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Yangfei Kongliu Formula (YKF) For Advanced Lung Cancer by Inhibiting Biological Behaviors Via Beclin-1 Promoting Autophay

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Abstract

Background: Traditional Chinese medicine Yangfei Kongliu Formula (YKF) has been proved to be effective in the treatment of advanced non-small-cell lung cancer (NSCLC). However, its molecular mechanism is poorly characterized. The aims of this study were further investigated the effect of YKF on NSCLC and biological behaviors of lung cancer cells.

Patients and Methods: Total 60 cases with advanced NSCLC with deficiency of both "qi and yin" were randomly divided into chemotherapy group and chemotherapy plus YKF group, evaluated for treatment outcomes with clinical symptoms, Karnofsky performance scale (KPS), progression-free survival (PFS) and RECIST. YKF was given to SD rats preparing YKF serum for cell culture. Autophagy with or without 3-methyladenine inhibition was analyzed by electron microscopy. Real-time fluore- scence quantitative PCR, RNAi and Western blotting were used to detect the related gene or protein expression. Cell proliferation, migration and invasion were evaluated by CCK-8 or Transwell assay, respectively.

Results: Compared with the chemotherapy group, the patients with NSCLC in the combined YKF group had obvious advantages in improving clinical symptoms and life quality, with significant prolonging survival time for 1-year (53.3 % *vs* 80 %, χ^2 =

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4.801, $P = 0.028$) and for 2-year (23.3 % *vs* 66.7 %, $\chi^2 = 11.380$, $P < 0.001$). YKF has up-regulated Beclin-1 and p62 expression, enhanced LC3 II/LC3 I ratio to promote autophagy, and inhibited biological behaviors of proliferation, migration and invasion of A549 cells *in vitro*. This should be a novel anti-cancer mechanism of YKF that had been confirmed by inhibited autophagy or silenced Beclin-1 expression, indicated that the chemotherapy plus YKF be reasonable in accordance with the principle of"Nourishing positive accumulation and eliminating self"for advanced lung cancer.

Conclusion: Combined YKF has obvious advantages for advanced lung cancer and inhibit biological behaviors via increasing Beclin-1 promoting autophagy.

Keywords: Yangfei Kongliu Formula (YKF), Traditional Chinese Medicine; Advanced Lung Cancer, Lung A549 cells; Autophagy, Beclin-1; LC3 II/I ratio; Biological Behavior

Introduction

Lung cancer remains the leading cause of cancer mortality in men and women in the worldwide [1,2]. About 90 % of lung cancer cases are caused by smoking and other factors such as radon gas, asbestos, air pollution exposures, chronic infections, multiple inherited and acquired mechanisms of susceptibility to lung carcinogenesis. Lung cancer is divided into two broad histologic classes, which grow and spread differently: small-cell lung cancer (SCLC) and non-small cell lung cancers (NSCLC). Treatment options for lung cancer include surgery, radiation therapy, chemotherapy, and targeted therapy [3]. Clinically, immunotherapy and gene therapy have not been popularized. Chemotherapy and radiotherapy often caused severe side effects. Operative treatment is to excise tumor from the body via clinical surgery, but it limits the minority of cancers. Therapeutic-modalities depend on several factors, including the type and stage of cancer [4]. Despite the improvements in diagnosis and therapy have been made, the responses to current standard therapies are poor except for the most localized cancers, and the prognosis for lung cancer is still unsatisfactory [5,6]. A better understanding of the autophagy and traditional Chinese medicine (TCM) pertinent to these challenging malignancies, might lead to the development of more efficacious and perhaps more specific drugs in lung cancer biology and therapeutic strategies including currently under clinical investigation [7,8].

In recent years, accumulating evidence of TCM can assist in the treatment of severe side effects caused by radiotherapy and chemotherapy, improve the response rate of radiotherapy and chemotherapy, and improve the life quality of patients [9]. In addition, some patients are unable to withstand the side effects of radiotherapy and chemotherapy, so they choose to treat cancer with Chinese herbal compound decoction and get a good therapeutic effect [10]. The evidences of clinical treatment, animal models and cell experiments showed that Chinese medicine could inhibit the occurrence, metastasis and angiogenesis of tumors, inhibit xenografted tumor growth, and affect the growth phenotype of tumor cells, including the biological behaviors of invasion and migration, survival, apoptosis and autophagy [11,12]. Autophagy is distinct from programmed cell death type I apoptosis and plays a pivotal role in maintaining cellular homeostasis during stress conditions by clearing damaged proteins, organelles, pathogens, or aggregates under physiological conditions. Also, autophagy disorder is involved in pathogenesis of lung cancer with related-proteins (Beclin-1, LC3, and p62) alterations [13,14].

Previous studies have shown that Yangfei Kongliu Formula (YKF) has been developed depending on the therapeutic principle of "Nourishing positive accumulation and eliminating self". The deficiency in both lung-qi, qi and yin based on the theory is the primary lung cancer syndrome [15]. YKF has be confirmed that could inhibit the growth and metastasis of Lewis lung cancer cells and cisplatin in tumor-bearing mice [16]. However, the inhibitory mechanism of YKF on NSCLC remains to be established. The objectives of this study were investigated the effect of YKF on cases with NSCLC and biological behaviors of lung cancer cells by analyzing the autophagy-related proteins (ARP, Beclin-1, LC3, and p62) expressions.

