Adiponectin facilitates the cell cycle, inhibits cell apoptosis and induces temozolomide resistance in glioblastoma via the Akt/mTOR pathway

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Abstract. Adiponectin (ADN) regulates DNA synthesis, cell apoptosis and cell cycle to participate in the pathology and progression of glioblastoma. The present study aimed to further explore the effect of ADN on temozolomide (TMZ) resistance in glioblastoma and the underlying mechanism of action. Glioblastoma cell lines (U251 and U87-MG cells) were treated with ADN and TMZ at different concentrations; subsequently, 3.0 μg/ml ADN and 1.0 mM TMZ were selected as the optimal concentrations for the experimental conditions. LY294002 (a PI3K inhibitor) was added to ADN or ADN + TMZ-treated glioblastoma cell lines. Cell growth rate was determined using the Cell Counting Kit-8 assay, the apoptotic rate and cell cycle were evaluated using Annexin V/propidium iodide and cell cycle assays, and p-Akt (Thr308), p-Akt (Ser473), Akt, p-mTOR, c-caspase 3, caspase 3, Bax, cyclin B1 and cyclin D1 expression was determined by western blotting. Adiponectin receptor (ADIPOR) 1 and ADIPOR2 were expressed in glioblastoma cell lines. The glioblastoma cell line growth rate was increased by ADN in a concentration- and time-dependent manner. ADN inhibited glioblastoma cell line apoptosis and facilitated cell cycle. Of note, ADN activated the Akt/mTOR pathway and the addition of LY294002 reversed the effect of ADN, indicating that ADN activated the Akt/mTOR pathway to suppress apoptosis and promote cell cycle in glioblastoma cell lines. Notably, TMZ inhibited glioblastoma cell line growth, promoted apoptosis and increased G_2 phase cell cycle arrest. However, the addition of ADN reversed the effect of TMZ in glioblastoma cell lines, disclosing that ADN induced TMZ resistance. Markedly, ADN-mediated TMZ resistance was further attenuated by LY294002, suggesting that ADN activated the Akt/mTOR pathway to induce TMZ resistance in glioblastoma cell lines. In conclusion, ADN activated the Akt/mTOR pathway to facilitate cell cycle, inhibit cell apoptosis and induce TMZ resistance in glioblastoma.

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Introduction

Gliomas are the most common type of primary brain tumor with a global prevalence of 42.8% among primary central nervous system tumors (1,2). According to the World Health Organization (WHO), gliomas can be classified into four grades and a higher grade reflects a higher degree of malignancy (3). Glioblastoma, a WHO grade IV glioma, is the most aggressive type of glioma and accounts for ~49% of malignant brain tumors (4,5). It is estimated that the annual incidence of glioblastoma is ~3.23 per 100,000 individuals (6). Temozolomide (TMZ) is the first-line chemotherapy for glioblastoma and acts by inducing DNA nucleotide mismatch, leading to glioblastoma cell damage, apoptosis and cell cycle arrest in the G_2/M phase (7-10). However, TMZ resistance develops in nearly 50% of patients with glioblastoma and is a contributor to poor prognosis (10-12). Therefore, exploring potential mechanisms that lead to TMZ resistance is worthwhile in improving the management of patients with glioblastoma.

TMZ resistance can be induced by the overexpression of O^6-methylguanine-DNA methyltransferase and/or deficiency in the DNA repair pathway (11). Recently, other mechanisms for TMZ resistance have been proposed (13). The Akt/mTOR pathway plays a fundamental role in glioblastoma pathology and progression, which regulates multiple cellular processes, such as cell survival, proliferation, angiogenesis, invasion and metastasis (14). Of note, this pathway also played a fundamental role in TMZ resistance in glioblastoma according to previous studies (15-17).

Adiponectin (ADN) is a protein consisting of 244 amino acids with a molecular weight of 28 kDa, which is secreted by adipocytes and serves as a pivotal mediator of insulin sensitivity, lipid metabolism and inflammation (18). Previous

studies report that ADN was closely involved in the pathology and progression of various types of cancer, including glioblastoma (19-22). For instance, one study indicated that ADN regulates pancreatic cancer cell growth through the β-catenin pathway (23). Regarding glioblastoma, another study illustrated that ADN regulates DNA synthesis, cell proliferation and cell cycle arrest to modulate glioblastoma progression (22). ADN also exerts fundamental effects on drug resistance in cancer cells (24) and ADN regulates the sensitivity of prostate cancer cells to doxorubicin (25). Another study proposed that ADN modulated sunitinib sensitivity by abrogating the PI3K/Akt/NF-κB pathway in renal cell carcinoma (26). Considering the involvement of ADN in glioblastoma progression and its ability to modulate drug resistance, it was hypothesized that ADN may also participate in TMZ resistance in glioblastoma.

The current study intended to explore the engagement of ADN in TMZ resistance in glioblastoma and the involvement of the Akt/mTOR pathway during this process.

Materials and methods

Cell culture and ADN treatment. U251 (cat. no. SCSP-559; National Collection of Authenticated Cell Cultures) and U87-MG (cat. no. TCHu138; National Collection of Authenticated Cell Cultures) cells were maintained in high-glucose Dulbecco's Modified Eagle's Medium (cat. no. SH30022.01; Hyclone; Cytiva) containing 10% fetal bovine serum (cat. no. SH30084.04; Hyclone; Cytiva) at 37˚C in the presence of 5% CO_2. They were subsequently treated with ADN (cat. no. 1065-AP-050; R&D Systems, Inc.) at concentrations of 0.1, 0.5, 1.0 3.0 and 10.0 μg/ml. Subsequently, the cell growth rate was determined by the Cell Counting Kit-8 (CCK-8) assay and the phosphorylated (p)-Akt (Thr308), p-Akt (Ser473), Akt and p-mTOR expression was analyzed by western blotting. The U87-MG cell line used in the present study was the American Type Culture Collection (ATCC) version, which is most probably a glioblastoma cell line of unknown origin. The authenticity of the U87-MG cell line was verified by Short Tandem Repeat (STR) Profiling. The STR Profiling results of the U87-MG cell line were as follows: Amelogenin: X; D5S818: 11, 12; D13S317: 8, 11; D7S820: 8, 9; D16S539: 12; vWA: 15, 17; TH01: 9.3; TPOX: 8; CSF1PO: 10, 11; D19S433: 15, 15.2; D21S11: 28, 32.2; D18S51: 13, 14, which were from the website of the supplier (https://www.cellbank.org.cn/search-detail.php?id=211).

