Co-expression of Cytoskeletal Protein Adducin 3 and CD133 in Neurospheres and a Temozolomide-resistant Subclone of Glioblastoma

MING-WAI POON*, JAMES TIN FONG ZHUANG*, STANLEY THIAN SZE WONG, STELLA SUN, XIAO-QIN ZHANG and GILBERTO KA KIT LEUNG

Department of Surgery, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong, R.O.C.

Abstract. *Background: Glioma stem cells are associated for temozolomide-resistance in glioblastoma. Adducin 3 (ADD3) is a cytoskeletal protein associated with chemoresistance but its role in glioblastoma has not been investigated. Materials and Methods: Using an in vitro model of glioblastoma cells with acquired temozolomide resistance (D54-MG-R), the expressions of ADD3 and cancer stem cell markers were compared to those in temozolomide-sensitive glioblastoma cells (D54-MG-S). Immunofluorescence staining was used to investigate the expression patterns of ADD3 and cancer stem cell markers in temozolomide resistance and neurospheres of glioblastoma. Results: Chemoresistant cells were found to have up-regulation of ADD3 and CD133 expression. A sub-population of D54-MG-R cells and glioma neurospheres exhibited coexpression of ADD3 with CD133. Conclusion: To our knowledge, this is the first report of a possible link between cytoskeletal protein expression, cancer stem cell phenotype and temozolomide resistance in human glioblastoma. This report lays the foundation for further investigation for ADD3 as a potential biomarker and therapeutic target in temozolomide-resistant glioma cells.*

Glioblastoma multiforme (GBM) is the most common and malignant form of primary brain tumor (1). The current standard treatment of GBM involves maximal surgical resection followed by chemoirradiation and adjuvant temozolomide (2). Despite this multimodality treatment, the prognosis of patients with GBM remains poor. Although temozolomide can provide significant survival benefit, intrinsic and acquired drug resistance is common and accounts for the majority of treatment failures (3). Glioma stem cells (GSCs) are thought to be at least partially responsible for temozolomide resistance in GBM, and have attracted intense research effort in recent years (4).

Cells use their cytoskeletons to move, polarize, divide and maintain organization within multicellular tissues. The cytoskeleton has three main structural components: microfilaments, intermediate filaments, and microtubules. Amongst these, actin is a highly conserved and essential building block of the cytoskeleton that is constantly remodeled by more than 100 different actin-binding proteins. The latter include adducins (ADDs), which are heteromeric proteins composed of different subunits referred to as alpha, beta and gamma. ADDs stimulate spectrin–actin binding to form meshworks beneath plasma membranes such as ruffling membranes (5). ADDs have been implicated in the development of chemoresistance in osteosarcoma (6, 7). Györffy *et al*. studied 30 cancer cell lines and reported correlations between ADD expression and drug resistance to paclitaxel, topotecan, doxorubicin and vinblastin (8). In glioma, the tetramer ADD3 has been implicated in tumor development, progression and migration (9); its role in temozolomide resistance, however, has not been investigated.

In this pilot study, we compared the expression level of ADD3 in temozolomide-resistant and temozolomide-sensitive GBM cells. We also investigated ADD3 co-expression with GSC markers in temozolomide-resistant GBM cells and glioma neurospheres. We hypothesized that the acquisition of temozolomide resistance and development of GSC phenotypes would be associated with up-regulation of ADD3 expression.

*These Authors contributed equally to this study.

Correspondence to: Dr. Gilberto K.K. Leung, Division of Neurosurgery, Department of Surgery, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Queen Mary Hospital, 102 Pokfulam Road, Hong Kong. (Hong Kong Postal Code: 999077), R.O.C. Tel: +852 22553368, Fax: +852 28184350, e-mail: gilberto@hku.hk

Key Words: Co-expression, cytoskeletal protein, adducin 3, cancer stem cell marker, CD133, neurospheres, temozolomide-resistant subclone, glioblastoma, NANOG.

Materials and Methods

Temozolomide-resistant GBM cells and neurosphere formation. Human GBM cells, D54-MG, (Duke University Medical Center, Durham, NC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1), supplemented with 10% fetal bovine serum (FBS) (all from GIBCO; Life Technologies, Inc., Carlsbad, CA, USA), and maintained in humidified incubators with 5% carbon dioxide and 95% air atmosphere. Temozolomide-resistant sub-clones were developed as previously described by our group (10). Briefly, temozolomide (TEMODAR; MSD, Whitehouse Station, NJ, USA) was dissolved in 100% dimethylsulphoxide (DMSO) to form 10 mM stocks, which were then diluted in DMEM/F12 to different working concentrations. Cells were treated with escalating concentrations of temozolomide using the pulse-treatment method. The temozolomide-resistant sub-clone developed was designated as D54-MG-R and the parental temozolomide-sensitive cells as D54-MG-S.

To obtain neurospheres, D54-MG-S cells were cultured in serum-free DMEM/F12 (1:1). After culturing for 3 to 4 days, neurospheres staining positively for prominin 1 (CD133) were generated.

Temozolomide-sensitivity test. Sulforhodamine B (SRB)-based *in vitro* toxicology assay kit (Sigma-Aldrich, Saint Louis, MO, USA) was used according to the manufacturer's instructions. Cells were seeded on 96-well plates. DMEM/F12 with different temozolomide concentrations (0, 125, 250, 500, 1,000, 2,000, 3,000 μM) were added for 72 h. Surviving cells were measured by absorbance at an optical density (OD) of 490 nm. The 50% inhibitory concentrations (IC_{50}) were calculated from the toxicity curves derived from three independent experiments.

RNA extraction and preparation. Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). *ADD3* mRNA was reversely transcribed into cDNA, using assay-specific primers according to the manufacturer's protocol (Applied Biosystems Ltd., Foster City, CA, USA). *ADD3* (Assay ID: Hs 00249895_m1) and *CD133* (Assay ID: Hs 01009261_m1) mRNA expression levels were detected by Taqman RNA probes in 7900 HT Faster Real-Time Polymerase Chain Reaction (PCR) System (Applied Biosystems Ltd.).

