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Original Article

Modulatory role of garlicin in migration and invasion of intrahepatic cholangiocarcinoma via PI3K/AKT pathway

Kun Xie, Jianze Nian, Xingyang Zhu, Xiaoping Geng, Fubao Liu

Department of General Surgery, The First Affiliated Hospital of Medical University of Anhui, Hefei 230022, Anhui, China

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Abstract: Increasing evidences have indicated the role of garlicin in inhibiting the progression of various tumors including glioma, pulmonary carcinoma and pancreatic carcinoma, via mediating cell apoptosis or cell cycle. The regulatory effect and related molecular mechanism of garlicin in intrahepatic cholangiocarcinoma, however, remained unknown. This study thus aimed to investigate this scientific issue. HCCC-9810 cell line was treated with serially diluted garlicin, followed by cell proliferation assay using MTT approach. Transwell migration and invasion assays were further employed the regulatory effect of garlicin. The expression level of p-AKT and AKT proteins in tumor cells was quantified by Western blot. The growth of tumor cells was significantly inhibited by high concentration of garlicin (> 1.5 μM). Lower concentration of garlicin showed dose-dependent inhibition of tumor cell invasion and migration. After using specific agonist IGF-1 (50 ng/mL) of PI3K/AKT signaling pathway, such facilitating effects of garlicin were depressed ($P < 0.05$). Western blotting showed significantly decreased phosphorylation level of AKT after treated with gradient concentrations of garlicin, while leaving the total AKT protein level unchanged. Garlicin may inhibit the invasion and migration of intrahepatic cholangiocarcinoma cells via inhibiting PI3K/AKT signaling pathway.

Keywords: Garlicin, PI3K/AKT signaling pathway, intrahepatic cholangiocarcinoma, tumor invasion, tumor migration

Introduction

As one active intergradient extracted from garlic, garlicin has multiple biological activities such as cancer prevention by inhibiting nitrate reductase, anti-tumor functions via macrophage potentiation, and the potentiation of anti-tumor drug sensitivity [1, 2]. Increasing evidences in recent years have indicated the inhibitory role of garlicin in the progression of various malignant tumors including glioma, gastric, pancreatic and hepatocyte carcinoma via modulating cell apoptosis or cell cycle, such as those in U87 and human gastric adenoma cell line SGC-7901 [1-4]. The relationship between garlicin and invasiveness of intrahepatic cholangiocarcinoma, however, has not been reported yet. This study thus aimed to investigate the effect of garlicin on invasion and migration of human intrahepatic cholangiocarcinoma cells, in an attempt to provide evidences for clinical treatment of intrahepatic cholangiocarcinoma.

Materials and methods

Cell culture

Human intrahepatic cholangiocarcinoma cell line HCCC-9810 (Cell Biology Institute, Shanghai, China) were kept in DMEM medium containing 10% fetal bovine serum (FBS) in a 37°C humidified chamber perfused with 5% CO_2.

MTT assay

Cells at log phase were added into 96-well plate (4×10^3/mL). Gradient concentrations of garlicin (0, 0.5 μM, 1.0 μM, 1.5 μM, 2.0 μM, 3.0 μM, 4.0 μM and 5.0 μM) were added for 24-hour continuous incubation, followed by the addition of 5 mg/mL MTT reagents (Beyotime, China). After 4-hour incubation, formanzan solvent was added to each well (0.1 mL per well). The crystal was completely dissolved by further 4-hour incubation. Absorbance value at 570 nm was measured by a microplate reader.

