Original Article
Ethyl acetate extracts of *Fructus Ligustri Lucide* induce cell apoptosis in human neuroglioma cell

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Abstract: Objective: Previous studies have shown that Fructus Ligustri Lucide (FLL) can be used to improve the tumor cells sensitivity to chemotherapeutics and promote cell death. However, the mechanism by which FLL mediate this effect is unclear. In the present study, ethyl acetate extracts of FLL induced cell apoptosis in human neuroglioma 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 neuroglioma cell 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 untreatment group. Moreover, human neuroglioma cells were exposed to the ethyl acetate extracts of FLL for 48 h, which resulted in an accumulation of cells in G2/Mphase. Apoptotic bodies were clearly observed in human neuroglioma cells that had been treated with FLL for 48 h and then stained with Hochest 33342. The expression of Cyclin B1, CDC2 and cdc25C were downregulated upon FLL treatment in human neuroglioma cells. The expression level of Cyclin B1, CDC2 and cdc25C was negatively correlated with the time of treatment by FLL. In contrast, p53, p21 and p16 were obviously upregulated by FLL treatment in a time-dependent manner. Conclusions: These results confirmed that FLL could induce apoptosis in human neuroglioma cells, the underlying molecular mechanisms, at least partially, through activation p21/p53 and suppression CDC2/cdc25C signaling in vitro.

Keywords: Fructus Ligustri Lucide, neuroglioma, CDC2/cdc25C, p53/p21

Introduction

The fruit of ligustrum lucidum (FLL) is a major herb for treating aged-related diseases. It is commonly prescribed in Traditional Chinese Medicine for detoxifying kidney and strengthening the bones [1, 2]. FLL also is useful in maintaining Ca balance and preventing age-related osteoporosis since it effective in improving Ca balance and increasing Ca retention in animal model [3, 4]. Besides balancing of the intracellular and extracellular calcium homeostasis, FLL serves a broader purpose, such as restraining the algal growth [5], ameliorating hypercalciuria and trabecular bone deterioration in diabetic mice [6], anti-oxidative [7] and anti-viral effects [8]. Studies alse show that FLL induces

apoptosis and cell senescence in human hepatoma cells [9] and glioma U87MG cell lines [10]. However, the pharmacological functions are rarely conducted in neuroglioma cell growth and anti-cancer efficacy.

In vitro studies, chemotherapy drugs can inhibit the proliferation of human glioma cells through cell cycle arrest and apoptosis [11]. Cell division cycle 2 (CDC2) is always overexpressed in malignant glioma cells and is correlated with chemosensitivity. Knockdown of CDC2 expression inhibits proliferation, enhances apoptosis, and increases chemosensitivity to temozolomide in glioblastoma cells [12]. The phosphorylation of cdc25C, a cycle regulatory protein, is involved in arresting effect of glioblastoma cells on the cell

cycle at G2/M phase [13]. Interestingly, inhibition of Hsp90 function by ansamycins causes downregulation of CDC2 and cdc25C and G2/M arrest in glioblastoma cell lines [14]. These findings indicate that cell-cycle regulatory proteins CDC2 and cdc25C paly an evident role to control the proliferation of human glioma cells. Moreover, p53 and p21 are an important tumor suppressor genes, and their alteration paly a role in the pathogensis and progression of human glioma [15, 16]. In U87 and U251 human glioma cell lines, activating p21/p53 signaling pathway inhibits glioma cell growth in vitro and in vivo by targeting epidermal growth factor [15, 17]. FLL-induced human glioma cells death through activation p21/p53 and suppression CDC2/cdc25C is unknown.

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

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 Anatomy, College of Basic Medical Sciences, Jilin University (Changchun, China). The dried and powdered (10 kg) crude plant was extracted with ethyl acetate 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 human neuroglioma H4 cells 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 streptomy-cin (Invitrogen) at 37°C in a humidified, 5% CO_2, 95% air atmosphere. The medium was replenished every day. Confluent cells were treated with various concentrations of FLL (0-30 mg/mL).

