Although dysregulation of hypoxia-inducible genes (particularly those regulated by HIF-2) is strongly linked to risk of RCC with *VHL* inactivation, HIF-dysregulation appears to be necessary but insufficient for *VHL*-related RCC tumorigenesis (Clifford et al., 2001; Raval et al., 2005). A number of HIF-independent functions have been ascribed to pVHL. In the context of *COL14A1* and *COL15A1* methylation it is interesting to note that analysis of *Caenorhabditis elegans* worms mutant for *vhl-1* demonstrated a clear HIF-independent link between pVHL and extracellular matrix function (Bishop et al., 2004). Recently, pVHL has been reported to regulate the p53 pathway (relevant to *RPRM*) and the Wnt/beta catenin pathway (downstream of *SFRP1*) by targeting beta catenin for proteasomal degradation (Chitalia et al., 2008).

The identification of frequently methylated RCC TSGs can highlight critical pathways that might be targeted for novel therapeutic interventions. In addition, the detection of methylated DNA in urine or serum might be used as a biomarkers for the diagnosis, staging or risk stratification of RCC (Battagli et al., 2003; Hoque et al., 2004; Urakami et al., 2006). *VHL* inactivation occurs early in tumorigenesis but, to date, has not been demonstrated to be a significant prognostic indicator (Smits et al., 2008). Thus, given the infrequency of mutations in other TSGs in RCC, detection of TSG methylation would appear to presents a promising strategy for prognostic biomarkers in RCC. However, only a few potential RCC methylation prognostic biomarkers have been reported (Breault et al., 2005; Costa et al., 2007; Christoph et al., 2006; Gonzalogo et al., 2004; McDonald et al., 2009; Yamada et al., 2006). We found that methylation of *BNC1*, *COL14A1* and *SFRP1* was each associated with significantly poorer patient survival. However for *SFRP1* this association was not independent of conventional prognostic factors (stage, size and grade). In multivariate analysis with conventional prognostic factors, methylation of *COL14A1* and *BNC1* were each significant predictors of poorer survival. Combining the *COL14A1* and *BNC1* methylation data identified *COL14A1* methylation as an independent prognostic factor with higher statistical significance than stage, size and grade.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

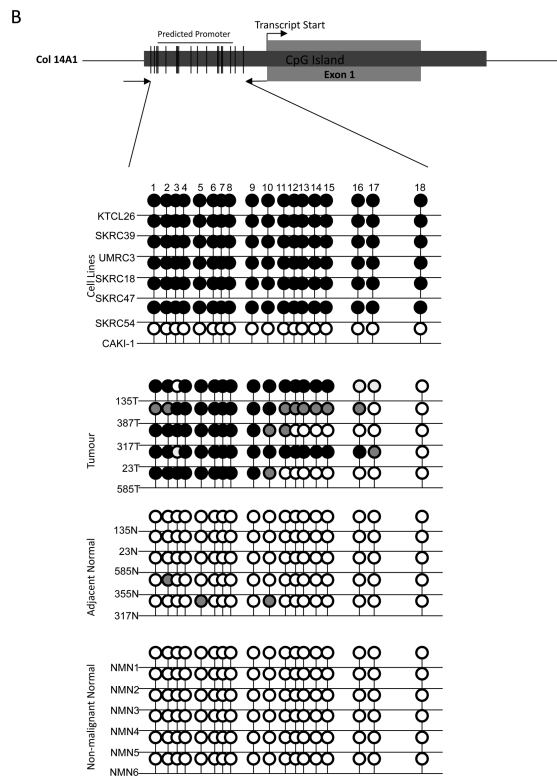
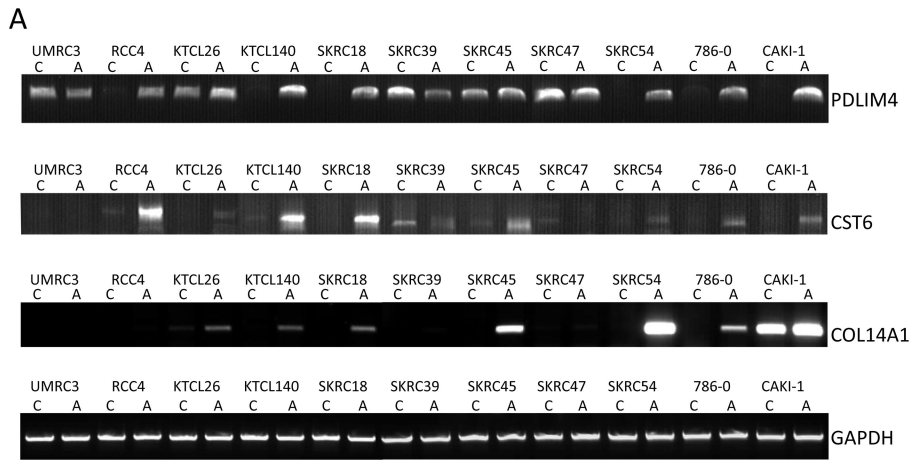
We thank Cancer Research UK for financial support.

