Diallyl disulfide selectively causes checkpoint kinase-1 mediated G_2/M arrest in human MGC803 gastric cancer cell line

HUI LING[1,2*], LI-FENG LU[1-3*], JIE HE[1,2], GUO-HUA XIAO[1,2], HAO JIANG[2] and QI SU[1,2]

[1]Key Laboratory of Tumor Cellular and Molecular Pathology (University of South China), College of Hunan Province, Cancer Research Institute and [2]Center for Gastric Cancer Research of Hunan Province, University of South China, Hengyang, Hunan 421001; [3]Department of Pathology, Hospital of Jiaonan Economic-Technological Development Area, Jiaonan, Shandong 266400, P.R. China

Received April 15, 2014; Accepted August 4, 2014

DOI: 10.3892/or.2014.3417

Abstract. Previous studies have shown that diallyl disulfide (DADS), a naturally occurring anticancer agent in garlic, arrested human gastric cancer cells (MGC803) in the G_2/M phase of the cell cycle. Due to the importance of cell cycle redistribution in DADS-mediated anticarcinogenic effects, we investigated the role of checkpoint kinases (Chk1 and Chk2) during DADS-induced cell cycle arrest. In the present study, the northern blot analysis showed that mRNA expression of for Chkl and Chk2 was unchanged. Notably, DADS induced the accumulation of phosphorylated Chk1, but not of Chk2, activated phospho-ATR (ATM-RAD3-related gene), and dowregulated CDC25C and cyclin B1 expression. Furthermore, CDC25C was immunoprecipitated by anti-Chk1 but not anti-Chk2. Results of the overexpression and knockdown studies, showed that Chk1 but not Chk2 regulated the DADS-induced G_2/M arrest of MGC803 cells. The overexpression of Chk1 resulted in significantly increased DADS-induced G_2/M arrest, increased DADS-induced Chk1 phosphorylation and inhibited CDC25C expression. Knockdown of Chk1 reduced DADS-induced G_2/M arrest and blocked the DADS-induced inhibition of CDC25C and cyclin B1 expression. These results suggested that Chk1 is important in DADS-induced cell cycle G_2/M arrest in the human MGC803 gastric cancer cell line. Furthermore, the DADS-induced G_2/M checkpoint response is mediated by Chk1 signaling through ATR/Chk1/CDC25C/cyclin B1.

Correspondence to: Professor Qi Su, Key Laboratory of Cancer Cellular and Molecular Pathology of Hunan Provincial University, Cancer Research Institute, University of South China, 28 West Changsheng Road, Hengyang, Hunan 421001, P.R. China
E-mail: suqi1945@163.com

Dr Hao Jiang, Center for Gastric Cancer Research of Hunan Province, University of South China, 28 West Changsheng Road, Hengyang, Hunan 421001, P.R. China
E-mail: jhao16859@163.com

*Contributed equally

Abbreviations: DADS, diallyl disulfide; ATR, ATM-RAD3-related gene; Chk1, checkpoint kinase 1; CDC25C, cell division cycle 25C; PBS, phosphate-buffered saline

Key words: diallyl disulfide, G_2/M phase, checkpoint kinase-1, gastric cancer

Introduction

Although the incidence of gastric cancer has been on the decrease in most industrialized nations over the past two decades, it remains the second leading cause of cancer-related mortalities worldwide. The incidence of this cancer type is highest in Japan, Korea, China, Latin America and Eastern Europe (1). In Western countries such as the USA the incidence is lower, with 21,000 new cases diagnosed each year. Despite recent advances in surgery and chemotherapy, the 5-year survival rate for gastric cancer patients remains extremely low (2). Therefore, identifying alternative factors that may reduce the initiation and promotion of gastric cancer is important to minimize the incidence and severity of this disease.

Diallyl disulfide (DADS), a main organosulfur component responsible for the diverse biological effects of garlic, exhibits a wide variety of internal biological activities and has shown potential as a therapeutic agent in various types of cancer (3-7). The antiproliferative property of DADS in cultured human colon tumor cells (HCT-116 and SW480), leukemic and prostate (PC-3) cancer cell lines is connected with its ability to arrest cells in the G_2/M phase (8-11). Changes in activity may also result from a combination of the quantity and activity of specific cellular proteins. For example, the hyperphosphorylation of p34(cdc2) kinase was increased by 15% following the exposure of colon cells to DADS, and DADS also reduced CDC25C protein expression (12). Ashra and Rao (13) demonstrated that the elevated phosphorylation of checkpoint kinase 1 (Chk1), decreased phosphorylation of Chk2, and decreased levels of CDC25C, 14-3-3 and cyclin B1 were the critical changes associated with the abrogation of the G_2/M checkpoint control.

Previously, we reported that DADS inhibits the growth of gastric cancer *in vitro* and *in vivo* (6,14) and that it induces cell cycle G_2/M arrest in human MGC803 gastric cancer cells (15). Decreased CDC25C expression is crucial in G_2/M

arrest following treatment with DADS (15). The aim of the present study was to investigate the underlying mechanism involved in the induction of G_2/M phase cell cycle arrest by DADS in the human MGC803 gastric cancer cell line, with special emphasis on its role in the key G_2/M checkpoint kinase-Chk1 and -Chk2. We showed that DADS inhibited the growth of BGC823 cells, which was associated with the phosphorylation of ATR (ATM-RAD3-related gene) and Chk1 while suppressing the expression of CDC25C and cyclin B1 (16). In the present study, DADS was shown to induce phosphorylation of ATR and Chk1 while suppressing the expression of CDC25C and cyclin B1 in the human MGC803 gastric cancer cell line. The results demonstrated that DADS selectively causes Chk1-mediated G_2/M arrest in this cell line. These observations contribute to understanding the mechanisms of the antitumor effect of DADS in gastric cancer cells, and indicate the potential of DADS for clinical development as a therapeutic drug to combat gastric cancer.

Materials and methods

Chemicals and reagents. DADS was purchased from Fluka Chemika (Ronkonkoma, NY, USA). Phospho-Chk1 (Ser345) antibody, phospho-ATR (Ser428) and ECL LumiGLO reagent were obtained from Cell Signaling Technology, Inc. (CST, USA). The phospho-Chk2 (Thr68) antibody, ATR antibody and anti-rabbit IgG (HRP-linked) Chk1 and Chk2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The CDC25C, cyclin B1 and β-actin antibodies were from Boster Company (Boster, China).

Cell line and cell culture. The human MGC803 gastric cancer cell line was obtained from the Cell Research Institute of the Chinese Academy of Science (China). The cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA), 100 μg/ml streptomycin and 100 U/ml penicillin G (Life Technologies) in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. The cells were suspended at a final concentration of $1x10^4$ cells/ml in RPMI-1640 medium containing 10% FBS in culture flasks. Reagents were added to each flask in various combinations and incubated at 37˚C in 5% CO_2.

