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Research article

A novel sensitizer reduces EGFR-TKI resistance by regulating the PI3K/Akt/mTOR pathway and autophagy

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ABSTRACT

Background: The incidence and mortality of lung cancer are high, and treatment with epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) is the preferred first-line treatment for patients suffering from non-small cell lung cancer (NSCLC) with EGFR mutations. However, EGFR-TKI resistance leads to treatment failure. Yifei-Sanjie pill (YFSJ) is a novel type of Chinese patent medicine for lung cancer. The development of YFSJ has progressed for more than 30 years; however, little is known about the molecular mechanisms associated with the inhibition of drug resistance.

Methods: In this study, flow cytometry and transcriptome sequencing were used *in vitro* to explore the anticancer effect of Yifei-Sanjie pill (YFSJ) on EGFR-TKI-resistant cell lines and to identify potential molecular mechanisms associated with the inhibition of drug resistance.

Results: We found that *in vitro*, YFSJ and YFSJ combined with gefitinib significantly reduced the viability of H1975 and H1650 cells, which is dose-dependent at 24 and 48 h. PI3K, Akt and mTOR were downregulated, while after 24 and 48 h of treatment with YFSJ alone and in combination with gefitinib, LC3A and LC3B were up-regulated in both cell lines.

Conclusion: YFSJ reduced the viability of EGFR-TKI-resistant cell lines, reducing resistance to gefitinib. This might be caused by a decrease in the PI3K/Akt/mTOR pathway and an increase in autophagy.

1. Introduction

Lung cancer is the leading cause of cancer death, and according to the Global Cancer Observatory, it has both high incidence and high mortality rates worldwide. Therefore, better treatments for lung cancer are urgently needed.

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Non-small cell lung cancer (NSCLC) is the most common type of lung cancer [1]. Mutations in epidermal growth factor receptor (EGFR) are known to cause carcinogenic mutations in NSCLC [2]. For patients suffering from NSCLC with EGFR mutations, EGFR-TKIs could minimize side effects and improve survival rates [3]. Therefore, the first-line treatment option for NSCLC patients with EGFR mutations is EGFR-TKIs [4].

However, as with chemotherapy drugs, EGFR-TKIs also have drug resistance problems. Patients who experience clinical benefits from EGFR-TKIs still experience disease progression, which leads to treatment failure, and the underlying cause is acquired drug resistance [5]. Currently, there is no effective reversal of drug resistance, which is problematic for the treatment of NSCLC. Therefore, effective reduction or management of acquired resistance to EGFR-TKIs is an important problem that needs to be solved in the treatment of NSCLC.

Traditional Chinese medicine (TCM) can be used to treat cancer effectively and reduce drug resistance in NSCLC [6,7]. Yi-Fei San-Jie pill (YFSJ), also known as the Yi-Qi Chu-Tan formula (YQCT), is a novel traditional Chinese patent medicine used to treat lung cancer. YFSJ originated from *Nanjing* in 206 BC-8 AD, and *Zhengzhi Huibu* originated in 1687 AD. *Nanjing* showed that if the lung Qi is deficient, it needs to be replenished. *Zhengzhi Huibu* mentions the lung as a reservoir of phlegm. Hence, for lung cancer patients, it is necessary to dispel phlegm and replenish lung Qi, which is the efficacy of YFSJ. It contains Panax quinquefolius L. (Xi Yang Shen), Fritillaria thunbergii Miq. (Zhe Bei Mu), Ranunculus ternatus Thunb. (Mao Zhua Cao), Bombyx Batryticatus (Jiang Can), Sarcandra glabra (Thunb.) Nakai (Zhong Jie Feng), Solanum nigrum L. (Long Kui), Cremastra appendiculata (D. Don) Makino (Shan Ci Gu), Pinellia ternata (Thunb.) Makino (Fa Ban Xia), and Ganoderma lucidum (G. lucidum Leyss. ex Fr.) (Ling Zhi) [8]. The name of the plant has been verified through the World Flora Online, and the main chemical components of the plant are ginsenoside Ro, ginsenoside Rb1, ginsenoside Rc, peimisine and peimine [9,10]. The Chinese medicine and main chemical components of this formula have been used in clinical cancer treatment for many years [11–25].

In vitro, our previous research revealed that YFSJ was able to inhibit the growth of NSCLC by triggering cell cycle arrest in the G1/S phase, which in turn synergistically reduced the viability and migration of tumor cells [8]. We also found that YQCT combined with gefitinib reduced EGFR-TKI resistance and improved anticancer effects [26]. *In vivo*, YQCT showed significant inhibitory effect on the mice with Lewis lung cancer and Pi-asthenia syndrome, which not only inhibited the growth and metastasis of tumors, but also effectively prolonged the survival of mice [27]. YFSJ can also alleviate cancer-related fatigue (CRF), enhance chemotherapy sensitivity and improve quality of life in lung cancer model mice [28]. In clinical trials, patients with advanced NSCLC received YQCT had a disease control rate (DCR) of 36.96 % [29]. YQCT prolonged overall survival (OS) [30], and YFSJ alleviated CRF in NSCLC patients [31].

