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Allicin inhibits the invasion of lung adenocarcinoma cells by altering tissue inhibitor of metalloproteinase/matrix metalloproteinase balance via reducing the activity of phosphoinositide 3-kinase/AKT signaling

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Abstract. Allicin, the main active principle associated with *Allium sativum* chemistry, has various antitumor activities. However, to the best of our knowledge, there is no available information to address the anti-invasive effect and associated mechanism in lung adenocarcinoma. In the present study, cell viability assay, cell adhesion assay, western blot analysis, Transwell migration and invasion assays and reverse transcription-quantitative polymerase chain reaction were performed. Allicin was identified to inhibit the adhesion, invasion and migration of lung adenocarcinoma cells in a dose-dependent manner, accompanied by decreasing mRNA and protein levels of matrix metalloproteinase (MMP)-2 and MMP-9. Conversely, the mRNA and protein levels of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 were increased in a dose-dependent manner. Furthermore, it was revealed that allicin treatment significantly suppressed the phosphorylation of AKT ($P<0.05$), but not the total protein expression of AKT. Combined treatment with LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K)/AKT signaling, and allicin led to the synergistic reduction of MMP-2 and MMP-9 expression, followed by an increase in TIMP-1 and TIMP-2 expression. The invasive capabilities of lung adenocarcinoma cells were also suppressed. However, insulin-like growth factor-1 (an activator of PI3K/AKT signaling) reversed the effects of allicin on cell invasion and expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. The present study concluded that allicin may inhibit invasion of lung adenocarcinoma cells by altering TIMP/MMP balance, via reducing the activity of the PI3K/AKT signaling pathway. This indicated that allicin may be recognized as an anti-invasive agent for lung adenocarcinoma treatment.

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Introduction

Lung cancer is the predominant reason of cancer-associated mortality in developed and developing countries. Lung adenocarcinoma is the most common histological type of lung cancer, and accounts for ~50% of all lung cancers (1). Although the management and treatment of surgery, radiotherapy and chemotherapy have improved, the therapeutic efficacy is poor. One of the major reasons is the lack of adequate treatment against lung adenocarcinoma invasion (2,3). Therefore, the development of novel adjuvant therapeutic strategies specifically targeting the progression of invasion is of critical importance for improving the prognosis of patients with lung adenocarcinoma.

Invasion occurs through a complex pathophysiological process involving multiple genetic alterations (4,5). Matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, play an important role in the invasion process of numerous malignant tumors by degrading the basement membrane and extracellular matrix (ECM) (6-8). Previous studies have suggested that MMP-2 and MMP-9 are associated with invasion in lung cancer (4,5). In addition, numerous studies have demonstrated that MMPs may be regulated by tissue inhibitor of metalloproteinase (TIMP) (6,9). Disturbing the balance of MMPs and TIMPs may affect the remodeling, formation and degradation of matrix protein and induce invasion of cancer cells.

The phosphoinositide 3-kinase (PI3K)/AKT signaling pathway performs a key role in the control of cell differentiation and proliferation. Previously, activation of the PI3K/AKT signaling pathway was suggested to be associated with the invasion of numerous tumor types, including prostate, ovarian, colon and breast cancers (10-13). In lung cancer, the PI3K/AKT signaling pathway is also considered as a crucial activator of intracellular signaling cascades in invasion progression and is useful as a therapeutic target for anticancer drug development.

Allicin, the main active principle associated with *Allium sativum* chemistry, possesses therapeutic potential, with antioxidant, anti-inflammatory and antitumor activities. Previous studies demonstrated that allicin may induce tumor cell apoptosis, inhibit the tumor cell cycle and regulate angiogenesis (14-17). Numerous mechanisms are involved in the biological activities of allicin, including unfolded protein response, p53-mediated autophagy and p38 mitogen-activated protein kinase/caspase-3 signaling (14-18). However, to the best of our knowledge, the effects and mechanisms of allicin on lung adenocarcinoma remain undefined. In addition, the role of allicin in inhibiting invasion has not been reported. In the present study, it was revealed that allicin may suppress migration and invasion of lung adenocarcinoma cells by altering TIMP/MMP balance via reduction of the activity of the PI3K/AKT signaling pathway *in vitro*.

