RESEARCH COMMUNICATION

Knockdown of Cdc25B in Renal Cell Carcinoma is Associated with Decreased Malignant Features

Xiu-Yue Yu[1], Zhe Zhang[1], Guo-Jun Zhang[2], Kun-Feng Guo[1], Chui-Ze Kong[1]*

Abstract

Cdc25 phosphatases are important regulators of the cell cycle. Their abnormal expression detected in a number of tumors implies that their dysregulation is involved in malignant transformation. However, the role of Cdc25B in renal cell carcinomas remains unknown. To shed light on influence on renal cell carcinogenesis and subsequent progression, Cdc25B expression was examined by real-time RT-PCR and western blotting in renal cell carcinoma and normal tissues. 65 kDa Cdc25B expression was higher in carcinomas than in the adjacent normal tissues (P<0.05), positive correlations being noted with clinical stage and histopathologic grade (P<0.05). To additionally investigate the role of Cdc25B alteration in the development of renal cell carcinoma, Cdc25B siRNA was used to knockdown the expression of Cdc25B. Down-regulation resulted in slower growth, more G2/M cells, weaker capacity for migration and invasion, and induction of apoptosis in 769-P transfectants. Reduction of 14-3-3 protein expression appeared related to Cdc25B knockdown. These findings suggest an important role of Cdc25B in renal cell carcinoma development and provide a rationale for investigation of Cdc2B-based gene therapy.

Keywords: Cdc25B - renal cell carcinoma – apoptosis- migration -invasion

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Introduction

Renal cell carcinoma of the clear cell types is the most common malignant tumor of the kidney, which has a poor prognosis (Leroy et al., 2007;Young et al., 2008). Approximately one-third of patients with renal cell carcinoma develop metastasis, and systemic treatment such as chemotherapy and cytokines has not proved to be effective. Some studies have identified some putative oncogenes involved in the carcinogenesis of renal cell carcinoma, but the molecular mechanisms regulating aggressive properties of renal cell carcinoma are still poorly understood (Girolami et al., 2002; Kardas et al., 2005).

Cdc25s are dual-specificity phosphatases that regulate cell cycle progression of eukaryotes and in response to DNA damage (Boutros et al., 2006). There are three Cdc25 isoforms in humans: Cdc25A, Cdc25B and Cdc25C. Cdc25A has a role in promoting the entry into S phase, and Cdc25A, -B and -C isoforms cooperate to drive entry into mitosis (Boutros et al., 2007). Cdc25B is activated in G2 phase and is responsible for the activation of cyclin A/Cdk2 in G2 phase (Goldstone et al., 2001) and initiation of cyclin B/Cdk1 activation and mitotic entry (Gabrielli et al., 1996; Karlsson et al., 1999; Loffler et al., 2006). It also has a unique function in recovery from a G2-phase checkpoint arrest (van Vugt et al., 2004). A number of mechanisms can regulate Cdc25B, including its expression, stability, localization, and its interaction with 14-3-3 protein. Cdc25B protein expression increases in G2 phase and peaks in metaphase, prior to its degradation upon mitotic exit (Gabrielli et al., 1996).

Cdc25B was identified as a novel miRNA-148a target which may confer a proliferative advantage in human pancreatic ductal adenocarcinoma (Liffers et al., 2011). Overexpression of Cdc25B, Cdc25C and phospho-Cdc25C (Ser216) in vulvar squamous cell carcinoma is associated with malignant features and aggressive cancer phenotypes (Wang et al., 2010). Taken together, it is suggested that Cdc25B protein might have a positive regulation of tumor progression. However, the effect of Cdc25B expression on renal cell carcinogenesis and subsequent progression and its intracellular mechanism is poorly understood.

In this study, to the roles of Cdc25B expression in the renal cell carcinogenesis and subsequent progression, we examined the expression of Cdc25B mRNA and protein in renal cell carcinoma, the adjacent normal tissues, and carcinoma cell line 769-P. Additionally, its expression in tissues was compared to clinicopathological parameters of carcinoma. The effect of Cdc25B knockdown on cell phenotype and 14-3-3 expression in renal cancer cells was also investigated. These results suggest that Cdc25B may play an important role in the pathogenesis of human renal cell carcinoma, and may help in designing effective therapy targeting the Cdc25B pathway to control renal cell

*[1]Department of Urology, The First Hospital of China Medical University, [2]Department of Hematology, Shengjing Hospital of China Medical University, Shenyang, China *For correspondence: kongchuize_cmuu@yahoo.com*

carcinoma growth and invasion.

