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The Potential Role of Adenylate kinase 2 As an Oncogene in Small Cell Lung Cancer

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의학과

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소세포폐암의 암유전자로서
Adenylate kinase 2의 역할 연구

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ABSTRACT

소세포폐암의 암유전자로서 Adenylate kinase 2의 역할 연구

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서론: 소세포폐암은 예후가 좋지 않으며, 현재까지 개발된 표적치료제가 없다. Adenylate kinase(AK) 2는 미토콘드리아 사이막 공간에서 발현되는 특별한 효소로서 AK계의 아이소엔자임 중 하나이다. AK2는 미토콘드리아 외의 중요한 기능을 가지고 있는데 이는 암을 생성하는데 있다. 이에 우리는 인간 소세포폐암 세포주를 가지고 AK2 유전자 녹다운이 종양세포의 증식에 미치는 영향에 대해 연구하였다.

방법: 4개의 인간 소세포폐암 세포주 (HCC-33, NCI-H889, NCI-H416, NCI-H417)를 사용하여 RNA-seq expression 검사와 western blot 분석을 시행하였다. 또한, HCC-33과 NCI-H417세포주를 가지고 세포 증식과 Soft-agar 콜로니 형성 분석을 시행하였다. NCI-H417 세포주로 누드마우스를 이용해 생체 내 종양 증식 분석을 진행하였다.

결과: western blot 분석에서 HCC-33 과 NCI-H417 소세포폐암 세포주에서 발현이 증가됨을 화인 하였다. HCC-33 과 NCI-H417 소세포폐암 세포주를 이용한 soft-agar 콜로니 형성 분석을 통해 AK2 녹다운이 세포의 증식과 콜로니 형성을 억제하였다. NCI-H417 세포주를 이용한 AK2 선택적 녹다운이된 종양세포를 접종한 누드마우스군과 종양세포를 접종한 누드마우스군의 종양의 부피를 비교한 결과 AK2 녹다운 군이 대조군에 비교하여 감소함을 확인하였다.

결론: 이번 실험을 통해 AK2가 소세포폐암의 진행에 중요한 역할을 하고 있으며, 향후 표적치료제로서 후보 암표지자가 될 수 있는 가능성을 확인 하였다. AK2의 발현이 실제 폐암환자에게 끼치는 영향과 종양형성을 유발하는 기전에 대한 연구가 필요하겠다.

I. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, accounting for 1.79 million deaths yearly (1). Small-cell lung cancer (SCLC) constitutes approximately 15% of all lung cancer cases. SCLC is predominantly associated with smoking and has a poor clinical prognosis owing to rapid tumor growth and tendency to metastasis (2). Owing to targeted therapy and immunotherapy, therapeutic advances have been made in the treatment of non-small-cell lung cancer (NSCLC) over the past decade. However, there is currently no proven molecularly targeted therapy for SCLC.

Adenylate kinase (AK) is a small, ubiquitous enzyme that controls the cellular energy metabolism and homeostasis. AK, a phosphotransferase, catalyzes the reversible interconversion of ATP + AMP ↔ 2ADP (3-5). There are nine isoenzymes of AK (AK1-AK9) in different intracellular compartments. Several studies have demonstrated that AK1, AK2, AK4, and AK6 play an essential roles in the initiation of cancer cell transformation, metabolic signaling, and metastatic potential (6-10). AK2 is a mitochondrial isoenzyme located in the intermembrane space that plays an important role in controlling cell apoptosis and proliferation of hematopoietic precursors (11, 12). Overexpression of AK2 in lung adenocarcinoma is associated with tumor progression and poor prognosis, and knock-down of AK2 suppresses the proliferation, migration, and invasion, while inducing the apoptosis and autophagy of cells (8). However, the role of AK2 in SCLC remains unclear.

In this study, we investigated the impact of AK2 knock-down on the proliferation of human SCLC cells.

