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Aclidinium bromide inhibits human glioma cell proliferation, migration and invasion and promotes apoptosis via the PI3K/AKT signaling pathway

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This study investigates the anti-cancer potential of Aclidinium bromide (INN) in glioblastoma. Glioblastoma cell lines U251 and U87 were treated with INN and its effects on cell migration and invasion were assessed by transwell migration and invasion assays., The effects of INN on proliferation and apoptosis were detected by CCK-8 kit and flow cytometry, and Western blotting determined anti-apoptotic proteins and signaling pathway changes. The results show that INN effectively suppressed proliferation, migration and invasion and induced apoptosis in U251 and U87 cells, respectively. Furthermore, the expression levels of the Bcl-2 anti-apoptotic protein was significantly decreased while Bax and caspase-3 expression were both increased in glioblastoma cells (all, $p<0.05$). Moreover, INN inactivated the PI3K/AKT signaling pathway by down-regulating the level of p-AKT, p-mTOR, P70 and CyclinD1 (all, $p<0.05$). In conclusion, our data suggests that INN could provide novel anticancer therapy in the treatment of glioblastoma.

Key words: aclidinium bromide, glioblastoma, proliferation, migration, invasion, apoptosis

Glioblastoma is the most common malignant and aggressive form of brain cancer in the central nervous system and it accounts for 15–20% of all primary intracranial tumors [1, 2]. Glioblastoma is a complex and heterogeneous disease at the genetic and epigenetic levels [3] and has strong therapy resistance, high recurrence, and rapid progression [4] leading to high morbidity and mortality. Although recent treatment of glioblastoma and surgical methods and equipment have improved [5] patients, particularly those in advanced clinical stages, usually have high relapse rates and poor prognosis with survival rate less than 10% [4]. Therefore, in order to improve the survival of patients with glioblastoma, it is urgent to understand the molecular mechanism of glioblastoma progression and to develop new therapeutic strategies.

Aclidinium bromide (INN) is a new inhaled long-acting muscarinic antagonist (LAMA) which was approved by the European Medicines Agency and the US Food and Drug Administration on July 24, 2012 [6]. As a maintenance treatment for chronic obstructive pulmonary disease (COPD), INN has high affinity for all 5 human muscarinic receptor subtypes (M1–M5) and lower potential for anticholinergic adverse events [7]. In addition, INN can be rapidly hydrolyzed to two major inactive metabolites in human plasma [8], and urinary excretion of the drug is therefore very low [9]. Although evidence confirms that INN can improve the life quality of patients with COPD [7], the functional role of INN in treatment of human glioblastoma still remains unknown.

This study applies INN to human glioblastoma U251 and U87 cells and determines its anti-tumor properties.

Materials and methods

Cell culture. Human glioblastoma cell lines U251 and U87 purchased from the Shanghai Academy of Sciences Cell Bank and the primary cultured HT22 cell line were cultured at 37 °C and 5% CO_2 in DMEM-F12 media with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). In the logarithmic phase, the cells were washed three times in PBS and then digested with trypsin enzyme. Medium was then added to terminate digestion and single cell suspension was planted in six orifice plates. Cell density reached 80% and the cells were exposed to INN (20 mM) for 24 hours. Negative controls were performed in DMSO (1:1000) diluted in medium.

Western blot. Proteins from U251 and U87 cells following treatment with INN or DMSO at the above-mentioned concentrations for 24 hours were extracted by RIPA buffer. Protein concentration was determined by BCA method. Proteins were then separated in sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gel and blotted onto a polyvinylidene difluoride membrane (PVDF). Membranes were blocked with 5% skim milk powder for 1 hour, followed by overnight incubation at 4 C with indicated antibodies. Membranes were rinsed with TBST and then incubated with secondary antibodies for 1 hour at room temperature. GAPDH was used as an internal control and ECL kit was performed for chemiluminescent detection. Experiments were repeated at least three times.

