Expression of glioma stem cell marker CD133 and O^6-methylguanine-DNA methyltransferase is associated with resistance to radiotherapy in gliomas

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Abstract. In the present study, we investigated the prognostic roles of the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene methylation status, the protein profiles of MGMT, and the glioma stem cell (GSC) marker CD133 in malignant glioma resistance to radiotherapy. The proliferation of glioma cells was assessed using a clonogenic survival assay and flow cytometry. CD133 expression was assessed in SHG-44-GSCs using RT-PCR and flow cytometry. *MGMT* exhibited resistance to radiation in the SHG-44-GSCs using siRNA transfection. The effects of the siRNA on mRNA and protein expression of *MGMT* in SHG-44-GSCs were detected using semi-quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting. *MGMT* methylation status, MGMT and CD133 expression profiles were assessed in 59 malignant glioma patients using methylation-specific polymerase chain reaction (MSP), and immunohistochemistry. *In vitro*, SHG-44-GSCs exhibited a characteristic resistance to radiation that was not observed in SHG-44 cells. This resistance was attributed to the unmethylated status of the *MGMT* promoter and to high expression levels of *MGMT* mRNA in the glioma cells. In these patients, the CD133 marker, but not *MGMT* promoter methylation or MGMT protein level, was associated with resistance to radiotherapy (n=59; hazard ratio=2.838; 95% CI, 1.725-7.597; p=0.001). The median progression-free survival (PFS) among patients with the CD133 marker was 14 months, whereas it was 35 months in patients without CD133 (p=0.001). Notably, co-expression of the methylated *MGMT* promoter and the CD133 marker was associated with the poorest outcome in patients with gliomas treated by radiotherapy; in these patients, PFS was 7 months. These results suggest that assessment of GSC MGMT and CD133 levels will guide future clinical targeted therapies and stratify glioma patient treatment regimens. High expression levels of the CD133 protein could be used as a predictor for poor survival in patients treated with radiotherapy.

Introduction

Adjuvant radiochemotherapy after surgery is the main therapeutic strategy for treating malignant gliomas. Agents currently used in chemotherapy rely on the alkylation of DNA at the O^6 position of guanine, an important step in the formation of mutations that lead to the apoptosis of cancer cells (1-3). However, radiochemotherapy resistance in malignant gliomas results in poor clinical outcome. A contributing factor to such resistance is O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme that reverses alkylation by removing promutagenic methyl groups from the O^6 position and transferring them to cysteine acceptor sites on the protein itself. Thus, a high level of *MGMT* gene expression increases drug resistance and radioresistance (4-6).

The epigenetic silencing of the *MGMT* gene by promoter methylation was reported to be associated with longer overall survival in patients with glioblastoma who, in addition to radiotherapy, received alkylating chemotherapy [carmustine (CAR) or temozolomide (TMZ)] (7,8). The low expression of MGMT caused by *MGMT* promoter methylation in gliomas decreases the DNA repair capacity of tumor cells and increases their sensitivity to radiation, alkylating agents, and camptothecin derivatives (9,10). These findings suggest that high levels of MGMT activity in cancer cells may elicit a resistant phenotype by blunting the therapeutic effect of radiochemotherapy, which in turn contributes to treatment failure.

Recent theories concerning cancer development suggest that a minority population of undifferentiated cancer cells may determine the biologic behavior of tumors, including resistance to radiochemotherapy. Such cancer stem cells are characterized by properties of self-renewal and proliferative differentiation potential, and they possess identifying cell-surface markers. Evidence has shown that glioma stem cells

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(GSCs), positive for the cell-surface glycoprotein CD133, are involved in radioresistance and were found to promote tumor progression in a glioblastoma animal model (11-16). Researchers speculate that CD133-positive (CD133^{+}) GSCs possess many properties of normal stem cells, including a long lifespan, relative quiescence, resistance to drugs and apoptosis, and an active DNA-repair capacity (17). More recently, resistance to radiotherapy and chemotherapy was shown to result in poor prognosis in CD133^{+} glioblastomas (18).

With the goal of identifying a molecular signature for the resistance of gliomas to radiotherapy and determining prognostic factors, we investigated whether the *MGMT* promoter methylation status and levels of MGMT and CD133 in gliomas are associated with radiotherapeutic clinical outcomes.

Materials and methods

Cell culture. The human glioblastoma cell line SHG-44 was purchased from Shanghai Life Sciences Research Institute, Cell Resources Center, Chinese Academy of Science and cultured in DMEM with high glucose (Invitrogen, Carlsbad, CA, USA) containing 10% FCS (Sigma, St. Louis, MO, USA). SHG-44 cells express S-100 and glial fibrillary acidic protein (GFAP). The cells were grown in stem cell-permissive DMEM/F12 media with 2% B27 (Gibco, Grand Island, NY, USA), human recombinant leukemia inhibitory factor (10 ng/ml; PeproTech, NJ, USA), basic fibroblast growth factor (20 ng/ml; PeproTech), epidermal growth factor (20 ng/ml; PeproTech), penicillin (100 units/ml; NCPC, Shijiazhuang, China), streptomycin (100 μg/ml; NCPC), and L-glutamine (2 mmol/l; Invitrogen). After 7-10 days, the gliospheres were counted under a microscope. These culture conditions enable tumor cells to retain their molecular characteristics, and are a current conventional research method for GSCs (9-11). Among the SHG-44 cells, SHG-44-GSCs were identified by characteristics that distinguished them as a glioma initiator or 'stem-like' cell subpopulation, including self-renewal, CD133-positive expression, and GFAP-negative expression.

