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Genistein Induced Apoptosis of Gastric Cancer Cell through Bcl-2 and Caspase-3 Regulation

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Abstract

Aim: To investigate the effects of genistein on cell apoptosis in the human gastric cancer cell BGC-823 and the potential regulation pathway involved in its apoptotic effect.

Methods: Effects of genistein on the proliferation, cell cycle progression and apoptosis of gastric cancer cells were examined in vitro. Western blotting were applied to analyze the proteins associated with the biological effects of genistein.

Results: Gastric cancer cell growth was attenuated by genistein treatment in dose- and time-dependent manner. Cell cycle progression analysis showed that apoptosis was enhanced in genistein treated cells and cell cycle arrest at G2/M phase. Increased apoptosis was accompanied by increased level of caspase-3 protein and decreased level of Bcl-2 protein.

Conclusions: Genistein induce the apoptosis of BGC-823 in dose- and time-dependent manner. This apoptosis may be mediated by down-regulating the apoptosis-associated Bcl-2 gene and up-regulating the expression of apoptosis-associated caspase-3 gene. As genistein was effective in vitro in promotion of apoptosis of gastric cancer cell by caspase-3 and Bcl-2 related apoptotic pathway, it might be a potential therapeutic agent against gastric cancer.

Keywords: Genistein; Human gastric cancer; Apoptosis; Caspase-3; Bcl-2

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Introduction

Gastric cancer, a daunting global problem, which accounts for 8% of the total number of cases of cancer and 10% of annual deaths from cancer, is the fourth leading cause of cancer-related death worldwide [1,2]. Despite substantial and accelerated research in the area of gastric cancer, it still remains the third most common cancer and the second most common cause of cancer mortality in Asia [3,4]. Until now, surgical resection has remained the frontline treatment for patients with early stage gastric cancer. Nevertheless, the majority of such patients have poor prognosis due to high rates of tumor recurrence as well as Lymph Node (LN) and systemic metastases. Therefore, there is a need to develop novel therapeutic approaches to improve the outcomes of patients with gastric cancer.

For centuries, nature has proven as a rich source of lead compounds for treatment of various ailments [5]. Genistein, a natural isoflavone extracted from soybean products, exerts varieties of biological functions including inhibiting microsomal lipid peroxidation, angiogenesis and inducing differentiation of numerous cell types [6-9]. It was first discovered that genistein was a potent inhibitor of the tyrosine-specific protein kinase activity of the epidermal growth factor receptor in 1987 [6]. Its structure is similar to that of human 17-β-estradiol causing estrogenic and/or anti-estrogenic effects [10]. Since then, numerous researchers have studied the possible use of genistein as a cancer chemopreventive agent based on the key role of protein tyrosine kinase inhibitors in cancer cell growth and apoptosis [11,12]. Recently in vitro studies conducted in different cancer cell lines confirmed that by affecting various cellular targets, genistein treatment repressed cancer cell proliferation, induced apoptosis and led to cell cycle arrest [13-16]. A survey of recent related literature [17] reveals that genistein might be related to induce the apoptosis of gastric cancer, but the precise mechanism of anti-tumor activity remains unknown.

Apoptosis, also described as programmed cell death, is a highly programmed cell death process. Selectively inducing apoptosis in cancer cells has been increasingly recognized as a promising therapeutic approach for many cancers. Two principal apoptosis signal transduction pathways, which were described as the intrinsic pathway (also known as the mitochondrial pathway) and extrinsic pathway (also known as the death receptor pathway), have been delineated that constitute the basic machinery for triggering apoptosis in mammalian cells [18]. Apoptosis is controlled by the Bcl-2 family of proteins and by Caspases, a family of cysteine proteases [19]. Apoptosis induced by these molecules can prevent carcinogenesis by eliminating damaged cells or inhibiting abnormal cell development. Therefore, the induction of cell cycle arrest and programmed cell death play crucial roles in the anticancer properties of many anticancer agents. Based on the researches above, we hypothesized that genistein might induce the apoptosis of the human gastric cancer cell BGC-823 through Bcl-2 and Caspase-3 regulation.

In the present study, we evaluated the effects of genistein on cell apoptosis in the human gastric cancer cell BGC-823. We also assessed the potential regulation pathway involved in its apoptotic effect.

