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Original Article

Ganoderma lucidum fruiting body extracts inhibit colorectal cancer by inducing apoptosis, autophagy, and G0/G1 phase cell cycle arrest in vitro and in vivo

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Abstract: Although previous studies have found that *Ganoderma lucidum* extracts have the ability to directly resist tumor proliferation and reduce metastasis and invasion, the effect of the extracts of *Ganoderma lucidum* fruiting body (GLE) on cancer is not clarified. This study intends to investigate the anticancer role of GLE on HCT116 colorectal cancer cells in vitro and in vivo. The effects of GLE on the proliferation, apoptosis, autophagy and cell cycle arrest of HCT116 cells were detected by cell counting kit-8 (CCK-8), flow cytometry, electron microscope, quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blot assay. Xenografted mouse models were used to evaluate the tumor growth inhibition effect of GLE in vivo. GLE could significantly inhibit the viability of four tumor cell lines (A549, SW1990, SKOV3 and HCT116) and HCT116 cells were more sensitive to GLE treatment with a half inhibitory concentration of 106 μg/mL. GLE treatment induced apoptosis of HCT116 cells by downregulating of the ratio of Bcl-2 to Bax and increasing cleaved caspase-3 and poly ADP-ribose polymerase (PARP) protein expression. Autophagy of HCT116 cells also increased after GLE treatment, as shown by observation of autophagosomes formation and altered protein expressions in the mTOR pathway. In addition, GLE treatment led to G0/G1 cell cycle arrest as evidenced by flow cytometry analysis and changes in cell-cycle-related gene expressions at the mRNA levels. Of note, in vivo evaluation indicated that GLE significantly inhibited tumor weight and tumor volume and decreased Ki67 expression. In summary, GLE has potential to be developed as an anticancer agent against colorectal cancer, and further evaluation is needed.

Keywords: *Ganoderma lucidum* fruiting body extracts, colorectal cancer, apoptosis, autophagy

Introduction

As one of the major public health problems, cancer poses a serious threat to people's life and health [1]. The incidence of colorectal cancer is up to 6.1%, making it the world's fourth most deadly cancer after lung cancer (11.6%), female breast cancer (11.6%) and prostate cancer (7.1%). Recently, due to the increasing screening methods and effective personalized precision therapies, the survival period of cancer patients has been greatly prolonged, and the quality of life has been significantly improved [2, 3]. Usually, surgery is the primary therapeutic measure for patients with early-stage cancer [4]. Radiation therapy and systemic treatment, including chemotherapy, targeted therapy, hormonal therapy and immunotherapy, are more suitable for late-stage and invasive cancer [5, 6]. However, the side effects associated with these treatment modalities such as pain, fatigue, emotional distress, fertility impairment and neurological sequelae, and subsequent cancers are of particular concern.

The fungus *Ganoderma lucidum* is widely used in both the traditional Chinese medicine and dietary supplements to promote health [7]. Researches indicated that *Ganoderma lucidum* had many functions, including anti-tumor, immune regulation and anti-oxidation, blood sugar lowering and liver protection [8-12].

Clinical studies have confirmed that *Ganoderma lucidum* and its extracts combined with tumor radiotherapy, chemotherapy and surgery can improve patients' clinical symptoms, immune function and tolerance, and quality of life, and reduce side effects caused by radiotherapy and chemotherapy [13-15]. Thus, *Ganoderma lucidum* and its extracts are among the best tumor adjuvant therapies. Moreover, the polysaccharide and triterpenoids are identified to be the two major active ingredients of *Ganoderma lucidum*. The water-extracted polysaccharides stimulate the immune system, and ethanol-extracted triterpenes directly targeted cancer cells [16]. Previously, we also investigated the effects of the immunomodulation and antitumor metastasis of the *Ganoderma lucidum* water extract on B16 melanoma and found that the water extract not only promoted the mouse's immunity but also inhibited tumor metastasis [17]. Recently, supercritical extraction of carbon dioxide was used for *Ganoderma lucidum* fruiting body, but the effect of the extracts of *Ganoderma lucidum* fruiting body (GLE) on cancer is not clarified.

To identify the anti-tumor activity of GLE and to find a more sensitive tumor cell, we conducted a preliminary screening in four different tumor cells lines, including human lung adenocarcinoma A549 cell line, human pancreatic cancer SW1990 cell line, human ovarian cancer SKOV3 cell line and human colorectal cancer HCT116 cell line by cell counting kit-8 (CCK8) assay. Next, we evaluated the effect of GLE on colorectal cancer cell line (HCT116 cell) in vitro including the induction of apoptosis, autophagy and cell cycle arrest and related molecular mechanisms. Finally, an animal model was also established to study whether GLE can suppress tumor growth in vivo. Our date suggest that GLE may be a potential agent against colorectal cancer.

