Grifola frondosa polysaccharides induce breast cancer cell apoptosis via the mitochondrial-dependent apoptotic pathway

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Abstract. *Grifola frondosa*, a type of food and medical fungus, has been shown to exhibit various pharmacological activities, including anticancer effects. As the most typical cancer diagnosed among female patients, breast cancer remains a huge concern threatening human health globally. In the present study, the anti-breast cancer effects of *Grifola frondosa* polysaccharides (GFPs) and the underlying mechanisms were investigated in MCF-7 and MDA-MB-231 cells, as well as in nude mice bearing MCF-7 tumor xenografts. GFPs exerted cytotoxic effects on the cells, as indicated by a decrease in cell viability, and an increase in the apoptototic rate, lactate dehydrogenase release and reactive oxygen species accumulation, inducing mitochondrial dysfunction. The increased expression of Bax, cleaved caspase-3 and caspase-8, and the reduced levels of B-cell lymphoma 2 (Bcl-2) and Bcl-extra large (Bcl-xL) were observed in the cells incubated with GFPs and in the tumor tissues of the mice treated with GFPs. Moreover, the GFPs significantly suppressed the phosphorylation of AKT/glycogen synthase kinase-3β and extracellular signal-regulated kinases in a time-dependent manner. Finally, the inhibition of MCF-7 tumor xenograft growth further confirmed the anti-breast cancer effects of GFPs. All these findings revealed that GFPs induced human breast cancer cell apoptosis via the mitochondrial-dependent apoptotic pathway, and provide experimental evidence to support the use of *Grifola frondosa* as a potential treatment for breast cancer.

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Introduction

According to the statistics, almost a quarter of female patients suffering from cancer are diagnosed with breast cancer (1). As the most typical type of cancer affecting women, even in an era with advanced medical applications, breast cancer remains a serious concernt and a threat to human health, causing significant morbidity and mortality (2). Several subtypes of breast cancer, each requiring different therapeutic regimens, limit the treatment options. The standard treatment for breast cancer is chemotherapy and radiotherapy; however, treatment outcomes are, in the most part, discouraging for patients (3). In this scenario, it is imperative to explore different alternative therapies or medicines with low toxicicity for breast cancer treatment.

Due to the significant cytotoxic activities and less adverse effects, herbal medicines have gradually become good candidates for cancer therapy (4). It has been proven that *Cordyceps militaris*, a folk tonic in Asia, displays pro-apoptotic properties in cells and tumor xenografts in C57BL/6 mice via mitochondrial-related pathways (5,6). As a type of food and medical fungus, *Grifola frondosa* has been studied for years, and amino acids, polysaccharides and amounts of trace elements have been found in its fruitbody. Since the first study on the anti-tumor effects of *Grifola frondosa* polysaccharide (GFP) in 1984, the structure and function of its polysaccharides have been gradually analyzed (7). Pharmacological analyses and clinical trials have demonstrated that the polysaccharide-enriched extract of *Grifola frondosa* exhibits various activities, including anti-tumor, immunomodulatory, and blood glucose and lipid regulating effects (8-10). A chemically sulfated polysaccharide purified from *Grifola frondosa* has also been shown to induce HepG2 cell apoptosis via the Notch 1-NF-κB pathway (11). However, few studies to date have reported the pro-apoptotic activities of GFP on breast cancer cells and the underlying mechanisms.

Apoptosis, an energy-dependent process, is regulated by various signals (12). During this process, cell shrinkage, chromatin condensation and DNA damage are observed (13). Mitochondrial apoptosis occurs gradually along with the depolarization of mitochondrial transmembrane potential (MMP; $\Delta\Psi m$), the abnormal expressions of B-cell lymphoma 2 (Bcl-2) family members, cytochrome *c* (Cyto *c*)

over-release and caspase-3 activation (14,15). The initiator caspase (caspase-8) controls the proteolytic maturation of caspase-3 (16). The accumulation of intracellular reactive oxygen species (ROS) is capable of inducing apoptosis by interacting with proteins related to mitochondrial dysfunction. On the other hand, the activation of AKT and extracellular signal-regulated kinases (ERKs) contributes to cell proliferation and apoptosis (17,18).

