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ORIGINAL RESEARCH

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NR3C2 inhibits glycolysis in non-small cell lung cancer cells by regulating the Akt pathway

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Abstract

Background: Uncertainty surrounds the function of Nuclear Receptor Subfamily 3 Group C Member 2 (NR3C2) in controlling glycolysis in non-small cell lung cancer (NSCLC) and its molecular relationship to the Akt (Protein kinase B) pathway. In a number of malignancies, NR3C2 has been identified as a tumor suppressor. This research attempts to show how NR3C2 inhibits the development of NSCLC. **Methods**: The expression of NR3C2 and its impact on patient survival in NSCLC were investigated using a public database. Following NR3C2 overexpression via plasmid transfection, cell viability was assessed using Cell Counting Kit-8 (CCK8) assays. The colony formation assay was used to evaluate cell proliferation. Adenosine Triphosphate (ATP) generation, glucose uptake, and lactate production were measured in cells using appropriate kits. Cell apoptosis was assessed by flow cytometry. Western blotting was employed to evaluate protein expression levels of NR3C2, cleaved- Poly ADP-ribosepolymerase (PARP), cleaved-caspase3, Glucose transporter 1 (GLUT1), hexokinase2 (HK2), p-Akt/Akt and p-mammalian target of rapamycin (mTOR)/mTOR. **Results**: Database analysis revealed reduced NR3C2 expression in tumor. Overexpression of NR3C2 resulted in increased apoptosis and impaired colony formation and cell survival, and inhibited glucose consumption, lactate production and ATP levels. Furthermore, apoptotic indicators cleaved-PARP and cleaved-caspase3 showed markedly increased protein levels, whereas p-Akt/Akt, p-mTOR/mTOR, GLUT1 and HK2 showed markedly decreased levels. **Conclusions**: NR3C2 suppresses NSCLC cell proliferation and glycolysis, potentially through the suppression of the Akt pathway.

Keywords

NSCLC; NR3C2; Proliferation; Glycolysis; Apoptosis; Akt

1. Introduction

Lung cancer remains the leading cause of cancer-related mortality and morbidity worldwide. Among its histological subtypes, non-small cell lung cancer (NSCLC) is the most prevalent. For individuals with advanced-stage disease, the five-year survival rate is approximately 5%. Despite significant research efforts over the past two decades to identify the molecular mechanisms driving cancer initiation, therapeutic options for NSCLC remain limited, and survival rates have shown only modest improvement [1]. Therefore, finding potential therapeutic targets is crucial for advancing NSCLC treatment.

Due to the low oxygen supply, glycolysis is essential for the metabolic adaptability of tumor cells during rapid development. It generates adenosine triphosphate (ATP), supporting tumor cell proliferation, invasion and metastasis, thereby promoting malignant progression. As a result, glycolysis-targeted therapies have emerged as an important strategy in the treatment of lung cancer [2].

The mineralocorticoid receptor (MR), a protein encoded by the nuclear transcription factor. Numerous cancer types have been found to benefit from the tumor suppressor function of Nuclear Receptor Subfamily 3 Group C, Member 2 (NR3C2), whose expression is frequently downregulated in malignancies. In colon cancer cells, NR3C2 expression is reduced, and its overexpression has been shown to inhibit cell proliferation, colony formation, migration and invasion. Furthermore, NR3C2 overexpression in colon cancer cells suppressed angiogenesis and inhibited the Akt/extracellular regulated protein kinases (ERK) signaling pathways [3]. Additionally, overexpression of NR3C2 resulted in decreased glucose consumption, lactate production, and ATP levels in these cells. The expression of key enzymes involved in glucose metabolism, such as HK2 and Recombinant Lactate Dehydrogenase A (LDHA), was significantly reduced at both mRNA and protein levels [4]. Downregulated NR3C2 expression is also seen in individuals with clear cell renal cell carcinoma, and it is associated with decreased overall, disease-specific and progression-free survival [5].

However, there is limited research on the role and mech-

anism of NR3C2 in NSCLC. This study aims to elucidate the tumor-suppressive function of NR3C2 in NSCLC, with a focus on its regulation of glycolysis and apoptosis through the Akt pathway.

