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Original Article

Fangchinoline induces cell apoptosis via the mitochondrial apoptotic pathway in gastric cancer cells

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Abstract: The aim of this study was to explore the effect of fangchinoline (FCL), a bioactive compound derived from traditional Chinese herb *Stephaniatetrandra S. Moore* (Fen Fang Ji), on the proliferation of gastric cancer cells, and to define the related mechanisms. MTT assay, PI staining/flow cytometry, Annexin V-PI staining and western blot assays were conducted to validate the effect of FCL in gastric cancer cells. Our data demonstrated that FCL significantly inhibited cell growth of human gastric cancer HGC-27 and SGC-7901 cells and stimulated cell cycle arrest at G0/G1 phase. Furthermore, FCL induced cell apoptosis in gastric cancer HGC-27 and SGC-7901 cells, and apoptosis induced by FCL was reversed by total caspase inhibitor Z-VAD-FMK or caspase 3 inhibitor Ac-DEVD-CHO. The following western blotting results indicated that FCL down-regulated the expression of Bcl-2, caspase 3, up-regulated the expression of Bax, cleaved caspase 3 and stimulated the release of cytochrome C from mitochondria into cell cytoplasm in gastric cancer cells. In addition, incubating gastric cancer cells with FCL resulted in the suppression of pAkt and pNF-kB. Collectively, these results suggest that FCL promotes apoptosis in gastric cancer cells via inhibition of mitochondrial capacity.

Keywords: Fangchinoline, gastric cancer, apoptosis

Introduction

Gastric cancer is the second leading cause of cancer-related death, and continues to be a major public health issue worldwide, especially in China [1, 2]. In most cases, the disease was diagnosed at the advanced stages. Accordingly, the survival rate of patients with advanced gastric cancer remains low even after the combination treatment with chemotherapy or radiotherapy [2, 3]. Therefore, identification of novel and effective anti-gastric cancer drugs with less toxic is of great interest.

Fangchinoline (FCL, $C_{37}H_{40}N_2O_6$) is an alkaloid that is isolated from traditional Chinese medicine *Stephaniatetrandra S. Moore* (Fen Fang Ji), and is widely used for the treatment of inflammatory diseases in Asian countries [4]. Additionally, FCL possesses many pharmacological properties, including anti-oxidation [5], neuroprotective [6], anti-hypertension [7] and anti-cancer activities [8-13]. It has been reported to inhibit the growth of lung cancer [10, 13], breast cancer [8, 9], prostate cancer [11] and glioma [12] cells. However, little research has been done on the effect of FCL on gastric cancer cells.

In this study, we aimed to explore the anti-gastric cancer function of FCL and investigated its possible molecular mechanisms. Our study demonstrated that FCL induces apoptosis in gastric cancer cells via inhibition of mitochondrial capacity, providing a potential drug agent for the treatment of gastric cancer clinically.

Materials and methods

Reagents

FCL was obtained from Shanghai Nature Standard Biotech. Co. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsinase were from the Gibco Life Tech. (Grand Island, NY). The Z-VAD-

FMK, Ac-DEVC-CHO, cytosol and mitochondrion proteins extraction assay, BCA protein assay reagents and goat-anti-rabbit/rat horseradish-peroxidase-conjugated (HRP) secondary antibodies were purchased from Beyotime Biotechnology (Shanghai, China). Dimethyl sulfoxide (DMSO), β-actin antibody and MTT were from Sigma (MO, USA). The bax, bcl-2, caspase 3, cleaved caspase 3, cytochrome C (Cyto-C), Cox IV, pAkt, Akt, pNF-kB and NF-kB antibodies were all from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

Cell lines were from the Shanghai cell bank of Chinese academy of sciences (Shanghai, China). The human gastric cancer SGC-7901 and HGC-27 cells were cultured in DMEM with 10% (v/v) FBS and 1% antibiotics at 37°C in a 5% CO_2 humidified atmosphere.

MTT assay

Cells were seeded in 96-well plates and incubated with the desired doses of FCL for 48 h. Then the cell viability was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions as reported [9].

Cell cycle assay

Cells were seeded in 6-well plates and treated with the desired doses of FCL for 24 h. The cells were collected and fixed in 70% ethanol at 4°C. The cells were washed with PBS twice, and resuspended in PBS containing 50 μg/mL RNase for 30 min, and stained with 100 μg/mL Propidium Iodide (PI) in the dark for 30 min. The changes in the cell cycle were evaluated by a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Cell apoptosis assay

Cells were seeded in 6-well plates at a density of 2×10^5 cells/well and treated with desired concentrations of FCL for 24 h. The cells were collected and incubated with 5 μl FITC-conjugated Annexin V and 5 μl PI for 30 min. Then the apoptotic cells were assessed using a FACS calibur flow cytometer (BD Bioscience, USA).

