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MGMT promoter hypermethylation correlates with a survival benefit from temozolomide in patients with recurrent anaplastic astrocytoma but not glioblastoma

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ABSTRACT

Aims: To investigate the correlation between O^6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation and benefit from temozolomide in patients with recurrent high-grade glioma.

Patients and methods: A real-time, quantitative, methylation-specific PCR assay was performed on archival tissue blocks from patients treated with temozolomide at the first recurrence.

Results: A subgroup of 38 patients who were chemotherapy-naive at recurrence was analysed (22 glioblastoma, 12 anaplastic astrocytoma [AA] and 4 anaplastic oligoastrocytoma [AOA]); none had 1p/19q loss. Among 10 (26%) patients with a hypermethylated MGMT promoter, none experienced disease progression within the first two treatment cycles compared with 12 of 28 (43%) patients with an unmethylated promoter ($p = 0.016$). By Cox multivariate analysis, tumour grade and MGMT promoter methylation correlated with time to progression ($p < 0.05$); MGMT promoter methylation correlated with superior overall survival in AA/AOA but not in glioblastoma.

Conclusions: MGMT promoter methylation predicted a survival benefit in patients with 1p/19q intact AA/AOA treated with temozolomide at recurrence.

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1. Introduction

Beyond tumour histology, molecular-genetic characterisation of gliomas provides valuable information regarding their natural history and sensitivity to chemotherapy. For example, heterozygous chromosomal deletion of 1p and 19q (most often caused by a [1;19][q10;p10] translocation) identifies a subgroup of low-grade and anaplastic gliomas, typically with oligodendroglial histology, which have a more favourable prognosis and appear to be the most sensitive to alkylating agents.[1–3] In addition, malignant transformation is characterised by genome-wide changes in DNA methylation patterns, an epigenetic mechanism by which tumour cells can control gene expression and silence tumour suppressor genes.[4–6] Epigenetic silencing of key DNA repair proteins can affect sensitivity to alkylating agents.

The cytotoxic effects of temozolomide (TMZ) are mediated by DNA methylation at the O^6 position of guanine as well as by an intact DNA mismatch repair pathway. The DNA repair protein O^6-methylguanine-DNA-methyltransferase (MGMT) repairs O^6-methyl adducts in the DNA.[7] Consequently, MGMT is a critical regulator of the cytotoxic effects of TMZ.[8] Hypermethylation of the MGMT promoter region can silence its expression and result in a deficiency in MGMT-mediated DNA repair. This is most frequently detected in high-grade glioma (HGG) and colorectal carcinomas.[9,10] Hypermethylation of the MGMT promoter in gliomas is associated with sensitivity to alkylating agents including nitrosoureas and TMZ.[9,11–13] The degree of MGMT promoter methylation can be investigated with the use of a methylation-specific PCR assay.[14,15]

In a randomised trial conducted by the European Organisation for the Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) in patients with newly diagnosed glioblastoma, treatment with radiotherapy (RT) plus concomitant TMZ followed by 6 cycles of adjuvant TMZ significantly improved overall survival compared with RT-alone.[16] A retrospective analysis of a subset of patients in this trial demonstrated that MGMT promoter methylation was significantly associated with a survival benefit from RT plus TMZ.[11] A similar correlation was also demonstrated in smaller phase II trials with TMZ and nitrosoureas.[17] The analysis of the EORTC/NCIC trial also suggested that a subgroup of patients with a methylated MGMT promoter who were treated with TMZ at recurrence (approximately 60% of patients in the RT-alone arm received salvage therapy with TMZ) had improved survival compared with patients with an unmethylated promoter (the overall survival curves only separated beyond the median).[16]

A number of prospective, multicentre, phase II studies have also shown that treatment with TMZ is effective in patients with recurrent HGG following primary treatment with surgery, radiotherapy and in some cases adjuvant nitrosourea-based chemotherapy.[18–21] However, no data are currently available regarding the predictive value of MGMT promoter methylation status in patients with recurrent HGG who are treated with TMZ at recurrence following surgery and radiation therapy. In this study, we investigated the correlation between MGMT promoter methylation status and the clinical outcome of patients who were treated with TMZ only at recurrence of anaplastic astrocytoma (AA), anaplastic oligoastrocytoma (AOA) or glioblastoma. Patients were treated with either the standard 5-d schedule or an extended daily dosing schedule. Extended daily dosing of TMZ has been shown to deplete MGMT activity in peripheral blood monocytes,[22] and encouraging data have been published on the activity of extended daily, dose-dense TMZ regimens in patients with recurrent HGG.[23–25]