2. Methods

2.1 Bioinformatics analysis

The Gene Expression Profiling Interactive Analysis (GEPIA) database was used to ascertain the expression of NR3C2 in lung adenocarcinoma and lung squamous cell carcinoma (http://gepia.cancer-pku.cn/). Utilizing the Kaplan-Meier Plotter database (https://kmplot.com/analysis/), the impact of NR3C2 expression on lung cancer patients' survival rates was examined.

2.2 Cell culture and transfection

The American Type Culture Collection (Manassas, VA, USA) provided the human lung cancer cell lines A549 and NCI-H520. Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin was used to cultivate cells at 37 °C in an incubator with 5% CO_2. Cells were seeded into 24-well plates (5×10^4 cells/well) until they reached 60–70% confluence. One microgram of the NR3C2 plasmid and two microliters of Lipofectamine 3000 were diluted in fifty microliters of Opti-MEM for transfection. After standing for 5 minutes, the two solutions were mixed and incubated for 15 minutes. The old medium was then aspirated, and 100 μL of the plasmid-lipid complex was added along with 400 μL of fresh medium. The media was swapped out for full growth medium after six hours. Cells were collected after 48 hours, and NR3C2 overexpression efficiency was verified by Western blot. A549 or NCI-H520 cells were transfected with NR3C2 overexpression vectors using Lipofectamine 3000.

2.3 Cell viability

2×10^3/well of A549 and NCI-H520 cells were planted onto 96-well plates. Ten microliters of CCK8 solution were added to each well after the 24-hour period, and the plate was then incubated for an extra hour. To evaluate cell viability, absorbance at 450 nm was measured with a microplate reader.

2.4 Colony formation

Cells were seeded into culture dishes and cultured for 10 to 15 days to perform colony formation assays. The culture medium was changed every 4–5 days. After 20 minutes of 4% paraformaldehyde fixation, the cells were rinsed with phosphate-buffered saline (PBS) and stained for 10 minutes with crystal violet solution. Colonies with more than fifty cells were tallied.

2.5 Assessment of glycolysis related indices

To determine the degree of glycolysis, three assay kits were used: one for measuring glucose absorption colorimetrically, another for measuring lactate generation, and a third for measuring ATP synthesis. All assays were performed according to the manufacturer's instructions.

2.6 Flow cytometry

As directed by the manufacturer, Annexin V Fluorescein (FITC)/Propidium Iodide (PI) labeling was used to evaluate apoptosis in A549 and NCI-H520 cells. The concentration of cells was set at 7×10^4 cells/mL. To resuspend the cells, 500 μL of Binding Buffer, 5 μL of Annexin V-FITC, and 5 μL of PI were added. Following a 15-minute dark incubation period at 4 °C, the samples were analyzed using flow cytometry.

2.7 Western blotting

10% Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used to separate the total protein from A549 and NCI-H520 cells, which was subsequently moved to nitrocellulose membranes. After blocking the membranes with 5% non-fat milk, they were treated with either rabbit anti-β-actin monoclonal antibodies or specific antibodies for the target proteins. After that, an anti-rabbit Immunoglobulin G (IgG) Horseradish Peroxidase (HRP)-conjugated secondary antibody was added to the membranes for incubation. Protein expression was normalized to β-actin. The following primary antibodies were used: NR3C2 (#DF13302, 1:1000, Affinity, Changzhou, China); HK2 (#DF6176, 1:1000, Affinity, Changzhou, China); GLUT1 (#AF5462, 1:1000, Affinity, Changzhou, China); cleaved-caspase3 (#AF7022, 1:1000, Affinity, Changzhou, China); cleaved-PARP (#AF7023, 1:1000, Affinity, Changzhou, China); p-Akt (#AF0016, 1:1000, Affinity, Changzhou, China); Akt (#AF0836, 1:1000, Affinity, Changzhou, China); p-mTOR (#AF3308, 1:1000, Affinity, Changzhou, China); mTOR (#AF6308, 1:1000, Affinity, Changzhou, China); β-actin (#AF7018, 1:3000, Affinity, Changzhou, China).