LY294002 treatment. LY294002 (a PI3K inhibitor; cat. no. ab120243; Abcam) was dissolved in dimethyl sulfoxide (cat. no. PHR1309; MilliporeSigma) for further treatment. Subsequently, U251 and U87-MG cells were treated with 10 μM LY294002 in combination with ADN for 24 h at 37˚C. Furthermore, the apoptotic rate was assessed by Annexin V/propidium iodide (AV/PI). The expression levels of cleaved caspase 3 (c-caspase 3), caspase 3 and Bax were evaluated by western blotting.

TMZ treatment. TMZ (cat. no. ab141055; Abcam) at concentrations of 0.1 and 1.0 mM were cultured with U251 and U87-MG cells, respectively. ADN with concentrations of 1, 2 and 3 μg/ml were cultured with cells in combination with TMZ at 0.1 and 1.0 mM at 37˚C for 24 h. The mixture of 1.0 mM TMZ, 3 μg/ml ADN and 10 μM LY294002 was also added to the cells. Finally, 1 mM TMZ or LY294002 was cultured with the cells in the presence of 3 μg/ml ADN at 37˚C for 24 h. Subsequently, the cell growth rate was assessed using CCK-8, the apoptotic rate and cell cycle were evaluated using AV/PI assay and cell cycle assay; the expression levels of c-caspase 3, caspase 3, Bax, cyclin B1 and cyclin D1 were determined by western blotting.

CCK-8 assay. The CCK-8 regent (cat. no. C0037; Beyotime Institute of Biotechnology) with an amount of 50 μl was added and cultivated with the cells for 1 h at 37˚C. Subsequently, the optical density value at 450 nm was measured with a microplate reader (Bio-Rad Laboratories, Inc.). The cell growth rate was calculated.

AV/PI and cell cycle assays. An AV/PI kit (cat. no. C1062S; Beyotime Institute of Biotechnology) was used to detect cell apoptosis. The cells were digested with trypsin (cat. no. SH30042.01; Hyclone; Cytiva) for 1 min at room temperature and re-suspended. Following rinsing with PBS, the cells were incubated with 5 μl AV and 5 μl PI at room temperature in the dark for 15 min at room temperature. Finally, the cells were measured with a CytoFLEX flow cytometer (Beckman Coulter, Inc.). The data were analyzed with FlowJo X (FlowJo LLC). The cells in quadrant 2 (Q2) and quadrant 4 (Q4) were considered as apoptotic cells.

For the cell cycle assay, the cells were harvested and re-suspended. Subsequently, they were fixed in 70% ethanol at 4˚C overnight. The cells were centrifuged (800 x g at 4˚C for 3 min) and collected and stained with PI solution (cat. no. ST1569; Beyotime Institute of Biotechnology) for 30 min at room temperature. A CytoFLEX flow cytometer (Beckman Coulter, Inc.) was applied to detect the cells.

Western blotting. The cells were lysed with radio immunoprecipitation assay buffer (cat. no. V900854; MilliporeSigma) and the protein solution was quantified with the bicinchoninic acid kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). The protein was denatured at 100˚C for 10 min following mixing with a loading buffer. Subsequently, 10-μg protein samples were loaded into a 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel for separation and transferred to the nitrocellulose membrane (cat. no. HATF00010; MilliporeSigma). The membrane was blocked with skimmed milk for 2 h at 37˚C, followed by incubation with primary and secondary antibodies overnight at 4˚C and secondary antibodies at 37˚C for 90 min. The primary antibodies used were for the following proteins: Adiponectin receptor (ADIPOR) 1 (cat. no. APR06109G; 1:1,000; Epitomics, Inc.), ADIPOR2 (cat. no. APG01582G; 1:500; Epitomics, Inc.), Akt (cat. no. 9272S; 1:1,000; Cell Signaling Technology, Inc.), p-Akt (Thr308) (cat. no. 9275S; 1:500; Cell Signaling Technology, Inc.), p-Akt (Ser473) (cat. no. 9271S; 1:500; Cell Signaling Technology, Inc.), c-caspase 3 (cat. no. 9661S; 1:500; Cell Signaling Technology, Inc.), caspase 3 (cat. no. 9662S; 1:1,000; Cell Signaling Technology, Inc.), Bax (cat. no. 2772S; 1:1,000; Cell Signaling Technology,