Materials and methods

Cells and chemicals

Ganoderma lucidum was obtained from the Ta-pieh Mountains forested area (800-1500 m above sea level) in Anhui Province, East China. The extract of *Ganoderma lucidum* fruiting body (GLE) was prepared by supercritical fluid carbon dioxide extraction and provided by Nanjing Zhongke Group Corp Ltd, which mainly engages in health food. A549, SW1990, SKOV3 and HCT116 were purchased from KeyGEN biotechnology company and cultured in DMEM, DMEM, DMEM and 1640 medium, respectively, containing 10% fetal bovine serum, penicillin (50 U/mL) and streptomycin (50 U/mL) in a humidified incubator with 5% CO_2 at 37°C. GLE powder was dissolved in ethanol at a total concentration of 20 mg/mL and diluted with medium when used. Cisplatin was used as a positive control.

Cell viability assay

The effect of GLE on the viability of A549, SW1990, SKOV3 and HCT116 cells was determined by CCK8 assay. Briefly, A549, SW1990, SKOV3 and HCT116 were seeded at a density of 2×10^3, 2×10^3, 2×10^3, 10^3 and 2×10^3 cells/well in a 96-well plate and allowed to grow for 12 to 24 h. Cells were then treated with GLE at various concentrations (0, 25, 50, 100, 150 and 200 μg/mL) and cisplatin (5 μg/mL) for 12 h, 24 h and 48 h. CCK8 working solution was prepared by mixing DMEM medium and CCK8 solution at a ratio of 10:1, and 10 ul mixed solution was added to the cultured cells and incubated for an additional 2 h at 37°C. Absorbance values were detected through a micro-plate reader at 562 nm.

Cell apoptosis analysis

A total of 7×10^5 HCT116 cells were cultured in 60 mm dishes and treated with GLE (0, 82, 106 and 136 μg/mL) and cisplatin (5 μg/mL) for 24 h and 48 h. Then HCT116 cells were harvested and suspended by a solution containing 195 μL binding buffer, 5 μL FITC-labeled annexin-V and 10 μL propidium iodide, followed by incubation without light for 20 minutes and examination of the apoptotic rate of HCT116 cells by flow cytometry (BD FACSCalibur, USA).

Electron microscopy

The GLE-treated HCT116 cells were assessed by electron microscopy to examine autophagy. In brief, the HCT116 cells were seeded in 10 cm dishes and treated with 0, 82, 106, 136 μg/mL GLE and 5 μg/mL cisplatin for 24 h. After discarding the culture medium, the cells were trypsinized and collected in a centrifuge. After centrifuging for 2 minutes at 2000 rpm, we discarded the supernatant, fixed the cells in the electron microscope fixative in the dark for 30

Table 1. Primer sequences used in RT-PCR

Gene	Primer sequence
GAPDH	5' TGGACCTGACCTGCCGT 3'
	5' TGGAGGAGTGGGTGTCGC 3'
Bax	5' CCCGAGAGGTCTTTTTCCGAG 3'
	3' CCAGCCCATGATGGTTCTGAT 3'
Bcl-2	5' ATTGTGGCCTTCTTTGAGTTCG 3'
	5' CATCCCAGCCTCCGTTATCC 3'
E2F1	5' CCAACTCCCTCTACCCTTGA 3'
	5' GTCTCCCTCCCTCACTTTCC 3'
CDK2	5' CAGGATGTGACCAAGCCAGT 3'
	5' TGAGTCCAAATAGCCCAAGG 3'
CDK4	5' CTGGACACTGAGAGGGCAAT 3'
	5' TGGGAAGGAGAAGGAGAAGC 3'
CDK6	5' GTGAACCAGCCCAAGATGAC 3'
	5' TGGAGGAAGATGGAGAGCAC 3'
Cyclin A2	5' ATGTCACCGTTCCTCCTTG 3'
	5' GGGCATCTTCACGCTCTATT 3'
Cyclin B1	5' GCCAATAAGGAGGGAGCAGT 3'
	5' ACCTACACCCAGCAGAAACC 3'
Cyclin E1	5' CAGCCTTGGGACAATAATGC 3'
	3' TTGCACGTTGAGTTTGGGTA 3'
P21	5' TTAGCAGCGGAACAAGGAGT 3'
	5' CGTTAGTGCCAGGAAAGACA 3'

minutes, and transferred them to 4°C storage. The cells were then treated with ethanol and embedded in resin. Thin section were cut with the aid of an ultramicrotome and observed by electron microscopy.

Cell cycle analysis

Because cisplatin is a cell-cycle non-specific drug, we only assessed the cell cycle arrest of GLE on HCT116 cells by flow cytometry. A total of 7×10^5 HCT116 cells were cultured in 60 mm plates and treated with GLE (0, 82, 106, 136 μg/mL) for 24 h and 48 h. Cells were harvested in PBS and fixed in ice-cold 70% ethanol for 24 h. Then, the cells were centrifuged at 2000 rpm for 5 minutes and washed once with PBS. Finally, the cells were stained with propidium iodide and measured by flow cytometry.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (RT-PCR) was utilized to analyze the mRNA expression of apoptosis related genes (Bcl-2, Bax) and cell cycle associated genes (E2F1, CDK2, CDK4, CDK6, Cyclin A2, Cyclin B1, Cyclin E1 and P21). HCT116 cells were maintained in 6-well plates at a density of 1.2×10^5 cell/well and treated with GLE for 24 h. The total RNA was extracted following the TRIzol method. The cDNA was synthesized from 1 μg of total RNA by using HiScript II Q RT SuperMix kit (Vazyme, Nanjing, China) and used for PCR amplifications by the SYBRGreen Supermix reagent (Bio-Rad, California, USA) in the 7500 real-time PCR system. The condition for PCR cycling was one cycle at 94°C for 4 minutes, 40 cycles of 95°C for 15 s and 60°C for 30 s. The gene expression data was normalized to GAPDH. Data were analyzed by the method of $2^{-\Delta\Delta Ct}$. Details of the primers are available in **Table 1**.