## References

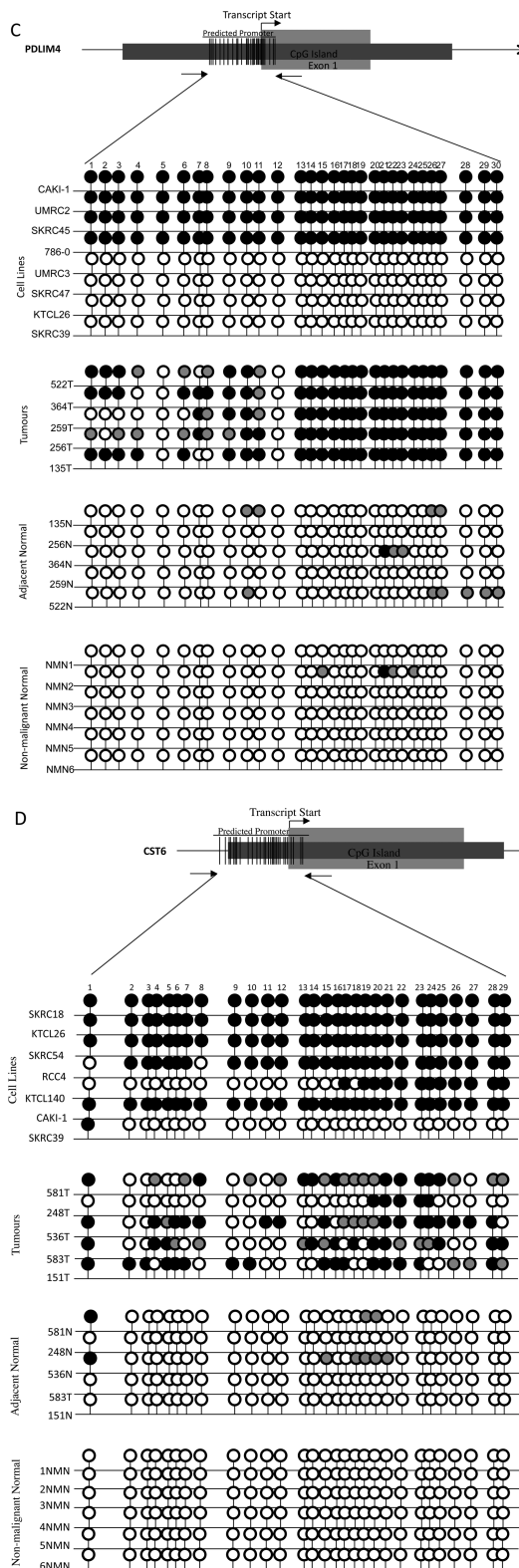
- Ai L, Kim WJ, Kim TY, Fields CR, Massoll NA, Robertson KD, Brown KD. *Cancer Res.* 2006; 66:7899–909. [PubMed: 16912163]
- Amenta PS, Scivoletti NA, Newman MD, Sciancalepore JP, Li D, Myers JC. *J Histochem Cytochem.* 2005; 53:165–76. [PubMed: 15684329]
- Awakura Y, Nakamura E, Ito N, Kamoto T, Ogawa O. *Oncol Rep.* 2007; 20:1257–63. [PubMed: 18949430]
- Battagli C, Uzzo RG, Dulaimi E, Ibanez de Caceres I, Krassenstein R, Al-Saleem T, Greenberg RE, Cairns P. *Cancer Res.* 2003; 63:8695–9. [PubMed: 14695183]
- Bellail AC, Hunter SB, Brat DJ, Tan C, Van Meir EG. *Int J Biochem Cell Biol.* 2004; 36:1046–69. [PubMed: 15094120]
- Bernal C, Aguayo F, Villarroel C, Vargas M, Diaz I, Ossandon FJ, Santibanez E, Palma M, Aravena E, Barrientos C, Corvalan AH. *Clin Cancer Res.* 2008; 14:6264–9. [PubMed: 18829507]
- Bishop T, Lau KW, Epstein AC, Kim SK, Jiang M, O'Rourke D, Pugh CW, Gleadle JM, Taylor MS, Hodgkin J, Ratcliffe PJ. *PLoS Biol.* 2004; 2:e289. [PubMed: 15361934]
- Boumber YA, Kondo Y, Chen X, Shen L, Gharibyan V, Konishi K, Estey E, Kantarjian H, Garcia-Manero G, Issa JP. *Cancer Res.* 2007; 67:1997–2005. [PubMed: 17332327]
- Brandan E, Retamal C, Cabello-Verrugio C, Marzolo MP. *J Biol Chem.* 2006; 281:31562–71. [PubMed: 16936287]
- Breault JE, Shiina H, Igawa M, Ribeiro-Filho LA, Deguchi M, Enokida H, Urakami S, Terashima M, Nakagawa M, Kane CJ, Carroll PR, Dahiya R. *Clin Cancer Res.* 2005; 11:557–64. [PubMed: 15701841]
- Bromme D, Kaleta J. *Curr Pharm Des.* 2002; 8:1639–58. [PubMed: 12132996]
- Chitalia VC, Foy RL, Bachschmid MM, Zeng L, Panchenko MV, Zhou MI, Bharti A, Seldin DC, Lecker SH, Dominguez I, Cohen HT. *Nat Cell Biol.* 2008; 10:1208–16. [PubMed: 18806787]
- Chowdhury S, Larkin JM, Gore ME. *Eur J Cancer.* 2008; 44:2152–61. [PubMed: 18829302]
- Christoph F, Weikert S, Kempkensteffen C, Krause H, Schostak M, Kollermann J, Miller K, Schrader M. *Clin Cancer Res.* 2006; 12:5040–6. [PubMed: 16951219]
- Clifford SC, Cockman ME, Smallwood AC, Mole DR, Woodward ER, Maxwell PH, Ratcliffe PJ, Maher ER. *Hum Mol Genet.* 2001; 10:1029–38. [PubMed: 11331613]
- Clifford SC, Prowse AH, Affara NA, Buys CH, Maher ER. *Genes Chromosomes Cancer.* 1998; 22:200–9. [PubMed: 9624531]
- Costa VL, Henrique R, Ribeiro FR, Pinto M, Oliveira J, Lobo F, Teixeira MR, Jerónimo C. *BMC Cancer.* 2007; 23:133. [PubMed: 17645803]
- Dahl E, Wiesmann F, Woenckhaus M, Stoehr R, Wild PJ, Veeck J, Knuchel R, Klopocki E, Sauter G, Simon R, Wieland WF, Walter B, Denzinger S, Hartmann A, Hammerschmid CG. *Oncogene.* 2007; 26:5680–91. [PubMed: 17353908]
- Dallol A, Forgacs E, Martinez A, Sekido Y, Walker R, Kishida T, Rabbitts P, Maher ER, Minna JD, Latif F. *Oncogene.* 2002; 21:3020–8. [PubMed: 12082532]
- Ehnis T, Dieterich W, Bauer M, Kresse H, Schuppan D. *J Biol Chem.* 1997; 272:20414–9. [PubMed: 9252349]
- Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. *Ann Oncol.* 2007; 18:581–92. [PubMed: 17287242]

