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Research Article

Mechanism of Jinlong Capsule (JLC) in Human Esophageal Squamous Cell Carcinoma (ESCC) via the MAPK Signal Pathway

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Abstract

Background and Objective: Human Esophageal Squamous Cell Carcinoma (ESCC) is one of the most common malignancies and the most frequent reason of cancer death around the world. Jinlong Capsule (JLC) is a Traditional Chinese Medicine (TCM) and has been used for several decades to treat cancers with significantly improved clinical outcomes as adjuvant therapy. In the present study, the effect of JLC on anti-proliferative, pro-apoptotic activities of human Eca-109 and EC9706 cells *in vitro* were investigated. **Materials and Methods:** The MTT assay was used to detect cell viability. Apoptosis analysis by flow cytometry was used to detect the percentages of the living cells as well as early, late apoptotic cells and necrotic cells. Ultrastructure observation using Transmission Electron Microscopy (TEM) was used to observe the ultrathin sections. Mitochondrial Transmembrane Potential (MTP) assessment, immunofluorescence staining and expression level of proteins by western blotting were detected its mechanism. **Results:** All the results showed that JLC could decrease proliferation and induce apoptosis in ESCC cells. Moreover, JLC might increase the expression of pro-apoptotic proteins caspase-8 and caspase-9 by activating the extrinsic and the intrinsic apoptotic pathways. The anti-apoptosis protein B-cell lymphoma-2 (Bcl-2) also could promote mitochondrial-mediated apoptosis by inducing Mitochondrial Outer Membrane Permeabilization (MOMP) in the study. Besides, the MAPK signal pathway took function in ESCC cells that treated with different concentrations of JLC. **Conclusion:** These findings suggested that JLC may be a promising agent worthy of being investigated for the treatment of human ESCC *in vitro*. The JLC holds the multi-targeted therapeutic effects of TCM.

Key words: Human esophageal squamous cell carcinoma, jinlong capsule, proliferation, anti-apoptosis, MAPK signal pathway

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Esophageal Squamous Cell Carcinoma (ESCC) is one of the most prevalent and aggressive malignant cancer. It's the eighth most commonly occurring cancer worldwide[1]. The overall survival rate ranges[2] from 15-20%. The overall five-year survival rate[3] is 9-40%. Multiple factors, such as, tobacco, alcohol, nutritional deficiencies, infectious agents were confirmed to have a relevant relationship to esophageal cancer[4]. Esophageal cancer exists in two main histological types, including adenocarcinoma and squamous cell carcinoma. The incidence of esophageal adenocarcinoma is increasing but ESCC is more predominant in East Asia. It's the highest mortality rates found in Eastern Asia[5]. In the clinic, measures of surgery, chemotherapy, radio therapy and neoadjuvant chemoradiotherapy are taken to prolong the survival rate of ESCC patients. However, ESCC has high metastatic potential and a worse prognosis[6]. Therefore, it is urgently required to identify a novel and in-depth study about the mechanism of ESCC cells to improve the treatments for patients with ESCC.

The mitogen activated protein kinase (Mitogen-activated protein kinases, MAPKs) signal transduction pathway exists in most of the cells[7] and plays a key role in the development and progression of cancer. It's a kind of serine/threonine protein kinase, including extracellular regulated protein kinase (ERK or p44/42), the p38 kinase and the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinases (JNK). Under the stimulation of extracellular signal, these pathways are activated to regulate cell growth, proliferation, differentiation, migration and apoptosis[8,9]. Recent studies have shown that the MAPKs pathway plays a critical role in the development of various tumors[10].

Traditional Chinese Medicine (TCM) is used widely to treat many diseases, including various cancers[11]. Jinlong Capsule (JLC, Chinese national patent number: Z10980041) as the modern Chinese medicine products, a new concept of modern medicine has gradually been recognized by the medical profession and the society. The JLC is a TCM preparation and multi-component is one feature of the main ingredient of modern medicine. It's composed of *Bungarus*, *Agkistrodon* and *Gecko*. *Gecko* can be reached through the network and reduce phlegm stasis, *Bungarus* and *Agkistrodon* have the function of dispelling wind and removing obstruction. The active compounds are extracted by the process of modern cryogenic and biochemical separation from raw animals. The JLC influences many cancers in the clinic treatments, such as pancreatic cancer[12], primary hepatic carcinoma[13], gastric cancer and so on but ESCC has not been reported yet. This study was designed to investigate the effect of JLC on the proliferation and apoptosis of Eca-109 and EC9706 cells, which may provide new hope for clinical tumor therapy.

