Genetic and epigenetic control of UNC5C expression in human renal cell carcinoma

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ABSTRACT

Inappropriate gene silencing and subsequent promiscuous activity define the transformation of many solid tumours including renal cell carcinoma (RCC). Here, we report that UNC5C, one of the Netrin-1 receptors, was frequently inactivated in RCC cell lines and primary tumours. UNC5C protein was expressed in the proximal convoluted tubules of the human kidney, the presumed origin of clear cell RCC (ccRCC) and papillary RCC (pRCC). Compared to paired adjacent non-malignant tissues, both UNC5C mRNA and protein expression were significantly down-regulated in RCC. Immunohistochemical analysis showed that UNC5C was inactivated in 94.3% of the samples and the loss of UNC5C occurred at the early stage of RCC. Methylation specific PCR showed that UNC5C promoter was methylated in two renal carcinoma cell lines. Pharmacologic demethylation alone or in combination with inhibition of deacetylation dramatically induced UNC5C expression. Furthermore, bisulfite genomic sequencing (BGS) confirmed that dense methylation existed in UNC5C promoter. In paired tumour samples, UNC5C methylation was observed in 12 out of 44 patients (27.3%). Moreover, we analysed the loss of heterozygosity (LOH) of UNC5C in renal cell carcinoma, the LOH was observed in 27 out of 44 patients (61.4%). Finally, restoration of UNC5C expression suppressed the colony formation of renal carcinoma cells. In addition, UNC5C inhibited tumour cell proliferation, migration and enhanced chemosensitivity to cisplatin and etoposide. Therefore, UNC5C acts as a tumour suppressor in RCC and is down-regulated in RCC. Loss of heterozygosity and DNA methylation contribute to the inactivation of UNC5C in renal cell carcinoma.

1. Introduction

Renal cell carcinoma is the most frequent primary malignancy in the adult kidney, accounting for 2–3% of all adult tumours.1 Clear cell (ccRCC, 70% of all RCC), papillary (pRCC, 10–15%), and chromophobe RCC (5%) constitute the majority of RCCs. Among them, both ccRCC and pRCC originate in the lining of the proximal convoluted tubules. Treatment failures in RCC patients are mainly due to its vascular dissemination and resistance to chemo- or radiotherapy. The silent and aggressive progression renders early RCC detection difficult; most patients are typically diagnosed at an advanced state, for...
which there are no effective therapies. Therefore, characterisation of the mechanisms underlying the tumourigenesis in RCC may provide promising targets for early diagnosis and further therapeutic intervention.

In recent years, emerging evidence suggests that RCCs arise from the accumulation of multiple genetic and epigenetic alterations. The most common genetic alteration in sporadic ccRCCs is inactivation of the von Hippel-Lindau (VHL) gene, due to loss of heterozygosity (LOH) at 3p26-25. Frequent methylation of the promoter CpG dinucleotide region of tumour suppressor genes (TSGs) has also been implicated in the pathogenesis of RCCs. A growing number of TSGs have been aberrantly methylated in ccRCCs including VHL, RASSF1A, p16, TIMP3, E-cadherin and β-catenin. Both RCCs themselves and established RCC cell lines are resistant to cytotoxic agents. This may be partly attributed to activation of survival pathways or inactivation of apoptotic pathway. For example, loss of VHL has been reported to be involved in activation of NFκB which provides survival signal for RCC. Up-regulation of Bcl-2 expression and down-regulation of pro-apoptotic proteins such as Bax, Bim, p53 in RCCs have been found to be associated with the development and poor prognosis of RCCs. The loss of apoptotic signals may be crucial to the pathogenesis of RCC as well as the resistance to radio- and chemotherapy.

UNC5C, one of the Netrin-1 receptors, belongs to the UNC5H functional dependence receptor family and has the ability to induce apoptosis in the absence of the Netrin-1 ligand. Like other members of Netrin-1 receptors, UNC5C was also thought to be a tumour suppressor. The loss of UNC5C expression was reported in colorectal cancer, gastric cancer, etc. Deletion of chromosome 4q, where UNC5C resides, has been observed in 50% of ccRCC, thus suggesting that UNC5C represents a critical tumour suppressor in ccRCC. Here we demonstrated that UNC5C was exclusively expressed in cells of the proximal convoluted tubules and frequently down-regulated in RCC, mainly due to LOH and tumour associated promoter methylation. Consistently, restoration of UNC5C expression in renal cancer cells inhibits the colony formation, cell proliferation, migration and enhances chemosensitivity.

