AdipoR2 Inhibits Human Glioblastoma Cells Growth Through the AMPK/mTOR Pathway

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Research

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Abstract

Background: AdipoR2belongs to seven transmembrane domains receptor family, which has been shown to have played an important role in the development of human tumor, but the underlying mechanisms are poorly understood. In this study, we found that AdipoR2 expression correlates with glioma grade. In addition, we also investigated the mechanisms behind the anti-proliferative effects of AdipoR2 in U251 cells (human glioma cell line) using the colony formation assay and WST-8 growth assay.

Methods: U251 cell line were cultured in vitro; Western Blotting was used to detect the expression of related proteins; Using Quantitative RT-PCR to detect AdipoR1and AdipoR2 expression; Detection of Cell cycle assay by flow cytometry; The gene expression profiles of glioma samples from CCGA were analyzed by Matlab and GSEA software.

Results: We found 648 upregulated genes and 436 downregulated genes correlated with AdipoR2 expression in 158 glioma samples. GSEA analysis suggested that AdipoR2 is a cell cycle–associated gene. Results of flow cytometry analysis indicated that AdipoR2 induced cell cycle G0/G1 arrest in U251 cells. Furthermore, we identified the AMPK/mTOR signaling axis to be involved in AdipoR2-induced cell cycle arrest.

Conclusions: Our results suggest that AdipoR2 may represent a novel endogenous negative regulator of GBM cell proliferation. These findings also suggested that AdipoR2may be a promising therapeutic target in GBM patients.

Introduction

Glioblastoma (GBM) is highly malignant and lethal cancerof the central nervous system. Recurrence is systematic and prognosis poor. Regardless ofsurgery combined with radiation therapy and chemotherapy, patients suffering from malignant glioma have a life-spanbetween 12–15 months after diagnosis[1, 2].Therefore, there is an urgent need to figure outprecise molecular mechanism involved in the pathogenesis of GBMand explore novel therapeutic strategies to treat this devastating disease.

Adiponectin (Acrp30), a 30-kDa component C1q-relatedprotein, is implicated in cancer development. Most of the biological effects of Acrp30 aremediated by its receptors (AdipoR1 and AdipoR2)belonging toseven transmembrane domains receptor family, which have been shown to have abnormal expression in various types of humancancer[3–5].The receptors are believed to be functionally distinct with respect to downstream pathway activation, but definitesignalingpathways involved in a particular tumorremain unclear.

Adenosine monophosphate-activated protein kinase (AMPK) acts as an important‘metabolic sensor’ which is activated by increases in adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio and/or adenosine diphosphate (ADP)/ATP ratio[6]. Since it plays an important role in the regulation of energyhomeostasis, AMPK is responsible for cancer cell proliferation and apoptosis. So, targeting AMPK

caninduce apoptosis and to inhibit cell proliferation[7].Phosphorylated AMPKsuppresses mammalian target of rapamycin (mTOR) signalingpath way[8], which plays a central role in the regulationof cell proliferation, differentiation and migration[9, 10], and may promotes tumorigenesis[6, 11]. So we can control cancer progression by modulating the AMPK/mTORpathway[7, 12, 13].Sugiyama M[14]et.al reported that Adiponectin inhibits colorectal cancer cell growth through the AMPK/mTOR pathway.However, there is no information on the relationship between AdipoRsand AMPK/mTORin central nervous systemtumors such as GBM.

Considering the inhibitory role of AdipoRsin differenttumors, we hypothesized thatAdipoRswould likely influenceGBM growth through the AMPK/mTOR pathway. In this study, we found that expression of AdipoR2 correlates with glioma grade, so we future investigated the biological effect of over-expressionAdipoR2treatment in U251 humancell lines.

Materials And Methods

Human tissue samples

Messenger RNA (mRNA) expression data for 158glioma samples was downloaded from the Chinese Glioma GenomeAtlas (CGGA) data portal (http://www.cgga.org.cn/portal.phpg).In total, 158 glioma samples included 48astrocytomas (As), 13oligodendrogliomas (Os), 8 anaplastic astrocytomas (AAs), 10 anaplastic oligodendrogliomas (AOs), 15 anaplastic oli-goastrocytomas (AOAs) and 64 tumors of glioblastoma multiforme (GBMs).Tissue sampleswere obtained from the Department of Neurosurgery in Xuzhou Central Hospital from 2012 to 2015. Of thesetissuesamples, three were normalbrain tissues (NBTs) and twelve(3 grade II, 4 grade III and 5 grade IV) were glioma samples. NBT samples were obtained from three patients who suffered severe brain trauma. This study was approved by the Medical ReviewBoard of Xuzhou Central Hospital.

Cell Culture

U251 cell line was purchased from Chinese Academy of Sciences Cell Bank and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) (all from Invitrogen, Carlsbad, USA) at 37 ˚C under a humidified atmosphere of 5% CO_2.

Reagents and transfection

The recombinant plasmid for pcDNA3.1 vector, which contains ORF of human AdipoR2 was chemically synthesized and purified by Genechem (Shanghai, China). The blank vector pcDNA3.1 was used as a negative control (NC). All plasmids were transfected into cells using Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) according to the manufacturer's instructions.Selective AMPK inhibitorcompound C (iAMPK) was purchased fromCalbiochem (La Jolla, CA).

Quantitative RT-PCR

RNA was extracted from tissues using TRIzol(Invitrogen). AdipoR1 and AdipoR2 (qRT-PCR) reactions were performedusing Fermentas reverse transcription reagents andSYBR Green PCR Master Mix (Applied Biosystems)according to manufacturer's protocols. GAPDH wasused for normalisation. Relativegene expression was calculated via $2^{-\triangle Ct}$ method.

WST-8 growth assay

U251 cells were seeded in 96-well culture plates at 2000 cells/well/100 μL. Cells were treated with AdipoR2 for 1–4 days. Then, tetrazolium monosodium salt WST-8 (Dojindo, Japan) was added (10 μL/well). After incubation for 2 h, the absorbance was determined using a microplate reader (Bio-Rad, USA) at 450 nm wavelength with the reference wavelength set at 630 nm.