Western blot assay

A total of 2×10^6 HCT116 cells were maintained in 100 mm dishes and exposed to GLE for 24 h. After washing cells with PBS, protein lysates were harvested by incubation of cells with ice-cold cell lysis buffer at 4°C for 30 minutes, centrifugation at 1,2000 rpm for 15 minutes at 4°C, mixing with sodium dodecyl sulfate (SDS) and denaturation by boiling at 100°C for 10 minutes. A BCA protein assay kit (Beyotime Biotechnology, Nanjing, China) was used to evaluate protein concentration. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were blocked in TBST containing 5% bovine serum albumin for 2 h at room temperature, and then incubated with primary antibodies (Bcl-2, Bax, caspase-3, cleaved caspase-3 (c-caspase-3), mTOR, phosphorylated mTOR (p-mTOR), atg5, atg7, beclin-1, LC3A/B, and GAPDH) overnight at 4°C. Last, after washing and incubating with second antibodies, membranes were subjected to a chemiluminescence imager to assess protein levels.

In vivo study

The evaluation of GLE in vivo was performed in xenografted mice models. This study was carried out in strict accordance the Institutional Animal Care and Use Committee guidelines and approved by the Animal Care Committee of Nanjing University. Male BALB/c nude mice were subcutaneously injected with 5×10^6 HCT116 cells in the right flank and divided into three groups (control, cisplatin and GLE groups).

