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Preliminary Study on Relationship Between Temozolomide Chemotherapy-Resistant Cells and Stem Cells in Gliomas

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ABSTRACT

AIM: To study the relationship between temozolomide (TMZ) chemotherapy-resistant cells and stem cells in gliomas.

MATERIAL and METHODS: The U251 glioma cell line was exposed to TMZ to generate TMZ-resistant colonies (U251/TMZ cell line) using the pulse drug method. The TMZ sensitivity of U251/TMZ and parental cells was examined using an MTT assay. The cell growth curve was drawn to show the growth of the two kinds of cells. Glioma stem cells (GSCs) were cultured and differentiated in vitro. Immunofluorescence assays were used to identify the expression of CD133, Nestin, and ABCG2 in U251/TM and U251 cells. Western blot analysis was used to analyse protein expression levels.

RESULTS: The U251/TMZ cell line was successfully cultured in vitro. The IC50 value of the U251/TMZ cell line is 8.1 times that of the parental U251 cell line (t=-63.28, p=0.00). The doubling time of U251/TMZ cells was long compared with the parental cells. GSC tumour spheres were successfully cultured in vitro, and they differentiated in medium containing serum. The expression of CD133, Nestin, and ABCG2 in U251/TMZ cells was significantly higher than that in the parental U251 cells (t=43.35, p=0.00; t=12.31, p=0.00; t=11.49, p=0.00). Immunofluorescence staining of CD133, Nestin, and ABCG2 was significantly higher in U251/TMZ than in the parental U251 cells (t=43.35, p=0.00; t=12.31, p=0.00; t=11.49, p=0.00). Moreover, Western blot results showed that CD133, Nestin, and ABCG2 expression was significantly higher in U251/TMZ cells than that in the parental U251 cells (t=17.76, p=0.00; t=18.78, p=0.00; t=6.19, p=0.00).

CONCLUSION: The U251/TMZ cell line has the biological characteristics of GSCs. The relationship between GSCs and chemotherapy-resistant cells has been preliminary proven to be partially overlapping, which can provide a new perspective when using appropriate cell subpopulations as targets for glioma.

KEYWORDS: Glioma, Temozolomide, Drug resistance, Neoplasm, Chemotherapy-resistance, Stem cells

INTRODUCTION

Glioma, which accounts for more than 80% of all malignant brain tumours, presents treatment problems in the field of neurosurgery. Chemotherapy, such as temozolomide (TMZ), is often recommended as an important method for the treatment of glioma (18). Its approval by the FDA in 1999 provided hope for patients with gliomas and their physicians. However, a series of studies have shown that temozolomide can only slightly prolong survival in patients (7,13). Glioma-resistant cells are the major causes of glioma treatment failure or recurrence (2). Therefore, temozolomide cannot likely improve the prognosis of patients with glioma.

As a new research area derived from stem cell research in recent years, glioma stem cell (GSC) theories have been proposed and shown good prospects. GSCs have been recognized as the basis for brain tumour formation, growth and metastasis. According to GSC theories, GSCs, which are isolated from glioma tissue, have strong resistance and are

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recognized as the major cause of glioma treatment failure or recurrence (4,11). In fact, using appropriate cell subpopulations as targets for glioma cells is critical. In this study, we preliminarily discussed the relationship between chemotherapy-resistant cells and stem cells in gliomas.

MATERIAL and METHODS

Cell Culture and Reagents

The U251 human glioma cell line was purchased from the Institute of Life Sciences Cell Bank (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% heat-inactivated foetal bovine serum (FBS, Gibco) in a humidified atmosphere containing 5% CO_2. Cell passaging occurred when the cells covered the bottom of the culture medium.

Low glucose medium, DMEM/F12 medium, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), B27 and N2 additives, DMSO, trypsin, etc. were purchased from Gibco (USA). CD133, Nestin, and ABCG2 antibodies were purchased from NeoMarkers (USA). CD133 (AC133), IgG (secondary antibody), and CY3 antibodies were purchased from Miltenyi (Germany). ECL chemiluminescent kits were purchased from Merck Millipore (Germany), and TMZ and thiazole salt (MTT) were purchased from Sigma (USA).

