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Lentivirus-mediated silencing of SCIN inhibits proliferation of human lung carcinoma cells

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ABSTRACT

SCIN (scinderin) is a calcium-dependent actin severing and capping protein. Homologue in zebrafish has been found to be related with cell death. In the present study, we found that SCIN is highly expressed in human lung cancer specimens. However, the role of SCIN in lung cancer has not yet been determined. To investigate the function of SCIN in lung carcinoma cells, we took advantage of lentivirus-mediated RNA interference (RNAi) to knockdown SCIN expression in two lung carcinoma cell lines A549 and H1299. Silencing of SCIN significantly inhibited the proliferation and colony formation ability of both cell lines in vitro. Moreover, flow cytometry analysis showed that knockdown of SCIN led to G0/G1 phase cell cycle arrest as well as an excess accumulation of cells in the sub-G1 phase. Furthermore, depletion of SCIN resulted in a significant increase in Cyclin B1, p21 and PARP expression, and a little decrease in Cyclin D1 expression. These results suggest that SCIN plays an important role in lung carcinoma cell proliferation, and lentivirus-mediated silencing of SCIN might be a potential therapeutic approach for the treatment of lung cancer.

1. Introduction

Lung cancer is one of the most common causes of cancer-related death. There were 1.6 million new cases and 1.38 million deaths due to lung cancer in 2008 (Ferlay et al., 2010). Data from National Cancer Institute shows that there are 226,160 new cases and 160,340 Americans were expected to die from lung cancer in 2012, about 28% of all cancer-related death. Many methods have been developed for the treatment of lung cancer; however, only about 15% patients could survive five years after diagnosis (Reddy et al., 2011; Bach et al., 2012). New therapeutic targets and methods are needed to be developed for the treatment of lung cancer.

Cytoskeleton plays an essential role in the mitosis, migration and invasion of carcinoma cells (Hall, 2009). Many cytoskeleton-associated proteins of lung cancer cells have been intensively investigated (Bernal et al., 1983; Chen et al., 2010, 2011; Chang et al., 2012; Niu et al., 2012). SCIN (scinderin) is a calcium-dependent actin filament serving and capping protein (Marcu et al., 1998). It could regulate vesicle transport and exocytosis through organizing the cytoskeleton underneath the plasma membrane (Dumitrescu Pene et al., 2005; Trifaro et al., 2008). It could also regulate cell differentiation through the MAP kinases P38 and ERK1/2 mediated signaling pathway (Nurminsky et al., 2007). Reduction of the expression of Scinlb, the homologue of SCIN in *zebrafish*, is found to be associated with increased cell death (Jia et al., 2009). Recent studies found that SCIN is abnormal in certain kind of carcinomas and is involved in many processes of cancer cells, such as proliferation and tumor resistance. SCIN is downregulated in megakaryoblastic leukemia cells and overexpression of SCIN could inhibit proliferation and tumorigenesis (Zunino et al., 2001). In the T cell lysis resistant tumor cells, SCIN is overexpressed and knockdown of SCIN could significantly attenuate the resistance to cytolytic T lymphocytes killing (Abouzahr et al., 2006). However, the function of SCIN in lung cancer is largely unknown.

In the present study, we found that SCIN is overexpressed in lung cancer specimens. To investigate the pathological function of SCIN in lung cancer, we employed lentivirus-mediated RNAi to suppress the expression of SCIN in two lung carcinoma cell lines A549 and H1299. We found that SCIN knockdown could reduce the proliferation and colony formation ability of lung carcinoma cells. Furthermore, SCIN silencing blocked cell cycle progression via upregulation of Cyclin B1 and p21, and induced cell apoptosis via cleavage of PARP. Taken together, our results suggest that SCIN is essential for the proliferation and tumorigenesis of lung cancer.

Abbreviations: SCIN, scinderin; MAP kinases P38, P38 mitogen-activated protein kinases; RNAi, RNA interference; cDNA, complementary DNA.

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2. Materials and methods

2.1. Immunohistochemistry (IHC)

Thirty-nine lung cancer specimens were collected for immunohistochemistry (28 males, 11 females; 10 specimens from patients younger than 50 years old, 29 from patients older than 50 years old). Sixteen normal lung specimens were used as control. All the above tissue samples were provided by the Department of Thoracic Surgery in First Hospital of China Medical University, and the patients signed an informed consent form conforming to the guidelines of the Declaration of Helsinki. The tissues were fixed by formalin and embedded by paraffin. Before immunostaining, the paraffin was removed from the samples. Samples were blocked and incubated with primary antibody against SCIN (Sigma, Cat# HPA024264; dilution 1:50) overnight at 4 °C and biotinylated secondary antibody for 30 min at room temperature. After reaction with streptavidin peroxidase conjugate for 10 min at room temperature, DAB staining was performed. All samples were counterstained with hematoxylin.

