

# Sijunzi Tang Enhances The Sensitivity of Lung Cancer Cells To Gefitinib Based On Glutamine Metabolism

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## Research

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# Abstract

**Background:** Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) has been the major bottleneck that limits the long-term clinical efficacy. Therefore, the exploration of novel strategies for the treatment of non-small cell lung cancer (NSCLC) is urgent. Sijunzi Tang (SJZ) has been usually used as a complementary therapy for cancer patients that can prolong their overall life. Nevertheless, its underlying mechanism when combined with EGFR-TKIs is not clear.

**Methods:** Here, the anti-tumor activity was evaluated by determining cell viability by using the Cell Counting Kit-8 (CCK-8). A high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method was established to simultaneously quantify nine metabolites in the glutamine metabolic pathway, such as glutamine, glutamate,  $\alpha$ -ketoglutaric acid, fumarate, succinate, citrate, *cis*-aconitate, alanine, and malate in PC-9/GR cells.

**Results:** The results showed that SJZ could enhance the sensitivity of lung cancer cells to gefitinib. The content of glutamine and glutamate in the SJZ/gefitinib group was significantly lower than that in the gefitinib group, while the concentration of other metabolites did not change significantly.

**Conclusion:** In summary, SJZ can reverse gefitinib resistance by modulating glutamine metabolism. Our results suggested that SJZ might be a potential resistance reversal herbal medicine and combined with gefitinib might be a promising strategy for the therapy of lung cancer. Also, the research will provide novel insights for the study of the mechanisms on Traditional Chinese medicine (TCM) prescriptions-reversed resistance of EGFR-TKIs, and scientific basis for the combined treatment of lung cancer with TCM and EGFR-TKIs.

## 1 Introduction

Lung cancer ranks as a leading cause of cancer-related deaths worldwide in the light of high mortality and poor survival, and most patients (84%) suffer from non-small cell lung cancer (NSCLC) <sup>1,2</sup>. The current practices recommend gefitinib as first line treatment to advanced NSCLC patients with an activated epidermal growth factor receptor (EGFR) mutation. Gefitinib, one of the first-generation EGFR tyrosine kinase inhibitors (TKIs), selectively and reversibly blocks the intracellular EGFR signaling pathway to suppress the growth, metastasis, and angiogenesis of tumors. Initially, the unprecedented survival benefits of gefitinib are impressive. While most of the patients treated with gefitinib ultimately develop acquired resistance after 9 to 14 months of treatment, rendering the overall survival of patients unsatisfactory <sup>3</sup>. The inevitable barrier limits the effectiveness of gefitinib and remains a pervasive challenge for long term treatment. For this reason, it is necessary for the development of new treatment strategies to overcome gefitinib resistance.

As a highly heterogeneous disease, NSCLC has many patterns of gene expression that are not dependent on a single signaling pathway. Several molecular mechanisms of gefitinib resistance have been identified in the past few decades, including secondary mutation of EGFR (T790M mutation), mesenchymal-

epithelial transition factor (MET) amplification, hepatocyte growth factor (HGF) overexpression, and genetic alterations<sup>4</sup>. However, in up to approximately 30% of cases, the mechanisms are still unexplained. It has been reported that glutamine metabolism is related to many oncogenes and tumor suppressors<sup>5-7</sup>. As confirmed in an emerging study, gefitinib is a potent inducer of cancer cell death by inhibiting the utilization of glutamine and reducing the production of Adenosine triphosphate (ATP) and glutathione (GSH) in PC-9 cells, but gefitinib has no effect on the production of ATP and GSH in gefitinib-resistant PC-9 cells, which even can use glutamine for proliferation<sup>8</sup>. The researchers speculated that restricting glutamine metabolism might be a potential strategy to delay gefitinib resistance.

In recent years, Chinese medicine has been considered as an alternative and complementary therapy in cancer treatment with approved curative effects, which effectively delay progression, extend survival, and reduce adverse reactions in cancer patients<sup>9-11</sup>. Si Jun Zi Tang (SJZ), a four-herb Chinese medicine formula, was first recorded in the "Prescriptions People's Welfare Pharmacy". SJZ is obtained by decocting *Panax ginseng* C.A.Mey., *Atractylodes macrocephala* Koidz., *Poria cocos* (Schw.) Wolf and *Nardostachys jatamansi* DC in an appropriate ratio<sup>1</sup>. It has been reported that SJZ plays a crucial role in preventing tumor recurrence and metastasis, improving life quality, and prolonging the overall survival time<sup>12</sup>. In our previous study, it has been confirmed that SJZ significantly enhances the inhibitory effect of gefitinib on tumor growth and metastasis in the Lewis lung carcinoma (LLC) mice model<sup>13</sup>. In addition, our results suggest that SJZ might enhance the sensitivity of gefitinib via the regulation of corresponding metabolites. However, the specific mechanism requires further elucidation.

Therefore, we propose that gefitinib resistance could be reversed by SJZ through the glutamine metabolism pathway. The cell viability was determined using the cell counting kit-8 (CCK-8) assay. A high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) method was established and validated to determine the alterations of metabolites in the glutamine metabolism pathway.