Cell proliferation assays. Cell proliferation was evaluated by the CCK-8 kit according to the manufacturer's protocol. The routine cultured cells were digested and counted and cell suspensions were plated at a density of 1,000 cells/well in 96-well culture plates and cultured with INN (20 mM) in a 5% CO_2 incubator. Every 24 h, 10μl CCK8 medium was added to the plates for 1.5 h at 37 °C. The optical density (OD) was measured at 450nm by ELISA reader and all experiments were performed in triplicate.

Transwell migration and invasion assay. To assess invasion ability, the upper chamber of 24-well polycarbonate membrane transwell chambers were coated with matrigel (1:6; diluted with serum-free DMEM). After standing at 37 °C for 4–6 hours, 500 μl serum-free medium was added to the lower chambers. 100 μl suspension containing 1×10^5 cells treated with INN for 24 hours was then added to the upper chamber and 500 μl Dulbecco modified eagle medium (DMEM) containing 10% FBS was added to the lower chamber. After overnight incubation, cells that failed to pass through the membrane were removed with cotton-tipped swabs, and cells migrated to the lower membrane surface were immobilized with 4% methanol for 30 min and stained with 0.1% crystal violet solution for 10 min. Five fields were randomly selected and quantified by microscopy. Similar to the invasive experiment, the transwell chamber in the migration test did not need to be treated with glue. The suspension contained 5×10^4 cells.

Apoptosis analysis. Cell apoptosis was analyzed by Annexin-V FITC staining. Briefly, U251 and U87 cells were seeded in 6-well plates and treated 24 h later with INN. After incubation of starvation condition for 24 h, the cells were harvested, trypsinized, washed with cold PBS at