Cell viability detection by CCK8

Human neuroglioma cells (1.0 × 10^4/well) were plated and treated in 96-well plates (three wells per group) with FLL (0-30 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. Human neuroglioma cells 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 end-labeling (TUNEL) method, which examines DNA-strand breaks during apoptosis by using BD ApoAlert™ DNA Fragmentation Assay Kit. Briefly, human neuroglioma cells were incubated with FLL for the indicated times. The human neuroglioma cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodiumcitrate. After being washed, the human neuroglioma cells 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

Human neuroglioma cells (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
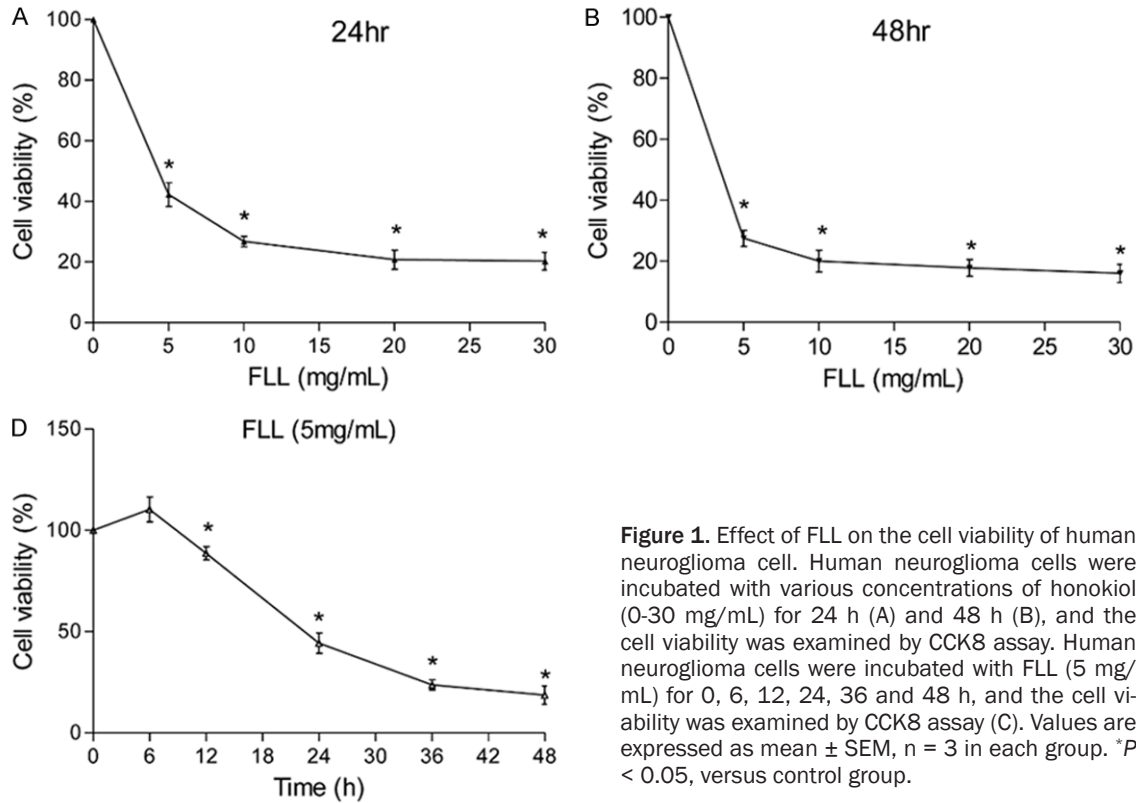
Figure 1. Effect of FLL on the cell viability of human neuroglioma cell. Human neuroglioma cells were incubated with various concentrations of honokiol (0-30 mg/mL) for 24 h (A) and 48 h (B), and the cell viability was examined by CCK8 assay. Human neuroglioma cells were incubated with FLL (5 mg/mL) for 0, 6, 12, 24, 36 and 48 h, and the cell viability was examined by CCK8 assay (C). Values are expressed as mean ± SEM, n = 3 in each group. *P < 0.05, versus control group.

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

Human neuroglioma cells were seeded into 6-well plates, and then treated with vehicle, DMSO or FLL (5 mg/mL) for 48 h, stained with 0.1 µg/ml hoechst 33342 (Sigma) for 5 min, then observed by fluorescence microscopy using appropriate filters for blue fluorescence.

