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# Allitridi induces apoptosis by affecting Bcl-2 expression and caspase-3 activity in human gastric cancer cells<sup>1</sup>

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**KEY WORDS** human gastric cancer cell line; apoptosis; diallyl trisulfide; Bcl-2; CPP32 protein

## ABSTRACT

**AIM:** To investigate the mechanism of allitridi-induced apoptosis in human gastric cancer cell line BGC823. **METHODS:** Growth inhibition by allitridi was analyzed using cell growth curve and MTT assay. Apoptotic cells were detected using staining with Hoechst 33342, and confirmed by flow cytometric analysis and DNA fragmentation analysis. The protein expression affected by allitridi was determined using Western blot. The activity of caspase-3 was measured using a fluorescence assay. **RESULTS:** Allitridi induced apoptosis, and then inhibited cells proliferation in human gastric cancer cell line BGC823. The protein level of Bcl-2 was decreased dramatically, while Bax and p53 were not significantly affected by allitridi. The expression and activity of caspase-3 started to increase after allitridi treatment for 72 h. **CONCLUSION:** Allitridi induced apoptosis through down-regulation of Bcl-2, and increased caspase-3 expression and its activity.

## INTRODUCTION

Gastric cancer is the second leading cause of cancer mortality in the world and is the leading cause of cancer mortality in China<sup>[1]</sup>. Numerous epidemiological investigations have demonstrated that an inverse correlation between gastric cancer incidence and garlic consumption<sup>[2-8]</sup>. For example, Linqu County has gastric cancer rate that was 15 times higher than that of Cangshan County in Shangdong Province<sup>[10]</sup>, and the death rate of gastric cancer in Linqu County was 7 times higher than that of Cangshan County<sup>[2]</sup>, even though

the two counties are within 200 miles of each other. The main reason is the difference of garlic consumption. That is, residents of Cangshan County consumed an average of 6000 g of garlic per year, while in Linqu County garlic consumption was less than 1500 g per year<sup>[2]</sup>. Similar observations had been made in Jiangsu Province of China and in Italy<sup>[4-7]</sup>. A double-blind factorial trial was also undertaken in China to investigate whether garlic extracts could prevent the progression of gastric precancerous lesions<sup>[9]</sup>.

At the same time, garlic and its derivatives have been used successfully in laboratory animals<sup>[11-14]</sup>. Organosulfur compound from garlic can suppress chemically induced carcinogenesis, such as colon, mammary, and skin, slow or prevent tumor growth.

More and more efforts have been made to reveal the biological and pharmacological activities of garlic. It has been reported that garlic and its derivatives possess various activities including enhanced immunocom-

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petence<sup>[15]</sup>, regulation gene expression associated with phase I and phase II enzymes<sup>[16-18]</sup>, suppression carcinogens-DNA adduct formation<sup>[19-21]</sup>, induction of cancer cell apoptosis<sup>[22-23]</sup>. Our present work aimed at investigating the effect of allitridi on human gastric cancer cell line BGC823, and further to elucidate its mechanism.

## MATERIALS AND METHODS

**Cell culture and drug treatment** Human gastric cancer cell line BGC823<sup>[24]</sup> was grown in complete medium containing DMEM (Dulbecco's modified Eagle's medium, Hyclone), 5 % (v/v) fetal bovine serum (Hyclone), benzylpenicillin (100 kU/L) and streptomycin (100 mg/L) at 37 °C in 5 % CO<sub>2</sub>. Diallyl trisulfide (DATS, 97.98 % purity) was ordered from Shanghai Hefeng Pharmacy Company (Shanghai, China), whose commercially name is allitridi. Cells grown up to 60 %-70 % confluence were treated with allitridi at a final concentration of 25 mg/L. Cells exposed to allitridi were refed with fresh complete medium and allitridi every day.

**Growth inhibition** Cell growth *in vitro* was determined using cell growth curve and MTT assay. For cell growth curve, cells were harvested by trypsinizing and the number of cells was counted by cell number counter. The effect on cellular proliferation was also measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the ability of live cells to utilize thiazolyl blue and convert it into dark blue formazan. Exponentially growing cells were seeded into a 96-well plate at 2000 cells per well. After allitridi stimulation, cells were treated with MTT (5 g/L, Sigma) for 4 h at 37 °C, and then dimethyl sulphoxide (Me<sub>2</sub>SO) was added into each well for 30 min. A 96-well microtitre plate reader (Pharmacia) was used to determine A<sub>570</sub>. All experiments were performed three times in triplicate.