MATERIALS AND METHODS

Preparation of JLC: The main components of JLC are *Bungarus* (*Bungarus multicinctus Blyth* in southern China, fresh whole body), *Agkistrodon* (*Agkistrodon acutus* (*Guenther*) in southern China, fresh whole body) and *Gecko* (*Hemidactylus frenatus Schlegel* in China, fresh whole body). Some studies found that the JLC contains physiological active peptides and the amino acid fingerprint. Seventeen kinds of amino acids were identified by UPLC, including histidine, serine, arginine, glycine, aspartic acid, glutamic acid, aspartate acid, alanine, proline, cystine, lysine, tyrosine, etc. The amino acid analysis of JLC was established by using 6-acetamido-4-hydroxy-2-methyl quinoline (AQC) as derivatizing agent. The JLC was purchased from Jiansheng Pharmaceutical Co., Ltd. (Lot. No. 160415, Beijing, China) and dissolved in the culture medium to configure the stock solution in 25 mg mL^{-1}, which was diluted to the suitable concentration in the following experiments. 5-fluorouracil (5 Fu) was the product of Xudong Haipu Pharmaceutical Co., Ltd. (Lot. No. 160724, Shanghai, China).

Cell line and reagents: Human ESCC Eca-109 and EC9706 cell lines were purchased from Shanghai aolushengwu Biotechnology Co., Ltd. (Shanghai, China). Roswell Park Memorial Institute (RPMI)-1640 medium and 0.25% trypsin were purchased from HyClone Laboratories, Inc. (Hyclone, Logan, USA). Fetal Calf Serum (FBS)were obtained from Gibco-BRL (Gaithersburg, MD, USA). About 0.25% trypsin-0.02% EDTA solution and Phosphate-Buffered Saline (PBS) were purchased from Gibco, Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Penicillin-Streptomycin Solution (100×) and Enhanced BCA Protein Assay Kit were purchased from Beyotime (China). Dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Chemical Co, (St. Louis, MO, USA). Primary antibodies against caspase-8 (cat. No. 9746; dilution, 1:1,000), caspase-9 (cat. No. 9504; dilution, 1:1,000), B-cell lymphoma-2 (Bcl-2, cat. No. 15071; dilution, 1:1,000), p38 (cat. No. 8690; dilution, 1:1,000), phosphorylated (p-) p38 (cat. No. 4511; dilution, 1:1,000), ERK (cat. No. 4695; dilution, 1:1,000), p-ERK (cat. No. 4370; dilution, 1:1,000) and β-actin (cat. No. 4970; dilution, 1:1,000) were the products of Cell Signaling Technology, Inc. (Beverly, MA, USA). The secondary antibody

(cat. No. G130321; dilution, 1:2,000) was the product of Hangzhou Huaan Biotechnology Co., Ltd., Hangzhou, China). Caspase-3 (cat. No. sc-7272) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, UK). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and mitochondrial membrane potential (MTP) assay kit were purchased from KeyGEN biotechnology Co., Ltd., (Nanjing, China). Other chemicals used were of analytical grade from commercial sources.

Cells culture: Human Eca-109 and EC9706 cells were cultured with RPMI-1640, supplemented with 10% FBS. All the cells were incubated at 37°C in a CO_2 regulated incubator in a humidified atmosphere. When cultures reached confluence, cells were detached with trypsin-EDTA solution and passaged. All the cells were used at logarithmic growth phase in the experiments.

MTT assay of cell viability: Eca-109 and EC9706 cells were respectively plated into 96 well culture plates at a density of 1×10^4 cells/well. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere overnight prior to treatment. After attachment, JLC was added at final concentrations in 0.05-3.2 mg mL^{-1} with six wells for each concentration. After 24, 48, 72 h incubation, 20 µL MTT (5 mg mL^{-1}) was added into each well at 37°C for 4 h. After the supernatant was discarded, 150 µL DMSO was added to dissolve the form azan crystals. Optical Density (OD) was measured at 490 nm with a microplate reader. The JLC concentration at which the cell viability was reduced by 50% (the 50% inhibitory concentration, IC_{50}) at 24, 48, 72 h of treatment was quantified by linear interpolation. The experiments were repeated in triplicate.

Apoptosis analysis by flow cytometry: According to the protocol of Annexin V-FITC/PI apoptosis detection kit, after 24 h incubation of Eca-109 and EC9706 cells with JLC-treated, negative control (without JLC) and positive control (5 FU) cells were collected and washed twice with ice-cold PBS. Then cells were in 250 µL binding buffer mixed with 5 µL Annexin V-FITC reagent and 5 µL Propidium Iodide (PI) reagent. After incubation for 30 min at room temperature in the darkness, flow cytometry was used to detect the percentages of the living cells as well as early, late apoptotic cells and necrotic cells.

