Original Article
Grifolin, a potent antitumour natural product inhibits the growth and invasion of gastric cancer cells in vitro

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Abstract: Grifolin, a natural biologically production, has been reported to have an anti-cancer effect in nasopharyngeal carcinoma and osteosarcoma. In this study, we found that grifolin also could inhibit the growth and invasion of gastric cancer cells in vitro. Our results demonstrated that grifolin suppressed proliferation and invasion by ERK1/2 pathway, and induced apoptosis in BGC823 and SGC7901 gastric cancer cell lines. Grifolin increased the release of cytochrome c accompanied by activation of caspase-3 and caspase-9. This result provided comprehensive evidence for grifolin's antitumor effect in gastric cancer in vitro, which might give a new approach for clinical treatment of gastric cancer.

Keywords: Gastric cancer, grifolin, antitumor, proliferation, apoptosis, invasion

Introduction

Gastric cancer (GC) is the second leading cause of cancer-associated mortalities worldwide, with an incidence of approximately 934,000 cases per year in East Asia [1]. At present, surgical resection and chemotherapy are the two major means of treating gastric cancer [2]. And over the last few decades, there have been significant advances in diagnostic and therapeutic approaches. However, the prognosis of GC still remains poor because of its high recurrence and metastasis [3]. Therefore, the explorations of novel drugs or new combination chemotherapies for gastric cancer treatment are urgently required.

In recent years, natural agents such as the pool of secondary metabolites isolated by fungi have attracted a great deal of chemopreventive and therapeutic interest. Grifolin is characterized by its broad pharmacological activity, high efficiency and low toxicity. At present, grifolin has shown multiple biological activities including inhibition of histamine release and NO production [4], antibiotic [5], anti-oxidation [6] and decrease of plasma cholesterol. A recent study has been demonstrated that grifolin possessed potential antitumor properties in vitro and in vivo. As a new anticancer drug, grifolin played an important role in the treatment of nasopharyngeal carcinoma [7] and osteosarcoma [8], but whether it has an antitumor effect in gastric cancer has not been reported. Accordingly, in this study, we are interested in the grifolin treatment in gastric cancer. Our results show that grifolin had a significant antitumor effect via the regulation of the proliferation, apoptosis, cell cycle, and invasion of human gastric cancer. Our finding gives a deep insight into the anti-tumor mechanisms of grifolin and will provide the theoretical basis for clinical treatment of gastric cancer.

Materials and methods

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Two human GC cell lines, BGC823 and SGC-7901, was purchased from Cell Bank of Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences, and maintained at 37°C in RMPI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco) in the presence of 5% CO_2. The medium was routinely changed every 2 days. The cells in logarithmic growth phase were used for experiments.

Table 1. List of primers and their applications

Primer	Sequences (5'-3')	Application
MEK1-assay-F	TGCCAGGCTGAACTACAGTA	qRT-PCR
MEK1-assay-R	CACAAGGCTCCCTCTCAGAC	qRT-PCR
MEKK3-assay-F	GATGGCAGAAGAACATTT	qRT-PCR
MEKK3-assay-R	ACCCATGTTCTCGCCATT	qRT-PCR
MEK5-assay-F	ATGCTGTGGCTAGCCCTTGG	qRT-PCR
MEK5-assay-R	GTAATATCTAGTAGTATGACC	qRT-PCR
CDKN2D-assay-F	GCCTTGCAGGTCATGATGTTTGGA	qRT-PCR
CDKN2D-assay-R	ATTCAGGAGCTAGGAAGCTGACCA	qRT-PCR
GAPDH-assay-F	CATCACCATCTTCCAGGAGCG	qRT-PCR
GAPDH-assay-R	TGACCTTGCCCACAGCCTT	qRT-PCR

Drug treatment

Grifolin (2-trans, trans-farnesyl-5-methylresorcinol) was provided by Kunming Institute of Botany, the Chinese Academy of Sciences (purity >99%, HPLC analysis) [9]. BGC823 and SGC-7901 cells in logarithmic growth phase were plated in 6-well plates until cell attachment then treated with grifolin at final concentrations of 10 µM and 50 µM for 48 hours. The cells treated with 0.1% DMSO were used as negative control.

Total RNA extraction and quantitative real-time (RT)-PCR

Total RNA was extracted from the BGC823 and SGC7901 cells using TRIzol (Invitrogen) reagent according to the manufacturer's recommended instructions. RNA purity and integrity were analyzed using an Agilent Bioanalyzer 2100 (Agilent Technologies).