This study aimed to investigate the anti-breast cancer effects of GFP in *in vitro* and *in vivo* models. We found that in MCF-7 and MDA-MB-231 cells, GFP induced apoptotic cell death related to mitochondrial function. GFP also significantly suppressed the growth of MCF-7 tumor xenografts in nude mice. Our data support the possible use of *Grifola frondosa* as a therapeutic agent for breast cancer therapy.

Materials and methods

Preparation of polysaccharides separated from Grifola frondosa. *Grifola frondosa* powder (100 g) was extracted twice with 10-fold double-distilled water (DD water) at 90˚C for 3 h. The protein existing in the extract was removed using Sevag reagent [v (n-butanol):v (chloroform) = 1:4, 50 ml]. Polysaccharides were collected via the alcohol precipitation method with 4-fold ethanol. The content of the total polysaccharides separated from *Grifola frondosa* was 65.2±1.05 mg/g.

Cell culture. The cell lines, MDA-MB-231 (human breast epithelial cell line; ATCC no. HTB-26) and MCF-7 (human breast carcinoma cell line; ATCC no. HTB-22), were maintained in Dulbecco's modified Eagle's medium (DMEM) medium, supplemented with a 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 g/ml streptomycin under a humidified atmosphere containing 5%/95% of CO_2/air at 37˚C. The cultured medium was refreshed every 3 days. Cell culture reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

MTT cell survival assay. The cells (5,000 cells/100 μl) were seeded into 96-well plates and incubated with GFPs at concentrations of 25, 50, 100, 200 and 400 μg/ml for 24 or 48 h. Subsequently, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) dissolved in phosphate-buffered saline (PBS) were added to each well. Following a 4-h incubation at 37˚C in the dark, the supernatant was aspirated, and then 100 μl DMSO were added. The absorbance was measured at a wavelength of 540 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Values were expressed as a percentage of those from the corresponding controls.

Analysis of lactate dehydrogenase (LDH) concentration and caspase-3 activation. The cells ($5x10^4$) were seeded into 6-well plates and treated with 50 and 200 μg/ml GFPs for 24 h. The LDH concentration in the culture medium was detected using a LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

The treated cells were collected and lysed with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA), and the protein concentration was examined using Bio-Rad protein assays. A caspase-3 colorimetric detection kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) was applied to detect caspase-3 activation. Values were expressed as a percentage of those from the corresponding controls.

Flow cytometric analysis of cell apoptosis. The cells were seeded into 6-well plates at $5x10^4$/well and treated with 50 and 200 μg/ml GFPs for 12 h. The treated cells were harvested and washed with PBS 3 times, and then suspended in binding buffer containing with 5 μl Annexin V-FITC (20 μg/ml) and 5 μl propidium iodide (PI; 50 μg/ml) (BD Biosciences, Franklin Lakes, NJ, USA). Following a 15-min incubation at room temperature in the dark, the apoptotic rate was analyzed using a flow cytometer (FC500; Beckman Coulter, Inc., Brea, CA, USA).

Detection of ROS. Following treatment with GFPs for 12 h at concentrations of 50 and 200 μg/ml, the cells were suspended and incubated with 10 μM dichlorodihydrofluorescein diacetate (DCFH-DA) for 10 min at 37˚C in the dark. After being washed with PBS 3 times, the intracellular ROS levels were determined using a flow cytometer (FC500; Beckman Coulter).

Detection of MMP. The cells were seeded into 6-well plates at $5x10^4$/well and treated with 50 and 200 μg/ml GFPs for 12 h. The cells were further incubated with 2 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Sigma-Aldrich) at 37˚C for 10 min. After being washed with PBS, the changes in fluorescent color were examined using a fluorescence microscope (x20 magnification; CCD camera, TE2000; Nikon, Tokyo, Japan).

MCF-7 tumor xenograft model. Six-week-old male BALB/c nude mice purchased from Weitong Lihua Laboratory Animal Technology Ltd. Co. (Beijing, China) were used in our *in vivo* experiments. The protocol was approved by the Animal Ethics Committee of Jilin University. The mice were housed in groups 3 per cage and maintained on a 12 h light/dark cycle at 23±1˚C with water and food available *ad libitum*.

