Aged black garlic extract inhibits HT29 colon cancer cell growth via the PI3K/Akt signaling pathway

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Abstract. Accumulating evidence indicates that aged black garlic extract (ABGE) may prove beneficial in preventing or inhibiting oncogenesis; however, the underlying mechanisms have not been fully elucidated. The present study aimed to investigate the effects of ABGE on the proliferation and apoptosis of HT29 colon cancer cells. Our results demonstrated that ABGE inhibited HT29 cell growth via the induction of apoptosis and cell cycle arrest. We further investigated the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signal transduction pathway and the molecular mechanisms underlying the ABGE-induced inhibition of HT29 cell proliferation. We observed that ABGE may regulate the function of the PI3K/Akt pathway through upregulating PTEN and downregulating Akt and p-Akt expression, as well as suppressing its downstream target, 70-kDa ribosomal protein S6 kinase 1, at the mRNA and protein levels. In conclusion, these findings suggest that the PI3K/Akt signal transduction pathway is crucial for the development of colon cancer. ABGE inhibited the growth and induced apoptosis in HT29 cells through the inhibition of the PI3K/Akt pathway, suggesting that ABGE may be effective in the prevention and treatment of colon cancer in humans.

Introduction

Colon cancer is currently one of the most common types of gastrointestinal cancer, with an incidence rate of ~10-15% among cancer patients. Surgical resection has been the standard treatment for colon cancer; however, the local recurrence rate following surgical resection is 20-50% (1). Chemotherapy is currently considered as the gold-standard treatment for cancer cell elimination. However, the majority of chemotherapeutic agents also destroy normal cells, resulting in the development of intolerable adverse reactions. The clinical outcome of chemotherapy has been disappointing, due to the severe side effects, which may include gastrointestinal reactions, bone marrow suppression, oral mucositis, hepatic damage and toxic effects on the urinary system (2-4). Therefore, there is a need for the development of low-toxicity anticancer agents. Numerous studies demonstrated that allicin, an organosulfur compound obtained from garlic, has the ability to promote the apoptosis of tumor cells, cause cell cycle arrest and improve the antioxidant activity (5,6). The phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) intracellular signaling pathway is frequently activated in cancer cells through numerous mutations and epigenetic changes. The PI3K/Akt signaling pathway is involved in a variety of processes underlying tumor cell proliferation, apoptosis and migration. The occurrence, development and outcome of tumor diseases are significantly associated with the PI3K/Akt signaling pathway (7-9) and recent scientific research has been focused on the PI3K/Akt signaling pathway (10). The development of inhibitors targeting different components of the PI3K pathway may result in novel targets for alternative cancer therapy. However, predicting the efficacy of these drugs is challenging and methods for therapy monitoring are required.

We previously reported the potential action of aged black garlic extract (ABGE) against gastric cancer by evaluating its effect on the inhibition of cell proliferation and induction of apoptosis in SGC-7901 human gastric cancer cells (10). Elucidating the effect of ABGE on HT29 cells may be of value in developing novel cancer treatment strategies. In this study, we investigated the activity of ABGE against HT29 colon cancer cells and its association with the PI3K/Akt signaling pathway.

Material and methods

Materials. FBS, DMEM, penicillin, streptomycin, trypsin/EDTA, trypan blue dye, MTT, PI, bicinchoninic acid working solution and the ECL Plus light-emitting kit were purchased from Sigma (St. Louis, MO, USA). Antibodies

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against Akt, PTEN and β-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Acrylamide and the protein assay kits were obtained from Bio-Rad (Hercules, CA, USA). Western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The p-Akt, 70-kDa ribosomal protein S6 kinase 1 (p70S6K1) and β-actin primers and the real-time kit were purchased from Bao Biological Engineering Co. (Dalian, China).

Preparation of ABGE. ABGE was provided by the Dalian Hua Valley Garlic Industry Co. (Dalian, China). The mashed aged black garlic was extracted with 95% ethanol for 24 h under mixing. Following precipitation, the cooled solution was filtered and evaporated under a reduced pressure at 47˚C to provide a residue. The extract was then filtered through a 0.22-μm membrane, dissolved in 0.9% saline to prepare a solution at a concentration of 1 g/ml and stored at -80˚C prior to use (10).

Cell culture. The HT29 human colon cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The HT29 cell line was cultured in DMEM containing 10% heat-inactivated FBS, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a humidified incubator with 5% CO_2. HT29 colon cancer cells in the logarithmic phase of growth were collected and inoculated in a 96-well culture plate, with $3x10^3$ cells per well. After 24 h, the cells were exposed to varied doses of ABGE (20, 50 and 100 mg/ml). The cells in the control group were treated with 0.1% DMSO. There were 3 wells per drug concentration group.