II. Material and Methods

RNA-sequencing (RNA-seq) and alignment

To evaluate the transcriptome features in lung cancer at the nucleotide-level, we performed RNA-seq analysis of poly(A+) RNAs isolated from normal lung cells and lung cancer cells. The short reads were aligned to the 10mm reference genome using TopHat, with up to two mismatches allowed. The unmapped reads were first trimmed to remove poly-A/T tails (repeats of [A/N] s or [T/N] s) from read ends/starts and then aligned to the reference genome. It is worth noting that we retained only the reads with at least 30 bp at both ends after trimming. Finally, 89.3% of short reads from normal lung cells and 89.8% of sequence reads from lung cancer cells were mapped to the reference genome using TopHat for APA analysis in the study.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay/proliferation assay

Cell viability was determined using the MTT assay. MTT assay was performed according to a the standard protocol. Following treatment, the cells were incubated with 10 μL MTT (1 mg/mL) in phosphate-buffered saline (PBS) in a 96-well plates for four hour at 37℃. Subsequently, the medium containing MTT was removed, and 100 μL dimethyl sulfoxide (DMSO) was added. The cells were incubated for an additional 10 min at 37℃ with gentle shaking. The absorbance was read on an ELISA plate reader (Tecan, Trading AG, Switzerland) with a 570-nm filter. The cell viability was calculated based on the relative color intensities of the treated and untreated samples.

Soft agar colony formation assay

Soft agar assays were performed on a 6-well plates. The base layer of each well consisted of 2 mL with final concentrations of 1×medium and 0.6% low-melting-point agarose (Duchefa, Haarlem, The Netherlands). The plates were chilled at 4℃ until they were solid. Next, a 1 mL growth agar layer was poured, consisting of 5×10^4 cells suspended in 1× medium and 0.3% low-melting-point agarose, and the plates were

again chilled at 4℃ until the growth layer congealed. An additional 1 mL of 1× medium without agarose was added on top of the growth layer. Cells cultures were maintained at 37℃ for 14 days and the media was replaced every three days. The total colonies were stained with 0.005% crystal violet (Sigma, St Louis, MO, USA) and counted in five random fields. Images were analyzed using the Image-Pro Plus 4.5 software (Media Cybernetics).

Immunoblotting

Cells were washed with 1× PBS and lysed in a lysis buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM Na3VO4, and 5 mM NaF). Protein concentrations were determined using a dye-binding microassay (Bio-Rad, Hercules, CA, USA). Equal concentrations of cell or tissue extracts were resolved using 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by the electrophoretic transfer of the protein bands onto a polyvinylidene difluoride membrane (PALL Life Sciences, NY, USA). The membranes were blocked for one hour with Tris-buffered saline-Tween (TBS-T; 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat milk and incubated with specific primary antibodies overnight at 4℃. The blots were washed with TBS-T four times for 15 min per wash and incubated for 1 h with the corresponding peroxidase-conjugated secondary antibodies (1:4000 dilution; Jackson Immuno Research Inc, West Grove, PA, USA). The blots were washed four times with TBS-T and developed using enhanced chemiluminescence detection system (iNtRON Biotechnology). The antibodies used for western blotting were rabbit anti-AK2 (ab166901; Abcam, Cambridge, UK) and mouse anti-β-actin (ab6276; Abcam, Cambridge, UK) antibodies.

Tumor formation in nude mice

Six-week-old male BALB/c nude mice (Orient Bio Inc. Seongnam, Korea) used in this study were housed in our pathogen-free facility, handled in accordance with the standard use protocols and animal welfare regulations, and subsequently used in this

study. siRNA-control-H417 cells (1×10^6 cells) and siRNA-AK2-H417 Cells (1×10^6 cells) were resuspended in serum-free Roswell Park Memorial Institute medium and injected subcutaneously into the left flanks of the BALB/c nude mice. Tumor volume was measured every other day using digital calipers and calculated according to the following formula: [length × (width) 2]/2. After three weeks of therapy, all mice were euthanized according to the institutional guidelines, and local tumors were resected and analyzed.

Statistical analysis

The results are expressed as the means ± standard deviation, unless otherwise noted. Student's t-test or analysis of variance was used to compare the tumor volumes between the two groups. Statistical significance was set at $P < 0.05$. All experiments were repeated at least three times, and the results of the representative experiments are shown.

III. Results

Overexpression of AK2 in human lung cancer

We studied and compared the expression levels of the AK2 using RNA-seq analysis of poly(A+) RNAs isolated from normal lung cells and lung cancer cells. A statistically significant increase in AK2 expression levels was observed in human lung cancer cells ($p < 0.001$) (Figure 1).

