β-elemene inhibits stemness, promotes differentiation and impairs chemoresistance to temozolomide in glioblastoma stem-like cells

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Abstract. Accumulating evidence indicates that glioblastoma stem-like cells (GSCs) are key factors in tumour development, recurrence and chemoresistance. The impairment of stemness and the enhancement of differentiation contributes to the weakening of radiation and chemotherapy resistance of GSCs. We previously found that β-elemene was an effective anti-glioblastoma agent and chemosensitizer. In this study, we examined the distribution of CD133+ cells in human glioblastoma tissues by immunohistochemistry. Following treatment with β-elemene, the formation of GSC spheres was investigated by manual counting, the proliferation of GSCs was measured with a Cell Counting Kit-8 (CCK-8) assay, and the dispersion of GSC spheres was observed with an inverted microscope. GSC spheres were treated with β-elemene, and the expression levels of CD133, ATP-binding cassette subfamily G member 2 (ABCG2) and glial fibrillary acidic protein (GFAP) were examined by western blotting. After treatment with β-elemene, the volumes and weights of GSC xenografts were measured, and the expression of CD133, ABCG2 and GFAP was evaluated through immunohistochemistry analysis. After treatment with β-elemene and temozolomide (TMZ), GSC viability was examined by the CCK-8 assay, and the volumes and weights of xenografts were measured. We found that CD133+ cells were assembled in some vascular walls and also sparsely distributed in other parts of glioblastoma tissues. β-elemene decreased the formation of GSC spheres, dispersed GSC spheres and inhibited the proliferation of GSCs *in vitro* and *in vivo*. In the GSC spheres and xenografts treated with β-elemene, the expression of CD133 and ABCG2 was significantly downregulated, and the expression of GFAP increased. Furthermore, the sensitivity of GSCs to TMZ was enhanced *in vitro* and *in vivo*. These results suggest that β-elemene impaired the stemness of GSC spheres, promoted their differentiation and sensitized GSCs to TMZ. β-elemene will hopefully become a valuable agent to enhance the effects of radiotherapy and chemotherapy.

Introduction

Glioblastoma is the most common and lethal type of primary brain tumours, accounting for approximately 52% of primary intracranial tumours. Glioblastoma is characterised by strong chemotherapy resistance and post-operative recurrence with a median survival of less than 12 months. Despite many advances in therapeutics over the past several years, the prognosis for patients with glioblastoma remains dismal (1-5).

In the past decade, accumulating evidence has indicated that glioblastoma stem-like cells (GSCs, also known as glioblastoma initiating cells) are at the root of tumour development, recurrence and drug resistance (6). GSCs are a small population (1-10%) of cells with some neural stem cell (NSC) properties. GSCs have limitless self-renewal capacity and express some stem cell hallmarks, such as CD133, ATP-binding cassette subfamily G member 2 (ABCG2) and nestin. Additionally, GSCs are multipotent and can differentiate into neurons, astroglial cells and oligodendroglial cells to thus sculpt a resulting tumour that is composed of a mixture of different cell types. GSCs also have the ability to initiate and drive tumour progression in animal models (7,8). These characteristics have been used to identify GSCs in many studies (9-11).

GSCs frequently show strong radioresistance and chemoresistance. It has been suggested that conventional post-operative chemotherapy and radiotherapy will kill most remaining cancer cells but will leave GSCs intact, resulting in the recurrence of glioblastoma. It has been reported that the radioresistance and chemoresistance of tumour stem cells (TSCs) is markedly decreased after their differentiation into normal tumour cells that display certain differentiation hallmarks (12,13). Thus, finding effective ways to promote GSC differentiation will hopefully limit the radiotherapy

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and chemotherapy resistance of GSCs and notably enhance therapeutic efforts.

For use as an effective antitumour medicine, elemene is extracted from Curcuma wenyujin as an essential oil mixture of β-, γ- and δ-elemene (14). β-elemene (molecular formula $C_{15}H_{24}$, molecular weight 204.34), the major active anticancer component in the elemene mixture, has strong anti-proliferative activity and induces apoptosis in various tumours, such as glioma, breast carcinoma and leukaemia (15-17). We previously found that β-elemene can inhibit the proliferation of glioblastoma cells and induce cell apoptosis *in vitro* and *in vivo* (17-21). In addition, β-elemene can promote the differentiation of Tca8113 human tongue squamous cancer cells and PLA801D human pulmonary giant cell carcinoma cells *in vitro* (22,23). Therefore, it was essential to illustrate the effects of β-elemene on GSC proliferation, differentiation and chemoresistance.

In this study, we illustrated the effect of β-elemene on some stem cell phenotypes of GSCs. We found that CD133^{+} cells were not only assembled in some vascular walls but were also sparsely localized to other zones of glioblastoma tissues. β-elemene inhibited the proliferation of GSCs *in vitro* and *in vivo*, decreased the formation of GSC spheres, dispersed GSC spheres and even resulted in the fragmentation and death of some sphere cells. β-elemene significantly downregulated the expression levels of CD133 and ABCG2 and increased the expression of the astroglial cell marker glial fibrillary acidic protein (GFAP) *in vitro* and *in vivo*. Additionally, treatment with β-elemene sensitized GSCs to temozolomide (TMZ). These results suggested that β-elemene could impair the stemness of GSCs and promote their differentiation, which may make it a useful tool to enhance the effects of radiotherapy and chemotherapy.

Materials and methods

Reagents and antibodies. β-elemene (98% purity) was obtained from Jingang Pharmaceutical Co. (Dalian, China). TMZ and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). The antibodies against CD133, ABCG2, GFAP, neuron specific enolase (NSE), and myelin basic protein (MBP), used in western blots and immunofluorescence assays, were purchased from Boster Co., Ltd. (Wuhan, China). Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), leukaemia inhibitory factor (LIF), N2, Accutase Cell Dissociation Reagent and Alexa Fluor 594 goat anti-rabbit IgG were from Invitrogen Corp. (Carlsbad, CA, USA). The antibody against GAPDH was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-CD133 and IgG1 isotype control antibodies used in flow cytometry analysis were purchased from Miltenyi Biotec (Bergish Gladbach, Germany). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. (Japan). Nude mice were provided by the Experimental Animal Center of The Academy of Military Medical Sciences. Primary glioblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclone, UT, USA) supplemented with 10% foetal calf serum, 50 IU/ml penicillin and 50 mg/ml streptomycin and grown at 37°C in a humidified atmosphere with 5% CO_2.

