SPANDIDOS PUBLICATIONS

Changes of the O6-methylguanine-DNA methyltransferase promoter methylation and MGMT protein expression after adjuvant treatment in glioblastoma

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Received November 13, 2009; Accepted January 18, 2010

DOI: 10.3892/or_00000760

Abstract. The aim of this study was to evaluate variations of O6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation and protein expression after adjuvant treatment in glioblastoma patients. Sixteen patients with a glioblastoma underwent 34 microsurgeries including 18 re-operations. After surgery, patients underwent follow-up with radiotherapy and chemotherapy (temozolomide, ACNU and cisplatin) between 2000 and 2008. To investigate *MGMT* methylation and MGMT expression, methylation-specific PCR (MSP) and immunohistochemical staining (IHC) were performed. The methylation status of the *MGMT* promoter was altered in five (27.8 %) of 18 re-operation specimens. In four specimens, the *MGMT* promoter was found to be methylated after primary surgery, but was found to be unmethylated on post-treatment samples. MGMT protein expression was altered in 15 (83.3%) of 18 cases. Fifteen specimens showed higher levels of protein expression as compared to previous samples and three samples demonstrated a similar expression pattern. After irradiation and exposure to steroid and temozolomide 6 and 24 h later, a methylated *MGMT* promoter and negative protein expression were seen in U343 glioblastoma cell lines which have methylated promoter and negative protein expression. Variations in *MGMT* promoter methylation and protein expression can occur after treatment. We suggest that changes of *MGMT* promoter methylation and protein expression might not be related to a direct effect of irradiation and exposure to steroid and temozolomide.

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Key words: chemotherapy, glioblastoma, immunohistochemistry, *MGMT* promoter methylation, MGMT protein, re-operation

Introduction

After recurrence of glioblastoma, treatment options for patients are limited. Most patients have already undergone irradiation and chemotherapy before recurrence. Available options include no further treatment, repeated resection, chemotherapy, stereotaxic radiosurgery and brachytherapy. Without further treatment, the median survival from recurrence is approximately 16 weeks (1,2). Repeat surgical resection may extend survival from 14 to 35 weeks and may be influenced by the preoperative Karnofsky performance score (KPS) and possibly by the use of postoperative chemotherapy (1,3).

The enzyme O6-methylguanine-DNA methyltransferase (MGMT) is responsible for the repair of DNA damage induced by alkylating agents that produce adducts at the position of O^6-guanine in DNA (4), such as BCNU, CCNU and temozolomide. For the treatment of glioblastoma, these alkylating agents cause cell death by forming cross-links between adjacent strands of DNA due to alkylation that occurs at the O^6 position of guanine. However, the DNA repair protein MGMT inhibits the cross-linking of double-stranded DNA by removing the alkylated lesions, leading to tumor cell resistance to chemotherapy (5-7). Therefore, adjuvant chemotherapy such as the use of temozolomide, which has been shown to be the most adapted drug used for in glioblastoma treatment, is limited by the action this enzyme, resulting in a very poor survival of glioblastoma patients (8-10).

The aim of this study was to evaluate potential alterations in the methylation status of the *MGMT* promoter and MGMT protein expression in recurrent glioblastomas as compared directly to matched primary glioblastomas from the same patient and to confirm if the direct effect of exposure to radiation, chemotherapeutic agent and steroid is related with gene expression in a glioblastoma cell line.

In a previous study, we focused on the role of the *MGMT* promoter methylation status in combination with MGMT protein expression in the response to chemotherapy (11). There was a trend toward longer overall survival in patients with a methylated promoter and no protein expression with a median survival of 20.07 months as compared to patients

Table I. Clinical data for 16 glioblastoma patients.

