

Original Article

Aqueous extracts of *Fructus Ligustri Lucide* induce gastric carcinoma cell apoptosis and G2/M cycle arrest

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Abstract: Objective: Previous studies have shown that *Fructus Ligustri Lucide* (FLL) can be used to anti-cancer. However, the mechanism by which FLL mediate this effect is unclear. In the present study, aqueous extracts of FLL induced cell apoptosis in human gastric carcinoma cell was investigated. Methods: The cell viability was detected by the CCK8 assay. The cell apoptosis was assessed by annexin V-PI double-labeling staining and hoechst 33342 staining. The protein expression of cell cycle regulators and tumor suppressors were analyzed by western blotting. Results: Treatment of human gastric carcinoma cells with FLL induced cell death in a dose-and time-dependent manner by using CCK8 assay. Consistent with the CCK8 assay, the flow cytometry results showed that the proportion of the early and terminal phase of apoptosis cells had gained after FLL treatment as compared to untreated group. Moreover, human gastric carcinoma cells were exposed to the aqueous extracts of FLL for 48 h, which resulted in an accumulation of cells in G2/M phase. Apoptotic bodies were clearly observed in human gastric carcinoma that had been treated with FLL for 48 h and then stained with Hoechst 33342. Treatment of gastric carcinoma cells with increasing doses of FLL and increasing durations significantly increased the protein expression of Bax and Caspase3, decreased the anti-apoptotic Bcl-2 level. The expression of CDC2 and cdc25C were downregulated upon FLL treatment in human gastric carcinoma. In contrast, p53 and p21 were obviously upregulated by FLL treatment in a concentration-dependent manner. Conclusions: These results confirmed that FLL could induce apoptosis in human gastric carcinoma, the underlying molecular mechanisms, at least partially, through activation p21/p53 and suppression CDC2/cdc25C signaling in vitro.

Keywords: *Fructus Ligustri Lucide*, gastric carcinoma, CDC2/cdc25C, p53/p21

Introduction

Fructus ligustri lucidi is commonly prescribed in Traditional Chinese Medicine for detoxifying kidney and strengthening the bones [1-4]. Besides balancing of bone metabolism, FLL serves a broader purpose, such as restraining the algal growth [5], anti-oxidative [6] and anti-viral effects [7]. Studies also show that FLL induces apoptosis and cell senescence in human hepatoma cells [8], colorectal carcinoma HCT-116 cell lines [9] and glioma U87MG cell lines [10]. However, the pharmacological functions are rarely conducted in gastric carcinoma growth and anti-cancer efficacy.

Cell cycle control is the major regulatory mechanisms of cell growth. Many chemotherapeutic drugs or Chinese Herbal Medicine arrest the

cell cycle and subsequently induce cell death [11-13]. The phosphorylation of cell division cycle 2 (CDC2) and cdc25C, cycle regulatory proteins, are involved in arresting effect of gastric carcinoma cells on the cell cycle at G2/M phase [12, 14]. CDC2 is always overexpressed in malignant carcinoma cells and is correlated with chemosensitivity [15]. Knockdown of CDC2 expression inhibits proliferation, enhances apoptosis, and increases chemosensitivity to temozolomide in glioblastoma cells [15]. These findings indicate that cell-cycle regulatory proteins CDC2 and cdc25C play an evident role to control the proliferation of carcinoma cells. In human gastric carcinoma BGC-823 cells, oroxylin A-treated can downregulate the expression of cyclin-dependent kinase 7 (CDK7), which is responsible for the low expression of cyclin B1 and CDC2 [16]. Similarly, gambogic acid-in-

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duced G2/M phase cell-cycle arrest via disturbing CDK7-mediated phosphorylation of CDC2/p34 in human gastric carcinoma BGC-823 cells [17]. However, FLL inhibitions cell-cycle progression in human gastric carcinoma cells is unknown.

FLL can induce cell apoptosis through upregulation the expression of tumor suppressor genes and activation cell apoptotic signaling pathway [8]. P53 and p21 are an important tumor suppressor genes, and their alteration play a role in the pathogenesis and progression of human gastric carcinoma [18, 19]. P53 is a transcription factor that up-regulating a number of important cell cycle-modulating genes such as p21, p38 and p16 [20, 21]. Part of the mechanism by which p53 blocks cells at the G2/M checkpoint involves inhibition of CDC2, the cyclin-dependent kinase required to enter mitosis. In the absence of CDC2 dephosphorylation by cdc25C, and also the direct phosphorylation of cyclin B, the accumulation of CDC2/cyclin B in the nucleus is prevented and entrance into mitosis is stalled [12]. In addition to controlling the cell cycle to regulate the cell growth process, several therapeutic and chemopreventive agents could eliminate cancerous cells by inducing apoptosis. Cell apoptosis is an intracellular suicide program executed by the activation of caspases, a family of cytoplasmic cysteine proteases. Caspase-3, caspase-8, and caspase-9, as key components of the apoptotic machinery, have been shown to be activated in apoptotic cells [22].