This study further delves into the underlying mechanism of YFSJ *in vitro* and aims to elucidate how YFSJ overcomes acquired resistance to EGFR-TKIs.

2. Materials and methods

2.1. Drugs, cells and reagents

YFSJ (Cat. #Z20190015000) was obtained from the First Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, Guangdong Province, PR China). Each packet of YFSJ was 8 g, which was equivalent to 16.4 g of herbal medicine [31].

Gefitinib was purchased from Selleck Chemicals (Houston, TX, USA).

NCI-H1650 (ATCC number: CRL-5883™, lot number 63633377, RRID: CVCL_1483) and NCI-H1975 (ATCC number: CRL-5908™, lot number 70000787, RRID: CVCL_1511) cells were purchased and authenticated from American Type Culture Collection (Rockville, MD, USA). Mycoplasma was not found in either cell line.

Roswell Park Memorial Institute-1640, fetal bovine serum, and the PureLink™ RNA Mini Kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and N-methyl dibenzo pyrazine methyl sulfate (PMS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The NEBNext® UltraTM RNA Library Preparation Kit was obtained from Illumina® (NEB, Massachusetts, USA). Nuclear factor fixation and permeabilization buffer was obtained from Biolegend (San Diego, CA, US).

The primary and secondary antibodies described below were purchased from Abcam (Cambridge, UK): recombinant anti-PI3 kinase p85 alpha for flow cytometry (ab191606), recombinant anti-AKT3 + AKT2 + AKT1 for flow cytometry (ab87540), recombinant anti-MAP1LC3A (ab52768), anti-LC3B (ab51520), goat anti-rabbit IgG H&L (ab150077).

2.2. Cell culture

H1650 and H1975 cells were seeded in 96-well trays at a density of 4×10^3 cells/well, or 2×10^5 cells/well in 6-well plates overnight. PRMI-1640 complete culture medium containing 10 % fetal bovine serum was used for cultivation, followed by cells in a 37 °C, 5 % CO_2 incubator.

2.3. Cell viability assay

A total of 4×10^3 H1650 or H1975 cells were cultivated in a 96-well tray overnight. Then, YFSJ, gefitinib, and YFSJ in combination with gefitinib were used to treat the cells at the appropriate concentrations for 24 h and 48 h. XTT (1 mg/ml) and 1.25 mM PMS were mixed at a ratio of 50:1, and then 50 μl of the mixture of XTT and PMS was added to each well at 37 °C in a 5 % CO_2 incubator for 4 h to

measure cell viability. Then, the optical density (OD) was measured at 490 nm.

2.4. The extraction, sequencing, data processing and functional annotation of RNA

H1975 cells (2×10^5) were cultivated in a 6-well plate for 24 h. Then, YFSJ, gefitinib, and YFSJ in combination with gefitinib were used to treat the cells at the appropriate concentrations for 24 h and 48 h. Total RNA was extracted from these cells by using the PureLink™ RNA Mini Kit. The quality and quantity of all the samples were analysed by using a bioanalyzer to ensure an RNA integrity number (RIN) > 7.0, and sequencing libraries were constructed using high-quality RNA. The construction of the libraries and transcriptome sequencing were performed by Novogene (Hong Kong, China). The NEBNext® UltraTM RNA Library Preparation Kit from Illumina® was used to formulate RNA libraries, and the Illumina HiSeq X platform was used for sequencing of non-stranded paired-end 150 bp reads. The RNA-Sequencing data is available from this follow link:https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125933.

2.5. Flow cytometry

2×10^5 cells were cultivated in each well of a 6-well plate and then treated for 24 h or 48 h. The cells were fixed and permeabilized using Nuclear Factor Fixation and Permeabilization buffer, and then labelled with primary antibodies and secondary antibodies. Then, the cells were acquired on a BD Accuri C6, and then the data were analysed using FlowJo (V10) software.

2.6. Statistical analysis

One-way ANOVA, LSD and Dunnett's T3 tests were used for the statistical analysis. The standard deviation (SD) is represented by the error bar. The data were presented as the means ± SDs. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered to indicate statistical significance.

3. Results

3.1. Effects of YFSJ on reducing drug resistance in NSCLC cells

H1975 is 1st and 2nd generation EGFR-TKI-resistant cell line, because it contains EGFR T790M mutation. And H1650 is EGFR-TKI-resistant because of PTEN-loss. In our study, an XTT assay was used to detect the effect of YFSJ on the viability of H1975 and H1650 cells. The results are shown in Fig. 1. XTT inhibited the viability of H1975 and H1650 cells, the effect is dose-dependent. At 1 mg/ml, the lowest dose showed effectiveness against both cell lines, and 2 mg/ml enhanced these effects but had no toxicity-like effects. Hence, YFSJ at 1 mg/ml and 2 mg/ml was selected as the subsequent dose because both concentrations of YFSJ could reduce cell viability in both cell lines at 24 h and 48 h.