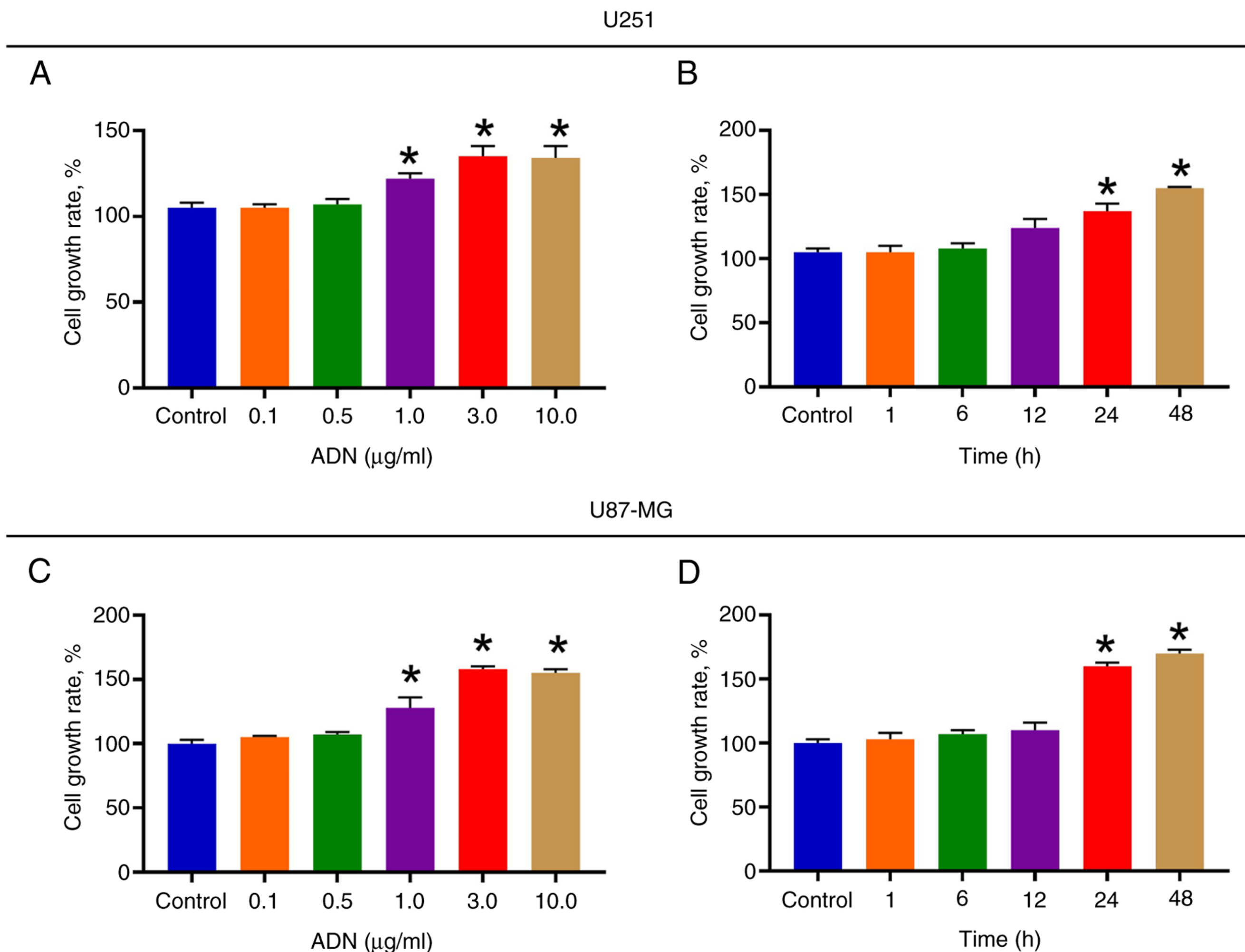

Figure 1. ADN facilitates U251 and U87-MG cell growth in a concentration- and time-dependent manner. (A) Effect of different concentrations of ADN on U251 cell growth rate. (B) Effect of 3.0 µg/ml ADN on U251 cell growth rate at different time points. (C) Effect of different concentrations of ADN on U87-MG cell growth rate. (D) Effect of 3.0 µg/ml ADN on U87-MG cell growth rate at different time points. *P<0.05 vs. control. ADN, adiponectin.

Inc.), p-mTOR (cat. no. 2983S; 1:500; Cell Signaling Technology, Inc.), cyclin B1 (cat. no. ab32053; 1:1,000; Abcam), cyclin D1 (cat. no. ab16663; 1:1,000; Abcam), β-actin (cat. no. 20536-1-AP; 1:4,000; Proteintech Group, Inc.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (cat. no. 10494-1-AP; 1:4,000; Proteintech Group, Inc.). Finally, the horseradish peroxidase-conjugated secondary antibody (cat. no. ab6721; 1:10,000; Abcam) was incubated with the membrane. The protein bands were visualized with an enhanced chemiluminescence kit (cat. no. 32209; Thermo Fisher Scientific, Inc.) and X-ray film. The gray value was quantified by Gene Tools 3.7 (SynGene).

Statistical analysis. The SPSS software (v.26.0; IBM Corp.) was used for statistical analysis. One-way ANOVA was applied for the comparison among groups and Tukey's test was employed for post-hoc comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

ADN increases growth and activates the Akt/mTOR pathway in U251 and U87-MG cells. Considering that ADN binds to its receptors to exert its functions, western blotting was applied to detect ADIPOR1 and ADIPOR2 in U251 and U87-MG cells. It was found that ADIPOR1 and ADIPOR2 were expressed in U251 and U87-MG cells, suggesting that these two types of cells possessed the basis for ADN to exert its functions (Fig. S1).

U251 cell growth rate was increased following treatment with 1.0, 3.0 and 10.0 µg/ml ADN compared with that noted in the control (all P<0.05; Fig. 1A). Subsequently, U251 cells were treated with 3.0 µg/ml ADN at different time points. It was found that following ADN treatment for 24 and 48 h, U251 cell growth rate was increased compared with that of the control (both P<0.05; Fig. 1B). Similarly, the effect of ADN on the U87-MG cell growth rate further indicated a concentration-dependent (Fig. 1C) and time-dependent (Fig. 1D) mode of action. It was noted that when the treatment duration was 24 h, ADN exhibited an optimal effect in promoting U251 and U87-MG cell growth. In addition, compared with the treatment duration of 24 h, the effect of ADN on U251 and U87-MG cell growth was not further promoted after 48 h (both P>0.05). Therefore, the treatment duration of ADN for 24 h was selected as the experimental condition for subsequent experiments.