2.8 Statistical analysis

The results are shown as means $\pm$ standard deviation (SD), and each experiment was run in triplicate. The data's normality was verified using the Shapiro-Wilk test. One-way Analysis of Variance (ANOVA) combined with Tukey's *post hoc* test was used to analyze comparisons between several groups. Using the Student's unpaired *t*-test, two groups were compared. For all statistical analyses, SPSS 22.0 software (International Business Machines Corporation, New York, NY, USA) was used.

3. Results

3.1 Downregulation of NR3C2 occurs in NSCLC cells

Evaluation of NR3C2 expression in lung squamous cell carcinoma and lung adenocarcinoma was done using The Cancer Genome Atlas (TCGA) data. The results revealed reduced NR3C2 expression in both lung adenocarcinoma (Fig. 1A) and lung squamous cell carcinoma (Fig. 1B). Additionally, the Kaplan-Meier database was utilized to investigate the effect of NR3C2 expression on patient survival. The results imply that patients' survival outcomes were better when their NR3C2 expression was higher (Fig. 1C).

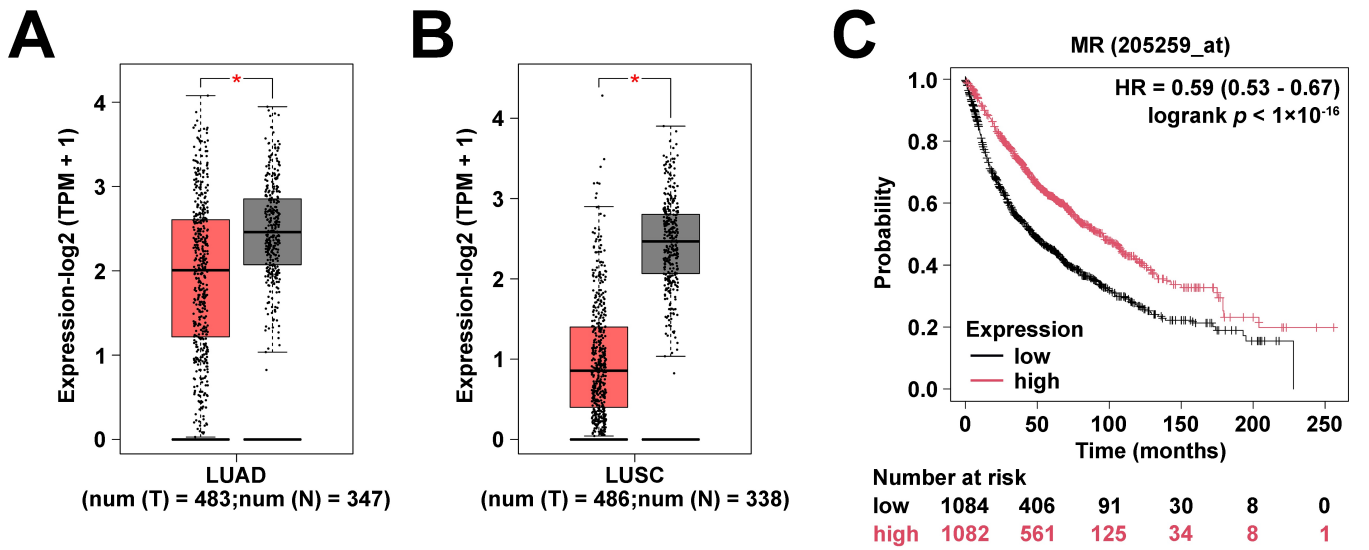

FIGURE 1. Downregulation of NR3C2 occurs in NSCLC cells. (A) An analysis of NR3C2 protein levels in lung adenocarcinoma was conducted utilizing the GEPIA database. (B) An analysis of NR3C2 protein levels in lung squamous cell carcinoma was conducted utilizing the GEPIA database. (C) Using the Kaplan-Meier database, the impact of NR3C2 expression on lung cancer patients' survival rate was evaluated. *$p < 0.01$. TPM: Transcripts Per Million; LUAD: Lung adenocarcinoma; LUSC: lung squamous cell carcinoma; MR: Mortality Rate; HR: Hazard Ratio; num (T): Number of Tumor samples; num (N): Number of Normal samples.

