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ORIGINAL ARTICLE

Genome-wide methylation analysis identifies epigenetically inactivated candidate tumour suppressor genes in renal cell carcinoma

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The detection of promoter region hypermethylation and transcriptional silencing has facilitated the identification of candidate renal cell carcinoma (RCC) tumour suppressor genes (TSGs). We have used a genome-wide strategy (methylated DNA immunoprecipitation (MeDIP) and whole-genome array analysis in combination with high-density expression array analysis) to identify genes that are frequently methylated and silenced in RCC. MeDIP analysis on 9 RCC tumours and 3 non-malignant normal kidney tissue samples was performed, and an initial short-list of 56 candidate genes that were methylated by array analysis was further investigated; 9 genes were confirmed to show frequent promoter region methylation in primary RCC tumour samples (*KLHL35 (39%)*, *QPCT (19%)*, *SCUBE3 (19%)*, *ZSCAN18 (32%)*, *CCDC8 (35%)*, *FBN2 (34%)*, *ATP5G2 (36%)*, *PCDH8 (58%)* and *CORO6 (22%)*). RNAi knockdown for *KLHL35*, *QPCT*, *SCUBE3*, *ZSCAN18*, *CCDC8* and *FBN2* resulted in an anchorage-independent growth advantage. Tumour methylation of *SCUBE3* was associated with a significantly increased risk of cancer death or relapse ($P = 0.0046$). The identification of candidate epigenetically inactivated RCC TSGs provides new insights into renal tumourigenesis.
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Introduction

Erroneous hypermethylation of CpG islands associated with gene promoters induces transcriptional silencing by multiple mechanisms involved in chromatin modification (Li *et al*., 2007). Since the identification of *RB1* tumour suppressor gene (TSG) inactivation by promoter hypermethylation 17 years ago (Ohtani-Fujita *et al*., 1993), it has become increasingly apparent that tumour suppressor promoter methylation has a significant role in the clonal evolution of cancer. For renal cell carcinoma (RCC), large-scale sequencing projects have revealed that, with the exception of *VHL* TSG, candidate TSGs are mutated in $<10\%$ of tumours (Dalgliesh *et al*., 2010), whereas a much larger number of TSGs are frequently silenced by cancer-specific promoter methylation. Indeed, several important RCC TSGs are frequently inactivated by promoter hypermethylation but rarely mutated; these include *RASSF1A* (Morrissey *et al*., 2001; Hogg *et al*., 2002, Morris *et al*., 2003), *SFRP1* (Dahl *et al*., 2007; Morris *et al*., 2010), *DAPK1* (Morris *et al*., 2003; Christoph *et al*., 2006) and *SPINT2* (Morris *et al*., 2005). These observations, in combination with the frequent difficulty of distinguishing 'driver' and 'passenger' mutations in human cancers, suggest that strategies to identify genes targeted by *de novo* promoter methylation can provide an efficient approach to identify novel RCC TSGS.

In the past decade, the tools available to those wishing to identify epigenetically silenced genes in cancer have developed rapidly. Initially, significant progress was made by functional epigenomic approaches using gene expression microarrays to study changes in gene expression following global demethylation of cancer cell line genomes (Yamashita *et al*., 2002; Sato *et al*., 2003; Lodygin *et al*., 2005). For RCC, this approach resulted in the identification of $\sim$14 candidate RCC TSGs (Morris *et al*., 2005, 2008, 2010; Ibanez de Caceres *et al*., 2006). However, the application of this strategy is limited, as evidenced by the observation that many of the genes that are upregulated in RCC cell lines do not show promoter methylation and that many genes that are methylated in cell lines are not methylated in primary tumours (Morris *et al*., 2008, 2010). The technique of methylated DNA immunoprecipitation (MeDIP) enables the isolation of the methylated DNA

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fraction from primary tumour DNA, which can then be analysed by high-density whole-genome microarray, thus allowing the direct analysis of genomic methylation patterns in primary tumours. Previously, no whole-genome methylation detection strategies were applied to the analysis of methylation in RCC. We have used MeDIP with comparative high-density whole-genome microarray analysis to identify differentially methylated regions in primary tumour DNA directly. We have combined these data with expression array data from RCC-derived cell lines that have been globally demethylated by treatment with 5-Aza-2′-deoxycytidine to increase the likelihood of identifying tumour-specific methylation that correlates to gene silencing. We have identified a number of genes that are frequently methylated in an RCC tumour-specific manner resulting in gene silencing. We have also identified a subset of these genes that have shown *in vitro* tumour suppressor activity.

Results

Identification of candidate silenced genes involved in RCC

DNA samples from nine clear cell RCC tumours and from three non-malignant kidneys were prepared by MeDIP, and the resulting methylated and unmethylated fractions were hybridised to Nimblegen HG18 whole-genome oligonucleotide arrays. Each gene is represented on the HG18 arrays by up to 14 probes grouped into 'peaks'. A 'peak score' of 2 equals a twofold enrichment of DNA in the methylation immunoprecipitation fraction. A twofold enrichment is the minimum level of methylation that we have accepted to merit further investigation.

To prioritise the identification of gene promoters that were frequently methylated in RCC tumours, we created a shortlist using the following criteria; there must be no methylation in any of the three non-malignant kidney samples, and at least 45% (4/9) of RCC tumours must have a peak score of $\geqslant 2$. Applying these criteria gave a shortlist of 574 'peaks', which related to 443 individual genes and open reading frames (Supplementary Table 2 shows a full list of genes that had peak scores in 4/9 primary tumours).

To reduce the list of genes to those in which promoter methylation is likely to be biologically relevant, we applied a further filter. We have previously analysed genome-wide expression changes in 11 RCC-derived cell lines following treatment with the demethylating agent 5-Aza-2′-deoxycytidine using Affymetrix U133 Plus-2 microarrays (Morris *et al*., 2010). We prioritised those genes from our MeDIP methylation array that also showed a significant reexpression ($\geqslant$8-fold change, see Morris *et al*. (2008)) in at least two cell lines. A total of 78 genes met this criteria, including two, *KRT19* and *EDNRB*, previously shown to be epigenetically silenced in RCC (Pflug *et al*., 2007; Morris *et al*., 2008) and two, *RARRES1* and *IRF7*, that have been shown to be infrequently methylated in RCC (Morris *et al*., 2008). Eleven candidate genes were not analysed further, as they had no CpG island at the predicted promoter region (from www.genome.ucsc.edu and Genomatix promoter inspector (www.genomatix.de)). X-chromosome and imprinted genes were also excluded ($n=8$), leaving 55 genes that have not previously been associated with epigenetic dysregulation in RCC as candidates for further analysis (see Figure 1 for a schematic of filtering criteria and Table 1 for a full list of candidate genes).