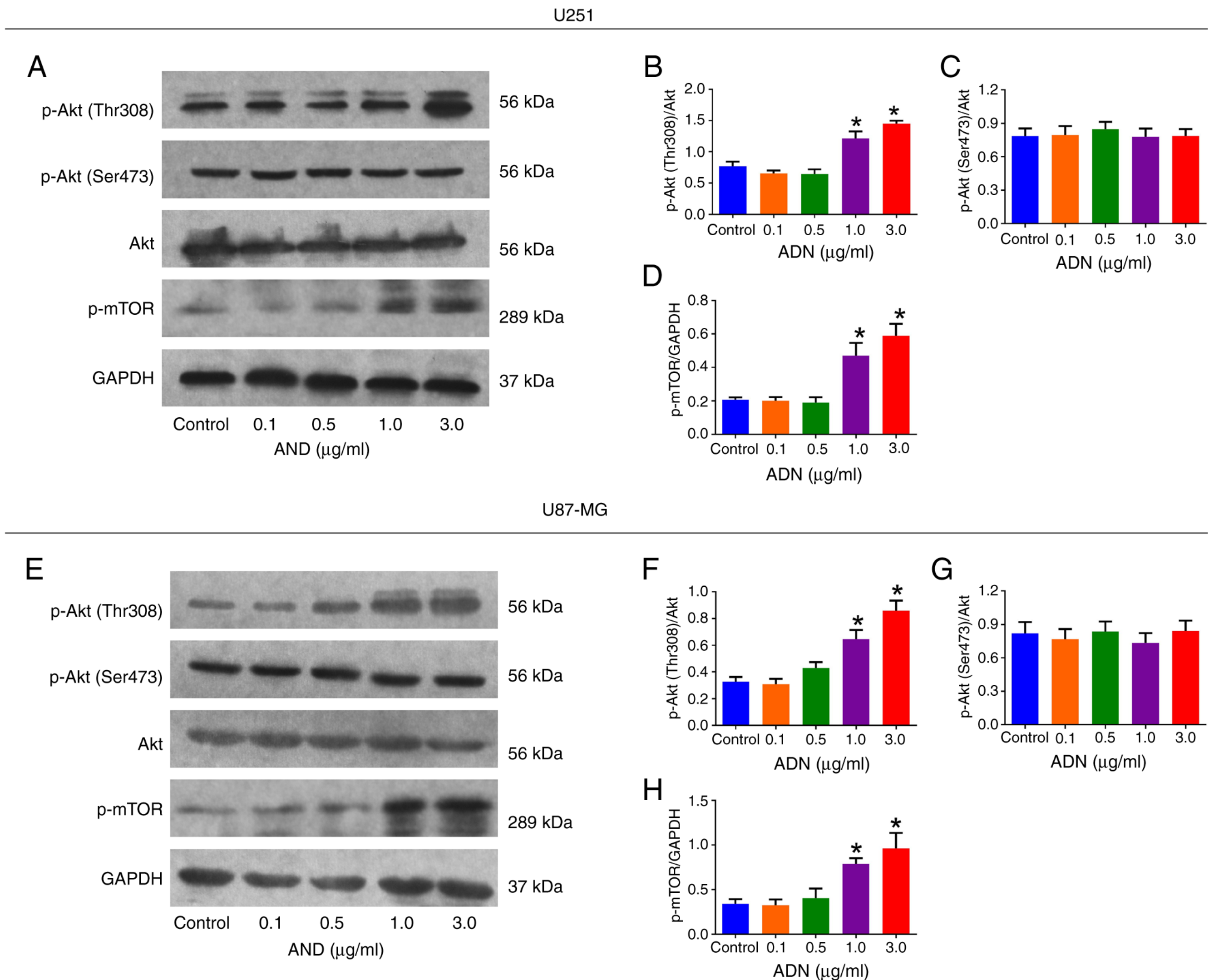

Figure 2. ADN activates the Akt/mTOR pathway in U251 and U87-MG cells. (A) Representative images of p-Akt (Thr308), p-Akt (Ser473), Akt, p-mTOR and GAPDH by western blotting in U251 cells. Effect of different concentrations of ADN on (B) p-Akt (Thr308)/Akt, (C) p-Akt (Ser473)/Akt and (D) p-mTOR/GAPDH in U251 cells. (E) Representative images of p-Akt (Thr308), p-Akt (Ser473), Akt, p-mTOR and GAPDH by western blotting in U87-MG cells. Effect of different concentrations of ADN on (F) p-Akt (Thr308)/Akt, (G) p-Akt (Ser473)/Akt and (H) p-mTOR/GAPDH in U87-MG cells. *P<0.05 vs. control. ADN, adiponectin; p-, phosphorylated.

According to western blotting analysis (Fig. 2A), p-Akt (Thr308)/Akt was elevated following 1.0 and 3.0 μg/ml treatment with ADN compared with that of the control in U251 cells (both P<0.05; Fig. 2B). However, p-Akt (Ser473)/Akt was not affected by ADN treatment at any concentration used in U251 cells (all P>0.05; Fig. 2C). p-mTOR/GAPDH was increased following treatment with 1.0 and 3.0 μg/ml ADN compared with those of the control in U251 cells (both P<0.05; Fig. 2D). The same trends were also noted in U87-MG cells (Fig. 2E-H).

Notably, when the concentration was 3.0 μg/ml, ADN indicated outstanding ability to activate the Akt/mTOR pathway and facilitate U251 and U87-MG cell growth. Therefore, 3.0 μg/ml ADN was selected as the experimental condition for subsequent experiments.

ADN activates the Akt/mTOR pathway to inhibit apoptosis in U251 and U87-MG cells. According to the AV/PI assay (Fig. 3A), the apoptotic rate was decreased by ADN compared with that of the vehicle (P<0.05); however, it was increased by ADN + LY294002 compared with ADN in U251 cells (P<0.05; Fig. 3B). western blotting (Fig. 3C) indicated that c-caspase 3/caspase3 (Fig. 3D) and Bax/GAPDH (Fig. 3E) were decreased by ADN compared with the vehicle (both P<0.05); however, they were elevated by ADN + LY294002 compared with ADN in U251 cells (both P<0.05). The same trends were noted in U87-MG cells (Fig. 3F-J).

ADN activates the Akt/mTOR pathway to increase U251 and U87-MG cells in the S phase. According to cell cycle assay results (Fig. 4A), the proportion of U251 cells in the S phase was increased by ADN treatment compared with the vehicle (P<0.05); however, it was reduced by ADN + LY294002 compared with ADN (P<0.05; Fig. 4B). Western blotting (Fig. 4C) indicated that cyclin B1/β-actin (Fig. 4D) and cyclin D1/β-actin (Fig. 4E) were increased following treatment with ADN compared with the vehicle (P<0.05); however, they were decreased by ADN + LY294002 compared with ADN in U251