- Foster K, Prowse A, van den Berg A, Fleming S, Hulsbeek MM, Crossey PA, Richards FM, Cairns P, Affara NA, Ferguson-Smith MA, et al. *Hum Mol Genet.* 1994; 3:2169–73. [PubMed: 7881415]
- Gonzalzo ML, Yegnasubramanian S, Yan G, Rogers CG, Nicol TL, Nelson WG, Pavlovich CP. *Clin Cancer Res.* 2004; 10:7276–83. [PubMed: 15534102]
- Gumz ML, Zou H, Kreinest PA, Childs AC, Belmonte LS, LeGrand SN, Wu KJ, Luxon BA, Sinha M, Parker AS, Sun LZ, Ahlquist DA, Wood CG, Copland JA. *Clin Cancer Res.* 2007; 13:4740–9. [PubMed: 17699851]
- Harris A, Harris H, Hollingsworth MA. *Mol Cancer Res.* 2007; 5:1241–5. [PubMed: 18171981]
- Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarr JR, Linehan WM, et al. *Proc Natl Acad Sci U S A.* 1994; 91:9700–4. [PubMed: 7937876]
- Hoque MO, Begum S, Topaloglu O, Jeronimo C, Mambo E, Westra WH, Califano JA, Sidransky D. *Cancer Res.* 2004; 64:5511–7. [PubMed: 15289362]
- Ibanez de Caceres I, Dulaimi E, Hoffman AM, Al-Saleem T, Uzzo RG, Cairns P. *Cancer Res.* 2006; 66:5021–8. [PubMed: 16707423]
- Latif F, Tory K, Gnarr J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L, et al. *Science.* 1993; 260:1317–20. [PubMed: 8493574]
- Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H. *Cancer Res.* 2005; 65:4218–27. [PubMed: 15899813]
- Mancini V, Battaglia M, Ditonno P, Palazzo S, Lastilla G, Montironi R, Bettocchi C, Cavalcanti E, Ranieri E, Selvaggi FP. *Urol Oncol.* 2008; 26:225–38. [PubMed: 18452811]
- Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. *Nature.* 1999; 399:271–5. [PubMed: 10353251]
- McRonald FE, Morris MR, Gentle D, Winchester L, Baban D, Ragoussis J, Clarke NW, Brown MD, Kishida T, Yao M, Latif F, Maher ER. *Mol Cancer.* 2009; 8:31. [PubMed: 19493342]
- Morris MR, Gentle D, Abdulrahman M, Clarke N, Brown M, Kishida T, Yao M, Teh BT, Latif F, Maher ER. *Br J Cancer.* 2008; 98:496–501. [PubMed: 18195710]
- Morris MR, Gentle D, Abdulrahman M, Maina EN, Gupta K, Banks RE, Wiesener MS, Kishida T, Yao M, Teh B, Latif F, Maher ER. *Cancer Res.* 2005; 65:4598–606. [PubMed: 15930277]
- Morris MR, Hesson LB, Wagner KJ, Morgan NV, Astuti D, Lees RD, Cooper WN, Lee J, Gentle D, Macdonald F, Kishida T, Grundy R, Yao M, Latif F, Maher ER. *Oncogene.* 2003; 22:6794–801. [PubMed: 14555992]
- Morrissey C, Martinez A, Zatyka M, Agathangelou A, Honorio S, Astuti D, Morgan NV, Moch H, Richards FM, Kishida T, Yao M, Schraml P, Latif F, Maher ER. *Cancer Res.* 2001; 61:7277–81. [PubMed: 11585766]
- Nomoto S, Kinoshita T, Kato K, Otani S, Kasuya H, Takeda S, Kanazumi N, Sugimoto H, Nakao A. *Br J Cancer.* 2007; 97:1260–5. [PubMed: 17968429]
- Ohki R, Nemoto J, Murasawa H, Oda E, Inazawa J, Tanaka N, Taniguchi T. *J Biol Chem.* 2000; 275:22627–30. [PubMed: 10930422]
- Qiu J, Ai L, Ramachandran C, Yao B, Gopalakrishnan S, Fields CR, Delmas AL, Dyer LM, Melnick SJ, Yachnis AT, Schwartz PH, Fine HA, Brown KD, Robertson KD. *Lab Invest.* 2008; 88:910–25. [PubMed: 18607344]
- Ramchandran R, Dhanabal M, Volk R, Waterman MJ, Segal M, Lu H, Knebelmann B, Sukhatme VP. *Biochem Biophys Res Commun.* 1999; 255:735–9. [PubMed: 10049780]
- Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. *Mol Cell Biol.* 2005; 25:5675–86. [PubMed: 15964822]
- Santra M, Reed CC, Iozzo RV. *J Biol Chem.* 2002; 277:35671–81. [PubMed: 12105206]
- Sato N, Fukushima N, Maitra A, Matsubayashi H, Yeo CJ, Cameron JL, Hruban RH, Goggins M. *Cancer Res.* 2003; 63:3735–42. [PubMed: 12839967]
- Sato N, Fukushima N, Matsubayashi H, Iacobuzio-Donahue CA, Yeo CJ, Goggins M. *Cancer.* 2006; 107:251–7. [PubMed: 16752411]
- Schaefer L, Tsalastra W, Babelova A, Baliova M, Minnerup J, Sorokin L, Grone HJ, Reinhardt DP, Pfeilschifter J, Iozzo RV, Schaefer RM. *Am J Pathol.* 2007; 170:301–15. [PubMed: 17200203]

