RESEARCH ARTICLE

Effects of Garlic Oil on Pancreatic Cancer Cells

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Abstract

Background: To investigate the preventive and therapeutic potential of garlic oil on human pancreatic carcinoma cells. Methods: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed to study the effects of garlic oil on three human pancreatic cancer cell lines, AsPC-1, Mia PaCa-2 and PANC-1. Cell cycle progression and apoptosis were detected by flow cytometry (FCM), staining with PI and annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI), respectively. Morphologic changes of pancreatic cancer cells were observed under transmission electron microscopy (TEM) after treatment with garlic oil at low inhibitory concentrations (2.5 μM and 10 μM) for 24 hours. Results: Proliferation of the AsPC-1, PANC-1, and Mia PaCa-2 cells was obviously inhibited in the first 24 hours with the MTT assay. The inhibition effect was more significant after 48 hours. When cells were exposed to garlic oil at higher concentrations, an early change of the apoptotic tendency was detected by FCM and TEM. Conclusion: Garlic oil could inhibit the proliferation of AsPC-1, PANC-1, and Mia PaCa-2 cells in this study. Moreover, due to programmed cell death, cell cycle arrest, or both, pro-apoptosis effects on AsPC-1 cells were induced by garlic oil in a dose and time dependent manner *in vitro*.

Keywords: Garlic oil - pancreatic carcinoma - AsPC-1 cells - proliferation - apoptosis

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Introduction

Pancreatic cancer, the fourth leading cause of cancer-related death, possesses a very poor prognosis and an extremely high death/incidence ratio of approximately 99%, which is not only due to its little possibility of surgical resection but also the increasing resistance to almost all chemotherapeutic agents (Lange et al., 2012; Jiang et al., 2012; Ren et al., 2012). Therefore multidisciplinary therapeutic approaches and new anti-cancer pharmaceuticals are necessary to prevent pancreatic cancer from occurring and to improve its outcome.

Recently, there has been encouraging progress, from a western perspective, in the cancer research field regarding the Chinese Herbal Medicines (CHM) as an effective therapeutic method to improve cancer survival, to increase tumor response, or to reduce chemotherapy toxicity and to keep cancer from recurring (Qi et al., 2010). For example, it has been demonstrated that garlic (allium sativum) possesses anti-mutagenic or anti-proliferative properties that can be used in anticancer interventions. Another report presents that garlic oil can exhibit significant protection against N-nitrosodiethylamine-induced hepatocarcinogenesis (Agarwal at al., 2007; Zhang at al., 2012). Thus, garlic oil is believed to be a promising cancer chemo-preventive constituent (Kim et al., 2011). It was reported that garlic contains water soluble and oil-soluble sulfur compounds. Recently, it has been gradually understood that the latter are responsible for anticancer effects exerting through multiple mechanisms such as: amelioration of oxidative stress and improvement of immune function (Butt at al., 2009), and inhibition of metabolic carcinogenic activation (Miroddi at al., 2011; Velliyagounder et al., 2012; Wang et al., 2012; Zhang et al., 2012)

Experiments in further had shown new mechanisms for the anticancer role of garlic oil such as by inducing apoptosis (Choi and Park, 2012; Wang et al., 2012), inhibiting differentiation, inhibiting tumor angiogenesis (Konkimalla et al., 2008), and reversing multidrug resistance (Arora et al., 2004). As compounds, it has been demonstrated that garlic oil has the ability to enhance the absorption of its main active component (Amagase et al., 2001).

However, at present, there is a dispute whether garlic oil can reduce risk of cancer (Rivlin at al., 2009). How garlic oil exhibits its effect on the tumor cells and its underlying mechanism are poorly understood (Agarwal at al., 2007; Zhang at al., 2012), especially the effect on pancreatic carcinoma. For this reason, the aim of this study is to investigate the effect of garlic oil on human pancreatic cancer cells (including AsPC-1, PANC-1, and Mia PaCa-2 cell lines) and its underlying mechanism in order to find a pathway for pancreatic cancer prevention and treatment considering its multidrug resistance.

