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Original Article

p42.3 promotes cell proliferation and invasion in human Renal-Cell Carcinoma

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Abstract: p42.3 is a tumor-specific gene and found to be over-expressed in many tumor cell lines and primary tumor tissues. It plays a significant role in neoplastic transformation and tumor progression. To date, the association between p42.3 and Renal-Cell Carcinoma (RCC) has not been reported. This study investigated the biological effects and mechanisms of p42.3 in RCC progression. In this study, we found that p42.3 is overexpressed in various kinds of RCC cells, and knockdown of p42.3 dramatically reduced cell proliferation and invasion *in vitro*. Our studies revealed that overexpression of p42.3 accelerates the epithelial-mesenchymal transition (EMT) progression and induces RCC cells proliferation and invasion. Further studies show that p42.3 may involve in activation of β-catenin and participate in RCC cell invasion. Combined, these data indicate that p42.3 contributes to promoting RCC cells proliferation and invasion through accelerates the EMT progression and β-catenin activation.

Keywords: p42.3, RCC, cell invasion, E-cadherin, β-catenin

Introduction

RCC is the most common carcinoma of all renal neoplasm, and it represents approximately to 90% of all kidney cancer cases. RCC accounts for 3~5% of adult overall types of cancer [1] and its morbidity and mortality have gradually increased in recent years [2]. Currently, the most effective treatment for localized RCC is surgical resection [3], while distant metastasis after curative treatment were seen in more than 30% of the cases [4]. And patients with distant metastasis only have a median survival time of 13 months [5]. Unfortunately, metastatic RCC is poor response to radiotherapy, chemotherapy and immunotherapy, and current treatments are not effective for this carcinoma [6]. Therefore, novel diagnostic and therapeutic markers are urgently needed to develop for this tumor.

p42.3 is also referred to as C9orf140. It was a newly discovered tumor-specific gene that was initially ascertained by the mRNA differential display technique [7]. It has been reported that p42.3 is overexpressed in many human tumor cell lines, primary tumor and embryonic tissues but not in normal tissues from adult organs. The expression of p42.3 is cell cycle-dependent at both mRNA and protein levels in GC cell lines [8]. Previous study showed that mir-29a may downregulate the expression of p42.3 and cell cycle may be arrested in G1 phase [9]. Moreover, as a cell cycle-dependent gene, p42.3 can accelerate the process of mitosis and induces the cells malignant transformation through regulation and control of CyclinB1 and Cdks, which are two key proteins involved in cell cycle regulation [10, 11].

Recent studies showed that p42.3 plays an important role in the process of oncogenic transformation of cells and accelerate cell's chromosome segregation [8]. Moreover, p42.3 can promote cell proliferation, cell migration and invasion in colorectal cancer (CRC). Thus far, although all these reports suggest that p42.3 may be a potential tumor biomarker in many tumor cells, there is no paper reporting the expression levels of p42.3 in RCC.

In order to elucidate the role of p42.3 in RCC progression, we investigated the expression levels of p42.3 protein in various kinds of human RCC cells and normal human renal tubular epithelial cells. We also investigated its biologic effects and mechanisms in RCC cells lines. The present study aimed to clarify whether or not p42.3 is related to the development and progression of RCC.

Materials and methods

Cell culture and transient transfection

The Ketr-3, 786-0, ACHN, OSRC and normal human renal tubular epithelial cell line HK-2 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The 786-0 cells and OSRC cells were cultured in RPMI 1640 (Thermo, NY, USA) containing 10% fetal bovine serum (FBS; GIBCO-BRL). The Ketr-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo) supplemented with 10% FBS. The HK-2 cells were cultured in DMEM/F12 (Thermo, NY, USA) containing 20% FBS. The ACHN cell lines were cultured in minimum essential medium (MEM; Thermo) containing 10% FBS. All the cell lines were maintained at 37°C in a 5% CO 2 incubator. The siRNA against p42.3 was purchased from Integrated Biotech Solutions (Shanghai, China). Cells were grown to 50-60% confluence before being transiently transfected with p42.3 siRNA using SiLentFect (Bio-RAD, CA, USA) according to the manufacturer's instructions. 5-6 h after transfection, the medium containing transfection reagents were removed and incubated in fresh medium. 48 h after transfection, cells were lysed for Western blotting, and subjected to wound healing assays, CCK-8 cell proliferation assay, Tranaswell assay.