Colony formation assay

U251 cells were seeded in six-well plates and cultured overnight. And thensubsequently, AdipoR2 or NCwas transfected into cells. After 48 h, 5×10^2 treated and untreated cells were independently plated onto 60 mm tissue culture plates. After incubation for 2 weeks, visible colonies were fixed with 4% methanol for 30 min and the stained with 0.1% crystal violet for 20 min. Colony-forming efficiency was calculated as the number of colonies/plated cells × 100%.

Cell cycle assay

After 48 h post-transfection, U251 cells were collected and fixed with 70% ethanol at -20˚C overnight. DNA was stained by incubating cells in 50 mg/mL propidium iodide (PI) (Sigma-Aldrich, USA) and 10 mg/mL RNase A(Boehringer-Mannheim, Germany) for 1 h at room temperature. The cells were then analyzed by FACScan(Becton- Dickinson, USA).

Western Blotting

Proteins were extracted inlysis bufferaccording to the manufacturer's protocol. Lysates were separated by SDS-PAGEand transferred to anitrocellulose membrane (Bio-Rad, USA).The membranes were incubated in blocking buffer.Themembranes were incubated with the AdipoR2 (Abcam, USA), AMPK, phosphorylated (Thr172) AMPK (p-AMPK), mTOR, phosphorylated (Ser2448) mTOR (p-mTOR),70-kDa ribosomal protein S6 kinase(S6K), phosphorylated(Thr421/Ser424) p70S6 kinase (pS6K), and S6 ribosomal protein (S6P), phosphorylated(Ser240/244) S6 ribosomal protein (pS6P)and GAPDH (CST, USA) primary antibodyat 4 °Covernight, respectively. Immunoreactivity was visualizedwith horseradish peroxidase-conjugated goat anti-rabbit antibody (Bioworld, USA). Protein bands were detected and imaged with ChemiDocXRS$^+$ gel imaging system (Bio-Rad, USA)and analyzed bydensitometric quantification using Image J software.

Gene set enrichment analysis with AdipoR2 expression

The gene expression profiles of glioma samples from CCGA were analyzed by GSEA[15]. Pearson's correlation was used to analyze the relationship between AdipoR2 and all identified genes with Matlabsoftware ($P < 0.001$). GSEA (http://www.broadinstitute.org/gsea/) analysis was used to identify pathway gene sets that are correlated with the AdipoR2 expression profile. For GSEA, AdipoR2 expression

was treated as a binary variable divided into low or high AdipoR2 expression and the cut-off point is 50%. As a metric for ranking genes in the GSEA, the difference between the means of samples with low and high AdipoR2 expressionwas used, and the other parameters were set by their default values.

Statistical analysis

Kaplan-Meier survival analysis was used to estimatethe survival distributions. The log-rank test was usedto assess the statistical significance between stratifiedsurvival groups with use of GraphPad Prism. The t test was used to determine differences in each 2-group comparison.All statistical analyseswere performed using Matlab 2012, SPSS software for Windows, or GraphPad Prism (GraphPad Software). All data are presented asmean ± standard error. A 2-sided P value of < 0.05 was regarded as significant.

Results

AdipoR2 expression correlates with glioma grade

First, we analyzed AdipoR2expression level in whole genome gene profiling of 158 glioma tissues. AdipoR2 expression significantly decreased in gradeIII-IVcompared with grade II(Fig. 1A), but no significant relationshipwas observed between AdipoR1 expression and glioma grade (The data not shown). As shown in Fig. 1B, we also investigated the prognostic value of AdipoR2 in 158 glioma samples by Kaplan–Meier survival analysis. The results indicated that patients with high AdipoR2 expression had longer mean overall survival (OS) compared with patients with low AdipoR2 expression.In addition, we also analyzed 15 samples of varying grades of glioma and normal brain tissues, and the results were consistent with the CGGA database (Fig. 1C). These findings suggest that AdipoR2 may play an important role in glioma development.

AdipoR2 inhibits U251 cell proliferation

Next, to explore the effects of AdipoR2 on U251 cell proliferation, U251 cells were treated with AdipoR2 for 1, 2, 3, and 4 days. Proliferation of these AdipoR2-treated cancer cells was then assessed using WST-8 assays. As shown in Fig. 2B, AdipoR2 significantly inhibited cell proliferation in a time-dependent manner. In addition, we also used a colony formation assay to determine the role of AdipoR2 in glioma cell proliferation. As shown in Fig. 2C, colony formation assay showed that the number of colonies in the experimental groups was obviously less than that in the NC group.

AdipoR2is a cell cycle–associated gene

To identify the mechanism of AdipoR2 involvementin glioma, we first screened differentially expressedgenes and found 648 upregulated genes and 436 downregulated genescorrelated with AdipoR2 expressionin 158 glioma samples.These genes will be called“AdipoR2-associated genes”(Stable1). GSEA was used to analysisthe pathways that were differentially expressed betweenpatients with high levels of AdipoR2 expression andthose with low levels of AdipoR2 expression. GSEA analysis revealed that AdipoR2 regulates genes primarily associated with cell cycle progression (Fig. 3A).

GSEA results indicated that AdipoR2is involved in cell cycle. To provide evidence to support this result, we evaluatedcell cycle distribution using flow cytometry. As shown in Fig. 3B, cell cycle assay indicated that U251cell lines transfected with AdipoR2 were notably arrested in the G0/G1 phase compared with NC group.

G0/G1arrest and anti-proliferative effects by AdipoR2 occurs in U251 cells via AMPK/mTOR pathway

The effect of AdipoR2 on the AMPK/mTOR signaling pathway was examined. Western blot analysesdepicted in Fig. 4A revealed that the expression level of S6K, pS6P, AMPK and mTORwas not significantly affected by AdipoR2 overexpression, where the phosphorylation of S6K, pS6P, AMPK and mTOR was obviously affected (Fig. 4A).