Growth assays in vitro. The processed cells were cultured for 24, 48 and 72 h. MTT solution (8 μl) was added to each well, followed by the addition of 100 μl DMSO after 4 h. The absorbance value was measured by ELISA detection instrument at 570 nm and a reference wavelength of 630 nm (Universal Microplate Spectrophotometer; Thermo Fisher Scientific, Inc., Rockford, IL, USA). Each experiment was repeated three times.

Flow cytometry for cell cycle analysis and apoptosis. The HT29 cells were treated with ABGE at different concentrations (20, 50 and 100 mg/ml, with 0/0.1% DMSO vehicle used as the control) for 24 h. The treated HT29 cells were detached in 2 ml of PBS with 2 mM EDTA, centrifuged at 15,000 x g for 5 min and resuspended in 250 μl of hypotonic fluorochrome solution (PBS, 50 μg PI, 0.1% sodium citrate and 0.1% Triton X-100) with RNase A (100 U/ml). The DNA content was analyzed by a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). All the events (n=20,000) were analyzed per sample and the cell cycle distribution and apoptosis were determined based on the DNA content and the sub-G1 cell population, respectively.

Western blot analysis. The treated HT29 cells were washed with ice-cold PBS and suspended in lysis buffer on ice for 30 min. The lysates were cleared by centrifugation at 15,000 x g for 15 min. Equal volumes of cell extracts (60 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with primary antibodies against human PTEN, Akt and β-actin, followed by horseradish-conjugated secondary antibodies. Anti-β-actin antibody was used as a loading control. Detection was performed using an ECL Plus light-emitting kit (Sigma).

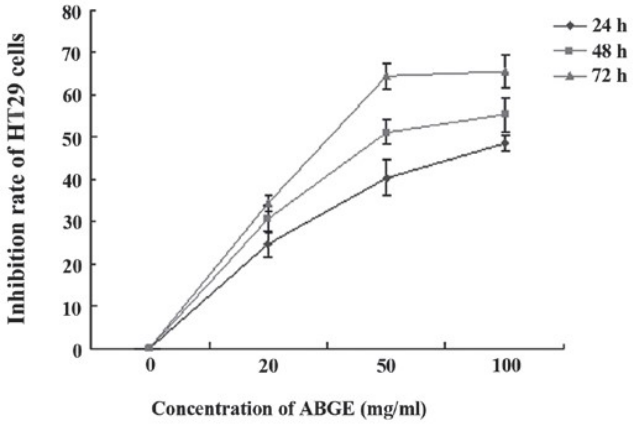

Figure 1. Effects of ABGE on the growth of HT29 cells in a time- and dose-dependent manner. HT29 cells were cultured for 24, 48 and 72 h in DMEM containing ABGE. The effects of ABGE on cell growth were determined by the MTT assay. The results are expressed as the means ± standard deviation from three independent experiments. Data were analyzed using the Student's t-test. The values between the control group and the cells treated with ABGE differed significantly (P<0.05). ABGE, aged black garlic extract.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from HT29 cells treated with different concentrations of ABGE (20, 50 and 100 mg/ml, with 0/0.1%DMSO vehicle as the control) for 24 h, using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions, and was quantified by spectrophotometry. The reverse transcription reaction was performed using total RNA. The primers were designed by Bao Biological Engineering Co. as follows: p-Akt mRNA: upstream, 5'-CAATTCCGGTCTGAGGAA-3' and downstream, 5'-CACATGGGAAGTGTTGTCTG-3'; and p70S6K1 mRNA: upstream, 5'-GGCAGTGATGGGCAACCT-3' and downstream, 5'-GGTCCAACCCTTACTTCAGCA-3'. The qPCR conditions included an initial denaturation for 30 sec at 95˚C, followed by 40 cycles of denaturation for 5 sec at 95˚C and annealing for 20 sec at 60˚C.

Statistical analysis. Data are expressed as the means ± standard deviation. The statistical analysis was conducted with SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA), using a two-sided Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of ABGE on the growth of HT29 cells. ABGE inhibited the growth of HT29 cells in a time- and dose-dependent manner (Fig. 1). The cell growth inhibition rate at ABGE concentrations of 20, 50 and 100 mg/ml was 24.6±4, 40.4±5 and 46.7±4%, respectively, at 24 h; 28.3±5, 48.1±4 and 55.2±3%, respectively, at 48 h; and 34.2±4, 56.1±6 and 63.9±5%, respectively, at 72 h (P<0.05).

