Combination with *cooling-heat* Chinese medicine Qing-kai-ling, not *warming-yang* Shen-fu, enhances the antitumor effect of gefitinib in resistant non-small cell lung cancer models in vitro and in vivo

Yan-chun Qu

The Second Affiliated Hospital of Guangzhou University of Chinese Medicine <https://orcid.org/0000-0003-4959-8050>

Ying Zou

Guangzhou Medical University Affiliated Cancer Hospital

Shuai Shi

Luoyang First People's Hospital

Yanjuan Zhu (✉ zyjsophy@gzucm.edu.cn)

<https://orcid.org/0000-0002-1956-7774>

Yi-hong Liu

The Second Affiliated Hospital of Guangzhou University of Chinese Medicine

Li-rong Liu

The Second Affiliated Hospital of Guangzhou University of Chinese Medicine

Xiao-hua Zheng

Conghua Hospital of Traditional Chinese Medicine

Hui-hui Chen

Zhuhai Hospital of Integrated Traditional Chinese and Western Medicine

Hai-bo Zhang

The Second Affiliated Hospital of Guangzhou University of Chinese Medicine

Research

Keywords: Traditional Chinese medicine, Treatment principle, Warming-yang treatment principle, Cooling-heat treatment principle, Non-small cell lung cancer, Epidermal growth factor receptor tyrosine kinase inhibitor

Posted Date: May 21st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-29063/v1>

Abstract

Background: Traditional Chinese Medicine (TCM) prescriptions should be decided according to the TCM treatment principle, and the warming-yang or cooling-heat should be the guide of treatment principle outline.

Methods: In order to identify which treatment principle, warming-yang or cooling-heat should be combined with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) in non-small cell lung cancer (NSCLC), we combined the typical warming-yang drug, Shen-fu (SF), and the typical cooling-heat drug, Qing-kai-ling (QKL) with gefitinib in resistant NSCLC models.

Results: The results demonstrated that QKL combined with gefitinib induced significantly increased cell viability inhibition and apoptosis in A549 cell line and significantly smaller tumor volume and lower tumor weight in H1975 xenograft transplanted nude mice. On the contrary, SF combined with gefitinib had significant antagonism effect on both cell viability inhibition and apoptosis in vitro, and on tumor weight in vivo. EGFR phosphorylation inhibition and the downstream PI3K/AKT and RAS/RAF/ERK pathway inhibition served an important role in the synergism effect between QKL and gefitinib in H1975 xenograft transplanted nude mice.

Conclusions: The present study indicated that cooling-heat TCM treatment principle may reverse or delay NSCLC resistance to EGFR-TKIs, and combination of them warrants further study.

Background

Epidermal growth factor receptor tyrosine kinase inhibitors (*EGFR*-TKIs) are the preferred treatment for intermediate to advanced stage non-small cell lung cancer (NSCLC) in patients with *EGFR* gene mutation. Despite the demonstrated benefits of EGFR-TKIs, not all patients respond to treatment. Moreover, even for the exclusively *EGFR*-mutant advanced NSCLC patients, the median progression free survival (PFS) was only 9–13 months for those who accepted first-line *EGFR*-TKI therapy¹. Approximately 50% of patients who respond well initially to TKIs develop resistance due to the occurrence of secondary mutation T790M in exon20 of the *EGFR* gene¹, and the third generation EGFR-TKIs have shown efficacy in these patients^{2,3}. For the other half of the patients resistant to EGFR-TKIs, no optimal therapy was available. Even for the patients with T790M mutation, most of them are not taking third generation EGFR-TKIs in the real world Chinese clinic, because of the high price. Therefore, effective therapies to delay the resistance of EGFR-TKIs are therefore required.