2.2. Cell culture

Human lung carcinoma A549 and H1299 cells and human embryonic kidney HEK293T cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). A549 and HEK293T cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. H1299 cells were cultured in RPMI1640 (Hyclone) supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Construction of SCIN shRNA lentivirus and cell infection

A 67 bp shRNA sequence (5′-CTAGCCGGGCAAGTGTCCTAAAGTGCAA ATTCAAGAGATTTGCACTTTAGGACACTTG CTTTTTAAT-3′) was designed to target human SCIN gene (NM_033128). A random shRNA sequence (5′-CTAGCCCGGTTCTCCGAACGTGTCACGTATCTCGAGATACGTGACACG TTCGGAGAATTTTTTTAAT-3′) was used as control. To rule out the possible off-target effect of shRNA, another shRNA sequence (5′- TGGAAAGG TAAAGATGCTAATCTCGAGATTAGCATCTTTACCTTTCCA-3′) against SCIN was used to get comparable results. All shRNAs were ligated into pFH-L vectors (Shanghai Hollybio, China). For lentivirus packaging, HEK293T cells were transfected with pFH-L-SCIN shRNA or control shRNA together with two helper plasmids (pVSVG-I and pCMVΔR8.92, Shanghai Hollybio, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two days after transfection, the supernatant containing packaged lentivirus was collected and passed through 0.45 μm filters. To infect A549 and H1299 cells, lentivirus particles were added to the culture medium at a multiplicity of infection (MOI) of 50. Cells were then cultured in the incubator at 37 °C. The efficiency of infection was determined by GFP percentage under fluorescence microscopy three days after lentivirus infection.

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was then retro-transcribed with oligodT using M-MLV reverse transcriptase (Promega). The expression level of SCIN was measured by qRT-PCR with primers: 5′-ATTGTGGAGGTTGATGTTGATG-3′ (forward) and 5′-AGTGGTGAGGTCTGGTAGTC-3′ (reverse). The primers of β-actin, used as endogenous control, were 5′-GTGGACATCCGCAAAGAC-3′ (forward) and 5′-AAAGGGTGTAACGCAACTA-3′ (reverse). The PCR reaction system was 10 μl 2× SYBR Premix Ex-Taq, 0.8 μl primers (2.5 μM), 5 μl cDNA and ddH_2O up to a final volume of 20 μl. Probe amplification was performed as follows: 1 min at 95 °C; 40 cycles of 95 °C for 5 s and at 60 °C for 20 s. The expression level of SCIN was determined by cycle threshold (Ct) normalized with that of β-actin using the $2^{-\Delta\Delta Ct}$ formula. Experiments were repeated at least three times.

2.5. Western blotting analysis

Four days after lentivirus infection, A549 and H1299 cells were washed by PBS and collected. Whole cell lysis was obtained using 2× SDS Sample Buffer (100 mM Tris–HCl (pH 6.8), 10 mM EDTA, 4% SDS, 10% glycine). Proteins were separated by SDS-PAGE and transferred to PVDF membrane. After the samples were incubated with primary antibodies overnight and secondary antibodies for 2 h at room temperature, the samples were visualized with enhanced chemiluminescence (Amersham). The following primary antibodies were used in this study: anti-SCIN (Sigma, Cat# HPA024264, dilution 1:500); anti-GAPDH (Proteintech Group, Inc., Cat# 10494-1-AP, dilution 1:100,000); anti-Cyclin D1 (Medical & Biological Laboratories Co., Ltd., Cat# MD-17-3, dilution 1:1000); anti-Cyclin B1 (Medical & Biological Laboratories Co., Ltd., Cat# K0128-3, dilution 1:1000); anti-p21 Waf1/Cip1 (12D1) (Cell Signaling Technology, Cat# 2947, dilution 1:200); and anti-PARP (Cell Signaling Technology, Cat# 9542, dilution 1:1000). The secondary antibodies are goat anti-rabbit IgG-HRP (Santa Cruz, Cat# SC-2054, dilution 1:5000) and goat anti-mouse IgG-HRP (Santa Cruz, Cat# SC-2005, dilution 1:5000). Experiments were performed in triplicate.

2.6. MTT assay

After lentivirus infection, A549 and H1299 cells were seeded into 96-well plates at a concentration of 2000 cells/well, respectively. At indicated time points (1, 2, 3, 4, 5 days), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. Then acidic isopropanol (10% SDS, 5% isopropanol and 0.01 M HCl) was added to stop the reaction and incubate at 37 °C for 10 min. The optical density was measured using a microplate reader at the wavelength of 595 nm. Experiments were repeated at least three times.

2.7. Colony formation assay

To determine the effect of SCIN in lung tumorigenesis in vitro, we performed colony formation assay according to the literature (Franken et al., 2006). Briefly, after lentivirus infection, 200 cells of A549 and H1299 cells were seeded into each well of 6-well plates, respectively. Cells were cultured in the 37 °C incubator for about 9 days until most single clones have more than 50 cells. Cells were washed by PBS, fixed by 4% PFA, and then stained with Giemsa. Images were captured by light/florescence microscopy. Experiments were performed at least three times.

2.8. Flow cytometry analysis

Cell cycle progression was analyzed by flow cytometry. In brief, four days after lentivirus infection, A549 and H1299 cells were collected, washed by PBS and fixed by 75% ethanol. Cells were stained with propidium iodide (PI) and RNase overnight at 4 °C. Samples were then analyzed by Cell Lab Quanta Beckman Coulter. Experiments were repeated at least three times.

2.9. Statistical analysis

The results of immunohistochemistry staining were evaluated by Chi-square test and the other data were evaluated by Student's t test and expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism 5 and $p < 0.05$ was considered to be statistically significant.