Western blot analysis

The cell lysates were collected and centrifuged at 15,000 g for 20 min at 4°C, and the concentration of the supernatants were determined by using the Bradford method. Then electrophoresed on SDS-polyacrylamide gels (12.5%) and transferred onto nitrocellulose membranes. After blocking for 2 h, membranes were incubated overnight at 4°C with the p42.3 antibody (1:1000, Abcam, Cambridge, UK), β-catenin antibody (1:200, Santa Cruze, CA, USA), E-cadherin antibody (1:200, Santa Cruze, CA, USA), N-cadherin antibody (1:200, Santa Cruze, CA, USA). Subsequently, the membranes were washed and incubated with goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody labelled with FITC (1:10000, Santa Cruze, CA, USA) for 2 h. Finally, the membranes were scanned after washed. The protein expression was normalized to an endogenous reference (β-actin) and relative to the control.

Cell proliferation assay

786-0/OSRC cells were seeded into 96-well culture plates (6 × 103 per well) and cultured for 24 h, then transfected with p42.3 siRNAs. Cell proliferation was evaluated by Cell Count Kit-8 (CCK-8, beyotime, China) at various time points 24 h, 48 h, 72 h and 96 h according to the manufacturer's instructions. The cells were incubated with 10 μl of CCK-8 in 100 μl medium per well at 37°C for 1 h, and then optical density was measured at 450 nm. Each experiment was performed in triplicate.

Wound-healing assay

After transfection with p42.3 plasmid, the 786-0/OSRC cells were cultured. A wound was made by dragging a yellow pipette tip along the centre of the plate. The distance between the cells bordering the wound was measured every 12 h. Images of cells were taken at the time indicated under the phase-contrast microscope with a digital camera.

Migration assay

A modified two chamber plates with a pore size of 8mm was used to determine 786-0 and OSRC cell migration. 1 × 105 786-0 and OSRC cells were seeded in serum-free medium in the upper chamber. After cultured for 24 h in a 37°C incubator, the cells were fixed in methanol and stained with leuco crystal violet. Cells in upper chamber were removed with a cotton swab and the number of cells which traversed the membrane was determined by counting the leuco crystal violet stained cells. Cells were counted under a microscope in five fields.

Invasion assay

A modified two chamber plates with a pore size of 8 mm was used to determine 786-0 and OSRC cell invasion. The transwell filter inserts were coated with Matrigel on the upper side (BD Biosciences, NJ, USA). 1 × 105 786-0 and OSRC cells were seeded in serum free medium in the upper chamber. After cultured for 48 h in