Materials and Methods

Patients' enrollment

A cohort of 60 patients with advanced NSCLC with deficiency of both "qi and yin" from the Nantong Hospital of Traditional Chinese Medicine, China, was investigated with written or

verbal consent. Diagnostic criteria of lung cancer was based on the guidelines for standardized diagnosis and treatment of lung cancer (2018). This study was approved by the Hospital Ethics Committee and performed in line with medical ethics of the Helsinki Declaration. No significant differences were found between the two groups before treatment, without drug allergy history, no serious heart disease, normal liver and kidney function, and blood parameters. Patients were randomly divided into chemotherapy group (n = 30) and combined YKF group (chemotherapy plus YKF, n = 30), based on the best supportive therapy. The chemotherapy group (15 males and 15 females, 68.2 ± 16.5 years old) with platinum-containing two-drug regimen, systemic chemotherapy for 4 cycles, one dose/per 3 weeks, and then given single drug for 2 cycles, one dose/per 4 weeks. The combined YKF group (15 males and 15 females, 68.2 ± 16.5 years old) with chemotherapy and YKF twice/per week to nourishing lung and controlling tumor growth. YKF consists of six components: 45 g of *Astragali Radix* (Huangqi), 15 g of *Panax Ginseng* (Renshen), 30 g of *Radix Glehniae* (Beishashen), 30 g of *Herba hedyotidis* (Baihuasheshecao), 30 g of *Agrimonia herba* (Xianhecao), and 30 g of *Coicis Semen* (Yiyiren).

Clinical observation

After treatment, the patients' clinical symptoms, Karnofsky performance scale (KPS), progression-free survival (PFS), and efficacy in the two groups were evaluated according to the new response evaluation criteria in solid tumors (RECIST guideline, Version1.1) [17]. The KPS was more than 60 and the survival time was more than 3 months, with complete response (CR) with disappearance of all target lesions, any pathological lymph nodes (whether target or non-target) must have reduction in short axis to less than target lesions, taking as reference the baseline sum diameters; partial remission (PR); no effect (NC); and progression disease (PD) with at least a 20 % increase in the sum of target lesion diameters, taking as reference the smallest sum on study including the baseline sum. In addition to the relative increase of 20 %, the sum must also demonstrate an absolute increase at least 5 mm including one or more new lesions; and stable disease (SD) with neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study.

YKF-contained serum preparation

YKF was purchased from the Nantong Hospital of Traditional Chinese Medicine, China, and decocted at 5-, 10-, and 15-times the equivalent human dose conversion according to the Experimental Methodology of Pharmacology of Chinese Materia Medica (3rd Edition). Male Sprague Dawley (SD) rats were randomly divided into 4 groups: control, 5-fold dose (5 ×, 3 g/mL of YKF), 10-fold dose (10 ×, 6 g/mL of YKF), and 15-fold dose (15 ×, 9 g/mL of YKF). Rats in the drug treatment groups received different doses of YKF, with twice/day (3 mL/time, morning and evening), and rats in the control group received an equal volume of 0.9 % NaCl solution. Blood was collected 2 h after the 6th gavage for preparing YKF-contained serum. After inactivation and sterilization, sera were stored at -20 °C before use. All procedures performed in accordance with the guidelines for experimental animals approved by the Animal Care and Use Committee of Nantong University, China.

Cell culture

Lung cancer A549 cells were obtained from the National Collection of authenticated cell cultures (Shanghai, China). Opti-MEM (GIBCO, USA) contained penicillin- streptomycin was used to culture A549 cells in a 6-well plate overnight. On the next day, the cells were cultured in the fresh complete culture medium with different doses of YKF-contained serum for 48 h. For autophagy analysis, 3-methyladenine (3-MA) was added to A549 cells to interfere with the formation of autophagosomes.

Electron microscopy detected autophagy

A549 cells after the YKF treatment for 48 h were digested, resuspended in phosphate buffer saline (PBS), washed 3 times with PBS after centrifugation, and added 2.5 % of glutaraldehyde along tube wall to fix cells overnight at 4 °C. The next day, the fixed cells were rinsed 3 times with PBS, then added 1 % osmium tetroxide at 4 °C for 30 min, successively added to cells for dehydration with 50 %, 70 %, 90 % and 100 % of acetone in order. Cells were mixed with diluted embedding agent for 30 min, replace with pure embedding agent at room temperature overnight, moved to capsule bottom and mixture with embedding solution, baked in oven at 60 °C to solidify into a hard block, cut into semi-thin sections (1 μm of thick), stained with sodium acetate and lead citrate, and observed under electron microscope with a bilayer membrane or with lysosomes fuse to form autophagosomes with a monolayer structure.