Northern blot analysis. RNA from the cells obtained at each time point was extracted with TRIzol (Invitrogen). Reverse transcription (RT) was performed with an RT kit (Promega, Madison, WI, USA). Total RNA (1 mg) was used as a template, and RT-generated cDNA encoding Chk1, Chk2 and β-actin (internal control) was amplified by PCR. Primer sequences were: forward 5'-CTG AAG AAG CAG TCG CAGTG-3' and reverse 5'-TTC CAC AGG ACC AAA CATCA-3' for Chk1; forward 5'-TCC GCT TGC TGA TGA TCT TTA TGG-3' and reverse 5'-GAC CTA CTC CTT GGG CTC GGC TAT-3' for Chk2; and forward 5'-CGT CAT ACT CCT GCTT-3' and reverse 5'-ATC TGG CAC CAC ACCT-3' for β-actin. The primers were designed using Primer Premier 5.0 software (Premier Company, Canada) and were synthesized by the Shanghai Sangon Biological Technology and Services Co., Ltd. (Shanghai, China). Amplification parameters were 95˚C for 5 min, and 30 cycles of 95˚C (30 sec), 60˚C (15 sec), 72˚C (60 sec) and 72˚C (60 sec) using 3 μl cDNA.

Northern blots were performed according to standard procedures (17). Total RNA (20-30 μg) was separated on 1.2% formaldehyde agarose gel and transferred to a polyamide membrane (Millipore, Billerica, MA, USA). cDNA oligonucleotide probes were labeled with α^{32}P-ATP using terminal deoxynucleotidyl transferase recombinant enzyme (Promega) as recommended by the manufacturer's instructions, followed by pre-hybridization (2 h) at 68˚C, heat denaturation (5 min) at 100˚C and hybridization (16 h) at 68˚C. The membrane was air-dried on blotting paper and then exposed to X-ray film by autoradiography at -70˚C for 24-48 h in a cassette containing an intensifying screen.

Western blot analysis. Human gastric cancer cells were cultured with or without DADS at the indicated concentrations for various periods of time, washed once with ice-cold phosphate-buffered saline (PBS), and lysed in a buffer consisting of 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol, 100 mM NaF and 1% Triton X-100. Protein concentrations in the lysates were measured with the Protein BCA sssay kit (Bio-Rad, Hercules, CA, USA). Protein lysate (30 μg) was subjected to 10% SDS-polyacrylamide gel electrophoresis and the proteins separated were transferred to polyvinylidene difluoride membranes (Millipore). To block non-specific binding, the membranes were incubated at room temperature for 1 h with 5% skim milk powder, followed by a 12 h incubation at 4˚C with an anti-serum containing antibodies against Chk1, Chk2, ATR, phospho-Chk1 (Ser345), phospho-Chk2 (Thr68), phospho-ATR (Ser428), CDC25C and cyclin B1. A peroxidase-conjugated secondary antibody (1:5,000 dilution) and ECL western blotting detection reagents were used to visualize the target proteins (ECL New England BioLabs, Ipswich, MA, USA), which were quantified with a BioImage Intelligent Quantifier 1-D (version 2.2.1; Nihon-BioImage Ltd., Japan).

Immunoprecipitation. Human gastric cancer cells were cultured with or without DADS at the indicated concentrations for various periods of time. The cells were characterized using a Seize (R) Classic Mammalian Immunoprecipitation kit (Piece Technology). Then cells were removed from the culture medium, washed once with PBS (0.1 M phosphate, 0.15 M NaCl, pH 7.2), harvested and lysed using lysis buffer of the M-PER reagent. The lysates were collected, transferred to a microcentrifuge tube and centrifuged at 13.000 x g for 5-10 min to separate the cell debris. The supernatants were transferred to another tube for subsequent analysis. Purified Chk1 or Chk2 antibody was added to the sample, followed by overnight incubation at 4˚C. The immune complex was added to the spin cup containing equilibrated protein G beads. Elution buffer (190 μl) was added to the spin cup and the samples underwent elution. Immunoprecipitated protein levels were determined by western blot analysis with the anti-CDC25C antibody and the ECL detection system.

Establishment of a stable MGC803 cell line expressing Chk1 or Chk2. PCR primers were designed and synthesized according to

human Chk1 or Chk2 cDNA sequences (GenBank), including *Bam*HI and *Hin*dIII restriction sites using these sequences: forward, 5'-CGG AAG CTT ATG GCA GTG CCC TTTG-3' and reverse, 5'-CGG CGA ATT CTC ATG TGG CAGGA-3' for Chk1; forward, 5'-CGC CAA GCT TAT GTC TCG GGA GTC-3' and reverse, 5'-CGG AAT TCT CAC AAC ACA GCA GCA CAC-3' for Chk2. The Chk1 or Chk2 gene was amplified from the plasmid expression vector pcDNA3.1(+)-Chk1 and pcDNA3.1(+)-Chk2 by PCR. The amplified Chk1 or Chk2 gene products and the plasmid vector pcDNA3.1(+) were digested with restriction endonucleases *Bam*HI and *Hin*dIII (Jingmei Biotech Co., Ltd., China), and linked with T4 DNA ligase (Shanghai Sangon Biological Technology and Services Co., Ltd.). The new recombinant plasmid vectors were transferred into competent DH5α bacteria to obtain a recombinant pcDNA3.1(+)-Chk1 or pcDNA3.1(+)-Chk2 vector, and subcloned PCR fragments were confirmed by DNA sequencing analysis (Shanghai Sangon Biological Technology and Services Co., Ltd.).

MGC803 cells were seeded in a 24-well plate and infected with a viral medium of 500 μl. Plasmid DNA (0.8 μg) in 50 μl non-serum Opti-MEM medium containing 2 μl Lipofectamine 2000 (Invitrogen Life Technologies) was subsequently added to each well. The mixtures were incubated for 5 h, and 3 ml fresh medium was added. On the day following infection, the cells were passaged at a 1:10 ratio into selective medium containing G418 (400 μg/ml). When cloned MGC803 cells became distinct, the drug concentration used for screening was reduced and the MGC803 cells were transferred into a new culture flask for cell growth. Chk1 or Chk2 protein expression was detected by western blot analysis.

RT-PCR. Chk1 or Chk2 mRNA was detected by RT-PCR according to the manufacturer's instructions. The primers for Chk1, Chk2 and β-actin were the same as those for northern blotting. Amplification parameters were 95˚C for 5 min, and 30 cycles of 95˚C (30 sec), 60˚C (15 sec), 72˚C (60 sec) and 72˚C (60 sec) using 3 μl cDNA. Standards were run in duplicate and samples in triplicate. The expression levels for all the genes analyzed were normalized to β-actin.

Chk1 or Chk2 siRNA transfection. Cells were seeded at $3x10^5$ cells/well in 6-well plates and allowed to grow overnight. The following day, the cells were left untreated, Lipofectamine-transfected, or transfected with Chk1 siRNA or Chk2 siRNA (both from Santa Cruz Biotechnology, Inc.). Transfections were performed using Lipofectamine 2000 and Opti-MEM-reduced serum medium (both from Santa Cruz Biotechnology, Inc.). The cells were transiently transfected with siRNA specifically targeting Chk1 or Chk2 (both from Santa Cruz Biotechnology, Inc.) at a final concentration of 100 nM using a transfection reagent for 24 h. Following transfection, the cells were left untreated or exposed to DADS for the indicated time. Cellular protein was extracted and subjected to western blot analysis of the targeted proteins (Chk1 and Chk2).