Following this, we assessed whether overexpression AdipoR2 induces cellcycle arrest and inhibits cell growth via the AMPK/mTOR pathway.Firstly, selective AMPK inhibitor compound C was used. U251 cells were treated withiAMPKand subsequently AdipoR2 plasmid was transfected tothese cells.The effects of overexpression AdipoR2 on cellcycle arrest of U251 cells were partially rescued by iAMPK(Fig. 4B).In addition, iAMPK treatmentsignificantly rescued AdipoR2-induced inhibition of cell growth in a time-dependent manner(Fig. 4C).These results indicatedthat AdipoR2-induced cellgrowth inhibition is mediated by activation of AMPK.

Discussion

GBMis the most common and deadly primary brain tumor-related rapid cell growth and resistance against apoptosis in adults[16]. Accumulatingevidence suggests that the expression ofAdipoRs(AdipoR1 and AdipoR2)was previously observed in humancancer tissues[17]. Porcile C et.al has demonstrated that AdipoR1 and AdipoR2 are often co-expressed in GBM samples, but the study did not compare the difference between AdipoRs(AdipoR1 and AdipoR2) and glioma grade[18]. In my study, we identified a significant negative association between AdipoR2 expression and glioma grade, but no statistically significant associations were observed between AdipoR1 expression and glioma grade.In addition, AdipoR2 were downregulated in human gastric cancer, endometrial adenocarcinoma[4, 19]. Knockdown of AdipoR2 relieved the suppressive effects of adiponectin on the growth of colon cancer cells[5].These studies had demonstrated that AdipoR2 functions as a novel regulatorof cell proliferation in varioushuman cancers.Consistent with these results, we also found that AdipoR2inhibited glioma cell proliferationand induced G0/G1 arrest in U251 cells.

AMPK is a ubiquitous serine/threonine protein kinase which has been found in the regulation ofcellular energy metabolism[6]. In addition, AMPK has also played a significant role of cell proliferation.mTOR-dependent activation of AMPKsignaling pathway can control cell growth in all eukaryotes and is deregulated in most human cancers[20].One mechanism by which AMPK controlsthe mTOR is by direct phosphorylation of its substrates.The 70-kDa ribosomal protein S6 kinase(S6K) and S6 ribosomal protein

(S6P) are included in thesignaling cascade downstream of mTOR. They are all activatedvia phosphorylation by mTOR[21].Mounting evidencesshow that dysregulation of AMPK/mTOR signaling pathway is associated with a variety of cancers[12, 14]. In our study, we found that AdipoR2inhibited glioma cell proliferation. However, the mechanisms throughwhich AdipoR2 affects cancer cells are not completelyelucidated. AdipoR2, whoseactivation results in the modulation of different protein kinasesincluding AMPK[22],are involved in Acrp30-modulate several metabolic processes, such asglucose and fatty acids metabolism[23]. Thus there is a possibility that AdipoR2 inhibits human glioblastoma cells growth through the AMPK/mTOR pathway. In line with our hypothesis, we found that AdipoR2 coulddownregulate the expression of p-AMPK. We also observedthe significant phosphorylation of mTOR, pS6K, and pS6P by the treatment with AdipoR2. In addition, Selective AMPK inhibitor compound C (iAMPK) significantly blocked the effect of AdipoR2-induced the cell cycle arrest of U251 cells. Taken together, these results led us to conclude that the AMPK/mTOR pathway is highly important for AdipoR2-induced anticancer effects.

Conclusions

we investigate the effect of AdipoR2overexpression on theglioma cell proliferation. This inhibiting effect is mediatedby AMPK-activated mTOR pathway. To our knowledge, this is also the first report to provide arationale for the implication of cross-linking between AdipoR2 and AMPK-mTOR signaling pathway in GBM.

Declarations

Acknowledgements

None.

Authors' contributions

Chen Jie, Wang Xuan and Han-DongFeng were in charge of the experiment.Ding-MaoHua, WangBo and Sun Fei analyzed the data and wrote the manuscript.ZhangHao and Jiang-DeHua designed the experiment. All authors read and approved the final manuscript.

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Availability of data and materials

All of the data and materials are available.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have given their consent for the manuscript to be published.

Competing interests

The authors report no conflicts of interest in this work.