U251/TMZ Cells Established by the Pulse Drug Method

The parental U251 cell line was plated into a 75 ml culture bottle and exposed to TMZ (120 μmol/L, Sigma-Aldrich, USA) to generate TMZ-resistant colonies (U251/TMZ cell line). After culture for 72 hours, the TMZ-containing medium was discarded and the cells were washed twice by PBS, after which the formed colonies were harvested by treatment with a trypsin solution. The obtained cells were grown as a monolayer culture until becoming confluent and were then subjected to another pulse treatment using the method described above. The screening cycle occurred once or twice each month. After developing and screening for 5 months, the surviving colonies were selected and established as U251/TMZ cell lines (21). The U251/TMZ cell lines were also treated with TMZ (120 μmol/L) and cultured for in medium for 3 days every two weeks to maintain the resistance.

MTT Assay, Drug Sensitivity and Cell Growth Curve

A cell suspension was prepared and the number of cells was adjusted to 5 × 10^4/mL. The cells were seeded in a 96-well plate (200 μL in each well) and allowed to grow for 24 h. Th cells were then treated with TMZ at various concentrations including 10, 50, 100, 200, 500, and 1000 μmol/L (5 wells for each concentration), while the control group was treated with 50% hydroglycol for 24 h. After culturing for 72 hours, 20 μl 0.5% MTT solution was added to each well and the plate was further incubated for 4 h at 37 °C. The formazan crystals were dissolved in 150 μL DMSO and the optical density (OD) was measured at 540 nm using a microplate reader (8). The half maximal inhibitory concentration (IC50) was calculated using the Probit program in SPSS 17.0. The resistance index (RI) was expressed as a percentage of the drug-resistant cell IC_{50} to the parental cell IC50.

U251/TMZ and parental cell suspensions (1×10^4/ mL) were plated into 6-well plates (3 wells per cell line, with 1 ml each in well). The total cells in each well were quantified every 24 hours for 1 week. The cell counts of three wells per time point per group were averaged.

GSC Culture and Differentiation in Vitro

Serum-free DMEM/F-12 (1:1), N2 additive (1:100), B27 (1:50) EGF (20 μg/L), bFGF (20 μg/L), 50 kU/L penicillin G and 50 kU/L streptomycin were added to stem cell media. The cells were grown in a standard tissue culture incubator with 5% CO_2 in humidified air at 37 °C. Serum-free media were changed twice a week (15).

The growth factors were removed from the stem cell media and DMEM medium (containing 10% FBS) was added to promote differentiation.

Immunofluorescence

Cells were seeded in a 24-well plate and fixed with 4% paraformaldehyde in PBS for 30 minutes at a room temperature. CD133, Nestin or ABCG2 monoclonal antibody was added and then incubated overnight at 4ºC. After being washed twice with PBS, the cells were incubated with CY3 for 2 hours in a 37ºC water bath (22). After three washes with PBS, the samples were examined with a fluorescence microscope. Each indicator was repeated three times with the same method.

Positive cells appeared as follows: red fluorescence for CD133 appeared in the cell membrane and red fluorescence appeared in the cytoplasm for CD133 with Nestin and ABCG2.

Western Blot

U251/TMZ and parental cells were adjusted to 1 × 10^6/mL and seeded in a 6-well plate (200 μL in each well). After culturing for 24 hours, the total proteins were extracted from the cells. Electrophoresis was conducted with a 12% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane, which was incubated for 2 hours with 5% skim milk powder; incubated overnight with monoclonal CD133, Nestin or ABCG2 antibodies; washed 3 times with PBS for 5 min/each; and then incubated with secondary antibody at 37°C for 2 hours (9). The images were developed using an ECL chemiluminescent kit. Nestin and ABCG2 identification was repeated three times with the same method.

Statistical Analysis

All data were analysed using the SPSS 17.0 statistical software. Measurement data were shown as the X ± S. The enumeration data was shown by the chi-squared test. Measurement data were compared with a t-test. $p<0.05$ indicated that a difference was statistically significant.

