Knockdown of ubiquitin-specific peptidase 39 inhibits the malignant progression of human renal cell carcinoma

YUAN XU\textsuperscript{1}, MEI-RONG ZHU\textsuperscript{2}, JING-YONG ZHANG\textsuperscript{1}, GUO-MIN SI\textsuperscript{1} and JIA-JU LV\textsuperscript{1}

\textsuperscript{1}Department of Urology, Shandong Provincial Hospital, Shandong University, Jinan, Shandong 250021; \textsuperscript{2}Department of Urology, Jinan Central Hospital, Shandong University, Jinan, Shandong 250013, P.R. China

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Abstract. Ubiquitin specific peptidase 39 (USP39) serves important roles in mRNA processing and is involved in tumorigenesis of multiple solid malignancies. However, the influence and underlying mechanism of USP39 on human renal cell carcinomas (RCC) remain to be elucidated. The current study investigated the functional roles of USP39 in human RCC cell lines. siRNA-mediated RNA interference was used to downregulate USP39 in RCC cells. CCK-8, wound healing and invasion assays were performed to assess the proliferative ability and metastatic potential. The cell cycle distribution and apoptosis were evaluated by flow cytometry. The activity of signaling pathways and the expression of cell cycle-related proteins were detected by western blot analysis. The siRNA-directed RNA interference targeting USP39 could effectively downregulate the expression level of USP39 in two RCC cell lines. Depletion of USP39 by siRNA significantly suppressed cell growth and decreased invasive capacity of RCC cells. Silencing of USP39 induced cell apoptosis and cell cycle arrest at G2/M phase. Additionally, the expression levels of apoptotic and G2/M phase-related proteins were notably decreased following depletion of USP39. Mechanistically, downregulation of USP39 blocked the activation of Akt and extracellular signal regulated kinase signaling pathways in RCC cells. These findings indicate that USP39 may serve as an oncogenic factor in RCC and could be a potential therapeutic candidate for human RCCs.

Introduction

Renal cell carcinoma (RCC) is the most frequent neoplasm of the adult kidney and accounts for almost 2-3% of all human malignancies (1). Among the three major different pathological subtypes of RCC, clear cell RCC is the predominant cancer type and accounts for almost 85% of all RCC (2). Currently, surgical resections are the only potentially curative treatments for patients with early-stage RCC (3). However, ~30% of RCC patients have developed distant metastases at the time of diagnosis, which indicates a relatively poor long-term prognosis (4). For RCC patients in late stages, conventional treatments are limited and therapeutic efficacy is also generally unsatisfactory (5). The tyrosine kinase inhibitors including sorafenib and sunitinib, which are available for advanced RCCs, also have limited effect in improving the 5-year survival rate of patients with RCC (6). Therefore, investigating the underlying molecular mechanisms responsible for RCC progression and screening out novel biomarkers for prognostic prediction of RCC patients are of great importance for RCC management.

It has been demonstrated that RNA splicing serves a critical role in eukaryotic gene expression (7). Increasing evidence has demonstrated that alterations of activities or multiple mutations of splicing-related elements could be involved in tumorigenesis and malignant progression (8,9). Ubiquitin specific peptidase 39 (USP39), also known as Sad1p in yeast and a 65 kDa SR-related protein in human, is implicated in the assembly of the mature spliceosome complex (10,11). Although USP39 is a member of the de-ubiquitylating enzyme family (12) and contains a central zinc finger domain and two ubiquitin C-terminal hydrolase domains, its de-ubiquitinating enzyme activity is completely deficient (10,13). A previous study demonstrated that USP39 could maintain the integrity of mitotic spindle checkpoint and supported cellular cytokinesis through the splicing of Aurora B and other mRNAs (13). Additionally, a growing number of researchers have reported the pivotal role of USP39 in cancer development and progression. Wang et al (14) identified that USP39 was significantly upregulated in breast cancer tissues when compared with normal breast tissues, indicating that USP39 may serve as a tumorigenic factor in this malignant tumor type. Upregulation of USP39 has also been identified to be involved in the tumorigenesis of human hepatocellular carcinoma (HCC) (15), medullary thyroid carcinoma (MTC) (16) and oral squamous...
cell carcinoma (17). A recent study indicated that increased expression of USP39 promoted the progression of prostate cancers by enhancing the transcriptional elongation and maturation of epidermal growth factor receptor (EGFR) mRNA, and predicted a poor outcome in patients with prostate cancer (18). However, the biological functions of USP39 in the development of human RCCs and its underlying molecular mechanisms remain to be elucidated.

