The Tumor-Suppressive Function of UNC5D and Its Repressed Expression in Renal Cell Carcinoma

Dan Lu1, Dong Dong1, Yu Zhou1, Min Lu2, Xue-Wen Pang1, Yan Li1, Xiao-Jun Tian3, Yu Zhang1, and Jun Zhang1

Abstract

Purpose: As a newly added member of the UNC5H receptors, the function of UNC5D/H4 in tumorigenesis remains poorly defined. The aim of this study was to examine the expression of UNC5D in primary renal cell carcinomas (RCC), analyze the mechanisms responsible for its downregulation in RCC, and assess its functional relevance to tumor growth and migration.

Experimental Design: Forty-four paired primary RCCs and corresponding adjacent noncancerous tissues were collected. The mRNA and protein expression level of UNC5D was assessed by reverse transcriptase-PCR, real-time PCR, or immunohistochemistry. Epigenetic alterations in UNC5D promoter and LOH in the UNC5D locus were also analyzed. Ectopic expression of UNC5D in renal cancer cells with silenced expression of UNC5D was used for analysis of the biologic functions of UNC5D.

Results: UNC5D expression was attenuated in multiple carcinoma cell lines including renal cancer cells. Similar reduction was also observed in primary RCC tissues as compared with paired adjacent noncancerous tissues. Methylation-specific PCR showed hypermethylation in UNC5D promoter in a significant proportion (18 of 44) of tumor tissue (40.9%). LOH of UNC5D was observed in 13 of 44 patients with RCCs (29.5%). Restoration of UNC5D expression in renal cancer cells significantly inhibited cell proliferation, anchorage-dependent and -independent growth, as well as migration and invasion, whereas knockdown of UNC5D promoted cell growth. Furthermore, ectopic expression of UNC5D induced G2–M cell-cycle arrest.

Conclusions: UNC5D is a functional tumor suppressor that is frequently downregulated in RCCs due to promoter hypermethylation and LOH. Clin Cancer Res; 1–10. © 2013 AACR.
Translational Relevance

Netrin-1 receptors—UNC5H family members play important roles in tumorigenesis. In this study, we found that UNCS5/H4, the newly added member of the UNC5H family, widely expressed in normal tissues, was frequently absent or attenuated in cancer cell lines and primary renal cell carcinoma (RCC). Promoter CpG methylation was responsible for this. Pharmacologic demethylation restored UNCS5 expression along with concomitant promoter demethylation. UNCS5 methylation was also frequently detected in primary RCCs. LOH also contributed to its downregulation in RCCs. Ectopic UNCS5 expression in silenced renal cancer cell line dramatically inhibited the growth of renal cancer cells through promoting G2–M cell-cycle arrest. Ectopic UNCS5 expression also inhibited the migration and invasion of renal cancer cells. These findings raise the possibility that UNCS5 might be a novel tumor suppressor in RCCs and serve as a potential diagnostic and therapeutic target for RCCs in the future.

by the ligand Netrin-1, these receptors induce apoptosis but inhibit apoptosis and provide a survival signal in the presence of the ligand. Through such a mechanism of action, these receptors may act as tumor suppressors (19). In line with this speculation, the receptor DCC shows reduced or lost expression in a variety of cancers (20). Another group of Netrin-1 receptors referred to as UNC5H (1-3/A-C) are also downregulated in a number of tumors. Overexpression of these receptors inhibits tumor cell anchorage-independent growth and invasion (21–23).

UNC5D/H4 is the most recently identified member of the UNC5H receptors (24). One study reported that UNCS5 is induced during DNA damage–mediated apoptosis and is a direct transcripational target of p53 (25). Several other studies showed that rearrangement of 8p with loss of distal 8p, where UNCS5 is located, is one of the most frequent genomic events in common epithelial cancers (26, 27). Nevertheless, the function of UNCS5 in tumorigenesis remains poorly defined.

