RESEARCH ARTICLE

Effect of Diallyl Trisulfide on Human Ovarian Cancer SKOV-3/DDP Cell Apoptosis

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Abstract

Aim: To investigate the effects of diallyl trisulfide (DT) on apoptosis of cisplatin (DDP)-resistant human epithelial ovarian cancer SKOV-3 cells (SKOV-3/DDP), and the role of p53 upregulated modulator of apoptosis (PUMA). Methods: SKOV-3/DDP cells were randomly divided into control, DT, DPP and DPP+DT groups, which were treated with DT or combined DT and DDP. All cells were incubated for 48 h. and apoptosis rates were assessed by flow cytometry. mRNA and protein expression of PUMA, Bax and Bcl-2 was determined by RT-PCR and Western blot assays, respectively. Results: Compared with control group, the apoptosis rates of SKOV-3/DDP cells in DT groups were obviously increased, with dose-dependence ($P < 0.05$), the mRNA and protein expressions of PUMA, Bax also being up-regulated ($P < 0.05$), while those of Bcl-2 were down-regulated ($P < 0.05$). Compared with DT groups, the apoptosis rate in the DDP+DT group was significantly increased ($P < 0.05$). After knockdown of PUMA with specific siRNA, the apoptosis rate of SKOV-3/DDP cells was obviously decreased ($P < 0.05$). Conclusion: DT can promote the apoptosis of SKOV-3/DDP cells with PUMA playing a critical role.

Keywords: Diallyl trisulfide - SKOV-3/DDP cell - PUMA - human ovarian cancer - apoptosis

Asian Pac J Cancer Prev, **14 (12)**, 7197-7201

Introduction

Ovarian cancer is one of the most common malignant tumors of the female productive tract. Its mortality is the highest in gynecological tumors (Chauhan et al., 2009; Rouzier et al., 2010). At present, chemotherapy is one of the most important methods to treat ovarian cancer. However, drug resistance is a major reason to cause reoccurrence of ovarian cancer and treatment failure. Thus, it is necessary to seek anti-drug resistance, nontoxic and effective drugs to treat ovarian cancer. Diallyl trisulfide (DT) is the main bioactive compound in garlic. Some studies have revealed that, DT can induce the cell apoptosis of many tumors such as lung cancer, breast cancer, colon cancer and skin cancer (Fleischauer and Arab, 2001; Mao et al., 2010; Altonsy and Andrews, 2011; Jeong et al., 2011; Tsubura et al., 2011; Li et al., 2012). In this study, cisplatin (DDP)-resistant human epithelial ovarian cancer SKOV-3 cells (SKOV-3/DDP) were treated with DT or combined DT and DDP. The effects of DT on apoptosis of SKOV-3/DDP cells and expressions of p53 upregulated modulator of apoptosis (PUMA), Bax and Bcl-2 were investigated. The role of PUMA in cell apoptosis induced by DT was explored. The objective was to give some new ideas for clinical drug treatment for ovarian cancer patients with resistance to DDP.

Materials and Methods

Groups and drug treatment

SKOV-3/DDP cells (Beijing Tumor Hospital, Beijing, China) were cultured, and then collected after logarithmic growth. All cells were randomly divided into control group, DT groups (treated with 10, 20 and 40 μg/ml DT (Shanghai Hefeng Pharmaceutical Co., Ltd., Shanghai, China), respectively), DDP group (treated with 5 μg/ml DDP (Shandong Luoxing Pharmaceutical Co., Ltd., Linyi, China)) and DT+DDP group (treated with 20 μg/ml DT and 5 μg/ml DDP). Experiments were performed in triplicate.

Hoechst33342 fluorescent staining assay

Cultured cells were seeded into 6-well plates and treated with drugs for 48 h, and then 10 μg/ml Hoechst33342 (Shanghai Shize Biological Technology Co., Ltd., Shanghai, China) was added, followed by

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Table 1. Sequences of Gene Primers

Primer	Sequences	Size (bp)
β-actin	5'-TATGACTTAGTTGCGTTACACC-3'	155
	5'-CCTTCACCGTTCCAGTTT-3'	
PUMA	5'-TCCTCAGCCCTCGCTCTCGC-3'	558
	5'-CCGATGCTGAGTCCATCAGC-3'	
Bax	5'-TTTGCTTCAGGGTTTCATCC-3'	434
	5'-GCCACTCGGAAAAAGACCTC-3'	
Bcl-2	5'-GTGGAGGAGCTCTTCAGGGA-3'	304
	5'-AGGCACCCAGGGTGATCAA-3'	

culture for 0.5 h. After the used medium containing Hoechst33342 was removed, plates were washed with PBS or medium in triplicate. Finally, cells were observed and photographed under fluorescence microscope.