Materials and methods

Reagents. Lipofectamine® 2000 reagents and fetal bovine serum (FBS) were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Allicin and specific activator of PI3K (insulin-like growth factor-1; IGF-1) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Specific inhibitor of PI3K (LY294002) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). MTT was purchased from Beyotime Institute of Biotechnology. Anti-phospho-AKT^{Ser473} (anti-p-AKT^{S473}) and anti-AKT were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-MMP-2, anti-MMP-9, anti-TIMP-1, anti-TIMP-2 and anti-β-actin were purchased from Santa Cruz Biotechnology, Inc. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (cat no. ZDR-5307; dilution, 1:2,000) and anti-mouse immunoglobulin (cat no. ZDR-5307; dilution, 1:2,000) were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

Cell lines and cell culture. The two lung adenocarcinoma cell lines A549 and H1299 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2.

Cell viability assay. The effects of allicin on cell viability were determined by an MTT assay. MTT was purchased from Beyotime Institute of Biotechnology. Cells (10^4 cells per well) were seeded onto 96-well plates, incubated overnight at 37˚C and then incubated in various concentrations of allicin (0, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0 and 20.0 μM) for 24 h. The medium was then removed and the MTT solution (0.5 mg/ml) was added to the cell culture. Following incubation for 4 h at 37˚C, the reaction was stopped by adding dimethyl sulfoxide (0.5 mg/ml). At the end, absorbance was measured spectrophotometrically at 570 nm (Bio-Tek ELX800UV; Omega Bio-Tek Inc., Norcross, GA, USA).

Cell adhesion assay. The 96-well plates were prepared for coating with 5 mg/ml fibronectin (Sigma-Aldrich; EMD Millipore, Billerica, MA, USA) and blocking with 1% bovine serum albumin (BSA) for 4 h. A549 and H1299 cells were incubated for 48 h with various concentrations of allicin (0, 5.0, 7.5 and 10.0 μM) at 37˚C. Cells (20,000 cells/well) were then allowed to attach to fibronectin coated plates for 1 h at 37˚C. The unattached cells were washed away with PBS. Attached cells were quantified by MTT assay.

Transwell migration and invasion assays. Cell invasion experiments were assayed using 6.5-mm Transwell chambers (8-μm pore size; Corning-Costar Inc., Corning, NY, USA). The filters were precoated with 1-2 mg/ml Matrigel (reconstituted basement membrane; BD Biosciences, Franklin Lakes, CA, USA). Cells were pretreated with 0, 5.0, 7.5 and 10.0 μM allicin or IGF-1 (50 ng/ml) or LY294002 (25 μM). Surviving cells in 100 μl of serum-free medium were seeded in the upper chamber. Medium supplemented with 10% FBS was added to the lower chamber as the chemoattractant. Following 24 h of incubation at 37˚C, the cells on the upper side were wiped with a cotton bud. The cells that had migrated into the lower compartment were fixed with methanol, stained with hematoxylin and eosin (Beyotime Institute of Biotechnology) and counted in 5 random fields of the insert under a light microscope (magnifcation, x200). A migration assay was performed as described for the invasion assay, but with a shorter incubation period (12 h) and no Matrigel coating.

Western blot analysis. The A549 and H1299 cells were extracted with radioimmunoprecipitation assay buffer [1 mg/ml phenylmethylsulfonyl fluoride, 1 Mm aprotinin, 1 mg/ml leupeptin, 1 mM EDTA, 150 mM NaCl, 0.25% Na-deoxycholate, 1 mg/ml pepstatin and 50 mM Tris-HCl (pH 7.4)] following treatment with various concentrations (0, 5.0, 7.5 and 10.0 μM) of allicin, IGF-1 or LY294002. Total proteins were quantified using the bicinchoninic acid method. Equal amounts of protein were separated on SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride membranes. Subsequent to blocking in 5% BSA for 1 h at room temperature, membranes were incubated overnight at 4˚C with antibodies against MMP-2 (dilution, 1:500; cat no. sc-13594), MMP-9 (dilution, 1:500; cat no. sc-12759), TIMP-1 (dilution, 1:500; cat no. sc-6832), TIMP-2 (dilution, 1:500; cat no. sc-365671), p-AKT^{S473} (dilution, 1:1,000; cat no. sc-33437), AKT (dilution, 1:1,000; cat no. sc-24500) or β-actin (dilution, 1:1,000; cat no. sc-10731). The membranes were then incubated with the appropriate HRP-conjugated goat anti-rabbit (cat no. ZDR-5307; dilution, 1:2,000) and anti-mouse immunoglobulin (cat no. ZDR-5307; dilution, 1:2,000) for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Inc.). The densitometry analysis was performed using Quality One analysis software (version 6.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from A549 and H1299 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), subsequent to treatment with various concentrations of allicin (0, 5.0, 7.5 and 10.0 μM). cDNA was synthesized