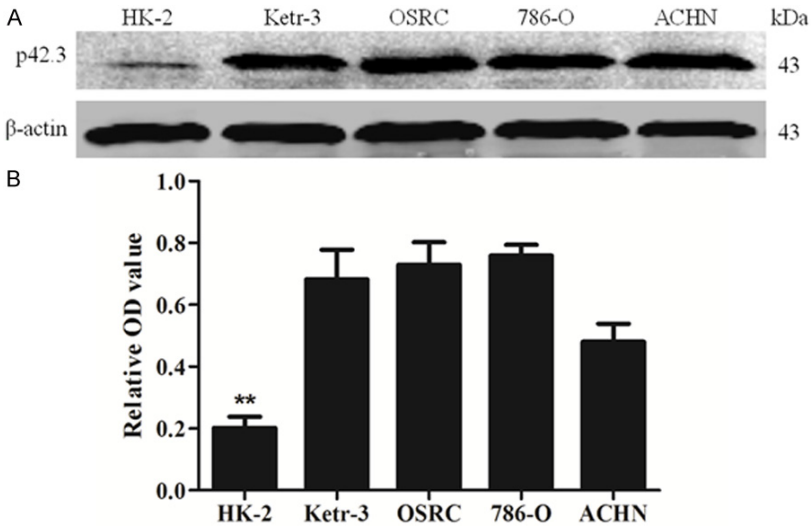

Figure 1. Protein Expression Level of p42.3 in HK-2 cells, Ketr-3 cells, OSRC cells, 7860 cells, and ACHN cells. A. Western blot analysis to evaluate the protein levels of p42.3 with p42.3 antibody, and β-actin served as a loading control. Results showed that p42.3 was overexpressed in RCC cells. B. Densitometric analysis of p42.3. The intensity of p42.3 was quantified by densitometry (software: Image J, NIH). Data are presented as mean ± SD (n = 3). **P < 0.01.

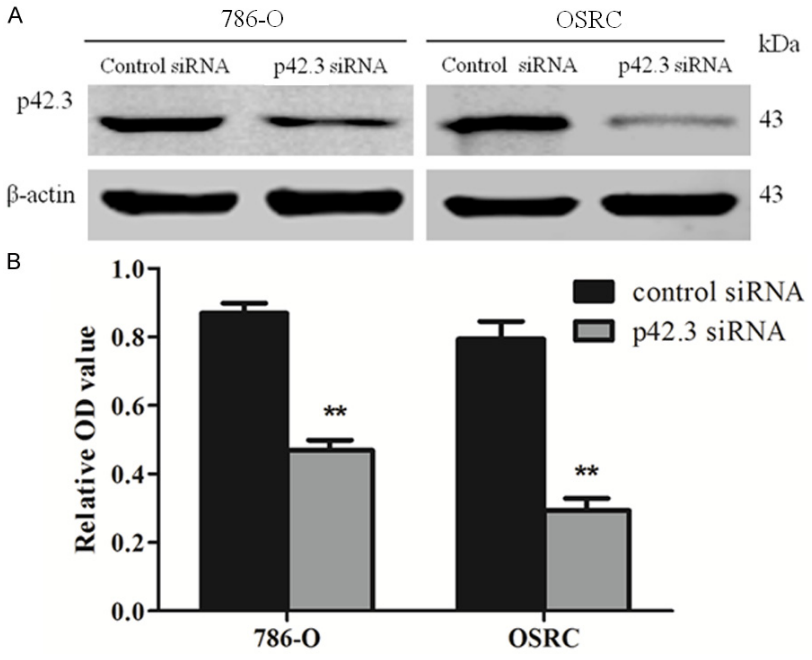

Figure 2. p42.3-specific siRNA knockdown of p42.3 in RCC cells. A. Downregulation of p42.3 protein in 786-O or OSRC cells treated with p42.3-specific siRNA. The whole cell lysates were extracted at 48 h after treatment with siRNA. Western blot analysis to evaluate the protein levels of p42.3 with p42.3 antibody, and β-actin served as a loading control. B. The intensity of p42.3 was quantified by densitometry (software: Image J, NIH). Data are presented as mean ± SD (n = 3). **P < 0.01.

a 37°C incubator, the cells were fixed in methanol and stained with leuco crystal violet. Cells in upper chamber were removed with a cotton swab and invasive cells at the bottom of the Matrigel were counted.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 software (SPSS). All values are expressed as means ± SD. Student's t-tests or one-way analysis of the variance (ANOVA) was used for determination of *P* values. All experiments were performed at least three times unless otherwise indicated. A *P*-value of < 0.05 was considered as statistically significant.