We studied the expression levels of AK2 in four SCLC cell lines (HCC-33, NCI-H889, NCI-H416, and NCI-H417). AK2 levels were measured via western blotting analysis and quantified using the ImageJ software. The expression levels of AK2 were increased in human SCLC HCC-33 and NCI-H417 cells (Figure 2).

Cell viability and proliferation

We observed the effects of AK2 on the proliferation and viability of human SCLC HCC-33 and NCI-H417 cells. The MTT assay was designed and studied. AK2 knock-down suppressed the proliferation of human SCLC HCC-33 and NCI-H417 cells (Figure 3).

Soft agar colony formation assay

The control vector and AK2 knock-down in human SCLC HCC-33 and NCI-H417 cells were assessed for colony formation in soft agar for 14 days. We confirmed that knock-down of AK2 suppressed the colony formation of human SCLC HCC-33 and NCI-H417 cells compared to the control ($p < 0.001$) (Figure 4).

Effect of the knock-down of AK2 on tumor formation in a nude mice H417 xenograft model

To observe the effects of AK2 on tumor formation, we injected control and AK2 knock-down H417 cells subcutaneously into the left flank of nude mice. Tumor volume

was measured using digital calipers every four days over for 40 days. In the AK2 knock-down H417 cells group, tumor volume was suppressed compared to that in the control group (89.4 mm^2 vs 831 mm^2; $p < 0.001$) (Figure 5).

IV. Discussion

Lung cancer is the most common cause of cancer-related deaths worldwide (13). In South Korea, the leading cancer site causing mortality in both men and women is the lungs (14, 15). In 2020, the mortality rate per 100,000 people in patients with lung cancer was 54.0 in men and 18.8 in women. Lung cancer is divided into NSCLC and SCLC according to the histological type. NSCLC, which accounts for 85% of all cases, includes squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma (16). The major cause of lung cancer is cigarette smoking, with the risk increasing with the age at initiation, intensity, and duration of smoking (17-20). In a meta-analysis of 34 studies, cigarette smoking increased the risk of lung cancer (risk ratio, 10.92; 95% CI, 8.28–14.40) (21). More than 90% of SCLC patients are current or past smokers. The incidence of SCLC in never-smokers is only 2% (22). Cigarette smoking has carcinogenic effects and results in extremely high mutation rates in the genomic profiles of SCLC (23). The incidence of lung cancer among never-smokers in South Korea is 36.4%, which is higher than observed in Western countries (10–15%) (24).

Lung cancer screening using low-dose computed tomography to detect early stage lung cancer improved lung cancer mortality in two large randomized controlled trials (the National Lung Screening Trial [NLST] and the Dutch-Belgian Randomized Lung Cancer Screening [NELSON] trial) (25, 26). NLST reported that lung cancer mortality in the low-dose computed tomography (LDCT) group was 20% lower than that in the chest radiography group ($p = 0.004$) (25). The NELSON trial showed that the cumulative rate ratio for lung cancer-related death was 0.76 ($p = 0.01$) compared with the control group (26). The *U.S. Preventive Sevices Task Force* (USPSTF) recommended annual lung cancer screening with LDCT for asymptomatic individuals aged 55–80 years with a 30-pack-year history of smoking or quitting within the past 15 years (27). Based on these trials, a national lung cancer screening program using LDCT in South Korea enrolled the high-risk group aged 55–74 years and current smokers with a smoking history of at least 30 pack-years (28).

Chest CT is the standard diagnostic modality for patients with suspected lung cancers. Tissue biopsy should be performed for lung tumors, lymph nodes, or metastatic sites by bronchoscopy for endobronchial tumors, endobronchial ultrasound transbronchial needle biopsy for suspected metastatic lymphadenopathy and central lung mass, percutaneous needle biopsy for peripheral lung tumors, and video-associated thoracoscopic surgery.