and RT-qPCR was performed in accordance with previously described protocols (19). The primers for human MMP-2 (forward, 5'-GGTTGTCTGAAGTCACTGCACAGT-3' and reverse, 5'-CTCGGTAGGGACATGCTAAGTAGAG-3'), MMP-9 (forward, 5'-GCTGGGCTTAGATCATTCCTCA-3' and reverse, 5'-CTGGCGACGCAAAAGAAGA-3'), TIMP-1 (forward, 5'-GAGAACCCACCATGGCCC-3' and reverse, 5'-TATCAGCCACAGCAACAACAGG-3'), TIMP-2 (forward, 5'-CCACCCAGAAGAAGAGCCTG-3' and reverse, 5'-CAG CGCGTGATCTTGCAC-3') and GAPDH (forward, 5'-CCT CCCGCTTCGCTCTCT-3' and reverse, 5'-CTGGCGACG CAAAAGAAGA-3') were used for RT-qPCR. The average expression level of genes was normalized to the reference gene GAPDH. Data analysis was performed using the $2^{-\Delta\Delta Cq}$ method (19).

Statistical analysis. Each experiment was repeated at least three times. Data are presented as the mean ± standard deviation. SPSS version 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) and Student's t-test were used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Allicin inhibits proliferation of lung adenocarcinoma cells. To determine the antitumor effect of allicin against lung adenocarcinoma cells, the ability of allicin to inhibit proliferation in lung adenocarcinoma A549 and H1299 cells was first examined by the cell viability assay. As shown in Fig. 1, cell proliferation was significantly reduced in A549 and H1299 cells following treatment with 15.0 and 20.0 μM allicin ($P<0.000$). However, no significant reduction in proliferation was observed when lung adenocarcinoma cells were treated with allicin at concentrations below 15.0 μM. Therefore, a concentration range of allicin <15.0 μM was selected for all subsequent experiments in order to *exclude* the effect of cellular cytotoxicity on invasion.

Allicin inhibits adhesion, migration and invasion of lung adenocarcinoma cells. Cell migration and invasion are critical events in the development of lung adenocarcinoma. The present study first examined the cell adhesion ability following incubation of A549 and H1299 cells with allicin. It was observed that allicin treatment decreased tumor cell adhesion to fibronectin in a concentration-dependent manner (Fig. 2A and B). The effect of allicin on migration and invasion was then examined in A549 and H1299 cells that were exposed to various concentrations of allicin for 12 h (cell migration) and 24 h (cell invasion). The number of migratory or invasive lung adenocarcinoma cells decreased in a dose-dependent manner (Fig. 2C and D). The aforementioned data demonstrated that allicin inhibited adhesion, migration and invasion of lung adenocarcinoma cells.

Allicin alters TIMP/MMP balance in lung adenocarcinoma cells. It has been demonstrated that MMPs, particularly MMP-2 and MMP-9, are involved in the invasion of lung cancer. Therefore, the present study then investigated whether allicin regulates the expression of MMP-2 and MMP-9. Allicin was revealed to dose-dependently inhibit MMP-2 and MMP-9 mRNA and protein levels in H1299 cells (Fig. 3). Previous studies indicated that the expression of MMPs was regulated by their endogenous tissue inhibitors (TIMPs) (7-9). Thus, the expression levels of TIMP-1 and TIMP-2 in H1299 cells were examined by RT-qPCR and western blot analysis following treatment with 0, 5.0, 7.5 and 10.0 μM of allicin for 48 h. It was identified that allicin upregulated the RNA and protein levels of TIMP-1 and TIMP-2 in H1299 cells in a concentration-dependent manner (Fig. 3). These results indicated that allicin regulates TIMP/MMP balance and stimulates H1299 cell invasion.

