

## Original Article

# Effect of silencing S-phase kinase-associated protein 2 on chemosensitivity to temozolomide of human glioma cells U251

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**Abstract:** Objective: To examine the effect of silencing SKP2 on chemosensitivity of human glioma cells U251 to temozolomide (TMZ). Methods: Adenoviruses harbouring shRNA targeting SKP2 (i.e. Ad-shSKP2) and non-targeting scrambled shRNA (i.e. Ad-shNC) were used to infect U251 cells. The transduced cells were then treated with TMZ. Cell viability after treatment was assayed using CCK8; while cell cycle and apoptosis were examined using flow cytometry. To study the effect of silencing SKP2 on autophagy in U251, we co-transduced the cells with Ad-mRFP-LC3 and Ad-shSKP2/Ad-shNC. The expression of autophagy marker LC3 after TMZ treatment was studied using microscopy and Western blotting assays. Results: The cytotoxicity of TMZ (i.e. 20-100  $\mu$ M) was more significantly seen in Ad-shSKP2-transduced U251 cells than in the Ad-shNC-transduced U251 cells. The  $IC_{50}$  values in shSKP2-U251 were significantly lower than those of the shNC-U251 ( $P < 0.05$ ). Both TMZ and Ad-shSKP2 alone increased apoptosis and promoted expression of LC3 in U251. Combined treatment of Ad-shSKP2 and TMZ further elevated apoptosis and LC3 expression. Conclusion: Silencing SKP2 in U251 cells increased chemosensitivity to TMZ that was accompanied with enhanced apoptosis and autophagy. Targeting SKP2 may be a potential approach to potentiate TMZ treatment in patients with glioma.

**Keywords:** Glioma, S-phase kinase-associated protein 2 (SKP2), temozolomide (TMZ), chemosensitivity, autophagy

## Introduction

Glioma is a commonly seen as tumour of the central nervous system. In general, the prognosis of glioma is poor, showing an unacceptable high rate of recurrence after treatment. Surgical intervention alone cannot be curative, so in many cases, radiotherapy and chemotherapy are administered as adjuvant therapies. Temozolomide (TMZ), an oral chemotherapeutic agent that can be permeable across the blood-brain barrier, is the first-line treatment for glioma [1]. However, despite its clinical usefulness, TMZ shows limited efficacy in prolonging the survivals of patients with glioma [2]. Treatment modalities that can potentiate the therapeutic efficacy of TMZ are therefore believed to benefit patients' outcomes [3].

S-phase kinase-associated protein 2 (SKP2) is a key mediator of ubiquitination. By targeting cell cycle control elements like p21 and p27 to protein degradation, SKP2 is involved in the

normal regulation of cell cycle entry and  $G_1/S$  transition [4, 5]. However, deregulated proteolysis by SKP2 is implicated in cancer biogenesis [6]. SKP2 was also shown to reactivate the PI3K-AKT signalling pathway, which has already been inhibited by PI3K inhibitors, in breast cancer [7]. The oncogenic functions of SKP2 were also demonstrated in hepatocellular carcinoma [8], colorectal cancer [9], papillary thyroid cancer [10], and gastric cancer [11]. In human glioma, the expression of SKP2 was found up-regulated, of which the overexpressed level was associated with a poor prognosis of patients [12, 13]. Silencing SKP2 suppressed the proliferation and invasive property of human glioma cells U251 *in vitro* [14]. Downregulation of SKP2 also resulted in growth arrest and apoptosis of T98G glioblastoma cells [15]. In view of these findings, the present study was aimed to further investigate the role of SKP2 in glioma by examining the silencing effect of SKP2 on cellular sensitivity to TMZ.

## Materials and methods

### Cell culture and reagents

Human glioma astrocytoma cells U251 were revived from the cell bank of our research institute and were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified incubator (37°C, 5% CO<sub>2</sub>). Adenoviruses harbouring shRNA targeting SKP2 gene (i.e. Ad-shSKP2) and shRNA of scramble sequence (i.e. Ad-shNc) were prepared and validated in a previous study (12). Both shRNA plasmids contained GFP open reading frame, allowing the transduction efficacy to be studied by GFP fluorescence microscopy. Adenovirus expressing RFP-LC3 fusion protein (i.e. Ad-mRFP-LC3) was obtained from Hanbio (Shanghai, China). The use of this fusion protein construct permitted the detection of LC3 level by measuring RFP signal using microscopy. TMZ was purchased from MedChem Express (Monmouth Junction, NJ, USA), and was prepared as a 0.1 M stock solution with dimethyl sulfoxide (DMSO). SKP2 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). LC3 antibody was obtained from Cell Signaling Technology (Danvers, MA, USA).

