Short hairpin RNA targeting AKT1 and PI3K/p85 suppresses the proliferation and self-renewal of lung cancer stem cells

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Abstract. The aim of the present study was to investigate the effect of short hairpin (sh)RNA targeting AKT1 and phosphatidylinositol 3-kinase (PI3K)/p85 on the proliferation and self-renewal of lung cancer stem cells (LCSCs). The recombinant adenovirus expression vector, which contained shRNA targeting open reading frames of AKT1 and PI3K/p85, was transfected into LCSCs. It was found that AKT1 and PI3K/p85 expression was upregulated in LCSCs compared with that in the primary lung cancer cells. Recombinant adenovirus vector rAd5-siAKT1-siPI3K/p85 significantly downregulated AKT1 and PI3K/p85 mRNA and protein expression in LCSCs. The downstream factors, proliferating cell nuclear antigen (PCNA) and cyclin D1 were also downregulated, while p53 was upregulated. Following silencing of AKT1 and PI3K/p85, cell proliferation, tumor sphere formation and tumor formation in NOD/SCID mice were also reduced. According to the present results, it was hypothesized that the PI3K/Akt signaling pathway is important in the self-renewal and proliferation of LCSCs, and that targeting the PI3K/Akt signaling pathway decreases the rate of tumor formation in vivo.

Introduction

Recent evidence has indicated that certain solid tumors, including brain gliomas (1,2) and breast (3), prostate (4), colon (5) liver (6) and lung cancer (7) contain a small population of cancer stem cells (CSCs), which have a high capacity for self-renewal, multilineage differentiation, inducing malignancy, drug-resistance and radiotherapy resistance, as well as recurrence and metastasis (8,9). These cells are responsible for tumor maintenance and metastasis. It is postulated that therapies for cancer that specifically target stem cell signaling pathways utilized by CSCs may be beneficial (10,11).

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway has been demonstrated to be involved in the regulation of cell proliferation and apoptosis, and is pivotal in the initiation and progression of malignancies, enhancing cell survival by stimulating cell proliferation and inhibiting apoptosis (12,13). More recent studies have identified that the PI3K/Akt signaling pathway is overactivated in several types of human cancer, including brain glioma (14), pancreatic cancer (15), lung cancer (16), and high expression of PI3K and p-Akt is often associated with a poor prognosis.

The overactivation of the PI3K/Akt signaling pathway has also been observed in several CSCs (17-19). However, there are no reports with regard to the correlation between the PI3K/Akt signaling pathway, and proliferation and self-renewal of lung cancer stem cells (LCSCs) in the English-language literature. In the present study, a cell population with a CD133+ phenotype was isolated from the single cell suspension of lung adenocarcinoma tissue using a magnetic-activated cell sorting (MACS) technique, and enriched by serum free cultures. AKT1 and PI3K/p85 were suppressed by RNA interference. The expression of proliferating cell nuclear antigen (PCNA), cyclin D1 and p53 were also detected by western blot analysis. The effects of AKT1 and PI3K/p85 on the self-renewal and proliferation of LCSCs were investigated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, sphere forming assay and xenograft formation assay. In addition, a cell cycle assay was also conducted using flow cytometry following AKT1 and PI3K/p85 silencing in LCSCs. The present study demonstrated that the PI3K/Akt signaling pathway is consistently overactivated in LCSCs. Additionally, it was revealed that the downregulation of AKT1 and PI3K/p85 suppressed the self-renewal and proliferation of LCSCs and decreased the rate of tumor formation in vivo.