The present study was focused on the potential role of UNCS5 in the development of RCCs. We first examined its expression in tumor versus adjacent noncancerous tissue and explored the mechanisms underlying its suppression in tumor tissue. Subsequently, the implication of UNCS5 downregulation in RCC was analyzed by monitoring altered cell behaviors following restoration of its expression in otherwise silenced cells. Data thus acquired support a tumor-suppressive function of UNCS5 in RCCs.

Materials and Methods

Cell lines, tumor specimens, and 5-aza-dC treatment

A series of cancer cell lines were used for this study, including 5 RCCs (786-O, A498, ACHN, Caki-1, and Os-RC-2), 2 bladder carcinoma, 5 hepatoma, 5 lung cancer, 4 gastrointestinal cancer, 5 leukemia, 1 prostate carcinoma, 1 breast cancer, 1 cervical carcinoma, 1 ovarian cancer, 2 melanoma, and 1 osteosarcoma cell line. Human immortalized embryonic kidney cell lines HEK293 and HEK293T were also used. Renal carcinoma and corresponding noncancerous tissues (n = 44) were obtained from the Peking University Third Hospital (Beijing, China) with patient’s consents and institutional ethics approval. All of the specimens were pathologically confirmed. For demethylation, cell lines were treated with 10 μmol/L of 5-aza-2′-deoxycytidine (5-aza-dC, Sigma-Aldrich) for 3 days with exchange of reagents and medium every 24 hours.

Reverse transcriptase PCR and real-time PCR

A human normal tissue cDNA panel was purchased from Clontech. Conventional PCR and quantitative real-time PCR were carried out as previously described (15). All primers used in this study are shown in Supplementary Table S1.

Immunohistochemistry and scoring

The tissue microarray (Shanghai Outdo Biotech Co., Ltd.) of 30 paired tumor and noncancerous tissue was incubated with anti-UNC5D antibody (Santa Cruz) at 1:100 dilution overnight at 4°C. The scoring method which combined intensity and percentage of positivity was previously described (28). Slides were scored by 3 reviewers, and discrepancies were resolved by a urological pathologist. Extent and intensity measures for each core were combined as weak (intensity weak), moderate (intensity moderate), and strong (intensity strong).

Methylation-specific PCR and bisulfate genomic sequencing

Methylation-specific PCR (MSP) and bisulfate genomic sequencing (BGS) analysis were conducted as described previously (29, 30). Amplified BGS PCR products were cloned into the pGEM-T Easy vector (Promega), and 10 random clones from each sample were sequenced.

LOH analysis with microsatellite markers

A set of 3 polymorphic microsatellite markers (D8S1750, D8S505, and D8S1803) were used to determine LOH at chromosome 8p12. gDNA from paired tissue samples and cell lines was amplified by PCR using fluorescently labeled primers for the indicated polymorphic microsatellite markers.

Transfection, infection, and Western blotting

The expression vectors (pCCL-Flag-UNC5D-FL, pCCL-Flag-UNC5D-DD, pCCL-Flag-UNC5D-AZU5) with the full-length or deleted mutants of UNCS5 were constructed. Adenoviruses were packaged by the Vector Gene Technology Company Limited. Lentiviral particles were produced by transfecting HEK293T cells with the pCCL vector and the packaging vectors psPAX2 and pMD2.G. The 786-O or A498 cells were infected with adenoviruses or lentiviruses. For Western blotting, primary antibodies used include anti-
washing with PBS, cell pellet was resuspended in PBS. Cells were collected and fixed with ice-cold ethanol. After incubation for another 16 hours, cells were harvested and replated. At indicated time points, infection was conducted essentially as the migration assay, except that the Transwell insert was coated with 30 μg of Matrigel (BD Biosciences) and the invasion time was extended to 24 hours.

**Results**

Quantitative data are presented as individual data plots or as mean ± SD. Statistical analyses were done with SPSS, version 13.0 (SPSS, Inc.). Comparisons of UNC5D gene expression levels between paired tumor and adjacent nonmalignant tissue samples were conducted using the Wilcoxon signed rank test. Differences between two independent groups were analyzed by the Student t test. The paired t test was done for comparison of differences within pairs. The χ² test was used to calculate differences in the patient’s age, gender, tumor stage, histologic grade, LOH, and methylation status between paired tumor and adjacent non-malignant tissue samples. P < 0.05 was considered significant.

