USP39 promotes colorectal cancer growth and metastasis through the Wnt/β-catenin pathway

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Abstract. In the present study, we first examined the expression of USP39 protein using tissue array containing 90 colorectal cancer (CRC) tissues and 9 clinical samples, and observed that it has significantly higher expression in cancer tissues as compared to the corresponding adjacent normal tissues. Also, we tested USP39 expression level in four CRC cancer cell lines and identified that it indeed had higher expression in all these CRC cell lines. In addition, its knockdown inhibited not only the cell growth of SW480 and HT29 cells, but also the cell migration and invasion. Further analysis of its molecular mechanism suggested that the expression of four crucial proteins of Wnt/β-catenin pathway, including β-catenin, TCF4, MMP2 and MMP9 was reduced as a result of USP39 knockdown. Taken together, all these findings demonstrated that USP39 protein plays an important role in the growth and metastasis of colorectal cancer mainly through Wnt/β-catenin pathway.

Introduction

Colorectal cancer (CRC) is one of the most prevalent tumors associated with cancer-related death worldwide (1). The development of drug resistance and relapse with refractory disease, further limits the 5-year survival of metastatic CRC patients to less than 10% (2). Aberrant alternate splicing commonly observed in cancer cells, has been implicated in the transformation of many types of cancers (3,4). For instance, many genes involved in the proliferation and invasiveness of cancer cells, are frequently alternatively spliced. These specific splice variants, which are often upregulated in many tumors, stimulate cell proliferation and migration and thus, contribute to the transformed phenotype (5). However, the roles of different spliced variants along with their splicing factors, involved in controlling the alternate splicing have not been completely appreciated in tumor development.

USP39 is one of the deubiquitinating enzymes (DUBs) which do not have ubiquitin protease activity due to lack of three important residues important for the protease activity (6). It encodes a conserved protein, U4/U6·U5 tri-snRNP, which has 65% overall homology with yeast Sad1p splice factor, and has been implicated in the assembly of mature spliceosomes. In vitro studies have indicated that Usp39 is essential for pre-mRNA splicing, but not for the stability of the spliceosome complex after its formation (7). USP39 has also been shown to be essential in the spindle assembly checkpoint and controls the Aurora B mRNA levels in U2OS cells (6). In addition, USP39 mutation in zebrafish induce G1/S arrest by rb1 splicing defect, and the e2f4 appears to be also the target of USP39. This mutation eventually contributes to adenohypophyseal sensitivity to rb1 and e2f4, and lead to pituitary tumorigenesis (8). Similarly, USP39 mutation in breast cancer cells, also implicate it as an oncogenic factor, and its downregulation induced apoptosis of the MCF-7 breast tumor cells (9). A study (10) has further demonstrated that USP39 overexpression enhanced the proliferation of prostate cancer cells, thereby suggesting that USP39 may also play a key role in prostate cancer development. All these studies have emphasized that USP39 may be a potential molecular target of cancer.

In the present investigation, we first explored the relationship between USP39 expression and colorectal cancer. Initial
results suggested that USP39 expression levels were higher in tumor tissues than in adjacent normal tissues, and thus, indicated that USP39 may act as a new target in colorectal cancer. To further verify our hypothesis, we tested USP39 expression in four different colorectal cancer cells, and later by knocking down its expression by RNA interference (RNAi) technology in SW480 and HT29 colorectal cancer cell lines, we investigated its role in in vitro cell invasion and migration. Our results indicated that USP39 inhibition suppressed the migration of colorectal cancer cells through regulation of Wnt/β-catenin pathway.

Materials and methods

Cell culture. Human CRC cell lines, LoVo, Caco2, SW480 and HT29 were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). LoVo cells were cultured in F-12K medium, and Caco2 cells in minimum essential medium (MEM), SW480 were cultured in L15 medium and HT29 cells in McCoy's 5A medium. The culture mediums of three cell lines were supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg/ml of streptomycin. The Caco2 cells, instead were cultured in medium and HT29 cells in McCoy's 5A medium. The culture oscillated for 30 min at room temperature, and later the absorbance at 490 nm was measured. The values were calculated by multiplying staining intensity with percentage of stained cells. The final staining scores were calculated according to the staining intensity and the percentage of cells stained. The final staining scores were calculated by multiplying staining intensity with percentage of stained cells.