Validation of methylation in candidate genes

PCR primers were designed to amplify the predicted promoter region for all 55 candidate genes from bisulphite-modified DNA (see Supplementary Table 1 for primer details). Direct sequencing of promoter regions from nine RCC cell lines and six normal kidney samples obtained from non-RCC patients was performed to confirm the results from the MeDIP analysis. Genes were selected for further investigation if methylation was present in $\geqslant$40% of cell lines and was absent in all 6 non-malignant kidney samples, as determined by sequencing and combined bisulphite and restriction

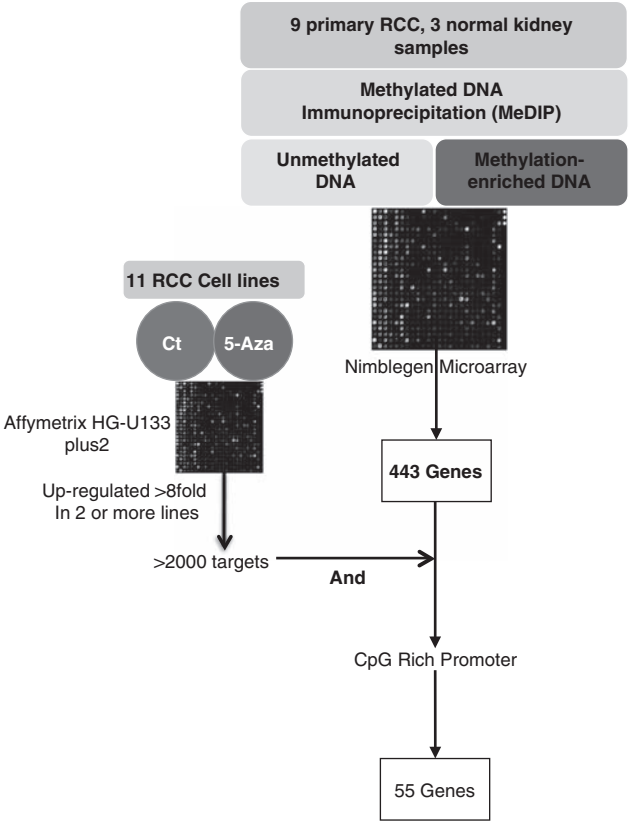

Figure 1 Schematic of method applied to shortlist candidate genes. Genes identified as methylated in primary RCC tumours and not in normal kidney DNA were compared with the differential expression of those genes in RCC-derived cell lines following culture in the demethylating agent 5-Aza-2′-deoxycytidine. Those genes that were determined methylated by MeDIP array and reexpressed in at least two cell lines were considered for further analysis (see main text for further details).

Table 1 Genes shortlisted by combining MeDIP array and cell line expression array analysis

Gene	*Accession no.*	*Location*	*Biological process*	*Cell line meth.*	*NM meth.*	*Tumour meth.*	*Adj norm. meth.*	*Mean MI*
ATP5G2	NM_005176	chr12: 52,345,211–52,356,376	Proton transport	9/9	0/12	36%	0/18	59%
PCDH8	NM_032949	chr13: 52,316,110–52,320,775	Cell adhesion	9/9	0/12	58%	0/18	44%
CORO6	NM_139115	chr17: 24,966,522–24,972,570		5/8	0/12	22%	0/18	31%
KLHL35	NM_001039548	chr11: 74,811,088–74,819,322	Protein binding	5/6	0/12	39%	0/18	39%
QPCT	NM_012413	chr2: 37,425,257–37,453,969	Proteolysis	8/9	0/12	19%	0/18	34%
SCUBE3	NM_152753	chr6: 35,290,168–35,326,587	Protein hetero-oligomerization	6/9	0/12	19%	0/18	25%
ZSCAN18	NM_001145542	chr19: 63287022–63321605	Regulation of transcription, DNA-dependent	6/9	0/12	32%	0/18	43%
CCDC8	NM_032040	chr19: 51,605,427–51,608,759		7/6	0/10	35%	0/18	52%
FBN2	NM_001999	chr5: 127,621,500–127,901,634	Anatomical structure morphogenesis	9/9	0/12	34%	0/18	31%
PTPLAD2	NM_001010915	chr9: 20,996,365–21,021,635		6/6	0/12	37%	4/18	
PKHD1L1	NM_177531	chr8: 110,443,882–110,612,676		7/9	2/12	49%		
ANK3	NM_020987	chr10: 61,458,165–61,819,494	Signal transduction	5/9	0/12	15%		
HMX1	NM_018942	chr4_random: 279,194–283,964	Negative regulation of transcription, DNA-dependent	2/2	0/12	15%		
P2RX5	NM_002561	chr17: 3,523,271–3,546,332	Ion transport	3/8	0/12	15%		
TF	NM_001063	chr3: 134,947,667–134,980,539	Ion transport	9/9	0/12	15%		
LRRC2	NM_024750	chr3: 46,531,882–46,596,576		7/8	0/12	14%		
PROM1	NM_006017	chr4: 15,578,947–15,694,721	Response to stimulus	7/9	0/12	11%		
EGR4	NM_001965	chr2: 73,371,566–73,374,337	Positive regulation of transcription	4/9	0/12	10%		
CELSR3	NM_001407	chr3: 48,648,900–48,675,352	Cell adhesion	4/8	0/10	10%		
BIK	NM_001197	chr22: 41,836,701–41,855,662	Induction of apoptosis	6/8	0/12	10%		
IGFBP2	NM_000597	chr2: 217,206,372–217,237,403	Regulation of cell growth	4/8	0/6	7%		
SLC6A2	NM_001043	chr16: 54,247,853–54,295,199	Transport	6/7	0/6	7%		
LYNX1	NM_023946	chr8: 143,842,760–143,856,641		6/8	0/6	5%		
DUOX2	NM_014080	chr15: 43,172,144–43,193,651	Oxidation reduction	9/9	0/6	5%		
CLDN7	NM_001307	chr17: 7,103,946–7,106,519	Calcium-independent cell-cell adhesion	9/9	0/6	0%		
DGKI	NM_004717	chr7: 136,724,925–137,182,149	ATP binding	9/9	0/6	0%		
ALDH2	NM_000690	chr12: 110,688,729–110,732,167	Response to hyperoxia	3/8	0/6	0%		
CACNB2	NM_201596	chr10: 18,469,612–18,870,694	Ion transport	4/8	0/6	0%		
HTR1A	NM_000524	chr5: 63,292,034–63,293,302	Signal transduction	4/9	0/6	0%		
SLC29A4	NM_153247	chr7: 5,289,087–5,310,230	Transport	9/9	0/6	0%		
MGC26733 (NRARP)	NM_001004354	chr9: 139,313,904–139,316,524	Multicellular organismal development	6/9	0/6	0%		
RHOD	NM_014578	chr11: 66,580,865–66,596,063	Rho protein signal transduction	6/9	0/6	0%		
PRRX2	NM_016307	chr9: 131,467,741–131,524,772	Regulation of transcription, DNA-dependent	6/9	0/6	0%		
HIST1H4L	NM_003546	chr6: 27,948,905–27,949,268	Chromatin organization	9/9	1/6			
WBSCR17	NM_022479	chr7: 70,235,725–70,816,520		9/9	3/6			
ADRA1A	NM_000680	chr8: 26,683,139–26,778,839	Apoptosis	7/8	2/6			
PENK	NM_006211	chr8: 57,516,069–57,521,147	Signal transduction	7/9	3/6			
SOX14	NM_004189	chr3: 138,966,269–138,967,086	Regulation of transcription	6/9	4/5			
HOXD3	NM_006898	chr2: 176,737,051–176,746,072	Regulation of transcription, DNA-dependent	9/9	4/4			
ANGPT2	NM_001147	chr8: 6,344,581–6,408,192	Cell differentiation	9/9	5/5			
GUCA2B	NM_007102	chr1: 42,391,679–42,394,082	Excretion	6/6	5/5			
TNFSF12	NM_003809	chr17: 7,393,099–7,401,931	Apoptosis	4/4	4/4			
SOX1	NM_005986	chr13: 111,769,914–111,774,021	Regulation of transcription, DNA-dependent	7/7	2/6			
DNAJA4	NM_018602	chr15: 76,343,542–76,361,593	Protein folding	9/9	6/6			
SLC9A8	NM_015266	chr20: 47,862,657–47,942,179	Transmembrane transport	2/8	0/6			
YPEL3	NM_001145524	chr16: 30,011,139–30,015,038		1/9	0/6			
SLC37A1	NM_018964	chr21: 42,792,811–42,874,619	Carbohydrate transport	0/9	0/6			
PARVA	NM_018222	chr11: 12,355,722–12,507,986	Cell adhesion	0/9	0/6			
LRRC6	NM_012472	chr8: 133,653,629–133,756,995		0/6	0/5			
FCHO1	NM_001161357	chr19: 17,719,527–17,760,377	Intracellular protein transport	0/9	0/6			
RASL11B	NM_023940	chr4: 53,423,252–53,427,759	Signal transduction	1/9	0/6	—		
RAB15	NM_198686	chr14: 64,482,285–64,508,628	Protein transport	0/7	0/6	—		
MAFF	NM_012323	chr22: 36,927,885–36,942,461	Regulation of transcription, DNA-dependent	0/4	0/5	—		

