ORIGINAL ARTICLE

MGMT Gene Silencing and Benefit from Temozolomide in Glioblastoma

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ABSTRACT

BACKGROUND

Epigenetic silencing of the *MGMT* (O^6-methylguanine–DNA methyltransferase) DNA-repair gene by promoter methylation compromises DNA repair and has been associated with longer survival in patients with glioblastoma who receive alkylating agents.

METHODS

We tested the relationship between *MGMT* silencing in the tumor and the survival of patients who were enrolled in a randomized trial comparing radiotherapy alone with radiotherapy combined with concomitant and adjuvant treatment with temozolomide. The methylation status of the *MGMT* promoter was determined by methylation-specific polymerase-chain-reaction analysis.

RESULTS

The *MGMT* promoter was methylated in 45 percent of 206 assessable cases. Irrespective of treatment, *MGMT* promoter methylation was an independent favorable prognostic factor ($P<0.001$ by the log-rank test; hazard ratio, 0.45; 95 percent confidence interval, 0.32 to 0.61). Among patients whose tumor contained a methylated *MGMT* promoter, a survival benefit was observed in patients treated with temozolomide and radiotherapy; their median survival was 21.7 months (95 percent confidence interval, 17.4 to 30.4), as compared with 15.3 months (95 percent confidence interval, 13.0 to 20.9) among those who were assigned to only radiotherapy ($P=0.007$ by the log-rank test). In the absence of methylation of the *MGMT* promoter, there was a smaller and statistically insignificant difference in survival between the treatment groups.

CONCLUSIONS

Patients with glioblastoma containing a methylated *MGMT* promoter benefited from temozolomide, whereas those who did not have a methylated *MGMT* promoter did not have such a benefit.

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EPIGENETIC SILENCING OF THE *MGMT* (O^6-methylguanine–DNA methyltransferase) gene by promoter methylation has been associated with longer overall survival in patients with glioblastoma who, in addition to radiotherapy, received alkylating chemotherapy with carmustine or temozolomide.[1,2] The *MGMT* gene is located on chromosome 10q26 and encodes a DNA-repair protein that removes alkyl groups from the O^6 position of guanine, an important site of DNA alkylation. The restoration of the DNA consumes the MGMT protein, which the cell must replenish. Left unrepaired, chemotherapy-induced lesions, especially O^6-methylguanine, trigger cytotoxicity and apoptosis.[3,4] High levels of MGMT activity in cancer cells create a resistant phenotype by blunting the therapeutic effect of alkylating agents and may be an important determinant of treatment failure.[5-10] Epigenetic silencing of the *MGMT* gene by promoter methylation is associated with loss of MGMT expression[11-13] and diminished DNA-repair activity. In the course of tumor development, gene silencing by DNA methylation is an early and important mechanism by which tumor-suppressor genes are inactivated.[14,15]

In a phase 2 evaluation of combined radiotherapy and temozolomide for newly diagnosed glioblastoma, we found that methylation of the *MGMT* promoter in the tumor was associated with longer survival.[2] In the current study, we investigated whether *MGMT* promoter methylation in glioblastoma is associated with a benefit from temozolomide treatment. We determined the *MGMT* promoter methylation status in tumor tissues from patients who were enrolled in a randomized trial that showed a survival advantage among patients treated with temozolomide and radiotherapy as compared with radiotherapy alone.[16]

METHODS

PATIENTS AND TREATMENT

Patients were enrolled in a randomized trial of chemoradiotherapy (temozolomide plus radiotherapy) versus radiotherapy alone (carried out by the European Organisation for Research and Treatment of Cancer and the National Cancer Institute of Canada [NCIC]) (EORTC trial 26981/22981 and NCIC trial CE.3).[16] Patients in the experimental group received the alkylating agent temozolomide (Temodal or Temodar, Schering-Plough) at a dose of 75 mg per square meter of body-surface area daily during standard fractionated radiotherapy (60 Gy) for 6 to 7 weeks and at a dose of 150 to 200 mg per square meter per day for 5 days of every 28-day cycle after radiotherapy, for up to six cycles. In the case of tumor progression, salvage or second-line therapy was administered at the investigators' discretion; most patients received additional chemotherapy. All patients provided written informed consent for molecular studies of their tumor, and the protocol was approved by the ethics committee at each center.