### U251 sensitivity towards TMZ

Single-cell suspension of U251 (1 × 10<sup>5</sup> cells/mL) was transduced with Ad-shSKP2 or Ad-shNc with a MOI of 20. Twelve hours after the transduction, U251 cells were treated with different concentrations of TMZ (10, 20, 40, 60, 80 and 100 μM) for 24, 48 and 72 hours. Cells treated with 1% DMSO served as the vehicle control. An empty well was also included for baseline measurement. Cell viability at the designated time points was determined using CCK8 assays, with the results read at an optical density of 450 nm. The sensitivity of U251 to TMZ was presented as the percentage of cell inhibition, which was calculated as  $(OD_{\text{vehicle control}} - OD_{\text{test sample}}) / (OD_{\text{vehicle control}} - OD_{\text{baseline}}) \times 100\%$ .

### Cell cycle and apoptosis analysis

U251 cells were transduced with Ad-shSKP2 or Ad-shNC. After 24 hours, transduced cells were treated with 100 μM TMZ or 1% DMSO for 48 hours, and then fixed with ice-cold 70% ethanol at 4°C overnight. The fixed cells were washed

with PBS, stained with propidium iodide (PI) solution (50 μg/mL PI, 100 μg/mL RNase A, 0.2% w/w Triton X-100) at 4°C for 30 minutes, and analysed by flow cytometry.

### Autophagy analysis

U251 cells (1 × 10<sup>5</sup> cells/mL) were seeded onto glass slides placed in 24-well plates and were transduced with Ad-mRFP-LC3 with a MOI of 50. The transduced cells were then further transduced with Ad-shSKP2 or Ad-shNC with a MOI of 20. After 24 hours, the transduced cells were treated with 100 μM TMZ or 1% DMSO for 24 hours. The treated cells were fixed with 4% paraformaldehyde, washed with PBS, and stained with 5 μg/mL DAPI. Expression of LC3, which is an autophagy marker, was studied by determining mRFP signal.

### Western blotting assays

Whole-cell lysate was prepared on ice using cell lysis buffer supplemented with protease inhibitors, and was subsequently resolved using SDS-PAGE. The resolved proteins were transferred onto PVDF membrane, which was then blocked with 5% non-fat milk at ambient temperature for 1 hour. The blocked membrane was incubated with antibody against SKP2 (1:500 dilutions), LC3 (1:1000 dilutions), and α-tubulin (1:1000 dilutions) at 4°C for overnight. After washing, the antibody-probed membrane was incubated with HRP-conjugated anti-rabbit (1:10000 dilutions) or anti-mouse (1:10000 dilutions) at room temperature for 2 hours. Signals were finally detected using ECL solution, with the image captured with the Bio-Rad imaging system.

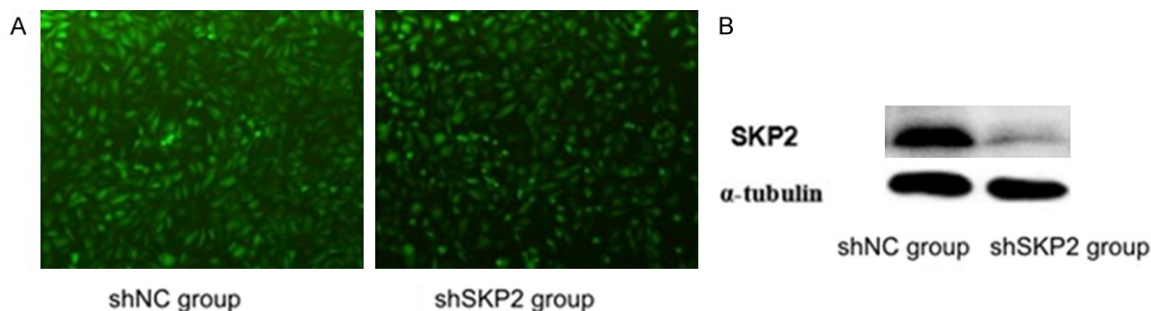
### Statistical analysis

All measurement data were presented as mean ± standard derivation ( $\bar{x} \pm S.D.$ ), and analysed using SPSS version 20. Differences between treatment groups were evaluated using t-test or one-way ANOVA whenever appropriate, with statistically significant difference indicated by P value < 0.05.