In this study, we intended to investigate the effect of FLL extracts on gastric carcinoma cell proliferation in vitro. The results showed that FLL extracts induced cell death through activation p21/p53 and suppression CDC2/cdc25C signaling in vitro. These data suggested that FLL extracts might be an effective adjuvant therapy drug for human gastric carcinoma.

Materials and methods

Preparation of FLL extract

Fructus Ligustri Lucidi (FLL) was obtained from Jilin province of China in Mar 2014 and authenticated according to a method listed in Chinese Pharmacopeia. A voucher specimen was deposited in Department of Gastroenterology, Shenzhen People's Hospital, the second affiliated

hospital of Jinan University (Shenzhen, China). The dried and powdered (10 kg) crude plant was extracted with water for two times, the preparation was filtered and concentrated under vacuum to produce a viscous residue at a yield of 30%, by weight of the starting materials.

Cell culture

The AGS and SGC-7901 gastric carcinoma cell were obtained from the Chinese Academy of Sciences (Institute of Shanghai Cell Biology and Chinese Type Culture Collection, China), and maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37°C in a humidified, 5% CO₂, 95% air atmosphere. The medium was replenished every day. Confluent cells were treated with various concentrations of FLL (0-10 mg/mL).

Cell viability detection by CCK8

AGS and SGC-7901 gastric carcinoma cell (1.0 × 10⁴/well) were plated and treated in 96-well plates (three wells per group) with FLL (0-10 mg/mL) for 24 or 48, respectively. 10 µL of CCK8 (Dojindo, Kumamoto, Japan) was added to the cells, and the viability of the cells was measured at 490 nm using an ELISA reader (BioTek, Winooski, VT, USA) according to the manufacturer's instructions.

Quantification of apoptosis by flow cytometry

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface of apoptotic cells. Cells were treated with vehicle or honokiol for indicated time intervals. After treatment, cells were washed twice with PBS (pH = 7.4), and re-suspended in staining buffer containing 1 µg/mL PI and 0.025 µg/mL annexin V-FITC. Double-labeling was performed at room temperature for 10 min in the dark before the flow cytometric analysis. AGS and SGC-7901 gastric carcinoma cell were immediately analyzed using FACScan and the Cellquest program. Quantitative assessment of apoptotic cells was also assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) method,

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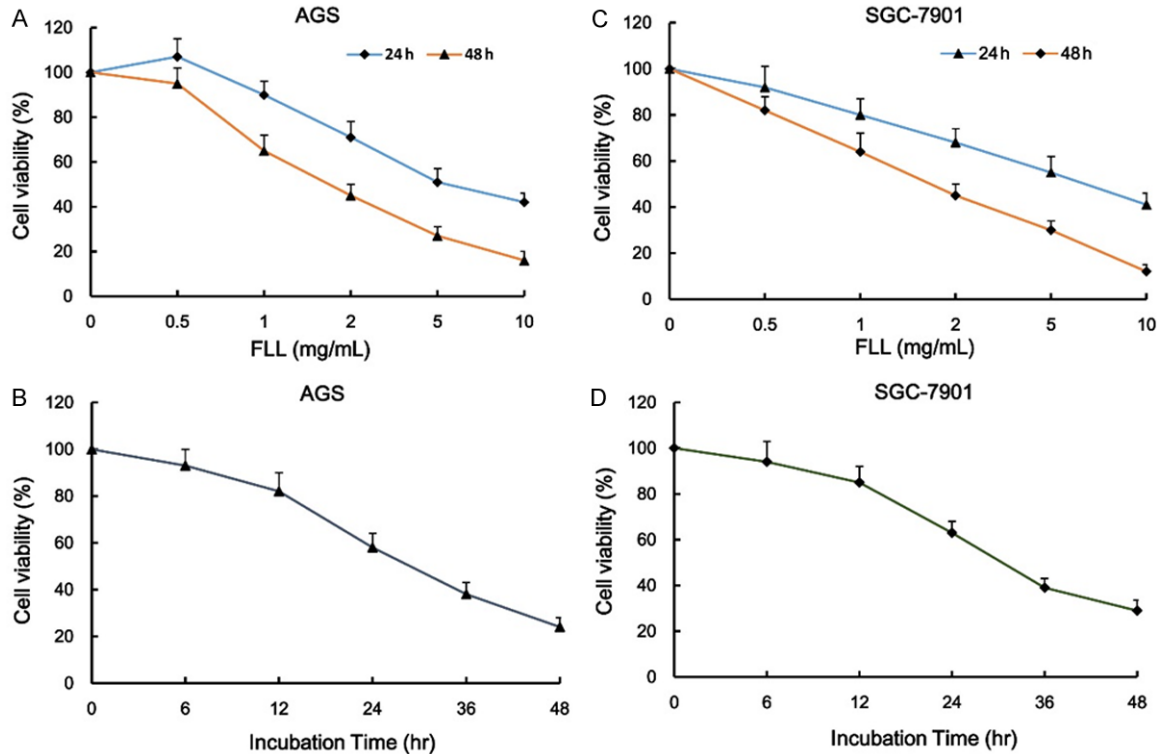