2. Materials and methods

2.1. Cell lines, tumour samples and 5′-aza-dC treatment

A series of cancer cell lines were used for this study including 5 RCC cell lines (Caki-1, Os-RC-2, A498, ACHN, 786-O). Human embryonic kidney cell line HEK 293T and proximal epithelial cell line HKC were also used in this study.

Primary renal carcinoma and their corresponding non-cancerous tissues (n = 44) were obtained through surgical resections at the Peking University Third Hospital with patient’s consents and institutional ethics approval. All of the specimens were pathologically confirmed.

The treatment of 5′-aza-2′-deoxycytidine (Aza) (Sigma, St. Louis, USA) has been described previously.

2.2. Reverse transcriptase-PCR and real-time PCR

Total RNA was isolated from cell lines and frozen stored tissue specimens by TRIZOL reagent (Invitrogen, Carlsbad, CA). RNA was reverse transcribed using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer’s instruction. Human normal tissue cDNA panel was purchased from Clontech. Quantitative real-time polymerase chain reaction (PCR) was performed using the Bio-Rad Real-Time PCR system with Power SYBR Green PCR master mix (Applied Biosystems). Each reaction was performed in duplicate, and negative controls were included in each experiment. The gene expression was quantified as the yield of the UNC5C relative to that of GAPDH. The ΔΔCT formula was used as described. All primers used in this study were included in Table S1.

2.3. Tissue microarray and immunohistochemistry

A tissue microarray (TMA) was purchased from Chaoying Biotechnology Company (Xi’an, China). According to the manufacturer’s instructions, sections were then incubated with anti-UNC5C antibody (Sigma–Aldrich) 1:200 diluted in PBS or with anti-N-cadherin antibody (Santa Cruz) 1:100 diluted in PBS overnight at 4 °C. Staining was evaluated by percent of tumour cell positivity and staining intensity (0% = 0, 1–10% = 1, 11–50% = 2, 51–80% = 3, 81–100% = 4; negative means 0% area staining, positive means 1–80% area staining).

UNC5C-positive normal human kidney tissue was used as the positive control.

2.4. Bisulfite treatment, sequencing and methylation-specific polymerase chain reaction

Genomic DNA was extracted from cell lines and tissue samples by using a commercial DNA extraction kit (Promega). Bisulfite treatment was as described. Detailed method for it was supplied in supplementary text. The bisulfite-treated DNA was amplified with the methylation specific primer set and sequencing primer set (Table S1) by PCR using hot-start GoTaq (Promega) for 40 cycles. The PCR products were cloned into the pGEM-T-Easy (Invitrogen) with 10 colonies randomly chosen and sequenced.

2.5. Microsatellite analysis

Genomic DNA (50 ng) from matched tumour and corresponding non-cancerous tissues were amplified by polymerase chain reaction (PCR) and using fluorescently labelled primers for the indicated polymorphic microsatellite markers on chromosomes 4q21–23 for UNC5C locus. The 44 renal carcinomas were investigated for LOH at the following 2 markers: C1 (nt 96,037,547–96,037,735) located telomerically and C2 (nt 96,304,362–96,304,495) located centromerically.

2.6. Cell transfection and Western blot analysis

Myc-tagged UNC5C expressing vector is a courtesy of Dr. Patrick Mehlen. Using it as the template, we amplified the full-length UNC5C and cloned it into a CMV vector with C-terminal Flag tag. The mock and UNC5C plasmid was transfected into A498 cell line by Lipofectamine 2000 (Invitrogen). After electrophoresis and transfer, the membrane was blocked and then incubated with anti-GAPDH (Proteintech Group, Inc.) or anti-Flag (Sigma–Aldrich) antibody overnight.
at 4 °C. IRDye-700-conjugated anti-Ig were used as secondary antibodies, and proteins were detected using the Odyssey Imaging System).

### 2.7. Cell proliferation and migration assay

Cell proliferation was measured with Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Japan), according to the manufacturer’s instructions. Each condition was repeated at least 3 times. All the cells were harvested at the designated times after treatment. For migration assay, cells were seeded on 8-μm-pore-size Transwell filter insert (Costar) coated with 10 μg/ml fibronectin (Sigma–Aldrich, St. Louis, MO). After 20 h of incubation at 37 °C, cells adherent to the upper surface of the filter were removed using a cotton swab. Cells were fixed with methanol and stained with crystal violet, and the number of cells at the bottom was counted. Data are from three experiments done in triplicate.

### 2.8. Soft-agar colony formation assay

Cells were transfected as above. Soft agar colony formation assay was performed following the previous protocol. A total of 5000 cells were mixed with 0.35% agarose and were set in each well of six-well plates which contains 0.5% agarose. After 2 weeks, colonies were stained with 0.005% crystal violet. Each treatment was triplicate.