Table 1 Continued

Gene	*Accession no.*	*Location*	*Biological process*	*Cell line meth.*	*NM meth.*	*Tumour meth.*	*Adj norm. meth.*	*Mean MI*
TST	NM_003312	chr22: 35,736,852–35,745,437	Cyanate catabolic process	0/9	0/5	—		
PLXNC1	NM_005761	chr12: 93,066,630–93,223,356	Cell adhesion	0/9	0/6	—		

Abbreviations: Adj Norm, non-tumour kidney tissue resected from the same kidney as the relative tumour; Meth, methylation; MI, methylation index; NM, non-malignant kidney.
All shortlisted genes were analysed for promoter region methylation in RCC-derived cell lines and non-malignant kidney tissue. Those that were frequently methylated in RCC cell lines and not in normal tissue were then analysed in primary tumours. Those CpG island regions that were methylated in primary tumours and not in the adjacent normal kidney were then analysed by bisulphite sequencing to provide details of methylation density within those regions as determined by mean methylation indices. Shading key: Green: methylation present in RCC cell lines and primary tumours, no methylation in non-malignant kidney tissue. Yellow: methylation in cell lines and $\leqslant$15% of primary tumours, no methylation in non-malignant kidney. Red: methylation present in non-malignant kidney tissue. Blue: methylation infrequent in RCC-derived cell lines ($\leqslant$25%).

analysis (CoBRA). In all, 33 promoter-GpG islands were frequently methylated in cell lines and not methylated in normal kidney tissue. The other CpG islands were either infrequently methylated in RCC cell lines ($<$40% $n=11$) or dense methylation ($\geqslant$5% CpGs analysed) was present in normal kidney tissue ($n=11$) (Table 1).

Promoter hypermethylation in primary RCC tumours

To determine whether the 33 candidate genes methylated in RCC cell lines were methylated in primary tumours, we performed CoBRA analysis in a further 6 normal kidney samples from patients with no history of RCC, as well as in 60 primary RCCs and 18 normal kidney samples matched to 18 primary RCCs.

PKHD1L1, although frequently methylated in primary RCC (50%), was also found to be methylated in 2/6 additional normal kidney samples (2/12 normal kidney samples tested) and was excluded from further investigation.

A two-stage protocol was used to determine methylation frequency in primary RCC. Initially, 20 primary RCC tumours were analysed; if the frequency of methylation was $>$15%, a further 40 RCCs were tested. Of the 33 genes analysed, 21 were not (*CLDN1, DGKI, ALDH2, CACNB2, HTR1A, SLC29A4, NRARP, RHOD* and *PRRX2*) or were infrequently ($\leqslant$15% of tumours; *BIK, HMX1, ANK3, ALOX15, LYNX1, DUOX2, PROM1, CELSR3, P2RX5, LRRC2, SLC6A2* and *TF*) methylated. No further analysis was carried out on these genes. Twelve gene promoters were methylated in $>$15% of the first 20 tumours analysed; however, after testing a further 40 RCCs, two genes (*IGFBP2* and *EGR4*) were methylated in $<$3% (1/40 tumours) of the additional samples and were not further investigated.

Nine gene promoters were frequently methylated ($>$15%); these were *ATP5G2* (36% of tumours methylated), *PCDH8* (58% of tumours methylated), *CORO6* (22% of tumours methylated), *KLHL35* (39% of tumours methylated), *QPCT* (19% of tumours methylated), *SCUBE3* (19% of tumours methylated), *ZSCAN18* (32% of tumours methylated), *CCDC8* (35% of tumours methylated) and *FBN2* (34% of tumours methylated). These gene promoters were not methylated in any non-tumour kidney samples resected from regions adjacent to the tumours ($n=18$). The *PTPLAD2* CpG island was methylated in 37% of primary tumours. However, methylation was also observed in 4/18 kidney samples restricted adjacent to the methylated tumours. Figure 2 shows representative CoBRA digest products.

To analyse the methylation status of the nine genes that are frequently methylated in an RCC tumour-specific manner in more detail, we carried out bisulphite sequencing in tumours that had been identified as methylated by CoBRA ($n=8$ tumours per gene). The mean methylation index (MI) for the genes analysed by sequencing ranged from 25 to 59% (*SCUBE3*, MI = 25%; *ZSCAN18*, MI = 43%; *CORO6*, MI = 31%; *FBN2*, MI = 31%; *ATP5G2*, MI = 59%; *QPCT*, MI = 34%; *CCDC8*, MI = 52%; *KLHL35*, MI = 39%; and *PCDH8*, MI = 44%). (Figure 3). Of the genes identified by this screen, only one, *DGKI,* has been shown to be mutated in RCC; this was in 1 tumour case out of 101 analysed (Dalgliesh *et al*., 2010).

Expression analysis of identified genes

Reverse transcriptase–PCR analysis of 22 pairs of tumours and corresponding non-tumour kidney complementary DNA confirmed that the genes identified by our screen were frequently silenced or downregulated. Transcripts were present in all tumour-matched normal kidney tissues and absent or significantly reduced ($>$5-fold reduction compared with the corresponding normal sample) in many tumour samples: *FBN2* (expression absent/reduced) in 55% of the 22 RCCs, *ATP5G2* (41%), *KLHL35* (41%), *PCDH8* (36%), *CCDC8* (23%), *QPCT* (41%), *SCUBE3* (45%), *ZSCAN18* (23%) and *CORO6* (41%). Tumours with absent/reduced expression were tested for gene methylation; for *FBN2*, *ATP5G2*, *KLHL35*, *PCDH8* and *CCDC8*, most tumours tested demonstrated methylation (67, 77, 67, 88 and 80%, respectively; Figure 4a). To further demonstrate that the presence of hypermethylated CpG islands was associated with the absence of each respective mRNA transcript, we carried out reverse transcriptase–PCR on methylated cell lines. Treatment with the demethylating agent 5-Aza-2′-deoxycytidine (5 μM) for 5 days restored gene expression (Figure 4b).