The PI3K/AKT signaling pathway is associated with the anti-invasive mechanism of allicin. Studies have shown that activation of PI3K/AKT signaling plays a vital role in the invasion process of lung cancer (12,20). Thus, the effect of allicin on the PI3K/AKT signaling pathway in H1299 cells was investigated. As shown in Fig. 4, allicin significantly suppressed the phosphorylation of AKT in a concentration-dependent manner ($P<0.05$). However, the total protein expression of AKT was not altered by allicin. To confirm whether the inhibitory effect of allicin on cell invasion and TIMP/MMP balance was associated with inhibition of the PI3K/AKT signaling pathway, H1299 cells were pretreated with or without PI3K inhibitor (LY294002, 25 μM) for 1 h, and then exposed to allicin (0 or 7.5 μM) for 48 h. It was identified that treatment with LY294002 and allicin significantly inhibited cell invasion ($P<0.015$; Fig. 5A), decreased MMP-2 ($P<0.002$) and MMP-9 ($P<0.000$) protein expression and increased TIMP-1 ($P<0.000$) and TIMP-2 ($P<0.000$) protein expression (Fig. 5B and C) compared with the allicin treated group. Furthermore, H1299 cells were pretreated with PI3K activator (IGF-1; 0 or 50 ng/ml) for 1 h and then exposed to various concentrations of allicin (0 or 7.5 μM) for 48 h. It was revealed that the effects of allicin on cell invasion and protein expression of MMP-2 ($P<0.001$), MMP-9 ($P<0.001$), TIMP-1 ($P<0.000$) and TIMP-2 ($P<0.000$) were significantly reversed by IGF-1 (Fig. 5D-F). These results revealed that allicin inhibited the invasion of lung adenocarcinoma cells by altering TIMP/MMP balance via regulation of PI3K/AKT signaling.

Discussion

Abnormal invasion of cancer cells is considered to be the crucial biological feature of cancer. The presence of invasion is the major reason of recurrence and mortality in patients with lung cancer (1,20). In the present study, allicin was found to inhibit lung adenocarcinoma cell adhesion, migration and invasion. It also provided evidence that the mechanism underlying the aforementioned effects was associated with altering TIMP/MMP balance, which was regulated by the PI3K/AKT signaling pathway. The present study shed light on the investigation of allicin in lung adenocarcinoma invasion.

Increasing studies have demonstrated that allicin exhibits a cytotoxic effect in several human cancer cells, including glioma U87, liver cancer G2 and gastric cancer MGC803 cells (15-17). These high specificities make allicin a promising anticancer agent for lung adenocarcinoma. In the present study, it was identified that treatment with allicin was able to

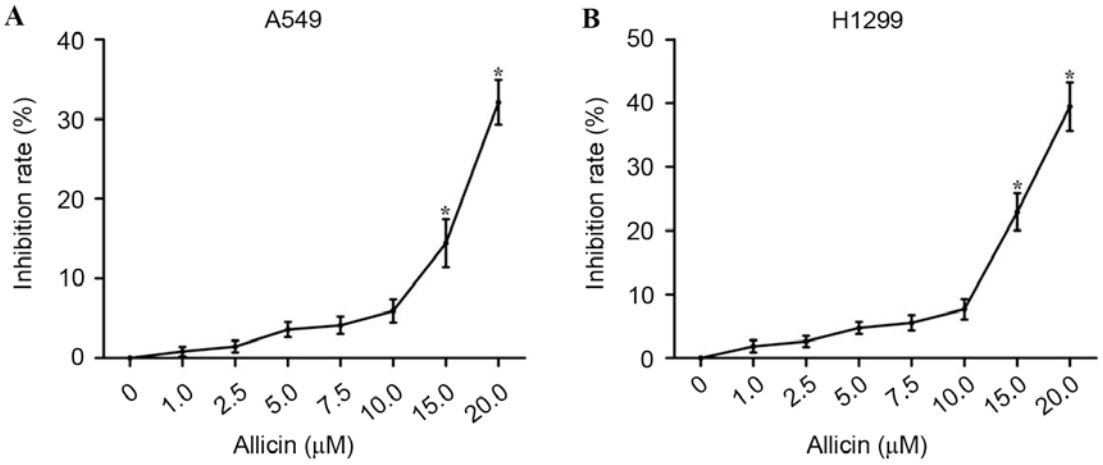

Figure 1. Effects of allicin on the proliferation of lung adenocarcinoma cells. (A) A549 and (B) H1299 cells were treated with different concentrations of allicin for 24 h. Cell viability was determined by the MTT method. Data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 compared with the 0 μM arctigenin group.