Mitochondrial transmembrane potential (MTP) assessment by JC-1 staining: The MTP was measured by a mitochondrial membrane potential assay kit, which was a sensitive fluorescent dye, as the probe. Eca-109 and EC9706 cells were plated into 6 well plates at a density of 8×10^3 cells/well. When growing to about 80%, cells were treated with JLC at various concentrations (0.05, 0.1, 0.2, 0.4 and 5 FU) at 37°C for 24 h. Then cells were washed by 1×PBS for twice and add 1 mL 1640 medium per hole. Added 2 mL JC-1 staining buffer (5×) and mixed, namely, JC-1 staining solution. JC-1 staining solution was added to mix completely and incubated at 37°C in the dark for 20 min. During the incubation period, according to the proportion of 4 mL distilled water (5×) per 1 mL JC-1 buffer, the right amount of JC-1 buffer solution (1×) was prepared and placed in the ice bath. After 37°C incubation, the supernatant was removed and washed with JC-1 buffer (1×) for 2 times. Added 2 mL RPMI-1640 medium and observed by fluorescence microscope immediately.

Ultrastructure observation using Transmission Electron Microscopy (TEM): Following the treatment with JLC, EC9706 cells were washed with PBS for 3 times, collected by centrifugation at 1500×g, 4°C, 5 min of conditions and fixed in 2.5% glutaraldehyde. Next step, these specimens were rinsed with 0.1 M PBS, fixed in 1% osmium tetroxide for 1-2 h and then dehydrated sequentially through a graded series of ethanol, processed for Epon™ embedding. A transmission electron microscope (CM100, Philips, Netherlands) was used to observe the ultrathin sections that post-stained with uranyl acetate and lead citrate.

Immunofluorescence staining: EC9706 cells were plated in six-well culture slides with 1640 medium. After cell attachment, cells treated with 0.05, 0.1, 0.2 and 0.4 mg mL^{-1} JLC for 24 h. Cells were washed three times with 1×PBS, fixed immediately in 4% paraformaldehyde for 15 min at room temperature, then washed with PBS, permeabilized with 0.5% Triton X-100 for 20 min, washed with PBS for three times. Dry with filter paper and specific binding sites were blocked by incubation with 3% Bovine Serum Albumin (BSA) for 30 min. After three times of washing with PBS, the sections were incubated with primary antibody against caspase-3 (dilution 1:500) at 4°C overnight, washed with PBS and incubated with secondary Alexa Fluor 633-conjugated goat anti-mouse antibody (1:1000) for 1h at 37°C, washed with PBS, incubated with Hoechst 33342 to stain the nucleus to avoid the light for 5 min, washed with PBS, remove all the slides

and covered with glycerol. Cells were visualized and images were captured with an inverted fluorescence microscope (Olympus, Japan).

Detect expression level of proteins by western blotting: Cells were treated with treatment groups (0, 0.05, 0.1, 0.2 and 0.4 mg mL^{-1} JLC) and positive control group (5 FU) for 24 h. Then cells were lysed to extract total cellular proteins. Determination of protein concentration by the Bicinchoninic acid method. SDS-PAGE was performed to separate protein (50 μg per hole). Protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was kept at room temperature for 2 h with Tris-Buffered Saline and Tween20 (TBST) containing 5% non fat milk. The corresponding primary antibody (1:1000) was incubated at 4°C overnight. The membrane was washed three times for 10 min each at room temperature in the TBST. The membrane was incubated at room temperature for 2 h to secondary antibody (1:2000). The ECL detection was used to visualize the proteins on a Molecular Imager ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Band intensity was quantified using the software Image J and expressed as relative intensity compared with β-actin that was used as a loading control.

Statistical analysis: All statistical analysis were analyzed by SPSS 16.0 software. The data are expressed as Mean±Standard Deviation (SD). Significance was evaluated using one-way analysis of variance in comparison of data of more than two groups and Student's t-test for of comparison data between two groups. In all the test, $p<0.05$ was regarded as a statistically significant difference.

RESULTS

Effect of JLC on ESCC cells growth: The anti-proliferative potential of JLC onEca-109 and Eca-9706 cells were measured by MTT assay. As shown in Fig. 1a-b, cells treated with various concentrations of JLC (0.05-3.2 mg mL^{-1}) exhibited a dose-dependent growth inhibition. The IC_{50} value for 109 and 9706 cells was determined as 0.6443 and 0.6779 mg mL^{-1}, respectively. It was decided that JLC exerted a negative activity against viability of human ESCC cells. Thus, this study used the treatment with 0.05-0.4 mg mL^{-1} JLC on Eca-109 and EC9706 cells for 24 h in subsequent experiments.