In 24 and 48 h observations, YFSJ combined with gefitinib significantly reduced the viability of both cell lines in a dose-dependent fashion (Fig. 2). These results indicate that combined treatment can attenuate resistance to gefitinib in NSCLC cells, especially at the 48h administration.

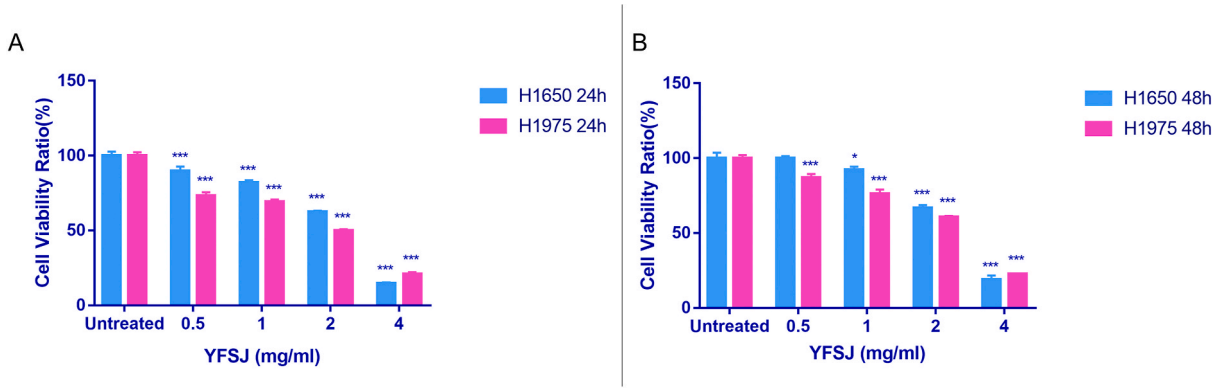

Fig. 1. Effects of YFSJ on EGFR-TKI-resistant cell lines. 4×10^3 cells were seeded into each well in 96-well trays overnight, followed by YFSJ at concentrations of 0.5 mg/ml, 1 mg/ml, 2 mg/ml or 4 mg/ml for (A) 24 h or (B) 48 h, respectively. Cells with only medium were labelled "Untreated". The value is expressed as a percentage of cell viability, which is the ratio of the viability of the treated groups to that of the untreated group. The data of each group was the values after subtracting the blank solvent's optical density.

3.2. Potential molecular pathway targets of YFSJ in NSCLC

In the EGFR-TKI resistance pathway, high- and low-dose YFSJ, gefitinib, and high- and low-dose YFSJ combined with gefitinib affected the PI3K-Akt, Jak-STAT and MAPK signalling pathways (Fig. 3). In the NSCLC pathway, the Ras, ErbB, MAPK, calcium, PI3K-Akt, cell cycle, and P53 signalling pathways were regulated by high and low doses of YFSJ, gefitinib, and high and low doses of YFSJ combined with gefitinib.

The PI3K/Akt pathway decreased in both the EGFR-TKI resistance pathway and the NSCLC pathway after treatment with YFSJ alone or YFSJ combined with gefitinib (Fig. 4). We therefore concluded that this pathway is an effective target through which YFSJ can reduce gefitinib resistance in NSCLC.

3.3. YFSJ decreased the PI3K/Akt/mTOR pathway and increased autophagy in NSCLC, it might be an mTOR inhibitor and autophagy agonist

Our previous studies indicated that YFSJ inhibited the expression of Akt and reduced the phosphorylation level of Akt in tumor cells, thereby inhibiting tumor growth [32,33]. Our sequencing analysis also showed that YFSJ affected the PI3K/Akt pathway; thus, the protein expression of phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt) and mammalian target of rapamycin (mTOR) in the PI3K/Akt/mTOR pathway was assessed by flow cytometry, and the results were shown in Fig. 5A–C. We found that YFSJ and YFSJ combined with gefitinib reduced the activity of the PI3K/Akt/mTOR pathway in both cell lines.

mTOR is a factor that negatively regulates autophagy [34], so we speculated that YFSJ could also affect autophagy. Microtubule-associated protein 1 light chain 3 (LC3) family proteins are the core proteins involved in autophagy. In the LC3 family, microtubule-associated protein 1 light chain 3α (LC3A) and microtubule-associated protein 1 light chain 3β (LC3B) are the main family