TCM therapies have been widely used in cancer, including NSCLC. Although some clinical trials have reported the efficacy of some TCM decoction or patent prescription in combination with *EGFR*-TKIs, most of them were with small samples and low quality⁴. Besides, the TCM treatment principles in these studies were even different. As a result, most of the TCM therapies in clinical practice are still based on doctors' experience, rather than evidence from clinical trials. A typical TCM treatment process should be

programmed as the following sequential steps, first diagnosing TCM syndrome types, then deciding the TCM treatment principle and finally writing out a TCM prescription. Although the syndrome types in the TCM theory are complicated, *Yin-cold (YC)* or *Yang-heat (YH)* type differentiation is believed as the outline of TCM syndrome type diagnosis according to the traditional book *Huangdi Neijing*. Therefore, the *warming-yang* or *cooling-heat* should be the guide of treatment principle outline⁵. We have demonstrated that *EGFR* gene mutated NSCLC patients are more likely with *YC* syndrome type⁶. Besides, the most common side effect of *EGFR*-TKIs was red acneiform rashes^{7–11}, with thirsty, red and dry tongue and yellow tongue coating, which are typical symptoms and signs for *YH* syndrome type. Therefore, *EGFR*-TKIs may affect with *warming-yang* influence according to TCM theory, and we do not know whether the *warming-yang* influence of *EGFR*-TKIs is a treatment effect or just a kind of side effect. If the *warming-yang* influence is a therapeutic effect, then TCM therapies with *warming-yang* principles may help to improve the efficacy of *EGFR*-TKIs, and vice versa.

In this study, we attempted to know which treatment principle, *cooling-heat* or *warming-yang*, should be combined with the *EGFR*-TKIs. We used the typical *warming-yang* drug, Shen-fu (SF), and the typical *cooling-heat* drug, Qing-kai-ling (QKL), because they are without known anticancer effect. SF injection has been used for nearly 30 years in China for patients with *YC*-syndrome type. The active components of SF injection are extracted from *Radix Ginseng* and *Radix Aconiti Carmichaeli*. QKL injection or oral solution has been widely used for the treatment of high fever or acute infection in clinical practice. It is prepared from *cholic acid*, *hyodeoxycholic acid*, *baicalin*, and active materials extracted from *Gardeniae Fructus*, *Bubali Cornu*, *Margaritifera Concha*, *Isatidis Radix*, and *Lonicerae Japonicae Flos*. The aim of the present study was to investigate the effect of SF or QKL when combining with gefitinib on resistant NSCLC models *in vitro* and *in vivo*.

Materials And Methods

Preparation of drugs

Gefitinib was kindly provided by AstraZeneca China. SF injection was purchased from Ya-an San-jiu pharmaceutical co., LTD (Sichuan, China), and QKL injection and QKL oral solution were purchased from Ming-xing pharmaceutical co., LTD (Guangzhou, China). The component herbs of SF decoction used in the *in vivo* experiments, prepared *Radix Aconiti Carmichaeli* and *Radix Ginseng*, were purchased from Kang-mei pharmaceutical co., LTD (Guangzhou, China). Seventy-five gram of *Radix Ginseng* and 150 g prepared *Radix Aconiti Carmichaeli* were mixed and first soaked in 1000 ml water for 30 minutes, and then boiled for 90 minutes. The liquid was filtered through a piece of medical gauze and the drugs were boiled once more with 800 ml water for 90 minutes. The liquid was filtered again and mixed with that from the first boiling. The solution was concentrated into 250 ml in the rotary evaporator (IKA®RV 10 Basic), with a concentration of 0.9 g/ml crude drug, and then stored at -80°C until use.

Reagents

4,5-dimethylthiazol-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) was purchased from MP Biomedicals (California, USA). Annexin V/ propidium iodide (PI) apoptosis Kit was purchased from MultiSciences(Lianke)Biotech Co., Ltd (Hangzhou, China). BCA protein assay kit was purchased from Thermo Fisher Scientific, Inc (ML, USA). Rabbit anti-human EGFR, phospho-EGFR (p-EGFR), AKT, p-AKT, ERK and p-ERK monoclonal antibodies (mAb) and horseradish peroxidase [HRP] conjugated anti-rabbit antibody were purchased from Cell Signaling Technology, Inc (MA, USA). Electro-Chemi-Luminescence (ECL) reagent was purchased from Millipore Corporation (MA, USA).