### 2.9. Statistical analysis

Comparisons of UNC5C methylation, LOH and gene expression levels between paired tumour and adjacent non-malignant tissue samples were performed using the Wilcoxon signed rank test. For cell proliferation assay, statistical significance was examined using paired t-tests. The chi-square test was used to calculate differences in gender of the patient, tumour stage and LOH status between adjacent normal tissues and tumour tissues. These analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL). P values <0.05 were considered statistically significant.

### 3. Results

#### 3.1. UNC5C is expressed in cells of proximal convoluted tubules

To test if UNC5C is involved in RCC development, we initially examined the expression profile of UNC5C in 16 normal adult tissues. RT-PCR showed that UNC5C was widely expressed in normal tissues including kidney (Fig. 1A). To further examine UNC5C expression in kidney, we detected UNC5C protein levels in the normal kidney tissues by immunohistochemistry. As shown in Fig. 1B, UNC5C was not uniformly expressed in the kidney. According to the four major characteristics for distinguishing distal and proximal tubules, by H&E staining and morphological analysis, we observed that UNC5C was exclusively expressed in cells of the proximal convoluted tubules. Proximal tubules are more eosinophilic in H&E staining, microvillus brush border is sloughed and can be seen in the lumen. Moreover, to verify this localisation, we also stained the normal kidney tissues by an N-cadherin specific antibody, as N-cadherin has been reported to be expressed in the proximal tubules. It clearly showed that UNC5C had similar expression pattern to N-cadherin (Fig. 1B). In conclusion,
UNC5C is expressed in the proximal tubules in which most RCCs originate.

3.2. UNC5C is down-regulated in renal carcinoma cell lines by hypermethylation

To verify this hypothesis that UNC5C acts as a tumour suppressor in RCC, we examined UNC5C mRNA expression in a variety of carcinoma cell lines. The RT-PCR results showed that UNC5C was undetectable in four RCC cell lines 786-O, Os-RC-2, A498 and ACHN. There was weak expression of UNC5C in Caki-1 cell line (Fig. 2A). Furthermore, UNC5C was down-regulated or silenced in other carcinoma cell lines.

The region spanning the putative promoter of UNC5C and exon 1 is a typical CpG island. We next analysed the methylation status of UNC5C in these cell lines by methylation specific PCR (MSP) primers against UNC5C promoter. As indicated in Fig. 2B, UNC5C was methylated in 2 RCC cell lines 786-O, A498 (out of the 5 RCC cell lines, 2/5, 40%). In addition, the methylation of UNC5C was also observed in leukaemia (Raji, Jurkat, 2/3), lung cancer cell lines (H460, H446, 2/3). There was no methylation of UNC5C in HEK-293T and SK-Mel-37 which showed relatively high expression of UNC5C. Further bisulfite genomic sequencing (BGS) confirmed MSP results (Fig. 2C). To determine whether methylation directly mediated UNC5C inactivation, the 786-O, Os-RC-2 and Jurkat cell lines were treated with the DNA demethylation agent Aza alone or with the histone deacetylase inhibitor TSA (Fig. 3D). After treatment, UNC5C expression was significantly increased in these cell lines along with an increase in unmethylated alleles and a decrease in methylated alleles (Fig. 2D).

3.3. UNC5C expression is down-regulated in primary renal cell carcinomas

We further investigated whether UNC5C was down-regulated in renal carcinomas by RT-PCR and quantitative real-time PCR. As shown in Fig. 3A and B, compared to adjacent non-malignant tissues, UNC5C mRNA was dramatically down-regulated or inactivation in 44 paired renal cancer samples ($p < 0.0001$).

Moreover, we confirmed the reduction of UNC5C in RCC at the protein level by immunohistochemistry. By several paired