3.2 NR3C2 inhibits the growth of NSCLC cells

Plasmids that overexpress NR3C2 were transfected into NCI-H520 and A549 cells, and Western blot analysis confirmed successful NR3C2 expression. The results demonstrated an increase in NR3C2 protein expression in the transfected groups, confirming the efficacy of the transfection (Fig. 2A). After successful transfection, cell viability was assessed at various time points using the CCK8 assay, and the colony formation was evaluated by crystal violet staining. The results showed that both cell viability (Fig. 2B) and colony formation (Fig. 2C) were significantly reduced in the NR3C2 overexpression group, indicating that NR3C2 inhibited cell proliferation.

3.3 NR3C2 suppresses glycolysis in NSCLC

To investigate the effect of NR3C2 on glycolysis in NSCLC, assays were performed to measure ATP content, glucose uptake, and lactate production in tumor cells. Additionally, GLUT1 and HK2 were among the glycolysis-related proteins whose expression was evaluated by Western blotting. NR3C2 overexpression was shown to significantly decrease lung cancer cells' ATP production (Fig. 3A), glucose uptake (Fig. 3B), and lactate generation (Fig. 3C) in lung cancer cells. Furthermore, the protein expression levels of HK2 and GLUT1 protein expression were also decreased (Fig. 3D), indicating that NR3C2 effectively suppresses glycolysis in NSCLC.

3.4 NR3C2 promotes apoptosis of lung cancer cells

Flow cytometry was used to assess the impact of NR3C2 on NSCLC apoptosis. The NR3C2 overexpression group's apoptosis rate significantly increased, according to the results (Fig. 4A). Additionally, Key indicators of apoptosis, cleaved-PARP and cleaved-caspase3, were measured for expression using Western blot analysis. The findings indicated that NR3C2 overexpression significantly increased the expression of these apoptotic markers (Fig. 4B). The above results suggest that NR3C2 induces apoptosis in NSCLC cells.

3.5 NR3C2 modulates the Akt pathway in NSCLC

Western blot analysis was used to determine the protein expression levels of p-Akt, Akt, p-mTOR, and mTOR in cells in order to validate the impact of NR3C2 on the Akt pathway in NSCLC. The findings demonstrated that p-Akt/Akt and p-mTOR/mTOR protein expression levels in lung cancer cells were significantly reduced upon NR3C2 transfection (Fig. 5), indicating that NR3C2 inhibits the Akt pathway in NSCLC.

4. Discussion

Small cell lung cancer and non-small cell lung cancer (NSCLC) are the two primary kinds of lung cancer that are distinguished by histology. About 80% of cases of lung cancer are NSCLC, making it the most common subtype [6]. Understanding the molecular pathways underlying NSCLC development is essential for the development of effective therapies and the identification of novel biomarkers. In recent decades, several key regulators involved in cancer cell proliferation, glycolysis and apoptosis in NSCLC have been identified. For example, overexpression of Ring Finger Protein 180 (RNF180) has been shown to reduce the tumorigenicity of NSCLC cells *in vivo* and inhibiting their growth and glycolytic activity [7]. Additionally, Ubiquitin Specific Peptidase 7 (USP7) has been reported to increase the glycolysis and survival of NSCLC cells [8].