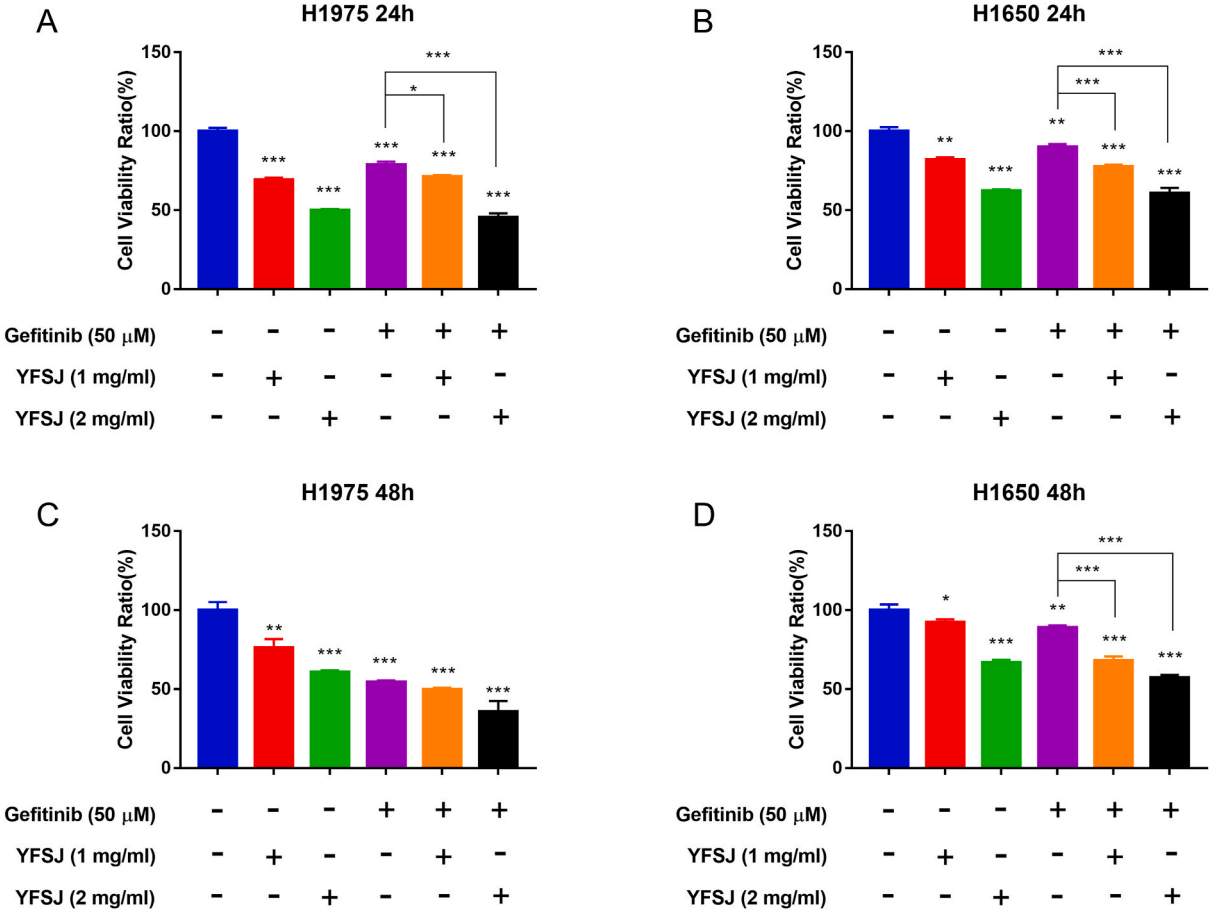

Fig. 2. Effects of YFSJ combined with gefitinib in EGFR-TKI-resistant cells. A total of 4 × 10^3 cells per well (A) H1975 or (B) H1650 cells were cultured overnight in 96-well trays and then treated with 50 μM gefitinib either with or without 1 mg/ml or 2 mg/ml YFSJ for 24 h. A total of 4 × 103 H1975 or (D) H1650 cells per well were cultured in 96-well trays overnight and then treated with 50 μM gefitinib with or without 1 mg/ml or 2 mg/ml YFSJ for 48 h. The group without medication was labelled "Untreated". The value is expressed as a percentage of cell viability, which is the ratio of the viability of the treated groups to that of the untreated group.