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Materials and Methods

Cell Culture

PANC-1, AsPC-1, Mia PaCa-2 human pancreatic cancer cell lines were given by Kumamoto University (Japan) as a gift. Cells cultured with DMEM (High Glucose) medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 units/ml penicillin, and 100μg/ml streptomycin (all from Sigma Aldrich, USA), at 37°C in an atmosphere of 5% CO_2, plated in 5× 10^4/cm^2 culture flasks. Logarithmically growing cells were treated with 0 μM, 2.5 μM, 10 μM, 25 μM, 50 μM, 100 μM and 200 μM garlic oil (Xuchang Yuanhua Biotechnology, China) respectively at 37°C.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) Assay for Cell Growth Inhibition Rate

PANC-1, AsPC-1, Mia PaCa-2 human pancreatic cancer cells were seeded at 5× 10^4/ml respectively in 96-well plates. After 24 and 48 hours of incubation with different concentrations of garlic oil, ranging from 0 μM to 200 μM, at 37°C in a medium without serum, 20 ul MTT (5 mg/ml) was added to each well. 4 hours later, the MTT solution was removed and 150ul dimethylsulfoxide (DMSO) was added to each well. Absorbance at 570nm corrected to 650 nm was measured with a microplate reader (Multiskan MK3; Pioneer Co; China). Cell growth inhibition rate was calculated from these results using the formula (=[OD (value of control group) - OD (value of essay group)]/OD (value of control group) × 100%).

Transmission Electron Microscopy (TEM) Analysis for Morphological Changes

AsPC-1 cells were seeded and grown at 5×10^7/ml in three flasks. Cells after treated with garlic oil (0, 2.5 μM, 10 μM) were harvested and washed with 1xPBS twice, and then added to 2.5% glutaraldehyde fixative for microtome sectioning using ultramicrotome (LKB-V; JEOL Co; Japan). TEM was performed with a Transmission Electron Microscope (JEM-2000EX; JEOL Co; Japan).

Propidium iodide (PI) Staining for Cell Cycle Distribution

24 hours after 0 μM, 2.5 μM, 10 μM, 50 μM, 100 μM garlic oil exposure, the cells were collected respectively, adjusted into single cell suspension and centrifuged at 500g for 3-5min. After discarding the supernatant, cells were washed twice with cold PBS and fixed with alcohol at 4°C for 24 h. Cells were suspended in 1ml of buffer (10^6/ml), washed three times with cold PBS, treated with RNase (0.25 g/L) for 30min at 37°C, and stained with PI (Nanjing KeyGen Biotech, China) for 30min at 37°C in the dark. Then cell cycle distribution was analyzed with flow cytometry (BD FACSAria II; BD Co; America) in 12 h.

Annexin V/PI Assay for Apoptosis Rate

Viability of cells were examined by Annexin V-FITC and PI (AV-PI) (Nanjing KeyGen Biotech, China) staining according to the manufacturer's protocol and then analyzed by flow cytometry. At the end of the incubation with garlic oil for 24 h, 1×10^6 AsPC-1 cells were collected and washed twice with cold 4°C PBS. The samples were resuspended in 1ml of 1×binding buffer mixed with 5μl of Annexin V-FITC and 5 μl of PI, and then incubated in the dark at room temperature for 15 minutes. Samples were then analyzed by flow cytometry immediately after staining (within 30 min).

Statistical analysis

SPSS software package (version 13.0, Chicago, IL, USA) was used for statistical analysis. Data was expressed as means±SD. The cell phases and apoptosis were compared using Paired-Simple T Test to analyze the significance of difference between different groups, respectively. The MTT results were compared using Analysis of Variance (ANOVA). $P<0.05$ was considered to be statistically significant.

Results

The reduction of cell density and promotion of cell-clustering by garlic oil

Under inverted phage contrast microscope, the control group cells exhibited a typical polygonal shape (Figure 1A). After exposing to garlic oil for 24h, cells began to experience a process of morphological changes, the majority of cells became rounded, small ones, some even aggregated into multicellular spheroids ones. The number of AsPC-1 cells was decreased significantly after exposing to garlic oil for 24h in a dose-dependent manner (Figure 1B, C, and D).