An amount of 0.1 ml ($1x10^8$ cells/ml) of MCF-7 cells at the mid-log phase was inoculated subcutaneously into the right flank of BALB/c nude mice. When the diameter of the tumor reached to 3-5 mm, the mice were divided into 2 groups (n=3 each) randomly, and orally treated with 0.5 g/kg GFPs or DD water every other day continuously for 2 weeks. During the GFP administration, the body weight and tumor dimension were measured. The equation of length x $(width)^2$ x 0.5 was applied to estimate the tumor volume (mm^3). All the mice were sacrificed via an injection of 200 mg/kg pentobarbital after the final treatment, and tumor tissues were dissected.

Western blot analysis. The MCF-7 or MDA-MB-231 ($2x10^5$ cells) were seeded into 6-well plates and exposed vaqrious concentrations of GFPs for the indicated periods of time. The cells and collected tumor tissues were lysed by RIPA buffer containing 1% protease inhibitor cocktail and 2% phenylmethanesulfonyl fluoride (PMSF) (both from Sigma-Aldrich). The bicinchoninic acid method was applied to detect the protein concentrations. Protein samples (40 μg) were separated on a 12% SDS-PAGE gel,

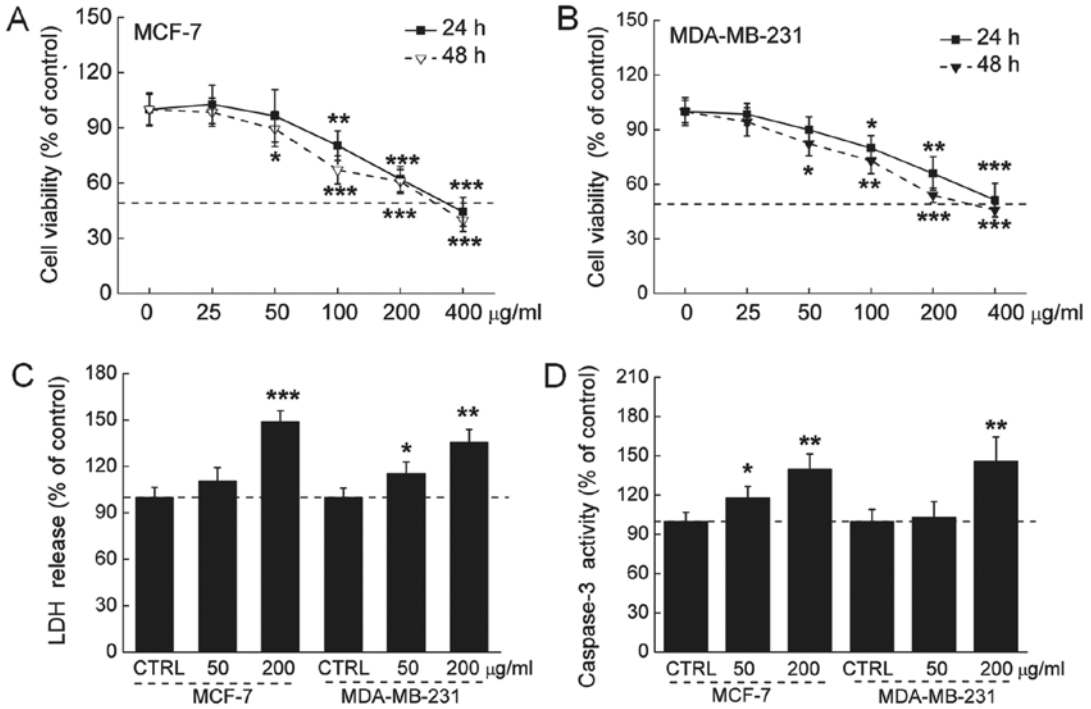

Figure 1. *Grifola frondosa* polysaccharides (GFPs) exhibit intracellular toxicity in MDA-MB-231 and MCF-7 cells. GFPs dose- and time-dependently suppressed cell viability after 24 and 48 h of incubation with the (A) MCF-7 and (B) MDA-MB-231. (C) In breast cancer cells, a incubation with the GFPs for 24 h enhanced LDH release and (D) caspase-3 activation. Data are expressed as the means ± SD (n=6 repeats in each group) and analyzed using a one-way ANOVA. *P<0.05, **P<0.01 and ***P<0.001 vs. controls.