No.	Age	Sex	Extent of removal (first OP)	Adjuvant treatment (radiotherapy and chemotherapy)	Reoperation	Survival
1	63	F	GTR	RT, recurrent temodal	1 (GTR)	1 y and 8 m
2	31	F	STR	RT, recurrent temodal	1 (STR)	2 y and 3 m
3	52	F	GTR	RT, recurrent temodal, ACNU-CPPD	1 (STR)	1 y and 2 m
4	52	M	GTR	RT, recurrent temodal	1 (GTR)	1 y and 1 m
5	59	F	GTR	RT, recurrent temodal, ACNU-CPPD	1 (GTR)	1 y and 5 m
6	53	F	GTR	RT, recurrent temodal	2 (GTR, STR)	1 y and 10 m
7	57	F	GTR	RT, recurrent temodal	1 (STR)	8 m
8	36	M	GTR	RT, recurrent temodal, ACNU-CPPD	1 (STR)	10 m
9	54	M	GTR	RT, recurrent temodal, ACNU-CPPD	2 (GTR, STR)	1 y and 7 m
10	53	M	GTR	RT, recurrent temodal	1 (STR)	3 y and 10 m (alive)
11	47	M	GTR	RT, recurrent temodal, ACNU-CPPD	1 (STR)	3 y and 10 m (alive)
12	68	F	GTR	RT, recurrent temodal	1 (GTR)	1 y and 10 m
13	66	M	GTR	CCRT with temodal	1 (STR)	9 m
14	67	M	GTR	RT, recurrent temodal	1 (GTR)	4 y and 1 m
15	35	F	GTR	CCRT with temodal, ACNU-CPPD	1 (GTR)	1 y (alive)
16	40	F	GTR	RT, recurrent temodal, ACNU-CPPD	1 (GTR)	3 y and 8 m (alive)

GTR, gross total removal; STR, subtotal removal; RT, radiotherapy; temodal, temozolomide; y, year; m, month.

with a methylated promoter and protein expression with a median survival of 12.80 months. This finding was statistically significant based on the use of multivariate analysis. Therefore, the status of a methylated *MGMT* promoter and positive protein expression (methylated-immunopositive) could serve as a good prognostic factor for glioblastoma patients. In this study, we have also analyzed the *MGMT* promoter methylation status in combination with MGMT protein expression.

Materials and methods

Patients and tissue collection. From 1995 to June 2008, surgery was performed in 155 patients with grade IV glioblastoma according to the WHO classification (12). Between 2000 and 2008, 16 patients were included in this study. These patients underwent initial surgery followed by radiotherapy (with a dose of 60 Gy) and temodal chemotherapy. Two of the 16 patients received concomitant-adjuvant temodal chemotherapy and 14 patients underwent recurrent temodal chemotherapy (Table I). Concomitant and adjuvant temodal chemotherapy were used at a dose of 75 mg/m^2/day during radiotherapy and then 150 mg/m^2/5 days for 4 weeks, followed by 200 mg/m^2/5 days for 4 weeks for a total of six cycles. Patients that underwent recurrent temodal chemotherapy received a dose of 200 mg/m^2/5 days for 4 weeks after recurrence. These 16 patients underwent 18 re-operations after recurrence. Seven patients received additional therapy after the above treatments with ACNU and CDDP. Institutional Review Board approval was obtained for the study.

Tumor samples were obtained from an initial resection and a later resection (a total of 34 operations). Specimens were formalin-fixed and were paraffin-embedded for histological studies and, in some cases, specimens were frozen and were stored at -80˚C until processing. A total of 34 cases were available for methylation-specific PCR (MSP) and immunohistochemical staining (IHC). Samples were collected from two different sites within the tumor for immunohistochemical staining. Higher expression patterns were chosen for the analysis of MGMT protein expression in patients who showed heterogeneous expression patterns.

Irradiation and exposure to steroid and temozolomide for the U343 glioblastoma cell line. The human glioblastoma cell line U343 was obtained from the Brain Tumor Research Center, University of California, San Francisco, CA USA. These cells have a methylated *MGMT* promoter and show negative expression of MGMT protein. Exponentially growing cells were exposed to γ radiation using a Gamma Cell 3000 Elan irradiator (MDS Nordion, Ottawa, Canada) at a dose rate of 5.0 Gy/min with doses of 2.5, 5, 7.5 and 10 Gy. Cells were also treated with temozolomide at a concentration of 5, 10, 15 and 20 μg/ml. Furthermore, cells were treated with the methyl prednisolone at a concentration of 5, 10, 15 and 20 μg/ml. Cells were treated with combined radiation (5 and 10 Gy) and temozolomide (10 and 20 μg/ml). After treatment, the *MGMT* promoter methylation status was determined by the use of methylation specific PCR and MGMT protein expression was determined by the use of Western blotting 6 and 24 h after exposure.