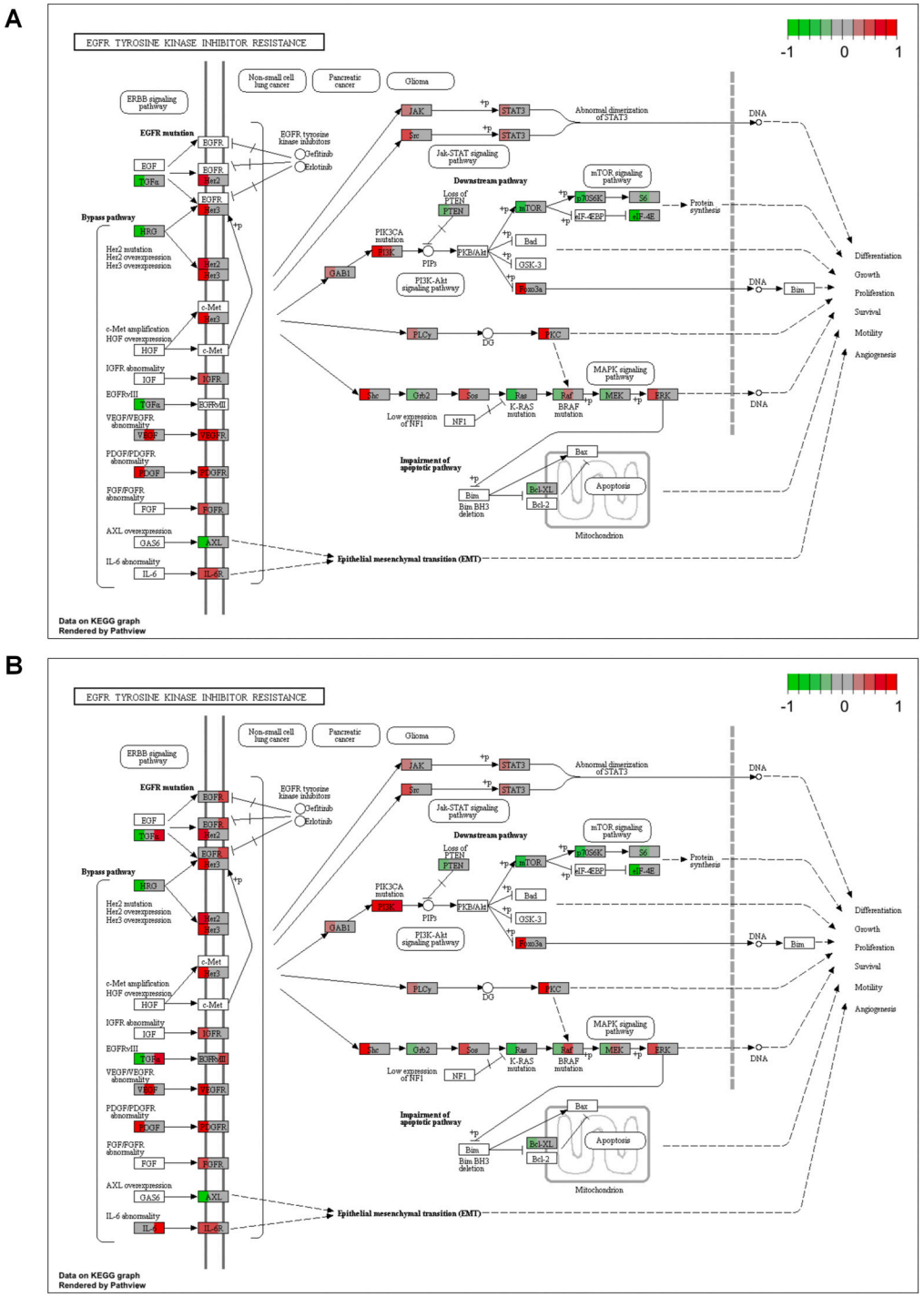

Fig. 3. Comparison of the changes in the expression of individual genes. In the EFGR-TKI resistance pathway, H1975 cells were treated with (A) 50 μM gefitinib, 1 mg/ml YFSJ or 1 mg/ml YFSJ with 50 μM gefitinib and (B) 50 μM gefitinib, 2 mg/ml YFSJ or 2 mg/ml YFSJ with 50 μM gefitinib. Red represents upregulation of the change in the scaled log fold, green represents downregulation of the change in the scaled log fold, and white or gray represents genes with no significant difference in expression. Each box representing gene families consisted of three sections: the left section shows the change in expression in cells treated with 50 μM gefitinib in comparison to that in untreated cells, the middle section shows the change in expression in cells treated with 1 mg/ml YFSJ or 2 mg/ml YFSJ in comparison to that in untreated cells, and the right section shows the change in expression in cells treated with 1 mg/ml YFSJ or 2 mg/ml YFSJ combined with 50 μM gefitinib in comparison to that in cells treated with 50 μM gefitinib.

