

# Acquisition of temozolomide resistance by the rat C6 glioma cell line increases cell migration and side population phenotype

YA XU<sup>1\*</sup>, XIANGCAI YANG<sup>1\*</sup>, SHUTING MEI<sup>1</sup>, YI SUN<sup>1</sup> and JIEJING LI<sup>1</sup>

Department of Clinical Laboratory, The Affiliated Hospital of KMUST, Medical Faculty, Kunming University of Science and Technology, Kunming, Yunnan 650032, P.R. China

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**Abstract.** Cancer stem cells are reportedly associated with drug resistance in glioma, but there are conflicting findings on the effects of cancer stem cells on drug resistance. The aim of the present study was to identify the underlying mechanisms of drug resistance in rat C6 glioma cells, through the use of Transwell invasion assays, flow cytometric and western blot analyses as well as immunohistochemical staining. The results revealed that acquisition of drug resistance by C6 cells enhanced migration ability *in vivo* and *in vitro*. Notably, drug resistance did not depend on the cancer stem cells of C6 cells, but on the increase of a side population phenotype. Blockade of the ABC transporter could increase sensitivity to temozolomide and temozolomide-induced apoptosis in C6 cells. Collectively, these data indicated that drug resistance of C6 cells was mediated by the side population phenotype rather than by cancer stem cells.

## Introduction

Glioma are the most aggressive brain tumors, arising from glial cells. Despite considerable advances in treatment, resistance and relapse of brain tumors remain a significant clinical challenge. Current treatments for glioma patients include surgery, radiotherapy and chemotherapy. Although a combined approach may eradicate most of a glioma mass, the ability of cancer cells to become concurrently resistant to different drugs remains a significant setback to successful chemotherapy. A clearer understanding of the underlying mechanisms of drug

resistance is required to identify novel therapeutic targets and improve treatment efficacy.

Cancer stem cells (CSCs) likely contribute to chemotherapy resistance (1). CSCs are a slow-dividing, small proportion of cancer cells with self-renewal, differentiation, and propagation abilities. CSCs were first conceptualized in human myeloid leukemias (2), where CD34<sup>+</sup> and CD38<sup>-</sup> cells could engraft severe combined immune-deficient (SCID) mice to produce large numbers of colony-forming progenitors with characteristics of stem cells (3). Following this, CSCs were identified in solid tumors including breast (4), prostate (5), pancreas (6), colon (7), brain (8), as well as other cancers. Brain tumor stem cells *in vitro* are able to form tumor spheres and express neural stem cell (NSC) markers CD133, nestin, Sox2, as well as others. *In vivo*, they can initiate tumors. Singh *et al* revealed that a CD133<sup>+</sup> cell subpopulation from human brain tumors exhibited stem cell properties *in vitro* and could initiate tumor development in SCID mouse brain (8,9). Conventional chemotherapy may eradicate most susceptible cells in a tumor, but leave the CSCs intact, resulting in development of resistance.

Although CSCs can self-renew, most are generally quiescent, spending most of their time in the G0 cell cycle phase. Since chemotherapeutic drugs are designed to target either the cell cycle or rapidly dividing cells, this contributes to the capacity of CSCs for drug resistance. A resistance to apoptosis, activation of detoxification mechanisms, and a capacity for DNA repair are also contributing factors (10-12). Glioma CSCs also exhibit active efflux of chemotherapeutic drugs through the cellular membrane (13), attributable to drug transportation such as occurs during overexpression of the adenosine triphosphate-binding cassette (ABC) superfamily. The ABC superfamily mainly includes P-glycoprotein [P-gp, also known as multidrug resistance protein 1 (MDR1)], multidrug resistance associated protein (MRP), and breast cancer resistance protein (BCRP). These transporters are able to actively efflux certain dyes out of cells. Due to the characteristics of ABC transport, flow cytometry is used to detect a side population (SP) on the basis of the ability of these cells to efflux Hoechst 33342 dye and a combination of surface marker expression. High levels of MDR1 RNA are often associated with resistance to chemotherapy in neuroblastoma, suggesting a contribution (14). Furthermore, high expression levels of ABC drug transporters are a unique feature of stem cells (15). The identification of ABC gene expression in CSCs has led to

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*Correspondence to:* Professor Jiejing Li, Department of Clinical Laboratory, The Affiliated Hospital of KMUST, Medical Faculty, Kunming University of Science and Technology, 157 Jinbi Road, Xishan, Kunming, Yunnan 650032, P.R. China  
E-mail: jjli@kmust.edu.cn

\*Contributed equally

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attempts to use this to isolate or characterize them. SP cells in glioma cell lines are able to form spheres, and have abilities of self-renewal, multi-lineage differentiation, and tumorigenicity, representing properties of CSCs (16). Stem cells are predominantly found in the SP fraction. The SP phenotype then is exploited to identify stem-like cells (17). Hirshmann-Jax *et al* first demonstrated that neuroblastoma SP cells are less sensitive to mitoxantrone (18). Moreover, the cancer progenitor-like cells isolated using SP have been revealed to be increased following treatment with temozolomide (TMZ), which indicates the existence of drug resistance in progenitor-like cells (19). CD133<sup>+</sup> CSCs from glioblastoma display significant resistance to conventional chemotherapeutic agents, which may be correlated with overexpression of drug resistance genes such as BCRP1 and DNA-mismatch repair genes such as MGMT, as well as genes related to inhibition of apoptosis in CD133<sup>+</sup> CSCs (20). Although various mechanisms involved in the drug resistance of glioma cells have been reported, their precise actions are still not fully understood.