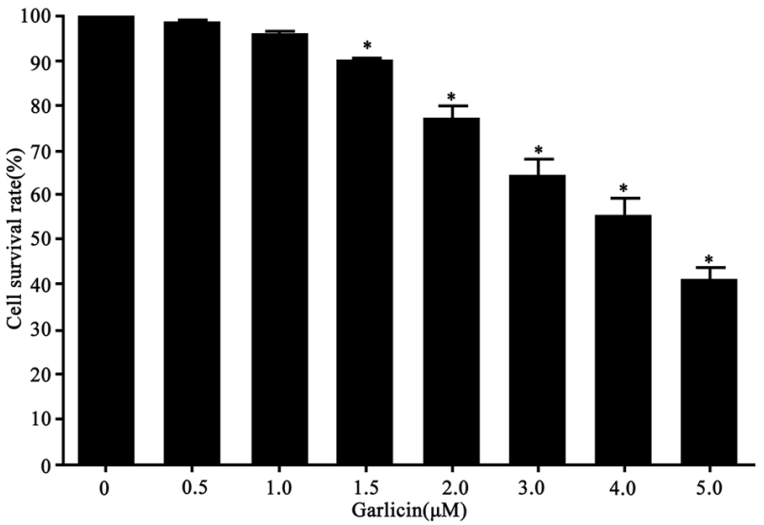

Figure 1. Proliferation of HCCC-9810 cells under different concentrations of garlicin. *$P < 0.05$ compared to control group.

Transwell migration assay

HCC-9810 cells were firstly pre-treated with garlicin (0, 0.5 μM and 1.0 μM) for 24 hours, followed by 12-hour incubation in serum-free medium. Trypsin was then added to stimulate the retraction of cytoplasm and disconnection of cell-to-cell adhesion. DMEM medium was then added to collect cells by centrifugation in order to remove serum-containing medium, followed by the addition of serum-free culture medium. Cell were seeded into the plate at 1 × 10^5 per well. Transwell chambers (Corning, US) were filled with serum-free medium to make the final volume up to 0.2 mL in each well. Culture medium 10% FBS was then added into each lower chamber, followed by 12-hour incubation at 37°C. Medium in the chamber was removed, and the chamber was rinsed by PBS. Upper layered cells on the basal membrane were removed, followed by crystal violet staining for 1 minute. Three randomly selected fields were counted for the number of migrated cells. Number of migrated cells = (Number of cells in all three fields)/3.

Transwell invasion assay

The membrane at the bottom of Transwell chamber was coated by 1:8 Matrigel (BD, US) followed by 1-hour incubation at room temperature. Different concentrations of garlicin (0, 0.5 μM and 1.0 μM) were used to pre-treated HCCC-9810 cells for 24 hours, followed by incubation in serum-free medium for 24 hours trypsin was then added to stimulate the retraction of cytoplasm and disconnection of cell-to-cell adhesion. DMEM medium was then added to collect cells by centrifugation in order to remove serum-containing medium, followed by the addition of serum-free culture medium. Cell were seeded into the plate at 1 × 10^5 per well. Transwell chambers (Corning, US) were filled with serum-free medium to make the final volume up to 0.2 mL in each well. Culture medium 10% FBS was then added into each lower chamber, followed by 12-hour incubation at 37°C. Medium in the chamber was removed, and the chamber was rinsed by PBS. Upper layered cells on the basal membrane were removed, followed by crystal violet staining for one minute. Three randomly selected fields were counted for the number of invaded cells. Number of invaded cells = (Number of cells in all three fields)/3.

Western blotting

Different concentrations of garlicin (0, 0.5 μM and 1.0 μM) were used to pre-treated HCCC-9810 cells for 24 hours. Total proteins were then extracted from culture HCCC-9810 cells. After quantification, proteins were separated by SDS-PAGE. Followed by blocking, the membrane was incubated with anti-phosph-AKT (1:1000, Santa Cruz) antibody, GAPDH (1:1000, Santa Cruz), MMP-2 (1:500; Santa Cruz) or MMP-9 (1:500) for overnight incubation. On the next day, anti-mouse IgG conjugated with horseradish peroxidase (1:2000) was employed for one hour incubation.

Statistical analysis

SPSS 19.0 software use used to collect all data. Measurement data were presented as mean ± standard deviation. Student t-test was used to compare means between groups. A statistical significance was defined when $P < 0.05$.