Figure 1. Effect of FLL on the cell viability of AGS and SGC-7901 gastric carcinoma cell. The AGS (A) and SGC-7901 (B) gastric carcinoma cell were incubated with various concentrations of FLL (0-10 mg/mL) for 24 h and 48 h respectively, and the cell viability was examined by CCK8 assay. The AGS (C) and SGC-7901 (D) gastric carcinoma cell were incubated with FLL (5 mg/mL) for 0, 6, 12, 24, 36 and 48 h respectively, and the cell viability was examined by CCK8 assay. Values are expressed as mean \pm SEM, n = 3 in each group.

which examines DNA-strand breaks during apoptosis by using BD ApoAlert™ DNA Fragmentation Assay Kit. Briefly, AGS and SGC-7901 gastric carcinoma cell were incubated with FLL for the indicated times. The AGS and SGC-7901 gastric carcinoma cell were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodium citrate. After being washed, the AGS and SGC-7901 gastric carcinoma cell were incubated with the reaction mixture for 60 min at 37°C. The stained cells were then analyzed with flow cytometer (FC500, Beckman Coulter, FL, USA).

Cell cycle assays

AGS and SGC-7901 gastric carcinoma cell (1.0×10^6 /well) were plated and treated in 6-well plates (three wells per group) with vehicle, DMSO or FLL (5 mg/mL) for 48 h. After treatment with FLL, the cells were harvested and subjected to the following assays. For the cell cycle assay, the cells were washed twice with ice cold PBS, fixed in 70% ethanol at 4°C overnight, incubated with 10 mg/mL RNase A

(Sigma-Aldrich) at 37°C for 30 min, and then incubated with 50 mg/mL propidium iodide (Sigma-Aldrich). Cell cycle distribution was assessed by flow cytometry (FC500, Beckman Coulter, FL, USA).

Hoechst 33342 staining analysis

The AGS and SGC-7901 gastric carcinoma cell were seeded into 6-well plates, and then treated with vehicle, DMSO or FLL (5 mg/mL) for 48 h, stained with 0.1 μ g/mL hoechst 33342 (Sigma) for 5 min, then observed by fluorescence microscopy using appropriate filters for blue fluorescence.

Western blotting

The AGS and SGC-7901 gastric carcinoma cell were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μ g of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules,

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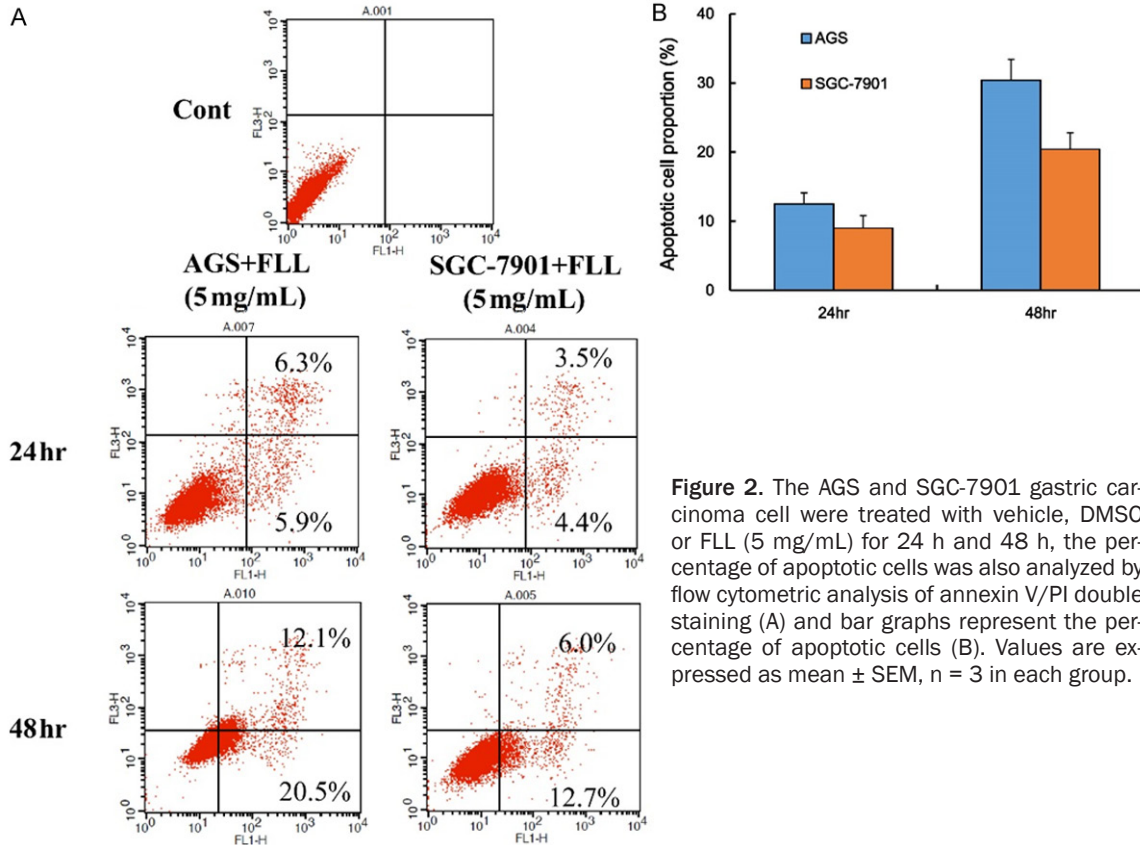