Notably, CD133<sup>+</sup> is not the only characteristic of CSCs. CD133<sup>-</sup> cells derived from six different human patients were tumorigenic when implanted into brains of nude rats and gave rise to CD133<sup>+</sup> cells in rats (21). In addition, when the ABC transporters ABCG2 (BCRP1), ABCB1 (MDR1), or ABCC1 are knocked out in mice, the mice remain viable, fertile, and have normal stem cell compartments (22-24). These results suggest that the SP phenotype is not necessary for the maintenance of CSCs. Moreover, not all cells in the SP compartment are stem cells, and non-stem cells often exhibit high expression of ABC transporters such as ABCG2 (BCRP1) and ABCB1 (MDR1) (23). Non-SP cells are able to generate SP cells and have tumor-initiating capacity as SP cells (25-28).

The rat C6 glioma cell line is a model for studying cell growth and invasion, and has been intensively studied for decades (29). However, the results in C6 cells are contradictory. Zhou *et al* revealed that only a small fraction (4.02%) of C6 cells were CSCs that could form tumor spheres in a simplified serum-free neural stem cell medium and express CD133 and nestin (30). In addition, Kondo *et al* suggested that only 0.4% of C6 cells could be considered CSCs in a complicated serum-free medium (31). Zheng *et al* revealed that most C6 cells were CSCs, although numerous cells were neither CD133<sup>+</sup> nor a side population (32,33). These conflicting findings underscore the need to improve methods of identifying CSCs and exploring the precise mechanisms of drug resistance in glioma cells. Therefore, C6 cells were used as an experimental model and their involvement in drug resistance was investigated.

## Materials and methods

**Cell culture.** Rat C6 glioma cells were provided by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Rat C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and pyruvate, with 10% fetal bovine serum plus antibiotics penicillin and streptomycin (serum was obtained from HyClone; GE Healthcare Life Sciences, while the other reagents were from Invitrogen; Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained in T25 tissue culture flasks at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Confluent

cells were harvested by washing in phosphate-buffered saline (PBS) followed by trypsinization (0.25% in EDTA) for subculture. For the generation of TMZ-resistant C6 subclones, parental C6 cells were treated with 35 μM TMZ for seven generations. During this period, dead cells were discarded and the remaining living cells were prepared to passage and the next exposure to TMZ. Resistant subclones were generated until there were no more dead cells. Verapamil hydrochloride (Sigma-Aldrich; Merck KGaA) 250 μM was used to block ABC transporters.

**Tumor sphere culture.** C6 cells were plated in 25-cm<sup>2</sup> tissue culture flasks at clonal density (2,500-5,000 cells/cm<sup>2</sup>) in serum-free medium containing 20 ng/ml epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) (both from Sigma Aldrich; Merck KGaA), and B27 supplement (50X; Gibco; Thermo Fisher Scientific, Inc.). Four days later, floating tumor spheres were observed and collected.

**Invasion assays.** Transwell membranes were precoated with 24 mg/ml Matrigel (R&D Systems, Inc.), and cells were incubated for 8 h. Cells on the seeded side were removed with a cotton swab, and cells adhering to the lower surface were fixed with 100% methanol at -20°C for 10 min, stained with 3% Giemsa solution at room temperature for 15 min and counted under a microscope in five randomly selected fields at a magnification of x200.

**Evaluation of C6-PL3 and C6 cell invasion in rats.** A rat glioma model was established to test the *in vivo* effect of C6 drug resistance on cell invasion. Six adult male Sprague-Dawley rats weighing between 250-300 g were used (three rats/group). Animals were maintained at a constant room temperature (24°C) and 50% relative humidity with free access to food and water under a 12-h light/dark cycle. They were anesthetized with intraperitoneal injection of sodium pentobarbital (Nembutal; 50 mg/kg) before surgery. Following administration of sodium pentobarbital, rats became ataxic, lost their righting reflex, had no responses to pain and eventually remained immobile. C6-PL3 and C6 cells were orthotopically injected to the right striatum of Sprague-Dawley rat brains through a pre-settled stainless-steel tube. Rat health and behaviour were monitored every 2 days. Rats were sacrificed by anesthesia overdose with intraperitoneal injection of sodium pentobarbital (Nembutal; 200 mg/kg) 20 days after injection and their brain tissues were removed and embedded immediately in optimal cutting temperature compound as previously described (34). Brain tissues were subsequently cut into 7-μm slices using a Leica CM1850 cryostat microtome. Slices were fixed with 95% ethanol and stained with hematoxylin and eosin (H&E; Sigma-Aldrich; Merck KGaA). After sealing with neutral mounting medium (Sigma-Aldrich; Merck KGaA), the slices were observed under an Olympus upright and inverted microscope (Olympus IX73PIF) at a magnification of x400.

