Am J Cancer Res 2021;11(4):1185-1205
www.ajcr.us /ISSN:2156-6976/ajcr0120746

Original Article

AKIP1 promotes glioblastoma viability, mobility and chemoradiation resistance via regulating CXCL1 and CXCL8 mediated NF-κB and AKT pathways

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Received August 20, 2020; Accepted January 19, 2021; Epub April 15, 2021; Published April 30, 2021

Abstract: This study aimed to investigate the interaction of A-kinase-interacting protein 1 (AKIP1) with C-X-C motif chemokine ligand (CXCL)1, CXCL2, CXCL8, and their effects on regulating glioblastoma multiforme (GBM) malignant behaviors. AKIP1 expression was modified by pcDNA and pGPH1 vectors in U-87 MG and U-251 MG cells. Subsequently, multiple compensative experiments were conducted via adding CXCL1, CXCL2 and CXCL8 in the pGPH1-AKIP1 (AKIP1 knockdown) transfected U-87 MG and U-251 MG cells, respectively. Furthermore, AKIP1, CXCL1/2/8 expressions in 10 GBM and 10 low-grade glioma (LGG) tumor samples were detected. AKIP1 was elevated in various GBM cell lines compared to normal human astrocytes. AKIP1 overexpression promoted U-87 MG and U-251 MG cell proliferation and invasion while inhibited apoptosis; and it enhanced chemoresistance to temozolomide (but not cisplatin) and radiation resistance; then AKIP1 knockdown showed the opposite effects. Meanwhile, AKIP1 positively regulated CXCL1/2/8, NF-κB pathway, AKT pathway and PD-L1 expression. Further multiple compensative experiments uncovered that CXCL1 and CXCL8 promoted proliferation, invasion, chemoradiation resistance, NF-κB pathway, AKT pathway and PD-L1 expression in U-87 MG and U-251 MG cells, also in pGPH1-AKIP1 (AKIP1 knockdown) transfected U-87 MG and U-251 MG cells; although CXCL2 exhibited similar treads, but its effect was much weaker. Besides, NF-κB pathway inhibitor and AKT pathway inhibitor attenuated the effect of CXCL1&CXCL8 on promoting GBM cell malignant behaviors. Clinically AKIP1 and CXCL1/8 were elevated in GBM compared to LGG tumor samples, and they were inter-correlated. AKIP1 promotes GBM viability, mobility and chemoradiation resistance via regulating CXCL1 and CXCL8 mediated NF-κB and AKT pathways.

Keywords: A-kinase-interacting protein 1, C-X-C motif chemokine ligand, glioblastoma multiforme, chemoradiation resistance, NF-κB and AKT pathways

Introduction

Glioblastoma multiforme (GBM) is the most challenging malignant primary brain tumor to treat, accounting for approximately 57% of all gliomas and 48% of all primary malignant central nervous system (CNS) tumors [1]. Based on the latest registry data report, GBM occurs in 4.23 cases per 100,000 populations every year, ranging from 2.00 per 100,000 populations in Asian/Pacific race to 4.71 per 100,000 populations in non-Hispanic white race [2]. The common treatment strategy for GBM is surgical resection followed by chemoradiotherapy; meanwhile, with the advancements of biological and medical technology, targeted therapy and immune therapy have been introduced recently [3, 4]. Despite of these achievements, the prognosis of GBM is still not obviously improved with 1-year survival rate of 41.4% and 5-year survival rate of 5.4% [2]. The heterogeneity, rapid progress and treatment resistance are key factors affecting the GBM prognosis, bring to the front that the importance of the deep understanding regarding GBM progression and treatment resistance is essential.

A-kinase-interacting protein 1 (AKIP1), initially observed in breast cancer and named as breast cancer-associated protein 3 (BCA3), is previously observed to be a key factor regulating NF-κB via multiple ways such as: binding to p65 and the cyclin D1 promoter in a neddylation-dependent way, as well as interaction with cAMP-dependent protein kinase (PKA) signaling pathway [5-7]. Subsequently, AKIP1 is further reported to be a tumor promoter and treatment resistance factor in cancers via multiple ways such as regulating AKT/GSK-3β/Snail pathway, VEGF pathway, Wnt/β-catenin/CBP pathway [8-11]. Importantly, AKIP1 is recently discovered to regulate the excretion of CXCL families such as C-X-C motif chemokine ligand (CXCL)1, CXCL2, CXCL8 to further promote cancer angiogenesis and growth; meanwhile, their interaction correlates with worse disease features and unsatisfied prognosis in both solid tumors and hematological malignancies [12-14]. Besides, NF-κB pathway which is directly modified by AKIP1, plays a critical role in PD-1/PD-L1 interaction through several ways such as its involvement in TCR-CD28 signaling [15-17]. Interestingly, CXCL members that are regulated by AKIP1, also closely interacts with NF-κB pathway, AKT pathway and are implicated in the regulation of immune checkpoint expressions [18-28]. Considering the above-mentioned potential interaction among AKIP1, CXCL1/2/8, NF-κB pathway, AKT pathway and PD-1/PD-L1 linkage, as well as their role in carcinogenesis, we hypothesized AKIP1, CXCL1, CXCL2 and CXCL8 might be involved in the GBM progression and treatment resistance. However, no related research has clarified this issue.

Therefore, this study aimed to investigate the effect of AKIP1, CXCL1, CXCL2 and CXCL8 on regulating GBM cell viability, mobility and chemoradiotherapy sensitivity, and their modifications on NF-κB pathway, AKT pathway and PD-L1 expression.

Methods

Cell culture

Human GBM cell lines U-251 MG (ECACC, UK), T98G (ATCC, USA) and U-87 MG (ATCC, USA) were cultured in Eagle's Minimum Essential Medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA). Human GBM cell line LN-229 (ATCC, USA) was cultured in Dulbecco's Modified Eagle Medium (Gibco, USA) containing 10% FBS (Gibco, USA). Normal human astrocytes (NHA) (Lonza, Switzerland) were cultured in AGM™ Astrocyte Growth Medium Bullet Kit™ (Lonza, Switzerland). All cell lines were contained in a 37°C, 5% CO_2 incubator. The expression of AKIP1 in cells (NHA as control) was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot.

Transfection detection

For overexpression, AKIP1 and negative control (NC) DNA fragment were cloned into pcDNA3.1 vector (Genepharma, China) to construct the pcDNA-AKIP1 plasmid and pcDNA-NC plasmid. For knock-down, Short hairpin RNA (ShRNA) targeting AKIP1 and NC shRNA were designed and cloned into pGPH1 vector (Genepharma, China) to construct the pGPH1-AKIP1 plasmid and pGPH1-NC plasmid. The pcDNA-AKIP1, pcDNA-NC, pGPH1-AKIP1 and pGPH1-NC plasmids were transfected into U-87 MG and U-251 MG cells with the application of Lipofectamine 3000 (Invitrogen, USA), respectively. After transfection, the cells were divided into pcDNA-AKIP1, pcDNA-NC, pGPH1-AKIP1 and pGPH1-NC groups accordingly.

Cell proliferation, apoptosis and invasion detection

At 24 hour (h) after transfection, the expression of AKIP1 in pcDNA-AKIP1, pcDNA-NC, pGPH1-AKIP1 and pGPH1-NC group cells was evaluated by RT-qPCR and western blot. At 0 h, 24 h, 48 h and 72 h post transfection, cell counting kit-8 (Dojindo, Japan) was applied to detect cell proliferation following the kit's instruction. At 48 h after transfection, Annexin V Apoptosis Detection Kit FITC (Invitrogen, USA) was used to assess cell apoptosis following the manufacturer's instruction. After transfection, Matrigel Basement Membrane Matrix (BD, USA) coated transwell chamber was used to detect cell invasion according to the methods described in the previous study [29].

Chemosensitivity detection

After transfection, cells in pcDNA-AKIP1, pcDNA-NC, pGPH1-AKIP1 and pGPH1-NC groups were treated with different concentrations of temozolomide (TMZ) (Sigma, USA) or cisplatin

(Sigma, USA) for 72 h. The concentration ranges of TMZ (Sigma, USA) and cisplatin (Sigma, USA) were 0-320 μM and 0-80 μM, respectively. The cell viability was evaluated by cell counting kit-8 (Dojindo, Japan) referring to the kit's protocol and the relative cell viability was calculated according to the methods described in the previous study (the viability of cells without treatment was set as 100%) [30]. Meanwhile, after 80 μM TMZ or 40 μM cisplatin treatment for 72 h, cell apoptosis detection was performed with Annexin V Apoptosis Detection Kit FITC (Invitrogen, USA) according to the kit's instruction.

Irradiation sensitivity detection

After transfection, cell irradiation was performed at room temperature by using Faxitron Cabinet X-ray System (Faxitron, IL), with a dose rate of 0.36 Gy/min at various doses (0, 2, 4, 6, and 8 Gy). Then cell viability was detected by cell counting kit-8 (Dojindo, Japan) and relative cell viability was calculated by setting the viability of cells without treatment as 100%. At the same time, cell apoptosis was detected with Annexin V Apoptosis Detection Kit FITC (Invitrogen, USA) according to the kit's instruction after cells being treated with 6 Gy irradiation.

CXCL1/2/8 expression, PD-L1 expression, NF-κB and AKT pathways detection

AKIP1 is reported to elevate the CXCL1, CXCL2, and CXCL8 expressions in the promotion of tumor growth in cancers other than GBM [12-14]. Meanwhile, the NF-κB pathway, AKT pathway and PD-L1 play important roles in the regulation of metastasis, chemosensitivity and radiation sensitivity by AKIP1 [7, 8, 31-34]. To evaluate whether CXCL1/2/8, PD-L1, NF-κB pathway, AKT pathway were regulated by AKIP1 in GBM, the protein expressions of CXC L1, CXCL2 and CXCL8 in cells were evaluated by Enzyme-linked immunosorbent assay (ELISA) 24 h after transfection, and the expressions of NF-κB p60, phoso-NF-κB p60 (p-NF-κB p60), AKT, phoso-AKT (p-AKT) and PD-L1 were assessed by western blot.

ELISA

At 24 h after transfection, cell supernatant was collected. Then, the CXCL1, CXCL2 and CXCL8 in supernatant was measured by ELISA. The Human CXCL1 ELISA Kit (Invitrogen, USA), Human CXCL2 ELSA Kit (R&D, USA) and Human CXCL8 ELSIA Kit (R&D, USA) were applied to perform ELISA. The detection was carried out following the kit's procedure.

Multiple compensative experiments

The pGPH1-NC and pGPH1-AKIP1 cells were constructed with the methods mentioned in "Transfection" subsection. Then, the pGPH1-NC and pGPH1-AKIP1 cells were incubated with 250 pg/ml CXCL1 (R&D, USA), 350 pg/ml CXCL2 (R&D, USA) and 400 pg/ml CXCL8 (R&D, USA), respectively. The cells were then divided into pGPH1-NC, pGPH1-AKIP1, pGPH1-NC& CXCL1, pGPH1-AKIP1&CXCL1, pGPH1-NC& CXCL2, pGPH1-AKIP1&CXCL2, pGPH1-NC& CXCL8, pGPH1-AKIP1&CXCL8 groups for analyses.

Furthermore, the pGPH1-NC and pGPH1-AKIP1 cells were incubated with 250 pg/ml CXCL1 (R&D, USA) plus 400 pg/ml CXCL8 (R&D, USA) simultaneously. And the cells were divided into pGPH1-NC, pGPH1-AKIP1, pGPH1-NC&CXCL1&CXCL8, pGPH1-AKIP1&CXCL1&CXCL8 groups for analyses.

Cell proliferation, cell apoptosis and cell invasion, chemosensitivity, radiation sensitivity were determined with methods mentioned above. At 48 h after incubation, the expressions of NF-κB p65, p-NF-κB p65, AKT, p-AKT and PD-L1 were detected by western blot.

LY and CAPE treatment

250 pg/ml CXCL1 (R&D, USA) plus 400 pg/ml CXCL8 (R&D, USA) was added to culture normal U-87 MG and U-251 MG cells. Then they were treated by 10 μM AKT pathway inhibitor (LY294002 (LY)) (Sigma, USA) and 50 μM NF-κB pathway inhibitor (caffeic acid phenethyl ester (CAPE)) (Sigma, USA), respectively, followed by detection of cell proliferation, apoptosis and invasion with methods mentioned above.

RT-qPCR

After the extraction of total RNA with TRIzol™ Reagent (Invitrogen, USA), the reverse transcription of cDNA was performed by the application of ReverTra Ace® qPCR RT Kit (Toyobo,

Table 1. Antibodies used in western blot

Antibody	Company	Dilution
Primary Antibody		
Rabbit polyclonal to AKIP1	Abcam (UK)	1:500
Rabbit monoclonal to NF-kB p65	Abcam (UK)	1:2000
Rabbit monoclonal to p-NF-kB p65	Abcam (UK)	1:1000
Rabbit polyclonal to AKT	Abcam (UK)	1:500
Rabbit polyclonal to p-AKT	Abcam (UK)	1:500
Rabbit monoclonal to PD-L1	Abcam (UK)	1:1000
Rabbit polyclonal to GAPDH	CST (USA)	1:1000
Secondary Antibody		
Goat Anti-Rabbit IgG-HRP	CST (USA)	1:3000

Japan). Then the qPCR was carried out using ReverTra Ace® qPCR RT Kit (Toyobo, Japan). The relative expression of AKIP1 was calculated using $2^{-\Delta\Delta Ct}$ with GAPDH serving as the internal reference. The primers sequences were listed as follows: AKIP1, forward primer: 5' AGAACATCTCTAAGGACCTCTACAT 3'; reverse primer: 5' TCCAGAATCAACTGCTACCACAT 3'; GAPDH, forward primer: 5' GACCACAGTCCATGCCATCAC 3'; reverse primer: 5' ACGCCTGCTTCACCACCTT 3'.