Western blot analysis. To analyze protein expression levels, 20 μg of total protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes by electroblotting. The membranes were blocked in 5% milk powder in Tris-buffered saline (Tris base 2.42 g/l, pH 7.6, NaCl 8 g/l) with 0.001% Tween 20 (TBS-T) at room temperature for 1 h. The membrane was probed overnight at 4˚C with antibodies against ADD3 (1:750; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), CD133 (1:1,000; Abcam, San Francisco, CA, USA), and the house keeping protein β-actin (1:2,000; Sigma-Aldrich). After 1 h of incubation with horseradish peroxidase-coupled secondary anti-rabbit or anti-mouse antibodies (1:1,000; Invitrogen-Zymed Laboratories, Carlsbad, CA, USA), specific antibody binding was detected by the enhanced chemiluminescence technique (GE Biosciences, Pittsburgh, KS, USA) and the Western Blotting Detection System (GE Biosciences).

Immunofluorescence staining. Cells were fixed with 2% paraformaldehyde/PBS (pH 7.2) (Invitrogen-Zymed Laboratories), and stained with the following primary antibodies: ADD3 (1:100; Santa Cruz Biotechnology), CD133 (1:100; Abcam), POU Class 5 Homeobox 1 (OCT3/4) and Nanog homeobox (NANOG) (1:200; Santa Cruz Biotechnology), and then with the corresponding secondary antibodies (1:1000; Invitrogen-Zymed). The cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen-Zymed), and visualized under fluorescence microscopy for the specific fluorescent signals from the specific staining by the specific antibodies. Images were captured and presented.

Statistical analysis. Data analysis was performed by SPSS for Windows version 17.0 (SPSS, Inc., Chicago, IL, USA). The *p*-values were calculated by the paired-sample *t*-test. *p*-Values of less than 0.05 were considered statistically significant. All *p*-values were two-sided.

Results

Temozolomide-resistant GBM model. We compared D54-MG-R cells with D54-MG-S cells in their responses to temozolomide treatment. The IC_{50} was around 1000 μM for D54-MG-S and 3000 μM for D54-MG-R cells (Figure 1). The resistance index (RI), calculated with the formula: RI=IC_{50} for D54-MG-R/IC_{50} for D54-MG-S, was around 3, indicating the successful development of a temozolomide-resistant subclone.

Up-regulated ADD3 expression in temozolomide-resistant cells. We measured the mRNA level of *ADD3* by real-time quantitative PCR (qPCR). *ADD3* expression was 6.47-fold higher in D54-MG-R than in D54-MG-S cells (p=0.041). (Figure 2A) To confirm up-regulation of expression, ADD3 protein level was measured by western blotting, we demonstrated a relative increase in D54-MG-R over D54-MG-S cells (Figure 2B).

Up-regulated CD133 expression in temozolomide-resistant cells. *CD133* mRNA expression, measured by qPCR, was significantly increased (by 2.38-fold) in D54-MG-R when compared to D54-MG-S cells (p=0.0032) (Figure 2C). Up-regulation of CD133 protein expression in D54-MG-R cells was confirmed with western blot analysis (Figure 2B).

Morphological changes with temozolomide-resistance development. Under light microscopy, the parental D54-MG-S cells exhibited typical spindle-shaped morphology. In D54-MG-R cells, we observed a sub-group of spherical cells with intense ADD3 expression that lacked typical astrocytic cytoplasmic extensions. When compared to the typical tumor cells that expressed ADD3 on the cell membrane of their cytoplasmic extensions, these spherical cells exhibited a condensed ADD3 expression pattern in the cell body (Figure 3A). The proportion of these spherical cells was significantly higher (2.76-fold) in D54-MG-R than in D54-MG-S subclones (Figure 3B).

Temozolomide-resistant cells co-expressed ADD3 and CSC markers. We then proceeded to study the characteristics of

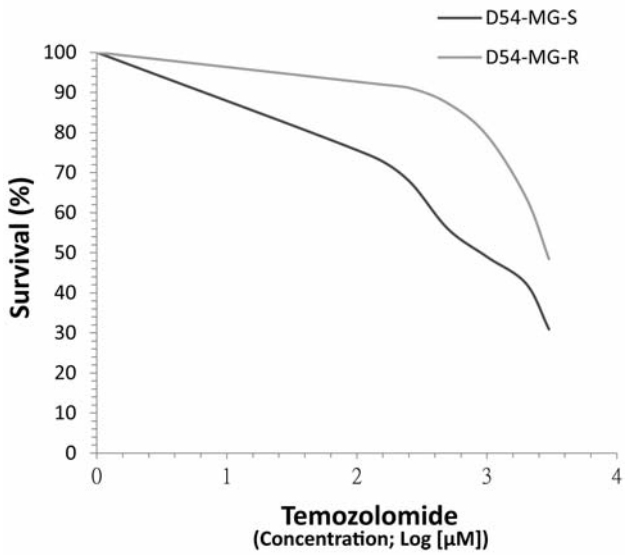


Figure 1. *Effect of temozolomide (TMZ) on glioma subclone D54-MG-S (D54-S) and glioma subclone D54-MG-R (D54-R). Sulphorhodamine assays showed a higher survival rate for the TMZ-resistant subclone than the parental TMZ-sensitive subclone when treated with temozolomide.*

these spherical cells in the D54-MG-R subclone by immunofluorescence staining. These cells were found to have strong expression levels of GSC markers CD133, OCT3/4 and NANOG (Figure 4A) Moreover, these putative CD133-positive and NANOG-positive GSCs also strongly co-expressed ADD3, suggestive of a possible association between up-regulation of ADD3 expression and the development of GSC phenotype in the temozolomide-resistant subclones (Figure 4B).

Expression of ADD3 in CD133$^+$ neurospheres generated from GBM cells. Next, we investigated neurospheres generated from glioma cells. Serum-free culture, upon >80% confluence, yielded neurospheres within 3 to 4 days (Figure 5A). These neurospheres were capable of regenerating monolayered culture the same as the original culture on re-plating under full-serum conditions (Figure 5B). These neuropsheres were CD133 immunofluorescence-positive. Coexpression of ADD3 and CD133 was also observed in double-stained images of these neurospheres (Figure 6).