Cell Culture

Human A549 NSCLC cells were obtained from the Cell Line Bank at the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). H1975 cells were obtained from the Cell Line Bank of the Macao University of Science and Technology (Macao, China). Cells were maintained in RPMI-1640 medium (Gibco; ThermoFisher Scientific, Inc., MA, USA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 0.5% penicillin-streptomycin sulfate (Gibco; Thermo Fisher Scientific, Inc.), and incubated at 37°C with 5% CO₂.

Cell Viability Assay

Human A549 cell was used in the *in vitro* experiments. MTT assay was used to measure cell viability. Briefly, cells were plated in 96-well culture plates at the density of 5×10^3 per well in complete medium. After 24 hours of incubation, cells were treated with gefitinib (20–100 µM), QKL injection and SF injection (0.6%-1.0%) alone or combination for 48 and 72 hours. Then the cells were incubated with 110 µl of 5 mg/ml MTT at 37°C for 4 h. After removing the medium, 150 µl dimethylsulfoxide (DMSO, Sigma, MO, USA) was added to each well. After shaking for 10 min, the absorbance at 570 nm was measured spectrophotometrically using a microplate reader (VICTOR X5, PerkinElmer). Each condition was duplicated in 6 wells. After removing the maximum and minimum absorbance to calculate the average, the cellular viability in each condition was expressed as the percentage of the average absorbance to that of control cells.

Apoptosis Assay

Apoptotic cell death was determined by the Annexin V/PI apoptosis Kit according to the manufacturer's instructions. Briefly, cells were plated in 6-well culture plates at a density of 1.5×10^6 cells/mL in complete medium. After 24 hours of incubation, cells were treated with gefitinib, QKL and SF injection alone or combination, with the concentration and exposure time according to the MTT assay results. After removing the medium, cells were trypsinized with EDTA free trypsin solution (Gibco; Thermo Fisher Scientific, Inc.), harvested and then resuspended in 500 µl Binding Buffer (1×) with 5 µl Annexin V-FITC

and 10 μ l PI. After incubation for 5 min at room temperature in the dark, the samples were analyzed with flow cytometer (Beckman Coulter FC500).

Western Blot Analysis

The cells were plated in 60 mm dishes at a density of 3.5×10^6 cells/ml in complete medium. After 24 hours of incubation, cells were treated with gefitinib, QKL and SF injection alone or combination, with the concentration and exposure time according to the MTT assay results. After the treatment, the cells were washed in PBS and lysed in lysis buffer. Protein concentrations were determined using the BCA protein assay kit. The samples corresponding to 30 μ g of protein were boiled for 8–10 min, resolved on an 8% denatured SDS-polyacrylamide gel, and transferred onto a PVDF membrane (Millipore, MA, USA). After blocking non-specific binding sites for 30 minutes using 5% skim milk, the membranes were incubated with rabbit anti-human EGFR, p-EGFR, AKT, p-AKT, ERK and p-ERK monoclonal antibodies for 2 hours at room temperature. After washed with TBST for 3 times, the membranes were incubated with HRP conjugated anti-rabbit antibody for 1 hour at room temperature. After washed with TBST for 3 times, visualization of the protein bands was accomplished using ECL reagent. ImageLab software (version 4.0) was used to calculate the expression of each protein, which was normalized by GAPDH.

Determination of antitumor effect in nude mice.

Female BALB/c nude mice (18–20 g) were obtained from the Laboratory Animal Center of Southern Medical University (Guangzhou, China, License NO. 44002100006205). The animals were kept in the Animal Center of Guangdong Provincial Hospital of Chinese Medicine (License NO. SYXK(yue)2013-0094), under a specific pathogen-free (SPF) condition with a 12 h light/dark cycle and freely accessed autoclaved standard food and water. Human NSCLC cells H1975 (3×10^7 /ml) were suspended in RPMI-1640 medium and 0.2 ml of the suspension was subcutaneously inoculated into the right forelimb of nude mice. Tumor growth was measured with the longest diameter (a) and the shortest diameter vertical to a (b). Tumor volume was calculated using the formula, $V = \frac{1}{6} \pi ab^2$.