The reduction of cell survival rate of pancreatic cancer cells by garlic oil in a dose-dependent manner

The anti-proliferative effect of garlic oil on AsPC-1, Mia PaCa-2, PANC-1 cells were determined by the MTT in vitro. It showed that garlic oil possessed an anti-proliferative activity in a dose and time-dependent manner. In AsPC-1 cell lines, 48 hour group showed more significant inhibition effect. However, in Mia PaCa-2 and PANC-1 cells, 48 hour groups only presented obvious

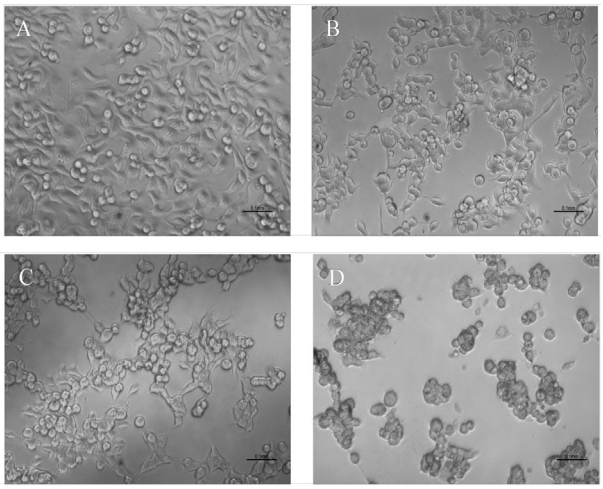

Figure 1. Cell Culture for 24 Hours after Exposing to Garlic Oil. Cells in 96-well plate were treated by garlic oil for 24h, and observed under inverted phage contrast microscope (x100). A) Exposed to 0 μM; B) Exposed to 10 μM; C) Exposed to 50 μM; D) Exposed to 100 μM

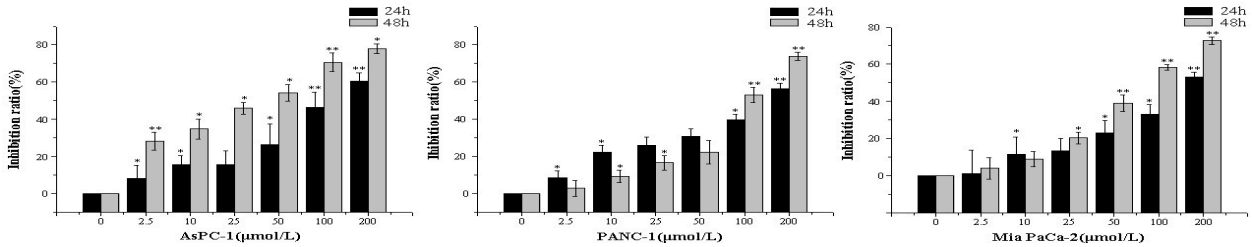

Figure 2. The Cell Inhibition Ratio of AsPC-1, PANC-1, Mia PaCa-2 Cells Exposed to Garlic Oil for 24 Hours and 48 Hours by MTT Assay. The changes of inhibition ratio at different garlic oil concentrations were determined after 24 hours and 48 hours. Comparing with the anterior garlic oil concentration: *P<0.05, or **P<0.01

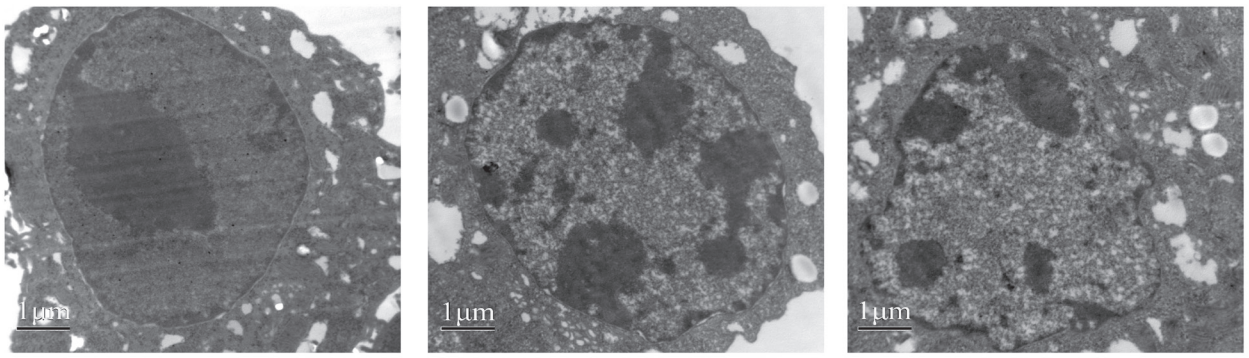

Figure 3. Transmission Electron Microscopy Showed Cell Morphologic Change of AsPC-1 under the Influence of Garlic Oil. A) Treated with 0 μM garlic oil; B) Treated with 2.5 μM garlic oil; C) Treated with 10 μM garlic oil