Real-time PCR and western blot analysis. Chk1 or Chk2 mRNA were detected according to the manufacturer's instruction. The used were: Chk1, forward 5'-CGG TAT AAT AAT CGT GAGCG-3' and reverse 5'-TTC CAA GGG TTG AGG TATGT-3'; Chk2, forward 5'-CTC GGG AGT CGG ATG TTGAG-3' and reverse 5'-GAG TTT GGC ATC GTG CTGGT-3'; and β-actin forward 5'-AAG AAG GTG GTG AAG CAGGC-3' and reverse 5'-TCC ACC ACC CTG TTG CTGTA-3'. Chk1 or Chk2 protein expression was detected by western blot analysis.

Cell cycle analysis. Cells were incubated in culture medium alone or in culture medium containing 30 mg/l DADS, at 37˚C for 12, 24, 36 and 48 h. The cells were harvested in cold PBS, fixed in 700 ml/l ethanol and stored at 4˚C for subsequent cell cycle analysis. Fixed cells were washed with PBS once and suspended in 1 ml propidium iodide-staining reagents (20 mg/l ribonuclease and 50 mg/l propidium iodide). Samples were incubated in the dark for 30 min prior to cell cycle analysis, and cell distribution in the various phases of the cell cycle was measured with a flow cytometer (Coulter EPICS XL; Beckman, Miami, FL, USA). The percentage of cells in the G_1, S and G_2/M phases was then calculated by CellQuest software on the flow cytometer (Coulter EPICS XL).

Statistical analysis. Results were analyzed by the SPSS 11.5 statistical software package. Data were expressed as means ± SD (standard deviation). Comparisons between different groups were made by one-way ANOVA or the Student's t-test. $P<0.05$ was considered to indicate a statistically significant result.

Results

Expression and activation of Chk1 and Chk2 induced by DADS. The expression of Chk1 and Chk2 mRNA associated with the cell cycle arrest of MGC803 cells was revealed by northern blotting. After treatment with 30 mg/l DADS for 12, 24, 36 and 48 h, the expression of Chk1 and Chk2 mRNA showed no significant changes when compared to the untreated cells ($p>0.05$).

Immunoblot analyses of phospho-Chk1 and Chk2 protein in MGC803 cells revealed that 30 mg/l DADS enhanced the levels of phospho-Chk1 protein in a time-dependent manner as compared with the untreated cells from 1 to 12 h ($p<0.05$). However, the phospho-Chk2 protein was not activated by DADS ($p>0.05$) (Fig. 1).

DADS regulates the protein expression levels in the G_2/M cell cycle. DADS (30 mg/l) inhibited the expression of the cell cycle-associated phosphatase CDC25C in MGC803 cells after 12 h, which was decreased by 81% after 48 h ($p<0.05$) (Fig. 2). As shown in Fig. 2A, the expression of cyclin B1 increased after 12 h of DADS treatment, and then decreased after 36 h, being reduced by 87.5% after 48 h ($p<0.05$).

ATR mediates checkpoint signaling through its downstream effect or Chk1 (14). As shown in Fig. 2B, 30 mg/l DADS treatment of MCC803 cells for 15 min to 2 h resulted in an increase in phospho-ATR expression, whereas no change was found in ATR expression.

The interaction between endogenous Chk1 and CDC25C was determined by co-immunoprecipitation using anti-Chk1 or anti-CDC25C. Western blot analysis showed that CDC25C was present in the immunoprecipitate of anti-Chk1 (Fig. 2C). Chk1 was also co-immunoprecipitated with

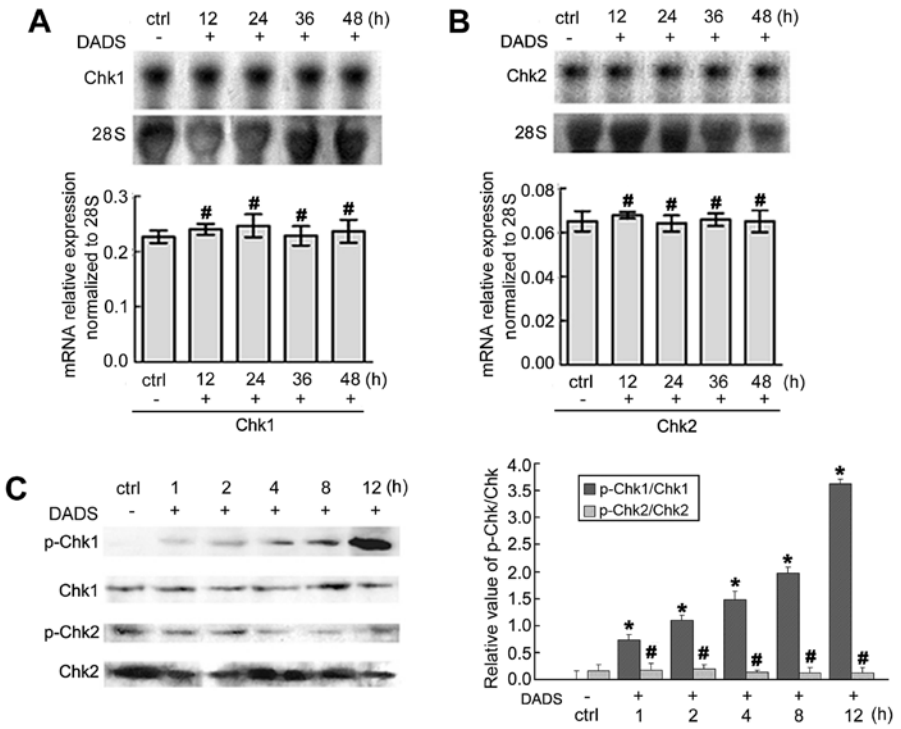

Figure 1. Northern and western blot analyses of Chk1 and Chk2 expression and phosphorylation induced by DADS. (A) Expression of Chk1 mRNA induced by DADS. There were no significant differences ($^{\#}$p>0.05 vs. 0 h control). (B) Expression of Chk2 mRNA induced by DADS. There were no significant differences ($^{\#}$p>0.05 vs. 0 h control). (C) Activation of Chk1 (*p<0.05 vs. 0 h control) and Chk2 ($^{\#}$p>0.05 vs. 0 h control) phosphorylation by DADS. The data are reported as means ± SD (n=3). Chk1, checkpoint kinase 1; DADS, diallyl disulfide.