Tumour specimens and primary cell cultures. Tumour tissues originated from 20 patients with glioblastoma (10 men and 10 women, 42±11.7 years, World Health Organization/WHO grade III-IV) undergoing surgical resection in the Department of Neurosurgery of the General Hospital of Shenyang Military. The cells of two of these patients (case 1, WHO grade III; and case 2, WHO grade IV) were cultured as primary glioblastoma cells. The study was approved by the Ethics Committee of the General Hospital of Shenyang Military and abided by the Declaration of Helsinki, with informed consent obtained from all study participants. Tumour samples were stored in sterile serum-free DMEM/F12 and processed within 0.5 h after resection. Tissues were cut into 1-mm^2 pieces, washed with PBS and digested with 0.25% trypsin at 37°C for 15 min. Cells were cultured in serum-containing DMEM/F12 after passage through a 70-μm strainer (BD Pharmingen, San Diego, CA, USA). Primary glioblastoma cells were resuspended in NSC medium (NSCM) containing DMEM/F12 medium, 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/ml LIF and N2 (1:100) for culturing GSCs.

Immunohistochemistry. Paraffin sections of glioblastoma tissues were obtained and deparaffinized. Antigen retrieval was performed with citrate buffer, pH 6.0 (Invitrogen). Non-specific sites were blocked by incubating sections with 5% BSA in a humidified chamber for 1 h at room temperature. The samples were incubated with 0.3% H_2O_2 for 15 min to block endogenous peroxidase activity and then labelled with different primary antibodies at proper concentrations (1:100 for CD133, ABCG2 and GFAP) for 1 h at room temperature. In subsequent steps, we used the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and DAB as a chromogen (Changdao Biotech, China). The nuclear counterstaining of sections was performed with hematoxylin. In every tissue, additional staining without primary antibody was performed in parallel as a negative control. The integrated optical density (IOD) was used to semi-quantitatively estimate the expression of CD133, ABCG2 and GFAP in Image-Pro Plus 6.0.

Determining the CD133 expression by flow cytometry analysis. Cells were digested into single cells with Accutase Cell Dissociation Reagent and labelled with anti-CD133 and control IgG isotype monoclonal antibodies (Miltenyi Biotec, San Diego, CA, USA). Flow cytometry analysis was conducted on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data acquisition and analysis were performed with CellQuest software (Becton-Dickinson).

GSC culture and passage. Primary glioblastoma cells were cultured in NSCM and formed cell spheres after 3 days. After forming spheres of at least 100 cells (culturing for approximately 5 days), cell spheres were digested to single cell suspension with Accutase Cell Dissociation Reagent and subcultured with NSCM in ultra-low attachment culture flasks (Corning, Inc., NY, USA).

Immunofluorescence and DAPI staining. In each well of 24-well plates, 2x10^{5} dissociated cells or 20 cell spheres were cultured on poly-L-lysine-coated cover slips for various times. Cells on cover slips were washed with PBS, fixed with methanol and then treated with 0.1% Triton X-100. After blocking
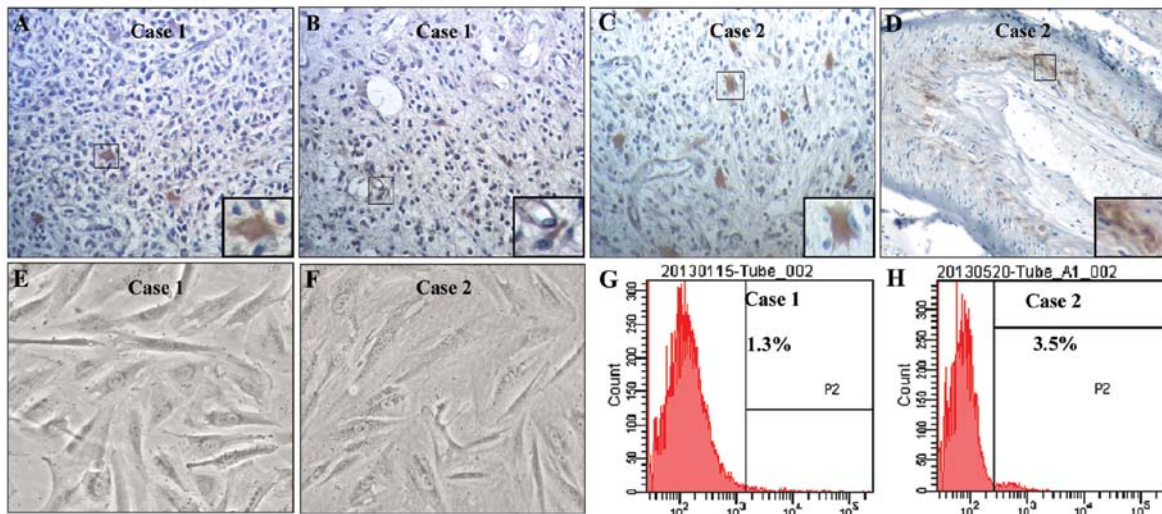
Figure 1. The distribution of CD133+ cells in human glioblastoma tissues and the proportion of CD133+ cells in primary glioblastoma cells. (A-D) CD133+ cells were not only assembled in some vascular walls but were also sparsely localized in other zones of glioblastoma tissues (x100). An individual tumour cell (inset black squares) was magnified to facilitate the interpretation of the staining pattern. (E and F) Primary glioblastoma cells were obtained from case 1 and case 2 (x200). (G and H) The proportions of CD133+ cells in primary glioblastoma cells were 1.3% for case 1 and 3.5% for case 2.

with 5% BSA, cells were incubated with proper dilutions of anti-CD133, GFAP, MBP or NSE antibodies at 4°C overnight. The primary antibodies were detected with Alexa Fluor 594 goat anti-rabbit IgG (dilution 1:500) for 40 min at 37°C in the dark. Cell nuclei were stained with DAPI, and immunofluorescent images were examined under a fluorescent microscope (Leica Microsystems, Germany).

Cell proliferation assay. Cell viability was evaluated with the CCK-8 assay after cells in exponential growth were cultured in a 96-well plate. Trypan blue staining confirmed >80% viability, and cells were treated according to the study design. Then, 10 μl of CCK-8 was added to each well, and the mixture was incubated for 4 h at 37°C. The optical density (OD) of each well was measured at 450 nm using a spectrophotometric microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Five replicate wells were used for each cell sample.

Western blotting. The cells were lysed with RIPA buffer [50 mM Tris-HCl (pH 7.4), 1.0% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 150 mM NaCl, 1 mM aprotinin, 1 mg/ml PMSF, 1 μg/ml pepstatin and 1 μg/ml leupeptin]. The concentrations of total protein in the cellular extracts were measured using the BCA assay kit from Keygen Biotech, Co., Ltd. (Nanjing, China). After separation in 10-12% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels, the proteins were transferred to nitrocellulose filter membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% BSA in Tris-buffered saline with Tween-20 at 4°C overnight and probed with various primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at 37°C for 3 h. The membranes were exposed to an ECL system (Amersham, Sweden), and fluorescence was detected by exposing the membrane to X-ray film. The results were scanned with the Image Quant 5.2 software (Amersham).