The complementary DNA (cDNA) was synthesized using an iScript cDNA Synthesis Kit (BIO-RAD) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Premix ExTaqTM II kit (TaKaRa). The conditions of qRT-PCR were as follows: 94°C for 10 s, 94°C for 5 s, 52°C for 30 s to anneal, 72°C for 15 s followed by 40 cycles. The detection was set at 62°C. PCR amplifications were performed in three duplicates for each sample. The relative RNA expression was calculated using the 2-ΔCt method. And the specific primers sequences were listed in **Table 1**.

Transwell invasion assay

Transwell invasion assay was performed using the Biocoat Matrigel Invasion Chamber (BD Biosciences) according to the manufacturer's recommended instructions. In brief, Cells were treated with the indicated concentration of grifolin for 24 h, and the cells were plated in in the upper chamber consisting of 8-mm membrane filter inserts coated with 0.75 mg/ml Matrigel (BD Biosciences) for 1 h. The bottom chamber contained RMPI-1640 medium with 10% FBS as an inducer of invasion. $4×10^4$ cells in serum-free media were placed inside each chamber. After 24 h, cells on the upper surface were removed, and those attached on the lower side of the membrane were fixed in buffered formalin and stained with crystal violet. Before counting under a microscope in five randomly selected fields.

Methyl thiazolyl tetrazolium (MTT) assay

BGC823 and SGC7901 cells were seeded in 96-well plates, cultured for 24 h and then treated with grifolin as described above. Cell viability was analyzed using MTT (Sigma) assay at the indicated time points as described previously [10]. In brief, 1 µl/well of MTT was added and incubated at 37°C for an additional 4 h. Then, the medium was discarded and cells were lysed in DMSO (150 µl/well). The absorbance at 490 nm was measured on a plate reader. Each experiment was performed in triplicate and repeated three times.

Cell cycle assay

Appropriate cells as earlier described were collected, washed and then suspended in PBS and fixed in 75% ethanol. The fixed cells were stained with propidium iodide (PI) supplemented with RNaseA (Sigma) and analyzed with a FACScan flow cytometer (BD Biosciences). Data were collected and analyzed with the ModFit software (BD Biosciences).