RESULTS

Successful Culture of U251/TMZ Cell Lines

In the early period, a small population of parental U251 cells survived and were passaged. After developing and screening

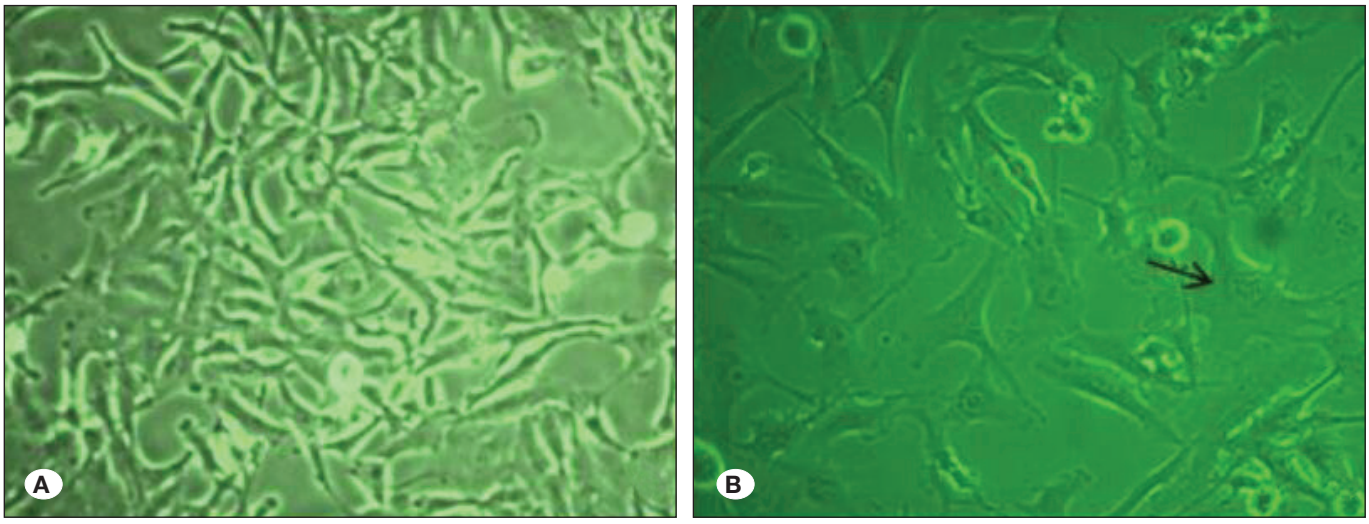

Figure 1: Morphological changes of resistant cell lines (100x). **A)** Parental glioma cells were mostly fusiform, with less giant cells; **B)** U251/TMZ cell lines had clear boundaries, with a little number of giant cells (→).

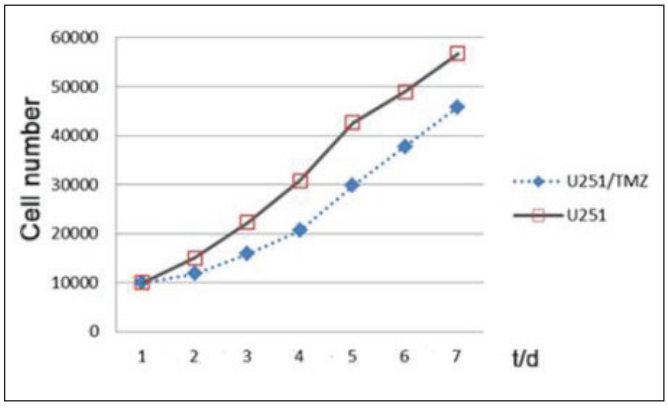

Figure 2: Cell growth curve U251/TMZ and parental U251 cells.

for 5 months, the surviving and stable colonies were selected and established as the U251/TMZ cell lines. The cell shapes of the parental glioma cells and drug-resistant (U251/TMZ) cells are shown in Figure 1A, B. The parental glioma cells were shaped as elongated fibres that were uniform in size, tightly packed, and adherent to the cytoderm (Figure 1A). Most of the drug-resistant (U251/TMZ) cell lines showed similar growth patterns to the parental cells (adhering to the cytoderm). However, there were a few deformed cells and newly generated giant cells. These cells also had a much clearer boundaries compared with the parental glioma cells (Figure 1B).

