RESEARCH COMMUNICATION

Cochinchina momordica Seed Extract Induces G2/M Arrest and Apoptosis in Human Breast Cancer MDA-MB-231 Cells by Modulating the PI3K/Akt Pathway

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Abstract

***Cochinchina momordica* seeds are a kind of traditional Chinese herb. In this study, anticancer activity and underlying mechanisms were investigated with an extract using human breast cancer MDA-MB-231 cells. The survival rate was reduced in a concentration- and time-dependent manner as assessed by MTT assay. After incubation for 48 h, typical apoptotic morphological changes were observed by Hoechst 33258 dye assay. Flow cytometry revealed that the treatment obviously induced G2/M arrest and apoptosis in MDA-MB-231 cells. Furthermore, western blotting demonstrated downregulation of protein expression of PI3K, Akt, NF-κB, Bcl-2, Cdk1 and cyclin B1, whereas Bax and caspase-3 were upregulated. Our results suggest that the extract induced cell cycle G2/M arrest and apoptosis in MDA-MB-231 cells by decreasing PI3K/Akt pathway. Therefore, we propose that ECMS has potential as a breast cancer chemotherapeutic agent.**

Keywords: *Cochinchina momordica* seeds - apoptosis - cell cycle - MDA-MB-231 cell - anticancer activity - PI3K/Akt

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Introduction

Breast cancer is a malignant tumor of the highest morbidity and mortality in the world although the treatments of breast cancer have been improved. Furthermore, the incidence of breast cancer is increasing at an alarming rate (Parkin et al., 2006; Barron et al., 2008). Apoptosis plays an important role in the organism development and immune defense machinery (Vaux et al., 1999; Hidalgo et al., 2003). It is a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells proliferated owing to various chemical agent inductions (Brown et al., 1999; Hengartner et al., 2000). Apoptosis is characterized by particular morphological changes, including cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation (Wyllie et al., 1980). Many researchers have demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which has become a principal mechanism for effective anticancer therapy (Kundu et al., 2005). As an important source, plants may produce potential chemopreventive or chemotherapeutic agents. A lot of therapeutic drugs in use are derived from plants, such as paclitaxel from *Taxus brevifolia* and vinblastine from *Catharanthus roseus* (Vickers, 2002; Bosch et al., 2004).

Cochinchina momordica seed, a kind of traditional Chinese herbs, is the dried ripe seed of Momordica cochinchinensis (Lour) Spreng. Medical use of *Cochinchina momordica* seed has been described in ancient Chinese medical literature from the Song Dynasty (AD 793) and currently included in the Chinese pharmacopeia (Chinese Pharmaceutical Codex Evaluation Committee, 2005). Chemical analysis shows that the extract of *Cochinchina momordica* seed (ECMS) are composed of compounds including momordin (Iwamoto M et al., 1985), eleostearic acid (Wang Wei et al., 2000), oleanolic acid (Murakami et al., 1966), momordic acid, glycoprotein (Yeung 1987; Stirpe Fiorenzo et al., 1990), sterol and momorcochin (Bolognesi et al., 1989). It has been proven effective in enhancing immune responses (Xiao et al., 2007; Rajput et al., 2007), antioxidative effects (Tsoi et al., 2007) and antiulcer activities (Kang et al., 2010). As a mainly active ingredient, ECMS has been used in the Chinese herbal anagraphy of "Fengling Mixture" which is an anticancer prescription approved by SFDA at 2005 (China FDA: 20053611). However, the anti-proliferative activities and the underlying mechanisms of ECMS have remained largely unknown. Therefore, we aimed at studying the effect of ECMS on cell proliferation, apoptosis and cell cycle in human breast cancer MDA-MB-231 cell line.

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We found that ECMS induces cell cycle G2/M arrest and apoptosis by decreasing PI3K/Akt signaling.

Materials and Methods

Materials and reagents

Cochinchina momordica seed was purchased from an herb market in China. Fetal bovine serum (FBS), RPMI 1640 medium, penicillin G, streptomycin and trypsin were obtained from the Invitrogen Corporation, USA. Dimethyl sulfoxide (DMSO) and ribonuclease (RNase) were purchased from Sigma Chemical, USA. The 3-(4, 5)-dimethylthiahiazo (-z-y1) -3, 5-di-phenytetrazoliumromide (MTT) was purchased from AMRESCO Inc., USA. Hoechst staining kit, Cell cycle analysis kit, Apoptosis analysis kit, BCA protein analysis kit and Propidium iodide (PI) agents were purchased from Beyotime, China. The PVDF membranes were purchased from Millipore Corporation, Massachusetts, USA. The polyclone antibodies to PI3K, Akt, NF-κB, caspase-3, Bax and Bcl-2 were purchased from Cell Signaling, USA.