Figure 2. The AGS and SGC-7901 gastric carcinoma cell were treated with vehicle, DMSO or FLL (5 mg/mL) for 24 h and 48 h, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (A) and bar graphs represent the percentage of apoptotic cells (B). Values are expressed as mean \pm SEM, n = 3 in each group.

CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies, Bax, Caspase3, Bcl-2, CDC2, cdc25C, p53 and p21 (Santa Cruz Biotechnology, CA, USA), at dilutions ranging from 1:500 to 1:2,000 at 4°C over-night. After three washes with TBST, membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG at a dilution of 1:10,000-1:20,000. After 1 hour incubation at 37°C, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β -actin signals to correct for unequal loading using the mouse monoclonal anti- β -actin antibody (Bioworld Technology, USA).

Statistical analysis

The data from these experiments were reported as mean \pm standard errors of mean (SEM)

for each group. All statistical analyses were performed by using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison test as a post test to compare the group means if overall $P < 0.05$. Differences with P value of < 0.05 were considered statistically significant.

Results

FLL inhibits cell growth and induces apoptosis

AGS and SGC-7901 gastric carcinoma cell viability were measured when cells were exposed to various concentrations of FLL (0-10 mg/mL) for 24 and 48 h. AGS and SGC-7901 gastric carcinoma cell were growth inhibited with FLL (**Figure 1A** and **1B**). The viabilities of gastric carcinoma cells treated with FLL were significantly lower than those of untreated group. As shown the growth curve in **Figure 1A**, the concentrations at which FLL inhibited AGS cell growth by 50% (IC_{50}) were 2 mg/mL and 5 mg/mL at 24 h and 48 h, respectively. The IC_{50} of growth inhibition of FLL for SGC-7901 was 2

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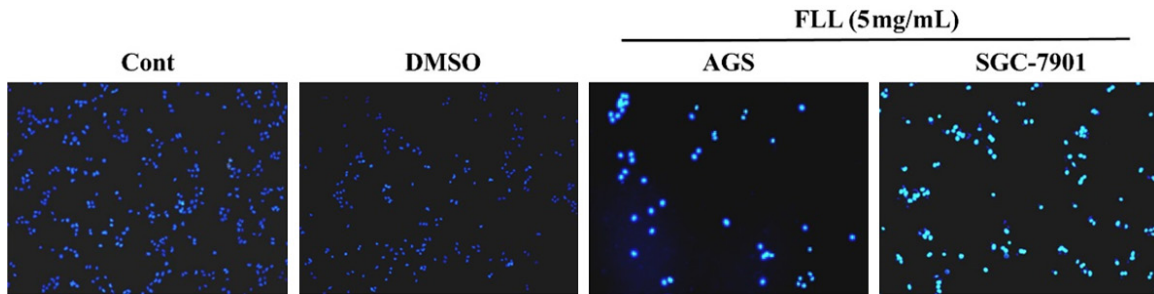


Figure 3. The AGS and SGC-7901 gastric carcinoma cell were treated with vehicle, DMSO or FLL (5 mg/mL) for 48 h. The morphologic changes in human neuroglioma cells were evaluated using hoechst 33342 staining.

Table 1. Effect of FLL on the distribution of the cell cycle in AGS cells

| FLL (mg/mL) | % of cell | | |
|-------------|-----------|-------|-------|
| | G0/G1 | S | G2/M |
| 0 | 51.52 | 19.26 | 29.22 |
| 0.5 | 50.63 | 20.70 | 28.67 |
| 1 | 46.34 | 18.92 | 34.74 |
| 2 | 41.65 | 19.36 | 38.99 |
| 5 | 33.09 | 20.31 | 44.60 |

Cells were incubated with various concentrations of FLL (0-5 mg/mL) for 48 h, and the percentage of cell cycle phase was analyzed by flow cytometry.

