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Apoptosis of human gastric cancer cells line SGC 7901 induced by garlic-derived compound S-allylmercaptocysteine (SAMC)

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Abstract. – BACKGROUND: Epidemiological and experimental carcinogenesis studies provide evidence that components of garlic (*Allium sativum*) have anticancer activity. Hepatocellular carcinoma is highly malignant and metastatic. Currently, there is no effective chemotherapy for patients with advanced Hepatocellular carcinoma leading to an urgent need to seek for novel therapeutic options.

AIM: To investigate the effect of cell growth, cell apoptosis of Garlic-Derived Compound S-Allylmercaptocysteine (SAMC) on Human Gastric Cancer Cells Line SGC 7901 cells.

MATERIALS AND METHODS: The SGC 7901 cells were cultured with different concentration's SAMC. Cell viability was detected by AO/EB staining. JNK and P38 pathway were assayed by PCR (*polymerase chain reaction*).

RESULTS: The best concentration of SAMC (300 μM) for induction SGC 7901 apoptosis was confirmed through cell viability. The PCR assay demonstrated that JNK and P38 pathway play important role in apoptosis of SGC 7901 cells.

CONCLUSIONS: This study indicated that SAMC can inhibit cell proliferation and induct apoptosis of SGC 7901 cells via JNK and P38 pathway.

Key Words:
Garlic, SAMC, JNK and P38 pathway, SGC 7901.

Introduction

Gastric cancer is the second leading cause of cancer-related deaths in the world, with an estimated ~760,000 cases of gastric cancer are diagnosed annually worldwide[1,2]. Gastric cancer is often asymptomatic, with only non-specific symptoms in its early stages. Thus, by the time symptoms occur, the cancer has generally metastasized to other regions of the body. The cause of gastric cancer remain unknown; however, it is more common in 55 years or older men. In addition, a high salt diet and low vitamin intake increases the risk of gastric cancer[3]. Garlic is a vegetable (*Allium sativum*) that belongs to the Allium class of bulb-shaped plants, which has been used as both food and medicine in many cultures for thousands of years, dating back to when the Egyptian pyramids were built. Today garlic is used to help prevent heart disease, high cholesterol, high blood pressure, and to boost the immune system[4-6]. Meanwhile, epidemiological and experimental carcinogenesis studies show evidence that components of garlic have anticancer activity[7-9]. Most of allyl sulfides of garlic, which are absorbed in gastrointestinal tract, were also reported to biotransform to the corresponding allylmercapto glutathione S-congugate after reacting with endogenous antioxidants, such as cysteine and reduced glutathione (GSH)[8,10]. One of these allylmercapto glutathione S-congugate, SAMC (S-allylmercapto-L-cysteine), was reported that showed inhibitory effect on tumorigenesis, but the mechanisms of which are poorly understood. In this study, the effects of SAMC on the growth of the human gastric cancer cells line, SGC 7901, were investigated.

Materials and Methods

Materials

SAMC (S-allylmercaptocysteine) are water-soluble components isolated from aged garlic extract and kindly provided by Wakunaga Pharmaceutical Co., Ltd. (Hiroshima, Japan). The molecular weight of SAMC is 193, According to the manufacturer's instructions, sterilized stock solutions of SAMC (5 mM) was made freshly in phosphate buffered saline (PBS), and stored in refrigerator at 4ºC.

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Cell Culture and Drug Treatment

Human Gastric Cancer Cells Line, SGC 7901 cells was obtained from the China General Microbiological Culture Collection Center (Beijing, China), and maintained in minimal RPMI (Roswell Parker Memorial Institute)-1640 medium (Gibco BRL, Gaithersburg, MD, USA) that contained 10% fetal bovine serum (FBS, Gibco, USA) and 1% antibiotic antimycotic (Gibco, USA), in an atmosphere of 5% CO_2 and 95% air at 37°C. SGC 7901 cells were seeded at a density of 1×104 cells per well in 24-well plates and incubated for 24 h. SAMC was dissolved in PBS and added to the culture media at various concentrations in the 0-400 μM range, and the cells were then incubated for 72 h.