- Schuppan D, Cantaluppi MC, Becker J, Veit A, Bunte T, Troyer D, Schuppan F, Schmid M, Ackermann R, Hahn EG. *J Biol Chem*. 1990; 265:8823–32. [PubMed: 2187872]
- Shames DS, Girard L, Gao B, Sato M, Lewis CM, Shivapurkar N, Jiang A, Perou CM, Kim YH, Pollack JR, Fong KM, Lam CL, Wong M, Shyr Y, Nanda R, Olopade OI, Gerald W, Euhus DM, Shay JW, Gazdar AF, Minna JD. *PLoS Med*. 2006; 3:e486. [PubMed: 17194187]
- Shridhar R, Zhang J, Song J, Booth BA, Kevil CG, Sotiropoulou G, Sloane BF, Keppler D. *Oncogene*. 2004; 23:2206–15. [PubMed: 14676833]
- Smits KM, Schouten LJ, van Dijk BA, Hulsbergen-van de Kaa CA, Wouters KA, Oosterwijk E, van Engeland M, van den Brandt PA. *Clin Cancer Res*. 2008; 14:782–7. [PubMed: 18245539]
- Suzuki M, Shigematsu H, Shames DS, Sunaga N, Takahashi T, Shivapurkar N, Iizasa T, Frenkel EP, Minna JD, Fujisawa T, Gazdar AF. *Br J Cancer*. 2005; 93:1029–37. [PubMed: 16234815]
- Takahashi T, Suzuki M, Shigematsu H, Shivapurkar N, Echebiri C, Nomura M, Stastny V, Augustus M, Wu CW, Wistuba II, Meltzer SJ, Gazdar AF. *Int J Cancer*. 2005; 115:503–10. [PubMed: 15700311]
- Urakami S, Shiina H, Enokida H, Hirata H, Kawamoto K, Kawakami T, Kikuno N, Tanaka Y, Majid S, Nakagawa M, Igawa M, Dahiya R. *Clin Cancer Res*. 2006; 12:6989–97. [PubMed: 17145819]
- Wang J, Zhang S, Schultz RM, Tseng H. *Biochem Biophys Res Commun*. 2006; 348:1261–71. [PubMed: 16919236]
- Wistuba II. *Curr Mol Med*. 2007; 7:3–14. [PubMed: 17311529]
- Yamada D, Kikuchi S, Williams YN, Sakurai-Yageta M, Masuda M, Maruyama T, Tomita K, Gutmann DH, Kakizoe T, Kitamura T, Kanai Y, Murakami Y. *Int J Cancer*. 2006; 118:916–23. [PubMed: 16152585]
- Yamashita K, Upadhyay S, Osada M, Hoque MO, Xiao Y, Mori M, Sato F, Meltzer SJ, Sidransky D. *Cancer Cell*. 2002; 2:485–95. [PubMed: 12498717]