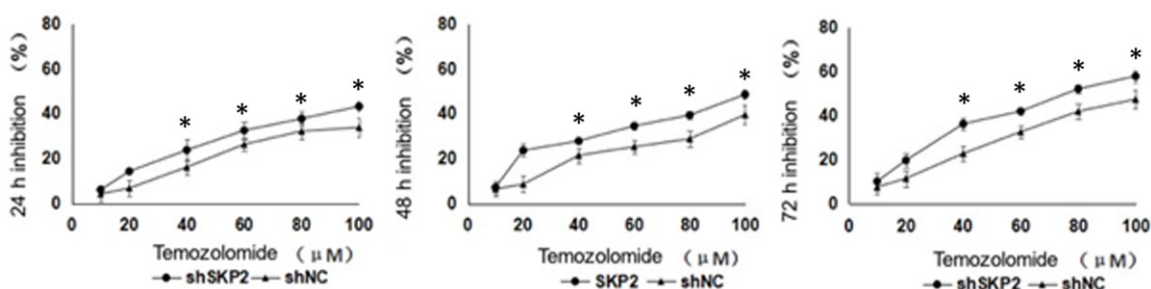
## Results

### Knockdown of SKP2 in U251

The transduction efficiency and SKP2 knockdown in U251 was assessed using GFP micros-



**Figure 1.** Transduction of human glioma cell U251 with Ad-shSKP2 (shSKP2 group) and Ad-shNC (shNC group). A. At 24 hours after transduction, the efficiency was examined by counting GFP-positive cell under microscope. B. At 48 hours after transduction, the down-regulation of SKP2 in shSKP2 group was examined using Western blotting assays. Shown is the representative set of data from 3 independent experiments.



**Figure 2.** Inhibitory effect of TMZ on Ad-shSKP2 or Ad-shNC-transduced U251 at time points of 24, 48, and 72 hours. Asterisks indicate significant differences in cell viability ( $P < 0.05$ ) between the two treatment groups. Shown is the representative set of data from 3 independent experiments.

copy and Western blotting assays, respectively (**Figure 1**). Twenty-four hours after transduction, successful transduction of U251 with the adenovirus was confirmed by the GFP signal in both Ad-shSKP2 and Ad-shNC transduced U251 (**Figure 1A**). The protein level of SKP2 in Ad-shSKP2-transduced cells was found significantly reduced compared to that of the Ad-shNC transduced, as shown by the Western blot of whole-cell lysate collected at 48 hours after transduction (**Figure 1B**).

#### Effect of SKP2 KD on TMZ sensitivity of U251

The inhibition of cell viability of Ad-shSKP2 and Ad-shNC-transduced U251 by TMZ was examined using CCK8 assay (**Figure 2**). At all time points tested, at the lowest concentration of TMZ (i.e. 10 μM), there were no significant differences in cell viability inhibition between Ad-shSKP2 and Ad-shNC-transduced U251. At higher concentrations of TMZ ( $\geq 20$  μM), TMZ treatment caused more significant death of Ad-shSKP2-U251 than Ad-shNC-U251. Treat-

ment of Ad-shSKP2-U251 with TMZ for 24, 48, and 72 hours resulted in  $IC_{50}$  of  $128 \pm 6$  μM,  $119 \pm 15$  μM, and  $75 \pm 4$  μM, respectively. Treatment of Ad-shNC-U251 with TMZ for 24, 48, and 72 hours resulted in  $IC_{50}$  of  $173 \pm 49$  μM,  $174 \pm 26$  μM, and  $111 \pm 10$  μM, respectively. The  $IC_{50}$  values in shSKP2-U251 were significantly lower than those of the shNC-U251 ( $P < 0.05$ ).