Preparation of mRFP-GFP-LC3 lentiviral vector

The pLVX-puro-mRGP-EGFP-LC3B lentiviral vector was prepared in 293 T cells. In brief, 293 T cells were seeded in a culture dish and cultured until 80 % confluence. A plasmid mixture of 5 μg of pLVX-puro-mRGP-EGFP-LC3B, 5 μg of psPAX2, and 5 μg of pMD2.G was dissolved in 600 μl of opti-MEM. Another mixture of 20 μl of lipo3000 and 600 μl of opti-MEM was prepared. The two-mixture solution was gently mixed, placed at room temperature for 20 min, and then added into the cell culture dish, cells were cultured for 48 h. The supernatant was collected to obtain a lentiviral vector and filtered through a 0.45 μm filter. The lentiviral vector was concentrated by super centrifugation and filtered with a 0.22 filter, and measured the virus titer.

Autophagy flux detection

For detecting autophagy flux in A549 cells, cells in the logarithmic growth phase were inoculated into well plates with 5×10^4 cells/well, placed in cell incubators overnight. Next day, each well was added with fresh culture solution with MOI = 20 virus/well. After infection for 24 h, the culture medium containing virus was removed. Then coincubation for 48 h, cells were fixed with paraformaldehyde and sealed. Green and red spots were observed with a confocal microscope and photograph.

Interfering plasmids & cell transfection

A549 cells at the logarithmic growth stage were washed once with sterile PBS and digested with trypsin. The cell pellet was resuspended in the complete culture medium. After the count, cells were seeded into 6-well plates at 3×10^5 times/well and placed in cell incubator overnight. Specific plasmids for targeting Beclin-1 gene sequences were constructed for Si-Beclin-1-1: sence 5'-AGAUACCGACUUGUUCCUUdTdT -3' and anti-sence 5'-AAGGAACAAGUCGGUAUCUdTdT-3'; Si-Beclin-1-2, sence 5'-GAGAUCUUAGAGCAAAUGAdTdT-3' and anti-sence 5'-UCAUUUGCUCUA AGAUCUCdTdT-3'; and Si-Beclin-1-3, sence 5'-GACCAUGCAAUGGUGGCU UdTdT-3' and anti-sence 5'-AAGCCACCAUUGCAUGGUCdTdT-3'. Si-negative -RNA as a control, sence 5'-UUCUCCGAACGUGUCACGUdTdT-3' and anti-sence 5'-ACGUGACACGUUCGGAGAAdTdT-3' were mixed with Lipofectamine 3000 (Biotend., Shanghai, China), and transfected to A549 cells, respectively. Cells were placed in cell incubator for 6 h, then change to complete culture medium for 48 h, and collected cells for further experiments.

Quantitative real-time PCR (qRT-PCR)

Purified Beclin-1 mRNA from cells with RNAiso Plus (Tri^{zol}, TAKANA, Japan) with lysed sufficiently, and its concentration was determined by a spectrophotometer. Two μg of RNAs were reversely transcribed into cDNA using PrimeScript™ RT Master Mix (Perfect Real Time, TAKARA, Japan), and then amplified with the Power SYBR Green PCR Master Mix (Thermo, USA) in the ABI ViiA7 fluorescent quantitative PCR instrument with pairs of primers: Beclin-1-h F: 5'-CCATGCAGGTGAGCTTC GT-3' and Beclin1-h R: 5'-GAATCTGCGAGAGACACCATC-3'. Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) was used for internal reference: GAPDH-h F: 5'-TGACAACTTTGGTATCGTGGAAGG-3' and GAPDH-h R 5'-AGGCAGGGATGATGTTCTGGAGAG-3'. The relative ratios of *Beclin-1* among different groups were calculated from Beclin-1 mRNA to GAPDH mRNA.

CCK-8 assay

Cells with 1×10^4 /per well were seeded in a 96-well plate and placed in an incubator overnight. The next day, cells were cultured with the complete medium contained with 10 % of YKF-contained serum for 24 h, 48 h and 72 h, respectively. After the incubation time, each well was added with 10 μl of CCK-8 solution, incubated for another 2 h, and each absorbance was measured at 450 nm.

Western blotting

Treated cells were washed with PBS, then lysed in Radio Immunoprecipitation Assay (RIPA, Beyotime, China) for 30 min on ice, collected supernatant after centrifugation, and quantified protein concentration by a bicinchoninic acid method (BCA, Beyotime, China). Total 50 μg of protein mixed with loading buffer was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred the protein from gels to polyvinylidene fluoride membrane. Nonspecific proteins were blocked by a 5 % of skimmed milk. Specific proteins were blotted by anti-Beclin-1, anti-p62, anti-LC3, and GAPDH (Proteintech, USA), visualized protein with Horseradish Peroxidase-labeled goat anti-mouse IgG (Beyotime, China) incubation and chemiluminescence with the ECL Kit (Millipore, USA).

Transwell assay

A549 cells were collected in the logarithmic growth phase, digested with trypsin, washed with PBS, resuspended on the serum-free medium, and then adjusted cell density to 4×10^5/mL. Transwell chambers with 8 μm of diameter were placed in a 24-well plate containing culture medium. Total 200 μl of cell suspension was added into the upper chamber and cultured for 48 h. Chamber was taken out, washed with PBS, and fixed with 4 % of paraformaldehyde for 20 min. Cells were stained with crystal violet solution and dyed purple. Chamber with cells was placed on slides, observed under a microscope, and photographed from three selected fields. Procedures for cell invasion ability analysis were the same as above except the chamber was added with Matrigel and 10^5 cells were added to the upper compartment of Transwell.