Western blot

Total protein was extracted from gastric cells by western blot buffer (Sangon Biotech, Shanghai, China). The mitochondrion and cytosol proteins were extracted according to the manufacturer's instructions. The proteins were evaluated using BCA protein assay. Then proteins were separated by the sodium dodecyl sulfate-polyacrylamide electrophoresis gel (SDS-PAGE) and transferred onto the nitrocellulose filter membrane. Thereafter, proteins on the nitrocellulose filter membrane were probed with corresponding primary monoclonal antibodies respectively at 4°C overnight, followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Finally, immunoreactive bands were detected with ECL-detecting reagents and optical density (OD) values were analyzed using ImageJ software.

Statistical analysis

Data was expressed as mean ± standard deviation (SD) of three independent experiments. Statistical analysis was performed using SPSS software (version 13.0). Between-group differences were evaluated using one way analysis of variance (ANOVA) with Dunnett's test and $P < 0.05$ was considered to be a significance level.

Results

FCL inhibits the proliferation of gastric cancer cells

To assess the pharmacological role of FCL (**Figure 1A**) in the proliferative property of gastric cancer cells, MTT assay was conducted to investigate the cell viability of both HGC-27 and SGC-7901 cells incubated with the designed concentrations of FCL for 48 h. As shown in **Figure 1B**, FCL exhibited obvious inhibitory effects on the cell proliferation of both HGC-27 and SGC-7901 cells in a dosage-dependent fashion. To validate the anti-proliferative effect of FCL on gastric cancer cells, the cell cycle distribution assay was further used in our study. Two gastric cell lines were incubated with the indicated doses of FCL for 24 h and the cell cycle was detected by PI staining and flow cytometry. As shown in **Figure 1C**, **1D**, the rate of G2/M phase reduced while the rate of G0/G1 phase increased in a dose-dependent man-

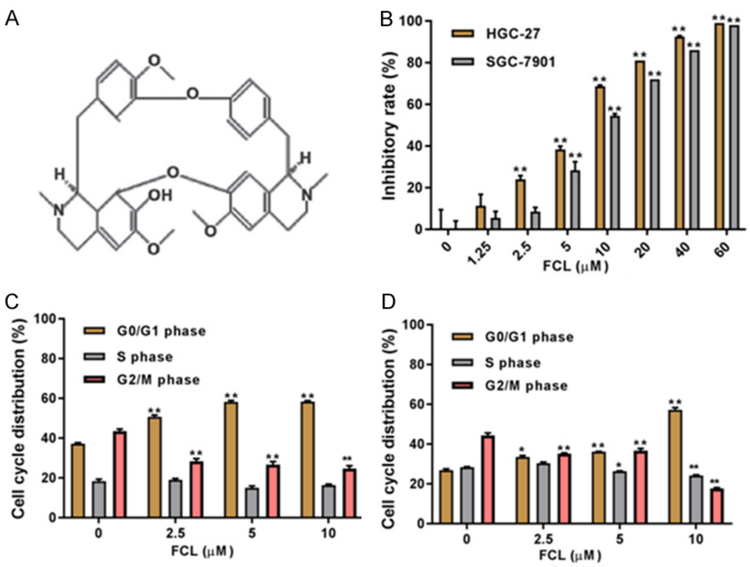

Figure 1. FCL suppresses gastric cancer cells proliferation. (A) Chemical structure of FCL. (B) HGC-27 and SGC-7901 cells were incubated with FCL at different concentrations for 48 h, the cell viabilities were assessed using MTT assay (n = 4). HGC-27 (C) and SGC-7901 (D) cells were treated with FCL at desired dosages (0, 2.5, 5 and 10 μM) for 24 h, the cell cycle distribution was determined by PI staining and flow cytometry analysis (n = 3). *P < 0.05, **P < 0.01, compared with the control group.