Materials and methods

LCSC isolation and cell culture. In our previous study (7), CD133+ cells were successfully isolated from the single cell suspension of lung adenocarcinoma tissue using a MACS technique, and enriched by serum free culture. The sorting
was verified with properties of LCSCs through experiments of self-renewal, multipotent differentiation capacity, drug resistance and tumorigenic capacity in vivo. LCSCs were harvested and cultured in serum-free Dulbecco’s modified Eagle’s medium (DMEM)-F12 (Beijing Beiruo Biotechnology Co., Ltd., Beijing, China) containing 50 µg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 100 µg/ml apo-transferrin (Sigma-Aldrich), 10 µg/ml putrescine (Sigma-Aldrich), 0.03 mM sodium selenite (Sigma-Aldrich), 2 µM prostegorone (Pure Chemistry Scientific, Inc., Sugarland, TX, USA), 0.6% glucose (LGM Pharma, Nashville, TN, USA), 5 mL HEPES (Nanjing Search Biotech Co., Ltd., Nanjing, China), 0.1% sodium bicarbonate, 0.4% bovine serum albumin (BSA; Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China), glutamine (Ameresco, Inc., Solon, OH, USA) and 1% penicillin and 1% streptomycin (Beijing Beiruo Biotechnology Co., Ltd.), as well as 20 ng/ml epidermal growth factor (EGF; PepTrogen, Rocky Hill, NJ, USA) and 10 ng/ml basic fibroblast growth factor (bFGF; PepTrogen) at 37˚C and 5% CO₂.

**Generation of rAd5-small interfering (si)AKT1-siPI3K/p85 RNAi lentiviruses** (Wuhan Boster Biological Engineering Co., Ltd.) After testing knockdown efficiencies of several shRNA constructs, the following shRNA oligonucleotides (rAd5-siAKT1-siPI3K-shRNA) were utilized: 5'-GGAGATCATGCAGCATCGC-3' [to target the 19 bp interference sequence of AKT1 gene (intervention sites: 1540-1558)] and 5'-GAAAGGAGGAAATAACAAA-3' [to target the 19 bp interference sequence of PI3K/p85 gene (intervention sites: 371-389)]. A non-specific shRNA (rAd5-siCtrl-shRNA) was also synthesized as a control, and each shRNA was cloned into a pGCL-GFP plasmid containing the U6 promoter and green fluorescent protein (GFP). The plasmids pDONR221 and pAd/CMV/V5-DEST were also included to provide the necessary packaging elements for lentivirus production. For viral transduction, shRNA lentiviral vectors at a multiplicity of infection (MOI) of 25 were added to dispersed LCSCs rapidly following plating. GFP fluorescence was measured 72 h post-transduction.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The primer sequences used included: Forward: 5'-GGCGCAGATGATCACCATCAC-3' and reverse: 5'-CTATGCCTCAGCAGCTCCA-3' for AKT1; forward: 5'-AGCATTAGGGACCTCACATTACA-3' and reverse: 5'-ACTGGAACACAGTCCATGCACATA-3' for PI3K/p85; and forward: 5'-CTGGACACCCAGCACAT-3' and reverse: 5'-GGCGATCCACAGGATCTA-3' for β-actin. The total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. cDNA was synthesized using a reverse transcription kit (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer’s instructions. cDNA (2.5 µl) was subjected to qPCR using SYBR-Green as a fluorescent reporter and 2.5X Real Master mix (Toyobo, Osaka, Japan). Specific gene primers (Wuhan Boster Biological Engineering Co., Ltd.) for AKT1, PI3K/p85 and β-actin, were amplified in separate reaction tubes. Threshold cycle numbers of triplicate reactions were determined using ABI-7500 software (v2.0; Invitrogen Life Technologies, Carlsbad, CA, USA) and averaged. Relative fold changes were calculated using the 2^−ΔΔCt method and standard curves were produced.

**Western blot analysis.** Cell extracts from the control group (untreated cells), Ad5-Control-shRNA group (cells infected with Ad5-Control-shRNA) and Ad5-siAKT1-siPI3K-shRNA group (cells infected with Ad5-siAKT1-siPI3K-shRNA), as well as primary lung cancer cells were collected, and protein samples (50 µg) were then electrophoresed on 12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Wuhan Boster Biological Engineering Co., Ltd.). Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and incubated with anti-AKT1 (sc-514032), anti-PI3K/p85 (sc-131324), anti-PCNA (sc-71858), anti-cyclin D1 (sc-70899) and anti-P53 (sc-377567) antibodies (diluted 1:1,000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. Membranes were incubated with 50 µl IgG/ hors eradish peroxidase secondary antibody (diluted 1:2,000, Wuhan Boster Biological Engineering Co., Ltd. Wuhan, China) for 2 h at room temperature after three washes with TBS-Tween-20 (TBST). Each membrane was also incubated with an anti-β-actin antibody (Santa Cruz Biotechnology Inc.) as a loading control. Membranes were washed three times with TBST and bound antibodies were detected using enhanced chemiluminescence (Beyotime, Jiangsu, China). Protein levels were quantitated by densitometry using Quantity One 4.6.2 software (Bio-Rad Laboratories, Munich, Germany).