Statistical analysis

SPSS Statistics version 21.0 (IBM, Armonk, NY, USA) was used for data analysis. Chi-square test was used for counting data, and mean ± standard deviation was used for measuring data. Difference between groups was performed by student *t*-test. Wilcoxon rank sum test was used for comparing symptom scores between groups or within groups, and Kaplan analysis was used for PFS, 1-year- or 2-year- survival. A P value less than 0.05 was considerately significant.

Results

Clinical observation of YKF on Patients with NSCLC

The comparative analysis of clinical symptoms, some biochemistries and tumor markers in the patients with NSCLC before and after treatment are shown in Table 1. Both the chemo-

Table 1: The improvement of clinical symptom score and biomarker alteration before and after treatment $\bar{x}$ (± SD)

	Chemotherapy			Combined YKF		
	Before	**After**	**D-value**	**Before**	**After**	**D-value**
Symptoms						
Cough	2.20±0.52	1.10±0.55**	1.10±0.79	2.30±0.47	0.50±0.51**	1.80±0.77△△
Phlegm blood	2.35±0.49	1.60±0.60**	0.75±0.72	2.45±0.51	0.70±0.47**	1.75±0.44△△
Breath shortness	2.20±0.62	1.00±0.73**	1.20±0.89	2.30±0.73	0.55±0.60**	1.75±0.97△
Chest/waist pain	2.35±0.48	1.40±0.50**	0.95±0.76	2.15±0.67	0.55±0.51**	1.60±0.75△△
Expectoration	2.55±0.51	1.45±0.60**	1.10±0.55	2.30±0.66	0.50±0.51**	1.80±0.70△△
Mental fatigue	2.65±0.49	1.75±0.64**	0.90±0.72	2.70±0.47	0.60±0.50**	2.00±0.65△△
Mouth thirsty	2.45±0.51	1.30±0.57**	1.15±0.59	2.40±0.50	0.65±0.49**	1.75±0.72△△
Night sweats	2.55±0.51	1.35±0.49**	1.20±0.52	2.81±0.41	0.70±0.57**	2.10±0.64△△
Total scores	19.30±2.49	10.95±1.28**	8.35±2.49	19.30±2.66	4.75±1.48**	14.55±2.50△△
Biomarkers						
ALT (U/L)	27.25±8.96	33.30±7.78*	-6.05±3.71	27.55±8.26	29.15±7.98	-1.60±2.04△△
Cr (μMol/L)	59.95±22.52	67.85±19.53	-7.90±8.29	60.00±20.07	62.30±18.90	-3.70±7.38△
CEA (ng/mL)	35.25±11.99	26.50±11.25*	8.75±6.58	36.80±10.89	20.20±5.76**	16.60±8.85△△
NSE (ng/mL)	18.65±5.82	15.03±4.00*	3.62±2.44	18.58±5.81	14.23±5.17*	4.35±2.51
CYFRA21-1 (ng/ml)	4.08±1.39	3.32±1.32	0.76±0.67	4.03±1.35	3.13±1.15	0.90±1.04
TK1 (ng/mL)	2.68±0.69	2.10±0.47*	0.58±0.35	2.79±0.65	2.32±0.63*	0.47±0.33

Compared with the before treatment group, $^*P < 0.05$, $^{**}P < 0.01$; Compared with the chemotherapy group, △ $P < 0.05$, △△ $P < 0.01$.

ALT, alanine transaminase; CEA, carcinoembryonic antigen; CR, creatinine; CYFRA21-1, cytokeratin fragment; NSE, neurone specific enolase; TK1, thymidine kinase 1; YKF, Yangfei Kongliu Formula

therapy and the combined YFK group improved the symptoms of patients with advanced NSCLC. After treatment, the KPS scores according to some clinical symptom in the combined YKF group were significantly improved ($P < 0.01$) more than those in the chemotherapy group. Because YFK takes with overall concept as its guiding ideology, takes the evidence examination for the advanced NSCLC treatment, and plays an integral regulatory role in traditional Chinese medicine, with statistically significant decreasing levels ($P < 0.05$) of CEA or NSE, and hepatocyte damage with lower ALT activity.

Enhancing survival rate of NSCLC

The comparative analysis of the short- and the long-term curative effects in patients with NSCLC between the two groups are shown in Table 2. The total short-term effective rate (CR + PR + SD) was 43.3 % (13 of 30) in the chemotherapy group, and 60.0 % (18 of 30) in the combined YKF group, with no significant difference between two groups (χ^2 =1.950, P = 0.584). However, the long-term survival rates were from 53.3 % (16 of 30) in the chemotherapy group up to 80 % (18 of 30) in the combined YKF group for 1-year survival (χ^2 = 4.801, P = 0.028) and from 23.3 % (7 of 30) in the chemotherapy group up to 66.7 % (20 of 30) in the combined YKF group for 2-year survival (χ^2 = 11.380, $P < 0.001$). These data indicated the combined YKF therapy would be useful to achieve better clinical efficacy, with less adverse reactions and longer survival rates for NSCLC patients.