Preparation of the extract

500 ml of 62% ethanol solution were added to 150 g (dry weight) of *Cochinchina momordica* seed flour and the extract was obtained from a cold soak for 24 h. Then the extract was refluxed and filtered for 1 h. Collect the filtrate and added 160 ml of 62% ethanol solution into the residue. Duplicate the above processes and combine the filtrates. Add the filtrates into the wet macroporous resin column. The full absorption in the resin after the resin three times, the volume of 30%, 50%, 70%, 90% concentration of ethanol was eluted. Combine and freeze dry the eluent for ECMS.

Cancer cell line and culture

Human breast cancer MDA-MB-231 cells obtained from Shanghai Institute for Biological Sciences, China, and cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 100 IU/ml penicillin, 100 mg/ml streptomycin, kept at 37°C in a humidified 5% CO_2 incubator. Cells were passaged every 2-3 days and always used at about 80-90% of the confluence at passages between 3 and 20 after defreezing. They were seeded into different culture plates or dishes at a corresponding density for the respective assays.

Cell proliferation assay

The cell proliferation was measured by MTT method. Briefly, MDA-MB-231 cells were collected and seeded in 96-well plates at a density of 5×10^3 cells/well, and treated with phosphate buffered saline (PBS), various concentrations of ECMS and positive control factor, 5-fluorouracil. After incubation for 24 h, 48 h or 72 h, the medium was removed and replaced with a fresh medium (180 μl/well). 20 μl of MTT solution (5 mg/ml) added to each well and the plates were incubated for additional 4 h at 37°C. Then, the medium was aspirated off, and 150 μl of DMSO were added to each well. The absorbance was read at 570 nm as test wavelengths using a microplate reader (TECAN Infinite 200). Cell viability was expressed as a percentage of the untreated cells.

Nuclear staining with Hoechst 33258

MDA-MB-231 cells were seeded in 24-well plates at a density of 5×10^3 cells/well, and incubated with ECMS (0.1, 0.2 and 0.4 mg/ml) for 48h. Cellular monolayer in 24-well plates was fixed and stained with DNA fluorochrome Hoechst 33258 for 20 min. After washed with PBS, the morphological features of apoptosis (including cellular nucleus shrinkage, chromatin condensation, intense fluorescence and nuclear fragmentation) were monitored by fluorescence microscopy (Zeiss, German).

Measurement of apoptosis by Annexin V-FITC/PI staining

Flow cytometry was used to quantitatively detect the apoptotic rate. Cells (5×10^3/well) were seeded into 6-well plates and exposed to ECMS at various concentrations (0.1, 0.2 and 0.4 mg/ml) for 48 h, and then harvested and washed with phosphate buffered saline (PBS). Staining went along with 195 μl bonding buffer containing 5 μl Annexin V-FITC in the dark at room temperature for 10 min, and then added 10 μl PI in the dark 4°C for 10 min. The apoptotic cells were analyzed with FACScan flow cytometry (BD FACSCalibur).

Analysis of cell cycle by PI staining

Cells (3×10^5/well) were seeded into 6-well plates and exposed to ECMS at various concentrations (0.1, 0.2 and 0.4 mg/ml) for 48 h, and then harvested and washed with PBS, fixed in 70% ethanol at 4°C. Staining went along with PBS containing 40 (g/ml RNaseA and 10 μg/ml PI in the dark at room temperature for 30 min. The cell cycle was measured using FACScan Flow cytometry (BD FACSCalibur).

Western blotting assay

When administration with ECMS (0.1, 0.2, 0.3 mg/ml) for 48 h, MDA-MB-231 cells were collected and homogenized in 200 μl RIPA lysis buffer. Cell lysate was centrifuged (12,000×g for 15 min at 4°C) and protein concentration was determined by the BCA protein assay kit. Equal amounts of protein were denatured and separated by 10% SDS-PAGE, then transferred onto PVDF membranes using a Bio-Rad miniprotein-III wet transfer unit. Nonspecific binding was blocked with 5% bovine serum albumin dissolved in TBST at room temperature for 1 h. Subsequently, the membranes were then washed three times and incubated with individual primary antibodies of PI3K, Akt, NF-κB, Bax, Bcl-2, Cdk1, Cyclin B1 and caspase-3 at 4°C over night. The membranes were incubated with the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature followed by three washings. The signals were detected with a chemiluminescence ECL reagent and quantified by densitometry using a gel visualizer (Alpha Innotech, CA, USA). GAPDH served as the loading control, and the results were expressed as the percentage of control.