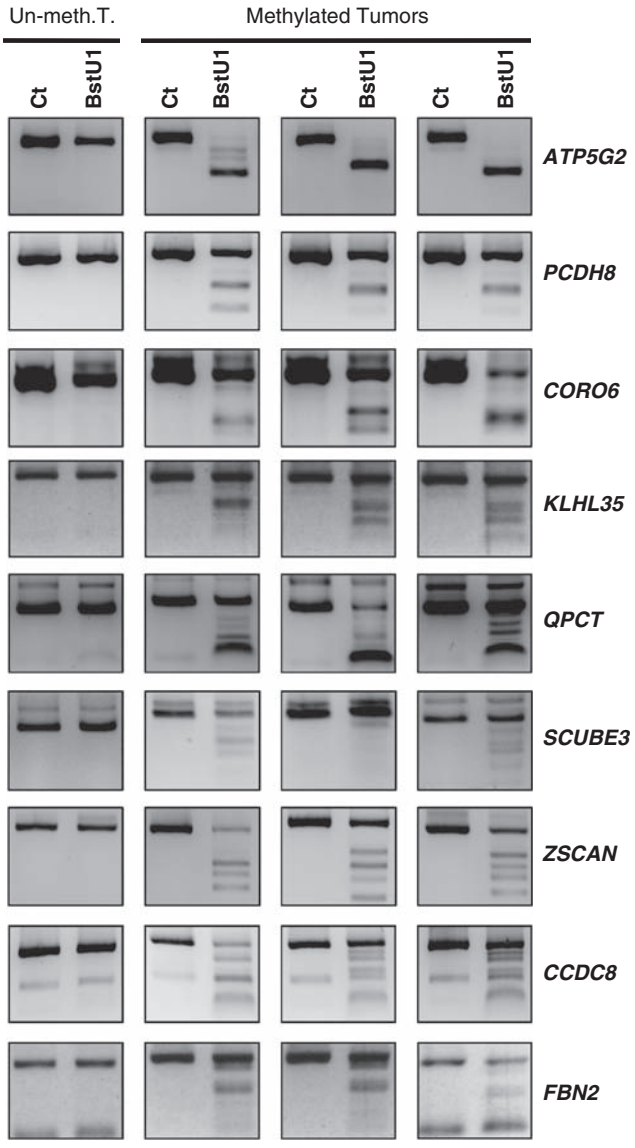

Figure 2 Representative CoBRA digests. Sixty sporadic RCC tumours were analysed for promoter methylation. Nine genes were frequently methylated: *ATP5G2* (36%), *PCDH8* (58%), *CORO6* (22%), *KLHL35* (39%), *QPCT* (19%), *SCUBE3* (19%), *ZSCAN18* (32%) *CCDC8* (35%) and *FBN2* (29%). The left panel is representative of tumours that were not methylated (un-meth T.), the other panels are representative of tumours that were methylated as determined by bisulphite-PCR product digestion with BstU1 (ct = PCR product, BSTU1 = PCR product digested with BSTU1).

Functional analysis of the tumour suppressor activity of epigenetically inactivated genes

To investigate whether the promoter region methylation and transcriptional silencing might promote tumourigenesis, RNAi was used to knock down the expression of the nine methylated genes in HEK293 cells. At 24 h after RNAi transfection, cells were seeded into 3% agar and colonies $>200\,\mu m$ were counted after 21 days. Transcript knockdown was determined by reverse transcriptase–PCR and western blotting, where appropriate antibodies were available (Supplementary Figure 1). Reduced expression of *ATP5G2*, *PCDH8* or *CORO6* did not result in a significant change in anchorage-independent growth. The number of resulting colonies $>200\,\mu m$ following reduced expression of *KLHL35* was 58% greater (s.d. = 10%, $P = 0.009$) than HEK293 cells transfected with a control RNAi oligo. Reduced expression of *QPCT* resulted in 69% more colonies $>200\,\mu m$ (s.d. = 3%, $P = 0.003$), *SCUBE3* reduction produced 71% more colonies $>200\mu m$ (s.d. = 14%, $P = 0.01$). Reduced expression of *ZSCAN18*, *CCDC8* and *FBN2* also significantly increased the number of anchorage-independent colonies $>200\,\mu m$ by 147 (s.d. = 17%, $P = 0.0003$), 154 (s.d. = 5%, $P = 0.0003$) and 205% (s.d. = 17%, $P = 0.003$), respectively (Figure 5). All experiments were carried out independently in triplicate.

Analysis of promoter methylation and patient survival/relapse

Kaplan–Meier analyses revealed no significant associations between the risk of cancer death/relapse and tumour methylation status for *ATP5G2* ($P = 0.5072$), *CCD8* ($P = 0.1682$), *CORO6* ($P = 0.4204$), *FBN2* ($P = 0.4922$), *KLH35L* ($P = 0.2477$), *PCDH8* ($P = 0.9912$), *QPCT* ($P = 0.2982$) and *ZSCAN* ($P = 0.5541$). However, methylation of *SCUBE3* was associated with a significantly increased risk of death ($P = 0.009$) and cancer death or relapse ($P = 0.0046$) (Figure 6).

Discussion

Previously, we and others had used functional epigenomic screens to identify epigenetically inactivated TSGs in RCC (Morris *et al*., 2005, 2008; Ibanez de Caceres *et al*., 2006). In contrast to high-throughput sequencing studies to detect genes mutated in RCC (Dalgliesh *et al*., 2010), epigenetic studies have identified at least 18 genes that are inactivated in >20% of RCCs (Morris *et al*., 2010 and references within), including *SPINT2* (Morris *et al*., 2005), *BNC1, CST6, PDLIM4, COL14A1* and *COL15A1* (Morris *et al*., 2010). However, to facilitate the identification of further RCC TSGs, we have used an approach that combined MeDIP with comparative high-density whole-genome microarray analysis and functional epigenomic expression data from RCC-derived cell lines treated with the demethylating agent 5-Aza-2′-deoxycytidine. Although this strategy also required a sequential prioritisation and analysis of genes to exclude those that were not frequently methylated in primary RCC, we were able to identify a further six genes that demonstrate promoter methylation in >30% of RCCs and three genes that had promoter methylation in ⩾19% of RCCs. We note that several genes (for example, *SFRP1* (Dahl *et al*., 2007; Morris *et al*., 2010), *DAPK1* (Morris *et al*., 2003; Christoph *et al*., 2006) and *SPINT2* (Morris *et al*., 2005)) that we and others have previously reported to be methylated in RCC were not identified by this strategy. This is likely to result from the promoter regions of these genes not being well covered by the HG18

methylation array and so suggests that further studies using higher-density arrays (or more-sensitive technologies) would lead to the identification of additional novel epigenetically regulated genes. Knockdown of six of these genes was also shown to increase anchorage-independent cell growth, providing direct functional evidence of tumour suppressor activity. The HEK293 cell line was used as an experimental model for the tumourigenicity assays, as all the target genes were expressed in this cell line (derived from Ad5-transformed embryonic kidney cells), and so the effect of each of the specific gene knockdowns could be evaluated in a consistent renal-derived cellular context. None of the nine genes have been previously reported to be methylated in RCC and, to the best of our knowledge, *CCDC8, ATP5G2, KLHL35, CORO6, ZSCAN18* and *SCUBE3* have not been previously reported to be methylated in neoplasia. *FBN2* has recently been reported to be epigenetically silenced in colorectal, oesophageal and non-small-cell lung cancers (Chen *et al*., 2005; Tsunoda *et al*., 2009; Yagi *et al*., 2010). *QPCT*, which encodes a glutaminyl cyclase (Fischer and Spiess, 1987; Pohl *et al*., 1991), is frequently methylated in malignant melanoma (Muthusamy *et al*., 2006). Functional *PCDH8* is frequently lost in breast cancer through both mutation and promoter methylation (Yu