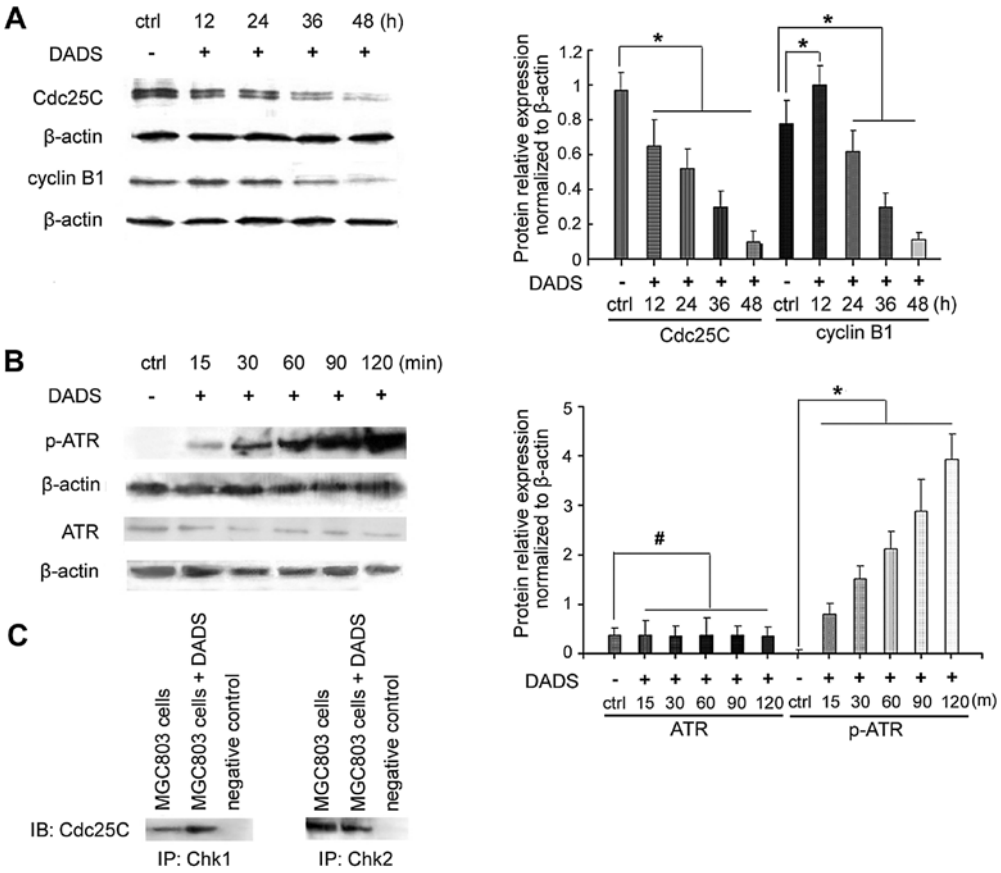

Figure 2. Effect of DADS on mitosis-associated proteins and the interaction between Chk1 or Chk2 with CDC25C in the MGC803 cell line. (A) Expression of CDC25C and cyclin B1 by western blotting. *p<0.05 vs. control. (B) ATR protein expression and phosphorylation by western blotting. *p<0.05 and $^{\#}$p>0.05 vs. control. (C) Expression of Chk1 and Chk2 in MGC803 cells by immunoprecipitation (IP) and western blotting. DADS, diallyl disulfide; Chk1, checkpoint kinase 1.

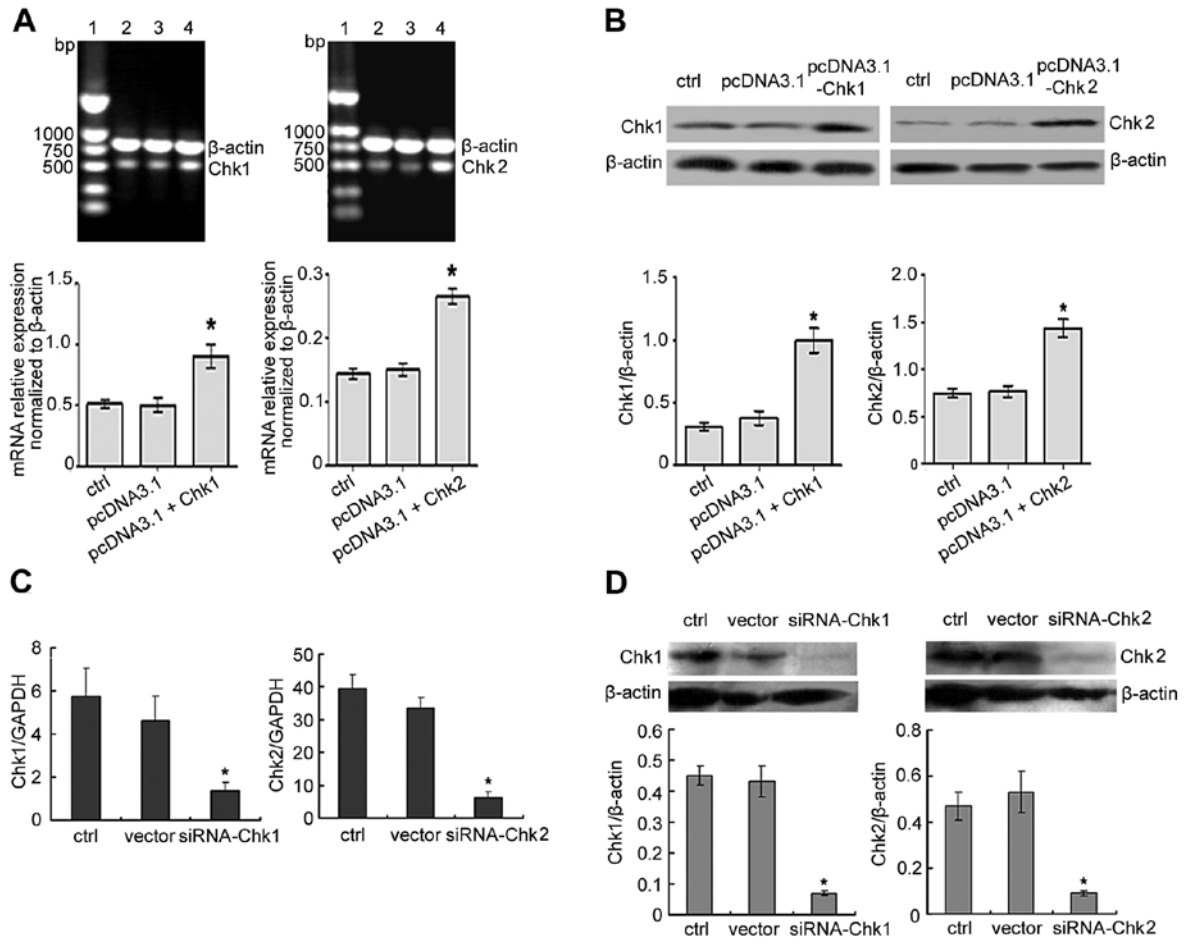

Figure 3. RT-PCR, qRT-PCR and western blot analysis of Chk1 or Chk2 expression levels in transgenic cell lines. (A) The mRNA expression Chk1 or Chk2 was significantly upregulated in the pcDNA3.1(+)-Chk1 or pcDNA3.1(+)-Chk2 transfection group. *P<0.05 compared to control and vector groups. There was no significant difference in the control and vector groups. (B) The Chk1 or Chk2 protein expression in different groups, with bar graphs showing the quantitative densitometric analysis of the protein amount of Chk1 or Chk2 normalized to β-actin. *P<0.05 compared to the the control and vector groups. (C) The mRNA expression of Chk1 or Chk2 was significantly downregulated in the knockdown groups. *P<0.05 compared to the control and vector groups. There was no significant difference in the control and vector groups. (D) The Chk1 or Chk2 protein expression in the knockdown groups was markedly decreased compared to the control and vector groups. *P<0.05 compared to the control and vector groups. Chk1, checkpoint kinase 1.

anti-CDC25C (Fig. 2C). Chk1 and CDC25C were not detected in the immunoprecipitate of rabbit IgG, which was used as a negative control. This result indicated that Chk1 may interact with CDC25C.