**Sphere-forming assay.** Cell extracts from the control group, Ad5-Control-shRNA group and Ad5-siAKT1-siPI3K-shRNA group were dissociated and cultured in 96-well plates in serum-free DMEM-F12 medium containing 50 µg/ml insulin, 100 µg/ml apo-transferrin, 10 µg/ml putrescine, 0.03 mM sodium selenite, 2 µM prostegorone, 0.6% glucose, 5 mM HEPES, 0.1% sodium bicarbonate, 0.4% BSA, glutamine and 1% penicillin and 1% streptomycin, as well as 20 ng/ml EGF and 10 ng/ml bFGF. Wells containing more than one cell or no cells were marked and dismissed from the statistical data. The cells were cultured under conditions of 5% CO₂ at 37°C for 10 days. The medium was replaced or supplemented with fresh growth factors twice a week. Wells that contained spheres were counted using inverted phase contrast microscopy (DMIL- PHL; Leica, Mannheim, Germany) and the percentage of cells with sphere-forming capacity was calculated.

**Proliferation assay.** The proliferation of the cells was detected using an MTT assay on days 1, 3, 5 and 7. Three groups were plated onto 96-well plates (2,000 cells in 0.2 ml cell culture medium/well). The cells were then incubated with 20 µl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 4 h prior to collection. The culture medium was finally removed, and 150 µl dimethylsulfoxide was added to the well. After shaking thoroughly for 10 min, the plates were read for absorbance in an enzyme immunoassay at 490 nm using an Automatic enzyme-linked immunity analyzer (Diasorin S.p.A, Italy). Six wells were analyzed for each group.

**Cell cycle phase distribution.** A total of 1x10⁶ cells from the control group, Ad5-Control-shRNA group and
Ad5-siAKT1-siPI3K-shRNA group were centrifuged at 375 x g for 5 min, resuspended in 0.2 ml phosphate-buffered saline and then fixed in 1 ml of 70% ethanol at 4˚C for 16 h. Subsequent to washing with PBS, the cells were incubated with 300 µl Green-DNA Dye (Nanjing Search Biotech Co., Ltd., Nanjing, China) at room temperature for 30 min. Cell cycle status was assessed by flow cytometry (Beckman Coulter, Brea, CA, USA). The relative proportions of cells in the G₀/G₁, S and G₂/M phases were analyzed, and the percentages of cells in each phase were calculated.

Tumorigenicity in NOD/SCID mice. Cells from the control group, Ad5-Control-shRNA group and Ad5-siAKT1-siPI3K-shRNA group were diluted in growth factor-containing medium alone prior subcutaneous injection. Serial dilutions of cells (10², 10³, 10⁴ and 10⁵ cells) were injected subcutaneously into the abdominal wall of 4-week-old NOD/SCID mice (5 mice/group; Beijing Vitalriver Experimental Animal Technical Co., Ltd., Beijing, China). Tumor size was measured using calipers and tumor volume was calculated using the equation V=πr²h (length x width x height). The mice were sacrificed 8 weeks following the subcutaneous injection. The present animal study was approved by the Ethics Committee of Taizhou People’s Hospital (Jiangsu, China) and was performed according to the Declaration of Helsinki.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). All experiments were performed at least three times and representative results are presented as the mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance and comparisons among groups were achieved using independent sample t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

AKT1 and PI3K/p85 expression in primary lung cancer cells and LCSCs. RT-qPCR assays were performed to detect AKT1 and PI3K/p85 mRNA levels. In these assays, AKT1 and PI3K/p85 mRNA abundance was higher in LCSCs compared with the primary lung cancer cells. Similar results from western blot assays using the same cell types confirmed the results. These results indicated that AKT1 and PI3K/p85 expression is upregulated in LCSCs (Fig. 1).