Materials and Methods

Cell lines and reagents

BGC-823, the human gastric cancer cell line, was provided by Cell Culture Stock Center of Central South University. BGC-823 cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY, USA) containing 15% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA), penicillin 100 U/ml, stretomycin 100 μg/ml in an atmosphere of 5% CO_2 and 95% humidified air at 37°C.

Genistein (>99% pure), was purchased from Senfu Biotechnology Co. (Xi'an, China). Genistein was first resolved in DMSO (Fisher Chemicals, Fair Lawn, NJ) to prepare 160 mmol/L store solution and then serially diluted to serials of concentration prior to experiments.

Measurement of cell viability

MTT assay was used to evaluate the cell viability according to the manufacturer's instructions. Briefly, 1×10^4 cells per well were plated into 96-well plates and incubated for 3 h. The cells were subsequently exposed to different concentrations of genistein (10, 20, 40, 60, 80 and 160 μmol/L) for 48 h. Each concentration was performed in triplicate. MTT reagent (Promega, Madison, WI) was added. After incubation for 4 h at 37°c, the absorbance, which is directly proportional to the number of viable cells in cultures, was measured at 570 nm by an automatic microplate reader (Epoch; Bio-Tek Instruments, Winooski, VT). The cell viability was expressed as a percentage value of control cells cultured with medium alone. The test was tripled and inhibition rate was calculated with formula:

Inhibition rate=1-($Treatment_{A570}$-$Blank_{A570}$)/($Control_{A570}$-$Blank_{A570}$) ×100% to produce inhibition curve and read out IC50 of genistein.

Flow cytometry for detection of cell cycle

Cells were plated in 35 mm dishes at concentrations determined to yield 60–70% confluence within 48 h and then treated with genistein in indicated concentrations (10, 20, 40, 60, 80 and 160 μmol/L) for 48 h. Both the adherent and floating cells were harvested, and the cells were resuspended in PBS, fixed with 70% ethanol at -20 overnight. The cells were first incubated with RNase A (20 U/mL, Sigma Co. St. Louis, MO) at 37 for 30 min and then labeled with propidium iodide (PI, Becton-Dickinson, San Diego, CA) and incubated at room temperature in the dark for 30 min. DNA content was then analyzed using a FACS can instrument equipped with FACS tation running cell Quest software (Becton-Dickinson). At least 10,000 cells were collected for each measurement in a triplicate experiment.

Flow cytometry for detection of apoptosis

BGC-823 cells were seeded in 24-well plates. After 24 h, the medium was changed and chemicals were added with indicated concentrations. After treatment for 48 h, all the adherent cells were collected with 0.05% trypsin, including the floating cells in the medium. Annexin-V (FITC) and propidium iodide (PI, Becton-Dickinson, San Diego, CA) were used for staining according to the manufacturer's instructions. Vehicle-treated cells were set for control. The double-stained cells were subsequently analyzed by a FACS Can to flow cytometer (Becton-Dickinson, Mountain View, CA). All experiments were processed independently three times. At least 10,000 cells were counted each time.

Caspase activity measurement

Caspase activities were measured by colorimetric assay (BioVision, Inc., Mountain View, California, US), according to the manufacture's recommendations. Briefly, BGC-823 cells, treated with genistein (10, 20, 40, 60, 80 mM) for 24 h, were harvested and incubated in ice-cold cell lysis buffer for 30 min on ice. The supernatants were collected and protein concentrations were determined using BCA Protein Assay Reagent (Pierce, Rockford, IL, US). Equivalent amount of proteins for each sample was incubated with interested caspase-9. After incubation at 37°c for 4 h, the protease activity was determined at 405 nm with microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, US).

Western blotting analysis

Western blot analysis to assess Bcl-2, Caspase-3 and Caspase-9 expression was performed as previously described [20]. Briefly, A total of 10^6 cells were sedimented and lysed for 15 minutes in ice-cold lyses buffer (0.1% SDS, 1% NP-40, 50mM HEPES, pH 7.4, 2mM EDTA, 100mMNaCl, 5mM sodium orthovanadate, 40 μM p-nitrophenyl phosphate, and 1% protease inhibitor mixture set I; Calbiochem). After removing the cell debris by centrifugation at 16200 g for 15 minutes, equal amounts of proteins were separated on a 12% SDS-PAGE, blotted onto a nitrocellulose membrane (GE Healthcare, Little Chalfont, United Kingdom) and blocked with 5% nonfat dry milk in PBS/Tween (0.05% Tween-20 in PBS). The following antibodies were used: Bcl-2 antibody (Santa Cruz, CA, USA), Caspase-3 antibody (Santa Cruz, CA, USA), Casepase-9 antibody (Santa Cruz, CA, USA). The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000). The immunoreactions were developed by Enhanced Chemiluminescence (ECL). β-actin expression was used as the loading control. The intensity of the specific immunoreactive bands was detected by Super Signal West Pico Substrate (Thermo-pierce, Rockford, IL) and quantified by densitometry using ImageJ 1.45 software. Data were supplied as a ratio of the β-actin for analyzing and plotting.

