

Original Article

Methylation status of the MGMT gene promoter fails to predict the clinical outcome of glioblastoma patients treated with ACNU plus cisplatin

Chul-Kee Park,^{1,2} Sung-Hye Park,^{3,6} Se-Hoon Lee,⁴ Chae-Yong Kim,¹ Dong-Wan Kim,⁴
Sun Ha Paek,^{1,6} Dong Gyu Kim,¹ Dae Seog Heo,^{2,4} Il Han Kim^{2,5} and Hee-Won Jung¹

Departments of ¹Neurosurgery, ³Pathology, ⁴Internal Medicine and ⁵Radiation Oncology, and ⁶Neuroscience Research Institute, Seoul National University College of Medicine, Seoul National University, and ²Cancer Research Institute, Seoul National University Hospital, Seoul, Korea

We analyzed the methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) promoter using a methylation-specific polymerase chain reaction (MSP) in glioblastoma patients treated with 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU) plus cisplatin followed by radiation therapy. Forty-eight patients with interpretable MSP results were included in this study. The MGMT promoter was methylated in 26 patients (54.2%, methylated group) and unmethylated in 22 patients (45.8%, unmethylated group). Comparison of clinical outcomes between the two groups revealed that the methylation status of the MGMT gene promoter was not a prognostic factor for overall survival ($P=0.516$) or a predictive factor for radiological response to ACNU plus cisplatin treatment ($P=0.529$). The most noteworthy explanation for the result is that the synergistic antitumor effects of ACNU and cisplatin resulting from inactivation of MGMT by cisplatin in MGMT active tumors offset the drug resistance.

Key words: glioblastoma, O6-methylguanine-DNA-methyltransferase, survival.

INTRODUCTION

O6-methylguanine-DNA methyltransferase (MGMT) is an enzyme in the DNA repair process that specifically

removes cytotoxic O6-alkylguanine adducts, thus mediating resistance to alkylating agents.¹ This DNA repair enzyme plays a role in maintaining the integrity of the DNA in normal cells but also protects tumor cells against alkylating and methylating chemotherapeutic agents, resulting in drug resistance.² There have been several studies suggesting the clinical value of MGMT evaluation in predicting benefit from alkylating agents in malignant glioma patients treated with nitrosourea as well as with temozolomide.³⁻⁷ However, there is also evidence denying a connection between the MGMT methylation status of gliomas and the clinical outcome when treated with nitrosourea.⁸⁻¹² There is also in vitro evidence indicating that the enhancement of the radiation response by temozolomide is independent of the epigenetically silenced MGMT gene.¹³

Previous MGMT analysis studies were performed using a variety of different techniques, and the best technique for analyzing MGMT status is a matter of debate. However, it is generally accepted that a methylation-specific polymerase chain reaction (MSP) evaluating the methylation status of the MGMT promoter is the best way to predict the MGMT expression of the tumor in a manner that also correlates with clinical outcome.¹⁴

Since nitrosoureas were introduced in the early 1970s, a large number of clinical trials have evaluated their role in glioblastoma and other malignant gliomas, and they have been used as a mainstay agent for the treatment of malignant gliomas.^{15,16} We previously reported the effectiveness and feasibility of preradiation chemotherapy with 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU) plus cisplatin in glioblastomas in

Correspondence: Sung-Hye Park, MD, PhD, Department of Pathology, Seoul National University College of Medicine, 101 Daehangno, Jongno-gu, Seoul, 110-744, Korea. Email: shparknp@snu.ac.kr

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the setting of a single arm phase II clinical trial.¹⁶ Based on this evidence, we have managed newly diagnosed glioblastoma patients using preradiation chemotherapy with an ACNU plus cisplatin protocol. Our hypothesis is that the methylation status of the MGMT promoter is associated with prognosis in glioblastoma patients treated uniformly with surgery, ACNU plus cisplatin chemotherapy and radiation therapy. We analyzed the methylation status of the MGMT promoter in tumor cells using MSP and determined its relationship with radiological response after ACNU plus cisplatin treatment and overall survival.

MATERIALS AND METHODS

Patients and management

Between January 2000 and December 2003, 77 newly diagnosed glioblastoma patients were treated in our institute with preradiation chemotherapy with ACNU plus cisplatin after surgery, followed by radiation therapy. Among them, proper tissue samples for MGMT promoter MSP analysis were available from 50 patients, who were included in this study. Interpretable results from the MGMT-promoter MSP were obtained in 48 of these 50 patients, and these results were analyzed. Patient data were based on information contained in hospital charts and radiological studies and were collected using a case record form approved by the institutional review board.

