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Research paper

Serum and glucocorticoid kinase 1 promoted the growth and migration of non-small cell lung cancer cells

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ABSTRACT

Serum and glucocorticoid kinase 1 (SGK1) has been reported to be up-regulated in non-small cell lung cancer (NSCLC). However, its functions in NSCLC remained unclear. Here, SGK1 was found to be up-regulated in NSCLC samples. Over-expression of SGK1 promoted the growth and migration of NSCLC cells, while down-regulation of SGK1 inhibited the growth, migration and metastasis of NSCLC cells. SGK1 promoted the phosphorylation of GSK3 beta and the accumulation of beta-catenin, up-regulation of the target genes downstream of beta-catenin/TCF signaling, and activating the transcriptional activity of beta-catenin/TCF complex. Collectively, SGK1 might promote the progression of NSCLC through activating beta-catenin/TCF signaling.

1. Introduction

Activation of the phosphoinositide-3-kinase (PI3K) pathway promoted cell growth and migration which ultimately leads to tumorigenesis (Chen et al., 2014; Papadatos-Pastos et al., 2015; Yang et al., 2014). Although AKT was found to be the major mediator of PI3K signaling, recent studies have suggested that other effectors might mediate PI3K signaling independent of AKT (Miller et al., 2011; Moniz and Vanhaesebroeck, 2013). For example, over-expression of AKT could not fully restore the malignant phenotypes induced by PDK1 (Raimondi and Falasca, 2011). Also, in a subset of tumors, activation of AKT correlated poorly with the constitutive mutation of PI3K catalytic subunit (Woenckhaus et al., 2007). These observations indicated the presence of other important effectors which mediated the PI3K signaling independent of AKT.

Serum and glucocorticoid kinase (SGK) family contains three members: SGK1, SGK2 and SGK3 (Bruhn et al., 2010). All of the three members shared 80% similarity in the amino acid sequence (Bruhn et al., 2010). SGK1 was found to be expressed in virtually all the tissues (Bruhn et al., 2010). With regard to the biological functions, SGK1 was reported as the regulator for ion channel activity (Naray-Fejes-Toth and Fejes-Toth, 2000; Pearce et al., 2000), transport and gene transcription (Loffing et al., 2006; Pearce et al., 2000). Although knocking out of SGK1 in mouse didn't lead to lethal phenotype, various physiological deficits were observed, suggesting the broad function of SGK1 (Borst et al., 2015). Regulation of the SGK1 could occur at different levels. Firstly, the transcription of SGK1 could be induced by the stimulus of serum, glucocorticoid and a set of growth factors, and inhibited by heparin (Cheng et al., 2014; Delmolino and Castellot, 1997). Moreover, phosphorylation of SGK1 by PI3K/PDK1 cascade leads to the activation of SGK, especially the phosphorylation of Thr256 of SGK1 (Kobayashi and Cohen, 1999; Park et al., 1999). Also, the degradation of SGK1 was regulated by Nedd4-2 ubiquitin ligase (Henry et al., 2003; Kim et al., 2014).

Recently, the functions of SGKs in the tumorigenesis have attracted much attention. The expression of SGK3 was positively correlated with the progression of breast cancer and promoted the tumorigenesis independent of AKT (Sahoo et al., 2005). Consistent with these observations, SGK3 was reported to promote the proliferation and survival of the liver cancer cells (Liu et al., 2014). Also, in prostate cancer, the expression of SGK3 was induced by androgen and promoted the growth of cancer cells by activating P70S6 kinase (Deng et al., 2012). Several studies have revealed the physiological functions of SGKs in the lungs. SGK1 was found to be involved in the pulmonary vascular remodeling (BelAiba et al., 2006). Besides, SGK played a very important role in the lung fluid absorption (Li et al., 2009). In the non-small cell carcinoma (NSCLC) clinical samples, the expression of SGK1 was significantly up-regulated (Abbruzzese et al., 2012). However, the functions of SGK1 in the tumorigenesis of NSCLC still remained unknown.