Lung cancer staging is performed through fluorodeoxyglucose-PET- CT and brain MRI to determine future treatment directions. The tumor-node-metastasis (TNM) classification should be used for staging of NSCLC. The eighth edition of lung cancer stage classification may predict survival and guide therapy more accurately than the former TNM system (29). SCLC are classified into limited and extensive stages. The limited stage of chemoradiation is confined to the ipsilateral hemithorax and regional lymph nodes, which can be included in a single tolerable radiation port (TNM stages I─III). The extensive stage of chemotherapy alone was confined to the tumors beyond the boundaries of a radiation port and included contralateral supraclavicular or hilar involvement, malignant pericardial or pleural effusions, and distant metastasis (TNM stage IV). (30-32). The 5-year relative survival rate for lung cancer from 2012 to 2018 in the U.S was 22.9%, which was higher for NSCLC (29.8% for adenocarcinoma, 22.5% for squamous cell carcinoma, and 18.6% for large-cell carcinoma) than for SCLC (7.2%) (33).

New concepts in cancer biology include clonal and subclonal evolution, driver mutations, epithelial-to-mesenchymal transition, field-to-cancerization, glutamine addiction, lung cancer stem cells, mutation signatures, neoantigens, spatial and temporal tumor heterogeneity, and immune evasion. Next-generation sequencing (NGS) of targetable driver genes is the standard of care for managing and treating of locally advanced or metastatic NSCLC. Common and targetable driver mutations and fusions in NSCLC include KRAS proto-oncogene (KRAS), epidermal growth factor receptor (EGFR), B-Raf proto-oncogene (BRAF), erb-b2 receptor tyrosine kinase 2 (ERBB)2, anaplastic lymphoma kinase (ALK), and ROS proto-oncogene 1 (ROS1) fusion, rearranged during transfection (RET) fusion (34, 35). Accoding to the National Comprehensive Cancer

Network (NCCN) guidelines for advanced or metastatic NSCLC, molecular and biomarker testing performed by NGS includes EGFR exon 19 deletion or L858R mutation, EGFR S768I, L861Q, and/or G719X mutation; EGFR exon 20 insertion mutation; ALK rearrangement; KRAS G12C mutation; ROS1 rearrangement; neurotrophic tyrosine receptor kinase (NTRK) 1/2/3 gene fusion; mesenchymal-epithelial transition exon 14 (METex14) skipping mutation, BRAF V600E mutation, RET rearrangement, and programmed death-ligand 1 (PD-L1) testing (36).

EGFR is a receptor-activated tyrosine kinase. EGFR mutations are the most common driver mutations in lung adenocarcinoma. The incidence of EGFR mutations in women than in men in never-smokers compared with ever-smokers. Midha et al. reported that the highest EGFR mutation frequency in the Asia-Pacific subgroup of lung adenocarcinoma was 47% and the lowest EGFR mutation frequency in the Oceania subgroup of lung adenocarcinoma was 12% (37). Common EGFR mutations included EGFR L858 mutation, insertion, and exon 19 deletion. EGFR tyrosine kinase inhibitors (TKI) are first-, second-, and third-generation TKIs. The first-generation EGFR-TKIs include gefitinib and erlotinib. The second-generation therapy was afatinib and dacomitinib. First- and second-generation EGFR-TKIs provide significant improvements in progression-free survival and overall survival compared with standard doublet chemotherapy in patients with metastatic lung adenocarcinoma with EGFR mutations (38-43). The third-generation EGFR-TKI osimertinib, which inhibits both mutant EGFR and T790M resistance mutations, showed superior efficacy compared with standard chemotherapy in patients with T790M mutations who progress after first-line therapy and has CNS efficacy compared with erlotinib or gefitinib in patients with untreated EGFR-mutated advanced NSCLC (44-50).

ALK mutations are the second most common driver mutations (51). The frequency of ALK rearrangements in patients with NSCLC is 3-7% (52). Patients with "ALK-positive" NSCLC are more commonly in young, never smokers, and have lung adenocarcinoma (53). The ALK inhibitor crizotinib showed superior efficacy in progression-free survival compared with first-line standard chemotherapy in patients with

ALK-positive NSCLC (54-56). However, crizotinib shows decreased penetration into brain and develops acquired resistance (57-59). Second-generation AKI inhibitors are ceritinib and alectinib (58, 60-62). Alectinib demonstrated improvement in progression-free survival and CNS progression compared to crizotinib in patients with advanced ALK-positive NSCLC. The third-generation ALK inhibitor was lorlatinib. Lorlatinib has longer progression-free survival than crizotinib as first-line chemotherapy in patients with advanced ALK-positive NSCLC (63, 64).