The study population consisted of 31 men and 17 women ranging in age from 20 to 69 years (mean, 49 years). All patients underwent surgical removal or biopsy sampling of their tumor. The histological diagnosis of glioblastoma was confirmed according to World Health Organization criteria.¹⁷ The preradiation chemotherapy of ACNU (40 mg/m²/day) and cisplatin (40 mg/m²/day) by continuous intravenous infusion for 72 h started 2–3 weeks after surgery and was repeated after 6 weeks. Two cycles were administered unless the patient showed progressive disease, unacceptable toxicity or refused further treatment. Before the second cycle of treatment, neurological examinations and MRI were performed. Patients who showed a radiological response or stable state without neurological deterioration were eligible for a second cycle of chemotherapy, and patients with tumor progression were referred for radiation therapy. In addition to tumor response, the treatment schedule was readjusted according to bone marrow, renal and hepatic function, as described previously.¹⁶ Radiation therapy was initiated 6 weeks after the last cycle of chemotherapy, with a total dose of 60 Gy, 1.8 Gy per fraction with five fractions per week. The target volume included the post-chemotherapy tumor volume and surrounding edema with a margin of 3 cm, as defined by contrast-enhanced MRI taken 5 weeks after the last

cycle of chemotherapy. Thirty-five patients completed the two cycles of ACNU plus cisplatin, and 13 patients underwent one cycle before radiation therapy. The survival time was defined as the time from the date of the surgery to the date of death. Post-surgical performance status, expressed using the Karnofsky performance scale, and the extent of resection, classified as complete and incomplete, were analyzed as possible compounding prognostic factors.

Tumor samples and MSP

The methylation status of the MGMT promoter was confirmed by MSP. After microscopic examination of HE-stained slides, we delineated the most representative areas. Tissue was dissected from paraffin blocks and put into polyethylene microtubes. After deparaffinization with xylene and alcohol, the blocks were dissolved in a lysis buffer solution containing proteinase K. For the MSP, purified DNA was modified by sodium bisulfite treatment using an EZ DNA methylation-Gold Kit™ (Catalog No. D5005; Zymo Research, Orange, CA, US). The primer sequences for the MGMT were as follows: methylated forward: 5' TTT CGA CGT TCG TAG GTT TTC GC 3', methylated reverse: 5' GCA CTC TTC CGA AAA CGA AAC G 3', unmethylated forward: 5' TTT GTG TTT TGA TGT TTG TAG GTT TTT GT 3', unmethylated reverse: 5' AAC TCC ACA CTC TTC CAA AAA CAA AAC A 3'. The annealing temperature was 64°C. The PCR products obtained were electrophoresed in 2% agarose gels and visualized under ultraviolet illumination after staining with ethidium bromide. CpGenome universal unmethylated (Catalog No. S7822; Chemicon, Temecula, CA, US) and methylated DNA sets (Catalog No. S 7821; Chemicon) were used as negative and positive controls respectively.

For the evaluation of the assay results, the products from the controls were examined first. The MGMT promoter fragments in the controls should be observed at 80 and 92 base pairs in the methylated DNA–methylated primer and unmethylated DNA–unmethylated primer combinations respectively. The methylated DNA–unmethylated primer and unmethylated DNA–methylated primer controls should not show any bands. If the control results were acceptable, patient samples were evaluated for the presence of amplification with the methylated and unmethylated primers. The results were interpreted as positive if MGMT promoter methylation was detected as a fragment of 80 base pairs observed on the gel, and negative if MGMT promoter methylation was not detected with the methylated primers.

Radiological response analysis

Radiological responses were assessed by comparing patients' post-surgical MRI taken within 48 h of the operation with the preradiation-therapy MRI obtained 5 weeks

after the last cycle of ACNU plus cisplatin treatment. The volume of enhanced tumor was measured 3-dimensionally in gadolinium-enhanced T1-weighted images using a volumetric program (Osiris® v4.0, Digital Imaging Unit, Informatics Center, University Hospital of Geneva, Geneva, Switzerland). The error associated with repeat measurements of the volume of the same MRI was within $\pm 2\%$. Response criteria proposed for 3-dimensional volume changes in solid tumors were used.¹⁸ A complete response (CR) was defined as total disappearance of all enhancing tumor, a partial response (PR) was defined as a 65% or more reduction in volume, stable disease (SD) was defined as no change or $< 65\%$ reduction or $< 40\%$ increase, and progressive disease (PD) was defined as $\geq 40\%$ increase in volume or the appearance of new lesions.

