

Drug screening study using glioma stem-like cells

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Abstract. Glioma is one of the most common malignant tumors affecting the central nervous system. We screened active drugs from 12 classes to provide experimental data for the study of human glioma. Serum-free suspension clone formation was used to induce the formation of glioma stem-like cells (GSLCs) from U251 cells. We screened active drugs from 12 classes and observed their effects on the number of tumor balls. The results revealed that serum-free suspension clone formation successfully induced the formation of GSLCs. Six active drugs [curcumin, chrysin, apigenin, luteolin, casticin and 8-bromo-7-methoxychrysin (BrMChR)] were identified by MTT assay. The drugs that affected the number of tumor balls were curcumin, luteolin, casticin and BrMChR (all $P < 0.05$). The GSLCs were obtained by serum-free clone suspension formation and exhibited the features of cancer stem cells.

Introduction

Glioma is one of the most common malignant tumors affecting the central nervous system. In the United States, 22,000 people per year are diagnosed with primary brain tumors (1). Despite a wide range of treatments, including surgery, radiotherapy and chemotherapy, the majority of therapies eventually fail (2). The characteristics of the disease are a short survival period and a high recurrence rate and mortality. Human glioma is defined by the World Health Organization as the most common and malignant type of central nervous system tumor. There is increasing evidence that a variety of cancers, including human glioma, are driven by certain tumor-initiating cells that retain stem cell-like properties. In previous years, researchers have proposed that glioma stem-like cells (GSLCs) not only have similar features to neural stem cells in a number of aspects but also have a certain relationship with embryology (3). Non-Chinese researchers consider GSLCs to be the cause of the occurrence and recurrence of glioma. GSLCs are also the main source of the tolerance of glioma to radiotherapy and

chemotherapy (4). Therefore, GSLCs have a guiding significance for individual programs of glioma clinical treatment. At present, there are three main methods for separating and identifying GSLCs: serum-free suspension clone formation (5), a CD133 immunomagnetic bead sorting method (6) and flow cytometry of the side population (SP) (7). Temozolomide (TMZ), a DNA-alkylating agent (8), has potent antitumor activity. TMZ is a commonly used neuro-oncology drug. Drug resistance limits the clinical therapeutic effect of this alkylating agent and is one of the primary reasons for the failure of glioma chemotherapy. Therefore, there is an urgent and important requirement for the discovery of new chemotherapy drugs with high selectivity, low toxicity and potent effects for use in the field of brain science.

It has been reported that the growth of tumor cells is inhibited by genistein (9), quercetin (10), resveratrol (11), curcumin (12), chrysin (13), apigenin (14), luteolin (15) and casticin (16). Studies have also shown that the acetoacetate extract of *Vitex negundo* seed (EVn-50) (17), neolignan (VB-1) (18) and 8-bromo-7-methoxychrysin (BrMChR) (19) are able to inhibit tumor cell growth and promote cell apoptosis. Vitexicarpin, an active component of *Vitex trifolia* (20) has been reported to induce the apoptosis of breast tumor cells. EFV-3 is an extract of *Fructus Viticis*. In our study, U251 cells were incubated with serum-free medium. Following the formation of neurosphere-like cells, the active drugs, which are from 12 different classes, were screened by MTT assay. We then observed the effects of these drugs on the number of tumor balls, to provide experimental data for the study of human glioma.

Materials and methods

Reagents. Dulbecco's minimum essential medium (DMEM) and serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium were obtained from Hyclone (Logan, UT, USA). Fetal calf serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Trypsin was purchased from Beyotime Biotech (Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Genview (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), genistein, quercetin, resveratrol, curcumin, chrysin, apigenin, luteolin and casticin were purchased from Sigma (St. Louis, MO, USA). EVn-50, VB-1, BrMChR and EFV-3 were gifts from Professor Jianguo Cao (Hunan Normal University College of Medicine). Typan blue, insulin, penicillin and streptomycin were purchased

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from Beijing Dingguo Changsheng Biotech Co., Ltd. (Beijing, China). Cell culture plates and ultra-low attachment plates were purchased from Corning Inc. (Acton, MA, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from Protein Specialists (Ness-Ziona, Israel). Genistein, quercetin, resveratrol, curcumin, chrysin, apigenin, luteolin, casticin, EVn-50, VB-1, BrMChR and EFV-3 were dissolved in DMSO. All drug solutions dissolved in DMSO were stored at -20°C .