MTT Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell viability and proliferation as described by Ho et al[11]. Cell suspension of 200 l was placed on each well of 96-well micro plates at the concentration of 1×104 cells/well. Cells in logarithmic growth phase, with SAMC containing complete RPMI-1640 media and corresponding controls were set simultaneously. 6 replicates were prepared for each treatment and cultured until 12, 24, 48, 60 and 72 h. After the addition of MTT 20 μl (5 mg/ml PBS)) each well, the cells were cultured for another 4 h. The supernatant was discarded. After the addition of 200 l DMSO (dymethylsulfoxide) in each well, the samples were incubated in the dark for 30 min, and then swirled for mixing. Absorbance A at 490 nm was measured using enzymatic reader. Experiments were repeated three times.

AO/EB Staining

Ten microliters of AO and EB (Sigma, Aldrich, St Louis, MO, USA) (both 2 mg/mL in ethanol) were added 98 into 3 ml cell suspension harvested from a well on a 6-well microplate, different samples were observed under a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) with the excitation wavelengths of 488 nm and 543 nm.

PCR

RNA was isolated from cell samples according to the protocol described by Chomczynski and Sacchi[12]. Primers (SBS Genetech, Mainland, China) were listed in Table I corresponding to the standards of O'Driscoll et al[13]. PCR was conducted according to the protocol described by Saiki et al[14]. PCR products were detected by 2.5% agarose gel electrophoresis. Real-time PCR was carried out using a Takara SYBR Green qPCR kit (Takara, Dalian, China). The PCR mixture, which contained 20 pmol of forward and reverse primers and 2 l of cDNA, was subjected to amplification with a DNA Engine Opticon 1 (Takara, Dalian, China). The cycles were set at 95°C for 10 min for preheating, followed by 40 cycles at 94°C for 15 sec, at 55°C for 30 sec and at 72°C for 30 sec. The amplicons were detected directly by measuring the increase in fluorescence caused by the binding of the SYBR Green I dye to gene-specific and amplified double-strand DNA using a DNA Engine Opticon 1. Following the completion of the PCR reaction, the temperature was raised from the annealing temperature to 95°C for melting curve analysis. The expression level was calculated by the 2^{-Ct} method and compared with the relative expression.

Statistical Analysis

Statistical analysis of the data were performed with a one-way ANOVA followed by the Tukey-Kramer honestly significant difference (HSD) test for the three sets of results. A *p*-value of less than 0.05 was considered significant. Statistical analyses were done with a JMP® Statistical Discovery Software (SAS Institute, Cary, NC, USA).

Results

Morphological Observation

Under normal circumstances, the adherent cells were elliptical shaped. In logarithmic phase, the passage cells will gradually adhere and grow. The cells of the control showed that cells were closely arranged with uniform size, as well as good vitality and refractivity (Figure 1A). The SAMC treated cells displayed atrophy and vacuoles, shrinkage, cell number decrease, and cell fragmentation took place, with blurred contour, the declined in cells connection, and even lysed into small pieces (Figure 1B). Apoptotic cells detached from adjacent normal cells, and are different in morphology. A typical morphological feature observed during apoptosis is the reduction of cellular volume, a feature that distinguishes this form of cell death from necrosis (Figure 1C).

Table I. Primer sequences used in RT-PCR and qPCR assay.