MTT assay. Human CRC cells (1x10^5 in 0.2 ml/well) in three replicates, were seeded in 96-well plate in the complete medium and cultured at 37°C for 1-5 days. The 100 µl (5 mg/ml) of MTT solution was added into each well and incubated at 37°C for 4 h. Next, the supernatant was removed and 150 µl of dimethyl sulfoxide (DMSO) was added per well. The plate was oscillated for 30 min at room temperature, and later the absorbance at 490 nm was measured. The values were calculated after background subtraction. All the MTT experiments were repeated at least three times.

USP39 knockdown. The lenti-shRNA vector system to knock down USP39 was constructed, packed and purified by Shanghai GeneChem, Co., Ltd., (Shanghai, China) and was later manipulated according to the protocol provided by the manufacturer.

Wound healing assay. CRC cells seeded in 6-well plates at a density of 10^5 cells/well, were incubated for 48 h. A 2-µl pipette tip was used to scratch a linear wound in the cell monolayer and cells were then allowed to grow in complete media. Images were taken at 0 and 48 h after wounding, respectively.

Transwell migration. Cell migration assay was performed using Boyden chambers (Transwell Costar; 6.5-mm diameter, 8-µm pore size) according to the manufacturer's instructions. Briefly, 3x10^5 cells were resuspended in 500 µl of serum-free medium and seeded in the upper chamber. In parallel, 800 µl of the complete medium containing 10% FBS was added into the lower chamber. Cells were allowed to migrate for 48 h. Later, migrated cells were stained with 1% crystal violet and the cell numbers were counted under a microscope. Six fields of view from each chamber were randomly selected for cell counting.

Quantitative real-time PCR. Total RNA was extracted from CRC cell lines using TRIZol (Invitrogen) following the manufacturer's instructions. qRT-PCR was performed as previously described (11). Briefly, 1 µg of total RNA was transcribed using random primers and PrimeScript reverse transcriptase (Takara Bio, Dalian, China). Quantitative PCR reaction for USP39 and GAPDH genes was carried out using SYBR-Green qPCR kit (Takara) on a fluorescent temperature cycler (Mx3000P real-time PCR system; Stratagene, La Jolla, CA, USA). The following primers were used to detect the expression of USP39 (F, 5'-CCAGCGATGGCAGCAGCTA-CG-3' and R, 5'-ACCACAA CGGAAACACG-3') and GAPDH (F, 5'-TGACCTCACAG CGACACCCA-3' and R, 5'-CACCCCTGCTCTGTAGCCA AA-3'). The PCR reaction was performed with the following parameters; denaturation at 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Finally, using GAPDH as an endogenous control, relative gene expression of USP39 was determined by comparative ∆Ct method using the Stratagene analysis software. The experiment was repeated at least three times.

Western blot analysis. CRC cells were lysed in RIPA lysis buffer (Tiangen Biotech, Co., Ltd., Beijing, China) and extracted proteins (20 µg) were separated on a 10% SDS-PAGE gel and then transferred to Immobilon-P polyvinylidene difluoride membrane (PVDF) (Millipore, Billerica, MA, USA). The expression of different proteins were visualized by chemiluminescence using ECL-Advance Western blotting detection kit (Millipore). The antibody dilutions used for USP39 (ab150393; Abcam) were 1:5,000; β-catenin (ab19452; Abcam) 1:1,000; TCF4 (ab60727; Abcam) 1:1,000; MMP2 (#4022; Cell Signaling Technology, Hitchin, UK) 1:2,000; MMP9 (#3852; Cell Signaling Technology) 1:2,000; GAPDH (ab8227; Abcam) 1:7,500; and goat anti-rabbit IgG H&R (Abcam) 1:6,000.