Transplantation of GSCs into nude mice and treatment of animals. GSC spheres were digested into single cells, and 1000 cells/animal (suspending in 0.1 ml DMEM/F12) were subcutaneously injected into the right shoulder region of 4-week-old female nude mice. The use of animals was approved by the Institutional Animal Care and Use Committee. Beginning with the 7th day after transplantation, various drugs were intraperitoneally injected. The volumes of tumours were measured every 3 days and calculated according to the formula: V = 1/2 x largest diameter x smallest diameter^2 (21). Tumours were weighed on the 21st day after transplantation.

Statistical analysis. The values are reported as the means ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using Student's t-test. Statistical significance was accepted at the level of P<0.05 between different groups, and P<0.01 was considered highly significant. Statistical analyses were performed with SPSS software (SPSS, Inc., Chicago, IL, USA).

Results

The expression of CD133 in human glioblastoma tissues and the proportion of CD133+ cells in primary glioblastoma cells. To investigate the distribution of CD133+ cells in human glioblastoma tissues, immunohistochemistry was performed with an anti-CD133 antibody. To examine the proportion of CD133+ primary glioblastoma cells, we cultured these cells from fresh glioblastoma tissues and performed flow cytometry using an anti-CD133 antibody. We found that CD133+ cells were not only assembled in some vascular walls but were also sparsely localized in other zones of glioblastoma tissues (Fig. 1A-D). Primary glioblastoma cells obtained from two glioblastoma samples (case 1 and case 2) had obvious cellular atypia and pathological nuclear fission (Fig. 1E and F). The proportions of CD133+ cells were 1.3% in case 1 (Fig. 1G) and 3.5% in case 2 (Fig. 1H).
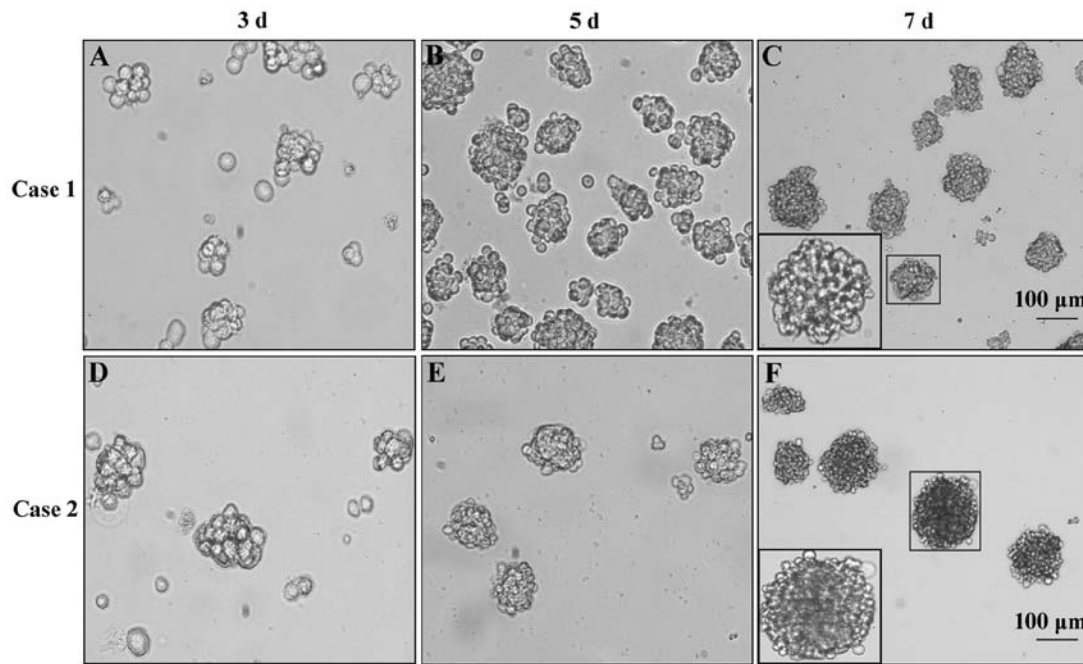
Figure 2. The formation of second-generation GSC spheres. (A) The second-generation GSC spheres of case 1 formed on the 3rd day and contained approximately 20 cells each. (B) Spheres of case 1 gradually expanded to 100 cells with a dense structure and high refractivity on the 5th day. (C) Spheres of case 1 continued to grow on the 7th day (the magnified sphere in black square). (D) The second-generation GSC spheres of case 2 formed on the 3rd day and contained approximately 30 cells each. (E) Spheres of case 2 gradually expanded to 100 cells with a dense structure and high refractivity on the 5th day. (F) Spheres of case 2 continued to grow on the 7th day (the magnified sphere in black square). Original magnifications x100. Scale bar, 100 μm.

The formation of second-generation GSC spheres. To obtain GSC spheres, we cultured primary glioblastoma cells in NSCM and observed cell sphere formation with an inverted microscope. The results showed that primary glioblastoma cells derived from case 1 and case 2 could form GSC spheres in NSCM. The second-generation GSC spheres formed on the 3rd day after passage and then grew gradually with a denser structure and higher refractivity (Fig. 2).

GSC spheres strongly express the NSC marker CD133 and show multipotent differentiation capacity. To test the expression of CD133 and the multipotent differentiation capacity in the second-generation GSC spheres, we cultured GSC spheres in DMEM/F12 with 10% foetal calf serum and examined CD133, GFAP (astrocyte marker), MBP (oligodendrocyte marker) and NSE (neuron marker) expression by immunofluorescence assays 0, 48 and 96 h after plating. We found that the second-generation GSC spheres strongly expressed CD133 with little expression of GFAP, MBP and NSE (Fig. 3, 0 h). During 48 h of adherent growth conditions in serum-containing DMEM/F12, GSC spheres gradually spread out. The expression of CD133 decreased, whereas the expression of GFAP, MBP and NSE increased with time. After 96 h, GFAP, MBP and NSE showed strong expression (Fig. 3). These results suggest that the GSC spheres we obtained possessed stem cell phenotypes and multipotent differentiation potential.

β-elemene inhibits the formation of GSC spheres, decreases the proliferation of GSCs and disperses cell spheres. To evaluate the effect of β-elemene on GSC sphere formation, cell spheres were digested into single cells. The cells were then plated at a density of 100 cells/well in 96-well plates and simultaneously treated with 100 μg/ml β-elemene for 72 h (untreated cells as control group). Finally, the number of cell spheres (containing at least 10 cells/sphere) was counted. To examine the effect of β-elemene on proliferation, 1000 GSCs and 10000 primary glioblastoma cells per well were independently plated in 96-well plates. These cells were then treated with 0 (control group), 50, 100, 150 or 200 μg/ml β-elemene for 24 h, and the CCK-8 assay was used to examine cell viability. To test the ability of β-elemene to dissociate GSC spheres, we added 200 μg/ml β-elemene to cell spheres for 12 h and observed sphere dispersion with an inverted microscope. The results showed that the number of GSC spheres that formed in the β-elemene group was less than in the control groups in both cases (Fig. 4A, P<0.01). The proliferation of both GSCs and primary glioblastoma cells (GC) (Fig. 4B) decreased dose-dependently in the presence of β-elemene. The degree of this viability loss was less for GSCs than for primary glioblastoma cells (Fig. 4B), which suggested that GSCs possessed stronger resistance to β-elemene than glioblastoma cells. β-elemene can disperse GSC spheres, and this can result in the fragmentation and death of some cells (Fig. 4C-F).