Flow cytometry

The apoptosis rate of SKOV-3/DDP cells was detected using Annexin V-FITC and PI combined staining in flow cytometry, which could distinguish different stages of cell apoptosis. After treatment with trypsinase solution without EDTA, cells were washed with PBS twice and centrifugated (2000 rp/min) for 5 min, then cells were collected and put into different tubes (1-5×10^5 cells/tube)

RT-PCR

mRNA expressions of PUMA, Bax and Bcl-2 in SKOV-3/DDP cells were detected by RT-PCR method. Total RNA was extracted from peripheral blood mononuclear cells with TRIzol reagent according to manufacturer's protocol, followed by synthesis of cDNA and amplification. The amplified products were detected by 1.5% agarose gel electrophoresis. Compared with band of β-actin (Santa Cruz Inc., California, USA), relative gene expressions of each group were calculated by Launch SensiAnsys Image Acquisition and Analysis Software. Sequences of gene primers were shown in Table 1.

Western blot assay

Expressions of PUMA, Bax and Bcl-2 protein in SKOV-3/DDP cells were detected by western blot assay. The cells were collected and total protein was extracted and quantitated by Bradford method. The lysates (30 μg of protein for each well) were electrophoresed on SDS-PAGE gel for 4 h before blotting onto PVDF membranes. The blots were incubated with primary antibodies of PUMA, Bax and Bcl-2 (1: 200 dilution, ABcam Inc., Massachusetts, USA) overnight at 4 oC, followed by washing and incubation for 1.5 h with horseradish peroxidase-conjugated secondary antibody (1: 2000 dilution, Beijing ZSGB-BIO Company, Beijing, China). After washing, the membranes were developed in ECL system. Protein expression levels were determined by analyzing the signals captured on membranes using Image-Pro Plus6.0 analyzer. β-actin was used as internal control.

Specific PUMA siRNA experiment

According to cDNA sequences of PUMA, specific PUMA siRNA primers (5'-GGGUCCUGUACAAUCUCAUTT-3', 5'-AUGAGAUUGUACAGGACCCTT-3') were designed and synthesized by Shanghai GenePharma Co., Ltd., Shanghai, China. PUMA siRNA was transfected into SKOV-3/DDP cells using LipofectamineTM2000 (Invitrogen Corp., California, USA). The effects of DT on SKOV-3/DDP cell apoptosis and protein expression were detected. There were four groups to be performed, including control group (cells were cultured with no-serum medium for 6 h, and then cultured with normal medium for 48 h), negative control (NC) group (the protocols were the same with control group, expect adding transfected siRNA products into medium), siRNA group (the protocols were the same with control group, expect adding transfected si-PUMA fragment I into medium) and siRNA+DT group (after transfection with si-PUMA fragments for 6 h, medium containing 20 μg/ml DT was used for culture for 48 h).