Generation of Chk1 or Chk2 overexpressing/knockdown MGC803 human gastric cancer cell line. To investigate the functional role of Chk1 or Chk2 in the cell cycle arrest of DADS-induced human MGC803 gastric cancer cell line, cell lines were produced with overexpressed or reduced levels of Chk1 or Chk2. Overexpression and knockdown were confirmed by RT-PCR, RT-qPCR and western blotting. Antibody labeling showed that levels of Chk1 or Chk2 protein were increased in overexpressed cells compared to the controls (Fig. 3A and B). By contrast, the knockdown cells showed reduced levels of Chk1 or Chk2 expression compared to controls in the MGC803 cell line (Fig. 3C and D).

Overexpression of Chk1 but not Chk2 promotes DADS-induced G_2/M arrest by activating phospho-Chk1 and decreasing CDC25C expression. We evaluated whether Chk1 and Chk2 expression enhanced DADS-induced cell cycle arrest in MGC803 cells. Flow cytometry revealed the proportion of cells in the G_2/M phase. FACS analysis indicated there were no difference in the G_2/M phase between pcDNA3.1(+)/Chk1-transfected cells or pcDNA3.1(+)/Chk2-transfected cells and untransfected control cells ($p>0.05$) (Fig. 4). However, 30 mg/l DADS induced a higher percentage of G_2/M phase cells in pcDNA3.1(+)/Chk1-transfected cells (41.9%, 48 h) than in untransfected control cells (25%, 48 h) ($p<0.05$) (Fig. 4). By contrast, DADS did not have this effect on pcDNA3.1(+)/Chk2-transfected cells. Thus, the results suggested that the overexpression of Chk1, but not Chk2, enhanced DADS-induced G_2/M arrest in MGC803 cells.

The effect of DADS on the expression and phosphorylation of Chk1 or Chk2 was also examined. Western blot analysis showed that Chk1 phosphorylation was increased in Chk1-transfected MGC803 cells in a time-dependent manner following treatment with DADS (30 mg/l) at 2, 4, 8 and 12 h ($p<0.05$). By contrast, Chk2 phosphorylation remain unchanged in Chk2-transfected MGC803 cells by DADS ($p>0.05$). DADS reduced the expression of CDC25C in pcDNA3.1(+)/Chk1-transfected cells (Fig. 4). After stimulation with 30 mg/l DADS for 12 h the CDC25C expression showed a significant decrease, and continued to decrease gradually

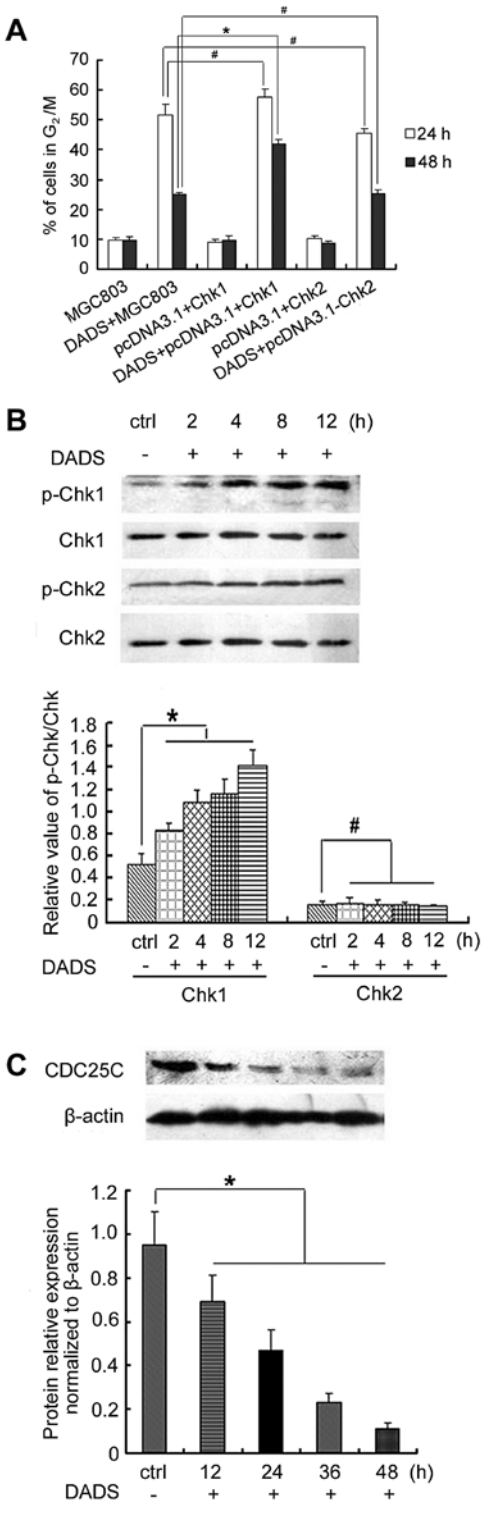

Figure 4. Effect of Chk1 or Chk2 overexpression on DADS-induced G_2/M arrest. (A) The changes in the percentage of MGC803 cells in G_2/M phases. *p<0.05 vs. (DADS+MGC803) group. #P>0.05 vs. (DADS+MGC803) group. (B) Level of phospho-Chk1 and -Chk2. *P<0.05 and #P>0.05 vs. control group. (C) Expression of CDC25C by pcDNA3.1(+)/Chk1-transfected MGC803 cells. *P<0.05 vs. control group. The data are reported as means ± SD (n=3). Chk1, checkpoint kinase 1; DADS, diallyl disulfide.

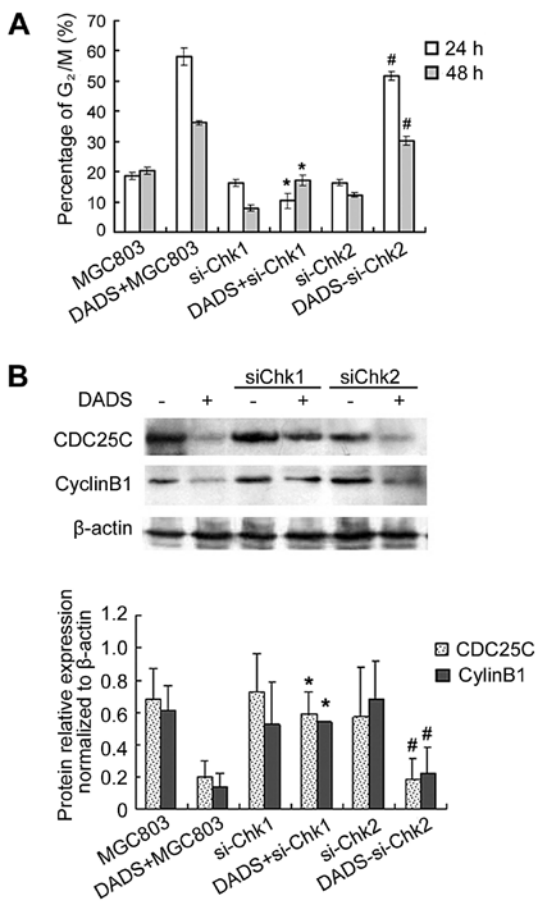

Figure 5. Effect of Chk1 or Chk2 knockdown on DADS-induced G_2/M arrest. (A) The changes in the percentage of MGC803 cells in G_2/M phases. *P<0.05 compared to (DADS+MGC803) group and #p>0.05 compared to (DADS+MGC803) group. (B) Expression of CDC25C and cyclin B1 in Chk1 or Chk2 knockdown MGC803 cell lines by western blotting. The data are reported as means ± SD (n=3). *P<0.05 compared to (DADS+MGC803) group and #P>0.05 compared to (DADS+MGC803) group. Chk1, checkpoint kinase 1; DADS, diallyl disulfide.