β-elemene decreases the expression of stem cell markers CD133 and ABCG2 and increases the expression of the differentiation marker GFAP in GSC spheres. To investigate the effect of β-elemene on the expression of GSC markers CD133 and ABCG2 and differentiation marker GFAP, GSC spheres were treated with 100 and 200 μg/ml β-elemene for 24 h, and western blot analysis was performed using specific antibodies. The results were semi-quantitatively estimated using Gel-Pro
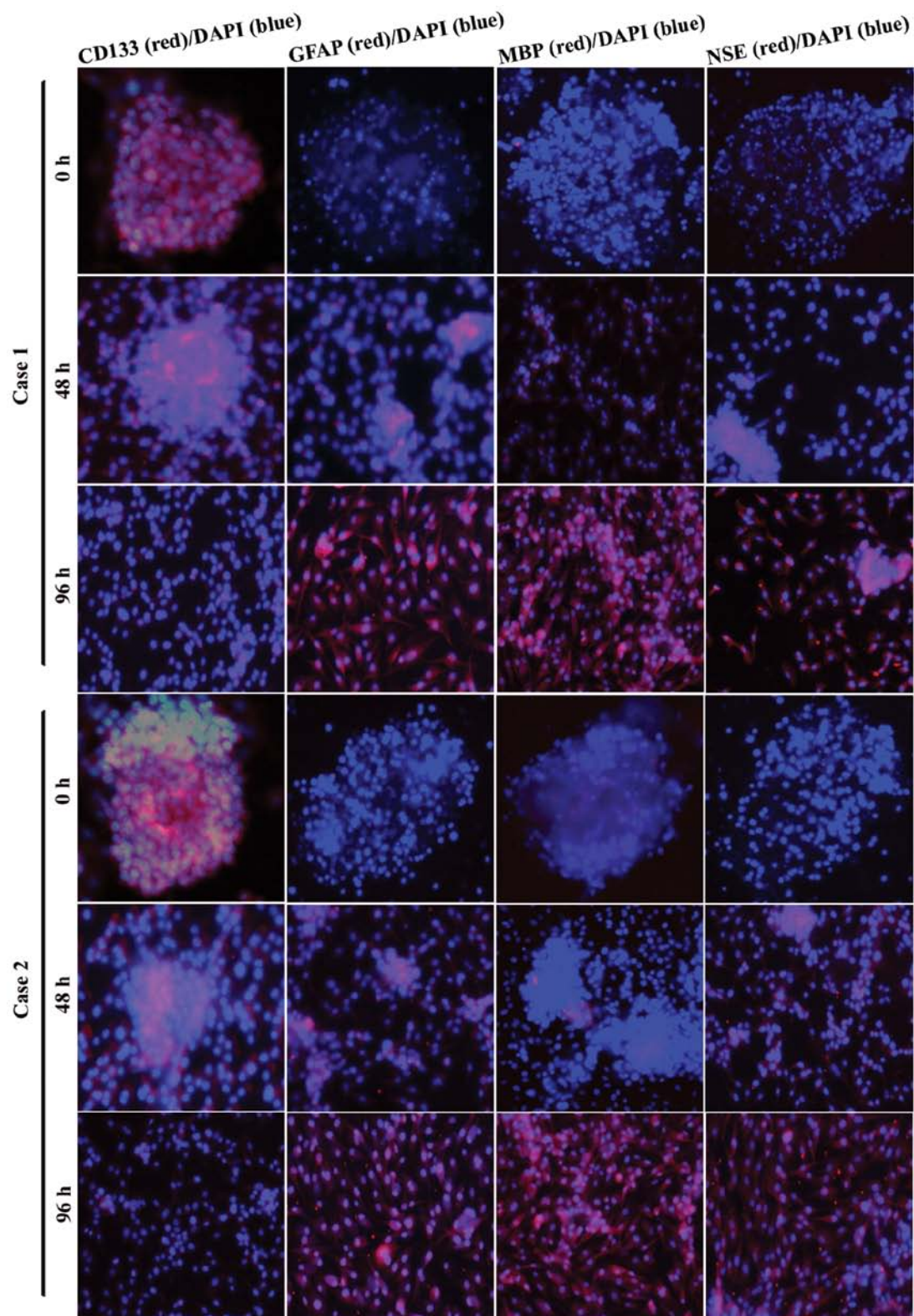
Figure 3. GSC spheres strongly expressed CD133 and showed multipotent differentiation potential. CD133, GFAP, MBP and NSE were examined in GSC spheres by immunofluorescence analysis 0, 48 and 96 h after plating. The results showed that CD133 was strongly expressed in the second-generation GSC spheres with little GFAP, MBP and NSE (0 h). GSC spheres adherently grew and gradually spread to the surroundings at 48 h. The expression of CD133 weakened, whereas the expression of GFAP, MBP and NSE increased with time. GFAP, MBP and NSE showed strong expression at 96 h. This result suggested that the GSC spheres we obtained possessed stem cell phenotypes and multipotent differentiation potential. Original magnifications x100.