The current study inhibited the expression of USP39 in human RCC cell lines by RNA interference (RNAi) technology and then assessed the cell growth, cell cycle, apoptosis, invasion and metastasis capacity of human RCC cell lines. The findings revealed that silencing of USP39 markedly suppressed RCC cell proliferation and invasion, and induced cell cycle arrest and apoptosis. In addition, depletion of USP39 suppressed the activation of Akt and extracellular signal regulated kinase (ERK) signaling pathway. Taken together, the data suggest that USP39 may be a promising prognostic biomarker and potential therapeutic target for human RCC.

**Materials and methods**

**Cell lines and cell culture.** The human RCC cell lines A498 and OSRC-2 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The A498 cell line was maintained in MEM media ( Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the OSRC-2 cell line was cultured in RPMI-1640 media ( Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂. The media were supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin.

**Cell transfection and gene silencing.** Synthetic small interfering RNAs (siRNAs; 5'-AAGTTGCTCCATGATATATCTC-3') targeting USP39 and negative control were purchased from Shanghai Biotend Biotechnology Co., Ltd. (Shanghai, China). The transfection of siRNAs was performed according to the manufacturer's protocol. Briefly, RCC cell lines were incubated in 12-well plate (4.5x10⁴ cells/well) at 37°C for 12 h and then transfected with siRNA using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). At 12 h following transfection, fresh medium was added to the plate wells and the RCC cells were maintained for subsequent experiments.

**Western blotting.** The cultured cells were lysed in Triton lysis buffer (Pierce; Thermo Fisher Scientific, Inc.) and centrifuged at 12,000 x g for 15 min at 4°C. Protein concentrations were detected by using the BCA assay kit (Thermo Fisher Scientific, Inc.) at 37°C overnight. The cells were subsequently transfected with siRNAs as aforementioned and maintained in 100 µl SYBR Premix Ex Taq (Takara Bio, Inc.) according to the manufacturer's instructions. The RT reaction was performed using 2 µg total RNA in a reaction mixture containing 2 µl oligo dT primers (50 µM), 1 µl dNTPs (10 mM), 0.5 µl RNasin, 0.5 µl M-MLV RT (RNase H-) and nuclease-free water in a total volume of 20 µl. The reaction mixture was incubated at 42°C for 30 min, 75°C for 15 min, and then cooled on ice in accordance with the M-MLV RT protocol. RT reagents were obtained from Promega Corporation (Madison, WI, USA). mRNA levels were determined by RT-qPCR using SYBR Premix Ex Taq (Takara Bio, Inc.) according to the manufacturer's instructions. All experiments were performed in triplicate, and the mRNA level of GAPDH was used as an endogenous reference control. qPCR was performed at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 10 min. The following primers were used: USP39 forward, 5'-GCC ATT GAA CTT AGC CAG GA-3'; GAPDH forward, 5'-GCCATTGAACTTAGCCAGGA-3'; GAPDH reverse, 5'-TGCGCTACCTGAGCCACAG-3' and reverse, 5'-AAG TGTTGCTAGGAGCAAT-3'. Forward and reverse primers were mixed and diluted to 2.5 µM. The PCR reaction mixture contained 0.8 µl primers, 5 µl cDNA (30 ng/µl), 10 µl 2X SYBR Premix Ex Taq (Takara Bio, Inc.) and 4.2 µl RNA-free water, in a total volume of 20 µl. The data were analyzed relative to controls. All assays were performed on an ABI 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each experiment was performed in triplicate, and GAPDH expression was used for normalization. Fold change relative to mean value was determined using the 2−ΔΔCq method (19).

**Cell proliferation assay.** The cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc. Kumamoto, Japan) as previously described (20). Briefly, 5,000 cells per well were seeded in triplicates into 96-well plates and were incubated at 37°C overnight. The cells were subsequently transfected with siRNAs as aforementioned and maintained in 100 µl fresh cultural medium following a 48 h incubation at 37°C. Subsequently, each well was mixed with 10 µl CCK-8 and incubated at 37°C for an additional 1 h, and the optical density values of each well were detected at an absorbance of 450 nm.
using a microplate reader (Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA).

**Cell invasion assay.** The *in vitro* cell invasion assay was performed using Transwell filter chambers (Costar; Corning Incorporated, Corning, NY, USA) according to manufacturer's protocols. Briefly, 1x10⁵ siRNA-transfected cells were suspended in DMEM containing 0.5% FBS and plated into the upper Matrigel-coated invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA). Then, 500 µl culture medium supplemented with 10% FBS was added to the lower chambers. The transmigrated cells were fixed and stained with crystal violet at 37°C for 1 h after 24 h invasion. The invaded cell number was counted and averaged in 6 randomly-selected fields using a Olympus IX73 inverted microscope (Olympus Corporation, Tokyo, Japan; magnification x200).