DNA EXTRACTION AND METHYLATION-SPECIFIC POLYMERASE CHAIN REACTION

Genomic DNA was isolated from one or two paraffin sections of glioblastoma tissue (Ex-Wax DNA Extraction Kit S4530, Chemicon) (proteinase digestion lasted a maximum of six hours). DNA was denatured with sodium hydroxide in a volume of 35 μl and subjected to bisulfite treatment in a volume of 360 μl (4.4 M sodium bisulfite and 20 mM hydroquinone) for five hours at 55°C and then purified (Wizard DNA Clean-Up System A7280, Promega). Unmethylated cytosine, but not its methylated counterpart, is modified into uracil by the treatment. The methylation-specific polymerase chain reaction (PCR) was performed in a two-step approach.[17] The results were confirmed in an independent experiment, starting with reisolation of DNA from the tumor. The PCR products were separated on 4 percent agarose gels. The investigators who selected and analyzed the glioblastoma samples were blinded to all clinical information.

STATISTICAL ANALYSIS

Overall and progression-free survival curves were estimated by the Kaplan–Meier technique and compared with use of the two-sided log-rank test. All treatment comparisons are presented on an intention-to-treat basis according to the randomized assignment. The Cox proportional-hazards model was fitted to assess the prognostic and predictive values of the methylation status of the *MGMT* promoter, the protocol treatment, and potential prognostic factors[18] that were found to be statistically significant in this population on the basis of univariate testing.

ORGANIZATION OF THE STUDY

This project was initiated and carried out without the involvement of a commercial sponsor. Dr. Hegi designed and supervised the translational study and wrote the manuscript, with input from the coauthors. Methylation-specific PCR was performed by Ms. Diserens. The statistical analysis was per-

formed by Mr. Gorlia. The clinical trial was designed and directed by Dr. Stupp, in collaboration with the EORTC and the NCIC Clinical Trials Group.

RESULTS

Methylation-specific PCR was performed on 307 of 573 glioblastoma specimens (53.6 percent) from patients enrolled at 66 of 85 participating centers (Fig. 1); adequate paraffin-embedded tumor tissue was not available from 266 patients. *MGMT* methylation status could be determined for 206 of the 307 tumors (67.1 percent), or 36.0 percent of the tumors from the overall study population. The success rate of methylation-specific PCR on paraffin-embedded tumor samples was highly variable and center-dependent. For centers with four or more testable samples, the median success rate was 75.0 percent (range, 0 to 100 percent). Treatment assignments among the 307 patients with evaluable tumor specimens was equally distributed, with 152 patients (49.5 percent) randomly assigned to radiotherapy alone and 155 (50.5 percent) randomly assigned to temozolomide and radiotherapy.

The subgroup of 206 patients in whom *MGMT* promoter methylation status could be determined was representative of the overall treatment population with respect to known prognostic factors and outcomes. However, the proportion of patients who had only a diagnostic biopsy specimen (and no debulking surgery) was smaller in the subgroup tested for *MGMT* promoter methylation than in the subgroup of patients in whom methylation status could not be determined (3.4 percent vs. 23.0 percent). Overall survival did not vary significantly according to whether or not the test was attempted (P=0.27 by the log-rank test) or whether or not the results were interpretable (P=0.23 by the log-rank test) (Fig. 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org). Of the 206 evaluated tumors, 92 (44.7 percent) had detectable *MGMT* promoter methylation, whereas 114 (55.3 percent) did not. The proportion of methylated tumors was similar in the two treatment groups (Table 1).

For the entire population of 206 patients for whom *MGMT* status could be evaluated, there was a significant difference, irrespective of treatment assignment, in overall survival between patients whose tumors had *MGMT* promoter methylation and those whose tumors did not (P<0.001 by the log-rank test) (Fig. 2). The hazard ratio for death was 0.45 (95 percent confidence interval, 0.32 to

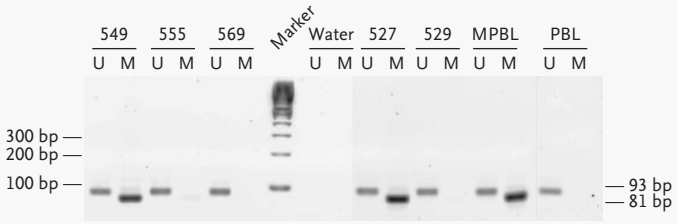

Figure 1. Methylation Status of the *MGMT* Promoter in Glioblastoma Biopsy Specimens, as Determined by a Nested Methylation-Specific PCR Assay.