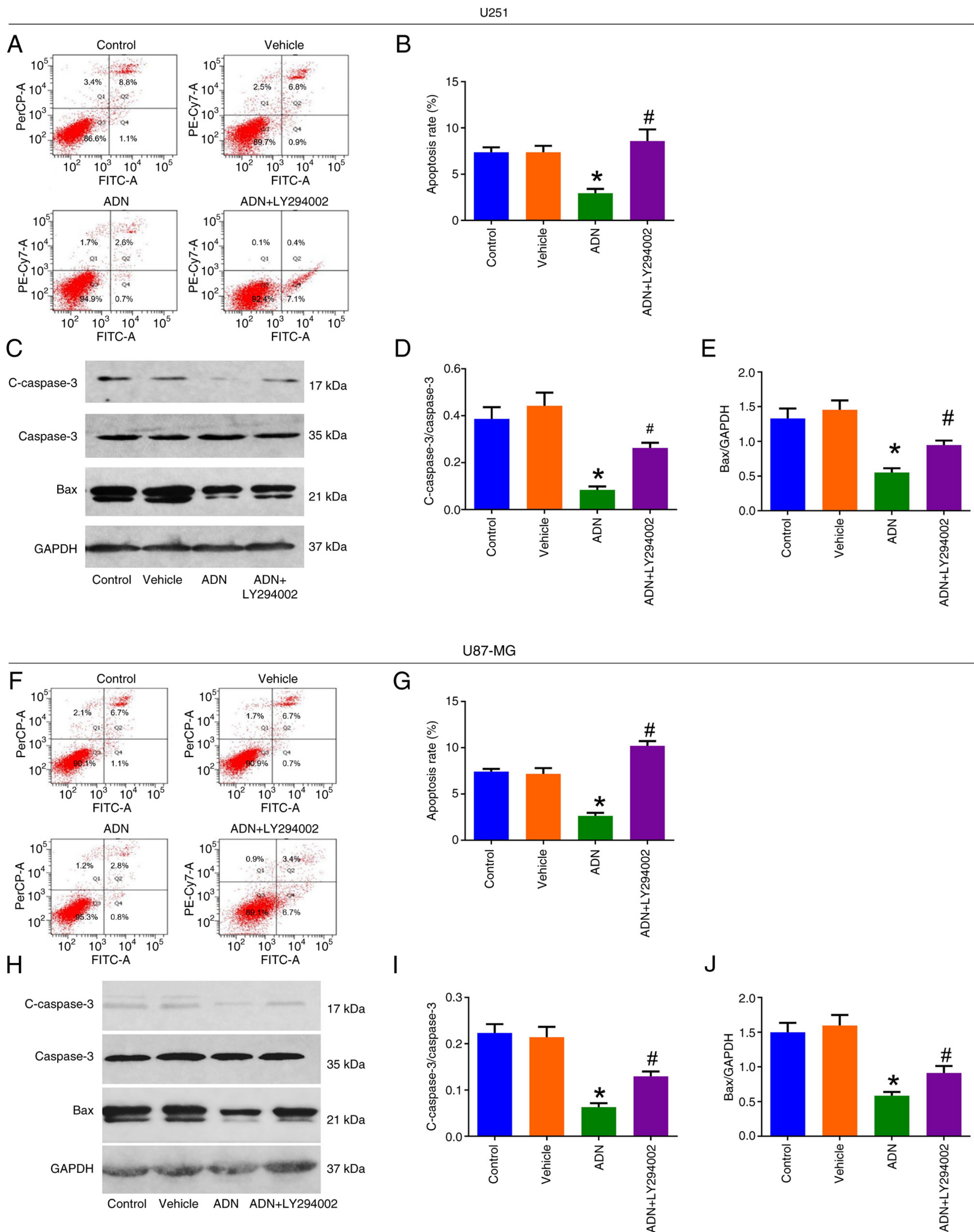

Figure 3. LY294002 attenuates the inhibition of ADN on U251 and U87-MG cell apoptosis. (A) Representative images of U251 cell apoptosis by AV/PI assay and (B) comparison of apoptosis rate among control, vehicle-treated, ADN-treated and ADN + LY294002-treated U251 cells. (C) Representative images of c-caspase 3, caspase 3, Bax and GAPDH in U251 cells by western blotting and comparison of (D) c-caspase 3/caspase 3 and (E) Bax/GAPDH among control, vehicle-treated, ADN-treated and ADN + LY294002-treated U251 cells. (F) Representative images of U87-MG cell apoptosis by AV/PI assay and (G) comparison of apoptosis rate among control, vehicle-treated, ADN-treated and ADN + LY294002-treated U87-MG cells. (H) Representative images of c-caspase 3, caspase 3, Bax and GAPDH in U87-MG cells by western blotting and comparison of (I) c-caspase 3/caspase 3 and (J) Bax/GAPDH among control, vehicle-treated, ADN-treated and ADN + LY294002-treated U87-MG cells. *P<0.05 vs. vehicle; #P<0.05 vs. ADN. ADN, adiponectin; c-caspase, cleaved caspase.