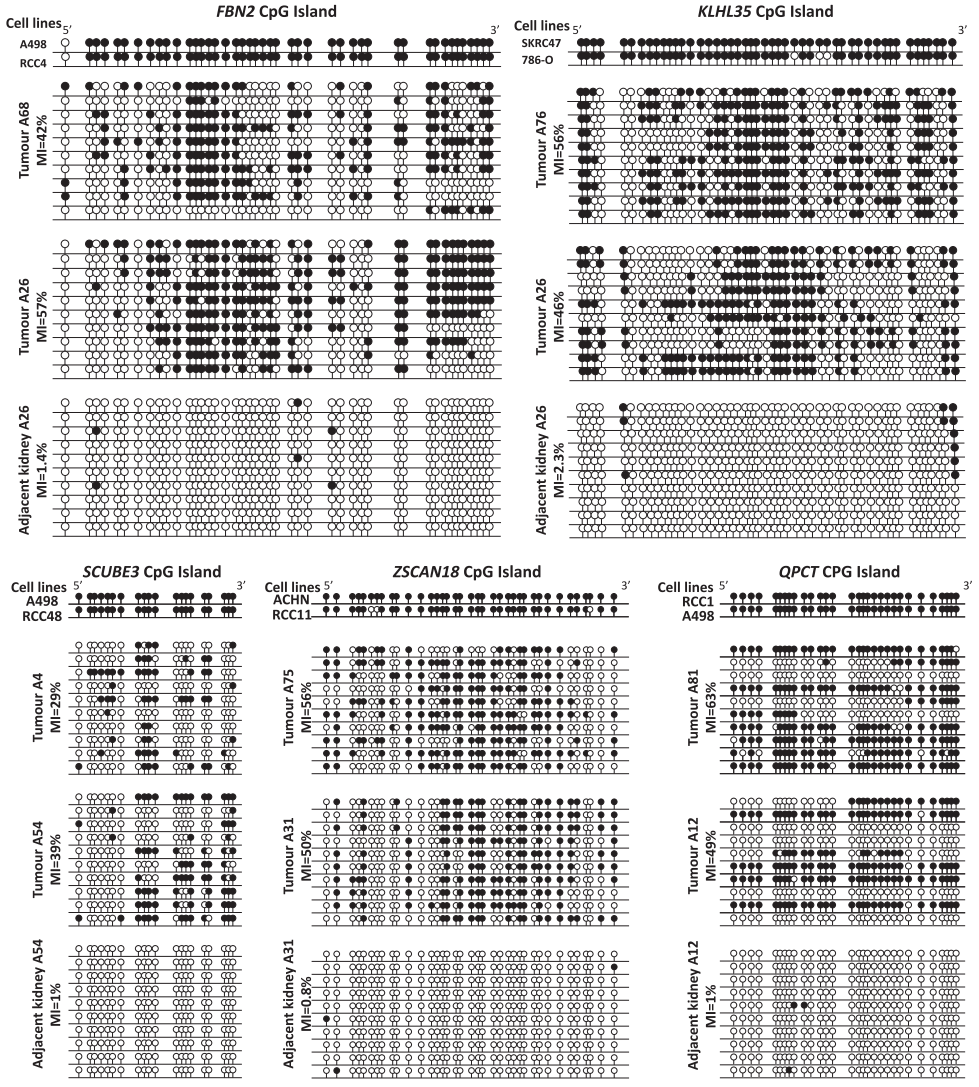

Figure 3 Bisulphite sequencing in tumours and non-malignant kidney resected adjacent to the tumour. Tumours that had been identified as methylated by CoBRA (eight tumours per gene) were analysed by cloning and sequencing bisulphite-PCR products to determine the extent of methylation within the region analysed by CoBRA. The mean MI for the genes analysed by sequencing ranged from 25 to 59%:SCUBE3, MI = 25%; ZSCAN18, MI = 43%; CORO6, MI = 31%; FBN2, MI = 31%, ATP5G2, MI = 59%; QPCT, MI = 34%; CCDC8, MI = 52%, KLHL35, MI = 39%; and PCDH8, MI = 44%). Methylation was absent in adjacent non-malignant kidney samples resected adjacent to the tumour (< 5%MI in all cases). Each circle represents one CpG; shaded circles indicate the presence of methyl-cytosine and clear circles indicate absence of methylation. Two cell lines (which were sequenced directly), two methylated tumours (10 clones of each) and one adjacent kidney tissue sample (10 clones) are shown for each of the nine genes. MI is defended as the total number of methylated CpG dinucleotides given as a percentage of all CpGs analysed. The mean MI is the average MI calculated for 10 clones per tumour.

