ORIGINAL RESEARCH PAPER

CircAKT3 inhibits glycolysis balance in lung cancer cells by regulating miR-516b-5p/STAT3 to inhibit cisplatin sensitivity

Yangling Xu · Tiantian Jiang · Changgang Wu · Yongqing Zhang

Abstract

Objective Lung cancer was one of the most deadly cancers around the world. Circular RNA AKT3 (CircAKT3) was highly expressed in lung cancer and could inhibit cell proliferation, but there were few studies on the mechanism of specific regulation of drug resistance. Therefore, we aimed to provide new ideas and perspectives for the role of circAKT3 in the mechanism of tumor resistance.

Methods The levels of circAKT3, miR-516b-5p and STAT3 in lung cancer tissues and cells were examined using quantitative real-time polymerase chain reaction (qRT-PCR) or western blot assays. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to examine the sensitivity of cells treated under different conditions to cisplatin (DDP). A glucose assay kit and lactate assay kit were used to assess glycolysis and lactate production of cells treated with different plasmids and 2-deoxy-glucose (2-DG). Western blot analysis was used to detect the expression level of the hypoxia-inducible factor (HIF-1α) in A549 and H1299 cells. Starbase 3.0 predicted a targeted relationship between circAKT3 and miR-516b-5p, STAT3 and miR-516b-5p, and the relationship was proved by a dual-luciferase reporter assay. Knockdown of circAKT3 was used to study the effects of circAKT3 on tumor development in vivo.

Results The levels of circAKT3 and STAT3 were upregulated, miR-516b-5p was downregulation in lung cancer tissues and cells. Functionally, circAKT3 knockdown improved cell sensitivity to DDP, and repressed glycolysis in lung cancer cells. Meanwhile, inhibition of HIF-1α-dependent glycolysis attenuated the circAKT3-induced increase of chemo-resistance in A549 cells. Mechanistically, miR-516b-5p was found to possess some binding sites with circAKT3. Noticeably, the inhibitory action of circAKT3 knockdown on DDP resistance and glycolysis was overturned through inhibitor of miR-516b-5p in lung cancer cells. Furthermore, besides, circAKT3 knockdown suppressed lung tumor cell growth by the miR-516b-5p/STAT3 axis in vivo.

Conclusions CircAKT3 inhibit cisplatin sensitivity of lung cancer cells at least partly through regulating miR-516b-5p/STAT3 axis-mediated glycolysis balance, providing a possible long noncoding RNA – targeted therapy for lung cancer.

Keywords circAKT3 · miR-516b-5p · STAT3 · Lung cancer · Glycolysis · Drug sensitivity · Cisplatin

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Abbreviations

CircAKT3	Circular RNA AKT3
qRT-PCR	Quantitative real-time polymerase chain reaction

| 2-DG | 2-Deoxy-glucose |
| HIF-1α | Hypoxia-inducible factor |

Introduction

Lung cancer is the most common and deadly malignancy in most countries, with the highest clinical mortality (Ho et al. 2018; Osuoha et al. 2018; Hasan et al. 2014). Advanced technologies such as high-throughput technologies had shown great potential and role in the diagnosis of lung cancer (Walter et al. 2018; Sørli et al. 2018). Early detection of lung cancer could be achieved by analyzing respiratory tissue samples or by using biomarkers in peripheral biological fluids, such as blood and urine. However, there was no research on the detailed mechanism of lung cancer, which severely restricted the clinical treatment of lung cancer and effective prevention of the disease. Hence, it is an urgent need to figure out novel biomarker and molecular mechanisms for elevating lung cancer treatment.

Circular RNA (circRNA) had a stable cyclic structure (Hu et al. 2019). Recent evidence showed the functional diversity of circRNAs, which could be sponge microRNA, interacted with protein or translated into small peptide (Klec et al. 2019). CircRNA was involved in the progression of different cancers. Song et al. proved that circRNA hsa-circRNA_101996 was involved in the regulation of increased cervical cancer, and promoted the proliferation of its cells (Song et al. 2019). Yu's research confirmed that circRNA-104718, a potential carcinogenic factor, boosted the development of liver cancer by regulating molecules of corresponding signaling pathways (Yu et al. 2019). Bi et al. (2018) reported that circRNA circRNA_102171 was a potential therapeutic target that mediated breast cancer progression by activating signaling pathways. Studies had reported that the circAKT3 could enhance the cisplatin sensitivity of gastric cancer, which might have great clinical significance (Huang et al. 2019). However, studies on the principle of circular RNA AKT3 in lung cancer and the drug sensitivity of lung cells had not been reported.

MicroRNAs (miRNAs) could negatively modulate target genes expression through binding to the 3′ untranslated regions (3′UTR) of mRNA containing complementary sequences (Zhao et al. 2018). Emerging evidence suggested that miRNAs were involved in numerous cytological processes, such as growth and apoptosis in breast cancer (Akbulut et al. 2019; Swellam et al. 2018). Previous documents presented that miR-516b-5p played an anti-tumor role in lung cancer (Zhu et al. 2017). Related studies had shown that miR-516b-5p could be used as one of the marker molecules for early pregnancy patients (Hromadnikova et al. 2017). However, studies on the development and drug sensitivity of miR-516b-5p in lung cancer were lacking.

Genes were carriers of genetic information, and proteins that are expressed were also performers of functions. STAT3 was found to be involved in the development of ischemic cardiac hypertrophy, and tumor-associated macrophage differentiation (Yuan et al. 2018; Kumar et al. 2016). Yu et al. reported that STAT3 was related to the drug resistance of non-cellular lung cancer (Dong et al. 2019). There was little research on the mechanism of STAT3 in the development of lung cancer and its drug sensitivity to lung cancer cells.

In the exploration, we evaluated the expression of circAKT3, miR-516b-5p and STAT3 in lung cancer tissues and cells. Besides, we also studied their effects on cell resistance and explored the regulatory network of circAKT3/miR-516b-5p/STAT3. Therefore, these findings might offer different insights on the exploring of lung cancer.

Materials and methods

Tissue collection

Twenty-eight paired lung cancer tissues and normal adjacent tissue were provided from the patients who had undergone surgery at Liaocheng Infectious Disease Hospital. These tissues were collected, promptly frozen in liquid nitrogen and then stored in a refrigerator at − 80 °C until the experiments were performed. All participants had signed the informed consents before collecting the tissues. All protocols in our research were endorsed by the Research Ethics Committee of Liaocheng Infectious Disease Hospital.

Cell culture and transfection

Two lung cancer cell lines (A549 and H1299) and pulmonary epithelial cells (BEAS-2B) were brought from BeNa Culture Collection (Beijing, China). These cells were maintained in Dulbecco's modified eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), penicillin (100 U/mL) and streptomycin (100 mg/mL) (Gibco). Then all cells were cultivated at 37 °C under a moist atmosphere with 5% CO_2.