Cell culture and treatment. The U251 cells were obtained from the College of Life Science of Hunan Normal University and were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin at 37°C in a humidified atmosphere of 5% CO_2 . When the cells were near to 80% confluence, they were passaged with 0.25% trypsin. The Ethics Committee of Hunan Normal University approved the study.

Formation of GSLCs. The U251 cells, near 80% confluence and in good condition, were digested with trypsin and then washed with PBS three times. The U251 cells were seeded in 6-well ultra-low attachment plates at 2×10^3 cells/ml in the serum-free stem cell culture medium (DMEM/F12) in the presence of 20 ng/ml EGF and bFGF, 4 $\mu\text{g/ml}$ insulin, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin at 37°C in a humidified atmosphere of 5% CO_2 . Fresh stem cell medium was added every 3 days. Cell growth and cloning ball formation was observed under an inverted microscope after 10 days. The cell culture medium was collected and centrifuged for 5 min at 800 rpm. The cells were digested with trypsin and incubated for 10 days as described above.

Screening active drugs with MTT assay. The U251 GSLCs were seeded in 96-well ultra-low attachment plates at a density of 5×10^3 cells/well and U251 cells were seeded in 96-well plates at the same density. After 24 h, the U251 GSLCs and U251 cells were treated with the 12 drugs. Each drug was tested in three concentrations (10, 20 and 40 μM) and each concentration was used in three parallel wells. After treatment of the cells for 48 h, 10 μl MTT was added to each well and the cells were cultured for 4 h at 37°C . DMSO (150 μl) was added to each well. The absorbance at a wavelength of 490 nm (A_{490}) was measured using an enzyme-linked immunosorbent instrument (ELx800, Bio-Tek, Winooski, VT, USA). The experiments were divided into zero setting, control and experimental groups. The relative cell proliferation inhibition rate (IR) = $(1 - \text{average } A_{490} \text{ of the experimental group} / \text{average } A_{490} \text{ of the control group}) \times 100\%$. The experiment was repeated three times.

Effect on the number of tumor balls. The U251 GSLCs were seeded in 6-well plates. When the cells were near 80% confluence, the active drugs were applied to treat the cells at a final concentration of 10 $\mu\text{mol/l}$. The cells were digested with trypsin after 48-h treatment and then washed with PBS three times. The cells were seeded in 96-well ultra-low attachment plates at a density of 5×10^3 cells/well. The drug-treated cells were seeded in 96-well ultra-low attachment plates at densities of 5×10^2 and 1×10^3 cells/well. Stem cell medium was added

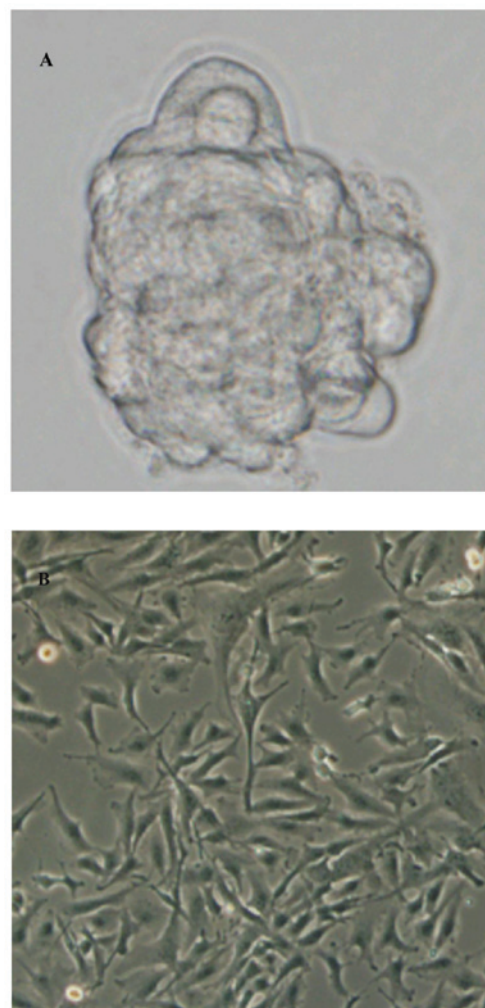


Figure 1. U251 cells and U251 glioma stem-like cells (GSLCs). (A) Serum-free suspension clone formation successfully induces the formation of U251 GSLCs; magnification, x40. (B) U251 cells were adherent to the plastic surface of the cell culture plates grown in DMEM medium containing 10% FBS; magnification, x10.

every 2 days. The numbers of tumor balls were counted after 7 days.