Materials and Methods

Cell culture

The human renal carcinoma cell line 769-P comes from Cell bank of Chinese Academy of Sciences, Shanghai, China. 769-P cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37 °C.

RNA silencing

Double-stranded siRNA oligomers were transfected into 769-P cells using Qiagen Attractene Transfection Reagent according to the manufacturer's instructions. Briefly, cells were seeded into 24-well plates at a density of 30,000 cells per well and grown for 12 h prior to transfection with human Cdc25B siRNA(5'-AAUCCUCCCUGUCGUCUGAAU-3') for 24h or 48 h (Vazquez-Novelle et al., 2010). The negative siRNA control was purchased from Santa Cruz.

Real-time -PCR

Total RNA was extracted from treated 769-P cells or tissues using Trizol (QIAGEN, Germany) according to the manufacturer's protocol. Total RNA was subjected to cDNA synthesis using the AMV transcriptase and random primers (Takara). Oligonucleotide primers for PCR were sense, 5'- CCCCTGGCCCTAGGTCGCTT-3' and anti-sense, 5'- AGAGCCGCTGGCACTTGCTG-3' for Cdc25B (208bp, 1484-1691, NM_004358.3), and sense, 5'-GCCTTCCGTGTCCCCACTGC-3' and anti-sense, 5'- CAATGCCAGCCCCAGCGTCA-3' for GAPDH (211bp, 796-1006, NM_002046.3). Real-time PCR was performed according to the protocol of SYBR Premix Ex TaqTM II kit.

Western blot

Denatured protein was separated on an SDS-polyacrylamide gel and transferred to Hybond membrane (Amersham), which was then blocked overnight in 5% skim milk in TBST. For immunobloting, the membrane was incubated for overnight at 4 °C with the rabbit antibody against Cdc25B (ab70927, abcam, Algeria; 1:100) or rabbit antibody against 14-3-3 (sc-33752, Santa Cruz, 1:200). Then, it was rinsed by TBST and incubated with IgG conjugated to horseradish peroxidase (DAKO, Carpinteria, CA93013, USA, 1:1000) for 2 hours. Bands were visualized by ECL-Plus detection reagents (Amersham). After that, membrane was washed with WB Stripping Solution (Pierce) for 15 minutes and treated as described above except mouse anti-β-actin antibody (Santa Cruz, 1:1000) as an internal control.

Subjects

Fresh renal cell carcinoma and paired normal tissues from the surgical resection were collected from the First Affiliated Hospital. None of patients underwent chemotherapy, radiotherapy treatment before operation. Patients' consent for the research use of tumor tissue was obtained, and the research protocol was approved by Ethical Committee at China Medical University.

Cell cycle analysis

The 3×10^6 cells were collected, washed by PBS twice and fixed in cold ethanol for 3 h. And then, cells were washed by PBS and incubated with RNase at 37 °C for 1 h, and then the cells was added by PI to 50 μg/ml and incubated at 4 °C in the dark for 30 min. Finally, the PI signal was examined by FACS.

Proliferation assay

Cell viability was measured by a 3-(4,5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. MTT solution was added to cells in 96 well plates to the final concentration of 0.5 mg/ml, and cells were incubated at 37 °C for 4 h. After removing culture media, 150 μl of dimethyl sulfoxide (DMSO) was added, and the optical density of each well was read at 590 nm.

Apoptosis assay by flow cytometry

Flow cytometry was performed with FITC and propidium iodid (PI)-labeled annexin V (556419, BD Pharmingen, USA) to detect phosphatidylserine externalization as an endpoint indicator of early apoptosis as the protocol recommends. The cells that were considered viable are both FITC Annexin V and PI negative while cells that were in early apoptosis are FITC Annexin V positive and PI negative, while cells that were in late apoptosis or already dead were both FITC Annexin V and PI positive.