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Figures

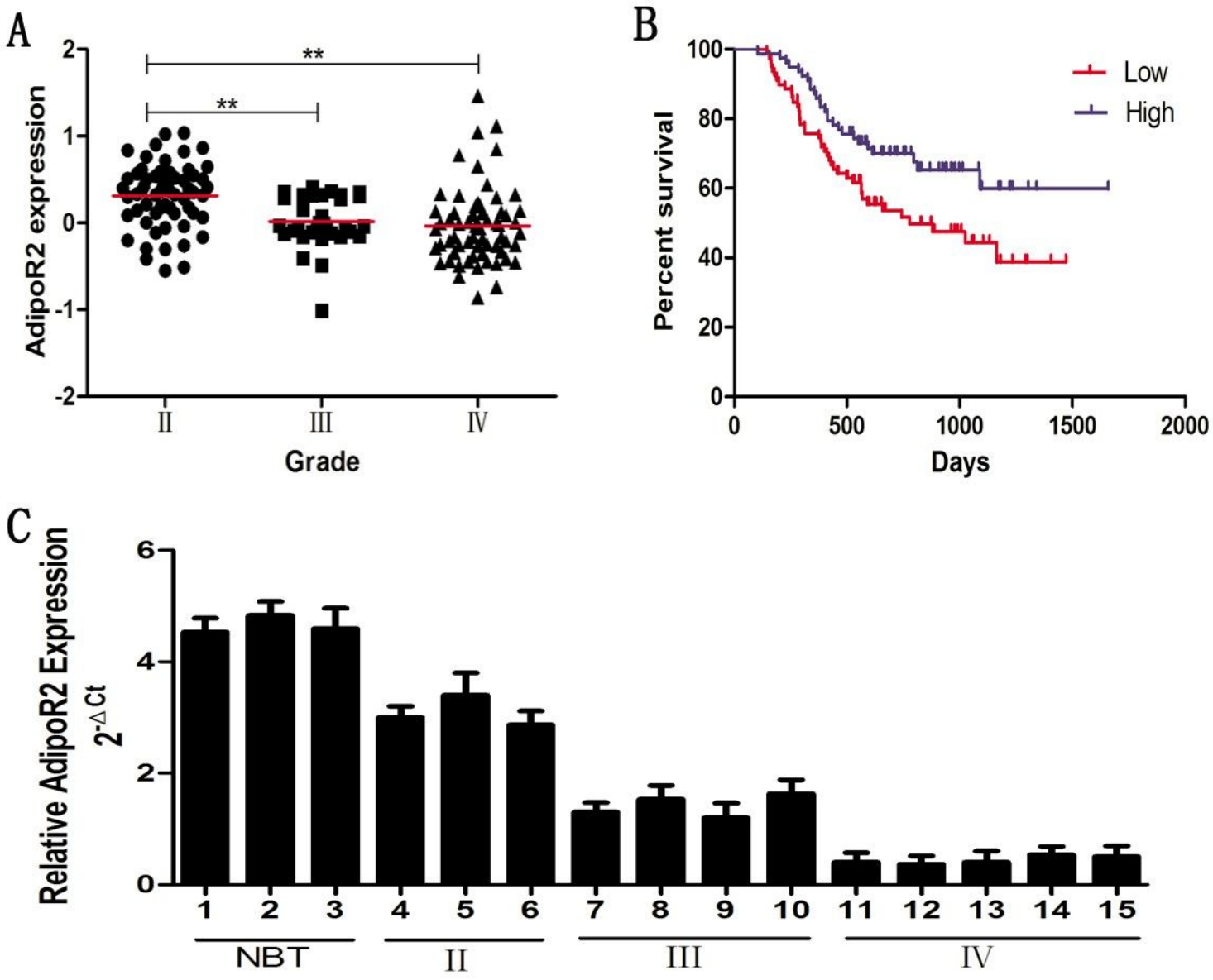
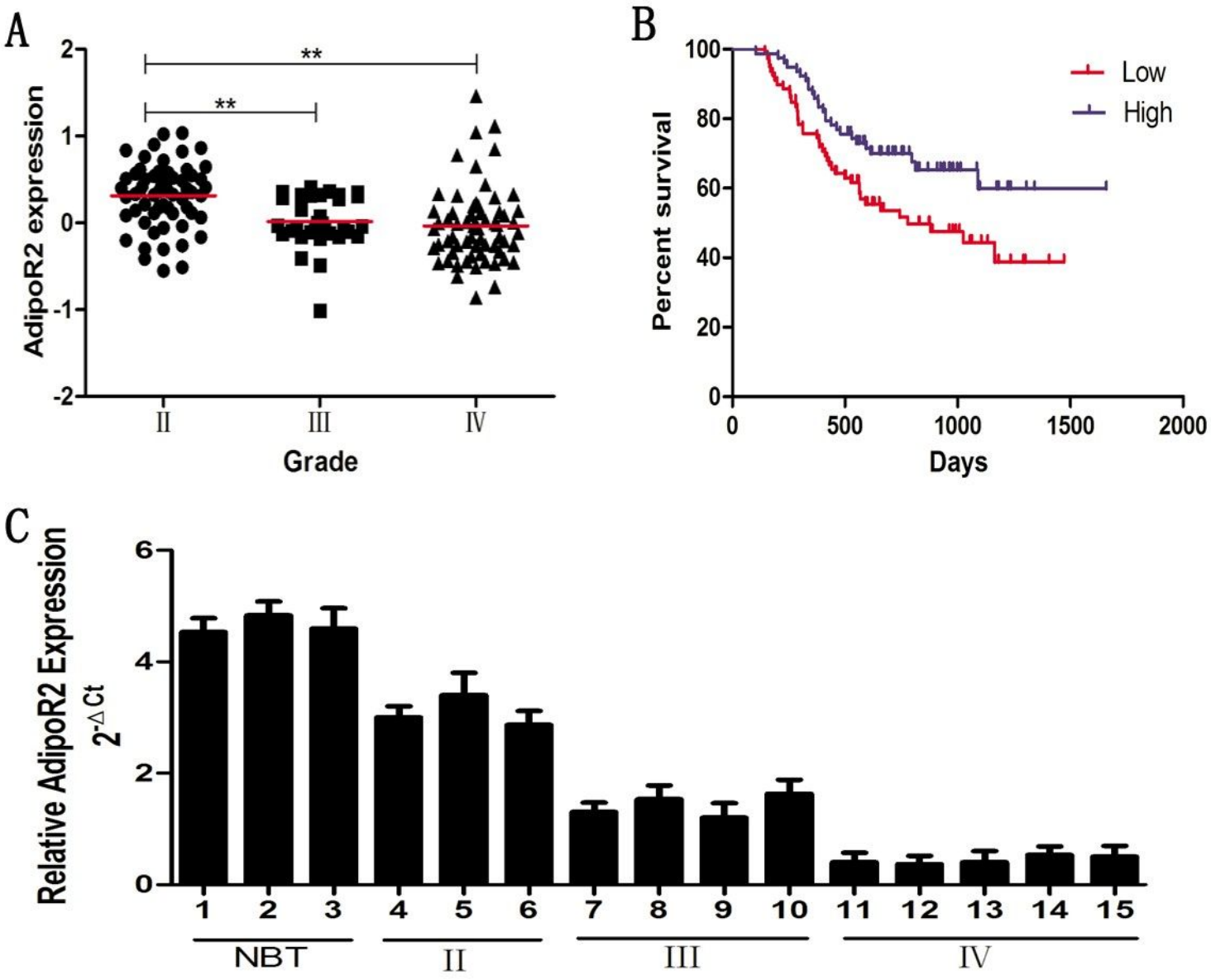


Figure 1

Clinical significance of AdipoR2 in glioma cases. (A) The levels of AdipoR2 were analyzed in glioma tissues of the CGGA glioma datasets. (B) Kaplan– Meier survival curves for AdipoR2 expression in glioma tissues of the CGGA glioma datasets. (C) qRT-PCR analysis shows decreased AdipoR2 levels in high grade glioma tissues compared with low grade glioma tissues and normal brain tissues. **P < 0.01, *P < 0.05.