Sensitivity Changes to TMZ and Cell Growth Curve

The U251/TMZ and parental U251 cell lines were treated with TMZ at various concentrations. The IC_{50} values were calculated using the Probit program in SPSS 17.0. The IC_{50} value of the U251/TMZ cell line (286.76 ± 8.36 μmol/L) was significantly different from the IC_{50} value of the parental U251 cell line (35.62 ± 2.97 μmol/L) (t=-63.28, p=0.00). The IC_{50} value of the U251/TMZ cell line was 8.1 times greater than that of the parental U251 cell line.

Growth curves are shown in Figure 2. The U251/TMZ and parental U251 cells showed different growth rates beginning on the third day. The U251/TMZ cells showed a slower growth rate and entered the logarithmic growth phase on the fourth day, and the slope of the growth curve was smaller than that of the parental cells. The doubling time of the U251/TMZ cells was long compared with the parental cells.

Successful Culture and Differentiation of GSCs in Vitro

The U251/TMZ cell lines (Figure 3A) were switched into stem cell media, and single cell division occurred in 3 days followed by the formation of small-diameter "neurosphere-like" tumour spheres (Figure 3B). Large numbers of larger "neurosphere-like" spheres formed within 7 days (Figure 3C). After approximately 12 days of culture, the formation of tumour spheres was observed and imaged under a phase-contrast microscope (Figure 3D).

DMEM (containing 10% FBS) was added to the tumour spheres to promote differentiation. Taking spheroids (MCSs) as the centre, the cell spheres gradually differentiated and developed pseudo-foot-like structures to adhere to the cytoderm (Figure 4A, B).

Immunofluorescence of U251/TMZ and U251 Cells

Red fluorescence appeared in the cell membrane for CD133. The cell body and protrusions of positive cells also presented red fluorescence, which also covered the nucleus. The cells were round or oval in shape and displayed aggregation (Figure 5A, B). Red fluorescence also appeared in the cytoplasm for CD133, indicating Nestin and ABCG2. The cells were also round or oval in shape and displayed aggregation (Figure 5C-F). Immunofluorescence results for the U251/TMZ and U251 cells are shown in Table I. The expression of CD133, Nestin, and ABCG2 was significantly higher in U251/TMZ cells than in the parental U251 cells (t=43.35, p=0.00; t=12.31, p=0.00; t=11.49, p=0.00).