Western blot

The total protein was extracted with RIPA Buffer (Sigma, USA). Protein concentration was assessed using a Pierce™ BCA Protein Assay Kit (Thermo, USA). After thermal denaturation, 20 μg protein was separated by TruPAGE™ Precast Gels (Sigma, USA) and transferred onto polyvinylidene fluoride membrane (PALL, USA). After blocking with 5% BSA (Sigma, USA), the membrane was incubated with primary antibodies at 4°C overnight. Subsequently, secondary antibody was applied to incubate with the membrane at 37°C for 1 h. Finally, a Novex™ECL Chemiluminescent Substrate Reagent Kit (Invitrogen, USA) was used to detect the immunoreactive bands. The antibodies applied in western blot were listed in **Table 1**.

Clinical samples and detections

After the ethics approval by our institution and informed consents obtained from patients or their families, 10 tumor samples from GBM patients and 10 tumor samples from age/gender matched low-grade glioma (LGG) patients were retrieved from Specimen Room. Then AKIP1 CXCL1, CXCL2 and CXCL8 expressions in tumor tissues were detected by immunohistochemistry (IHC), then scored by a semiquantative method according to a previous study [14]. The antibodies information were as follows: Rabbit AKIP1 Polyclonal Antibody (1:50 dilution, Invitrogen, USA); Rabbit CXCL1 Polyclonal Antibody (1:100 dilution, Invitrogen, USA); Rabbit CXCL2 Recombinant Monoclonal Antibody (1:50 dilution, Invitrogen, USA); Rabbit CXCL8 Polyclonal Antibody (1:50 dilution, Invitrogen, USA); horseradish peroxidase-conjugated Goat anti-Rabbit IgG (H+L) secondary antibody (1:10000 dilution, Invitrogen, USA).

Statistical analysis

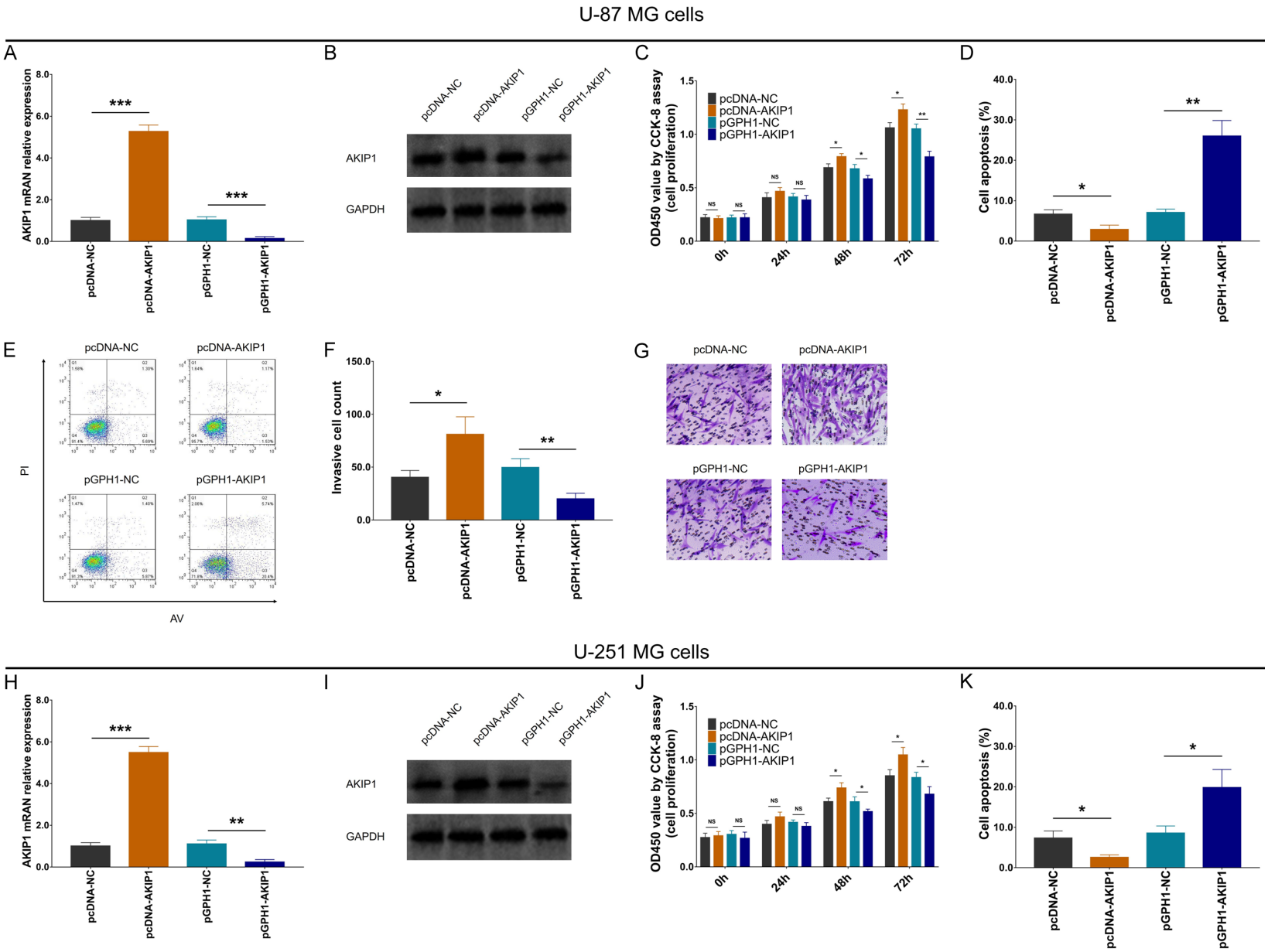
Data was described as mean with standard deviation (SD). GraphPad Prism Software version 7.02 (GraphPad Software Inc., USA) was used for data analyses and graph plotting. One-way ANOVA followed by Dunnett's multiple comparisons test was used to determine comparisons between control and other groups. One-way ANOVA followed by Tukey's multiple comparisons test was used to determine multiple comparisons among groups. Unpaired t test was used to compare data between GBM and LGG patients. Pearson correlation test was used to analyze the correlation among indexes in GBM patients. *P* value <0.05 was considered as significant. *P* value >0.05, <0.05, <0.01, and <0.001 were respectively marked as no significance (NS), *, **, and ***.

Results

The effect of AKIP1 modification on GBM proliferation, apoptosis and invasion

AKIP1 expression was elevated in GBM cell lines compare to control cell line (NHA) (Supplementary Figure 1A, 1B). After transfection of pcDNA and pGPH1 vectors, AKIP1 was increased in pcDNA-AKIP1 group compared to pcDNA-NC group, while decreased in pGPH1-AKIP1 group compared to pGPH1-NC group in both U-87 MG cells (**Figure 1A**, **1B**) and U-251 MG cells (**Figure 1H**, **1I**).

Importantly, U-87 MG cell proliferation (**Figure 1C**), cell invasion (**Figure 1F**, **1G**) were enhanced, while cell apoptosis rate (**Figure 1D**, **1E**) was reduced in pcDNA-AKIP1 group compared to pcDNA-NC group; oppositely, U-87 MG cell proliferation (**Figure 1C**), cell invasion (**Figure 1F**,


U-87 MG cells
A
AKIP1 mRAN relative expression
pcDNA-NC
pcDNA-AKIP1
pGPH1-NC
pGPH1-AKIP1
B
AKIP1
GAPDH
C
OD450 value by CCK-8 assay (cell proliferation)
0h
24h
48h
72h
D
Cell apoptosis (%)
E
PI
AV
F
Invasive cell count
G
U-251 MG cells
H
I
J
K

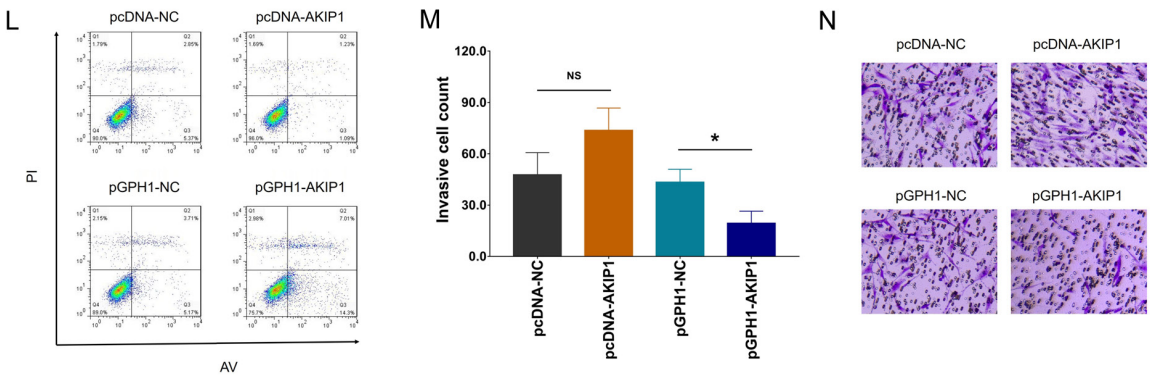


Figure 1. Cell proliferation, apoptosis and invasion after transfection. AKIP1 mRNA expression (A) and protein expression (B) among groups in U-87 MG cells; Cell proliferation (C), cell apoptosis rate (D, E), invasive cell count (F, G) among groups in U-87 MG cells. AKIP1 mRNA expression (H) and protein expression (I) among groups in U-251 MG cells; Cell proliferation (J), cell apoptosis rate (K, L), invasive cell count (M, N) among groups in U-251 MG cells. AKIP1, A-kinase-interacting protein 1; NC, negative control; AV, Annexin V; PI, prodium Iodide.

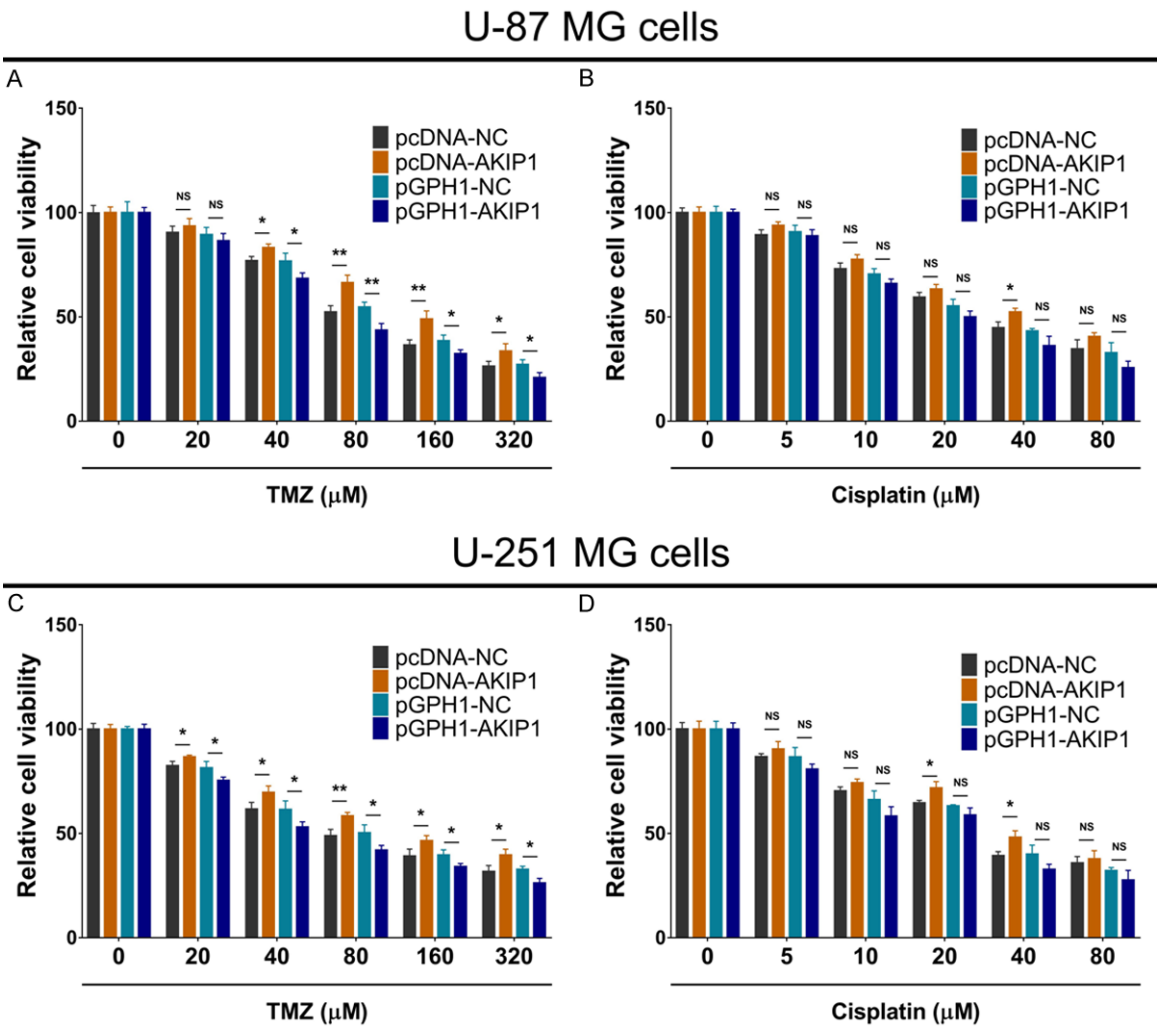


Figure 2. Relative cell viability under chemotherapy after transfection. Relative cell viability under 0-320 μM TMZ treatment (A) or 0-80 μM cisplatin treatment (B) among groups in U-87 MG cells. Relative cell viability under 0-320 μM TMZ treatment (C) or 0-80 μM cisplatin treatment (D) among groups in U-251 MG cells. AKIP1, A-kinase-interacting protein 1; NC, negative control; TMZ, temozolomide.