JLC induces apoptosis in ESCC cells: Apoptosis analysis tested by flow cytometry. It revealed an increasing change in the apoptotic rate in treatment groups with higher JLC concentration compared with the control group (Fig. 2a, b). The cells in the lower-right and upper-right quadrants of the FACS histogram showed JLC could induce cell apoptosis (Fig. 2c, d). Western blot analysis was used to detect the expression level of proteins of Bcl-2, caspase-3, caspase-8 and caspase-9. As shown in Fig. 2e and f, after 24 h incubation, compared with the negative control, the expression level of Bcl-2 in JLC treatment group decreased and the expression of caspase-3, caspase-8 and caspase-9 increased in a dose-dependent manner. These results illustrated that JLC promotes the apoptosis of Eca-109 and EC9706 cells by both increasing the expression of pro-apoptotic protein caspase-3, caspase-8, caspase-9 and reducing the level of anti-apoptotic protein Bcl-2 (Fig. 2g, h).

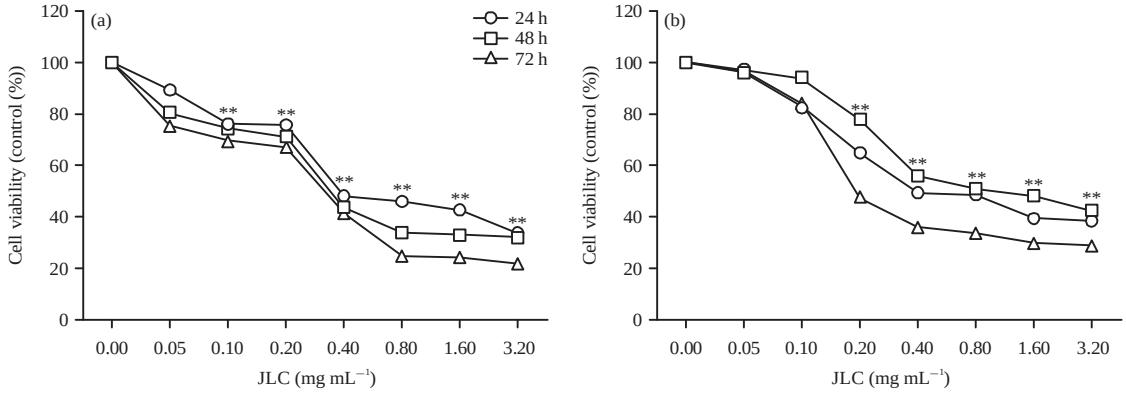

Fig. 1(a-b): JLC treatment inhibited the viability of Eca-109 and EC9706 cells by the MTT assay. Cells were treated with 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg mL^{-1} JLC for 24, 48 and 72 h, (a-b) Dose and time-dependent curve of inhibition rate of JLC on Eca-109 and EC9706 cells. Date were presented as the Means±SD of three independent experiments performed