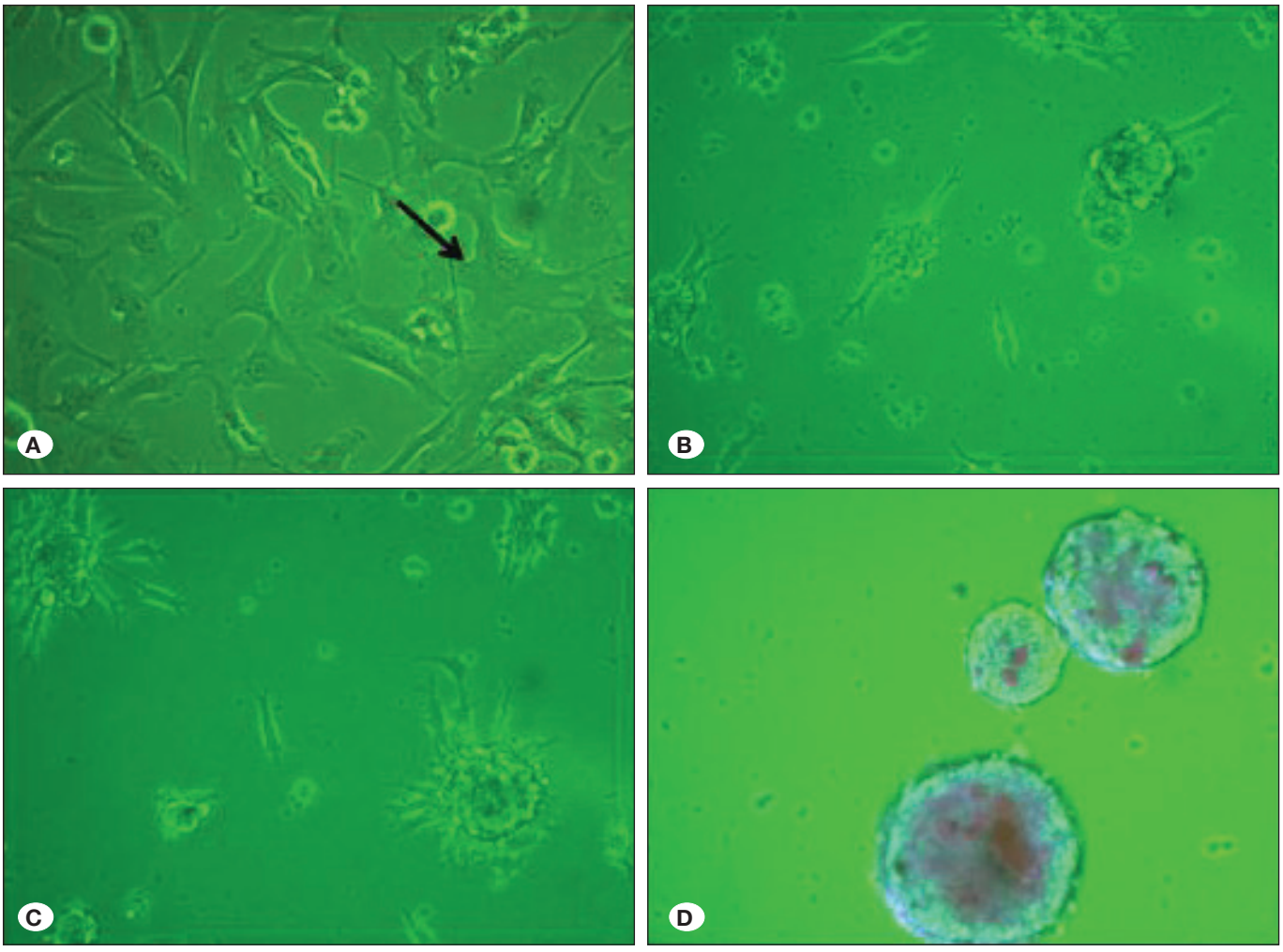

Figure 3: Morphological changes of GSC spheres. **A)** U251/TMZ cell lines had clear boundaries, with a little number of giant cells (→) (100x). **B)** After cultured for 3 days, single cell division occurred, followed by the formation of small-diameter "neurosphere-like" tumor spheres (40x). **C)** Large numbers of bigger "neurosphere-like" spheres formed within 7 days (40x). **D)** After approximately 12 days of culture, the formation of tumor spheres was observed (10x).