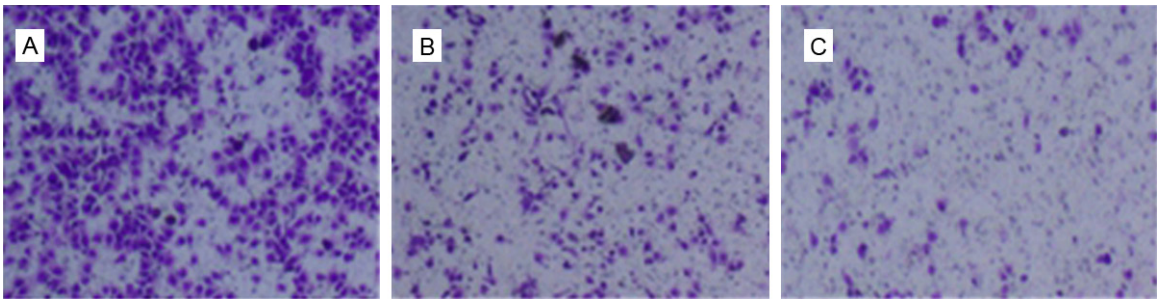

Figure 2. Cell migration under garlicin. A. Control group; B. 0.5 μM garlicin; C. 1.0 μM garlicin.

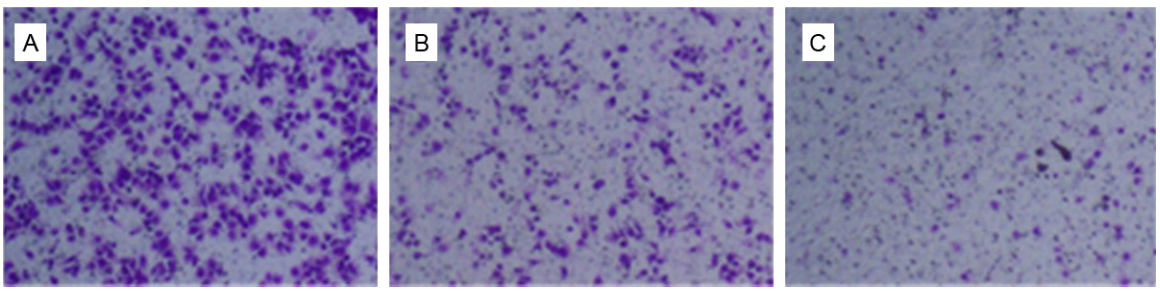

Figure 3. Cell invasion statuses. A. Control group; B. 0.5 μM garlicin; C. 1.0 μM garlicin.

Results

Effect of garlicin on HCCC-9810 cell proliferation

Using MTT assay, we measured the survival rate of HCC-9810 cells under different concentrations of garlicin. Results showed that garlicin with concentrations higher than 1.5 μM significantly depressed the survival of tumor cells ($P < 0.05$, **Figure 1**). In the following experiments, we thus used 0.5 μM and 1.0 μM garlicin to study its effects on cell migration and invasion, as it can minimize the interference from proliferative effect.

Effects of garlicin on cell migration

Using Transwell assay, we found that both 0.5 μM and 1.0 μM garlicin significantly suppressed the number of migrated cells per field compared to control group (Control: 623.00 ± 36.45; 0.5 μM garlicin: 168.00 ± 26.13; 1.0 μM garlicin: 39.00 ± 11.00; $P < 0.05$; **Figure 2**). These results suggested the inhibitory effect of garlicin on migration ability of HCCC-9810 cells.

Cell invasion ability under garlicin

We further tested the invasiveness of HCCC-9810 cells after 24-hour treatment using garlicin. Results showed significantly depressed invasive cell numbers after applying gradient concentrations of garlicin (Control: 574.00 ± 31.32; 0.5 μM garlicin: 154.00 ± 22.98; 1.0 μM garlicin: 36.00 ± 17.11; $P < 0.05$; **Figure 3**), suggesting the inhibition of HCCC-9810 cell invasiveness by garlicin.

Mechanism of garlicin in regulating HCCC-9810 cell proliferation and migration

We further tested the expression of AKT after 24-hour treatment using different concentrations of garlicin. Results showed decreased phosphorylated AKT protein level with increased concentrations of garlicin, while leaving total AKT protein level intact (**Figure 4**).