The small interfering RNA against-circAKT3 (si-circAKT3) and its negative control (si-NC), the mimic or inhibitor of miR-516b-5p (miR-516b-5p or anti-miR-516b-5p) and their negative control (miR-NC or anti-NC) were bought from Ribobio (Guangzhou, China). The pcDNA-STAT3 overexpression plasmid (pcDNA-NEAT1) and its negative control (pcDNA-NC), pcDNA-circAKT3 overexpression plasmid (pcDNA-circAKT3) and its negative control (pcDNA-NC) were bought from Sangon Biotech (Shanghai, China). According to the recommendations, the cells were transfected with the Lipofectamine 2000 reagent (Invitrogen).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from tissues or cells utilizing the Trizol reagent (Invitrogen) according to the standard instructions, and then the first strand complementary DNA (cDNA) was synthesized by Taq-Man Reverse Transcription Kit (for circAKT3 and STAT3) or TaqMan microRNA Reverse Transcription Kit (for miR-516b-5p) (Applied Biosystems, Foster City, CA, USA).The qRT-PCR was carried out using the SYBR Select Master Mix for CFX (Invitrogen) on CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The sequences of primers in this study were as follows: circAKT3 (Forward, 5′-TCCAAATAAACGCCTTGGTGG-3′; Reverse, 5′-CCTCAGAGAACACCCGCTCT-3′), miR-516b-5p (Forward, 5′-TCGTACCGTGAGTAATAATGCG-3′), STAT3 (Forward, 5′- CAGCAGCTTGACA-CACGGTA-3′; Reverse, 5′- AAACACCAAAGTGG-CATGTGA-3′), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Forward, 5′-CGCTCTCTGC TCCTCCTGTTC-3′; Reverse, 5′- ATCCGTTGA

CTCCGACCTTCAC-3′); U6 (forward, 5′-TCCGG GTGATGCTTTTCCTAG-3′, reverse, 5′-CGCTT CACGAATTTGCGTGTCAT-3′). The gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method, and normalized relative to U6 and GAPDH. And each experiment was conducted in triplicate.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assays

MTT assay was performed to detect cell ability based on the manufacturer's instructions. In brief, A549 and H1299 cells (5×10^3 cells/well) were plated into 96-well plates and incubated for 48 h. 20 μL MTT solution (Thermo Fisher, Wilmington, DE, USA) was added into each well with a final concentration of 0.5 mg/mL. After incubating for 4 h at room temperature, 150 μL DMSO was added into each well. The absorbance at 570 nm was determined under a microplate reader. The whole experiment was repeated three times.

Detection of glucose consumption and lactate production

Glucose consumption was determined by a glucose assay kit (Biovision, Mountain View, CA). A lactate Assay kit (Biovision) was used to evaluate lactate production as the instructions described. A549 and H1299 cells (5×10^4 cells/well) were seeded into 24-well plates and treated with si-circAKT3, pcDNA-circAKT3, miR-516b-5p + pcDNA-circAKT3, or matched controls, or treated with 20 mM 2-DG for 24 h. After 48 h, supernatant of cell culture medium was collected. Three independent experiments were carried out for each assay.

Western blot assay

Cells were collected and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing phenylmethanesulfonyl fluoride (PMSF; Beyotime) to obtain total proteins. Then, bicinchoninic acid (BCA) protein assay kit (Beyotime) was used for determination of protein concentration. An equal amount (40 μg) of protein was resolved by 8%-10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After that, the gels were transferred to polyvinylidene

fluoride (PVDF) membranes (0.2 μm, Beyotime). Next, all membranes were blocked using the 5% non-fat milk (Sangon Biotech) and then probed with the following primary antibodies: anti-HIF-1α (1:2000, EP1215Y, Abcam, Cambridge, UK), circ-STAT3 (1:500, EP2147Y, Abcam) and GAPDH (1:2000, ab37168, Abcam). Subsequently, all membranes were maintained in HRP-conjugated anti-rabbit/mouse IgG (Sangon Biotech). Finally, all protein bands were observed by the enhanced chemiluminescence (ECL) system (Millipore, Billerica, MA, USA). The protein abundances were normalized by GAPDH, and ImageJ software was applied to evaluate the density of bands. Each experiment was conducted in triplicate.

Dual-luciferase reporter assay

Potential binding sites of miR-516b-5p and circAKT3 or STAT3 were provided by starBase v3.0. The circAKT3 or STAT3-3′UTR fragment containing putative or mutated miR-516b-5p binding site was synthesized and cloned into the pmirGlO luciferase reporter vector (Promega, Madison, WI, USA), namely circAKT3-WT, STAT3-WT, circAKT3-MUT or STAT3-MUT. The miR-516b-5p or miR-NC and reporter plasmid were co-transfected into A549 and H1299 cells. At 48 h post-transfection, dual-luciferase reporter assay system (Promega) was employed to assess the luciferase activity, followed by normalizing to Renilla luciferase activity. We repeated this same experiment three times.

Murine xenograft model in vivo

To measure the impact of circAKT3 in vivo, we injected 1×10^6 A549 cells with sh-circAKT3 in a volume of 20 μL into 5-week-old female nude mice (NU/NU Crl: NU-Fox1nu, Charles River Laboratories; Sulzfeld, Germany). And, 1×10^6 cells were re-suspended in phosphate-buffered saline (PBS; 1:1 mixed with matrigel, Corning), and subcutaneously injected into the left flank of mice. Tumor volume was measured every 7 days, and animals were executed before tumors got to the diameter of 10 mm. Tumors volumes were computed by the equation V (mm^3)= (width)^2 × length/2. Our research was endorsed by the Animal Care and Use Committee of Liaocheng Infectious Disease Hospital and carried out following

guidelines of the national animal protection and ethics institute. The mice inoculated with cells were executed after 5 weeks, and assessed the weight of tumors and measure the expression of circAKT3.

Statistical analysis

Experimental data were analyzed by GraphPad Prism (GraphPad, La Jolla, CA, USA) and presented by mean ± standard deviation (SD). Two different groups were compared by Student's t-test. For more groups, the one-way analysis of variance (ANOVA) was utilized to evaluate the difference. Pearson's correlation coefficient was utilized to confirm the correlation between circAKT3 or STAT3 and miR-516b-5p in BC tissues. Every test was executed at least three times independently. $P < 0.05$ represented statistical significance.

Results

CircAKT3 was upregulated in lung cancer tissues and cells

To explore the role of circAKT3 in lung cancer, its expression level was examined by qRT-PCR. Data suggested that circAKT3 level was increased in lung tumor tissues (n = 28) compared with normal tissues (n = 28) (Fig. 1a). Furthermore, our results further proved that circAKT3 expressed at the high level in lung cancer cell lines (A549 and H1299) relative to pulmonary epithelial cells (BEAS-2B) (Fig. 1b). These data suggested the involvement of circAKT3 in the progression of lung cancer.