## 2 Materials And Methods

### 2.1 Chemicals and reagents

Methanol, acetonitrile, and formic acid (Fisher Scientific, Loughborough, UK) were used HPLC-grade. Ultrapure water was prepared by the Milli-Q plus ultrapure water system (Millipore, Bedford, USA). Alanine was obtained from National Institutes for Food and Drug Control (Beijing, China). Glutamine, glutamate, and  $\alpha$ -ketoglutaric acid were obtained from Shanghai Aladdin Bio-Chem Technology Co., LTD (Shanghai, China). Fumarate, succinate, citrate, *cis*-aconitate, and malate were obtained from Harveybio (Beijing) Gene Technology Co., LTd (Beijing, China). L-Glutamic Acid-d5 (Glu-d5) was purchased from Toronto Research Chemicals (Toronto, Canada). Phosphate buffer saline (PBS) was acquired from BasalMedia (Shanghai, China). Fetal bovine serum (FBS), DMEM culture medium, penicillin, and streptomycin were obtained from Gibco (Gibco, USA).

## 2.2 Cell culture

PC-9 acquired gefitinib-resistant (PC-9/GR) cells were purchased from Hunan Fenghui Biotechnology Co., Ltd. (Hunan, China), which were cultured in DMEM medium (Gibco, USA), which contains 800 ng/mL gefitinib with the 10% (v/v) FBS. In addition, 100 U/mL penicillin and 100 g/mL streptomycin (Beijing Dingguo Co., Ltd., Beijing, China) are also necessary, and then placed at a temperature of 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

## 2.3 Cell viability assay

The inhibitory effects of gefitinib on cell proliferation were evaluated by CCK-8 (Dojindo, Kumamoto, Japan). PC-9/GR were cultured in 96-well plates at a density of  $1 \times 10^4$  cells per well for 24h and then treated with gefitinib and/or SJZ at the indicated concentrations. After incubating for 48 h, the cells were washed twice with PBS and incubated with CCK-8 working solution for 1 h at 37°C, according to the manufacturer's protocol. Through iMark™ Microplate Absorbance Reader (Molecular Devices, Sunnyvale) measuring absorbance at 450 nm.

## 2.4 Standard stock solutions, calibration standards, and quality control samples

Glutamate, glutamine, alanine, *cis*-aconitate,  $\alpha$ -ketoglutaric acid, fumarate, succinate, citrate, malate, and glu-d5 were dissolved in 0.1% formic acid in water to obtain a concentration of 5 mg/mL standard stock solutions. Putting stock solutions of all analytes and IS at -20°C for further study. The working solutions of four analytes such as glutamate, glutamine, alanine, and *cis*-aconitate were determined in ESI+ mode, and the other working solution of five analytes such as  $\alpha$ -ketoglutaric acid, fumarate, succinate, citrate, and malate were measured in ESI- mode. Stock solutions of the analytes were prepared by step wisely diluting with cell extraction solvent to generate the calibration standards at 6-12 different concentrations. For the investigation of precision and stability, quality control (QC) samples were prepared by using separate aliquot of the stock solution in accordance with the same way but independently from the calibrators. The levels of lower limits of quantification (LLOQ), and QCs were shown in Table 2. All standard and QC solutions contain and all measured samples contain internal standard (IS) (173.2 ng/mL). All of them were consisted of methanol-water (80:20, v/v).

## 2.5 Sample collection and preparation

PC-9/GR cells were seeded in a six-well culture plate (Corning) at a density of  $5 \times 10^5$  cells/well. Harvesting the cells when the cells are 80-90% confluence, discarding the medium, and washing twice with cold PBS, then using liquid nitrogen quenched. Cells were lysed with cold extraction solvent and by using a cell scraper to harvest. Transfer the extract to a 1.5 mL test tube (from Eppendorf) and place it in a refrigerator at -80°C for later use. Centrifuge the mixture at 14,000 rpm (4°C, 15 minutes) to remove cell

debris and collect the supernatant. Transfer the supernatant to a vial for analysis and aliquot and store it at -80°C. The content of each analyte is quantified by the protein concentration of the cell pellet.