Gene	Primer sequence	Tm (°C)	Cycle	Fragment size (bp)
Ras	F 5' TTGACGTTGCTCCACGCTAT 3' R 5' ATCGCCACTTTCTGACCGTT 3'	58.8	30	244
Raf 1	F 5' AATGGGCAGCCGTTAGGAAA 3' R 5' GCCCAATACGACCAAATCAGAG 3'	59.2	30	160
ERK1	F 5' TGCAACCGGGAAGGAAATGA 3' R 5' GCCCAATACGACCAAATCAGA 3'	59	30	182
Elk-1	F 5' GCCTCAACTTTCAGGGGGAG 3' R 5' TCCCGTGAAGTCCAGGAGAT 3'	61	30	216
JNK	F 5' ATGTTGCAACCGGGAAGGAA 3' R 5' CGCCCAATACGACCAAATCAGA 3'	56	30	190
C-jun	F 5' GCTGCTCTGGGAAGTGAGTT 3' R 5' TTTCTCTAAGAGCGCACGCA 3'	52	30	219
P38	F 5' GAAAGCCTGCCGGTGACTAA 3' R 5' GCCCAATACGACCAAATCAGAGA 3'	58	30	150
P53	F 5' CCAGTCACAGCAGCACAGAT 3' R 5' ACCGTCTCGGTTTTCACTGC 3'	57.5	30	215
Bcl 2	F 5' GAATGGGCAGCCGTTAGGAA 3' R 5' CCCAATACGACCAAATCAGAGA 3'	57	30	168
Bax	F 5' AGGGTGTAAAACGCAGCTCA 3' R 5' AGGGTGTAAAACGCAGCTCA 3'	58.7	30	202
fas	F 5' AGGGACTGCACAGTCAATGG 3' R 5' CCATGTTCACATCATGTCCTTCA 3'	60	30	156
Procaspase-3	F 5' TCCTTCCTGGGTATGGAATCCT 3' R 5' GCTCAGTAACAGTCCGCCTA 3'	59	30	153
GAPDH	F 5' CCCGTTGCTGTCGCCCGTTC 3' R 5' GCCTTGACCGTGCCGTGGAA 3'	60	30	149

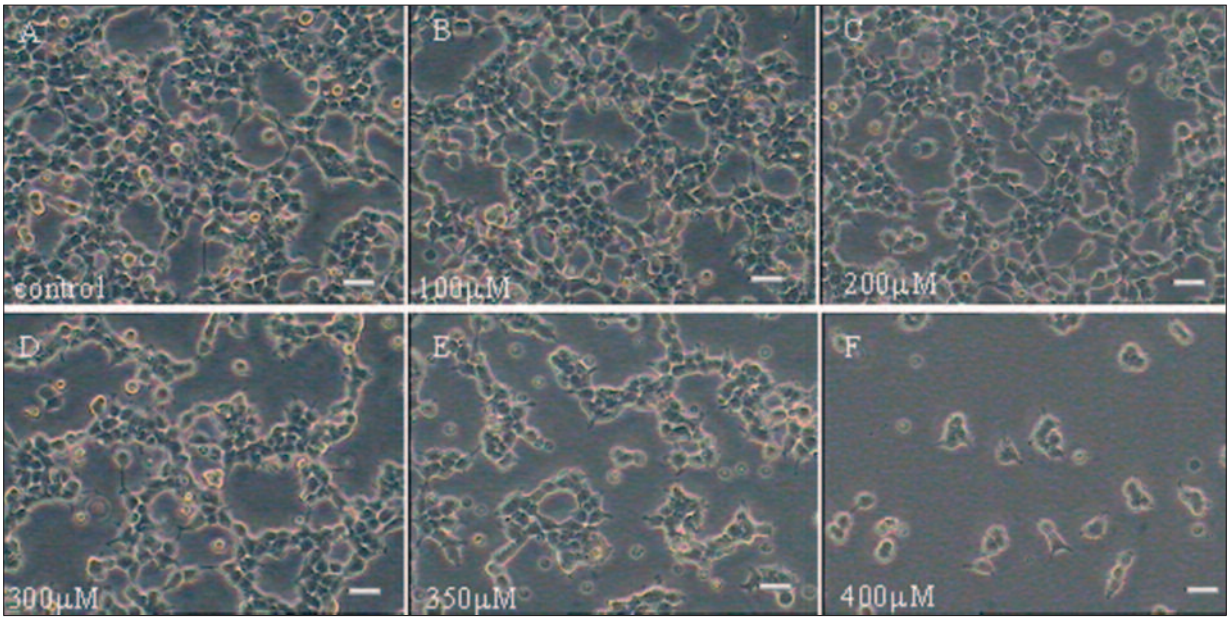

Figure 1. Morphological observation of SGC 7901 cells by inverted phase contrast microscope. Morphology of SGC 7901 cells at 48 h after treatment with SAMC. ***(A)*** Control; and SGC 7901 cells treated with SAMC of ***(B)*** 100 μM; ***(C)*** 200 μM; ***(D)*** 300 μM; ***(E)*** 350 μM and F 400 μM (bar = 50 m).