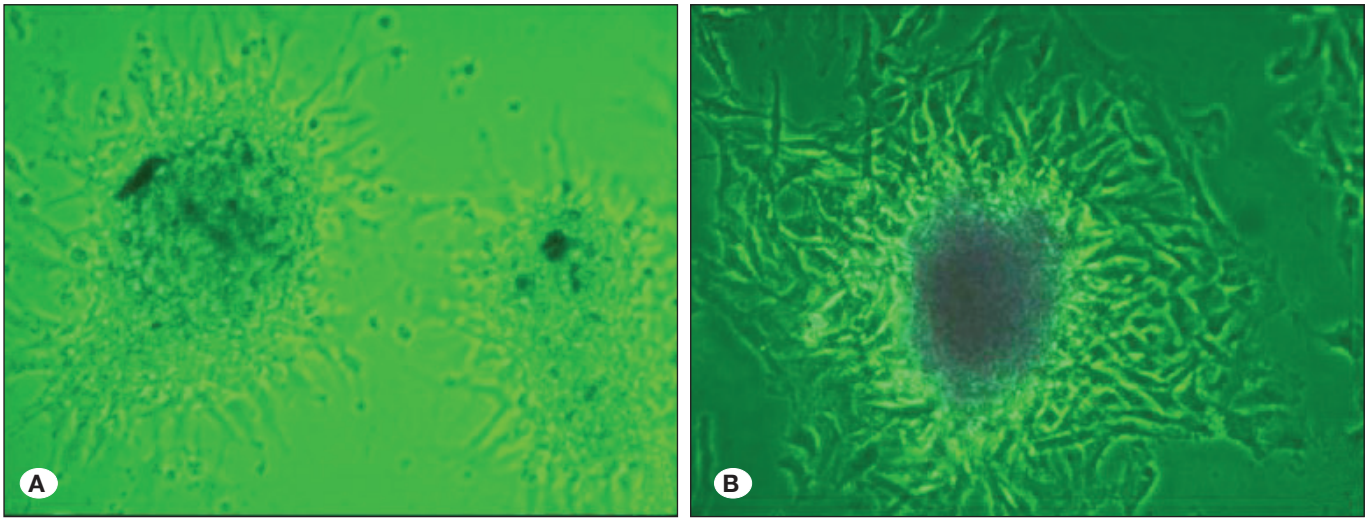

Figure 4: Differentiation of Glioma Stem Cells (40x). **A)** Tumor spheres was added in DMEM medium and allowed to grow for 24 hours. Most cell spheres defferentiated and grew pseudo-foot-like adherent to the cytoderm. **B)** After cultured for 48 hours, more clumps cells eptended outward.