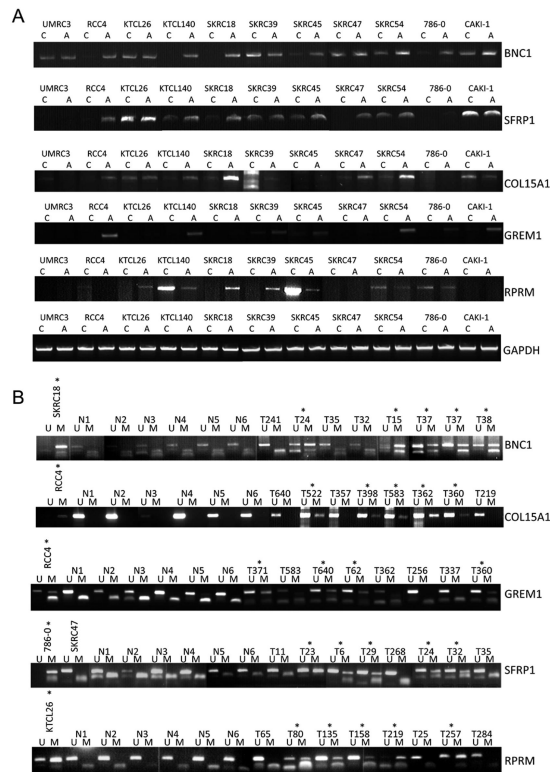




**Figure 1.**  
**A, RT-PCR analysis of *PDLIM4*, *CST6* and *COL14A* expression. *PDLIM4* expression was restored (SKRC18, SKRC54, CAKI-1)/up-regulated (RCC4, KTCL140, 786-0) in 6 of**

11 cell lines following treatment with the demethylating agent, 5-Aza-2'-deoxycytidine. *CST6* expression was restored (KTCL26, SKRC18, SKRC54, 786-0, CAKI-1)/up-regulated (RCC4, KTCL140, SKRC45) in 8/11 cell lines. Global demethylation restored expression of *COL14A* in 5/11 cell lines (KTCL140, SKRC18, SKRC45, SKRC54, 786-0).