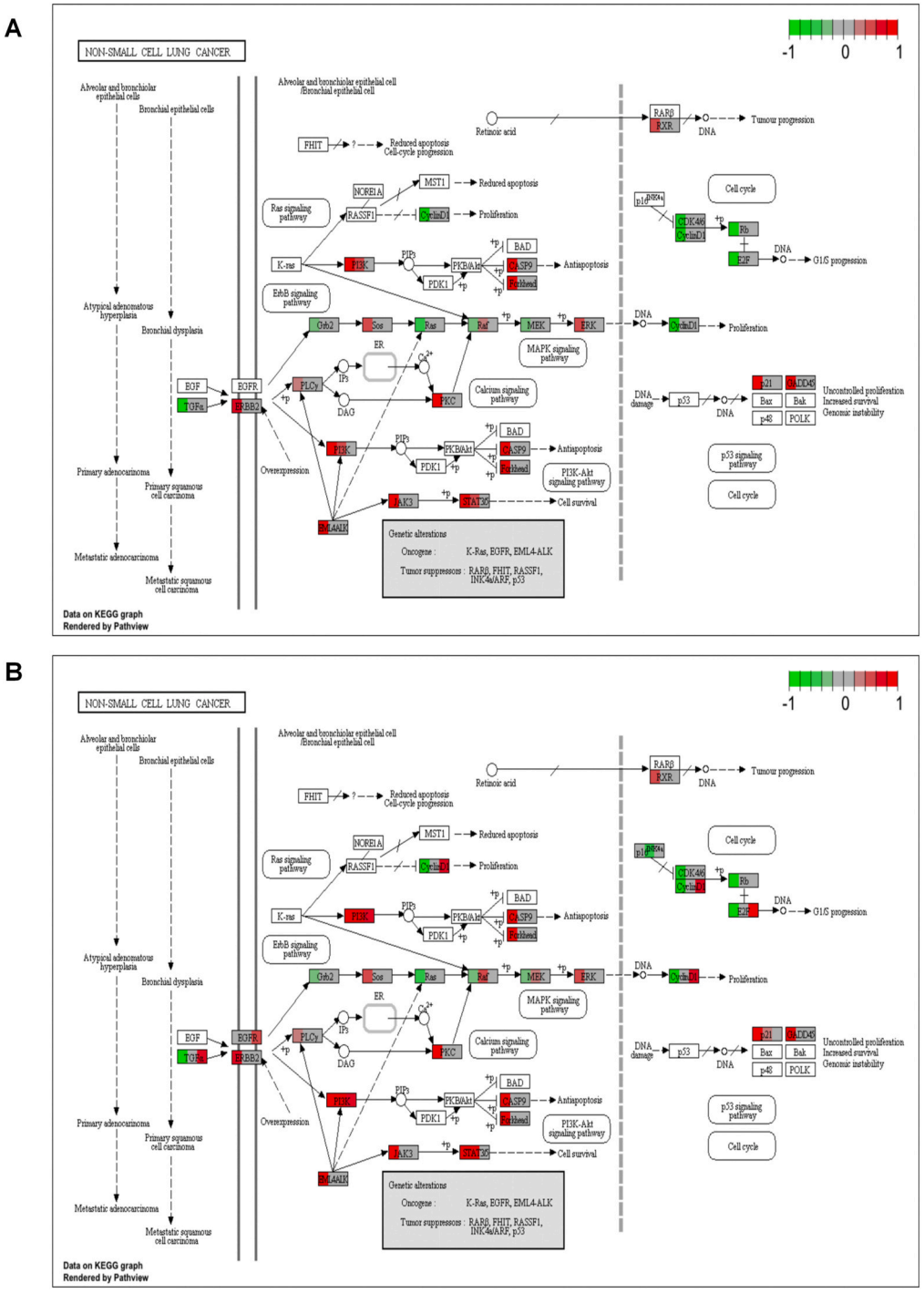

Fig. 4. Comparative analysis of individual gene expression changes. In the NSCLC pathway, H1975 cells were treated with (A) 50 μM gefitinib, 1 mg/ml YFSJ or 1 mg/ml YFSJ together with 50 μM gefitinib and (B) 50 μM gefitinib, 2 mg/ml YFSJ or 2 mg/ml YFSJ together with 50 μM gefitinib. Red represents upregulation of the change in the scaled log fold, green represents downregulation of the change in the scaled log fold, and white or gray represents genes with no significant difference in expression. Each box representing gene families consisted of three sections: the left section shows the change in expression in cells treated with 50 μM gefitinib in comparison to that in untreated cells, the middle section shows the change in expression in cells treated with 1 mg/ml YFSJ or 2 mg/ml YFSJ in comparison to that in untreated cells, and the right section shows the change in expression in cells treated with 1 mg/ml YFSJ or 2 mg/ml YFSJ combined with 50 μM gefitinib in comparison to that in cells treated with 50 μM gefitinib.