**Apoptosis analysis** Cell apoptosis was identified by dual fluorescence staining with Hoechst 33342 and PI, flow cytometric analysis, and DNA fragmentation analysis.

For morphological examination of apoptosis, single cell suspensions were stained with Hoechst 33342 (10 mg/L, Sigma) and PI (5 mg/L, Sigma). One drop of the stained cell suspension was placed on a microscope slide and observed under a fluorescence microscopy (Olympus RX400).

Moreover, apoptosis was determined by flow cytometry analysis. Cells treated with allitridi as well as control cells were washed with 1×PBS twice and fixed with 70 % ethanol. After being centrifuged, cells were stained with PI (50 mg/L, Sigma) to investigate apoptosis by FACScan instrument (Beckman).

After lysed by lysis solution (Tris-Cl 10 mmol/L, pH 7.6; NaCl 100 mmol/L; edetic acid 10 mmol/L; 0.5 % SDS), cells were treated with RNase A (10 g/L, Sigma) at 37 °C for 3 h and subsequently with proteinase K (20 g/L, Sigma) at 37 °C overnight. The DNA fragments were then precipitated with 2.5 volumes of ethanol. DNA fragmentation was visualized by electrophoresis in 1.5 % agarose at 45 V for 2 h.

**Western blot** Cells were lysed by lysis buffer [Tris-Cl 50 mmol/L, pH 6.8; dithiothreitol (DTT) 100 mmol/L; 2 % sodium dodecyl sulfate (SDS); 10 % glycerol] and total protein was extracted. Of 180 μg protein samples were separated through 12 %-15 % polyacrylamide-sodium dodecyl sulfate gels (SDS-PAGE), and transferred onto nitrocellulose membrane. Immunoblotting was performed using the monoclonal antibody Bcl-2 (sc-7382, Santa Cruz), monoclonal antibody p53 (sc-99, Santa Cruz), monoclonal antibody Bax (sc-7480, Santa Cruz) and the polyclonal antibody caspase-3 (sc-7148, Santa Cruz), polyclonal antibody Actin (sc-1616, Santa Cruz). The secondary antibodies (anti-mouse and anti-rabbit) were from Amersham Pharmacia Biotechnology, and the secondary antibody (anti-goat) from Santa Cruz Biotechnology. The detection of specific protein binding was performed with the enhanced chemiluminescence Western blot detection system (Amersham Pharmacia Biotech).

**Caspase-3 activity assay** The activity of caspase-3 was measured using a fluorescence assay according to the manufacturer's instructions (Clontech). In brief, cells were lysed in chilled cell lysis buffer on ice for 10 min. The lysates were centrifuged at 20 000×g for 3 min at 4 °C. The supernatants were incubated with caspase-3 substrate, DEVD-AFC, at 37 °C for 1 h. The fluorescence of free AFC, generated as a result of cleavage of the DEVD-AFC bond, was monitored continuously with a Shimadzu RF-5000 Spectrofluorometer at excitation and emission wavelengths of 400 nm and 505 nm, respectively. Enzyme activity was calculated according to the formula provided by the manufacturer. The experiment was performed in triplicate.

**Statistical analysis** Data were expressed as

mean  $\pm$  SD and analyzed by one-way ANOVA and LSD test.  $P < 0.05$  was considered significant.

## RESULTS

**Growth Inhibition of BGC823 cell exposed to allitridi** Cell viability was significantly decreased by allitridi treatment in time- and dose-dependent manner (Fig 1A). Cell viability started to decrease 24 h after treatment of allitridi 25 mg/L and the ratio of growth inhibition is 91.85 % after 96 h (Fig 1B and 1C).