When the tumors reached the size over 150 mm^3 , the mice were randomly divided into 6 groups (n = 10): control (saline solution 0.2 ml), gefitinib (1 mg, in 0.2 ml saline solution), QKL oral solution (0.25 ml), SF decoction (0.2 ml), gefitinib + SF, and gefitinib + QKL. The compounds were administered orally once a day for consecutive 21 days. Tumor volume and body weight of the mice were measured every 3 days during the administration period. The daily dosage of each drug for nude mice (with average weight of 20 g) was obtained based on the daily dosage for humans in clinical and the human-mouse transfer formula: Animal dose = Human dose $\times$ (HKm/AKm), where HKm and AKm represent the Km factor of human (37) and mouse (3)¹². The daily dosages for humans (with average weight of 60 kg) are 250 mg of gefitinib, 60 ml of QKL oral solution, and 45 g crude drug of SF decoction.

Immunohistochemistry

For immunohistochemical staining, paraffin-embedded tumor tissues were applied. The sections were deparaffinized in xylene and rehydrated with graded alcohol. Hydrogen peroxide (3%) was applied to block endogenous peroxidase activity and then boiled in 0.01M citrate buffer (pH 6.0) twice with an autoclave. After blocking non-specific binding sites using normal goat serum (Boster Biological Technology Co., Ltd, California, USA), tissue sections were incubated with rabbit anti-human EGFR (1:100), p-EGFR (1:100), AKT (1:200), p-AKT (1:50), ERK (1:200) or p-ERK (1:100) monoclonal antibodies at 4°C overnight. After washed with phosphate buffer saline (PBS) for 3 times, the sections were incubated with HRP conjugated anti-rabbit antibody for 30 minutes at 37°C, and the peroxidase reaction was developed with diaminobenzidine substrate kit (Zhongshan Golden Bridge-Bio, Beijing, China). Hematoxylin (Dingguo Changsheng Biotechnology CO., Ltd, Beijing, China) was then used for nucleus staining. Image-ProPlus 5.0 software was used to calculate the ratio of integrated optical density (IOD) to area (IOD/Area).

Statistics

Statistical analysis was performed using SPSS 19.0 statistical software (SPSS, Inc., Chicago, USA). The in vitro experiments were performed three times, independently. All data were presented as the mean $\pm$ standard deviation (SD). Differences between groups were assessed by two-tailed t test, one-way analysis of variance or analysis of variance for repeated measuring data and least significant difference (LSD)-t test was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference. q value method was used to evaluate the combination effect of gefitinib and QKL/SF, and it was calculated using the equation: $q = EAB / (EA + EB - EA \times EB)$, where EA and EB were the inhibition effect of gefitinib and QKL/SF, respectively. EAB represented an observed value of combined effect, and $(EA + EB - EA \times EB)$ represented an expected value of combine effect. A q value of 1.15 or more is considered synergism, $q < 0.85$ as antagonism and the value between 0.85 and 1.1.5 is considered as additive effect¹³.

Results

Cell viability in vitro

We first screened the concentration of gefitinib. According to the MTT assay, 45 μ M of gefitinib for A549 was used in the experiments with 48 hours of drug exposure, and 35 μ M for A549 was used in the cells exposed to the drugs for 72 hours. As shown in Fig. 1, in A549 cell lines, the 0.9% and 1.0% of QKL injection combined with gefitinib induced significantly increased viability inhibition after 72 hours treatment compared with gefitinib alone. However, the q value indicated only additive not synergism effect ($0.85 < q < 1.15$). There were no significant viability differences between gefitinib alone and SF + gefitinib treated A549 cells. According to the maximum combination effect and minimum effect of QKL or SF injection alone in MTT assay, 35 μ M of gefitinib and 0.9% of SF or QKL injection with 72 hours in A549 was used in the apoptosis and western blot experiments.