Western blotting

The human neuroglioma cells were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 µg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, 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, Cyclin B1, CDC2, p-CDC2, cdc25C, p-cdc25C, p53, p21, p16 and p38 (Santa Cruz Biotechnoogy, 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 GAPDH signals to correct for unequal loading using the mouse monoclonal anti-GAPDH antibody (Bioworld Technology, USA).

Statistical analysis

The data from these experiments were reported as mean ± 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
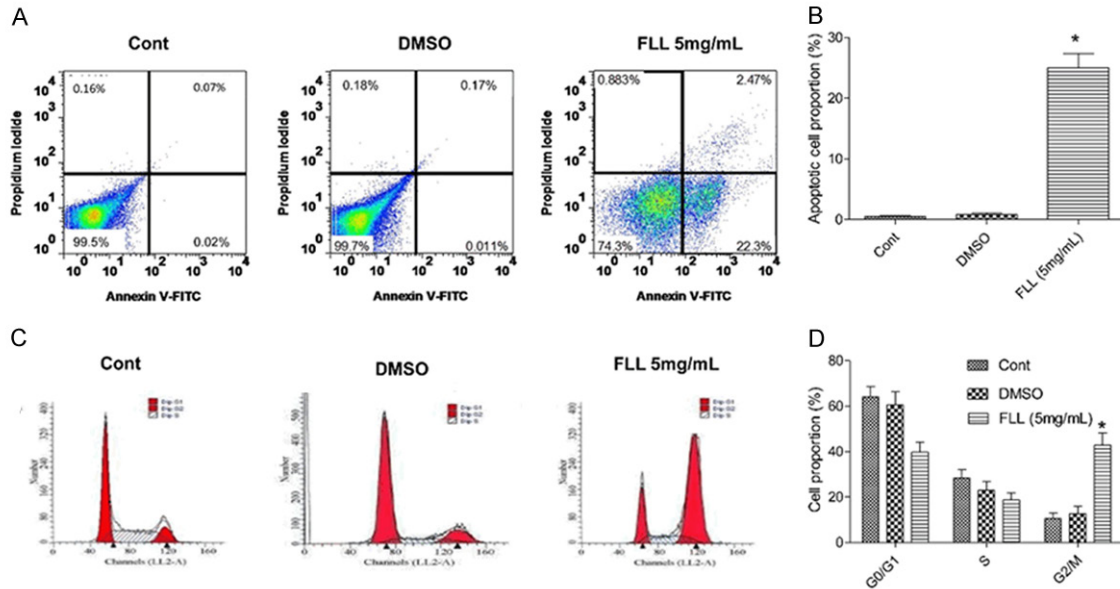
Figure 2. Human neuroglioma cells were treated with vehicle, DMSO or FLL (5 mg/mL) for 48 h, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (A) and bar graphs represent the percentage of apoptotic cells (B). The population of cell cycle phase in a 48 h exposure to FLL (5 mg/mL) was analyzed by flow cytometry analysis (C) and bar graphs represent the percentage of cell cycle phase (D). Values are expressed as mean ± SEM, n = 3 in each group. *P < 0.05, versus control group.
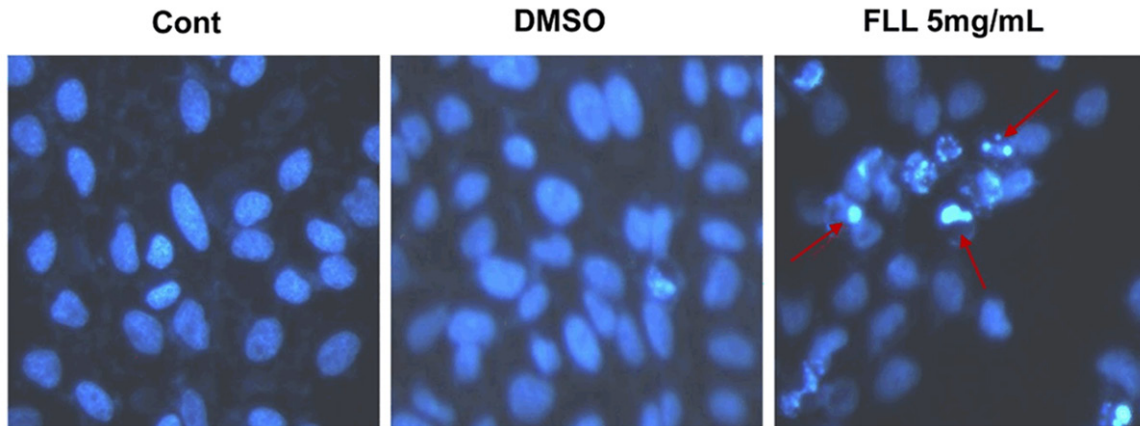

