Reduced SPOCK1 expression inhibits non-small cell lung cancer cell proliferation and migration through Wnt/β-catenin signaling

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Abstract. – OBJECTIVE: Accumulating evidence suggests that SPARC/osteonectin, cwcv, and kazal-like domain proteoglycan 1 (SPOCK1) contributes to the initiation and progression of human cancers. However, little is known about the function mechanisms of SPOCK1 in non-small cell lung cancer (NSCLC). The aim of this study was to investigate the molecular mechanism of SPOCK1 in NSCLC.

PATIENTS AND METHODS: The expression levels of SPOCK1 in NSCLC tissues and cell lines were analyzed by qRT-PCR and Western blotting. The proliferative activity of NSCLC cells was determined by MTT and colony formation assays. The transwell assay was used to examine the cell migration and invasive ability. To study the impact of SPOCK1 on Wnt/β-catenin signaling, we further performed Western blotting for related proteins in this pathway.

RESULTS: We observed that the expression of SPOCK1 at both protein and mRNA levels was also increased in human NSCLC tissues and cell lines. Functionally, down-regulation of SPOCK1 in NSCLC cells markedly suppressed cell proliferation, colony formation, migration and invasion in vitro. Mechanistically, we found that the activation of Wnt/β-catenin pathway was suppressed by SPOCK1 silencing.

CONCLUSIONS: The expression of SPOCK1 served as a tumor promoter, possibly through the Wnt/β-catenin signaling pathway in NSCLC. Targeting SPOCK1 could be a potential therapeutic strategy in NSCLC.

Key Words: SPOCK1, NSCLC, Wnt/β-catenin, Proliferation, Metastasis.

Introduction

Lung cancer is the leading cause of cancer death worldwide, responsible for more deaths than esophageal, colon, and bone tumors1-2. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers1. Despite recent advances in clinical and experimental oncology, the overall survival time of NSCLC patients has not improved dramatically3-5. The poor prognosis of NSCLC patients was due to the high rate of tumor recurrence and metastasis6. Therefore, it is of great urgency to elucidate the molecular mechanisms underlying NSCLC and develop new therapeutic strategies.

SPARC/osteonectin, cwcv and kazal-like domains proteoglycan 1 (SPOCK1), an important oncogene, encodes a matricellular glycoprotein belonging to a novel Ca2+-binding proteoglycan family7. It has been reported that SPOCK protein family has a similar N-terminus, follistatin-like domain and C-terminus, and its function may be involved in cell proliferation, differentiation, and apoptosis8,9. Recently, SPOCK1 was found to be abnormally expressed in various tumors10,11. Growing in vitro and in vivo assay revealed that SPOCK1 acted as a positive regulator in progression of tumor growth and metastasis12-14. Noteworthy, the up-regulation of SPOCK1 was reported in lung cancer by Miao et al15. However, its function mechanisms in NSCLC are still unclear and need to be investigated.

The Wnt/β-catenin signaling pathway has been reported to be aberrantly activated in various types of cancers, including NSCLC16,17. The up-regulated transcriptional activities of the β-catenin were found in NSCLC tissues and cell lines18. Moreover, altered activity of the Wnt/β-catenin signaling pathway was also associated with EMT and cancer metastasis19-20. Evidence indicated that many tumor-related genes exerted its role by the regulation of Wnt/β-catenin signaling pathway21-22. Thus, the association between SPOCK1 and Wnt/β-catenin signaling pathway was worthy to be investigated. Therefore, the aims of the present study were to investigate the expression pattern of SPOCK1 and its role on
NSCLC metastasis in vitro, as well as to determine its influence in activation of Wnt/β-catenin signaling pathway.

**Patients and Methods**

**Patient Samples**

NSCLC tissues and paired adjacent non-tumor corresponding tissues were obtained from 18 patients diagnosed as NSCLC between 2015 and 2016 in the Department of Thoracic Surgery, Chinese PLA General Hospital. None of these 18 patients received neoadjuvant or adjuvant chemotherapy before operation. Samples were stored and transported in liquid nitrogen. Written informed consent was obtained from all patients, and research protocols were approved by the Clinical Research Ethics Committee of Chinese PLA General Hospital.

**Cell Culture and Transfection**

We cultured human normal lung epithelial cells line NLEC and three human NSCLC cell-lines (A549, HCC827 and 95D) in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were maintained at 37°C incubator at 5% CO₂. SPOCK1 siRNA (si-SPOCK1) and scrambled siRNA (si-NC) were purchased from Life Technologies (Waltham, MA, USA). The transient or stable transfection was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions.