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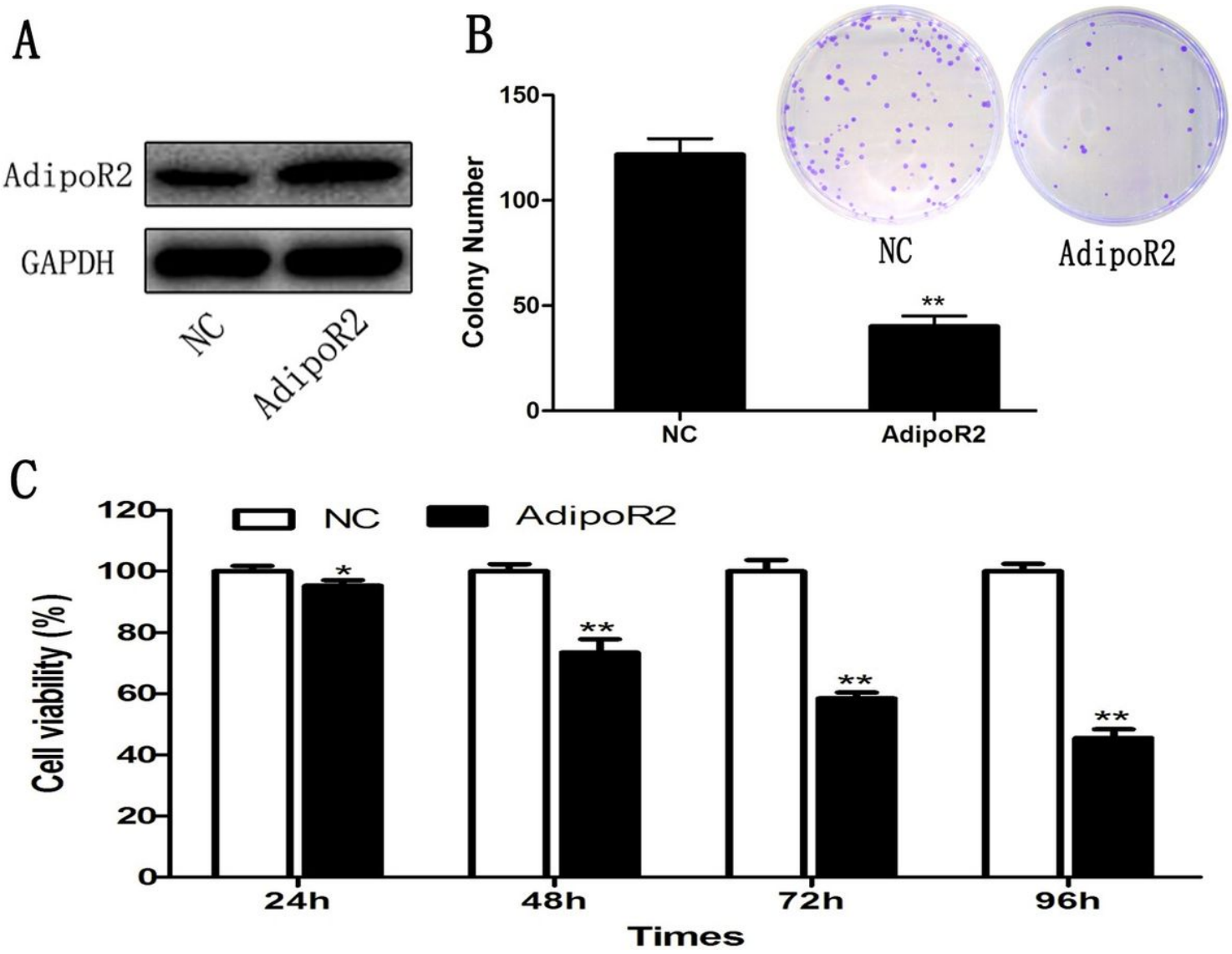
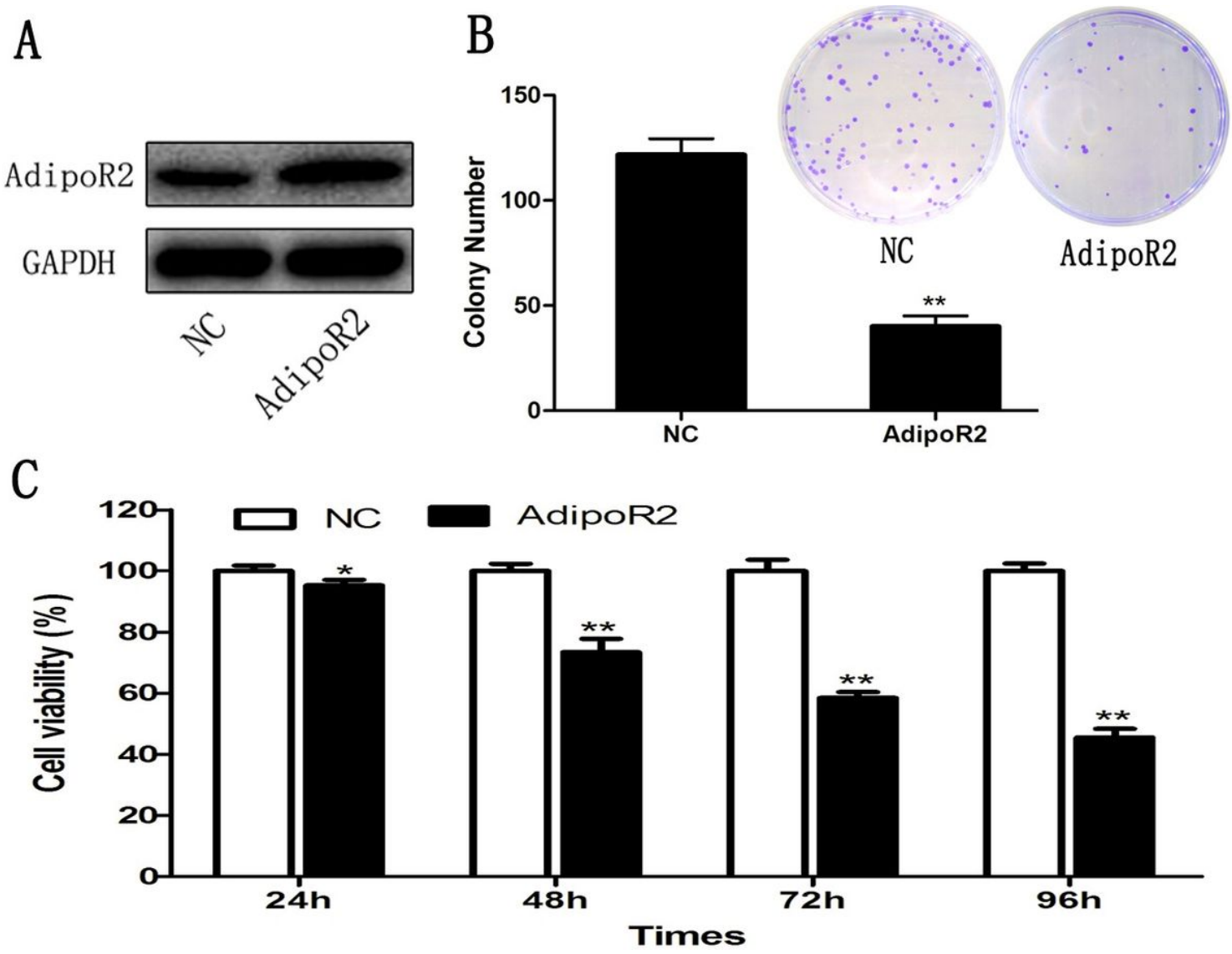