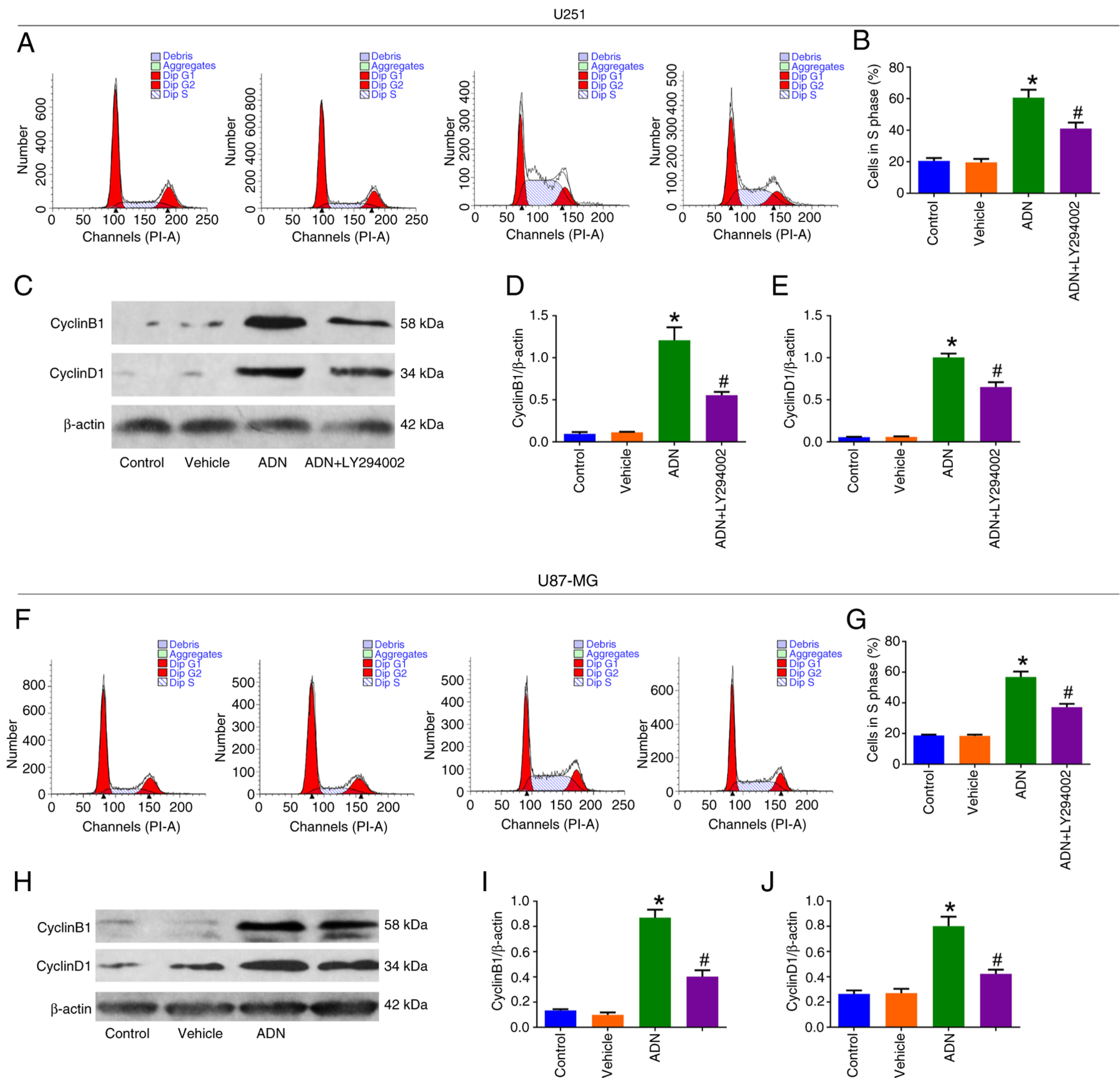

Figure 4. LY294002 attenuates ADN-induced U251 and U87-MG cells in the S phase. (A) Representative images of U251 cell cycle analysis by cell cycle assay and (B) comparison of the proportion of cells in the S phase among control, vehicle-treated, ADN-treated and ADN + LY294002-treated U251 cells. (C) Representative images of cyclinB1, cyclinD1 and β-actin in U251 cells by western blotting and comparison of (D) cyclinB1/β-actin and (E) cyclinD1/β-actin among control, vehicle-treated, ADN-treated and ADN + LY294002-treated U251 cells. (F) Representative images of U87-MG cell cycle analysis by cell cycle assay and (G) comparison of the proportion of cells in the S phase among control, vehicle-treated, ADN-treated and ADN + LY294002-treated U87-MG cells. (H) Representative images of cyclinB1, cyclinD1 and β-actin in U87-MG cells by western blotting and comparison of (I) cyclinB1/β-actin and (J) cyclinD1/β-actin among control, vehicle-treated, ADN-treated and ADN + LY294002-treated U87-MG cells. *P<0.05 vs. vehicle; #P<0.05 vs. ADN. ADN, adiponectin.

cells (P<0.05). Similar trends were noted in U87-MG cells (Fig. 4F-J).

ADN facilitates TMZ resistance in U251 and U87-MG cells. Different concentrations of TMZ were applied to treat the glioblastoma cell lines. It was noted that treatment with 1.0 mM TMZ indicated an optimal effect on inhibiting glioblastoma cell line growth; therefore, this concentration was selected as the optimal treatment condition for the subsequent step. In addition, it was found that only 3.0 µg/ml ADN+1.0 mM TMZ increased glioblastoma cell line growth compared with 1.0 mM TMZ (both P<0.05; Fig. S2A and B). Therefore, 3.0 µg/ml ADN and 1.0 mM TMZ were selected as the conditions for subsequent experiments.

The AV/PI assay (Fig. 5A) suggested that the apoptotic rate was enhanced by TMZ compared with vehicle (P<0.05); however, it was reduced by ADN+TMZ compared with TMZ in U251 cells (P<0.05; Fig. 5B). Western blotting analysis (Fig. 5C) revealed that the ratio of c-caspase 3/caspase 3 (Fig. 5D) and the ratio of Bax/GAPDH (Fig. 5E)