DNA from normal peripheral blood lymphocytes (PBL) was used as a control for the unmethylated *MGMT* promoter (U), enzymatically methylated DNA from PBL (MPBL) served as a positive control for the methylated *MGMT* promoter (M), and water was used as a negative control for the PCR. A 100-bp marker ladder was loaded to estimate molecular size, as shown on the left scale; the sizes of PCR products are indicated on the right scale. Glioblastoma numbers 549 and 527 contain a methylated promoter, whereas 555, 569, and 529 harbor only an unmethylated promoter. The nested PCR approach renders the analysis highly sensitive, while allowing it to retain the specificity that results in the detection of unmethylated *MGMT* promoter in all specimens that may also contain DNA derived from infiltrating lymphocytes, blood vessels, or contaminating normal tissue.

Table 1. Effect of *MGMT* Promoter Methylation Status on Survival, According to Random Treatment Assignment.*

Promoter Status and Outcome	Radiotherapy (N=100)	Temozolomide plus Radiotherapy (N=106)
Methylated *MGMT* promoter		
No. of patients	46	46
Progression-free survival		
Median duration (mo)	5.9 (5.3–7.7)	10.3 (6.5–14.0)
Rate at 6 mo (%)	47.8 (33.4–62.3)	68.9 (55.4–82.4)
Hazard ratio for death	1.00	0.48 (0.31–0.75)
Overall survival		
Median duration (mo)	15.3 (13.0–20.9)	21.7 (17.4–30.4)
Rate at 2 yr (%)	22.7 (10.3–35.1)	46.0 (31.2–60.8)
Hazard ratio for death	1.00	0.51 (0.31–0.84)
Unmethylated *MGMT* promoter		
No. of patients	54	60
Progression-free survival		
Median duration (mo)	4.4 (3.1–6.0)	5.3 (5.0–7.6)
Rate at 6 mo (%)	35.2 (22.5–47.9)	40.0 (27.6–52.4)
Hazard ratio for death	1.00	0.62 (0.42–0.92)
Overall survival		
Median duration (mo)	11.8 (9.7–14.1)	12.7 (11.6–14.4)
Rate at 2 yr (%)	<2†	13.8 (4.8–22.7)
Hazard ratio for death	1.00	0.69 (0.47–1.02)

* Numbers in parentheses are 95 percent confidence intervals.
† None of the patients in this subgroup were followed up for two years.

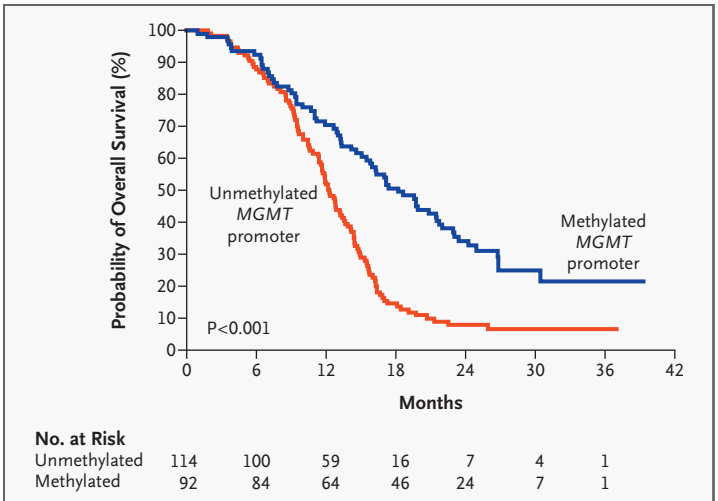

Figure 2. Kaplan–Meier Estimates of Overall Survival, According to *MGMT* Promoter Methylation Status.