SAMC-Induced Reduction of SGC 7901 cells Viability

Viability of SAMC-treated SGC 7901 cells was first assessed using the MTT assay. We found that SAMC decreased tumor cell viability in a dose- and time-dependent manner (Figure 2). However, the cell death was observed when the concentration of SAMC was higher than 400 M.

AO/EB (Acridine Orange/Ethidium Bromide) staining

SGC 7901 were treated under SAMC for different concentration, and then cultured for 48 h. AO/EB staining was performed to visualize chromatin distribution and to evaluate the integrity of membrane via their binding with DNA and RNA and permeability differences (AO permeable, whereas EB impermeable). From the photographs it could be observed that the cells radiated experienced chromatin aggregation and nuclei condensation, typical morphological events in apoptosis (Figure 3).

JNK and P38 Pathway assay Through PCR

Cellular total RNA was isolated after 0, 12, 24, 48 and 72 h of incubation with SAMC respectively, and RT-PCR and real time PCR were performed members of JNK (Jun N-terminal kinases) and P38 pathway, including Ras (Rat sarcoma), ERK1 (extracellular signal-regulated kinase 1), JNK, P38, C-jun, Elk-1 and P53, and members of apoptosis pathway, including Bcl2 (B-cell lymphoma leukemia 2), fas, Bax, procaspase 3. The PCR assay indicated that after incubation with SAMC, the specific genes of JNK and P38 pathway, apoptosis pathway, including Ras, ERK 1, JNK, P38, Elk-1 fas, Bax, procaspase 3 and P53 were detected, and gene expression level showed a time-lapse increase, however, the gene expression of Bcl 2 and c-Jun showed a time-lapse decrease (Figures 4 and 5).

Discussion

Epidemiological studies suggest that the consumption of garlic or its derivatives may have preventive effects for several types of cancer including stomach, colorectal, and prostate cancers Antiproliferative effects of garlic-derived and other allium related compounds[15,16]. In this study, we have demonstrated a novel anticancer effect of garlic derived compounds-SAMC, and provided possible mechanisms responsible for their anti-invasive effect. Our findings suggest that the induction of apoptotic cell death by SAMC occurred via the JNK and P38 pathway that activates apoptosis pathway.

JNKs and p38 pathway are activated by environmental and genotoxic stresses and have key roles in inflammation, as well as in tissue homeostasis, as they control cell proliferation, differentiation, survival and the migration of specific cell types[17-22]. JNK and p38 pathways regulate