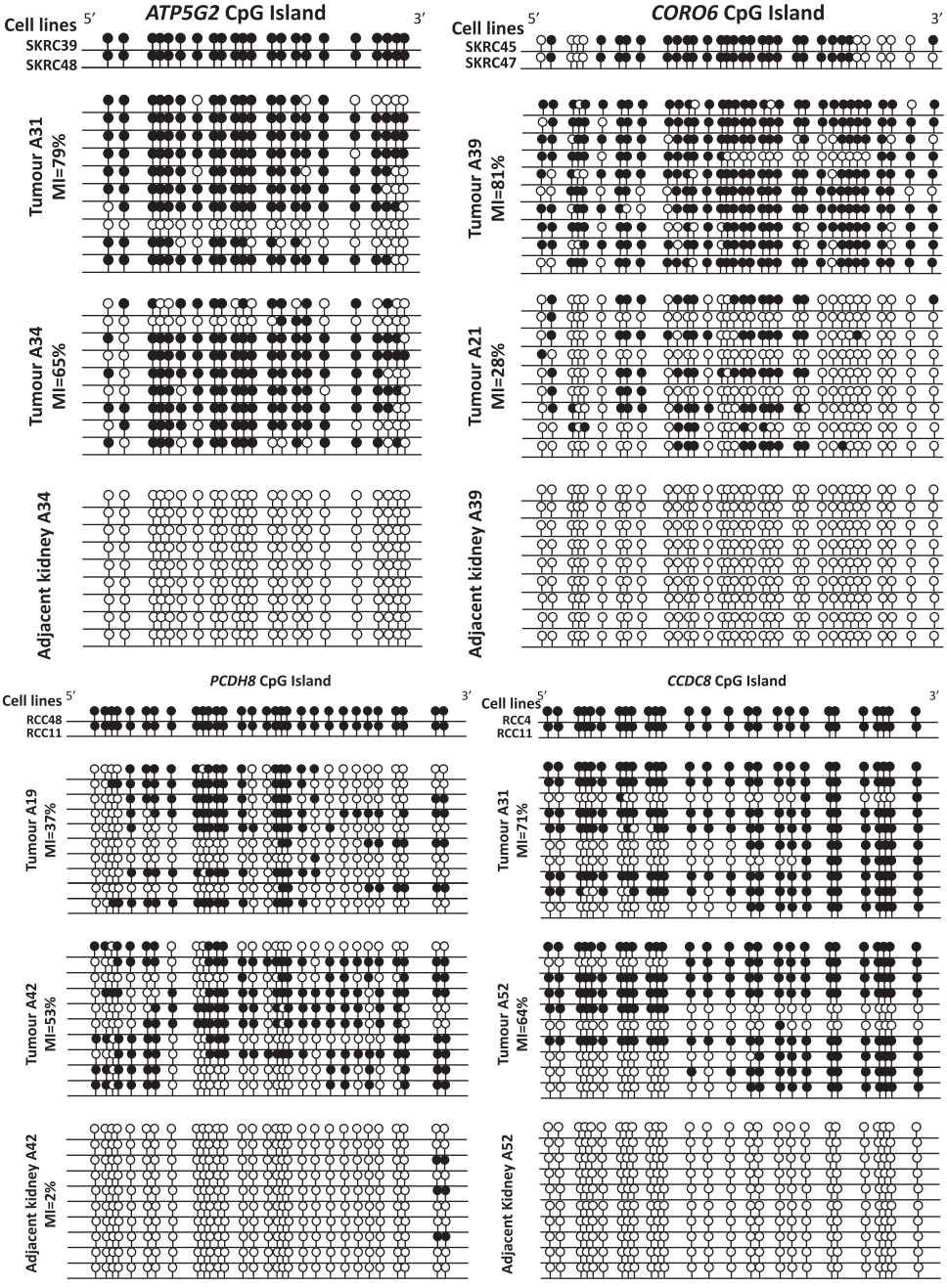

Figure 3 Continued.

et al., 2008), and also methylated in mantle cell lymphoma (Leshchenko *et al*., 2010).

FBN2 and the related gene *FBN1* encode large modular extracellular matrix glycoproteins, which are the key components of human microfibrils (Zhang *et al*., 1994). The microfibrillopathies, Marfan's syndrome and congenital contractural arachnodactyly, result from dominant mutations in *FBN1* and *FBN2*, respectively (Robinson and Godfrey, 2000). There is increasing evidence that these molecules regulate TGF-β signalling. The binding of TGF-β-bound large latency complex to fibrillins has two roles; it renders TGF-β inactive, facilitating fine control of TGF-β activity. It also concentrates TGF-β to specific locations, thus regulating the biological response to TGF-β (Annes *et al*., 2003). *FBN1* mutations in Marfan's syndrome result in the excess activation of TGF-β (Chaudhry *et al*., 2007). However, less is known about the role of Fibrillin-2. Loss of Fibrillin-2 in RCC may contribute to a malignant phenotype by contributing to the dysregulation of the complex network of signalling pathways regulated by TGF-β. Loss of large extracellular matrix proteins may also give angiogenic and metastatic advantages to RCC.

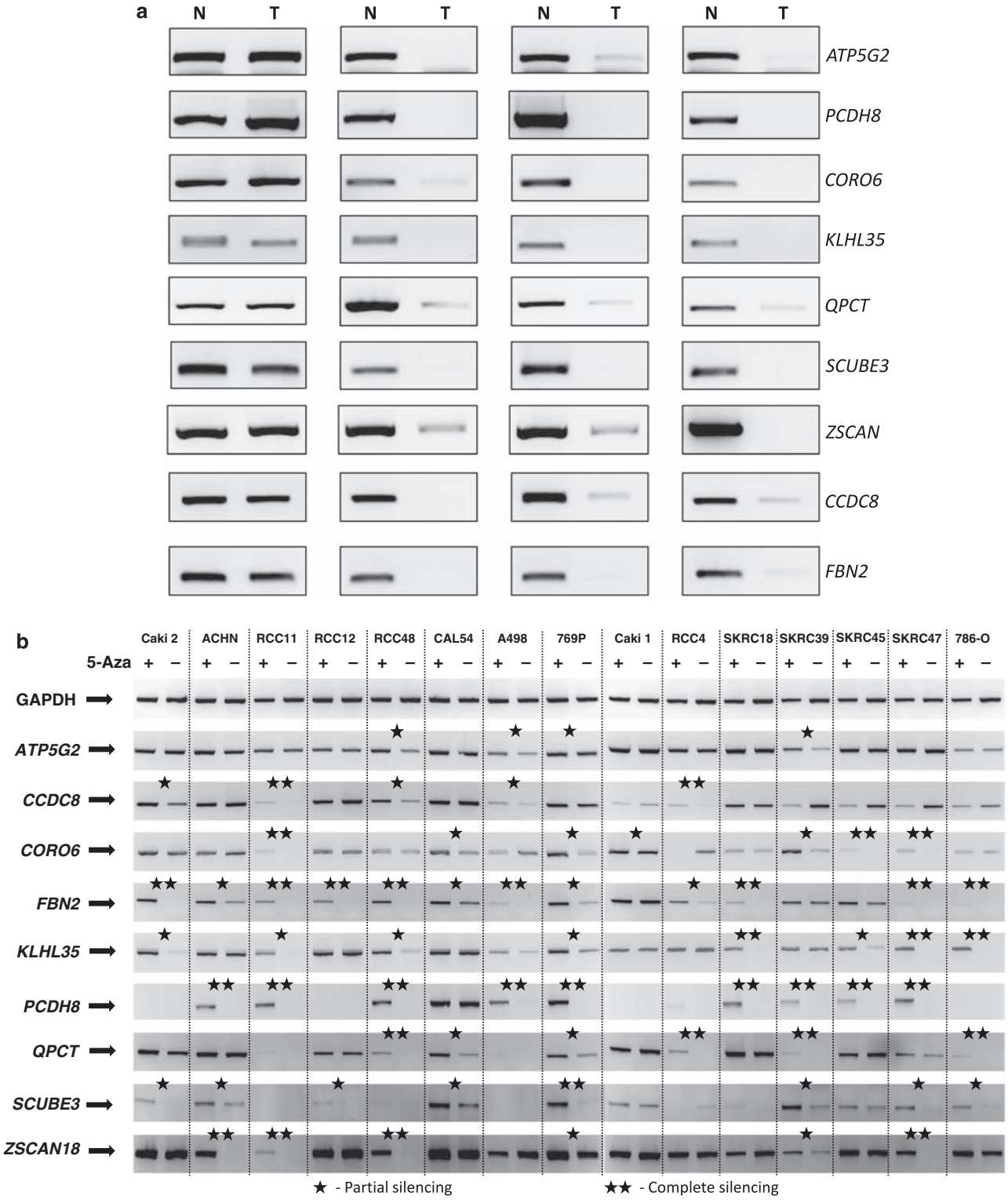

Figure 4 Candidate genes are frequently silenced in primary tumours. (**a**) Reverse transcriptase–PCR analysis of primary tumours (T) and non-malignant kidney resected adjacent to the tumour (N) showed that transcripts were present for all candidate genes in normal kidney found adjacent to tumours, and were frequently silenced or significantly reduced in tumours (see main text for details). (**b**) Reexpression of candidate genes in RCC cell lines following global demethylation by the addition of 5-Aza-2′-deoxycytidine to the growth medium (5-Aza). Transcript absence correlated to methylation in the CpG island region analysed. Demethylation resulted in the reexpression of silenced transcripts (single star indicates low level of expression before 5-Aza-2′-deoxycytidine treatment and increased expression afterward. Double star indicates complete silencing of transcript, followed by reexpression after 5-Aza-2′-deoxycytidine treatment).