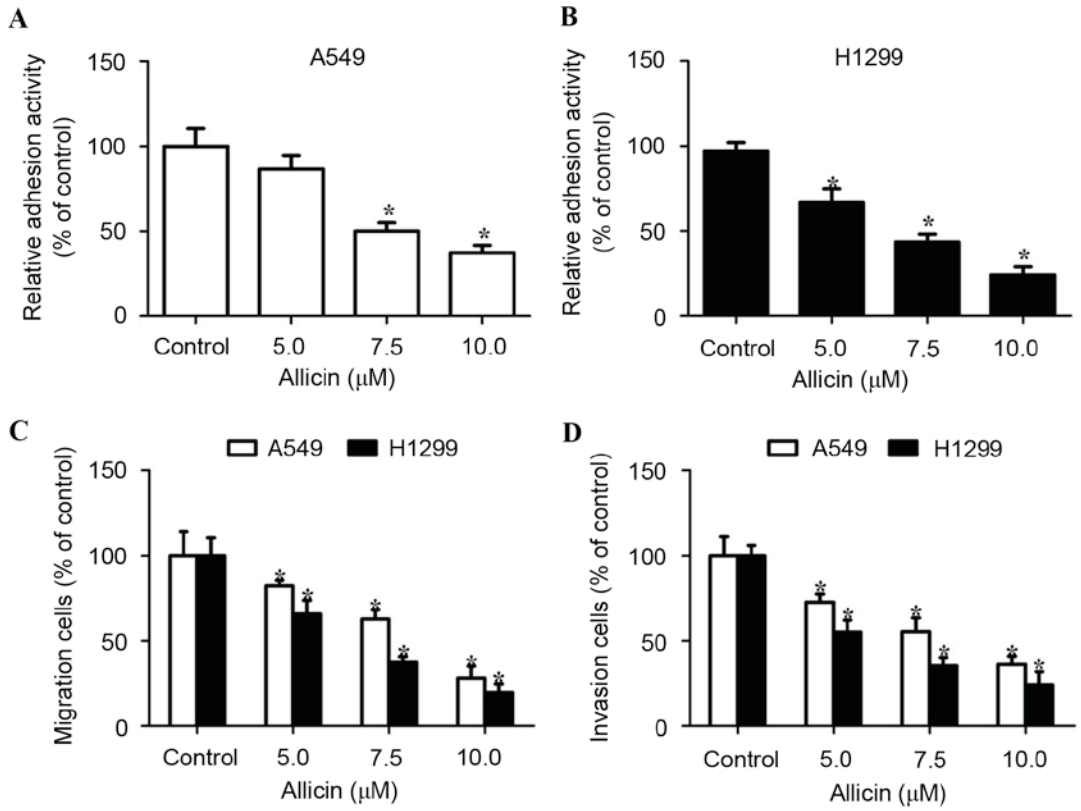

Figure 2. Effect of allicin on the adhesion, migration and invasion of lung adenocarcinoma cells. (A) A549 and (B) H1299 cells were incubated for 24 h with various concentrations of allicin. Cells were seeded onto 96-well plates coated with fibronectin. After 1 h, the adhered cells were analyzed by MTT assay. The adhesion rate was expressed as a percentage of the control (0 μM). (C) A549 and H1299 cells were seeded in the upper wells without coating of Matrigel, and treated with various concentrations of allicin. After 12 h, cells on the bottom side of the filter were fixed, stained and counted. The migration rate was expressed as a percentage of the control (0 μM). (D) A549 and H1299 cells were seeded in the upper wells with coating of Matrigel, and treated with various concentrations of allicin. After 24 h, cells on the bottom side of the filter were fixed, stained and counted. The invasion rate was expressed as a percentage of the control (0 μM). Data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 compared with controls.

suppress cell viability, indicating that allicin also possesses cytotoxicity against lung adenocarcinoma. To the best of our knowledge, there are only a small number of studies on the association between allicin and the invasion of cancer cells. Thus, the role of allicin in the invasion of lung adenocarcinoma cells was also analyzed. It was revealed that allicin is associated with decreased adhesion, migration and invasion of lung adenocarcinoma cells. This indicated that allicin may suppress invasiveness in lung adenocarcinoma.

