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Original Article

Alkannin inhibits growth and invasion of glioma cells C6 through IQGAP/mTOR signal pathway

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Abstract: Objective: This study aims to explore the effect of alkannin on the growth and invasion of glioma cells and its mechanism. Methods: The effects of alkannin on the growth and invasion of glioma cells were detected with MTT assay, clone forming test and transwell assay. The effects of alkannin on the cell cycle were detected with flow cytometry assay. The changes of cyclin, MMPs and IQGAP/mTOR signal pathway related proteins were detected with western blotting methods. Results: Alkannin (1 μM, 3 μM and 10 μM) can significantly inhibit the growth, proliferation, migration and invasion of glioma cells C6 with dose dependent. Alkannin can block cell cycle in G1 phase with the increased concentration, which was related with the down-regulation of cyclinA1, cyclinA2 and cyclinD1 expression. Alkannin can also down-regulate the expression of MMP 2, MMP 9 and IQGAP. Alkannin has no effect on mTOR but can inhibit the phosphorylation of mTOR. Conclusions: Alkannin can inhibit the growth and invasion of glioma cells C6 through IQGAP/mTOR signal pathway.

Keywords: Alkannin, glioma cells, MMPs, invasion, MTT assay, flow cytometry assay

Introduction

Glioma is the most common intracranial malignant tumor in human and account for about 35%-60% of brain tumors. It often invades the normal brain tissue showing aggressive growth and forms satellite tumor group around the primary tumor. It has high recurrence rate after operation resection and poor prognosis [1]. Glioma is still not completely cured with the development of neurosurgery operation therapy, gene therapy, immunotherapy, chemotherapy and radiotherapy technology and the survival period is less than 1 year [2-4].

IQGAP is a cytoskeleton remodeling protein and play an important role in the proliferation, migration and invasion of tumor cells [5, 6]. The expression of IQGAPl was significantly higher in glioma than that in the normal tissue [6, 7]. Wang reported that IQGAP1 can promote cellular proliferation through CDC42-mTOR signal pathway in NIHT3 cells [8, 9]. High expression of IQGAP1 in the tumor cells can also stimulate the growth and invasion of Hela, HepG2 and HIT-T15 cells through promoting the interaction of mTOR and AKT [10, 11]. These results suggested that IQGAP1/mTOR signal pathway affected the proliferation and invasion of tumor cells, we speculated that this pathway was also in glioma cells.

Lithospermum erythrorhizon belongs to boraginaceae perennial herb and is a traditional chinese medicinal herbs. Alkannin extracted from its root has the activity of proliferation inhibition of tumor cells. Alkannin can inhibit invasion ability of gastric cancer cell SGC7901 by reducing the secretion of MMP2 and MMP7 proteins [12, 13]. We speculated that alkannin can also inhibit the growth and invasion of glioma cells. Therefore, we explored the effect of alkannin on the growth and invasion of glioma cells and its mechanism in this study.

Materials and methods

Cell culture

Rat glioma cell lines C6 was obtained from ATCC (American Type Culture Collection). The cells were grown in DMEM supplemented with 5% fetal bovine serum. They were cultured at 37°C with 5% CO_2. They were divided into Alkannin group and control group.

MTT assay

MTT assay was performed using 96-well plate according to the manufacturer's manual. Cells

Table 1. Inhibition of alkannin on proliferation of glioma cells C6 ($\bar{x}$ ± SD)

group	Dose (μM)	Inhibition rate (%)		
		24 h	48 h	72 h
Control	0	0.1 ± 0.02	3.0 ± 0.3	3.1 ± 0.5
Alkannin	1	3.5 ± 0.8*	14.5 ± 2.1**	14.3 ± 2.4**
	3	6.5 ± 0.5*	23.7 ± 3.6**	23.8 ± 3.8**
	10	10.7 ± 1.9**	35.3 ± 5.1**	35.2 ± 4.3**

*P < 0.05, **P < 0.01 vs. control.