**Wound-healing assay.** The cells were incubated in 12-well plates to reach confluence at 37°C. Then, the cells were maintained in culture medium containing 0.1% FBS for another 24 h and the wound was scratched with a sterile plastic pipette tip in the center of the cell monolayer. Following three washes with phosphate buffer saline (PBS) to remove any floating cells, the cells were maintained in culture medium supplemented with 0.5% FBS. At the indicated time points, images of cells were captured using an inverted light microscope (Olympus Corporation, Tokyo, Japan) and the migration rates were determined by calculating the distance of wound via ImageJ software (version 1.49; National Institutes of Health, Bethesda, MD, USA).

**Cell cycle assay.** The cells were maintained in culture medium until the cell confluence reached ~80%. Then the cells were washed with PBS and fixed in ice-cold 75% ethanol at -20°C for 12 h. The fixed cells were collected and washed with PBS twice, then stained with binding solution containing 50 mg/ml propidium iodide (PI; cat. no. P1304MP; Invitrogen; Thermo Fisher Scientific, Inc.) and 0.5 mg/ml RNase A for 30 min at room temperature in the dark. Following incubation, the cells were re-suspended in PBS and the cell cycle distribution was analyzed by using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and BD CellQuest Pro Software (version 5.1; BD Biosciences).

**Cell apoptosis assay.** Cell apoptosis were determined by using the Annexin V-FITC Apoptosis Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Annexin V/PI double staining was analyzed by flow cytometry within 1 h. Briefly, ~2x10⁵ cells were collected and re-suspended in 300 µl 1X binding buffer containing 5 µl Annexin V and 5 µl PI for 30 min in the dark. The apoptotic rate was quantified and results were presented as the percentage of apoptotic cells at early stage (Annexin V-positive and PI-negative) and late stage (Annexin V/PI-double positive) using BD CellQuest Pro software (version 5.1; BD Biosciences).

**Statistical analysis.** All data presented are expressed as mean ± standard deviation. The Student's t-test was used to determine statistical significance. Data analysis was performed using SPSS version 16 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Silencing of USP39 inhibits cell proliferation of RCC cells.** To determine the functional roles of USP39 in RCC, the expression of USP39 in two RCC cell lines (A498 and OSRC-2) was inhibited using a siRNA-based knockdown approach. The knockdown efficiency of USP39 was verified by western blotting and RT-qPCR (Fig. 1A and B). To determine whether USP39 siRNA-mediated gene silencing may influence RCC cell proliferation and CCK-8 assays were performed. A498 cells treated with siRNA targeting USP39 (siUSP39) exhibited a significantly reduced cell proliferation when compared with control or cells treated with scrambled siRNA (siCon) (P<0.01; Fig. 1C). Following incubation for 48 h, the relative proliferation rate of USP39-silenced A498 cells was reduced when compared with control and siCon-transfected cells. Similarly, silencing of USP39 also suppressed cell proliferation rates of OSRC-2 cells under normal culture conditions (Fig. 1D), confirming that silencing of USP39 impaired cell proliferative capacity in RCC cells.

**Depletion of USP39 suppresses the migratory and invasive capacity of RCC cells.** It has been demonstrated that RCC cells are characterized by a marked distant metastatic potential, which primarily relies on cancer cell invasion and migration (4). Due to the oncogenic role of USP39 in tumor progression, the present study investigated whether USP39 is required for RCC cell invasion and migration to occur, using wound-healing assays to identify cell migration. Depletion of USP39 contributed to markedly reduced cell migration, as revealed by the smaller healed wound area in A498 and OSRC-2 cells transfected with siUSP39 at indicated time points (Fig. 2A and B). Matrigel invasion assays were also used to determine the effect of USP39 on cell invasive ability. As expected, downregulation of USP39 markedly attenuated cell invasive capacity when compared with control or siCon-transfected cells (Fig. 2C and D). These data suggested that knockdown of USP39 inhibited cell migration and invasion in RCC cells.