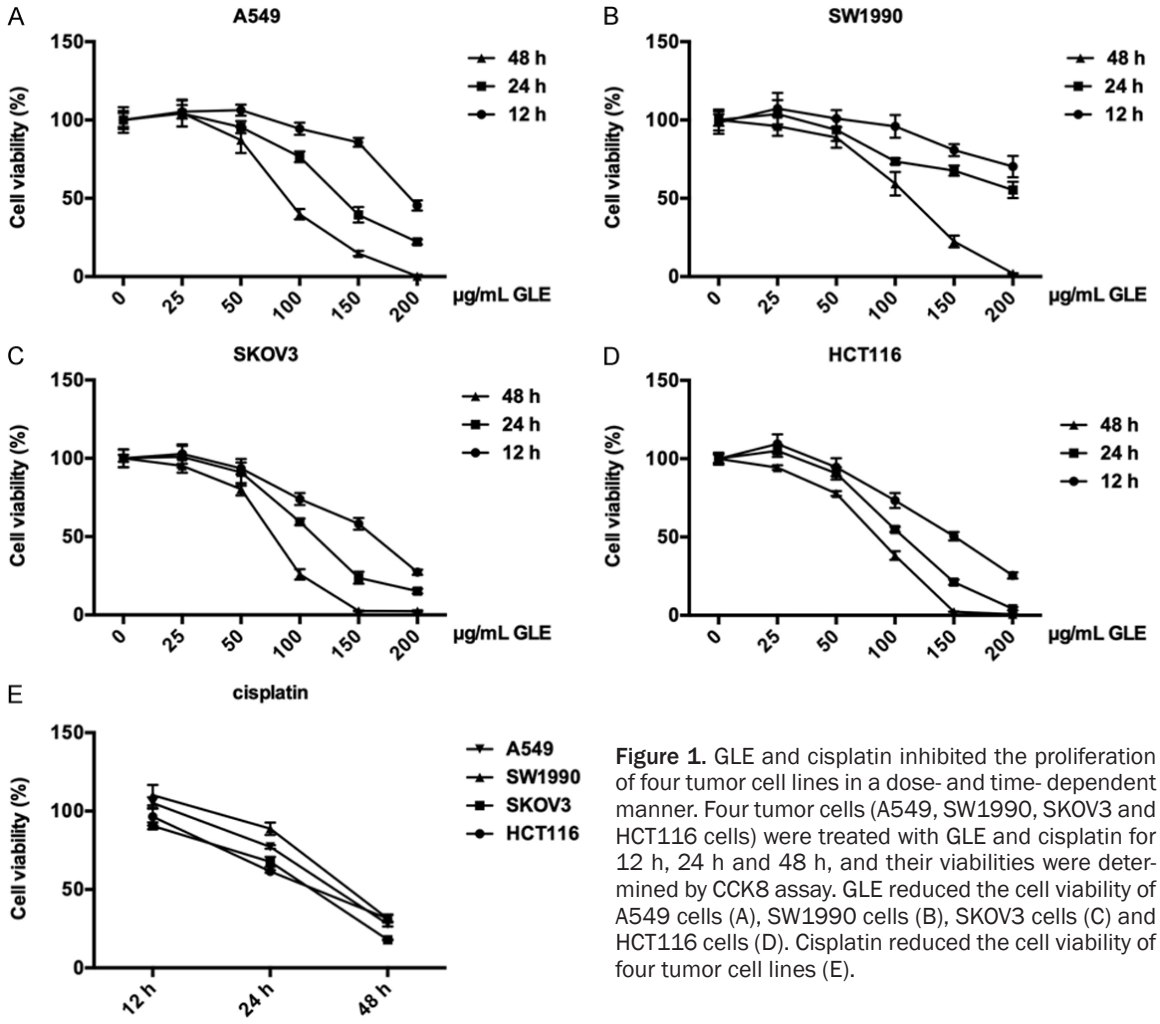

Figure 1. GLE and cisplatin inhibited the proliferation of four tumor cell lines in a dose- and time- dependent manner. Four tumor cells (A549, SW1990, SKOV3 and HCT116 cells) were treated with GLE and cisplatin for 12 h, 24 h and 48 h, and their viabilities were determined by CCK8 assay. GLE reduced the cell viability of A549 cells (A), SW1990 cells (B), SKOV3 cells (C) and HCT116 cells (D). Cisplatin reduced the cell viability of four tumor cell lines (E).

Cisplatin was dissolved in PBS solution containing 5% ethanol and intraperitoneally injected at 10 mg/kg every other two days; GLE was given at 100 mg/kg by gavage administration every day, while the control group were given ethanol (5%) dissolved in PBS by gavage administration every day. Treatments were performed from the second day of tumor inoculation. Every two days, we used the Vernier caliper to measure the length (L) and width (W) of the tumor and calculate the volume according to the formula V (volume) = $(LW^2)/2$. At the 24th day, the mice were sacrificed and tumors were harvested for assessment of tumor growth and other investigation.

Immunohistochemical staining

Immunohistochemical analysis was performed on histological sections of paraffin-embedded slides. After deparaffinizing in xylene and hydrating in a descending alcohol series, slides were soaked in 3% H_2O_2 for 10 minutes to block endogenous peroxidase. Next, the slides were washed before being exposed to the antigen retrieval system; then, the slides were covered with 5% goat serum at room temperature for 1 h. The the primary Ki67 antibody (Abcam, Cambridge, UK) was dropped onto the slides, and they were incubated at 4°C overnight. The next day, the slides were washed with PBS 3 times for 5minutes each time and incubated with the rabbit secondary antibody (Abcam) for 1 h at room temperature. The slides were then stained with 3.3'-diaminobenzidine (Beyotime Biotechnology, Nanjing, China) for 5 minutes. Finally, the slides were counterstained with hematoxylin (CST, Boston, USA) and observed under a microscope.

Statistics

All experiments were repeated in triplicate. The differences between groups were analyzed by