Figure 2

AdipoR2 is a cell cycle–associated gene. (A) GSEA analysis of gene ontology terms showed that genes correlated with AdipoR2 were involved in cell cycle progression in glioma patients. (B) After treatment with AdipoR2, U251 cells were used for cell cycle analyses with propidium iodide staining and flow cytometry analysis. **P < 0.01, *P < 0.05.



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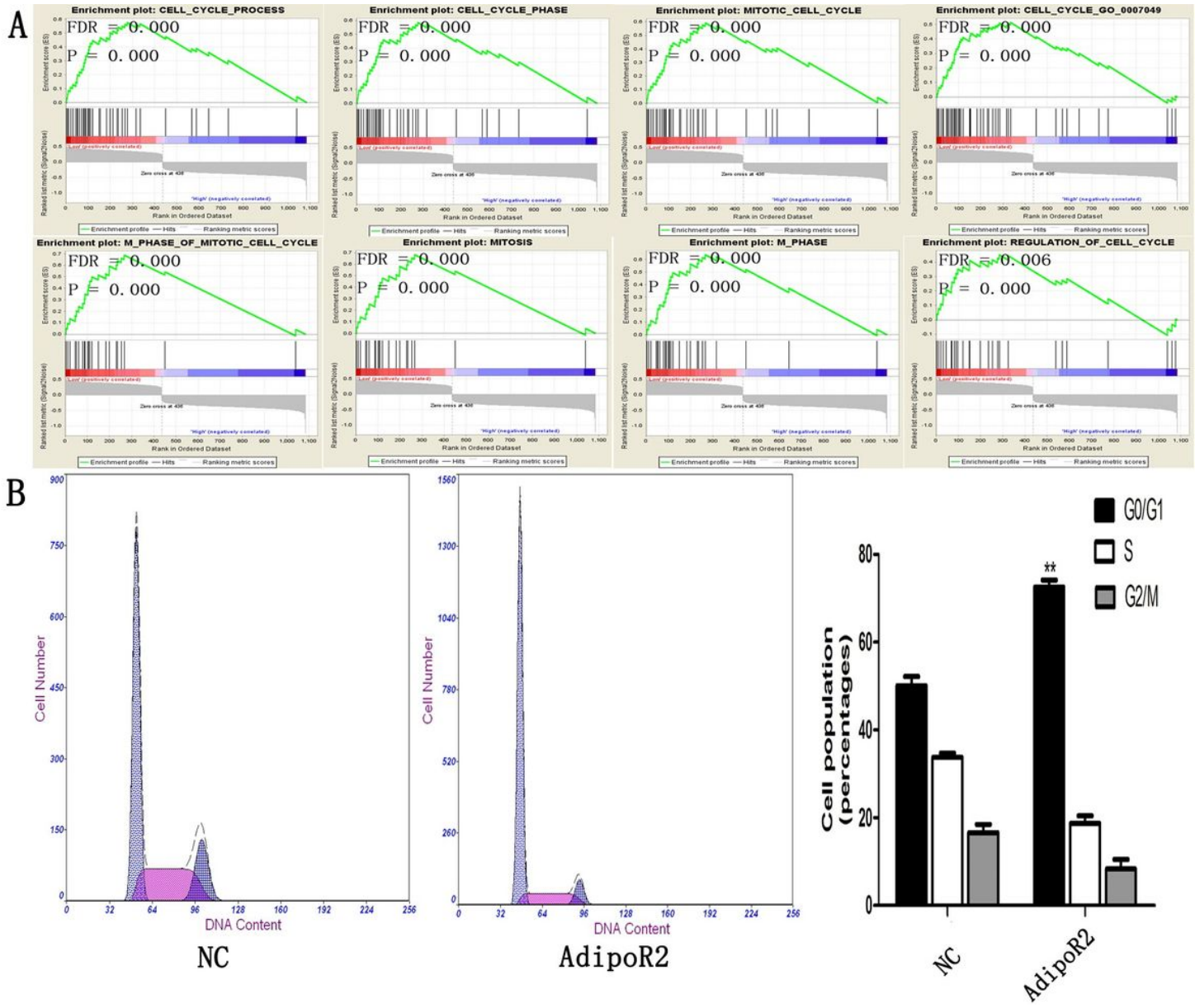
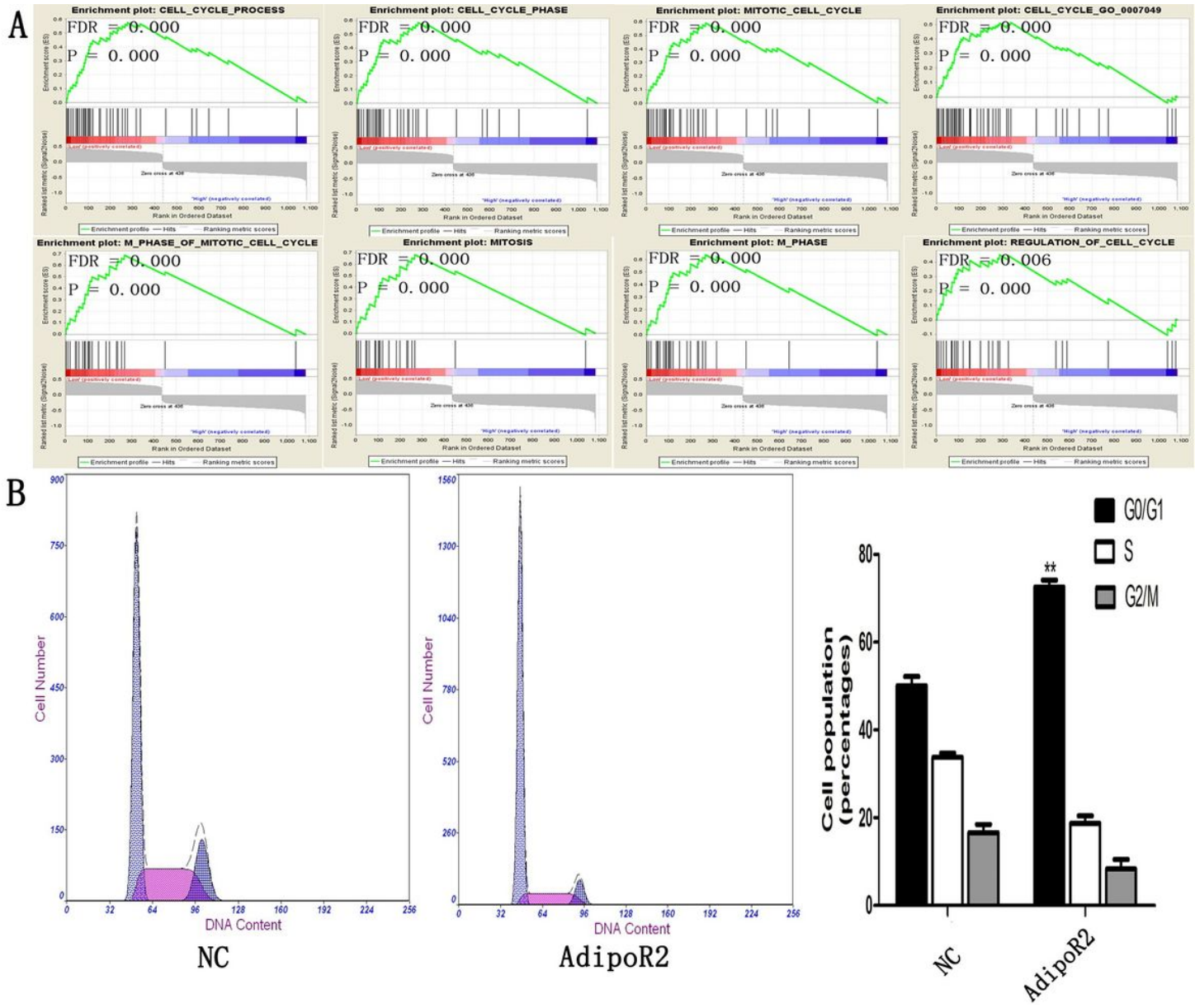