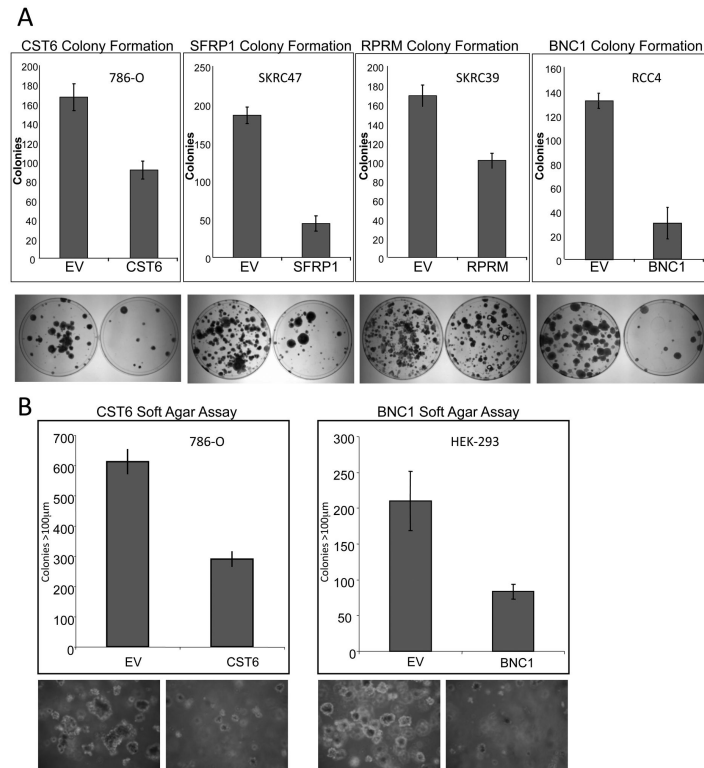
**B-D, Bisulphite sequencing analysis of *PDLIM4*, *CST6* and *COL14A1* 5' CpG island regions.** Primers were designed to amplify the predicted promoter region of the 5' CpG island of *PDLIM4*, *CST6* and *COL14A*. The schematic represents the region sequenced; vertical lines indicate individual CpGs. Sequencing indicated methylation in RCC cell lines correlated with gene silencing/ down-regulation: black circle; complete methylation; grey circle; partial methylation; empty circle; no methylation. Bisulfite sequencing of RCC tumours and adjacent normal tissue showed frequent methylation in tumours and infrequent methylation in normal tissue (see results for details). Analysis of normal tissue derived from non-malignant kidneys indicated no CpG island methylation, with the exception of *PDLIM4* in which 3 CpGs were partially and 1 CpG was fully methylated in 1 non-malignant sample (NMN2). T=Tumour, N= normal tissue adjacent to tumour, NMN= non-malignant normal tissue. Representative sequencing traces are shown in supplementary fig. 1.



**Figure 2.**

**A, RT-PCR analysis of *BNC1*, *SFRP1* and *COL15A1*, *GREM1*, and *RPRM* expression.** *BNC1* expression was restored (RCC4, KTCL140, SKRC18)/up-regulated (SKRC45, 786-0) in 5 of 11 cell lines following treatment with the demethylating agent, 5-Aza-2'-deoxycytidine. *SFRP1* expression was restored (RCC4, SKRC47)/up-regulated (KTCL140, SKRC18, SKRC45) in 5/11 cell lines. *COL15A1* expression was restored (RCC4)/up-regulated (KTCL140, SKRC18, SKRC47, SKRC54) in 5/11 cell lines. *GREM1* expression was restored (RCC4, SKRC140, SKRC54, CAKI-1)/up-regulated (SKRC39) in 5/11 cell lines following treatment with the demethylating agent, 5-Aza-2'-deoxycytidine. Global demethylation restored expression of *RPRM* in 3/11 cell lines (KTCL26, SKRC18, SKRC39).