The difference in survival between patients with a methylated *MGMT* promoter (92 patients, 65 of whom died) and those with an unmethylated *MGMT* promoter (114 patients, 105 of whom died) was highly significant (P<0.001 by the log-rank test), indicating that the *MGMT* methylation status has prognostic value. In the group of patients with a methylated *MGMT* promoter, there was a risk reduction of 55 percent (hazard ratio for death, 0.45; 95 percent confidence interval, 0.32 to 0.61), as compared with the group with an unmethylated *MGMT* promoter.

0.61) among those with *MGMT* promoter methylation, a result that corresponds to a 55 percent decrease in the risk of death in this subgroup. The median overall survival among patients with methylation was 18.2 months (95 percent confidence interval, 15.5 to 22.0), as compared with 12.2 months (95 percent confidence interval, 11.4 to 13.5) among those without methylation.

When both treatment assignment and *MGMT* promoter methylation status were considered, the longest median overall survival, 21.7 months, was observed among patients with promoter methylation who were assigned to receive both temozolomide and radiotherapy (Table 1). Their two-year survival rate was 46.0 percent, as compared with 22.7 percent among those with *MGMT* promoter methylation who were assigned to radiotherapy alone. Kaplan–Meier estimates of overall survival in these two subgroups were significantly different (P=0.007 by the log-rank test) (Fig. 3A).

By contrast, among patients whose tumors were not methylated at the *MGMT* promoter, the difference in overall survival favoring the temozolomide-plus-radiotherapy group was only marginally significant (P=0.06 by the log-rank test) (Fig. 3A); the median survival was 12.7 months among those assigned to temozolomide and radiotherapy and 11.8 months among those assigned to radiotherapy, with 2-year survival rates of 13.8 percent and less than 2 percent, respectively (Table 1). The interaction between the magnitude of the treatment effect and *MGMT* promoter methylation status with respect to overall survival was not statistically significant, according to the Cox proportional-hazards model (P=0.29) (Table 2). However, this result was not unexpected, since neither the clinical trial nor this study was powered to test the interaction.

In addition, a probable confounding factor in the analysis of overall survival was the administration of temozolomide or other alkylating chemotherapy as salvage or second-line treatment after disease progression. More than 70 percent of the patients in the radiotherapy group received salvage chemotherapy; 59.7 percent received temozolomide. In the temozolomide-plus-radiotherapy group, 57.8 percent received second-line chemotherapy; 24.6 percent were retreated with temozolomide. We therefore analyzed progression-free survival relative to *MGMT* promoter methylation status and treatment assignment (Fig. 3B and Table 1).

In the group of patients whose tumors contained a methylated *MGMT* promoter, those who received temozolomide and radiotherapy had a median progression-free survival of 10.3 months, as compared with 5.9 months for patients who received radiotherapy alone (P=0.001). Among the patients whose tumors contained an unmethylated *MGMT* promoter, those who received temozolomide and radiotherapy had a median progression-free survival of 5.3 months, as compared with 4.4 months for patients who were treated with radiotherapy alone (P=0.02) (Fig. 3B). The relatively long overall survival despite the short progression-free survival among patients with a methylated *MGMT* promoter who were assigned to receive only radiotherapy indicates that salvage therapy at the time of recurrence has some efficacy in this subpopulation.

To analyze further the influence of the methylation status of the *MGMT* promoter, we performed a multivariate analysis with the use of the Cox proportional-hazards model, stratified according to treatment group and including known clinical prognostic factors (Table 2). The methylation status of the *MGMT* promoter (P<0.001) and the score on the