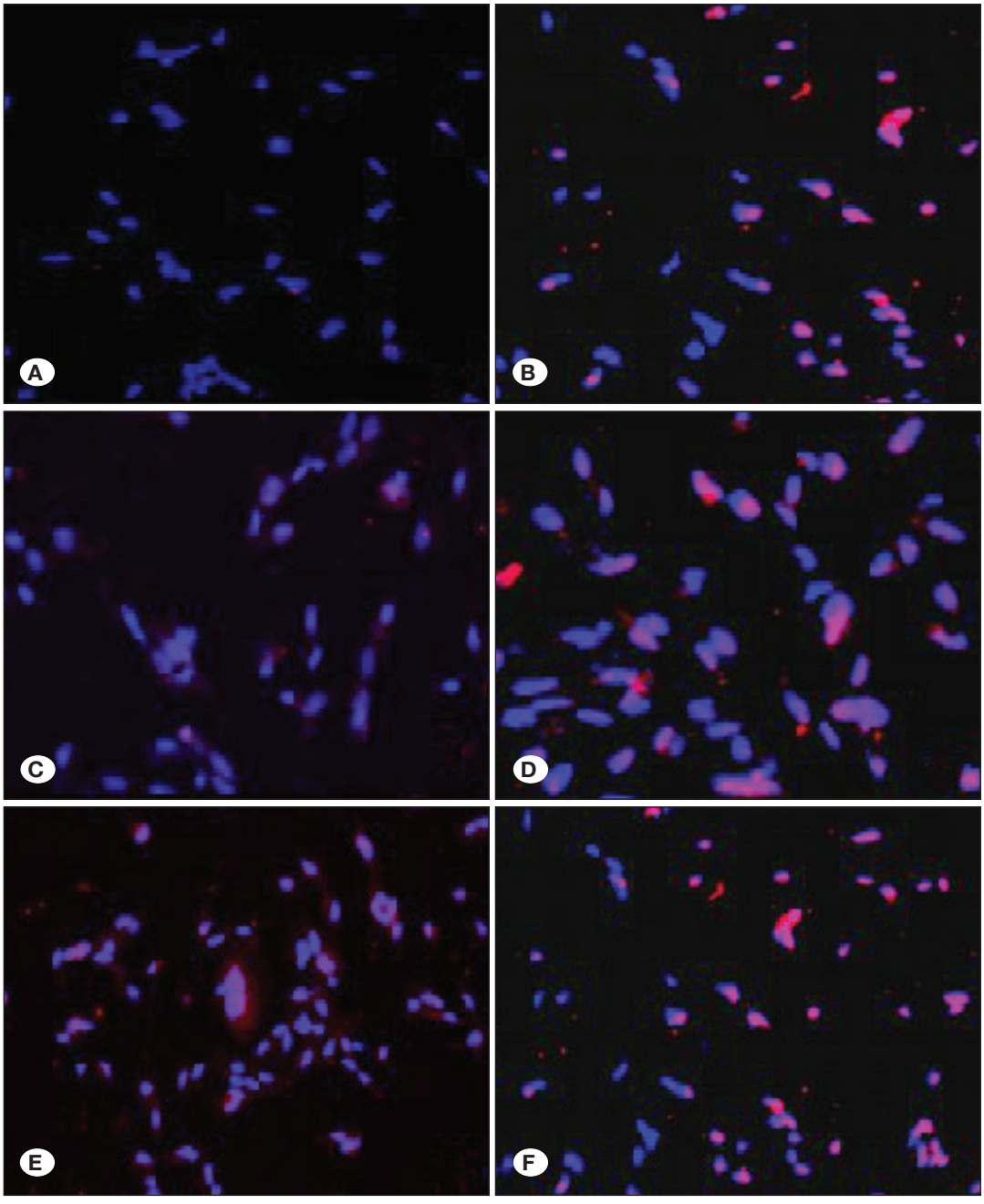

Figure 5: Immunofluorescence of CD133, Nestin, and ABCG2 expression in U251/TMZ and U251 cells (400x).
A) Expression of CD133 in U251 cells.
B) Expression of CD133 in U251/TMZ cells.
C) Expression of Nestin in U251 cells.
D) Expression of Nestin in u251/TMZ cells.
E) Expression of ABCG2 in U251 cells.
F) expression of ABCG2 in U251/TMZ cells.

Western Blot for CD133, Nestin, and ABCG2 Expression in U251/TMZ and U251 Cells

Western blot results from U251/TMZ and U251 cells are shown in Table II and Figure 6. The expression of CD133, Nestin, and ABCG2 was significantly higher in U251/TMZ cells than in the parental U251 cells (t=17.76, p=0.00; t=18.78, p=0.00; t=6.19, p=0.00).

DISCUSSION

Chemotherapy-Resistant Cell Culture

The establishment of drug-resistant cell lines in vitro commonly includes conventional methods such as continuous drug exposure and pulse drug treatments (20). The cell models established by these two methods are very different in terms of their biological characteristics and morphological structures (5). Cell models established using the continuous drug exposure method are induced into acquiring drug resistance by slowly increasing the concentration of chemotherapeutic drugs. High drug resistance is acquired in this method. Cell morphology and subcellular structure are often greatly changed. In contrast, cell models established by pulse drug treatments are screened cells with strong drug resistance developed through high doses and short-time treatment of cells with chemotherapy drugs. It is believed that the administration

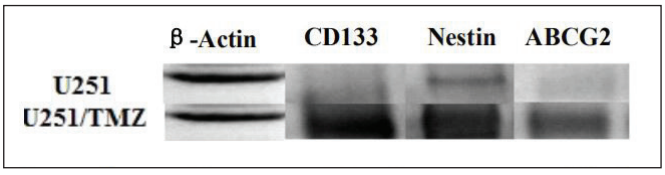

Figure 6: Western blot results of CD133, Nestin, and ABCG2 expression in U251/TMZ and U251 cell.