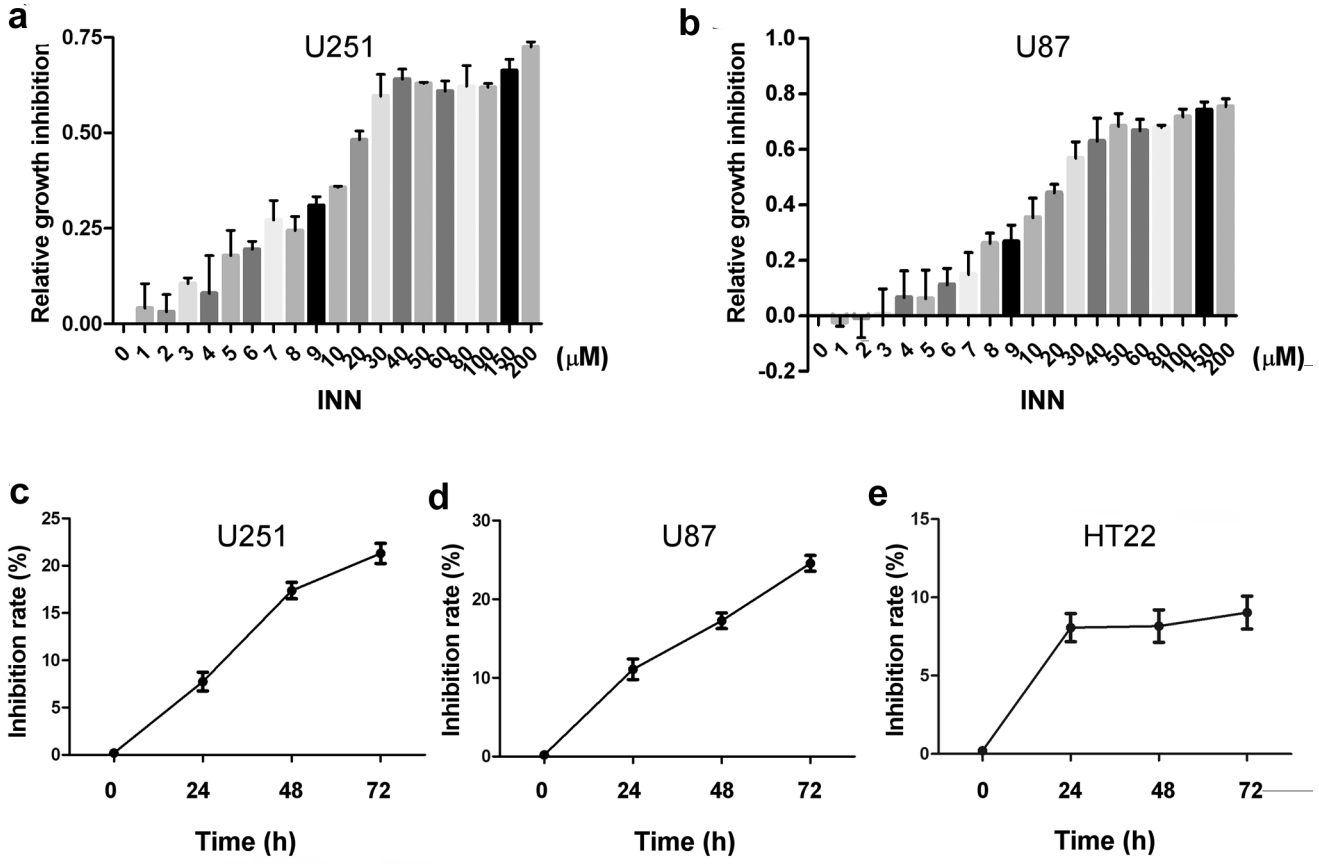

Figure 1. INN inhibits glioma cell proliferation. U251 (a), U87 (b) cells were treated with increasing concentrations of INN for 96 h. Both cell lines (c, d) as well as HT22 (e) cells were assessed for growth inhibition with 20 mM INN using CCK-8 assay at 24 h, 48 h, and 72 h.

4 °C and centrifuged at room temperature. The cells were then re-suspended in Binding Buffer 1X and stained with Annexin-V FITC and PI for 10 min in the dark at room temperature. The samples were analyzed by Flowjo.

Statistical analysis. Statistical analyses were performed with SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA), and data is expressed as mean ± standard deviation (SD). Differences between two groups were determined by t test, and $p<0.05$ was considered statistically significant.

Results

INN reduced proliferation of U251 and U87 cells. In order to investigate the disease-resistant activity of INN, we evaluated the effects of INN on cell growth of U251 and U87 cell lines after 24 h, 48 h and 72 h by CCK-8. As shown in Figures 1A and 1B, INN inhibited the growth of human U251 and U87 cells in a dose dependent manner at 96 hours, and the IC50 values in U251 and U87 cells were 18 and 20 mM, respectively. Therefore, INN at a final concentration of 20 mM was collected for subsequent experiments. The effect of INN on cell proliferation indicated that INN is cytotoxic to U251 and U87 cells and inhibits cell proliferation *in vitro* at 24 h, 48 h and 72 h (Figures 1C and D).

To explore the effect of the concentration of 20 mM INN on normal cells, we assessed the proliferation ability of HT22. The result showed that the concentration of INN had no significant effect on the proliferation of normal cells (Figure 1C).

INN inhibited migration and invasion of U251 and U87 glioblastoma cells. To further demonstrate the potential roles of INN on U251 and U87 cell migration and invasion, we performed transwell assays. The results showed that the INN-treated cell number passing the membrane was significantly lower than that of the control group in U251 and U87 cells (both $p<0.01$; Figures 2A, B). Similarly, the invasion assay revealed that the invasive ability of U251 and U87 cells treated with INN was significantly decreased compared to controls (both, $p<0.01$). The combined data suggests that INN is involved in the migration and invasion of glioblastoma cells, and treatment by INN inhibits glioblastoma cell migration and invasion.