Tumor samples and patient characteristics. We studied tumor samples from 59 patients with primary malignant gliomas who were admitted to Nanjing Brain Hospital, Nanjing Medical University, from March 2003 to July 2008. The patients were 37-76 years (median age 57 years); 36 were male and 23 were female. Tumor specimens were frozen immediately after resection and stored at -80°C until isolation of genomic DNA was performed. The specimens were then placed in 10% formalin for the histopathological assay. According to the 2007 World Health Organization diagnostic criteria, among the 59 patients, 26 had anaplastic astrocytoma (grade III) and 33 had glioblastoma multiforme (GBM, grade IV). Patients received only standard-fractionated radiotherapy (60 Gy total dose; 2 Gy x 5 days/week for 6 weeks) after surgery. All patients provided written informed consent, and the protocol was approved by the Ethics Committee of the Nanjing Brain Hospital.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the SHG-44 cells and their GSCs using TRIzol (Invitrogen). RNA (1 μg) was used for RT-PCR using oligo(dt)$_{20}$. Complementary DNA (cDNA) for RT-PCR was performed with β-actin as an endogenous control. The primer sequences of the *MGMT* gene (Shanghai Sangon Biological Engineering Technology Service, Shanghai, China) were 5'-TTCGACTTGGTACTTGGA-3' (forward primer) and 5'-GGGCTGGTGGAAATAGGC-3' (reverse primer); 241 bp. The primer sequences of β-actin (Invitrogen) were 5'-TATCGGACGCCTGGTTAC-3' (forward primer) and 5'-CTGTGCCGTTGAACTTGC-3' (reverse primer); 139 bp. PCR reactions consisted of an initial denaturing at 95°C for 5 min followed by 40 cycles at 95°C for 60 sec, 55°C for 60 sec, and 72°C for 60 sec, and a final extension at 72°C for 10 min after the last cycle. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The target bands were analyzed by density using a Vista Fluor Imager SI (Molecular Dynamics, CA, USA).

Small interfering RNA (siRNA) transfection. The two sequences of *MGMT* siRNA were designed and manufactured by Shanghai GenePharma, and corresponded to two separate coding regions: region 1, 5'-AAGCTGGAGCTGTCTGGTTGT-3' (nucleotides 52-71), and region 2, 5'-AAGGTTGTGAAATTCGGAGAA-3' (nucleotides 310-330). The sequences of both regions are unique to *MGMT*, as indicated by a sequence search. The negative control siRNA, a scrambled siRNA duplex, was used. SHG-44-GSCs in an exponential growth phase were plated in 6-cm plates at $5x10^4$ cells/plate, grown for 24 h, and then transfected with the *MGMT* siRNA duplex and the control scrambled siRNA duplex using Lipofectamine™ 2000 (Invitrogen), or only Lipofectamine™ 2000 separately according to the manufacturer's instructions. Transfected cells were incubated at 37°C for 60 h before testing for transgene expression. Total RNA was extracted from the cell lines using a TRIzol kit (Invitrogen). Messenger RNA (mRNA; 100 ng) was transcribed to cDNA using a cDNA Synthesis kit (Fermentas, USA). Semi-quantitative real-time PCR was used to evaluate the expression of MGMT (232 bp) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 238 bp) as a control. Primers used for MGMT were 5'-TGGAGCTGTCTGGTTGTGAG-3' (sense) and 5'-CTGGTGAACGACTCTTGCTG-3' (antisense) and for GAPDH primers were 5'-GAGTCAACGGATTTGGTCGT-3' (sense) and 5'-TTGATTTTGGAGGGATCTCG-3' (antisense). All primers were designed and manufactured by Shanghai Sangon Biological Engineering Technology Services. After an initial denaturation at 95°C for 10 min, the samples were subjected to 35 cycles of RT-PCR [95°C for 10 sec, annealing temperature 67°C (MGMT) and 56°C (GAPDH) for 15 sec, and 72°C for 15 sec], and after the 35th cycle, 65°C for 5 min. All PCR reactions were performed in triplicate and a negative control was included, which contained primers with no DNA. All PCR products were electrophoresed onto a 1% agarose gel and the images analyzed.

Western blot analysis. Total cellular proteins from the cell lines were extracted using Bio-Rad protein assay reagents according to the manufacturer's instructions. Protein (20 μg) was separated by 10% SDS-PAGE and transferred onto a 0.45 μm nitrocellulose membrane (Trans-Blot, Bio-Rad,

Shanghai, China) by electroblotting. The membranes were blocked in 5% milk powder in 0.02% Tween PBS (TPBS) for 30 min and probed with antibodies, including mouse monoclonal anti-MGMT (1:1000, clone 16200; Chemicon International, Temecula, CA, USA), mouse monoclonal anti-β-actin (1:4000, clone C4; Chemicon International) overnight at 4°C, and the horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 goat anti-mouse HRP; Biomeda, Foster City, CA, USA) and were diluted in Tris-buffered saline with Tween-20 (TBST)/5% milk for 1 h at room temperature. Quantitative assessment was carried out by densitometry using Quantity One 1-D analysis software (Bio-Rad Shanghai).

Clonogenic survival assay. After exposure to increasing doses of radiation (0, 0.5, 1, 2, 3, 4, 6, 8, 10 and 20 Gy), incubation of SHG-44 cells and their GSCs continued for 24 h. Plating efficiencies of the cell lines were initially determined. The survival fraction at each radiation dose was calculated, taking plating efficiency into consideration. Colonies were defined as having >50 cells. Each set of experiments was performed in duplicate.

Flow cytometric analysis. Nucleated SGH-44-GSCs (10^7) were added per 80 μl of PBS buffer, 20 μl of FcR blocking reagent with CD133/2-PE (1:100 mouse monoclonal, clone 293C3; Miltenyi Biotec GmbH, Germany). These were mixed well, refrigerated for 10 min in the dark at 4-8°C, and analyzed by flow cytometry and fluorescence microscopy.