MMPs, particularly MMP-2 and MMP-9, control cell-cell and cell-matrix interactions. Normally, TIMPs specifically combine with MMPs and keep their activity in a dynamic

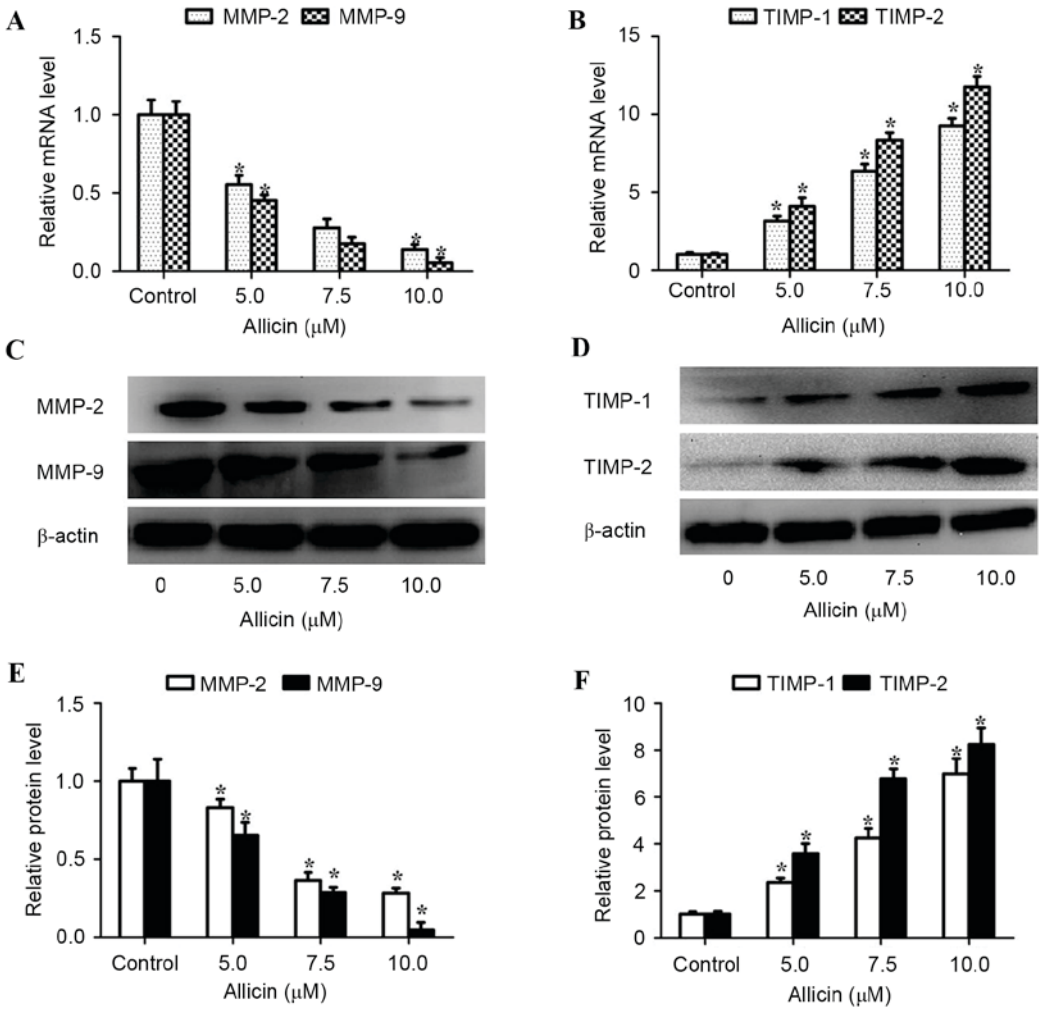

Figure 3. Allicin alters TIMP/MMP balance in H1299 cells. H1299 cells were treated with 0, 5.0, 7.5 and 10.0 μM allicin for 48 h and the mRNA levels of (A) MMP-2 and MMP-9, and (B) TIMP-1 and TIMP-2 were assayed by reverse transcription-quantitative polymerase chain reaction and normalized to GAPDH. H1299 cells were incubated with various concentrations of allicin (0, 5.0, 7.5 and 10.0 μM) for 48 h. The protein levels of (C) MMP-2, MMP-9, (D) TIMP-1 and TIMP-2 in cell lysates were analyzed by western blot analysis and band intensity was quantified by densitometry and normalized to β-actin for (E) MMP-2 and MMP-9 and (F) TIMP-1 and TIMP-2. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05, compared with control (0 μM). MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