Effect of INN on glioblastoma cell apoptosis. Subsequently, we investigated whether the INN was related to induction of glioblastoma apoptosis, and the number of apoptotic cells was assessed by the Annexin V-FITC/PI as described above. Figure 3A shows that the percentage of apoptotic U251 cells treated with INN increased compared

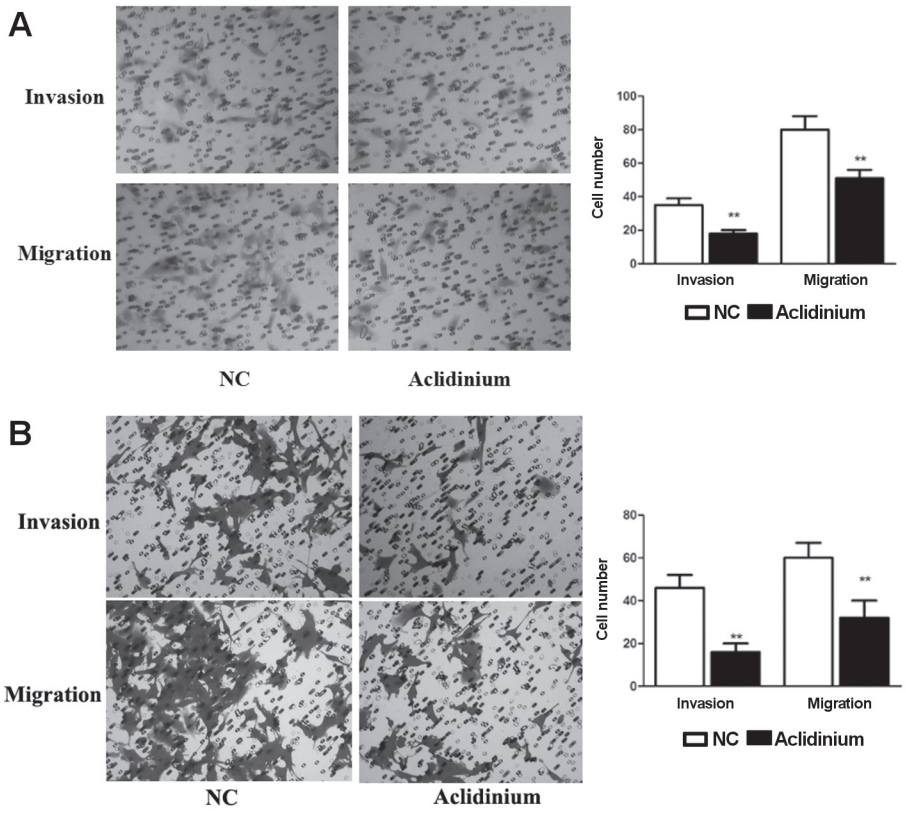

Figure 2. Transwell migration and invasion assay. INN inhibits U251 (A) and U87 (B) cells migration and invasion compared to the controls. **p<0.01.

to control cells (23.78% versus 5.69%). Similarly, treatment with INN significantly induced apoptotic response in U87 cells compared to controls (27.80% versus 13.16%; Figure 3B). Furthermore, in order to further confirm the apoptotic effect induced by INN, we also tested Bcl2/Bax and caspase-3 proteins related to apoptosis. Western blotting analysis demonstrated that INN treatment decreased the expressions of Bcl-2, but increased the expression of Bax and caspase-3 in U251 and U87 cells (Figures 3C, D – all $p<0.05$). Collectively, INN induced apoptosis of glioblastoma cells by regulating the expression of apoptosis-related proteins.