Inactivation of TP53 and RB1, which regulates cell cycle progression, is frequently observed in SCLC cells (23). Notch signaling in SCLC is both tumor-suppressive and pro-tumorigenic (65). The subtypes of SCLC (SCLC-A, SCLC-N, SCLC-P, and SCLC-Y) are defined by four transcription factors (achaete-scute homologue 1, neurogenic differentiation factor 1, yes-associated protein 1, and POU class 2 homeobox 3) have been previously described (66).

SCLC does not have any targeted therapy for treatment compared with the molecularly selected targeted therapies in available for NSCLC (e.g., EGFR, ALK, RET, NTRK, ROS1, BRAF). SCLC has intra-tumoral and inter-tumoral heterogeneity, which results in the emergence of treatment resistance (2, 67, 68). Emerging therapeutic targets in SCLC include antitumor immunity, cell cycle, DNA damage repair pathways, growth and survival signaling pathways, and epigenetic regulators. The addition of immune checkpoint inhibitors (durvalumab or atezolizumab) to standard chemotherapy (platinum and etoposide) as first-line therapy in patients with extensive-stage SCLC significantly improves both overall survival and progression-free survival (69, 70). However, pembrolizumab plus standard chemotherapy did not significantly prolong overall survival (71). The response to immune checkpoint inhibitors, which enhances the activity of T cells against cancer cells in patients with SCLC, is limited to 15% (2, 72, 73). BCL-2, a key regulator of apoptosis, is overexpressed in SCLC cells (74). Navitox (ABT-263) is a potent and selective inhibitor of BCL-2. A phase I/II study of vistusertib (AZD2014), which is a mechanistic target of rapamycin inhibitor combined with navitox for treatment in patients with relapsed SCLC, is currently ongoing (NCT03366103).

Enhacer of zeste homolog 2 (EZH2) promotes chemoresistance by epigenetically silencing schlafen family member 11. Inhibition of EZH2 may prevent the chemoresistance of tumor cells to chemotherapeutic drugs in SCLC (75). The EZH2 inhibitor (DS-3201b) and irinotecan for patients with recurrent SCLC are currently being tested in phase I/II clinical trials (NCT03879798). In our study, AK2 knock-down in SCLC cells inhibited the tumor growth *in vivo*. Further studies are required to determine the migration and invasion of SCLC cells *in vitro* and for the survival analysis of AK2, and signaling pathways.

AK is the essential, ubiquitous enzyme in cell energy metabolism and homeostasis. AK in the cellular phosphotransfer system catalyzes the reversible interconversion of ATP + AMP ↔ 2ADP (4, 76-78). There are nine isoenzymes of adenylate kinase (AK1-AK9) in different intracellular compartments. AK1, AK5, AK7, and AK8 are detected in cytosol, AK6 is in the nucleus, and AK9 is found in a free diffusion between the cytosol and the nucleus. AK2, AK3, and AK4 are in the mitochondrial matrix (3, 11). In several studies, AK1, AK2, AK4, and AK6 were associated with cancer formation and metastasis in the controls of cancer metabolism, metabolic signaling, and cell migration and invasion (6, 7, 9, 79-87).

AK2 abundantly present in the mitochondrial intermembrane space of the liver, heart, skeletal muscle, kidneys, and lungs, and plays an essential role in mitochondrial energy metabolism, which is directly linked to inflammation, neurodegenerative diseases, and cancer (88, 89). Kim et. al. reported that downregulation of AK2 levels enhances cell proliferation and induces tumorigenesis in breast cancer cells showing high levels of phospho-fas-associated protein with death domain Ser194 (85). However, in our study, we found that human lung cancer cells overexpressed AK2.

Our data demonstrate that AK2 knock-down in SCLC cells decreased the cell proliferation and colony growth in soft agar, and selective silencing of the AK2 group reduced the tumor volume compared with the control group at 40 days after tumor cell injection. Recently, several studies have demonstrated the role of AK2 in lung adenocarcinoma. Liu et. el. showed that overexpression of AK2 in lung adenocarcinoma

is associated with advanced tumor stage and poor prognosis, and knock-down of AK2 in lung adenocarcinoma cells inhibits the tumor growth, migration, and invasion, while inducing cell apoptosis and autophagy (8). In a separate study, Cai et al. reported that AK2 knock-down suppresses cancer cell proliferation and induces cancer cell apoptosis in lung adenocarcinoma. In addition, AK2 regulates tumor cell metastasis by regulating the epithelial-mesenchymal transition by activating TGF-β/Smad pathway *in vitro* and *in vivo* (79). These studies indicate that AK2 plays an essential role in lung adenocarcinoma progression and metastasis and is a promising agent for the molecularly targeted therapy of lung adenocarcinoma.