2. Patients and methods

2.1. Study design, patients and tumour material

This retrospective study was conducted by a single university hospital centre (Universitair Ziekenhuis [UZ] Brussel). The primary objective was to evaluate the correlation between the degree of MGMT promoter methylation and tumour response and survival, following treatment with TMZ (Temodal, Schering-Plough Labo NV, Heist-op-den-Berg, Belgium) in chemotherapy-naive patients with HGG at the first recurrence following surgery and RT. The secondary objective was to evaluate the correlation of MGMT promoter methylation with patient demographic characteristics and World Health Organisation (WHO) tumour grade.

Tumour material was collected from patients who had been treated for a recurrent HGG at one of 5 Belgian institutions. Formalin-fixed and paraffin-embedded archival tumour specimens were centralised at the UZ Brussel Department of Pathology. Patients participated either in a compassionate-use program for TMZ[26] or in a prospective multicentre study of dose-dense TMZ for recurrent AA or AOA.[24] Additional patients who were treated with TMZ at recurrence were identified at two centres. Patients received treatment with oral TMZ 200 mg/m^2/d for 5 consecutive days of every 28 days (i.e. standard schedule) or 100 mg/m^2/d for 21 consecutive days of every 28-d cycle (i.e. 21/28-d schedule). Criteria for re-treatment and patient evaluation were as previously published.[24,27] All patients were evaluated by gadolinium-enhanced magnetic resonance imaging (Gd-MRI) of the brain for every 2 treatment cycles. Approval for this study was obtained from the institutional ethical committee of the UZ Brussel. Clinical data were retrieved either from the case report forms for those patients participating in a clinical trial or from the hospital patient records.

2.2. Extraction, quantification and modification of tumour DNA

Before extracting DNA from the tumour tissue, the region with the highest proportion of malignant cells was delineated by an expert neuropathologist (A.M.). Tissue sections were cut from formalin-fixed and paraffin-embedded tumour tissue, deparaffinated and dehydrated. Nucleic acid extraction from paraffin-embedded tissue samples was performed by the phenol/chloroform method.

2.3. Real-time, quantitative, methylation-specific PCR

Real-time, quantitative, methylation-specific PCR (QMSP) was performed by OncoMethylome Sciences SA (OMS; Liège,

Belgium) as previously described.[28] OMS, in collaboration with Hegi and colleagues, conducted a side-by-side comparison of QMSP by the OMS assay and the gel-based assay.[11,15] A lack of MGMT promoter methylation was defined as a test result (1000 × MGMT:β actin ratio) <5, whereas a test result >12 correlated with promoter hypermethylation.[11,14,29] Accordingly, MGMT:β-actin ratios between 5 and 12 were considered indeterminate (i.e. grey zone).

2.4. *Fluorescent in situ hybridisation (FISH) for 1p36 and 19q*

For the evaluation of possible loss of heterozygosity (LOH) of 1p and 19q chromosomal arms, we used the locus specific microsatellite instability (LSI) 1p36/LSI 1q25 and the LSI 19q13/19p13 Dual Colour Probe Sets. Sections of 4–6 μm were cut from the formalin-fixed and paraffin-embedded glioma tissue, and slides were prepared using a kit from Vysis (division of Abbott Laboratories, Des Plaines, IL) including LSI 1p36/LSI 19q13/19p13 dual-colour probes according to the manufacturers' recommendations. Signals for the 1p or 19q and control probes were counted, and the ratio was determined. Tumours were considered to have LOH for 1p or 19q when those ratios were below the cut-off value of 0.80 (Mayo Reference Services: 'Fluorescence In Situ Hybridisation (FISH) for 1p/19q Deletion in Gliomas #80029'; December 2002).

2.5. *Statistical analysis*

Cross-table statistics were calculated with a 2-sided Fisher exact test. Progression-free and overall survival was calculated from the date of the first day of TMZ administration until disease progression, death or last follow-up (for censored cases) according to the Kaplan–Meier method using SPSS statistical software (release 7.5, 1996; SPSS Inc., Chicago, IL). The survival and the proportion of patients who were free of clinical disease progression at 6, 12 and 24 months were calculated with a 95% confidence interval (CI) based on Kaplan–Meier estimates. Cox's proportional hazard models were used to analyse the correlation between MGMT promoter methylation and the patient and tumour baseline characteristics.