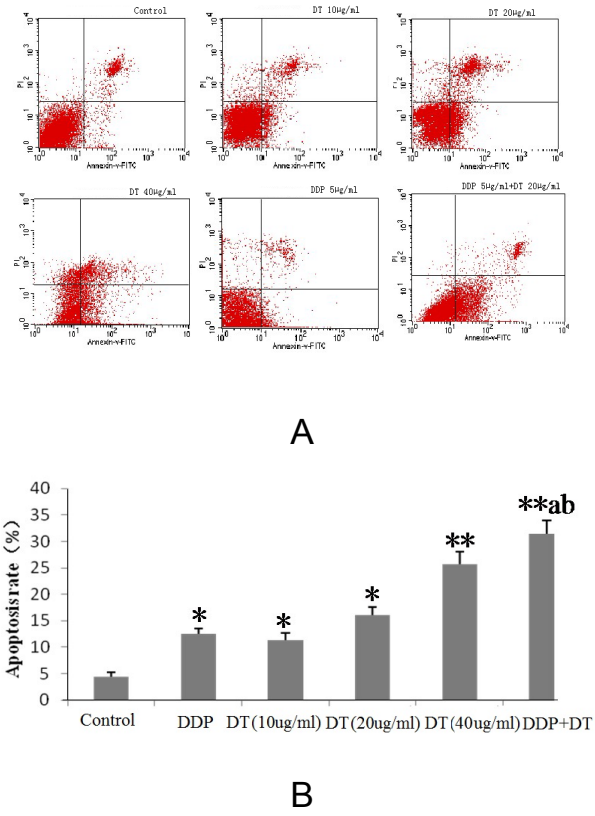


Figure 1. Effect of DT on Apoptosis of SKOV-3/DDP Cells. *$P < 0.05$, **$P < 0.01$ compared with control group; $^aP < 0.01$ compared with DDP group; $^bP < 0.05$ compared with 20 μg/ml DT group

Statistical analysis

All results were presented as mean±SD. Statistical analysis was performed using SPSS 17.0 software. One-way analysis of variance was used for comparison among different groups. $P < 0.05$ was considered as statistically significant.

Results

Effect of DT on apoptosis of SKOV-3/DDP cells

Effect of DT on apoptosis of SKOV-3/DDP cells in different groups were shown in Figure 1A and B. After treatment with DT and DDP for 48 h, compared with

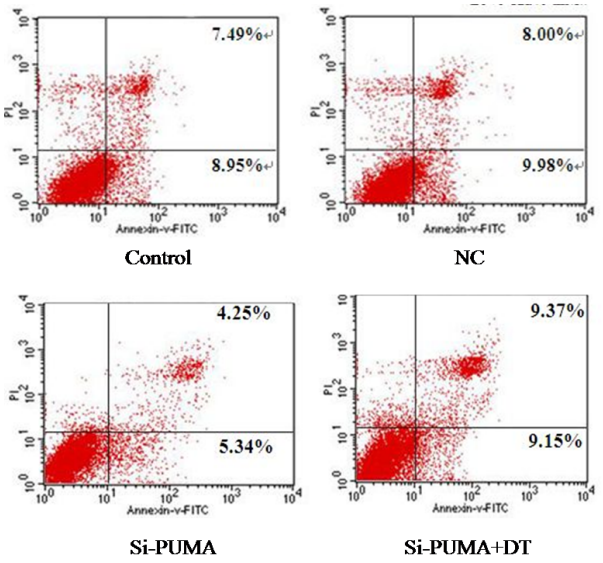


Figure 2. Effects of Specific PUMA siRNA on SKOV-3/DDP Cell Apoptosis Induced by DT

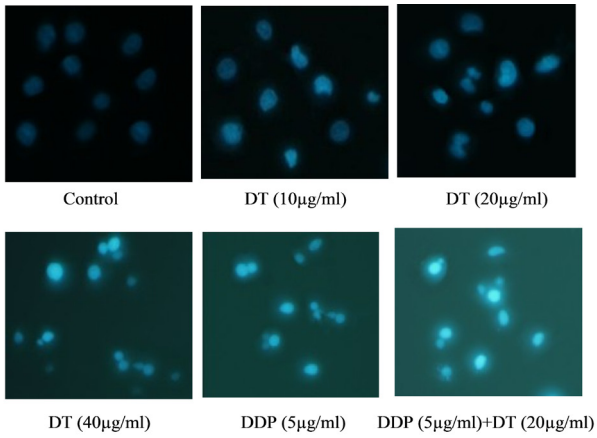


Figure 3. Morphological Changes of SKOV-3/DDP Cells. (Hoechst 33342 fluorescent staining, ×1000)

control group, the apoptosis rates in three DT groups, DDP group and DDP+DT group were significantly increased ($P < 0.05$ or $P < 0.01$). Compared with DDP group and DT groups, the early apoptosis rate of DDP+DT group was obviously increased ($P < 0.01$). This indicated that, the treatment effect of combined DDP and DT was better than that of DT or DDP alone.