Flow cytometric analysis of apoptosis

For detection of apoptosis, appropriate adherent cells were collected, washed and suspended in cold PBS for analysis. Apoptosis was detected using the Alexa Fluor® 647/7-AAD apoptosis kit (BioLegend) according to the manufacturer's recommended instructions. Data were assessed by flow cytometry (BD Biosciences).
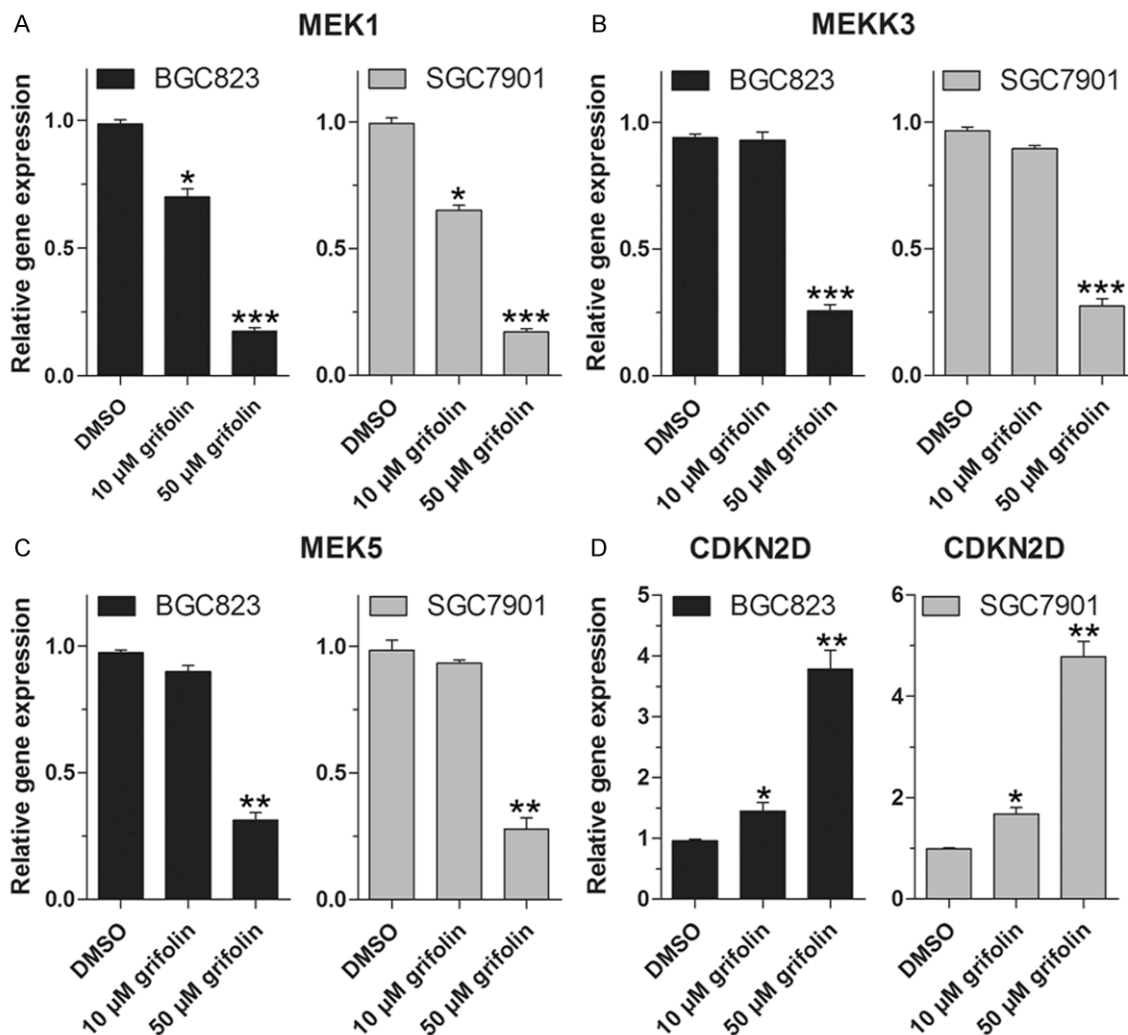
Figure 1. Effects of grifolin on gene expression of GC cells. Expression profiles of genes in BGC823 and SGC7901 cells treated with grifolin (10 and 50 µM) was detected using qRT-PCR. The cells treated with 0.1% DMSO were as negative control in all these experiments above. Data are at least three independent experiments and are shown as mean ± SD (*P<0.05, **P<0.01, ***P<0.001 as compared with control group).

Western-blot

Western blotting was carried out as previously described [11]. Briefly, Cells were collected and lysed with complete cell lysis (Beyotime) with protease inhibitors cocktail (Rocha). Identical quantities of proteins was loaded and separated on SDS-PAGE, transferred to polyvinyl dene fluoride membranes (Millipore), and then incubated with appropriate antibodies, which are against ERK1/2 (Cell Signaling Technology), p-ERK1/2 (Cell Signaling Technology), ERK5 (Cell Signaling Technology), p-ERK5 (Cell Signaling Technology), cytochrome c (Cell Signaling Technology) and GAPDH (Abcam). The immuno-reactive bands were visualized using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL) and obtained using a CCD camera system (Tanon). The density of which was measured by Image J.

Assay for cytochrome c release

The assay of release of cytochrome *c* from mitochondria was carried out as described previously [7, 9]. In brief, Mitochondrial and cytosolic fractions were prepared by resuspending cells in ice-cold buffer (pH 7.5, 20 mM Hepes-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 20 µg/ml leupeptin, 10 µg/ml aprotinin, and 250 mM sucrose). Cells were passed and homogenized through a nee-
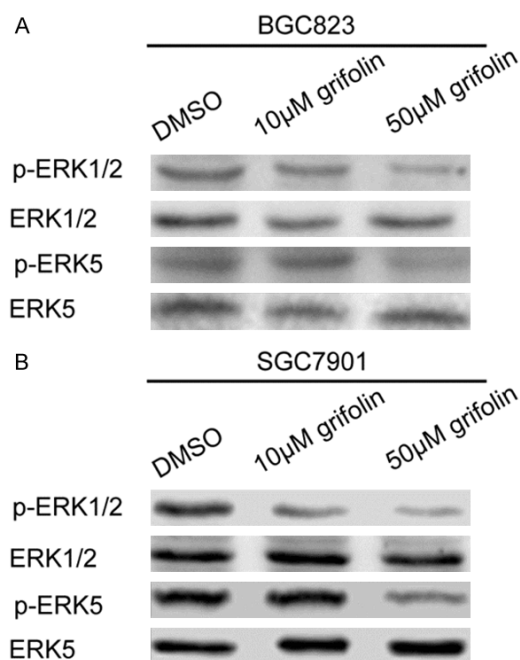
A