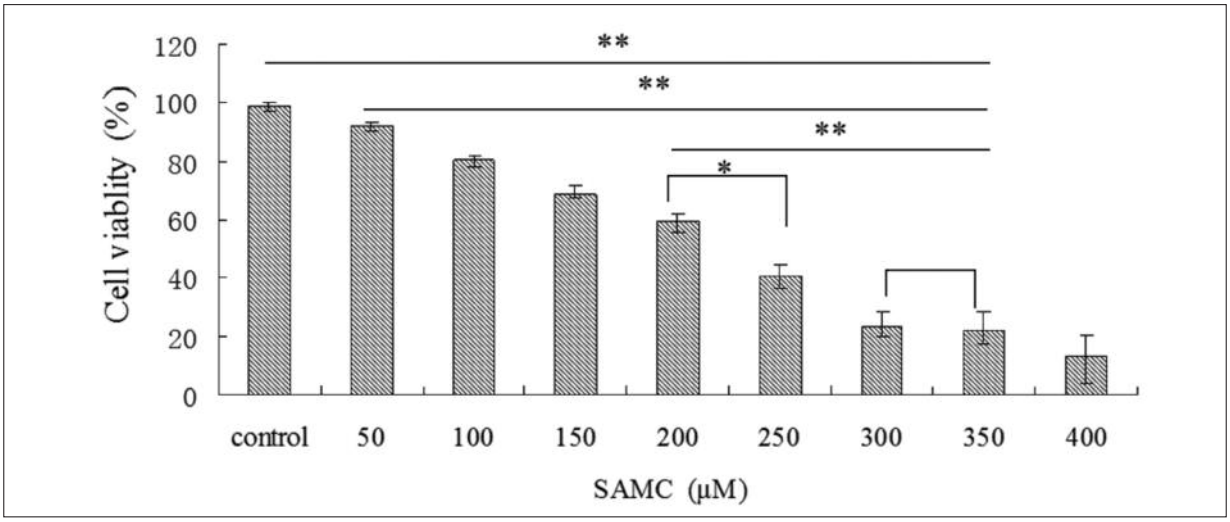

Figure 2. Decreased cell viability by S-allylmercapto-L-cysteine (SAMC) in SGC 7901 cells. After treatment with SAMC for 48 h, the cell viability was assessed by MTT staining. Results are expressed as the percent change of the control condition (medium without SAMC) in which the cells were grown. Statistical significance to corresponding controls is marked with (*) ($p < 0.05$) and (**) (I < 0.01). Data represent the mean values of five replicates, with bars indicating SEM.

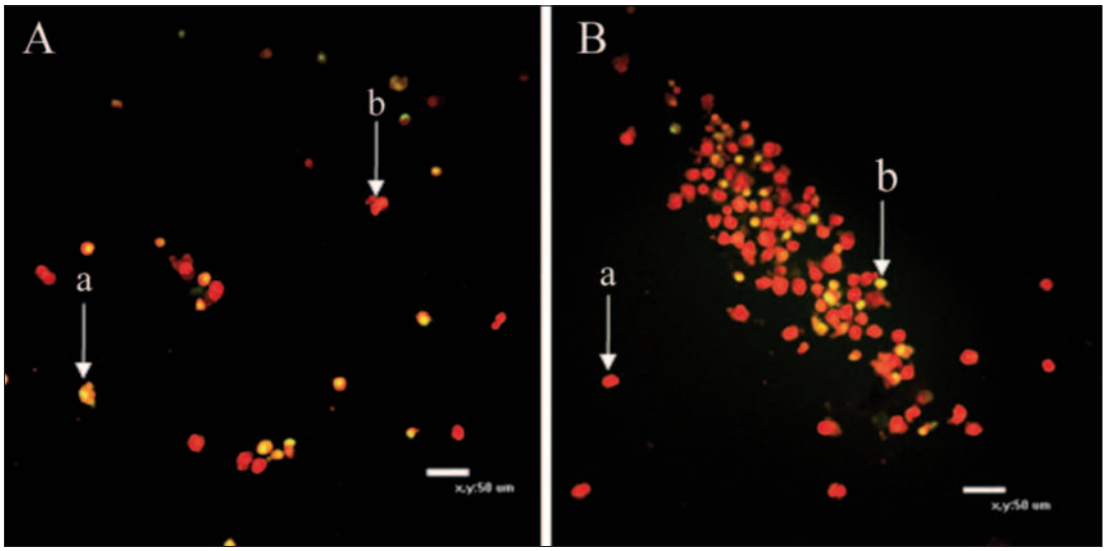

Figure 3. Morphological observation of SGC 7901 cells using AO/EB double staining by confocal microscopy. ***(A)*** Morphology of SGC 7901 cells at 24 h after treatment with SAMC (300 μM). ***(B)*** Morphology of SGC 7901 cells at 48 h after treatment with SAMC (300 μM). Arrow a, viable apoptotic cells; Arrow b, non-viable apoptotic cells. Scale bars = 50 μm.