**Ethical statement.** Methods involving live animals were carried out in accordance with the guidelines and regulations enacted and enforced by the Chinese National Ministry of Science and Technology as well as the National Ministry of Health. All experimental protocols were approved by the Institutional Lab

Animal Ethics Committee at Kunming University of Science and Technology (Kunming, China).

**Flow cytometry.** BCRP1 and MDR1 ABC transporters allow the active efflux of Hoechst 33342 dye, which is a feature that defines pluripotential SPs. To determine whether drug resistance in C6-PL3 cells was involved in the SP phenotype, cells were stained with Hoechst 33342 fluorescent dye. Verapamil, a calcium channel blocker and non-specific inhibitor of ABC transporters, can inhibit SP generation. To analyze SP production, harvested C6 cells were adjusted to  $10^6$  cells/ml in pre-warmed DMEM containing 2% fetal bovine serum and 10 mM HEPES buffer, and incubated for 90 min in a 37°C water bath with Hoechst 33342 at 2.5  $\mu$ g/ml final concentration prepared from 1 mg/ml stock of the dye dissolved in water (Invitrogen; Molecular Probes; Thermo Fisher Scientific, Inc.) and with intermittent mixing. Control cells were treated with verapamil (50  $\mu$ M final concentration, in 95% ethanol; from Sigma-Aldrich; Merck KGaA). At the end of incubation, the cells were centrifuged at 300 x g for 5 min under refrigeration and then adjusted to  $3 \times 10^6$  cells/ml in PBS containing 2% fetal bovine serum and 10 mM HEPES.

Apoptosis was assessed using an Annexin V Apoptosis Detection kit (BD Biosciences) according to the manufacturer's instructions. Fluorescence intensity was measured using a BD FACS Vantage SE flow cytometer. Original data were analyzed using WinMDI 2.9 software (Windows Multiple Document Interface for Flow Cytometry) and presented in the form of dot plots, with fluorescein isothiocyanate (FITC)-conjugated Annexin V as the x-axis and PI (propidium iodide) as the y-axis.

**Western blot analysis.** Cells were prepared using a RIPA Lysis Buffer (product no. P0013B; Beyotime Institute of Biotechnology) in the presence of a proteinase inhibitor cocktail (cOmplete™, mini; Roche Diagnostics), then centrifuged at 4°C for 15 min at 13,201 x g. Protein concentration was assessed using the BCA protein assay kit (Applygen Technologies, Inc.). Proteins (50  $\mu$ g) were separated on a 12% SDS polyacrylamide gel and transferred to a 0.20- $\mu$ m PVDF membrane (EMD Millipore). Membranes were blocked at room temperature with 5% BSA for 2 h and incubated with the following primary antibodies: Nestin (dilution 1:2,000; cat. no. 33475), Sox2 (dilution 1:1,000; 3579), Bmi-1 (dilution 1:2,000; cat. no. 6964), BCRP1 (dilution 1:2,000; cat. no. 42078) and MDR1 (dilution 1:2,000; cat. no. 13342; all from Cell Signaling Technology, Inc.) and  $\beta$ -actin (dilution 1:5,000; product no. A5316; Sigma-Aldrich; Merck KGaA) for overnight at 4°C. Detection was carried out using HRP-conjugated secondary antibodies (cat. no. 7076, anti-mouse IgG, 1:10,000; and cat. no. 7074, anti-rabbit IgG, 1:10,000; all from Cell Signaling Technology, Inc.) for 2 h at room temperature and enhanced chemiluminescence substrate (GE Healthcare).

**Immunofluorescence.** C6-PL3 and C6 cells were cultured on Lab-Tek chamber slides (Sigma-Aldrich; Merck KGaA) for 24 h, then fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.4% Triton X-100 for 15 min at room temperature. Cells were then blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) for 1.5 h and incubated with nestin primary antibodies (dilution

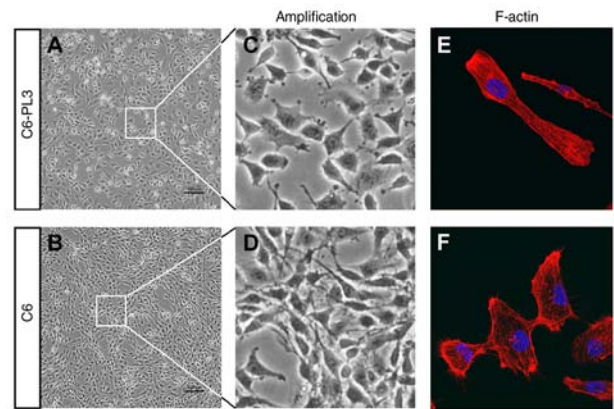


Figure 1. Phase-contrast microscopy indicated morphological changes in (A and C) temozolomide-resistant rat glioma cells (C6-PL3) and (B and D) parental rat glioma cells (C6 cells). Representative images of (E) C6-PL3 cells and (F) C6 cells under F-actin staining. Images of F-actin were obtained at a magnification of x400.