1G) were attenuated, while cell apoptosis rate (**Figure 1D**, **1E**) was promoted in pGPH1-AKIP1 group compared to pGPH1-NC group. These indexes among groups showed similar treads in U251 MG cells as well (**Figure 1J-N**), except for no difference of cell invasion between pcDNA-AKIP1 group and pcDNA-NC group. These data indicated that AKIP1 promoted GBM proliferation and invasion but inhibit its apoptosis.

The effect of AKIP1 modification on GBM chemosensitivity to TMZ and cisplatin

The relative viability was enhanced in pcDNA-AKIP1 group compared to pcDNA-NC group, while reduced in pGPH1-AKIP1 group compared to pGPH1-NC group in U-87 MG cells under 40-320 μM TMZ treatment (**Figure 2A**). However, no difference of relative viability was observed among these four groups in U-87 MG cells under various concentrations of cisplatin treatment (**Figure 2B**), except for that relative viability was enhanced in pcDNA-AKIP1 group compared to pcDNA-NC group of U-87 MG cells under 40 μM cisplatin treatment. In addition, the above findings were also validated in another GBM cell line (U-251 MG cells) (**Figure 2C**, **2D**).

Subsequently, we detected cell apoptosis rate in each group after TMZ and cisplatin treatment with IC_{50} concentration, respectively.

Then we found that under 80 μM TMZ treatment, both U-87 MG cell and U-251 MG cell apoptosis rates were inhibited in pcDNA-AKIP1 group compared to pcDNA-NC group, while promoted in pGPH1-AKIP1 group compared to pGPH1-NC group (**Figure 3A**, **3B**, **3E** and **3F**). However, under 40 μM cisplatin treatment, both U-87 MG cell and U-251 MG cell apoptosis rates were of no difference between pcDNA-AKIP1 group and pcDNA-NC group, but was elevated in pGPH1-AKIP1 group compared to pGPH1-NC group (**Figure 3C**, **3D**, **3G** and **3H**).

These data suggested that AKIP1 enhanced GBM resistance to TMZ but not cisplatin.

The effect of AKIP1 modification on GBM radiation sensitivity

The relative viability was enhanced in pcDNA-AKIP1 group compared to pcDNA-NC group, while reduced in pGPH1-AKIP1 group compared to pGPH1-NC group, in U-87 MG cells underwent 4-10 Gy radiation treatment and in U-251 MG cells underwent 2-10 Gy radiation treatment (**Figure 4A**, **4D**). Furthermore, under 6 Gy (IC_{50} dose) radiation treatment, cell apoptosis was repressed in pcDNA-AKIP1 group compared to pcDNA-NC group, while enhanced in pGPH1-AKIP1 group compared to pGPH1-NC group (**Figure 4B**, **4C**, **4E** and **4F**). These data implied that AKIP1 promoted GBM resistance to radiation treatment.

The effect of AKIP1 modification on CXCL1/2/8, NF-κB, AKT and PD-L1 expressions in GBM

In U-87 MG and U-251 MG cells, CXCL1 and CXCL8 were both elevated in pcDNA-AKIP1 group compared to pcDNA-NC group, while were reduced in pGPH1-AKIP1 group compared to pGPH1-NC group (**Figure 5A**, **5C**, **5E** and **5G**). However, CXCL2 was increased in cDNA-AKIP1 group compared to pcDNA-NC group, while was decreased in pGPH1-AKIP1 group compared to pGPH1-NC group in U-251 MG cells (**Figure 5F**) but not in U-87 MG cells (**Figure 5B**). These indicated that AKIP1 positively regulated CXCL1, CXCL2 (weak effect) and CXCL8 in GBM.

Furthermore, in U-87 MG (**Figure 5D**) and U-251 MG (**Figure 5H**) cells, p-NF-κB p65, p-AKT and PD-L1 expressions were all raised in pcDNA-AKIP1 group compared to pcDNA-NC group, but reduced in pGPH1-AKIP1 group compared to pGPH1-NC group. These indicated that AKIP1 activated NF-κB and AKT pathways, meanwhile increased PD-L1 expression.

Interaction of CXCL1/2/8 with AKIP1 knockdown on GBM proliferation, apoptosis and invasion

In order to further evaluate the interaction of AKIP1 with CXCL1/2/8 and their effects on regulating GBM malignant behaviors as well as chemoradiation sensitivity, multiple compensative experiments were conducted.

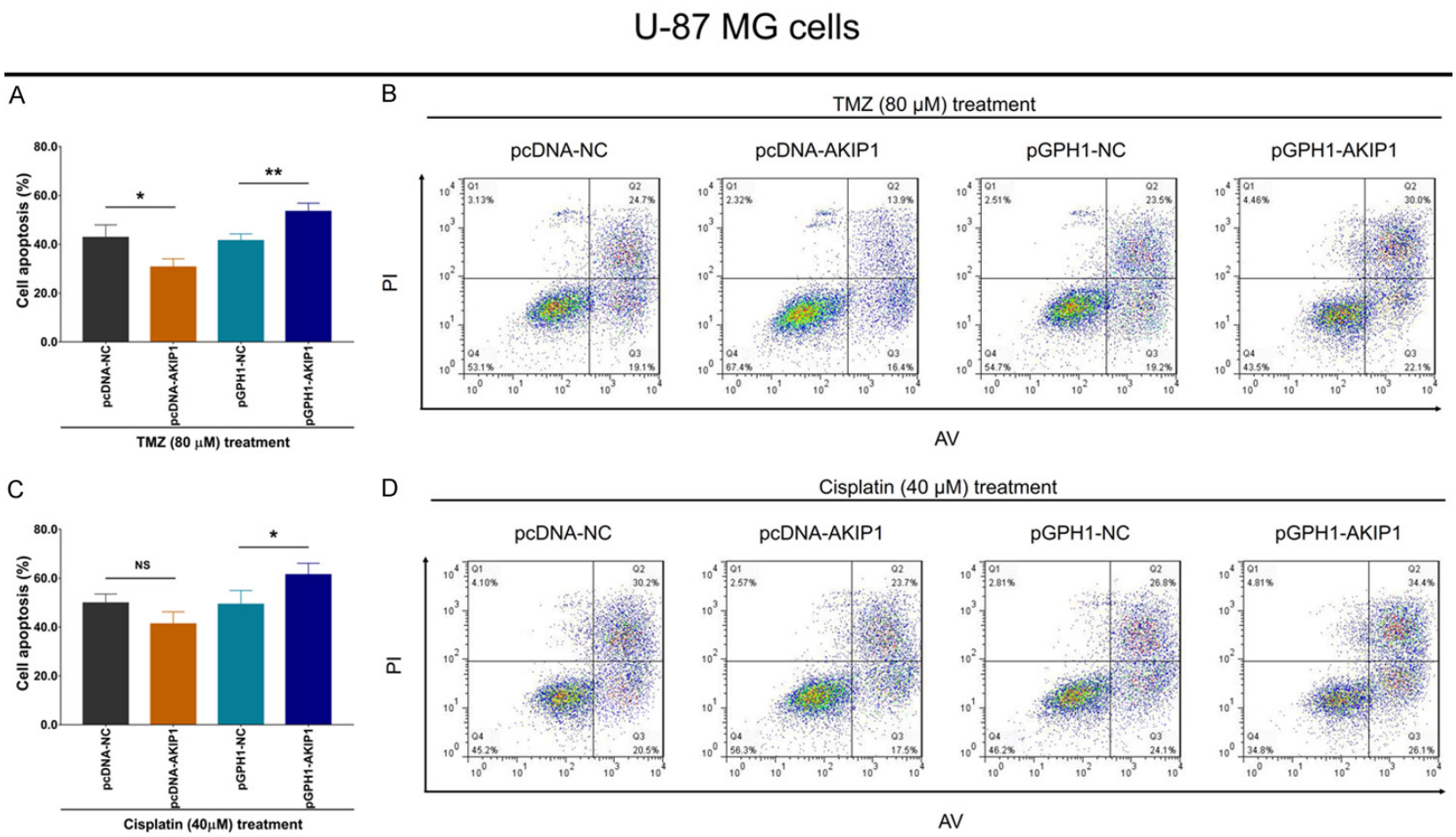
In both U-87 MG (**Figure 6A-C**) and U-251 MG (**Figure 6D-F**) cells, CXCL1 and CXCL8 enhanced cell proliferation, inhibited apoptosis, while CXCL2 did not affect them. Furthermore, in pGPH1-AKIP1 treated U-87 MG (**Figure 6A-C**) and U-251 MG (**Figure 6D-F**) cells, CXCL1, CXCL2 and CXCL8 all promoted cell proliferation, while CXCL1 and CXCL8 but not CXCL2 reduced cell apoptosis.

As to invasion ability, in both U-87 MG (**Figure 7A**, **7B**) and U-251 MG (**Figure 7C**, **7D**) cells, CXCL1 and CXCL8 promoted cell invasion, while CXCL2 did not work. In addition, in pGPH1-AKIP1 treated U-87 MG (**Figure 7A**, **7B**) and U-251 MG (**Figure 7C**, **7D**) cells, CXCL1 and CXCL8 also accelerated cell invasion, but CXCL2 could not.

Findings from compensative experiments indicated that AKIP1 regulated GBM proliferation, apoptosis and invasion via stimulating CXCL1 and CXCL8.

Interaction of CXCL1/2/8 with AKIP1 knockdown on GBM chemoradiation sensitivity

In U-87 MG cells (**Figure 8A-C**), CXCL1 and CXCL8 increased relative cell viability under 80 μM TMZ treatment, 40 μM cisplatin treatment, or 6 Gy radiation treatment; while CXCL2 only increased relative cell viability under 80 μM TMZ treatment, but not 40 μM cisplatin treatment, or 6 Gy radiation treatment. In pGPH1-AKIP1 treated U-87 MG cells (**Figure 8A-C**), CXCL1 only increased relative cell viability under 80 μM TMZ treatment, but not 40 μM cisplatin treatment or 6 Gy radiation treatment; CXCL2 did not affect the relative cell viability under 80 μM TMZ treatment, 40 μM cisplatin


U-87 MG cells
A
Cell apoptosis (%)
80.0
60.0
40.0
20.0
0.0
*
**
pcDNA-NC
pcDNA-AKIP1
pGPH1-NC
pGPH1-AKIP1
TMZ (80 μM) treatment
B
TMZ (80 μM) treatment
pcDNA-NC
pcDNA-AKIP1
pGPH1-NC
pGPH1-AKIP1
PI
AV
Q1 3.13%
Q2 24.7%
Q4 53.1%
Q3 19.1%
Q1 2.32%
Q2 13.9%
Q4 67.4%
Q3 16.4%
Q1 2.51%
Q2 23.5%
Q4 54.7%
Q3 19.2%
Q1 4.46%
Q2 30.0%
Q4 43.5%
Q3 22.1%
C
Cell apoptosis (%)
80.0
60.0
40.0
20.0
0.0
NS
*
pcDNA-NC
pcDNA-AKIP1
pGPH1-NC
pGPH1-AKIP1
Cisplatin (40μM) treatment
D
Cisplatin (40 μM) treatment
pcDNA-NC
pcDNA-AKIP1
pGPH1-NC
pGPH1-AKIP1
PI
AV
Q1 4.10%
Q2 30.2%
Q4 45.2%
Q3 20.5%
Q1 2.57%
Q2 23.7%
Q4 56.3%
Q3 17.5%
Q1 2.81%
Q2 26.8%
Q4 46.2%
Q3 24.1%
Q1 4.81%
Q2 34.4%
Q4 34.8%
Q3 26.1%

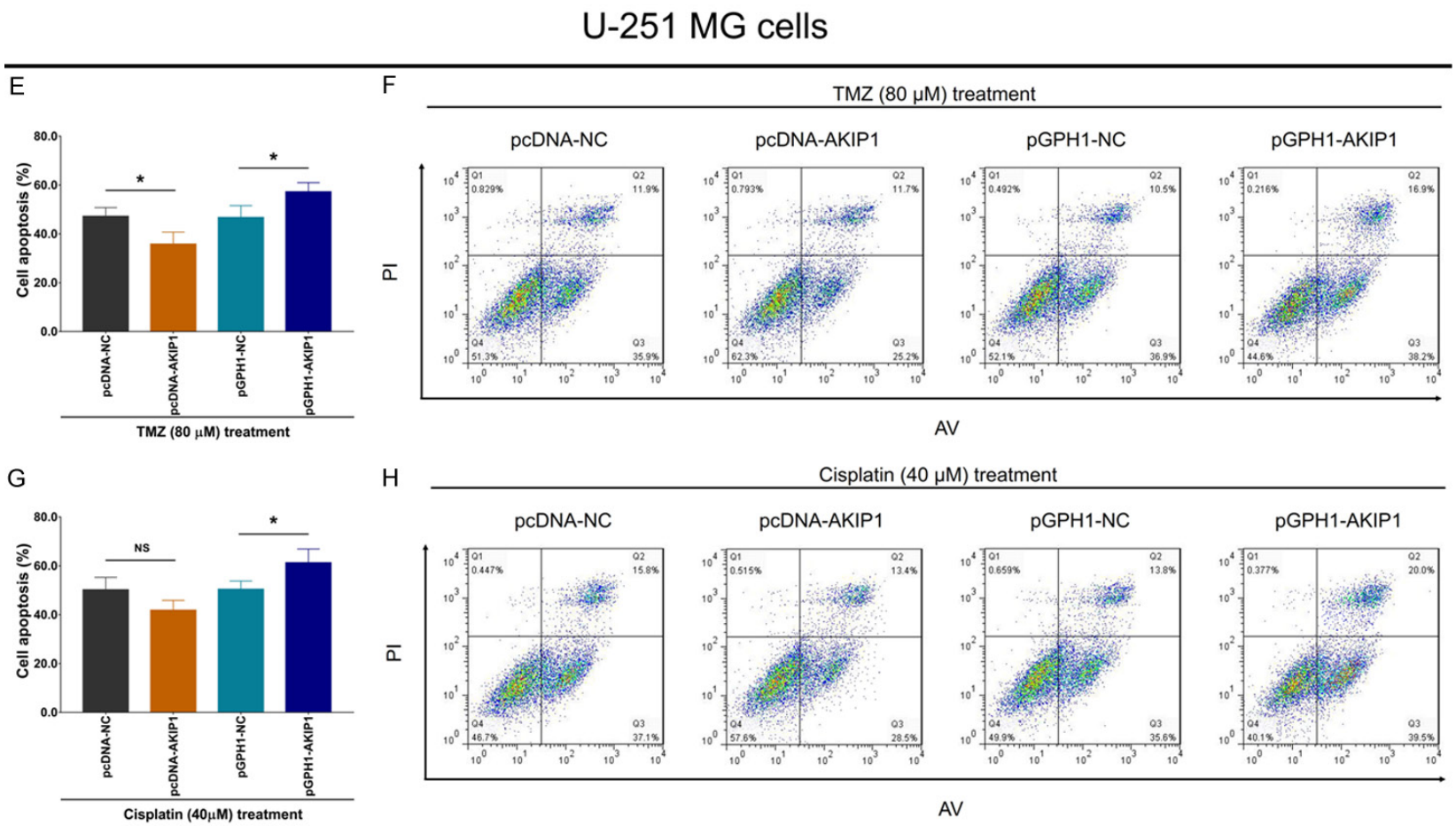


Figure 3. Cell apoptosis rate under chemotherapy after transfection. Cell apoptosis rate under 80 μM TMZ treatment (A, B) or 40 μM cisplatin treatment (C, D) among groups in U-87 MG cells. Cell apoptosis rate under 80 μM TMZ treatment (E, F) or 40 μM cisplatin treatment (G, H) among groups in U-251 MG cells. AKIP1, A-kinase-interacting protein 1; NC, negative control; TMZ, temozolomide; AV, Annexin V; PI, prodium Iodide.