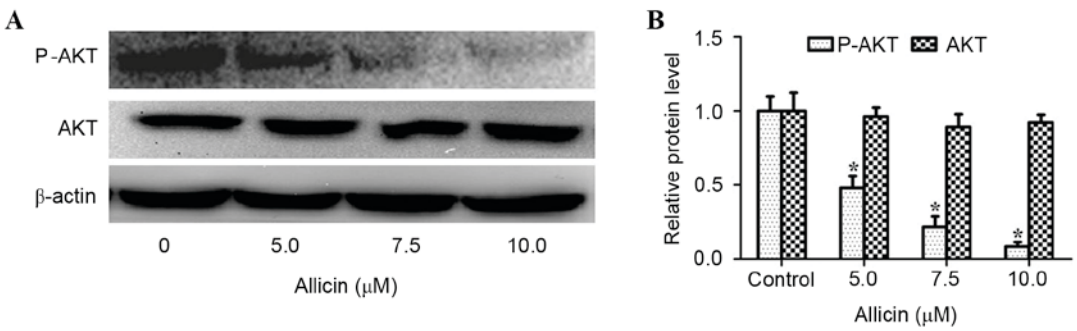

Figure 4. Effects of allicin on the PI3K/AKT signaling pathway. (A) H1299 cells were incubated with various concentrations of allicin (0, 5.0, 7.5 and 10 μM) for 48 h. The protein levels of P-AKT and AKT in cell lysates were analyzed by western blot analysis. (B) The band intensity was quantified by densitometry and normalized to β-actin. Values are expressed as the mean ± standard deviation of three independent experiments. *P<0.05, compared with the control (0 μM). P-AKT, phospho-AKT.

balance. However, once this balance is broken, invasion and metastasis of cancer cells is induced (6-9). The imbalance between TIMPs and MMPs has been recognized as the main mechanism for promoting the invasive processes of lung cancer. Hu *et al* reported that hypoxia may affect the invasiveness of lung cancer cells by regulating MMP-9 and TIMP-2 expression (21). Ylisirnio *et al* demonstrated that serum MMP-2, MMP-9, TIMP-1 and TIMP-2 were associated with the