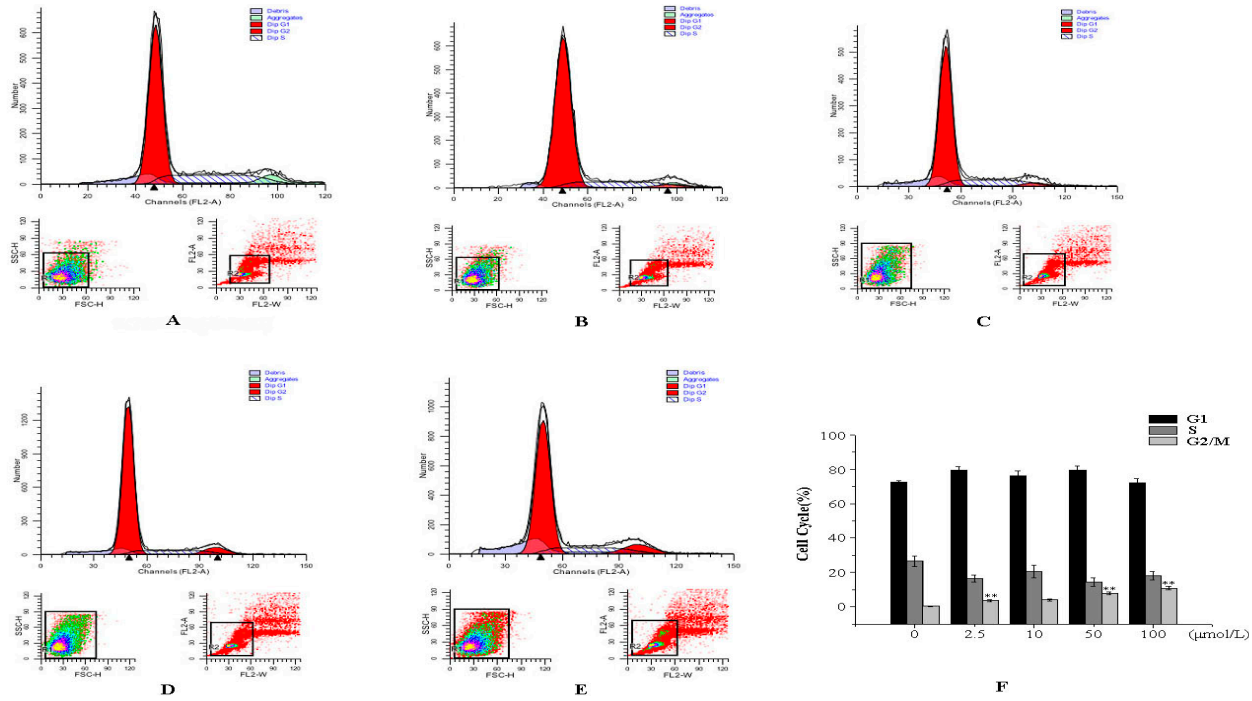

Figure 4. An Accumulation of G2/M Phages in AsPC-1 Cells after Garlic Oil Treatment was Observed at 24 Hours Using Flow Cytometer Analysis. A) Exposed to 0 μM; B) Exposed to 2.5 μM; C) Exposed to 10 μM; D) Exposed to 50 μM; E) Exposed to 100 μM; F) The percentage of cell cycle in each garlic oil concentration. Horizontal axis presents drug concentration, and vertical axis presents the percentage of cell cycle. Comparing with the anterior concentration in G_2/M phages: **P<0.01

inhibitory effect in higher concentrations of garlic oil (Figure 2). These results indicated that garlic oil could induce pancreatic cancer cells apoptosis or damage. The difference was statistically significant (P<0.05).