SHG-44 cells and their GSCs were exposed to increasing doses of radiation (0, 2, 4, 8, 10 and 20 Gy). After 48 h, Annexin V-FITC binding and propidium iodide (PI) double staining was performed according to the manufacturer's protocol (Caltag, Burlingame, CA, USA), and cells were analyzed by flow cytometry (FACScan; Becton-Dickinson, Franklin Lakes, NJ, USA). The excitation wavelength during flow cytometry was 488 nm. FITC fluorescence was detected with a passband filter at a wavelength of 515 nm, and another filter with a wavelength >560 nm was used to detect PI. In the two-variable flow cytometric scatter plot, the lower left quadrant indicated living cells (FITC⁻/PI⁻), the right upper quadrant showed necrotic cells (FITC⁺/PI⁺), and the apoptotic cells (FITC⁺/PI⁻) were shown in the lower right quadrant. Cell cycle analysis was also conducted using flow cytometry according to the manufacturer's protocol. The treated cells were stained with PI and counted using flow cytometry. The total number of cells of each sample and the percentages of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle were determined using standard ModiFit and Cell Quest software (Becton-Dickinson).

Immunohistochemistry and immunocytochemistry. The expression of CD133 and MGMT proteins in sections of formalin-fixed, paraffin-embedded tissue was determined by immunohistochemistry using rabbit polyclonal anti-CD133 antibody (clone ab19898, 1:20; Abcam, Cambridge, UK) and mouse monoclonal anti-MGMT antibody (clone MT3.1, 1:50; Neomarkers, Fremont, CA, USA). Prior to the incubation of primary antibodies, heat-mediated antigen retrieval was performed. Secondary antibody (anti-mouse/rabbit IgG) and the EnVision™ Plus Polymer HRP (mouse/rabbit)

IHC kit (Lab Vision, Fremont, CA, USA) were used in the following procedures. Diaminobenzidine (DAB) was used as a chromogen. Counterstaining was performed with Mayer's hematoxylin. As a negative control, the primary antibody was omitted. Endothelial staining was used as an internal positive control. SHG-44 cells and their GSCs were fixed, washed, and pre-blocked prior to incubation with primary antibodies for CD133 and MGMT. Cells were then stained with EnVision™ Plus Polymer HRP (mouse/rabbit) secondary antibodies, and the nuclei were counterstained with Mayer's hematoxylin.

The number of positive-stained tumor cells was counted under a microscope. CD133 was located on the cell membrane and MGMT was located in the cell nuclei and the cytoplasm. The immunoreactivity of CD133 or MGMT protein was evaluated semi-quantitatively according to the percentage of positive-stained cells: <10% was regarded as negative (-), 10-25% as low reactivity (+), >25-50% as moderate reactivity (++), and >50% as high reactivity (+++).

Methylation-specific polymerase chain reaction (MSP). Promoter methylation of the *MGMT* gene was measured using MSP. DNA was modified with sodium bisulfite using the EZ DNA Methylation-Gold kit™ (Zymo Research, CA, USA). MSP was performed with MGMT primers specific for either methylated or modified unmethylated DNA. Primer sequences for the methylated reaction were 5'-GTTTCGGATATGTTGGGATAGTTC-3' (forward primer) and 5'-AACACCTAAAAAACACTTAAAACGC-3' (reverse primer); 261 bp. For the unmethylated reaction, the primers were 5'-GTTTTGGATATGTTGGGATAGTTTG-3' (forward primer) and reverse 5'-ACACTAAAAAACACTTAAAACACA-3' (reverse primer); 262 bp. PCR conditions were as follows: 96°C for 10 min then 40 cycles at 94°C for 30 sec, 59°C for 40 sec, and 70°C for 40 sec, and finally 10 min at 70°C. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV illumination. DNA from lymphocytes treated with SssI methyltransferase (New England Biolabs, Ozyme, St-Quentin-Yvelines, France) was used as a positive control for methylated alleles of *MGMT*.

Statistical analysis. All data were analyzed using Statistical Package for the Social Sciences (SPSS) 13.0 statistical software (Chicago, IL, USA). The χ^2 test for homogeneity, and independent t-tests where appropriate, were used to compare the clinicopathological factors of patients or glioma cells with and/or without the *MGMT* gene and CD133 expression. The probabilities of survival were estimated according to the Kaplan-Meier method. The significance of differences among subclasses was determined using the log-rank test. Partial correlation analyses were conducted using the Spearman rho test. The significance level was set at $p < 0.05$.