*p<0.05, **p<0.01, as compared with the untreated control

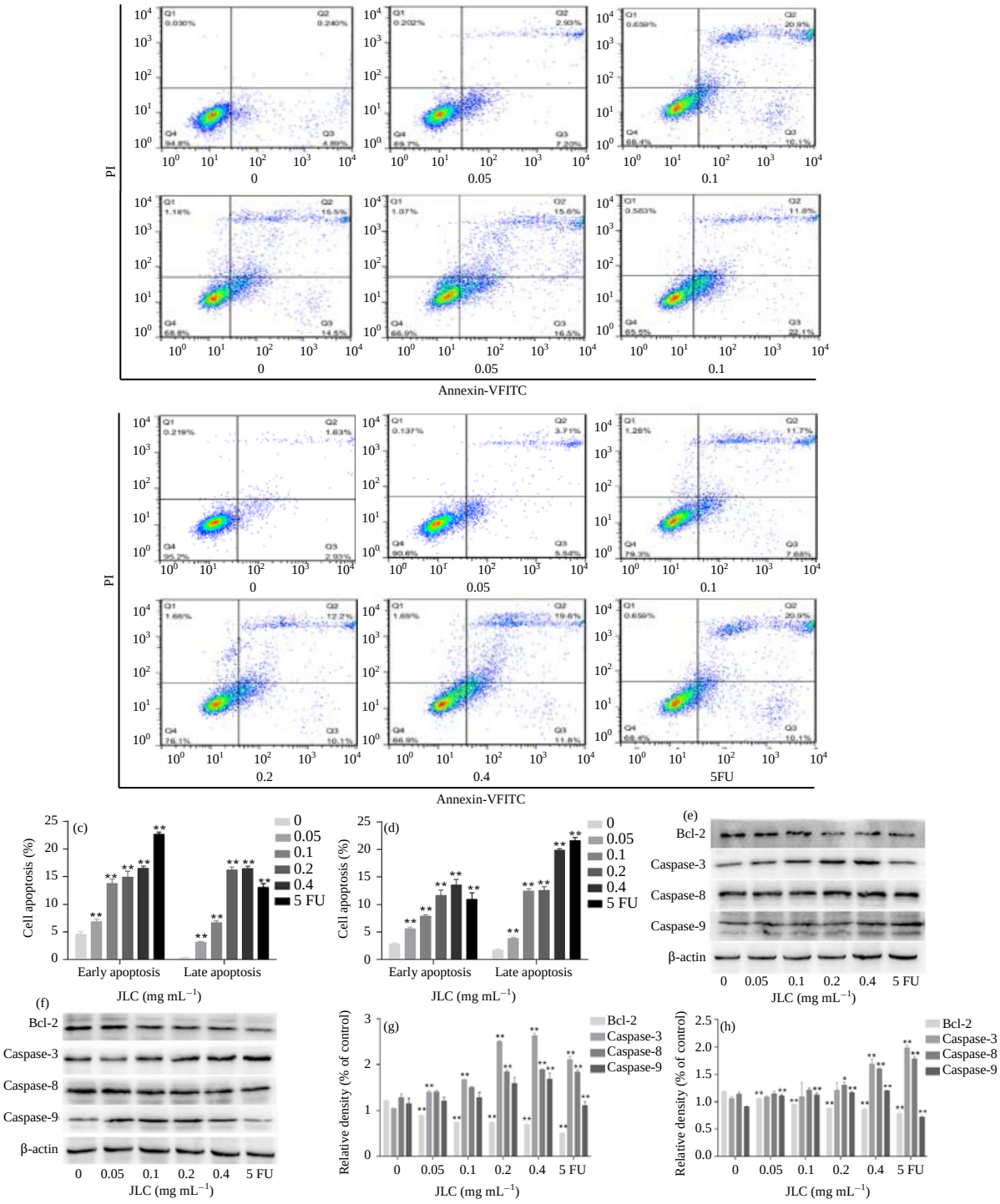

Fig. 2(a-h): Apoptotic effects of JLC on Eca-109 and EC9706 cells. JLC induced the apoptosis was determined using Annexin V-FITC/PI double staining and flow cytometry, (a-b) The cells in the lower-right and upper-right quadrants of the FACS histogram represent apoptotic cells. Significant apoptotic effects of JLC on Eca-109 and EC9706 cells, (c-d) Intuitive quantitative chart analysis including early and late apoptosis in each group, (e-f) Changes in expression levels of apoptosis-associated Bcl-2, caspase-3, caspase-8 and caspase-9 proteins in Eca-109 and EC9706 cells after 24 h treatment with JLC. JLC changed the expression levels of apoptosis-associated protein and (g-h) JLC of western blotting was normalized against the expression of β-actin and is expressed as the Mean±standard deviation

n = 3; *p<0.05, **p<0.01, as compared with the untreated control

Effects of JLC on MTP in human ESCC cells: The change of cell mitochondrial membrane potential can be easily detected by the transition from red fluorescence to green fluorescence of JC-1. At the same time, JC-1 can also be used as a marker for early detection of apoptosis from the red fluorescence to green fluorescence. When the mitochondrial membrane potential is high, JC-1 can gather in the mitochondrial matrix to form the J-aggregates, which can produce red fluorescence. However, when the mitochondrial membrane potential is low, JC-1 can't gather in the mitochondrial matrix and the JC-1 monomer can produce green fluorescence. So it can detect the change of mitochondrial membrane potential by the fluorescent color. The relative proportion of red and green fluorescence were used to measure mitochondrial depolarization ratio. From the Fig. 3a and b, green fluorescence significantly increased with the change of JLC concentration compared to the negative control, which revealed that JLC caused damage to the MTP in Eca-109 and EC9706 cells. This experiment was performed a total of three independent times.

JLC changes the ultrastructures of human ESCC cells: Under an electron microscope, EC9706 cells were found with no necrosis or apoptosis and relatively had intact organelles and nucleolus chromatin and tumor in the control group. However, cells with JLC treatment for 24 h showed obvious apoptotic phenomenon, including cell necrosis, cell shrinkage, nuclear collapse, fragmentation and chromatin condensation (Fig. 4a and b). Besides, JLC affected the expression level of apoptosis-related proteins.

Immunofluorescence analysis: The antigen antibody reaction has high specificity, so when the antigen and antibody reacted, just know that one of the factors and another factor can be found. Immunofluorescence technique labeled fluorescent dye in antibody or antigen that will not affect the activity of antigen and antibody. Corresponding antigen or antibody after binding show a specific fluorescence under a fluorescence microscope. From Fig. 5, JLC significantly increased the expression of caspase-3 in human EC9706 cells in all treated groups compared to the control.