BGC823

B

SGC7901

Figure 2. Grifolin inhibits the phosphorylation of ERK1/2 and ERK5. BGC823 (A) and SGC7901 (B) cells were treated with 10 or 50 µM grifolin as indicated. Cell lysates were prepared and examined by Western blotting analysis with antibodies against ERK1/2, p-ERK1/2, ERK5, and p-ERK5. The experiments were repeated three times with similar results.

dle 15-20 times. Unlysed cells and nuclei were pelleted by centrifugation for 5 min at 1,000 g, and the supernatants were collected and centrifuged at 12,000 g for 15 min. The mitochondrial pellets were resuspended in lysis buffer. Supernatants were transferred to new tubes and centrifuged again at 12,000 g for 15 min, and the resulting supernatants, the cytosolic fractions, were separated. The protein from the cytosolic fraction of each sample was used to perform western blotting with anti-cytochrome c antibody.

Caspase-3/9 fluorescent assay

Cells with appropriate treatment were collected and washed with PBS and then resuspended in lysis buffer (pH7.5, 25 mM HEPES, 5 mM MgCl$_2$, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 10 mg/ml pepstatin A and 10 mg/ml leupeptin). Cell lysates were centrifuged at 12,000 g for 5 min, and supernatants, containing 50 mg of protein were incubated with 50 mM Ac-DE-VD-AMC (a specific substrate for caspase-3) or Ac-LEHD-AMC (a specific substrate for cas-

pase-9) at 37°C for 1 h. The fluorescence of AMC was measured using a spectrofluorometer (Hitachi F-4500) with excitation at 360 nm and emission at 460 nm (Promega CaspACE2 Assay System).

Statistical analysis

All experiments were performed independently for at least three times. All data are presented as the mean values ± standard deviations (SD) of each group. Statistically significant differences were calculated by two-tailed Student's *t*-test using SPSS software (version 19.0). The graphs were generated with GraphPad Prism 5.0.

Result

Expression profiles of genes reveal the grifolin potential antitumor effect in GC

Several previous researches have shown that grifolin could inhibit the growth and induced significant apoptosis of many types of cancer cell lines via regulating the expression of related genes [7, 12]. To initially determine whether it also has a potential antitumor effect on GC cells, we assessed the expression levels of related genes in two human GC cell lines, BGC823 and SGC7901, treated with different concentrations of grifolin using qRT-PCR. As presented in **Figure 1A**, the expression levels of MAPK signaling pathways related genes, MEK1 were remarkably decreased both in 10 µM and 50 µM grifolin treatment group, but a significant decrease of the MEKK3 and MEK5 expression were only observed at the high dose (50 µM) (**Figure 1B**, **1C**). Many researches have reported that the grifolin anti-cancer effect is closely relevant to the activation of MAPK pathways. Therefore, this finding implied that grifolin might influence the GC metastasis via inhibiting the MAPK pathway. Additionally, cyclin-dependent kinase 4 inhibitor2D (CDKN2D), a cell-cycle regulator, was significantly upregulated, which suggests that grifolin might regulate the cell-cycle by changing the expression of CDKN2D (**Figure 1D**).