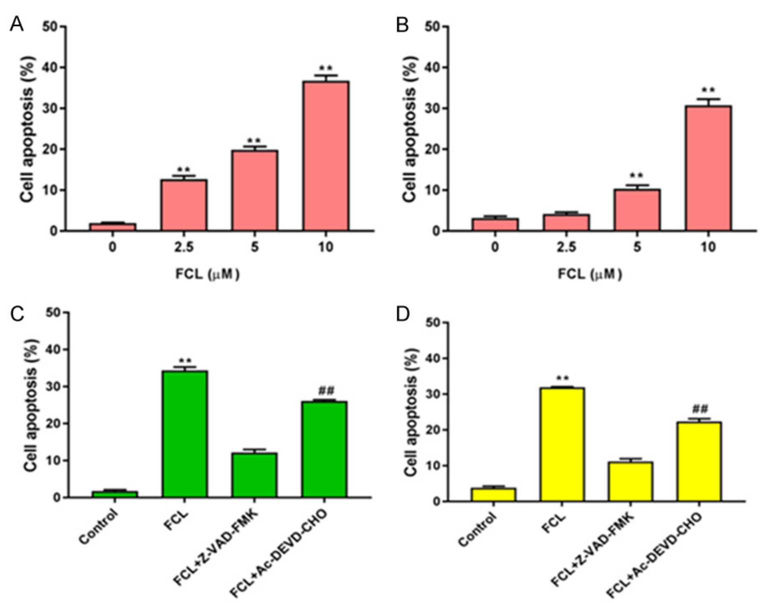

Figure 2. Pro-apoptotic effect of FCL on gastric cancer cells. HGC-27 (A) and SGC-7901 (B) cells were treated with FCL at desired dosages (0, 2.5, 5 and 10 μM) for 48 h, the cells were stained with Annexin V-FITC/PI and detected by flow cytometry and statistically analyzed. (C) HGC-27 cells were pretreated with Z-VAD-FMK or Ac-DEVC-CHO for 2 h and then treated with or without FCL for 48 h, the cell apoptosis was determined. (D) SGC-7901 cells were pretreated with Z-VAD-FMK or Ac-DEVC-CHO for 2 h and then treated with or without FCL for 48 h, the cell apoptosis was tested. **P < 0.01, compared with the control group; ##P < 0.01, compared with the FCL group.

ner after incubation with FCL in two gastric cancer cell lines. These data suggested that FCL obviously inhibited the cell proliferation of gastric cancer cells *in vitro*.

FCL induced cell apoptosis in gastric cancer cells

To investigate whether FCL mediated gastric cancer cells growth inhibition was associated with cellular apoptosis, FCL treated gastric cancer cells were stained with FITC-Annexin V and PI, and the results showed that the apoptotic cancer cells were significantly increased in a concentration-dependent manner after the incubation of FCL in both HGC-27 and SGC-7901 cells (**Figure 2A**, **2B**). Additionally, the caspase inhibitor Z-VAD-FMK and caspase 3 inhibitor Ac-DEVD-CHO were also used to further confirm the apoptosis induction of FCL in gastric cancer cells. As shown in **Figure 2C**, FCL induced cell apoptosis was almost reversed by caspase inhibitor Z-VAD-FMK, and caspase 3 inhibitor Ac-DEVD-CHO partly inhibited the apoptotic promoting property of FCL in gastric cancer HGC-27 cells. The similar patterns were also observed in another gastric cancer cell line SGC-7901 (**Figure 2D**). These results demonstrated that FCL induced typical apoptosis in human gastric cancer cells.

To further verify the proapoptotic activity of FCL on gastric cancer cells, western-blot was performed to explore the expression of apoptosis related proteins including Bcl-2, Bax, caspase 3 and cleaved caspase 3. As shown in **Figure 3A**, **3B**, after treating with various

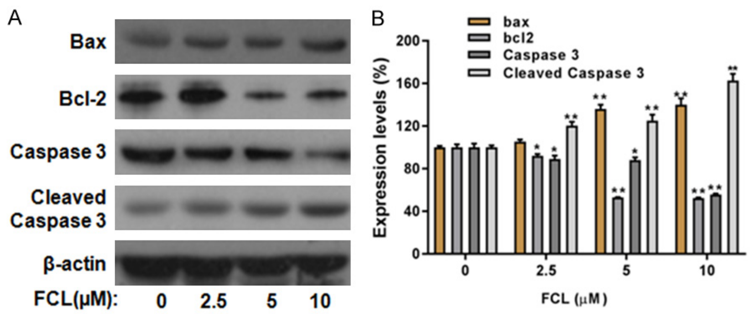

Figure 3 Effect of FCL on the expression of apoptosis associated proteins. A. Cells were treated with FCL for 48 h; then, the total proteins were extracted and subjected to Western blot analysis using antibodies against bax, bcl-2, caspase 3 and cleaved caspase 3, and β-actin was used an internal control. B. Data were expressed as mean ± SD (n = 3). *P < 0.05, **P < 0.01, compared with the control group.