and then electroblotted onto nitrocellulose membranes (0.45 μm; Bio Basic, Inc., Markham, ON, Canada). The membranes were incubated at 4˚C overnight with Bcl-2 (MABC573), Bcl-extra large (Bcl-xL; MAB4625), Bax (AB2915), cleaved caspase-3 (AB3623), cleaved caspase-8 (AB1879), and phosphorylated (p)-AKT (05-1003) (all from Merck Millipore, Darmstadt, Germany), total (t)-AKT (ab126811) and p-glycogen synthase kinase-3β (GSK-3β) (ab75745) (both from Abcam, Cambrige, UK), T-GSK-3β (PK1111) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ABS16) (both from Merck Millipore) at dilution of 1:1,000. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h at room temperature. Band detection was performed using enhanced chemiluminescence (ECL) detection kits (GE Healthcare Life Sciences, Chalfont, UK). The intensity of the bands was quantified using ImageJ software.

Statistical analysis. Data are expressed as the means ± standard deviation (SD) and analyzed by a one-way analysis of variance (ANOVA) followed with Dunn's test using SPSS software (SPSS, Inc., Chicago, IL, USA). The IC_{50} values are calculated using SPSS 16.0 software (IBM Corporation, Armonk, NY, USA). A value P<0.05 was considered to indicate a statistically significant difference.

Results

Intracellular toxic effects of GFPs on breast cancer cells. The 24-h IC_{50} values of the GFPs were 335 and 412 μg/ml, and the 48-h IC_{50} values of the GFPs were 295 and 348 μg/ml in the MCF-7 and MDA-MB-231 cells, respectively (Fig. 1A and B). The release of LDH was increased during cell death. An approximately 47 and 32% LDH over-release was observed in the 200 μg/ml GFP-treated MCF-7 and MDA-MB-231 cells (P<0.01; Fig. 1C). The activation of caspase-3 serves as a marker of cell apoptosis. We found that the GFPs at 200 μg/ml enhanced caspase-3 activation by almost 35 and 43% in the MCF-7 and MDA-MB-231 cells, respectively (P<0.01; Fig. 1D).

In addition, incubation with the GFPs (50 μg/ml) for 12 h led to approximately 22 and 21% of the MCF-7 and MDA-MB-231 cells, respectively to become apoptotic (Fig. 2A). Furthermore, oxidative stress, particularly, the overproduction of intracellular ROS, leads to cellular dysfunction and apoptosis (19). In this study, following incubation with the GFPs for 12 h at 200 μg/ml, a 50 and 26% increment in intracellular ROS levels was noted in the MCF-7 and MDA-MB-231 cells, respectively compared with the controls (Fig. 2B). All these data confirmed that GFPs exerted cytotoxic effects on the MCF-7 and MDA-MB-231 cells.

GFPs cause mitochondrial dysfunction. Mitochondrial function plays a central role during cell apoptosis (20). As indicated by the reduced ratio of red to green fluorescence by JC-1 staining, treatment with the GFPs for 12 h at concentrations of 50 and 200 μg/ml significantly decreased MMP in the MCF-7 and MDA-MB-231 cells, compared with untreated cells (Fig. 3A). Furthermore, the increased expression levels of Bax, cleaved caspase-3 and caspase-8, and the reduced levels of Bcl-2 and Bcl-xL were observed in the MCF-7 and MDA-MB-231 cells following incubation with the GFPs for 24 h GFPs at concentrations of 50 and 200 μg/ml (Fig. 3B).