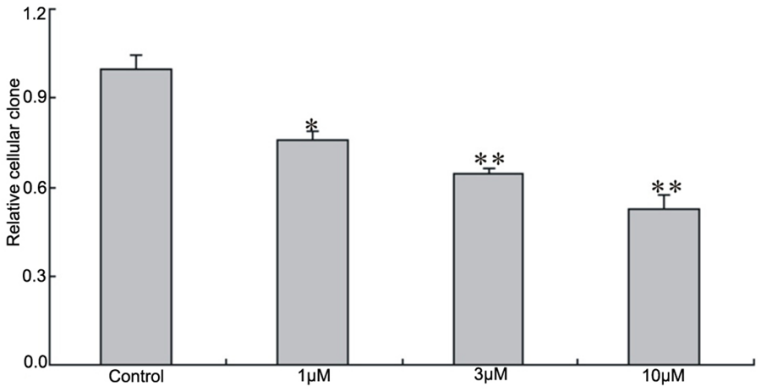

Figure 1. The effects of alkannin on the number of cell clone in glioma cells C6. *P < 0.05, **P < 0.01.

were cultured at 37°C with 5% CO_2 for 24 h and 1 μM, 3 μM and 10 μM of alkannin was added to culture for 48 h. Then they were incubated with MTT for 4 h, and then the insoluble substance dissolved in DMSO. The OD values were determined at 570 nm. The inhibition rate of cellular proliferation was calculated.

Clone forming test

The cells with concentration of 8 × 10^3/ml were cultured in 6-well plate at 37°C with 5% CO_2 for 24 h and 1 μM, 3 μM and 10 μM of alkannin was added to culture for 48 h. The newly formed colony in 6-well plate were fixed with 10% formaldehyde for 10 min and stained with 0.1% crystal violet. Staining solution was discarded gently and the 6-well plate was washed and dried. They were observed under inverted optical microscope and counted.

Transwell assays

The cells with concentration of 8 × 10^3/ml were cultured in 6-well plate at 37°C with 5% CO_2 for 24 h and 1 μM, 3 μM and 10 μM of alkannin was added to culture for 48 h. The matrigel was diluted with cooling DMEM to the concentration of 1 mg/ml. 100 μl of it was added in the bottom center of upper chamber of transwells and incubated at 37°C, 200 μl DMEM was added in each well. The above cells were placed in the upper chamber and DMEM with 5% FBS was added in the lower chambers. They were incubated at 37°C with 5% CO_2 for 24 h. The upper chamber was removed after incubation and cleansed the filter side of the upper chamber with a cotton swab. The filter was fixed with 4% formaldehyde for 10 min and stained with 0.1% crystal violet. Gently cut the filter from the chamber and count the cells that have migrated through the filter pores from the underside of the filter in 5 high-power fields per insert and average values afterwards.

Detection of the cell cycle

The cells with concentration of 8 × 10^3/ml were cultured in 6-well plate at 37°C with 5% CO_2 for 24 h and 1 μM, 3 μM and 10 μM of alkannin was added to culture for 48 h. The cells were collected and cell cycles were detected according to the manual of cell cycle detection kit. They were analyzed by flow cytometry.

Western blotting

The cells were collected after being treated for 48 h. Total proteins were lysed with RIPA lysis buffer and extracted to quantify using BAC protein assay kit according to the protocol. They were analyzed with SDS-PAGE electrophoresis. Then they were electro-transferred to the PVDF membrane. The membrane containing the proteins was used for immunoblotting with required antibodies. The protein bands were scanned and quantified, β-actin was used as internal control.

Statistical analysis

The data were expressed as $\bar{x}$ ± SD and analyzed using SPSS17.0 software. The variance

Table 2. The effect of alkannin glioma cells C6 cell cycle ($\bar{x}$ ± SD)

group	Dose (μM)	G1 (%)	S (%)	G2 (%)
Control	0	11.11 ± 2.12	33.92 ± 4.02	54.97 ± 5.07
Alkannin	1	37.96 ± 1.11**	23.53 ± 2.06*	38.51 ± 4.01*
	3	42.41 ± 3.67**	22.47 ± 2.9*	35.13 ± 3.8**
	10	44.53 ± 5.22**	22.01 ± 2.11*	33.45 ± 2.9**

*P < 0.05, **P < 0.01 vs. control.

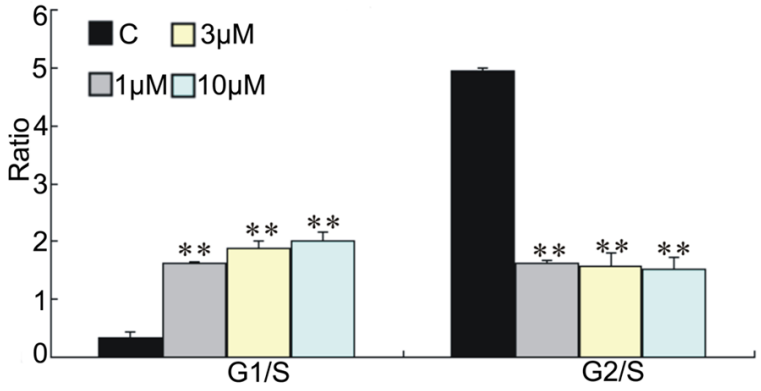

Figure 2. The effects of alkannin on the ration of G2 and G1 in glioma cells C6. **P < 0.01.