Statistical analyses. The data shown are presented as the mean ± standard deviation (SD) of three independent experiments. All statistical analyses were performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA) and P<0.05 was considered statistically significant.
Results

**USP39 expression is upregulated in CRC tumor tissues and correlates with poor patient overall survival.** The USP39 expression was examined in human CRC tissue array samples by immunohistochemical staining. The array included 90 CRC patient tissue samples including cancer and corresponding adjacent normal tissues. Among these, 48 tissues samples were from male patients, while 42 were from female patients, with a median age of 71 years (range, 24-90 years). As shown in Table I and Fig. 1A, USP39 had strong expression in CRC tumor tissue samples, as 89 of these showed a score of ≥2. In contrast, adjacent normal tissue samples displayed dramatically reduced USP39 expression, compared with cancer tissues (P<0.001). In addition, USP39 expression was not associated with other factors. However, Kaplan-Meier survival curves and the log-rank test survival analysis indicated that CRC patients with high USP39 expression had significantly poor overall survival (P<0.05), as shown in Fig. 1B. Additionally, the tissue sample analysis of 9 colorectal patients, also revealed significantly higher mRNA and protein levels of USP39 in tumor tissues than corresponding adjacent normal tissues, as shown in Fig. 2.

**USP39 knockdown inhibits growth of human CRC cells.** Next, we examined the USP39 protein expression in four human CRC
cell lines, LoVo, Caco2, SW480 and HT-29, using western blot analysis, as shown in Fig. 3A. Our results indicated that indeed all these cell lines have elevated expression of USP39. Next, we knocked down the USP39 expression in these CRC cells and analyzed its effects on their growth, using MTT assay. As shown in Fig. 3B and C, USP39 knockdown significantly inhibited the growth of HT-29 and SW480 cells, as compared to control knock down (NC). However, the LoVo and Caco2 cells, did not show any significant differences in their growth (data not shown).

Table I. The expression of USP39 in human colorectal cancer tissue array.

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A tissue microarray was stained with anti-human USP39 antibody. The staining intensity was scored on a scale of five as follows: negative (0+), weak (1+), moderate (2+) and strong (3+). None means that the site is lost in the section.
Figure 3. Expression of USP39 and MTT assay in CRC cell lines. (A) Protein level of USP39 in CRC cell lines. (B) MTT assay to test the cell proliferation in SW480/NC and SW480/KD cells. USP39 knockdown inhibited the growth of SW480. (*P<0.05, **P<0.01). (C) MTT assay to test the cell proliferation in HT29/NC and HT29/KD cells. USP39 knockdown inhibited the growth of HT29. (*P<0.05, **P<0.01).

Figure 4. USP39 knockdown inhibits the migration and invasion of CRC cells in vitro. (A) Cell migration was determined by wound healing in SW480 cells transfected by shRNA and negative control. (B) Cell migration was determined by wound healing in HT29 cells transfected by shRNA and negative control. (C) Cell invasion was determined by Transwell assay in SW480 cells transfected by shRNA and negative control. (D) Cell invasion was determined by Transwell assay in HT29 cells transfected by shRNA and negative control.
USP39 knockdown inhibits migration and invasion of CRC cells. To assess the USP39 contribution to CRC cell (HT-29 and SW480 cells) migration and invasion, we performed the scratch wound healing assay and Matrigel coated Transwell invasion assay. As shown in Fig. 4A and B, the scratch was about half closed after 48 h in control knockdown cells, while the USP39 knocked down cells had very little effect on the rate of wound closure. Similarly, USP39 knockdown also reduced the cell invasion of both CRC cell types in comparison to the control knockdown, as shown in Fig. 4C and D.

USP39 knockdown in migration and invasion involved the Wnt signaling pathway. Wnt signaling pathway and its modulators play a significant oncogenic role in CRC development and progression (12-14), we tested the effect of USP39 knockdown on proteins related to Wnt/β-catenin pathway and its downstream factor matrix metalloproteinases (MMPs) (15,16). Our results showed that USP39 knockdown significantly reduced the expression of β-catenin, TCF4, MMP2 and MMP9 proteins, as shown in Fig. 5.