3. Results

3.1. *Molecular testing of tumour samples*

Archival tumour blocks with WHO grade III or IV glioma tissue were collected from 84 patients. Sufficient tumour material for DNA extraction and testing by QMSP was obtained in 64 patients primarily from blocks obtained at initial diagnosis of HGG, and an informative QMSP test result (methylated, unmethylated or grey zone) was obtained in 55 patients (Table 1). The MGMT promoter was hypermethylated in 14 of 55 (25%) patients with an informative test result, including in 8 of 35 (23%) patients with GBM and in 6 of 20 (30%) patients with AA/AOA.

Among 16 patients with AA or AOA (all with a predominant astrocytoma histology), tumours were characterised for LOH at chromosome 1p36 and 19q. None of these gliomas had significant LOH at either 1p or 19q (ratio of 1p/1q and 19p/19q, respectively: 0.97, range 0.81–1.15; and 0.95, range 0.87–1.03).

Table 1 – Results of MGMT promoter methylation testing by real-time, quantitative methylation-specific PCR

	Patients, *n* (%) (N = 84)
Sufficient tumour material	64 (76%)
Informative test results	55 (65%)
Methylated	14 (17%)
Unmethylated	38 (45%)
Grey zone	3 (4%)
Invalid	9 (11%)
Insufficient material	20 (24%)

MGMT = O^6-methylguanine-DNA-methyltransferase; PCR = polymerase chain reaction.

3.2. *Patients and treatment disposition*

To investigate the correlation between the MGMT promoter methylation status and the outcome of treatment with TMZ at recurrence, a homogeneous subgroup of 38 patients was selected (22 patients with glioblastoma and 16 patients with AA or AOA). All these patients had been treated with surgery (either complete or partial resection) followed by standard RT at initial diagnosis of HGG, and none had received chemotherapy as part of their initial treatment. Therefore, all patients included in this analysis were chemotherapy-naive (with respect to systemic chemotherapy) at the time of recurrence, and they all received TMZ between May 1999 and September 2004. All 38 patients completed at least 2 cycles of TMZ after which time they were evaluated for response (clinical and Gd-MRI). Their baseline characteristics are summarised in Table 2.

Twenty-seven patients were treated with the standard 5/28-d regimen, and they received a median of 3 cycles (range, 2–30 cycles). Eleven patients (all with AA or AOA) were treated with the 21/28-d dose-dense regimen, and they received a median of 5 cycles (range, 1–12 cycles). Eight (21%) patients had an objective response, 18 (47%) patients had stable disease and 12 (32%) patients had progressive disease.

3.3. *Correlation between MGMT methylation status and response to TMZ*

Among the 38 patients included in the correlative analysis, 10 (26%) had a methylated MGMT promoter and 28 (74%) had an unmethylated promoter. Methylation of the MGMT promoter was significantly correlated with the absence of disease progression within the first 2 treatment cycles (2-sided Fischer exact test, $p = 0.016$). None of the 10 patients with a methylated promoter progressed during the first 2 treatment cycles compared with 12 of 28 (43%) patients with an unmethylated promoter. Among patients with an unmethylated promoter, 11 of 21 (52%) patients who were treated with the standard 5/28-d regimen progressed within the first 2 cycles compared with 1 of 7 (17%) patients treated with the 21/28-d regimen; albeit only patients with AA or AOA were treated with the 21/28-d regimen.

Table 2 – Characteristics of patients included in the correlative analysis

Characteristic	N = 38
Sex (M/F)	27/11
White ethnicity	38
Median age at study entry, years (range)	55 (23–80)
Previous treatment for LGG	
Surgery	7
Radiotherapy	4
Histopathology at study entry	
Glioblastoma	22
AA	12
AOA	4
Tumour localisation	
(Sub)cortical	36
Deep (thalamus, corpus callosum)	2
Surgery for HGG	
Complete resection	21
Partial resection	17
Postoperative RT for HGG	
Yes	35
No	3[a]
Surgery for recurrence HGG	6
Postoperative Gliadel Wafer	3
KPS at start of temozolomide	
100	3
90–60	35

LGG = low-grade glioma; AA = anaplastic astrocytoma; AOA = anaplastic oligoastrocytoma; HGG = high-grade glioma; RT = radiotherapy.

a These 3 patients had received prior RT at the diagnosis of LGG.