Table 2. Effect of FLL on the distribution of the cell cycle in SGC-7901 cells

| FLL (mg/mL) | % of cell | | |
|-------------|-----------|-------|-------|
| | G0/G1 | S | G2/M |
| 0 | 46.61 | 26.64 | 26.75 |
| 0.5 | 46.90 | 25.29 | 27.81 |
| 1 | 43.22 | 25.50 | 31.28 |
| 2 | 40.84 | 26.32 | 32.84 |
| 5 | 35.71 | 24.13 | 39.98 |

Cells were incubated with various concentrations of FLL (0-5 mg/mL) for 48 h, and the percentage of cell cycle phase was analyzed by flow cytometry.

mg/mL and 7.5 mg/mL at 24 h and 48 h, respectively (**Figure 1B**). Treatment of gastric carcinoma cells with FLL induced cell growth inhibition in a dose-dependent manner by using CCK8 assay. To evaluate the time-dependent effect of FLL on the cell viability, the AGS and SGC-7901 cells were exposed to 5 mg/mL FLL for various times. As shown in **Figure 1C** and **1D**, the cell viability was significantly decreased after 6 h of FLL treatment. We next investigated whether FLL induced cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. Consistent with the CCK8 assay, the results showed that growth inhibition was accompanied with an increase in apoptotic cells, as determined by flow cytometry (**Figure 2A** and **2B**). The proportion of the early and terminal phase of apoptosis cells had gained after FLL (5 mg/mL) treatment as compared to non-treatment group (**Figure 2A** and **2B**). Moreover, the results showed that the proportion of apoptosis cells was significantly increased after treatment with FLL (5 mg/mL) for 48 h compared with the 24 h treatment in AGS and SGC-

7901 gastric carcinoma cell lines. In order to detect whether AGS and SGC-7901 cells treated with FLL were undergoing apoptosis, DNA fragmentation analysis was detected by hoechst 33342 staining. After treatment with FLL for 48 h, a typical DNA ladder pattern of internucleosomal fragmentation at 5 mg/mL FLL was also observed (**Figure 3**).

FLL effects on cell cycle distribution

Cells were exposed to different concentrations of FLL for 48 h, and the cell-cycle distribution was determined via a flow cytometry assay. The results showed that treatment with FLL increased the cell population in the G2/M phase in a dose-dependent manner and an accordingly decreased in the G1 phase populations in AGS gastric carcinoma cell lines (**Table 1**). The similarly results were observed in SGC-7901 gastric carcinoma cell lines (**Table 2**). Treatment with 1 mg/mL FLL for 48 h increased the percentage of cells in the G2/M phase from 29.22% (control group) to 34.74%, and treatment for the same duration of time with 5 mg/mL FLL further increased the G2/M population

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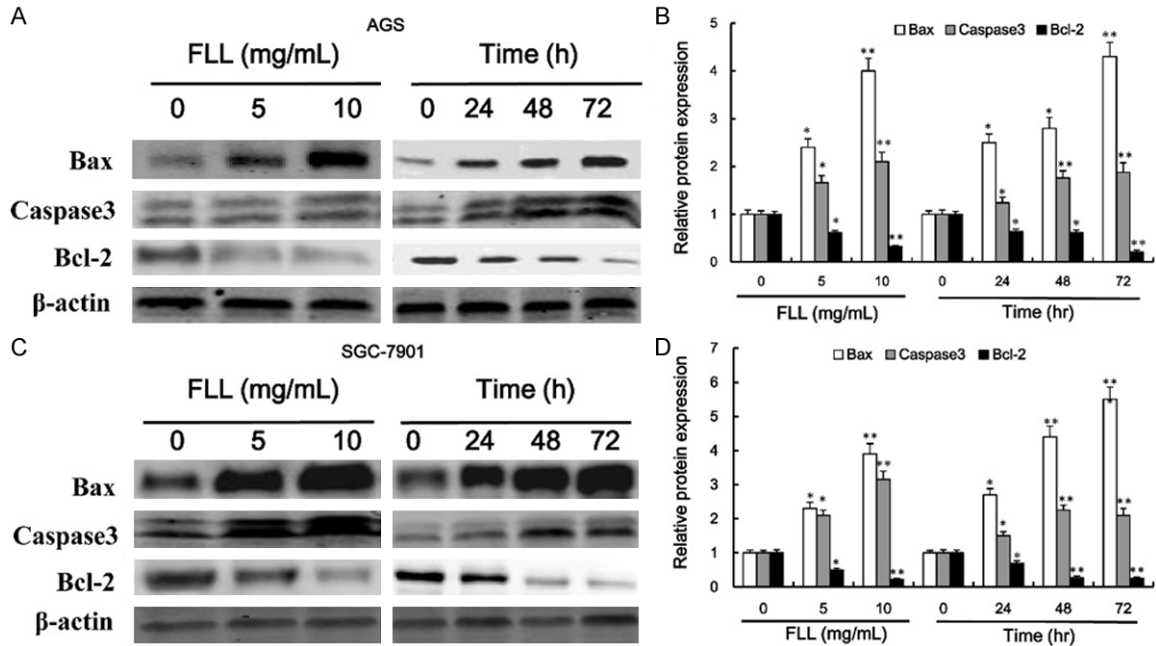