Results

Expression of p42.3 in RCC cells

Western blot analysis was used to investigate the expression of p42.3 in different kinds of RCC cells and normal human renal tubular epithelial cells HK-2. p42.3 protein expression levels were examined in Ketr-3 cells, OSRC cells, 786-O cells, ACHN cells and HK2 cells, and the level of p42.3 was much lower in normal cells than in tumor cells ($P < 0.01$) (**Figure 1A**, **1B**). We chose 786-O cells and OSRC cells, which expressed higher level of p42.3 by comparison with other RCC cells, for further studies.

p42.3 was silenced by p42.3-specific siRNA

To investigate the function of p42.3 in RCC progression, the RCC cell lines 786-O cells and OSRC cells were transfected with p42.3-specific siRNA. After two days of transfection, the efficiency of p42.3 silencing was determined by

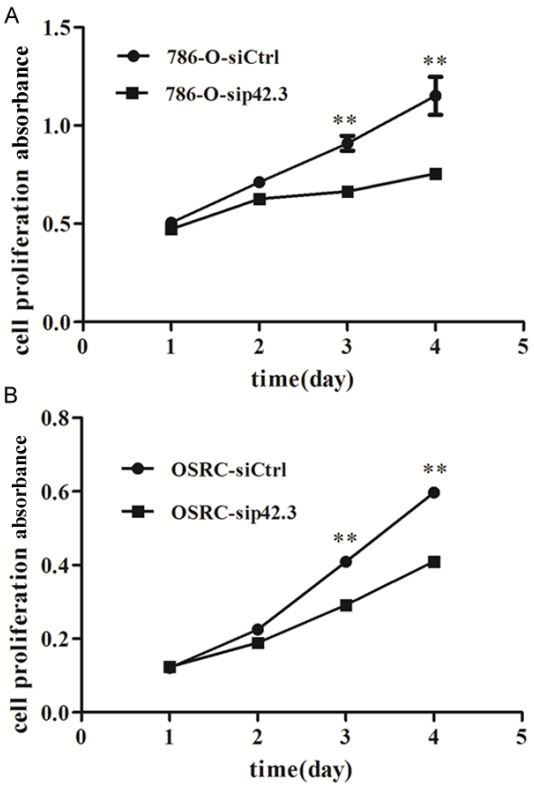

Figure 3. Effects of p42.3 knockdown on the Proliferation of RCC Cells. A, B. CCK-8 assay was performed in 786-O and OSRC cells after transfection of p42.3 siRNA for 24, 48, 72 and 96 h. Data are presented as mean ± SD (n = 3). **P < 0.01.

analysis of p42.3 protein levels. Our result showed that the p42.3-siRNA was effective in reducing the expression of p42.3 protein (**Figure 2A**, **2B**).

Knockdown of p42.3 gene inhibits cell proliferation in RCC cells in vitro

CCK-8 assay was performed in p42.3-knockdown 786-O cells, OSRC cells and negative control cells with the absorbance values detected at 24, 48, 72 and 96 h respectively. The cell proliferation had no significant difference between the p42.3-knockdown cells and negative control cells within 24 h and 48 h ($P > 0.05$), while the difference between two groups were statistically significantly different within 72 h and 96 h ($P < 0.01$) (**Figure 3A**, **3B**), prompting that p42.3 overexpression cells have stronger proliferative capability.

The cell migration and invasion inhibitory effects of p42.3 knockdown in RCC cells

Since the expression of p43.2 is increased significantly in RCC cells compared with normal renal cells, we detected the further involvement of p42.3 in the migration and invasion of human RCC cells. In cell wound-healing assay, less wound closure was observed in p42.3 knockdown RCC cells when compared with the negative control groups (**Figure 4A**, **4D**), indicating that p42.3 may have a significant effect on cell invasion in RCC cells. We further confirmed the properties of p42.3 by transwell cell invasion assays, and showed that p42.3 knockdown significantly decreased the migration and invasive ability of RCC cells (**Figure 4E**, **4H**). These data further suggest that p42.3 play a vital role in cell invasion of RCC cells.