Results

Formation of GSLCs. The U251 cells were adherent to the plastic surface of the cell culture plates (Fig. 1B), while the human brain glioma cell lines grew in a globular form in the serum-free stem cell culture medium and successfully formed U251 GSLCs (Fig. 1A). The U251 GSLCs gathered into spherical cell masses of various sizes, suspended in the stem cell medium. The clone balls comprised dozens to hundreds of cells, which had a strong refraction. However, there remained a small number of cells grown in an adherent state. With the prolongation of the time of incubation, we observed the tumor balls gradually becoming larger, with a very clear cell shape and outline, smooth edges, no distinct processes and regular shape. The cells were passaged once every 10 days and the growth process was repeated. The U251 GSLCs had the capacity to proliferate and grow and were able to be passaged continuously.

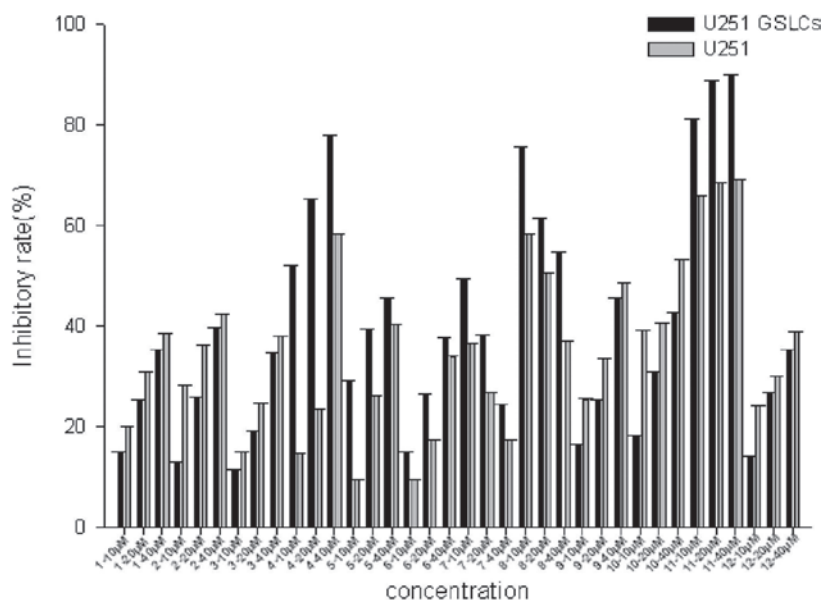


Figure 2. MTT assay screening of active drugs. 1, genistein; 2, quercetin; 3, resveratrol; 4, curcumin; 5, chrysin; 6, apigenin; 7, luteolin; 8, casticin; 9, EVn-50; 10, VB-1; 11, BrMChR; 12, EFV-3. MTT assay showed that the inhibition rate of active drugs from 6 classes to U251 GSKs is higher than U251 cells, including curcumin, chrysin, apigenin, luteolin, casticin and BrMChR. GSKs, glioma stem-like cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; EVn-50, acetoacetate extract of *Vitex negundo* seed; VB-1, neolignan; BrMChR, 8-bromo-7-methoxychrysin; EFV-3, extract of *Fructus Viticis*.