Cell migration and invasion assays

Cell migration or invasion was done using Bio-coat cell migration chambers (Becton Dickinson, Bedford, MA). Inserts containing 8-μm–pore-size filters were coated with fibronectin (100 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) for migration or Matrigel (320 μg/ml; BD PharMingen, Bedford, MA) for invasion assay. After filter blockage with 1 mg/ml bovine serum albumin for 1 h, treated cells in 0.1% bovine serum RPMI-1640 were added. After incubated for 24 h at 37 °C, cells that had migrated through the filter pores were fixed in methanol and stained with Giemsa dye, and counted in 10 random fields (200×).

Statistical analysis

Statistical evaluation was performed using Spearman's correlation test to analyze the rank data and Fisher's exact test to compare the different rates. SPSS 13.0 software was applied to analyze all data and $P<0.05$ was considered statistically significant.

Results

Cdc25B expression in renal cell carcinoma tissue samples

Among 20 cases of frozen bladder samples, Cdc25B mRNA level was greater in carcinoma than the adjacent normal tissues by real-time RT-PCR ($P<0.05$, Figure 1A). Western blot detected that 65kDa Cdc25B protein bands were stronger in carcinoma than matched adjacent normal

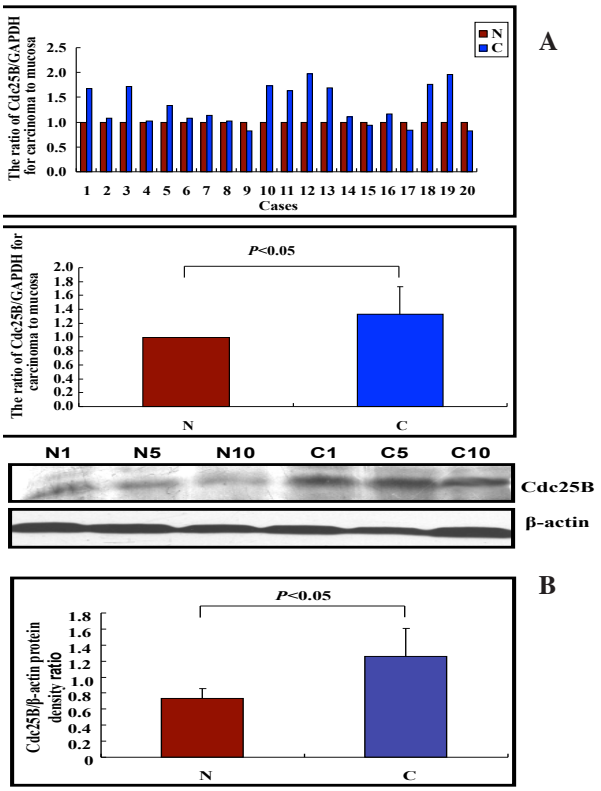

Figure 1. Cdc25B Expression in Renal Cell Carcinoma and Matched Normal Tissues. Cdc25B cDNA was amplified by real-time RT-PCR with GAPDH as an internal control and densometric analysis showed Cdc25B mRNA level was higher in carcinoma than paired normal tissues (P<0.05, A). There was also an obvious increase of Cdc25B expression (65 kDa) in the tissue lysates of renal cell carcinoma compared with matched normal tissues with β-actin (43 kDa) as an internal control (P<0.05, B). Note: N, normal tissues; C, cancer

Table 1. Relationship Between Cdc25B Expression by Western Blot and Clinicopathological Features of Renal Cell Carcinoma

linicopathological features	n	Cdc25B expression by western blot -	+	PR (%)	p value
Groups					<0.05
renal cell carcinoma	70	18	52	74.3	
Normal tissue	20	11	9	45	
Clinical stage					<0.05
I	21	17	4	19	
II	29	15	14	48.3*	
III~IV	20	8	12	60*	
Histopathologic Grade					<0.05
1	18	15	3	16.7	
2	27	15	12	44.4#	
3~4	25	11	14	56#	
Recurrence					>0.05
-	55	35	20	36.4	
+	15	10	5	33.3	

PR, positive rate; *Significantly different from clinical stage I; #Significantly different from histopathologic grade 1

tissues (P<0.05, Figure 1B), and in addition, the results showed that 70 cases of renal cell carcinoma in 52 cases (74.3%) were positive (Table 1).