Table 2: Comparative analysis of short- and long- term curative effect between chemotherapy and combined groups (%)

	Short-term curative effect (%)			**Long-term curative effect (%)**		
Group n	CR	PR	NC	PD	1-year-survival	2-year-survival
Chemotherapy 30	2 (6.7)	7 (23.3)	4 (13.3)	17 (56.7)	16 (53.3)	7 (23.3)
Combined YKF 30	3 (10.0)	8 (26.7)	7 (23.3)	12 (40.0)	24 (80.0) △	20 (66.7) △

CR: complete response; NC: no effect; PD: progression disease; PR: partial remission; YKF: Yangfei Kongliu Formula

*Compared with the control group, △$P < 0.05$

YKF promotes autophagy of A549 cells

The effect of YKF on A549 cells treated with different doses of YKF-contained serum for 48 h are shown in Figure 1. The cell viability was significantly inhibited by progressively higher doses of YKF-contained serum, but no significant differences were found among the three groups, and higher dose hadn't showed better ability to inhibit cell activity (left panel, Fig.1A). So, the 5 × YKF-contained serum was selected for further study. After the cells were exposed for 24 h, 48 h and 72 h, the cell viability was marketed decreasing in a time-dependent manner (right panel, Fig.1B). The protein LC3 II/LC3 I ratio was used to observe the autophagosome formation and autophagy (Fig.1C). The A549 cells were treated with three doses of YKF-contained serum for 48 h, the alterations of autophagy related LC3 II/I ratio (Fig.1D), p62, and Beclin-1 expressions (Fig.1E) were analyzed to evaluate YKF on the effect of A549 cell autophagy. Compared with the control, significantly increasing LC3 II/I, p62 and Beclin-1 expressions were confirmed in the A549 cells with YKF, and indicated that YKF might induce autophagic death in lung cancer cells.

Confirmation of YKF promoting autophagy

Representative electron photomicrographs of autophagosomes after A549 cells with the YKF or autophagy inhibitor (3-MA) are shown in Figure 2. Compared with the control without YKF (Fig.2A), YKF promoted the autophagy of A549 cells and the number of autophagosomes was obviously increased under the electron microscope (Fig.2B). The LC3 puncta between of YKF treated A549 cells with or without 3-MA were compared to further confirm the autophagy-promoting role of YKF that induced the increasing of LC3 puncta (Fig.2C), and more increase was abolished after 3-MA treated, suggesting that YKF exert a promoting autophagy impact on cancer cells.

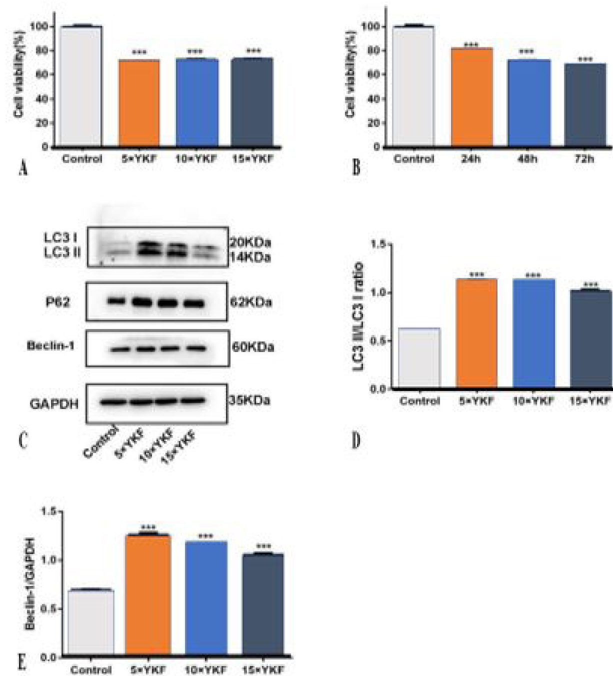

Figure 1: YKF promoting lung cancer A549 cell autophagy.

A**,** the CCK-8 viability after A549 cells treated with 5 ×, 10 ×, or 15 × YKF-contained serum for 48 h; **B**, A549 cells with 5 × YKF-contained serum for different times; **C**, the LC3 I, LC3 II, P62, and Beclin-1 protein by Western blotting; **D**, the relative ratio of LC3II/I; and **E**, the relative ratio of Beclin-1. **YKF**, Yangfei Kongliu Formula $^{***}P < 0.001$ compared with control.

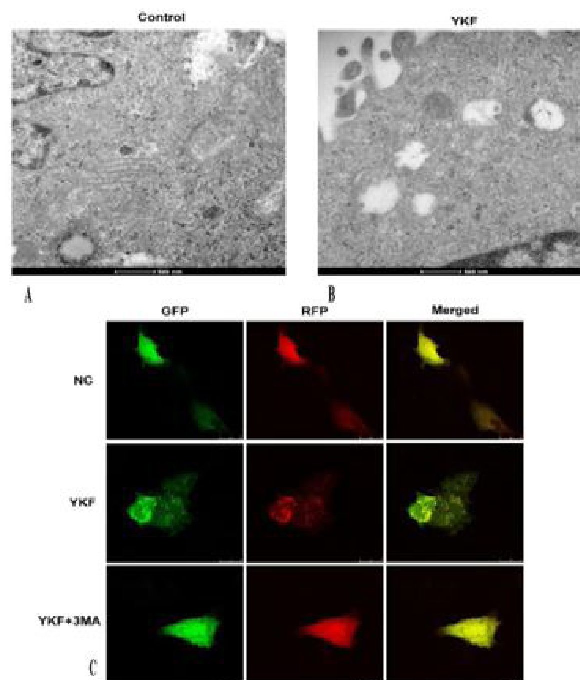

Figure 2 :YKF-induced autophagy or 3-MA inhibition on A549 cells.