**Frequent inactivation of UNC5D by CpG methylation in tumor cell lines**

Bioinformatics analysis revealed that the UNC5D gene harbor a typical DNA sequence fulfilling the criteria for a CpG island in a region covering the promoter, exon 1, and the beginning of intron 1 (Fig. 2A). Given the importance of epigenetic mechanisms in the regulation of gene expression, we sought to determine whether DNA methylation contributed to the silenced expression of UNC5D in cancer cells. Indeed, MSP analysis showed that the UNC5D promoter was methylated in 2 of 4 RCC cell lines, 2 of 2 colon cancer cell lines, 2 of 3 lung cancer cell lines, 1 of 1 cervical cancer cell line, and 1 of 2 leukemia cell lines (Fig. 2B). In contrast, no methylation of the UNC5D promoter was found in the immortalized epithelial cell line HEK293. We also confirmed UNC5D methylation by high-resolution
BGS of CpG sites within the CpG island, including 43 CpG sites analyzed by MSP. Again, high levels of DNA methylation were detected in 786-O, A498, SW480, SW620, and HeLa cells but not in HEK293 cells (Fig. 2B). To show that CpG methylation is functionally associated with UNC5D silence, several carcinoma cell lines were treated with the DNA demethylation reagent 5-aza-dC. Such treatment restored UNC5D expression (Fig. 2C, left), which was accompanied by a decrease in methylated promoter alleles and an increase in unmethylated alleles. Furthermore, the demethylation was confirmed by BGS analysis (Fig. 2C, right). These results support that DNA methylation constitutes one of the major mechanisms responsible for the downregulation or inactivation of UNC5D.

Loss or reduced expression of UNC5D in primary RCC

The study was extended to primary tumors, with a focus on RCCs. RT-PCR and quantitative real-time PCR analysis revealed that UNC5D mRNA expression was markedly reduced or inactivated in a large proportion of RCC samples (Fig. 3A and B). Specifically, 32 of 44 tumor tissues showed more than 2-fold reduced expression in comparison with paired noncancerous tissue. Among them, 18 showed more than 10-fold reduced expression. We further examined UNC5D protein expression in a total of 30 paired tissue sections by immunohistochemistry. Strong UNC5D staining was shown in noncancerous renal tissue, mainly in the epithelium. On the contrary, UNC5D signal was absent or barely detectable in cancer tissue (Fig. 3C). The immunohistochemical staining was further scored by taking into consideration of both staining intensity and percentage of cells showing positive staining. The percentage of each group with strong, weak, or moderate expression level was calculated of noncancerous versus paired cancer tissue. As shown in Fig. 3D, compared with noncancerous tissue, UNC5D protein was dramatically downregulated in RCC tissue. We also analyzed the association between clinicopathologic features and the expression of UNC5D and observed no correlation between loss or reduction of expression of

Figure 1. UNC5D expression is downregulated in multiple carcinoma cell lines. A, ICGC data analysis of UNC5D expression in multiple cancer tissues. x-axis, BIC, breast invasive carcinoma; CA, colon adenocarcinoma; GM, glioblastoma multiforme; KRCCC, renal clear cell carcinoma; LA, lung adenocarcinoma; LSCC, lung squamous cell carcinoma; PC, pancreatic cancer; RA, rectal adenocarcinoma; y-axis, log2 scale normalized data for mRNA expression. B, UNC5D expression profile in human adult tissues. C, RT-PCR analysis of UNC5D expression pattern in multiple cancer cell lines.
UNC5D and the parameters including age, gender, tumor stage, and histologic grade (data not shown).