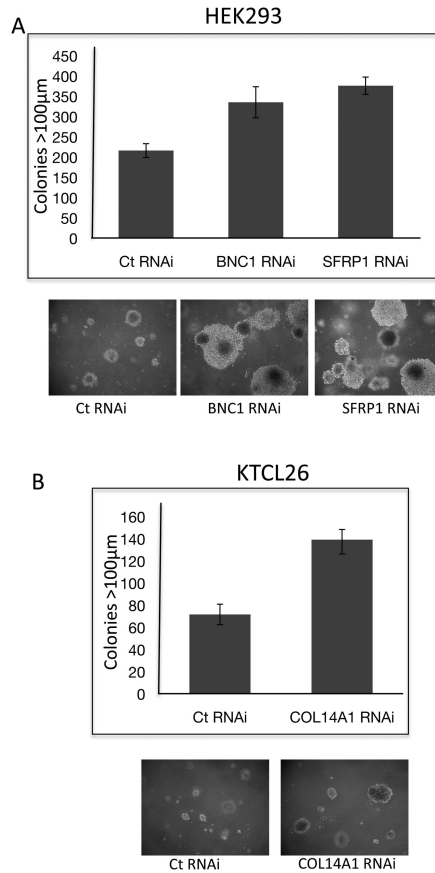
**B, Methylation-specific PCR analysis of *BNC1*, *SFRP1* and *COL15A1*, *GREM1*, and *RPRM*.** MSP analysis showed frequent (34-53%) promoter methylation in tumours, infrequent or absent methylation in adjacent tissue and no methylation in samples derived from non-malignant normal tissue (see results for details). RCC tumour cell lines were used for each MSP assay as positive controls for methylated and unmethylated DNA. Representative samples are shown. M=product derived from methylation-specific primers; U= products derived from unmethylated specific primers, T= tumour sample, N= non-malignant sample, \*=methylated tumours. MSP and USP product are of a similar size. Smaller bands are primer dimmers.



**Figure 3.**

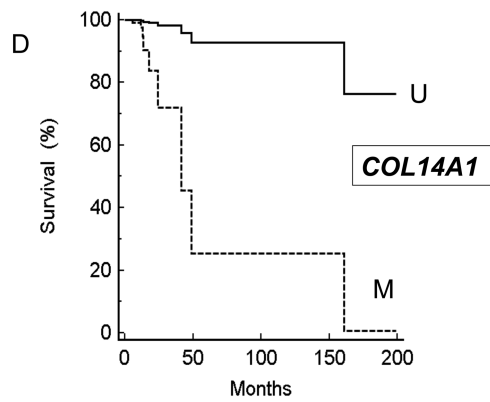
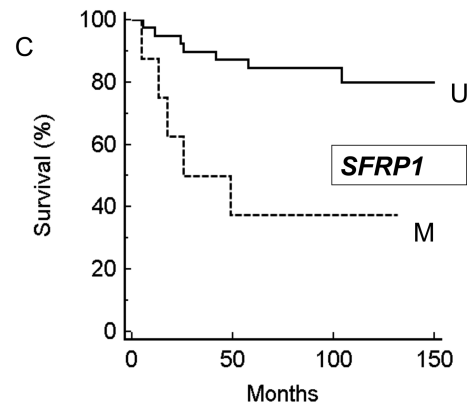
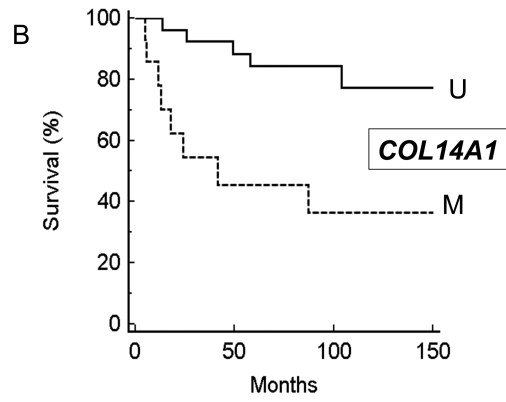
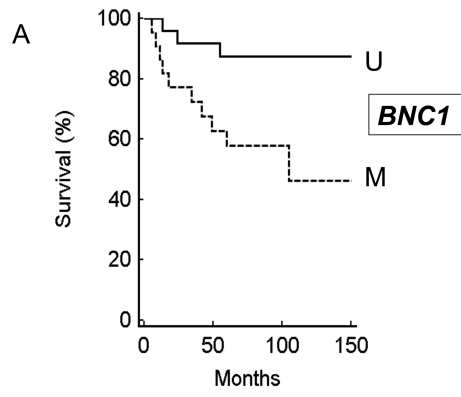
**A, Re-expression of CST6, SFRP1, RPRM and BNC1 in RCC cells results in growth suppression.** Exogenous re-expression of selected genes in RCC cell lines that do not express their respective genes resulted in a significant reduction of *in vitro* colony formation compared to RCC lines transfected with an empty vector (EV). Equal amounts of empty vector or pCDNA3.1-wt*CST6*, or pCDNA3.1-wt*SFRP1*, or pCDNA3.1-wt*RPRM*, or pCDNA3.1-wt*BNC1* were transfected into 786-O (*CST6*), SKRC47 (*SFRP1*), RCC4 (*BNC1*) or SKRC39 (*RPRM*) cells. Each experiment was done in triplicate. There was a statistically significant reduction of colonies in each of the re-expression experiments ( $p=0.003$ ,  $p<0.0001$ ,  $p<0.0001$  and  $p=0.001$  respectively). Shown below each graph are representative plates showing reduction of colonies following gene re-expression.