Grifolin inhibited the activation of ERK1/2 and ERK5

It has been reported that ERK1/2 and the ERK5 pathways plays a crucial role in cancer
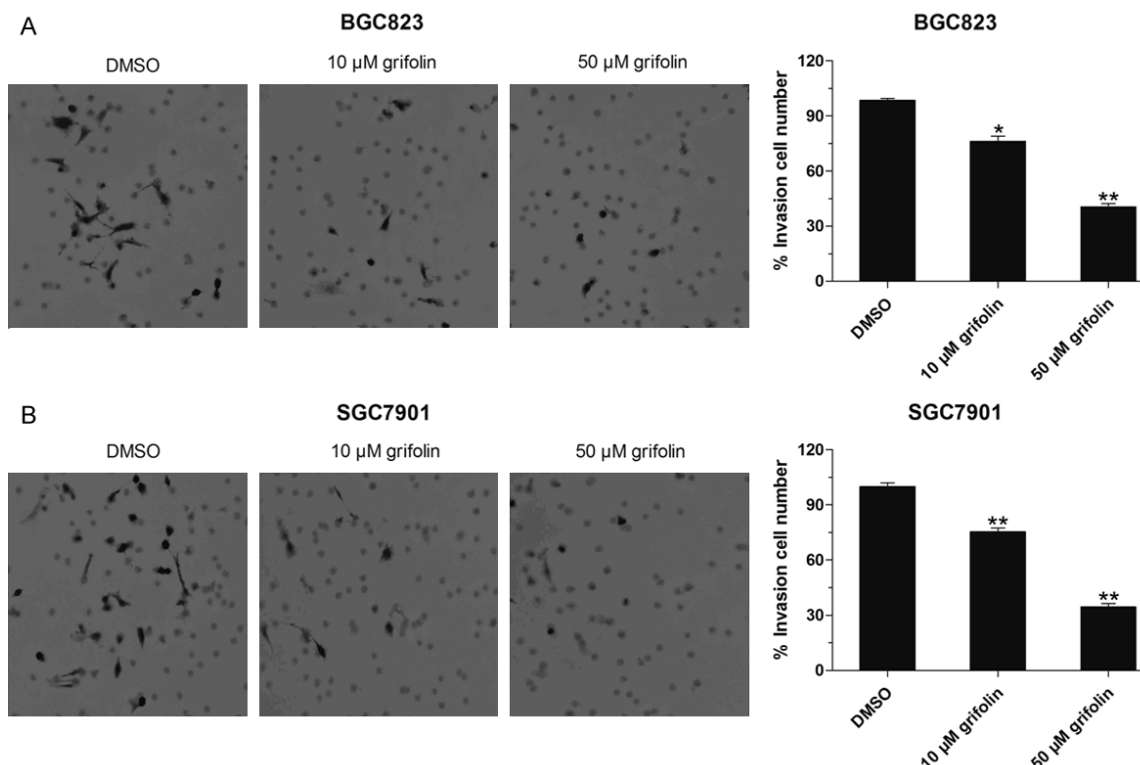
Figure 3. Effects of grifolin on cell invasion. The invasiveness of GC cells treated with grifolin was measured by transwell assay. In these experiments above, the cells treated with DMSO were adopted as negative control. Data are at least three independent experiments and are shown as mean ± SD (*$P<0.05$, **$P<0.01$ as compared with control group).

biology, which could be activated by MEK1 and MEK5, respectively [7]. And MEK5 is specific for ERK5 and is directly activated by MEKK3 [7, 13]. Therefore, to further determine if the inhibition of MEK1 and MEKK3, MEK5 was associated with the activation of ERK1/2 and MEK5, we analyzed the phosphorylation status of ERK1/2 and ERK5. The western blot assays showed that p-ERK1/2 was remarkably downregulated both in the low dose and the high dose of grifolin treatment groups (10 µM and 50 µM), while a significant decrease in ERK5 activity was found only at the high dose of grifolin (50 µM) (**Figure 2**).

Grifolin suppresses cell invasion

Constitutively active ERK1/2 signaling has been shown to lead to increased tumor invasion [14-18], thus we further investigated the potential anti-metastatic activity of grifolin in GC cells by transwell assay. As shown in **Figure 3**, the invasiveness of BGC823 and SGC7901 treated with grifolin were suppressed

compared with negative control cells. It means that grifolin could inhibit the invasion of GC cells via ERK1/2 pathway.

