Article

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Gefitinib Induces Apoptosis in NSCLC Cells by Promoting Glutaminolysis and Inhibiting the MEK/ERK Signaling Pathway

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Background: Over 80% of lung cancer cases constitute non-small cell lung cancer (NSCLC), making it the most prevalent type of lung cancer globally and the leading cause of cancer-related deaths. The treatment of NSCLC patients with gefitinib has demonstrated promising initial efficacy. However, the underlying mechanism remains unclear. This study aims to investigate how gefitinib affects the mitogen-activated protein kinase kinase (MEK)/extracellular regulated protein kinases (ERK) signaling pathway-mediated growth and death of NSCLC cells.
Methods: In this study, the NSCLC cell line A549 was cultured *in vitro* and divided into a control group and a gefitinib group. The viability of the A549 cells was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Flow cytometry was employed to detect apoptosis in A549 cells, and the expression of glutamate dehydrogenase (*GDH1*) mRNA in these cells was determined using real-time quantitative PCR (RT-PCR). Western blotting was utilized to evaluate the protein expression levels of key components in the MEK/ERK signaling pathway, including phospho-MEK1/2, MEK1/2, phospho-ERK1/2, and ERK1/2. Additionally, intracellular glutamine content in A549 cells was measured using a colorimetric method.
Results: In contrast to the control group, the proliferation of A549 cells, the transcription level of glutamate dehydrogenase (*GDH1*), the intracellular glutamine content, and the protein expression levels of phospho-MEK1/2 and phospho-ERK1/2 were significantly lower in the gefitinib group. Moreover, apoptosis markedly increased.
Conclusions: Gefitinib expedites apoptosis and diminishes proliferation in the NSCLC cell line A549 by downregulating the epidermal growth factor receptor (EGFR)/MEK/ERK signaling pathway. This effect is accomplished by fostering the expression of *GDH1* to augment glutaminolysis in A549 cells.

Keywords: gefitinib; MEK/ERK pathway; glutaminolysis; NSCLC; apoptosis

Introduction

Lung cancer stands as the most widespread malignant tumor worldwide, bearing the highest burden of cancer-related fatalities [1]. Non-small cell lung cancer (NSCLC) constitutes the predominant subtype, accounting for roughly 85% of cases [2]. Epidemiological studies indicate that epidermal growth factor receptor (*EGFR*) mutations are detectable in approximately 30–40% of NSCLC patients [3].

EGFR serves as a key regulator of cell proliferation, survival, and metabolism [4]. Currently, the primary treatment option for EGFR-mutated NSCLC patients is EGFR-Tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib, crizotinib, afatinib, and AZD9291, which effectively inhibit cancer cell growth, enhance patient quality of life, and prolong survival [5]. Among these options, gefitinib is widely used as a first-line therapy for EGFR-mutated NSCLC patients; nevertheless, the evolution of drug resistance limits its clinical usefulness. Despite the development of second- or third-generation tyrosine kinase inhibitors, drug resistance remains a significant obstacle in NSCLC treatment. Numerous researchers have attempted to address this issue and proposed various solutions; however, the outcomes have fallen short of expectations [6–8]. This leads us to contemplate whether resolving drug resistance necessitates tracing its origins. In addition to investigating classical resistance mechanisms, should we explore alternative pathways?

The energy metabolism of tumor cells has been a prominent subject of research due to its crucial role in tumor growth and metastasis, which heavily relies on adaptive cellular metabolism [9]. In response to the demanding tumor microenvironment, cancer cells modify their metabolic processes to fulfill their unique energy needs. In addition to glucose, tumor cells also require glutamine as a nourish-

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ment agent. When malignant cells experience glutamine deprivation after cell death, this condition is referred to as "glutamine addiction" because the tumor cells rely more on glutamine to meet their energy demands for development [10,11]. Given that glutamine metabolism represents a metabolic pathway that may be effectively targeted, it emerges as a promising target for cancer treatment [12]. In many malignancies, glutamine metabolism, a significant part of glutamine metabolism, is also essential for cell proliferation, signaling, and metabolism in cancer cells [13]. Glutamate dehydrogenase (GDH1) stands out as a key enzyme involved in glutamine metabolism [14]. This enzyme facilitates the entry of glutamine into the tricarboxylic acid cycle, producing ATP and NADPH while also regulating reactive oxygen species levels and glutathione homeostasis [15].