### Apoptosis occurred in allitridi-treated cells

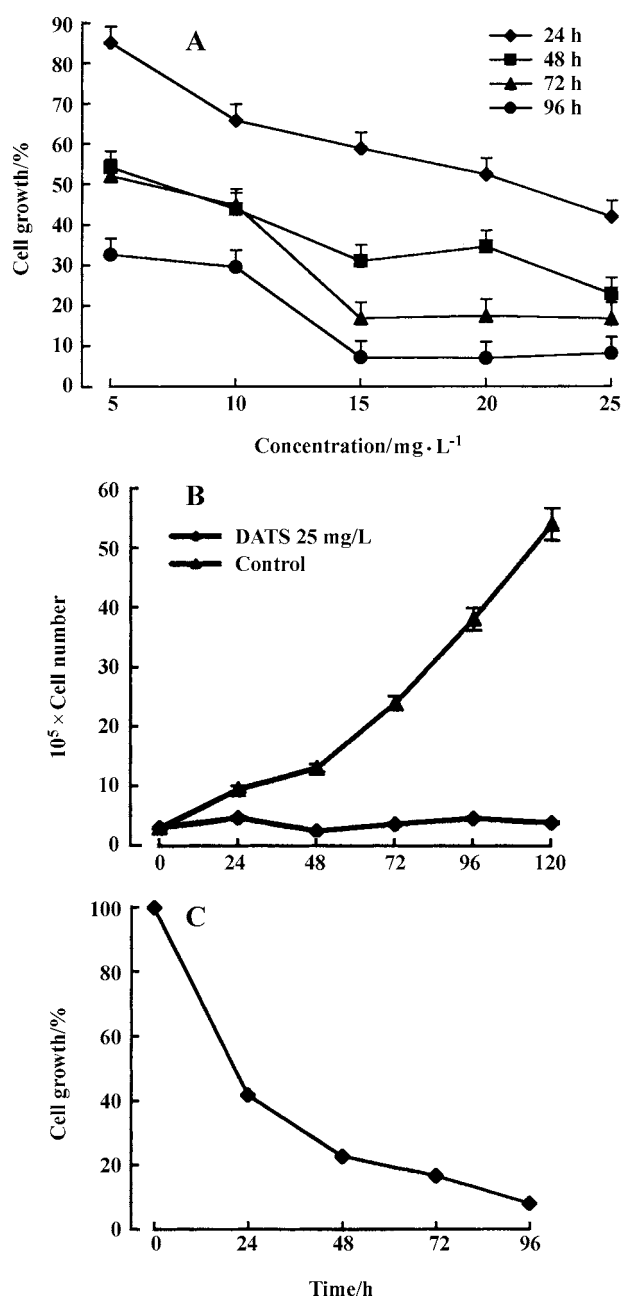
Staining of the cells with Hoechst 33342 and PI revealed that allitridi induced nuclear chromatin condensation and nuclear fragmentation (Fig 2A). Sub-G1 fraction was detected by flow cytometric analysis (Fig 2B). Moreover, agarose gel (1.5 %) electrophoresis also showed that treatment of cells with allitridi induced internucleosomal DNA fragmentation in the form of a laddering pattern (Fig 2C).

**Expression of Bcl-2, Bax, and p53 proteins** To further elucidate the mechanisms of allitridi-induced apoptosis in BGC823 cells, The protein levels of three key apoptosis-linked gene products, p53, Bcl-2, and Bax were measured. Compared with control cells, allitridi-treated cells decreased dramatically Bcl-2 protein expression but no change of Bax and p53 proteins. The ratio of Bcl-2 to Bax decreased, which began from 24 h and kept up to 72 h following allitridi treatment (Fig 3).

**Effect on the expression and activation of caspase-3 kinase** Procaspase-3 (32 kDa) present usually in normal cells. Only activated caspase-3 was into two units, 17 kDa and 12 kDa. However, only procaspase-3 and the subunit (17 kDa) were detected by Western blot. We found that the increased protein levels of procaspase-3 and the p17 fragment of active caspase-3 were detected after allitridi treatment for 72 h (Fig 4). Caspase-3 activity started to increase 72 h after allitridi treatment and was 5-fold higher than that of control cells at 96 h (Fig 4B).