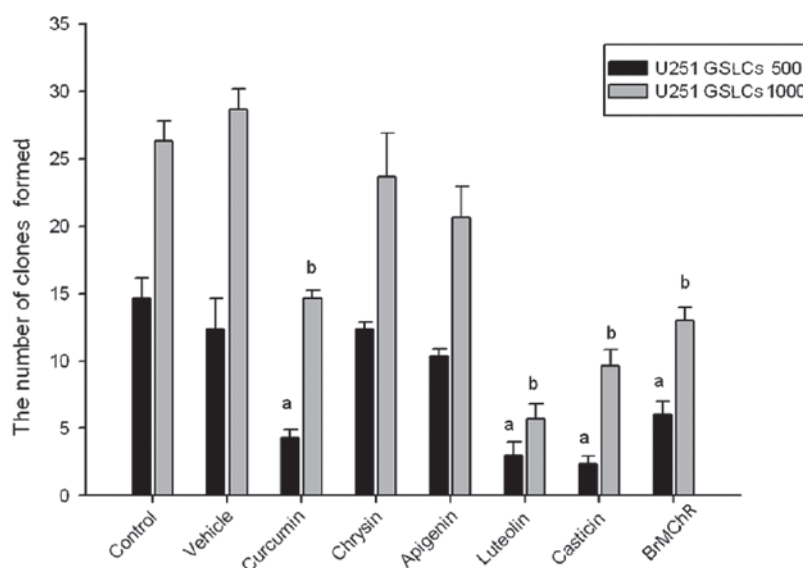


Figure 3. The effect of different drugs on the number of tumor balls. Four drugs affected the number of tumor balls, specifically curcumin ($P<0.05$), luteolin ($P<0.05$), casticin ($P<0.05$) and BrMChR ($P<0.05$), compared with the control group. ^a $P<0.05$ vs. control U251 GSKs 500; ^b $P<0.05$ vs. control U251 GSKs 1000. GSKs, glioma stem-like cells; BrMChR, 8-bromo-7-methoxychrysin. GSKs 500, 500 U251 GSKs seeded in 96-well ultra-low attachment plates; GSKs 1000, 1000 U251 GSKs seeded in 96-well ultra-low attachment plates.

MTT assay screening of active drugs. The inhibitory rates of drugs from 12 different classes on tumor stem cell-like cells and tumor cells were compared by MTT assay. The results revealed that 6 of the drugs had higher inhibitory rates on the U251 GSKs than on the U251 cells; these drugs were curcumin, chrysin, apigenin, luteolin, casticin and BrMChR (Fig. 2).

Effect on the number of tumor balls. As shown in Fig. 3, U251 GSKs treated with 6 active drugs were seeded in 96-well ultra-low attachment plates. The number of tumor balls in the

control samples gradually increased over 7 days. The number of tumor balls was decreased by treatment with 4 of the drugs after 7 days, specifically curcumin, luteolin, casticin and BrMChR; all $P<0.05$ compared with the control group.

Discussion

Glioma is a common type of adult malignancy with high morbidity and mortality. Although clinical treatments with surgery, chemotherapy, radiotherapy and biological therapy, are presently used, the tumors recur without exception (21-24).

The disease progresses rapidly following recurrence and seriously threatens the life and health of the patients (25,26). Within the past decades, accumulating evidence from a number of biological systems, including the blood (27), breast (28) and brain (6,29), has indicated that the transformation of cancer stem-like cells may induce the formation of tumors. One novel treatment strategy under investigation is to make cancer stem-like cells differentiate into non-dividing cells. If successful, patients with brain tumors would be able to lead a normal life. The tumor would stop growing due to the terminal differentiation of the cells (30). A number of sorting technologies for stem cell-like cells, including immunomagnetic beads and SP sorting methods, are becoming widely used in the study of cancer stem cell-like cells. However, these sorting methods have a number of limitations.

In our study, serum-free suspension clone formation successfully induced the formation of U251 GSLCs. Purified U251 GSLCs could be suspended in serum-free medium. Active drugs of 6 classes (curcumin, chrysin, apigenin, luteolin, casticin and BrMChR) were identified by the screening of drugs from 12 classes by MTT assay. Four of the drugs were able to affect the number of tumor balls; these were curcumin, luteolin, casticin and BrMChR (all $P < 0.05$).

We have preliminarily identified methods for culturing stem cell-like cells and used the cells in the screening of active drugs. The immunofluorescence identification of relevant molecular markers of GSLCs and animal experiments *in vivo* are subjects of our future studies. Only by performing these studies are we likely to find the most active drug, to contribute further to the investigation of human glioma.

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