analyzer 4.0 software and illustrated graphically. We found that the expression levels of CD133 and ABCG2 were significantly downregulated, while the expression of GFAP was increased by β-elemene in a dose-dependent manner (Fig. 5). These results suggested that β-elemene impairs the stemness of GSC spheres and promotes their differentiation.
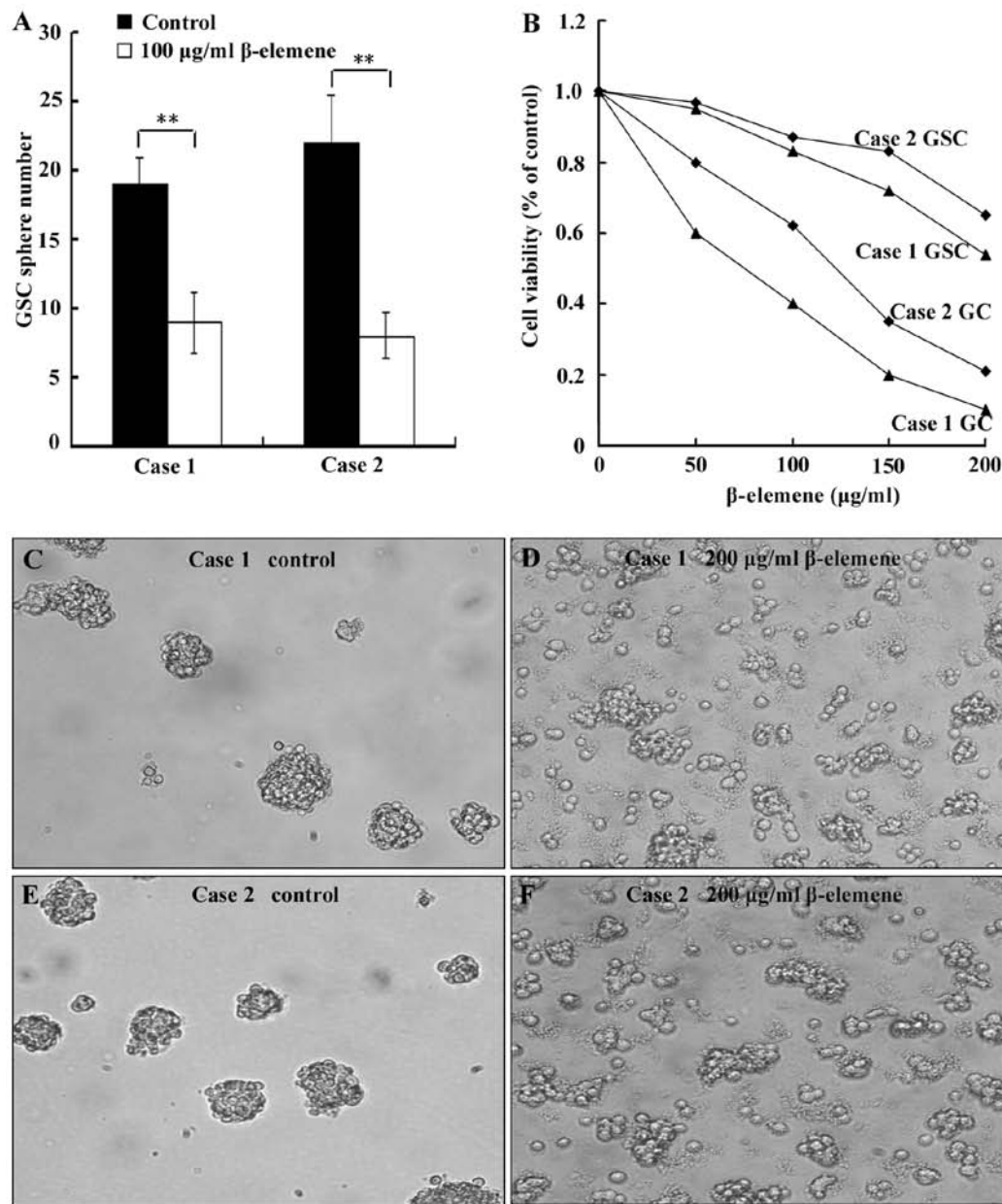
Figure 4. β-elemene inhibits the formation of GSC spheres, decreases the viability of GSCs and disperses cell spheres. (A) The number of GSC spheres that formed in the β-elemene group was less than in the control groups in both cases. (B) The proliferation of both GSCs and primary glioblastoma cells (GC) decreased dose-dependently in the presence of β-elemene. The degree of viability loss was less in GSCs than in primary glioblastoma cells, which suggested that GSCs are more resistant to β-elemene than glioblastoma cells. (C-F) GSC spheres were dispersed, and partial cells fragmented and died after β-elemene treatment in both cases. Values are shown as the means ± SD (*P<0.05, **P<0.01).

β-elemene suppresses the development of tumours in nude mice transplanted with GSCs. To evaluate the effect of β-elemene on GSC tumours *in vivo*, we subcutaneously injected GSCs into the flank of nude mice and then intraperitoneally injected NaCl or 50 mg/kg β-elemene for 1 week. The tumour volumes were measured every 3 days. Points are the means ± SD of tumour volume in each group (n=6). The results showed that a significant inhibition of tumour volume was observed in the β-elemene-treated group compared with the control group from the 9th day after transplantation (the 3rd day after intraperitoneal injection) to the 21st day, as shown in Fig. 6A and B. On the 21st day, tumours were dissected and measured. The tumour weights of the β-elemene group were significantly lower

than those of the control group, as shown in Fig. 6C and D. These results suggested that the development of tumours was suppressed by β-elemene in GSC-bearing nude mice.