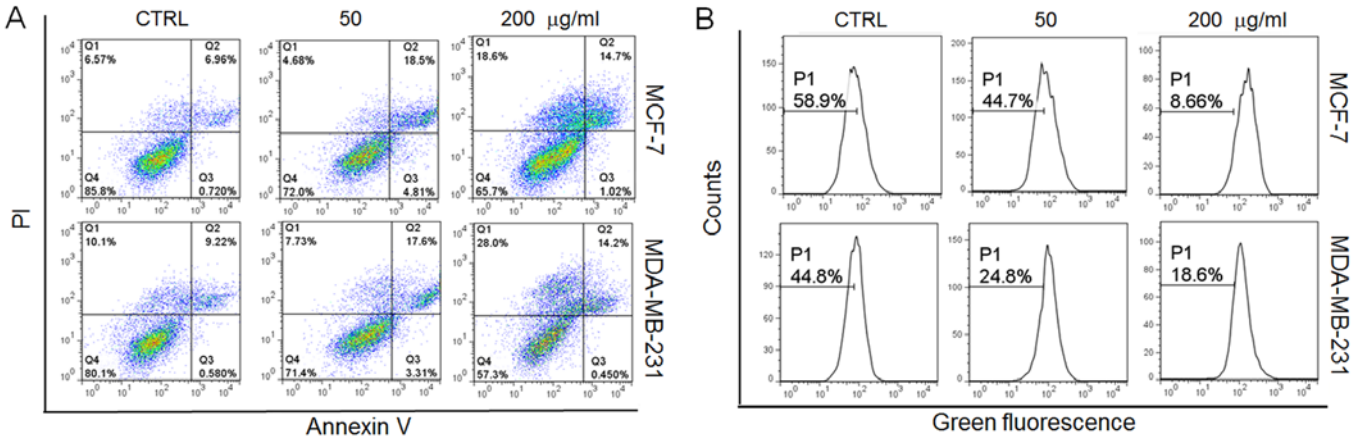

Figure 2. Incubation for 12 h with *Grifola frondosa* polysaccharides (GFPs) at concentrations of 50 and 200 μg/ml strongly enhanced the (A) apoptotic rate and (B) intracellular reactive oxygen species (ROS) levels in MDA-MB-231 and MCF-7 cells. The experiments were repeated 3 times.