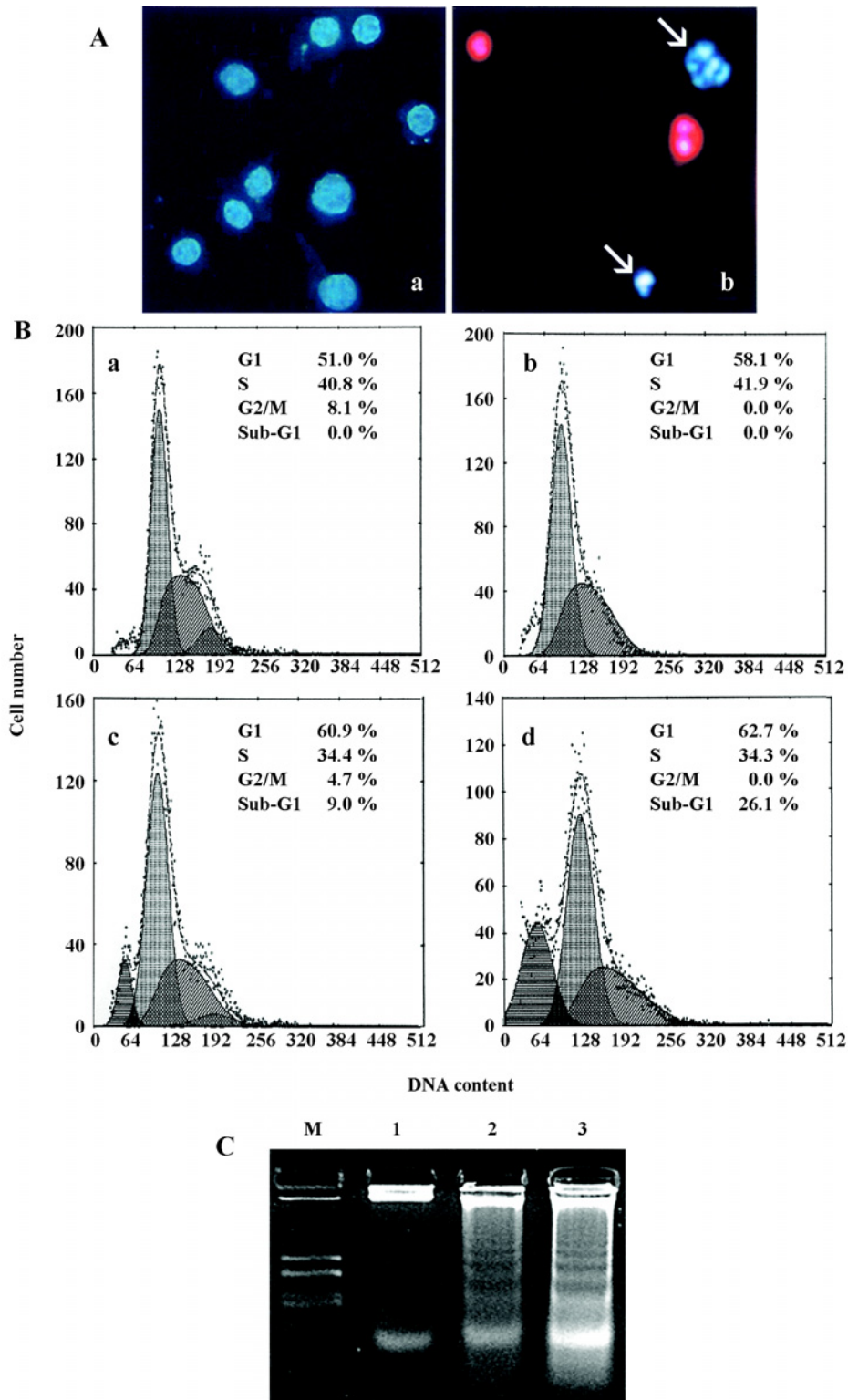
## DISCUSSION

In this study we explored the efficacy of allitridi for its antiproliferative activity against human gastric cancer cell line BGC823, and found that allitridi-induced apoptosis and suppressed cellular proliferation in BGC823 cells. Garlic contains several active organosulphur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), ajoene,