**RNA Extraction and Quantitative Real-Time RT-PCR**

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA (2 lg) was reverse-transcribed using reverse transcription system (Promega, Madison, WI, USA). The expression of SPOCK1 was detected by RT-Real-time PCR. The following primers were used for qPCR: SPOCK1, 5’-CAACTGCTTGTTCCCAGAGG-3’ (sense) and 5’-GCCAATGACTTCCCTACCCA-3’ (antisense). GAPDH, 5’-GGAGCGAGATCCCTCCAAAAT-3’(sense); GAPDH, 5’-GGCTTGGTCATACCTTCTCATGG-3’(antisense). Data were expressed as fold changes relative to GAPDH calculated based on the following formula: \( RQ = 2^{-\Delta\Delta Ct} \).

**Proliferation Analysis**

Cell viability and proliferation were determined by cell counting and MTT assay (Promega, Madison, WI, USA). Cells were seeded in 96-well plates at a density of \( 4 \times 10^3 \) cells/well in 100 μl cell culture medium. Cell proliferation was measured once every 24 h. MTT (20 ml, 5 mg/ml) was added into each well at the indicated time point and incubated for 4 h at 37°C. All experiments were performed in quadruplicate.

**Colony Formation Assay**

48 h after transfection, cells were trypsinized and plated on 6-well plates at a density of 300 cells/well and cultured for 10 days. Fresh medium was added to each well every 3 days. The number of colonies was analyzed using ImageJ software. These experiments were performed in triplicate.

**Wound Healing Assay**

Wound-healing assays was performed to evaluate the cell migratory capacity of NSCLC cells in each group. We transfected the cells with si-SPOCK1 and si-NC; they were seeded 1 × 10⁶ in six-well plates and cultured for 24 h with mitomycin. Then, an artificial “wound” was created by scratching a confluent cell monolayer of cells using 200 μl pipette tip. The cell motility in terms of wound closure was measured by photographing.

**Transwell Assay**

Cell migration and invasion were performed using chambers (BD Biosciences, Franklin Lakes, NJ, USA) inserted to 24-well plate. A549 and HCC827 cells were transfected with si-SPOCK1 or si-NC, and 48 h later detached with 0.25% trypsin-EDTA. The lower chamber was filled with media with 10% serum. After 24 h of incubation, the medium was removed, and the filter membrane was fixed with 4% formalin for 1 h. After the inserts were rinsed, cells were counted and photographed at high magnification using an inverted microscope. For invasion assay, the transwell chamber was coated with matrigel.

**Western Blot Analysis**

Proteins were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer (Beyongtime, Beijing, China). The obtained protein was dissolved in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membranes. After blocking with 5% non-fat milk, the membrane was probed with
primary antibodies against human SPOCK1 and Wnt related proteins, followed by horseradish peroxidase (HRP)-linked secondary antibodies. Finally, immunoreactive protein bands were detected with enhanced chemiluminescence (ECL) system.

**Statistical Analysis**

Statistical analysis was performed using SPSS software, version 19.0 (IBM, Armonk, NY, USA). GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used to plot all graphs. Differences/correlations between groups were calculated using Student’s t-test. \( p < 0.05 \) was defined as statistical significance.

**Results**

**SPOCK1 was Upregulated in NSCLC Tissues and Cell Lines**

We initially examined the expression of SPOCK1 mRNA and proteins in human NSCLC tissues and matched adjacent non-tumor tissues. As shown in Figure 1A and 1B, we found that the expression level of SPOCK1 mRNA and proteins in NSCLC tissues was significantly higher than that in adjacent non-tumor tissues \((p < 0.05)\). Subsequently, we detected the levels of SPOCK1 mRNA and proteins in NSCLC cell lines by PCR and Western blot. The results indicated that expression of SPOCK1 mRNA and proteins in three NSCLC cell lines were significantly increased compared with that in NLEC cells (Figure 1C and 1D). Those results revealed that SPOCK1 may play a positive role in NSCLC progression.