Methylation-specific PCR (MSP)

Bisulfite modification. DNA methylation patterns in the CpG islands of the *MGMT* gene were determined by chemical modification of unmethylated, but not methylated, cytosines to uracil, according to the protocol described by Herman *et al*

MSP and IHC for 16 patients.

No. of patients	1st Operation		2nd Operation		3rd Operation	
	MSP	IHC	MSP	IHC	MSP	IHC
1	M	1	M	2		
2	M	1	U	2		
3	M	1	M	3		
4	M	1	M	2		
5	M	1	U	3		
6	M	1	M	2	U	2
7	U	2	M	3		
8	U	1	U	3		
9	U	1	U	2	U	3
10	U	2	U	3		
11	M	1	M	2		
12	M	1	M	2		
13	M	2	U	2		
14	M	1	M	2		
15	U	2	U	2		
16	M	1	M	2		

MSP, methylation specific PCR; IHC, immunohistochemical staining; M, methylated; U, unmethylated.

(13). Briefly, total DNA (3 μg) in a volume of 50 μl was denatured by a reaction with NaOH (final concentration, 0.2 M) for 10 min at 37˚C. Then, 30 μl of 10 mM hydroquinone (Sigma, St. Louis, MO, USA) and 520 μl of 3 M sodium bisulfite (Sigma) at pH 5 that were both freshly prepared were added to the reaction and were mixed. The samples were then incubated under mineral oil at 50˚C for 16 h. Modified DNA was purified using Wizard DNA purification resin in accordance with the instructions supplied by the manufacturer (Promega, Madison, WI, USA) and DNA was eluted in 50 μl of water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and was used immediately or was stored at -80˚C.

PCR amplification. PCR uses primers specific for either methylated or modified unmethylated DNA (13). Previously reported specific primers for the unmethylated reaction, 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' (upper primer) and 5'-AACTCCACACTCTTCCAAAAACAAAACA-3' (lower primer) and for the methylated reaction, 5'-TTTCGACGTTCGTAGGTTTTCGC-3' (upper primer) and 5'-GCACTCTTCCGAAAACGAAACG-3' (lower primer), were used. The annealing temperature was 59˚C. Low-quality DNA yielding uncertain PCR results was discarded. DNA extracted from normal lymphocytes was treated *in vitro* with *Sss*I methyltransferase (New England Biolabs, Beverly, MA, USA) and was used as a positive control for methylated alleles of *MGMT*. DNA from normal lymphocytes was used as a negative control for methylated alleles of *MGMT*. After 34 cycles of PCR reactions were performed, PCR products were loaded on a 4% agarose gel, stained with ethidium bromide, electrophoresed for 40 min, and DNA bands were then visualized using UV light.

Immunohistochemical staining. Formalin-fixed and paraffin-embedded specimens were cut at a thickness of 5 μm. In deparaffinized sections, endogenous peroxidase was blocked with 3% H_2O_2 in methanol. Antigen retrieval was performed in a pressure cooker for 30 min in Tris buffer. Non-specific binding was blocked in 10 min using protein-blocking buffer. Sections were washed in phosphate-buffered saline (PBS) and were incubated with anti-MGMT antibody (clone MT3.1, Abcam, Cambridge, MA, USA) (dilution: 1/25) overnight at 4˚C . The samples were then incubated with a secondary antibody (biotinylated link) for 10 min, streptavidin-HRP for 10 min and diaminobenzidine (DAB), which was used as a chromogen. All chemicals were obtained from the LSAB system (DakoCytomation; Dako, Glostrup, Denmark). Counter-staining was performed with Mayer's hematoxylin. As a negative control, the primary antibody was omitted. Endothelial staining was used as an internal positive control. Immunoreactivity was quantified by counting the stained tumor nuclei expressed as percentage of the positive cells. Endothelial cells and perivascular lymphocytes were excluded from the positive cell counting. Fifteen to 20 fields per specimen were analyzed at x200 magnification.