Figure 3. Human neuroglioma cells were treated with vehicle, DMSO or FLL (5 mg/mL) for 48 h. The morphologic changes in human neuroglioma cells were evaluated using hoechst 33342 staining.

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

Cell growth inhibition

Human neuroglioma cell viability was measured when cell was exposed to various con-

centrations of FLL (0-30 mg/mL) for 24 and 48 h. The viabilities of human neuroglioma cell treated with FLL were significantly lower than those of untreatment group. Treatment of human neuroglioma cell with FLL induced cell death in a dose-dependent manner by using CCK8 assay (**Figure 1A** and **1B**). As shown the growth curve in **Figure 1A** and **1B**, the concentrations at which FLL inhibited cell growth by 50% (IC_{50}) were 5 mg/mL and 2.5 mg/mL at 24 h and 48 h, respectively. To evaluate the time-
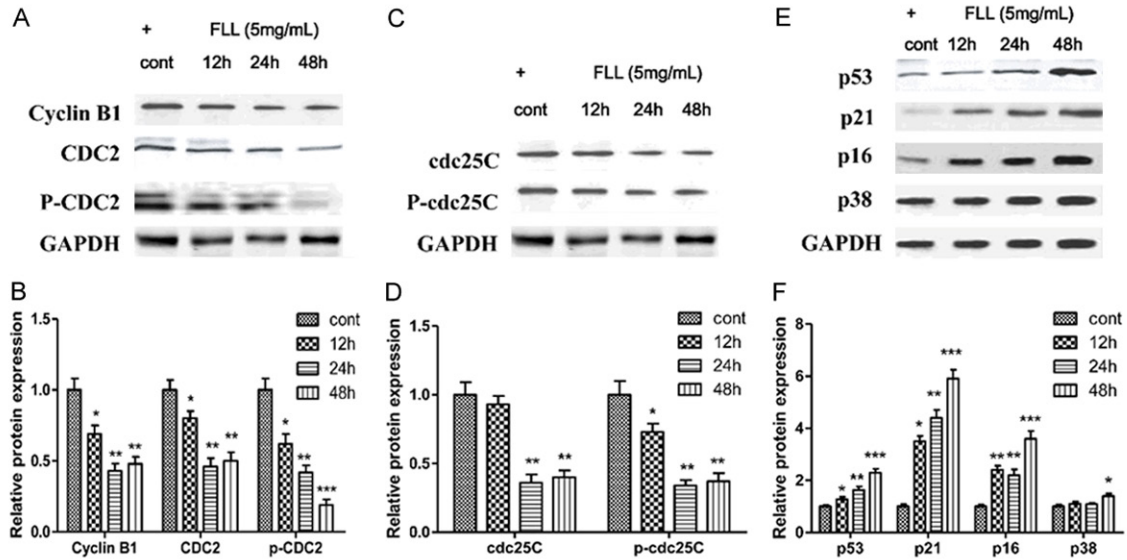
Figure 4. Effects of FLL on G2/M checkpoint proteins and tumor suppressors. Human neuroglioma cells were incubated with FLL (5 mg/mL) for 0, 6, 12, 24, 36 and 48 h and subjected to western blot analysis using the antibodies indicated. The expression levels of Cyclin B1, CDC2 and p-CDC2 were determined by western blot (A). Protein bands were quantified, normalized to GAPDH protein bands (B). The expression levels of cdc25C and p-cdc25C were determined by western blot (C) and densitometric analyses (D). The expression levels of p53, p21, p16 and p38 were determined by western blot (E) and densitometric analyses (F). Values are expressed as mean ± SEM, n = 3 in each group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group.