## 2.6 Chromatography and mass spectrometry conditions

A Triple Quad™ 4500 Triple Quadrupole Tandem Mass Spectrometer (SCIEX, Japan) with an electrospray ionization source (ESI). The specific detection of analytes is achieved through multiple reaction monitoring (MRM). MS/MS method development is accomplished by injecting a standard solution (1 µg/mL) directly into the mass spectrometer. The tuning parameters of the ion spray voltage are 5500 V (ESI+), -4500 V (ESI-); other tuning parameters are the same as the positive and negative ion modes, and the parameters are as follows: temperature, curtain gas, ion source gas 1 (atomizer) and 2 (Turbo ion spray) were set to 550°C, 25 psi, 55 psi respectively. The specific precursor/product ion pairs, collision energy (CE), and declustering potential (DP) of the analyte are shown in Table 1. Liquid chromatography was carried out on a liquid chromatography system (SCIEX, Japan). Chromatographic retention was achieved on an ACQUITY HSS T3 column (2.1 × 100 mm, 1.8 µm) with a column temperature at 40°C. The flow rate was 0.30 mL/min. Solvent A in the mobile phase is composed of 0.1% formic acid in water, and solvent B is composed of 0.1% formic acid in acetonitrile. The total elution time of positive ionization mode was 11 min and the gradient was as follows: 0-0.5 min, 0% B; 0.5-5 min, 0% - 10% B; 5-5.5 min, 10% - 95% B; 5.5-6.5 min, 95% B; 6.5-6.9 min, 95% - 0% B; 6.9-11 min, 0% B. The total elution time of negative ionization mode was 12 min and the gradient was as follows: 0-0.5 min, 0% B; 0.5-5 min, 0% - 10% B; 5-6 min, 10% - 95% B; 6-8 min 95% B; 8-8.5 min, 95% - 0% B; 8.5-12 min, 0% B. Each sample injection was 3 µL. All the above data acquisition and analysis are done by Analyst® software version 1.6.3 (SCIEX, Japan) and MultiQuant™ MD software version 3.0.3 (SCIEX, Japan).

## 2.7 Therapeutic drug treatments

After culturing the PC-9/GR cells for 24 hours, they were cultured in a medium containing Sijunzi tang, Gefitinib, Sijunzi tang/gefitinib and complete medium according to the aforementioned conditions for 48 hours, respectively. Then the cells are processed according to the previously described steps.

## 2.8 Method validation

The calibration curve of the analyte is obtained by plotting the relationship between the peak area ratio of each analyte to IS and the concentration of the analyte. Linearity is measured by the correlation coefficient (r). Using MultiQuant™ software (version 3.0.3) to integrate the peak area. Theoretically, the requirement of limit of detection (LOD) is 3. LOQ is defined as a S/N ratio of 10. LLOQ is the lowest concentration of an analyte that can be accurately quantified in a sample. Since the 9 analytes are endogenous, cell lysates are used as a biological matrix. The accuracy of QC samples is expressed as relative standard deviation (RSD). The stability is tested by evaluating the concentration change of analytes in the QC sample after preparation. The prepared QC sample was tested five times at room temperature for 12 hours, and then analyzed.

## 2.9 Statistical analysis

The experimental results were analyzed using Graphpad Primer 8.0 statistical software. One-way analysis of variance (one-way ANOVA) was used for the comparison of measurement data among multiple groups, and the t-test was used for comparison between the two groups. When  $P < 0.05$  indicates a significant difference, and  $P < 0.01$  indicates a very significant difference.

## 3 Results

### 3.1 SJZ enhanced the sensitivity of lung cancer cells to gefitinib

To investigate the effect of SJZ on reversing gefitinib resistance, choosing PC-9/GR cells as representative gefitinib-resistant lung cancer cells. After PC-9/GR cells were pretreated 48h with SJZ or gefitinib or a combination of them, the viability of cell lines was evaluated using CCK-8. Gefitinib and SJZ inhibited tumor cell growth in a dose-dependent manner as shown in Figure 1A and B. The  $IC_{50}$  values of gefitinib and SJZ on PC-9/GR cells were  $4.81 \mu\text{g}/\text{mL}$  and  $7.81 \text{mg}/\text{mL}$ , respectively. When the concentration of gefitinib was lower than  $0.780 \mu\text{g}/\text{mL}$ , more than 90% of the cell viability was not affected. Thus, three concentrations, 0.0159, 0.112, and  $0.780 \mu\text{g}/\text{mL}$  were used in the following experiments. Subsequently, as  $1.62 \text{mg}/\text{mL}$  SJZ combined with the above three concentrations of gefitinib, SJZ significantly enhanced the inhibitory effects of gefitinib on cell proliferation (Supplementary Figure S1). Furthermore, the results of JinZhengJun  $Q$  value analysis showed that  $0.0159 \mu\text{g}/\text{mL}$  gefitinib plus  $1.62 \text{mg}/\text{mL}$  SJZ had a synergistic inhibitory effect because  $Q$  value was 1.17.  $Q$  value was calculated using the JinZhengjun Method to evaluate the combination efficacy,  $Q > 1.15$ , named as synergy,  $Q = 0.85 \sim 1.15$ , named as an additive effect, and  $Q < 0.85$ , named as antagonism effect<sup>14</sup>. Interestingly, when the SJZ concentration was reduced to  $1.089 \text{mg}/\text{mL}$  combined with gefitinib,  $0.0159$  and  $0.112 \mu\text{g}/\text{mL}$  gefitinib had a synergistic inhibitory effect except for  $0.780 \mu\text{g}/\text{mL}$ , and the  $Q$  values were 1.26 and 1.29 (Figure 1C and D). In particular, we continued to reduce the concentration of SJZ to  $0.0159 \text{mg}/\text{mL}$  in the next experiment, which did not affect more than 90% of cell viability. The results demonstrated that  $0.0159$  and  $0.112 \mu\text{g}/\text{mL}$  gefitinib had a synergistic inhibitory effect except for  $0.780 \mu\text{g}/\text{mL}$  (Supplementary Figure S2).

