SHORT REPORT
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Atractylenolide-II inhibits proliferation and migration of glioblastoma cells

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ABSTRACT: Glioblastoma (GBM) is the most malignant and lethal brain tumor in adults, with limited therapeutic options and dismal prognosis. Atractylenolide-II (AT-II) is a major bioactive compound from *Atractylodes macrocephala* Koidz. (Baizhu in Chinese), with anti-inflammatory and anti-tumor activities. However, the anti-tumor effects of AT-II on GBM cells remain unclear. In the present study, the cytotoxicity of AT-II on GBM cells was analyzed using CCK-8 assay, and it revealed that the cell viability was inhibited by AT-II in a dose-dependent manner. The results of Transwell migration assay indicated that AT-II treatment significantly inhibited the cell migration. Furthermore, the cell cycle arrest at G0/G1 phase in AT-II treated cells was presented in the flow cytometry data. Consistently, real-time PCR and western blotting revealed a remarkable decrease of the CCNA and CCNB expression upon AT-II treatment. Moreover, the phosphorylation of both extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) was triggered by AT-II. Collectively, these results suggested that MAPK signaling was involved in AT-II induced cell cycle arrest, contributing to the inhibition of cell viability and migration of GBM cells.

KEYWORDS: atractylenolide II, glioblastoma, cell cycle, mitogen-activated protein kinase

INTRODUCTION

Glioblastoma (GBM) is recognized as the most frequent and aggressive brain tumor, with poor prognosis and a median survival of less than 15 months [1]. The first-line treatment for GBM is surgical resection followed by radiotherapy and temozolomide chemotherapy [2]. Despite the standard of care that provides temporary relief, GBM is eventually recurrent and acquired resistance. Moreover, GBM represents a challenge owing to the heterogeneity and cellular stratification, which enable tumor initiation, extensive proliferation and invasiveness [3, 4]. As a result, it is urgent to develop potential therapeutic agents.

Atractylodis macrocephalae Koidz. (Baizhu in Chinese), a valuable traditional medical herb, has been used for the treatment of gastrointestinal dysfunction, osteoporosis, obesity, and cancer [5]. Atractylenolide II (AT-II) is a sesquiterpene compound isolated from *Atractylodes macrocephala* Koidz., and it exhibited anti-inflammatory and anti-tumor activities, protecting radiation damage and improving hyperlipidemia [6–8]. AT-II presented inhibitory effects on several tumor cells, including cells of melanoma, colon tumor, gastric carcinoma, and breast cancer [9–12]. Previous studies indicated that AT-II induced apoptosis of prostate cancer cells via JAK/STAT3 signaling [13]. However, the anti-tumor effects of AT-II on GBM cells remain unclear.

In this study, the inhibitory effects of AT-II on the cell viability and migration of GBM cells were analyzed. The cell cycle distribution and mitogen-activated protein kinase (MAPK) signaling were further investigated.

MATERIALS AND METHODS

Cell culture

Human GBM cell lines U-87 and U-251 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). DMEM (Hyclone, Beijing, China) and supplemented with 10% FBS (Hyclone) and 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA) were utilized to culture the GBM cell lines. These cells were maintained in a 37 °C incubator (5% CO_2). The cells were treated with AT-II at indicated concentrations, and DMSO was used as a control.

Cell viability assays

The cell viability assays were carried out using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Shanghai, China) in 96-well plates. The U-87 and U-251 cells were seeded into each well at a density of 3000 cells per well; and then, incubated at 37 °C overnight. Following treatment with AT-II (50, 100, 150, 200 μM) for 24 h, the cells of each well were incubated with 10 μl CCK-8 at 37 °C for 1 h. Microplate reader

(Bio-Rad, Hercules, CA, USA) was used to measure the optical density (OD) at 450 nm with a reference wavelength of 630 nm.

Transwell migration assay

The U-87 and U-251 cells resuspended in serum-free medium were seeded into the upper Transwell chamber (diameter: 6.5 mm, pore size: 8 μM; Corning, Tewksbury, MA, USA) at a density of 1×10^4 cells/well, and complete medium was added into the lower chamber. Following incubation for 24 h, transmigrated cells were fixed with 4% polyoxymethylene, and 0.1% crystal violet (Beyotime Biotechnology, Shanghai, China) was utilized to stain cells. Images were obtained using a CKX31 microscope (Olympus, Tokyo, Japan).