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**Fig. 2 – Down-regulation of UNC5C in renal cell carcinoma cell lines and up-regulation of UNC5C by demethylation agent treatment.** (A) UNC5C mRNA expression profile in multiple cell lines. (B) Methylation specific PCR analysis of UNC5C promoter methylation in multiple carcinoma cell lines. (C) Sequence of UNC5C promoter. The transcription start was indicated as an arrow. MSP primers were also shown in the figure. Bisulfite genomic sequencing of the UNC5C promoter in some cell lines. Each row represents individual allele of UNC5C promoter analysed. Filled box indicates methylated, open box indicates unmethylated. (D) Pharmacologic demethylation with Aza alone or combined with TSA induced UNC5C expression in methylated and silenced carcinoma cell lines (real-time PCR). MSP analysis of UNC5C promoter methylation in 786-O cell line before or after treatment with demethylation agents. """"$p < 0.0001$."""
tissue specimen available, we found that UNC5C protein expression was lost or very weak in RCC, in contrast to the strong staining for UNC5C in paired non-malignant tissues (Fig. 3C). In order to analyse the UNC5C protein expression in large-scale RCC samples, we examined the UNC5C expression in a tissue microarray including 70 clear cell renal carcinoma (ccRCC) tissues and 10 normal tissues. UNC5C was undetectable in 66 tumour tissues (66/70, 94.28%). There was weak expression of UNC5C in 4 tumour tissues (4/70, 5.71%) (Fig. 2). In contrast to that, 8 normal tissues (8/10, 80.00%) exhibited strong staining for UNC5C, 2 normal tissues exhibited weak expression. Further statistical analysis revealed that loss of expression of UNC5C did not correlate with any histological parameters including age, gender, tumour stage and histological grade (Table S2). This suggested that the inactivation or down-regulation of UNC5C expression occurred at the early stage of the RCC. The loss of UNC5C expression may be an important initiation step for RCC development.

3.4. UNC5C promoter is methylated in primary renal cancer tissues

We also analysed the UNC5C methylation status in 44 primary RCC samples and paired adjacent non-malignant renal tissues. Of 44 tumour samples, 12 (27.3%) showed methylation in the UNC5C promoter (Fig. 4A), as compared with no methylation in the corresponding normal tissues. The methylation-specific PCR results were confirmed by direct sequencing of CpG islands in the UNC5C promoter region (Fig. 4A). We also evaluated the relationship of UNC5C methylation with clinicopathological features in patients with RCC (Table 1). All methylated specimens we detected were in histological grade 2 (P = 0.002). No correlation with age, gender, tumour stage was observed by statistical analysis. Although not statistically significant, it exhibited the trend that more methylation of UNC5C occurred at higher tumour stages (stages 2 and 3) (Table 1, Fig. 4D).

3.5. UNC5C down-regulation correlates with LOH

As shown above, promoter methylation may partly contribute to the inactivation of UNC5C in RCC. Human UNC5C is located at chromosome 4q21–23. To search for the other mechanism that may explain for its down-regulation in RCC, we determined the frequency of allelic losses in two markers C1 and C2inside on chromosome 4q21–23 that had been described. C1 maps closely to the gene, C2inside is inside UNC5C gene. Of the 44 specimens investigated, 27 (61.4%) cases showed allelic imbalance in at least one of the markers (Fig. 4B and C).
We further examined that LOH and methylation of UNC5C occur concurrently, versus independently of one another. We found that only one case (3.1%) underwent simultaneous methylation and LOH of UNC5C. Independent promoter methylation or allelic imbalance of UNC5C occurred in 20.5% (9/44) and 52.3% (23/44) RCCs, respectively. Taken together,

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77.3% cases showed one of the genetic or epigenetic alterations in RCCs (Table 1).

3.6. Restoration of UNC5C expression suppresses the colony formation, cell proliferation, migration and enhances the chemosensitivity

Finally, we investigated the effects of ectopic UNC5C expression on cell behaviour. As shown in Fig. 5A, A498 cells with restoration of UNC5C exhibited low proliferation rate compared to vector control. Besides, UNC5C inhibited the colony formation of 786-O and A498 cells (Fig. 5B). Additionally, UNC5C inhibited the cell migration of A498 cells (Fig. 5C). Because UNC5C belongs to the dependence receptor family, we further tested that if it can regulate apoptosis sensitivity. A498 cells were transfected with control or UNC5C expression vector and exposed to conventional chemotherapeutic agents, cisplatin or etoposide at various concentrations for up to 48 h. We found that restoration of UNC5C expression can enhance chemosensitivity to cytotoxic agents (Fig. 5D).

4. Discussion

UNC5Hs were first suggested as putative tumour suppressors by Thiebault et al. They found that expression of human UNC5H family members was down-regulated in multiple cancers including colorectal, breast, ovary, uterus, stomach, lung, or kidney cancers. Recent studies specifically about UNC5C demonstrated that UNC5C was down-regulated in colorectal and gastric cancers. However, the expression profile of UNC5C and the possible control mechanism in renal cell carcinoma are poorly understood. By RT-PCR and immunohistochemistry, we demonstrated that UNC5C was expressed in human normal kidney. Intriguingly, its expression was restricted to proximal tubules. There was no expression of UNC5C in the distal tubules and glomerulus. ccRCC and pRCC account for the majority of RCC. Both of them arise from the proximal tubules. Given the information above, UNC5C may be a tumour suppressor in RCC.