Figure 3

AdipoR2 inhibited U251 cells proliferation. (A) Western blot results for AdipoR2 protein levels in U251 cells treated with AdipoR2. (B) WST-8 assays were conducted on U251 cells after treatment with AdipoR2. (C) Colony formation assay was performed on U251 cells after treatment with AdipoR2. **P < 0.01, *P < 0.05.



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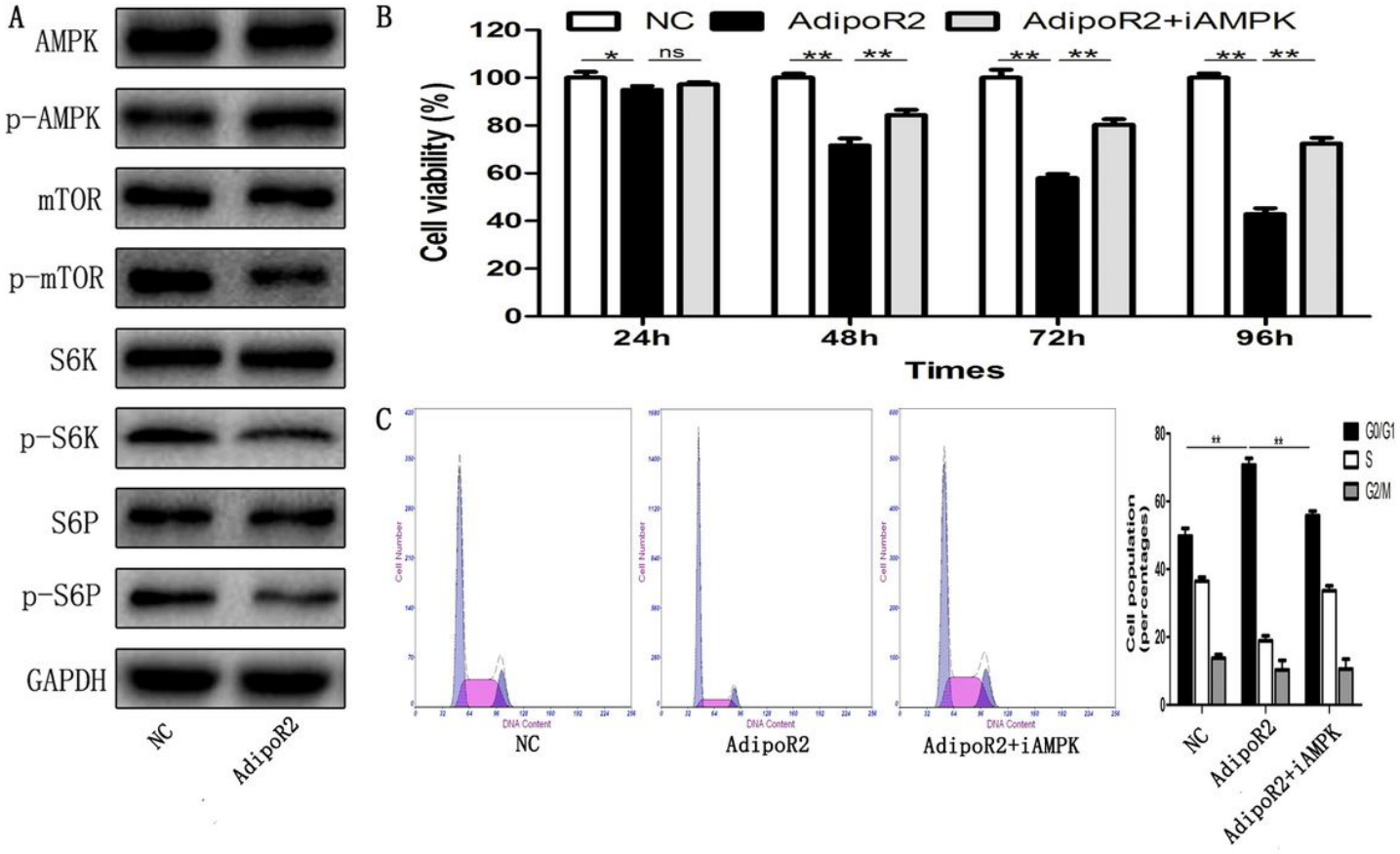
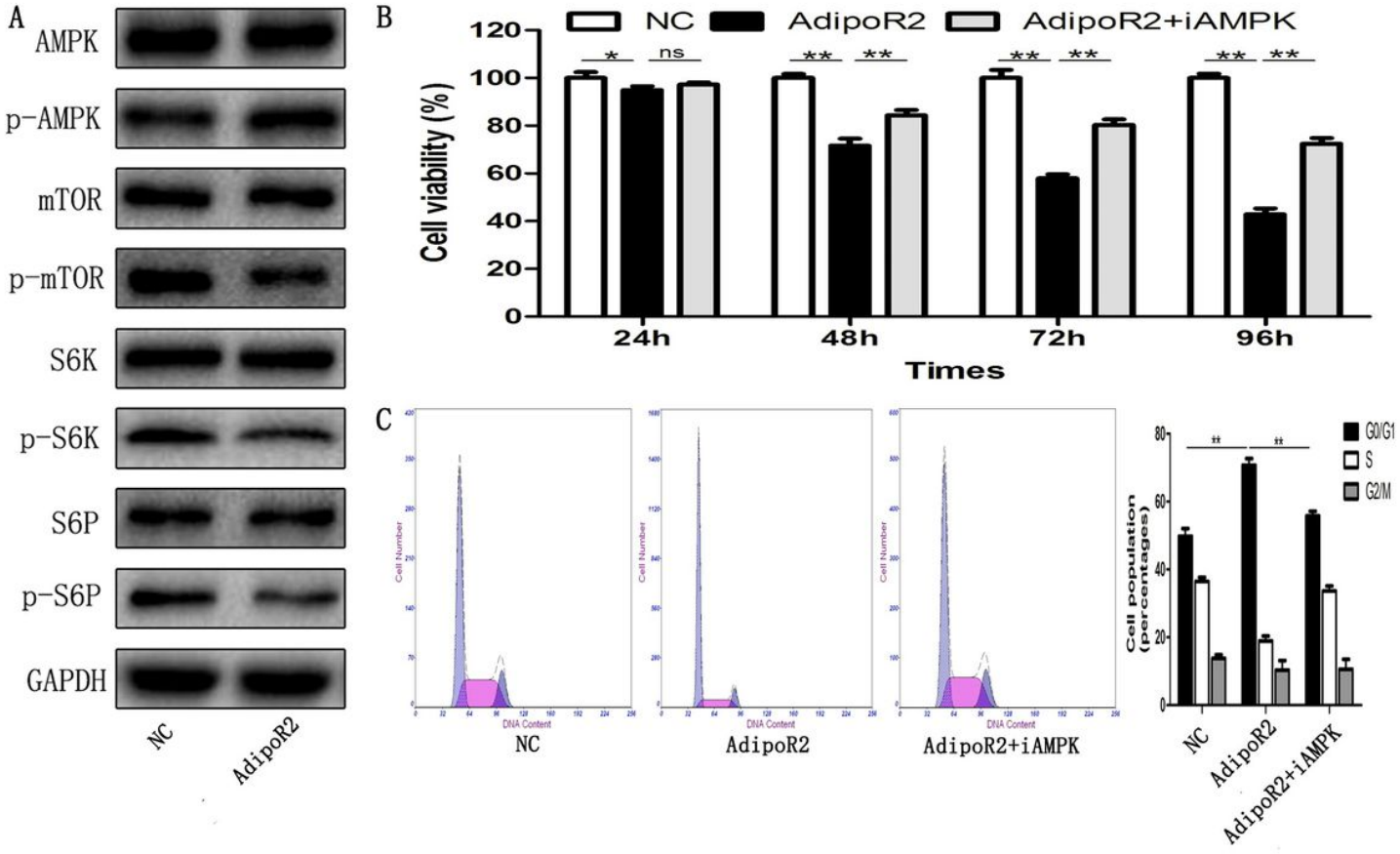


Figure 4

Evaluation of involvement of the AMPK-mTOR signaling pathway in AdipoR2-induced G0/G1 arrest (A) Western blot analysis for AMPK, p-AMPK, mTOR, p-mTOR, S6K, pS6K, S6P, pS6P protein levels in U251 cells after treatment with AdipoR2. (B, C) Rescue experiment performed by introducing iAMPK into U251 cells in the presence or absence of AdipoR2. **P < 0.01, *P < 0.05.



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