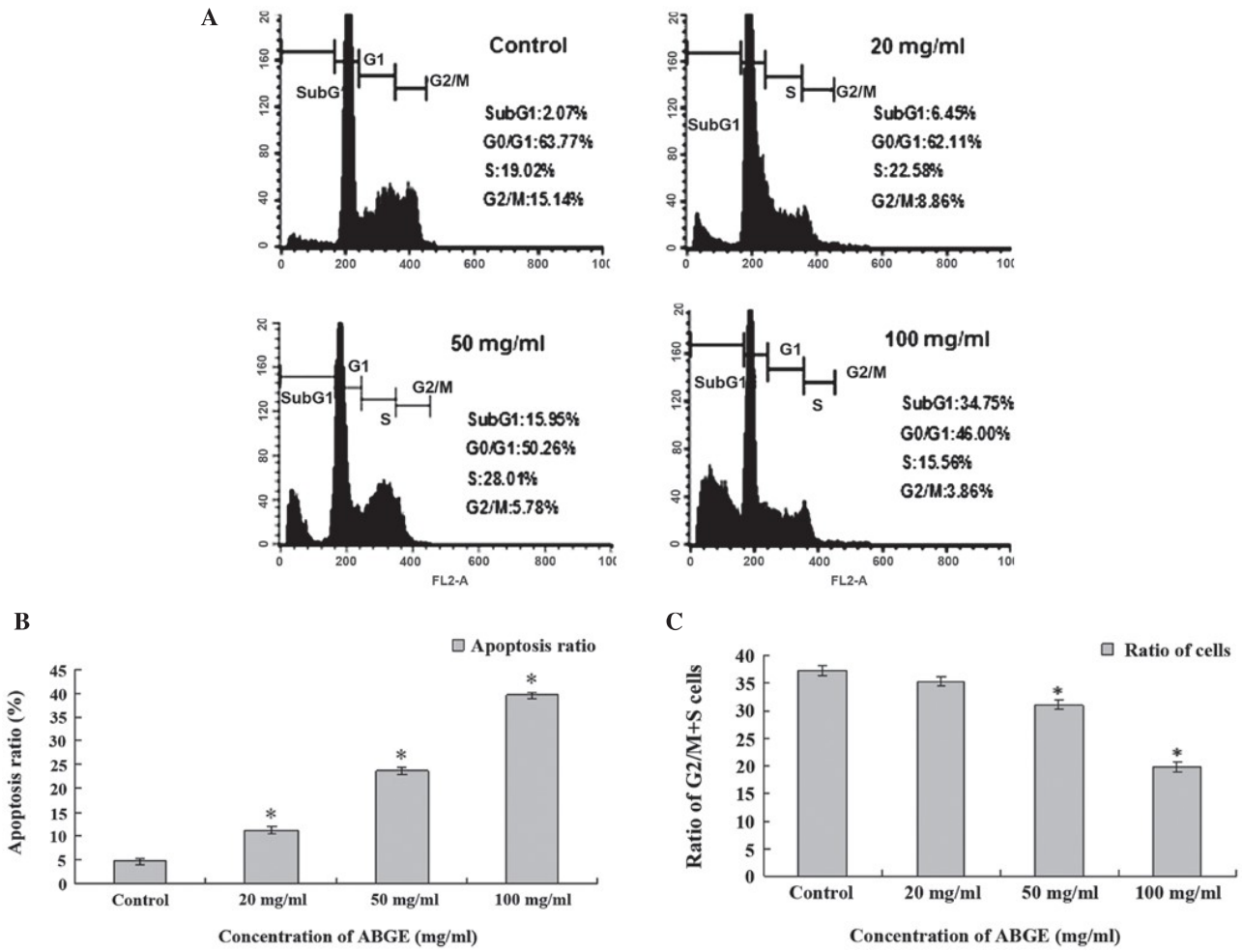

Figure 2. Effects of ABGE on the cell cycle and apoptosis of HT29 cells *in vitro*. The HT29 cells were treated with varied concentrations of ABGE (20, 50 and 100 mg/ml, with 0/0.1% DMSO vehicle as the control) for 24 h. The cells were harvested, stained with PI and analyzed by flow cytometry. (A) Representative results of the alteration of the cell cycle and apoptosis assessment by Annexin V/PI single-staining assay. ABGE (B) induced a statistically significant increase in the apoptosis of HT29 cells and (C) interfered with the G2/M+S phase of the cell cycle. The results are expressed as the means ± standard deviation from three independent experiments. Data were analyzed using the Student's t-test. *P<0.05, compared to the control group. ABGE, aged black garlic extract.