Grifolin suppresses cell proliferation and cell cycle

Previous researches have shown that grifolin could suppress cell proliferation and induce cell-cycle arrest in G1 phase in nasopharyngeal carcinoma. And our preliminary experiment also showed that CDKN2D, an important cell-cycle regulator, was remarkably upregulated. Therefore, to further investigated its effect in the growth of GC cells, BGC823 and SGC7901 were treated with grifolin and then analyzed for cell viability by MTT assay, and cell-cycle alteration by flow cytometry. As expected, the MTT assays revealed that grifolin resulted in a significant decrease of cell viability at 24 and 48, and 72 h, respectively (**Figure 4A**, **4B**), and the cell cycle assays also indicated that grifolin caused an accumulation of cells in G1. Both in BGC823 and SGC7901 cells, the percentage of
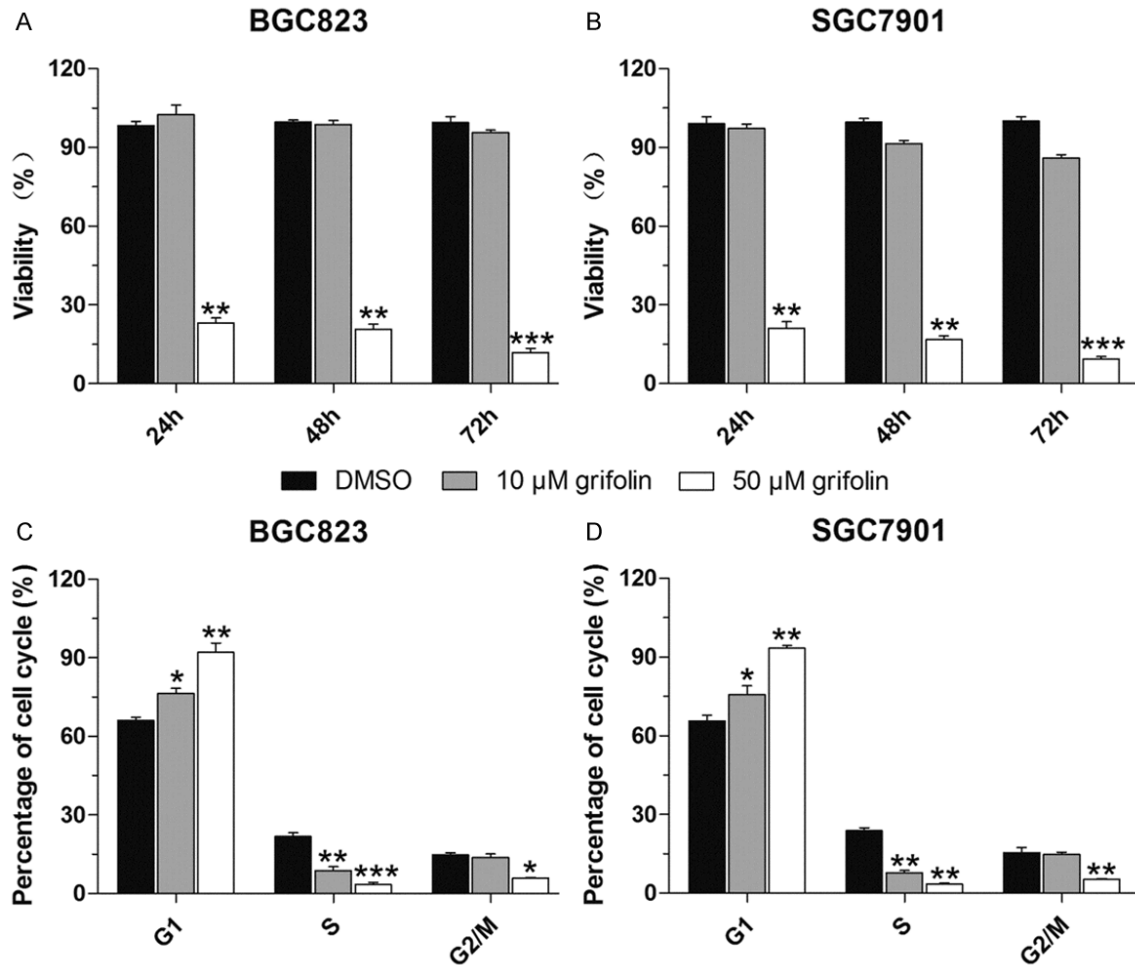
Figure 4. Effects of grifolin on cell proliferation and cell cycle. A, B. Cell viability was measured using the MTT assay. BGC823 and SGC7901 cells were treated with grifolin for 24, 48, and 72 h and then were collected for assays. C, D. BGC823 and SGC7901 cells treated with grifolin were collected and stained with PI and the cell cycle distribution was analyzed with flow cytometer. In these experiments above, the cells treated with DMSO were adopted as negative control. Data are at least three independent experiments and are shown as mean ± SD (*P<0.05, **P<0.01 and ***P<0.001 as compared with control group).

G1 cells increased from around 65% in the control to around 92% in populations treated with 50 µM grifolin; consequently, fewer cells progressed to S phase, from around 25% in the untreated group to around 3.5% in the treated group, and the percentage of G2/M cells decreased from around 15% to around 5%. This finding indicated that cell cycle progression was dramatically blocked in G1 when GC cells were treated with grifolin (**Figure 4C**, **4D**).