We also compared the distribution of ADD3-positive and CD133-positive cells before and after neurosphere formation. Nuclear staining with DAPI confirmed similar cell numbers under the two conditions. In culture before neurosphere formation, CD133 expression was found to be evenly distributed across the monolayered culture. In contrast, following neurosphere generation, we observed neurosphere-like cell masses in the culture. On immunofluorescence

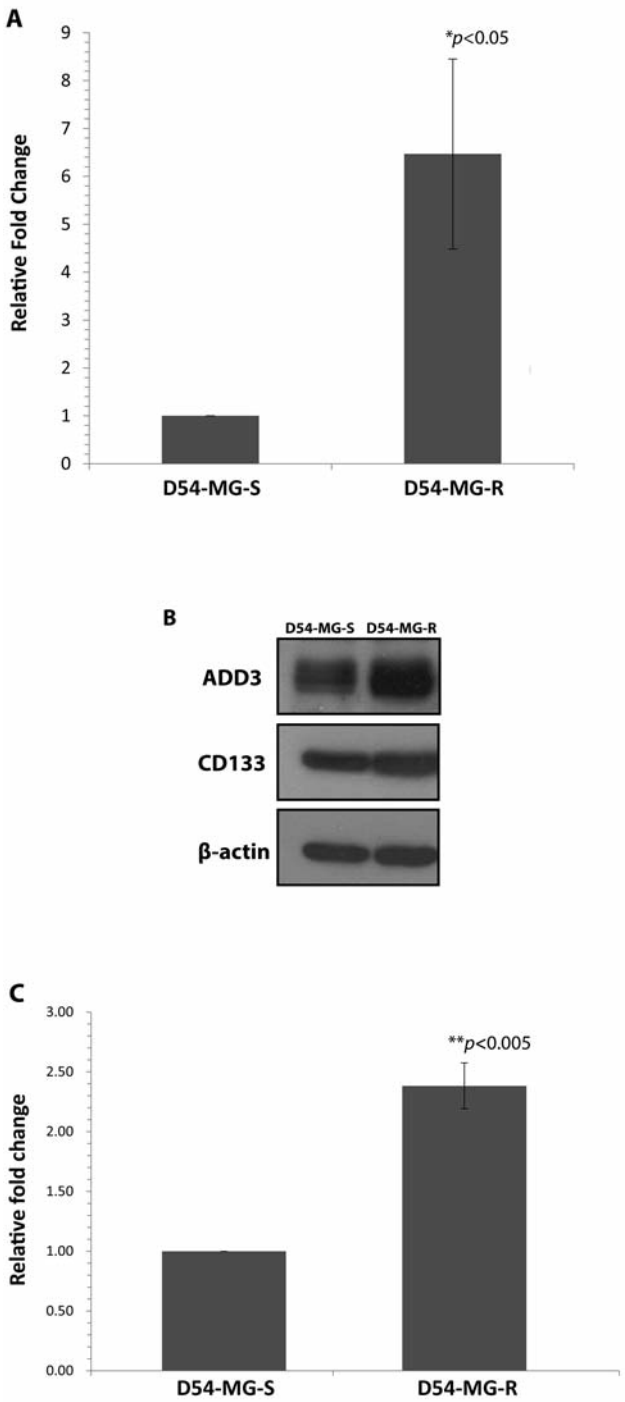
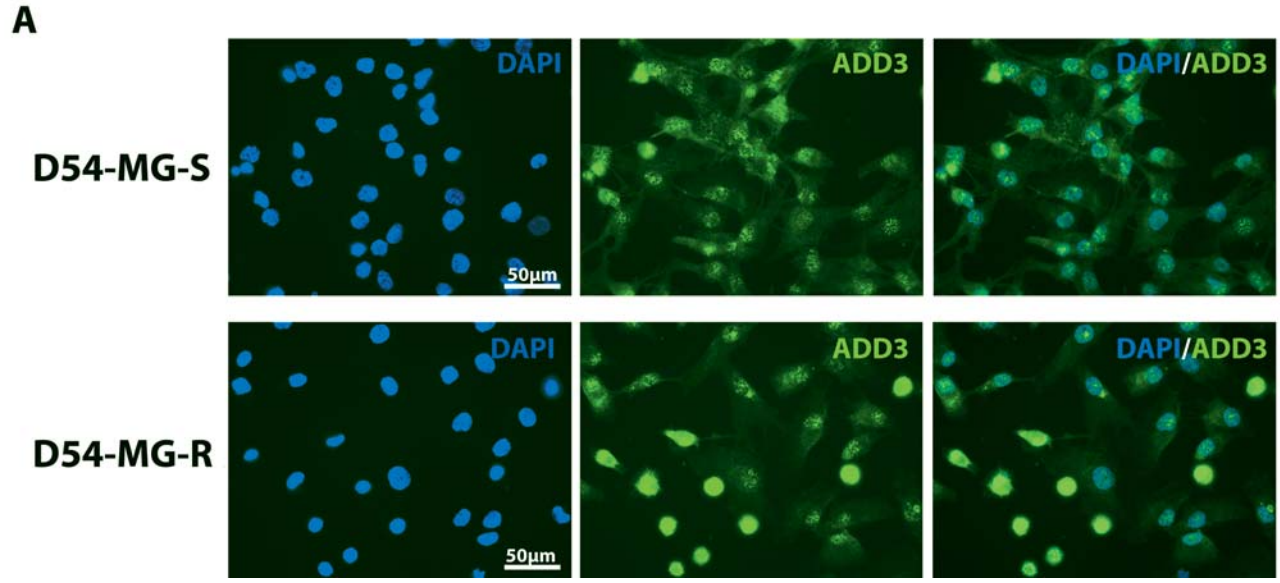


Figure 2. *mRNA and protein expression of the cytoskeletal protein adducin 3 (ADD3) and the glioma stem cells marker CD133 in subclones of glioblastoma cells. A: Real-time reverse transcriptase polymerase chain reaction (RT-PCR) showed up-regulation of ADD3 expression in the chemoresistant D54-MG-R subclone when compared with the chemosensitive D54-MG-S subclone. B: Western blot analysis showed increased levels of ADD3 and CD133 proteins in D54-MG-R subclone when compared with the D54-MG-S subclone. C: Real-time RT-PCR showed up-regulation of CD133 expression in the D54-MG-R subclone when compared with the D54-MG-S subclone.*