Knockdown of p42.3 decelerates EMT process and decreases activation of β-catenin

We then discussed the mechanisms of p42.3 signaling in RCC progression. In contrast, knockdown of p42.3 significantly enhanced the expression of E-cadherin and reduced the expression of N-cadherin in RCC cells by western blot analysis, suggesting that epithelial-mesenchymal transition (EMT) initiation may be mediated by p42.3. Furthermore, we detected the expression levels of β-catenin in RCC cells. Interestingly, Western blot analysis showed that knockdown of p42.3 expression significantly decreased the expression of β-catenin, indicating that p42.3 may be connected with the activation of β-catenin and acceleration of EMT process (**Figure 5A**, **5C**).

Discussion

p42.3 gene was initially discovered from synchronized human GC cell line BGC-823 by Xu in 2007. p42.3 gene is located at 9q34.3 of human chromosome, and its cDNA contains an open reading frame of 552bp. Previous studies pointed out that p42.3 is a cycle-dependent gene and it can regulate many cycle-dependent proteins and accelerate mitotic progression. As an oncogene, p42.3 has been shown to be overexpressed in GC cells, HCC cells and CRC cells [8, 12, 13], and these cells and tissues with p42.3 overexpression often have stronger multiplication abilities and invasive abilities. Recent studies [14] showed that the expression of p42.3 is regulated by the cooperation of STAT5, EZH2 and β-catenin. However, the biological effects and molecular mechanisms underlying overexpression of p42.3 in RCC progression are still not clear. To the best of our knowledge, this is the first report demonstrat-