Grifolin induces cell apoptosis in GC cells

In addition, we also investigated its effect on cell apoptosis. BGC823 and SGC7901 cells were treated with 10 µM and 50 µM grifolin for

6 h, 12 h and 24 h, and then were collected for flow cytometric analysis. As described in **Figure 5A**, the apoptosis rates of both BGC823 and SGC7901 cells were dramatically increased. In the meanwhile, we examined the release of cytochrome C from mitochondria to cytosol, which was reported to be involved in apoptosis process induced by various apoptotic inducer. As shown in **Figure 5B**, treatment with 50 µM grifolin for 24 h induced the translocation of cytochrome c from the mitochondria to cytosol in two GC cell lines. Moreover, we further investigated the effects of grifolin treatment on the activation of caspases-9 and caspase-3, which play crucial roles in apoptotic pathway. We used caspase fluoresce assays to evaluate the acti-
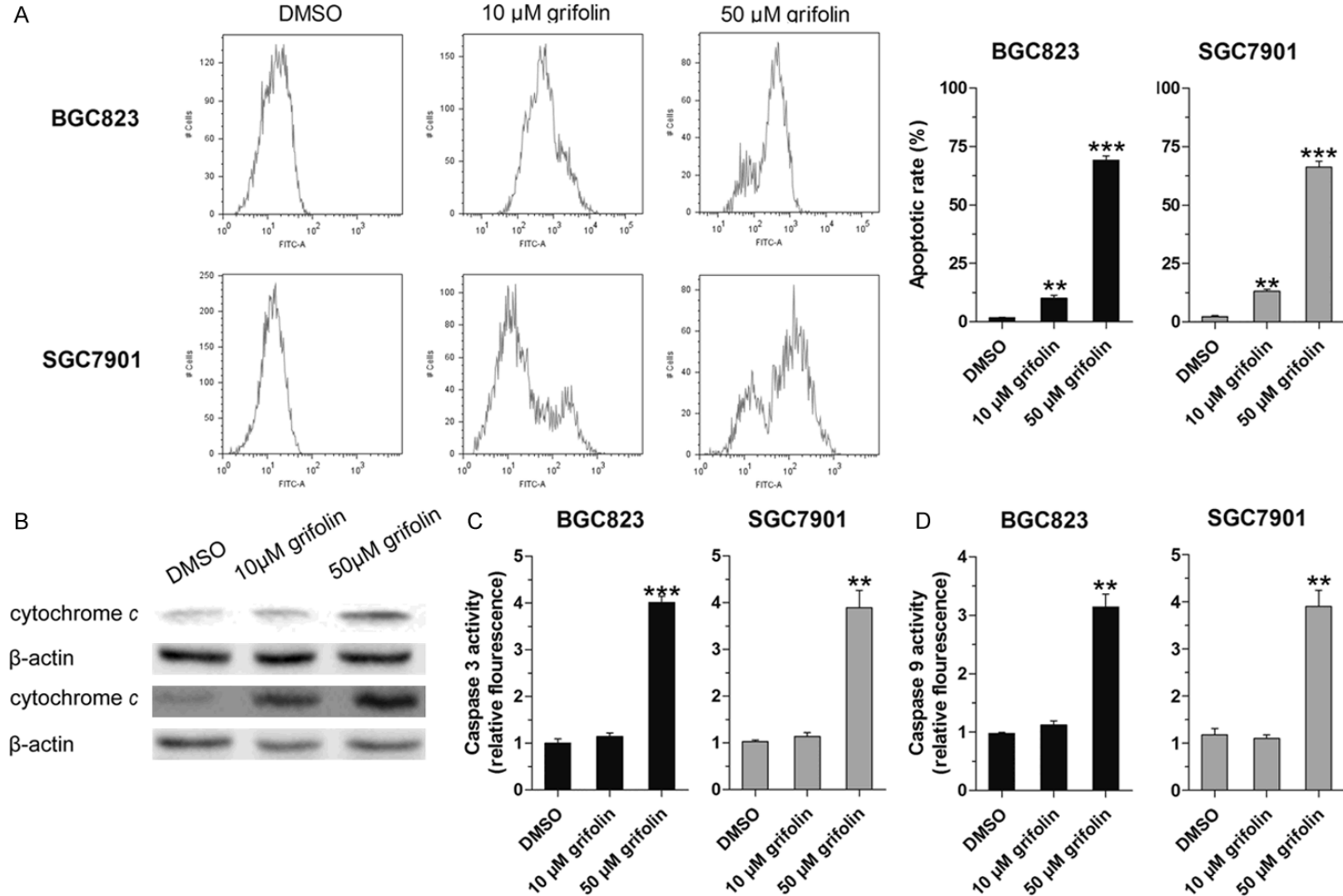
Figure 5. Effects of grifolin on cell apoptosis. (A) BGC823 and SGC7901 cells treated with grifolin for 48 h and the apoptosis rate was examined using flow cytometric analysis. (B) The release of cytochrome c was examined using western blot assay. β-actin was used as a reference. (C, D) The activation of caspase 3 (C) and caspase 9 (D) were determined using caspase-3/9 fluorescent assay. The results show the means ± SD from at least 3 separate experiments (*P<0.05, **P<0.01, ***P<0.001 as compared with control group).