Efficiency of adenovirus transfection. For viral transduction, shRNA lentiviral vectors at an MOI of 25 were added to dispersed LCSCs shortly following plating. After three days, the GFP expression of the LCSCs confirmed that the lentiviral infection was achieved, compared with the corresponding white
field image of the same cell population. RT-qPCR and western blot assays were performed to detect AKT1 and PI3K/p85 mRNA and protein levels in control, Ad5-Control-shRNA and Ad5-siAKT1-siPI3K-shRNA groups. The results showed that the expression levels of AKT1 and PI3K/p85 mRNA and protein were significantly decreased in the Ad5-siAKT1-siPI3K-shRNA group, compared with the control or Ad5-Control-shRNA groups. The results demonstrated the high knockdown efficiency of Ad5-siAKT1-siPI3K-shRNA in vitro (Fig. 2).

PCNA, cyclin D1 and p53 protein expression in control, Ad5-Control-shRNA and Ad5-siAKT1-siPI3K-shRNA groups. Western blot assays were performed to detect PCNA, cyclin D1 and p53 protein levels. In these assays, expression levels of PCNA and cyclin D1 were significantly decreased in the Ad5-siAKT1-siPI3K-shRNA group, compared with the control or Ad5-Control-shRNA groups. While expression of p53 was significantly increased in the Ad5-siAKT1-siPI3K-shRNA group, compared with control or Ad5-Control-shRNA groups (Fig. 3).

AKT1 and PI3K/p85 knockdown decreases the proliferation rate of LCSCs. The Ad5-siAKT1-siPI3K-shRNA group exhibited a low proliferation rate when compared with the rate in the control group or Ad5-Control-shRNA groups beginning the fifth day after seeding (Fig. 4).

AKT1 and PI3K/p85 knockdown induces the arrest of the cell cycle of LCSCs. The cell cycle distribution was assessed using flow cytometry. The results revealed a marked arrest in the G0/G1 phase in the Ad5-siAKT1-siPI3K-shRNA group relative to the control group or Ad5-Control-shRNA group (67.15±3.31% vs. 57.89±2.86% and 60.22±2.29%; P<0.01, respectively) (Fig. 5).

AKT1 and PI3K/p85 knockdown slows the sphere-forming ability of LCSCs. The control, Ad5-Control-shRNA and Ad5-siAKT1-siPI3K-shRNA groups were examined for their ability to form new spheres after being initially cultured as a single cell. After 10 days, 49±5.4 and 51±4.9% of wells with a single cell derived from the control group and the Ad5-Control-shRNA group formed a novel set of spheres, while only 14±2.7% of wells with a single cell derived from the Ad5-siAKT1-siPI3K-shRNA group was able to form spheres. These results indicate that the downregulation of AKT1 and PI3K/p85 leads to the loss of self-renewal in LCSCs (Fig. 6).
AKT1 and PI3K/p85 knockdown decreases tumorigenic potential in LCSCs in NOD/SCID mice. The control, Ad5-Control-shRNA and Ad5-siAKT1-siPI3K-shRNA cells were injected subcutaneously into NOD/SCID mice in a limiting dilution experiment (i.e., $10^2$, $10^3$, $10^4$ and $10^5$ cells). The results showed that rAd5-siAKT1-siPI3K cells were associated with a decreased level of tumorigenicity relative to that of the control or Ad5-Control-shRNA groups. In order to
Table I. Incidence of tumors of control group, Ad5-Control-shRNA group and Ad5-siAKT1-siPI3K-shRNA group cells serially transplanted into NOD/SCID mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^2$</td>
</tr>
<tr>
<td>Control</td>
<td>2/5</td>
</tr>
<tr>
<td>Ad5-Control-shRNA</td>
<td>2/5</td>
</tr>
<tr>
<td>Ad5-siAKT1-siPI3K-shRNA</td>
<td>0/5</td>
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shRNA, short hairpin RNA; si, small interfering.