The apoptosis promotion of AsPC-1 cell by garlic oil under the detection by transmission electron microscopy

Under the observation of transmission electron microscope, normal AsPC-1 cells were round and regular, with abundant organelles and normal double-membrane nuclei (Figure 3A). After exposing to garlic oil for 24 hours, early stage apoptosis could be observed both in 2.5 μM (Figure 3B) and 10 μM (Figure 3C) garlic oil group. It showed that nuclear membrane was domed outward with a sharp angle, and the nuclei chromatin was concentrated and clustered on the inner border of

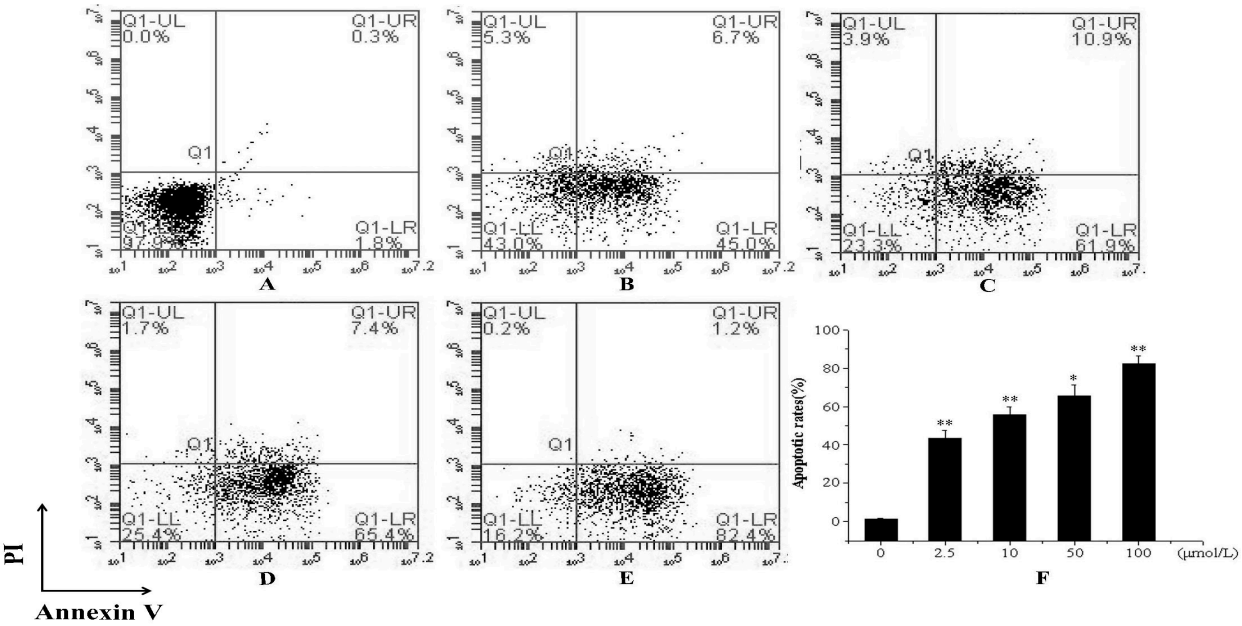

Figure 5. Apoptosis of AsPC-1 Cells Induced by Garlic Oil was Detected in a Concentration-dependent Manner at 24 Hours. A) Exposed to 0 μM; B) Exposed to 2.5 μM; C) Exposed to 10 μM; D) Exposed to 50 μM; E) Exposed to 100 μM; F) The different apoptosis rate of cells treated with each concentration. Comparing with the anterior garlic oil concentration: **P*<0.05, or ***P*<0.01

karyotheca. The endoplasmic reticulum became dilated in the inner segment.

An accumulation of G_2/M phages in AsPC-1 cells analyzed by flow cytometry

The effect of garlic oil on AsPC-1 cell proliferation was evaluated by flow cytometry at 24 hours, through measuring the distribution of cells in different phases of the cell cycle. It was showed that percentages of G1 phase decreased from 72.66% to 72.57%, while the percentages of G_2/M phase increased from 0.45% to 10.15%. Therefore, the cell cycle was dramatically arrested at G_2/M phase comparing with the control groups. On the other hand, the shift of cell distribution above was in a remarkable concentration-dependent manner (Figure 4). In conclusion, these results suggested that garlic oil possessed a prominent ability to inhibit cell proliferation and induce apoptosis in AsPC-1 cells, the difference had statistical significance (*P*<0.05).