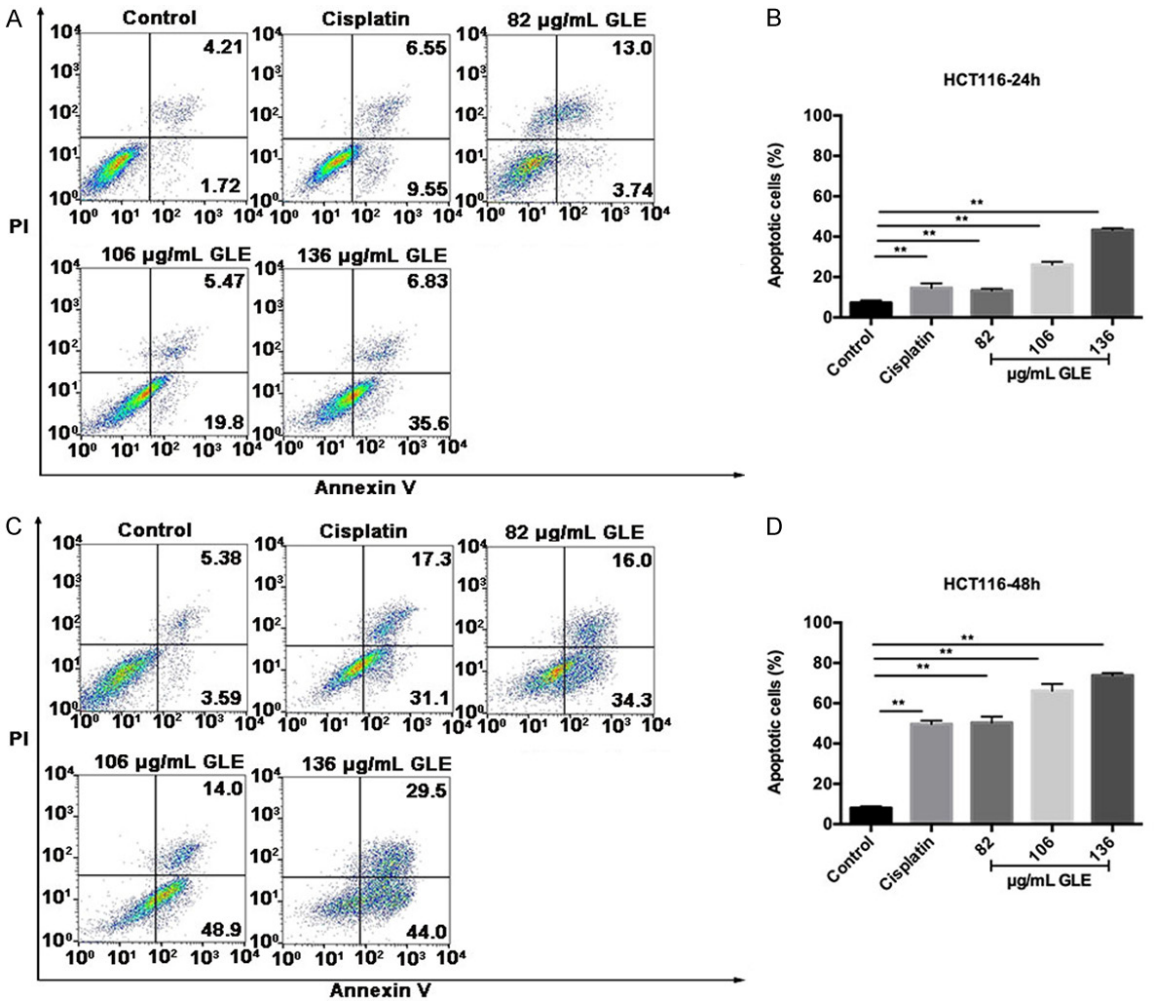

Figure 2. Effect of GLE on apoptosis in HCT116 cells. HCT116 cells were treated with cisplatin (5 μg/mL) and GLE (82, 106 and 136 μg/mL) for 24 h and 48 h, and the apoptotic rates were detected by Annexin V/PI double staining assay. Two-parameter (dual color fluorescence) dot plots (A and C) were obtained via flow cytometric analysis. Bar graphs (B and D) depict mean ± SD of three replicates. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Student's *t*-test for two group comparison or one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple group comparisons, and the significant difference was achieved at $^{*}P < 0.05$ and $^{**}P < 0.01$.

Results

GLE inhibits the proliferation of tumor cells

To evaluate the effect of GLE on the viability of tumor cells, we treated A549, SW1990, SKOV3 and HCT116 cells with different concentrations of GLE (25, 50, 100, 150 and 200 μg/mL) and cisplatin for various treatment durations (12, 24 and 48 h). The 5 μg/mL concentration of cisplatin was used as a positive control. As shown in **Figure 1**, GLE treatment resulted in a dose- and time-dependent reduction in cell viability. The half inhibitory concentrations (IC_{50}) for A549, SW1990, SKOV3 and HCT116 cells at 24 h were 136, 257, 119 and 106 μg/mL, respectively. Therefore, the HCT116 cell line was chosen for subsequent experiments.

GLE induces apoptosis and autophagy of HCT116 cells

The impact of GLE on the HCT116 cells was first evaluated by Annexin V/PI staining. As shown in **Figure 2**, the percentage of the apoptotic HCT116 cells increased with the increase time and concentration of GLE. Moreover, the apoptosis was further confirmed by the