1:400, cat. no. 556309; BD Transduction Laboratories™) at 4°C overnight. They were subsequently incubated with PE-conjugated secondary antibodies (cat. no. sc-516191; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h and labeled with DAPI (Sigma-Aldrich; Merck KGaA) for 5 min to identify cell nuclei. Fluorescence was detected using an Olympus IX81SIF-3 laser confocal scanning microscope.

**Statistical analysis.** All experiments were repeated at least three times prior to statistical analysis. All experimental data are presented as the mean  $\pm$  SEM. Differences between samples were analyzed using a two-tailed Student's t-test and regarded as statistically significant at  $P < 0.05$ .

## Results

**Acquisition of TMZ resistance affects cell morphology and enhances cell migration.** It was revealed that C6-PL3 cells had shorter extrusions and irregular cell shapes (Fig. 1A-D), as well as reduced cell-to-cell contact. This suggests that TMZ resistance may interfere with migration and invasion abilities of C6 cells. A Transwell assay indicated that the invasive ability of C6-PL3 cells was significantly increased compared to that of C6 cells (Fig. 2A and B). In allografted rats with C6-PL3 and C6 glioma cells, it was revealed that more C6-PL3 glioma cells infiltrated normal rat brain tissues than C6 cells (Fig. 2C-H). C6-PL3 glioma cells frequently infiltrated into normal brain tissues, and <20% of all selected regions had a clear boundary between tumor and normal brain tissue. While for C6 cells, >80% of all selected regions had a clear boundary between tumor and normal brain tissue (Fig. 2I). These results indicated that the acquisition of TMZ resistance by glioma cells can affect cell morphology and enhance cell invasion ability, reflecting a more aggressive phenotype.

**Acquisition of TMZ resistance by C6 cells is not associated with CSCs.** Previous clonal and population analyses have indicated that most C6 cells are CSCs (32). Given that CSCs may contribute to treatment resistance, it was investigated whether the aggressive phenotype of C6-PL3 was associated