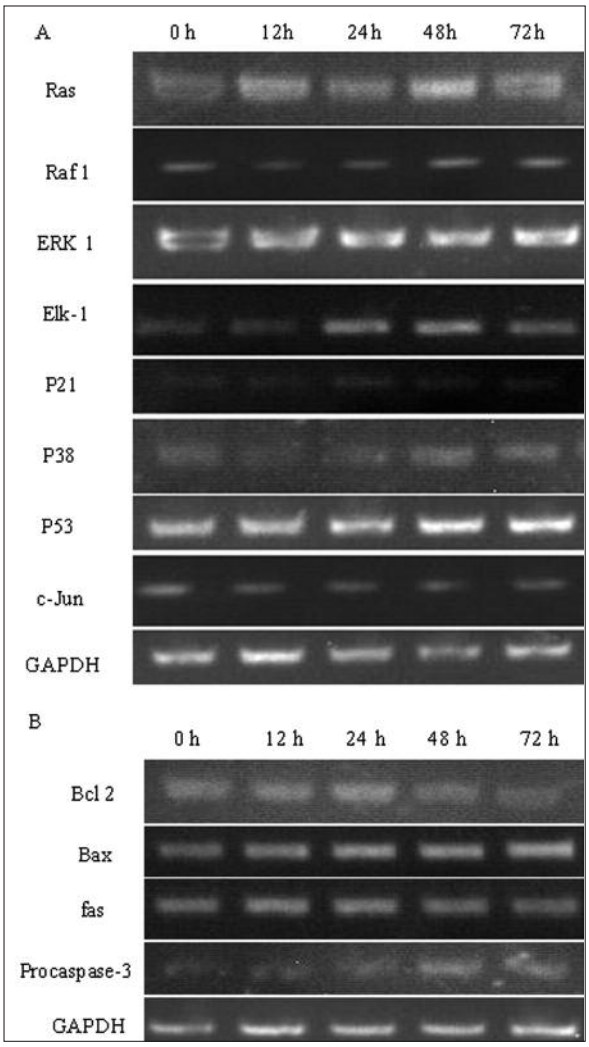

Figure 4. RT-PCR analyses expression of JNK and P38, apoptosis pathway members. ***A,*** The JNK and P38 pathway members expression of SGC 7901 cells at 48 h after treatment with SAMC (300 μM). ***B,*** The apoptosis pathway members expression of SGC 7901 cells at 48 h after treatment with SAMC (300 μM).