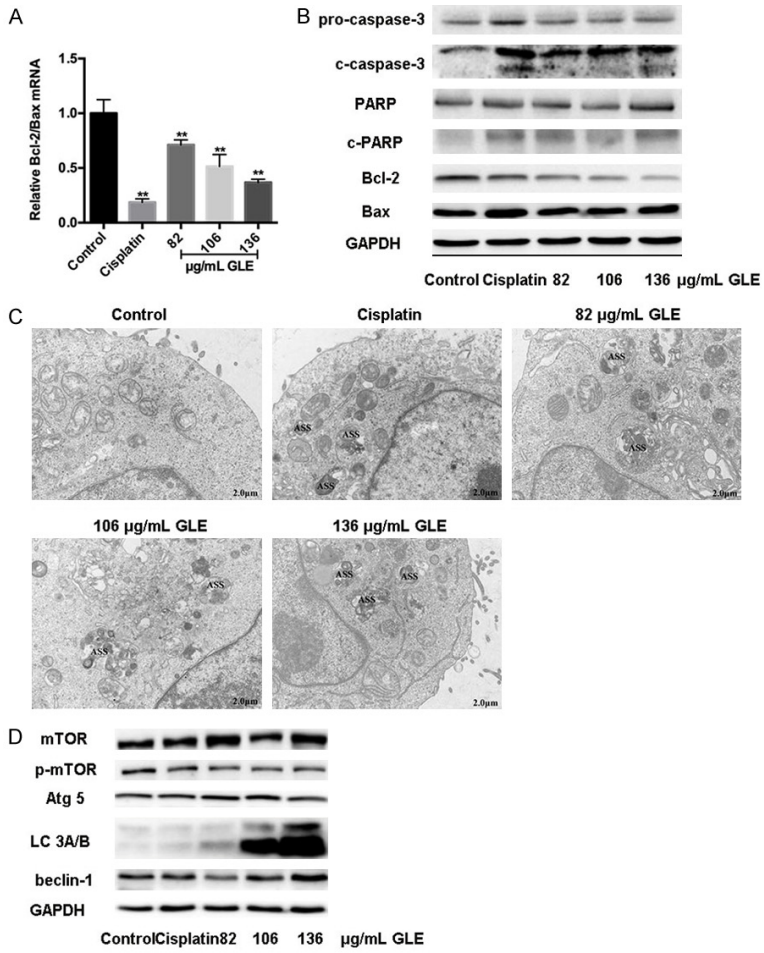

Figure 3. GLE treatment changed the levels of apoptosis-related genes and induced autophagy of HCT116 cells. HCT116 cells were treated with cisplatin (5 μg/mL) and GLE (82, 106 and 136 μg/mL) for 24 h and 48 h, and the related protein expressions and autophagy was examined by Western blot experiment and electron microscope. A. GLE treatment decreased the ratio of Bcl-2 to Bax on mRNA level. B. Protein levels of pro-caspase-3, cleaved caspase-3, PARP, cleaved PARP and GAPDH after GLE (0, 82, 106, 136 μg/mL) and cisplatin (5 μg/mL) treatment for 24 h. C. Electron microscope images of GLE and cisplatin treated HCT116 cells. D. Proteins levels of mTOR, p-mTOR, atg5, atg7, LC3A/B, beclin-1 and GAPDH after GLE (0, 82, 106, 136 μg/mL) and cisplatin (5 μg/mL) treatment for 24 h. ASS: autophagosome.

decreased ratio of Bcl-2 to Bax on both the mRNA and protein levels (**Figure 3**). The apoptosis-inducing effect was partly through the caspase-3 pathway, as evidenced by increased cleaved-caspase-3 and cleaved PARP and decreased total PARP protein expression without impact on pro-caspase-3 protein expression (**Figure 3**). Cisplatin might promote apoptosis of HCT116 cells by increasing the expression of total and cleaved caspase-3 and PARP (**Figure 3**). Furthermore, GLE and cisplatin also triggered the formation of autophagosomes in the HCTT16 cells, indicative of autophagy (**Figure 3C**). To confirm autophagy, the expression of autophagy-associated proteins was examined. The results showed that GLE caused an upsurge of Beclin-1 and the ratio of LC3B to LC3A (**Figure 3D**) and decreased p-mTOR but increased atg5 and total mTOR protein expression (**Figure 3D**). The data indicated that the autophagy-induced effect of GLE was partly through the mTOR pathway.

GLE causes the G0/G1 arrest of HCT116 cells

To investigate the effect of GLE on the cell cycle distribution of HCT116 cells, flow cytometry was used. Results showed that GLE caused a remarkable increase in the percentage of G0/G1 phase cells from upon 38.7% to 42.3%, 51.3%, and 57.8% after treatment with 82, 106 and 136 μg/mL GLE, respectively (**Figure 4A**, **4B**). Moreover, increased cell cycle arrest correlated tightly with the reduction of E2F-1, CDK2, CDK4, CDK6, Cyclin A2, Cyclin B1 and Cyclin E1, as well as the increase of P21 gene expression at the mRNA level (**Figure 4C**).

GLE restrains tumor growth in vivo

Considering the above, we sought to examine the anticancer effects of GLE in vivo in xenografted mice models. The results showed that the tumor weight and volume were significantly inhibited by cisplatin and GLE at the dosage of 10 and 100 mg/kg respectively (**Figure 5**). Moreover, cisplatin and GLE treatment also decreased the expression of Ki67 compared with the control group (**Figure 5D** and **5E**). In addition, consistent with the previous results, GLE treatment also decreased the ratio of Bcl-2 to Bax and changed the expressions of cell