Effects of ABGE on the G0/G1 phase of the cell cycle and apoptosis of HT29 cells. We investigated the effects of different concentrations of ABGE on the apoptosis of HT29 cells. The flow cytometric analysis confirmed that ABGE at concentrations of 20, 50 and 100 mg/ml achieved a dose-dependent induction of apoptosis. Additionally, ABGE at 20, 50 and 100 mg/ml induced the apoptosis of HT29 cells at 24 h with an apoptosis ratio of 11.21±0.86, 23.77±0.78 and 39.56±0.59%, respectively (P<0.05). Moreover, ABGE (20, 50 and 100 mg/ml) achieved a dose-dependent induction of G0/G1 cell cycle arrest and a G2/M+S decrease (Fig. 2). Our results demonstrated that different concentrations of ABGE, as well as other apoptosis-related proteins, inhibited colon cancer cell growth through the induction of apoptosis and cell cycle arrest.

Effects of ABGE on the expression of Akt and PTEN in HT29 cells. The effect of ABGE treatment on the expression of Akt and PTEN was assessed by western blot analysis. Compared to the control group, the Akt protein level in the HT29 cells treated with ABGE was lower, whereas the PTEN protein level was higher (Fig. 3). The western blot analysis revealed that treatment with ABGE at different concentrations for 24 h significantly reduced the levels of Akt and increased the levels of PTEN in HT29 cells.

Effects of ABGE on the expression of p-Akt and p70S6K1 in HT29 cells. We investigated the effects of ABGE on p-Akt and its downstream target, p70S6K1, in HT29 cells by qPCR. The qPCR analysis demonstrated that treatment with ABGE at different concentrations for 24 h significantly reduced the mRNA levels of p-Akt and p70S6K1 in HT29 cells (Fig. 4).

Discussion

Garlic has been long recognized for its medicinal properties and has been used for sterilization and anti-inflammation throughout history. Allicin, the main active ingredient of garlic, is currently extensively investigated for antitumor therapy (11). Black garlic is a type of fermented garlic. Numerous *in vivo* and *in vitro* studies demonstrated that aged black garlic possesses

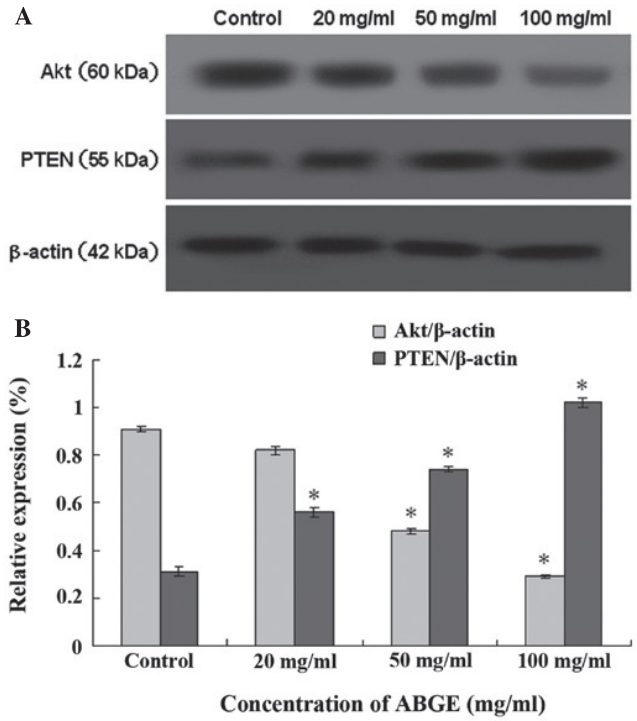

Figure 3. Effects of ABGE on (A) Akt protein expression and (B) PTEN protein expression in HT29 cells treated with various concentrations of ABGE (20, 50 and 100 mg/ml, with 0/0.1% DMSO vehicle as the control) for 24 h. The cells were harvested and analyzed by western blotting. Anti-β-actin was used as the loading control. The results are expressed as the means ± standard deviation from three independent experiments. Data were analyzed using the Student's t-test. *P<0.05, compared to the control group. ABGE, aged black garlic extract.