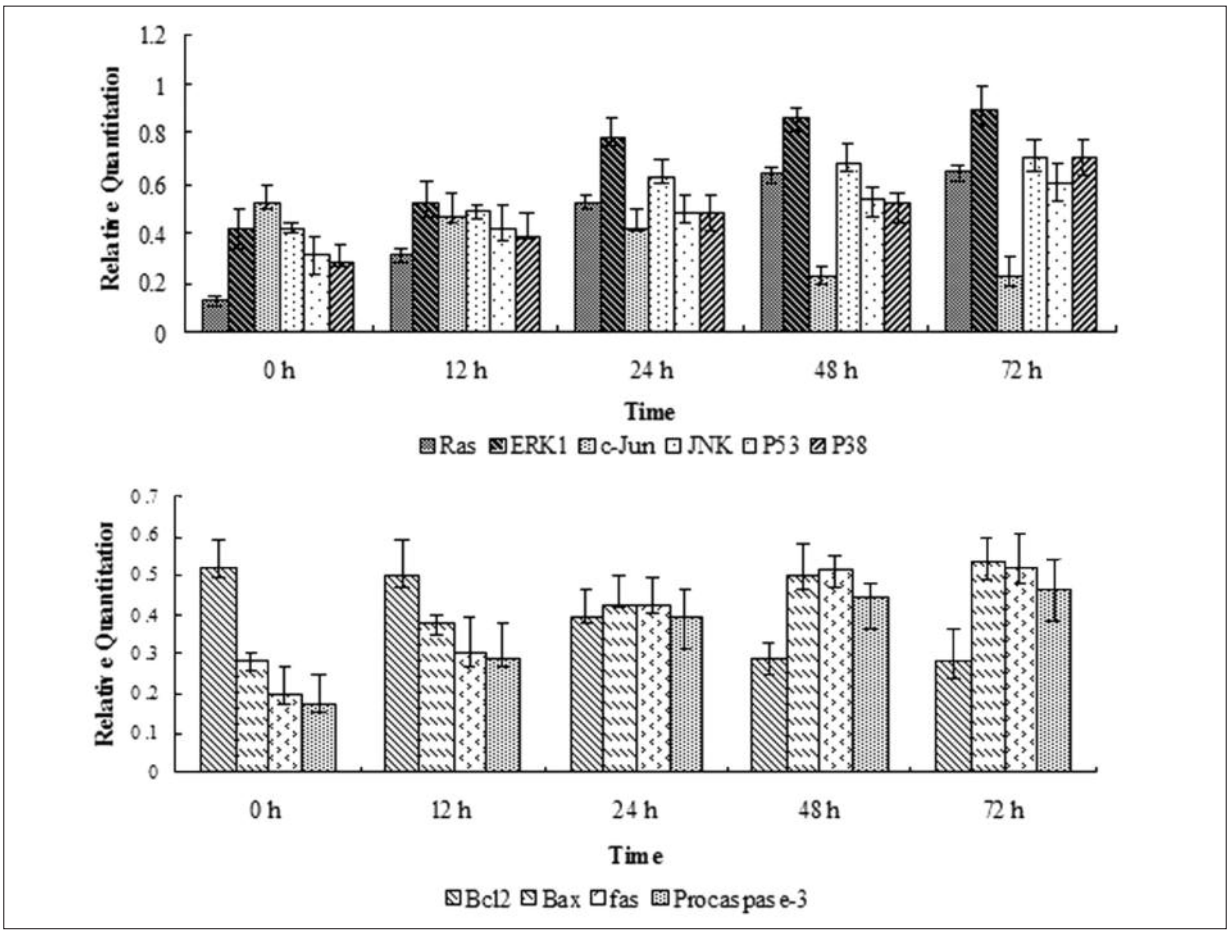

Figure 5. Real time PCR analyses expression of JNK and P38, apoptosis pathway members. ***A,*** The JNK and P38 pathway members expression of SGC 7901 cells at 48 h after treatment with SAMC (300 μM); ***B,*** The apoptosis pathway members expression of SGC 7901 cells at 48 h after treatment with SAMC (300 μM).

cell cycle progression at different transition points by both transcription-dependent and transcription-independent mechanisms. In addition, both pathways modulate the cellular programmes for cell survival and differentiation, with profound effects on the development of various cancers. Depending on the stimuli and duration of JNK activation, the cellular response has diverse outcomes, which range from the induction of apoptosis to increased survival and altered proliferation. A role for JNKs in cell survival is well established, although the proposed underlying mechanisms are controversial[19]. As cytoplasmic injection of cytochrome crescued the apoptotic defects of JNK-deficient fibroblasts, JNK pro-apoptotic functions were proposed to be mediated by the mitochondrial pathway[23]. P38 pathway can negatively regulate cell cycle progression both at the G1/s and the G2/M transitions by several mechanisms, including the downregulation of cyclins, upregulation of cyclin-dependent kinase (CDK) inhibitors and modulation of the tumour suppressor P53 pathway[24,25]. Therefore, JNK and P38 pathway can regulate the apoptosis of cells through activation P53 pathway.

Conclusions

In our study, we have provided evidence that JNK and P38 pathway plays an important role in the early phase of apoptosis induced by SAMC, and that this effect is independent of the G2-M cell cycle arrest induced by this compound. And demonstrated that the garlic derived compounds-SAMC, is able to activate JNK and P38 pathway in invasive cancer cells, suggesting that they may also be effective agents for the treatment of invasive cancers.

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