Figure 4. Effects of FLL on apoptosis-related protein expression. The AGS (A) and SGC-7901 (C) gastric carcinoma cell were incubated with FLL (5 or 10 mg/mL) for 24 and 48 h, the expression levels of Bax, Caspase3 and Bcl-2 were determined by western blotting. Protein bands were quantified in AGS (B) and SGC-7901 (D) gastric carcinoma cell, normalized to β -actin protein bands. Values are expressed as mean \pm SEM, $n = 3$ in each group. * $P < 0.05$, ** $P < 0.01$ versus control group.

to 44.60% in AGS gastric carcinoma cell lines (Table 1). In SGC-7901 gastric carcinoma cell lines, treatment with 1 mg/mL FLL for 48 h increased the percentage of cells in the G2/M phase from 26.75% (control group) to 31.28%, and treatment for the same duration of time with 5 mg/mL FLL further increased the G2/M population to 39.98% (Table 2). Overall, these results suggested that the inhibition of growth observed in response to FLL was associated with the G2/M arrest of the cell cycle.

Effect of FFL on the protein expression of Bax, Caspase3 and Bcl-2

The CCK8 assay and flow cytometry results showed that FLL could induce apoptosis in gastric carcinoma cell. The apoptotic response was further investigated by measuring apoptosis-related proteins expression. Western blotting analysis showed that treatment of AGS and SGC-7901 cells with increasing doses of FLL and increasing durations significantly increased the pro-apoptotic Bax level, decreased the anti-apoptotic Bcl-2 level (Figure 4A-D). In addition, the effects of FLL treatment on the expression of cleaved caspase-3 were also examined. As

shown in Figure 4A-D, the protein expression of caspase-3 was increased with increasing doses and increasing durations. Therefore, our data suggested that FLL regulation apoptosis-related proteins expression could induced gastric carcinoma cell apoptosis.

FLL downregulates the protein levels of CDC2 and cdc25C

To evaluate the potential molecular mechanism by which FLL causes a G2/M arrest, we analyzed the steady-state levels of proteins involved in the G2/M checkpoint. The results found that CDC2 and cdc25C were downregulated upon FLL treatment in AGS and SGC-7901 cells with increasing doses of FLL (Figure 5A-D). The expression level of CDC2 and cdc25C was negatively correlated with the concentrations of treatment by FLL.

FLL upregulates the protein levels of p53 and p21

Significant changes in the protein levels of tumor suppressors were observed in AGS and SGC-7901 cells with FLL-treated. As shown in

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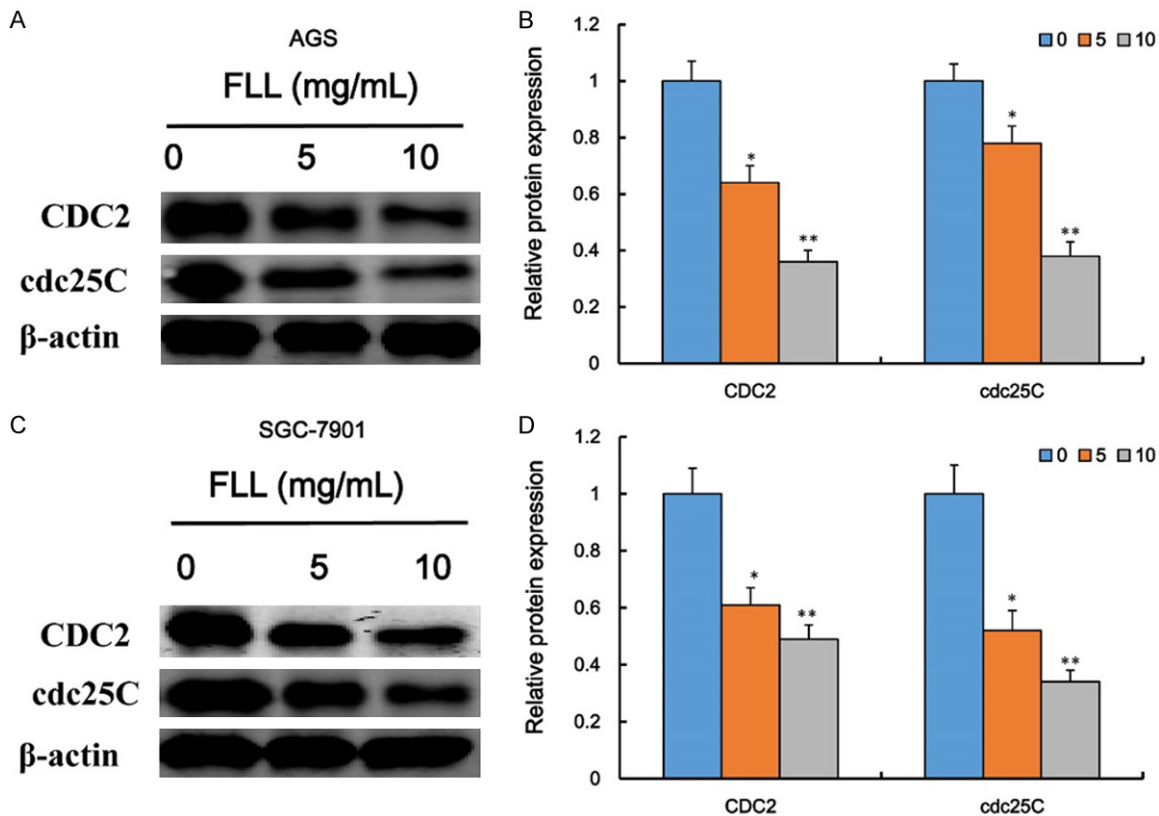