in a time-dependent manner (p<0.05). A high Chk1 expression was able to increase G_2/M arrest induced by DADS in MGC803 cells, and the Chk1/CDC25C pathway was involved in DADS-induced G_2/M arrest.

Knockdown of Chk1 but not Chk2 inhibits DADS-induced G_2/M arrest by inhibiting expression of CDC25C and cyclin B1. After MGC803 cells were treated with 30 mg/l DADS for 24 h, G_2/M cells increased to 51.5% (24 h) and 25% (48 h), which was significantly higher than those of the untreated control group. However, siChk1 transfection markedly reduced the proportion of DADS-induced G_2/M cells, which showed that the G_2/M phase ratio of siChk1-transfected cells was significantly reduced after treatment with 30 mg/l DADS compared to the untransfected cells, particularly in the 24 h treatment group, which was reduced from 58.1 to 10.4%. These results demonstrated that DADS-induced G_2/M arrest was significantly inhibited by siChk1, but that siChk2 transfection had no effect on DADS-induced G_2/M ratio in MGC803 cells (Fig. 5).

Western blotting showed that although DADS reduced the expression of CDC25C and cyclin B1 in untransfected cells, inhibition of CDC25C and cyclin B1 expression treated by DADS was blocked by Chk1 gene silencing (p<0.05). By contrast, Chk2 gene silencing did not have this effect (Fig. 5). These results suggested that Chk1 gene silencing abrogated G_2/M arrest induced by DADS in the MGC803 cell line, and

that the Chk1/CDC25C/cyclin B1 pathway was involved in the G_2/M arrest induced by DADS.

Discussion

The focus of the present study was to understand the mechanisms responsible for activation of the G_2/M checkpoint in response to DADS treatment of human gastric cancer cells. Previous studies have shown that DADS, the major component of cooked garlic, suppresses the proliferation of cancer cells in, for example, gastric and colon cancer, and leukemic cells (8-11,16). However, the mechanisms by which DADS inhibits the proliferation of cancer cells remain to be determined, since little is known about DADS-induced G_2/M arrest.

Cell cycle arrest and apoptosis are two important mechanisms involved in anticancer drug treatment (18,19). In a previous study, we reported that DADS-induced G_2/M arrest and differentiation of MGC803 cells involved activation of the p38 MAP kinase and ERK1/2 signaling pathway (6,15). Although these phenomena occurred in a time-dependent manner, the G_2/M arrest-inducing mechanisms were not investigated in depth.

Many compounds have been developed to inhibit specific checkpoint components, particularly checkpoint kinase 1 (Chk1), an active transducer kinase, at the S and G2 checkpoints, rendering it a target for rational anticancer drug development (20,21). The hyperactive cell cycle Chk1 and Chk2 play a pivotal role in the DNA damage response including radiation and chemotherapy, and the therapy targeting Chk1 gene may be a novel method of treating glioblastoma (22). Chk1 is required for the intra-S phase and G_2/M checkpoints in the cell cycle, and plays a critical role in maintaining genomic stability and transducing the DNA damage response (23). The duration of Chk1-activated G_2/M cell cycle arrest determines the level of autophagy following teh DNA mismatch repair (MMR) processing of these nucleoside analogs (24). 2-(3-Methoxyphenyl)-6,7-methylenedioxoquinolin-4-one (MMEQ) induced G_2/M arrest through the promotion of Chk1, Chk2 and CDC25C in TSGH8301 cells (25). Pabla *et al* reported that Chk1-S, an N-terminally truncated alternative splice variant of Chk1, is an endogenous repressor and regulator of Chk1. During DNA damage Chk1 is phosphorylated, which disrupts Chk1-Chk1-S interaction and results in free active Chk1 to arrest the cell cycle (26). Deletion of a single Chk1 allele compromises G_2/M checkpoint function, which is not further affected by Chk2 depletion (27). Loratadine directly damages DNA and activates Chk1, thereby promoting G_2/M arrest, making cells more susceptible to radiation-induced DNA damage. Additionally, loratadine downregulates total Chk1 and cyclin B, abrogating the radiation-induced-G_2/M checkpoint and allowing cells to re-enter the cell cycle despite the persistence of damaged DNA (28). In the present study, no change was observed in the the mRNA and protein expression of Chkl and Chk2 following treatment with DADS. However, DADS induced the accumulation of phosphorylated Chk1 and ATR, while downregulating the expression of CDC25C and cyclin B1 expression, without Chk2 phosphorylation. Furthermore, CDC25C was found to be immunoprecipitated by anti-Chk1 but not anti-Chk2. In order to clarify the function of Chk1 and Chk2 in DADS-induced G_2/M arrest in the MGC803 cell line, this cell line was generated with increased and decreased levels of Chk1 or Chk2 protein. We assessed the cell cycle G_2/M arrest in these cells. In the MGC803 cell lines tested, increasing the levels of Chk1 increased the percentage of G_2/M phase cells, demonstrating that Chk1 is required for DADS-induced G_2/M arrest. Conversely, decreasing the levels of Chk1 decreased DADS-induced G_2/M phase cells, suggesting that Chk1 is involved in DADS-induced G_2/M arrest in the MGC803 cell line. By contrast, Chk2 overexpression or knockdown had no significant effect on DADS-induced G_2/M arrest in the MGC803 cell line. These results show that Chk1 is crucial in DADS-induced G_2/M arrest in MGC803 cells.

Pectenotoxin-2 induces G_2/M phase cell cycle arrest in human breast cancer cells, while ATM- and Chk1/2-mediated phosphorylation of CDC25C plays an important role in G_2/M arrest (29). Activation of the G_2/M checkpoint involves a series of signaling events, including activation of ATR and Chk1 kinases and inhibition of Cdc2/cyclin B activity (31). Serine (Ser)/threonine (Thr) protein phosphatase 2A (PP2A) has an important role in G_2/M checkpoint activation in response to γ-irradiation (IR). Specific PP2A inhibition abrogates the IR-induced activation of ATR and Chk1 kinases, as well as the phosphorylation of Cdc2-Tyr15, and attenuates IR-induced G_2/M arrest (30), an important role of the DNA damage response mediated by ATR-Chk1 in p53/p21(Waf/CIP1) activation and downstream G_2/M arrest during treatment with gambogic acid (GA) (31).