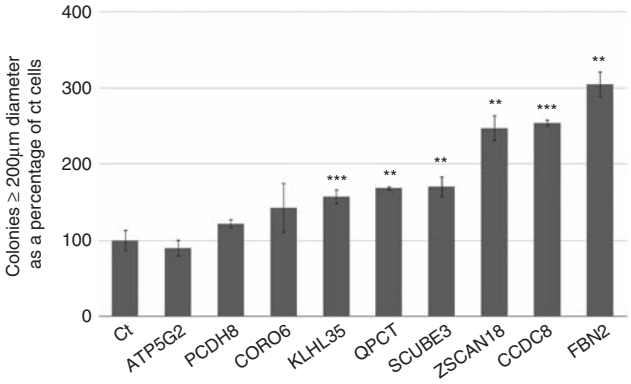

Figure 5 Knockdown of expression of *FBN2*, *CCDC8*, *ZSCAN18*, *SCUBE3, QPCT* or *KLHL35* increases anchorage-independent growth potential. An RNAi-induced reduced expression of *FBN2*, *CCDC8*, *ZSCAN18*, *SCUBE3, QPCT* or *KLHL35* in HEK293 cells resulted in the growth of significantly more colonies $>200\,\mu m$ in diameter compared with cells transfected with a control RNAi oligo when seeded at the same density into soft agar ($^{**}P<0.01$, $^{***}P<0.001$). Each gene knockdown experiment was repeated three times.

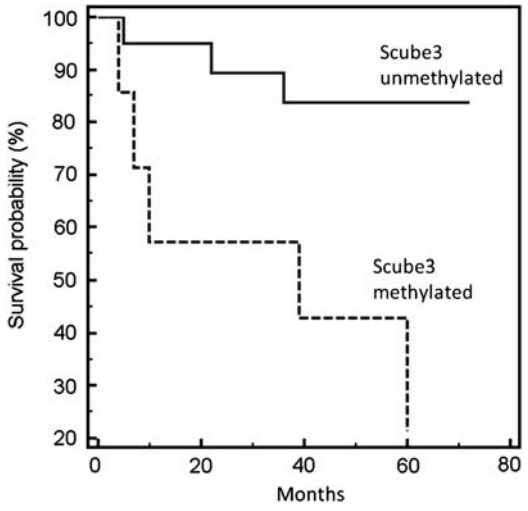

Figure 6 Analysis of promoter methylation and patient survival/relapse. Kaplan–Meier analyses revealed that methylation of *SCUBE3* was associated with a significantly increased risk of cancer death or relapse ($P=0.0046$).

There is increasing evidence that protocadherins can function as tumour suppressors. Protocadherins 10 and 20 are epigenetically silenced in nasopharyngeal and lung cancers (Imoto *et al*., 2006; Ying *et al*., 2006), and recently it has been reported that *PCDH8* is methylated in mantle cell lymphoma and breast cancer (Yu *et al*., 2008; Leshchenko *et al*., 2010). Yu *et al*. found that reexpressing wild-type PCDH8 in a breast cancer cell line inhibited cell migration. Although we did not find that silencing of *PCDH8* increased the anchorage-independent growth potential of kidney cells, the role of protocadherins, and their mechanism of action, in renal tumourigenesis is likely to merit further investigation.

QPCT encodes a glutaminyl cyclase that converts precursor glutaminyl peptides to their bioactive pyroglutaminyl peptide forms (Fischer and Spiess, 1987; Pohl *et al*., 1991). Loss of expression of members of this family of proteins has been observed in a number of tumour types, including QPCT itself in melanoma (Muthusamy *et al*., 2006) and phaeochromocytomas (Thouënnon *et al*., 2007). *SCUBE* (Signal peptide CUB EGF-like domain-containing protein) genes encode a small group of secreted plasma membrane-associated proteins characterised by an *N*-terminal signal peptide sequence, multiple EGF (epidermal growth factor) domains, a large spacer region containing multiple *N*-linked glycosylation sites and a *C*-terminal CUB (Complement subcomponent C1r/C1s EGF-related sea Urchin protein, Bone morphogenetic protein1) domain (Grimmond *et al*., 2000). Little is known about SCUBE3; however, it is plausible to suggest that it may have an antitumourigenic role, in a similar manner to proteins such as gremlin (Morris *et al*., 2010), in maintaining correct TGFβ signalling. In addition, we note that methylation of *SCUBE3* was associated with a significantly increased risk of cancer death/relapse.

The identification of frequent methylation of *ATP5G2,* which encodes a mitochondrial ATP synthase subunit *C* (Dyer and Walker, 1993), in sporadic RCC is of interest as it is becoming increasingly apparent that mitochondrial dysregulation may have a significant role in the pathology of a number of tumour types, including RCC. Two common characteristics of tumours can both be related to errors in normal mitochondrial function. These are an increase in cellular energy production and the introduction of reactive oxygen species into the cellular environment, which, in turn, can induce a hypoxic response (for a review see Hüttemann *et al*. (2008)). Other genes encoding metabolic processes such as the *succinate dehydrogenases* (*SDH*) and *fumarate hydratase* (*FH*) have previously been shown to be inactivated in familial RCC (Morris *et al*., 2003, 2004; Ricketts *et al*., 2008).

Very little is known about the function of the *CORO6* gene product (coronin-6), but the other members of the coronin family are actin-binding proteins that have been shown to function in cell motility, vesicle trafficking and cell division (Roadcap *et al*., 2008). Whether CORO6 has similar activity is unclear, but such fundamental cell functions are often dysregulated in cancer. Similarly, the function of *KLHL35*, *CCDC8* and *ZSCAN18* gene products is not well characterised, but we note that RNAi-induced downregulation of these transcripts resulted in some of the most significant growth advantages we observed in the anchorage-independent growth assay (to our knowledge there are no previous reports of a growth advantage to a non-cancer cell line following the knockdown of these genes and also of *SCUBE3*, *QPCT* and *FBN2*). These genes merit further investigation to determine their role in RCC. It will also be of interest to determine whether these genes are dysregulated in a broader range of tumours.

More than 200 000 new cases of kidney cancer are diagnosed in the world each year (Bray *et al*., 2002), and

although, if detected early, partial or radical nephrectomy is an effective treatment, many patients present with advanced disease. The response of metastatic RCC to conventional chemotherapy is poor, but characterisation of the molecular pathology of RCC can provide a basis for developing novel therapeutic approaches. This is exemplified by the *VHL* TSG paradigm in which (a) inactivation of *VHL* is the most common event in sporadic clear cell RCC (Latif *et al*., 1993; Foster *et al*., 1994; Herman *et al*., 1994; Clifford *et al*., 1998); (b) *VHL* inactivation leads to stabilisation of HIF-1 and HIF-2 transcription factors and activation of hypoxic response genes that drive renal tumourigenesis (Maxwell *et al*., 1999); and (c) inhibitors (for example, tyrosine kinase inhibitors such as sorafenib and sunitinib) of HIF target pathways are active in the treatment of metastatic RCC (Chowdhury *et al*., 2008). Hence, identification of frequently inactivated RCC TSGs can provide a basis for novel therapeutic interventions. Strategies to identify epigenetically inactivated TSGs therefore represent an important approach to elucidating the molecular pathogenesis of RCC and, furthermore, the detection of methylated RCC TSG DNA in urine or serum might be used as biomarkers for the diagnosis, staging or risk stratification of RCC (Battagli *et al*., 2003; Hoque *et al*., 2006; Urakami *et al*., 2006). Although confirmation is required, our findings suggest that *SCUBE3* methylation status could be a prognostic marker in RCC.