We further replenished the PI3K/AKT signaling pathway agonist IGF-1 (at 50 ng/mL) in addition to 0 or 0.5 μM garlicin to treat tumor cells. 24 hours later, both migration and invasion abilities of HCCC-9810 cells were further tested. Transwell migration assay showed significantly increased number of migrated cells after replenishing IGF-1, when compared to those cells treated with 1.0 μM garlicin only (Control: 532.00 ± 29.87; 0.5 μM garlicin: 186.00 ± 25.09; 1.0 μM garlicin + 50 ng/mL IGF-1: 519.05 ± 31.10; $P < 0.05$ compared to garlicin group; **Figure 5**). Therefore, the inhibitory effect

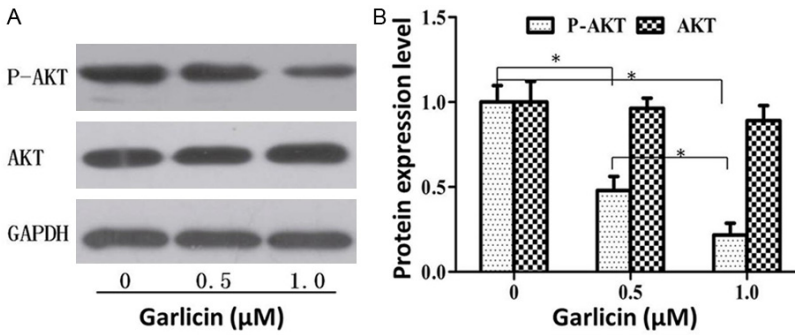

Figure 4. AKT protein expression level in garlicin treated cells. A. Representative Western blotting bands of p-AKT (top panels); total AKT (middle panels) and GAPDH (lower panels). B. Quantitative protein expression relative levels.

of garlicin on tumor cell migration was eliminated by IGF-1, suggesting that garlicin exerted its effects via PI3K/AKT signaling pathway.

Cell invasion assay obtained consistent results, as the number of invasive cells per field was significantly increased after replenishing 50 ng/mL IGF-1 (Control: 504.00 ± 22.36; 0.5 μM garlicin: 167.00 ± 19.04; 1.0 μM garlicin + 50 ng/mL IGF-1: 528.93 ± 18.11; $P < 0.05$ compared to garlicin group; **Figure 6**). These results suggested the depressed inhibitory effect on tumor cell invasion by PI3K/AKT signaling pathway agonist IGF-1.

Discussion

As one of the most common malignant tumors in liver, intrahepatic cholangiocarcinoma owns unfavorable prognosis, mainly due to the high rate of tumor invasion and metastasis [5-7]. Therefore, the development of novel drugs targeting invasiveness of intrahepatic cholangiocarcinoma is of critical importance for improving patients' prognosis. Recently various evidences have supported the significant inhibitory role of garlicin on both occurrence and progression of dozens of tumors including glioma, pulmonary carcinoma and pancreatic cancer [1-4]. The correlation between garlicin and intrahepatic cholangiocarcinoma, however, remained poorly understood. This study thus utilized different concentrations of garlicin to treat HCCC-9810 cells, and found significant inhibition on tumor cell growth with garlicin concentration higher than 1.5 μM. Our results thus suggested the cytotoxicity of garlicin on intrahepatic cholangiocarcinoma cells, and can inhibit tumor growth.

Currently few studies have been performed regarding the correlation between garlicin and tumor cell invasiveness. Previous reports have indicated the inhibitory effects of garlicin on the invasion and/or adhesion of hepatocyte carcinoma [8] and glioma [9]. This study utilized garlicin to treat intrahepatic cholangiocarcinoma cells, on which Transwell invasion and migration assays were performed. Results showed significant inhibitions on both tumor cell invasion and migration by increasing concentrations of garlicin, as consistent with previous studies.

Tumor cell invasion and metastasis is a complicated process involving multiple genetic factors [10-14]. Amount of studies confirmed the hyper-active status of PI3K/AKT signaling pathway in multiple malignant tumors including cervical cancer, pancreatic carcinoma, gastric cancer and esophageal cancer, along with important roles in tumor invasion and metastasis [14-20]. PI3K/AKT signaling pathway can modulate the expression of cytoskeleton proteins, thus altering cell morphology and cell motility. In tumor cells, the activation of PI3K/AKT signal can cause the relevant change of cell morphology, indicating certain correlation between signaling pathway and tumor invasion [11-14]. The expression of p-AKT has been found to be related with clinical indexes for invasiveness of endometrial cancer such as TNM stage. The significant elevation of p-AKT in those tumors with high metastatic potency suggested the facilitating role of PI3K/AKT signaling activation on tumor invasion [11]. The pharmaceutical antagonist of PI3K/AKT signaling pathway, LY294002, can significantly inhibit the invasiveness of prostate cancer cell lines including LNAcP, PC-3 and DU145, along with decreased expression of uPA and MMP-9 [11]. PI3K/AKT signaling pathway was also found to modulate GSK3β-phosphorylation induced cell migration and invasion [12]. Moreover, PI3K/AKT signal can regulate the invasion of pulmonary carcinoma cell A459, featured with the up-regulation of VEGF, suggesting the critical role of VEGF