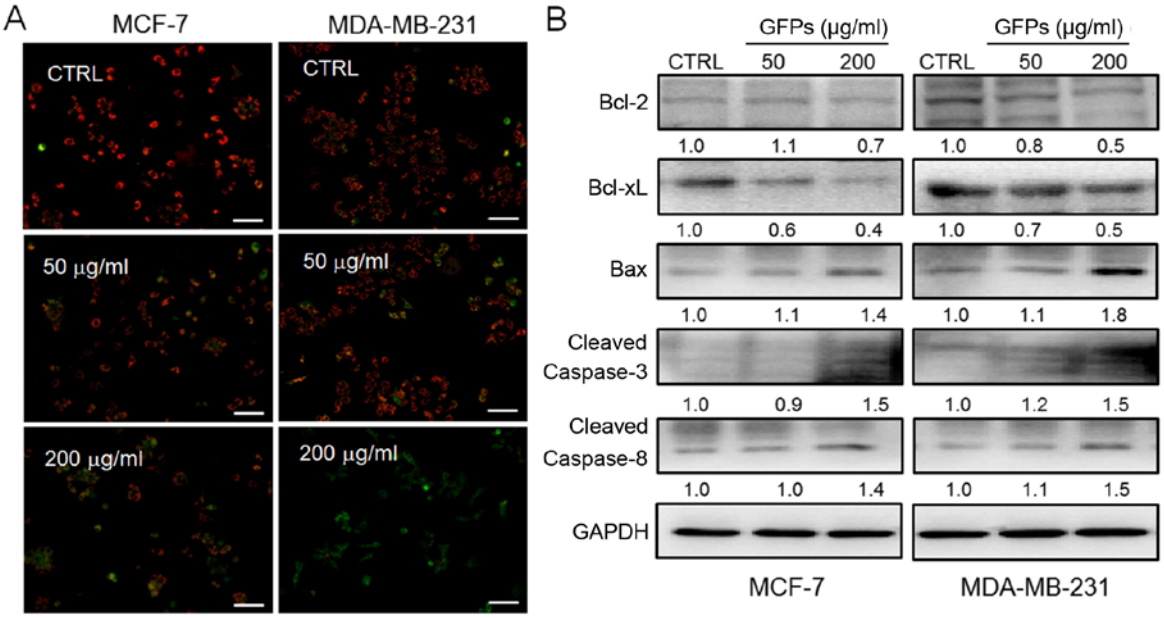

Figure 3. A 12-h incubation with *Grifola frondosa* polysaccharides (GFPs) at concentrations of 50 and 200 μg/ml caused mitochondrial dysfunction in MDA-MB-231 and MCF-7 cells. (A) The dissipation of $\Delta\Psi$m was observed detecting by JC-1 staining (x10 magnification; scale bar, 100 μm). The experiment were repeated 3 times. (B) GFPs dose-dependently reduced the expression of B-cell lymphoma 2 (Bcl-2) and Bcl-extra large (Bcl-xL), and enhanced the levels of Bax, cleaved caspase-3 and caspase-8. The average fold of band intensity compared to related controls was marked respectively (n=6 repeats in each group).

The activation of AKT/GSK-3β and ERK is involved in GFP-mediated cytotoxicity in breast cancer cells. It has been reported that the activation of AKT/GSK-3β and ERK participate in cell proliferation, survival and even apoptosis (21,22). The GFPs time-dependently suppressed the phosphorylation of AKT and GSK-3β from 0.5 to 3 h in the breast cancer cells incubated with 200 μg/ml of GFPs, particularly at 1 and 3 h (Fig. 4). In addition, incubation with 200 μg/ml GFPs significantly inhibited the activation of ERK from 1 and 3 h in the MCF-7 and MDA-MB-231 cells (Fig. 4).

GFPs inhibits the growth of MCF-7 tumor xenografts. GFP administration at 0.5 g/kg significantly suppressed the growth of MCF-7 tumor xenografts from the 8th day to the end of the experiment (P<0.05; Fig. 5A and C). Compared with the controls, GFPs decreased the tumor size by almost 42% on the 14th day (P<0.01; Fig. 5C). The GFPs did not to influence the body weight of the mice compared with the untreated mice, which suggested limited aggressive side-effects (Fig. 5B). Additionally, compared with the controls, in the tumor tissues from the treated mice, GFPs increased the expression levels of Bax, cleaved caspase-3 and caspase-8, and suppressed the expression levels of Bcl-2 and Bcl-xL (Fig. 5D).

Discussion

In the present study, the potential anti-tumor effects of GPFs on breast cancer were successfully confirmed in MCF-7 and MDA-MB-231 cells, and tumor-bearing nude mice. The GFPs exerted cytotoxic effects on the breast cancer cell lines, as evidenced by a decrease in cell viability, and an increase in LDH release, ROS accumulation and caspase-3 activation, as

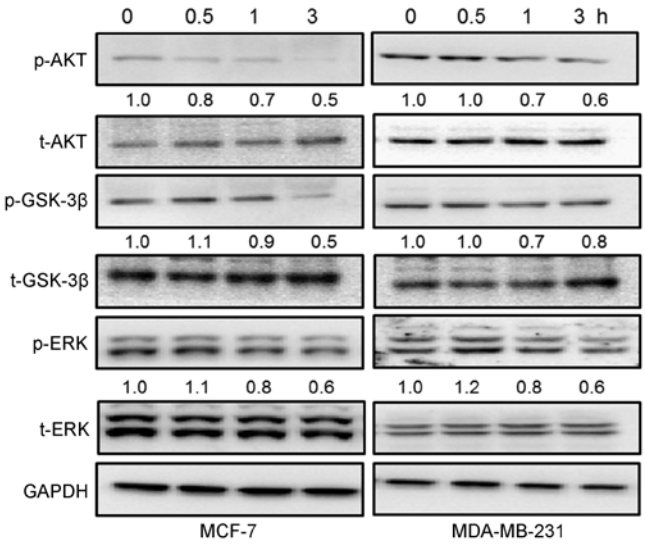

Figure 4. The activation of AKT/glycogen synthase kinase-3β (GSK-3β) and extracellular signal-regulated kinase (ERK) contributes to *Grifola frondosa* polysaccharide (GFP)-mediated apoptotic cell death. GFPs (200 μg/ml) reduced the expression levels of phosphorylated (p)-AKT, p-GSK-3β and p-ERK from 0.5 to 3 h. The average fold of band intensity compared to the related controls was marked respectively (n=6 repeats in each group).

well as the induction of cell apoptosis and mitochondrial apoptotic alterations. The suppressed phosphorylation of AKT/GSk-3β and ERK, related to mitochondrial function, revealed the possible mechanisms involved.