### 3.2 Optimization of chromatography and mass spectrometry conditions

Methanol and acetonitrile are widely used in chromatographic separations. In our experiments, acetonitrile provided higher selectivity and elution strengths than methanol in the light of chromatographic retention mechanisms and behaviors.  $5 \text{mM}$  ammonium acetate resulted in a significant decrease in the analyte response and high ionic strength might lead to poor ESI performance and ion-ion suppression. Therefore, 0.1% formic acid was selected to improve ionization efficiency and optimize the flow rate and gradient elution to ensure symmetrical peak shapes. Each analyte and IS was first infused into the mass spectrometer and their molecule were used for method development. Using transitions to check the sensitivity and specificity of each analyte in biological samples. The highest

sensitivity of fumarate, succinate, citrate, malate,  $\alpha$ -ketoglutaric acid was achieved in ESI- mode, while glutamate, glutamine, alanine, *cis*-aconitate, and IS were acquired in ESI+ mode. Moreover, the fewer MRM transitions were detected at one time, the higher intensity of each MRM transition. The typical MRM chromatograms of 9 metabolites and IS are shown in Figure 2. All compounds were determined and analyzed to obtain specific and sensitive MRM transition ion pairs. In addition, CE and DP conditions were optimized to ensure each analyte had the highest sensitivity. Stable isotopelabeled IS(SIL-IS) was used to avoid ionization inhibition or enhancement of analytes in the complex biological matrix by ESI. The selected ion pairs and the optimal parameters for analyte detection (identified by Analyst® Software Version 1.6.3) are shown in Table 1.

Table 1  
Multiple reaction monitoring conditions of each analyte in the ESI+ and ESI- mode.

Analyte	Precursor ion (m/z)	Product ion (m/z)	CE (eV)	DP (Volt)	Ion mode
Glutamine	147.3	84.2	31	25	positive
Glutamate	148.2	84	31	24	positive
Alanine	90.2	44.1	22.06	13.98	positive
<i>Cis</i> -aconitate	175.1	138.9	21.02	13.43	positive
Glu-d5	153.2	88	26.13	22.14	positive
$\alpha$ -ketoglutaric acid	145	100.9	-31.31	-19.08	negative
Fumarate	115.1	71.2	-21.61	-17.81	negative
Succinate	116.8	72.9	-24.21	-21.73	negative
Citrate	190.9	111.1	-26.26	-20.76	negative
Malate	133	114.9	-28.03	-22.23	negative

### 3.3 Methods validation

The linearity, correlation coefficient, LLOQ, and LQC of quantitative compounds are shown in Table 2 and Table 3 respectively. The results showed that within a certain linear range, the correlation coefficients are all above 0.99, which means that the concentration has a good linear relationship with the peak area. The LLOQ was in the range of 8.5-4065ng/ml.

**Table2** Calibration range, linearity ( $R^2$ ), linear equation for analyses.

Analyte	Calibration range (ng/mL)	R <sup>2</sup>	Linear equation
Glutamine	122-24476	0.99421	y=1.05379x+0.06531
Glutamate	28-2788	0.99878	y=0.68609x+0.03642
Alanine	126-25140	0.99577	y=0.15034x+0.01507
<i>Cis</i> -aconitate	8.5-8670	0.99627	y=0.60309x+0.00133
α-ketoglutaric acid	131-6557	0.99358	y = 1.82426 x -0.16580
Fumarate	100-20119	0.99333	y = 1.24195 x + 0.01483
Succinate	39-7714	0.99312	y = 8.22447 x + 0.50098
Citrate	27-5476	0.99278	y=10.87762x+0.04339
Malate	167-8333	0.99027	y = 6.44047 x + 0.49843

**Table 3** The LLOQ and the three levels of QC for analytes of glutamine metabolism.

Analyte	Concentration (ng/mL)			
	LLOQ	LQC	MQC	HQC
Glutamine	122	367	11014	19580
Glutamate	28	288	836	2788
Alanine	126	377	11313	25140
<i>Cis</i> -aconitate	8.5	130	3901	6936
α-ketoglutaric acid	131	131	1311	6557
Fumarate	100	302	9054	16095
Succinate	39	116	3471	6171
Citrate	27	82	2464	4381
Malate	167	167	36589	65048

HQC, high quality control; LQC, low quality control; MQC, medium quality control

## 3.4 Application to the study of glutamine metabolism in lung cancer cells

The altered cellular metabolism was recently reconsidered as a core hallmark of cancer, which is also closely associated with drug resistance. The method has been developed to quantify the metabolites related to glutamine metabolism pathway in PC9/GR cells. The concentration changes of 9 metabolites were summarized in Figure 3. Compared with the model group and SJZ group, the concentration of glutamine and glutamate in PC-9/GR cells decreased in the SJZ combined with the gefitinib group, however, alanine and *cis*-aconitic acid had no significant change. Additionally, fumarate, succinate, citrate,  $\alpha$ -ketoglutaric acid had no significant change among the groups. Compared with the model group, the concentration of malate was lower in the combined with gefitinib group.