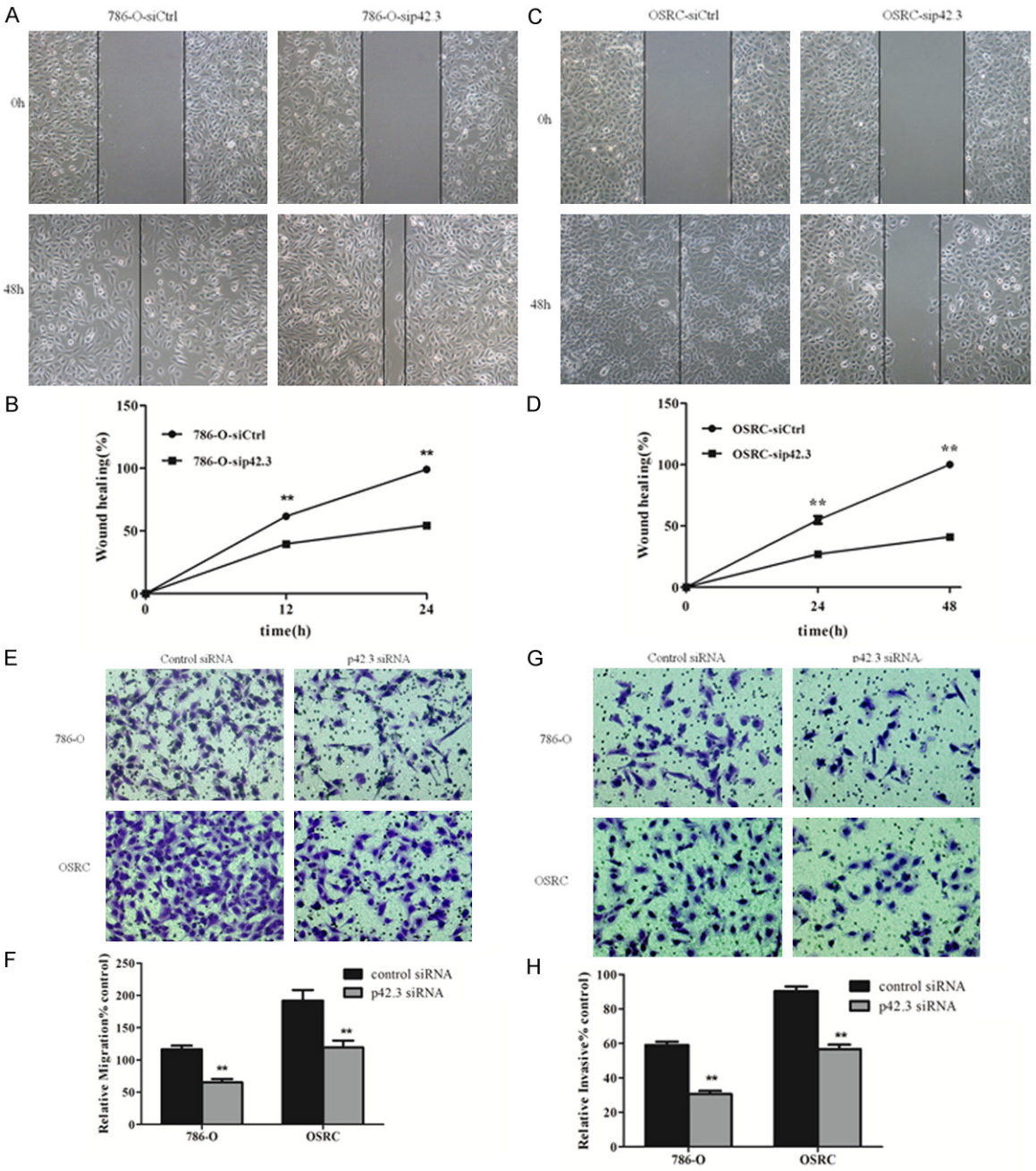

Figure 4. Effects of p42.3 Knockdown on the Migration and Invasive Ability of RCC Cells. A-D. Wound healing assays were performed in 786-O/OSRC cells after transfection of p42.3 siRNA. E-H. Transwell Matrigel metastasis and invasion assays were performed in 786-O/OSRC cells transfected with p42.3 siRNA. Cells were observed under a light microscope and photographed (× 200 magnification). Cells were counted from five random microscopic fields per insert in triplicate. The migrated cell numbers were normalized to that of the control group. Data are shown as mean ± SD from three separate experiments. **P < 0.01.

ing that p42.3 might play some potential roles in the development and progression of RCC.

The major characteristics of tumor cells are proliferation in an uncontrolled manner and ability of distant metastasis [15]. Our studies have showed that knockdown of p42.3 could inhibit cell proliferation in RCC cells by CCK-8 assay. In wound-healing assay and Transwell cell invasion assay, p42.3 knockdown could reduce cell metastasis and invasion in RCC cells. And the results are in agreement with