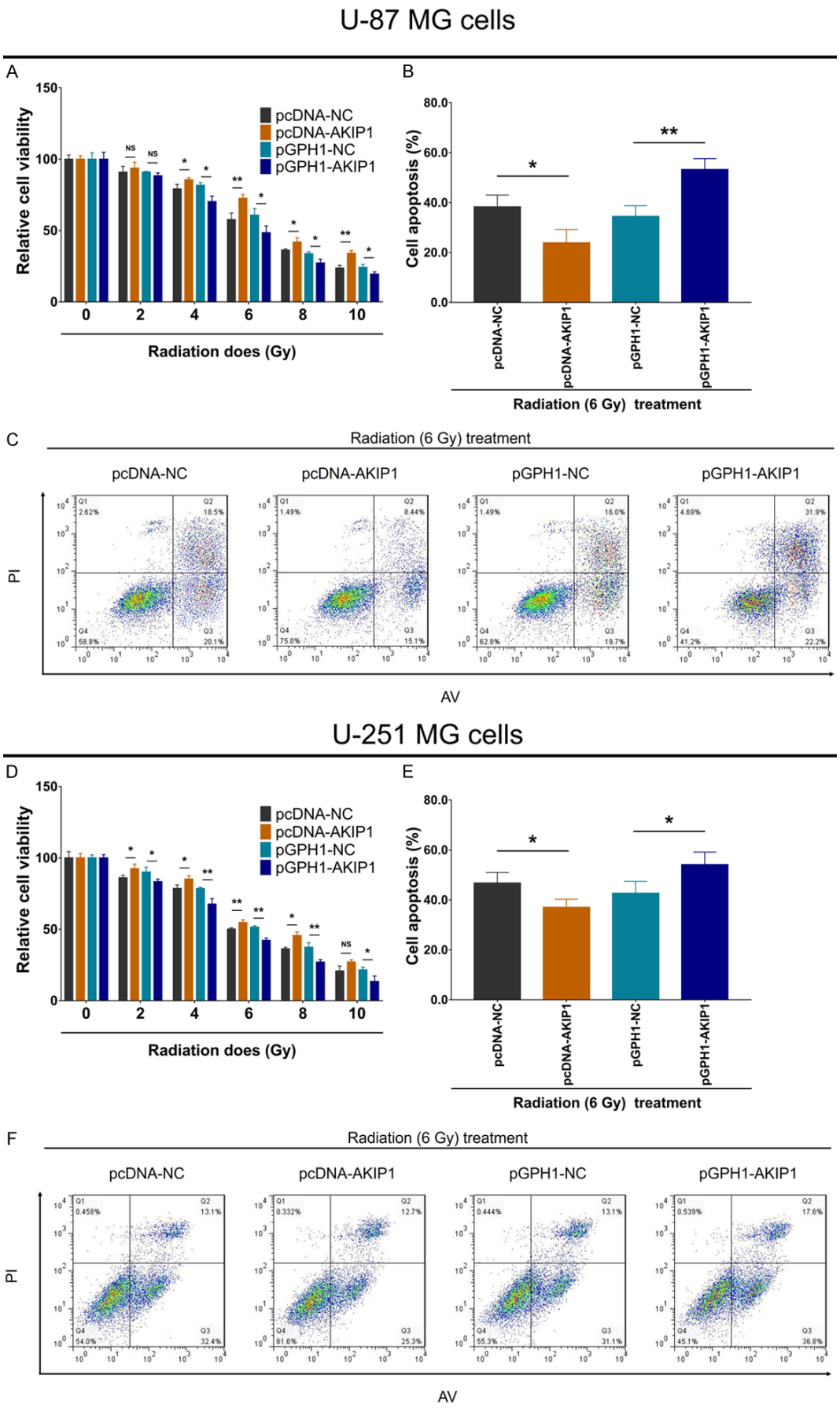

U-87 MG cells
A
Relative cell viability
pcDNA-NC
pcDNA-AKIP1
pGPH1-NC
pGPH1-AKIP1
Radiation does (Gy)
B
Cell apoptosis (%)
Radiation (6 Gy) treatment
C
Radiation (6 Gy) treatment
pcDNA-NC
pcDNA-AKIP1
pGPH1-NC
pGPH1-AKIP1
PI
AV
U-251 MG cells
D
E
F

Figure 4. Relative cell viability and cell apoptosis rate under radiation therapy after transfection. Relative cell viability under 0-10 Gy radiation treatment (A), cell apoptosis rate under 6 Gy radiation treatment (B, C), among groups in U-87 MG cells. Relative cell viability under 0-10 Gy radiation treatment (D), cell apoptosis rate under 6 Gy radiation treatment (E, F), among groups in U-251 MG cells. AKIP1, A-kinase-interacting protein 1; NC, negative control; TMZ, temozolomide.

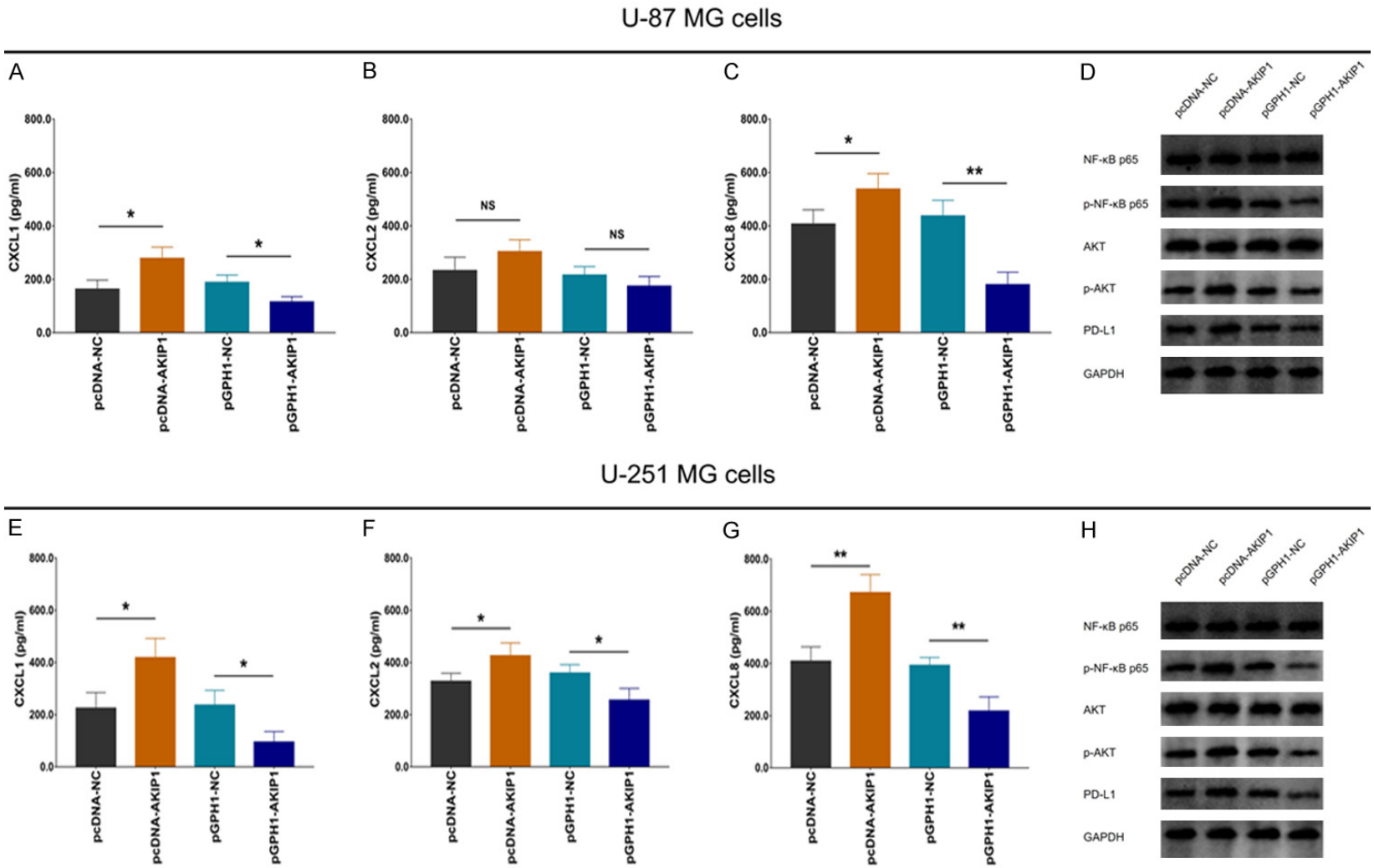
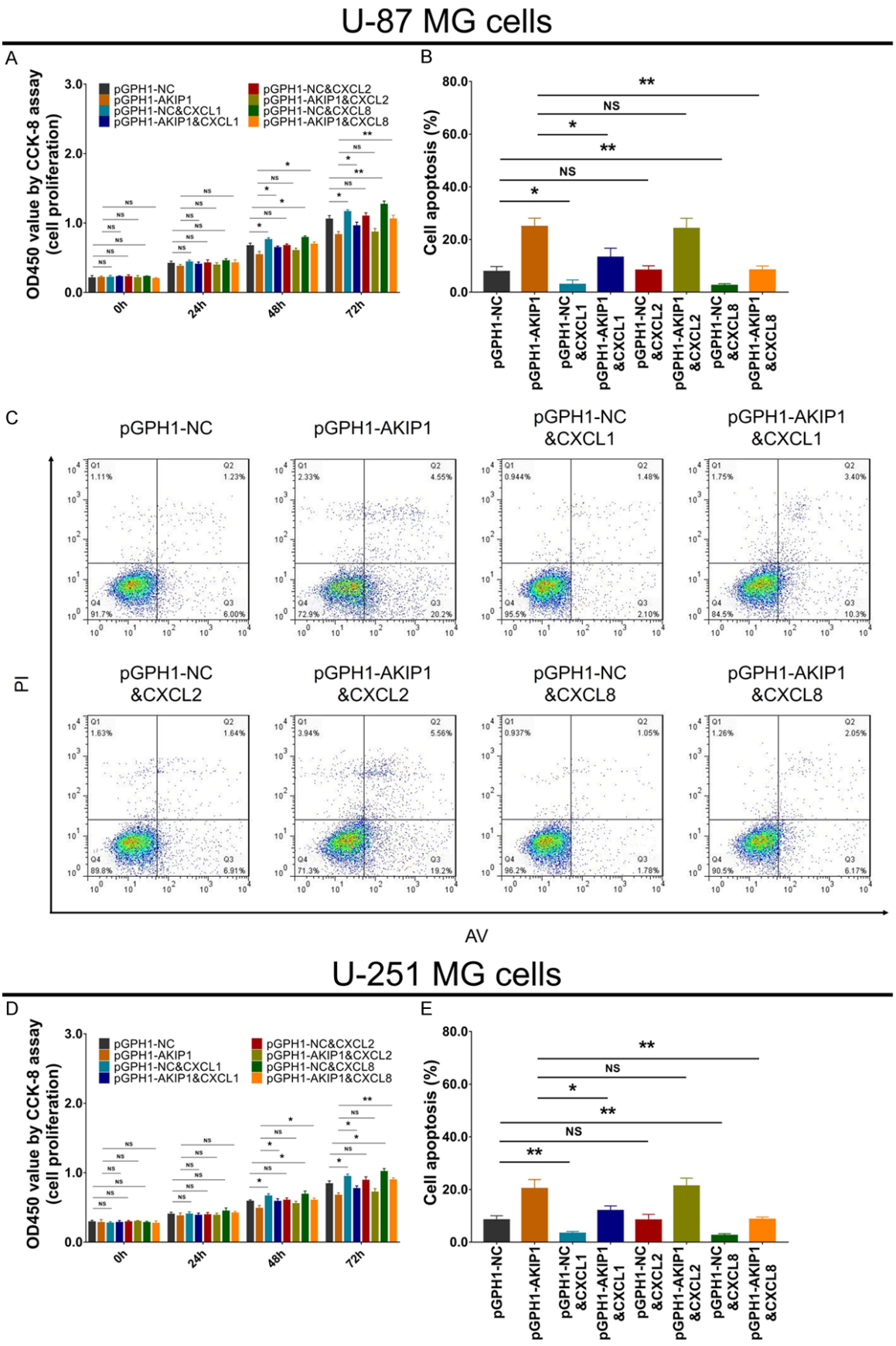


Figure 5. CXCL1/2/8, NF-κB, AKT and PD-L1 expressions after transfection. Supernatant CXCL1 (A), CXCL2 (B), CXCL8 (C) levels, and cell NF-κB p65, p-NF-κB p65, AKT, p-AKT, PD-L1 (D) expressions among groups in U-87 MG cells. Supernatant CXCL1 (E), CXCL2 (F), CXCL8 (G) levels, and cell NF-κB p65, p-NF-κB p65, AKT, p-AKT, PD-L1 (H) expressions among groups in U-251 MG cells. AKIP1, A-kinase-interacting protein 1; NC, negative control; CXCL, C-X-C motif chemokine ligand.