### Discussion

The experimental results reveal that SJZ can increase the sensitivity of PC-9/GR to gefitinib. Different concentrations of SJZ, the inhibition rate of PC-9/GR varies between 5.55-26.17%, combined with the resistance concentration of gefitinib, had a synergistic inhibitory effect on PC-9/GR because the CI values are all lower than 0.9. Moreover, as the concentration of SJZ decreased, the synergistic effect was stronger. The results indicated that the synergistic effect was the strongest under the condition that neither SJZ nor gefitinib affected the viability of more than 90% of cells.

A recent study showed that gefitinib resistance is mainly caused by glutamine related metabolic pathways when compared with gefitinib-sensitive cells<sup>15</sup>. Further studies have shown that gefitinib inhibits the utilization of glutamine in gefitinib sensitive cells and reduces the production of ATP and GSH, thereby inducing cancer cell death. However, gefitinib has no effect on the ATP and GSH of drug-resistant cells, and drug-resistant cells can use glutamine for proliferation. To further clarify how SJZ reverses gefitinib resistance, we measured PC-9/GR cells after administering SJZ, gefitinib, and SJZ/ gefitinib by HPLC-MS/MS technology. Changes in the content of related metabolites in the glutamine metabolic pathway. Based on this, we measured the content changes of 9 metabolites including glutamine. Related metabolites in the glutamine metabolic pathway are polar compounds, which have small reservations on the conventional column. We found that waters ACQUITY HSS T3 column (2.1×100 mm, 1.8 $\mu$ m) could achieve satisfactory separation, which is reliable in terms of determining the metabolites efficiently. Our research results show that the content of glutamine and glutamate in the combined administration group was significantly lower than gefitinib group, but there was no significant change compared with the model group; and when glutamine was converted to  $\alpha$ -ketoglutaric acid after entering the tricarboxylic acid (TCA) cycle, the content of fumarate, succinate, citrate, malate, *cis*-aconitate, and alanine did not change significantly among the administration groups, which suggests that SJZ is through down-regulation of glutamine and glutamate content to reverse gefitinib resistance. Glutamine is a non-essential amino acid. Studies have shown that the expression level of glutamine in lung cancer tissues increases, especially in NSCLC<sup>16</sup>. Glutamine makes many

contributions in redox homeostasis, mating energy, macromolecule synthesis, and cancer cell signaling<sup>17</sup>. In glioblastoma cells, increased glutamate levels can provide a fuel cycle for TCA<sup>18</sup>. Glutamine is catabolized by GLS1 to produce glutamate, and then glutamate is produced by the action of glutamate dehydrogenase or glutamate related aminotransferases, such as alanine aminotransferase and aspartate aminotransferase. Glutamate is converted to  $\alpha$ -ketoglutaric acid and  $\text{NH}_4^+$ ,  $\alpha$ -ketoglutaric acid provides a carbon source for the synthesis of various biological precursors in the TCA cycle, which are essential for the survival and proliferation of tumor cells. Although cancer cells performed obvious Warburg effect, they can still maintain a complete TCA cycle and gradually become more dependent on glutamine metabolism, which makes it possible for cancer cells to use TCA cycle intermediates as a precursor to biosynthetic pathway<sup>19,20</sup>. Most of the  $\alpha$ -nitrogen produced by the degradation of glutamine is secreted from the cell in the form of ammonia and alanine, and the  $\alpha$ -nitrogen will be mainly made use of maintaining the intracellular amino acid pool. The results of the study indicate that the amino group derived from glutamate secretion is necessary for the use of glutamine as a precursor for angioplasty and the production of NADPH<sup>19</sup>. By stimulating the decomposition of glutamine, transforming cancer cells become glutamine addiction to maintain strong cell proliferation., A higher ratio of glutamate to glutamine is associated with prolonging overall survival in breast cancer patients<sup>21</sup>. The enhanced glutamine metabolism pathway helps to increase the survival rate of cancer cells. Compared with the loss of glutamine catabolism ability, gefitinib-resistant cells still make use of glutamine as a response to gefitinib. In addition, when circulating glutamine levels were reduced, VM-M3 cells proliferation was significantly inhibited and long-distance transfer in vitro and in vivo was also inhibited<sup>22</sup>. In short, the reduction of glutamine level helps to inhibit cell growth, which also shows that SJZ can reverse the resistance of gefitinib by reducing the concentration of glutamine and increase the resistance of gefitinib-resistant cells to gefitinib. Taken together, our research shows that SJZ can reverse gefitinib resistance by reducing the concentration of glutamine and glutamate. SJZ regulates the changes in the content of glutamine and glutamate in the glutamine metabolism pathway. The recognition that alterations in glutamine metabolism plays a significant role in the development and progression of cancer has driven continuous efforts to exploit this metabolic changes to treat cancer<sup>23</sup>. However, how SJZ regulates the targets on the glutamine metabolism pathway to affect the content of glutamine and glutamate, thereby reversing gefitinib resistance, is still unclear. Therefore, the mechanism of how SJZ regulates the pathway of glutamine metabolism needs further study. This study will provide some inspiration for the treatment of gefitinib resistance in NSCLC patients, as well as strategies to reverse the resistance of targeted drugs.