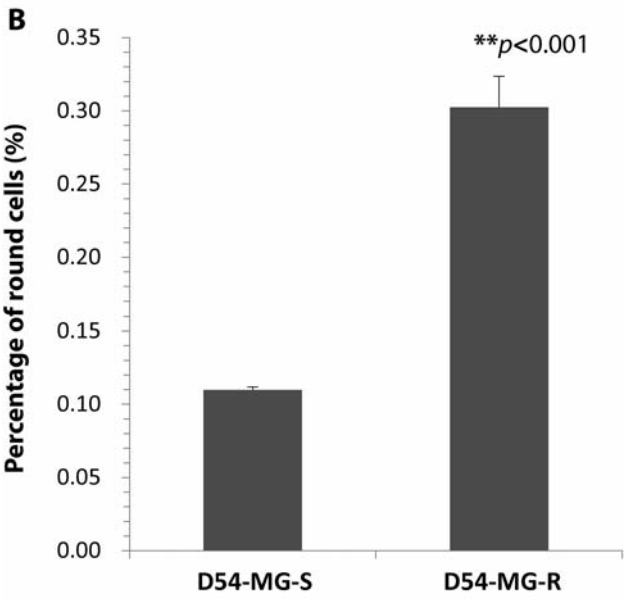


Figure 3. *Immunofluorescence staining in the D54-MG-R glioma subclone and appearance of spherical cells. A: Immunofluorescence staining demonstrated the presence of spherical glioma cells (arrow) in the D54-MG-R subclone. These cells also exhibited a condensed pattern of adducin 3 (ADD3) expression. B: Graphical representation of the percentage of putative glioma stem cells that expressed ADD3 was 2.76-fold higher in the D54-MG-R subclone than in the D54-MG-S subclone.*

staining, high expression of CD133 was found to be concentrated in these cell masses, while reduced expression was found at the periphery. We further observed that ADD3 expression followed a pattern corresponding that of CD133-positivity (Figure 7).

Discussion

ADD is expressed in normal human brain cells (11). Recent evidence suggests that ADD may play a role in cell motility by acting as a target of calcium-dependent pathways (12). ADD contains the major phosphorylation site for protein kinase C (PKC), and PKC-dependent phosphorylation has been shown to cause changes in cytoskeletal-dependent processes, such as tumor migration (13). MicroRNA-145, which down-regulates *ADD3* expression, has also been shown to reduce adhesion and invasion of GBM cells, suggesting that ADD3 may contribute to the infiltrative behavior of glioma (14). Dysregulation of ADD has been implicated in the development of chemoresistance in a variety of human malignancies, including lung cancer (15), osteosarcoma (7), prostate cancer (16), and multidrug-resistant gastric carcinoma (17). In the present study, we found, to our knowledge for the first time, that up-regulation of ADD3 expression was associated with the development of temozolomide-resistance in GBM.

We also observed changes in the subcellular distribution of ADD3 with the development of temozolomide-resistance. Fowler *et al*. described changes in ADD localization that paralleled an increase in growth potential and de-differentiation in renal tumors (18). The implications of these findings were unknown, and future work may explore the localization of ADD in glioma of different malignancy grades. Interestingly, Huang *et al*. conducted gene expression