A crucial step in G_2/M phase arrest was reported as Cdc2 and CDC25C activated by Chk1/2 in response to DNA damage (32-34). The arresting effect of gossypin on the cell cycle in G_2/M phase was involved in the phosphorylation of CDC25C tyrosine phosphatase via the activation of Chk1 (35). Human CDC25C is one of the central targets and regulators of the G_2/M checkpoint mechanisms activated in response to DNA injury (36). CDC25C is thought to be the major effector of the G_2/M DNA damage checkpoint kinase, Chk1 and/or Chk2, which triggers the cyclin B1/CDK1 complex (37,38). The present study has demonstrated that DADS reduced the expression of CDC25C in pcDNA3.1(+)/Chk1-transfected cells. After stimulation with 30 mg/l DADS for 12 h CDC25C expression showed a significant decrease, and this expression continued to decrease gradually with time. Thus, a high expression of Chk1 was able to increase G_2/M arrest induced by DADS in MGC803 cells, and the Chk1/CDC25C pathway was involved in DADS-induced G_2/M arrest. Furthermore, inhibition of the expression of CDC25C and cyclin B1 treated with DADS was blocked by Chk1 gene silencing. By contrast, Chk2 gene silencing did not have this effect. These results suggest that Chk1 gene silencing abrogated G_2/M arrest induced by DADS in MGC803 cells, and that the Chk1/CDC25C/cyclin B1 pathway was involved in the G_2/M arrest induced by DADS. These results are important in determining the mechanism of action of DADS and its future clinical use.

The subsequent degradation of CDC25C in response to DADS was mediated by Chk1. Chk1 and Chk2 were not directly responsible for the inhibition of DNA synthesis induced by DADS. However, Chk1 negatively regulated the entry of DADS-treated cells into mitosis. These findings suggest that DADS stimulates Chk1 to initiate a G_2/M cell cycle checkpoint. Furthermore, Chk1 acts to coordinate the cell cycle with

DNA synthesis, thus preventing premature mitotic entry in DADS-treated cells. Co-immunoprecipitated Chk1 or Chk2 was detected by anti-Chk1 or anti-Chk2 immunoprecipitation followed by anti-CDC25C immunoblotting. DADS treatment enhanced the binding activity of Chk1 with CDC25C in MGC803 cells, however, it did not influence the binding activity of Chk2 with CDC25C. This finding confirms that DADS induces G_2/M arrest through the interaction of Chk1 and CDC25C in MGC803 cells.

There are two possible explanations for how DNA synthesis may be arrested: either directly mediated by checkpoint activation, or by other mechanisms that activate other checkpoints, for example chain termination.

Compounds induced the activation of ATR as well as Chk1 protein, suggesting that the compound caused effective DNA damage. During DNA damage, Chk1 is activated by ataxia telangiectasia and Rad3-related (ATR)-mediated phosphorylation. Chk1 is a key regulator of checkpoint signaling in the unperturbed cell cycle and the DNA damage response. Ataxia telangiectasia [(ATR) and Rad-3-related)] protein kinases are important in the cell response to DNA damage (39). Once the DNA damage has occurred, checkpoint effector proteins such as Chk1 and Chk2 are phosphorylated and activated by ATR, leading to cell cycle arrest in G1, S, G2 and M phases. As radio- and chemotherapy activate the checkpoints, it can be expected that abrogating the DNA damage checkpoints, particularly G_2/M, by certain agents would sensitize DNA damage, leading to the death of cancer cells (40). ATR is capable of specifically phosphorylating Chk1 (37). Another important finding of the present study was that ATR is required for the maximal phosphorylation of Chk1 checkpoint proteins following DADS treatment. However, ATR only phosphorylates Chk1 (Ser345), and not Chk2. This substrate specificity may reflect the initial oxidative DNA strand breaks. Furusawa *et al* demonstrated that Chk1 plays a crucial role in the apoptosis and regulation of cell cycle progression in human leukemia Jurkat cells under hyperthermia induced by heat stress (HS) (41). The ATR-Chk1 pathway was preferentially activated under HS. Inhibition of ATR and Chk1 also abrogated G_2/M checkpoint activation by HS. In addition, the efficiency of Chk1 inhibition on G_2/M checkpoint abrogation and apoptosis induction was confirmed in the adherent HeLa, HSC3 and PC3 cancer cell lines, suggesting that the targeting of Chk1 can be effective in solid tumor cells (41).

The results of the present study have shown that DADS induces G_2/M arrest in MGC803 gastric adenocarcinoma cell lines. These effects may be related to the activation of phospho-checkpoint kinase-1. The DADS-induced G_2/M checkpoint response is mediated by Chk1 signaling through ATR/Chk1/CDC25C/cyclin B1 and is independent of Chk2.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant nos. 30600285 and 81071966), the Key Project of Hunan Province Science and Technology Plan (no. 2012SK2012), the Platform Open Innovation Fund Project for Hunan Province Universities (grant no. 12K093), the Foundation of the Construct Program of the Key Discipline in Hunan Province of China [no. (2011)76], and the Aid Program for Science and Technology Innovative Research Team in University of South China.