V. Conclusion

Lung cancer is the leading cause of cancer-related deaths worldwide. Numerous studies have led to advances in lung cancer treatment. Lung cancer shows tumor heterogeneity. NGS testing identified driver mutations in NSCLC. Several studies have been conducted on driver mutation-targeted therapies. NCCN guidelines of NSCLC recommended NGS testing for EGFR mutations. ALK rearrangements, KRAS mutations, ROS1 rearrangements, NTRK, METex14 skipping mutations, BRAF V600E mutations, RET rearrangements, and PD-L1. Targeted therapies for driver mutation-driven NSCLC have demonstrated superior efficacy in terms of progression-free survival and overall survival compared with standard chemotherapy. However, SCLC has a poor clinical prognosis and no currently proven molecularly targeted therapy.

AK2 is an isoenzyme of the AK family and a special enzyme expressed in the mitochondrial intermembrane space. AK2 may have significant extra-mitochondrial functions, particularly in tumorigenesis. We investigated the impact of AK2 knock-down on the proliferation of human SCLC cells.

Western blotting analysis was performed using four human SCLC cell lines (HCC-33, NCI-H889, NCI-H416, and NCI-H417). Cell proliferation, soft-agar colony formation assays, and *in vivo* tumor growth assays were conducted using NCI-H417 cells.

Western blotting analysis revealed higher AK2 protein levels in HCC-33 and NCI-H417 cells. AK2 protein expression in HCC-33 and NCI-H417 cells transduced with control siRNA and two different AK2 siRNA designs (siRNA1 and siRNA2). AK2 silencing decreased NCI-H417 cell proliferation and colony growth in soft agar. Selective silencing of AK2 reduced tumor volume compared with NCI-H417 cells transfected with control-siRNA, as measured 40 days after tumor cell injection.

We have demonstrated that AK2 plays an essential role in SCLC progression *in vivo* via the knock-down of AK2 and can be a potential target for molecularly targeted therapy. However, further studies on the detailed mechanisms and prognostic significance of SCLC are essential.

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Figure 1. RNA-sequencing expression analysis

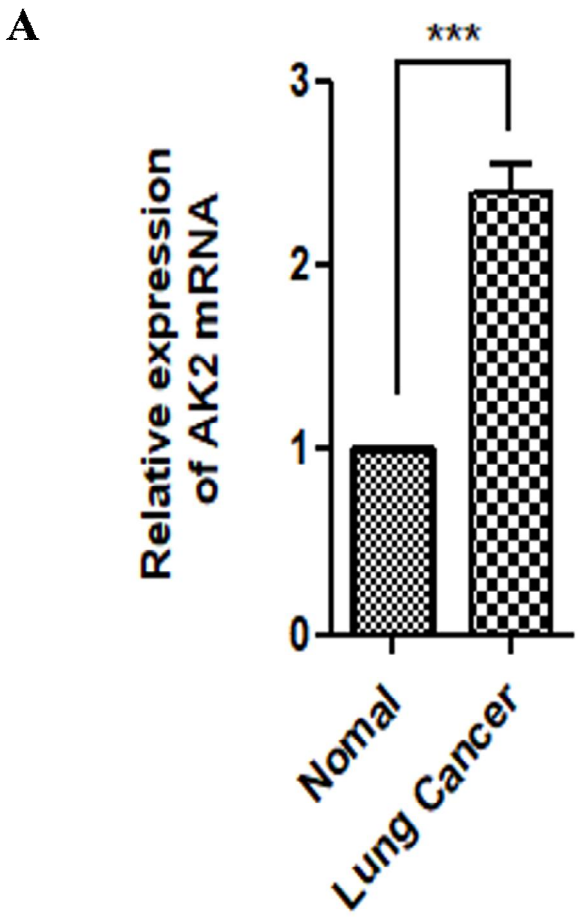

Figure 1. RNA-sequencing expression analysis.
Overexpression of adenylate kinase (AK2) in the human lung cancer cells. Relative expression levels of AK2 in normal lung and lung cancer cells are shown in panel A.