JLC inhibits the ERK/p38MAPK signaling pathway: To determine whether the ERK/p38MAPK associated-proteins played an important role in mediating JLC-induced apoptosis, western blot was used to detect the levels of ERK, p38, p-ERK

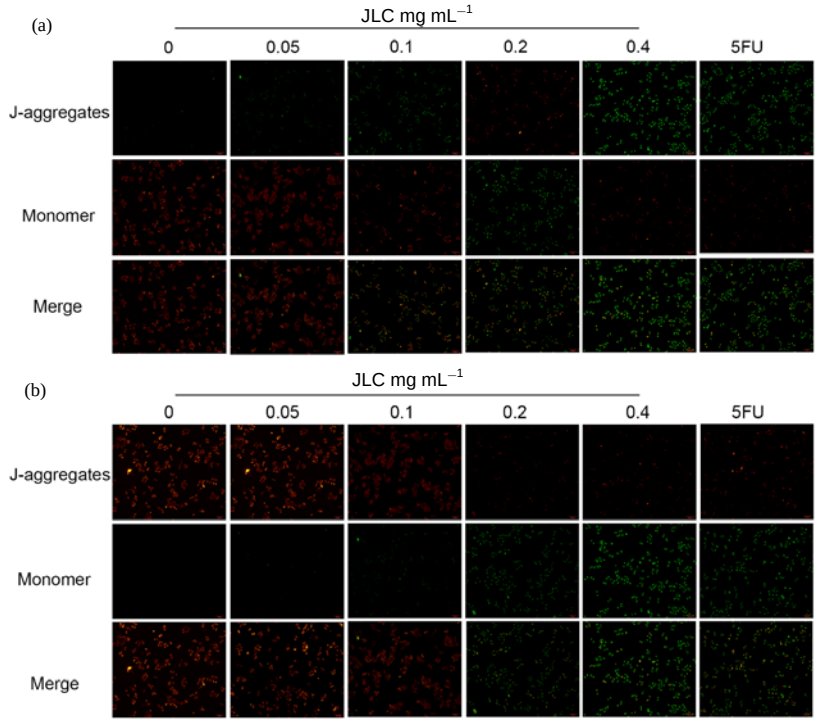

Fig. 3(a-b): JLC's Effect on Eca-109 and EC9706 cells mitochondria. EC9706 cells were treated by JLC at various concentrations (0, 0.05, 0.1, 0.2 and 0.4 mg mL^{-1}) for 24 h and the MTP was determined by JC-1 staining, (a-b) JLC significantly decreased cell mitochondrial membrane potential, indicating JLC-induced apoptosis was mediated through the mitochondrial pathway. Images were obtained from the Fluorescence Inversion Microscope system (magnification, ×100)

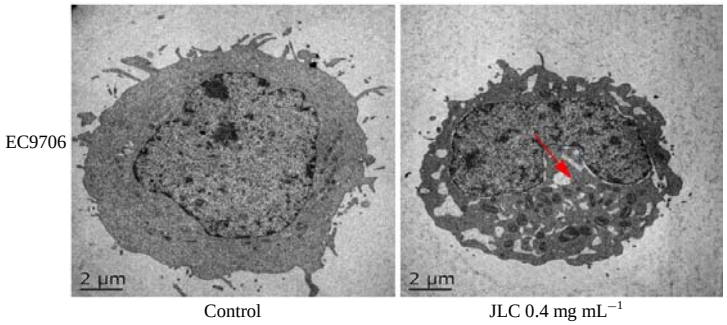

Fig. 4: Observation of ultrastructural changed under transmission electron microscope in JLC-treated groups. Under an electron microscope, EC9706 cells was found with no necrosis or apoptosis and relatively had intact organelles and nucleolus chromatin and tumor in the control group. However, cells with JLC treatment for 24 h showed obvious apoptotic phenomenon, including cell necrosis, cell shrinkage, nuclear collapse, fragmentation and chromatin condensation