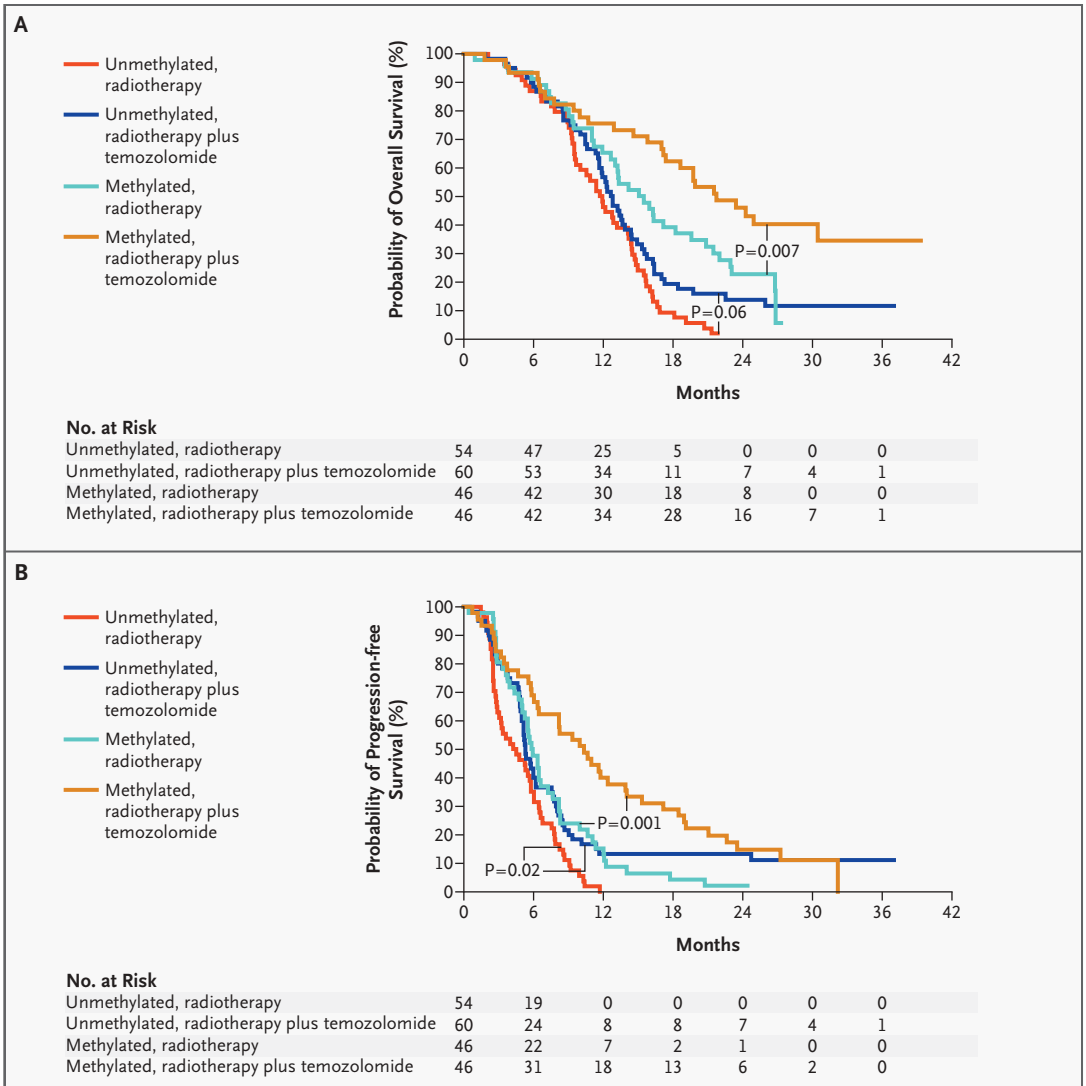

Figure 3. Kaplan–Meier Estimates of Overall and Progression-free Survival, According to *MGMT* Promoter Methylation Status and Random Assignment to Temozolomide plus Radiotherapy or Radiotherapy Alone.

The Kaplan–Meier estimates for overall survival indicate that the group of patients with a methylated *MGMT* promoter who were randomly assigned to temozolomide and radiotherapy (46 patients, 40 of whom had progression and 27 of whom died) had a 49 percent risk reduction (hazard ratio for death, 0.51; 95 percent confidence interval, 0.31 to 0.84), as compared with the group with a methylated *MGMT* promoter who were randomly assigned to radiotherapy only (46 patients, 45 of whom had progression and 38 of whom died) (Panel A). An unmethylated *MGMT* promoter and random assignment to temozolomide and radiotherapy (60 patients, 53 of whom had progression and 52 of whom died) yielded a risk reduction of 31 percent (hazard ratio for death, 0.69; 95 percent confidence interval, 0.47 to 1.02), as compared with an unmethylated *MGMT* promoter and random assignment to radiotherapy only (54 patients, all of whom had progression and 53 of whom died). In order to display a possible effect of salvage treatment on overall survival, in particular in the group of patients with a methylated *MGMT* promoter who were randomly assigned to radiotherapy alone. Kaplan–Meier curves are also shown for progression-free survival (Panel B) in a similar manner.