Apoptosis in vitro

Synergism effect of QKL injection and gefitinib on apoptosis were seen in the A549 ($q = 1.40$) cell line, with significantly increased apoptosis rate in the QKL + gefitinib treated cells compared with the gefitinib treated cells (Fig. 2). On the contrary, although the differences in apoptosis rates were not significant between the SF + gefitinib and gefitinib alone treated cells, q value method showed antagonism effect of SF injection and gefitinib in A549 ($q = 0.75$).

Antitumor activity in vivo

In H1975 xenograft transplanted nude mice, QKL oral solution, gefitinib, and QKL + gefitinib inhibited tumor growth, with significantly smaller tumor volume and lower tumor weight compared with those in control group ($p < 0.05$, Fig. 3). Differences in tumor volume and weight among SF decoction, SF + gefitinib and control group were not significant. Tumor volume and weight in QKL + gefitinib group were even significantly lower than in gefitinib group ($p < 0.05$). Q value according to the inhibition rate in tumor weight indicated synergism effect between QKL and gefitinib ($q = 1.19$). Although the differences in tumor volume and weight between SF + gefitinib and gefitinib groups were not significant, q value shown antagonism effect between SF and gefitinib ($q = 0.50$).

EGFR Pathway Protein Expression

In the experiments *in vitro*, the protein expression levels of p-EGFR were significantly lower in gefitinib, gefitinib + SF, and gefitinib + QKL groups in A549 cell line, comparing with control groups (Fig. 4A). The differences among the three groups were not significant. No significant differences were seen in EGFR, AKT/p-AKT, or ERK/p-ERK protein expression among A549 groups.

In H1975 xenograft transplanted nude mice, p-EGFR, AKT/p-AKT and p-ERK protein levels were significantly lower in gefitinib + QKL treated group, comparing with control group (Fig. 4B). Differences in EGFR and AKT/p-AKT protein levels were also significant between gefitinib alone and gefitinib + QKL treated groups. These data suggested that EGFR phosphorylation inhibition and the downstream PI3K/AKT and RAS/RAF/ERK pathway inhibition served an important role in the synergism effect between QKL oral solution and gefitinib in H1975 xenograft transplanted nude mice. Although EGFR protein level in gefitinib + SF group was significantly increased, comparing with control group, there were no significant differences in p-EGFR or downstream protein levels. Therefore, mechanism of antagonism effect between SF decoction and gefitinib was not clear.

Discussion

EGFR-TKIs are the preferred treatment for intermediate to advanced stage NSCLC in patients with *EGFR* gene mutation. However, the median PFS was only 9–13 months even for the exclusively *EGFR*-mutant advanced NSCLC patients. More effective therapies are therefore required. Traditional Chinese medicine,

which is popular in Chinese and East Asian societies, may be a potential effective strategy to delay the resistance to EGFR-TKIs.

The present study demonstrated that in gefitinib resistant *in vitro* and *in vivo* models, *cooling-heat* TCM prescription and gefitinib had synergism effect, while *warming-yang* prescription was antagonistic to gefitinib. Some clinical studies also indicated that TCM prescriptions or decoction with *cooling-heat* treatment principle may increase the efficacy of first-generation EGFR-TKIs. In Yang X's study, the TCM treatment principle of the decoction was strengthening vital *qi* and cooling cancer toxicity, especially cooling *heat*, and TCM decoction combining with gefitinib significantly prolonged progression free survival and overall survival¹⁴. In He W's meta-analysis, which analyzed the Chinese studies published from 2000 to 2016, only 6 studies reported increased efficacy from TCM when combining with EGFR-TKIs. Four of these 6 studies used specific TCM patent prescription or decoction, in which 3 were with *cooling-heat* treatment principle⁴. On the other hand, a case report by Sung-Wook Hwang et al. reported that inappropriate TCM herbs, mainly Ginseng with warming-yang effect, induced resistance to gefitinib whereas withdrawing of the herbs caused sensitivity again to gefitinib¹⁵. All these studies, together with our findings, suggested that the observed *warming-yang* effect of EGFR-TKIs may be a kind of side-effect even in TCM theory, and should be combined with *cooling-heat* treatment principle TCM therapies.