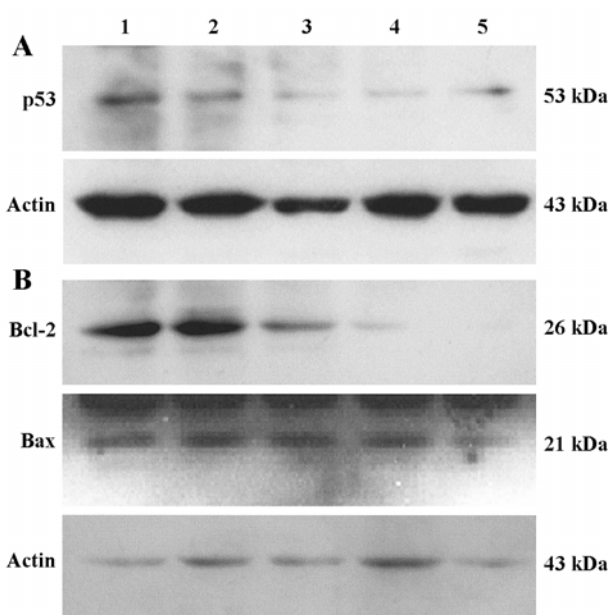


**Fig 1.** Effects of allitridi on BGC823 cells growth. (A) MTT assay. Cells were treated with various concentration of allitridi. After treated at various time (24, 48, 72, and 96 h), viable cells were exposed to MTT. (B) Cell growth curve. Cells were treated with allitridi 25 mg/L. At designed time (1, 2, 3, 4, and 5 d) cells were harvested and counted. The number of control cells ( $3 \times 10^5$ - $54 \times 10^5$ ) increased, but the number of allitridi-treated cells ( $3 \times 10^5$ - $3.9 \times 10^5$ ) remained unchanged. (C) MTT assay. Cells were treated with allitridi 25 mg/L. At various time (24, 48, 72, and 96 h), viable cells were exposed to MTT. The ratio of cell growth by allitridi decreased from 41.85 % (24 h) to 8.15 % (96 h).

*S*-allylmercaptocysteine (SAMC). Up to now some studies about DADS<sup>[32]</sup>, ajoene<sup>[36]</sup>, and SAMC<sup>[23]</sup> had been



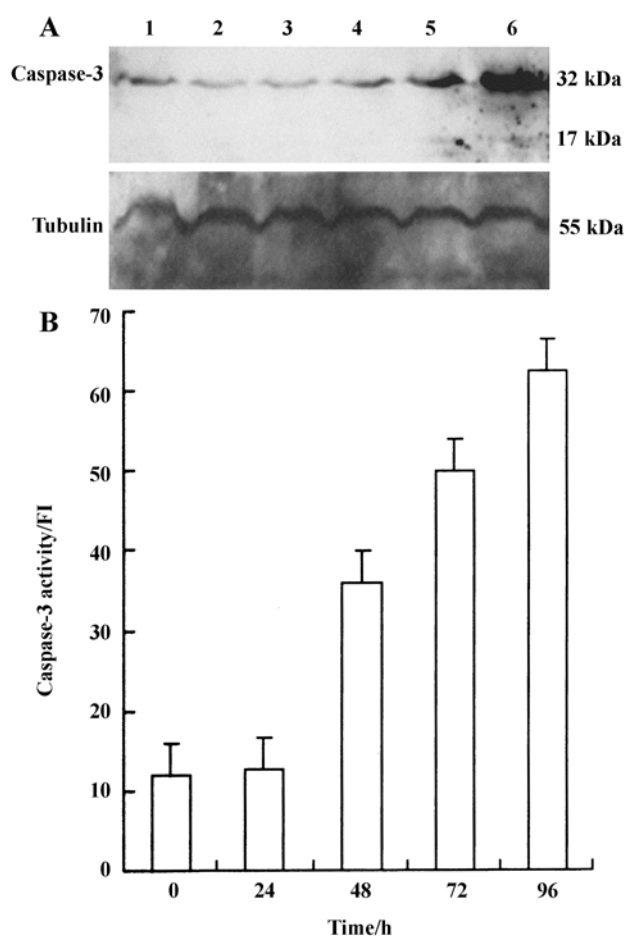
**Fig 2.** Allitridi-induced apoptosis. (A) Nuclear morphological analysis. Cells were treated with allitridi 25 mg/L for 96 h and stained with Hoechst 33342 and PI. Stained cells were observed by a fluorescence microscopy. Potential apoptotic nuclei are indicated by arrows. a: control; b: allitridi 25 mg/L treated. (B) Flow cytometric assay. Sub-G1 peak appeared in cells exposed to allitridi. Sub-G1 peaks were indicated by arrows. a: control; b: allitridi 25 mg/L treated for 48 h; c: allitridi 25 mg/L treated for 72 h; d: allitridi 25 mg/L treated for 96 h. (C) Internucleosomal DNA fragmentation. M: marker; Lane 1: control; Lane 2: allitridi 25 mg/L treated for 48 h; Lane 3: allitridi 25 mg/L treated for 72 h.



**Fig 3.** Effect of allitridi on expression of apoptosis relative proteins. allitridi down-regulated Bcl-2 expression but did not change expression of Bax and p53 significantly. The sizes of molecular weight markers are shown on the right, and the names of proteins are shown on the left, respectively. Lane 1: control; Lane 2: allitridi 25 mg/L treatment for 12 h; Lane 3: 24 h; Lane 4: 48 h; Lane 5: 72 h.

demonstrated to induce apoptosis and inhibit proliferation of various cell types (leukemia, colon, breast, and lung cancer cells). Here we first report that allitridi (containing DATS, purity 97.98 %) induced apoptosis and suppressed cellular proliferation in human gastric cancer cells. Our previous study had shown a primary data of allitridi-induced apoptosis<sup>[25]</sup>, but the present study did focus on its molecular mechanism. And we assumed that expect for the blockage of cell cycle (data not shown), the inhibitory effect of allitridi on BGC823 cell growth may be in part due to the induction of apoptosis.