A: the control electron photomicrographs of A549 cells (magnification, 13000 ×); **B:** the electron photomicrographs of autophagosomes in A549 cells treated with YKF for 48 h (magnification, 13000 ×); **C:** the pretreated A549 cells with or without 1 mM 3-MA exposed to YKF for an additional 48 h (magnification, 800 ×), and LC3 puncta were carried out by immunofluorescence technique. GFP, green fluorecent protein; 3-MA, 3-methyladenine; RFP, red fluorecent protein; YKF, Yangfei Kongliu Formula.

YKF promoting autophagy associated with Beclin-1

The up-regulation of Beclin-1 expression plays a pivotal role in cell autophagy, and YKF promoting the autophagy of lung cancer cells associated with Beclin-1 alteration are shown in Figure 3. After the A549 cells transfected by si-Beclin1-1, si-Beclin1-2, si-Beclin1-3 and control plasmids, the Beclin-1 expression were significantly silenced by the specific si-Beclin1-2 (Fig.3A) that could inhibit the proliferation of A549 cells (Fig.3B). Silencing Beclin-1 could decrease YKF-induced LC3II/I ratio and p62 expression level (Fig.3C). Similarly, adding 3-MA also might achieve the same effect as Beclin-1 silencing (Fig.3B), and inhibiting LC3II/I ratio and p62 expression (Fig.3C), suggesting that YKF induce A549 cell autophagy depends on Beclin-1 and reverse by both Beclin-1 silencing and 3-MA.

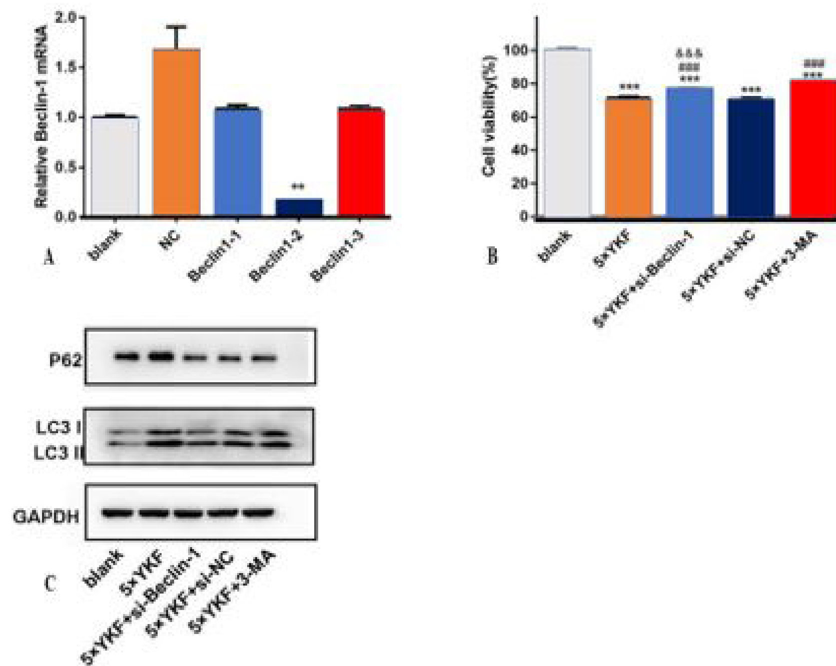

Figure 3: Silencing Beclin-1 reversed autophagy promoting effect of YKF on cancer cells.

A, the Beclin-1 iRNA plasmids were transfected to A549 cells and the si-Beclin-1-2 plasmid was screened with specific inhibiting Beclin-1 expression; B, the effects of si-Beclin-1-2 and 3-MA on cell viability of the A549 cells treated with YKF; C, the effects of si-Beclin-1-2 and 3-MA on cell autophagy related-protein expression of P62 and LC3B I/II. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with blank. $^{\#\#\#}P < 0.001$ compared with 5 × YKF. $^{\&\&\&}P < 0.001$ compared with 5 × YKF + si-NC. 3-MA, 3-methyladenine; YKF, Yangfei Kongliu Formula

YKF inhibiting migration or invasion of cancer cells

The anti-tumor properties or effects of YKF-contained serum on A549 cell migration or invasion are shown in Figure 4. Compared with the blank group (Fig.4A), 5 × YKF serum caused a significant decrease in migratory cell number (Fig.4B). Then, added YKF serum to Beclin-1 silenced A549 cells (Fig.4C). Compared with the YKF serum or negative control cells (Fig.4D), the Beclin-1 silenced A549 cells migrated more. Besides, compared with YKF serum or negative control cells, si-Beclin-1, autophagy inhibited A549 cells by 3-MA treatment (Fig.4E) migrated more. These data indicated that YKF could inhibit A549 cell migration via affecting autophagy and Beclin-1(Fig.4F).