Figure 5. Effects of FLL on G2/M checkpoint proteins. The AGS and SGC-7901 gastric carcinoma cell were incubated with FLL (5 or 10 mg/mL) and subjected to western blot analysis using the antibodies indicated. The expression levels of CDC2 and p-CDC2 were determined by western blot in AGS (A) and SGC-7901 (C) gastric carcinoma cell. Protein bands were quantified in AGS (B) and SGC-7901 (D) gastric carcinoma cell, normalized to β -actin protein bands. Values are expressed as mean \pm SEM, $n = 3$ in each group. * $P < 0.05$, ** $P < 0.01$ versus control group.

Figure 6A-D, p53 and p21 were obviously upregulated by FLL treatment in a dose-dependent manner. These results indicated that FLL might induce gastric carcinoma cell death through activation tumor suppressors signaling pathway.

Discussion

There are mostly sparse reports of the anticancer activity of FLL on human tumor, especially on human gastric carcinoma. However, as a kind of Traditional Chinese Herb, FLL has multiple functions. It has been widely used to its characteristics of medicine and food [23]. Previous research has shown that FLL extracts can suppress tumor growth, such as hepatocellular carcinoma [8], human glioma [10] and human colorectal carcinoma [24]. Several active compounds in FLL, ursolic acid, oleanolic acid and ligustroflavone, show much better anticancer effect than crude extract of FLL

[25]. Moreover, aqueous extracts of FLL enhance the sensitivity of human colorectal carcinoma DLD-1 cells to chemotherapy drugs, the findings suggest that FLL has a potential of being an appealing agent for auxiliary chemotherapy in treatment of human colorectal carcinoma [24]. These studies indicate that FLL may possess a potential efficiency in treating human gastric carcinoma.

In the present study we made several important observations. First, according to the CCK8 assay, hoechst 33342 staining and Annexin V-PI double-labeling staining, FLL induced cell death through an apoptotic mechanism, the proportion of the early and terminal phase of apoptosis cells had significantly gained, and we found that it induced G2/M-phase arrest in human gastric carcinoma. Next, FLL-treatment caused the downregulation of both CDC2 and cdc25C in human gastric carcinoma. CDC2 is the cyclin-dependent kinase responsible for