**Frequent UNC5D promoter methylation and LOH in primary RCC**

We next investigated whether promoter methylation also contributed to the attenuated or lost expression of UNC5D in primary RCCs. Among the 44 paired samples, 18 (40.9%) showed markedly increased methylation of the UNC5D promoter in tumor in contrast to that only 5 samples showed very weak methylation in the adjacent noncancerous tissue (Fig. 4A). BGS analysis confirmed that the UNC5D promoter was markedly methylated in primary RCCs but not in paired noncancerous renal tissues (Fig. 4B).
As noted above, promoter methylation may be the main contributor to the inactivation of \textit{UNC5D} in RCCs, although possibly there are other reasons for this loss. Human \textit{UNC5D} is located at chromosome 8p12. Through the analysis of chromosomal rearrangement, we found that in breast cancer there were 2 types of translocation occurring in 8p12 where \textit{UNC5D} is located, which included ampli-
con-to-amplicon translocation and inverted orientation (data not shown). Therefore, to search for other mechan-
isms that may explain the reduced expression of \textit{UNC5D} in RCCs, we determined the frequency of allelic losses in 3
markers (D8S1083, D8S505, and D8S1750). Of the 44 specimens investigated, 13 (29.5\%) cases showed allelic
imbalance in at least one of the markers (Fig. 4C and D).

Statistical analysis showed no correlation of methylation or LOH with age, gender, tumor stage, or histologic grade.
Although it was not statistically significant, a trend in which most methylation of \textit{UNC5D} occurred at tumor stage pT1 (Supplementary Table S2) was found. A relatively good correlation between \textit{UNC5D} expression level and its methylation or LOH status was observed in several randomly
chosen samples (Supplementary Table S3). In addition, in \textit{UNC5D}-negative cells without methylation modification such as NCI-H460, K562, and Os-RC-2, allelic imbalance in at least one marker was also observed (data not shown). Therefore, LOH may be indeed implicated in the loss of \textit{UNC5D} expression in these cell lines.

\textbf{\textit{UNC5D} inhibits proliferation and clonogenicity of RCC lines}

The frequent inactivation of \textit{UNC5D} in cancer cell lines and primary renal carcinoma tissue suggests a potential role of \textit{UNC5D} in tumorigenesis. To test this possibility, 786-O and A498, 2 RCC lines which showed no \textit{UNC5D} expression with a heavily methylated \textit{UNC5D} promoter were infected with \textit{UNC5D} expressing viruses and mon-
itored for changes in cell behavior. Enforced expression of \textit{UNC5D} significantly inhibited the proliferation of both
786-O (Fig. 5A) and A498 cells (data not shown). Colony formation in monolayer culture or soft agar was found to be
dramatically reduced in 786-O cells, compared with mock cells (Fig. 5B and C). Cell-cycle analysis with 786-O

![Figure 4. Promoter hypermethylation and LOH contribute to the downregulated expression of \textit{UNC5D} in primary RCCs. A, \textit{UNC5D} promoter methylation was assessed by MSP in primary RCCs and adjacent noncancerous tissues. Results from 6 representative pairs are shown. B, detailed BGS analysis of the \textit{UNC5D} promoter in 2 representative paired tissues. C, representative results of LOH status on \textit{UNC5D} in RCC and adjacent noncancerous tissue. Top, the illustration of adjacent noncancerous tissues; bottom, the tumor tissues which present a deletion. D, summary of LOH analysis for 44 noncancerous/tumor pairs in the \textit{UNC5D} locus, showing noninformative (homozygous) markers (white squares), retained markers (shaded squares), and markers with LOH (black squares). Each vertical line indicates a single patient and cases having LOH at the \textit{UNC5D} flanking markers are indicated by \textbf{\textit{\textdagger}}.](image-url)
cells revealed a decrease in G0–G1 phase and a concurrent increase in G2–M phase (Fig. 5D). To more closely examine cell-cycle progression, 786-O cells were synchronized at G2–M phase with nocodazole. Enforced expression of UNC5D delayed the re-entry of G2–M cells into a new cycle following removal of nocodazole (Fig. 5D). Consistent with G2–M cell-cycle arrest, cyclin B1 and A expression level increased in UNC5D overexpressed cells (Fig. 5E). In contrast, UNC5D-ADD lost the ability to induce cell-cycle arrest (data not shown) and abolished the inhibition on cell proliferation and colony formation (Fig. 5F–H). These data support that the death domain of