Effects of specific PUMA siRNA on SKOV-3/DDP cell apoptosis induced by DT

After knockdown of PUMA expression with specific siRNA, SKOV-3/DDP cells were treated with 20 μg/ml DT for 48 h. Figure 2 showed that, compared with control group, both the apoptosis rates at early and late stages in si-PUMA group were significantly reduced ($P < 0.05$). Furthermore, compared with si-PUMA group, the apoptosis rate at late stage in si-PUMA+DT group was increased ($P < 0.05$). This result implied that, PUMA could promote the apoptosis of SKOV-3/DDP cells.

Changes of morphology

The morphology of SKOV-3/DDP cells was observed under fluorescence microscope. Figure 3 showed that,

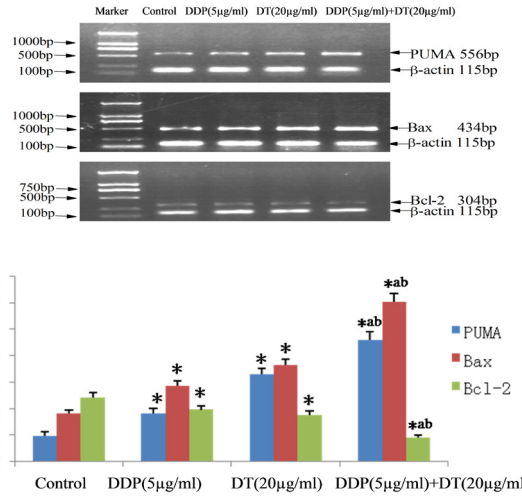
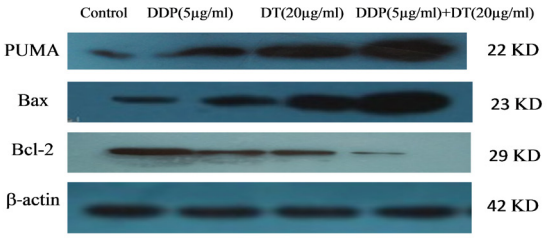


Figure 4. Expressions of PUMA, Bax and Bcl-2 mRNA in SKOV-3/DDP Cells. *$P < 0.05$ compared with control group; [a]$P < 0.01$ compared with DDP group; [b]$P < 0.05$ compared with 20 μg/ml DT group



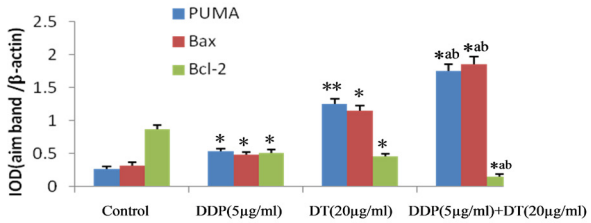


Figure 5. Expressions of PUMA, Bax and Bcl-2 protein in SKOV-3/DDP Cells. *$P < 0.05$ and **$P < 0.01$ compared with control group; [a]$P < 0.01$ compared with DDP group; [b]$P < 0.05$ compared with 20 μg/ml DT group

morphological changes of cells were obvious in DT groups after treatment for 48 h, presenting karyopyknosis, chromatin condensation and crescent shape around karyotheca. In 40 μg/ml DT group, with increase of DT dose, the nuclei were darker gradually, with obvious karyorrhexis. This indicated that, DT could induce SKOV-3/DDP cell apoptosis. However, there was not obvious morphological change in DDP group. In DT+DDP group, karyorrhexis was more obvious, and nuclei presented different sizes and irregular morphology. These results further demonstrated that, combined DT and DDP could significantly promote the apoptosis of SKOV-3/DDP cells.

Expressions of PUMA, Bax and Bcl-2 mRNA in SKOV-3/DDP cells

Figure 4 showed that, compared with control group, mRNA expressions of PUMA and Bax were up-regulated in DDP group and DT group, with down-regulation of Bcl-2 mRNA expression ($P < 0.05$ or $P < 0.01$).