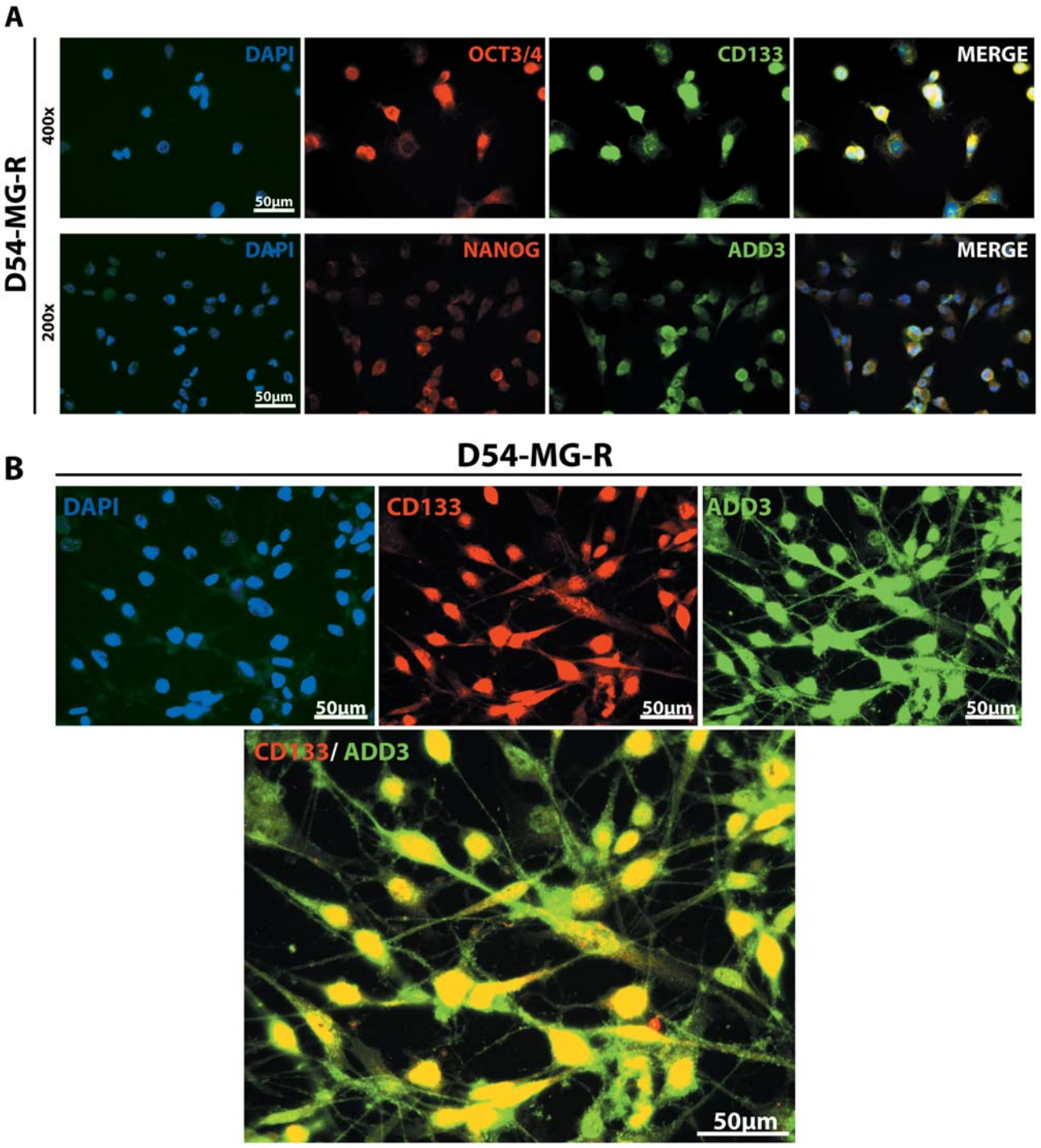


Figure 4. *Immunofluorescence staining of stemness markers prominin 1 (CD133), POU class 5 homeobox 1 (OCT3/4), nanog homeobox (NANOG) and adducin 3 (ADD3). A: Immunofluorescence studies showed strong expression of CD133, OCT 3/4, and NANOG in the D54-MG-R subclone. NANOG was coexpressed with ADD3. B: CD133-positive showed co-expression with ADD3.*

profiling on low-grade gliomas, and reported down-regulation of *ADD3* in tumors when compared with normal brain tissues (19).

We identified a sub-group of temozolomide-resistant cells with distinct morphological features and strong CD133 and ADD3 co-expression. CD133, also known as

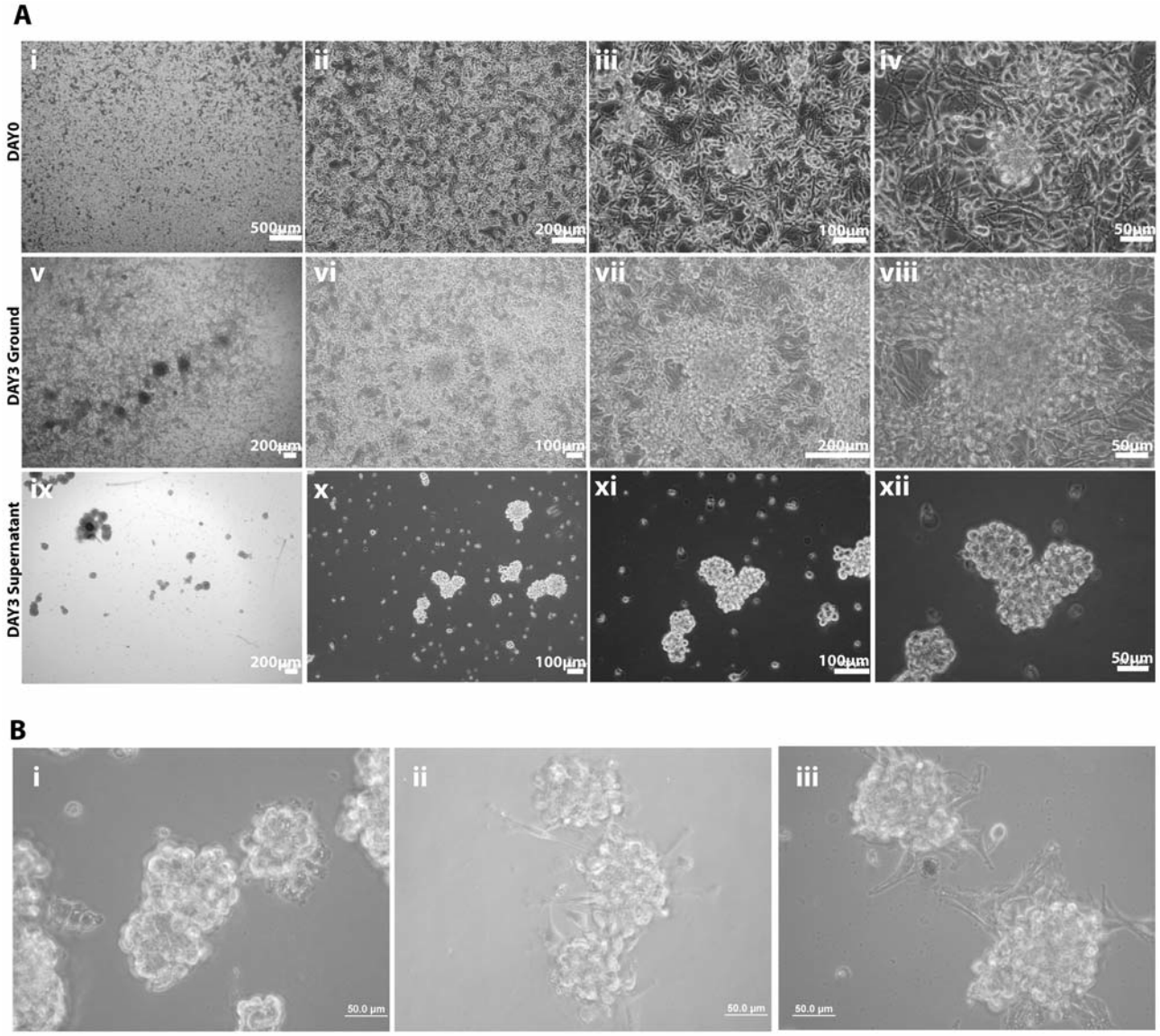


Figure 5. *A: Glioma neurosphere formation and dual-growth pattern. When cultured in serum-supplemented medium, glioma cells grew as adherent monolayered culture (i); in serum-free medium, they grew as single cells suspended in the medium after 6 h and as small tumor spheres after 24 h (ii); larger tumor spheres were observed after 72 h (iii & iv) and 96-168 h (v-viii) on the parental culture; neurospheres were observed in the supernatant (ix-xii). B: After the spheres were re-seeded in serum-supplemented medium, tumor spheres began to form monolayered culture after 1 h (i); by 6 h, cytoplasmic extensions were observed (ii); and after 24 h (iii), the cells became attached, with a morphology similar to that of the original culture.*

prominin-1, is a GSC marker characteristically down-regulated during cell differentiation (20). It is widely used to isolate GSCs (21). The latter are characterized by their abilities for self-renewal, sustained proliferation (22), tumor initiation and propagation. Singh *et al*. demonstrated that $CD133^+$ glioma cells were capable of producing tumors in immunodeficient mice at a much lower cell number than were $CD133^-$ cells (23). Clinically, the proportion of $CD133^+$ cells and their topological organization were significant prognostic factors (24), and CD133 expression has been implicated in the development of chemoresistance (25). $CD133^+$ cells were also found to have high expression of O6-methylguanine-DNA methyltransferase and anti-apoptosis proteins (26), as well as multidrug resistance genes in GBM (27, 28). We surmise that a possible link may exist between ADD3 expression, GSC selection and the development of temozolomide resistance. Clinically, these putative GSCs may represent tumor cells that can give