No significant correlation was observed between MGMT promoter methylation status and any baseline variables, including WHO tumour grade at diagnosis, age at study entry, sex, history of low-grade glioma or tumour localisation. Absence of disease progression within the first 2 treatment cycles was significantly correlated with age <50 years ($p = 0.04$) and female sex ($p = 0.008$), and a non-significant trend was observed for the correlation with WHO grade III tumours ($p = 0.178$) and treatment with the 21/28-d regimen ($p = 0.121$).

3.4. *Correlation of baseline covariates with time to progression*

Documentation of disease progression on Gd-MRI of the brain was available for 36 of 38 patients. Two patients stopped TMZ treatment in the absence of documented disease progression because of an intercurrent illness. Median time to progression (TTP) for this population was 4.8 months (95% CI: 2.7, 7.0 months). By multivariate Cox regression analysis (both forward and backward-looking) only WHO tumour grade ($p < 0.001$) and MGMT methylation status ($p = 0.057$ in the forward-looking regression and $p = 0.047$ in the backward-looking regression) were independent prognostic variables for TTP. Time to progression by MGMT promoter methylation status is shown in Fig. 1A.

When the importance of MGMT promoter methylation status was assessed within the subgroup of patients with

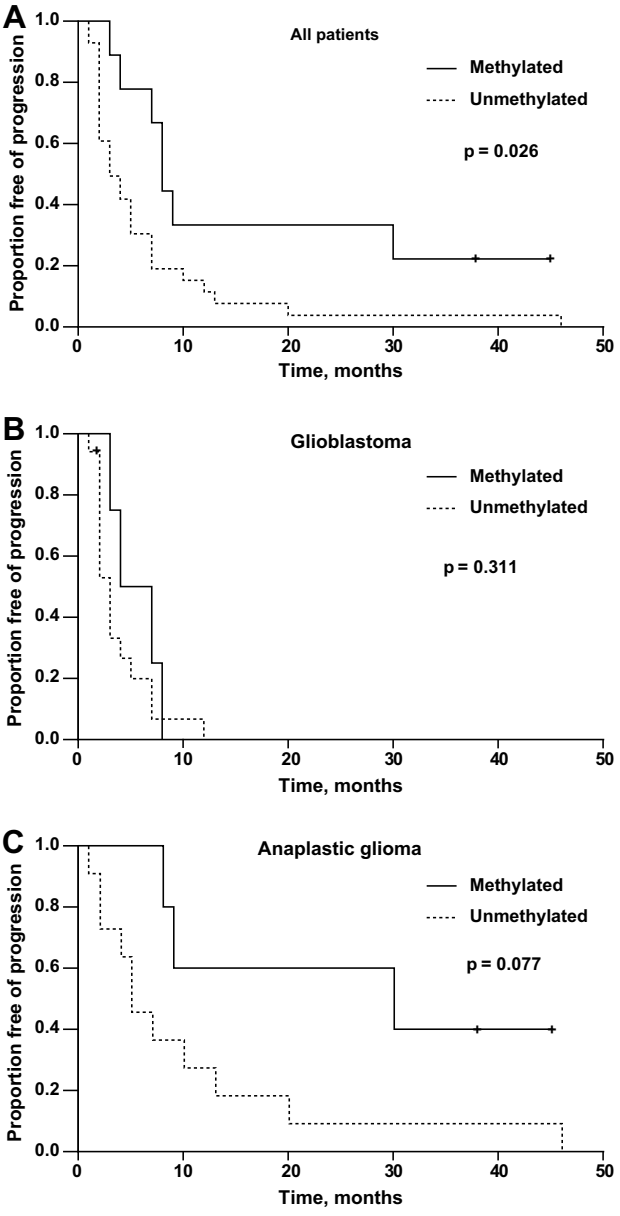

Fig. 1 – Kaplan–Meier estimate of time to disease progression based on O^6-methylguanine-DNA-methyltransferase promoter methylation status for all patients (panel A; $n = 38$) and by histology (glioblastoma; $n = 22$ [panel B]; anaplastic astrocytoma/anaplastic oligoastrocytoma; $n = 16$ [panel C]).