vation of caspase-9 and caspase-3 (**Figure 5C**, **5D**). The result also consist with the effect of grifolin on GC cells, that the activation of both caspase-9 and caspase-3 were obviously increased.

Discussion

Gastric cancer, the most common primary malignancy [19], is still a huge challenge to public health. Current treatments include surgical resection, embolization, ablation, and chemotherapy, which are limited by toxicity, side effects and so on. Therefore, there is a growing research studies aiming to find more effective antitumor drugs, which may prevent carcinogenesis, curtail its progression, or even cure the disease.

Researches increasingly show that natural dietary constituents would strongly influence the potential for disease outcome [20, 21]. Medicinal mushrooms have a long history of use in traditional oriental health care and therapy [22]. Grifolin attract more attention for its antitumor effect in some types of cancer cells, including nasopharyngeal carcinoma [7] and osteosarcoma [8]. In this study, our results provide strong evidence that grifolin could be able to inhibit the growth of GC cells in vitro via inhibiting cell proliferation, inducing cell-cycle arrest in G1 phase, suppressing cell invasion and invasion, and promoting cell apoptosis.

The MAPK pathways serve to coordinate key cellular processes. The ERK1/2 pathway and the BMK1/ERK5 pathway play key roles in the regulation of multiple biological activities, including cell proliferation, differentiation, cell-cycle transition, and survival. ERK1/2 can be activated by MAPK/ERK kinase 1/2 (MEK1/2) [23], while ERK5 (BMK1), a recently identified member of the mammalian MAPK family, is not activated by MEK1 or MEK2, but by MEK5 [24], which is directly activated by MEKK3 [13]. The ERK1/2 pathway can regulate the expression of cyclin D1, which is responsible for the G1/S transition [25]. Inhibition of the ERK1/2 pathway blocks the proliferation of many cell types in G1 [26-28]. Similarly, ERK5 is required for the G1-to-S cell-cycle transition, and decreased ERK5 expression will inhibit the proliferation arrested the cell cycle in G1 of epithelial cells [29]. At present, the bulk of the evidence suggests that constitutive activation of the ERK1/2 pathway contributes to tumorigenesis, or cancer growth, and increases the cell death threshold [30]. Recent reports have demonstrated that grifolin could suppress cell proliferation and cell-cycle mainly by inhibits the phosphorylation and kinase activity of ERK1/2, but not ERK5 in the human nasopharyngeal carcinoma cell line CNE1 [7]. Our study also consisted with this concept, that the phosphorylation of ERK1/2 was down-regulated at doses, while ERK5 activity was inhibited only at the highest dose (50 μM). This result showed that the ERK5 pathway was less sensitive to grifolin than the ERK1/2 pathway, which was consistent with previous research in nasopharyngeal carcinoma. Accordingly, we proposed that grifolin suppressed cell proliferation and regulated cell-cycle via ERK1/2 pathway in GC cells.

Mitochondria play an important role in regulating many cellular functions. During apoptosis, the permeability of mitochondrial membrane increased, leading to the release of cytochrome c to cytosol and the released cytochrome c binds to Apaf-1, and then this compound activates caspase-9 and caspase-3. In this study, the release of cytochrome c, activation of caspase-9 and caspase-3, were observed after grifolin treatment. These data indicated that release of cytochrome c mediated caspases activation is involved in the apoptotic effects of grifolin in GC cells. Our results are consistent with previous report which showed that grifolin induced cytochrome c and caspases dependent apoptosis in other several cancer cell lines [7].

Collectively, all these results suggested that grifolin could effectively inhibit the cell proliferation, apoptosis and invasion in gastric cancer cells. This is the first report to demonstrate its potential anti-cancer effect in GC cells, and the results may provide a theoretical support for the application of grifolin in clinical treatment of gastric cancer.

Disclosure of conflict of interest

None.

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