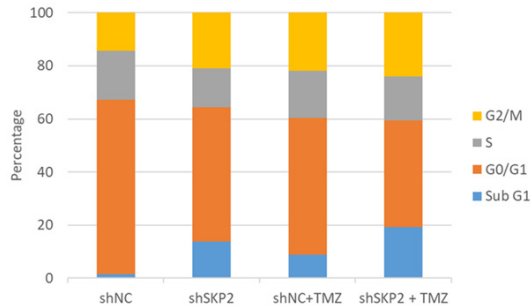
#### Effect of SKP2 KD and TMZ on U251 cell cycle and apoptosis

Cell cycle and apoptosis of shRNA-treated U251, either alone or in combination with TMZ, was examined using flow cytometry (**Table 1**; **Figure 3**). Compared to Ad-shNC-U251, Ad-shSKP2-U251, TMZ-treated Ad-shNC-U251 and TMZ-treated Ad-shSKP2-U251 displayed significant increase in sub- $G_1$  phase ( $P < 0.05$ ), which was accompanied with substantial decrease in  $G_0/G_1$  ( $P < 0.05$ ). Notably, SKP2 KD combined with TMZ treatment resulted in a higher portion of sub- $G_1$  than in SKP2 KD and TMZ treatment alone ( $P < 0.05$ ).

**Table 1.** Distribution of cell cycle phases of U251 receiving different treatments

	SUB	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
shNC	1.53 ± 1.05	65.63 ± 1.80	18.57 ± 2.12	14.27 ± 2.70
shSKP2	13.73 ± 1.90 <sup>a</sup>	50.67 ± 1.42 <sup>a</sup>	14.63 ± 2.04	21.80 ± 1.80 <sup>a</sup>
shNC+TMZ	8.77 ± 1.33 <sup>a,b</sup>	51.63 ± 3.44 <sup>a</sup>	17.67 ± 1.11	21.93 ± 3.69 <sup>a</sup>
shSKP2+TMZ	19.23±1.40 <sup>a,b,c</sup>	40.13 ± 4.69 <sup>a,b,c</sup>	16.63 ± 1.25	25.00±3.47 <sup>a</sup>

a, *P* < 0.05 compared to shNC group; b, *P* < 0.05 compared to shSKP2 group; c, *P* < 0.05 compared to shNC+TMZ group.



**Figure 3.** Effect of Ad-shSKP2 and TMZ on cell cycle and apoptosis was studied using flow cytometry. Shown is the representative set of data from 3 independent experiments.

*Effect of SKP2 KD and TMZ on autophagy marker expression*

The red signal of mRFP was seen in most Ad-mRFP-LC3-transduced U251 (**Figure 4A**). Compared to those co-transduced with Ad-shNC, U251 co-transduced with Ad-shSKP2 or treated with TMZ presented more mRFP-positive counts (*P* < 0.05). Ad-shSKP2 transduction combined with TMZ treatment further increased the number of mRFP-positive cells compared to other groups (*P* < 0.05) (**Figure 4B**). LC3-I is the cytosolic form of LC3. It is conjugated to phosphatidylethanolamine to form LC3-II. LC3-II is then recruited to autophagosomal membranes. Therefore, LC-II serves as the marker of autophagy. Western blot also revealed the combined treatment with Ad-shSKP2 and TMZ led to a substantial increase in LC3-II protein (**Figure 4C**).

**Discussion**

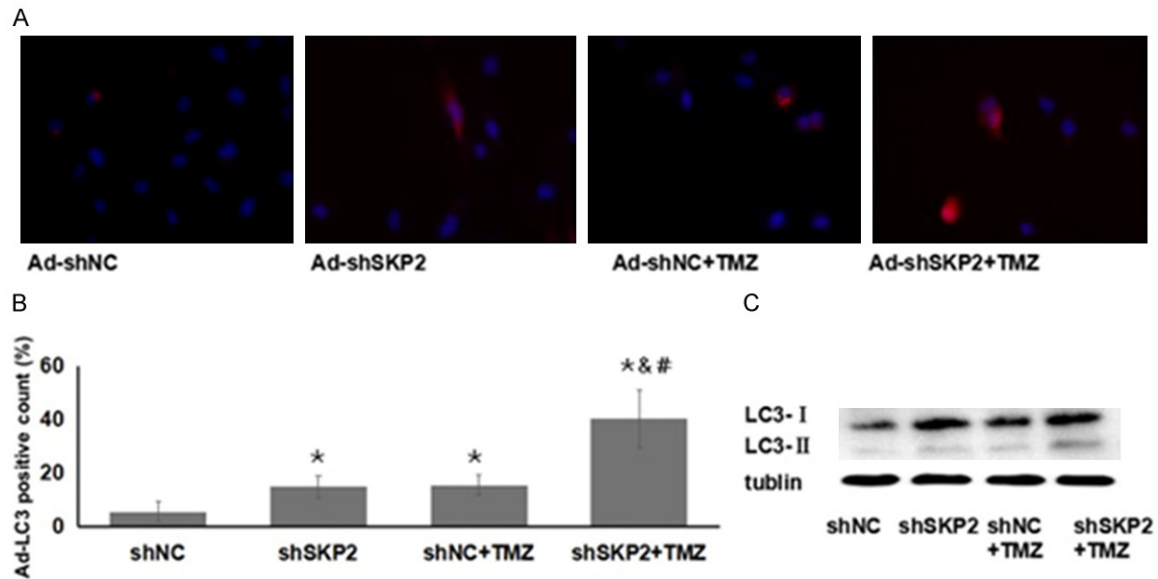
TMZ is an alkylating agent that kills cancer cells by inhibiting DNA synthesis through incorporation of base mismatches. However, cancer cells can overcome DNA methylation and hence