The immunoreactivity of MGMT protein was evaluated semi-quantitatively by estimation of the fraction of positive cells, and a level <5% was regarded as negative expression (grade 1), a level <50% was regarded as a moderate degree of expression (grade 2) and a level of ≥50% was regarded as a high degree of expression (grade 3). When we compared the correlation between *MGMT* promoter methylation and protein expression, the immunoreactivity was divided into

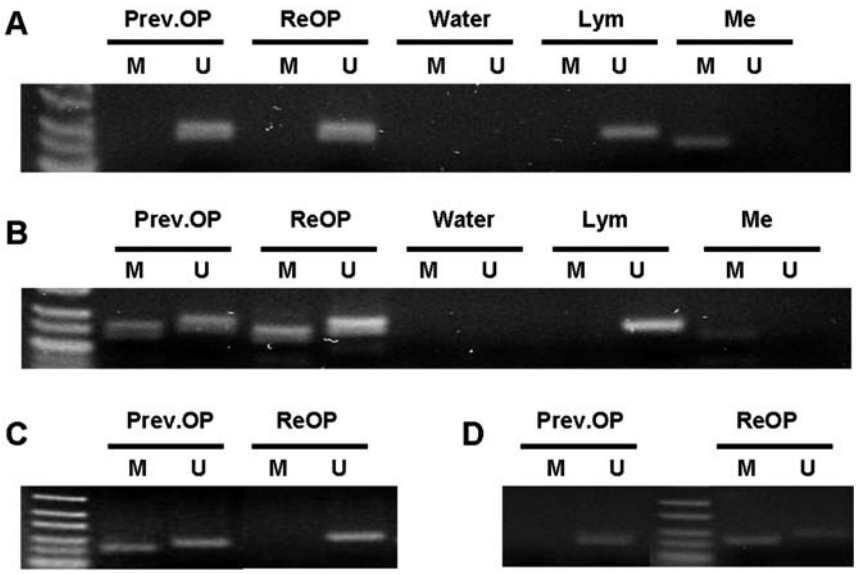

Figure 1. Changes of the *MGMT* promoter status were determined by MSP in 16 glioblastoma patients with 18 re-operations. (A) and (B) There were no changes for five cases of an unmethylated *MGMT* promoter and for eight cases of a methylated *MGMT* promoter. (C) and (D) There were changes as determined by the use of MSP from a methylated to an unmethylated *MGMT* promoter for four cases (22.2%) and from an unmethylated to methylated *MGMT* promoter in one case (5.6%). (Prev.OP, previous operation; ReOP, re-operation; Lym, lymphocyte; Me, positive control for methylation; M, methylated promoter; U, unmethylated promoter).

two groups: immunonegative (grade 1, Gr I) and immunopositive (grade 2 and grade 3, Gr II and Gr III). Only nuclear staining was considered for grading. All immunohistochemical analyses were carried out blinded to the clinical information.

Western blot analysis. Cells were lysed in a lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, 1% NP-40, 1 mM PMSF and 1 mg/ml protease inhibitor cocktail). Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Then, 20 μg of whole cell lysates were separated by the use of 15% SDS-PAGE and the separated proteins were transferred to a polyvinylidene difluoride membrane (Pall Corp., East Hills, NY, USA). Subsequently, the membrane was incubated for 2 h at room temperature in a solution of TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) supplemented with 5% non-fat dry milk, and membranes were probed overnight at 4˚C with mouse anti-MGMT (1:200). The bound antibodies were visualized with a secondary anti-mouse antibody (1:80000, Jackson Immunoresearch, West Grove, PA) conjugated with horseradish peroxidase using enhanced chemiluminescence reagents (ECL, Amersham Biosciences, Amersham, UK). ß-actin was used as an internal control.

Results

Changes of MGMT methylation and MGMT protein expression in samples from 16 glioblastoma patients that had undergone 18 re-operations. For 16 patients, matched primary and recurrent tumors were available for *MGMT* promoter methylation analysis and protein expression analysis. The results are summarized in Table II. The *MGMT* promoter was initially methylated in 11 patients and was unmethylated in five patients. After 18 re-operations to remove recurred tumors, there were no changes for eight cases with a methylated *MGMT* promoter and for five cases with an unmethylated *MGMT* promoter (Fig. 1). However, there were changes as determined by MSP from a methylated to unmethylated status for four cases (22.2%) and from an unmethylated to methylated status in one case (5.6%). MGMT protein expression initially showed grade 1 expression in 12 patients and grade 2 expression in four cases. There were alterations in 15 (83.3%) of 18 cases after a re-operation. Fifteen cases showed a higher level of expression as compared with the previous samples, and three cases showed similar expression patterns (Figs. 2 and 3).