Results

Radioresistance in glioma cells and GSCs in vitro. The CD133/2-PE positive rate was 97.9% in SHG-44-GSCs as determined by flow cytometry. Exposure to increasing doses of radiation resulted in decreased cell survival in the SHG-44-GSCs and SHG-44 cells. The survival fraction
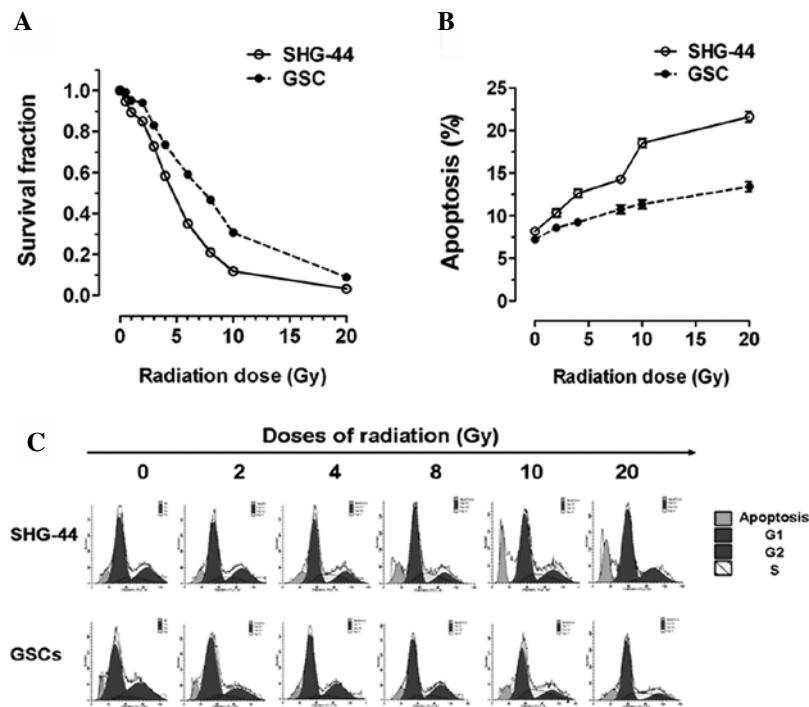
Figure 1. Dose response of cell survival and apoptosis induced by radiation in SHG-44 cells and GSCs. SHG-44 cells and GSCs were exposed to increasing doses of radiation (0-20 Gy). (A) Cell viability was assessed using a clonogenic survival assay. (B) Annexin V-PI apoptosis and (C) cell cycle analyses were conducted using flow cytometry. Values are shown as the mean ± SE (n=3). p<0.01, GSC vs. SHG-44 cells.
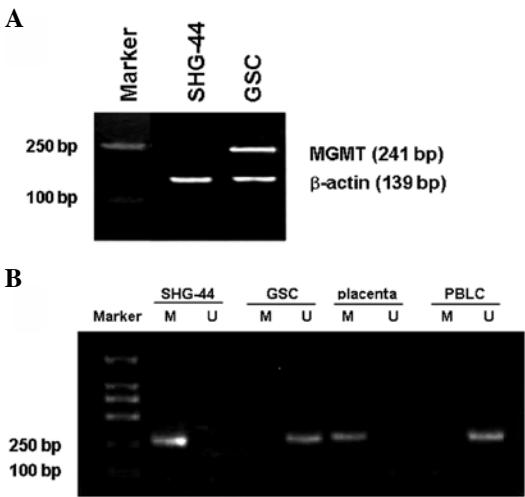

Figure 2. The M*GMT* gene and the *MGMT* promoter methylation status in gliomas. (A) Expression of *MGMT* mRNA in SHG-44 cells and GSCs. The level of mRNA was detected by RT-PCR, and β-actin was used as an internal control. (B) The methylated *MGMT* promoter in SHG-44 cells and SHG-44-GSCs. The methylation status was tested using a nested methylation-specific PCR assay. M denotes the presence of methylated genes, and U denotes the presence of unmethylated genes. Methylated DNA from placenta served as a positive control for the methylated *MGMT* promoter, and DNA from normal peripheral blood lymphocytes (PBLC) was used as a control for the unmethylated *MGMT* promoter.

There was no significant difference in the cell cycle phase (Fig. 1C) between SH-G44-GSCs and SHG-44 cells.

MGMT expression and MGMT promoter methylation status are associated with resistance to radiotherapy in glioma cells. To determine whether MGMT expression and *MGMT* promoter methylation are associated with glioma resistance to radiotherapy, we measured the level of *MGMT* mRNA and the *MGMT* promoter methylation status in glioma cells. Positive expression of *MGMT* mRNA was present in SHG-44-GSCs but not in SHG-44 cells (Fig. 2A). Additionally, the *MGMT* promoter in glioma cells was methylated, whereas it was unmethylated in SHG-44-GSCs (Fig. 2B). Together with the results shown in Fig. 1A and B, our data suggest that the unmethylated status of the *MGMT* promoter and high expression of MGMT contribute to resistance to radiotherapy in SHG44-GSCs.

The expression of *MGMT* was lower in SHG-44-GSCs treated with siRNA *MGMT* as compared to cells that were not treated (Fig. 3A). The survival fraction of the SHG-44-GSCs without treatment with siRNA *MGMT* was significantly higher than that of SHG-44-GSCs with siRNA *MGMT* treatment (p<0.01) for the same radiation dose (Fig. 3B).

Presence of the CD133 marker, but not MGMT promoter methylation or the MGMT protein level, is associated with resistance to radiotherapy in glioma patients. Our *in vitro* results indicated that the presence of the unmethylated *MGMT* promoter and high expression of *MGMT* mRNA were associated with radioresistance. The next step was to determine whether these conditions exist in glioma patients. Of the 59 patients studied, the MGMT promoter was methylated in 33

of SHG-44-GSCs was significantly higher than that of the SHG-44 cells (p<0.01; Fig. 1A). The Annexin-V apoptosis assay revealed that the apoptotic rate of GSCs was significantly lower than that of the SHG-44 cells (p<0.01; Fig. 1B), indicating that SHG-44-GSCs possess resistance to radiation.
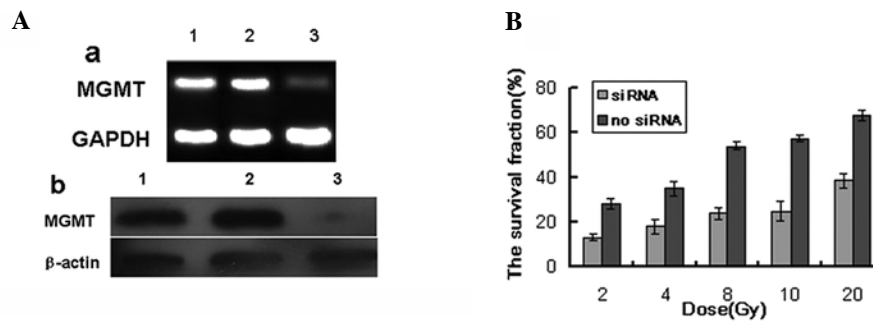
Figure 3. Knockdown of *MGMT* reduces *MGMT* expression of SHG-44-GSCs. (A) a RT-PCR of the *MGMT* expression in SHG-44-GSCs infected with a Lipofectamine control, siRNA-scrambled control, and siRNA-MGMT. (b) Western blotting showing the expression level of *MGMT* under Lipofectamine control, siRNA-scrambled control, and siRNA-*MGMT* in SHG-44-GSC. β-actin was used as a loading control. A representative result of two independent experiments is shown. 1, Lipofectamine control; 2, siRNA-scrambled; 3, siRNA-*MGMT*. GAPDH was used as an internal control. (B) The survival fraction of SHG-44-GSCs without siRNA *MGMT* was significantly higher than that of SHG-44-GSCs with siRNA *MGMT* ($p < 0.01$) for the same radiation dose.
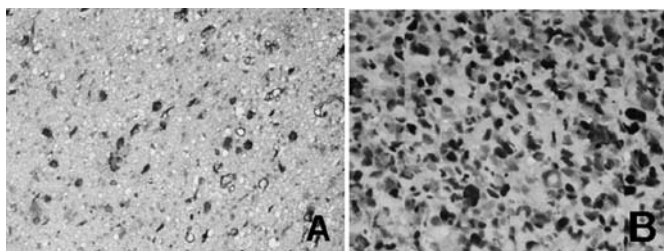