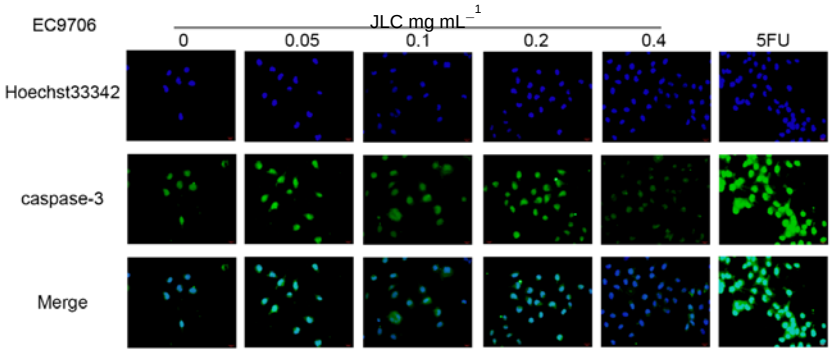

Fig. 5: EC9706 cells were seeded in 6-wells plate and incubated in RPMI-1640 mediums supplemented with 10% FBS. At each time EC9706 cells were fixed and caspase-3 expression (green) was detected using secondary Alexa Fluor 633-conjugated goat anti-mouse antibody. The nuclei were stained with Hoechst33342 (blue). Scale bar: 20 μm. Representative results from three replicates. JLC significantly increased the expression of caspase-3 in human EC9706 cells in all treated groups compared to the control and showed a specific fluorescence under a fluorescence microscope

and p-p38MAPK. Subsequent to treatment with various JLC for 24 h, there was an obvious increase in the expression of p38 and p-p38 MAPK in Eca-109 and EC9706 cells, in a dose-dependent manner. However, the expression of ERK unchanged and p-ERK decreased with 0.4 mg mL^{-1} JLC (Fig. 6a, b). This indicated that JLC may inhibit the proliferation of Eca-109 and EC9706 cells by mediating the phosphorylation of ERK and increasing the activity of p38 and p-p38 MAPK (Fig. 6c, d).

DISCUSSION

In the present study, JLC caused a significant decrease in the viability of Eca-109 and EC9706 cells at concentrations of 0.05-0.4 mg mL^{-1}. The results suggested that JLC may have a specific anticancer activity in human ESCC. The result of Annexin V-FITC/PI double staining by flow cytometry illustrated that the JLC can promote apoptosis of Eca-109 and EC9706 cells in a dose- and time-dependent manner which was consistent with the MTT assay. Those showed that promoting apoptosis may be one of the mechanisms involved in the suppression of human ESCC cells proliferation by JLC. The JLC has been demonstrated to inhibit different malignant cancer activity in recent studies[14-16]. Besides, JLC can induce apoptosis in malignant cancer cells[12]. Investigations have shown that cell apoptosis played an important role in the complex process of carcinogenesis of human cancers[17].

Apoptosis is natural way of removing aged cells from the body. It is the process of dying in physiological or pathological conditions under control by the gene, which is still a hot research topic[18]. To future research molecular mechanism of apoptosis, which is complicated and influenced by many factors, it assess some proteins related with apoptosis, including pro-apoptosis proteins and anti-apoptosis proteins.

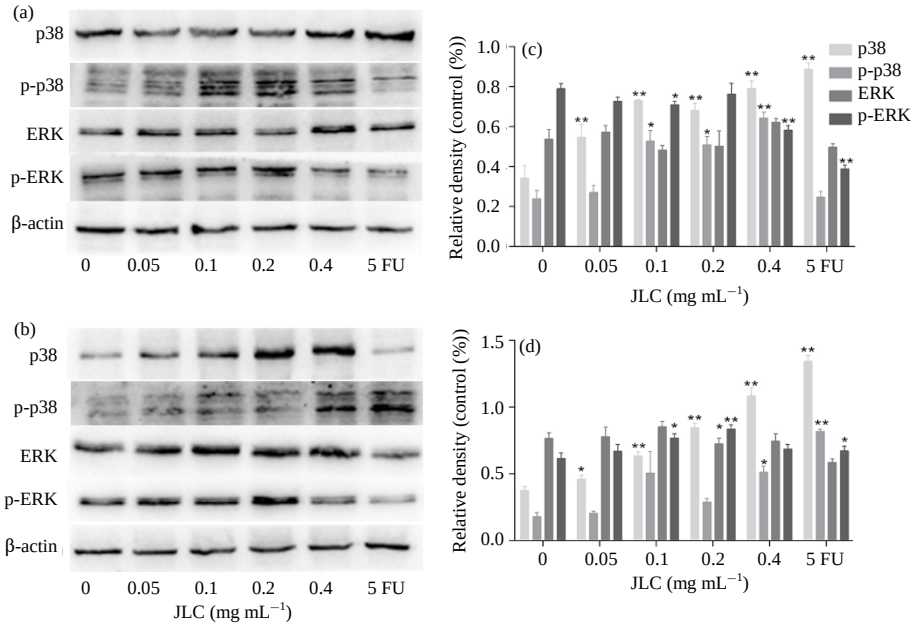

Fig. 6(a-d): Western blotting was used to detect the levels of ERK, p38, p-ERK and p-p38 to determine whether the ERK/p38MAPK signaling pathway plays an important role in mediating JLC-induced apoptosis, (a-b) Following JLC treatment, there was an obvious increase in the expression of p38 and p-p38 in Eca-109 and EC9706 cells. However, the expression of ERK unchanged and p-ERK decreased, (c-d) Histogram indicated that JLC may inhibit the proliferation of Eca-109 and EC9706 cells by mediating the phosphorylation of ERK and increasing the activity of p38 and p-p38. JLC of western blotting was normalized against the expression of β actin and is expressed as the Mean±Standard deviation
n = 3; *p<0.05, **p<0.01, as compared with the untreated control