Statistical analysis

Overall survival curves were estimated by the Kaplan–Meier technique and compared using the two-sided log-rank test. The Cox proportional-hazards model was fitted to assess the prognostic value of the methylation status of the MGMT promoter and potential prognostic factors such as age, performance status, and the extent of resection. For analyzing group differences and responses according to the methylation status of the MGMT promoter, the chi-square test and Student's *t*-test were used for parametric comparisons. Statistical significance was accepted at probability values of < 0.05 . These statistical analyses were performed with the aid of SPSS software (version 10.5; SPSS, Inc., Chicago, IL, US).

RESULTS

Methylation status of the MGMT promoter

Representative results of MGMT-promoter MSP assays are shown in Figure 1. Of the 48 patients whose samples were analyzed, the MGMT promoter was methylated in 26 (54.2%, methylated group) and unmethylated in 22 (45.8%, unmethylated group). The clinical characteristics of the methylated and unmethylated groups are summarized in Table 1. There were no significant differences in the distribution of age, extent of resection and performance status between the two groups.

Methylation status of the MGMT promoter and overall survival

Overall survival curves are presented in Figure 2. The Kaplan–Meier survival curves showed that the methylated group did not have a longer survival time than the unmethylated group did ($P = 0.516$). No difference was seen in the median survival time between the methylation (17.0

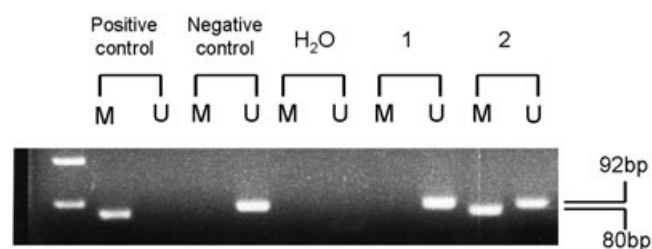


Fig. 1 Representative results of methylation-specific PCR assays. Lanes U and M indicate the presence of unmethylated and methylated DNA, respectively. The negative control for methylation was normal lymphocyte DNA and normal lymphocyte DNA treated with SssI methylase was the positive control. Water was the negative control for the PCR. The lanes marked 1 show the results from a patient without methylation and those in lanes 2 the results from a patient with methylation.

months, 95% CI: 12.0–21.9) and unmethylated (17.0 months, 95% CI: 10.1–23.9) groups. Estimated survival rates at 1 and 2 years in the methylation and unmethylated groups were similar (76.9% vs 72.7% and 30.8% vs 31.8% respectively). Multivariate analysis using a Cox proportional-hazards model including possible prognostic factors such as age, extent of resection, performance status as well as the methylation status of the MGMT promoter also did not show any significant relationships with overall survival (Table 2).

Methylation status of the MGMT promoter and radiological response

Table 3 shows the tumor response rate results and the tumor control rate for the methylation and unmethylated groups as determined from MRI taken before and after ACNU plus cisplatin treatment. Although small superiorities in response rates (50% vs 41%) and control rates (81% vs. 68%) were observed in the methylated group, their differences were statistically insignificant ($P = 0.529$ and $P = 0.316$, respectively).

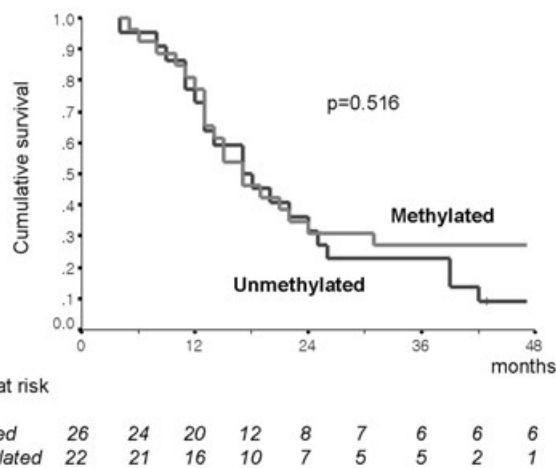
DISCUSSION

Many attempts to correlate the MGMT activity of tumor cells with clinical outcome in patients with high-grade gliomas treated with nitrosourea-based chemotherapy have been made previously, but the results have been controversial. Nakasu *et al.* reported the results of immunohistochemical staining of neoplastic cells from 51 patients with high-grade gliomas who were treated with radiation therapy followed by ACNU chemotherapy, and showed a significant benefit in median survival in the MGMT-negative group (23 months) over the MGMT-positive group (14 months).⁵ Tanaka *et al.* found a reverse correlation between MGMT mRNA copy number as measured by