AA/AOA or glioblastoma separately (univariate analysis), MGMT promoter methylation was more strongly correlated with TTP in the subgroup of patients with AA or AOA (Fig. 1C) than in patients with glioblastoma (Fig. 1B), but the correlation no longer reached statistical significance ($p = 0.077$ for AA/AOA versus $p = 0.311$ for glioblastoma). When the influence of MGMT promoter methylation was analysed within the subgroups of patients treated with the 5/28-d versus 21/28-d TMZ regimen, a significant correlation was found only in patients treated with the 5/28-d regimen ($p = 0.047$) but not in patients treated with the 21/28-d regimen ($p = 0.508$).

3.5. Correlation of baseline covariates with overall survival

Data on overall survival (all-cause and disease-specific mortality) were available for all 38 patients. Four patients were alive at the time of analysis and were censored at the date of last follow-up, and 34 patients had died. The cause of death was progression of disease in all but 1 patient (with a methylated MGMT promoter). Median overall survival was 8.2 months (95% CI: 6.0, 10.2 months) from initiation of TMZ for recurrent disease. By multivariate Cox regression analysis (both forward- and backward-looking), only WHO tumour grade was significantly associated with survival ($p < 0.001$).

When MGMT promoter methylation was analysed within the AA/AOA and glioblastoma subgroups separately, MGMT promoter methylation was significantly associated with improved overall survival in patients with AA or AOA ($p = 0.040$) but not in patients with glioblastoma ($p = 0.864$) (Fig. 2). In the Cox multivariate analysis, MGMT promoter methylation remained of borderline significance in the AA/AOA subgroup ($p = 0.059$). When the influence of MGMT promoter methylation on overall survival was analysed within the subgroups of patients treated with the 5/28-d or 21/28-d regimen separately, no significant correlation was found.

3.6. Analysis of overall survival from diagnosis of high-grade glioma

We also analysed the influence of baseline variables on overall survival from diagnosis of HGG (all-cause mortality) for all 52 patients from whom definitive MGMT test results (i.e. methylated or unmethylated) were available except for 1 patient with a low-grade glioma and 1 patient with an anaplastic oligodendroglioma (Table 3). In Cox multivariate analysis, only WHO tumour grade was significantly associated with survival ($p = 0.001$).

When the correlation of MGMT promoter methylation with overall survival was analysed within the subgroup of 18 patients with AA or AOA and 32 patients with glioblastoma separately, MGMT promoter methylation was associated with improved overall survival in the AA/AOA subgroup ($p = 0.028$) but not in the glioblastoma subgroup ($p = 0.699$) (Fig. 3). In Cox multivariate analysis, MGMT promoter methylation was retained as the only significant variable ($p = 0.011$) in the AA/AOA subgroup. The TMZ regimen used showed no significant correlation with overall survival.