Statistical analysis

The data were expressed as means (standard errors of

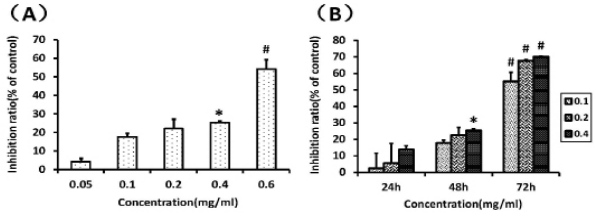

Figure 1. The Proliferative Inhibition Effects of ECMS on Human Breast Cancer MDA-MB-231 Cells. Cell survival was evaluated by MTT assay at a wavelength of 570 nm. (A) Incubation with ECMS at different concentrations for 48 h. (B) Administration with ECMS for 24 h, 48 h and 72 h. The inhibition ratio was expressed as the optical density ratio of the treatment to the control. Each value represents means ± SD in three independent experiments. *p<0.05 vs. control group, #p<0.01 vs. control group

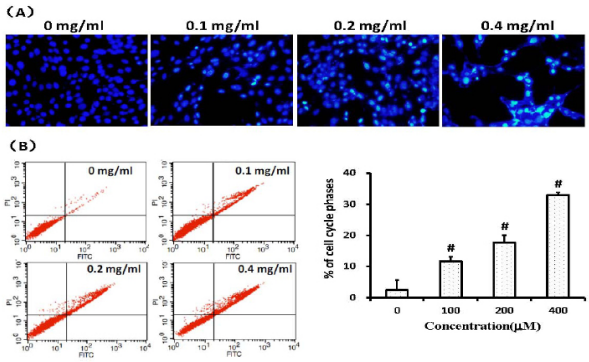

Figure 2. ECMS Induced Apoptosis of Human Breast Cancer MDA-MB-231 Cells. Typical apoptotic morphology was observed in MDA-MB-231 cells treated with ECMS (0.1, 0.2, and 0.4 mg/ml) for 48 h. (A) Cells stained with Hoechst 33342 were imaged with a fluorescence microscope (200'). Condensed and fragmented nuclei with a bright stain were considered as apoptotic cells. (B) Apoptotic cells were analyzed by flow cytometry with Annexin V-FITC and PI staining. Early apoptotic cells (Annexin-V+) displayed in the lower right quadrant and late apoptotic cells (Annexin-V+ and PI+) were shown in the upper right quadrant. Dead cells were shown in the upper left quadrant (PI+). The percentages of apoptotic cells were indicated by Annexin-V+ cells shown as means ± SD from three independent experiments. *p<0.05 vs. control group, #p<0.01 vs. control group

mean ± SD). The differences were analyzed by one-way analysis of variance (ANOVA) and Tukey's Studentized Range test. The differences were considered significant at p<0.05.

Results

Effect of ECMS on MDA-MB-231 cell proliferation

MDA-MB-231 cell survival rates were assayed by MTT method at 570 nm. After incubate with ECMS (0.05, 0.1, 0.2, 0.4 and 0.6 mg/ml) for 48 h, the inhibition rates of cells were increased from 4.5 ± 1.5% to 54.3 ± 5.1% (Figure 1A). When treated with ECMS (0.1, 0.2, 0.4 mg/ml) for 24 h, 48 h and 72 h, the inhibition rates were elevated from 2.4 ± 9.4% to 55.3 ± 5.4%, 5.6 ± 11.9% to 67.6 ± 0.9%, and 13.8 ± 2.4% to 70.2 ± 0.6%, respectively (Figure 1B). The 50% inhibit concentration (IC50) was 65 μg/ml at 72 h. The results showed that ECMS dramatically decreased the proliferation of MDA-MB-231 cells in a

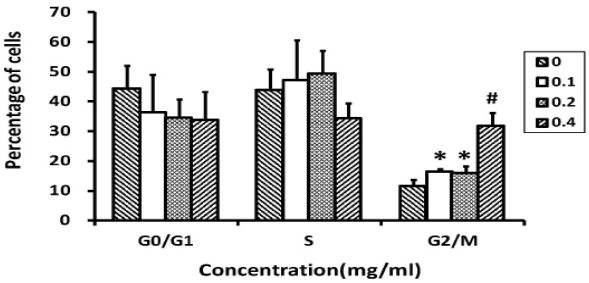

Figure 3. Effect of ECMS on Cell Cycle Progression of Human Breast Cancer MDA-MB-231 Cells. After treated with ECMS at doses of 0.1, 0.2 and 0.4 mg/ml for 48 h, MDA-MB-231 cell cycle distribution analysis by flow cytometry. Each value represents means ± SD in three independent experiments (n=3). *p<0.05 vs. control group, #p<0.01 vs. control group

dose- and time-dependent manner (p<0.01).