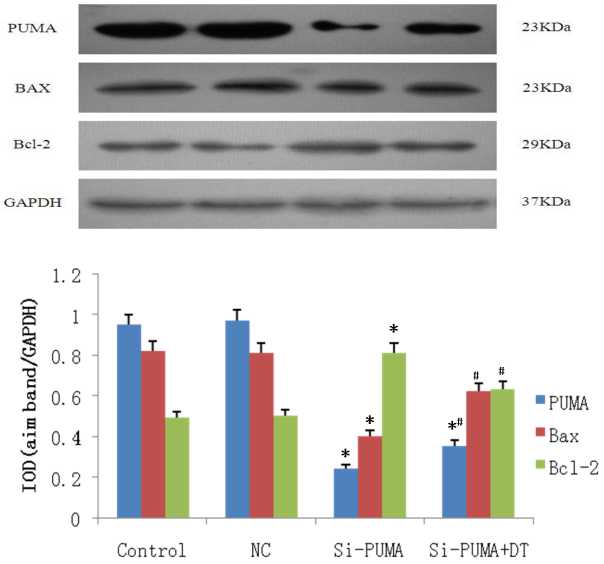


Figure 6. Expressions of PUMA, Bax and Bcl-2 Protein in SKOV-3/DDP Cells after Knockdown of PUMA. *P< 0.05 compared with control group; #P < 0.05 compared with Si-PUMA group

Moreover, compared with DDP group or DT group, the mRNA expressions of PUMA and Bax in DDP+DT group were increased, and the mRNA expression of Bcl-2 was decreased. This indicated that, DT could regulate the mRNA expressions of PUMA and Bax. Furthermore, combined DT and DDP had more obvious effects on genes expression.

Expressions of PUMA, Bax and Bcl-2 protein in SKOV-3/DDP cells

As shown in Figure 5, compared with control group, the protein expressions of PUMA and Bax were up-regulated and that of Bcl-2 was down-regulated in DDP group and DT group ($P < 0.05$ or $P < 0.01$). Compared with DDP group or DT group, the protein expressions of PUMA and Bax in DDP+DT group were up-regulated, and that of Bcl-2 was down-regulated. These results indicated that DT could regulate the protein expressions of PUMA and Bax, and combination of DT and DDP had a stronger effect.

Expressions of PUMA, Bax and Bcl-2 protein in SKOV-3/DDP cells after knockdown of PUMA

Figure 6 showed that, the levels of PUMA and Bax protein in si-PUMA group were significantly lower than control group ($P < 0.05$), and that of Bcl-2 protein was significantly increased in si-PUMA group ($P <0.05$). The levels of PUMA and Bax protein in si-PUMA+DT group were significantly lower than control group ($P < 0.05$), but significantly higher than si-PUMA group ($P < 0.05$). Expressions of Bcl-2 protein in si-PUMA+DT group were down-regulated significantly ($P < 0.05$), compared with si-PUMA group ($P < 0.05$). Additionally, there was no significant difference of PUMA, Bax and Bcl-2 protein level between control group and NC group. This indicated that, knockdown of PUMA could lead to decrease of Bax protein level and increase of Bcl-2 protein level, which could be inhibited by treatment with DT.

Discussion

Chemotherapeutic drugs exert anti-cancer effect mainly through inducing cell apoptosis. There are some reasons to severely obstruct drugs clinical application, like tumor cells resistance to drugs and toxic side effect. Now, it's generally considered that classic pathways of cell apoptosis include mitochondrial pathway, endoplasmic reticulum pathway and death signal receptor pathway. Mitochondrial apoptotic pathway is a close correlation with Bcl-2 gene family (Xiao and Singh, 2006; Gong et al., 2012; Tu et al., 2012). Mechanisms of DT anti-tumor effect are related to antioxidation, inducing apoptosis and so on (Oommen et al., 2004; Kim et al., 2011; Na et al., 2012; Wang et al., 2012; Yu et al., 2012; Chandra-Kuntal et al., 2013; Yi and Su, 2013). Hassan (2004) has found that allicin could enhance the apoptotic effect of chemotherapeutic drugs through up-regulating the expression of Bcl-2 and Caspase-3 in human leukemia CD4 positive cells. Zhang et al. (2007) have reported that combined treatment of allicin with paclitaxel has a synergistic inhibiting effect on growth of gastric cancer cell lines, and induction of apoptosis through enhancing Bax expression and decreasing bcl-2 expression, interestingly, there was the same effect of allicin in prostatic cancer (Chen et al., 2012). Our results indicated that allicin inhibited the proliferation of SKOV-3/DDP cells via mediating the expressions of PUMA, Bax and Bcl-2, moreover, combined allicin with DDP had a synergistic inhibiting effect on proliferation of SKOV-3/DDP cells. Furthermore, we explored the molecular mechanism of tumor cell apoptosis induced by DT through mitochondria initiated apoptotic pathway. Results suggested that DT induced cell apoptosis through up-regulating the expression of PUMA, which could increase Bax expression and reduce Bcl-2 expression.