Cell cycle analysis

To determine cell cycle distribution, the U-87 and U-251 cells were cultured in 6-well plates and treated with AT-II at 100 μM for 24 h. The cells were collected and fixed with 70% ethanol at 4 °C overnight, incubated with RNase at 37 °C for 30 min, and stained with propidium iodide (PI) for 30 min in the dark. The cell cycle distribution was determined using a flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

RNA extraction and real-time PCR

Total RNAs were extracted using RNAiso Plus (Takara, Dalian, China), and cDNA was synthesized using PrimeScript RT reagent Kit (Takara) according to the manufacturer's instruction. Real-time PCR was conducted on ABI 7500 Real-Time PCR System using PowerUp SYBR Master Mix (Applied Biosystems, Shanghai, China). Relative expression levels were analyzed using the ΔΔCT method. GAPDH was used as an internal control.

Western blotting

Cell lysates were prepared using RIPA lysis buffer (Beyotime Biotechnology), and the concentration of total protein was quantified using the BCA Protein Assay Kit (Takara). Samples containing equal amounts of proteins were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with 5% skimmed milk and incubated with primary antibodies (1:2000) at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibodies (1:5000, Proteintech). Immunoblot bands were visualized using GE (Amersham) ECL Prime Western Blotting (GE Healthcare, Shanghai, China). CCNA antibody (8202-1-AP), CCNB antibody (28603-1-AP), GAPDH antibody (60004-1-Ig), HRP-conjugated Goat Anti-Rabbit IgG (SA00001-2), and HRP-conjugated Goat Anti-Mouse IgG (SA00001-1) were purchased from Proteintech (Wuhan, China). The following antibodies: p38 (8690), p-p38 (4511), ERK (4695), and p-ERK (4370) were purchased from Cell Signaling Technology (Shanghai, China).

Statistical analysis

The data were presented as mean ± SD. The statistical analysis was determined using Student's t-test for 2 groups and one-way ANOVA for more than 2 groups. $*p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

AT-II inhibited cell viability and migration of GBM cells

To determine the anti-tumor effects of AT-II on GBM cells, CCK-8 and Transwell assays were used to detect cell viability and migration. Following treatment with various concentrations of AT-II (50, 100, 150, 200 μM) for 24 h, the results of CCK-8 assay showed that AT-II significantly inhibited cell viability of the U-87 and U-251 cells in a dose-dependent manner (Fig. 1A,B). As shown in Fig. 1C-E, the migration ability of U-87 and U-251 cells was remarkably inhibited by AT-II treatment at 100 μM compared with the control group. The proliferation and the migration of cancer cells are major drivers for tumor progression. They are also important indicators for evaluating prognosis and efficacy of drug treatment. It was reported that AT-II exhibited inhibitory effect on lung cancer cell and B16 melanoma cells by reducing cell viability and migration, which was consistent with our results. These data indicated that AT-II might be a potential candidate for GBM treatment.

AT-II induced cell cycle arrest at G0/G1 phase

Cell cycle is a sequence of tightly-controlled molecular events ensuring accurate DNA replication and cell division. Appropriate control of the cell cycle facilitates the transition from quiescence (G0) to cell proliferation and ensure the fidelity of the genetic transcript through cell cycle checkpoints [14]. However, owing to abrogation of cell cycle checkpoints, cancer cells represent dysregulation of cell cycle, which is a potential carcinogenic mechanism resulting in sustain uncontrolled cell proliferation and division [15]. Several studies reveal that therapeutic agents with anti-cancer effects can induce cell cycle arrest to inhibit proliferation of tumor cells [16–19]. AT-II was previously found to induce cell cycle arrest in cells of gastric cancer, prostate cancer and melanoma [11, 13, 19]. In accordance with these findings, we showed that AT-II treatment induced cell cycle arrest at G0/G1 phase in the U-87 and U-251 cells (Fig. 2A-C).

The activity of cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitor are required for cell cycle progression. To confirm the potential mechanisms, we detected cyclins and found that the mRNA and protein levels of CCNA and CCNB were significantly decreased