Table I: Immunofluorescence Results of CD133, Nestin, and ABCG2 Expression in U251/TMZ and U251 Cells

Group	CD133	Nestin	ABCG2
U251	0.039 ± 0.010	0.128 ± 0.034	0.247 ± 0.061
U251/TMZ	0.653 ± 0.042	0.622 ± 0.122	0.708 ± 0.108
t	45.350	12.310	11.490
p	**0.000**	**0.000**	**0.000**

Table II: Western Blot Results of CD133, Nestin, and ABCG2 Expression in U251/TMZ and U251 Cells

Group	CD133	Nestin	ABCG2
U251	1.480 ± 0.530	0.830 ± 0.157	0.110 ± 0.300
U251/TMZ	99.820 ± 12.370	79.080 ± 9.310	71.300 ± 25.800
t	17.760	18.780	6.190
p	**0.000**	**0.000**	**0.000**

of pulse drug treatments is similar to the administration of clinical chemotherapy courses (6,12). To reduce the adverse effects of clinical chemotherapy and improve the clinical efficacy, high doses of short-term administration are often recommended to rapidly reach peak blood concentration. Then, the second chemotherapy course is conducted a few days after the initial withdrawal. The similarity is helpful when studying the causes of clinical resistance. In this study, U251/TMZ cell lines were established by the pulse drug method. The biological characteristics showed significant changes. The IC_{50} value of the U251/TMZ cell lines was 8.1 times greater than the parental U251 cell lines, and the doubling time of the U251/TMZ cells was long compared with the parental cells.

Changes in Drug Resistance in U251/TMZ Cells

The use of chemotherapeutics frequently leads to the development of the clinically relevant phenomenon multidrug resistance (MDR). This drug resistance results in large part from the overexpression of members of the ATP-binding cassette (ABC) superfamily of membrane transporters. One of the basic mechanisms involved in MDR involves the elevated expression and/or activity of several ATP-binding cassette superfamily members (ABC transporters), which are normally responsible for the efflux of xenobiotics or secondary metabolites outside the cell (3,17). ABCG2 expression characteristics are significantly associated with the chemotherapy effects on malignant tumours. High expression indicates poor chemotherapy effects and low expression indicates relatively good chemotherapy effects (14,19). In this study, the expression of ABCG2 was significantly higher in U251/TMZ cells than in the parental cells, indicating an increase in drug resistance. This acquired resistance of U251/TMZ cell lines makes treatment complicated and varied.

Relationship Between Chemotherapy-Resistant Cells and Stem Cells

In recent years, GSC theories have been recognized as the basis for brain tumour formation, growth and recurrence. GSCs are recognized as acquiring drug resistance mechanisms and have strong resistance. Before this, glioma-resistant cells were recognized as the major cause of glioma treatment failure or recurrence. In this study, we assumed a relationship between GSCs and chemotherapy-resistant cells (U251/TMZ cells) as follows: 1) they are the same cells; 2) the cell types partially overlap; and 3) the cell types contain one another.

As was shown in the immunofluorescence results for U251/TMZ and U251 cells, the expression of CD133, Nestin, and ABCG2 was significantly higher in U251/TMZ cells than in the parental U251 cells. Specifically, a large and increasing number of drug-resistant cells demonstrated stem cell characteristics. However, not all of these cells showed stem cell characteristics. As was proven above, there were several factors leading to glioma resistance. Chemotherapy resistance associated with gliomas appears to be a very complex biological process, with multiple mechanisms and multi-factor characteristics (1,10,16). Meanwhile, not all GSC cells had high drug resistance. Some GSCs were relatively sensitive to chemotherapeutic agents.

CONCLUSION

The relationship between GSCs and chemotherapy-resistant cells is partially overlapping. Specifically, among chemotherapy-resistant glioma cells, most cells are GSCs. However, a small portion of non-tumour stem cells remains. In the GSC subcellular population, the majority of cells showed drug resistance, but a small portion of cells were relatively sensitive to chemotherapeutic drugs. Additionally, in the course of treatment, both the biological characteristics of GCSs and the small portion of non-tumour stem cells that are resistant to drugs should be given more attention. The biological characteristics of this small portion of cells and whether they can be converted into GCSs still require further study.

AUTHORSHIP CONTRIBUTION

Study conception and design: WC

Data collection: XM, XD

Analysis and interpretation of results: ZX

Draft manuscript preparation: XM

Critical revision of the article: XM

All authors (XM, XD, ZX, WC) reviewed the results and approved the final version of the manuscript.

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