Figure 4. MGMT and CD133 protein expression in gliomas visualized using immunohistochemistry. (A) Glioma cells exhibited expression of CD133, a transmembrane protein, which is visible as yellow-brown granules in the cell membrane and cytoplasm by Envision immunohistochemistry (x200). (B) Expression of MGMT protein in glioblastoma cell nuclei by Envision immunohistochemistry (x200). The positive expression of MGMT protein is visible as yellow-brown granules in cell nuclei and/or the cytoplasm.

Table I. Correlation between *MGMT* promoter methylation and MGMT protein expression among patients with gliomas.

MGMT protein	*MGMT* promoter methylation M	U	Total	Spearman	p-value
+	8	22	30		
-	25	4	29	γ=-0.600	<0.01
Total	33	26	59		

M, methylation; U, unmethylation.

(55.93%; Table I), and high expression of MGMT protein was detected in 30 (50.85%; Table I and Fig. 4). The expression of MGMT protein was negatively correlated with *MGMT* promoter methylation (γ=-0.600, p<0.01; Table I). However, neither the *MGMT* promoter methylation status nor the expression of MGMT protein was associated with the grade of the tumor (p=0.605), age (p=0.149), or gender (p=0.316) of the patients.

We also examined the role of CD133 protein, a tumor stem cell marker, in glioma resistance to radiotherapy. Of the 59 patients, 24 (40.68%) were positive for CD133 protein expression (Fig. 4). In addition, there was no significant difference between patients with methylated *MGMT* promoter and positive expression of CD133, and unmethylated *MGMT* promoter cases without CD133 expression (41.67 vs. 34.29%, χ²=3.340, p=0.068; Table II). Notably, the expression of CD133 protein was positively correlated with the expression of MGMT protein (γ=0.331, p=0.01), but it was not associated with the *MGMT* promoter methylation status (γ=-0.238, p=0.07; Table II). These results suggest that high expression of CD133 protein, but not *MGMT* promoter methylation or MGMT protein, may be predictive of resistance to radiotherapy in glioma patients.

CD133 marker is an independent prognostic factor for poor outcome in radiotherapy-treated glioma patients. To

Table II. Correlation of *MGMT* promoter methylation and MGMT protein expression with CD133 expression in glioma stem cells

Group	*MGMT* promoter methylation M	U	MGMT protein +	-
CD133+	10	14	17	7
CD133-	23	12	13	22
χ²	3.340		6.466	
p-value	0.068		0.011	
Spearman's ρ				
r	-0.238		0.331	
p-value	0.07		0.01	

M, methylated; U, unmethylated.

further confirm the roles of *MGMT* promoter methylation and MGMT and CD133 protein in resistance to radiotherapy, we used a Kaplan-Meier survival analysis to estimate the progression-free survival (PFS) of the patients. The median PFS was 17 months for patients with methylated gliomas and
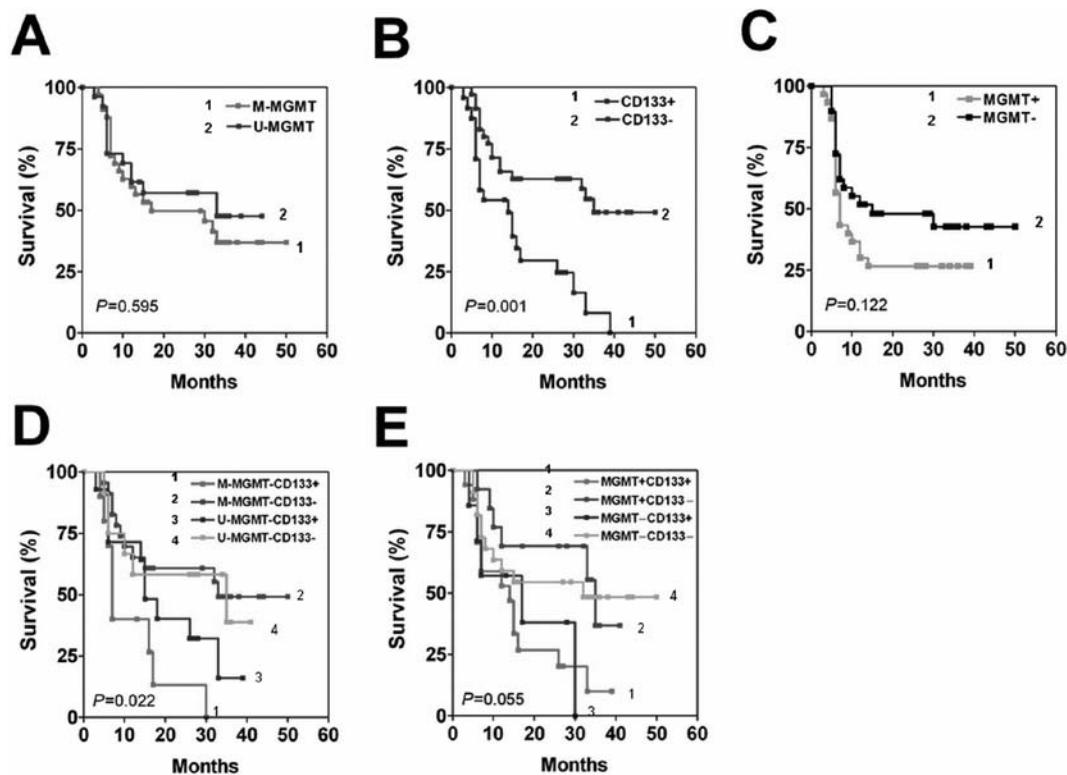
Figure 5. Relationships between glioma-derived *MGMT* promoter methylation status, MGMT, and the CD133 marker vs. survival among the glioma patients. Kaplan-Meier survival estimates of the 59 patients treated with radiotherapy segregated into groups according to the *MGMT* promoter methylation status (A), positive vs. negative expression of CD133 (B) and MGMT protein (C), and MGMT methylation status or MGMT protein stratified by CD133 expression (D and E, respectively). The p-value of the log-rank test is shown for the two groups. M, MGMT, methylated MGMT; U, MGMT, unmethylated MGMT.