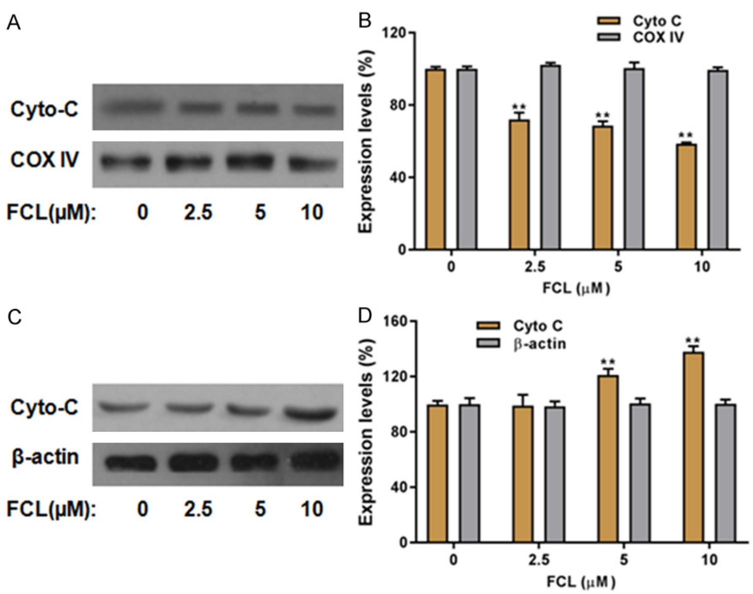

Figure 4. Effects of FCL on the release of Cytochrome C. A, B. Gastric cells were treated with FCL for 48 h, the mitochondrion proteins were extracted and subjected to Western blot analysis using antibodies against cytochrome C, and Cox IV was used internal controls of mitochondrion. C, D. Cells were treated with FCL for 48 h, the cytosol proteins were extracted and subjected to Western blot analysis using antibodies against cytochrome C, and β-actin was used internal controls of cytosol. Data were expressed as mean ± SD (n = 3). **P < 0.01, compared with the control group.

doses of FCL for 48 h, the protein expression of Bcl-2 and caspase 3 markedly decreased dosage dependently, while the expression of Bax and cleaved caspase 3 significantly increased in a dose-dependent manner in gastric cancer cells. Accounting for the close relationships between Bcl-2 and Bax with mitochondria, it is likely that mitochondrial disturbance may participate in FCL induced cell apoptosis via affecting the expression of Bcl-2 and Bax.

Effects of FCL on the release of cytochrome C

Release of cytochrome C (Cyto-C) plays a pivotal role in the mitochondria-mediated apoptosis pathway [14, 15]. To gain further insight into the mitochondrial pathway of apoptosis, we assessed the Cyto-C levels in both mitochondria and cytoplasm. As shown in **Figure 4A-D**, the protein expression of Cyto-C in the mitochondria was significantly decreased while the expression of Cyto-C in the cytoplasm was markedly increased after treating with FCL in a concentration-dependent manner. These data suggested that FCL promoted the release of Cyto-C from mitochondria into cell cytoplasm.

Effects of FCL on the expression of cell signaling proteins in gastric cancer cells

To determine whether activation of Akt and NF-kB was related to the FCL induced cell apoptosis, we further evaluated the effect of FCL on cellular signaling. As shown in **Figure 5A**, **5B**, the expression levels of Akt and NF-kB phosphorylation were both significantly decreased after treating with FCL in gastric cancer HGC-27 cells, indicating that Akt and NF-kB phosphorylation repression may play important roles in FCL mediated cell apoptosis.

Discussion

FCL is a major bioactive compound of the natural herb *Stephaniatetrandra S. Moore*, and possesses multiple biological activities such as anti-oxidation [5], neuroprotective [6], anti-