U-87 MG cells
A
OD450 value by CCK-8 assay (cell proliferation)
pGPH1-NC
pGPH1-AKIP1
pGPH1-NC&CXCL1
pGPH1-AKIP1&CXCL1
pGPH1-NC&CXCL2
pGPH1-AKIP1&CXCL2
pGPH1-NC&CXCL8
pGPH1-AKIP1&CXCL8
0h
24h
48h
72h
B
Cell apoptosis (%)
pGPH1-NC
pGPH1-AKIP1
pGPH1-NC &CXCL1
pGPH1-AKIP1 &CXCL1
pGPH1-NC &CXCL2
pGPH1-AKIP1 &CXCL2
pGPH1-NC &CXCL8
pGPH1-AKIP1 &CXCL8
C
pGPH1-NC
pGPH1-AKIP1
pGPH1-NC &CXCL1
pGPH1-AKIP1 &CXCL1
pGPH1-NC &CXCL2
pGPH1-AKIP1 &CXCL2
pGPH1-NC &CXCL8
pGPH1-AKIP1 &CXCL8
PI
AV
U-251 MG cells
D
OD450 value by CCK-8 assay (cell proliferation)
E
Cell apoptosis (%)

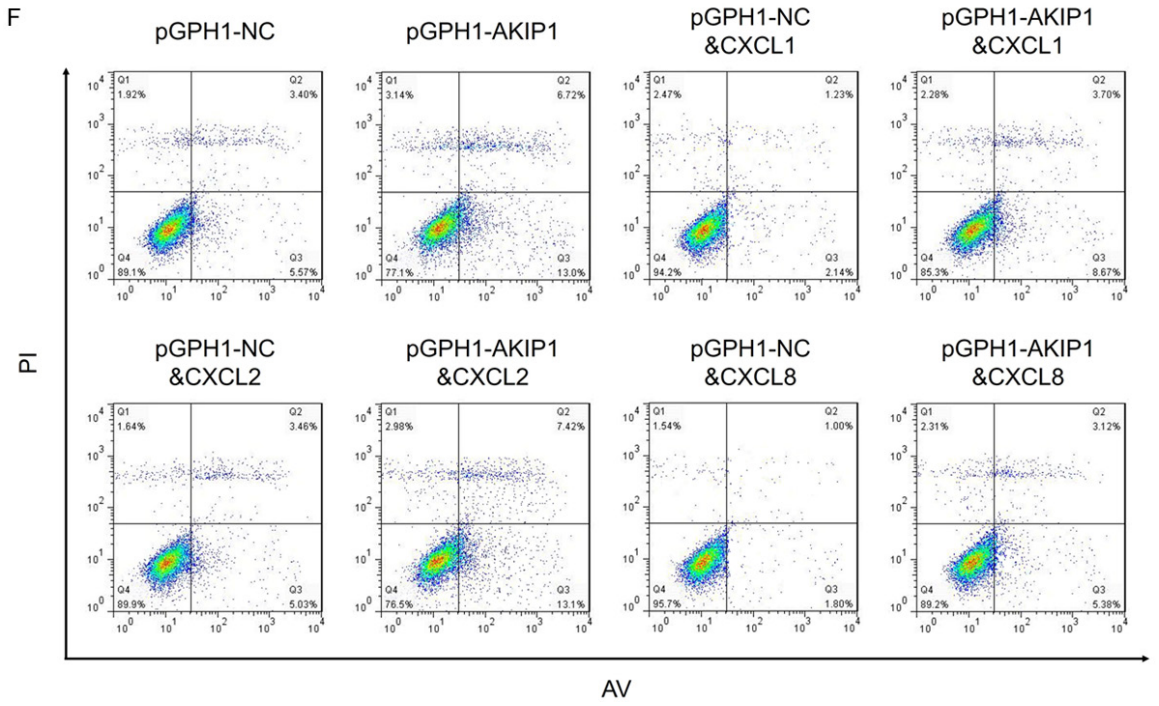


Figure 6. Cell proliferation and apoptosis in multiple compensative experiments. Cell proliferation (A) and cell apoptosis rate (B, C) among groups in U-87 MG cells. Cell proliferation (D) and cell apoptosis rate (E, F) among groups in U-251 MG cells. AKIP1, A-kinase-interacting protein 1; NC, negative control; CXCL, C-X-C motif chemokine ligand; AV, Annexin V; PI, prodium Iodide.

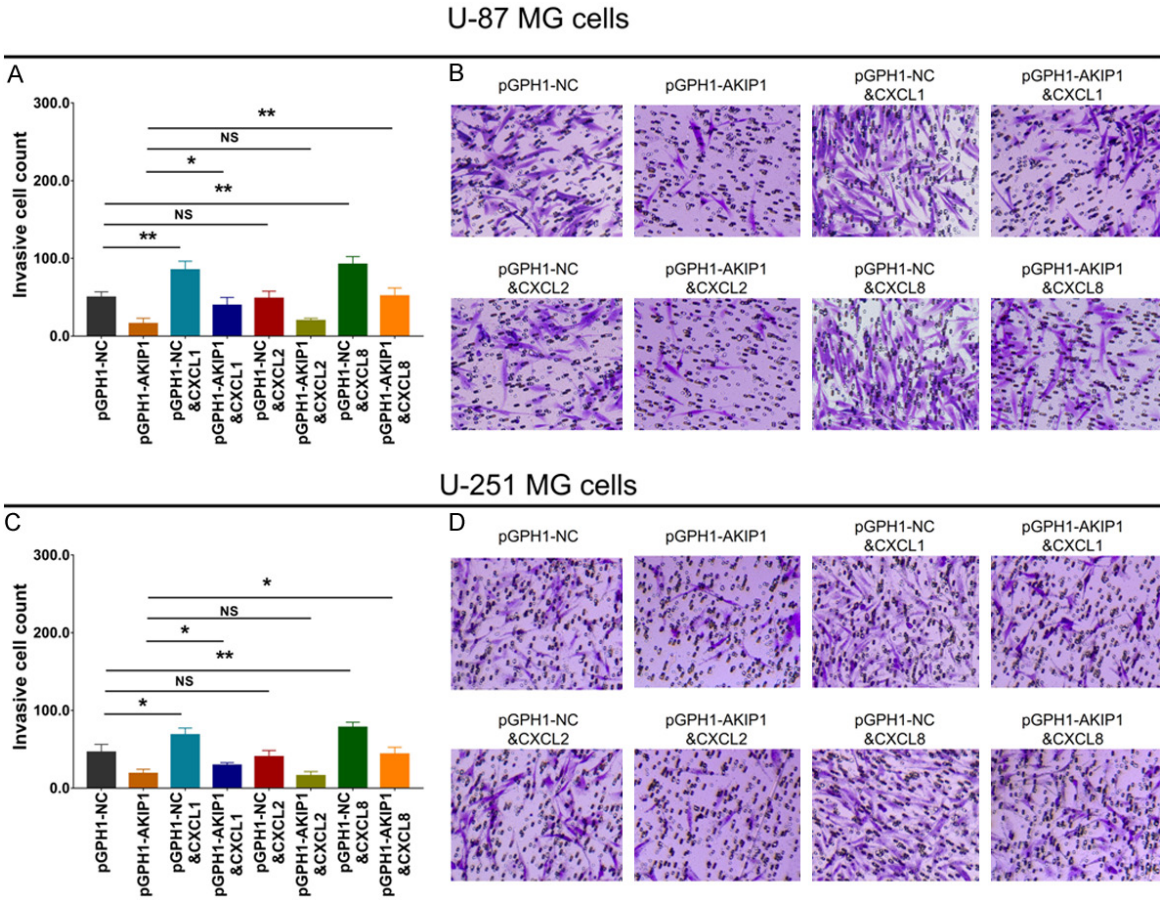


Figure 7. Cell invasion in multiple compensative experiments. Invasive cell count (A, B) among groups in U-87 MG cells. Invasive cell count (C, D) among groups in U-251 MG cells. AKIP1, A-kinase-interacting protein 1; NC, negative control; CXCL, C-X-C motif chemokine ligand.

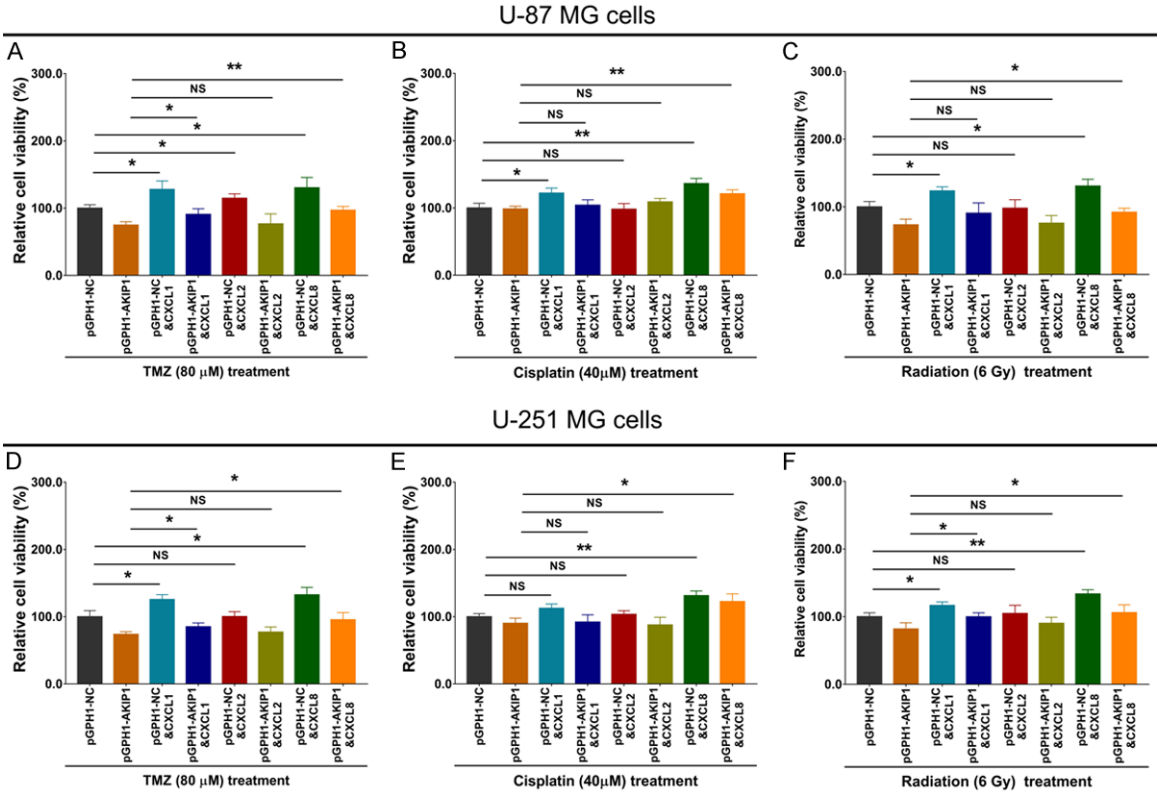


Figure 8. Chemoradiation sensitivity in multiple compensative experiments. Relative cell viability under 80 μM TMZ treatment (A), or 40 μM cisplatin treatment (B), or 6 Gy radiation treatment (C) among groups in U-87 MG cells. Relative cell viability under 80 μM TMZ treatment (D), or 40 μM cisplatin treatment (E), or 6 Gy radiation treatment (F) among groups in U-251 MG cells. AKIP1, A-kinase-interacting protein 1; NC, negative control; CXCL, C-X-C motif chemokine ligand; TMZ, temozolomide.

treatment or 6 Gy radiation treatment; notably, CXCL8 increased relative cell viability under 80 μM TMZ treatment, 40 μM cisplatin treatment, and 6 Gy radiation treatment. As to in U-251 MG cells, the related findings were similar as in U-87 MG cells (**Figure 8D-F**). At the same time, the related findings in pGPH1-AKIP1 treated U-251 MG cells were similar as in pGPH1-AKIP1 treated U-87 MG cells (**Figure 8D-F**); however, the effects of CXCL1, CXCL2 and CXCL8 were less.

Interaction of CXCL1/2/8 with AKIP1 knockdown on NF-κB, AKT and PD-L1 expressions in GBM

In both U-87 MG (**Figure 9A**) and U-251 MG (**Figure 9B**) cells, CXCL1 and CXCL8 increased p-NF-κB p65, p-AKT and PD-L1 expressions, while CXCL2 could not. Furthermore, in pGPH1-AKIP1 treated U-87 MG (**Figure 9A**) and U-251 MG (**Figure 9B**) cells, CXCL1 and CXCL8 also elevated p-NF-κB p65, p-AKT and PD-L1 expressions, but CXCL2 lacked effect. As for the detailed molecule mechanism between NF-κB/AKT and PD-L1 expression, it has been disclosed by Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/) that NF-κB directly regulates PD-L1 expression, while AKT indirectly regulates PD-L1 expression via activating IKK and subsequent NF-κB (Supplementary Figure 2). These data of compensative experiments suggested that AKIP1 regulated NF-κB pathway, AKT pathway and PD-L1 expression via stimulating CXCL1 and CXCL8.