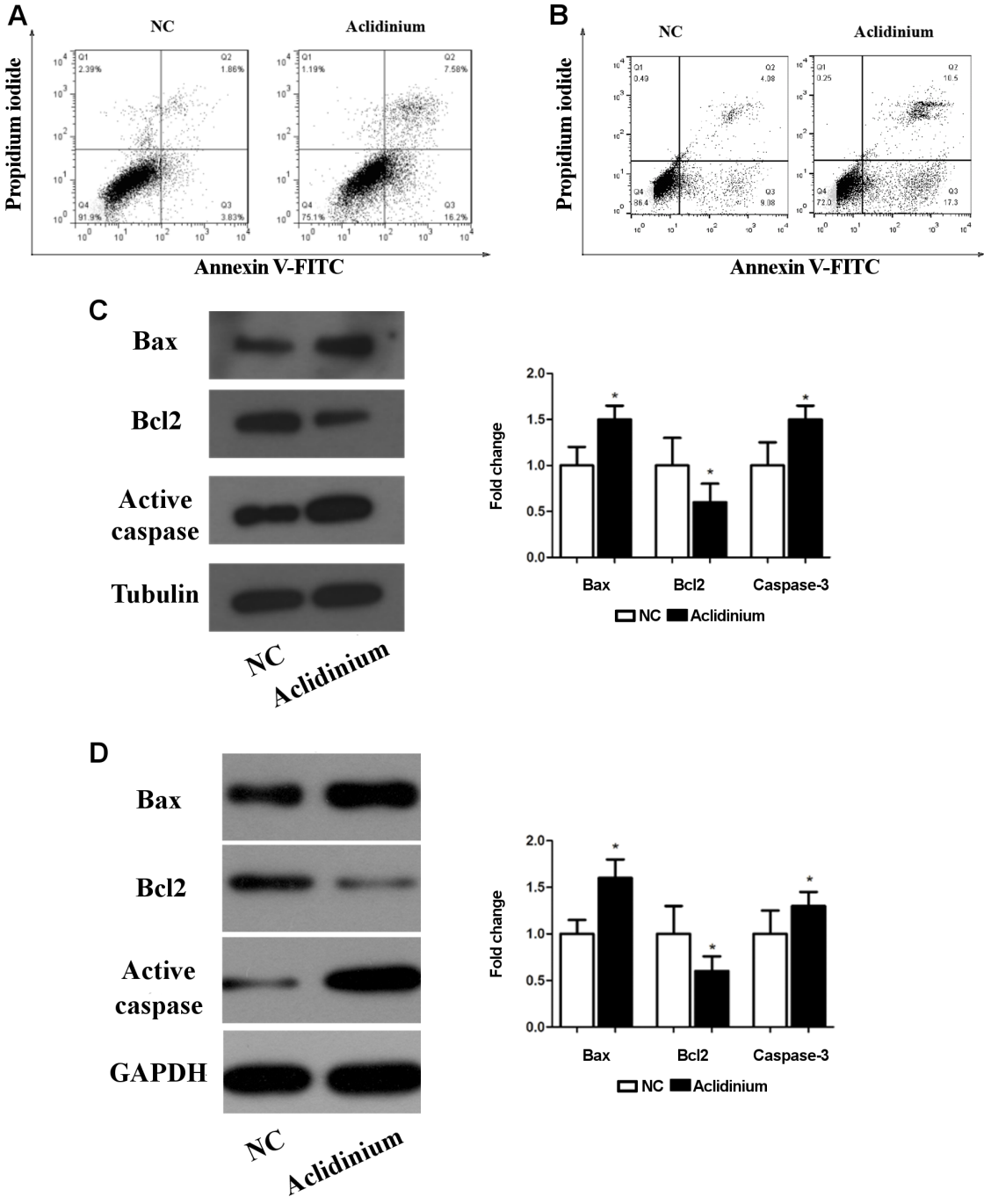

Figure 3. Effects of INN on cell apoptosis in glioblastoma cells. Effect of INN on U251 (A) and U87 (B) cell lines apoptosis detected by flow cytometry. The protein levels Bcl-2, Bax and caspase-3 of detecting using western blotting on U251 (C) and U87 (D) cell lines. *$p<0.05$.

INN inhibited the activation of the PI3K/AKT signal pathway of glioblastoma cells. Previous studies showed that the PI3K signaling pathway was involved in tumor progression with essential AKT and mTOR proteins, and that this has important roles in tumor cell proliferation and metastasis. Based on this knowledge, we speculated that INN regulated glioblastoma through the PI3K/AKT signal pathway. As shown in Figure 4A and 4B, INN treatment significantly down-regulated the level of p-AKT and p-mTOR (both, $p<0.05$) in U251 and U87cell lines. More importantly, the down-stream proteins of P70 and CyclinD1 were significantly decreased in cells treated with INN compared to controls.