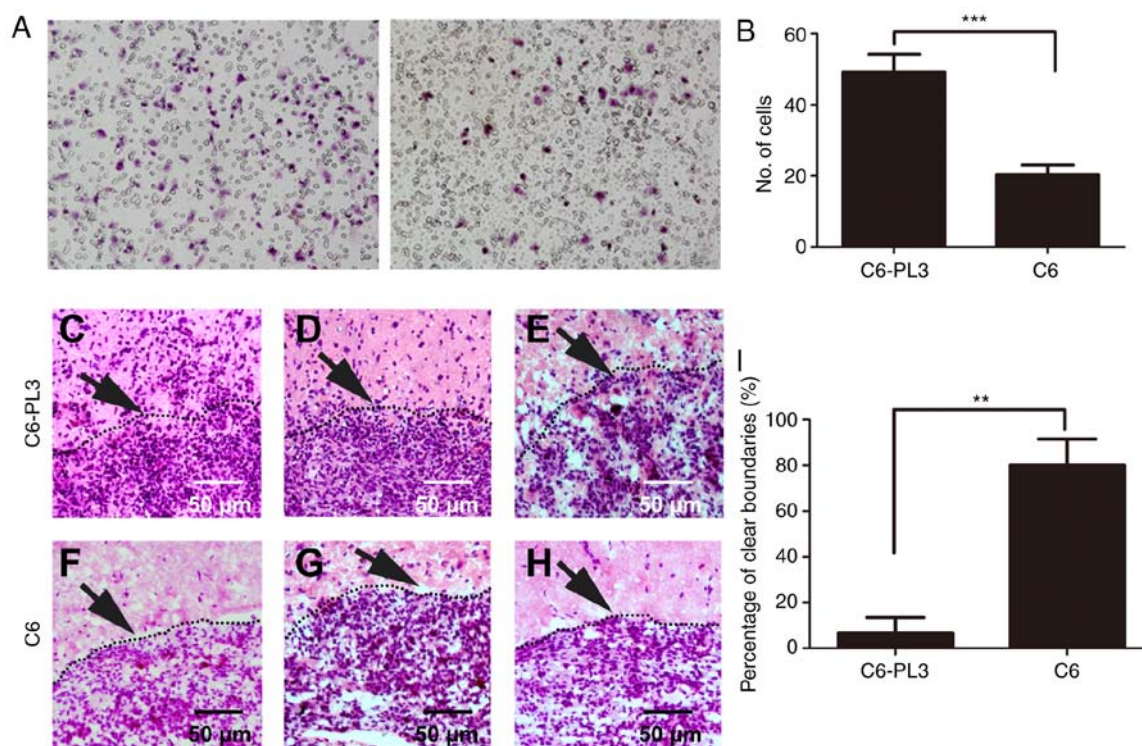


Figure 2. Drug resistance in parental rat glioma cells (C6 cells) induced promotion of cell invasion. (A) Transwell assays were carried out for TMZ-resistant rat glioma cells (C6-PL3) and C6 cells. Images of cells stained with Giemsa invading through Matrigel in a representative experiment are presented, and results of 15 different regions in three independent experiments (five regions per experiment) are summarized in (B) with an x200 magnification. (C-H) Rats were allografted with C6-PL3 and C6 cells for 20 days. Brain slices were collected and stained. Representative images of brain slices collected from different rats are presented, with arrows indicating C6 glioma cells. Dotted lines indicate the boundary between tumor and normal brain tissue. (I) Quantitative results from both groups (three rats/group) are summarized. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . TMZ, temozolomide.

with CSC properties of parental C6 cells. CSCs are able to grow *in vitro* in aggregates called tumor spheres and maintain an undifferentiated state expressing stem cell markers such as nestin (35). Notably, when C6-PL3 and C6 cells were cultured in serum-free culture medium, most of the cultured C6 cells formed tumor spheres after 10 days, while most of the C6-PL3 cells did not, and grew as flattened cells in a scattered pattern (Fig. 3A). Western blotting indicated that C6-PL3 and C6 cells exhibited different expression patterns of stem cell markers. SOX2 were expressed in both cell lines, while nestin and BMI1 were expressed only by C6 and C6-PL3 cells, respectively (Fig. 3B). Immunofluorescence staining confirmed that nestin was expressed only in C6 cells (Fig. 3C). These results indicated that most C6 cells were endowed with CSC properties, consistent with previous research (32,33). Conversely, these results also indicated that the acquisition of TMZ resistance by C6 cells was not associated with CSC.

*Acquisition of TMZ resistance by C6 cells is attributable to SP phenotype.* The active efflux of drugs, which requires the action of the ABC transporters, has been reported to contribute to drug resistance in glioma. Expression of BCRP1 and MDR1, which are ABC transporters in C6 cells, indicated that MDR1 expression occurred only in C6-PL3 cells (Fig. 4A). In addition, neither cell line expressed BCRP1. Flow cytometric analysis indicated that the C6 glioma cells contained 1.92% SP cells, while 74.6% of C6-PL3 cells were SP cells. Nearly all SP cells of both lines could be abolished using verapamil, which indicated that the selected populations were genuine SP cells

(Fig. 4B). This data raises the possibility that the acquisition of TMZ resistance by C6 cells may be attributed to the SP phenotype.

*Verapamil increases TMZ-induced tumor sphere formation and cell apoptosis in C6-PL3 and C6 cells.* To further confirm whether SP phenotype was associated with the drug resistance of C6-PL3 cells, cells were treated with TMZ for 24 h and verapamil was added for another 24 h. The results indicated that TMZ could significantly decrease the number of C6 cells, but not C6-PL3 cells. Verapamil treatment also restored tumor sphere formation, a CSC trait, in C6-PL3 (Fig. 5A). While C6-PL3 cells remained viable under TMZ treatment, ABC transporter blockade by verapamil led to a restored ability of tumor sphere formation. These results indicated that ABC transporters play a critical role in the TMZ-resistance of glioma cells, but not CSC. Verapamil blockade of ABC transporters yielded increased sensitivity to TMZ, as well as TMZ-induced apoptosis. The average apoptosis rate was 4.79 and 81.61% respectively (Fig. 5B and C). These findings indicated that ABC transporters mediated sensitivity to TMZ in glioma C6 cells.

## Discussion

Numerous glioma patients are subject to drug resistance, relapse, and an overall short survival. Details of the mechanisms that govern relapse and drug resistance remain unresolved. Brain CSCs have been revealed to be resistant to standard-of-care