Bcl-2 protein, as an anti-apoptotic protein, can prolong cell survival cycle and inhibit cell apoptosis[19-21]. In this study, Western blot results showed that the JLC could reduce the expression of Bcl-2 protein in humanEca-109 and EC9706 cells and may trigger off the cells apoptosis. In multicellular organisms, cell apoptosis has two different central mechanisms, the extrinsic (death receptor-mediated) and the intrinsic (mitochondrial-mediated) apoptotic pathways[22]. In the process of intrinsic apoptosis, the lessening of mitochondrial membrane potential (MTP) is an early signal of event of apoptosis[23,24]. The JLC caused the loss of MTP, increased the activities of caspase-9 in a dose-dependent manner. Subsequently, the activated caspase-9 promotes activation of caspase-3, resulting in apoptosis of the tumor cells. In contrast, for the extrinsic apoptosis signaling pathway, various cell Death Receptors (DR) are stimulated following binding to the corresponding ligands[25]. In most cases, this results in the activation of caspase-8. Activated caspase-8 binds to the secondary adaptor protein, which leads to the cleavage of caspase-3 and cell apoptosis directly[26]. Caspase-3 is in the downstream target of cell apoptosis, involved in apoptosis execution, which cleaves essential cellular components to orchestrate the proteolysis of the cell[27]. The result of western blot showed that the expression of caspase-8, caspase-9 and caspase-3 protein had a significant difference between each group. Furthermore, JLC affected the expression of caspase-3 in human Eca-109 and EC9706 cells in the immunofluorescence analysis which also contributed to the promoting of cell apoptosis. Besides, MAPK signal pathway was explored to further understand the mechanism of human ESCC cells induced by JLC. The ERK is activated by growth factor and cell proliferation and survival play a key role in inhibiting the activity of ERK. It can reduce the expression of c-myc in cells and promote cell apoptosis[28]. More and more evidences support that p38 can function as a tumor suppressor and the activation of p38MAPK enhances apoptosis. Upon activation, p38 proteins can translocate from the cytosol to the nucleus where it mediates cellular responses through affecting phosphorylation of its downstream transcription factors[29,30]. From the results, it was observed that activation of p38MAPK and accompanying decrease in a p-ERK level coincided with a decrease in Bcl-2 and increase the expression of apoptosis proteins in Eca-109 and EC9706 cells. In summary, JLC significantly decreased cell viability and induced apoptosis, which suggested that JLC had a selective and potent pro-apoptotic effect on Eca-109 and EC9706

cells *in vitro*. Besides, JLC may increase the expression of pro-apoptotic protein caspase-8 and caspase-9 by activating the extrinsic and the intrinsic apoptotic pathways. The anti-apoptosis protein Bcl-2 also can promote mitochondrial-mediated apoptosis by inducing mitochondrial outer membrane permeabilization (MOMP)[31] in this study. All of these mutually can affect caspase-3, synergistically promoting the apoptosis of human Eca-109 and EC9706 cells. The results of western blotting suggested that the possible mechanism of JLC on ESCC involves the MAPK signal pathway.

CONCLUSION

These findings suggest that JLC may be a promising agent worthy of being investigated for the treatment of human ESCC *in vitro*. In addition, this study illustrated that JLC as a compound preparation, holds the multi-targeted therapeutic effects of traditional Chinese medicine. It believed that this study only reveals part of the molecular mechanisms involved in JLC's anti-tumor function. Therefore, more researches required to study the pathogenesis and relevant novel treatments about JLC is critical for each type of cancer.

SIGNIFICANCE STATEMENT

Jinlong Capsule (JLC) is a Traditional Chinese Medicine (TCM) and has been used for several decades to treat ESCC with significantly improved clinical outcomes as adjuvant therapy. However, the specific molecular mechanisms underlying the anti-tumor activities of JLC in ESCC are not fully investigated. The JLC could decrease proliferation and induce apoptosis in ESCC cells. The JLC may increase the expression of pro-apoptotic protein caspase-8 and caspase-9 by activating the extrinsic and the intrinsic apoptotic pathways. The MAPK signal pathway took function in ESCC cells that treated with different JLC. Therefore, JLC showed potent therapeutic and anti-tumor effects on ESCC. These findings demonstrated that JLC can be considered as a promising candidate in ESCC treatment.

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