β-elemene downregulates the expression of CD133 and ABCG2 and upregulates the expression of GFAP in GSC-transplanted nude mice. To evaluate the effect of β-elemene on the expression of stem cell markers and differentiation markers *in vivo*, immunohistochemistry was performed using antibodies against CD133, ABCG2 and GFAP on the aforementioned tumour tissues of GSC-transplanted nude mice in β-elemene and NaCl groups (Fig. 7A). Image-Pro Plus 6.0 software was applied to calculate the IOD of each group (the same selected
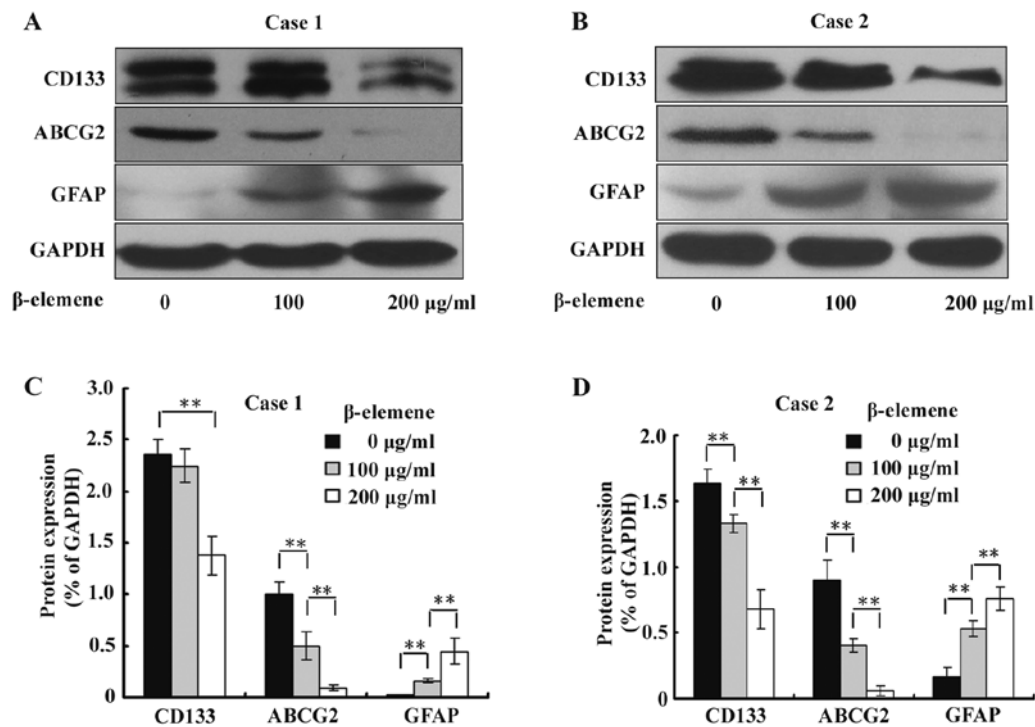
Figure 5. The expression of CD133 and ABCG2 is decreased, and the expression of GFAP is increased by β-elemene in GSC spheres. (A and B) β-elemene downregulated the expression of CD133 and ABCG2 and upregulated the expression of GFAP dose-dependently in case 1 and case 2. (C and D) The results of (A) and (B) were semi-quantitatively estimated using Gel-Pro Analyzer 4.0 software and illustrated in a polygram. The results are representative of three independent experiments, and values shown are the means ± SD (*P<0.05, **P<0.01).
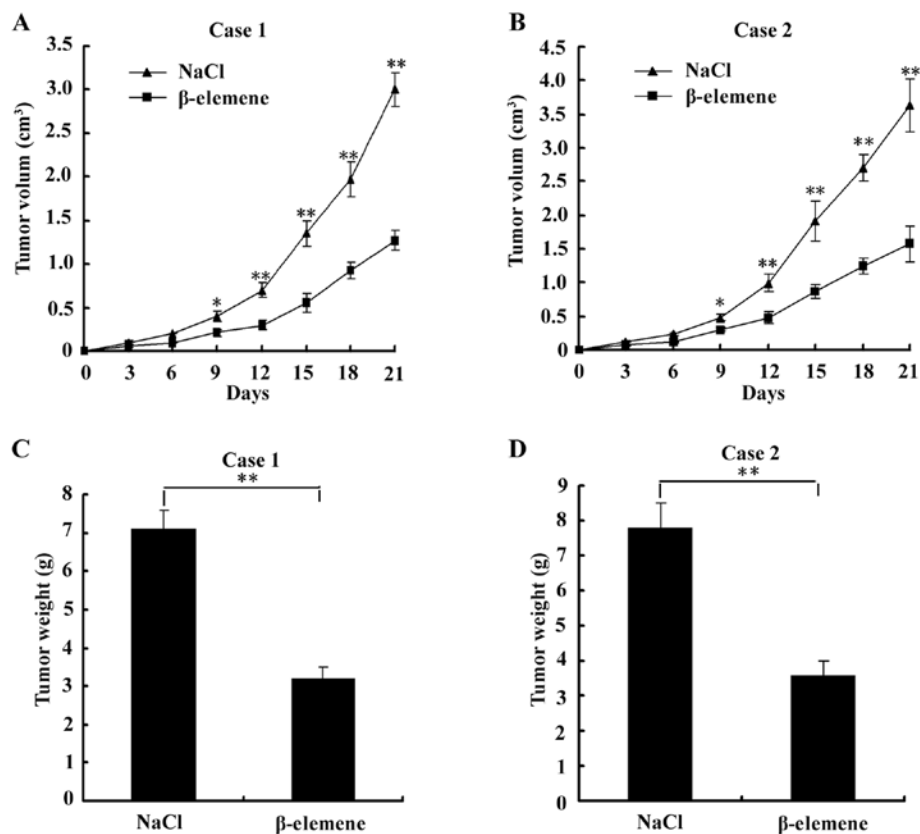

Figure 6. The development of tumours in nude mice transplanted with GSCs is suppressed by β-elemene. The nude mice transplanted with GSCs received an intraperitoneal injection of NaCl or 50 mg/kg β-elemene for 1 week, and the tumour volumes were measured every 3 days. (A and B) In contrast to the control group, the remarkable suppression of tumour growth was observed for the whole period from the 9th day after transplantation (the 3rd day after intraperitoneal injection) to the 21st day in both cases 1 and 2. (C and D) The weights of tumours in the β-elemene group on the 21st day after transplantation were significantly lower than those of the control group in both cases 1 and 2. Values shown are the means ± SD (*P<0.05, **P<0.01).
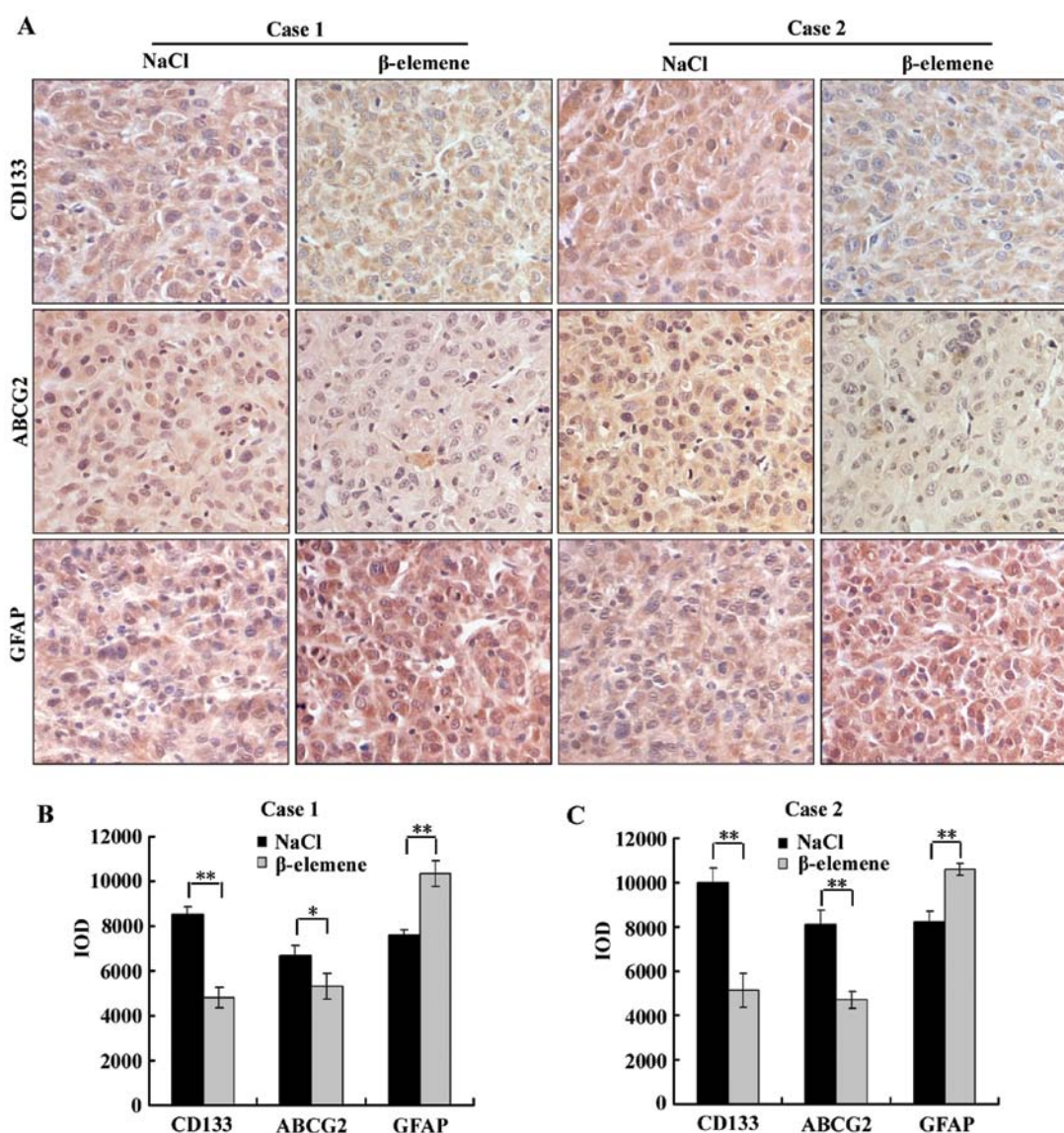
Figure 7. β-elemene decreases the expression of CD133 and ABCG2 and increases the expression of GFAP *in vivo*. (A) After the tumour tissues from GSC-transplanted nude mice treated with β-elemene or NaCl in the previous experiment were prepared following routine protocols, sample slides were probed with antibodies against CD133, ABCG2 and GFAP. (B and C) The IOD of each group was analysed using Image-Pro Plus 6.0 software (the same selected areas and magnifications in all the images) and illustrated by a vertical bar chart. The IODs for CD133 and ABCG2 were lower and the IOD for GFAP significantly higher in the β-elemene group than in the NaCl group. Serial sections stained with control antibodies were negative (data not shown). Values shown are the means ± SD (*P<0.05, **P<0.01). Original magnifications x200.