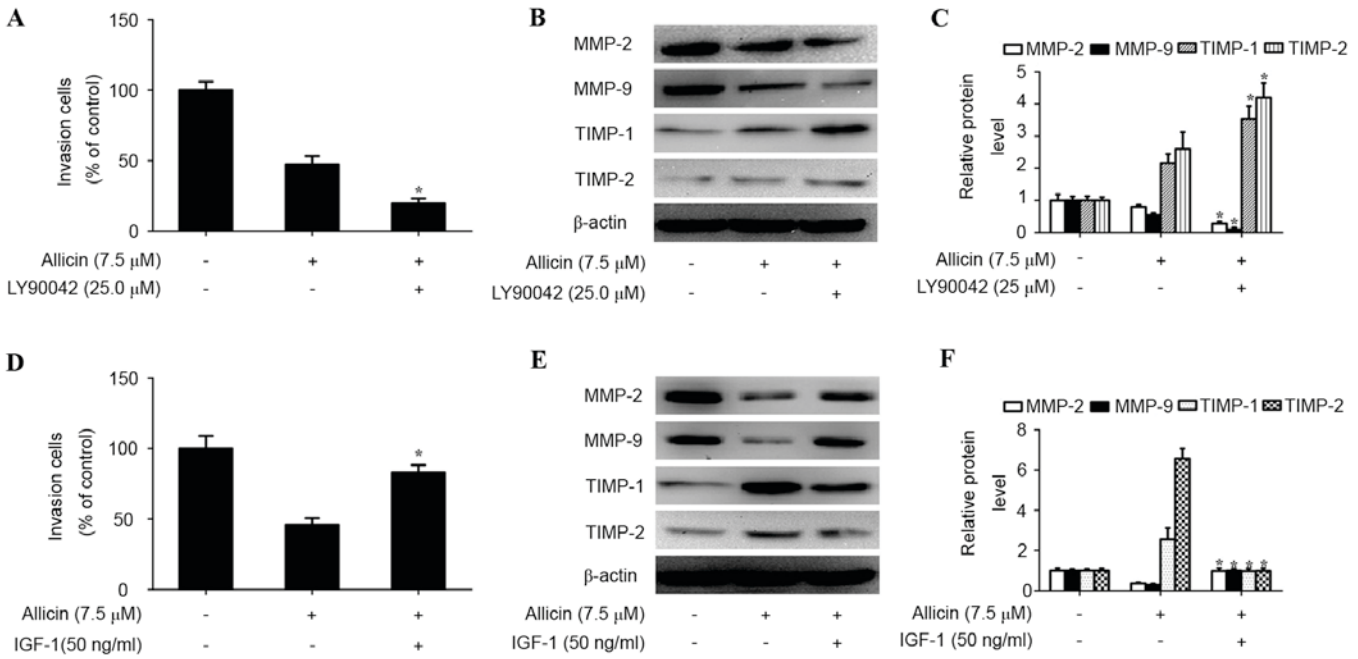

Figure 5. Involvement of the PI3K/AKT pathway in allicin-induced TIMP/MMP imbalance and cell invasion. (A) Cells were pretreated with LY294002 (25 μM) for 1 h and then incubated in the presence or absence of allicin (7.5 μM) for 48 h. Cellular invasiveness was measured using the Transwell invasion assay. The invasion rate was expressed as a percentage of control. (B) The protein levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 were analyzed in treated H1299 cells by western blotting. (C) Values are expressed as the mean ± SD of three independent experiments. *P<0.05, compared with the allicin treated group. (D) Cells were pretreated with IGF-1 (50 ng/ml) for 1 h and then incubated in the presence or absence of allicin (7.5 μM) for 48 h. Cellular invasiveness was measured using the Transwell invasion assay. The invasion rate was expressed as a percentage of the control. (E) The protein levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 were analyzed in treated H1299 cells by western blot analysis. (F) Band intensity was quantified by densitometry and normalized to β-actin. Values are expressed as the mean ± SD of three independent experiments. *P<0.05 compared with the allicin treated group. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; IGF-1, insulin-like growth factor-1; SD, standard deviation; PI3K, phosphoinositide 3-kinase.

clinical outcome of patients with lung cancer and may serve as prognostic markers (22). To explore the possible mechanism of allicin in the inhibition of lung adenocarcinoma invasion, the effects of allicin on the expression level of MMP-2, MMP-9, TIMP-1 and TIMP-2 were investigated. It was revealed that allicin dose-dependently downregulated mRNA and protein levels of MMP-2 and MMP-9 and then enhanced mRNA and protein levels of TIMP-1 and TIMP-2 in a dose-dependent manner. These data indicated that allicin may cause inhibition of lung adenocarcinoma invasion by inducing an imbalance of expression between MMPs (MMP-2 and MMP-9) and TIMPs (TIMP-1 and TIMP-2).

Previous studies established that the PI3K/AKT pathway is activated in numerous tumors, including breast cancers, pituitary adenoma and prostate cancer (10,13). An increasing number of studies have shown that the PI3K/AKT signaling pathway may modulate the expression of MMPs, as well as TIMPs, to promote the degradation of ECM proteins, and this mechanism was essential for invasion of human tumors, including lung cancer (13,23). To the best of our knowledge, allicin has been confirmed to inhibit the PI3K/AKT signaling pathway in the HepG2 cell line; however, it remains unknown whether such an effect also exists in lung adenocarcinoma (17). Therefore, the effect of allicin on the PI3K/AKT signaling pathway was investigated in H1299 cells. The results demonstrated that allicin may decrease the phosphorylation of AKT in H1299 cells, whereas no significant changes were observed in the total protein expression of AKT. In addition, allicin combined with LY294002 (an inhibitor of PI3K) significantly reduced H1299 cell invasion (P<0.05) and was accompanied by upregulation of TIMP-1 and TIMP-2 and downregulation of MMP-2 and MMP-9. Whereas, in H1299 cells, the PI3K/AKT signaling activator (IGF-1) reversed the effect produced by allicin on invasion, as well as the protein expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. These findings indicated that the regulation of cell invasion and TIMP-1, TIMP-2, MMP-2 and MMP-9 expression by allicin occurred via the suppression of the PI3K/AKT signaling pathway.

In summary, to the best of our knowledge, the present data demonstrated for the first time that allicin inhibits the invasion of lung adenocarcinoma cells by altering TIMP/MMP balance, via reducing the activity of the PI3K/AKT signaling pathway. Allicin may be recognized as an anti-invasive agent for lung adenocarcinoma treatment.

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