ECMS induced apoptosis of MDA-MB-231 cells

Morphologic changes in the nucleus representing cellular apoptosis were assessed by staining with the membrane-permeable DNA binding dye Hoechst 33258. MDA-MB-231 cells were treated with different concentrations of ECMS (0, 0.1, 0.2, 0.4 mg/ml) for 48 h and were analyzed by Hoechst 33258 staining. As showed in Figure 2A, normal cells exhibited regular and round shaped nuclei with a pallid blue, whereas apoptotic cells were characterized by the condensation and the fragmentation of nuclei with bright fluorescence. Flow cytometry analysis was used to quantify the apoptotic MDA-MB-231 cells after treatment with ECMS (0, 0.1, 0.2, 0.4 mg/ml) for 48 h. The proportion of apoptotic cells was increased from 2.5 ± 3.1% to 33.0 ± 0.8% (Figure 2B). The results revealed that ECMS induced apoptosis in MDA-MB-231 cells in a dose-dependent manner (p<0.01).

Effect of ECMS on MDA-MB-231 cell cycle

After the administration with ECMS (0.1, 0.2 and 0.4 mg/ml) for 48h, MDA-MB-231 cell cycle was detected by flow cytometry. As compared to the control, G2/M phases of MDA-MB-231 cells were elevated from 11.7 ± 2.1% to 31.9 ± 4.4%, whereas those at G0/G1 and S phases were decreased (Figure 3). The results indicated that ECMS blocked the cell cycle at G2/M phases in MDA-MB-231 cells in a dose-dependent manner.

Effect of ECMS on expressions of Bcl-2 family proteins

To further elaborate on the possible mechanism underlying ECMS-induced apoptosis, we tested the effect of ECMS on the protein expressions of Bcl-2, Bax and caspase-3. Western blotting analysis revealed that the Bcl-2 expression was obviously decreased, whereas the Bax expression was slightly increased (Figure 4A). The caspase-3 protein level was notably increased as compared to GAPDH control (Figure 4B). These results indicated that ECMS up-regulation of the Bax/Bcl-2 ratio and caspase-3 expression in a dose-dependent manner.

ECMS decreases Cyclin B1 and Cdk1 expressions

To investigate the mechanism underlying cell cycle arrest induced by ECMS, we tested the effect of ECMS

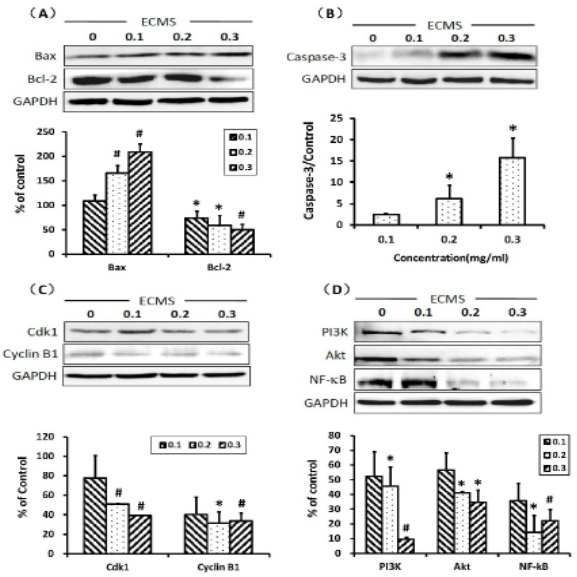

Figure 4. Effect of ECMS on the Protein Expression Levels in Human Breast Cancer MDA-MB-231 Cells. The relative quantity of the protein expression was measured by western blotting against the internal control GAPDH. (A) The protein expression of Bcl-2 and Bax. (B) The protein expression of caspase-3. (C) The protein expression of Cdk1 and Cyclin B1. (D) The protein expression of PI3K, Akt and NF-kB. Data expressed as mean ± SD of more than three different experiments, *p<0.05 vs. control group, #p<0.01 vs. control group

on Cyclin B1, Cdk1 levels. As showed in Figure 4C, western blotting analysis revealed that ECMS decreased the protein levels of Cyclin B1 and Cdk1 via a dose-dependent manner.