Glutamine, boasting the highest concentration among amino acids in the bloodstream, serves as a supplier of nitrogen and carbon for cells [16,17]. Engaged in various biological activities, it contributes to cellular redox balance, protein synthesis, purine synthesis, and the citric acid cycle [18]. Metabolic analysis of lung cancer tissues has revealed elevated levels of glutathione (GSH) compared to healthy lung tissues [19]. This GSH accumulation enhances the superantioxidant capacity of malignant cells and plays a pivotal role in their sensitivity to radiotherapy and chemotherapy [20]. The increased glutamine metabolism in cancer cells leads to the build-up of related metabolites capable of activating downstream signaling pathways of EGFR, including the mechanisms of rapamycin, extracellular regulated protein kinases (ERK)1/2, and signal transducer and activator of transcription 3 (STAT3) [10,17,21]. This phenomenon significantly contributes to reducing the sensitivity of NSCLC patients to gefitinib. Based on these findings, it is speculated that limiting glutamine intake could enhance the sensitivity of NSCLC cells to gefitinib and impede the development of drug resistance.

Materials and Methods

Cell Culture

The A549 cells were procured from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in Ham's F-12K medium (21127022, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (FSD500, Excell Bio, Suzhou, China) and 1% penicillin-streptomycin solution (C0222, Beyotime, Shanghai, China). Regular testing for short tandem repeat (STR) identification and mycoplasma contamination was conducted on all cells. The results confirmed the absence of mycoplasma contamination.

Cell Proliferation Assay

To assess the effect of gefitinib on the viability of NSCLC, A549 cells were treated with either dimethyl sulfoxide (DMSO, HY-Y0320, MedChemExpress, Monmouth Junction, NJ, USA) as a control or gefitinib at a final concentration of 25 µmol/L (ZD1839, MedChemExpress, Monmouth Junction, NJ, USA) for 24 hours. Cell viability was determined using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (C0009S, Beyotime, Shanghai, China), and the optical density of the solution was measured at a wavelength of 570 nm using a microplate reader (Berthold, Württemberg, Germany).

Cell Apoptosis Assay

To assess the effect of gefitinib on apoptosis in NSCLC, A549 cells were treated with either DMSO or a final concentration of 25 µmol/L gefitinib. After 24 hours, 3×10^5 cells were collected, quantified using an Annexin V-FITC kit (C1062L, Beyotime, Shanghai, China), and subjected to flow cytometric analysis (Invitrogen, Carlsbad, CA, USA).

Western Blot Analysis

To investigate the mitogen-activated protein kinase kinase (MEK)/ERK signaling pathway, A549 cells were treated with a final concentration of 25 µmol/L gefitinib or DMSO at specific time points. Total protein was extracted from each group, and the amount of protein was determined using a BCA protein reagent kit (WB6501, NCM Biotech, Suzhou, China). The target proteins were detected using the following primary antibodies: MEK1/2 (1:1000; #8727), phospho-MEK1/2 (1:1000; #9154), and phospho-ERK1/2 (1:2000; #8544) purchased from CST; ERK1/2 (1:2000; #ab17942) and β-actin (1:2000; #ab8226) obtained from Abcam, Cambridge, UK. The ratio of the target band to the β-actin band was used to quantify the level of protein expression.

Real-Time Quantitative PCR for GDH1 Levels

To evaluate glutamine metabolism, we examined *GDH1* gene expression. Total RNA was extracted from A549 cells using TRIzol reagent (9108, Takara, Osaka, Japan). Subsequently, reverse transcription and amplification were conducted using real-time quantitative PCR (RT-PCR), and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Table 1.

Table 1. Primers used in RT-PCR.

Primers	Sequence (5′→3′)	
GDH1	Forward	AGTTCCAAGACAGGATATCGGG
	Reverse	TCAGGTCCAATCCCAGGT
GAPDH	Forward	GGTCTCCTCTGACTTCAACA
	Reverse	GTGAGGGTCTCTCTCTTCCT

RT-PCR, real-time quantitative PCR; *GDH1*, glutamate dehydrogenase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

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Glutamine Levels were Detected by Colorimetry

To assess glutamine metabolism, we measured intracellular glutamine levels. 1×10^6 A549 cells were harvested and homogenized in 0.2 mL of physiological saline solution. The homogenate was then centrifuged at 10,000 ×g for 15 minutes at 4 °C, and the supernatant was collected. Subsequently, the collected supernatant was filtered through a 50 kD filtration tube via centrifugation. The filtrate was collected, and the optical density of each well was measured at a wavelength of 450 nm using a microplate reader (Berthold, Württemberg, Germany).