Figure 2. Expression levels of adenylate kinase 2 (AK2) in four small cell lung cancer cell lines.

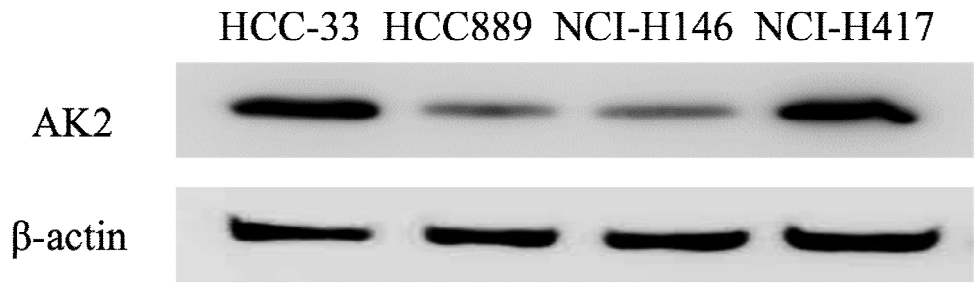

Figure 2. Expression levels of adenylate kinase 2 (AK2) in four small cell lung cancer cell lines.

AK2 protein levels were measured via western blotting analysis and quantified using the ImageJ software.

Figure 3. Knock-down of AK2 suppresses the cell proliferation of human small cell lung cancer HCC-33 and H417 cells.

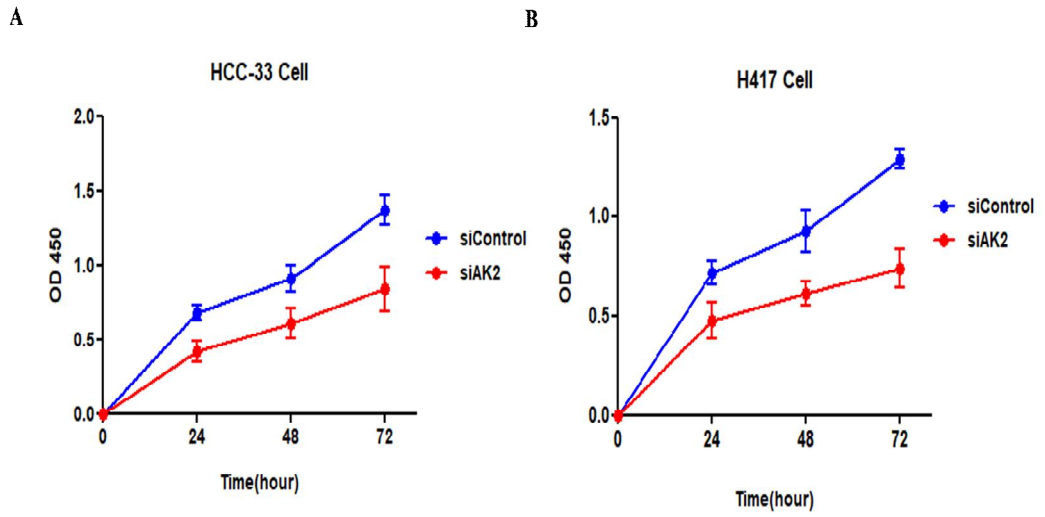

Figure 3. Knock-down of AK2 suppresses the cell proliferation of human small cell lung cancer HCC-33 and H417 cells. Cell proliferation of HCC-33 and H417 cells with si-control and si-AK2 was observed using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Figure 4. Knock-down of AK2 suppresses the soft agar colony formation of human small lung cancer HCC-33 and H417 cells.