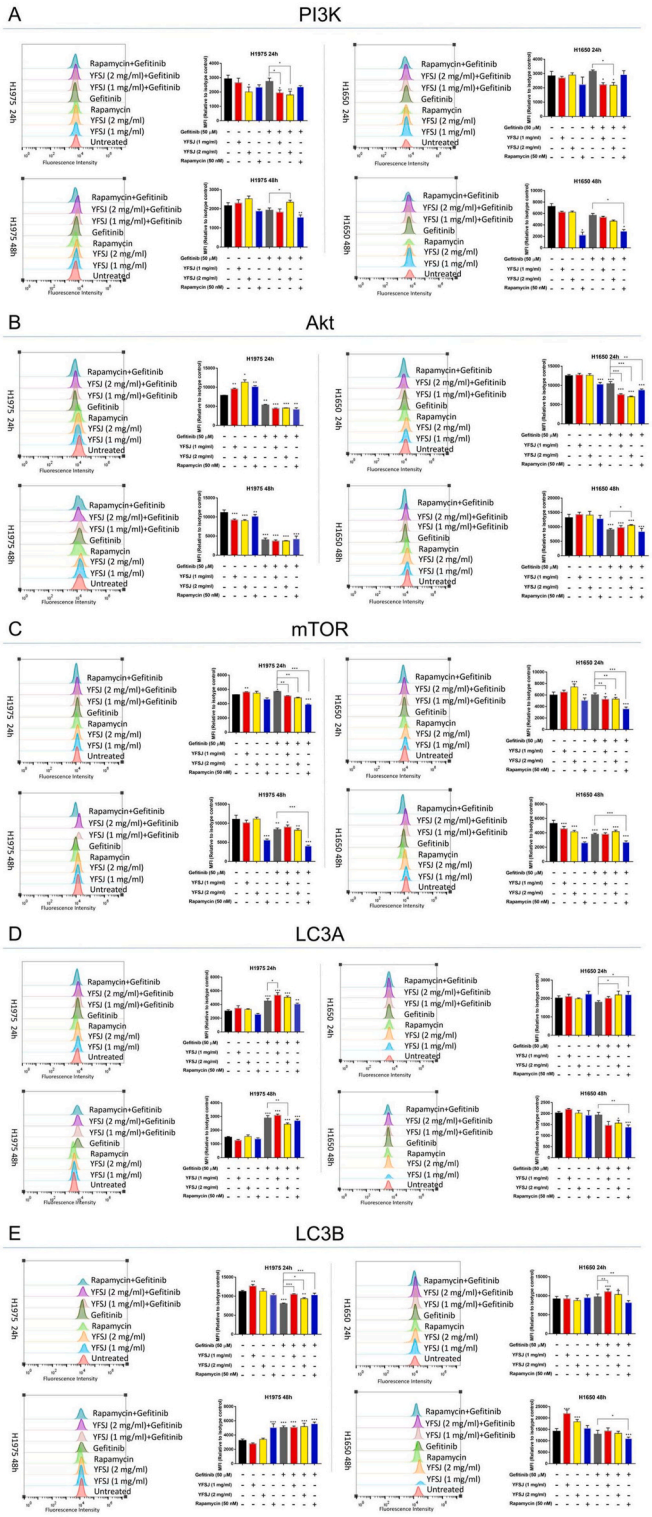

Fig. 5. Effects of treated groups on the PI3K/Akt/mTOR pathway and autophagy. Before being labelled for (A) PI3K, (B) Akt, (C) mTOR, (D) LC3A and (E) LC3B, the cells were cultivated with 50 μM gefitinib with or without 50 nM rapamycin or 1 mg/ml or 2 mg/ml YFSJ for 24 h or 48 h. The left panels show representative histograms, while the right panels show the MFI of the stained cells.

members [35]. Hence, we detected LC3A and LC3B protein expression at 24 h and 48 h (Fig. 5D–E). The results showed that autophagy was upregulated by YFSJ and YFSJ in combination with gefitinib in H1975 and H1650 cells.

Since the PI3K/Akt/mTOR pathway and autophagy account for most EGFR-TKI resistance, we determined whether reducing PI3K/Akt/mTOR signalling and promoting autophagy could decrease EGFR-TKI resistance. Rapamycin was used as an mTOR inhibitor and autophagy agonist. As shown in Fig. 5, YFSJ combined with gefitinib significantly decreased the PI3K/Akt/mTOR pathway and increased autophagy in H1975 and H1650 cells, and these effects were similar to those of rapamycin. This indicated that YFSJ might be an mTOR inhibitor and autophagy agonist.

4. Discussion

Gefitinib is a small molecule inhibitor of EGFR that has been widely used in NSCLC patients with EGFR mutations, although many of these patients benefit from gefitinib treatment, acquired resistance caused by secondary EGFR mutations and activation of alternative pathways [36], greatly reducing the effectiveness of gefitinib treatment. There is no expert consensus on how to reduce gefitinib resistance worldwide. Therefore, the urgent need to find a solution to the problem of gefitinib resistance is to improve its effectiveness in clinical application.