## Conclusion

we found that SJZ can enhance the sensitivity of lung cancer cells to gefitinib. The content of glutamine and glutamate in the SJZ/gefitinib group was significantly lower than that in the gefitinib group, while the concentration of other metabolites did not change significantly. Therefore, SJZ can reverse gefitinib resistance by modulating glutamine metabolism. Our findings will provide novel insights for the mechanisms study on Traditional Chinese medicine (TCM) prescriptions-reversed resistance of EGFR-

TKIs. These results may also have implications in exploration of novel therapeutic avenues of treating lung cancer.

## Abbreviations

EGFR-TKIs epidermal growth factor receptor tyrosine kinase inhibitors

NSCLC non-small cell lung cancer

SJZ Sijunzi Tang

CCK-8 Cell Counting Kit-8

HPLC-MS/MS high performance liquid chromatography-tandem mass spectrometry

TCM traditional Chinese medicine

LLC lewis lung carcinoma

PBS phosphate buffer saline

FBS fetal bovine serum

PC-9/GR PC-9 acquired gefitinib-resistant

QC quality control

LLOQ lower limits of quantification

ESI electrospray ionization source

CE collision energy

DP declustering potential

RSD relative standard deviation

S/N signal-to-noise ratio

IS internal standard

SIL-IS stable isotopelabeled IS

TCA tricarboxylic acid cycle

MET mesenchymal–epithelial transition factor

HGF hepatocyte growth factor

ATP adenosine triphosphate

GSH glutathione

MRM multiple reaction monitoring

HQC high quality control

LQC low quality control

MQC medium quality control

LOD limit of detection

## **Declarations**

### **Authors' contributions**

Zhihong Wang: Investigation, Data curation, Formal analysis, Methodology, Validation Writing – original draft, Visualization. Shiyuan Wang: Conceptualization , Writing - review & editing. Xia Liu: Resources, Formal analysis. Bo Cao: Formal analysis. Mingyu Zhang: Resources, Writing - review & editing. Chunyu Li and Guohui Li: Conceptualization, Supervision, Project administration, Writing - review & editing, Funding acquisition.

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### **Availability of data and materials**

All the data used to support the findings of this study are available from the corresponding author upon reasonable request.

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no competing interests.