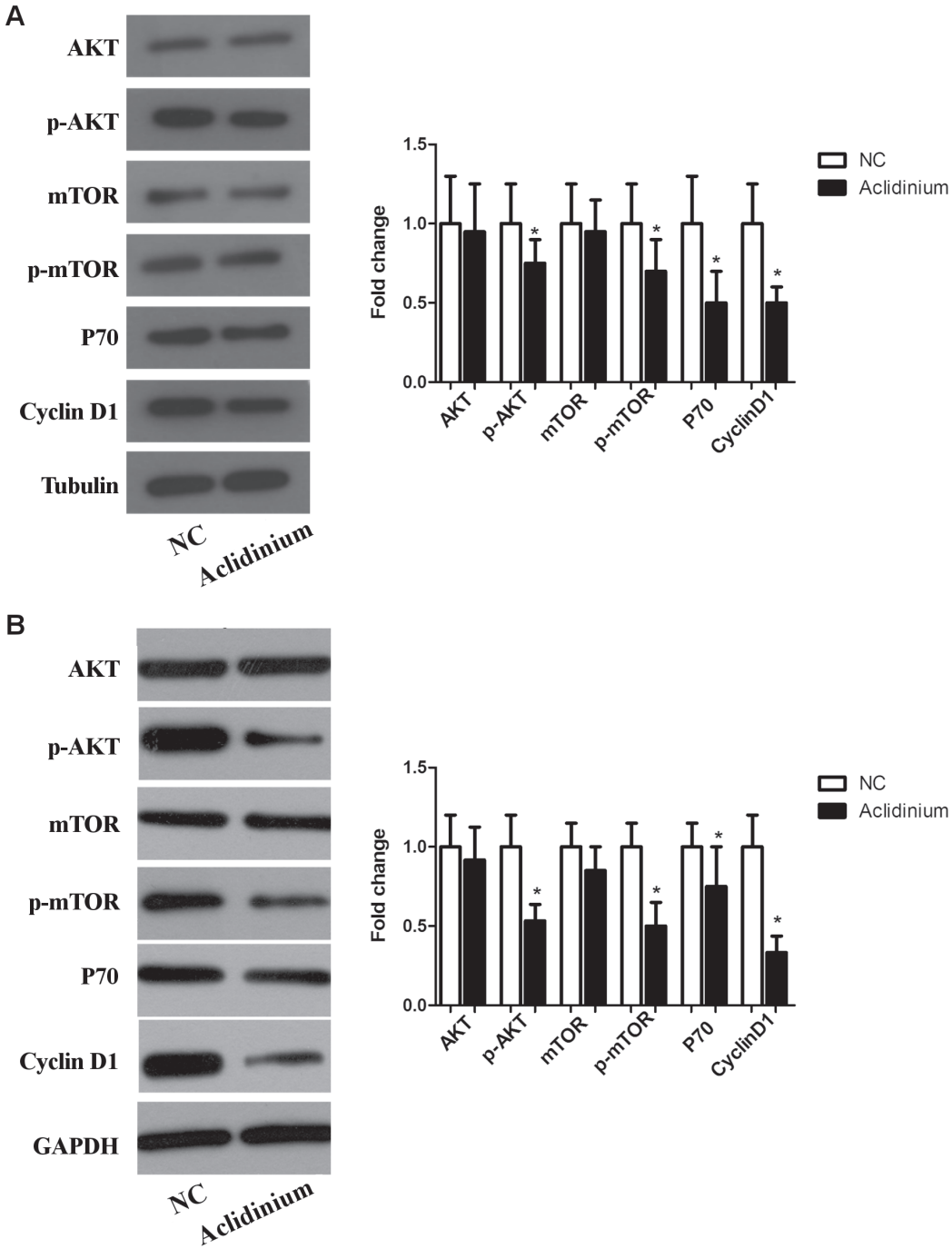

Figure 4. INN negatively regulates PI3K/AKT signaling pathway. The expression of AKT, p-AKT, mTOR, p-mTOR, P70 and Cyclin D1 were detected by western blotting on U251 (A) and U87 (B) cell lines. *p<0.05.