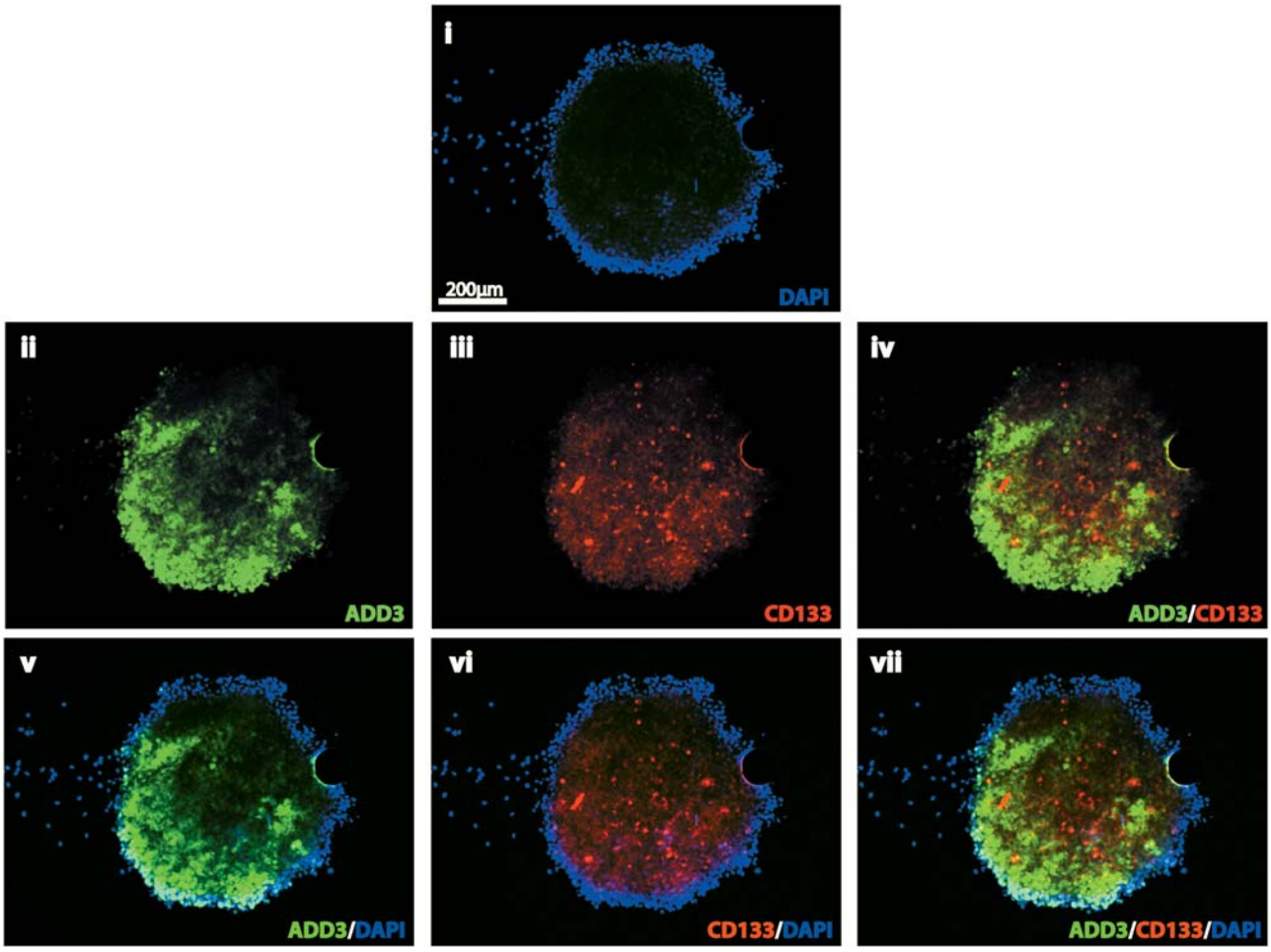


Figure 6. *Immunofluorescence staining of glioma neurospheres showed co-expression of adducin 3 (ADD3) and prominin 1 (CD133) i: 4',6-diamidino-2-phenylindole (DAPI); ii: ADD3; iii: CD133; iv: ADD3 and CD133; v: ADD3 and DAPI; vi: CD133 and DAPI and vii: ADD3, CD133 and DAPI.*

rise to recurrent disease. Further functional studies are required to investigate the mechanistic roles of ADD3 as an effector and biomarker of cancer stemness, and its role as a potential therapeutic target.

Our results, however, must be interpreted with caution as the use of CD133 as a GSC marker has been questioned, and the functional role of CD133 in the development of chemoresistance has not been fully delineated (29). Our *in vitro* model did not include the effect of radiation, which is commonly given with temozolomide in the clinical setting. GBM is characterized by the presence of multiple deregulated signaling pathways, and the microenvironment and pharmacokinetics of temozolomide in our model may differ from those in patients (30). Our $CD133^+$ neurospheres were isolated under serum-free conditions, without growth factor supplementation.

Conclusion

ADD3 is known to be associated with chemoresistance in many types of human cancers. The present study provides new information on a possible association between ADD3 expression and the development of temozolomide-resistance in human GBM. Temozolomide-resistant subclones of GBM cells which expressed CD133 and other cancer stem cell markers and $CD133^+$ neurospheres strongly co-expressed ADD3, suggestive of a possible link between cytoskeletal protein expression, cancer stem cell phenotype and temozolomide resistance. Our findings may direct future studies on the mechanistic roles of ADD3 and other cytoskeletal proteins in response to cancer drugs and their potential uses as novel therapeutic targets and biomarkers.