We also tried to find out the mechanism of the synergism or antagonism effect of QKL or SF to gefitinib. However, we only demonstrated that EGFR phosphorylation inhibition served an important role in the synergism effect between QKL oral solution and gefitinib in H1975 xenograft nude mice. Interestingly, H1975 cells are refractory to EGFR-TKIs due to the presence of the T790M mutation in EGFR¹⁶, which is the mechanism of 50% of the EGFR-TKIs resistant patients¹. The T790M mutation results in steric hindrance of binding of gefitinib to the ATP-kinase-binding pocket¹⁶. Our data suggested that QKL induced the EGFR phosphorylation re-inhibition and downstream PI3K/AKT and RAS/RAF/ERK pathway re-inhibition of gefitinib. This re-inhibition in T790M mutant H1975 may resulted from steric re-binding of gefitinib to the ATP-kinase-binding pocket, because downstream pathway re-inhibition was not observed in A549 (with *K-ras* not *EGFR* mutation¹⁷). Therefore, protein domain structure analyses warrant further researches.

The limitation of this present study was that we used only one drug to present each of the treatment principle. Expansion of the observed synergism effect of QKL and antagonism effect of SF with gefitinib to the effect of *cooling-heat* or *warming-yang* treatment principle should be very careful. Based on our initial finding, there are some TCM issues what warrant further research. First, we can further use other TCM patent prescriptions or decoction to present the *cooling-heat* or *warming-yang* treatment principle. If similar phenomena would be observed again, our hypothesis would be confirmed. Secondly, since synergism effect of QKL with gefitinib in tumor growth and apoptosis has been demonstrated *in vitro* and *in vivo*, reversing the resistance to gefitinib, effect on epithelial-mesenchymal transition (EMT) and metastasis warrants further researches, which should also been transferred to clinical validation. All

these researches will be meaningful for guiding the principle of TCM therapies in combination with *EGFR*-TKIs, or for finding new ways to delay the resistance to EGFR-TKIs.

Conclusions

In conclusion, we found that QKL, with *cooling-heat* TCM treatment principle, increased efficacy of gefitinib in refractory models, while *warming-yang* SF acted as antagonist to gefitinib. The influence of other drugs with *cooling-heat* or *warming-yang* TCM treatment principle warrant further study.

Abbreviations

TCM: Traditional Chinese Medicine; EGFR-TKIs: epidermal growth factor receptor tyrosine kinase inhibitors; NSCLC: non-small cell lung cancer; SF: Shen-fu; QKL: Qing-kai-ling; PFS: progression free survival; YC: Yin-cold; YH: Yang-heat; PI: propidium iodide; p-EGFR: phospho-EGFR; mAb: monoclonal antibodies; HRP: horseradish peroxidase; ECL: Electro-Chemi-Luminescence; PBS: phosphate buffer saline; SPF: specific pathogen-free; HKm: the Km factor of human; AKm: the Km factor of animal; IOD: integrated optical density; SD: standard deviation; LSD: least significant difference; EMT: epithelial-mesenchymal transition.

Declarations

Acknowledgements

Not applicable.

Authors'contributions

Yan-juan Zhu and Hai-bo Zhang designed the study; Yan-chun Qu and Yan-juan Zhu wrote the main manuscript text; Ying Zou, Shuai Shi, Xiao-hua Zheng, Hui-hui Chen performed the cell and animal experiments; Yan-chun Qu, Yi-hong Liu, Li-rong Liu analysed the data and prepared the figures. All authors read and approved the final manuscript.