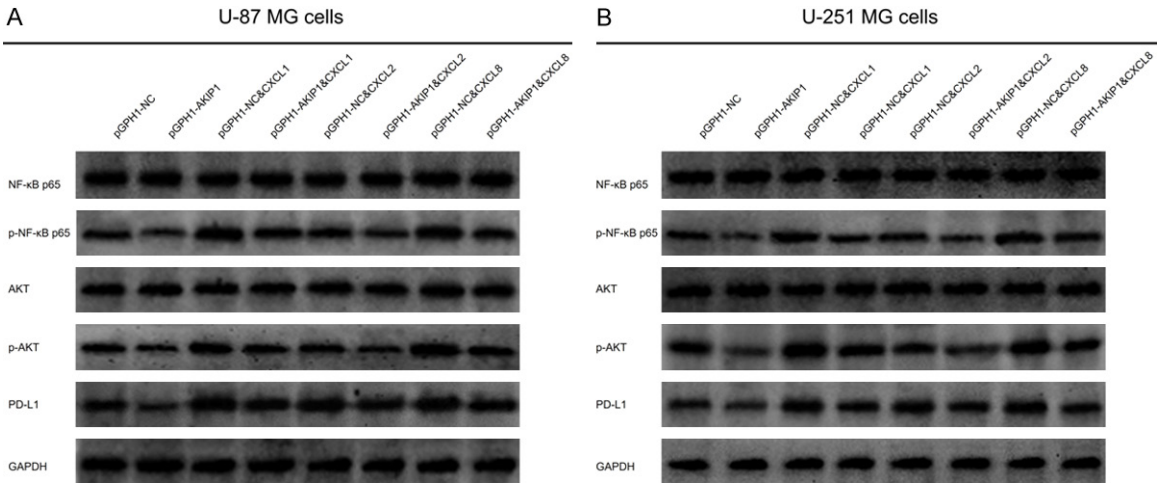


Figure 9. NF-κB, AKT and PD-L1 expressions in multiple compensative experiments. NF-κB p65, p-NF-κB p65, AKT, p-AKT, PD-L1 expressions among groups in U-87 MG cells (A) and U-251 MG cells (B). AKIP1, A-kinase-interacting protein 1; NC, negative control; CXCL, C-X-C motif chemokine ligand.

Validation of CXCL1 plus CXCL8 attenuating AKIP1 knockdown effect on GBM activities

CXCL1 plus CXCL8 simultaneous treatment greatly promoted GBM cell proliferation and invasion, as well as NF-κB and AKT pathways, while reduced cell apoptosis in pGPH1-AKIP1 treated U-87 MG (Supplementary Figure 3A-F) and U-251 MG (Supplementary Figure 3G-L) cells. Meanwhile, CXCL1 plus CXCL8 simultaneous treatment also increased relative cell viability under 80 μM TMZ, 40 μM cisplatin or 6 Gy radiation treatment in both pGPH1-AKIP1 treated U-87 MG (Supplementary Figure 4A-C) and U-251 MG (Supplementary Figure 4D-F) cells. Notably, pGPH1-AKIP1 did not affect relative GBM cell viability under cisplatin treatment (Supplementary Figure 4B, 4E). These data verified that AKIP1 regulated GBM viability, mobility and chemoradiation resistance via regulating CXCL1 and CXCL8.

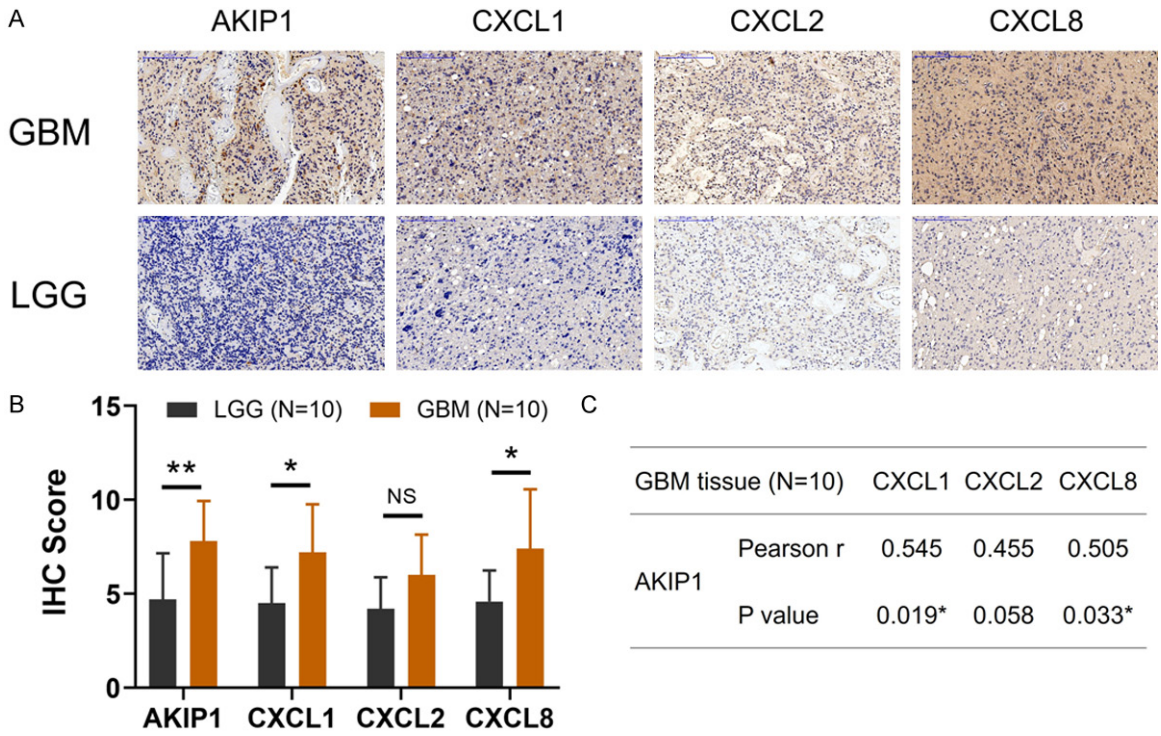
LY and CAPE treatment crippled the effect of CXCL1&CXCL8 on GBM malignant behaviors

Both AKT pathway inhibitor (LY) and NF-κB pathway inhibitor (CAPE) reduced proliferation and invasion while enhanced apoptosis in CXCL1&CXCL8 treated U-87 MG cells (Supplementary Figure 5A-E) and U-87 MG cells (Supplementary Figure 5F-J). Combining the above all data, it was speculated that AKIP1 promoted GBM malignant behaviors and chemoradiation resistance via regulating CXCL1 and CXCL8 mediated NF-κB and AKT pathways.

Correlation of AKIP1 with CXCL1/2/8 in GBM clinical tumor tissues

In order to further validate the interaction of AKIP1 with CXCL1/2/8, we further detected them in the GBM tumor samples and LGG tumor samples with the IHC examples presented in **Figure 10A**. Then we found that AKIP1, CXCL1 and CXCL8 were elevated in GBM tumor samples compared with LGG tumor samples; while CXCL2 showed a trend to be higher in GBM tumor samples compared with LGG tumor samples but without statistical significance (**Figure 10B**). Furthermore, AKIP1 was positively correlated with CXCL1 and CXCL8 in GBM tumor tissues, while only showed a trend to be correlated with CXCL2 but without statistical significance (**Figure 10C**).

In addition, we referred to GEPIA database (http://gepia.cancer-pku.cn/) to validate our findings, which was a newly developed interactive web server for analyzing the RNA sequencing expression data from the TCGA and the GTEx projects, using a standard processing pipeline. The findings were shown in Supplementary Figure 6, which observed that AKIP1, CXCL1, CXCL8 (but not CXCL2) were elevated in tumor tissue compared to non-tumor tissue of various cancers including GBM (Supplementary Figure 6A-D); furthermore,



GBM tissue (N=10)		CXCL1	CXCL2	CXCL8
AKIP1	Pearson r	0.545	0.455	0.505
	P value	0.019*	0.058	0.033*

Figure 10. AKIP1 and CXCL1/2/8 expressions in GBM and LGG clinical tumor samples. Examples of IHC images about AKIP1, CXCL1, CXCL2, CXCL8 expressions in GBM and LGG clinical tumor samples (A). Comparison of AKIP1, CXCL1, CXCL2, CXCL8 expressions between GBM and LGG clinical tumor samples (B). Correlation of AKIP1 with CXCL1, CXCL2 and CXCL8 in GBM tumor samples (C). AKIP1, A-kinase-interacting protein 1; CXCL, C-X-C motif chemokine ligand; GBM, glioblastoma multiforme; LGG, low-grade glioma; IHC, immunohistochemistry.

tumor AKIP1 positively correlated with CXCL1 and CXCL8 but not CXCL2 in GBM (Supplementary Figure 6E-G). These data further validated our study findings.

Discussion

GBM is the most deteriorative tumor among all primary brain tumors with an extremely pejorative prognosis, whose median survival time is only 12-15 months, and the 5-years survival rate only approximately 5% [1, 2, 35]. So as to improve the prognosis of GBM, several novel drugs have been introduced recently and now under clinical trials, such as cilengitide, bevacizumab, cediranib, enzastaurin, rindopepimut, etc. [36-40]. Encouragingly, PD-1/PD-L1 immune-checkpoint inhibitors are also proposed to treat GBM which shows some improvements on patients' survival [41-43]. However, the prognosis of GBM is still bad mainly due the quick progress, treatment resistance and recurrence [1, 2, 44]. In order to shed light on the novel mechanisms and treatment targets of GBM, we performed the current study to investigate the effect of AKIP1 on GBM cell malignant behaviors and chemoradiotherapy sensitivity as well as CXCL1/2/8; meanwhile, to further explore their regulation on NF-κB pathway, AKT pathway and PD-L1 expression.

AKIP1 is firstly discovered as a factor interacting with the N-terminal 30 residues of PKA [45]; then it's recognized as an important modifier of NF-κB signaling through its relationship with p65 and PKA [7, 46]. Furthermore, due to its multiple phosphorylation sites and functional domains, AKIP1 interacts with multiple proteins, through which it functions in various biological processes and disease development/progression [47]. Recently, AKIP1 is also reported to be a key carcinogenic factor not only in solid tumors but also in hematological malignancies. For instance, AKIP1 enhances cervical cancer epithelial-mesenchymal transition and metastasis through activating PI3K/AKT pathway [48]. Meanwhile, AKIP1 activates Wnt/β-catenin/cyclic AMP response element-binding

protein (CBP) pathway then accelerates hepatocellular carcinoma relapse [10]. Furthermore, AKIP1 overexpression could stimulate AKT/GSK-3β/Snail signaling and facilitates breast cancer metastasis [8]. Interestingly, AKIP1 also correlates with induction therapy response and disease progression in acute myeloid leukemia [49].

In our present study, AKIP1 overexpression was observed to promote GBM proliferation, invasion and inhibit apoptosis, while AKIP1 knockdown showed opposite trends. These could explained by: (1) AKIP could directly interacts with p65 and PKA to stimulate NF-κB pathway to exasperate these GBM malignant behaviors [7, 12, 45], which was further validated by our subsequent experiment data that AKIP1 activated NF-κB pathway in GBM; (2) AKIP1 might modify the AKT pathway to stimulate GBM viability and mobility [48], which was then validated by our subsequent experiments that AKIP1 activated AKT pathway in GBM; (3) AKIP1 would bind with multiple cancer-related proteins via its massive phosphorylation sites and functional domains, then enhanced the GBM malignant behaviors. Whereas, this hypothesis needed future works for validation [47]. Furthermore, our study also found that AKIP1 overexpression repressed GBM sensitivity to TMZ treatment and radiation treatment, while AKIP1 knockdown presented the reversed trends. The possible explanations might be that AKIP1 modified several treatment-resistance pathways such as NF-κB, AKT, β-catenin signaling pathways to inhibit GBM sensitivity to chemoradiation treatment [10, 45, 48].

As to the potential downstream factors of AKIP1, CXCL families have been introduced recently. For example, AKIP1 expedites cervical cancer viability and mobility *in vitro*, enhances angiogenesis and tumor growth *in vivo* via interaction with CXCL1, CXCL2, CXCL8 and NF-κB pathway [11]; meanwhile, AKIP1 intercorrelate with CXCL1, CXCL2, and is implicated in prostate cancer treatment outcomes [14]; Furthermore, AKIP1 also relates to CXCL1, CXCL2, which exhibits potential to be predictor for disease progression and survival [13]. More encouragingly, CXCL families are well-known for their tumor promoting roles in numerous cancers including GBM. For instance, CXCL1 promotes GBM proliferation and radiation resistance via activating NF-κB signaling [21]; CXCL2 upregulation is related to unfavorable outcomes in GBM; CXCL8 interacts with NF-κB pathway then involves in the treatment response and recurrence of GBM [22-24].