Silencing of circAKT3 enhanced the sensitivity of lung cancer cells to cisplatin and inhibited glycolysis

As mentioned above, abnormally high expression of circAKT3 might participate in the development of cancer. Therefore, we knocked down the circAKT3 expression in lung cancer cells. The transformation efficiency and effect of si-circAKT3 were verified by qRT-PCR. The data reported that the expression of cirAKT3 was reduced in lung cancer cells (A549 and H1299) when transfected with si-cirAKT3 (Fig. 2a, b). To investigate whether circAKT3 is involved in
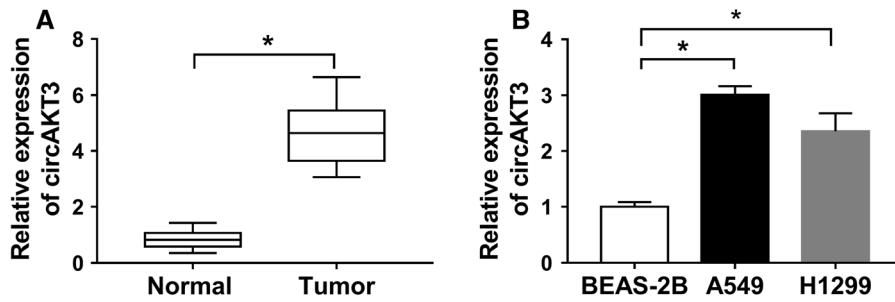
Fig. 1 CircAKT3 was highly expressed in the lung cancer tissues. **a** The expression level of circAKT3 in normal tissues and tumor tissues was detected by qRT-PCR. **b** The expression level of circAKT3 in normal cells (BEAS-2B) and tumor cells (A549 and H1299) was detected by qRT-PCR *$P < 0.05$
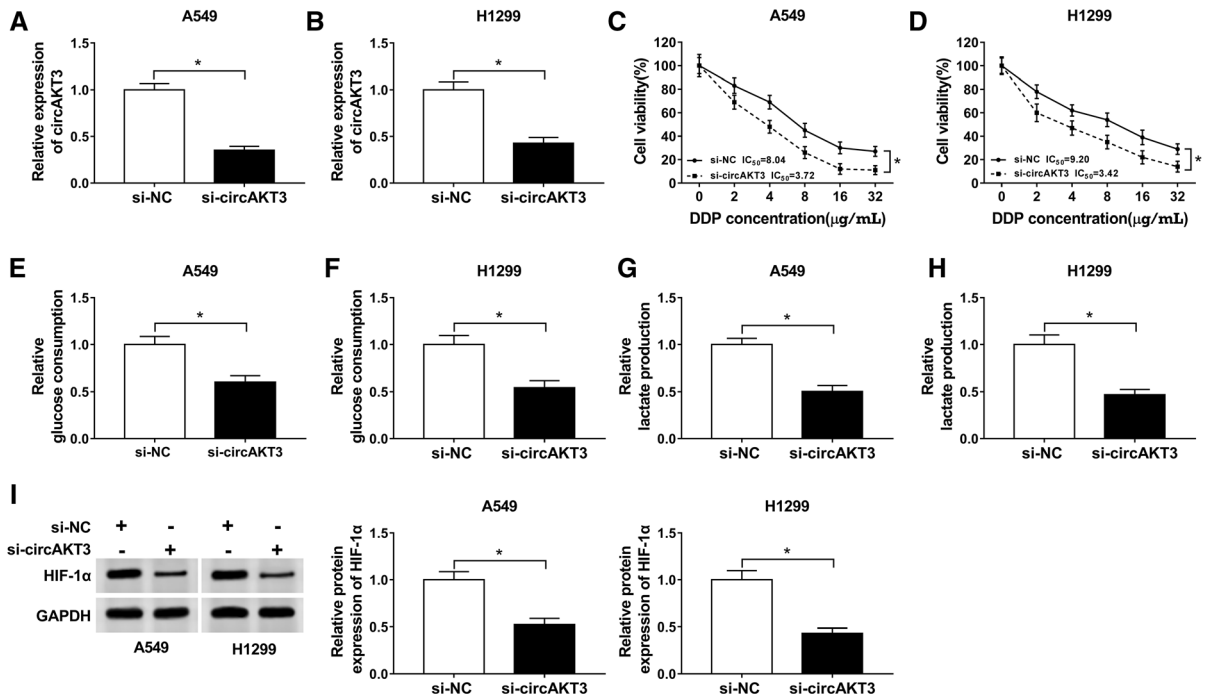

Fig. 2 Knockdown circAKT3 increased the sensitivity of lung cancer cells to cisplatin and inhibited glycolysis. **a**, **b** The expression of circAKT3 in lung cells transfected with si-circAKT3, as well as matched controls, was assessed by qRT-PCR. **c**, **d** Cell proliferation at different DDP concentrations (0, 2, 4, 8, 16, 32 μg/mL) was evaluated by MTT assay. **e**, **f** The kit was used to detect glucose consumption of cells transformed with si-circAKT3 and the control group. **g**, **h** The kit was used to detect lactate production of cells transformed with si-circAKT3 and the control group. **i** The level of HIF-1α in cells transfected with si-circAKT3 and matched controls was evaluated by western blot. *$P < 0.05$

cisplatin resistance in lung cancer cells, we analyzed the cellular activity of lung cancer cells transfected with the si-circAKT3 plasmid using MTT. The data of the research performed that the activity of the cells gradually decreased as the concentration of DDP increased (0, 2, 3, 8, 16, 32 μg/mL). The IC_{50} (8.04) of lung cancer cell A549 transformed with si-NC was higher than that of lung cancer cells transformed with

si-circAKT3 ($IC_{50} = 3.72$) (Fig. 2c). The cell viability tendency of lung cancer cell H1299 under different concentrations of DPP (0, 2, 3, 8, 16, 32 μg/mL) was the same as that of A549 cells. The lung cancer cell H1299 transfected with si-circAKT3 had a significantly lower IC_{50} (3.42) than cells transformed with si-NC ($IC50 = 9.20$) (Fig. 2d). The above studies demonstrated that circAKT3 might be involved in

DDP resistance in lung cancer cells. It was believed that abnormality in glycolysis contributed to the development of cancer (Bhattacharya et al. 2014). We analyzed the impacts of circAKT3 knockdown on the glycolytic metabolism in lung cancer cells (A549 and H1299). As showed in Fig. 2e–h, the deficiency of circAKT3 led to a decrease in glucose consumption, and lactate production in A549 and H1299 cells compared to control group. Hypoxia-inducible factor 1α (HIF-1α) was a transcription factor which controlled a vast array of gene products to participate in energy metabolism and glycolysis (Zhou et al. 2012). We discussed whether circAKT3-mediated regulation of glycolysis was dependent on HIF-1α. Western blot experiments reported that the protein level of HIF-1α was obviously declined in si- circAKT3-transfected A549 and H1299 cells relative to si-NC-transfected cells (Fig. 2i). From what has been discussed above, our data showed that circAKT3 knockdown weakened HIF-1α-dependent glycolysis.