References

1. Steigman SA, Kunisaki SM, Wilkins-Haug L, Takoudes TC and Fauza DO: Optical properties of human amniotic fluid: implications for videofetoscopic surgery. Fetal Diagn Ther 27: 87-90, 2010.
2. Kunisaki C, Makino H, Kimura J, *et al*: Impact of lymphovascular invasion in patients with stage I gastric cancer. Surgery 147: 204-211, 2010.
3. Arunkumar R, Sharmila G, Elumalai P, *et al*: Effect of diallyl disulfide on insulin-like growth factor signaling molecules involved in cell survival and proliferation of human prostate cancer cells in vitro and in silico approach through docking analysis. Phytomedicine 19: 912-923, 2012.
4. Lai KC, Kuo CL, Ho HC, *et al*: Diallyl sulfide, diallyl disulfide and diallyl trisulfide affect drug resistant gene expression in colo 205 human colon cancer cells in vitro and in vivo. Phytomedicine 19: 625-630, 2012.
5. Yi L, Ji XX, Tan H, *et al*: Involvement of Mcl1 in diallyl disulfide-induced G2/M cell cycle arrest in HL-60 cells. Oncol Rep 27: 1911-1917, 2012.
6. Ling H, Zhang LY, Su Q, *et al*: Erk is involved in the differentiation induced by diallyl disulfide in the human gastric cancer cell line MGC803. Cell Mol Biol Lett 11: 408-423, 2006.
7. Yi L and Su Q: Molecular mechanisms for the anti-cancer effects of diallyl disulfide. Food Chem Toxicol 57: 362-370, 2013.
8. Song JD, Lee SK, Kim KM, *et al*: Molecular mechanism of diallyl disulfide in cell cycle arrest and apoptosis in HCT-116 colon cancer cells. J Biochem Mol Toxicol 23: 71-79, 2009.
9. Liao QJ, Su J, He J, Song Y, Tang HL and Su Q: Effect of diallyl disulfide on cell cycle arrest of human colon cancer SW480 cells. Ai Zheng 28: 138-141, 2009.
10. Dasgupta P and Bandyopadhyay SS: Role of di-allyl disulfide, a garlic component in NF-κB mediated transient G2-M phase arrest and apoptosis in human leukemic cell-lines. Nutr Cancer 65: 611-622, 2013.
11. Arunkumar A, Vijayababu MR, Srinivasan N, Aruldhas MM and Arunakaran J: Garlic compound, diallyl disulfide induces cell cycle arrest in prostate cancer cell line PC-3. Mol Cell Biochem 288: 107-113, 2006.
12. Knowles LM and Milner JA: Diallyl disulfide inhibits $p34^{cdc2}$ kinase activity through changes in complex formation and phosphorylation. Carcinogenesis 21: 1129-1134, 2000.
13. Ashra H and Rao KV: Elevated phosphorylation of Chk1 and decreased phosphorylation of Chk2 are associated with abrogation of G2/M checkpoint control during transformation of Syrian hamster embryo (SHE) cells by Malachite green. Cancer Lett 237: 188-198, 2006.
14. Xiang SL, Xiao XL, Ling H, *et al*: Antitumor effect of diallyl disulfide on human gastric cancer MGC803 cells xenograft in nude mice. Ai Zheng 24: 940-944, 2005 (In Chinese).
15. Yuan JP, Wang GH, Ling H, *et al*: Diallyl disulfide-induced G2/M arrest of human gastric cancer MGC803 cells involves activation of p38 MAP kinase pathways. World J Gastroenterol 10: 2731-2734, 2004.
16. Ling H, Wen L, Ji XX, *et al*: Growth inhibitory effect and Chk1-dependent signaling involved in G_2/M arrest on human gastric cancer cells induced by diallyl disulfide. Braz J Med Biol Res 43: 271-278, 2010.
17. Shioya K, Michaux C, Kuenne C, Hain T, *et al*: Genome-wide identification of small RNAs in the opportunistic pathogen *Enterococcus faecalis* V583. PLoS One 6: e23948, 2011.
18. Dickson MA and Schwartz GK: Development of cell-cycle inhibitors for cancer therapy. Curr Oncol 16: 36-43, 2009.
19. Call JA, Eckhardt SG and Camidge DR: Targeted manipulation of apoptosis in cancer treatment. Lancet Oncol 9: 1002-1011, 2008.
20. Bucher N and Britten CD: G2 checkpoint abrogation and checkpoint kinase-1 targeting in the treatment of cancer. Br J Cancer 98: 523-528, 2008.
21. Gong QF, Liu EH, Xin R, Huang X and Gao N: 2ME and 2OHE2 exhibit growth inhibitory effects and cell cycle arrest at G2/M in RL95-2 human endometrial cancer cells through activation of p53 and Chk1. Mol Cell Biochem 352: 221-230, 2011.
22. Wu J, Lai G, Wan F, *et al*: Knockdown of checkpoint kinase 1 is associated with the increased radiosensitivity of glioblastoma stem-like cells. Tohoku J Exp Med 226: 267-274, 2012.

23. Hu W, Zong Q, John-Baptiste A and Jessen B: Transient knock down of checkpoint kinase 1 in hematopoietic progenitors is linked to bone marrow toxicity. Toxicol Lett 204: 141-147, 2011.
24. Zeng X and Kinsella TJ: BNIP3 is essential for mediating 6-thioguanine- and 5-fluorouracil-induced autophagy following DNA mismatch repair processing. Cell Res 20: 665-675, 2010.
25. Hsu SC, Yu CC, Yang JS, *et al*: A novel synthetic 2-(3-methoxyphenyl)- 6,7-methylenedioxoquinolin-4-one arrests the G2/M phase arrest via Cdc25c and induces apoptosis through caspase- and mitochondria-dependent pathways in TSGH8301 human bladder cancer cells. Int J Oncol 40: 731-738, 2012.
26. Pabla N, Bhatt K and Dong Z: Checkpoint kinase 1 (Chk1)-short is a splice variant and endogenous inhibitor of Chk1 that regulates cell cycle and DNA damage checkpoints. Proc Natl Acad Sci USA 109: 197-202, 2012.
27. Niida H, Murata K, Shimada M, *et al*: Cooperative functions of Chk1 and Chk2 reduce tumour susceptibility in vivo. EMBO J 29: 3558-3570, 2010.
28. Soule BP, Simone NL, DeGraff WG, Choudhuri R, Cook JA and Mitchell JB: Loratadine dysregulates cell cycle progression and enhances the effect of radiation in human tumor cell lines. Radiat Oncol 5: 8, 2010.
29. Moon DO, Kim MO, Nam TJ, Kim SK, Choi YH and Kim GY: Pectenotoxin-2 induces G_2/M phase cell cycle arrest in human breast cancer cells via ATM and Chk1/2-mediated phosphorylation of cdc25C. Oncol Rep 24: 271-276, 2010.
30. Yan Y, Cao PT, Greer PM, *et al*: Protein phosphatase 2A has an essential role in the activation of γ-irradiation-induced G2/M checkpoint response. Oncogene 29: 4317-4329, 2010.
31. Rong JJ, Hu R, Song XM, *et al*: Gambogic acid triggers DNA damage signaling that induces p53/p21$^{Waf1/CIP1}$ activation through the ATR-Chk1 pathway. Cancer Lett 296: 55-64, 2010.
32. Bonnet J, Mayonove P and Morris MC: Differential phosphorylation of Cdc25C phosphatase in mitosis. Biochem Biophys Res Commun 370: 483-488, 2008.
33. Reinhardt HC and Yaffe MB: Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. Curr Opin Cell Biol 21: 245-255, 2009.
34. Zegerman P and Diffley JF: DNA replication as a target of the DNA damage checkpoint. DNA Repair 8: 1077-1088, 2009.
35. Shi L, Chen J, Wang YY, *et al*: Gossypin induces G2/M arrest in human malignant glioma U251 cells by the activation of Chk1/Cdc25C pathway. Cell Mol Neurobiol 32: 289-296, 2012.
36. Aressy B and Ducommun B: Cell cycle control by the CDC25 phosphatases. Anticancer Agents Med Chem 8: 818-824, 2008.
37. Chaudhary P, Sharma R, Sahu M, Vishwanatha JK, Awasthi S and Awasthi YC: 4-Hydroxynonenal induces G_2/M phase cell cycle arrest by activation of the ataxia telangiectasia mutated and Rad3-related protein (ATR)/checkpoint kinase 1 (Chk1) signaling pathway. J Biol Chem 288: 20532-20546, 2013.
38. Duan J, Yu Y, Li Y, *et al*: Toxic effect of silica nanoparticles on endothelial cells through DNA damage response via Chk1-dependent G2/M checkpoint. PLoS One 8: e62087, 2013.
39. Matsuoka S, Ballif BA, Smogorzewska A, *et al*: ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316: 1160-1166, 2007.
40. Nishida H, Tatewaki N, Nakajima Y, *et al*: Inhibition of ATR protein kinase activity by schisandrin B in DNA damage response. Nucleic Acids Res 37: 5678-5689, 2009.
41. Furusawa Y, Iizumi T, Fujiwara Y, *et al*: Inhibition of checkpoint kinase 1 abrogates G2/M checkpoint activation and promotes apoptosis under heat stress. Apoptosis 17: 102-112, 2012.