**B, Re-expression of CST6 or over-expression of BNC1 inhibits anchorage-independent growth.** A, clones of 786-O-pCDNA3.1 and 786-O-pCDNA3.1-wt*CST6* or HEK293-pCDNA3.1 and HEK293-pCDNA3.1-wt*BNC1* were seeded at the same density into soft agar and incubated for 5 weeks. 786-O clones not expressing *CST6* (pCDNA3.1) produced many large (>100 µm) colonies. In contrast, 786-O clones expressing exogenous wt*CST6* did not grow as robustly. Three independent experiments showed a 53% reduction of large colonies ( $p=0.0001$ ) after 5 weeks of incubation. Similarly exogenous over-expression of *BNC1* resulted in 54% fewer large colonies ( $p=0.001$ ). Below each graph is shown a representative image of clones after 5 weeks of incubation (X100 magnification).



**Figure 4.** **Knock-down of expression of BNC1, SFRP1 or COL14A1 increases anchorage-independent growth potential.** **A.** RNAi-induced reduced expression of *BNC1* or *SFRP1* in HEK-293 cells resulted in the growth of significantly more colonies >100µm in diameter compared to cells transfected with a control RNAi oligo when seeded at the same density into soft agar ( $p < 0.0001$  in both cases). **B.** RNAi-induced reduced expression of *COL14A1* in KTCL26 cells resulted in the growth of significantly more colonies >100µm in diameter compared to cells transfected with a control RNAi oligo when seeded at the same density into soft agar ( $p < 0.0001$ ).





**Figure 5.**

Panel A: Kaplan Meier survival analysis for BNC1 methylation status and Survival (M=methylated U=Unmethylated); Panel B: Kaplan Meier survival analysis for COL14A1 methylation status and survival (M=methylated U=Unmethylated); panel C: Kaplan Meier survival analysis for BNC1 methylation status and survival (M=methylated U=Unmethylated). Panel D: Cox proportional hazard analysis for COL14A1 methylation status (analysis also incorporates tumour stage, Grade and size and BNC1 methylation status).

A summary of genes identified in this study and their frequency of promoter methylation in RCC-derived cell lines, sporadic tumours, kidney tissue resected from an area adjacent to the tumour and kidney tissue from patients with no kidney cancer.

**Table 1**

Gene Symbol	Accession no.	Gene Name	Loci	Cell line methylation frequency	Tumour methylation frequency	Adjacent kidney methylation frequency	Non-malignant kidney methylation frequency
<i>COL15A1</i>	NM_001855	Collagen, type XV, alpha 1	9q22	7/9	35/65 (53%)	9/30 (30%)	0/6
<i>GREM1</i>	NM_204978	Gremlin-1	15q13	7/11	11/27(41%)	7/29 (24%)	0/6
<i>COL14A1</i>	NM_021110	Collagen, type XIV, alpha 1	8q24	7/11	18/41 (44%)	1/20 (5%)	0/6
<i>PDLIM4</i>	NM_003687	PDZ and LIM domain 4	5q31	5/10	13/30 (43%)	0/22 (0%)	1/6
<i>RPRM</i>	NM_019845	Reprimo, TP53 dependant G2 arrest mediator	2q23	4/9	23/52 (44%)	8/44 (18%)	0/6
<i>SFRP1</i>	NM_003012	Secreted frizzled-related protein 1	8p11	5/10	20/58 (34%)	0/20 (0%)	0/6
<i>CST6</i>	NM_001323	Cystatin E/M	11q13	8/11	28/61 (46%)	4/35 (11%)	0/6
<i>BNC1</i>	NM_001717	Basonuclin 1	15q25	5/11	27/59 (46%)	1/20 (5%)	0/6