**Knockdown of SPOCK1 Inhibited the Proliferation of NSCLC Cells**

We next study the function of SPOCK1 through loss-of-function approaches. SPOCK1 was knocked down (by using si-SPOCK1 and si-NC) in A549 and HCC-827 (Figure 2A). Cell growth was measured by MTT assay. Our data indicated that the decreased expression of SPOCK1 significantly inhibited cell proliferation of A549 and HCC-827 (Figure 2B). Further colony formation assays also revealed that SPOCK1 silencing si-
Significantly inhibited colony formation abilities (Figure 2C). These data suggested that knockdown of SPOCK1 could inhibit NSCLC cell proliferation in vitro.

**Knockdown of SPOCK1 Inhibits the Migration and Invasion of NSCLC Cells**

To investigate the role of SPOCK1 on NSCLC cell migration and invasion, wound-healing and transwell assays were performed. As shown in Figure 3A, we found that knockdown of SPOCK1 in all three cells led to retarded wound closing compared to NC groups. Moreover, the result of transwell assay indicated that knockdown of SPOCK1 inhibited the migration and invasion abilities of the A549 and HCC-827 cells, respectively (Figure 3B and 3C). These data suggested that knockdown of SPOCK1 could inhibit NSCLC cell metastasis in vitro.

**SPOCK1 Silencing Inhibited Wnt/β-Catenin Signaling**

Wnt/β-catenin signaling pathway has been demonstrated to play important roles in the NSCLC progression. Thus, we hypothesized that SPOCK1 might play a relevant role to Wnt/β-catenin signaling. To test our hypothesis, we performed Western blot analysis to investigate the effects of SPOCK1 knockdown on this signaling pathway. Our results indicated that the protein levels of β-catenin, c-myc and cyclin D1 were significantly decreased in both A549 and HCC-827 cells transfected with si-SPOCK1, while E-cadherin expression was up-regulated (Figure 4A and 4B). These results indicated that the Wnt/β-catenin signaling pathway might be involved in the SPOCK1-induced proliferation and metastasis of NSCLC cells.

**Discussion**

We showed that SPOCK1 mRNA and proteins were significantly upregulated in NSCLC tissues and cell lines. Functional assay indicated that knockdown of SPOCK1 inhibited NSCLC cell proliferation, migration and invasion. These results revealed that SPOCK1 may act as an oncogene in NSCLC. As an important oncogene, the effects of SPOCK1 have been reported in va-
SPOCK1 and non-small cell lung cancer

Promoter by activating the PI3K/AKT pathway. Of note, the influence of SPOCK1 in PI3K/AKT pathway was also confirmed in colorectal cancer cells. Chen et al. reported that forced SPOCK1 expression promotes the invasion and metastasis of gastric cancer through Slug-induced EMT. Miao et al. showed that the significant up-regulation of SPOCK1 was found in NSCLC tissues, and overexpression of SPOCK1 was significant-

Figure 3. Down-regulation of SPOCK1 inhibited the migration and invasion of NSCLC cells. (A) Cell migration was determined by a wound-healing assay in the A549 and HCC827 cells transfected with the si-SPOCK1 or si-NC. (B,C) Transwell assays of A549 and HCC827 cells after treatment with si-SPOCK1 or si-NC. The relative ratio of invasive/migrated cells per field is shown. *p < 0.05, **p < 0.01.

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ly correlated with shorter disease-free survival. Those data suggested SPOCK1 as value biomarker, which can be targeted for the treatment of NSCLC patients.

In order to explore the underlying mechanisms of SPOCK1 in NSCLC, our attention focused on Wnt/β-catenin signaling pathway, which is one of the most common signaling abnormalities occurring in human cancer. Previous studies have reported that several genes could regulate the activation of Wnt/β-catenin signaling pathway. For instance, Chen et al. found that fibulin-5 served as a metastasis suppressor in lung cancer by suppressing Wnt/β-catenin signaling. Yang et al. showed that DEPDC1B was able to activate Wnt/β-catenin signaling to suppress migration and invasion of NSCLC cells. In the present study, we silenced SPOCK1 in NSCLC cells and found the protein levels of β-catenin, c-myc and cyclin D1 were decreased, while the protein levels of E-cadherin were increased. Those results indicated that SPOCK1 may exert its tumor-primitive role by regulating Wnt/β-catenin signaling pathway.

**Conclusions**

We demonstrated that SPOCK1 was increased in NSCLC tissues and cell lines. Furthermore, our data also provided clear evidence that SPOCK1...
served as a tumor promoter, possibly through regulating Wnt/β-catenin signaling pathway. Thus, SPOCK1 could potentially serve as a novel therapy target for NSCLC.

**Conflict of Interest**
The Authors declare that they have no conflict of interest.

**References**