Discussion

In our previous study, we have shown that USP39 promotes hepatocellular carcinoma (11), however, herein we first demonstrated that USP39 expression in colorectal cancer tissues was higher as compared with corresponding adjacent normal tissues. In addition, we also reported that USP39 was highly expressed in multiple CRC cell lines, and series of in vitro functional experiments revealed that it promotes the growth, migration and invasion of CRC cell lines. Knockdown of USP39 inhibited not only the growth of HT29 and SW480 cells, but also their migration and invasion ability. Further mechanistic insight into its function at molecular level indicated that USP39 might be mediating these effects through regulation of some of the proteins involved in Wnt/β-catenin pathway, as Wnt/β-catenin pathway has been shown to influence the growth and metastasis of CRC.

Wnt signaling is a crucial mechanism involved in the regulation of cell proliferation, differentiation and morphogenesis (17). Numerous studies have suggested that the abnormal activation of Wnt/β-catenin signaling pathway have a significant role in human tumorigenesis, especially colorectal cancer (18). β-catenin which is the key molecule in this pathway, is a multifunctional protein (19), and acts as a key transcriptional factor in the Wnt pathway. It widely participates in the regulation of cell proliferation and migration (20,21). In the present study, we observed a significant effect of USP39 knockdown on β-catenin levels, consistent with reduced growth and migration.

MMP2 and MMP9 are proteins expressed on many tumor cells, and act as potential indicators of invasion or metastasis in colorectal tumors (22-24). These MMPs are regulated by Wnt/β-catenin signaling pathway (25,26). Activation of cytokine signaling and cell recruitment in the areas of silica dust deposition can lead to excessive disruption of extracellular matrix, which is associated with the secretion of matrix metalloproteinases (MMPs). In the development of pulmonary fibrosis, MMPs specifically cleave important constituents of extracellular matrix (ECM) such as gelatin and collagen, and therefore damage the structure of the alveolar wall (22). It has been reported that β-catenin mediated expression of matrix metalloproteinases (MMPs) enhance epithelial-mesenchymal transition (27). The key feature of tumor invasion and metastasis is degrading extracellular matrix, and MMPs take part in its degradation, and hence is related closely with the tumor occurrence and development (28). Thus, decreased expression of MMP2 and MMP9 due to USP39 knockdown, coincide with role of USP39 in promoting growth and metastasis in CRC tumor cells.

The treatment of colorectal cancer affecting millions of people worldwide each year, is confronted by the challenges of metastasis, relapse and drug resistance (29). Molecular targeted therapies have become an alternative therapeutic approach, and are being used as a combination therapy with standard chemotherapy agents. For example, the recombinant monoclonal antibody bevacizumab against VEGF-A, and the chimeric monoclonal antibody cetuximab against EGFR have been recently approved for clinical use in combination with 5-FU and irinotecan, respectively, to treat metastatic colorectal cancer (30,31). Thus, identifying novel molecular targets would provide additional options for effective treatment, and the present study suggests that USP39 can be a tentative target for CRC treatment.

In summary, the present study identified that USP39 expression is not only upregulated in CRC patients, but also correlates with their overall survival. In addition, it is involved in regulation of CRC cells growth, migration and invasion. In terms of mechanistic understanding, USP39 effects are mediated through Wnt/β-catenin pathway, as its inhibition significantly reduced the expression of many proteins of this pathway. Thus, we conclude that USP39 promotes growth and metastasis of colorectal cancer mainly through the Wnt/β-catenin pathway.

Acknowledgements

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Figure 5. Mechanisms of USP39 regulate the growth of tumor cells. Western blot analysis was carried out to detect the expression of some important molecules including β-catenin, TCF4, MMP2 and MMP9. These proteins were decreased after USP39 knockdown.
References


4. Ladomery M: Aberrant alternative splicing is another hallmark of Natural Medicines (no. SKlNMKF201606).