Figure 5. UNC5D inhibits renal cancer cell growth. 786-O cells were infected with mock or UNC5D-expressing adenoviruses (A–E) or lentiviruses (F–H) and monitored for growth using various assays. Each assay was repeated at least 3 times. A, cell proliferation was analyzed using CCK-8. Results are presented as absorbance (OD) at 450 nm for triplicate wells from 1 representative experiment. The inset shows protein expression of UNC5D and GAPDH. B and C, colony formation of 786-O cells in monolayer culture (B) or in soft agar (C). D, UNC5D induced G2–M arrest in 786-O cells. Cell cycle was analyzed at different time points after removal of nocodazole in synchronized or unsynchronized 786-O cells by flow cytometry. Representative histograms (left) and the percentage of cells at different phases (right) are shown. E, Western blot analysis of cell cycle hallmarks in synchronized 786-O cells after nocodazole removal. F, schematic structure of the full-length and the deletion mutants of UNC5D protein. G&H, UNC5D-ADD abolished the inhibitory function on cell proliferation (G) and the colony formation of 786-O cells in monolayer culture (H). I and J, knockdown of UNC5D expression in PC3 cells increased cell proliferation (I) and colony formation (J). * *, P < 0.01; ***, P < 0.001.
UNC5D is essential for the tumor-suppressive function of UNC5D. In addition, knockdown of UNC5D expression in PC3 cells promoted cell proliferation and colony formation (Fig. 5I and J).

**UNC5D inhibits cell migration and invasion**

We also explored the impact of UNC5D on another important aspect of tumorigenesis, migration, and invasion. First, scratch wound-healing assay was engaged to assess the motility of 786-O cells. Wound closure was found to be retarded for UNC5D overexpressing 786-O cells (Fig. 6A). Next, cell migration was tested using the Transwell assay. Enforced expression of UNC5D led to a 2- to 3-fold reduction in the number of 786-O cells crossing over the filter (Fig. 6B). Similar inhibitory effect was observed on cell invasion in a Matrigel assay (Fig. 6C). We also obtained similar results with A498 cells (data not shown). Moreover, UNC5D-ADD abolished its effects on cancer cell migration (Fig. 6D). These data suggest a suppressive function of UNC5D on tumor cell migration and invasion.

**Discussion**

UNC5D is a new member of the UNC5H family. Limited information is currently available concerning its biologic function. In this report, we show that UNC5D expression is frequently reduced or lost in renal cancer tissues and in a whole variety of tumor lines. Moreover, enforced expression of UNC5D in expression-silenced cells inhibits cell growth and migration.

Both epigenetic and genetic mechanisms are implicated in the downregulation of UNC5D. In cancer lines, UNC5D silencing correlated well with hypermethylation of a CpG island in the promoter region. Moreover, pharmacologic demethylation was able to restore UNC5D expression, supporting a direct link between promoter methylation and UNC5D downregulation. High levels of promoter methylation were also observed in about 41% of primary renal cancer tissues. Previous studies have indicated that, compared with other major malignancies, RCC shows a very low frequency of DNA methylation. It is true for most of the TSGs such as VHL, p16/CDKN2A, p14/ARF, APC, and UNC5C (9–12). However, there are several tumor suppressors, including SFRP1, SFRP2 and SEMA3B, whose expression is mainly regulated by epigenetic mechanisms (32–34). UNC5D apparently belongs to this category. As a large proportion of RCC cancer tissues exhibited hypermethylation in the UNC5D promoter, this epigenetic change should constitute a major mechanism for the suppression of UNC5D in RCCs. In addition, LOH occurred at the UNC5D locus in approximately 30% of renal cancer tissues. Of note, UNC5D maps to 8p12. Loss of chromosome arm 8p together with amplification of proximal 8p have been commonly identified in a variety of epithelial cancers including breast, bladder, colon, pancreatic cancer, and others (26, 27, 35, 36). As such, LOH may represent another important mechanism for the downregulation of UNC5D. Most recently, a genome-wide associated study has identified UNC5D as one of candidate genes...
associated with colon cancer predisposition (37). It will be interesting to see whether gene polymorphism also affects UNC5D expression.