Table 3. The effect of alkannin on migration and invasion in glioma cells C6 ($\bar{x}$ ± SD)

group	Dose (μM)	migration	invasion
Control	0	99 ± 9.01	99 ± 8.02
Alkannin	1	75.23 ± 2.96*	85.67 ± 3.05
	3	63.67 ± 3.78**	72.33 ± 2.52**
	10	46.00 ± 4.00**	60.00 ± 5.57**

*P < 0.05, **P < 0.01 vs. control.

analysis ANOVA and t-test were conducted for comparison among groups. P < 0.05 was considered statistical significance.

Results

Proliferative inhibition of alkannin on glioma cells C6

As shown in **Table 1**, inhibitory effect of alkannin on glioma cells C6 was enhanced with the increase of its concentration, the inhibition rate reached the maximum when the concentration was 10 μM. The inhibitory effect was also enhanced with the increase of administration time and they were consistent after administration for 48 h and 72 h. So we selected 48 h as experimental time in this study.

The effects of alkannin on clone formation of C6 cells

The effects of alkannin on clone formation of glioma cells C6 were shown in **Figure 1**. Alkannin significantly inhibited the clone formation of glioma cells C6 with the increase of its concentration when comparing with control group (P < 0.01).

The effects of alkannin on cell cycle of glioma cells C6

As shown in **Table 2** and **Figure 1**, cells in G1 phase increased (from 11.11 ± 1.75% to 44.53 ± 5.8%) and cells in G2 phase decreased (from 54.97 ± 6.9% to 33.45 ± 3.98%) significantly with the concentration increased (P < 0.01). In control group, the ratio of G2/S cells decreased while the ratio of G1/S cells increased with the concentration increased (P < 0.05, **Figure 2**). These data suggested that alkannin can make the glioma cells C6 arrest in the G1 phase.

The effects of alkannin on migration and invasion of glioma cells C6

The transwell results were showed in **Table 3**. It showed that the number of cells migrated into the lower chambers decreased significantly in alkannin group than that of control group (P < 0.05). The number of cells into the lower chambers decreased gradually with the concentration of alkannin increased, there was statistical significance when the concentration of alkannin was 3 μM and 10 μM (P < 0.01). These results suggested that alkannin could inhibit the migration and invasion ability of glioma cells C6 and in a dose-dependent manner.

The effects of alkannin on the expression of IQGAP and mTOR in glioma cells C6

As shown in **Figure 3**, compared with control group, alkannin could inhibit the expression of

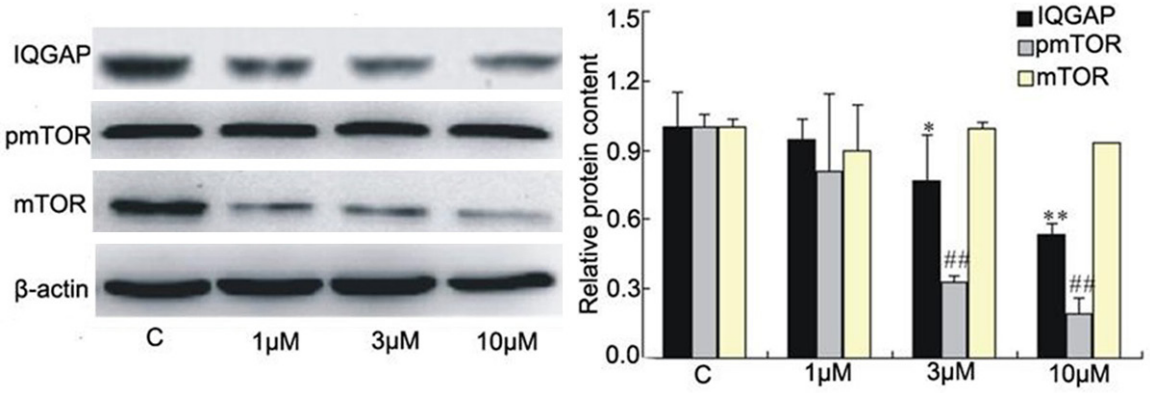

Figure 3. The effects of alkannin on expression of IQGAP and mTOR in glioma cells C6. *P < 0.05, **P < 0.01, ##P < 0.01.