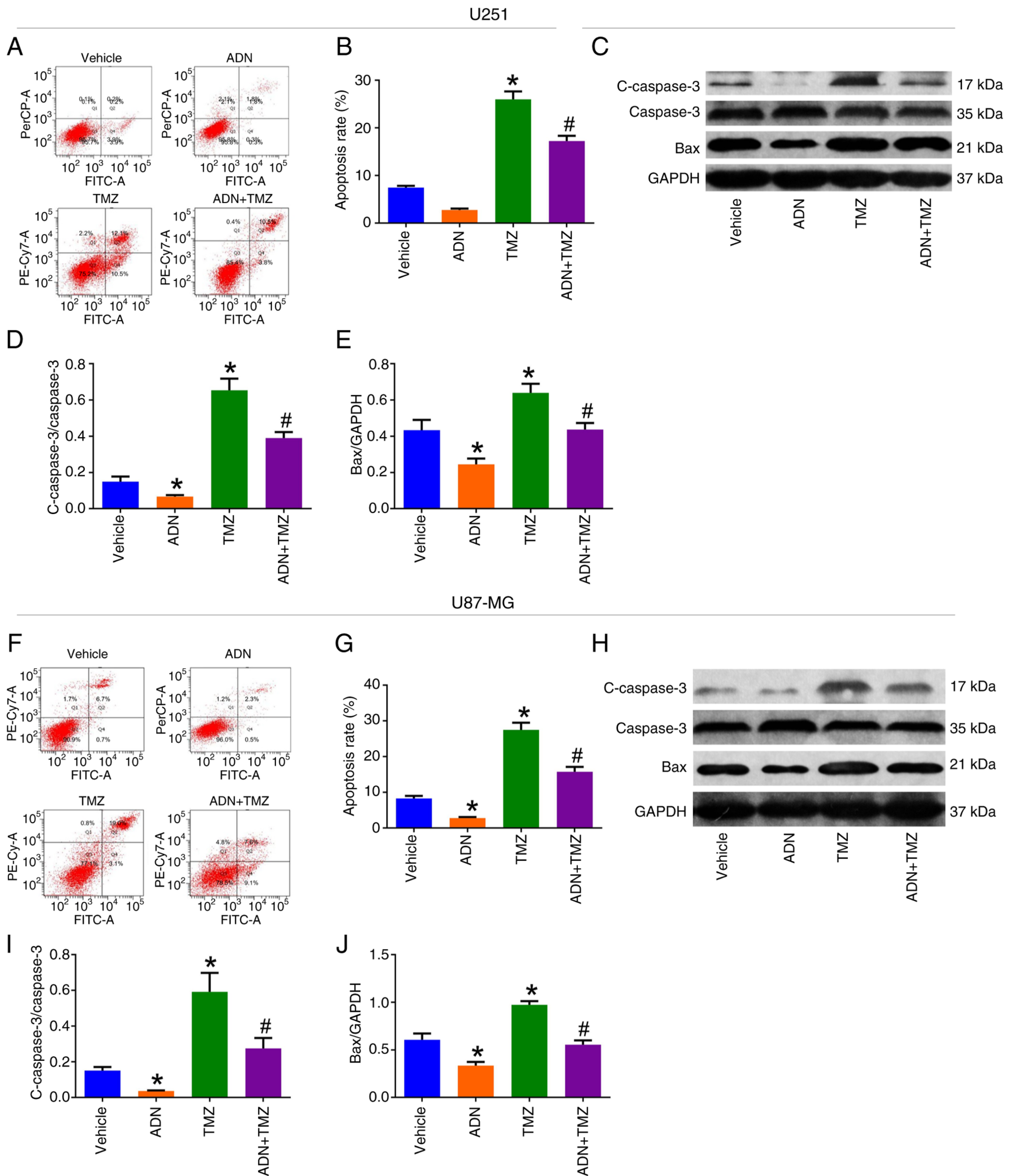

Figure 5. ADN attenuates the promotion of TMZ on U251 and U87-MG cell apoptosis. (A) Representative images of U251 cell apoptosis by AV/PI assay and (B) comparison of apoptosis rate among vehicle-treated, ADN-treated, TMZ-treated and ADN+TMZ-treated U251 cells. (C) Representative images of c-caspase 3, caspase 3, Bax and GAPDH in U251 cells by western blotting and comparison of (D) c-caspase 3/caspase 3 and (E) Bax/GAPDH among vehicle-treated, ADN-treated, TMZ-treated and ADN + TMZ-treated U251 cells. (F) Representative images of U87-MG cell apoptosis by Annexin V/PI flow cytometry and (G) comparison of apoptosis rate among vehicle-treated, ADN-treated, TMZ-treated and ADN + TMZ-treated U87-MG cells. (H) Representative images of c-caspase 3, caspase 3, Bax and GAPDH in U87-MG cells by western blotting and comparison of (I) c-caspase 3/caspase 3 and (J) Bax/GAPDH among vehicle-treated, ADN-treated, TMZ-treated and ADN+TMZ-treated U87-MG cells. *P<0.05 vs. vehicle; #P<0.05 vs. TMZ. ADN, adiponectin; TMZ, temozolomide; c-caspase, cleaved caspase.

were increased by TMZ compared with vehicle (all P<0.05); however, they were reduced by ADN+TMZ compared with TMZ in U251 cells (both P<0.05). The same trends were noted in U87-MG cells (Fig. 5F-J).

The cell cycle assay (Fig. 6A) suggested that the proportion of U251 cells in the G_2 phase was increased by TMZ compared with that of the vehicle group (P<0.05), whereas it was decreased in the ADN+TMZ group compared with that

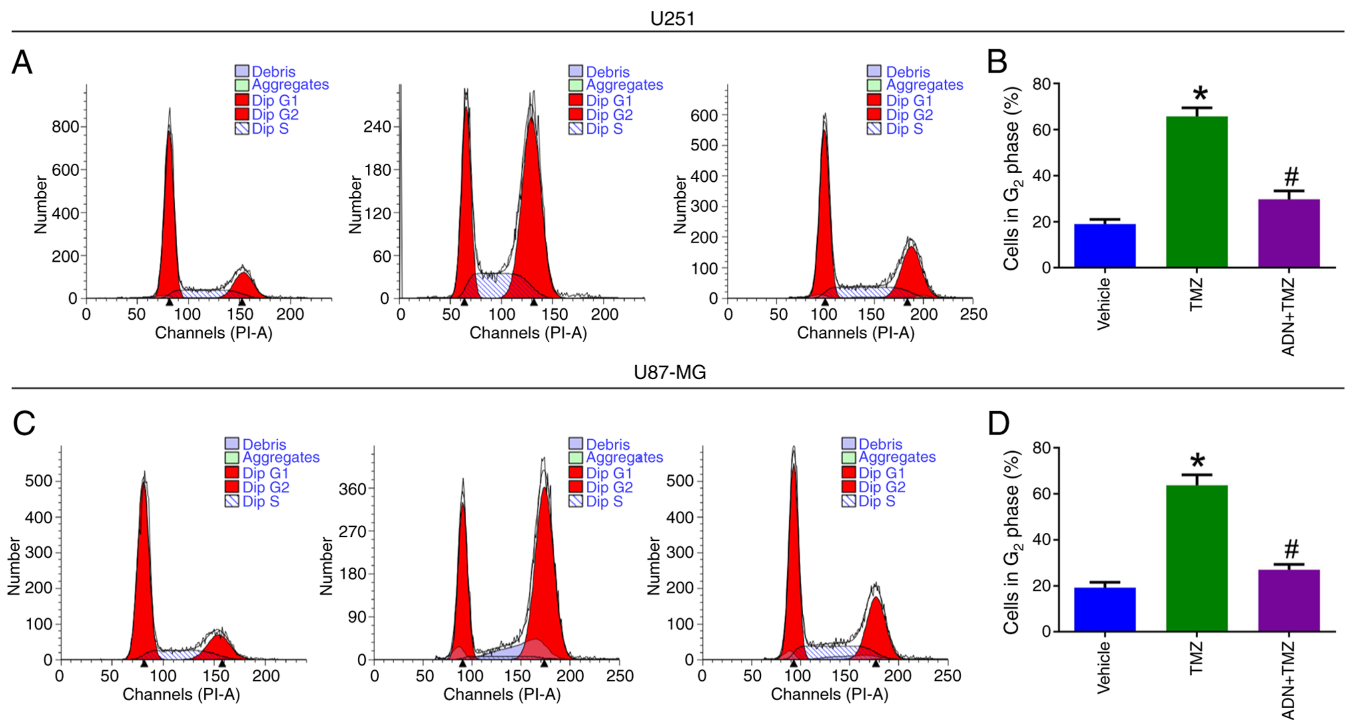

Figure 6. ADN attenuates TMZ-induced U251 and U87-MG cell cycle arrest in the G_2 phase. (A) Representative images of U251 cell cycle analysis by cell cycle assay and (B) comparison of the proportion of cells in the G_2 phase among vehicle-treated, ADN-treated, TMZ-treated and ADN +TMZ-treated U251 cells. (C) Representative images of U87-MG cell cycle analysis by cell cycle assay and (D) comparison of the proportion of cells in the G_2 phase among vehicle-treated, ADN-treated, TMZ-treated and ADN + TMZ-treated U87-MG cells. *P<0.05 vs. vehicle; #P<0.05 vs. TMZ. ADN, adiponectin; TMZ, temozolomide; c-caspase, cleaved caspase.

noted in the TMZ group (P<0.05; Fig. 6B). The same trends were noted in U87-MG cells (Fig. 6C and D).

ADN activates the Akt/mTOR pathway to facilitate TMZ resistance in U251 and U87-MG cells. LY294002 was added to further explore the underlying mechanism of ADN involved in TMZ resistance. It was found that the U251 cell growth rate was reduced by TMZ + ADN + LY294002 compared with TMZ+ADN (P<0.05; Fig. S2A). The same trends were discovered in U87-MG cells (Fig. S2B).