Beta-catenin/TCF signaling played very important functions in the carcinogenesis by regulating a set of cell growth and migration-related genes. GSK3 beta phosphorylated beta-catenin and promoted the degradation of beta-catenin (Clevers and Nusse, 2012). Activation

Abbreviations: SGK1, serum and glucocorticoid kinase 1; NSCLC, non-small cell lung cancer; PI3K, phosphoinositide-3-kinase; ATCC, American Type Culture Collection; FBS, fetal bovine serum; GFP, green fluorescence protein; PBS, phosphate-buffered saline.

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of beta-catenin/TCF signaling was very common in the progression of NSCLC (Carotenuto et al., 2014; Tseng et al., 2014). Although previous report has shown that SGK1 regulated the beta-catenin/TCF signaling by promoting the phosphorylation of GSK3 beta in vascular smooth muscle cells, whether SGK1 regulates the beta-catenin/TCF signaling in NSCLC cells and the molecule mechanism is not fully understood (Zhong et al., 2014). In this study, we examined the roles of SGK1 in NSCLC and studied the regulation of beta-catenin/TCF signaling by SGK1.

2. Materials and methods

2.1. Cell culture

NSCLC cell lines (A549 and H23) were purchased from ATCC (American Type Culture Collection). A549 and H23 cells were cultured in PRMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria), 10 units/ml penicillin-G and 10 mg/ml streptomycin. A549 and H23 cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Clinical samples

40 NSCLC tissues and paired non-cancerous tissues were collected from patients who received surgery for NSCLC at Shanghai General Hospital. All of the patients agreed with this study. NSCLC tissues and paired non-cancerous tissues were stored at −80 °C.

2.3. Plasmid construction and transfection

SGK1 cDNA was inserted into the expression vector pcDNA 3.1-myc. The SGK1 expression vector and empty pcDNA3.1 were transfected into H23 and A549 cells using Lipofectamine 2000 reagent (Invitrogen). After the selection with G418, the resistant cells were pooled and further confirmed the expression of exogenous SGK1 by Western blot.

2.4. Luciferase reporter assay

Cells were plated at a subconfluent density and co-transfected with 0.05 μg of the reporter plasmid, 0.5 μg si con or siSGK1 plasmid, and 0.05 μg of Renilla luciferase pRL-TK as an internal control for transfection efficiency. 24 h later, cells were treated with Wnt 3a recombinant protein for 8 h and then cell lysates were prepared. The reporter activity was measured using the dual-luciferase reporter assay system (Promega). Transfections were performed in triplicate and repeated three times to ensure reproducibility.

2.5. RNA extraction, reverse transcription and real-time PCR analysis

RNA was extracted from NSCLC tissues and matched non-cancerous tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 2 μg of total RNA was reversely transcripted to cDNA with the reverse transcription kit (Promega, Madison, WI), following the manufacturer's instructions. The primer pair used for amplification of the human SGK1 gene was as follows: forward primer, 5′-GGTAAACA AACAAAGACATGAATG-3′, and reverse primer, 5′-ATGAACATGACTCGTT CTCC-3′. As an internal standard, a fragment of human beta-actin was amplified by PCR using the following primers: forward primer, 5′-GATC ATTGCTCCTCCTGAGC-3′, and reverse primer, 5′-ACTCCT GCTTGCTGAT CCAC-3′. Amplification reactions were performed in a 15 μl volume of the LightCycler-DNA Master SYBR Green I mixture from Roche Applied Science as follows: with 10 pmol of primer, 2 mM $MgCl_2$, 200 μM dNTP mixture, 0.5 units of *Taq* DNA polymerase and universal buffer. All of the reactions were performed in triplicate in an iCycler iQ System (Bio-Rad), and the thermal cycling conditions were as follows: 95 °C for 3 min; 40 cycles at 95 °C for 30 s, 58 °C for 20 s, and 72 °C for 30 s; 72 °C for 10 min. In each reaction, the threshold cycle number (C_T) was determined for both the target gene (SGK1) and the control gene (beta-actin) with the iCycler software and the mean C_T for the three reactions was calculated. The $\triangle C_T$ or the C_T of the housekeeping gene subtracted from the C_T of the target gene was plotted for each sample.