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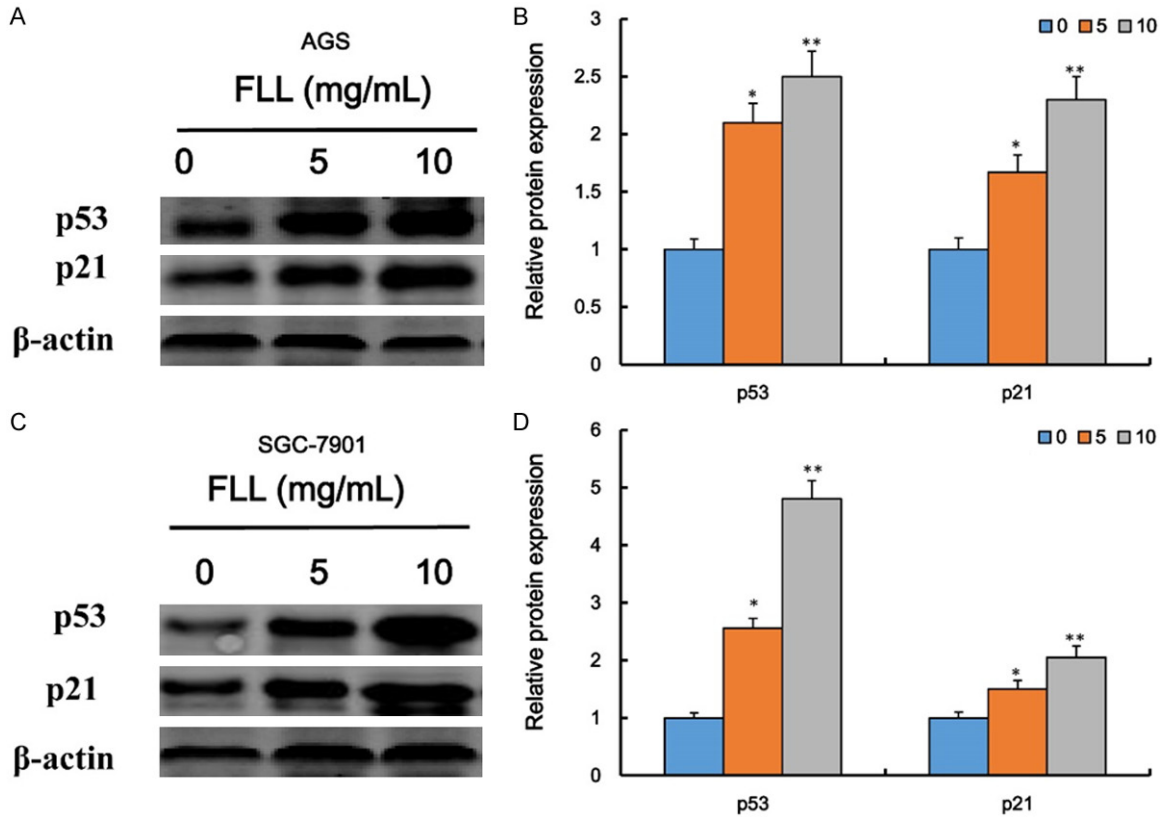


Figure 6. Effects of FLL on tumor suppressors. The AGS and SGC-7901 gastric carcinoma cell were incubated with FLL (5 or 10 mg/mL) and subjected to western blot analysis using the antibodies indicated. The expression levels of p53 and p21 were determined by western blot in AGS (A) and SGC-7901 (C) gastric carcinoma cell. Protein bands were quantified in AGS (B) and SGC-7901 (D) gastric carcinoma cell, normalized to β -actin protein bands. Values are expressed as mean \pm SEM, $n = 3$ in each group. * $P < 0.05$, ** $P < 0.01$ versus control group.

the entry and exit from G2 and mitosis. It forms a complex with cyclin B1 or cyclin A. It is known that cell cycle dysregulation is a hallmark of tumor cells. Regulation of proteins that mediate critical events of the cell cycle may be a useful antitumor target. A number of Cdks and Cdk inhibitors have been isolated, and have been shown to regulate the events of the cell cycle [11, 26]. In gastric carcinoma AGS cells, tanshinone II A inhibitions the expression CDC2, cyclin A and cyclin B1 induces G2/M phase arrest [14]. Interestingly, water extract of Strychni Semen-mediated G2/M arrest is found to be associated with up-regulation of cyclin A, CDC2, tumor suppressor p53 and cyclin dependent kinase in AGS cell [11]. This is a totally opposite results to our study. Next, FLL-treated could upregulate Bax and caspase3 expression and downregulate Bcl-2 expression. FLL regulation apoptosis-related proteins expression could induce gastric carcinoma cell apoptosis.

Furthermore, human neuroglioma cell exposure to FLL could upregulate the expression of p53 and p21. Previous studies show that p21 is identified as a protein suppressing cyclin activity and is originally considered as a negative regulator of the cell cycle and a tumor suppressor [27]. At present, p21 is known to be involved in regulation of fundamental cellular programs, such as cell proliferation, differentiation, migration, senescence, and apoptosis [28]. The p53 pathway has been reported to be involved in histone acetyltransferase inhibitor (HATi) II induces apoptosis in glioma cell lines, and upregulation of p53 signaling pathway-related genes in HATi II-treated cells is confirmed by quantitative RT-PCR and Western blotting [29]. P53 transcriptional function is considered as a marker of large-scale differently expressed genes, which are involved in cell cycle arrest [30]. Reprimo appears to induce cell cycle arrest by inhibiting CDK1 activity and nuclear

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translocation of the CDC2/cyclin B1 complex, and may be involved in regulation of p53-dependent G2 cell cycle arrest [31].

To the best of our knowledge, this was the first study to demonstrate that FLL induced cell apoptosis in AGS and SGC-7901 gastric carcinoma cell lines, the underlying molecular mechanisms, at least partially, through activation p21/p53 and suppression CDC2/cdc25C signaling. In view of the results of this experiment, it seemed reasonable to highlight the possibility of FLL in the clinical treatment of gastric carcinoma.

Disclosure of conflict of interest

None.

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