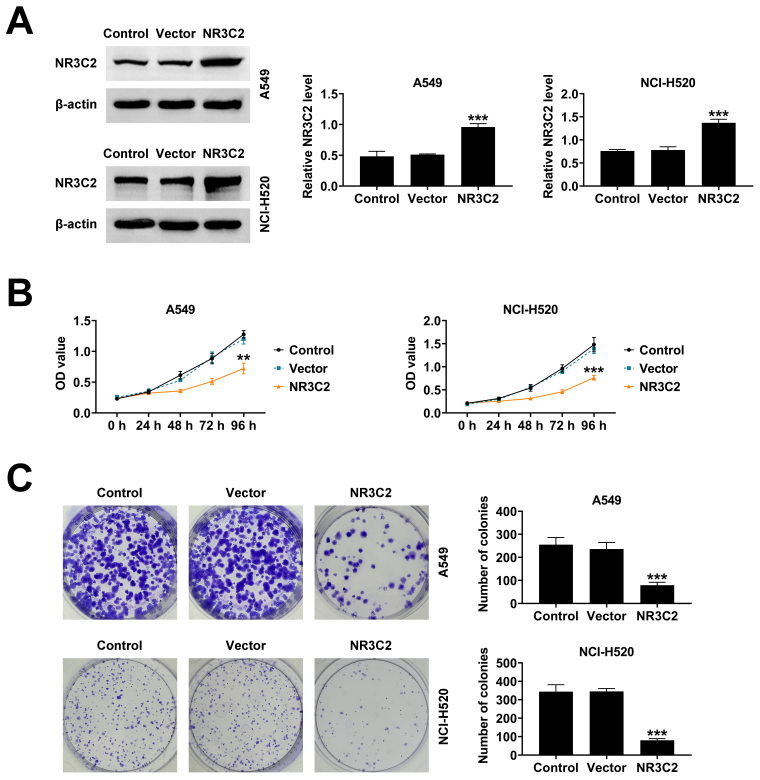

FIGURE 2. NR3C2 inhibits the growth of NSCLC cells. (A) Western blot analyses of NR3C2 protein expression level in cells after transfection with the NR3C2 overexpression plasmid. (B) A549 and NCI-H520 cell viability effects of NR3C2 as identified by CCK8. (C) Quantification of colony formation by crystal violet staining. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control. NR3C2: Nuclear Receptor Subfamily 3 Group C Member 2; OD: Optical Density.

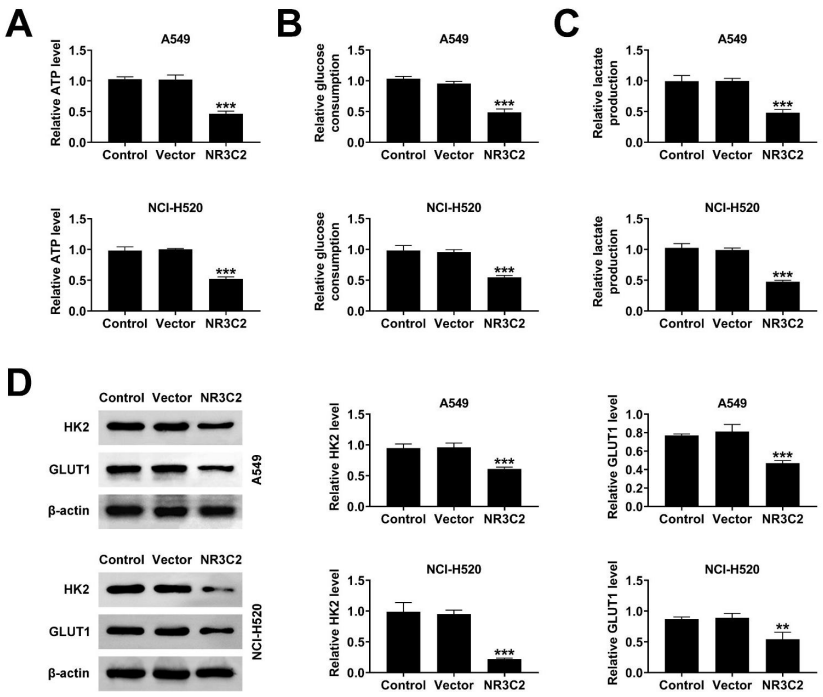

FIGURE 3. NR3C2 suppresses glycolysis in NSCLC cells. (A) A549 and NCI-H520 cells' ATP levels were examined using an ATP kit. (B) Glucose colorimetric kit was used to detect the effect of NR3C2 on glucose consumption. (C) Lactate detection kit was used to detect the effect of NR3C2 on lactate levels. (D) Analysis of NR3C2's impact on HK2 and GLUT1 protein expression using Western blot. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control. NR3C2: Nuclear Receptor Subfamily 3 Group C Member 2; ATP: adenosine triphosphate; HK2: hexokinase2; GLUT1: Glucose transporter 1.