Data Analysis

GraphPad Prism 9 (version 9.5.0) software (GraphPad Software, Inc., San Diego, CA, USA) was utilized to perform *t*-tests for data comparisons. The data were represented using the mean and standard deviation (SD). A statistically significant result was defined as $p < 0.05$.

Results

Gefitinib Inhibits the Proliferation of A549 Cells

MTT assays revealed a significant reduction in cell viability among A549 cells treated with gefitinib compared to the control cells (Fig. 1), indicating that gefitinib inhibited *in vitro* cell proliferation.

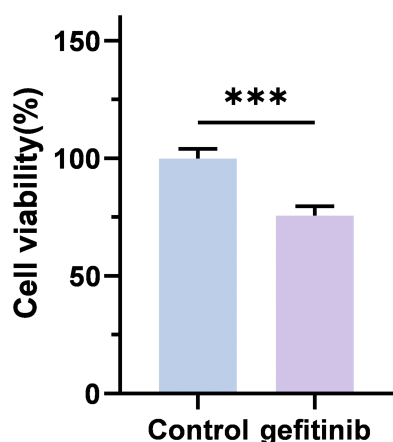

Fig. 1. Effect of gefitinib on the viability of A549 cells. A549 cells were treated with gefitinib (25 µmol/L) for 24 hours. ***$p < 0.001$.

Gefitinib Promotes Glutamine Decomposition in A549 Cells

Next, we examined the effect of gefitinib on glutamine metabolism. A549 cells treated with gefitinib exhibited a significant reduction in intracellular glutamine content (Fig. 2A) and a notable increase in *GDH1* transcript levels (Fig. 2B) compared to those in the control group. In cancer cells, glutamine serves as a crucial substrate that is converted into α-ketoglutarate (α-KG), subsequently entering the tricarboxylic acid (TCA) cycle. This metabolic process provides cancer cells with carbon and nitrogen sources for energy production while maintaining cellular redox balance and facilitating fatty acid synthesis [22,23]. GDH1 acts as the primary enzyme in cancer cells, converting glutamate to α-KG, thereby enabling the entry of glutamine-derived carbon into the TCA cycle [24]. These findings suggest that gefitinib promotes glutamine metabolism in A549 cells.

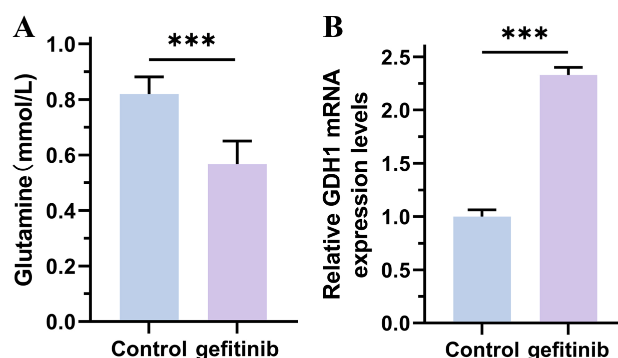

Fig. 2. Effect of gefitinib on glutamine decomposition in A549 cells. A549 cells were treated with gefitinib (25 µmol/L) for 24 hours. Glutamine (A) was quantified using a Glu assay kit. ***$p < 0.001$. Glutamate dehydrogenase (*GDH1*) expression (B) was determined through real-time quantitative PCR (RT-PCR) analysis.

Gefitinib Inhibits the MEK/ERK Signaling Pathway in A549 Cells

The downstream gene *GDH1* of the MEK/ERK signaling pathway enhances cellular proliferation, which significantly contributes to the progression of NSCLC [25]. Therefore, we investigated the potential involvement of the MEK/ERK signaling pathway in the antitumor effects of gefitinib. Western blot analysis revealed lower expression levels of phosphorylated MEK1/2 and ERK1/2 following gefitinib treatment (Fig. 3A–E). These results indicate that gefitinib inhibits the MEK/ERK signaling pathway and impedes the growth of NSCLC cells.