At first, we analysed the expression pattern of UNC5C in RCC. We found that UNC5C was dramatically down-regulated or inactivated in renal cancer samples both at mRNA and protein level. Notably, down-regulation of UNC5C protein expression occurred at the early stage of the development of RCC. This suggested that down-regulation of UNC5C may be a prerequisite for renal cancer development, moreover, it may serve as a diagnostic marker for early RCCs.

Searching for the mechanism underlying the down-regulation of UNC5C in RCC, we determined the methylation status of UNC5C promoter in renal cancer cell lines and renal cancer samples. UNC5C was methylated in 786-O and A498 (2 out of the 5 RCC cell lines, 40%). And treatment of DNA demethylation agents increased the expression of UNC5C. In primary RCCs, 27.3% (12/44) tumour samples showed methylation in the UNC5C promoter, as compared with no methylation in the corresponding non-malignant tissues. This data suggested that methylation of UNC5C promoter may contribute to the down-regulation or inactivation of UNC5C in RCC. The higher methylation rate in RCC cell lines than primary tumours observed in our study might be explained by the

Fig. 5 – Restoration of UNC5C expression suppressed renal cancer cell proliferation, colony formation, migration and enhanced chemosensitivity. (A) Cell proliferation was determined by CCK8 in the A498 cell line. The expression of UNC5C in A498 was detected by Western blot. (B) Soft agar colony formation assay. Colonies (>50 cells) were counted 2 weeks after seeding. (C) The migrative property of the cells was investigated by Transwell chamber. (D) The chemosensitivity of A498 cells to etoposide and cisplatin was measured by CCK8. *P < 0.05; **P < 0.001.
introduction of additional genetic and epigenetic alterations into cancer cell lines during the establishment or maintenance process.

The incidence of UNCSC methylation in RCCs was similar to that in gastric cancers (25%) and lower than that in colorectal cancers (78%). Although tumour suppressors TIMP3, RASSF1A, DAL-1 exhibited relatively higher methylation incidence in ccRCC. It has been speculated that DNA methylation changes are rather rare events in ccRCC in comparison with other major malignancies. Incidences of methylation in representative tumour suppressor genes including VHL, have been observed in a male infant manifesting early onset hepatoblastoma (HBL). The transition of follicular B cell lymphoma to diffuse large cell lymphoma was accompanied by 4q21-q23 deletion. In colorectal cancers, depending on different microsatellite markers used, the frequency of LOH at the UNC5C locus was relatively low (25-39%). In our study, we chose the C1 and C2 inside markers that had been verified for the LOH analysis. C1 was closer to the UNC5C gene than markers used in Ref. C2 inside was inside the UNC5C gene. In our study we observed a relatively high occurrence of LOH at the UNC5C locus in RCC, i.e. 27 (61.4%) cases showed allelic imbalance in UNC5C locus. This is agreement with previous report that 4q loss may occur in 50% of ccRCC. LOH may also be another mechanism that account for the loss expression of UNC5C in renal cancer cell lines.

The most common genetic alteration for RCC is inactivation of VHL. The LOH of a VHL allele is detected in 90% sporadic RCC. In our study, the loss of UNC5C allele displayed 61.4% frequency. It may represent another important tumour suppressor that is frequently inactivated in RCC and predisposed to tumourigenesis of RCC.

Dependence receptors share the ability that in the absence of the ligand, they can induce apoptosis. This may confer them to negatively regulate cell growth when unbound to the ligand. By restoration of UNC5C expression, we found that UNC5C inhibited cell proliferation and the colony formation of renal cancer cells. This is consistent with previous study that UNC5Cs inhibit anchorage-independent growth. The loss of pro-apoptotic molecules may be associated with the resistance of RCCs to cytotoxic agents. We observed that restoration of UNC5C expression can enhance the chemosensitivity to etoposide and cisplatin. This function of UNC5C has not been described elsewhere. This feature is similar to earlier studies about the roles of other proapoptotic molecules like Bax, Bim, p53 in RCC.

To our knowledge, this is the first report that systematically analyses the expression and regulation of UNC5C in RCC. UNC5C is down-regulated or inactivated in RCC both at the mRNA and protein level. It may be a prerequisite for RCC development. LOH and promoter methylation account for the down-regulation or inactivation. Restoration of UNC5C expression in renal cancer cells inhibits the colony formation, cell proliferation, migration and enhances chemosensitivity to cytotoxic agents. The understanding of UNC5C in RCC adds to the full portrait of the development and regulation of RCC. It may provide potential useful diagnostic or therapeutic values for RCC. More studies are needed to elucidate whether UNC5C can serve as an early diagnostic marker for RCC.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data


References