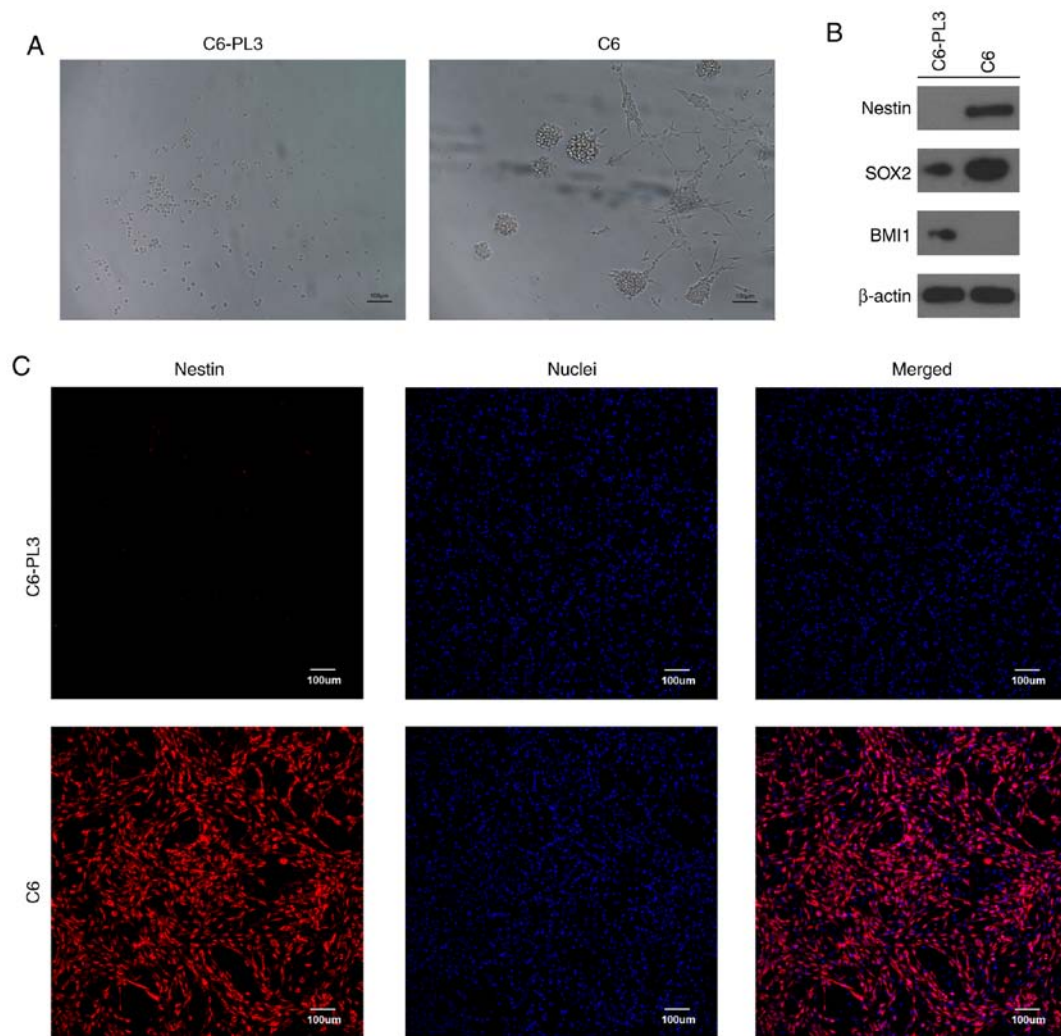


Figure 3. TMZ-resistant rat glioma (C6-PL3) cells do not possess typical properties of cancer stem cells. (A) The ability of C6-PL3 cells and parental rat glioma (C6) cells to form tumor spheres was observed under phase-contrast microscopy. (B) Expression of Nestin, SOX2, BMI1, and  $\beta$ -actin in C6-PL3 and C6 cells was analyzed using western blotting. (C) Expression of nestin in C6-PL3 and C6 cells was analyzed using immunofluorescence. TMZ, temozolomide.

chemotherapy (36). Exploring the potential role of CSCs in drug resistance in glioma, and other mechanisms of resistance, may be applicable in identifying novel therapeutic targets.

The present results revealed that the acquisition of TMZ resistance permitted C6-PL3 cells to gain a more significant invasive phenotype, in accordance with a previous demonstration. Growing evidence indicates a close link between metastatic potential and therapeutic resistance in gliomas (37,38). Wang *et al* revealed that TMZ-resistant glioma cells enhanced cell migration capacity and acquired epithelial-mesenchymal transition (EMT)-like changes (37). It was revealed that the acquisition of TMZ resistance by C6 cells was not associated with CSCs, but attributable to the SP phenotype and increased expression of MDR1. This result is in agreement with a previous finding which identified MDR1 as the most relevant ABC transporter in drug resistance of rat C6 glioma cells (39). In addition, the *BCRP1* gene is a conserved feature of stem cells from a wide variety of sources, and serves as a molecular determinant of the SP phenotype (13,40). The discrepancy between the present results and other studies may be due to species difference and measurement limitations. In the present study, rat C6 glioma cells were used, since they are

a well-established *in vitro* cancer cell line; it is possible that these differ from primary human glioma cells in genotype and gene expression pattern. In addition, emerging evidence suggests that the ABC transporters play a more active role in cell biology, such as exporting endogenous metabolites as well as signaling molecules (41). The present results also indicated that blocking the ABC transporter could increase sensitivity to TMZ and TMZ-induced apoptosis in C6 cells, which further supports the role of ABC transporters on drug resistance development. Further research effort should be devoted to the effects of ABC transporters and SP phenotype in chemotherapy, which may lead to new therapeutic targets and better anticancer strategies.