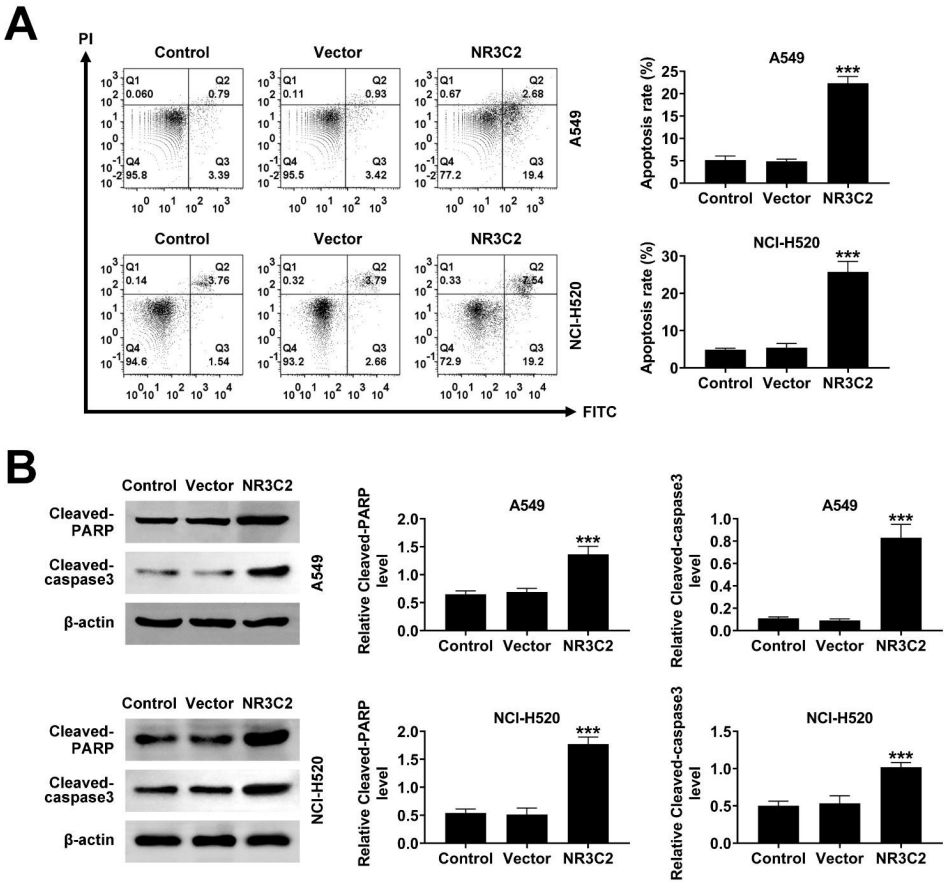

FIGURE 4. NR3C2 promotes apoptosis of lung cancer cells. (A) Effect of NR3C2 on the apoptosis rate of A549 and NCI-H520 cells detected by flow cytometry. (B) Western blot analyses was used to detect the protein expressions of cleaved-PARP, cleaved-caspase3. ***$p < 0.001$ *vs*. control. NR3C2: Nuclear Receptor Subfamily 3 Group C Member 2; PI: Propidium Iodide; FITC: Fluorescein; PARP: Poly ADP-ribosepolymerase.