Generally, the results suggest that INN inhibits glioblastoma progression by deregulating the PI3K/AKT signal pathway.

Discussion

As commonly found in intracranial tumors with relatively high rates of recurrence, glioblastoma has poor response to conventional surgery, radiotherapy, chemotherapy and targeted therapy, and produces profound side effects. Although more and more anti-tumor drugs have been investigated, most are expensive and have adverse reactions. More importantly, most are restricted in clinical application [10–14]. Therefore, it would be helpful to discover new drugs with low toxicity, efficacy and stability for the treatment of glioblastoma. In this study, for the first time, we found that INN could inhibit glioblastoma cell migration and invasion and promote their apoptosis. Moreover, we also demonstrated that INN was able to inactivate the PI3K signaling pathway.

While INN has been a valuable new therapy in the treatment of COPD, this is the first investigation of its glioblastoma anti-tumor effects. Abnormal cell proliferation is a major factor in the development of tumors, and therefore the inhibition of cell proliferation could be an effective anti-tumor therapy. Although dose-dependent INN can reduce fibroblast proliferation [15], there have been only a few reports in glioblastoma cells, but this study confirms that INN significantly inhibited cell proliferation in U251 and U87 cells.

Previous studies have shown that migration and invasion are the main biological characteristics related to tumor malignancy, including in glioblastoma [16–18]. Moreover, they are complex processes and major contributors to poor prognosis. Alagha et al [19] revealed that some LAMAs showed anti-inflammatory effects in inhibiting the migration of alveolar neutrophils.

In accordance with previous studies of INN in COPD, our data demonstrated that INN has an inhibitory effect on the migration and invasion of U251 and U87 glioma cells. The combined results indicate that INN inhibits the development of glioblastoma by inhibiting glioma cell migration and invasion.

Research demonstrates that there are lots of factors leading to apoptosis in cells, and anti-tumor drugs are one of the external factors [20]. According to our study, the results revealed INN could induce apoptosis, decrease expression of Bcl-2, and increase expression of Bax and caspase-3. Bcl-2, Bax and caspase-3 are apoptosis-related genes which have important roles in the regulation of apoptosis. As important members of the Bcl-2 protein family, Bcl-2 and Bax are important factors in the intrinsic apoptotic pathway [21, 22]. The Bcl-2 protein, in particular, inhibits cell apoptosis through multiple mechanisms [23] and caspase-3 is also an essential terminal caspase in apoptosis execution [21].

Accumulating evidence demonstrates that the PI3K/AKT pathway is a crucial signaling pathway in cancer development [24–27], and this is significantly activated in glioma [28, 29]. In glioma, the PI3K/AKT signaling pathway plays a crucial role in the induction of proliferation, migration and invasiveness [30]. Therefore, inhibition of PI3K/AKT signaling presents a potential approach for anti-tumor therapy. In this study, we assessed the effects of INN on the PI3K/AKT signaling pathway to further explore the molecular mechanisms INN employs to inhibit the proliferation, invasion and migration of U251 and U87 cells. The results indicated that INN reduces the level of p-AKT and p-mTOR. More importantly, the down-stream proteins of P70 and CyclinD1 were down-regulated after INN treatment. In fact, aberrant activation of Akt is frequently observed in glioma [31], and mTOR regulates multiple cell growth by controlling mRNA translation and metabolism [32]. Inhibition of mTOR therefore leads to regional apoptosis [33].

In conclusion, our research suggests that INN can inhibit proliferation, migration and invasion. INN can also promote apoptosis of human glioma cells via the PI3K/AKT signaling pathway, and it may therefore serve as a successful new anti-cancer drug in treating patients with glioma. While this is a distinct possibility, additional studies are still required to investigate INN's effects *in vivo*.

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