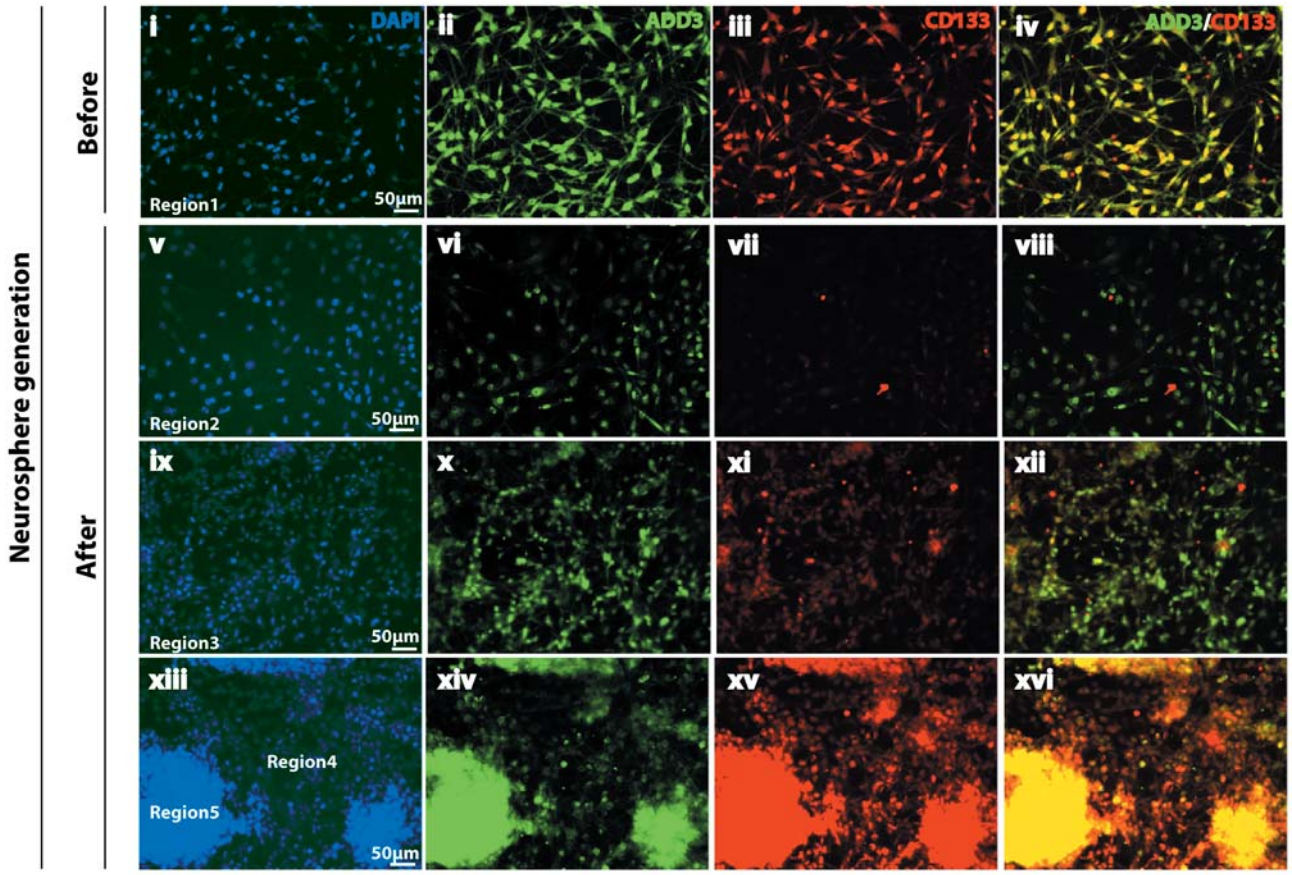


Figure 7. *Differential patterns of expression were observed before and after neurosphere formation. The expression of adducin 3 (ADD3) co-localized with prominin 1 (CD133). Region 1 (i-iv): before neurosphere formation; Region 2 (v-vii): after neurosphere formation, at the periphery of the culture distant from the neurosphere-like cell masses; Region 3 (ix-xii): near neurosphere-like cell masses; Region 4 (xiii-xvi): in between neurosphere-like cell masses; and Region 5 (xiii-xvi): at the neurosphere-like cell masses. Before neurosphere generation (Region 1), CD133 expression was evenly distributed. After neurosphere generation (Regions 2-5), CD133 expression was concentrated at the neurosphere-like cell masses (Region 5) and reduced expression was observed at the periphery (Region 2). ADD3 expression had a similar pattern, co-localizing with CD133.*

Conflicts of Interest

The Authors have no conflicts of interest.

Acknowledgements

The Authors would like to thank Dr. W.M. Lui and Dr. K.N. Hung for their valuable advice and support. They also thank Dr. Stephen Yinw Cheng for his technical support on the parameter settings of immunofluorescence imaging.

References

1 Dolecek TA, Propp JM, Stroup NE and Kruchko C: Cbtrus statistical report: Primary brain and central nervous system tumors diagnosed in the united states in 2005-2009. Neuro Oncol *14 Suppl 5(v)*: 1-49, 2012.

2 Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E and Mirimanoff RO: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med *352(10)*: 987-996, 2005.

3 Zhang J, Stevens MF, Laughton CA, Madhusudan S and Bradshaw TD: Acquired resistance to temozolomide in glioma cell lines: Molecular mechanisms and potential translational applications. Oncology *78(2)*: 103-114, 2010.

4 Cho DY, Lin SZ, Yang WK, Lee HC, Hsu DM, Lin HL, Chen CC, Liu CL, Lee WY and Ho LH: Targeting cancer stem cells for treatment of glioblastoma multiforme. Cell Transplant *22(4)*: 731-739, 2013.

5 Gardner K and Bennett V: Modulation of spectrin-actin assembly by erythrocyte adducin. Nature *328(6128)*: 359-362, 1987.

6 Mintz MB, Sowers R, Brown KM, Hilmer SC, Mazza B, Huvos AG, Meyers PA, Lafleur B, McDonough WS, Henry MM, Ramsey KE, Antonescu CR, Chen W, Healey JH, Daluski A, Berens ME, Macdonald TJ, Gorlick R and Stephan DA: An expression signature classifies chemotherapy-resistant pediatric osteosarcoma. Cancer Res *65(5)*: 1748-1754, 2005.