Statistical analysis

All the experiments were carried out in triplicate and data are presented as mean ± SD. Statistical analysis was performed using SPSS software (Release 17.0, SPSS Inc.). The difference between two groups was analyzed by the Student's *t*-test. A value of p <0.05 was considered as statistical significance.

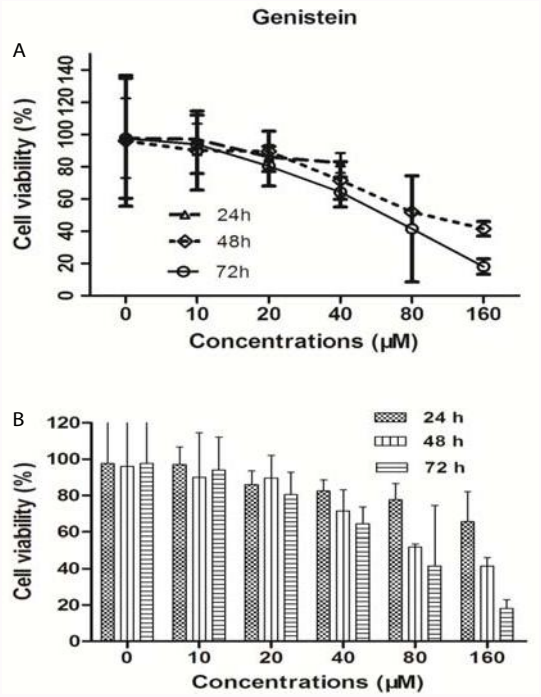

Figure 1: Effects of genistein on BGC-823 gastric cancer cell proliferation.

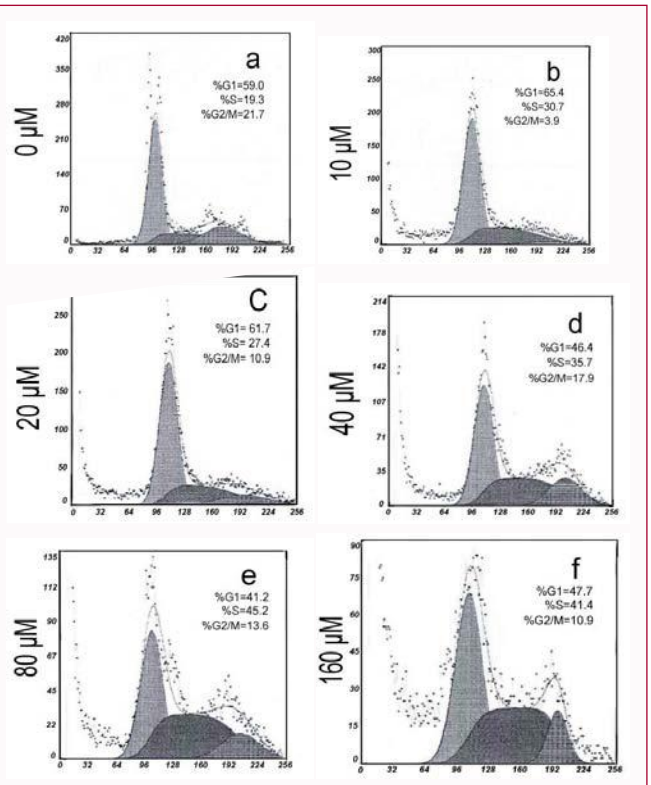

Figure 2: Effect of genistein on cell cycle arrest in BGC-823 cells. Flowcytometry analysis indicated a G2/M phase arrest in the BGC-823 cells after genistein (10 μM) treatment for 48 h. (A)0μM; (B)10μM; (C)20μM; (D)40μM; (E)80μM; (F)160μM.