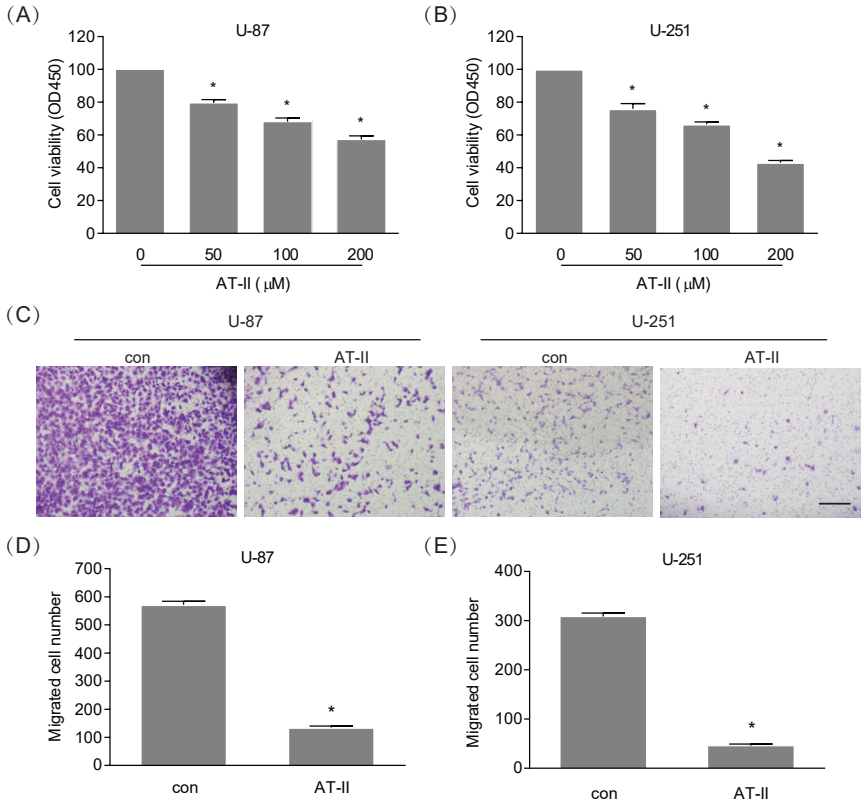

Fig. 1 Effects of AT-II on cell viability and migration. (A, B) The U-87 and U-251 cells were treated with different concentration of AT-II, and CCK-8 assays were used to measure cell viability. (C) The U-87 and U-251 cells were treated with AT-II at 100 μM for 24 h. Cell migration was measured using Transwell migration assay. (D, E) Migrated cell number was quantified using ImageJ software. The bar indicates 100 μm. $*p < 0.05$, compared with control, $n = 3$.

upon AT-II treatment (Fig. 2D-H), suggesting that AT-II-induced cell cycle arrest by downregulation of CCNA and CCNB. However, the definite mechanisms are still unclear. CCNA and CCNB were required for entering mitosis, and their expression were increased in various types of cancer. The downregulation of CCNA and CCNB can lead to tumor regression. It is reported that cell cycle arrest is the most prominent outcome of the tumor suppressor p53 activation [20]. Upon p53 activation, cell cycle proteins including CCNA, CCNB and CDK1 are transcriptionally downregulated [21]. Additionally, studies show that transcriptional repression of these cell cycle regulators by p53 requires the CDK inhibitor p21/CDKN1A [22]. These data suggested that AT-II might induce cell cycle arrest by silencing CCNA/CCNB-CDK family complex, via activation of tumor suppression gene and upregulation of CDK inhibitor.

AT-II activated MAPK signaling in GBM cells

MAPK family plays a pivotal role in cellular responses, including cell proliferation, differentiation, survival, transformation and cell cycle. Phosphorylation of MAPK inhibits the CDK-activating phosphatases to regulate cell cycle transitions [23]. To investigate the mechanisms of cell cycle progression induced by AT-II, phosphorylation of MAPK was determined using western blotting. As presented in Fig. 3, AT-II treatment significantly increased phosphorylation of ERK and p38 MAPK in the two cell types.

Recent data suggest that ERK activation promotes cell cycle arrest in response to oncogenic hyperproliferation signals [24]. In addition, it is reported that active phenolic compound hispolon induces cell cycle arrest of hepatocellular carcinoma cells via suprression of CCNA, CCNE, CDK2 and overexpression of p21, p27, accompanied by activation of ERK [25]. p38 MAPK is emerging as an important regulator that induces proliferative arrest by p53 activation and upregulation of CDK inhibitor p16 [26]. Studies also illustrate that p38 phosphorylates tumor suppressor Retinoblastoma (RB) to increase RB-E2F transcription factor affinity, downregulate gene expression, delay cell cycle progression, and prevent proliferation of cancer cells [27]. Moreover, p38 is considered as a tumor suppressor that targets p57Kip2 CDK inhibitor to repress CCNA-