Apoptosis plays an essential role as a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells. Inducing cancer cell apoptosis can inhibit their proliferation and then is a potential anti-tumor method. It is well known that apoptosis involves cellular nuclear changes. Nuclear condensation and nuclear fragmentation are common features of apoptotic cells<sup>[26]</sup>. And DNA fragmentation has also been observed in cells undergoing apoptosis. This cleavage produces ladders of DNA fragments that are the size of integer multiples of a nucleosome length (180-200 bp)<sup>[27]</sup>. Due to their characteristic patterns



**Fig 4.** Effect of allitridi on caspase-3 protein expression and its activity. (A) caspase-3 protein expression. The specific cleavage of caspase-3 was observed 72 h after treatment with allitridi. Lane 1: control; Lane 2: allitridi 25 mg/L treatment for 12 h; Lane 3: allitridi 25 mg/L treatment for 24 h; Lane 4: allitridi 25 mg/L treatment for 48 h; Lane 5: allitridi 25 mg/L treatment for 72 h; Lane 6: 96 h. (B) caspase-3 activity. Mean $\pm$ SD. <sup>b</sup> $P$ <0.05, <sup>c</sup> $P$ <0.01 vs control.

revealed by agarose gel electrophoresis, these nucleosomal DNA ladders are widely used as biochemical markers of apoptosis. In addition, loss of DNA may occur as a result of the shedding of apoptotic bodies containing fragments of nuclear chromatin. Thus, apoptotic cells often show a deficit in DNA content, and when stained with a DNA-specific fluorochrome can be recognized by the "sub-G1" peak on DNA content histogram<sup>[28]</sup>. In our studies, nuclear condensation and fragmentation, Sub-G1 peak and DNA fragmentation were detected. Thus, it is obvious that allitridi can induce BGC823 cell apoptosis.

To further investigate its molecular mechanism, we measured the protein levels of three key apoptosis-linked gene products, p53, Bcl-2 and Bax in allitridi-

treated BGC823 cell, which are known to regulate the cell death/survival. The ability of different garlic compounds to affect these proteins was previously reported. For example, Z-ajoene led to caspase-dependent Bcl-2 cleavage<sup>[37]</sup>, while diallyl disulfide induced apoptosis by a p53-dependent manner<sup>[32,38]</sup>. Our data showed that allitridi, another garlic compound, significantly decreased Bcl-2 expression, and did not change Bax and p53 expression in BGC823 cells. Thus, we suggest that allitridi can decrease the ratio of Bcl-2 to Bax by a p53-independent manner. The ratio of Bcl-2 to Bax within a cell is critical to determining its survival or death<sup>[29,30]</sup>. The low ratio of Bcl-2 to Bax might contribute to the initiation of apoptosis in allitridi-treated BGC823 cells. In cells exposed to allitridi, decreased Bcl-2 protein resulted in Bax in relatively excess and forming Bax-Bax homodimer, which makes cells susceptible to apoptosis. Incidentally, the decreased Bcl-2 may have no ability to block apoptosis induced by allitridi.

Caspases are central components of the cell death machinery<sup>[33-35]</sup>. Of these, caspase-3 is a central executioner of caspases and its activation is critical to apoptosis, which directly cleaves various proteins, resulting in morphological and biochemical changes and then leading to apoptosis. Our data showed that allitridi increased caspase-3 protein expression and activity. Recently, some reports also showed an enhanced caspase-3 activity by different garlic compounds in various cell types<sup>[31,39]</sup>. It is caspase-3 activation that may contribute to DNA fragmentation and nuclear morphologic changes. Based on these findings, we hypothesize that allitridi may affect the open of mitochondrial permeability transition pore, subsequently results in the release of cytochrome c and followed by the increased activation of caspase-3. And we have been working to further investigate the mechanism of allitridi-induced apoptosis.

Taken together, these results demonstrate that allitridi is an effective agent in suppressing human gastric cancer cell proliferation and suggest that growth inhibition may be partly due to its induction of apoptosis through down-regulation of Bcl-2 and increased caspase-3 activity. From epidemiological investigations and laboratory experiments, we suggest, allitridi may serve as potential targets for future drug or therapeutic developments for prevention and treatment of gastric cancer.

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