It was confirmed that acquisition of TMZ resistance by C6 cells was not due to CSCs. Most C6 cells were capable of forming tumor spheres, but did not exhibit an SP phenotype, which is recommended for CSC verification. Based on the present results, an SP phenotype may not be necessary for CSC development in C6 cells. Further study should clarify whether drug resistance in cancer is due to CSCs or SPs alone. In addition, current theories vary as to the characteristics of CSCs. Adding to the controversy is evidence that terminally differentiated

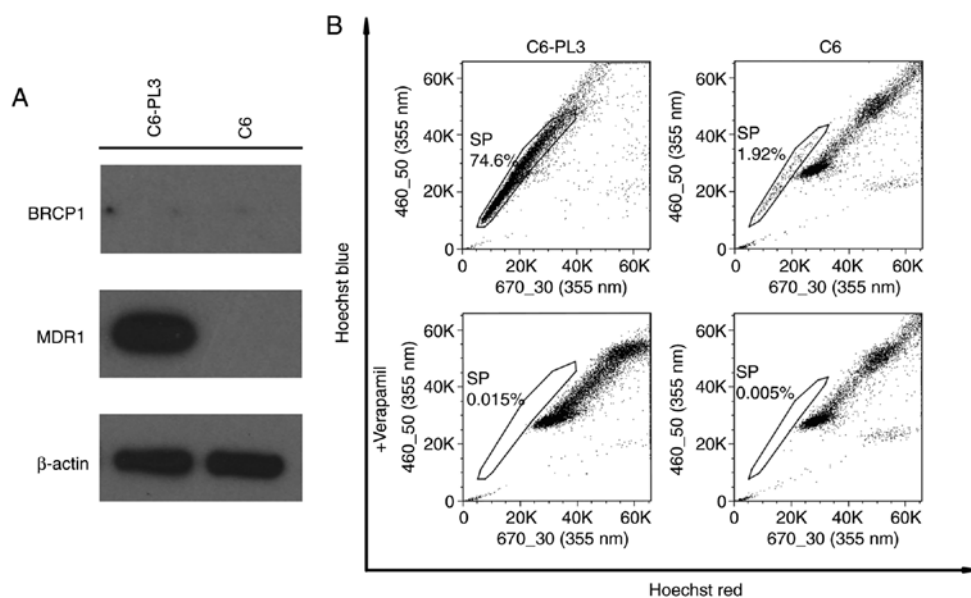


Figure 4. Most TMZ-resistant rat glioma (C6-PL3) cells are SP cells. (A) The expression of BRCP1, MDR1, and  $\beta$ -actin in C6-PL3 and parental rat glioma (C6) cells was analyzed using western blotting. (B) C6-PL3 and C6 cells treated with or without 50  $\mu$ M verapamil were labeled with the Hoechst 33342 and analyzed using flow cytometry. TMZ, temozolomide; SP, side population.