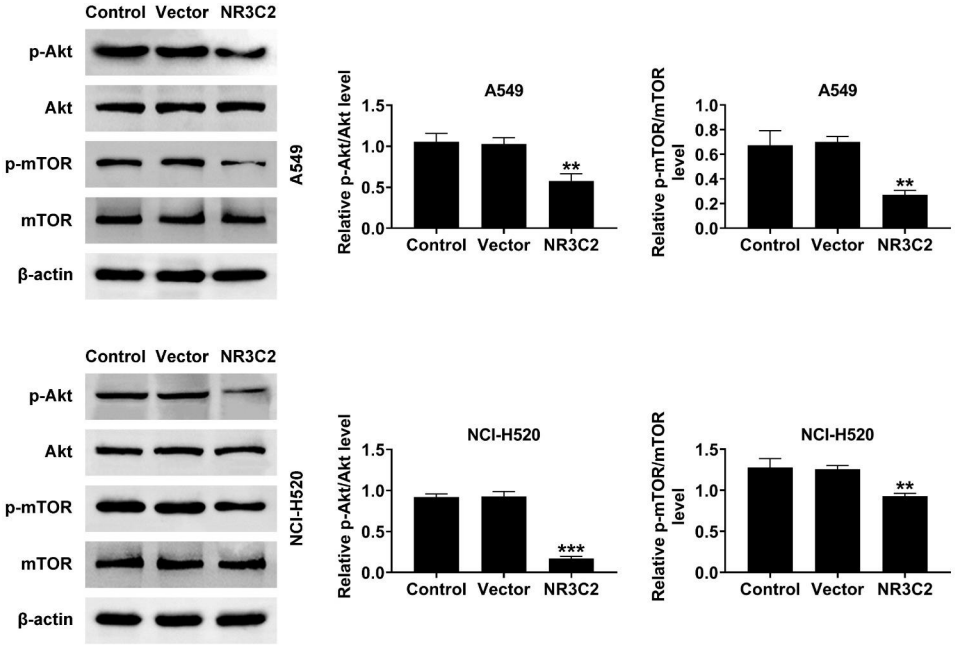

FIGURE 5. NR3C2 modulates the Akt pathway in NSCLC cells. Western blot analyses was used to detect the protein expressions of p-Akt, Akt, p-mTOR and mTOR in A549 and NCI-H520 cells. **$p < 0.01$, ***$p < 0.001$ *vs*. control. NR3C2: Nuclear Receptor Subfamily 3 Group C Member 2; mTOR: mammalian target of rapamycin; Akt: Protein kinase B.

Recent studies have shown that NR3C2 is implicated in the malignant progression of various human cancers. In oral squamous cell carcinoma, NR3C2 expression is downregulated, and regulating its expression can affect tumor growth, colony formation, invasion, and migration [9]. Targeting NR3C2, which is expressed at low levels in liver cancer, has been shown to inhibit the proliferation and migration of liver cancer cells [10]. This investigation revealed that NSCLC tissues have decreased NR3C2 expression and that NR3C2 could cause cell apoptosis and inhibit proliferation in NSCLC cells.

Aerobic glycolysis, a hallmark of malignant tumors, is a complex process influenced by both genetic and epigenetic alterations. As the primary energy source for tumor cell metabolism, aerobic glycolysis plays a crucial role in the regulation of carcinogenesis [11]. Therefore, targeting glycolysis in NSCLC represents an effective strategy to inhibit tumor progression. In pancreatic cancer, patients with high NR3C2 expression in their tumor tissues showed reduced levels of HK1, HK2 and LDHA. NR3C2 inhibits the expression of these enzymes, thereby preventing glucose uptake and lactate production [12]. The results of this study showed that NR3C2 significantly reduced ATP levels, glucose consumption and lactate production in NSCLC cells. Additionally, NR3C2 inhibited the expression levels of glycolysis-related proteins GLUT1 and HK2, further confirming its role in suppressing glycolysis in NSCLC.

Research has shown that the Akt pathway promotes glucose uptake by activating GLUT1. HK2, one of the five known hexokinase isoforms, is a key glycolytic effector whose activity or expression are regulated by the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. HK2 catalyzes the phosphorylation of glucose, marking the initial committed step in glucose metabolism. It has been demonstrated that Akt activation increases HK2 expression and inhibiting HK2 can reduce lung tumor growth [13]. In line with these findings, the present study showed that NR3C2 overexpression inhibited the Akt pathway and the expression of downstream glycolysis-related factors, including HK2 and GLUT1.

Recent literature suggests that NR3C2 is a potential target for NSCLC. NR3C2 dramatically reduces the proliferative, invasive and migratory abilities of NSCLC tumor cells [14]. Consistent with these studies, this study confirmed that NR3C2 inhibits the proliferation of NSCLC cells. In addition, this study also confirmed that NR3C2 suppresses glycolysis of NSCLC cells and induces apoptosis, offering promising targets and data for potential targeted therapy in NSCLC. However, this study does have some limitations. Due to practical constraints, tumor tissue was not collected for testing NR3C2 expression, and animal experiments were not conducted. These limitations will be addressed in future research to strengthen and expand upon the findings of this study.

5. Conclusions

NR3C2 is lowly expressed in non-small cell lung cancer. Overexpression of NR3C2 inhibits the growth and glycolysis of non-small cell lung cancer cells and promotes cell apoptosis, which may be related to its inhibition of the Akt pathway. This provides a potential therapeutic target for lung cancer research.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

YQG—designed the study and carried them out. YQG, YG, XYS—supervised the data collection; prepared the manuscript for publication and reviewed the draft of the manuscript. YQG, YG—analyzed the data; interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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