Also, the effect of YKF-contained serum on A549 cell invasion ability was examined (Fig. 4, A1 ~ F1). Compared with thee blank group (Fig.4A1), the YKF caused a significant decrease in the invaded cell number (Fig.4B1). Next, YKF serum was added to the Beclin-1 silenced A549 cells. Compared with the YKF serum or negative control cells (Fig.4C1)), the si-Beclin-1-2, Beclin-1-2 silenced A549 cells invaded more. Besides, compared with YKF serum or negative control cells (Fig.4 D1), si-Beclin-1, autophagy inhibited A549 cells by 3-MA treatment (Fig.4E1) invaded more, suggesting that YKF could inhibit A549 cell invasion via affecting autophagy and Beclin-1 expression (Fig.4F1).

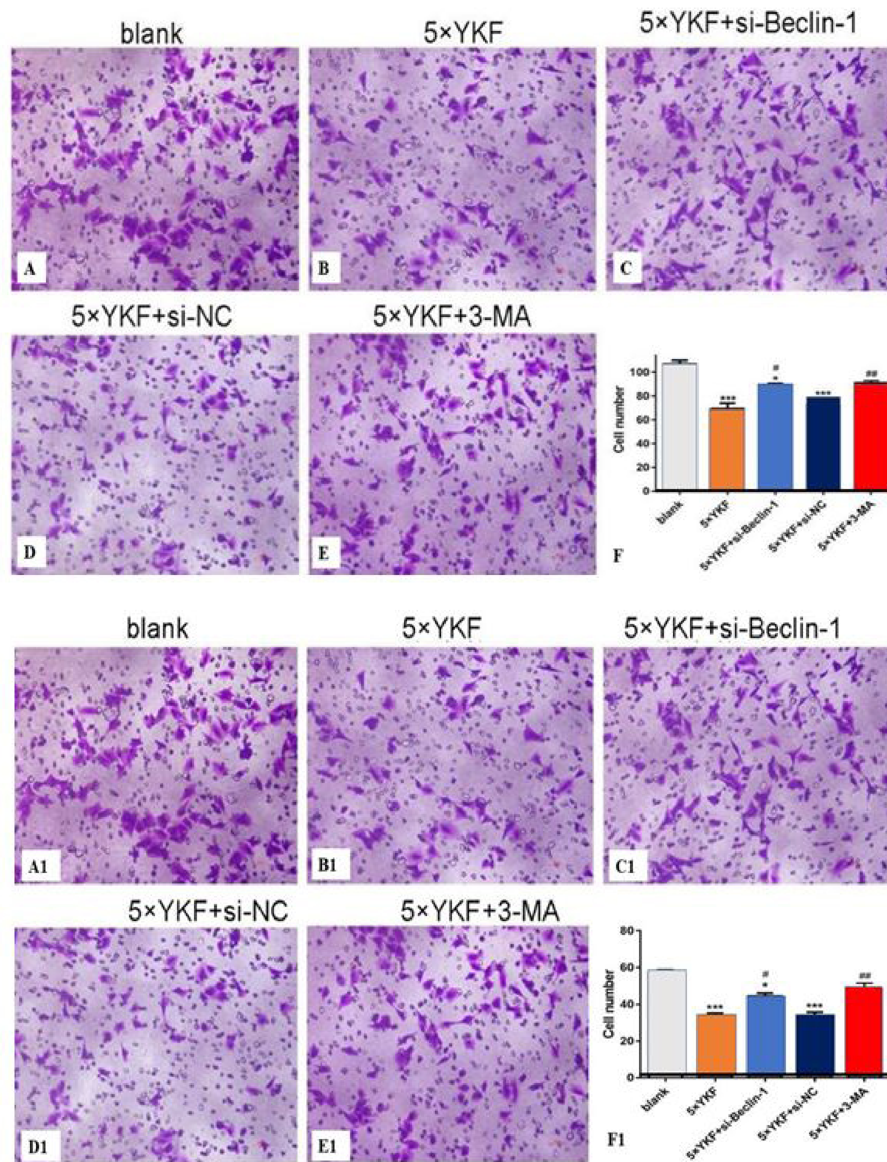

Figure 4: Silencing Beclin-1 reverses YKF inhibiting cell migration or invasion.

Representative photomicrographs of migratory cells (**A** ~ **F**) or invaded cells (**A1** ~ **F1**) cells were counted in three independent experiments (n = 3). **A**, the A549 cells as a blank control; **B**, the A549 cells with YKF; **C**, the A549 cells with YKF plus specific si-Beclin-1-2; **D**, the A549 cells with YKF plus si-Beclin-1 negative control; **E**, the A549 cells with YKF plus 3-MA; **F**, the comparative analysis of A549 migratory cell numbers among the different groups; **A1**, the A549 cells as a blank control; **B1**, the A549 cells with YKF; **C1**, the A549 cells with YKF plus specific si-Beclin-1-2; **D1**, the A549 cells with YKF plus si-Beclin-1 negative control; **E1**, the A549 cells with YKF plus 3-MA; **F1**, the comparative analysis of A549 invaded cell numbers among the different groups. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with blank. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ compared with 5 × YKF. **3-MA**, 3-methyladenine; **YKF**, Yangfei Kongliu Formula.