7 Walters DK, Steinmann P, Langsam B, Schmutz S, Born W and Fuchs B: Identification of potential chemoresistance genes in osteosarcoma. Anticancer Res *28(2A)*: 673-679, 2008.

8 Gyorffy B, Surowiak P, Kiesslich O, Denkert C, Schafer R, Dietel M and Lage H: Gene expression profiling of 30 cancer cell lines predicts resistance towards 11 anticancer drugs at clinically achieved concentrations. Int J Cancer *118(7)*: 1699-1712, 2006.

9 van den Boom J, Wolter M, Kuick R, Misek DE, Youkilis AS, Wechsler DS, Sommer C, Reifenberger G and Hanash SM: Characterization of gene expression profiles associated with glioma progression using oligonucleotide-based microarray analysis and real-time reverse transcription-polymerase chain reaction. Am J Pathol *163(3)*: 1033-1043, 2003.

10 Sun S, Wong TS, Zhang XQ, Pu JK, Lee NP, Day PJ, Ng GK, Lui WM and Leung GK: Protein alterations associated with temozolomide resistance in subclones of human glioblastoma cell lines. J Neurooncol *107(1)*: 89-100, 2012.

11 Citterio L, Tizzoni L, Catalano M, Zerbini G, Bianchi G and Barlassina C: Expression analysis of the human adducin gene family and evidence of add2 beta4 multiple splicing variants. Biochem Biophys Res Commun *309(2)*: 359-367, 2003.

12 Matsuoka Y, Li X and Bennett V: Adducin: Structure, function and regulation. Cell Mol Life Sci *57(6)*: 884-895, 2000.

13 Kiley SC, Clark KJ, Duddy SK, Welch DR and Jaken S: Increased protein kinase c delta in mammary tumor cells: Relationship to transformtion and metastatic progression. Oncogene *18(48)*: 6748-6757, 1999.

14 Rani SB, Rathod SS, Karthik S, Kaur N, Muzumdar D and Shiras AS: Mir-145 functions as a tumor-suppressive RNA by targeting sox9 and adducin 3 in human glioma cells. Neuro Oncol *15(10)*: 1302-1316, 2013.

15 Langer W, Sohler F, Leder G, Beckmann G, Seidel H, Grone J, Hummel M and Sommer A: Exon array analysis using re-defined probe sets results in reliable identification of alternatively spliced genes in non-small cell lung cancer. BMC Genomics *11*: 676, 2010.

16 Pascal LE, Vencio RZ, Page LS, Liebeskind ES, Shadle CP, Troisch P, Marzolf B, True LD, Hood LE and Liu AY: Gene expression relationship between prostate cancer cells of gleason 3, 4 and normal epithelial cells as revealed by cell type-specific transcriptomes. BMC Cancer *9*: 452, 2009.

17 Heim S and Lage H: Transcriptome analysis of different multidrug-resistant gastric carcinoma cells. In Vivo *19(3)*: 583-590, 2005.

18 Fowler L, Everitt J, Stevens JL and Jaken S: Redistribution and enhanced protein kinase c-mediated phosphorylation of alpha- and gamma-adducin during renal tumor progression. Cell Growth Differ *9(5)*: 405-413, 1998.

19 Huang H, Colella S, Kurrer M, Yonekawa Y, Kleihues P and Ohgaki H: Gene expression profiling of low-grade diffuse astrocytomas by cdna arrays. Cancer Res *60(24)*: 6868-6874, 2000.

20 Beier D, Hau P, Proescholdt M, Lohmeier A, Wischhusen J, Oefner PJ, Aigner L, Brawanski A, Bogdahn U and Beier CP: Cd133(+) and cd133(–) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res *67(9)*: 4010-4015, 2007.

21 Wu A, Oh S, Wiesner SM, Ericson K, Chen L, Hall WA, Champoux PE, Low WC and Ohlfest JR: Persistence of cd133+ cells in human and mouse glioma cell lines: Detailed characterization of gl261 glioma cells with cancer stem cell-like properties. Stem Cells Dev *17(1)*: 173-184, 2008.

22 Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL and Wahl GM: Cancer stem cells--perspectives on current status and future directions: Aacr workshop on cancer stem cells. Cancer Res *66(19)*: 9339-9344, 2006.

23 Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD and Dirks PB: Identification of human brain tumour initiating cells. Nature *432(7015)*: 396-401, 2004.

24 Zeppernick F, Ahmadi R, Campos B, Dictus C, Helmke BM, Becker N, Lichter P, Unterberg A, Radlwimmer B and Herold-Mende CC: Stem cell marker cd133 affects clinical outcome in glioma patients. Clin Cancer Res *14(1)*: 123-129, 2008.

25 Pallini R, Ricci-Vitiani L, Banna GL, Signore M, Lombardi D, Todaro M, Stassi G, Martini M, Maira G, Larocca LM and De Maria R: Cancer stem cell analysis and clinical outcome in patients with glioblastoma multiforme. Clin Cancer Res *14(24)*: 8205-8212, 2008.

26 Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, Lu L, Irvin D, Black KL and Yu JS: Analysis of gene expression and chemoresistance of cd133+ cancer stem cells in glioblastoma. Mol Cancer *5*: 67, 2006.

27 Nakai E, Park K, Yawata T, Chihara T, Kumazawa A, Nakabayashi H and Shimizu K: Enhanced mdr1 expression and chemoresistance of cancer stem cells derived from glioblastoma. Cancer Invest *27(9)*: 901-908, 2009.

28 Shervington A and Lu C: Expression of multidrug resistance genes in normal and cancer stem cells. Cancer Invest *26(5)*: 535-542, 2008.

29 Wang J, Sakariassen PO, Tsinkalovsky O, Immervoll H, Boe SO, Svendsen A, Prestegarden L, Rosland G, Thorsen F, Stuhr L, Molven A, Bjerkvig R and Enger PO: Cd133 negative glioma cells form tumors in nude rats and give rise to cd133 positive cells. Int J Cancer *122(4)*: 761-768, 2008.

30 Mao H, Lebrun DG, Yang J, Zhu VF and Li M: Deregulated signaling pathways in glioblastoma multiforme: Molecular mechanisms and therapeutic targets. Cancer Invest *30(1)*: 48-56, 2012.

Received September 11, 2015
Revised October 9, 2015
Accepted October 16, 2015