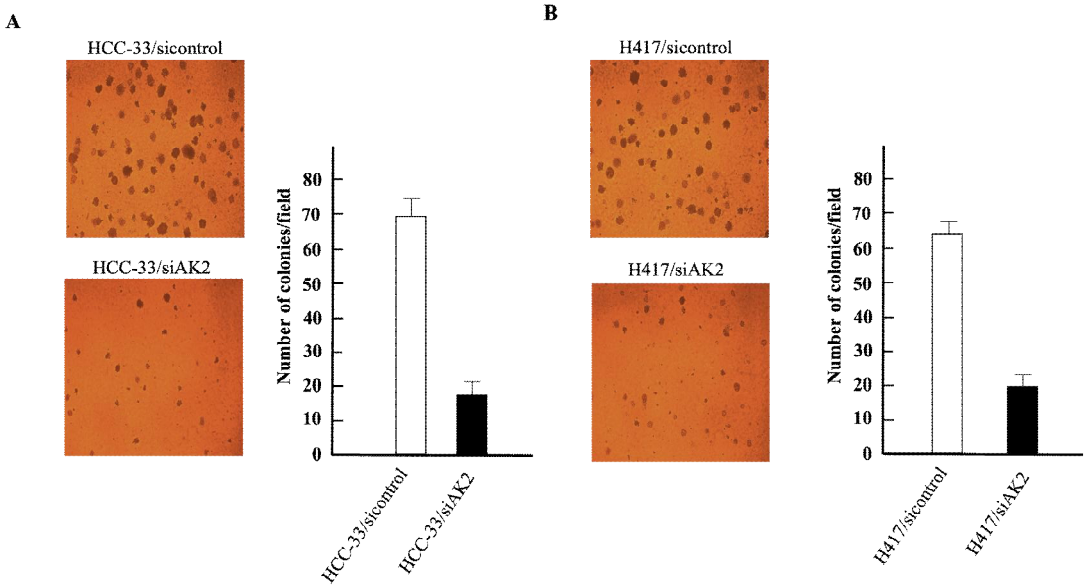

Figure 4. Knock-down of AK2 suppresses the soft agar colony formation of human small lung cancer HCC-33 and H417 cells.

(A) siControl and siAK2 HCC-33 cells were assessed for colony formation in soft agar for 14 days. (B) siControl and siAK2 H417 cells were assessed for colony formation in soft agar for 14 days. Data represent the mean cell numbers from five fields, $p < 0.001$, compared to the control.

Figure 5. Effects of AK2 knock-down on the tumor growth cells of H417 cells in a nude mice xenograft model.

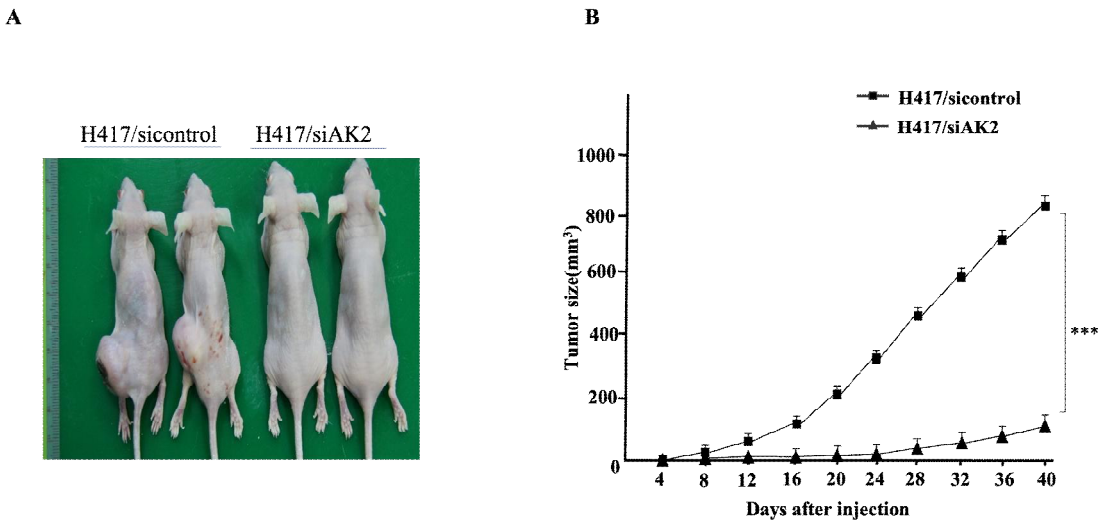

Figure 5. Effects of AK2 knock-down on the tumor growth cells of H417 cells in a nude mice xenograft model.

(A) sicontrol and siAK2 H417 cells were injected subcutaneously into the left flank of nude mice, respectively (n= 2).

(B) Tumor volumes were measured every four days for 40 days.