Previous RCC epigenetic studies (Breault *et al*., 2005; Morris *et al*., 2005, 2010; Christoph *et al*., 2006; Ibanez de Caceres *et al*., 2006; Yamada *et al*., 2006; Costa and Drabkin, 2007; McRonald *et al*., 2009) have identified candidate RCC TSGs that have functional roles in key pathways commonly dysregulated in cancer biology. Interestingly, many of the genes identified in this study, which were shown to be frequently methylated or to have *in vitro* growth suppressor activity, would not have been chosen as obvious candidate genes (none were represented in the 3544 genes sequenced in RCC by Dalgliesh *et al*. (2010)). One advantage of unbiased genome-wide approaches, such as that used in this study, is the potential to uncover novel genes and pathways that can be targeted for further investigation.

Materials and methods

Patients and samples

DNA from up to 69 primary RCCs (~80% clear cell, 20% non-clear cell) and matched adjacent macroscopically normal renal tissue and normal renal tissue from six patients undergoing non-cancer renal surgery (mean age 57 years, range from 23 to 79 years) were analysed. Nine tumour DNAs were used for MeDIP array analysis. Twenty tumour DNA samples were used to confirm initial array results and a further 40 were used to follow-up positive candidate genes (total = 69). 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 KTCL26, RCC4, UMRC2, UMRC3, SKRC18, SKRC39, SKRC45, SKRC47, SKRC54, 786-O, Caki-1, CAL54, RCC48, RCC1, RCC12, Caki-2, A498, ACHN and 769-P were routinely maintained in DMEM (Invitrogen, San Diego, CA, USA) supplemented with 10% FCS at 37 °C in 5% CO_2. The demethylating agent 5-Aza-2′-deoxycytidine (Sigma-Aldrich, Dorset, UK) was freshly prepared in dd H_2O and filter sterilised. Cell lines were plated in 75-cm^2 flasks in DMEM medium supplemented with 10% FCS at differing densities, depending on their doubling time, to ensure that both control and 5-Aza-2′-deoxycytidine-treated lines reached ~75% confluency at the point of RNA extraction. After 24 h, cells were treated with 5 μM 5-Aza-2′-deoxycytidine. The medium was changed 24 h after treatment and then changed again after 72 h. RNA was prepared 5 days after treatment using RNABee (AMS Biotechnology, Oxford, UK). Total RNA from all 19 cell lines +/− 5-Aza-2′-deoxycytidine was isolated using RNA-Bee reagent following the manufacturer's instructions (AMS Bio), followed by purification using RNeasy Minicolumns (Qiagen, Crawle, UK). Complementary RNA probes from KTCL26, RCC4, UMRC2, UMRC3, SKRC18, SKRC39, SKRC45, SKRC47, SKRC54, 786-O and Caki-1 cell lines were prepared using the Affymetrix protocol and hybridised to HG-U133 plus2 GeneChip oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA). Array hybridisation and data production were carried out by the CRUK Paterson Institute Microarray Service (http://bioinformatics.picr.man.ac.uk/mbcf/). Complementary RNA probes for CAL54, RCC48, RCC1, RCC12, Caki-2, A498, ACHN and 769-P lines were prepared using the Illumina method and hybridised to Human WG-6 geneChip arrays (Illumina, Little Chesterford, UK).

MeDIP DNA preparation and analysis

MeDIP was performed as per the suggested protocol of Nimblegen (Roch-Nimblegen, Madison, WI, USA). RNA-free genomic DNA was extracted from RCC cell lines and tumours by standard methods. A measure of 6 μg of each DNA sample was digested overnight at 37 °C with 24 U of MseI (supplemented with 100 ng/μl BSA). The reactions were stopped by heating at 65 °C for 20 min, and the success of the reactions was verified by running a small aliquot of the digested DNA on an agarose gel. DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and 1.25 μg of DNA was diluted in TE buffer to a final volume of 300 μl. The DNA was denatured at 95 °C for 10 min and 60 μl (250 ng) was removed for use as control (input) DNA. A volume of 60 μl of 5xIP buffer (100 mM Na phosphate pH7.0, 5 M NaCl, 10% Triton X-100) and 1 μg of IP antibody (mouse monoclonal anti-5-methylcytidine, Eurogentec, Southampton, UK) were added to the remaining DNA, and the mixture was incubated overnight at 4 °C with gentle rotation. Protein A agarose beads (24 μl) were prewashed twice in PBS/0.1% BSA, and resuspended in 24 μl 1xIP buffer; this 50% slurry was added to the DNA/antibody mixture and incubated for 2 h at 4 °C with gentle rotation. The beads were washed three times with 1xIP buffer and resuspended in 250 μl digestion buffer (1 M Tris–HCl pH 8.0, 0.5 M EDTA, 10% SDS; filter sterilised). A volume of 7 μl of proteinase K (at 10 mg per ml) was added to the above mixture, and incubation was carried out overnight at 55 °C. DNA was purified by phenol/chloroform extraction and ethanol precipitation (the latter with the addition of 20 μg glycogen to facilitate visualisation of the DNA pellet). A volume of 10 ng of input DNA and 10 ng of IP DNA were subjected to whole-genome amplification with the WGA2 kit (Sigma), and purified using the Qiaquick PCR purification

kit (Qiagen). A measure of 4 μg of input and IP DNA was sent to Nimblegen Laboratories, differentially labelled and applied to the HG18 RefSeq Human Promoter Array.

RT–PCR conditions
PCR cycling conditions consisted of 5 min at 95 °C, followed by 30 cycles of 45 s of denaturation at 95 °C, 45 s of annealing at 55–60 °C and 45 s of extension at 72 °C. Semiquantitative analysis of expression was carried out using LabWorks software (Ultraviolet products, Upland, CA, USA). (RT–PCR primers and conditions are available on request).

Bisulphite modification
A volume of 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 sulphonated by incubation in 3.12 M sodium bisulphite (pH 5.0; Sigma)/5 mM hydroquinone (Sigma) in a thermocycler (Thermo-Hybaid, Ashford, UK) for 20 cycles of 30 s at 99 °C and 15 min at 50 °C. The sulphonated DNA was recovered using the Wizard DNA cleanup system (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The conversion reaction was completed by desulphonating 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, Munich, Germany). Primers used to amplify these regions from bisulphite-modified DNA can be found in Supplementary Table 1. Promoter region methylation in cell lines was identified by direct sequencing of bisulphite-PCR products as described previously (Morris *et al*., 2005). CoBRA was carried out by digesting bisulphite-PCR products with BstU1. Promoter methylation analysis of tumour DNA was carried out by cloning bisulphite-PCR products into pGEM (Promega), followed by sequencing of individual clones using primers to T7 or M13.

Anchorage-independent growth assay
RNAi 'silencer select' oligos against KLHL35 (s49143), FBN2 (s5049), ATP5G2 (s1781), SOX14 (s15948), CORO6 (s39713), CCDC8 (s228331), PCDH8 (s10114), SCUBE3 (s48237), ZSCAN18 (s35299) and QPCT (s24500) or 'Silencer select' control oligo no. 1 (Ambion, Austin, TX, USA) was transfected into HEK293 cells using Interferin reagent (Polyplus, Illkirch, France) following the manufacturer's instructions. After 24 h incubation, cells were seeded into 2 ml DMEM in 10% FCS and 3% agar. Cells were maintained by addition of 200 μl of DMEM in 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 24 h before efficiency of knockdown was assessed by reverse transcriptase–PCR and western blotting (Supplementary Figure 1).

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

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)