CircAKT3 was able to target miR-516b-5p in lung cancer cells

Starbase3.0 was used to predict the potential targets of circAKT3. The data showed the potential binding sites for miR-516b-5p on circAKT3, as presented in Fig. 3a. To further confirm the relationship between circAKT3 and miR-516b-5p, luciferase reporter assay was conducted. The studies reported that miR-561b-5p overexpression weakened the luciferase activities of circAKT3-WT reporter vector in A549 and H1299 cells, but had no visible inhibitory effect on circAKT3-MUT reporter vector (Fig. 3b, c). QRT-PCR experiments verified that the expression level of miR-516b-5p was lower in lung cancer tissues and cells when compared with their respective controls (Fig. 3d, e). Our study demonstrated a negative correlation between the expression of circAKT3 and miR-516b-5p (Fig. 3f). The above results suggested that the impact of circAKT3 and miR-516b-5p in lung cancer cells was likely to be reversed.

CircAKT3 regulated glycolysis of lung cancer cells by targeting miR-516b-5p

To explore the relationship between circAKT3 and miR-516b-5p, lung cancer cells were treated with si-NC, si-circAKT3, si-circAKT3 + anti-miR-NC and si-circAKT3 + anti-miR-516b-5p. QRT-PCR data indicated that miR-516b-5p expression was upregulated in si-circAKT3-introduced A549 and H1299 cells, which was reversed by anti-miR-515b-5p (Fig. 4a, b). Moreover, the role of circAKT3 in DDP sensitivity was further explored by MTT assay. Results showed that the cell viability in DDP concentration (0, 2, 4, 8, 16, 32 ug/mL) of cells transfected with si-circAKT3 cells (IC_{50} = 4.34) was decreased compared with the negative control group (IC_{50} = 10.38). Cells co-transfected with si-circAKT3 and trans-516b-5p (IC_{50} = 7.46) showed that the cells treated with si-circAKT3 had a significantly higher cell viability than the control group (IC_{50} = 4.25) (Fig. 4c). The same was true for the overall situation in H1299 (Fig. 4d). Further investigation of the knockdown of circAKT3 on glycolysis, knockdown of circAKT3 reduced glucose consumption and lactic acid formation, but anti-miR-516b-5p reversed its effect (Fig. 4e, f). The expression of HIF-1α protein was also consistent. Western blot analysis presented that anti-miR-516b-5p reversed the inhibition action of circAKT3 knockdown on HIF-1α protein level in lung cancer cells (Fig. 4i, j). The results of the above studies indicated that circAKT3 could bind to miR-516b-5p and regulated lung cancer cell activity and HIF-1α-dependent glycolysis by regulating mir-516b-5p expression.

STAT3 bound to miR-516b-5p, and both circAKT3 and miR-516b-5p adjusted the expression of STAT3 in lung cancer cells.

Starbase3.0 predicted that miR-516b-5p might combine with 3′-UTR of STAT3 mRNA, a glycolysis-related gene (Fig. 5a). Dual-luciferase reporter assay showed that miR-516b-5p overexpression weakened the luciferase activities of STAT3-WT reporter vector in A549 and H1299 cells, but had no visible inhibitory effects on STAT3-MUT reporter vector (Fig. 5b, c). The results of qRT-PCR displayed that STAT3 mRNA expression in lung cancer tissues and cells was evidently upregulated when compared with the healthy tissues and cells (Fig. 5d, f). The data of the western blot experiment indicated that the expression of STAT3 protein was consistent with mRNA expression in lung cancer tissues and cells (Fig. 5e, g). In addition, miR-516b-5p and pcDNA-STAT3 overexpression plasmids were transformed into lung cancer
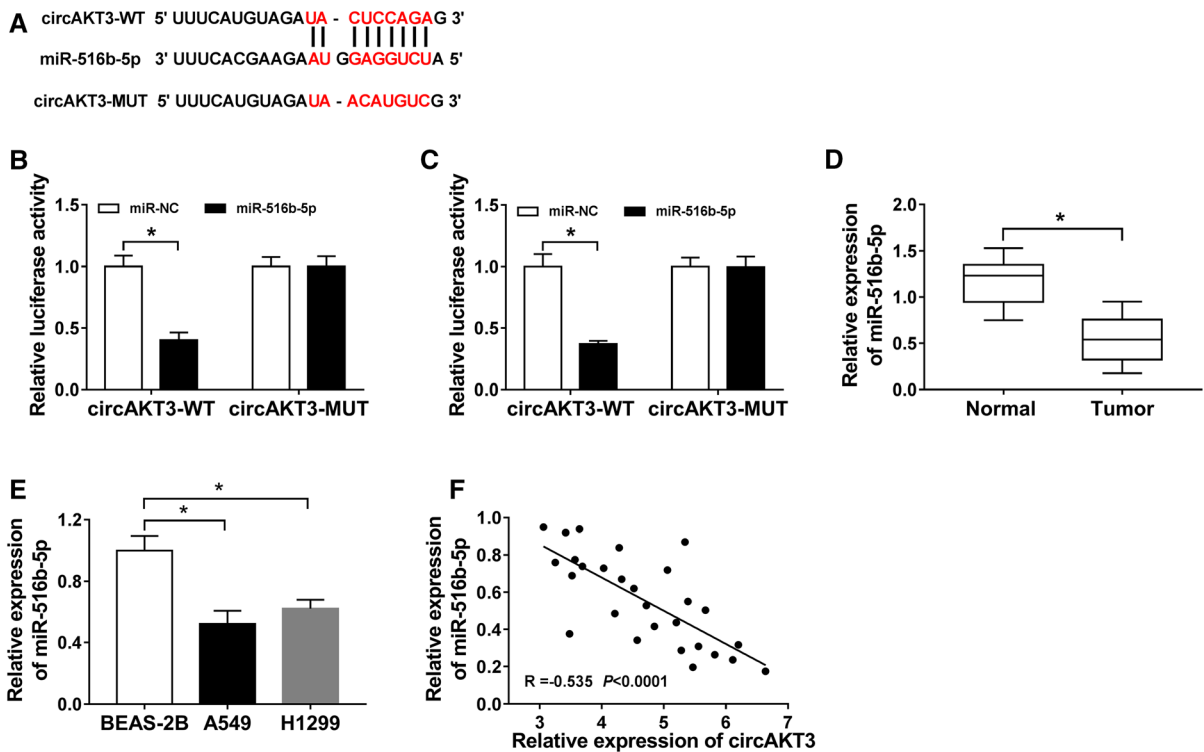
Fig. 3 CircAKT3 targeted and regulated miR-516b-5p in lung cells. **a** The putative binding sites between circAKT3 and miR-516b-5p were predicted by Starbase3.0. **b**, **c** The luciferase activity in lung cells co-transfected with miR-516b-5p and circAKT3 WT or circUBAP2 MUT was checked. **d** The level of miR-516p-5p in lung cancer tissues and its control group was evaluated by qRT-PCR. **e** The level of miR-516p-5p in lung cancer cells and its control group was evaluated by qRT-PCR. **f** The correlation between circAKT3 and miR-516b-5p in lung cancer tissues was verified using Pearson's correlation coefficient. *$P < 0.05$

cells, respectively. Experiments have shown that the high expression of miR-516b-5p apparently reduced STAT3 level, whereas the re-introduction of pcDNA-STAT3 reversed this inhibition to some extent (Fig. 5i, j). Meanwhile, the expression level of miR-516b-5p was negatively correlated with STAT3 in lung cancer tissues (Fig. 5h). Besides, the relationship between circAKT3 and STAT3 was further studied. It was demonstrated that knockdown of circAKT3 could significantly inhibit the mRNA and protein expression of circSTAT3, which was reversed by STAT3 over-expression plasmid in lung cancer cells (Fig. 5l, m). CircAKT3 was positively correlated with the expression of STAT3 (Fig. 5k). The above studies demonstrated the targeting relationship between miR-516b-5p and STAT3, and the fact that STAT3 could be regulated by miR-516b-5p.