Table 1 Comparison of clinical characteristics of the patients in the methylated MGMT promoter group ($n = 26$) and unmethylated MGMT promoter group ($n = 22$)

	MGMT promoter status		P value
	Methylated ($n = 26$)	Unmethylated ($N = 22$)	
Mean age	49.7 ± 12.4 years	48.5 ± 13.2 years	0.757
Extent of resection			
Complete resection	9 (34.6%)	7 (31.8%)	0.838
Incomplete resection	17 (65.4%)	15 (68.2%)	
Functional status			
KPS 80 or more	22 (84.6%)	17 (77.3%)	0.516
KPS less than 80	4 (15.4%)	5 (22.7%)	

MGMT, O6-methylguanine-DNA methyltransferase; KPS, Karnofsky performance scale

**Fig. 2** Overall survival expressed in Kaplan–Meier curves, showing statistically insignificant differences between the methylation and unmethylated groups (log rank test, $P = 0.516$). Number of patients at risk is shown at the bottom.**Table 2** Results of the Cox proportional-hazard analysis of overall survival

Variable	P value	Hazard ratio	95% CI
Age	0.100	1.025	0.995–1.055
Complete resection	0.509	1.266	0.628–2.552
KPS 80 or more	0.400	0.719	0.333–1.551
MGMT promoter methylation	0.518	0.813	0.434–1.523

MGMT, O6-methylguanine-DNA methyltransferase; KPS, Karnofsky performance scale

real-time RT-PCR and the response rate after ACNU treatment in patients with high-grade glioma.⁶ Esteller *et al.*, using an MSP method, reported that the methylation of the MGMT promoter in gliomas was a useful predictor of the responsiveness of the tumors and overall survival in patients with high-grade glioma treated with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).³ In contrast, there are many reports denying that MGMT has any prognostic value in cases of high-grade gliomas. Kamiryo *et al.* analyzed the methylation status of the MGMT promoter by

Table 3 Tumor response rates and control rates after ACNU plus cisplatin treatment

	MGMT promoter status		P-value
	Methylated ($n = 26$)	Unmethylated ($N = 22$)	
Response			
CR + PR	13 (50%)	9 (41%)	0.529
SD + PD	13 (50%)	13 (59%)	
Control			
CR + PR + SD	21 (81%)	15 (68%)	0.316
PD	5 (19%)	7 (32%)	

MGMT, O6-methylguanine-DNA methyltransferase; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease

MSP in 116 high-grade gliomas in patients treated with radiation therapy and ACNU-based chemotherapy.⁹ They found that it had significant predictive value for overall survival and progression-free survival in anaplastic astrocytoma patients, but no relationship was observed in glioblastoma patients.⁹ Belanich *et al.* reported that no correlations were found between low MGMT expression in tumor cells, determined by immunofluorescence assay, and longer overall survival or progression-free survival in 167 primary brain tumor patients, including 99 patients with glioblastomas treated with radiation therapy and BCNU.⁷ Jaeckle *et al.* also did not find any statistically significant correlation between low MGMT expression in tumor cells, as assessed by immunofluorescence assay, and longer survival in 64 patients with high-grade glioma treated with radiation therapy and BCNU.¹⁹ Blanc *et al.* studied methylation of the MGMT promoter in 44 glioblastomas using an MSP method and found that there was no relationship between the methylation status of the MGMT promoter and overall survival and response to BCNU.⁸ Brell *et al.* observed a correlation between MGMT protein expression and survival in glioblastoma patients, but they could not find any correlation between the methylation status of the MGMT promoter and survival period.²⁰ Rodriguez *et al.* evaluated MGMT status in a group of 50 glioblastoma patients using both immunohistochemistry and