become resistant to TMZ with their DNA repair machinery [16-18]. The present study demonstrated for the first time that silencing SKP2 could potentiate the cytotoxicity of TMZ on human glioma cell U251. The increase in TMZ sensitivity was attributed to apoptosis (as shown by

increases in sub-G<sub>1</sub> phase) and autophagy (as revealed by upregulations in LC3-II protein) induced by SKP2 silencing. Our findings might provide some clues into new treatment modalities that can enhance the therapeutic efficacy of TMZ in glioma treatment.

Many chemotherapeutic drugs including TMZ eradicate cancer cells by inducing apoptosis and autophagy [19]. However, in glioma, due to the presence of blood brain barrier, TMZ concentration may not reach the level sufficient for apoptosis induction. In addition, many PTEN mutant gliomas can overcome TMZ-induced apoptosis by activating AKT signalling [20]. Therapeutic approaches that sensitize glioma to chemotherapeutics are therefore urgently needed. Targeting SKP2 was shown to suppress glioma cells *in vitro*. The present study further showed that silencing SKP2 in glioma would enhance the sensitivity of cancer cells to TMZ because TMZ IC<sub>50</sub> of Ad-shSKP2-transduced U251 was significantly lower than that of the Ad-shNC-transduced. Indeed, the role of SKP2 in drug sensitivity has been studied in different types of cancers. In paclitaxel-resistant prostate cancer, destabilization and degradation of SKP2 by a novel selenonucleoside was recently shown to recover the drug sensitivity *in vitro* and *in vivo* [21]. In tamoxifen-resistant breast cancer cell line MCF-7, the induction of apoptosis by a plant extract was attributed to the suppression of SKP2 [22]. The implication of SKP2 in chemoresistance in lung cancer was also reported [23].

Induction of autophagy by TMZ has been accepted as a therapeutic approach for treatment of glioma [3]. This study clearly showed that an equivalent amount of TMZ resulted in higher autophagy marker LC3 expression in Ad-shSKP2-transduced than in Ad-shNC-



**Figure 4.** Effect of Ad-shSKP2 and TMZ on autophagy marker expression was examined. A. The red signal of LC3 of different treatment groups at 48 hour after transduction was examined under the microscope. Red, mRFP of LC3; Blue, DAPI; magnification,  $\times 400$ . B. The percentage of L3-positive cells in each treatment group was determined and compared. \* $P < 0.05$  compared to shNC group; # $P < 0.05$  compared to shNC+TMZ. C. The up-regulation of LC3-II by the combined treatment of shSKP2 and TMZ was examined using Western blotting assays. Shown is the representative set of data from 3 independent experiments.

transduced U251. SKP2 is a key signalling mediator of ubiquitination. In addition to its substantial roles in determining cell cycle and apoptosis, SKP2 is an important component of AMPK-SKP2-CARM1 signalling cascade for transcriptional regulation of autophagy. Down-regulation of SKP2 could elevate CARM1, ultimately leading to autophagy [24].

Whether autophagy sensitizes glioma to TMZ or vice versa remains to be studied. Previous published works showed that enhancing autophagy in glioma would promote cell chemosensitivity to TMZ [3, 18, 25]; however, there were studies illustrating that chemosensitivity to TMZ was increased by inhibiting autophagy [26, 27]. Nevertheless, our data collectively revealed that silencing SKP2 could sensitize glioma cells to TMZ that was accompanied with an increase in autophagy marker expression. Studies will be conducted to decipher the underlying mechanism by which SKP2 regulates TMZ chemosensitivity in glioma.

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#### Disclosure of conflict of interest

None.

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