During apoptosis, which is a physiological suicide process, mitochondrial function plays a central role (23). The functional loss of the mitochondria is related to the dissipation of MMP (20), which was noted in this study following incubation with the GFPs for 12 h. The reduced Bcl-2 and Bcl-xL levels, and enhanced Bax expression levels were also observed in the GFP-treated cells. The Bcl-2 family, located in the outer mitochondrial membrane, serves as an important index in mitochondrial-mediated apoptosis (24). Moreover, in this study, the accumulation of intracellular ROS was observed in the cells treated with the GFPs. The overproduction of ROS causes oxidative stress, further resulting in mitochondrial apoptosis and cellular dysfunction. It has been reported that ROS accumulation is responsible for the opening mitochondrial permeability transition pore (mPTP), which leads to mitochondrial depolarization, matrix solutes loss and Cyto *c* release (25). Taken together, the effects of GFPs on mitochondrial function are involved in its anti-breast cancer effects.

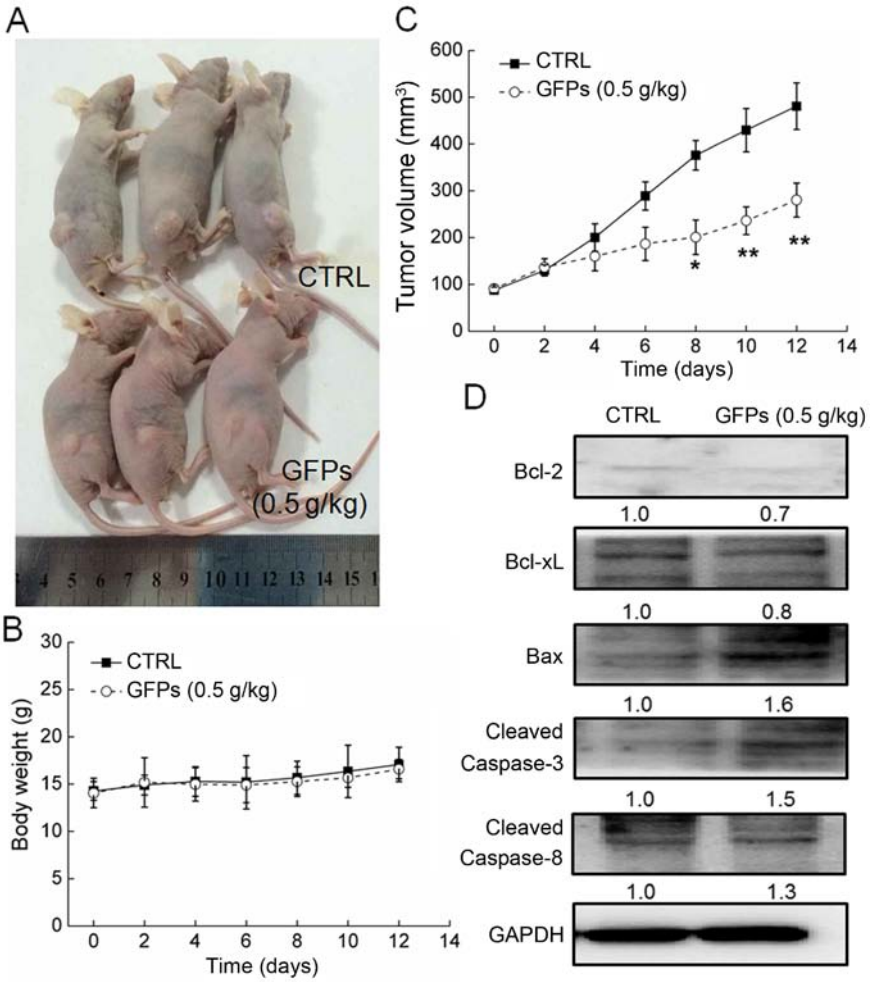

Figure 5. *Grifola frondosa* polysaccharides (GFPs) suppress the growth of MCF-7 tumor xenografts in nude mice. Male BALB/c athymic nude mice bearing MCF-7 tumors were treated for 14 days. (A) MCF-7 tumor xenograft growth between GFP-treated and untreated nude mice. (B) GFPs exerted no significant effects on body weight among the experimental mice. (C) Growth curves of MCF-7 tumor xenografts in GFP-treated and untreated mice. Tumor sizes were measured every 2 days. Data are expressed as the means ± SD (n=3) and analyzed using a one-way ANOVA. *P<0.05 and **P<0.01 vs. controls. (D) GFPs downregulated the anti-apoptotic proteins and upregulated the pro-apoptotic proteins in tumor tissues from treated mice. The average fold of band intensity compared with the untreated mice was marked respectively (n=3).

On the other hand, mitochondria control the intrinsic pathway of apoptosis, and during this process, MMP ignites caspases and other catabolic enzyme activation (26). Caspases are considered as inactive pro-enzymes and will be activated via proteolytic cleavage (27). Caspase-8, located mostly in the mitochondria, undergoes dimerization, and then cleaves itself to its fully activated form (28), which further leads to the cleavages of effector caspases in the cytosol (caspase-3) (29). Caspase-3, amplifying the initiation signals from caspase-8, plays a central role in activating the apoptotic program via regulating other caspases and some vital proteins (30), and