MSP, but neither approach showed a predictive value for overall survival.¹²

The controversial results regarding the prognostic significance of MGMT protein expression and MGMT promoter methylation in high-grade glioma patients may be explained by several reasons. First, the various methods used for the measurement of MGMT activity in tumor cells might have led to inconsistent results among studies. There are reports showing an inconsistent correlation between aberrant MGMT promoter methylation and loss of MGMT protein expression.^{12,20,21} In addition, discrepancies in results between assays on frozen and paraffin samples have been observed.²² Immunohistochemical staining has merits in that it is a method for measurement of functional proteins at the endpoint of regulation, as well as being fairly easy technically and generally available. Nevertheless, it has shortcomings in that confused results are produced if MGMT positive inflammatory cells have infiltrated the tumor, and it cannot predict the MGMT induction by radiation therapy or chemotherapy.^{5,23} Methylation-specific PCR of MGMT has advantage that it detects the functional status of gene expression, even if alkyltransferase is absent, and it has been clearly advocated as the mainstay of clinical evaluation of MGMT status in glioblastomas.^{2,24} However, MSP also has shortcomings in that it is a highly sensitive qualitative technique, and a positive result is observed even if cells that carry MGMT promoter hypermethylation represent only a minor portion of the tumor.^{20,25} This means that it cannot fully reflect the heterogeneity in MGMT status of the tumor, which can frequently be seen in high-grade gliomas.^{8,23,26} A major problem is that the discordant results for immunohistochemical staining, enzyme activity assays and MSP prevent the use of these assays interchangeably.²⁶ Second, as observed in the report by Kamiryo *et al.*, there might be pitfalls in analyzing glioblastomas and other high-grade gliomas together as a single entity.⁹ The discrepancy in determining the prognostic value of MGMT status between glioblastoma and other high-grade gliomas may have originated from the degree of tumor cellularity, as considerable contamination of the MGMT status results from non-tumor cells might occur if the tumor cellularity is low. Third, many patients in previously reported studies received nitrosourea after radiation therapy, and it was difficult to establish whether MGMT expression was a predictive factor in nitrosourea treatment or merely a prognostic factor.²⁷ Careful interpretation of study results is needed to assess the predictive value of MGMT expression for alkylating agent treatment, and large intergroup prospective studies using standardized techniques are required for proper validation.

Few studies have evaluated the impact of MGMT on the radiological response to chemotherapy.¹⁴ In this study, the methylation status of the MGMT gene promoter was not a

prognostic factor for overall survival of glioblastoma patients treated with ACNU plus cisplatin followed by radiation therapy or a predictive factor for radiological response to ACNU plus cisplatin treatment. It is true that this study is not free from weak points such as retrospective analysis, a single institution series and the above-mentioned pitfalls in MSP. However, the homogeneous group of glioblastoma patients and the neoadjuvant application of ACNU plus cisplatin before radiation therapy in this study population provided more reliable assessment of the influence of MGMT activity on nitrosourea treatment.

Hypotheses explaining the lack of difference in clinical outcomes between the methylation and unmethylated groups shown in this study can be proposed. One is that there may be an unknown nitrosourea resistance mechanism in addition to MGMT.⁹ The other is that the dose schedule for ACNU administration in this series resulted in sufficient MGMT depletion to overcome drug resistance in the unmethylated group. Another is that regulation of MGMT expression is a rather complex phenomenon, and the simple expectation of abnormal methylation of the promoter being a determinant factor may not be true, and effects on other associated genes or post-translation modification of MGMT may have distorted the results.^{20,25,28–30} However, the most likely explanation for the result of this study is the effect of the combination of cisplatin with ACNU. Previous studies have indicated that cisplatin can inactivate or attenuate MGMT in tumor cells and MGMT inactivators can enhance the toxicity of cisplatin.^{31–33} Therefore cisplatin plus ACNU might have improved antitumor activity, even in the unmethylated group, so that their MGMT-related drug resistance is offset. The excellent median overall survival of 17.0 months and the response rate of 41% observed in the unmethylated group support such reasoning. This possible synergistic effect of cisplatin and alkylating agents extends the strategies for glioblastoma treatment and is currently being evaluated.^{34–38}

The methylation status of the MGMT promoter failed to predict the clinical outcome of glioblastoma patients treated with ACNU plus cisplatin. Among the plausible explanations for this result, the synergistic antitumor effects of ACNU and cisplatin resulting from inactivation of MGMT by cisplatin in MGMT active tumor is noteworthy. However, controversies regarding the predictive value of MGMT in high-grade glioma treatment, including the correct method for MGMT-status evaluation, remain to be solved in the future.

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