The correlation between the methylation status of the MGMT promoter and MGMT protein expression after radiotherapy and chemotherapy. To evaluate the correlation between MGMT expression and the methylation status of the *MGMT* promoter, we performed both methylation-specific PCR (MSP) and immunohistochemical staining (IHC) for 16 samples after an initial operation and for 18 samples after a re-operation. *MGMT* promoter methylation was detected in 20 cases, while 14 tumors lacked *MGMT* promoter methylation. Of the 20 cases with promoter methylation, 10 (50%) cases were immunonegative and 10 (50%) cases exhibited a moderate to high degree of immunopositivity. Of 14 cases having an unmethylated promoter, there were 2 (14.3%) immunonegative cases and 12 cases (85.7%) with immunopositivity. A correlation between the methylation status of the *MGMT* promoter and MGMT protein expression was observed in 22 (64.7%) of 34 samples.

The changes of the methylation status of the MGMT promoter combined with MGMT protein expression after radiotherapy and chemotherapy. For the 16 initial operations, the cases were divided into four groups: methylated-immunonegative (n=10, 62.5%), methylated-immunopositive (n=1,

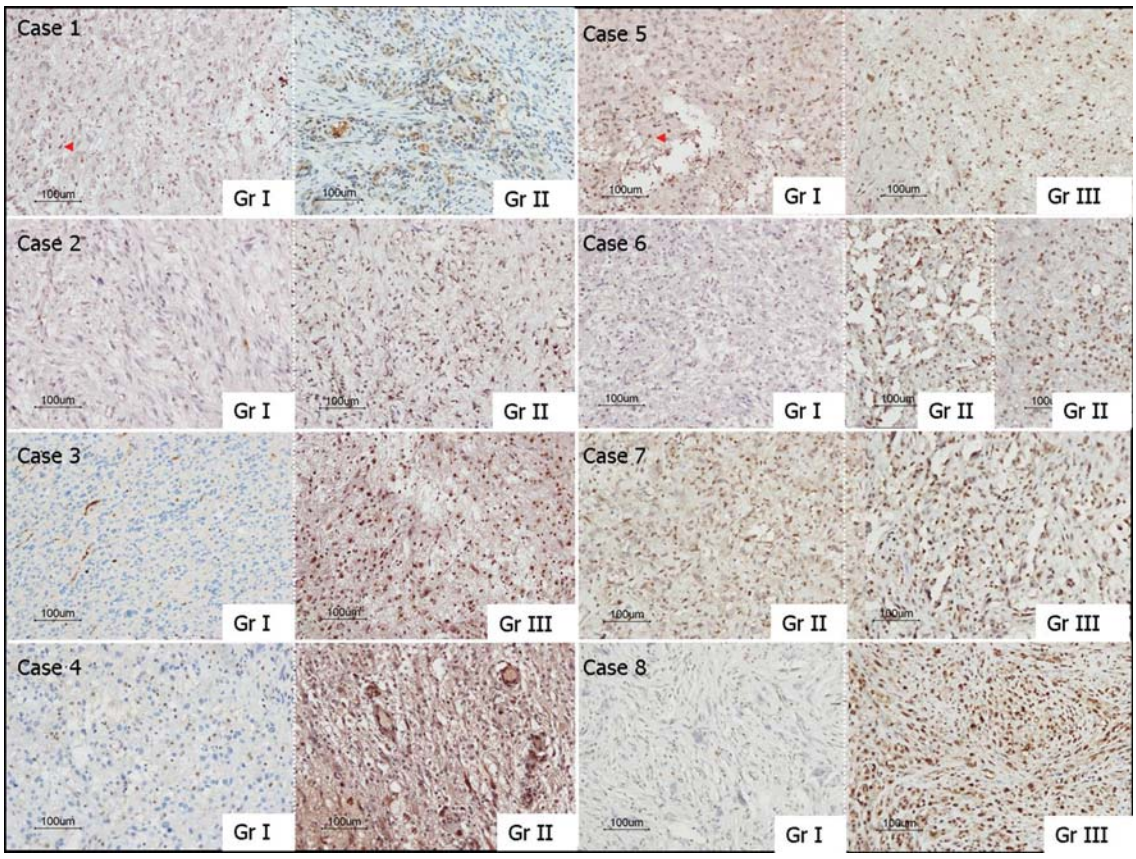

Figure 2. Change of MGMT protein expression as determined by the use of IHC in eight patients. The immunoreactivity of the MGMT protein was evaluated semi-quantitatively by estimation of the fraction of positive cells. A level <5% was regarded as negative expression (Gr I), a level <50% was regard as a as moderate degree of expression (Gr II), and a level ≥50% was regarded as a high degree of expression (Gr III) (red arrowhead, staining of lymphocytes).

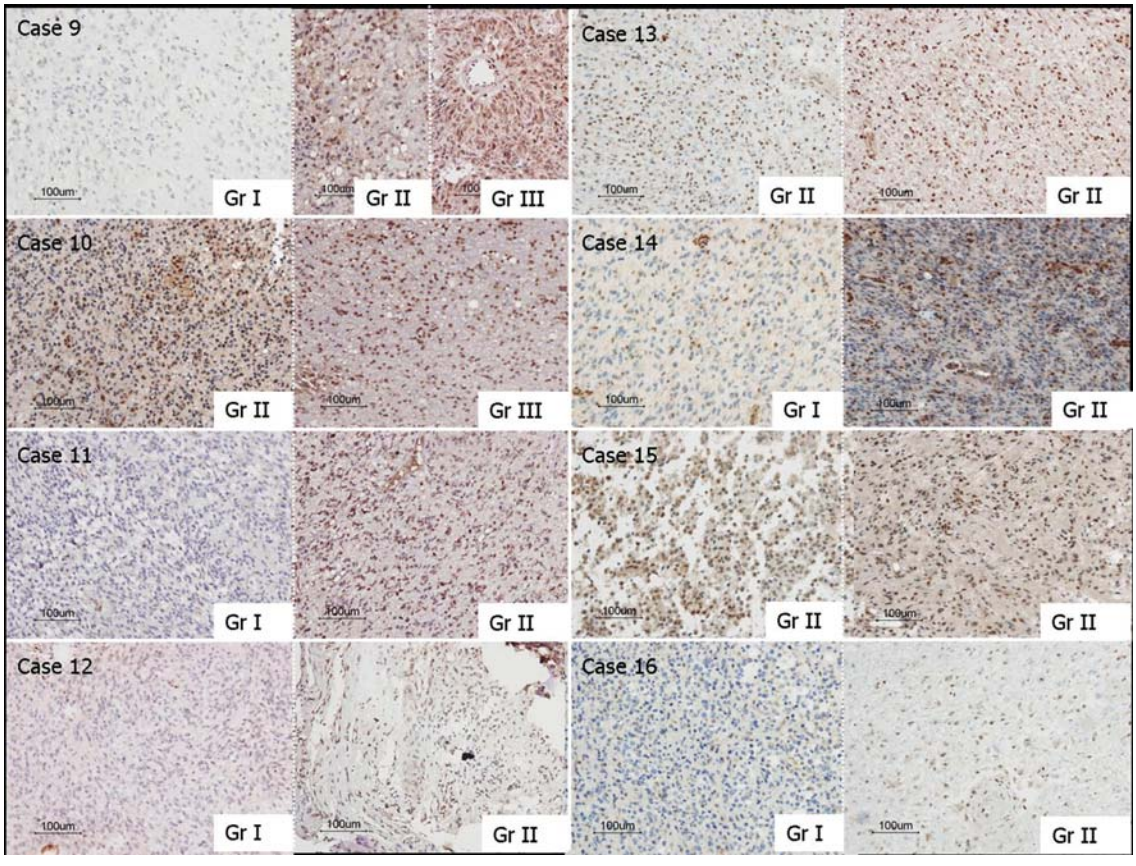

Figure 3. Change of MGMT protein expression as determined by the use of IHC in the subsequent eight patients.

Table III. *MGMT* promoter methylation status combined with MGMT protein expression.