**Downregulation of USP39 impairs cell cycle progression at the G2/M phase.** In order to identify the underlying mechanism of USP39-mediated cell proliferation, the cell cycle phases of RCC cells following USP39 depletion were assessed using flow cytometry. As presented in Fig. 3A, the cell population in the G0/G1 phase was markedly reduced from 56.0±2.1% in the control or 53.7±2.4% in siCon-treated cells to 42.0±1.7% in siUSP39-transfected cells. By contrast, the cell percentage of cells in the G2/M phase was increased from 12.3±1.2% in the control cells to 24.3±2.6% in siUSP39-treated cells. Similarly, it was also determined that ~66.7±1.5% of cells were in the G0/G1 phase in siUSP39-transfected OSRC-2 cells, which was markedly lower compared with control cells (74.2±2.5%) and siCon-treated cells (74.5±2.8%; Fig. 3B). As aforementioned, an increase in cell percentage of G2/M-phase was observed in siUSP39-transfected cells when compared with control cells. In mammalian cells, the G2/M transition primarily depends...
on the activity of the cyclin B1/cyclin-dependent kinase (CDK) 1 complexes. Therefore, the expression levels of the G2/M phase-related cyclin proteins following USP39 depletion were determined. Western blotting revealed that silencing of USP39 reduced the expression levels of cyclin B1, D1 and E1 in RCC cells (Fig. 3C). The findings suggested that knockdown of USP39 induced G2/M phase arrest in RCC cells, implying that the proliferation suppression may be associated with impaired cell cycle progression.

**Knockdown of USP39 induces apoptosis in RCC cells.** To further investigate the effect of USP39 on cell apoptosis, Annexin V/PI double staining was performed on RCC cells transfected with siCon or siUSP39. According to the Annexin V/PI plots from gated cells, the percentages of early apoptotic cells (Annexin V⁺/PI⁻) and late apoptotic cells (Annexin V⁺/PI⁺) were determined. Silencing of USP39 markedly increased the populations of apoptotic cells (early and late apoptosis), when compared with the control group (Fig. 3D and E). To support these findings, the expression levels of several apoptosis-associated proteins, such as caspase-3, -8, -9, and PARP, were subsequently detected by western blotting in the RCC cells following USP39 silencing. The cells transfected with siUSP39 exhibited increased expression levels of cleaved caspase-3, -8, and -9, and PARP, which represent the active forms (Fig. 3F). Together, the data indicated that depletion of USP39 could induce cell apoptosis and alter the expression profiles of pro-apoptotic proteins in RCCs.

**Knockdown of USP39 blocked the Akt and ERK signaling pathways in RCC cells.** The underlying mechanism by which USP39 promotes cell growth and cancer progression was investigated. Due to the essential role of extracellular ERK and Akt signaling pathways in the maintenance of malignant cell survival and proliferation (21), the present study investigated whether USP39 silencing may inhibit the activation of these two signaling pathways. Therefore, the phosphorylated forms of Akt and ERK in RCC cells transfected with siCon or siUSP39 were analyzed using western blotting. Knockdown of USP39 contributed to marked inhibition of Akt phosphorylation at the Ser473 site and ERK phosphorylation at the Thr202/Tyr204 when compared with the siCon transfection groups (Fig. 4). These findings suggested that expression of USP39 may promote cancer progression by activating the Akt and ERK signaling axis.

**Discussion**

At present, molecular-targeted therapies are a promising option for patients with unresectable RCC (22). Previous studies in molecular biology have contributed to an increased understanding of the underlying molecular mechanisms of RCC tumorigenesis (1,23). Novel therapeutic approaches against specific targets in RCC have demonstrated promising clinical activity in RCC patients (24). To the best of our knowledge, the present study provides novel evidence that USP39, a spliceosome factor, may have a critical role in RCC progression and metastasis.

It has been previously established that USP39 is an essential component of the spliceosome, which directly participates in the pre-mRNA splicing of several oncogenes including Aurora B and RB1 (13). This indicates that USP39 may have a growth-promoting function by controlling the process of mRNA splicing. In line with previous observations, the expression level of USP39 has been associated with cell proliferation in multiple malignancies (14-17). The present study demonstrated for the first time, to the best of the authors' knowledge, that silencing of USP39 via siRNA significantly suppressed RCC cell proliferation in vitro.
Previous studies have demonstrated that inhibition of USP39 via siRNA may block the cell cycle distribution of human MTC and HCC cell lines (15,16) and this conclusion was further supported by the findings of the present study. The whole cell cycle is divided into three periods: Interphase (including G1, S and G2 phases), the mitotic (M) phase and cytokinesis. As USP39 has been identified to be involved in the maintenance of spindle checkpoint and cytokinesis, the present study investigated the functional role of USP39 in the mitotic (M) phase during cell division. Flow cytometric analysis revealed that the cell cycle of RCC cells was impeded at the G2/M phase following silencing of USP39, which was in accord with a previous study on USP39 in prostate cancer cells (18). In cells with a nucleus, the cell cycle process is regulated by cyclin proteins that may directly activate CDKs (25). Among these family members, cyclin B1 has been identified to partner...
with CDK1 and form the CDK1/cyclin B kinase complex, which facilitates the entrance into mitosis, thus promoting the G2/M phase transition (26). Therefore, a USP39-mediated G2/M phase arrest of RCC was also identified to be accompanied with the downregulation of cyclin B1 expression in the present study. It was also observed that depletion of USP39 decreased the cell proportions of RCC cells in the G1 and S phases. Unlike cyclin B1, cyclin D1 and cyclin E1 are pivotal regulatory subunits of CDK2/4/6 and are able to interact with these kinases to promote the M/G1 and G1/S phase transitions (27,28). The expression levels of cyclin D1 and cyclin E1 were reduced in siUSP39-transfected RCC cells, confirming the reduced cell percentages of USP39-silenced RCC cells observed in the G1 and S phases. Therefore, it is possible to that depletion of USP39 may suppress RCC cell proliferation by inducing cell cycle arrest at G2/M phase. The present study determined that knockdown of USP39 had a significant pro-apoptotic effect in human RCC, which may partly account for the inhibition of RCC cell proliferation. A previous study demonstrated that cell apoptosis is a highly regulated and controlled biological process (29). Intrinsic and extrinsic pathways may initiate cell apoptosis by activating multiple apoptosis-associated proteins, including caspase families. Once these proteases or enzymes are activated, the intracellular components are degraded and cells were programmed to die in a controlled manner (30). PARP is another important