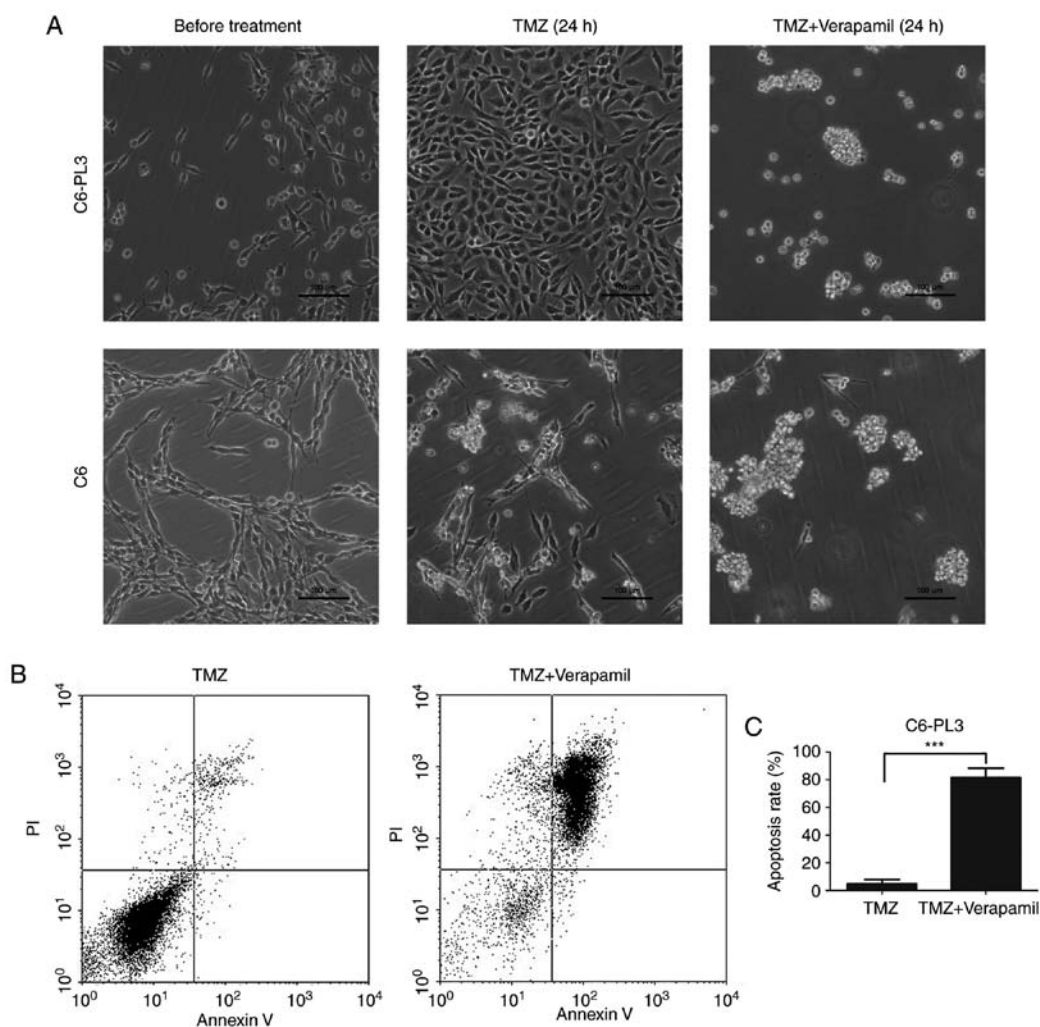


Figure 5. Verapamil increases TMZ-induced tumor sphere formation and apoptosis in TMZ-resistant rat glioma (C6-PL3) and parental rat glioma (C6) cells. (A) Tumor sphere formation in C6-PL3 and C6 cells was observed using phase-contrast microscopy after TMZ and verapamil treatment. (B and C) After treatment, apoptosis was detected using flow cytometry. The average apoptosis rate of TMZ treatment was 4.79%, while after adding the verapamil, the average apoptosis rate was 81.61%. \*\*\*P<0.001. TMZ, temozolomide.

cancer cells can de-differentiate into pluripotent CSCs (42). Ideally, a perfect enrichment method for CSCs would be based on a property that defines an essential CSC feature as their capacity for self-renewal and exact recapitulation of the original tumor. However, there is currently no such method in existence for any cell type. Numerous transcription factors or structural proteins essential for normal NSPC function also mark glioma CSCs, including SOX2 (43), OLIG2 (44), Myc (45), BMI1 (43), and nestin (46). Various potential cell surface markers such as CD133 (43) and CD44 (20) have also been suggested for identifying CSCs. However, it is not likely that any marker would be uniformly informative for CSCs, possibly due to the presence of multiple populations of stem cells in most tissue types due to the inherent adaptability of cancer cells. In addition to marker expression, growth as neurospheres in serum-free medium (47) or efflux of fluorescent dyes (31) have also been widely used to characterize glioma CSCs. Normally, neurosphere culture is carried out in conditioned medium without added serum, EGF receptor, or FGF receptor (48). However, this selection process fails to recapitulate the heterogeneity of the original tumor. In addition, it has been reported that the majority of spheres originate from progenitor cells with limited self-renewal potential, rather than from stem cells (48). Another approach to enrich CSCs is the use of flow cytometry to identify SPs which contain CSCs, but not all SPs isolated from different cell lines are self-renewing, reflecting species-specific challenges of this enrichment method (49-51). At present, the most reliable method in validating a putative CSC fraction is their transplantation into immunodeficient mice. An animal experiment of longer duration or injecting cells along with Matrigel could significantly increase the frequency of existing tumor-initiating cells (52,53). Collectively, these findings indicated further study of additional criteria to validate CSCs in C6 cells, as stringent standards are urgently required to help interpret various investigations into CSCs.

The present findings revealed that the acquisition of drug resistance by C6 cells conferred increased migration ability. Additionally, acquisition of drug resistance was not mediated by the properties of C6 CSCs, but by and increase in SP phenotype. Blocking the ABC transporter could increase tumor sensitivity to temozolomide and temozolomide-induced apoptosis in C6 cells. In summary, drug resistance of C6 cells was revealed to be dependent on ABC transporters rather than CSCs. These findings support a potential therapeutic application of ABC transporters in glioma, and suggest a more accurate choice of experimental model.

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### Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

### Authors' contributions

XY and JL designed the experiments. XY, YX, SM, YS performed the experiments. XY, YX and JL analyzed the data and wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

Methods involving live animals were carried out in accordance with the guidelines and regulations enacted and enforced by the Chinese National Ministry of Science and Technology as well as the National Ministry of Health. All experimental protocols were approved by the Institutional Lab Animal Ethics Committee at Kunming University of Science and Technology (Kunming, China).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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