Groups	1st Operation	Re-operation
Methylated-Immunonegative	10	0
Methylated-Immunopositive	1	9
Unmethylated-Immunonegative	2	0
Unmethylated-Immunopositive	3	9
No. of patients	16	18

6.3%), unmethylated-immunonegative (n=2, 12.5%) and unmethylated-immunopositive (n=3, 18.7%) (Table III). After 18 re-operations, the cases were divided into four groups: methylated-immunonegative (n=0), methylated-immunopositive (n=9, 50%), unmethylated-immunonegative (n=0) and unmethylated-immunopositive (n=9, 50%).

The direct influence of the MGMT promoter methylation status and protein expression with irradiation and exposure to steroid and temozolomide for the U343 glioblastoma cell line. After irradiation at a dose of 2.5, 5, 7.5 and 10 Gy or with treatment with temozolomide at a concentration of 5, 10, 15 and 20 μg/ml, a methylated *MGMT* promoter and negative protein expression were seen in U343 cells 6 and 24 h later. These cells were also treated with combined γ radiation (at a dose of 5 and 10 Gy) and temozolomide (10 and 20 μg/ml). After treatment, U343 cells also demonstrated the presence of a methylated *MGMT* promoter and negative protein expression 6 and 24 h later (Fig. 4). After exposure with methyl prednisolone at a concentration of 5, 10, 15 and 20 μg/ml, there was no change in the *MGMT* promoter status and MGMT protein expression.

Discussion

The prognosis for glioblastoma remains poor with a median survival time between 9 and 12 months. The use of adjuvant chemotherapy in addition to surgery and radiation therapy, in particular the use of alkylating agents that have been shown to be the most adapted drug types, have been shown to have a beneficial effect for the treatment of gliomas (10). Pharmacogenetic research to predict response to chemotherapy has not been fully explored. Until recently, relevant prognostic factors were considered to be age, Karnofsky performance status, extent of removal or the use of radiotherapy (14). Many genetic abnormalities are involved in gliomagenesis, and relevant mechanisms of resistance to common drugs used in the treatment of glioblastoma have been identified (15). MGMT is responsible to repair DNA damage induced by alkylating agents that produce adducts at the position of O^6-guanine in DNA, such as BCNU, CCNU and temozolomide (13). It has been reported that loss of MGMT expression results from promoter methylation of the gene (12,16,17) and many studies have suggested that promoter methylation in glioma is a useful predictor of response to treatment with alkylating agents. Many investigators have studied the use of *MGMT* promoter methylation status as well as MGMT protein expression as clinical biomarkers for routine diagnostic purposes (18-23). Based on these findings, *MGMT* promoter methylation is considered as a promising molecular factor predictive of chemotherapy response and longer survival.

While the value of *MGMT* promoter methylation as a predictive marker has been demonstrated following an initial biopsy, few studies have examined potential alterations in the methylation status of the *MGMT* promoter after recurrence.