The pro-apoptosis effects of garlic oil on AsPC-1 cells using staining with Annexin V-FITC/PI

Flow cytometry analysis with Annexin V-FITC/PI staining was undertaken to determine the effect of garlic oil on AsPC-1 apoptosis. The lower-right (LR) area was the Annexin V^+/PI^- portion, which represented the apoptotic fraction. In garlic oil free-medium group, there were rarely viable apoptotic cells, while in garlic oil group, the apoptotic rates were 45.0% (2.5 μM), 57.5% (10 μM), 65.4% (50 μM), and 82.4% (100 μM), respectively (Figure 5). These results indicated that apoptotic cells were gradually increased in a concentration-dependent manner.

Discussion

Pancreatic cancer patients have been suffering from limited treatment options due to late diagnosis, poor drug tolerance, and multi-drug resistance to almost all the current drug treatments, which was considered to be the most serious problem at present (Guo et al., 2013; Won et al., 2013; Szepeshazi et al., 2013). Therefore, it is significant for us to seek an alternative therapeutic medicines which can effectively prevent the disease and even eradicate the progression and metastasis of pancreatic cancer.

From the MTT assay and microphotographs, we found that garlic oil could remarkably inhibit the proliferation of each cell line of pancreatic carcinoma, including AsPC-1, Mia PaCa-2 and PANC-1, with minimum doses. Besides, the suppression effect of garlic oil acquired an most prominent exertion on AsPC-1 cell line probably due to the its high proliferation ratio and malignance. After exposing to garlic oil, nearly all tumor cells showed a shrinking and clustering tendency instead of spreading along with proliferation, demonstrating that garlic oil may prevent and even stop pancreatic cell from transferring and metastasis.

Our results showed that garlic oil possessed a prominent ability to inhibit cell proliferation of AsPC-1 cells, depending on an accumulation of G_2/M phages in AsPC-1 cells analyzed by FCM. Moreover, garlic oil presented a prominent ability to induce cell apoptosis using staining with Annexin V-FITC/PI, confirming that the underling mechanism of anti-proliferation was mainly mediated by an induction of apoptosis, also proved by our further investigation of TEM and FCM. The above results were similar to the earlier report that garlic oil is effective in the reduction of anti-proliferative gene and modulation of apoptosis-associated cellular proteins in non small cell lung cancer cells (Hong et al., 2000).

However, in our study, it was discovered firstly that garlic oil had a pancreatic cell cytotoxicity with cell

cycle blockade that occurs particularly in the G_2/M phage. The above results were different from a report that demonstrating the effect on gastric adenocarcinoma was through inhibition of cyclin E expression or inhibition of the TGFalpha autocrine and paracrine loops (Liang at al., 2007) .

For the main components of garlic oil, we propose that the active effectors maybe were related to the diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS) and others (Wu et al., 2002; Calvo-Gómez et al., 2004; Shin et al., 2010; Shin and Cha et al., 2010). Evidence showed that DADS and DATS suppress the growth of multiple cancer types both in vitro and in vivo (Powolny and Singh, 2008; Herman-Antosiewicz et al., 2010; Lee et al., 2011; Altonsy and Andrews, 2011; Altonsy et al., 2012; Wang et al., 2012). Further study would be necessary to confirm that in the following experiments.

Taken together, our study is the first to demonstrate the in vitro therapeutic effect of garlic oil on human pancreatic cancers, including AsPC-1, Mia PaCa-2 and PANC-1 cell lines, suggesting that garlic oil is a potential therapeutic drug for pancreatic cancer. And also, the results could further enrich the garlic oil's anti-tumor spectrum. Garlic oil can inhibit AsPC-1 cells growth through anti-proliferation and induce apoptosis. However, which exactly component of garlic oil plays an main role is still unknown, DADS or DATS, or others? what is the more or deep underlying signal transduction mechanism? Does it still have effect both in vivo and among different pancreatic cancer patients? Lots of questions need to be resolved. While a great of profits have been acquired from the widely use of garlic and garlic oil itself as an health care product, in the same time, no data has reported significant side effect within appropriate dosage. For this reason, it will be beneficial and necessary for further study to reveal the underlying mechanism and study the effect in vivo.

Acknowledgements

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