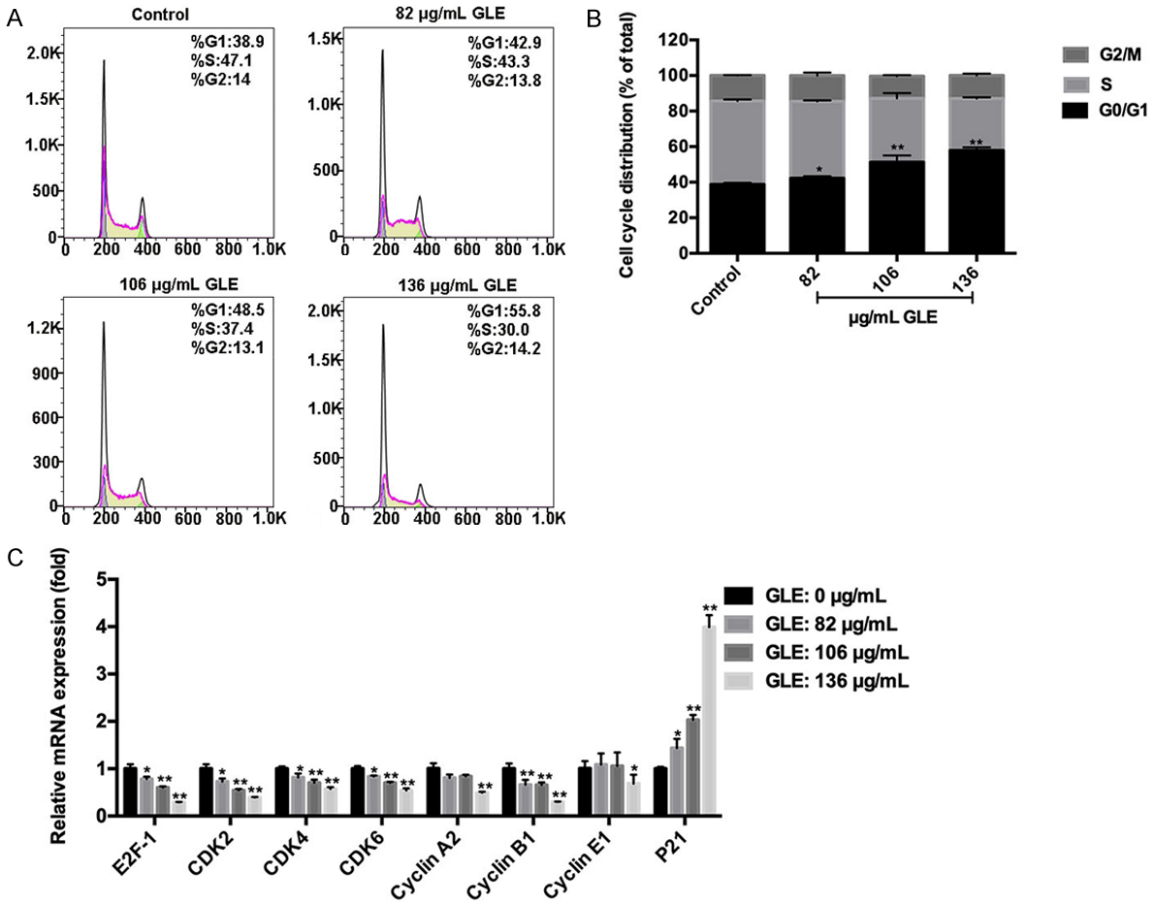

Figure 4. Effect of GLE on cell cycle distribution and cell cycle related genes expression in HCT116 cells. HCT116 cells were treated with GLE (82, 106 and 136 μg/mL) for 24 h, and the cell cycle distribution and related gene expression on mRNA level were detected by PI staining and RT-PCR assay, respectively. A. Flow cytometry images of the cell cycle in HCT116 cells upon exposure to GLE at 0, 82, 106, 136 μg/mL for 24 h. B. Quantified histograms of the effect of GLE on HCT116 cell cycle distribution. C. The mRNA expression of cell cycle related genes in HCT116 cells. $^{*}P < 0.05$ and $^{**}P < 0.01$.

cycle arrest-related genes on the mRNA level (**Figure 6**).

Discussion

Colorectal cancer ranks third and second, respectively, among men and women in terms of incidence, and ranks fourth and third among men and women in terms of mortality [1]. In particular, an increased incidence has been a trend in younger patients, as they are not a target population for routine CRC screening, which makes colorectal cancer comes to be one of the most challenging cancers [18]. Due to the lack of screening and delayed diagnosis, many people have reached metastatic colorectal cancer (mCRC) upon receiving treatment, and the mCRC patients only have a median overall survival of approximately 24-30 months [19]. Currently, plant-derived anticancer agents have attracted remarkable attention in the recent years due to their minimal toxic effects. As such, 5-fluorouracil (5-FU)-based chemotherapy combined with plant-based traditional Chinese medicines improved the tumor response rate of patients compared with 5-FU regimens alone [20]. Herein, the anticancer effects of GLE were examined. By CCK8 assay, we found that HCT116 cells were more sensitive to GLE treatment, and thus the following research was focused on HCT116 cells.

Apoptosis, a form of programmed cell death, can be triggered by the caspase-mediated extrinsic or intrinsic pathways. The Bcl-2 family, consisting of anti-apoptotic members (Bcl-2, Bcl-W and Bcl-xL) and pro-apoptotic members (Bax, Bok, Bak, Bid and Bad), participate in the