Results

Genistein inhibits BGC-823 cell proliferation

To examine the growth inhibitory effect of genistein, BGC-823 cells were treated with different concentrations (10, 20, 40, 60, 80 and 160 μM) of genistein for different periods of time (24, 48 and 72 h). MTT assay for cell viability was performed. As showed in (Figure 1), the cell viability was dose-dependently reduced in gastric cancer cell BGC-823 after genistein treatment at indicated concentrations in a time-dependent manner. The results of MTT assay showed that the significant suppression of BGC-823 was induced by genistein treatment with concentration of 40 μM above after 48 h. The genistein respectively inhibited BGC-823 cells with IC50 value of 247.3 μmol/L for 24 h, 84.2 μmol/L for 48 h and 65.3 μmol/L for 72 h.

Genistein promotes apoptosis and induces cell cycle arrest in BGC-823 cells

Since cell viability reduction appeared in the MTT assay, we further detected the possibility of apoptosis occurrence in genistein-treated BGC-823 cells using flow cytometry. BGC-823 cells were treated with different concentrations (10, 20, 40, 60, 80 and 160 μM) of genistein for 48 h. Apoptotic cells were determined by flow cytometry using Annexin V/Propidium Iodide (PI) double labeling. The Annexin V stain assay, an event typically associated with apoptosis, was used to evaluate phosphatidylserine externalization from the inner to the outer lipid layer of the plasma membrane [20]. As is described in (Table 1), the ratio of the apoptotic cells (Annexin V-FITC-positive) in BGC-823 was significantly increased in a dose-dependent manner when treated with genistein. We also examined the effect of genstein on the cell cycle progression of BGC-823. As is showed in (Figure 2), cell cycle analysis showed that exposure to genistein (10 μM) for 48 h induced a significant proportion of cells accumulated at the G2/M phase.

Table 1: Cell cycle and apoptotic rate of genistein treated BGC-823 cells.

Group	Cell cycle (%)			Apoptotic rate (%)
	G0/G1	S	G2/M	
0 μmol/L	59.0	19.3	21.7	3.36
10 μmol/L	65.4	30.7	3.91	13.6
20 μmol/L	61.7	27.4	10.9	15.4
40 μmol/L	46.4	35.7	17.9	19.5
80 μmol/L	41.2	45.2	13.6	26.7
160 μmol/L	47.7	41.4	10.9	40.6

*The ratio of the apoptotic cells (Annexin V-FITC-positive) in BGC-823 cells was significantly increased in a dose-dependent manner when treated with genistein.

Inhibition of Bcl-2 protein enhances the apoptosis-inducing activity of genistein

Based on the results above, we further investigated the potential effects of genistein on gastric cancer cell. Since Bcl-2 protein served as the key player in the death receptor pathway of apoptosis [21], we investigated the effects of genistein on this regulatory protein. We observed that genistein decreased Bcl-2 expression in gastric cancer cells in Western blotting assay (Figure 3A), which suggest that genistein promotes gastric cancer cells apoptosis via Bcl-2 protein regulating pathway. As the caspases cascade has been demonstrated to be involved in apoptosis signal transduction and execution [7,14,22], we further examined the expression of apoptotic protein caspase-3 and caspase-9 in this study. It showed that caspase-3 and caspase-9 expression were increased in Western blotting assay by different concentration of genistein possessed (Figure 3B). This suggested that genistein induce the gastric cancer cell apoptosis by activing the caspase-3. From all the results above, caspase-3 might correlate negatively with Bcl-2 protein in the apoptosis of BGC-83 cell.