Relationship between Cdc25B expression and clinicopathological parameters of renal cell carcinoma

The Cdc25B expression of renal cell carcinoma was

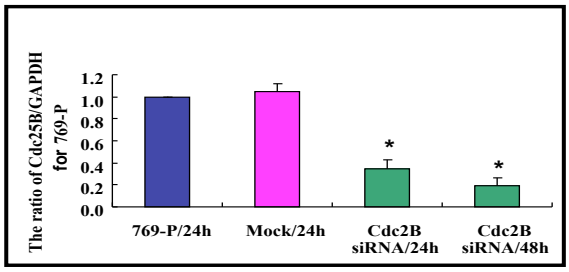

Figure 2. Cdc25B mRNA Level after Cdc25B siRNA Transfected Cell Line 769-P. After transfection of Cdc25B siRNA for 24 h or 48 h , Cdc25B mRNA level was reduced in 769-P cells by real-time RT-PCR. Note: *P < 0.05, compared with 769-P and mock for 24 h . Results are representative of 3 different experiments, and data is expressed as mean ± SE

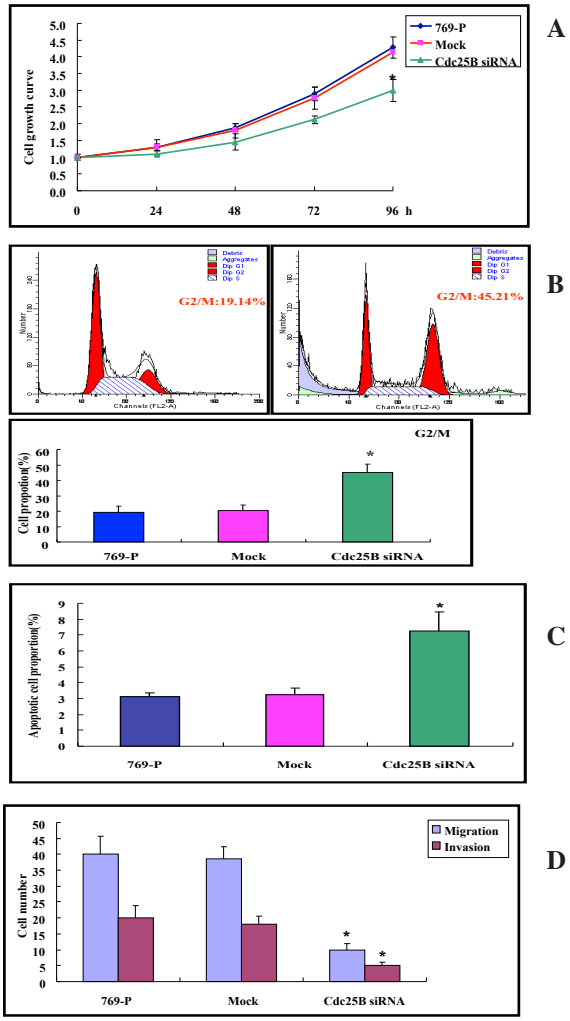

Figure 3. Cdc25B Knockdown makes 769-P Cells Display Different Cell Phenotypes. The Cdc25B siRNA transfectants showed a slower growth (A), more G2/M cells (B) in comparison with control. There was an apoptosis-induced effect of down-regulated Cdc25B expression in 769-P transfectant, evidenced by Annexin V assay (C). Cdc25B siRNA -treated 769-P had a weaker ability of migration and invasion (D). Note: *P < 0.05, compared with 769-P and mock for 48 h. Results are representative of 3 different experiments, and data is expressed as mean ± SE

significantly higher than normal tissue adjacent to cancer by the use of Western blot. As summarized in Table 1, there was a positive correlation between Cdc25B expression and clinical stage or histopathologic grade of renal cell

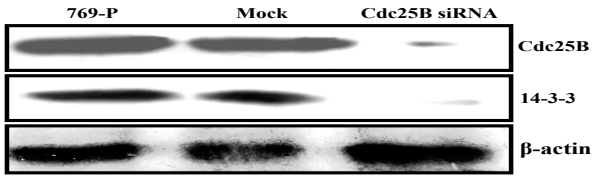

Figure 4. Cdc25B Knockdown Causes the Expression Alteration of 14-3-3 in Renal Cell Carcinoma Cells. After transfection of Cdc25B siRNA for 48 h , the protein expression of Cdc25B and 29 kDa 14-3-3 was reduced in 769-P cells by Western blot. Note: Results are representative of 3 different experiments, and data is expressed as mean ± SE

carcinoma (P<0.05). In contrast, the relationship of Cdc25B was not linked to recurrence (P> 0.05).