33 months for patients with unmethylated gliomas (χ^2=0.282, p=0.595). The hazard ratio was 1.209 (95% CI, 0.587-2.530; Fig. 5A, Table III). The median PFS was 7 months for the MGMT protein-positive cases and 15 months for the MGMT protein-negative cases (χ^2=2.395, p=0.122). The hazard ratio was 1.592 (95% CI, 0.865-3.445). No significant difference was found between these two groups. These results imply that neither the *MGMT* promoter methylation status nor MGMT protein expression affected the outcome and prognosis of radiotherapy.

The median PFS among patients with CD133 expression was 14 months, whereas it was 35 months among those without CD133 expression (χ^2=11.569, p=0.001). The hazard ratio was 2.838 (95% CI, 1.725-7.597, p=0.001; Fig. 5B, Table III). Overall, there was a significant difference in the PFS between the groups with positive and negative expression of CD133 protein, and these groupings were stratified according to *MGMT* promoter methylation status (χ^2=9.634, p=0.022; Fig. 4C). The shortest PFS, 7 months, was observed among patients with both *MGMT* promoter methylation and CD133 expression (Fig. 5C, Table III). The hazard ratio was 3.319 (95% CI, 1.830-18.57, p=0.003).

The PFS in patients with MGMT and CD133 protein co-expression was significantly shorter than in patients with MGMT- and CD133-negative expression (14 vs. 32 months). The hazard ratio was 2.090 (95% CI, 0.9982-5.406, p=0.050; Fig. 5D, Table III). However, co-expression of MGMT and CD133 was not correlated with the PFS of the patients (χ^2=7.59, p=0.055). These results further confirm that high expression of

CD133 protein is an independent prognostic factor for poor outcome in radiotherapy-treated glioma patients.

Discussion

Malignant gliomas are known for their resistance to chemora-diotherapy. This resistance has been attributed to DNA-repair deficiency and to the particular biological behavior of GSCs, which contribute to clinical recurrences and poor outcomes (18). In the present study, we found that the unmethylated status of the *MGMT* promoter and high expression of *MGMT* mRNA contributed to resistance to radiotherapy of GSCs *in vitro*. However, in glioma patients, the CD133 marker, but not *MGMT* promoter methylation or MGMT, was associated with resistance to radiotherapy. These findings provide important information for guiding novel treatment strategies and designing individualized therapy for glioma patients. Our results also support the concept that the tumor stem cell phenotype is involved in the resistance of gliomas to radiotherapy.

In this study, GSCs demonstrated more potent radioresistance than glioma cells. The underlying mechanisms of radioresistance may be associated with the biological characteristics of GSCs, such as resistance to apoptosis and hyperactivity to proliferation (19-21), which are similar to the mechanisms of resistance to chemotherapeutic agents (17,18,22). Additionally, our finding that GSCs can lack *MGMT* promoter methylation or a high level of *MGMT* mRNA expression, may help explain the DNA repair dysfunction in radiation-induced DNA damage.

Table III. Analysis of the prognostic characteristics for 59 patients with gliomas.

Groups	n	Median PFS (month)	Hazard ratio (95% CI)	χ^2	p-value
Methylation status					
Methylated	33	17	1.209 (0.5872 -2.530)	0.282	0.595
Unmethylated	26	33	-	-	-
CD133 expression					
CD133$^+$	24	14	2.838 (1.725-7.597)	11.569	0.001
CD133$^-$	35	35	-	-	-
MGMT protein expression					
MGMT$^+$	30	7	1.592 (0.865-3.445)	2.395	0.122
MGMT$^-$	29	15	-	-	-
Methylation status and CD133 expression				9.634	0.022
Methylated, CD133$^+$	10	7	3.319 (1.830-18.57)	8.895	0.003
Methylated, CD133$^-$	23	33	-	-	-
Unmethylated, CD133$^+$	14	15	1.681 (0.626-4.907)	1.142	0.285
Unmethylated, CD133$^-$	12	35	-	-	-
MGMT and CD133 protein				7.590	0.055
MGMT$^+$, CD133$^+$	17	14	2.807 (1.182-7.480)	5.363	0.021
MGMT$^+$, CD133$^-$	13	35	-	-	-
MGMT$^-$, CD133$^+$	7	17	1.855 (0.611-7.795)	1.445	0.229
MGMT$^-$, CD133$^-$	22	32	-	-	-

PFS, progression-free survival; 95% CI, 95% confidence interval.