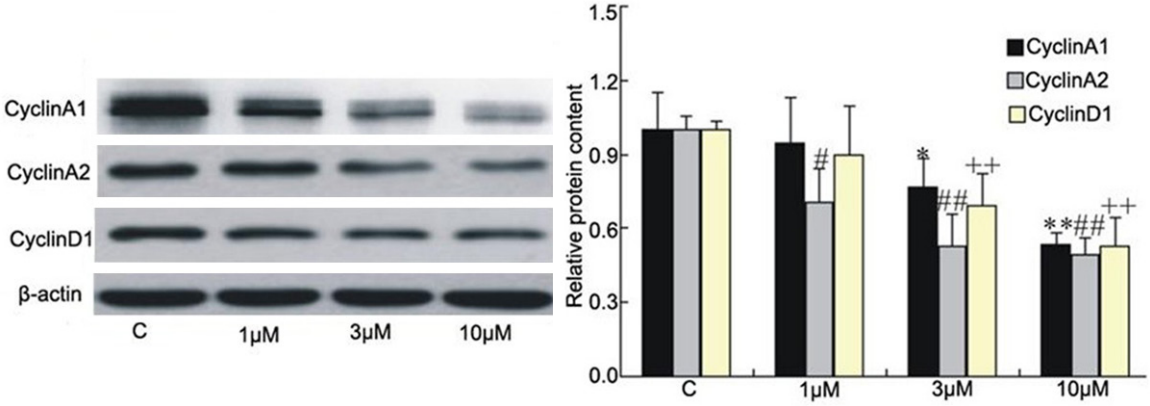

Figure 4. The effects of alkannin on expression of cyclin in glioma cells C6. *P < 0.05, **P < 0.01, #P < 0.05, ##P < 0.01, ++P < 0.01.

IQGAP significantly with the concentration of alkannin increased ($P < 0.05$). Alkannin has no effect on the expression of mTOR but can inhibit the phosphorylation of mTOR.

The effects of alkannin on the expression of cyclinA1, cyclinA2 and cyclinD2 in glioma cells C6

The results were shown in **Figure 4**. Compared with control group, alkannin could inhibit the expression of cyclinA1, cyclinA2 and cyclinD2 significantly with the concentration of alkannin increased ($P < 0.05$).

The effects of alkannin on the expression of MMPs in glioma cells C6

The results were shown in **Figure 5**. Compared with control group, alkannin could inhibit the expression of MMP 2 and MMP 9 significantly with the concentration of alkannin increased ($P < 0.05$).

Discussion

The inhibitory effects of alkannin on proliferation and invasion of glioma cells C6

In this study we found that alkannin could significantly inhibit the proliferation of glioma cells C6 with dose dependent, but the inhibition concentration was different from that in other tumor cells [14, 15]. This may be due to the different sensitivity to drugs of different tumor cells. It indicated that alkannin could inhibit the growth of glioma cells C6 in a certain dose range. Migration and invasion of malignant tumor is an important cause of death. Invasion inhibition can improve the survival rate effectively. We found that alkannin could inhibit the

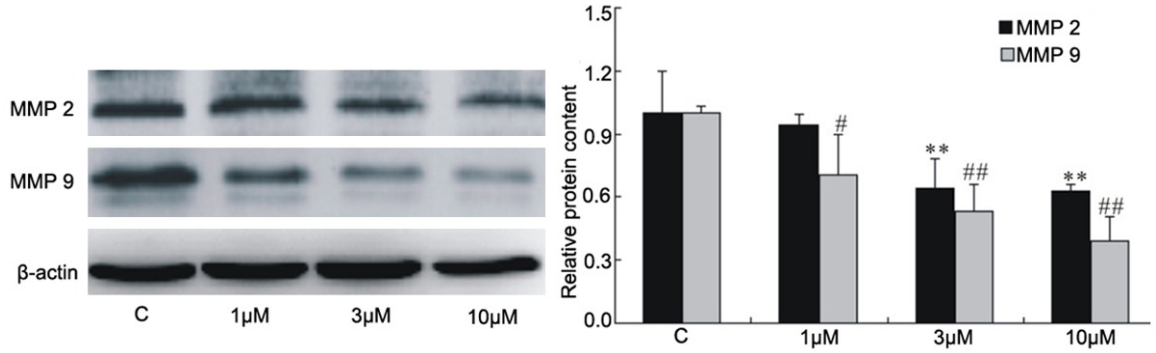

Figure 5. The effects of alkannin on the expression of MMPs in glioma cells C6. **P < 0.01, #P < 0.05, ##P < 0.01.

migration and invasion ability of glioma cells C6 and in a dose-dependent manner.