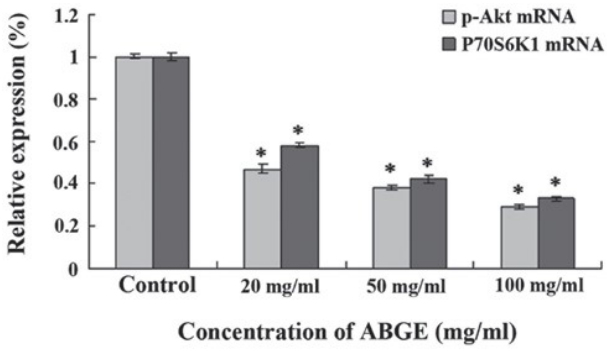

Figure 4. Effects of ABGE on p-Akt and p70S6K1 protein expression in HT29 cells. The values are expressed as means ± standard deviation. Data were compared with the control group and analyzed using the Student's t-test. Significant decreases in the mRNA expression levels were observed for p-Akt (n=3) and p70S6K1 (experiments were repeated in triplicate). *P<0.05 compared to the control group. ABGE, aged black garlic extract.

strong antioxidant and anticancer properties and may inhibit the proliferation of a variety of tumor cell lines by altering the cell cycle and inducing apoptosis (12-14). However, the underlying mechanisms have not been fully elucidated. A previous epidemiological investigation indicated that the development and progression of colon cancer is a complex, multistep and multifactorial process (15). The PI3K/Akt pathway is involved in intracellular signal transduction and mediates numerous cellular processes, including cell proliferation, migration and apoptosis. Therefore, in the present study, we hypothesized that the PI3K/Akt pathway is crucial for the development of colon cancer and ABGE treatment may be associated with induction of the PI3K/Akt pathway.

The PI3K/Akt signal transduction pathway plays an important biological role in the occurrence of apoptosis that is mediated through the regulation of apoptosis-related genes. PI3K/Akt signaling is deregulated through a variety of mechanisms, including overexpression or activation of growth factor receptors, mutations of the PI3K gene and mutation/amplification of the Akt gene (16).

Akt is a downstream target of PI3K and it is a 56-kDa serine/threonine kinase triggered by a lipid second messenger, phosphatidylinositol-3,4,5-trisphosphate, which is generated by PI3K. Akt may be phosphorylated to p-Akt by pyruvate dehydrogenase kinase-1, which is distributed across the cell membrane. p-Akt inactivates multiple effector molecules of apoptosis through a variety of mechanisms, promoting the proliferation and metastasis of tumor cells.

PTEN, the negative regulator of PI3K signaling, exhibits decreased expression in various types of cancer and may be downregulated through several mechanisms, including mutation, loss of heterozygosity, methylation, aberrant expression of regulatory microRNA and protein instability. The PTEN gene is an important effector molecule in the tumor signaling pathway, with the ability to inhibit growth and phosphatase activity in cancer cells. Low expression of PTEN may be associated with non-effective inhibition of the activation of Akt, resulting in the overactivation of the PI3K/Akt pathway. PTEN loss and Akt overexpression may significantly affect the progression of several advanced human cancers, including melanoma and breast, prostate and renal cancers (17-19).

p70S6K is the direct substrate of mTOR and forms downstream components of the PI3K/Akt signaling pathway. p70S6K was shown to be a signaling molecule, which is involved in the regulation of a series of physiological processes in protein synthesis. Once activated, p70S6K1 was shown to promote the reconstruction of actin filaments (20-22). Therefore, the abnormal activation of the PI3K/Akt pathway in cancer cells may promote their migration and proliferation.

Apoptosis is the outcome of a gene expression cascade and is regulated by several gene products. In the present study, treatment with ABGE resulted in marked inhibition of cell growth, induction of apoptosis and cell cycle arrest in HT29 colon cancer cells. To further elucidate the mechanisms underlying the ABGE-induced growth suppression in HT29 cells, we investigated its effect on Akt, PTEN, p-Akt and p70S6K expression. The results revealed that ABGE modulated the PI3K/Akt signaling pathway in HT29 cells through the upregulation of PTEN and the downregulation of Akt expression and the reduction of p-Akt and p70S6K1 expression at the mRNA level.

By investigating the correlation between the PI3K/Akt signaling pathway and the growth inhibitory effect of ABGE on HT29 cells, as well as the underlying mechanisms, we concluded that the PI3K/Akt signaling pathway is critical for the development of colon cancer.

Our present data suggest that ABGE may be of therapeutic and/or adjuvant therapeutic value in the treatment of colon

cancer, possibly via the modulation of the PI3K/Akt signaling pathway, the upregulation of PTEN and the downregulation of Akt and p-Akt expression. However, as ABGE may confer resistance to targeted drugs, further assessment of ABGE in clinical trials is required.

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