areas and magnifications in all images), and then statistical analysis was performed. The IOD of each group is illustrated in a vertical bar chart (Fig. 7B and C). We found in this assay that the expression levels of CD133 and ABCG2 in the β-elemene group were significantly lower than in the NaCl group, and the expression level of GFAP in the β-elemene group was higher than in the NaCl group in both cases 1 and 2. These results suggest that β-elemene decreased the expression of CD133 and ABCG2 but increased the expression of GFAP *in vivo*.

Treatment with β-elemene sensitizes GSCs to TMZ-induced cytotoxicity. As reported, GSCs were usually more resistant to TMZ cytotoxicity than normal glioblastoma cells (21). To determine whether treatment with β-elemene sensitizes GSCs to TMZ, GSC spheres were digested into single cells and then

plated at a density of $5x10^3$ cells/well in 96-well plates. Cells were organized into the following three groups: control (untreated), TMZ (treated with 6 µg/ml TMZ for 24 h) and combination (treated with 6 µg/ml TMZ and 100 µg/ml β-elemene for 24 h). After treatment with the drugs, CCK-8 assays were performed to examine cell viability. Additionally, 18 nude mice were injected with GSCs to create tumours and divided into the following three groups (6 mice/group): NaCl (negative control, intraperitoneally injected with 50 mg/kg NaCl for 1 week), TMZ (intraperitoneally injected with 33 mg/kg TMZ for 1 week) and combination (intraperitoneally injected with 33 mg/kg TMZ and 50 mg/kg β-elemene for 1 week). The volumes of tumours were measured and calculated according to the formula: V = 1/2 x largest diameter x smallest diameter^2, every 3 days, and tumours were weighed on the 21st day. The results showed that
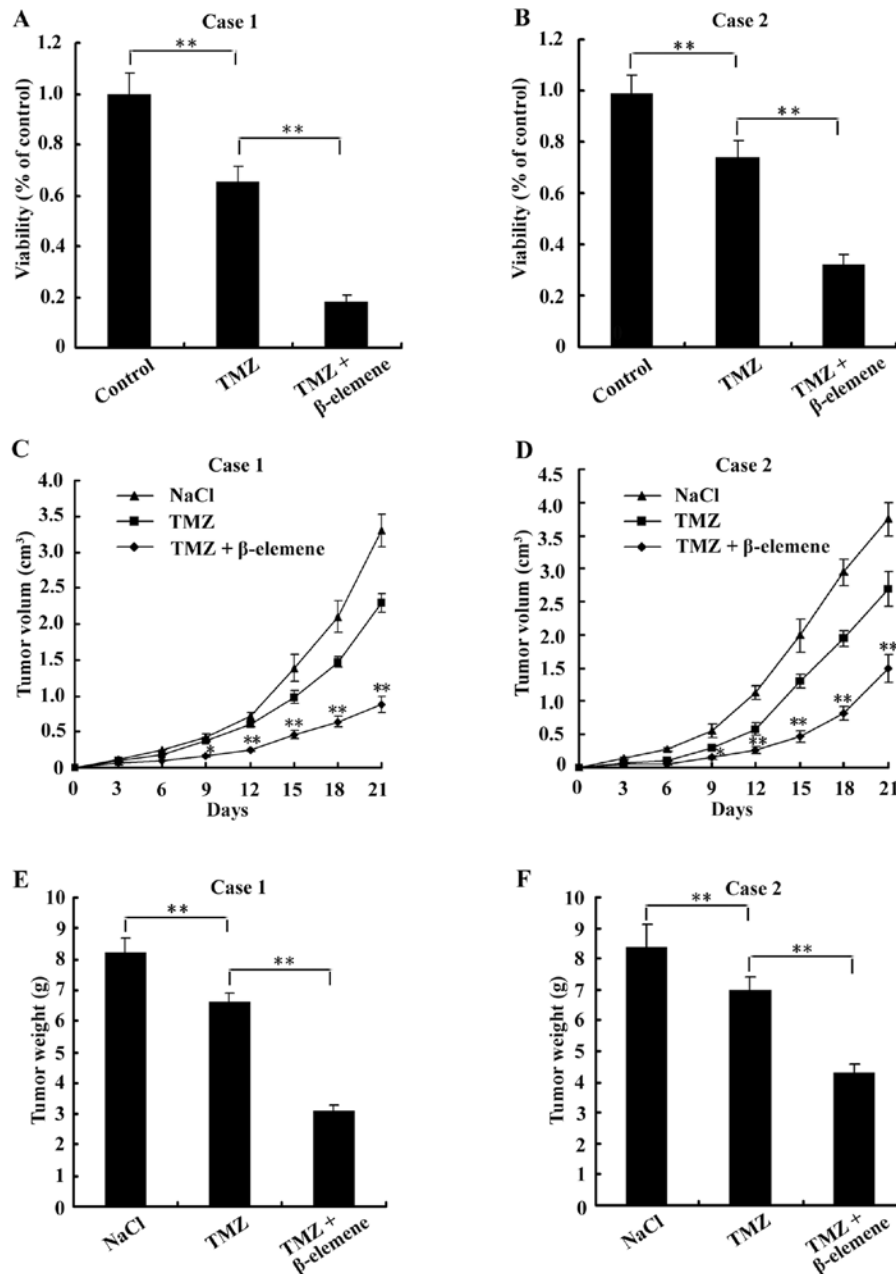
Figure 8. β-elemene increases the sensitivity of GSCs to TMZ-induced cytotoxicity *in vivo* and *in vivo*. (A and B) GSC spheres of case 1 and case 2 were digested into single cells and then plated in 96-well plates. CCK-8 assays were performed after treatment with the drugs. Cell viability was lower in the combination group than in either the control or TMZ groups. (C and D) The tumour volumes of nude mice were less in the combination group than in either the control or TMZ groups in case 1 and case 2. (E and F) The weights of tumours were less in the combination group than in either the control or TMZ groups in case 1 and case 2. These findings suggest that treatment with β-elemene sensitized GSCs to TMZ-induced cytotoxicity *in vitro* and *in vivo*. Values shown are the means ± SD (*P<0.05, **P<0.01).