The effects of Cdc25B knockdown on growth, cycle, apoptosis, migration and invasion of 769-P cells and the expression of 14-3-3

After transfected with Cdc25B siRNA for 24h or 48h, the mRNA level of Cdc25B was significantly decreased in 769-P cells (P<0.05, Figure 2). There was a lower growth of the Cdc25B siRNA transfectants than the maternal cells 769-P Group and 769-P transfected by control siRNA group (named Mock), evidenced by MTT experiments (P<0.05, Figure 3A). Cell cycle analysis indicated that the number of cells treated with Cdc25B siRNA in G2/M phase increased (P<0.05, Figure 3B). There was a high level of apoptosis in 769-P cells after Cdc25B knockdown, evidenced by Annexin-V (P<0.05, Figure 3C). Additionally, down-regulated Cdc25B expression could suppress migration and invasion (P < 0.05, Figure 3D). After the transfection of Cdc25B siRNA, the expression of 14-3-3 was decreased by western blot (Figure 4).

Discussion

A body of evidences indicated that Cdc25B abnormal expression detected in some tumors implies that its dysregulation is involved in malignant transformation. Cdc25B was identified as a novel miRNA-148a target. Cdc25B was down-regulated at the protein level in miR-148a overexpressing IMIM-PC2-cells, and in transiently transfected pancreatic cell lines, as well as in human pancreatic ductal adenocarcinoma (Liffers et al., 2011). Cdc5B plays a minor role in the pathogenesis and/or progression of vulvar carcinoma, which was associated with malignant features and aggressive cancer phenotypes, but not independently correlated to prognosis (Wang et al., 2010). In contrast, we for the first time examined Cdc25B expression in renal cell carcinoma samples by real-time RT-PCR and Western blot. It was found that a positive correlation of Cdc25B protein expression with Clinical stage or Histopathologic Grade of renal cell carcinoma. However, we have not found the relationship between Cdc25B and recurrence of renal cell carcinoma.

Up-regulation of CDC25B expression has been documented in some human cancer, however, the relationships with the alteration of the molecular mechanisms that lead to oncogenesis still remain unclear. To address this issue, we used 769-P cells with Cdc25B siRNA to knockdown the Cdc25B protein. Ablation of Cdc25B function by microinjection of specific antibodies blocks cell cycle progression in Hs68 cells by inhibition of entry into mitosis (Lammer et al., 1998). In contrast, our results provide evidence that Cdc25B knockdown could reduce 769-P cells proliferation, induce apoptosis, make more cells stay in G2/M phase, and block the ability of migration and invasion.

Cdc25B is regulated at the level of its expression, stability and activity (Gabrielli et al., 1996; Lindqvist et al., 2004; Kieffer et al., 2007). Its activity is regulated through the interaction of the catalytic C terminal with the regulatory N terminal (Gabrielli et al., 1997), and this interaction is stabilized by the 14-3-3 dimer binding to specific sites in the each domain (Forrest et al., 2001; Giles et al., 2003). Here, with these studies in mind, we also investigated the relationship between Cdc25B and 14-3-3 in renal cell carcinoma. The results showed that 14-3-3 protein reduction appeared with Cdc25B protein knockdown in 769-P cells, but the direct molecular mechanism need to be further discussed by more experiments.

To the best of our knowledge, this study is the first to describe the possible role of Cdc2B protein in cell phenotypes of renal cell carcinoma, which may play the regulating role through the interaction with 14-3-3 adaptor protein. However, our data do not demonstrate a direct connection between modulations of Cdc25B signaling. These require further detailed study.

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