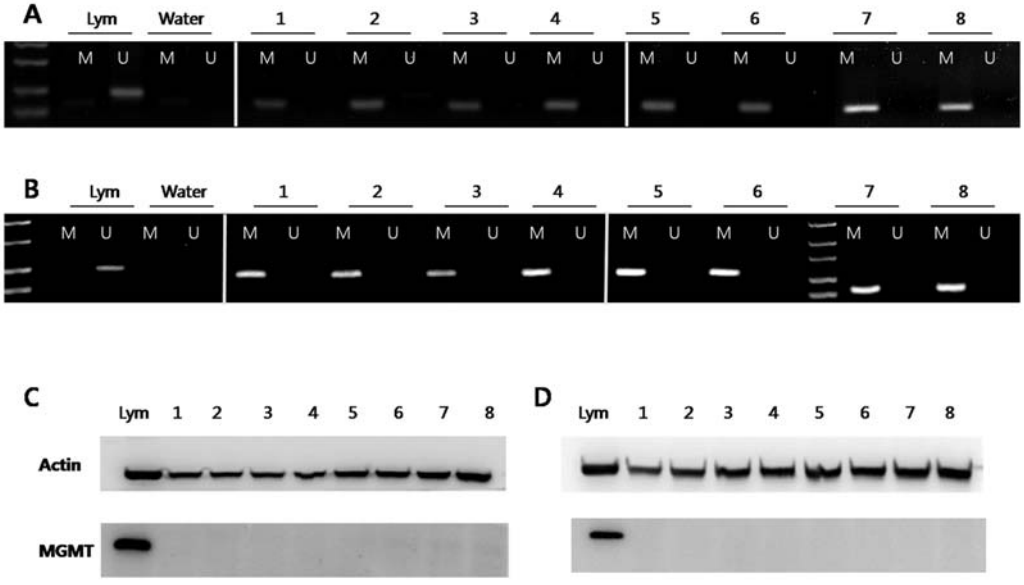

Figure 4. *MGMT* promoter status and protein expression with irradiation and exposure to steroid and temozolomide for the U343 glioblastoma cell line. (A) MSP 6 h later, (B) MSP 24 h later, (C) Western blotting 6 h later, (D) Western blotting 24 h later. (1, radiation 5 Gy and temozolomide 10 μg/ml; 2, radiation 5 Gy and temozolomide 20 μg/ml; 3, radiation 10 Gy and temozolomide 10 μg/ml; 4, radiation 10 Gy and temozlomide 20 μg/ml; 5, steroid 5 μg/ml; 6, steroid 10 μg/ml; 7, steroid 15 μg/ml; 8, steroid 20 μg/ml).

showed that variations in *MGMT* promoter methylation and MGMT protein expression could occur within the same tumor after treatment (17). Our study demonstrated that changes in *MGMT* promoter methylation and MGMT protein expression could also occur before and after adjuvant treatment. There were changes as seen by the use of MSP from a methylated to unmethylated status in four cases (22.2%) and from an unmethylated to methylated status in one case (5.6%). For MGMT protein expression, 15 cases (83.3%) showed higher levels of protein expression as compared with previous samples and three cases showed similar expression patterns after tumor recurrence. There are a number of potential explanations for these changes, including regional variations within a tumor, a direct influence on methylation by treatment, selection of unmethylated cell populations by treatment and further dedifferentiation of a tumor (17).

A few studies have investigated regional variations for the *MGMT* promoter methylation status and MGMT protein expression (24,25). It has been previously reported that the methylation status is homogeneous in tumors, and MGMT expression is very similar in different areas of the same tumor; however, there is no correlation between the two features (26). In our previous study (11), we agreed that the methylation status of the *MGMT* promoter was homogeneous in all locations, but various expression levels of the MGMT protein were observed. Therefore, samples were collected from two different sites within a tumor for immunohistochemical staining. The higher expression patterns were chosen for the analysis of MGMT protein expression in patients who showed different expression patterns with heterogeneity.

The selection of a significant predictor for patient outcome remains controversial as the degree of correlation between the methylation status of the *MGMT* promoter and MGMT protein expression might not be very high. Whereas methylation is clearly involved in the inactivation of the *MGMT* gene in numerous tumors and cancer cell lines, regulation of MGMT expression is a more complex phenomenon in which abnormal methylation of the promoter is not the only determining factor (5,16,23,26-29). In our study, a correlation between the methylation status of the *MGMT* promoter and MGMT protein expression was observed in 22 (64.7%) of 34 samples. Despite correlations that were not always strong, some investigators have reported on the prognostic significance of immunohistochemically assessed MGMT expression in glioblastoma (23). In our previous study (11), there was a trend toward longer overall survival in patients with a methylated *MGMT* promoter and no protein expression as compared to other reported findings. In this study, when we analyzed the changes of methylation status of the *MGMT* promoter combined with the expression of the MGMT protein after adjuvant treatment, 10 of 16 cases (62.5%) showed a methylated *MGMT* promoter with no protein expression; no cases with these two features were identified after 18 re-operations. Based on this finding, we suggest that recurred glioblastomas might have a poor prognosis.

To investigate the direct influence of treatment, we checked the *MGMT* promoter methylation status and protein expression in U343 cells after exposure of radiation, steroid and temozolomide. There was no change in the *MGMT* promoter status and MGMT protein expression. Therefore, we suggest that changes of *MGMT* promoter methylation and protein expression might not be related to a direct effect of radiation exposure and chemotherapy.

Acknowledgements

This study was supported by a grant (CRI09056-1) from Chonnam National University Hospital Research Institute of Clinical Medicine. We have no personal financial or institutional interest in any of the drugs, materials or devices described in the study.

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