Considering the regulation network among AKIP1 and CXCL families (especially CXCL1, CXCL2 and CXCL8), as well as their involvements in carcinogenic pathways such as NF-κB, AKT, β-catenin, etc. [7, 10-14, 22-24, 45, 47, 48], we further analyzed their interactions in GBM and effects on GBM malignant behaviors. Interestingly, we observed that AKIP1 positively regulated CXCL1, CXCL2 and CXCL8 expressions in GBM, while the latter three did not affect AKIP1 expression. These might due to the regulation network of AKIP1 on pathways such as NF-κB, AKT and so on, and CXCL1, CXCL2 as well as CXCL8 are closely related to these pathways, thus, AKIP1 positively regulated these three chemokines [7, 10-14, 22-24, 45, 47, 48]. Furthermore, we observed the addition of CXCL1, CXCL2 (the effect was weak) and CXCL8 respectively reversed the effect of AKIP1 knockdown on GBM cell behaviors to some extents by compensative experiments, which suggested that AKIP1 modified GBM malignant behaviors via promoting CXCL1, CXCL2 and CXCL8. These findings might benefit from (1) The inter-correlation of AKIP1 with CXCL1, CXCL2 and CXCL8 in cancers; (2) The modification of CXCL1, CXCL2 and CXCL8 on several critical oncogenic pathways including NF-κB, AKT, etc. [21-28]. In addition, we also discovered that AKIP1 modified GBM chemoradiation sensitivity via promoting CXCL1, CXCL2 and CXCL8, which might due to their inter-regulation relationship and the effects of CXCL families on cancer treatment sensitivities [25, 27, 28]. These findings were subsequently validated by our clinical explorations with GBM patients, which exhibited that AKIP1 expression positively correlated with CXCL1, CXCL2 (weak correlation), CXCL8 in GBM tumor tissues, and they were all elevated in GBM tissue samples compared to LGG tissue samples.

In addition to the above findings, we also tried to investigate the regulated pathways (that involved in the tumor progression and treatment regulation) by AKIP1, CXCL1, CXCL2 and CXCL8, which showed that AKIP1 overexpression activated NF-κB pathway and AKT path-

way, while its knockdown inactivated these pathways; besides, CXCL1, CXCL2 (the effect was weak) and CXCL8 treatments also stimulated these two pathways, then their addition would attenuate the regulation of AKIP1 knockdown on these two pathways as well. These findings were not surprising which were in accordant with previous studies about AKIP1, CXCL1, CXCL2 and CXCL8 on regulating NF-κB and AKT pathways in other cancers apart from GBM [7, 12, 21-28, 45, 48]. Besides, due to the implication of CXCL families with PD-1/PD-L1 linkage [20], as well as the involvement of pathways regulated by AKIP1 and CXCL families in PD-1/PD-L1 linkage [15-17], we also detected whether GBM PD-L1 expression was regulated by AKIP1, CXCL1, CXCL2 and CXCL8. We observed that AKIP1 overexpression increased while AKIP1 knockdown decreased PD-L1 expression in GBM, and CXCL1, CXCL2 (Weak effect) and CXCL8 additions could elevated PD-L1 expression in both treatment-naïve GBM and AKIP1 knockdown treated GBM. These findings might provide more evidence for AKIP1 as a potential treatment target in GBM.

In conclusion, AKIP1 promotes GBM viability, mobility and chemoradiation resistance via regulating CXCL1 and CXCL8 mediated NF-κB and AKT pathways.

Acknowledgements

This work was supported by the Natural Science Foundation of Heilongjiang Province (LH-2020H044), the First Clinical College of Harbin Medical University Foundation (2013Lx03).

Disclosure of conflict of interest

None.

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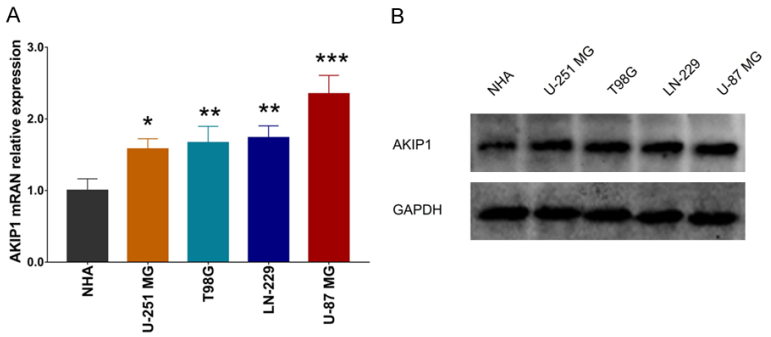
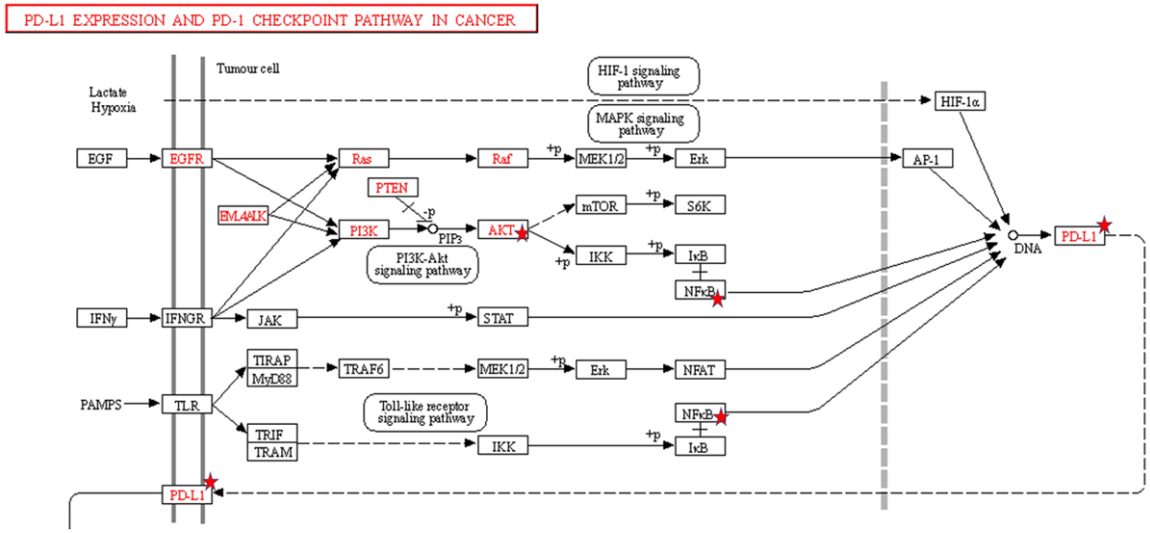
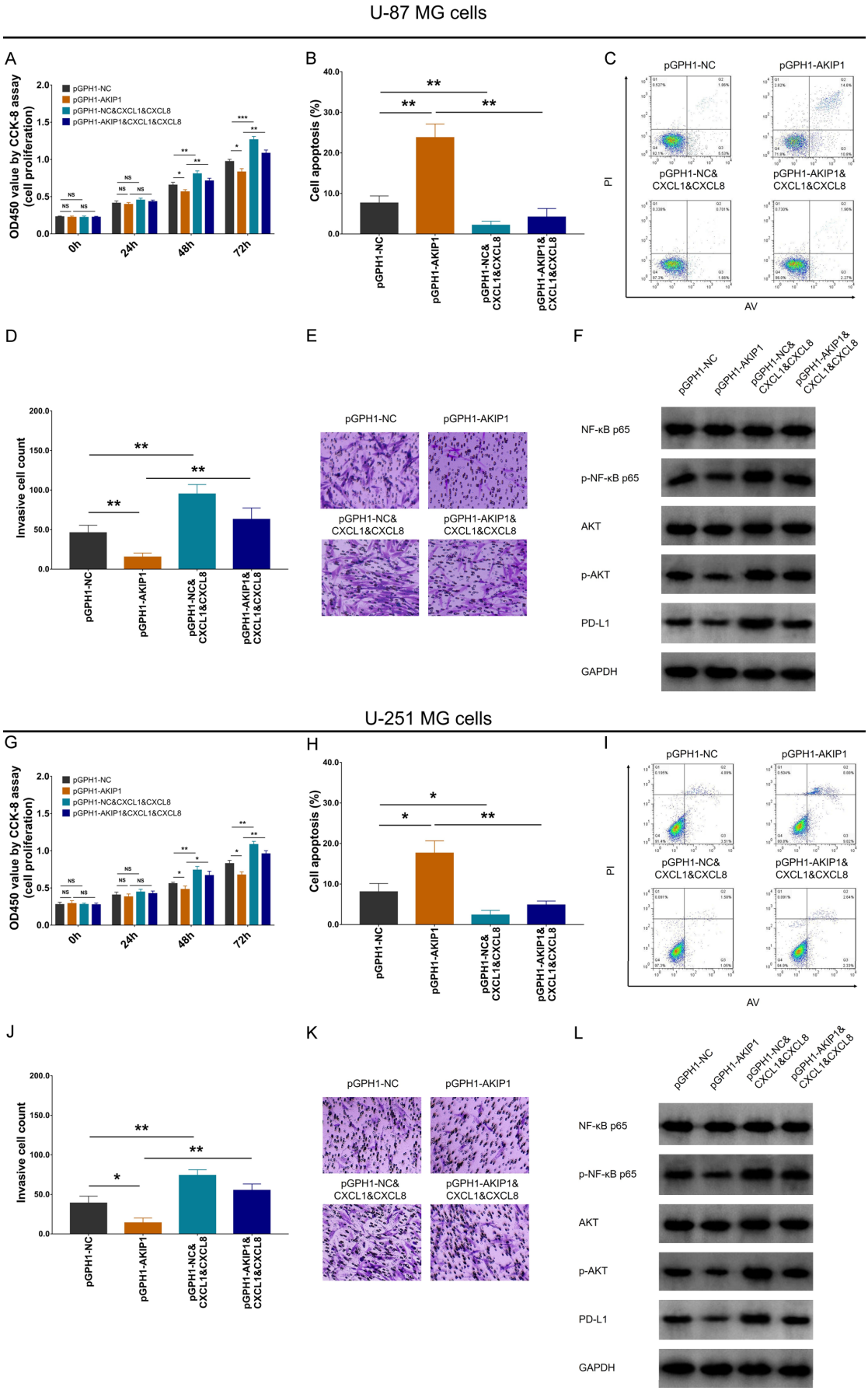


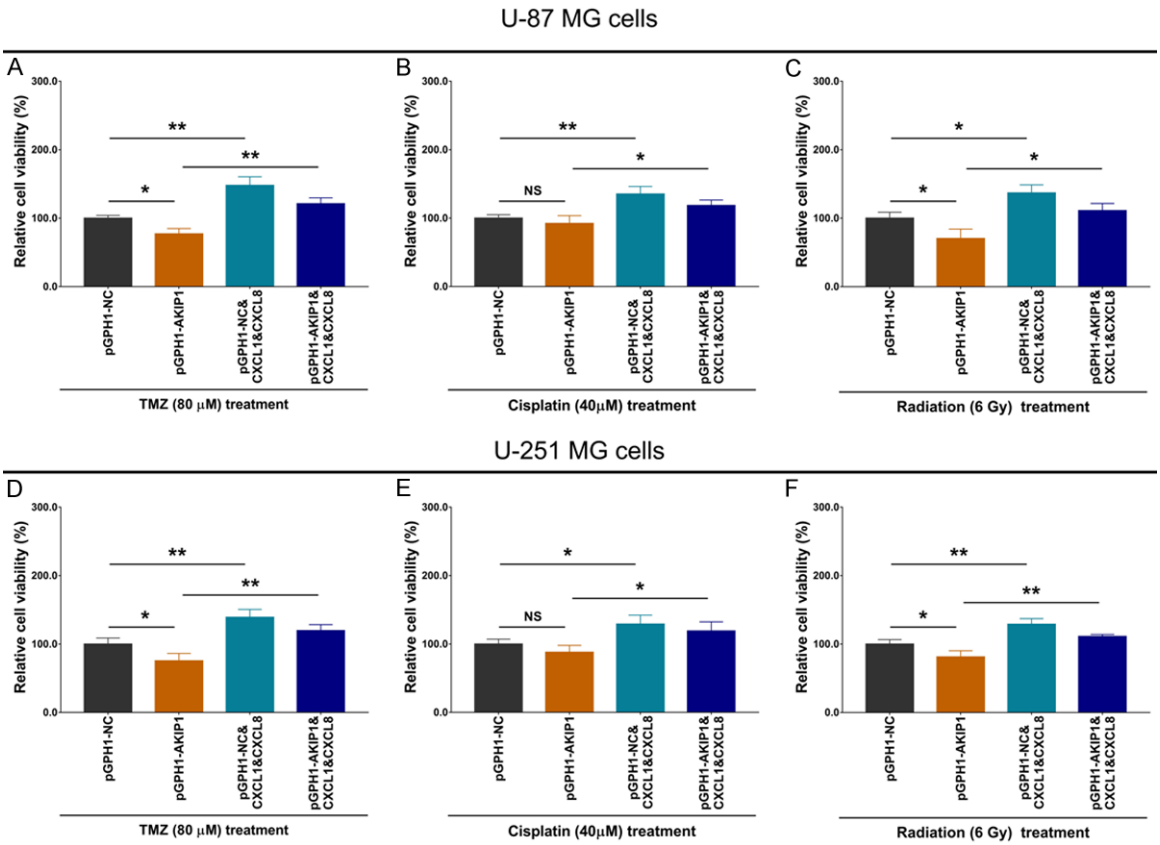
Supplementary Figure 1. AKIP1 expression in GBM cell lines. AKIP1 mRNA expression (A) and protein expression (B) between GBM cell lines and NHA cells (control cell line). AKIP1, A-kinase-interacting protein 1; GBM, glioblastoma multiforme.



Supplementary Figure 2. Interaction between NF-κB/AKT and PD-L1 expression in cancer from Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/). The red stars represented the NF-κB, AKT or PD-L1 in this regulation network.