Of the 59 patients with gliomas, 24 exhibited CD133 expression with radioresistance, and their survival was significantly shorter than patients who lacked the CD133 marker. It has been speculated that radioresistance and rapid repair of radiation-induced DNA damage are related to the characteristics of GSCs (self-renewal capacity and hyperproliferation) and to the difference between the intracellular and extracellular microenvironments in CD133$^+$ tumor cells (23-26).

Bao *et al* (9) reported that CD133$^+$ subpopulations contribute to glioma radioresistance. Their colony formation assay confirmed that CD133$^+$ cells were more resistant to irradiation treatment than CD133$^-$ cells. CD133$^+$ cells (i.e., GSCs) may be a source of tumor recurrence after radiation. Studies have revealed that GSCs contribute to radioresistance through a preferential checkpoint response and DNA repair and that they are associated with high expression of autophagy-related proteins, such as ATG5, ATG12, and LC3 (9,27,28). It has been reported that the proportion of CD133$^+$ cells was an independent risk factor for tumor regrowth and time to malignant progression in WHO grade II and III tumors (26). Our results also showed that the presence of CD133$^+$ cells can be regarded as an independent indicator for resistance to radiotherapy and poor prognosis. Thus, the CD133 marker may be a target for the biological treatment of human gliomas.

Several studies have reported that CD133$^+$ GSCs with high expression of *MGMT* mRNA contribute to the resistance to alkylating agents and poor prognosis. Conversely, *MGMT* promoter methylation induced loss of the expression of *MGMT* in GSCs, thereby increasing chemosensitivity to alkylating agents and improving prognosis (29,30). In our study,

MGMT promoter methylation status and MGMT expression in 59 patients with gliomas did not influence the outcome of radiotherapy and prognosis. In contrast, Rivera *et al* (31) found that in 225 patients with newly diagnosed GBM who received only radiotherapy following resection, methylation of the *MGMT* promoter was correlated with an improved response to radiotherapy, and unmethylated tumors were twice as likely to progress during radiation treatment (31). Taken together, this suggests that *MGMT* promoter methylation is a better prognostic factor for patients with GBM who received radiotherapy alone, but not for all types of gliomas. However, in the present study MGMT expression was significantly higher in CD133$^+$ cells than in CD133$^-$ cells, indicating that MGMT may be involved in the resistance of CD133$^+$ stem cells to radiotherapy.

A surprising finding was that the PFS among patients with both *MGMT* promoter methylation and CD133 expression was significantly shorter than in patients with CD133 expression alone. This result suggests that CD133$^+$ GSCs still retain various important functions despite methylation of the *MGMT* promoter and that other mechanisms may also be involved in the radioresistance of CD133$^+$ stem cells.

Many studies have reported the effect of the *MGMT* promoter methylation status on the success of chemotherapy. Hegi *et al* (32) found that the *MGMT* promoter was methylated in 45 of 206 cases. Stupp *et al* (33) found that the addition of TMZ to radiotherapy for newly diagnosed glioblastoma resulted in a clinically meaningful and statistically significant survival benefit with minimal additional toxicity. Furthermore, for patients with newly diagnosed glioblastoma who were randomly assigned to receive either standard radiotherapy

or identical radiotherapy with concomitant TMZ followed by up to six cycles of adjuvant TMZ, the methylation of the *MGMT* promoter was the strongest predictor for outcome and benefit from TMZ chemotherapy (34). Brandes *et al* (35) also reported that after the administration of TMZ concomitant with and adjuvant to radiotherapy in patients with newly diagnosed glioblastoma, the pattern of and time to recurrence were strictly correlated with *MGMT* methylation status. These patients with tumors, with a methylated *MGMT* promoter who were all treated with TMZ, had a significantly longer median survival than those with an unmethylated *MGMT* promoter (32-35). Together with our negative findings, this indicates that *MGMT* promoter silencing is an important factor that is related to better prognosis in glioma patients treated with chemotherapy, but not radiotherapy alone.

The positive expression of the *MGMT* gene is associated with shorter survival and poor prognosis in patients with gliomas. Kondo *et al* (4) reported that CD133$^+$ GSCs with a high level of MGMT expression showed drug resistance to alkylating agents such as TMZ, CAR, and etoposide (VP-16). The percentage of CD133$^+$ cells and the drug resistance and radioresistance increased in recurrent GBM, possibly due to the increased expression level of breast cancer-resistant protein 1 (BCRP1), MGMT, and inhibitor of apoptosis protein (IAP) in CD133$^+$ cells (10).

Recent studies have demonstrated that CD133$^+$ emerged as a predictor for poor survival in patients treated with chemo-radiotherapy. Murat *et al* (18) reported that the *HOX* marker, which comprises CD133, was an independent prognostic factor in multivariate analysis, adjusted for the *MGMT* methylation status and age. This report is consistent with our current findings. However, the factors that influence the prognosis of patients with CD133$^+$ gliomas are intricate. We found that high expression of MGMT played a dominant role in the resistance to radiotherapy in CD133$^+$ GSCs, but we found no significant correlation between *MGMT* methylation and resistance to radiotherapy in glioma cells. This may be due to the great heterogeneity of the samples and the insufficient sample size, although our current study demonstrated that the expression of CD133 and MGMT was not correlated with the grade of glioma or the gender and age of the patients. However, our results at the cellular level are consistent with those of the majority of previous studies (10).

In summary, identification of CD133 expression after surgery may be of merit in estimating the clinical prognosis of glioma patients, and it may be a useful predictor of the responsiveness of tumors to subsequent radiotherapy in patients with gliomas. CD133 may be used as a marker to predict resistance to radiotherapy and poor prognosis, and it could be a target for the biological treatment of human gliomas. In some circumstances, MGMT may also contribute to glioma radioresistance.