Gefitinib Promotes Apoptosis of A549 Cells

Evading apoptosis is a critical hallmark of cancer, promoting cellular proliferation [26]. Therefore, it is essential to examine the apoptotic status of human NSCLC cells following gefitinib treatment. Flow cytometry was performed to assess the rate of apoptosis. The results indicated a significantly higher percentage of apoptotic A549 cells in

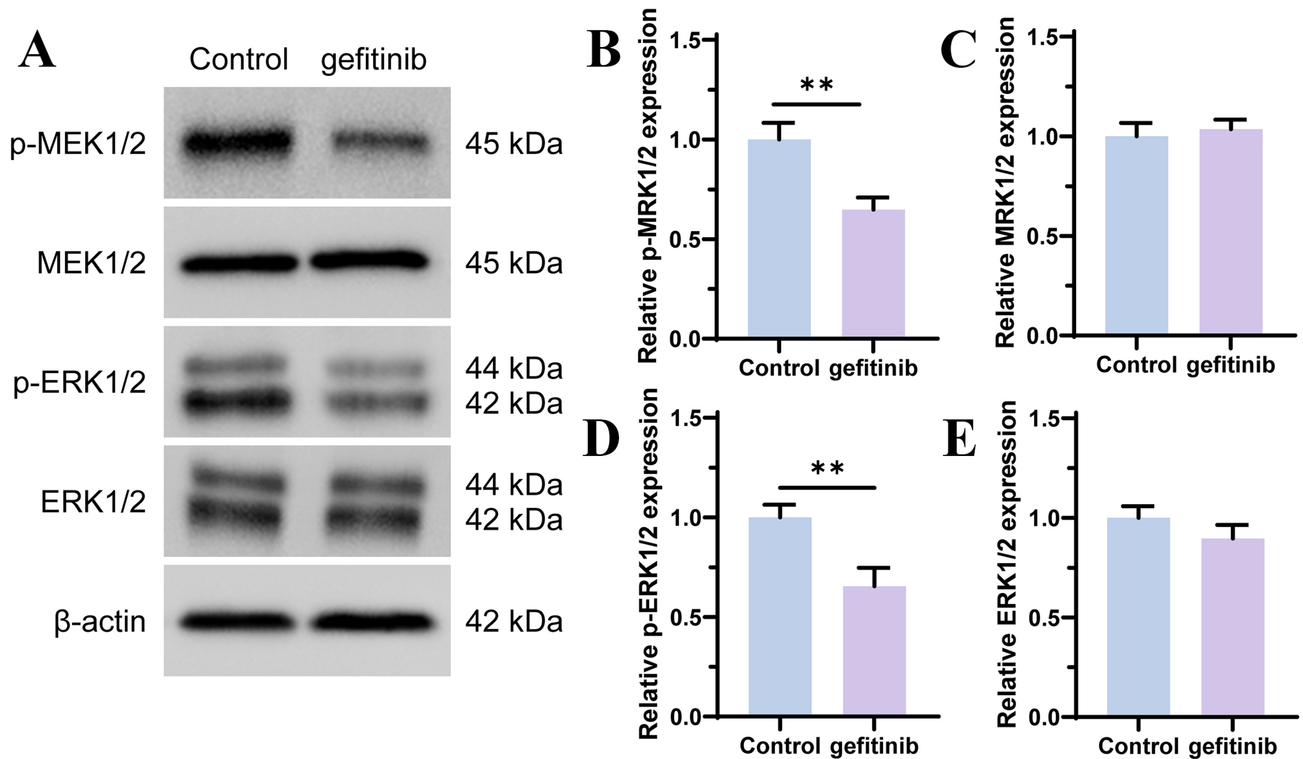

Fig. 3. The effect of gefitinib on MEK1/2, phospho-MEK1/2, ERK1/2, and phospho-ERK1/2 expression in A549 cells. A549 cells were treated with gefitinib (25 µmol/L) for 24 hours. phospho-MEK1/2 (A,B), MEK1/2 (A,C), phospho-ERK1/2 (A,D), and ERK1/2 (A,E) were detected via western blotting. $**p < 0.01$. MEK, mitogen-activated protein kinase kinase; ERK, extracellular regulated protein kinases.