Table 2. Results of Analyses with the Cox Proportional-Hazards Models.*

Variable	Prognostic-Factor Model		Predictive-Factor Model	
	P Value	Hazard Ratio (95% CI)	P Value	Hazard Ratio (95% CI)
MGMT promoter methylation and temozolomide plus radiotherapy (vs. no methylation or radiotherapy)	NA	NA	0.29	0.71 (0.37–1.35)
Temozolomide plus radiotherapy (vs. radiotherapy)	NA	NA	0.06	0.68 (0.45–1.02)
MGMT promoter methylation (vs. no methylation)	<0.001	0.41 (0.29–0.57)	0.001	0.49 (0.32–0.76)
Age (continuous)	0.47	1.01 (0.99–1.02)	0.36	1.01 (0.99–1.03)
Mini–Mental State Examination score (continuous increments)	0.007	0.94 (0.89–0.98)	0.004	0.93 (0.89–0.98)
Use of corticosteroids at randomization (vs. nonuse)	0.07	1.41 (0.97–2.04)	0.08	1.39 (0.96–2.00)

* CI denotes confidence interval, and NA not applicable.

Mini–Mental State Examination (P=0.007) emerged as significant independent prognostic factors. The adjusted hazard ratio of 0.41 (95 percent confidence interval, 0.29 to 0.57) for *MGMT* promoter methylation was consistent with the unadjusted hazard ratio of 0.45 (95 percent confidence interval, 0.32 to 0.61).

DISCUSSION

We found that *MGMT* promoter methylation is associated with a favorable outcome after temozolomide chemotherapy in patients with newly diagnosed glioblastoma. Our data suggest that the methylation status of the *MGMT* promoter may have prognostic value and, in addition, may be a clinically relevant predictor of benefit from temozolomide chemotherapy. Despite the survival benefit associated with temozolomide among patients with a methylated *MGMT* promoter, the overall survival curves for temozolomide and radiotherapy and for radiotherapy alone remain similar for the first nine months of follow-up. This suggests that *MGMT* methylation, though important, is not the sole factor determining outcome. Lack of mismatch-repair has also been shown to render tumors resistant to alkylating agents, even in the absence of MGMT.[4] Additional mechanisms and predictive factors are likely to be relevant and need to be identified.

Diagnostic *MGMT* testing requires sufficient and optimally preserved tumor tissue. The best results with methylation-specific PCR are obtained with cryopreserved tumor specimens, thus avoiding fixation-related deterioration of the quality of tumor DNA. Other methods, such as immunohistochemistry or activity testing, may not be reliable, since MGMT expression is prone to induction by glucocorticoids, ionizing radiation, and genotoxic agents[19,20] when the *MGMT* promoter is not methylated.

Determination of MGMT promoter methylation status by methylation-specific PCR may allow the selection of patients most likely to benefit from temozolomide treatment; patients whose tumors are not methylated at the *MGMT* promoter appear to derive little or no benefit from the addition of temozolomide to radiotherapy. For these patients, alternative treatments with a different mechanism of action or methods of inhibiting MGMT should be developed.[21,22] Our findings may be applicable to other solid tumors commonly treated with alkylating agents, such as melanoma, but possibly also to lung and breast cancer and lymphoma. Stratification according to *MGMT* promoter methylation status may be considered in future trials in which temozolomide or other alkylating agents are used.

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CLINICAL TRIAL REGISTRATION

The *Journal* encourages investigators to register their clinical trials in a public trials registry. The members of the International Committee of Medical Journal Editors plan to consider clinical trials for publication only if they have been registered (see N Engl J Med 2004;351:1250-1). The National Library of Medicine's www.clinicaltrials.gov is a free registry, open to all investigators, that meets the committee's requirements.