Effects of ECMS on PI3K/Akt pathway

In order to better elucidate the molecular basis of ECMS induced G2/M arrest and apoptosis, we investigated the expression of PI3K, Akt, NF-κB. As showed in Figure 4D, the levels of PI3K, Akt and NF-κB proteins dose-dependently decreased in response to ECMS.

Discussion

Cochinchina momordica seed has been used as an herbal medicine for the treatment of different diseases in China for thousands of years. Here, we reveal that ECMS was able to decrease the survival rate of human breast cancer cell line in a dose- and time-dependent manner. Furthermore, we found that the anti-proliferative potential mechanism of ECMS involved in G2/M arrest and induction of apoptosis in MDA-MB-231 cells. Apoptosis is an important regulator in developmental processes, maintenance of homeostasis and in elimination of the damaged cells. The Bcl-2 protein family is a key regulator of the apoptotic pathway (Oltersdorf et al., 2005; Thees et al., 2005). The Bcl-2 family includes two subfamilies: one is anti-apoptotic protein such as Bcl-2, the other is pro-apoptotic proteins such as Bax. Many anticancer agents induced apoptosis by targeting the proteins of Bcl-2 family and the ratio of Bax/Bcl-2 played a critical role in determining whether cells will undergo apoptosis (Gupta et al., 2002; Emi et al., 2005). The caspase family is an executioner of apoptosis, in which caspase-3 cascade is the key point (Heimlich et al., 2004). In our study, by examining the effects of ECMS on Bax, Bcl-2 and caspase-3, we found that ECMS increased pro-apoptotic Bax and caspase-3 expression and decreased anti-apoptotic Bcl-2 expression, leading to up-regulation of the ratio of Bax/Bcl-2 and subsequently inducing caspase-dependent apoptosis. This might be one of the molecular mechanisms through which ECMS induces apoptosis in MDA-MB-231 cells.

The induction of cell cycle arrest is also a common mechanism proposed for the cytotoxic effects of anticancer-drug as well as apoptosis. The cell cycle arrest can trigger proliferation inhibition and apoptosis in cancer cells (Pu et al., 2002; Chao et al., 2004). During cell cycle, the G2/M checkpoint is a potential target for cancer therapy. It prevents DNA-damaged cells from entering mitosis and allows for the repair of DNA damage (Wang et al., 2009). The G2/M checkpoint is controlled by Cdk1 and Cyclin B1 (Dash et al., 2005). Our studies showed that treating MDA-MB-231 cells with ECMS resulted in decreased expression of Cdk1 and Cyclin B1, suggesting that decreasing of Cdk1 and Cyclin B1 expression might be the molecular mechanism through which ECMS induced G2/M arrest.

PI3K/Akt is one of the most important signaling pathways in regulating cell growth, proliferation and apoptosis, and Akt is a major downstream target of PI3K (Kauffmann-Zeh et al., 1997). This pathway was aberrantly activated in various types of cancer (Vivanco et al., 2002). PI3K/Akt promotes cell survival by activating NF-κB signaling pathway (Romashkova JA et al., 1999) and also participates in the regulation of Bcl-2 family proteins (Asnaghi et al., 2004). Recent studies have shown that some anticancer-drugs could induce G2/M arrest and apoptosis accompanying down-regulation of Akt (Katayama et al., 2005; Weir et al., 2007). NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells), a transcription factor protein complex, can modulate the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation and suppression of apoptosis (Burstein and Duckett, 2003; Luo et al., 2005b; Basseress and Baldwin, 2006; Dutta et al., 2006; Cilloni et al., 2007; Jost and Ruland, 2007; Melisi and Chiao, 2007). Aberrant regulation of NF-κB is involved in cancer development and progression as well as in drug resistance. Inhibitors of NF-κB mediate effects potentially leading to antitumor responses or greater sensitivity to the action of antitumor agents. In our study, we found that treatment of MDA-MB-231 cells with ECMS reduced the protein expression of PI3K, Akt and NF-κB in a dose-dependent manner. Meanwhile, we also found that ECMS treatment down-regulated Cdk1/Cyclin B1, Bcl-2 protein and up-regulated Bax and caspase-3. In summary, our results indicated that ECMS induced G2/M arrest and apoptosis possibly by down-regulating PI3K/Akt and NF-κB signaling in human breast cancer MDA-MB-231 cells.

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