Figure 3. Downregulation of USP39 impaired cell cycle progression at G2/M phase and induced apoptosis in RCC cells. (A) Cell cycle distribution of RCC cells transfected with siCon or siUSP39 was analyzed by flow cytometric assay with PI staining. Representative fluorescence-activated cell sorting histograms of RCC cell cycle are presented. (B) Quantification of the percentage of RCC cells at different cell cycle phases (G0/G1, S and G2/M; *P<0.05, **P<0.01). Experiments were performed in triplicate and data are presented as the mean ± standard deviation. (C) The expression levels of cyclin B1, cyclin D1 and cyclin E1 proteins in RCC cells following transfection with siCon or siUSP39 was determined by western blot analysis. GAPDH protein was used as an internal control. (D) Cell apoptosis assay of RCC cells transfected with siCon or siUSP39 was analyzed by flow cytometric assay with Annexin V/PI staining. Representative images of RCC cells are shown. (E) The percentage of apoptotic cells in RCC cells following transfection with siCon or siUSP39 was assessed (**P<0.01, ***P<0.001). Experiments were performed in triplicate and data are presented as the mean ± standard deviation. (F) The expression levels of common apoptosis-related proteins (caspase-3, 8, 9, PARP and their cleaved forms) in RCC cells following transfection with siCon or siUSP39 was detected by western blot analysis. GAPDH protein was used as an internal control. USP39, ubiquitin specific peptidase 39; si, small interfering; RCC, renal cell carcinomas; Con, control; PI, propidium iodide; FASC, PARP, poly ADP ribose polymerase.

Figure 4. Knockdown of USP39 blocked the Akt and ERK signaling pathways in RCC cells. Expression levels of Akt, p-Akt (ser473), ERK, and p-ERK (Thr202/Tyr204) in RCC cells transfected with siCon or siUSP39 were determined by western blot analysis. GAPDH protein was used as an internal control. USP39, ubiquitin specific peptidase 39; p, phosphor; ERK, extracellular signal regulated kinase; RCC, renal cell carcinomas; si, small interfering; Con, control.
protein that has the ability to induce programmed cell death. As PARP may be inactivated by caspase cleavage, the cleaved form of PARP is also considered to be the biomarker of apoptosis (31). In the present study, the expression levels of cleaved-caspase-3 -8 and -9, and PARP were elevated in RCC cells following depletion of USP39, confirming the increased cell apoptotic rates in USP39-silencing RCC cells.

In conclusion, the possible mechanisms by which USP39 regulates a series of biological processes was investigated. Huang et al (18) identified EGFR to be a downstream target of USP39, whereas knockdown of USP39 suppressed the transcriptional elongation and maturation of EGFR mRNA. In addition, highly conserved signaling pathways including mitogen-activated protein kinase/ERK and PI3K/Akt axes are of great importance in internalizing the effects of external growth factors and of membrane tyrosine kinases, and have a key role in multiple cellular processes including cell division, apoptosis and mRNA transcription (32,33). The present study determined that silencing of USP39 impaired the activation of the ERK and Akt pathways, indicating the relevance between USP39 expression and the activities of the ERK and Akt pathways. Therefore, specific molecular inhibitors targeting these two pathways may be effective therapeutic treatments for RCC patients with high expression levels of USP39.

References