MiR-516b-5p/STAT3 regulated the glycolysis balance of cells and improved the sensitivity of cells to DDP.

To study the impact of miR-516b-5p and STAT3 on DDP sensitivity, lung cells were transfected with miR-516b-5p and its negative control (miR-NC), or pcDNA-STAT3 and pcDNA-NC. MTT assay results showed that the cell viability of cells transfected with miR-516b-5p ($IC_{50} = 3.10$) was lower than that of the negative control group with the increase of DDP concentration (0, 2, 4, 8, 16, 32 μg/mL)($IC_{50} = 9.02$). The cell viability co-transfected with miR-516b-5p and pcDNA-STAT3 ($IC_{50} = 3.48$) was lower than that of the si-circAKT3 group ($IC_{50} = 4.25$) (Fig. 6a). The same was true for the overall situation in H1299 (Fig. 6b). Meanwhile, the data reported that overexpression of miR-516b-5p inhibited glucose consumption and lactic acid formation in lung cancer cells, but
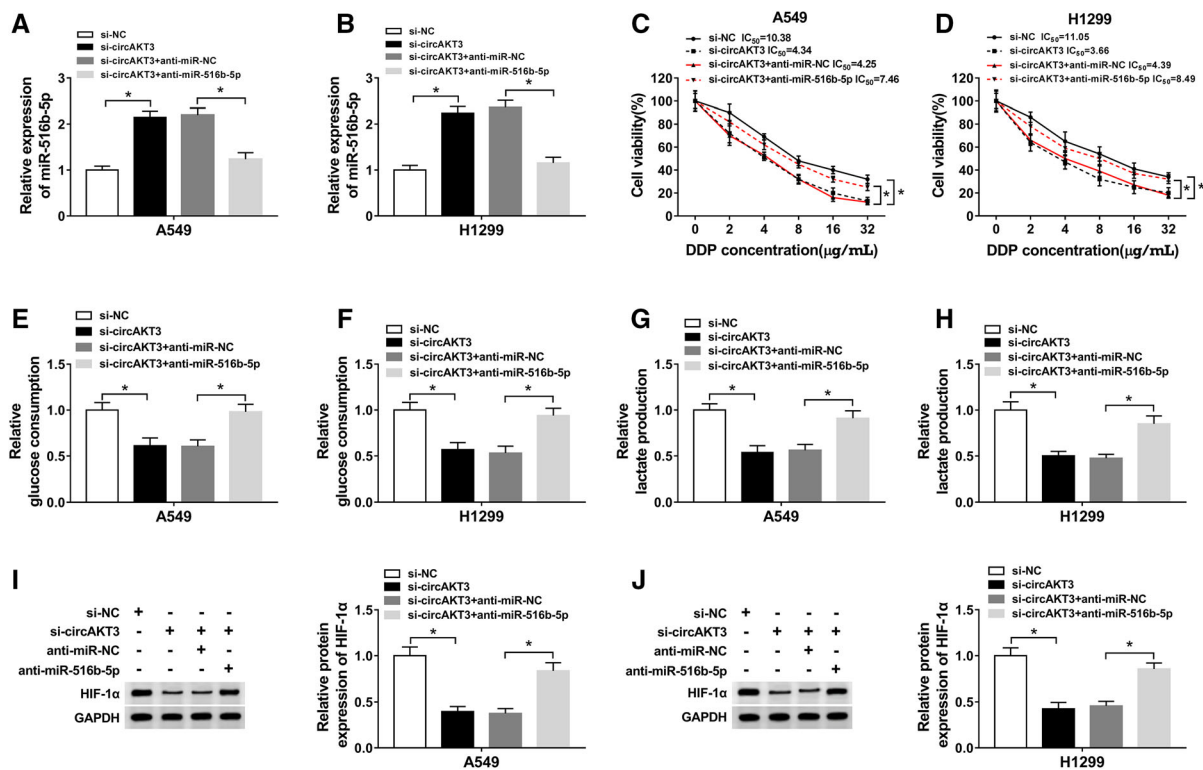
Fig. 4 CircAKT3/miR-516b-5p regulated glycolysis of lung cancer cells in lung cancer. Lung cells treated with anti-516b-5p, si-circAKT3, or corresponding negative controls. **a**, **b** The expression level of miR-516b-5p was checked by qRT-PCR. **c**, **d** Cell viability at different DDP concentrations was assessed by MTT assay. **e**, **f** The kit was used to detect glucose consumption

of cells transformed with si-circAKT3 + anti-miR-516p-5p or si-circAKT3 and the control groups. **g**, **h** The kit was used to detect lactate production of cells transformed with si-circAKT3 + anti-miR-516b-5p or si-circAKT3 and the control group. **e**, **f** The protein level of HIF-1α in lung cancer cells was checked by western blot. *$P < 0.05$

the inhibition was reversed by overexpression of STAT3 (Fig. 6c–f). The expression of HIF-1α was inhibited by miR-516b-5p, but overexpression of STAT3 reversed this inhibition (Fig. 6g, h).

Inhibition of circAKT3 inhibited HIF-1α-dependent glycolysis-induced A549/H1299 cell resistance, and tumor development in vitro

To study the impact of glycolysis on circAKT3-induced chemo-resistance in A549, the lung cancer cells were transfected with 20 mM 2-DG. 2-DG exposure inhibited the glucose consumption (Fig. 7a, e) and lactate production (Fig. 7b, f) in A549 cells when compared to control cells. Meantime, the HIF-1α protein level was inhibited by 2-DG treatment in A549 cells (Fig. 7c, g). 2-DG administration abated the positive impact of circAKT3 overexpression on the viability of A549 cells, as verified by MTT assay,

showing that inhibition of HIF-1α-dependent glycolysis attenuated the circAKT3-induced increase of chemo-resistance in A549 cells (Fig. 7d, h).