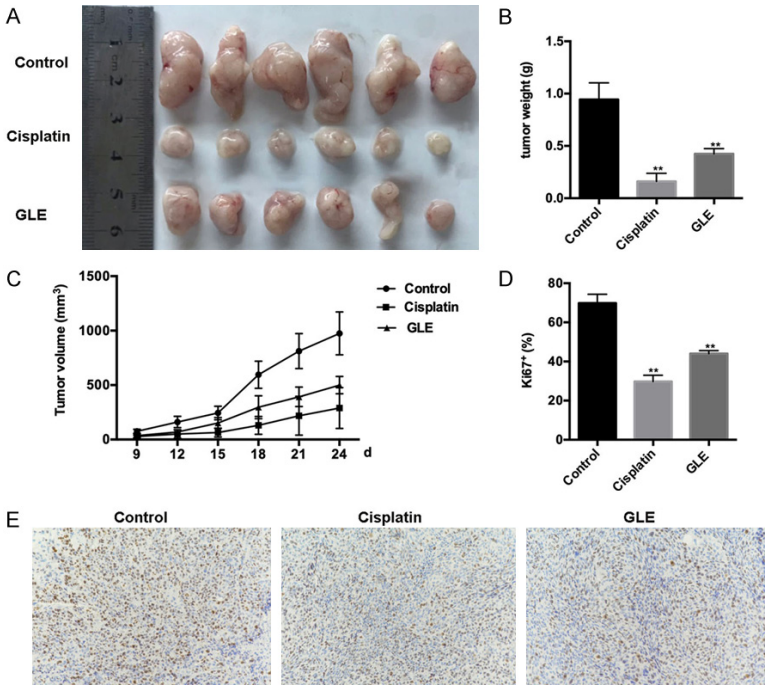

Figure 5. In vivo tumor growth inhibition by GLE and cisplatin. Male BALB/C nude mice were subcutaneously injected with 5 × 10^6 HCT116 cells and treated with cisplatin and GLE from the second day, and the tumors were harvested at the 24 h day. (A-C) GLE and cisplatin significantly inhibited tumor formation in xenografted mice models. (D) The cell proliferation in xenografted mice models was significantly impaired by GLE and cisplatin treatment, as detected by immunohistochemistry using Ki67. (E) Statistical analysis of Ki67 expression in (D). $^{*}P < 0.05$ and $^{**}P < 0.01$.

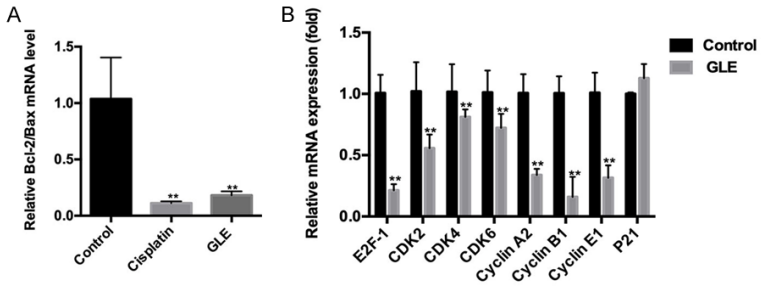

Figure 6. Expression of apoptosis and cell cycle related genes in xenografted mice models. Total RNA was obtained from tumor tissues by TRIzol method, and gene expressions on mRNA level were detected by RT-PCR assay. A. GLE and cisplatin treatment decreased the ratio of Bcl-2 to Bax in vivo. B. GLE treatment changed the expression of cell cycle related genes in vivo. $^{*}P < 0.05$ and $^{**}P < 0.01$.

regulation of apoptosis and determine whether a cell undergoes apoptosis. In addition, caspases play a central role in apoptosis as they are both the initiators (caspase-2, -8, -9 and -10, primarily responsible for the beginning of the apoptotic pathway) and the executors (caspase-3, -6 and -7, responsible for the definite cleavage of cellular components) of cell death [21]. Autophagy, characterized as a multistep lysosomal degradation pathway that supports nutrient recycling and metabolic adaption, has been implicated as a process that regulates cancer [22]. Recently, modulating autophagy has emerged as a potential therapeutic measure for certain cancer types. Some autophagy inducers such as metformin and spermidine have been shown to enhance cancer therapies in clinical trials [23]. Excessive activation and sustained cell proliferation are essential features of tumors, and thus induction of cell cycle arrest can effectively inhibit tumor growth. As a new kind of target, cyclin dependent kinases (CDKs) are receiving increasing attention, and CDK inhibitors such as palbociclib and flavopiridol have been used clinically [24].

In this study, we found that GLE induced the apoptosis of HCT116 cells through decreasing the ratio of Bcl-2 to Bax and upregulating the protein level of cleaved caspase-3 and PARP. Autophagy of HCT116 cells was also increased, as evidenced by electron microscopy observation and changes in autophagy-related protein expression. In addition, GLE caused arrest of HCT116 cells at the G0/G1 check point and thereby halted their growth. Because of the potential anticancer activity of GLE in vitro, we sought to know the antiproliferative effects of GLE in vivo and found that GLE could inhibit the growth of xenografted tumors, indicative of the potential of GLE in the treatment of colorectal cancer.

In summary, it is concluded that GLE inhibited the proliferation of HCT116 cells by inducing apoptosis, autophagy and cell cycle arrest. In

addition, GLE also inhibited the tumor growth in vivo. As such, GLE might be a promising, reliable treatment for colorectal cancer and warrants further investigation.

Acknowledgements

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Disclosure of conflict of interest

None.

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