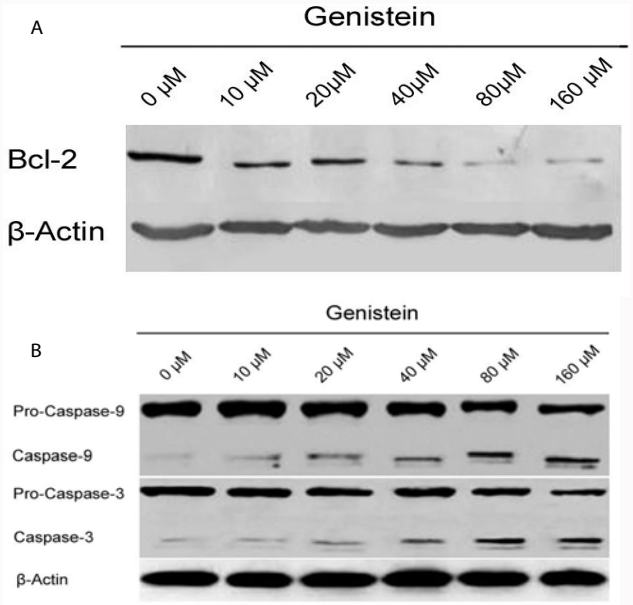

Figure 3: Western blotting analysis of bcl-2, caspases-3 and caspases-9 expression in different concentrated genistein treatment BGC-823 cell lines. A) Western blotting indicated that genistein decreased bcl-2 expression in gastric cancer cells. B) Western blotting indicated that caspase-3 and caspase-9 expression were increased by different concentration of genistein possessed BGC-823 cell lines.

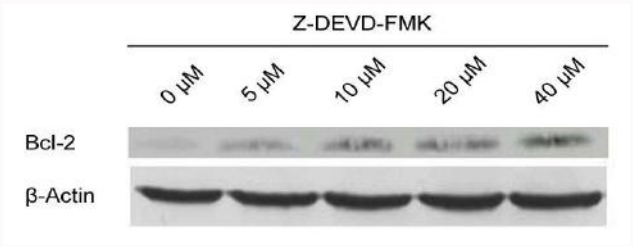

Figure 4: Effect of caspase-3-specific inhibitor, Z-DEVD-FMK, on up-regulation of bcl-2 expression on BGC-823 cells.
Western blotting indicated that bcl-2 expression was significantly increased by Z-DEVD-FMK.

Inhibition of caspase-3 activity upregulate the expression of Bcl-2 protein

Caspase-3 represents one of the key proteases responsible of cleavage of PARP and subsequent apoptosis [23,24]. It has been reported that caspase-3 can inactivate the Bcl-2 during the apoptosis [25,26]. To further evaluate the correlation of caspase-3 and Bcl-2 protein in the apoptosis of gastric cancer cell, we use a general and potent inhibitor of caspase-3, Z-DEVD-FMK. As showed in (Figure 4), Bcl-2 expression was significantly increased by Z-DEVD-FMK, indicating that caspase-3 was involved in the inactivation of Bcl-2 protein during the apoptosis of BGC-823 cell.

Inhibition of Bcl-2 protein activity upregulate the expression of caspase-9

The Bcl-2 protein probably serves to prevent the activation of the caspase subfamily in response to apoptotic stimuli. To further investigate whether the Bcl-2 is involved in the inactivation of caspase-9 in the gastric cancer cell, we use a general inhibitor of Bcl-2, ***HA14-1***. As is shown in (Figure 5), caspase-9 expression was significantly increased by ***HA14-1***, indicating that Bcl-2 protein was involved in the inactivation of caspase-9 in the apoptosis of BGC-823 cell.

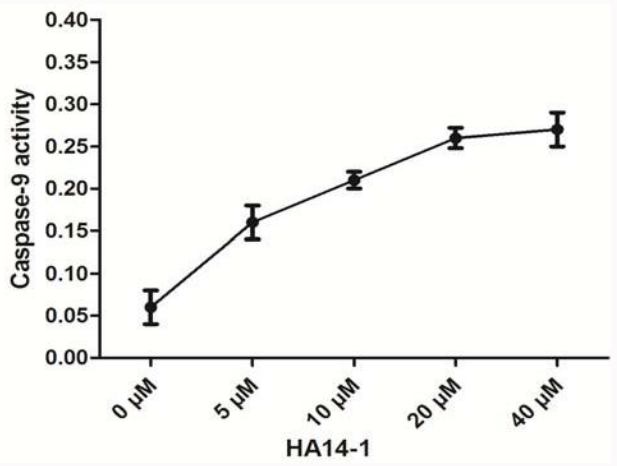

Figure 5: Effect of bcl-2 inhibitor, HA14-1 on up-regulation of caspase-9 expression on BGC-823 cells.
Western blotting indicated that caspase-9 expression was significantly increased by **HA14-1.**