Knockout of circAKT3 could inhibit the growth of lung cancer in vivo

For exploring the anti-cancer effect of circAKT3 silence, A549 cells, transfected with sh-circAKT3 or sh-NC were used to establish xenograft model in vivo. After cell injection for 5 weeks, tumor volume and weight were decreased in sh-circAKT3 group compared to the sh-NC (Fig. 8a, b). At the same time, circAKT3 expression was notably downregulated in the sh-circAKT3 group compared to sh-NC group (Fig. 8c). Moreover, the expression of miR-516p-5p was increased in the sh-circAKT3 group when compared with that in sh-NC group (Fig. 8d). However, the protein expression and mRNA and protein of
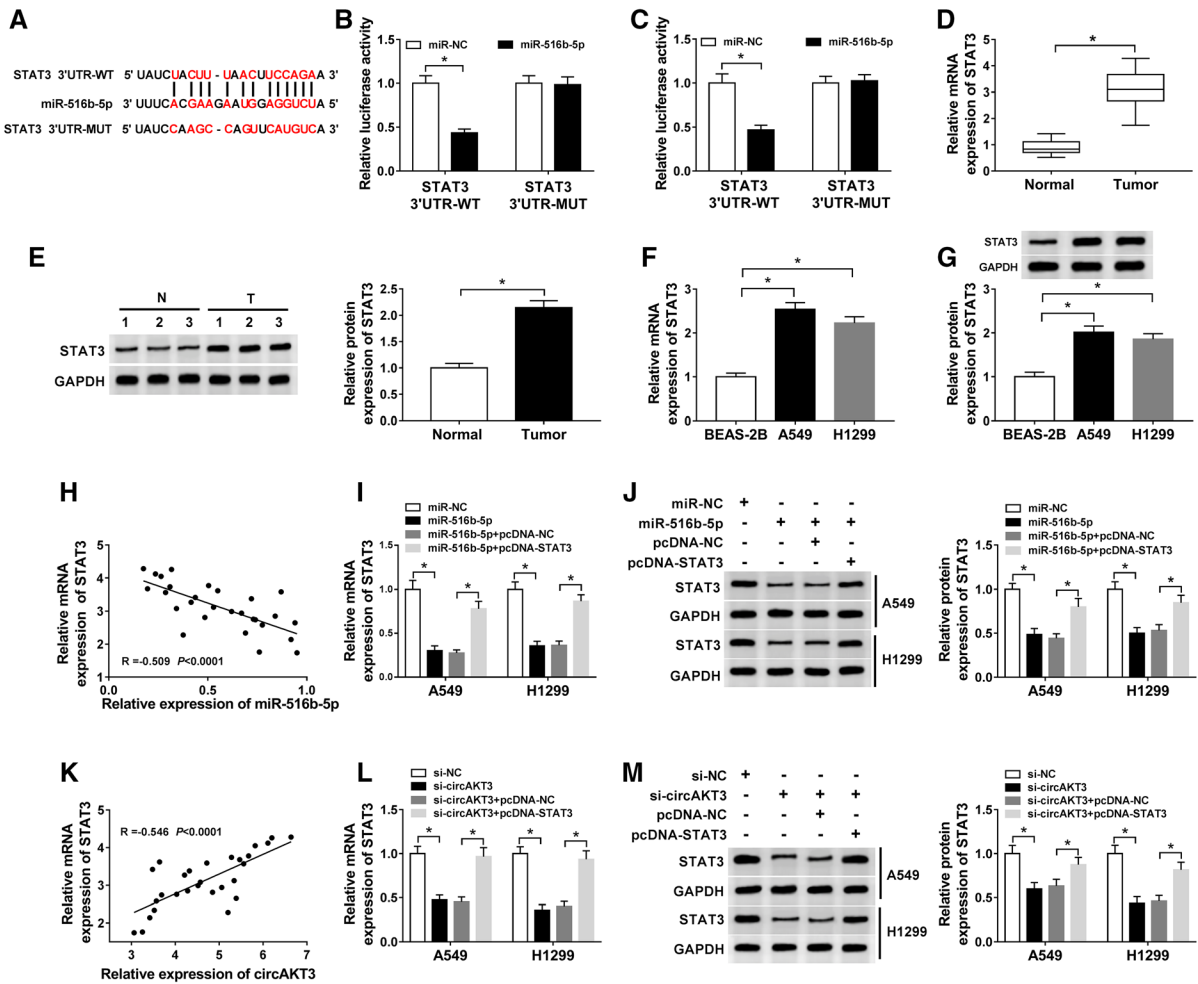
Fig. 5 STAT3 had targeted binding sites of miR-516b-5p, and circAKT3 and miR-516b-5p could regulate the expression of STAT3. Lung cells (A549 and H1299) were transfected with miR-516b-5p, pcDNA-STAT3, or corresponding negative controls. **a** The putative binding sites between STAT3 and miR-516b-5p were predicted by Starbase3.0. **b, c** The luciferase activity in lung cells co-transfected with miR-516b-5p and pcDNA-STAT3 WT or STAT3 MUT was checked. **d, e** Both mRNA level and protein level of STAT3 in lung cancer tissues was evaluated by qRT-PCR and western blot assays. **f, g** qRT-PCR and western blot assays were carried out to examine the mRNA level and protein level of STAT3 in BEAS-2B, A549 and H1299 cells. **h** The correlation between STAT3 mRNA and miR-516b-5p in lung cancer cells was determined using Pearson's correlation coefficient. **i** The level of STAT3 mRNA was evaluated in A549 and H1299 cells transfected with miR-NC, miR-516b-5p, miR-516b-5p + pcDNA-NC and miR-516b-5p + pcDNA-STAT3. **j** The level of STAT3 protein was assessed in transfected A549 and H1299 cells. **k** The correlation between STAT3 and circAKT3 in lung cancer cells was determined using Pearson's correlation coefficient. **l** The level of STAT3 mRNA was measured in A549 and H1299 cells transfected with si-NC, si-circAKT3, si-circAKT3 + pcDNA-NC and si-circAKT3 + pcDNA-STAT3. **m** STAT3 protein level in transfected A549 and H1299 cells was evaluated. *$P < 0.05$

STAT3 were reduced in the sh-circAKT3 group compared to sh-NC group (Fig. 8e, f). The above results suggested that circAKT3 knockout might help reduce the development of lung cancer by regulating miR-516b-6p/STAT3.