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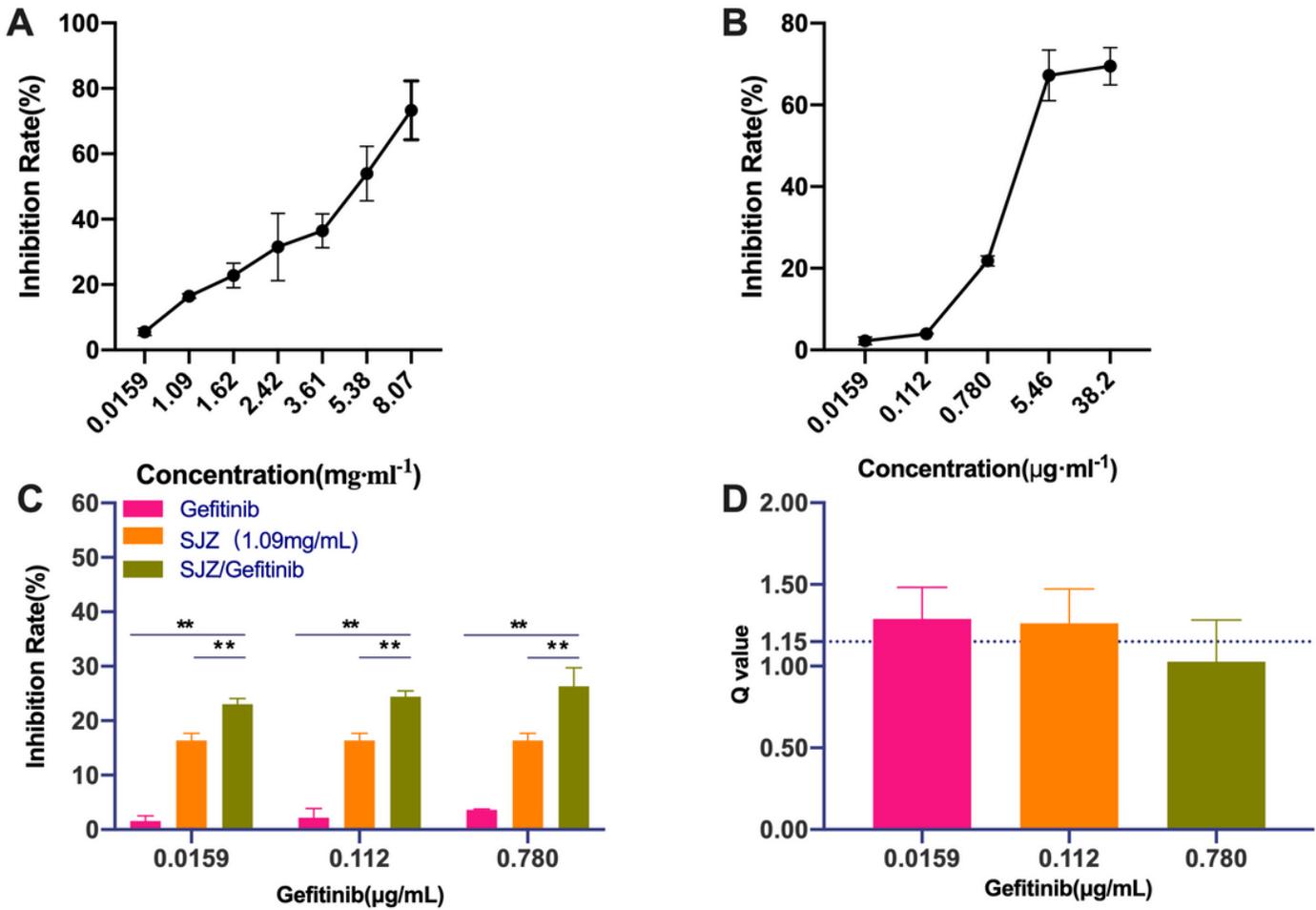
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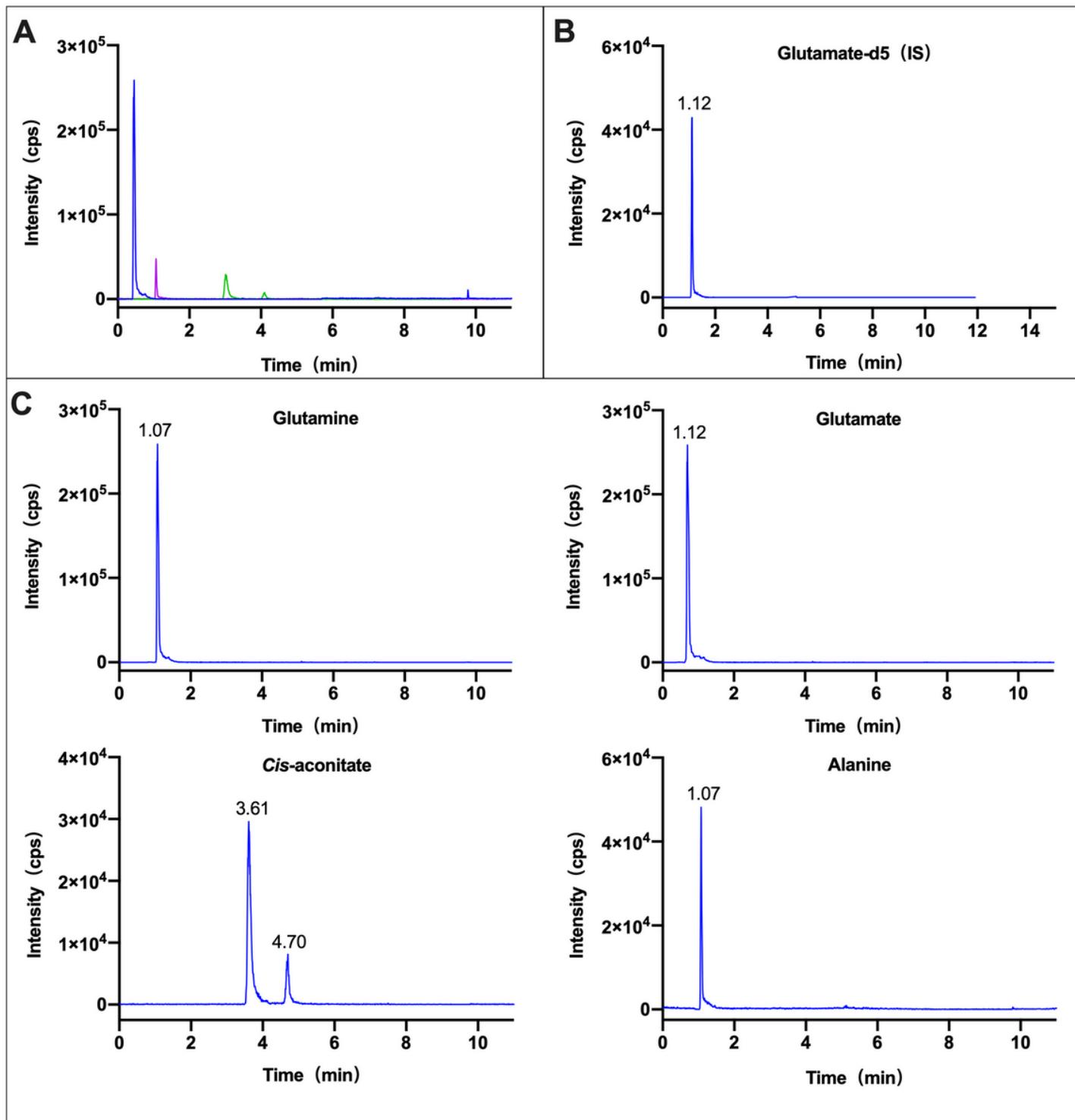
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## Figures