The reduced or lost expression of UNC5D in cancer cells suggests a tumor-suppressive activity for UNC5D. We showed that restoration of UNC5D expression in renal cancer cell lines suppressed cell proliferation and anchorage-independent and -independent growth. Enforced UNC5D expression also displayed a profound inhibitory effect on cell mobility, leading to a reduced capacity of migration and invasion.

RCC is notorious for its resistance to chemo- or radiotherapy due to its insensitive to cell death induction (38). Similarly, although UNC5D indeed induced apoptosis in some cell lines (25 and our unpublished data), it did not show this ability in renal cancer cells (data not shown). Here we showed that UNC5D exerted the tumor-suppressive function mainly by cell-cycle arrest in renal carcinoma. UNC5D induced G2–M cell-cycle arrest with the upregulation of G2–M hallmark cyclin B1. To date, there is no related reports about UNC5A-C. Further studies will extend to test whether cell-cycle arrest is a common event induced by UNC5Hs or a unique feature for UNC5D. Moreover, the growth-inhibitory effect of UNC5D was mainly mediated by the death domain. The involvement of death domain containing receptors or signal molecules in cell-cycle control is extensively studied. For example, one Netrin-1 receptor DCC induced G2–M arrest by inhibition of Cdk1 (39). Dependence receptor p75NTR suppresses the proliferation of human gastric cancer cells by cell-cycle arrest (40). The nuclear death domain protein p84N5 activates a G2–M cell-cycle checkpoint (41). FasL or TRAIL signaling by death receptors inhibited cell death due to a cell-cycle arrest in S- or G2–M (42), etc. Death domain is well known as the platform for interaction with other death domain containing proteins or signaling proteins. Intriguingly, by Scansite motif analysis, we found that Thr788 of UNC5D within the DD, facilitating the binding of RAIDD and caspase-2 which is phosphorylated by ATM kinase on Thr788 within the DD, facilitating the binding of RAIDD and caspase-2 activation (43). In future, identification of signal pathways initiated by UNC5D which lead to cell-cycle arrest will be very meaningful.

In summary, our findings highlight the potential of UNC5D as a TSG in RCCs, which may serve as a potential diagnostic and therapeutic target for RCC intervention. It also extends the current understanding of the general roles of UNC5H receptors in tumorigenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Zhang, J. Zhang
Development of methodology: D. Lu, D. Dong
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Lu, D. Dong, Y. Zhou
Analysis and interpretation of data (e.g., statistical analysis, biosimulation, computational analysis): D. Lu, D. Dong, Y. Zhou, M. Lu, Y. Zhang, J. Zhang
Writing, review, and/or revision of the manuscript: D. Lu, D. Dong, Y. Zhang, J. Zhang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Lu, X.-W. Pang, Y. Li, X.-J. Tian, J. Zhang
Study supervision: Y. Zhang, J. Zhang

Acknowledgments
The authors thank Dr. Mike McNutt for his careful proofreading of the manuscript.

Grant Support
This work received support from the National Basic Research Program of China (2011CB946103), Beijing Municipal Natural Science Foundation (7122104), and the National Natural Science Foundation of China (81072395).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 17, 2012; revised March 18, 2013; accepted April 3, 2013; published OnlineFirst April 15, 2013.

References
Clinical Cancer Research

The Tumor-Suppressing Function of UNC5D and Its Repressed Expression in Renal Cell Carcinoma

Dan Lu, Dong Dong, Yu Zhou, et al.

*Clin Cancer Res*  Published OnlineFirst April 15, 2013.

**Updated version**  Access the most recent version of this article at:

doi:10.1158/1078-0432.CCR-12-2978

**Supplementary Material**  Access the most recent supplemental material at:

http://clincancerres.aacrjournals.org/content/suppl/2013/04/17/1078-0432.CCR-12-2978.DC1

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.