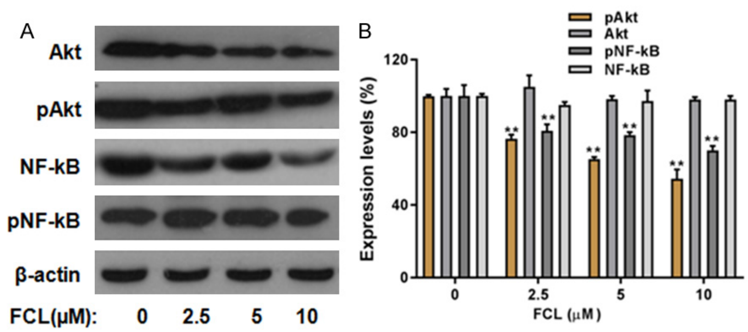

Figure 5. Effects of FCL on the expression of cell signaling proteins in gastric cancer cells. A. Cells were treated with FCL for 48 h, the total proteins were extracted and subjected to Western blot analysis using antibodies against pAkt, Akt, pNF-kB, NF-kB, and β-actin was used an internal control. B. The expression of pAkt, Akt, pNF-kB and NF-kB, was statistically analyzed. **P < 0.01, compared with the control group.

hypertension [7] and anti-inflammation activities [4]. Recently, more and more studies have demonstrated that FCL exhibits anti-cancer effect through inhibiting cell cycle arrest, inducing cell apoptosis and promoting cell autophagy in several types of cancers [8-13]. However, little is known about the effect of FCL on gastric cancer. Tian F and colleagues has shown that FCL suppressed the cell growth by inhibiting PI3K/AKT signaling pathway in gastric cancer cells using one gastric cancer cell line [16]. The anti-gastric cancer activity of FCL and the associated molecular mechanisms have not yet been clarified. In this study, we explored the effect of FCL on gastric cancer HGC-27 and SGC-7901 cell lines, and observed that FCL obviously suppressed proliferation of gastric cancer cells. Subsequently, we found that the cell growth inhibition of FCL was associated with cell cycle arrest at G0/G1 phase and induction of cell apoptosis. Moreover, we demonstrated that the stimulation of cell apoptosis by FCL was mediated by protein expression regulation of Bcl-2 family proteins such as Bcl-2 and Bax. In addition, Akt and NF-kB also played pivotal roles in FCL mediated cell apoptosis in gastric cancer cells.

Apoptosis refers to the programmed death of cells controlled by genes for maintaining the stability of the internal environment [17]. One of the main characteristics of cancer cells is resistant to cell apoptosis, providing a new strategy for anti-tumor drugs discovery. It is generally known that cellular apoptosis is regulated by two key pathways including the mitochondrial-mediated (intrinsic) and death receptor-mediated (extrinsic) pathways [18]. Caspase 3 is a family member of cysteinyl proteases, and its activation is an indicator of cell apoptosis initiation [19]. Activation of caspase 3 is something that these two apoptotic pathways generally have in common. In this study, FCL treatment resulted in the decrease of pro-caspase 3 and the increase of cleaved caspase 3, and the administration of specific inhibitors of caspase or caspase 3 both reversed the cell apoptosis induced by FCL in gastric cancer cells, demonstrating that FCL exerts cell apoptosis induction potential in gastric cancer cells. The mitochondrial apoptosis pathway is mostly controlled by the interplay between members of the Bcl-2 protein family. Bcl-2 is the central regulator belonging to the Bcl-2 family and plays a negative regulation during cellular apoptosis [20]. Bax protein, also a member of the Bcl-2 family, binds to Bcl-2 protein to promote cell apoptosis [20, 21]. In the procession of mitochondrial apoptosis pathway, the cellular apoptotic signals cause the release of cytochrome C into the cytosol from mitochondria, the cytochrome C then binds to Apaf-1, pro-caspase-9 and ATP/dATP to form apoptosomes, which activate pro-caspase 3, initiate the caspases cascade reaction and lead to apoptosis [22, 23]. In the present study, FCL administration decreased the expression of Bcl-2 and increased the expression level of proapoptotic protein Bax, and FCL also stimulated the release of cytochrome C from mitochondria into cell cytoplasm in gastric cancer cells, suggesting that FCL repressed cell growth through inducing mitochondria-mediated apoptosis in gastric cancer cells.

It has been demonstrated that activation of Akt or NF-kB could cause resistance to cellular apoptosis [24, 25]. The expression of Bcl-2 was suppressed after the silencing of NF-kB [24, 26]. Therefore, Akt or NF-kB signaling plays important roles in the cellular apoptosis. We found that the administration of FCL resulted in a significant decrease of pAkt and pNF-kB dose-dependently, which led to the down-regulation of Bcl-2.

In conclusion, FCL promotes apoptosis via the mitochondrial apoptotic pathway in gastric cancer cells. Therefore, FCL may be a potential therapeutic drug candidate for the treatment of gastric cancer.

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Disclosure of conflict of interest

None.

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