Many studies have revealed that PUMA played an important role in chemotherapeutic drugs by inducing tumor cells apoptosis (Wang et al., 2006; Jiang et al., 2006; Zhang et al., 2008), for example, treatment of DDP caused renal tubular epithelial cells apoptosis and necrosis, even renal injury, and meanwhile, there was an increase of PUMA expression (Jiang et al., 2006) in a time-dose dependence after treated with DDP. Liu and Wan also reported that allicin up-regulated the expression of PUMA to induce LoVo cells apoptosis (Liu and Wan, 2012). Once PUMA expression was inhibited, allicin had little effect on inducing cell apoptosis. Wang et al. (2008) found the same phenomenon in LoVo cells after treatment of green tea phlyphenols. Some studies demonstrated that after treatment of prostate cancer cells with wogonin, PUMA protein is increased, which release cytochrome C from mitochondria so as to activate caspases cascade reaction (Lee et al., 2008). We used RNAi technology to knockdown PUMA and find that apoptosis rate of SKOV-3/DDP cells was obviously decreased in si-PUMA transfection group, compared with treatment of DT after si-PUMA transfection. Moreover, si-PUMA significantly down-regulated the protein expression of PUMA and Bax more than control group, but up-regulated Bcl-2 expression more than control group. Compared with

si-PUMA group, PUMA and Bax levels were mildly up-regulated and Bcl-2 expression was down-regulated. Thus, in this study, combined allicin with DDP had a better inhibiting effect on SKOV-3/DDP cells apoptosis. PUMA is a pro-apoptotic member of the BH3-only subgroup of the Bcl-2 family (Lee et al., 2008; Wang et al., 2008; Liu and Wan, 2012; Huang and Wan, 2013). This implied that PUMA plays a critical role in SKOV-3/DDP cells apoptosis induced by DT and would probably become a new target for DT to treat tumors.

Acknowledgements

This work was supported by Key Science and Technology Programs of Jiang Xi Province (No. 2009JX01081).

References

Altonsy MO, Andrews SC (2011). Diallyl disulphide, a beneficial component of garlic oil, causes a redistribution of cell-cycle growth phases, induces apoptosis, and enhances butyrate-induced apoptosis in colorectal adenocarcinoma cells (HT-29). *Nutr Cancer*, **63**, 1104-13.

Chandra-Kuntal K, Lee J, Singh SV (2013). Critical role for reactive oxygen species in apoptosis induction and cell migration inhibition by diallyl trisulfide, a cancer chemopreventive component of garlic. *Breast Cancer Res Treat*, **138**, 69-79.

Chauhan SC, Kumar D, Jaggi M (2009). Mucins in ovarian cancer diagnosis and therapy. *J Ovarian Res*, **2**, 21-9.

Chen M, Li B, Zhao X, et al (2012). Effect of diallyl trisulfide derivatives on the induction of apoptosis in human prostate cancer PC-3 cells. *Mol Cell Biochem*, **363**, 75-84.

Fleischauer AT, Arab L. Garlic and cancer: a critical review of the epidemiologic literature (2001). *J Nutr*, **131**, S1032-40.

Gong Hui,Wan HF,Tu S, et al (2012). Effect of taurine on expression of gene GRP78 and Caspase-12 of the renal tissue at rats in ischemia reperfusion injury. *J Nachang Univ (Natural science)*, **36**, 166-71 (Chinese).

Hassan HT (2004). Ajoene (natural garliccompound) : A new anti-leukaemia agent for AML therapy. *Leuk Res*, **28**, 667-71.

Huang W, Wan FS (2013). New progress of PUMA protein structure and function. *Chin J Cancer Prev Treat,* **20**, 873-76 (Chinese).

Jeong EL, Ryung AL, Kwang HK, et al (2011). Induction of apoptosis with diallyl disulfide in AGS gastric cancer cell line. *JKSS*, **81**, 85-95.