Acknowledgements

References

1. Pegg AE: Repair of O(6)-alkylguanine by alkyltransferases. Mutat Res 462: 83-100, 2000.
2. Coulondre C and Miller JH: Genetic studies of the lac repressor. IV. Mutagenic specificity in the lacI gene of *Escherichia coli*. J Mol Biol 117: 577-606, 1977.
3. Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 8: 286-298, 2007.
4. Kondo T, Setoguchi T and Taga T: Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci USA 101: 781-786, 2004.
5. Salmaggi A, Boiardi A, Gelati M, *et al*: Glioblastoma-derived tumorospheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. Glia 54: 850-860, 2006.
6. Beier D, Röhrl S, Pillai DR, *et al*: Temozolomide preferentially depletes cancer stem cells in glioblastoma. Cancer Res 68: 5706-5715, 2008.
7. Esteller M, Garcia-Foncillas J, Andion E, *et al*: Inactivation of the DNA-repair gene *MGMT* and the clinical response of gliomas to alkylating agents. N Engl J Med 343: 1350-1354, 2000.
8. Hegi ME, Diserens AC, Godard S, *et al*: Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. Clin Cancer Res 10: 1871-1874, 2004.
9. Bao S, Wu Q, McLendon RE, *et al*: Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444: 756-760, 2006.
10. Liu G, Yuan X, Zeng Z, *et al*: Analysis of gene expression and chemoresistance of CD133$^+$ cancer stem cells in glioblastoma. Mol Cancer 5: 67, 2006.
11. Singh SK, Hawkins C, Clarke ID, *et al*: Identification of human brain tumour initiating cells. Nature 432: 396-340, 2004.
12. Yuan X, Curtin J, Xiong Y, *et al*: Isolation of cancer stem cells from adult glioblastoma multiforme. Oncogene 23: 9392-9400, 2004.
13. Singh SK, Clarke ID, Terasaki M, *et al*: Identification of a cancer stem cell in human brain tumors. Cancer Res 63: 5821-5828, 2003.
14. Hemmati HD, Nakano I, Lazareff JA, *et al*: Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci USA 100: 15178-15183, 2003.
15. Wang J, Sakariassen PØ, Tsinkalovsky O, *et al*: CD133-negative glioma cells form tumors in nude rats and give rise to CD133-positive cells. Int J Cancer 122: 761-768, 2008.
16. Beier D, Hau P, Proescholdt M, *et al*: CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res 67: 4010-4015, 2007.
17. Dean M, Fojo T and Bates S: Tumour stem cells and drug resistance. Nat Rev Cancer 5: 275-284, 2005.
18. Murat A, Migliavacca E, Gorlia T, *et al*: Stem cell-related 'self-renewal' signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. J Clin Oncol 26: 3015-3024, 2008.
19. Singh SK, Clarke ID, Hide T and Dirks PB: Cancer stem cells in nervous system tumors. Oncogene 23: 7267-7273, 2004.
20. Nakano I and Kornblum HI: Methods for analysis of brain tumor stem cell and neural stem cell self-renewal. Methods Mol Biol 568: 37-56, 2009.
21. Kamesaki S, Kamesaki H, Jorgensen TJ, *et al*: bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. Cancer Res 53: 4251-4256, 1993.
22. Kitada S, Pedersen IM, Schimmer AD and Reed JC: Dysregulation of apoptosis genes in hematopoietic malignancies. Oncogene 21: 3459-3474, 2002.
23. Lin H: The stem-cell niche theory: lessons from flies. Nat Rev Genet 3: 931-940, 2002.
24. Piccirillo SG, Reynolds BA, Zanetti N, *et al*: Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature 444: 761-765, 2006.

25. Calabrese C, Poppleton H, Kocak M, *et al*: A perivascular niche for brain tumor stem cells. Cancer Cell 11: 69-82, 2007.
26. Zeppernick F, Ahmadi R, Campos B, *et al*: Stem cell marker CD133 affects clinical outcome in glioma patients. Clin Cancer Res 14: 123-129, 2008.
27. Curman D, Cinel B, Williams DE, *et al*: Inhibition of the G2 DNA damage checkpoint and of protein kinases Chk1 and Chk2 by the marine sponge alkaloid debromohymenialdisine. J Biol Chem 276: 17914-17919, 2001.
28. Lomonaco SL, Finniss S, Xiang C, *et al*: The induction of autophagy by γ-radiation contributes to the radioresistance of glioma stem cells. Int J Cancer 125: 717-722, 2009.
29. Cheng JX, Liu BL and Zhang X: How powerful is CD133 as a cancer stem cell marker in brain tumors? Cancer Treat Rev 35: 403-408, 2009.
30. Bredel M and Zentner J: Brain-tumour drug resistance: the bare essentials. Lancet Oncol 3: 397-406, 2002.
31. Rivera AL, Pelloski CE, Gilbert MR, *et al*: MGMT promoter methylation is predictive of response to radiotherapy and prognostic in the absence of adjuvant alkylating chemotherapy for glioblastoma. Neuro Oncol 12: 116-121, 2010.
32. Hegi ME, Diserens AC, Gorlia T, *et al*: *MGMT* gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352: 997-1003, 2005.
33. Stupp R, Mason WP, van den Bent MJ, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-996, 2005.
34. Stupp R, Hegi ME, Mason WP, *et al*: Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10: 459-466, 2009.
35. Brandes AA, Tosoni A, Franceschi E, *et al*: Recurrence pattern after temozolomide concomitant with and adjuvant to radiotherapy in newly diagnosed patients with glioblastoma: correlation with MGMT promoter methylation status. J Clin Oncol 27: 1275-1279, 2009.