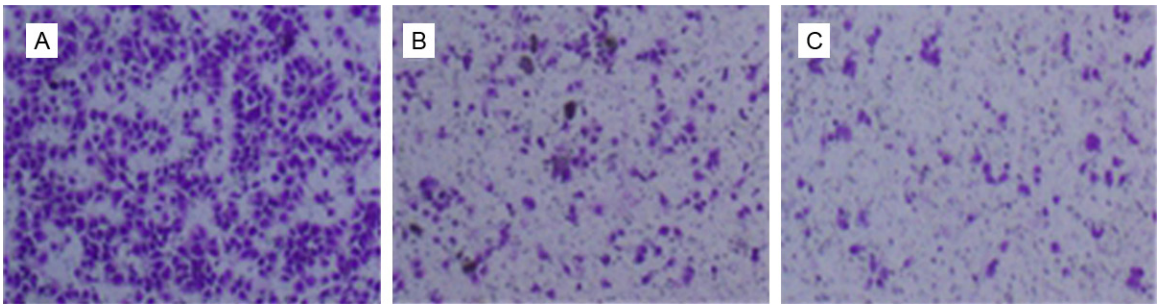

Figure 5. HCCC-9810 cell migration after using IGF-1 and garlicin. A. Control group; B. 1.0 μM garlicin + 50 ng/mL IGF-1; C. 0.5 μM garlicin.

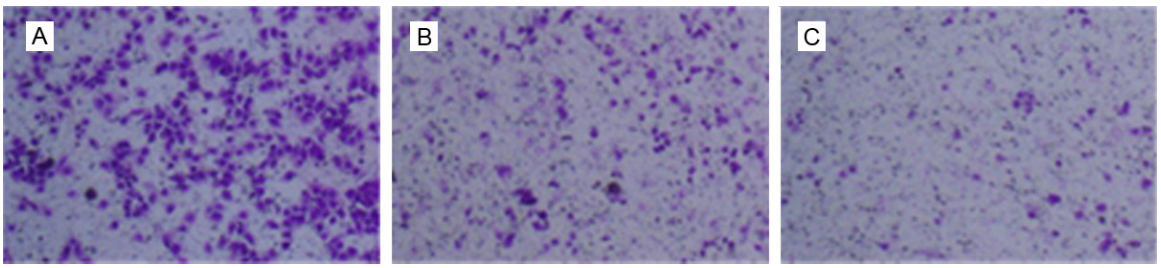

Figure 6. HCCC-9810 cell invasion after using IGF-1 and garlicin. A. Control group; B. 1.0 μM garlicin + 50 ng/mL IGF-1; C. 0.5 μM garlicin.

as the downstream target of PI3K/AKT in mediating cancer invasion and metastasis [14]. In a further study on nude mice, NF-κB was found to be related with PI3K/AKT signaling pathway to regulate intracellular expression of E-cadherin and Snail for invasiveness of breast cancer cells [15]. Western blotting in this study showed depressed phosphorylation level of AKT after treating with garlicin, while leaving total AKT protein level unchanged. We also found the elimination of inhibitory effect on tumor cells after treatment using PI3K/AKT signaling pathway agonist. All these results suggested the possible involvement of garlicin in mediating invasion and migration of intrahepatic cholangiocarcinoma via PI3K/AKT signaling pathway.

In summary, this study demonstrated the antitumor potency of garlicin, possibly via mediating PI3K/AKT signaling pathway activity. Further studies, however, are required to investigate the downstream factor of PI3K/AKT signal, due to the complicated process involving in the invasion and migration of tumor cells.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Fubao Liu, Department of General Surgery, The First Affiliated Hospital of Medical University of Anhui, 218 Jixi Road, Hefei 230022, Anhui, China. Tel: +86-551-62923191; Fax: +86-551-62923191; E-mail: nsifh-fisty112@163.com

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