Jiang M, Wei Q, Wang J, et al (2006). Regulation of PUMA-alpha by p53 in cisplatin-induced renal cell apoptosis. *Oncogene*, **25**, 4056-66.

Kim SH, Bommareddy A, Singh SV (2011). Garlic constituent diallyl trisulfide suppresses x-linked inhibitor of apoptosis protein in prostate cancer cells in culture and in vivo. *Cancer Prev Res (Phila)*, **4**, 897-906.

Lee DH, Kim C, Zhang L, et al (2008). Role of p53, PUMA and Bax in wogonin-induced apoptosis in human cancer cells. *Biochem Pharmacol*, **75**, 2020-33.

Li W, Tian H, Li L, et al (2012). Diallyl trisulfide induces apoptosis and inhibits proliferation of A549 cells in vitro and in vivo. *Acta Biochim Biophys Sin (Shanghai)*, **44**, 577-83.

Liu C, Wan FS (2012). Pro-apoptotic function of PUMA in human tumor. *Chemistry of life*, **32**, 23-8.

Mao ZG, Deng SH, Chen PS, et al (2010). The role of PUMA in apoptosisof colorectal cancer induced by allicin. *Chinese Journal of Hepatology*, **19**, 394-6 (In Chinese).

Na HK, Kim EH, Choi MA, et al (2012). Diallyl trisulfide induces apoptosis in human breast cancer cells through ROS-mediated activation of JNK and AP-1. *Biochem Pharmacol*, **84**, 1241-50.

Oommen S, Anto RJ, Srinivas G, et al (2004). Allicin (from garlic) induces caspase mediated apoptosis in cancer cells . *Eur J Pharmacol*, **485**, 97-103

Rouzier R, Bergzoll C, Brun JL, et al (2010). The role of lymph node resection in ovarian cancer: analysis of the Surveillance, Epidemiology, and End Results (SEER) database. *BJOG*, **117**, 1451-8.

Tsubura A, Lai YC, Kuwata M, et al (2011). Anticancer effects of garlic and garlic-derived compounds for breast cancer control. *Anticancer Agents Med Chem*, **11**, 249-53.

Tu S, Liu ZQ, Fu JJ, et al (2012). Inhibitory Effect of P53 Up-regulated Modulator of Apoptosis Targeting SiRNA on Hypoxia/Reoxygenation-induced Cardiomyocyte Apoptosis in Rats. *Cardiology*, **122**, 93-100.

Wang HC, Pao J, Lin SY, Sheen LY (2012). Molecular mechanisms of garlic-derived allyl sulfides in the inhibition of skin cancer progression. Ann N Y Acad Sci, **1271**, 44-52.

Wang X, Wang R, Hao MW, et al (2008). The BH3-only protein PUMA is involved in green tea polyphenol-induced apoptosis in colorectal cancer cell lines. *Cancer Biol Ther*, **7**, 902-8.

Wang XY, Li M, Wang JD, et al (2006). The BH3-only protein, PUMA, is involved in oxaliplatin-induced apoptosis in colon cancer cells. *Biochem Pharmacol*, **71**, 1540-50.

Xiao D, Singh SV (2006). Diallyl trisulfide, a constituent of processed garlic, inactivates Akt to trigger mitoehondrial translocation of BAD and caspase-mediated apoptosis in human prostate cancer cells. *Carcinogenesis*, **27**, 533-40.

Yi L, Su Q (2013). Molecular mechanisms for the anti-cancer effects of diallyl disulfide. *Food Chem Toxicol*, **57**, 362-70.

Yu CS, Huang AC, Lai KC, et al (2012). Diallyl trisulfide induces apoptosis in human primary colorectal cancer cells. *Oncol Rep*, **28**, 949-54.

Zhang JS, Cui J, Ding LJ, et al (2007). The inhibiting effect of combinationof allicin with paclitaxel on proliferation of gastric cancer cells and promotion of apoptosis. *Chin J General Surg*, **16**, 341-5 (Chinese).

Zhang YW, Liu XH, Ma HX, et al (2008). Apoptosis induced effect of transfected pum a geneon gastric carcinoma cell line SGC-7901 and its mechanisms. *Bull Chin Cancer*, **17**, 137-41 (Chinese).