dependent effect of FLL ethyl acetate extracts on the cell viability, the human neuroglioma cells were exposed to 5 mg/mL FLL ethyl acetate extracts for various times. As shown in **Figure 1C**, the cell viability was significantly decreased after 12 h of FLL treatment, although a slight up-regulation of cell proliferation was observed at 6 h. 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 the proportion of the early and terminal phase of apoptosis cells had gained after FLL treatment as compared to untreatment group (**Figure 2A** and **2B**). To gain insights into the mechanism of the antiproliferative activity of FLL, its effect on cell-cycle distribution was determined via a flow cytometry assay. As shown in **Figure 2C** and **2D**, human neuroglioma cells were exposed to 5 mg/mL FLL ethyl acetate extracts for 48 h, which resulted in an accumulation of cells in G2/Mphase. FLL caused a 3-fold enrichment of cells in G2/M phase and was accompanied by a decrease in G0/G1 phase cells compared to control group. These results suggested that the effects of FLL

suppressed human neuroglioma cell proliferation, at least in part, through delay in the G2/M transition. As shown in **Figure 3**, apoptotic bodies were clearly observed in human neuroglioma cells that had been treated with FLL for 48 h and then stained with Hochest 33342. These results were consistent with the Annexin V assay and cell cycle analysis, and confirmed that FLL could induce apoptosis in human neuroglioma cells.

Effects of FLL on cell cycle regulated proteins and tumor suppressors

To evaluate the potential molecular mechanism by which FLL causes a G2/M arrest, we analysed the steady-state levels of proteins involved in the G2/M checkpoint. The results found that Cyclin B1, CDC2 and cdc25C were downregulated upon FLL treatment in human neuroglioma cells. The expression level of Cyclin B1, CDC2 and cdc25C was negatively correlated with the time of treatment by FLL. However, the expression level of Cyclin B1, CDC2 and cdc25C had no obvious difference between 24 h and 48 h of FLL treatment (**Figure 4A-D**). We simultaneously discovered that the downregulation of both p-CDC2 and p-cdc25c was sustained over time.

Significant changes in the protein levels of tumor suppressors were observed in human neuroglioma cells with FLL-treated. As shown in **Figure 4E** and **4F**, p53, p21 and p16 were obviously upregulated by FLL treatment in a time-dependent manner. The protein expression of p38 was significantly increased after 48 h of FLL treatment (**Figure 4E** and **4F**). These results indicated that FLL might induce cell death through activation tumor suppressors signaling pathway.

Discussion

There are mostly sparse reports of the anticancer activity of FLL on human nerve tumor, especially on human neuroglioma. However, as a kind of Traditional Chinese Herb, FLL has multiple functions. It has been widely used to its characteristics of medicine and food [18]. The anticancer activity of FLL is widely employed in treatment for various cancers, such as hepatocellular carcinoma [9], human glioma [10] and human colorectal carcinoma [19]. Serveral active compounds in FLL, ursolic acid, oleanolic acid and ligustroflavone, show much better anticancer effect than crude extract of FLL [20]. 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 [19].

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 neuroglioma cell. Next, FLL-treatment caused the downregulation of both CDC2 and cdc25C in human neuroglioma cell. CDC2 is the cyclin-dependent kinase responsible for the entry and exit from G2 and mitosis. It forms a complex with cyclin B1 or cyclin A. It can be inactivated by wee-1 and myt-1 when it is phosphorylated in T15 [21]. In human esophageal cancer cells, jaridonin results in G2/M phase arrest through upregulation phosphorylation of cdc25C and activation of checkpoint kinases

Chk1 and Chk2 [22]. 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 [16]. At present, p21 is known to be involved in regulation of fundamental cellular programs, such as cell proliferation, differentiation, migration, senescence, and apoptosis [23]. 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 [15]. P53 transcriptional function is considered as a marker of large-scale differently expressed genes, which are involved in cell cycle arrest [24]. Reprimo appears to induce cell cycle arrest by inhibiting CDK1 activity and nuclear translocation of the CDC2/cyclin B1 complex, and may be involved in regulation of p53-dependent G2 cell cycle arrest [25].

To the best of our knowledge, this was the first study to demonstrate that FLL induced cell apoptosis in human neuroglioma H4 cells, 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 neuroglioma.

Disclosure of conflict of interest

None.

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