The effects of alkannin on the expression of IQGAP and mTOR in glioma cells C6

IQGAP1 is an important effect factor of GTP enzyme in the Rho family, and is a cytoskeleton remodeling associated protein. It can affect the proliferation and invasion of tumor cells by regulating PI3K/AKR signal pathway. Decreased expression of IQGAP1 could inhibit the invasion of human breast cancer cells [16]. Overexpression of IQGAP1 stimulated ERK signal pathway and promoted the invasion of colorectal cancer cells [17]. Overexpression of IQGAP1 also appeared in malignant glioma, lung cancer, gastric cancer and breast cancer and was related with the prognosis of cancer [1, 18-20]. mTOR is an effector protein in downstream of the PI3K/AKT signal pathway and control the translation of mRNA. mTOR inhibitors have significant anti-tumor effects on liver cancer, pancreatic cancer and glioma [21-23]. The high expression of IQGAP1 in liver cancer cells promoted the interaction between mTOR and AKT through the support role and stimulated the growth and invasion of liver cancer cells [11]. In this study, we also found overexpression of IQGAP in glioma cells and significant phosphorylation of mTOR, vigorous proliferation and invasion. Alkannin could reduce the expression of IQGAP and phosphorylation of mTOR in glioma cells, which suggested that alkannin could inhibit the proliferation and invasion of glioma cells C6 by IQGAP1/mTOR signal pathway.

The effects of alkannin on the cell cycle of glioma cells C6

The uncontrollable cell cycle can lead to unlimited proliferation of cells. Glioma often had excessive activation of cell cycle protein, which leads to cell cycle out of control and resistance to apoptosis [24, 25]. CyclinD1 is a cell cycle proteins which is closely related to the G1 phase of the cell cycle and proliferation and adhesion of tumor cells [26, 27]. It is reported that when the cell cycle arrested in G1 phase decreased expression of cyclinA, cyclinD1 and CyclinE significantly inhibited the proliferation of glioma cells [28]. After knockout of cyclinD1 gene in human pulmonary artery smooth muscle cells they were blocked at G1/S phase and the proliferation decreased [29]. In this study we found that in control group most cells arrested in G2 phage with over-expression of cyclinD1 cyclinA1 and cyclinA2, while cells were blocked in G1 phase with decreased expression of cyclins after treatment of alkannin. These suggested that alkannin could inhibit the proliferation of glioma cells C6 by regulating the expression of cyclins. Previous studies found that the expression of IQGAP was associated with the expression of cyclins [30, 31], whether mTOR was activated or not affected the expression of cyclins [32, 33]. We found that alkannin could regulate the expression of cyclins and affect the proliferation of glioma cells C6 by IQGAP/mTOR signal pathway.

The effects of alkannin on the MMPs of glioma cells C6

MMP 2 is a zinc-dependent proteolytic enzymes secreted by many cells, it can degrade the components of extracellular matrix and basement membrane and destruct local tissue structure, play an important role in the development and invasion of tumor [34]. MMP 9 can promote the invasion of tumor cells to the surrounding normal tissue and promote invasion and diffusion of tumor [35]. MMP 2 and MMP 9 highly

expressed in human glioma cells and played an important role in the development, invasion and metastasis process of glioma and positively correlated with the metastasis and invasion of tumor [36-38]. MMP 2 and MMP 9 inhibitor could reduce the invasive ability of glioma cells [39]. We found that alkannin could inhibit the expression of MMP 2 and MMP 9 with dose dependent. IQGAP was confirmed to involve the pseudopodia activity of cells to regulate invasion [40]. Knockout of MMP 2 could affect the secretion of MMPs by Akt/mTOR signal pathway [41]. The expression of MMP9 was down regulated by AMPK-TSC-mTOR [42] and Akt (PKB)/mTOR [43] signal pathway to inhibit invasion in glioma cells. We found that alkannin could regulate the expression of MMPs by IQGAP/mTOR signal pathway to affect invasion.

Conclusions

In this study we found that alkannin could significantly inhibit the growth, proliferation and invasion of glioma cells with the increased concentration, which may be related with the down-regulated expression of cyclinA1, cyclinA2 and cyclinD1 and inhibited secretion of MMP 2 and MMP 9 by IQGAP/mTOR signal pathway.

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Disclosure of conflict of interest

None.

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