Discussion

With the continuous breakthroughs in molecular biology and biochemistry, a great progress in the treatment of lung cancer have been constantly made [18,19]. There is no doubt that standard treatment, such as surgery, radiotherapy, chemotherapy, targeted therapy [20]. TCM has been widely accepted as the mainstream form of comple- mentary and alternative therapy that benefits NSCLC patients [21,22]. According to pathological process, it has been reported that lung cancer can be inhibited by TCM through potential mechanisms through inhibiting cancer metastasis, including promoting apoptosis or autophagy, and inhibiting epithelial mesenchymal transition and angiogenesis [9,23,24]. Systematic reviews of TCM in adjuvant therapy for NSCLC concluded that has significant advantages in terms of efficacy, safety, and low cost by improving patients' quality of life and extending survival rates like YKF [15]. In this study, the clinical efficacy and survival rate of YKF for advanced NSCLC patients were confirmed and further analyzed the alterations of ARP expression to explore the effect of YKF on the biological behavior of lung cancer cells.

Anti-cancer TCM prescriptions in clinical practice for lung cancer as one of complementary or alternative medicines have been used with beneficial effects on improving the life quality of patients [25,26]. TCM prescription YKF with some principal components from Astragali Radix (Huangqi), Panax Ginseng (Renshen) and so on were useful to enhance patients' immunity or inhibit HGF and its receptor for indirect anti-tumor role [9,15,16]. In the present study, good anti-tumor effects were confirmed by the data of the combined YKF group, with significantly improvement of patients' clinical symptoms. Although no significantly different between two groups was found in the short-term curative effect in the patients with YKF. But the long- term curative effect of YKF could prolong the survival time and improve the life quality of patients. Because lung cancer cells escape the recognition of the immune system, using YFK takes with overall concept as its guiding ideology, takes the evidence examination for the advanced NSCLC treatment, and plays an integral regulatory role in TCM, with significantly decreasing levels of CEA or NSE, and hepatocyte damage with lower ALT activity. However, the relationship between YKF and ARP in lung cancer hasn't been identified, and possibility regulatory mechanism of YFK via autophagy is worth further study.

As a type II programmed cell death, autophagy plays an important role in cancer by regulating ARP, which inhibits tumorigenesis at early stage and promotes tumor progression at late stage [27]. Once cancer at late stage, autophagy is a dynamic degradation and circulatory system that contributes to survival or growth, and promotes invasion and metastasis of cancer [28]. Among ARP, Beclin-1 is the core component of the Class III PI3K complex required for autophagy [29,30], LC3 is required for autophagy formation to bind to membrane lipid phospholipid ethanol- amine, and P62 (also known as Sqstm1) is the key molecule for autophagy by interacting with LC3 [31,32]. Their changes were associated with tumor formation, promotion and resistance to cancer therapy [33, 34], and the down-regulating Beclin-1, LC3 and P62 expressions in cancerous tissues were associated with poor prognosis or malignant progression [23,32]. In this study, YKF was discovered to increase LC3 II/I, P62 and Beclin-1 expression, and autophagic death occurred in lung cancer A549 cells, further experiments confirmed the autophagic promotion of YKF, specific Beclin-1 at mRNA transcription intervention by si-Beclin-1-2 plasmid in vitro or inhibition of autophagosome formation with 3-MD. Those data for the first time indicated that YKF not only inhibit cell proliferation but also promote the expression of Beclin-1, p62, and LC3 II/I and formation of autophagosomes

As suggested by previous study, autophagy has a complex dual role in tumor survival or cell death [28]. It is still complicated whether the regulation of autophagy is beneficial in eliminating cancer. However, many studies have shown that autophagic death of cancer cells can be induced by natural drugs or pharmaceutical ingredients, such as SH003 (a herbal formulation) [35], Kaempferol (a flavonoid) [36], and Polyphyllin VI (a saponin from Trillium tschonoskii Maxim) [37,38]. There are several TCM or herbal extracts in inducing lung cancer cells autophagic death [9,21]. In this study, YKF could effect on the migration and invasion of lung cancer cells except of inhibiting cell proliferation. YKF plays an important role in inhibiting biological behaviors of lung cancer cells that was useful to control the progression of prolonging the survival time of advanced NSCLC patients.

In conclusion, this is the first report to investigate YKF anti-cancer with a novel mechanism of the promoting autophagy for advanced NSCLC based on clinical and basic studies. Using the chemotherapy plus YKF, the long-term curative effect could prolong the survival time and improve the life quality of NSCLC patients. Also, there was a strong effective anticancer property of YKF in lung cancer cells with Beclin-1-dependent autophagy. The novel findings are promising, and initial evidence confirmed that YKF via the promoting autophagy should provide new mechanism insight into effectively therapy for lung cancer.

However, further work should be to accumulate more clinical cases and explore combining traditional prescription YKF with multi-targeting strategies for lung cancer therapy in the future.

Author Contributions

Shen SJ, Chen X & Yao M contribute equally to this work and wrote draft; Shen SJ & Jiang SJ perform experiments & analyze clinical data; Sai WL & Wang L perform statistical analysis; Shi Y & Yao DF are the guarantors & supervise the manuscript. All authors approved the final version of the manuscript.

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Conflict of Interest Statement

The authors declare none conflicts of interest.

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