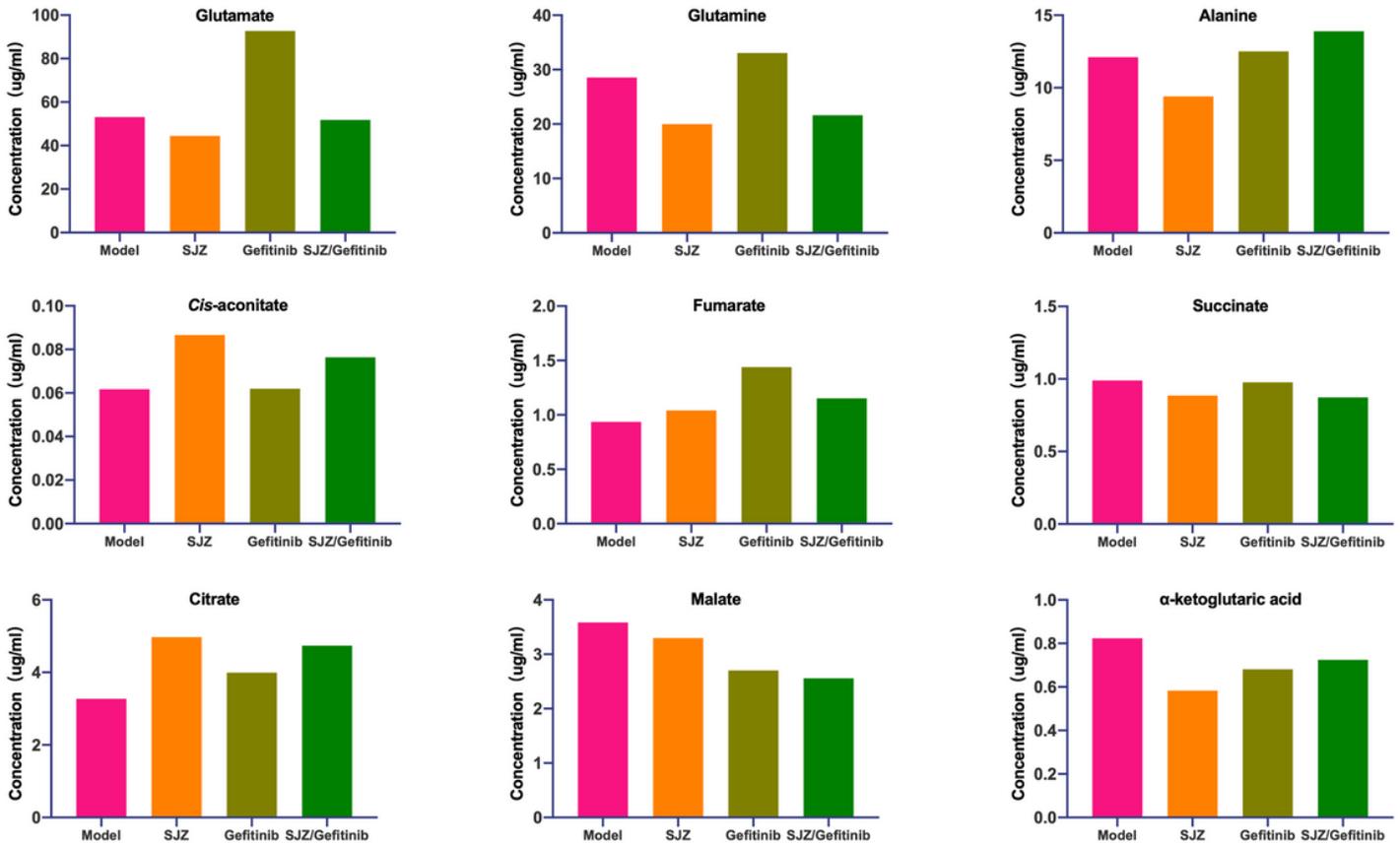
**Figure 1**

Gefitinib(A) and SJZ(B) inhibit cell proliferation in PC-9/GR cells. PC-9/GR cells were treated with SJZ and/or gefitinib at the indicated concentrations for 48 h. Cell inhibition was measured by CCK-8, and IC50 values were calculated (C). The concentration of SJZ was 1.089 mg/mL, but the concentration of gefitinib was indicated respectively (D). JinZhengJun Q value of the combination of SJZ and gefitinib. Cell viability was measured by CCK-8. The utility of the drug combination is additive when  $Q = 0.85 \sim 1.15$ , synergistic when  $Q > 1.15$  and antagonistic when  $Q < 0.85$ .



**Figure 2**

Representative chromatograms of a standard sample containing glutamate, glutamine, cis-aconitate, alanine respectively, and internal standard (IS) (A). Extracted ion chromatograms of IS, Glu-d5 (B). Extracted ion chromatograms of glutamate, glutamine, cis-aconitate, alanine from the standard sample (C).



**Figure 3**

The concentration of fumarate, succinate, citrate, malate,  $\alpha$ -ketoglutaric acid, glutamate, glutamine, alanine, cis-aconitate in model, SJZ, gefitinib, SJZ/Gefitinib group.

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