Discussion

Current studies suggest that drug resistance mechanisms in tumor cells have a close association with CSCs, which may lead to resistance to radiation and chemotherapy through the following mechanisms: i) The majority of CSCs (such as leukemia stem cells and liver stem cells) are in the G0 phase. Due to their slow growth and primarily dormant state, CSCs are resistant to cell cycle-specific agents (20-22). ii) Various types of CSCs overexpress membrane ABC transporter and drug resistance genes, including P-glycoprotein, encoded by the ABCB1 gene, multidrug resistance-related protein 1 and breast drug resistance protein 2, encoded by the ABCG2/MXR gene. These proteins can pump chemotherapeutic drugs out of the cell, decreasing the intracellular concentration and weakening their cytotoxic effects on cancer cells; thus, resulting in drug resistance (23-25). iii) CSCs express DNA repair protein and thymidylate synthase excision repair cross-complementation group 1 at a high level, increasing the capacity of cancer cells for repair following antitumor treatment, and thereby increasing resistance to anticancer drugs (26). iv) In comparison to normal stem cells, signaling pathways associated with self-renewal and proliferation in CSCs are often over-activated, becoming sources for tumor recurrence and metastasis (27,28).

Although there have been few studies of the PI3K/AKT signaling pathway in stem cells, a previous study has confirmed that this pathway is important in the proliferation of breast cancer stem cells (18). Gong et al (29) found that the proliferation of osteosarcoma cancer stem cells was significantly decreased by the PI3K inhibitor LY294002 in a dose- and time-dependent manner, suggesting that the PI3K/AKT signaling pathway is an important mediator of osteosarcoma cancer stem cell proliferation. Targeted inhibition of this pathway can effectively inhibit the proliferation of cancer stem cells (29). Sunayama et al (30) used rapamycin in combination with LY294002 to treat glioma stem cells and showed that proliferation and the self-renewal capacity of these cells were diminished, as was tumorigenicity in vivo. Yang et al (19) successfully extracted bronchioloalveolar stem cells (BASCs) from a mouse model of K-ras-driven cancer. Following treatment with a PI3K/AKT pathway inhibitor, migration and proliferation of BASCs were reduced and the PTEN gene was inactivated.
Currently, to the best of our knowledge, there are no reports demonstrating the involvement of the PI3K/AKT signaling pathway in the regulation of self-renewal, proliferation, and differentiation of human LCSCs. In the present study, shRNA technology was used to downregulate the expression of AKT1 and the PI3K/p85 subunit in LCSCs and changes were detected in cell proliferation in three groups. LCSC proliferation slowed and the cell doubling time increased when AKT1 and PI3K/p85 were silenced, suggesting that the PI3K/AKT signaling pathway promoted LCSC proliferation. In addition, the self-renewal capacity of LCSCs was analyzed through LCSC sphere formation. The results showed that the self-renewal capacity of LCSCs in the rAd5-siAKT1-siPI3K-shRNA group was lower than in the control group or Ad5-Control-shRNA group, indicating an important role for the PI3K/AKT signaling pathway in LCSC self-renewal.

To further investigate the mechanism by which the PI3K/AKT pathway controls LCSC proliferation, gene expression of PCNA, cyclin D1 and p53 was examined in three groups of LCSCs. PCNA is a nuclear protein closely associated with cell proliferation, while p53 was the first identified tumor suppressor gene. Diminished or absent expression of p53 is an important mechanism for promoting tumorigenesis. PCNA and p53 are closely related to each other. Cyclin D1 is highly expressed in a number of different cancer cells, promoting the G1/S transition and stimulating tumor growth (32). In the present study, downregulation of AKT1 and PI3K/p85 expression was accompanied by decreased expression of cyclin D1 and PCNA, and increased expression of p53. These results show that silencing of AKT1 and PI3K/p85 can result in cell cycle arrest at the G1/S phase, while S phase cells are significantly decreased, resulting in inhibition of cell proliferation.

The present study also showed that LCSC tumorigenicity in the rAd5-siAKT1-siPI3K-shRNA group was significantly reduced, compared with the control groups or Ad5-Control-shRNA group, but was still present in the treated cells, implying that LCSCs retained part of their self-renewal capacity when AKT1 and PI3K/p85 had been silenced. It was speculated that silencing of AKT1 and PI3K/p85 blocks LCSCs from entering the cell cycle, inhibits the self-renewal rate of LCSCs and reduces the number of stem cells with tumorigenic properties by causing asymmetric division.

In conclusion, AKT1 and PI3K were overexpressed in LCSCs. Silencing of AKT1 and PI3K/p85 gene transcription reduced cell proliferation, tumor sphere formation and tumour development in NOD/SCID mice. This study provides a potential novel therapeutic strategy for the treatment of drug resistance and recurrence of lung cancer.

References