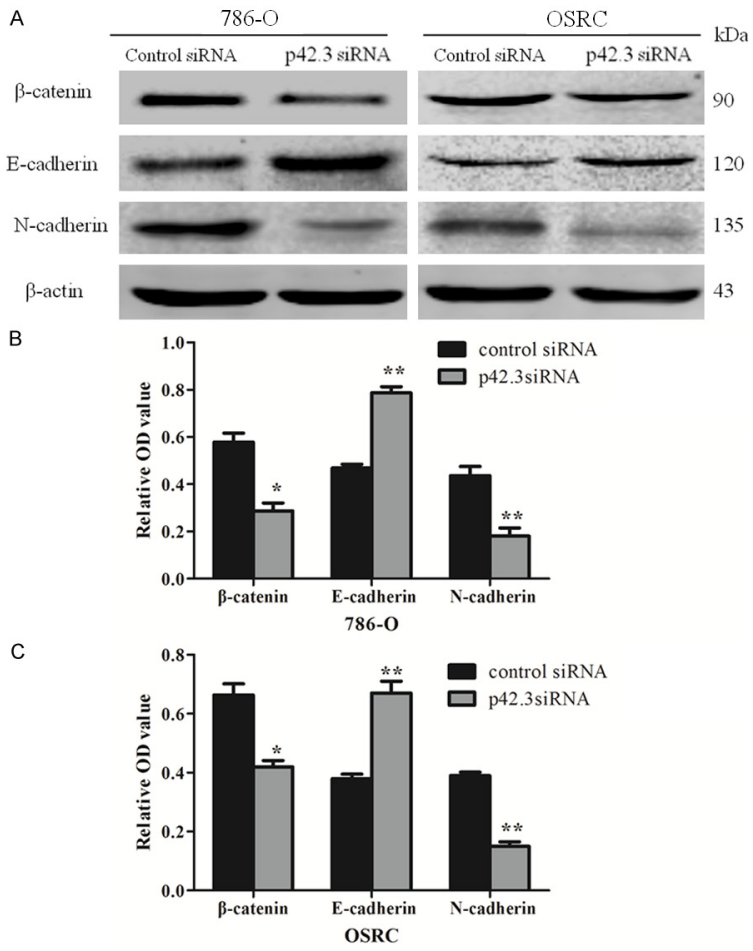

Figure 5. Knockdown of p42.3 facilitates the Process of EMT and Induces the Activation of β-catenin. A. Efficient expression of E-cadherin, N-cadherin and β-catenin are shown by western blot. Cells were transfected with p42.3 siRNA. Two days after transfection, western blot was used to analyze the prepared cell lysates. Results showed that the expression level of E-cadherin protein in p42.3 knockdown RCC cells was increased while that of N-cadherin protein was decreased. Moreover, the expression of β-catenin was enhanced in p42.3 knockdown RCC cells. B, C. Densitometric analysis of E-cadherin and β-catenin. The intensity of E-cadherin, N-cadherin, β-catenin were quantified by densitometry (software: Image J, NIH). Data are presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01.

that p42.3 can promote cell proliferation and induce cell migration and invasion in RCC cell lines.

EMT is an essential event for embryonic development, tissue regeneration and tumorigenesis [16, 17]. The biological process of EMT can confers metastasis potential to epithelial cell-derived tumor cells [18, 19]. Down-regulation of E-cadherin expression is an important step in the initiation of neoplasm metastasis and a fundamental event in EMT [20]. As one of the calcium-dependent cell adheion molecule, E-cadherin is mainly expressed in epithelial tissues. It is an important tumor suppressor gene and loses of it reduces the cell adhesion strength in tissues, which resulting in the increase of cell motility [21-25]. Our study showed that the expression of E-cadherin was significantly down-regulated since knockdown of p42.3 *in vitro*. In addition, N-cadherin is a mesenchymal marker of EMT [26, 27], and up-regulation of N-cadherin was also detected in p42.3 knockdown RCC cells in our studies. Importantly, expression of β-catenin was reduced by knockdown of p42.3, indicating that p42.3 is involved in the progression of renal cell carcinoma and it may be connected with the acceleration of EMT process and activation of β-catenin. Since the previous studies indicated that β-catenin may be responsible for overexpression of p42.3 in CRC [14], we can predict that β-catenin may mediate the expression of p42.3 in RCC cells, and p42.3 may involve in the activation of β-catenin in turn.

In summary, our study shows that the expression of p42.3 is significantly increased in human renal cell carcinoma. Strikingly, the overexpression of p42.3 in RCC may promote the development of tumor through accelerating the EMT progression and activating β-catenin. The clear molecular mechanisms of how p42.3 promotes RCC cell proliferation, cell migration and invasion need further investigation. Our data suggest that p42.3 is a potential target under cancer treatment and might be used as a diagnostic marker during clinical treatment of Human Renal-Cell Carcinoma.

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Disclosure of conflict of interest

None.

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