cell viability was lower in the combination group than either the control or the TMZ groups (Fig. 8A and B). In the *in vivo* experiments, we found that both the volumes (Fig. 8C and D) and the weights (Fig. 8E and F) of tumours were less in the combination group than in either the control or TMZ groups. These findings suggest that β-elemene increased the sensitivity of GSCs to TMZ-induced cytotoxicity.

Discussion

It was reported that CD133$^+$ cells tend to distribute around microvessels in glioblastoma tissues (24,25). In this study,

we found that CD133$^+$ cells were not only assembled in some vascular walls (smaller cells) but also sparsely localized in other parts of glioblastoma tissues (larger cells). This difference in the cell distribution may be due to individual variability between the samples.

It has been suggested that GSCs are the source of malignant tumour development, recurrence and chemoresistance. Conventional chemotherapy will only kill the bulk of normal glioblastoma cells in a tumour but will leave the drug-resistant GSCs intact, resulting in tumour recurrence. A representative property of GSCs is the ability to form neurospheres in special NSCM. Thus, the effects of drugs on GSCs can be conve-

niently evaluated by measuring cell proliferation, the ability to form neurospheres and the degree of neurosphere dispersion (26-29). We previously found that β-elemene can significantly reduce the size of tumours and prolong the life-span of patients without serious side effects and that it can inhibit glioblastoma growth through the activation of GMFβ-MKK3/6-p38 and the downregulation of phosphorylated ERK1/2 *in vitro* and *in vivo* (17-21). In this study, we found that β-elemene inhibited the proliferation of GSCs *in vitro* and *in vivo*, decreased the formation of GSC spheres, and dispersed GSC spheres, which resulted in the fragmentation and death of some sphere cells. β-elemene also suppressed the ability of GSCs to form xeno-grafts in nude mice. This is the first report of the effects of β-elemene on GSCs.

Human CD133 (prominin-1), a 120-kDa cell-surface protein, has been accepted as the standard marker for the identification of GSCs, although it may not be exclusive or the most ideal marker for this cell type (30-32). ATP-binding cassette subfamily G (ABCG) is a membrane pump that consumes ATP to excrete endogenous small molecules, such as cholesterol, ions and peptides, across cell membranes. ABCG also plays a pivotal part in detoxification and protection against xenobiotics by expelling drugs from the cell. NSCs and TSCs have been shown to express high levels of ABCG, which is normally inactive in more mature cells. It was shown that a major member of the ABCG family, ABCG2 (also known as BCRP1), is an important resistance-related molecule and GSC marker (8,33-36). The capacity for multipotent differentiation is a representative characteristic of GSCs. The expression of the astroglial cell marker GFAP was low in NSCs and GSCs and gradually increased during the cellular differentiation process. Therefore, the increase in the expression of GFAP generally indicated an enhancement of GSC differentiation. In this study, we found that the expression levels of CD133 and ABCG2 were significantly downregulated, whereas the expression of GFAP was increased by β-elemene in both GSC spheres *in vitro* and in nude mouse xenografts *in vivo*. These results suggested that β-elemene could impair the stemness of GSC spheres and promote their differentiation into normal glioblastoma cells.

TMZ is the most commonly used chemotherapeutic agent in glioblastoma treatment. It achieves its antitumour effect primarily by methylating the O6 position of guanine. TMZ can kill most glioblastoma cells and improves the overall survival and the progression-free survival of patients with glioblastoma undergoing surgical resection and radiotherapy (37-40). However, GSCs in glioblastoma tissues show incred-ible chemoresistance to TMZ treatment. It was reported that GSCs can survive even in the presence of 200 μM TMZ (13,41,42). The maximum TMZ concentrations in the plasma of patients are between 27 and 50 μM and are only 0.5-10 μM in cerebrospinal fluid (21,43,44), which suggests that it is virtu-ally impossible to use TMZ to eliminate GSCs. Therefore, enhancing the sensitivity of GSCs to chemotherapeutic drugs is crucial for decreasing neoplasm recurrence and improving long-term prognosis. β-elemene is not only an antitumour agent but is also a chemosensitizer in the treatment against glioblastoma. β-elemene could partially reverse the multidrug resistance to adriamycin in SGC7901/Adr human gastric carcinoma cells (45). Similarly, the synergistic anticancer

effects of β-elemene and cisplatin were observed in human laryngeal carcinoma-bearing nude mice and ovarian carci-noma cells (46,47). We previously found that β-elemene can sensitize U87 human glioblastoma cells to cisplatin *in vitro* (19). In this study, we conducted experiments *in vitro* and *in vivo* and found that β-elemene sensitized GSC spheres to TMZ-induced cytotoxicity and enhanced the anti-proliferative effects of TMZ on nude mouse xenografts.

However, the chemosensitization mechanism of β-elemene remains unclear. We speculate that the following aspects may be involved. First, the differentiation of GSCs into normal glioblastoma cells induced by β-elemene improved the sensitivity of GSCs to antitumour drugs. Second, β-elemene downregulated the expression of the resistance-related protein ABCG2, which was likely to increase the intracel-lular accumulation of chemotherapeutics. Finally, the p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases 1 and 2 (ERK1/2)/BCL-2 signalling pathways are closely related to the drug resistance of various tumours (17-21). We previously reported that β-elemene could activate the GMFβ-MKK3/6-p38 pathway and inhibit the ERK1/2/BCL-2 pathway, and this may also be a potential molecular mechanism underlying chemosensitization with β-elemene.

In conclusion, β-elemene impaired the stemness of GSC spheres, promoted their differentiation and sensitized GSCs to TMZ-induced cytotoxicity *in vitro* and *in vivo*. β-elemene will hopefully become a valuable agent to enhance the effects of radiotherapy and chemotherapy and improve the long-term prognosis of patients with glioblastoma.

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