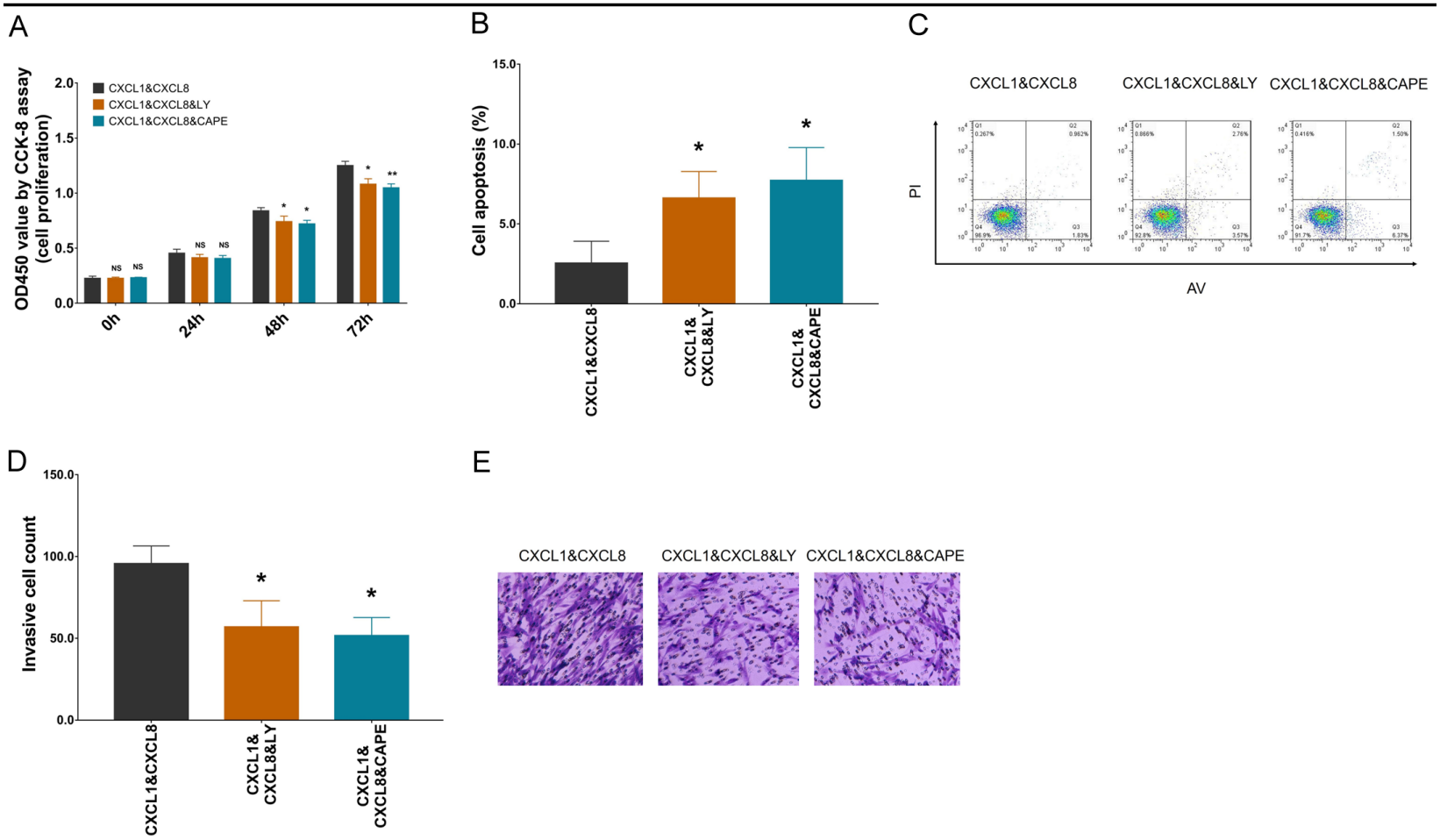

U-87 MG cells
A
OD450 value by CCK-8 assay (cell proliferation)
pGPH1-NC
pGPH1-AKIP1
pGPH1-NC&CXCL1&CXCL8
pGPH1-AKIP1&CXCL1&CXCL8
0h
24h
48h
72h
B
Cell apoptosis (%)
pGPH1-NC
pGPH1-AKIP1
pGPH1-NC& CXCL1&CXCL8
pGPH1-AKIP1& CXCL1&CXCL8
C
pGPH1-NC
pGPH1-AKIP1
pGPH1-NC& CXCL1&CXCL8
pGPH1-AKIP1& CXCL1&CXCL8
PI
AV
D
Invasive cell count
E
F
NF-κB p65
p-NF-κB p65
AKT
p-AKT
PD-L1
GAPDH
U-251 MG cells
G
H
I
J
K
L

Supplementary Figure 3. CXCL1&CXCL8 attenuated the effect of AKIP1 knockdown on GBM malignant behaviors. Cell proliferation, apoptosis and invasion, as well as NF-κB p65, p-NF-κB p65, AKT, p-AKT, PD-L1 expressions among groups in U-87 MG cells (A-F) and U-251 MG cells (G-L), respectively. AKIP1, A-kinase-interacting protein 1; CXCL, C-X-C motif chemokine ligand; GBM, glioblastoma multiforme.



Supplementary Figure 4. CXCL1&CXCL8 attenuated the effect of AKIP1 knockdown on Chemoradiation sensitivity. Relative cell viability under 80 μM TMZ treatment, or 40 μM cisplatin treatment, or 6 Gy radiation treatment among groups in U-87 MG cells (A-C) and U-251 MG cells (D-F), respectively. AKIP1, A-kinase-interacting protein 1; NC, negative control; CXCL, C-X-C motif chemokine ligand; TMZ, temozolomide.

U-87 MG cells


A
OD450 value by CCK-8 assay (cell proliferation)
CXCL1&CXCL8
CXCL1&CXCL8&LY
CXCL1&CXCL8&CAPE
0h
24h
48h
72h
B
Cell apoptosis (%)
CXCL1&CXCL8
CXCL1& CXCL8&LY
CXCL1& CXCL8&CAPE
C
CXCL1&CXCL8
CXCL1&CXCL8&LY
CXCL1&CXCL8&CAPE
PI
AV
D
Invasive cell count
CXCL1&CXCL8
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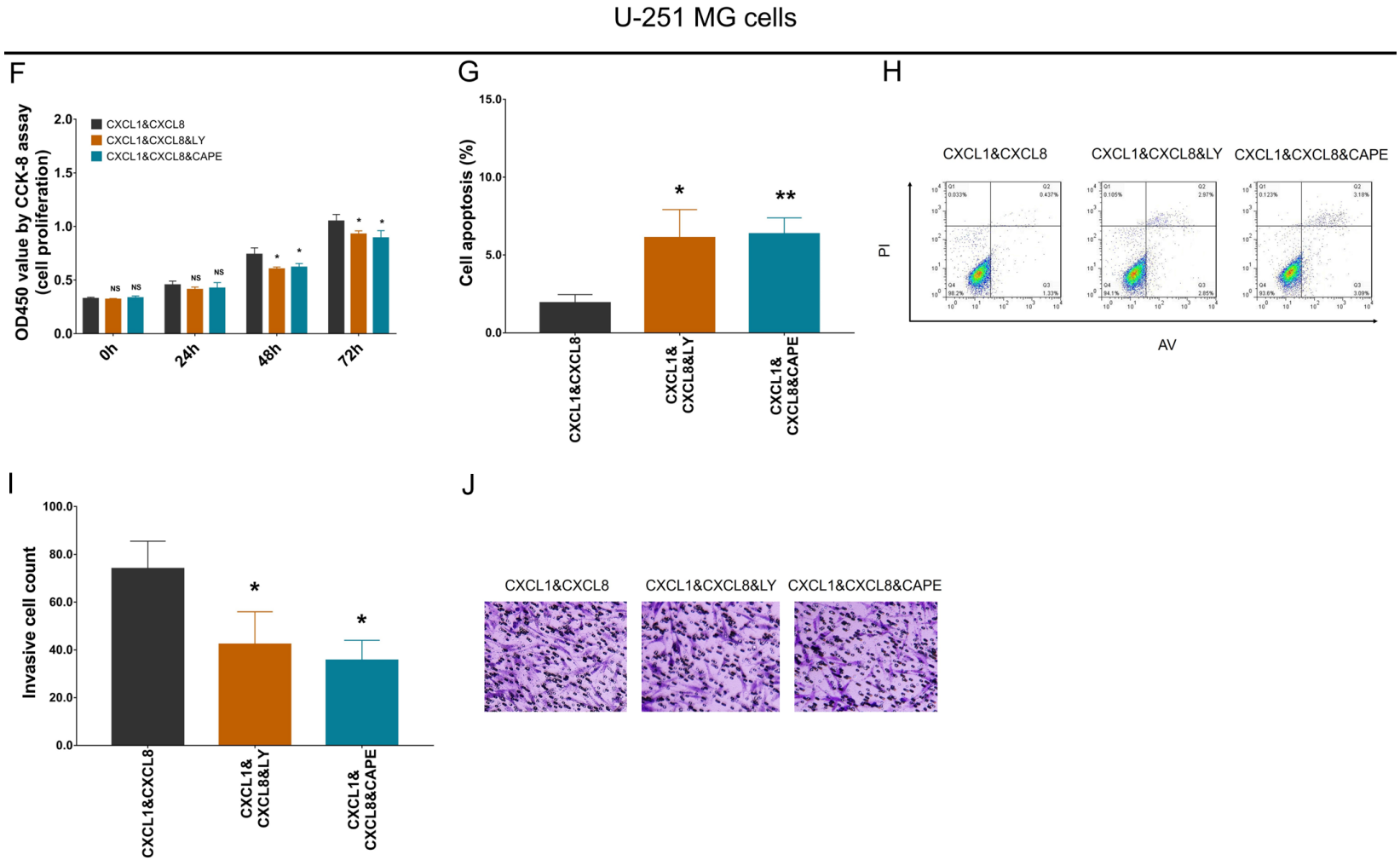
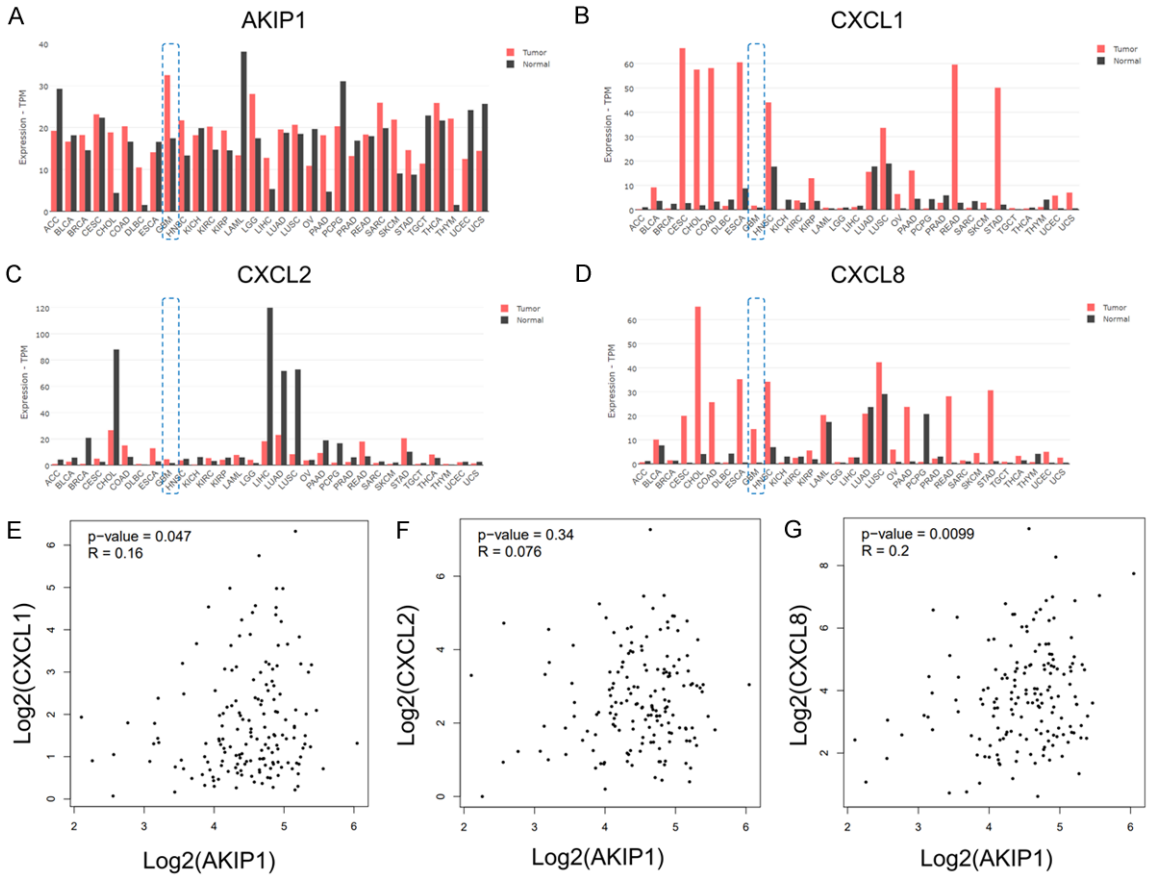
CXCL1& CXCL8&CAPE
E
CXCL1&CXCL8
CXCL1&CXCL8&LY
CXCL1&CXCL8&CAPE



Supplementary Figure 5. NF-κB pathway inhibitor and AKT pathway inhibitor impaired the effect of CXCL1&CXCL8 on GBM malignant behaviors. Cell proliferation, apoptosis and invasion among groups in U-87 MG cells (A-E) and U-251 MG cells (F-J), respectively. CXCL, C-X-C motif chemokine ligand; GBM, glioblastoma multiforme.



Supplementary Figure 6. AKIP1, CXCL1/2/8 expressions via GEPIA database. AKIP1 (A), CXCL1 (B), CXCL2 (C) and CXCL8 (D) expressions in tumor tissue compared to non-tumor tissue in cancers including GBM. Correlation of AKIP1 with CXCL1 (E), CXCL2 (F) and CXCL8 (G) in tumor tissue of GBM. AKIP1, A-kinase-interacting protein 1; CXCL, C-X-C motif chemokine ligand; GBM, glioblastoma multiforme.