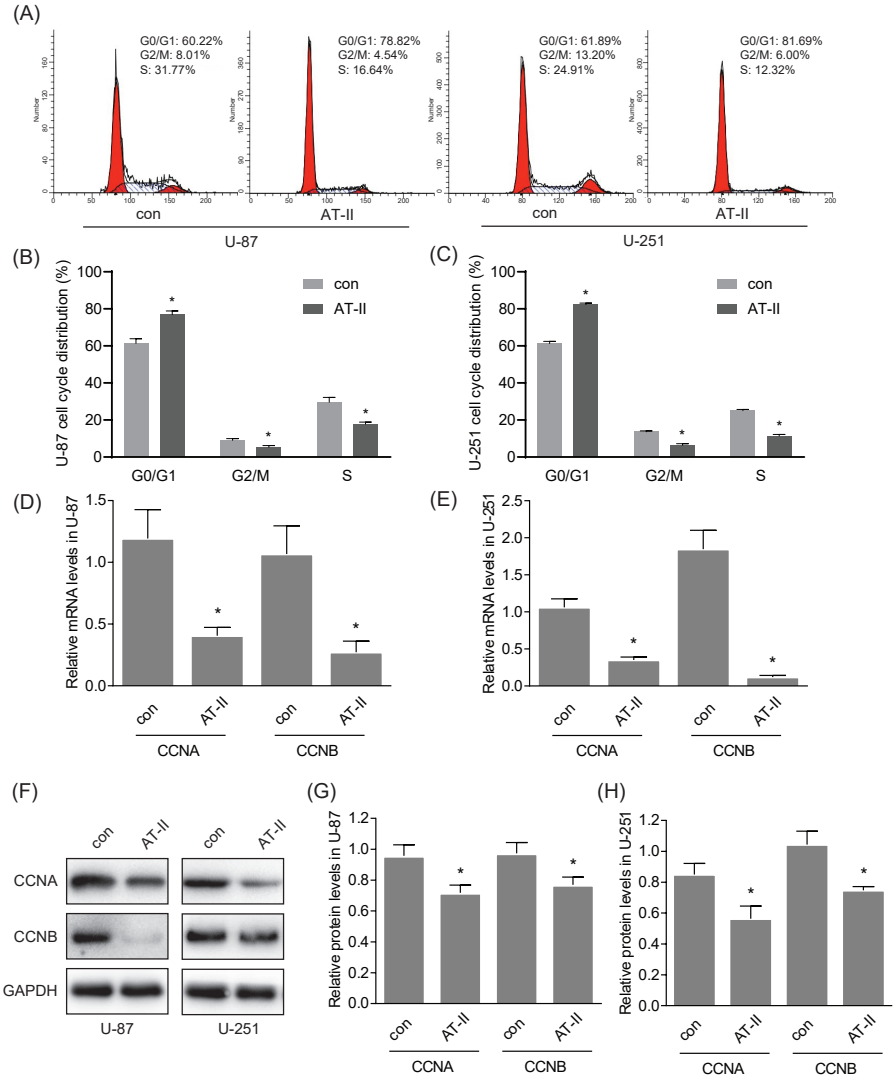

Fig. 2 Effects of AT-II on cell cycle arrest. The U-87 and U-251 cells were treated with AT-II at 100 μM for 24 h. (A-C) Cell cycle distribution was determined using Flow cytometry. (D, E) The mRNA levels of CCNA and CCNB in the U-87 and U-251 cells were detected using real-time PCR. (F) Western blotting assays were used to measure the expression of CCNA and CCNB in U-87 and U-251 cells. (G, H) Band densities of CCNA and CCNB protein in the U-87 and U-251 cells were analyzed using ImageJ software. $*p < 0.05$, compared with control, $n = 3$.

CDK2 activity and, hence, arrest the cell cycle [28]. Combination with our study, we hypothesized that ERK and p38 MAPK phosphorylation by AT-II might target CDK inhibitor to repress CCNA/CCNB-CDK family activity, inducing cell cycle arrest at G0/G1 phase, and inhibiting the proliferation and migration of GBM cells.

CONCLUSION

The present study indicated that AT-II is a candidate agent for the treatment of GBM. The findings demonstrated that AT-II treatment remarkably inhibited the viability and migration of GBM cells. Induction of cell cycle arrest by AT-II was found in GBM cells. Furthermore, MAPK signaling was involved in the inhibitory effects of AT-II.

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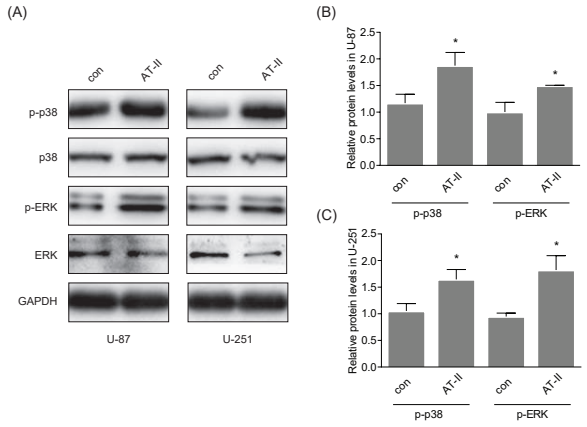

Fig. 3 Effects of AT-II on phosphorylation of MAPK. (A) Following AT-II treatment in the U-87 and U-251 cells, phosphorylation of ERK and p38 MAPK was measured using western blotting. (B) Band densities of p-ERK and p-p38 protein were analyzed using ImageJ software. $*p < 0.05$, compared with control, $n = 3$.

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