Discussion

Lung cancer, as a clinical disease with high morbidity and mortality (Bircan et al. 2018), has not stopped its clinical research. However, the specific mechanism analysis was still unknown, which brings great difficulties to the prevention and treatment of lung cancer.
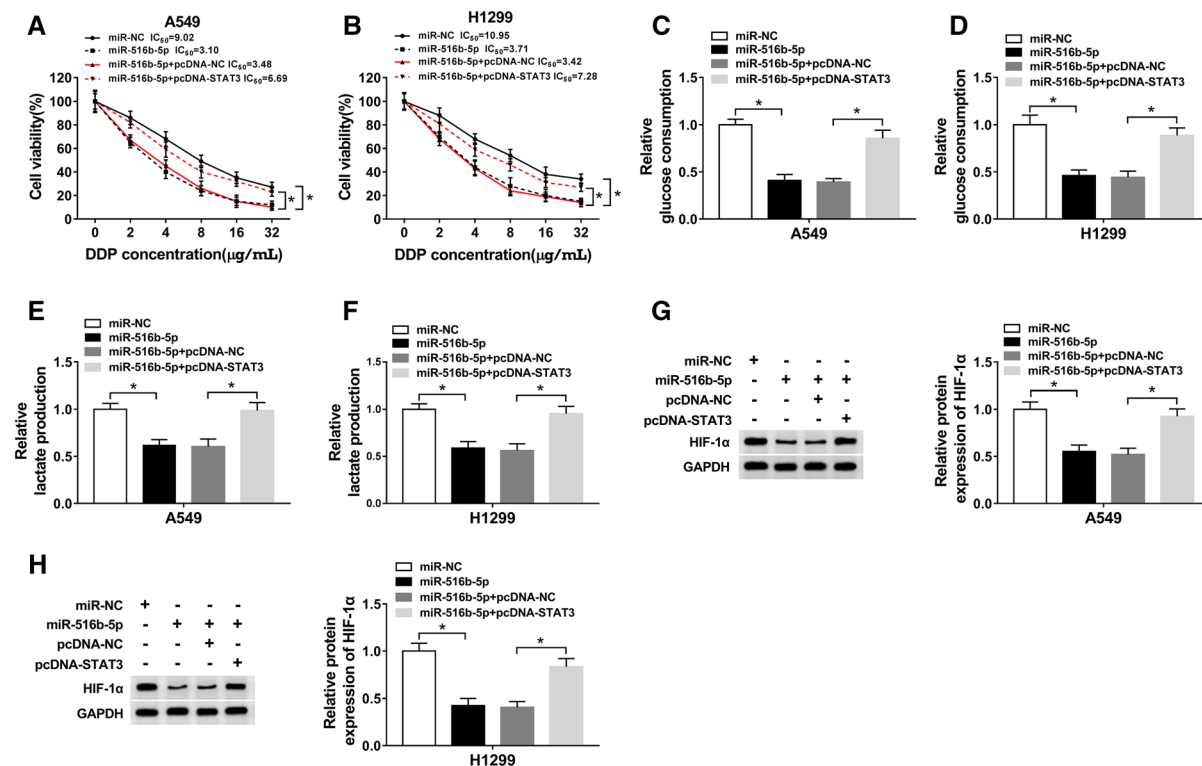
Fig. 6 MiR-516b-5p/STAT3 could regulate the glycolytic balance of cells and increase the sensitivity of cells to cisplatin. Lung cells (A549 and H1299) were transfected with miR-516b-5p, pcDNA-STAT3, or corresponding negative controls. **a**, **b** Cell viability at different DDP concentrations was assessed by MTT assay. **c**, **d** The kit was used to detect glucose consumption of cells transformed with pcDNA-STAT3 + miR-516p-5p or miR-516b-5p and the control groups. **e**, **f** The kit was used to detect lactate production of cells transformed with pcDNA-STAT3 + miR-516p-5p or miR-516b-5p and the control groups. **g**, **h** The protein level of HIF-1α in lung cancer cells was checked by western blot. $*P < 0.05$

CircRNAs were verified to regulate the progression of many cancers. Bi et al. (2018) confirmed that circRNA_102171 regulated papillary thyroid carcinoma by activating related signaling pathways. Liu et al. (2018) confirmed that the circular RNA hsa_circRNA_103809 was involved in lung cancer progression. Our study confirmed the high expression of circAKT3 in clinical tissues and cells, indicating that it played an essential impact in lung cancer. To prove the conjecture, we carried out a series of studies. Studies found that knockdown of circAKT3 could decrease the resistance of lung cancer cells to DDP. Not only that, t knocking down circAKT3 also inhibited glycolysis. It was proved that circAKT3 might play an essential impact on the progression of lung cancer.

Many literatures reported that microRNA played an important role in many disease methods (Gheytanchi et al. 2017; Li et al. 2019; Masood et al. 2017). Our

study demonstrates that circAKT3 could target miR-516b-5p. Moreover, in lung cancer cells and tissues, the expression of miR-516b-5p was also lower than that of normal tissues. The studies proved that miR-516b-5p might also be involved in the regulation of lung cancer progression. We confirmed that miR-516b-5p overturned the effect of circAKT3 knockdown on cells. It was suggested that miR-516b-5p might have the function of promoting DDP drug sensitivity and attenuating glycolysis in lung cancer.