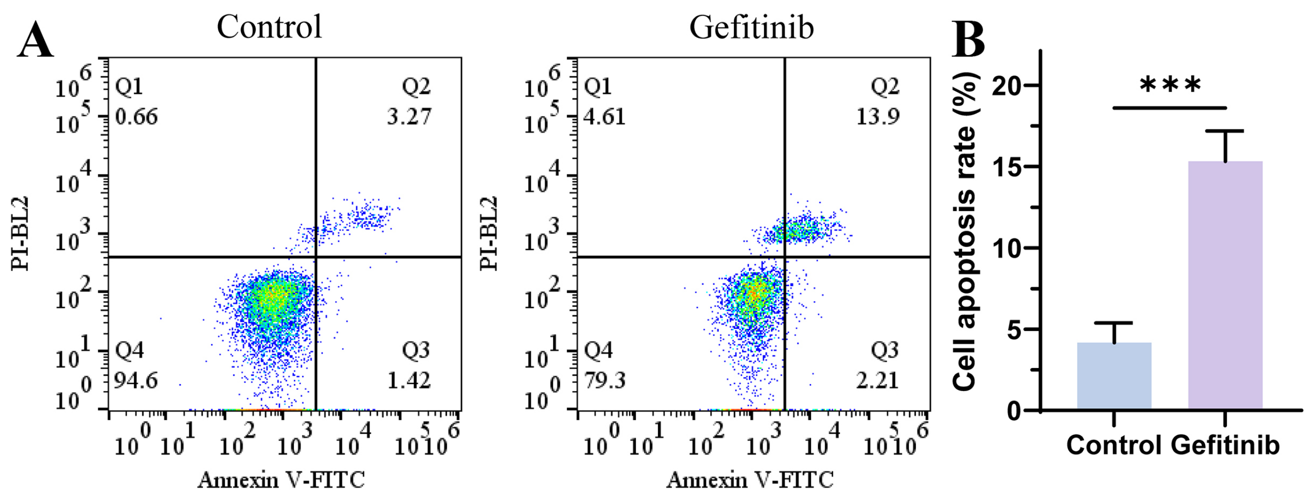

Fig. 4. The effect of gefitinib on apoptosis in A549 cells. A549 cells were treated with gefitinib at a concentration of 25 µmol/L for 24 hours. Flow cytometry analysis was performed to measure cell apoptosis (A,B) in the respective experimental groups. $***p < 0.001$.

the gefitinib-treated group compared to the control group (Fig. 4A,B). These findings underscore the ability of gefitinib to induce apoptosis in NSCLC cells.

Discussion

A growing body of research has highlighted the close connection between aberrant tumor metabolism and tumor cell resistance [27]. In addition to glucose, tumor cells also require glutamine as a vital source of nourishment. When malignant cells experience glutamine deprivation following cell death, it is termed "glutamine addiction" because tumor cells increasingly rely on glutamine to meet their energy demands for growth and development [10,11]. Lung cancer cells, in particular, become heavily reliant on glu-

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tamine to fulfill their energy requirements for rapid proliferation [28]. However, the precise mechanisms underlying the oncogenic alterations in glutamine metabolism remain unclear. In this study, we found that gefitinib inhibited glutamine uptake and induced apoptosis in NSCLC cells by suppressing the EGFR/MEK/ERK signaling pathway and promoting *GDH1* transcription.

One of the main reasons for resistance to gefitinib is the emergence of secondary mutations in EGFR and related signaling pathways. Among these pathways, the MEK/ERK signaling pathway stands out as a primary downstream pathway of EGFR, promoting tumor development and inhibiting apoptosis when activated [29]. In this study, we demonstrated that gefitinib has the capacity to inhibit NSCLC cell proliferation and induce apoptosis. Furthermore, we observed that gefitinib suppresses the phosphorylation of MEK1/2 and ERK1/2 in A549 cells.

GDH1, a downstream gene of the MEK/ERK signaling pathway, plays a crucial role in enhancing cellular proliferation, thereby significantly contributing to the progression of NSCLC [25]. Additionally, *GDH1* exhibits an antiapoptotic effect on lung cancer cell lines lacking LKB1, a gene essential for metastasis [30]. Loss of LKB1 in lung cancer patients is associated with tumor metastasis and shortened survival [30]. Knocking down *GDH1* has been shown to increase the sensitivity of LKB1-deficient cells, promote induction of tumor cell apoptosis, and reduce the potential for metastasis [30]. However, it's noteworthy that the GDH1 product, α-KG, preserves the apoptotic resistance of LKB1-deficient cells [30]. Our study revealed that gefitinib promotes *GDH1* transcription and intracellular glutamine decomposition in A549 cells.

Conclusions

In conclusion, our cellular experiments demonstrated that gefitinib inhibits the growth of NSCLC cells and promotes their apoptosis. The anticancer effect of gefitinib is associated with the suppression of the EGFR/MEK/ERK signaling pathway and the enhancement of *GDH1* transcription and intracellular glutamine decomposition.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

YKZ, HXL, XYL and LQL designed the study and performed the experiments. YKZ and LQL collected the data. HXL and XYL analyzed the data. YKZ, HXL and XYL prepared the manuscript. All authors participated in drafting the manuscript and critically revising it for important intellectual content. All authors read and approved the final manuscript. All authors are responsible for all aspects of the work to ensure that questions relating to the accuracy or completeness of any part of the work are appropriately addressed.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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