Discussion

The actions of ADN are mediated via classical receptors, which belong to the seven transmembrane domains receptor family, such as ADIPOR1 and ADIPOR2 (27-29). Notably, ADIPOR1 and ADIPOR2 are closely involved in the pathology and progression of glioblastoma (30,31) and several studies have reported that they are expressed in various tumor tissues, including glioma (22,32-34). According to a previous study, ADIPOR1 and ADIPOR2 are expressed in 83% (25/30) of human glioma tissues; in addition, 70% of glioma tissues indicated co-expression of both receptors, which were also expressed in glioblastoma cell lines (22). In line with this previous study (22), the present study also discovered that ADIPOR1 and ADIPOR2 were expressed in U251 and U87-MG cells, suggesting that these two glioblastoma cell lines possessed the basis for ADN to exert its functions. Subsequently, it was noted that ADN could facilitate glioblastoma cell line growth and cells in the S phase, while inhibiting apoptosis. A possible reason might be that ADN can bind to ADIPOR1 and ADIPOR2 to regulate several downstream pathways, such as the adenosine monophosphate-activated protein kinase (AMPK)/sirtuin 1, AMPK/mTOR and extracellular signal-regulated kinase (ERK)1/2 pathways to accelerate glioblastoma cell line proliferation, while inhibiting apoptosis (22,30,31).

The Akt/mTOR pathway, a key regulator of multiple cellular processes, is involved in the pathology and progression of various types of cancer (14,35-37). Regarding glioblastoma, previous studies have indicated that the Akt/mTOR pathway regulates cell proliferation, apoptosis, metastasis, autophagy and cell cycle arrest (38-40). Notably, a previous study discovered that Akt phosphorylation can be regulated by ADN, which is responsible for glioblastoma progression (22). The current study showed that ADN activated the Akt/mTOR pathway in glioblastoma cell lines, which was consistent with a previous study (22). It should be clarified that Ser473 and Thr308 are two phosphorylation sites of Akt (41). However, it was found that ADN could only lead to Akt phosphorylation at Thr308, while it could not induce its phosphorylation at Ser473 in glioblastoma cell lines. It is to notable that the addition of LY294002 reversed the effect of ADN on glioblastoma cell line apoptosis and cells in the S phase. The findings suggested that ADN might activate the Akt/mTOR pathway to facilitate glioblastoma progression.

TMZ is the first-line chemotherapy for patients with glioblastoma, whereas drug resistance to TMZ remains a challenging problem and is a cause of poor prognosis (42,43). U251 and U87-MG cell lines are widely used cell lines to establish TMZ-resistant glioblastoma cell models according to previous

studies (44-48). Guided by these previous studies (44-48), U251 and U87-MG cell lines we also applied to explore the effect of ADN on TMZ resistance in glioblastoma. It was found that ADN reversed the effect of TMZ on apoptosis, cell cycle arrest and growth in glioblastoma cell lines, which indicated that it contributed to TMZ resistance in glioblastoma. However, the underlying mechanisms responsible for ADN-induced TMZ resistance have not been reported by previous studies. The Akt/mTOR pathway was found to be aberrantly activated in primary glioblastoma samples and its activation could impair the efficacy of TMZ treatment (16,49-51). Therefore, it was further explored whether this pathway is involved in the regulation of ADN on TMZ resistance in glioblastoma. Notably, it was discovered that the addition of LY294002 reversed the effect of ADN on TMZ resistance in glioblastoma cell lines. Therefore, ADN might induce TMZ resistance by activating the Akt/mTOR pathway in glioblastoma.

The current study observed that ADN facilitated glioblastoma progression and induced TMZ resistance by activating the Akt/mTOR pathway. However, the underlying mechanisms of ADN's regulation of the Akt/mTOR pathway were unclear. According to a previous study, it was hypothesized that ADN might bind to its receptors (ADIPOR1 and ADIPOR2) to initiate the activation of PI3K, which further activated the Akt/mTOR pathway, thereby promoting glioblastoma progression and TMZ resistance (22). However, this hypothesis should be validated by further experiments.

It should be clarified that this study applied 0.1, 0.5, 1.0, 3.0 and 10.0 μg/ml as the concentration gradient of ADN to treat U251 and U87-MG cell lines. The setting of these concentrations of ADN was exploratory in nature. Initially, the present study tried to follow the experience of a relevant previous study, which used 0.0025, 0.025 and 0.25 μg/ml as the concentration gradient of ADN to treat U251 and U87-MG cell lines (22). However, after applying similar concentrations of ADN (0.1 and 0.5 μg/ml), it was found that U251 and U87-MG cell line growth was unaffected. Consequently, it was decided to use higher concentrations of ADN (1.0, 3.0 and 10.0 μg/ml) to treat these cell lines. It is hoped that the concentration gradient of ADN set in the present study can provide a reference for future researchers embarking on similar investigations.

Recently, several studies have revealed the potential mechanisms regarding glioblastoma progression and relapse, which are conducive to improving the management of glioblastoma patients (52-54). Nevertheless, the prognosis of glioblastoma patients is unsatisfactory and one of the contributors is TMZ resistance, which is developed in ~50% of glioblastoma patients (10). Therefore, investigating potential mechanisms contributing to TMZ resistance is crucial to improving the prognosis of patients with glioblastoma. The current study found that ADN was responsible for glioblastoma progression and TMZ resistance. The findings provided perspectives that ADN and its downstream Akt/mTOR pathway might be potential therapeutic targets to reverse TMZ resistance in patients with glioblastoma. Meanwhile, considering the involvement of ADN in glioblastoma progression and TMZ resistance, ADN may serve as a potential marker for patients with glioblastoma receiving TMZ. Therefore, the detection of ADN by enzyme-linked immunosorbent assay and reverse transcription PCR might assist in predicting the prognosis of patients with glioblastoma receiving TMZ. Overall, the findings might be conducive to enhancing the management of patients with glioblastoma. To achieve this goal, further *in vivo* experiments are required to validate the effect of ADN on glioblastoma progression and TMZ resistance. Further clinical studies are warranted to verify the potential of ADN to serve as a biomarker for patients with glioblastoma receiving TMZ.

Several limitations of this study should be noted. i) The underlying mechanism of the regulation of ADN on the Akt/mTOR pathway should be investigated by further experiments. ii) The glioblastoma cell lines used in this study included U251 and U87-MG cell lines, which could not fully represent the glioblastoma. Further studies could consider applying primary glioblastoma cells or other glioblastoma cell lines, such as A172, LN229 and LN18 cell lines, to validate the findings of this study. iii) Further *in vivo* experiments were required to confirm the engagement of ADN in glioblastoma progression and TMZ resistance.

In conclusion, the present study demonstrated that ADN activated the Akt/mTOR pathway to facilitate cell cycle, inhibited cell apoptosis and induced TMZ resistance in glioblastoma.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JY and JH contributed to the conception and design of the study. PS and FL were responsible for the acquisition of data. KH, JW and YC were responsible for analysis and interpretation of the data. SS contributed to the methodology of the overal study design and wrote the first draft of the manuscript. WM contributed to the interpretation of the data and manuscript revision.. JY and JH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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