it is important for cell death in a tissue-, cell type- or death stimulus-specific manner (31). In this study, in MCF-7 and MDA-MB-231 cells, and MCF-7 tumor xenografts, GFPs significantly enhanced the expressions of cleaved caspase-3 and caspase-8, which revealed that the anticancer activity of GFPs was associated with the regulation on caspase activation, which further targets the mitochondria.

AKT signaling is responsible for cell proliferation and apoptosis, which regulates apoptotic proteins including Bcl-2 family members GSK-3β (21). The reduced phosphorylation of AKT activates its downstream GSK-3β, which promotes Bax activation (32). As previously reported, GSK-3β mediates the release of cytochrome *c* into the cytosol, and its activated form helps to open mPTP (33). Via the AKT/GSK-3β- and ROS-dependent mitochondrial-mediated pathway, 18β-glycyrrhetinic acid induces the apoptosis of pituitary adenoma cells (23). Furthermore, the ERK pathway has been reported to be a target for cancer therapy, which is hyper-acted in human tumors (34,35). p-ERK, an active form, inhibits pro-apoptotic signals via the modulation of numerous substrates (22). In our study, the GFPs strongly suppressed the phosphorylation of AKT/GSK-3β and ERK in the MCF-7 and MDA-MB-231 cells, and this suppression may be involved in the GFP-mediated anti-tumor effects. Furthermore, ERK has been shown to exert positive regulatory effects on Bcl-2 and Bcl-xL expression, and ERKs/Bcl-2 have been confirmed as potential targets for cancer cell apoptosis (36,37). Previous studies have indicated that AKT contributes to the maintenance of mitochondrial integrity, which also affects Bcl-2 expression (38,39). Collectively, the downregulation of AKT/GSK-3β and ERK activation contributes to GFP-induced mitochondrial apoptosis.

The anti-breast cancer effects of GFPs were successfully confirmed in *in vitro* and *in vivo* experiments. GFPs reduced cell viability, enhanced the apoptotic rate, increased the ROS and caspase-3 intracellular levels, and caused LDH over-release, as well as MMP dissipation and the abnormal expression of pro-apoptotic proteins. The suppressed activation of ERK and AKT/GSK-3β in the GFP-incubated cells was responsible for mitochondrial dysfunction. All these findings reveal that the mitochondrial-dependent apoptotic pathway contributes to GFP-induced cytotoxicity in the MCF-7 and MDA-MB-231 cells, which provides pharmacological evidence to support the use of GFPs as a potential chemotherapeutic agent.

Acknowledgements

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