There were many studies on the disease correlation of STAT3 (Sirkisoon et al. 2018; Yamauchi et al. 2018). Solis et al. (2018) demonstrated that transcription factor STAT3 could regulate the immunophenotypic regulation of monocytes-macrophages from M1 to M2. Cheng et al. (2018) reported that in colorectal cancer, abnormal STAT3 expression increased the survival of cancer stem-like tumor balls. Our study confirmed the targeting relationship between STAT3
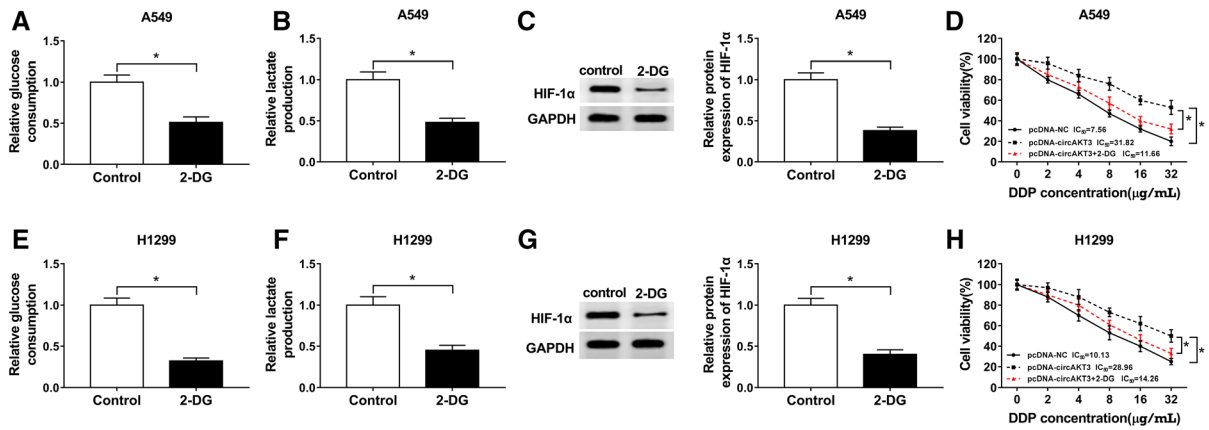
Fig. 7 Inhibition of circAKT3 inhibited HIF-1α-dependent glycolysis induced A549/H1299 cell resistance to 2-DG. **a** The glucose consumption of A549 cells under 2-DG (20 mM) and the control group. **b** The lactate production of A549 cells under 2-DG (20 mM) and the control group. **c** Western blot was used to measure the expression of HIF-1α in A549 cells under 2-DG (20 mM) and the control group. **d** Cell viability of A549 cells treated with pcDNA-circSTAT3 or 2-DG was assessed by MTT assay. **e** The glucose consumption of H1299 cells under 2-DG (20 mM) and the control group. **f** The lactate production of H1299 cells under 2-DG (20 mM) and the control group. **g** Western blot analysis was used to detect the expression of HIF-1α in H1299 cells under 2-DG (20 mM) and the control group. **h** Cell viability of H1299 cells treated with pcDNA-circSTAT3 or 2-DG was assessed by MTT assay. *$P < 0.05$
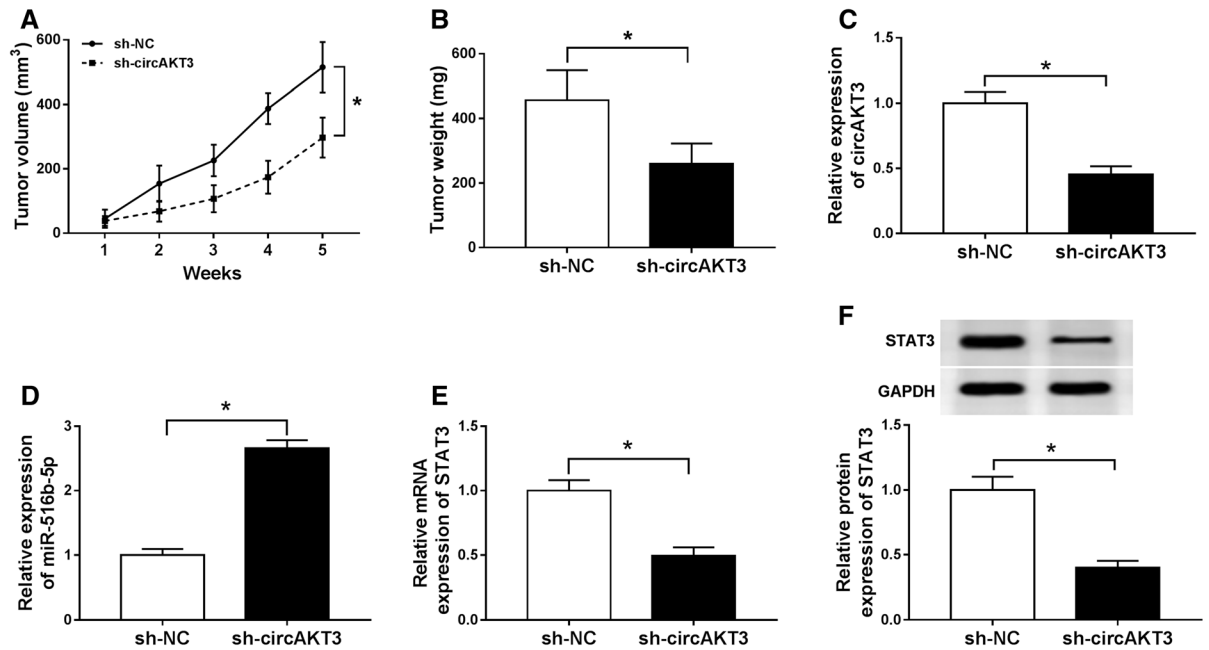

Fig. 8 Knockdown of AKT3 could inhibit the growth of lung cancer in vivo. **a** Volume analysis of xenograft tumors. **b** Weight analysis of xenograft tumors. **c** The levels of circAKT3 protein in xenograft tumors were quantified by qRT-PCR and western blot. **d** The level of miR-516b-5p in xenograft tumors treated with A549 cells stably expressing sh-circAKT3 or sh-NC was quantified by qRT-PCR. **e** The level of STAT3 mRNA in xenograft tumors treated with A549 cells stably expressing sh-circAKT3 or sh-NC was quantified by qRT-PCR. **f** The level of STAT3 protein in xenograft tumors treated with A549 cells stably expressing sh-circAKT3 or sh-NC was verified by western blot. *$P < 0.05$

and miR-516b-5p and also confirmed the upregulated expression of STAT3 in lung cancer tissues and cells than that of normal tissues and cells. In-depth studies confirmed that both circAKT3 and miR-516b-5p could adjust the expression of STAT3, which in turn regulates glycolysis and DDP sensitivity. To further investigate the function of circAKT3, circAKT3 was overexpressed. Our study demonstrated that overexpression of circAKT3 significantly reduced drug resistance in lung cancer cells. Further experiments also confirmed that knockdown of circAKT3 could reduce the expression of STAT3 and increase the expression of miR-516b-5p, thereby alleviating the progress of lung cancer in vivo.

Conclusions

Our entire study confirmed that circAKT3 knockdown increased the sensitivity of lung cancer cells to cisplatin and inhibited glycolysis through targeting the miR-516b-5p/STAT3 axis. In addition, the
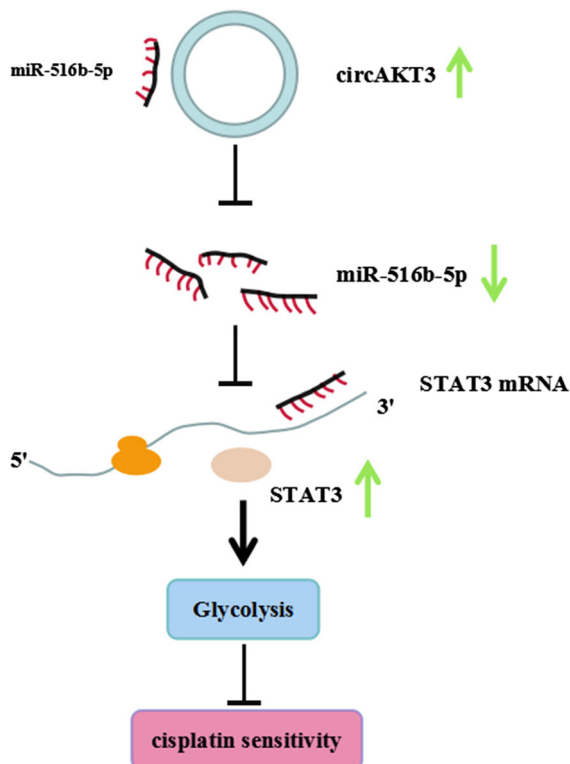
regulatory function of circAKT3/miR-516b-5p/STAT3 axis on the sensitivity of lung cancer cells to cisplatin was mediated by regulating glycolysis (Fig. 9). These findings provided new insights and ideas for clinical prediction and treatment of lung cancer, and might become a key to making breakthrough progress in the future.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no financial conflicts of interest.

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Fig. 9 The schematic representation of circAKT3/miR-516b-5p/STAT3 in lung cancer cells

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