Discussion

Epidemiologic studies indicate that the consumption of soy-containing diets is associated with a lower incidence of many human tumors and isoflavones have been proven to be the major active components in soybased diets [27,28]. Genistein is proved to be one of the most active flavonoids in soybeans. Accumulating evidence suggests that genistein alters a variety of biological processes in estrogen-related malignancies, such as hepatocellular carcinoma, colon carcinoma, breast and prostate cancers [8,9,28,29]. Furthermore, genistein has also been shown to induce a wide spectrum of cancer cell apoptosis [16,30,31]. However, the molecular mechanism of genistein in the prevention of gastric cancer remains unclear. This study presents data showing that genistein, one of the most active flavonoids, attenuated intro proliferation of human gastric cancer BGC-823 cells by inducing the apoptosis and this apoptosis might be mediated by down-regulating the apoptosis-associated Bcl-2 gene and up-regulating the expression of apoptosis-associated caspase-3 gene. These results imply that an adjuvant therapy with genistein may have potential therapeutic benefits in gastric cancer.

Our results suggest that the enhanced apoptosis and suppressed cell cycle progression may be the mechanisms of action of genistein on gastric cancer BGC-823 cell line. These results are consistent with the previous reports on other tumor cell lines [14-16,30]. HB Zhou et al reported that genistein inhibited cell proliferation and induced apoptosis in primary gastric cancer cells and that these events were related to the down-regulating the apoptosis-associated bcl-2 gene and up-regulating the expression of apoptosis-associated Bax genes [17]. As a radical regulator of apoptosis, Bcl-2, which is known to regulate apoptotic pathways and protect against cell death, is negatively related to malignant level and prognosis of gastric cancer patients [32]. It is encouraging that, consistent with previous studies, we have demonstrated that genistein exerted anticancer effects partially in a Bcl-2 dependent manner. Furthermore, genistein arrested BGC-823 cells at the G2/M phase in a dose-dependent manner. This was correlated to its effect of triggering the downregulation of Bcl-2. And these results were further substantiated by the accompanied increases

in the expression of the caspases proteins.

Caspases(Cysteine Aspartate Specific Proteases) are a group of cysteine proteolytic enzymes produced by the cells of living organisms [33]. With regard to the length of the prodomain, caspases are divided into two subfamilies: Those with a long prodomain are pro-inflammatory and apoptosis initiators; and those with a short prodomain are "executioner caspases". During apoptosis, several effector proteases such as caspase-3 mediate the deliberate disassembly of the cell into apoptotic bodies. These downstream caspases are activated through proteolytic cleavage by either caspase-8 or caspase-9, two upstream initiator caspases. A survey of researches favour the concept that the Bcl-2 family proteins function upstream of caspase activation and prevents apoptosis by suppressing caspase activity. It is now widely accepted that Bcl-2 functions to prevent the activation of caspases implicated in the execution phase of apoptosis (caspase-3-like enzymes) rather than being attacked and/or inactivated by these proteases [22,25,34,35]. Several lines of evidences show that Bcl-2 family proteins are involved in controlling the release of cytochrome c from the mitochondria to activate caspase-9 [34,36,37]. Others reported that caspase-3, as a major apoptotic executor, can eliminate Bcl-2 protein *in vitro* [38,39]. However, the biochemical connection between the Bcl-2 and caspases has been elusive, especially in gastric cancer.

Indeed, our analysis on between caspase-3 inactivation and Bcl-2 expression following applying Z-DEVD-FMK in gastric cancer cell showed that Bcl-2 expression was significantly increased by Z-DEVD-FMK, indicating that caspase-3 was involved in the inactivation of Bcl-2 protein during the apoptosis of BGC-823 cell. Furthermore from the results of the present study, caspase-9 expression was significantly increased by HA14-1, indicating that Bcl-2 protein was involved in the inactivation of caspase-9 in the apoptosis of BGC-823 cell. All the results above imply that Bcl-2 might not only inactivate caspcase-3 but also being inactivated by caspase-9 in the genistein-induced apoptosis in gastric cancer BGC-823 cells.

Conclusion

Genistein induce the apoptosis of BGC-823 in dose- and time-dependent manner. This apoptosis may be mediated by down-regulating the apoptosis-associated Bcl-2 gene and up-regulating the expression of apoptosis-associated caspase-3 gene. As genistein was effective *in vitro* in promotion of apoptosis of gastric cancer cell by caspase-3 and Bcl-2 related apoptotic pathway, it might be a potential therapeutic agent against gastric cancer.

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