Funding Sources

This work was supported by the Foundation for Chinese Medicine and Technology Researches of Guangdong Provincial Hospital of Traditional Chinese Medicine (YK2013B2N07, 2017KT1332), and Wu jieping medical foundation (320.6750.15085).

Availability of data and materials

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

Ethics approval and consent to participate

The animal experiment protocol was approved by Animals Research Committee of Guangdong Provincial Hospital of Chinese Medicine (No.2014027).

Consent for publication

The manuscript is approved by all authors for publication.

Declaration of conflicting interests

All the authors listed have approved this manuscript. The authors declare that we have no conflict of interest.

Author details

¹Oncology Department, Guangdong Provincial Hospital of Traditional Chinese Medicine, No. 111 Dade Road, Yuexiu District, Guangzhou 510120, China. ²Oncology Department, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, No.111 Dade Road, Yuexiu District, Guangzhou 510120, China. ³The Second Clinical Medical College of Guangzhou University of Chinese Medicine, No. 12 Jichang Road, Baiyun District, Guangzhou 510405, China. ⁴Guangdong Provincial Key Laboratory of Clinical Research on Traditional Chinese Medicine Syndrome, No. 111 Dade Road, Yuexiu District, Guangzhou 510120, China. ⁵Department of Integrated Traditional and Western Medicine, Affiliated Cancer Hospital & Institute of Guangzhou Medical University, No.78 Hengzhigang Road, Yuexiu District, Guangzhou 510635, China. ⁶Department of Respiration Medicine, Luoyang First People's Hospital, No.88 Zhongzhou East Road, Luoyang 471000, China. ⁷Department of Respiration Medicine, Conghua Hospital of Traditional Chinese Medicine, No.21, North road, Jiekou street, Conghua district, Guangzhou 510900, China. ⁸Zhuhai Hospital of Integrated Traditional Chinese and Western Medicine, No.208 Yuehua Road, Gongbei, Zhuhai 519000, China.

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Figures

Figure 1

MTT assay indicated that Qingkailing enhanced the cell viability inhibition of gefitinib in A549. Data are presented as the mean $\pm$ standard deviation of three independent experiments. * $P < 0.05$ vs. gefitinib alone. SF, Shenfu injection; QKL, Qingkailing injection.

Figure 2

Apoptosis assay indicated that Qingkailing combining with gefitinib increased the apoptosis rate in A549, with synergism effect ($q=1.40$). Shenfu and gefitinib had antagonism effect in A549 ($q=0.75$). Data are presented as the mean $\pm$ standard deviation of three independent experiments. * $P<0.05$ vs. gefitinib alone. SF, Shenfu injection; QKL, Qingkailing injection.

Figure 3

Qingkailing (QKL) oral solution, gefitinib, and QKL+gefitinib inhibited H1975 tumor growth in nude mice, with significantly smaller tumor volume and lower tumor weight compared with those in control group. Combining with QKL enhanced the efficacy of gefitinib, with synergism effect ($q=1.19$), while Shenfu decoction shown antagonism effect to gefitinib ($q=0.50$). Data are presented as the mean $\pm$ standard deviation. * $P<0.05$ and ** $P<0.01$ vs. control. ▲ $P<0.05$ vs. gefitinib alone. SF, Shenfu decoction; QKL, Qingkailing oral solution.

Figure 4

Western blot analysis didn't show clear mechanism of combination effect between gefitinib and Shenfu /Qingkailing injection in A549 cell line in vitro (4A). Data are presented as the mean $\pm$ standard deviation of three independent experiments. Immunohistochemistry analysis indicated that EGFR phosphorylation inhibition and the downstream PI3K/AKT and RAS/RAF/ERK pathway inhibition served an important role in the synergism effect between QKL oral solution and gefitinib in H1975 xenograft transplanted nude mice (4B). Data are presented as the mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control. $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$ and $\blacktriangle\blacktriangle\blacktriangle P < 0.001$ vs. gefitinib alone. SF, Shenfu injection (in vitro) or decoction (in vivo); QKL, Qingkailing injection (in vitro) or oral solution (in vivo).