2.6. Western blot

The cells were washed with PBS and then lysed in RIPA buffer. Cell lysates were centrifuged at 10,000 *g* (4 °C for 20 min). Protein concentrations were determined using Bradford reagent (Sigma) according to the manufacturer's instructions. Equal amounts of total cellular protein were mixed with loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 2% beta-mercaptoethanol and bromophenol blue), boiled for 5 min, and subjected to 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% fat-free dry milk for 1 h at room temperature and incubated overnight with primary antibodies in TBST with 1% bovine serum albumin. After washing with TBST, the membranes were further incubated for 1 h at room temperature with corresponding horseradish peroxidase-conjugated secondary antibody in appropriate dilution and then washed five times with the same buffer. The immunoreactive protein bands were visualized by ECL kit (Pierce). Antibodies to beta-catenin, c-Myc, CyclinD1, Snail and phosphorylated GSK3 beta were purchased from Cell Signaling Technology. Antibodies to GAPDH and SGK1 were purchased from Santa Cruz Biotechnology.

2.7. Knocking down the expression of SGK1

We used FG12 lentiviral vector to knock down the expression of SGK1 in A549 and H23 cells. Besides producing double-stranded RNA, FG12 vector independently expressed green fluorescence protein (GFP). To construct the hairpin siRNA expression cassette, complementary DNA oligonucleotides for siRNA of SGK1 (si SGK1) or mutated sequence as control (si con) were synthesized, annealed, and inserted into FG12. Two SGK1 siRNA constructs were used as follows: SGK1 siRNA 1# (highlighted sequence was the complementary sequence with SGK1 mRNA), 5′-ACCG**GGAGGATGGGTCTGAACG**TTCAAGAGA**CGTTCAGACCCA TCCTCC**TTTTTTGGATCCC-3′ and 5′-TCGAGGGATCCAAAAAAGAG**GGAGGA TGGGTCTGAACG**CTCTTGAA**CGTTCAGACCCATCCTCC**-3′; and SGK1 siRNA 2# (highlighted sequence was the complementary sequence with SGK1 mRNA), 5′-ACCG**GGTGCTTCATAAGCAGCC**TTCAAGAGA**GGCTGCTTATGA AGCACC**TTTTTTGGATCCC-3′ and 5′-TCGAGGGATCCAA AAAG**GGTGCTTC ATAAGCAGCC**CTCTTGAA**GGCTGCTTATGAAGCACC**-3′; si con vector (highlighted sequence was the random sequence as control that was not related to SGK1 mRNA), 5′-ACCG**GTACATAGGGACGTAACG**TTCAA GAGACGTTA**CGTCCCTATGTACC**TTTTTGGATCCC-3′ and 5′-TCGAGGGATC CAAAAAG**GTACATAGGGACG**TAACGTCTCTTGAA**CGTTACGTCCCTATGT AC**-3′. FG12 vector with si SGK1 or si con was transfected into HEK293T cells, and the virus with SGK1 siRNA or si con was harvested from culture medium. The harvested virus was purified by centrifugation at 25,000 *g* (4 °C, 150 min), and appropriate amounts of virus were used to infect A549 and H23 cells. After 3 days of infection, the GFP-positive cells were sorted by flow cytometry (BD Biosciences), which all stably expressed si SGK1 or si con.

2.8. Apoptosis and cell cycle analysis

1.0×10^5 cells were plated into 6-well plate. 24 h later, cells were harvested and fixed with 75% ethanol solution at 4 °C over night. After centrifugation at 3000 *g* for 5 min, the cells were re-suspended with a solution containing RNAse and PI, incubated at 37 °C for 1 h and then examined using flow cytometry (BD Biosciences).