This study showed that YFSJ and YFSJ combined with gefitinib inhibited the viability of gefitinib-resistant cells, suggesting that YFSJ could reduce resistance to EGFR-TKIs. We then found that YFSJ could downregulate the PI3K/AKT/mTOR pathway and upregulate the autophagy pathway *in vivo* and *in vitro*. Using rapamycin, we showed that YFSJ might be an mTOR inhibitor and autophagy agonist.

One of the main downstream pathways of EGFR is the PI3K/Akt/mTOR pathway, and activation of this pathway inhibits tumor growth [37]. MTOR is the main negative regulator of autophagy [38]. The PI3K/Akt/mTOR pathway promotes cell survival and prevents excessive autophagy [39], indicating that inhibiting this pathway may promote autophagy [40]. Autophagy has dual effects on cancer since it can both suppress tumors and has oncogenic effects [41]. EGFR-TKI-induced acquired resistance may result from autophagy loss or the inability to enhance autophagic flux above basic levels [42], suggesting that autophagy upregulation may reduce resistance to EGFR-TKIs. Our previous studies indicated that YFSJ could decrease the growth of EGFR-TKI-resistant tumors *in vivo*, which may affect the endoplasmic reticulum stress (ER stress) response by increasing the expression of CHOP/GADD153 and GADD34 (Li, Y.B., Yang, J. M et al., 2014, Sun, L. L, Fang, R. M et al., 2014). CHOP is important for regulating autophagy and can induce autophagy related to ER stress [43], while CHOP can induce the transcription of several autophagy-related genes (ATGs). In addition, an imbalance in autophagy may also serve as a feedback mechanism that triggers ER stress [44]. Thus, we conclude that the upregulation of autophagy may be the main mechanism by which YFSJ reduces gefitinib resistance in NSCLC.

Interestingly, in our transcriptome sequencing, in addition to the PI3K/Akt pathway, YFSJ also altered the MAPK pathway. MAPK is downstream of EGFR [45] and induces the development of autophagy [46]. Thus, we hypothesized that YFSJ may also downregulate the MAPK pathway. This supposition extends a novel research field of YFSJ.

It is important to know that there is a great difference between the experimental *in vitro* and *in vivo*. The cellular microenvironment *in vitro* is impossible to fully reproduce the complex conditions involved in human pharmacokinetics, so the dose of gefitinib used in clinical practice has limited reference and guiding value for this experiment. As for whether there is any difference between the dosage of 50 μM gefitinib used in this experiment and in the clinic, and whether it will cause off-target effects, it needs to be further explored and verified. Our research focuses on early response, but we are also fully aware of the indispensable value of long-term assessment. With an eye to future work, we plan to conduct an exposure experiment of up to 72h to complement existing findings and rigorously validate the continued role of YFSJ in drug-resistant cell lines.

5. Conclusion

In conclusion, YFSJ combined with gefitinib can significantly reduce cell viability in gefitinib-resistant NSCLC cell lines by decreasing the activity of the PI3K/Akt/mTOR pathway and increasing autophagy. Our study demonstrated that YFSJ is a novel gefitinib sensitizer, an mTOR inhibitor and an autophagy agonist.

CRediT authorship contribution statement

Jue Zhang: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Zhipeng Qu:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Data curation. **Xi Xiao:** Investigation. **David L. Adelson:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Funeng Wang:** Writing – review & editing, Funding acquisition. **Aisheng Wei:** Writing – review & editing, Funding acquisition. **Yuka Harata-Lee:** Writing – review & editing, Supervision. **Jian Cui:** Writing – review & editing, Supervision, Methodology. **Dongying He:** Methodology, Investigation. **Le Xie:** Supervision, Methodology. **Lingling Sun:** Supervision, Conceptualization. **Jing Li:** Investigation. **Zijing Huang:** Investigation. **Thazin Aung:** Writing – review & editing, Supervision, Methodology. **Hong Yao:** Writing – review & editing. **Lizhu Lin:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Availability of data and materials

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

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Lizhu Lin reports financial support was provided by Ministry of Science and Technology of the People's Republic of China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

EGFR	Epidermal growth factor receptor
EGFR-TKI	Epidermal growth factor receptor-tyrosine kinase inhibitor
NSCLC	Non-small cell lung cancer
YFSJ	Yifei-Sanjie pill
TCM	Traditional Chinese medicine
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
OD	Methyl dibenzo pyrazine methyl sulfate (PMS), Optical density
SD	Standard deviation
PI3K	Phosphatidylinositol 3-kinase
Akt	Protein kinase B
mTOR	Mammalian target of rapamycin
LC3	Microtubule-associated protein 1 light chain 3
LC3A	Microtubule associated protein 1 light chain 3α
LC3B	Microtubule-associated protein 1 light chain 3β
MFI	Mean fluorescent intensity
ER stress	Endoplasmic reticulum stress
ATGs	Autophagy-related genes

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