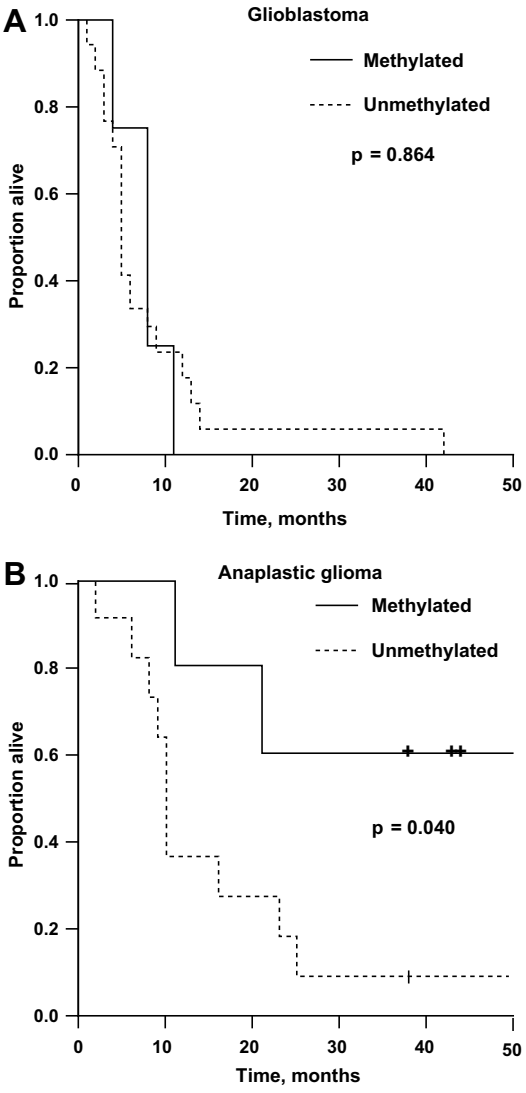

Fig. 2 – Kaplan–Meier estimate of overall survival from initiation of temozolomide for recurrent disease based on O^6-methylguanine-DNA-methyltransferase promoter methylation status and by tumour histology (glioblastoma, $n = 22$ [panel A]; anaplastic astrocytoma/anaplastic oligoastrocytoma, $n = 16$ [panel B]).

4. Discussion

In this retrospective analysis, we investigated the role of MGMT promoter methylation as a predictor of clinical outcome in patients with 1p/19q intact AA/AOA or with glioblastoma treated with TMZ at the first recurrence. Among the clinical and molecular-genetic baseline variables assessed, only WHO tumour grade and MGMT promoter methylation status correlated with sensitivity to TMZ. These findings are consistent with the results reported by Ishii et al.,[30] who also found a positive correlation between MGMT promoter methylation and response to TMZ therapy in patients with recurrent malignant gliomas. Moreover, in this study, MGMT promoter methylation correlated with improved time to progression and overall survival in patients with AA/AOA but not in patients with glioblastoma.

When interpreting the absence of a significant correlation in the glioblastoma subset, one needs to consider that these patients have a homogeneously poor prognosis (<10% of patients who are treated with surgery and RT at diagnosis survive for >2 years).[31] Our findings are not discordant with those of the EORTC/NCIC trial.[11] In that study, among the patients randomised to RT-alone who were subsequently treated with TMZ at recurrence, the overall survival curves according to MGMT promoter methylation status were separated only beyond the median. Given our small sample size, such a small difference in survival would be difficult to demonstrate statistically. To date, no molecular marker has demonstrated prog-

Table 3 – Analysis of survival according to baseline variables

Baseline variables		Overall survival from TMZ at recurrence		Overall survival from diagnosis of HGG	
	TTP (n = 38)	All cause mortality (n = 38)	Disease-specific mortality (n = 38)	All patients (n = 50)	Patients studied for TTP (n = 38)
WHO tumour grade (AA/AOA versus glioblastoma)	0.001	0.001	0.001	<0.001	<0.001
MGMT (meth versus unmeth)	0.026	0.086	0.059	0.088	0.063
TMZ regimen (5/28 versus 21/28d)	0.034	0.026	0.032	0.001	0.002
Sex (M versus F)	0.491	0.552	0.424	0.558	0.969
Localisation (frontal versus other)	0.326	0.863	0.701	0.722	0.667
Prior LGG (Y versus N)	0.139	0.044	0.043	0.015	0.041
Surgery for HGG (complete versus partial)	0.434	0.619	0.430	0.548	0.775
KPS (100% versus 90–60%)	0.903	0.549	0.575	0.500	0.781
Age (>50 year versus <50 year at TMZ)	0.020	0.015	0.024	0.009	0.036
Surgery at relapse (Y versus N)	0.333	0.404	0.420	0.068	0.052
First- versus second-line TMZ	_	_	_	0.694	_

Note: p values calculated using a 2-sided log rank test on Kaplan–Meier survival estimates. Patients who were not treated with radiation therapy at diagnosis of HGG were omitted.
TMZ = temozolomide; HGG = high-grade glioma; TTP = time to progression; AA = anaplastic astrocytoma; AOA = anaplastic oligoastrocytoma; MGMT = O^6-methylguanine-DNA-methyltransferase; LGG = low-grade glioma; KPS = Karnofsky performance score.

nostic value in recurrent glioblastoma. Prognosis at the first recurrence seems to be determined more so by clinical features such as age, Karnofsky Performance Score and corticosteroid use.[32,33] Therefore, the identification of a small but real difference in overall survival based on MGMT promoter methylation is unlikely in our small population of glioblastoma patients with a median survival of only 5 months (95% CI: 3.5–6.5) from the recurrence. In contrast, the survival of patients with AA or AOA is much more heterogeneous (median, 11 months; 95% CI: 0–22.7 from recurrence); hence the potential of a true predictive factor to discriminate patients with significantly different survival outcomes is greater.

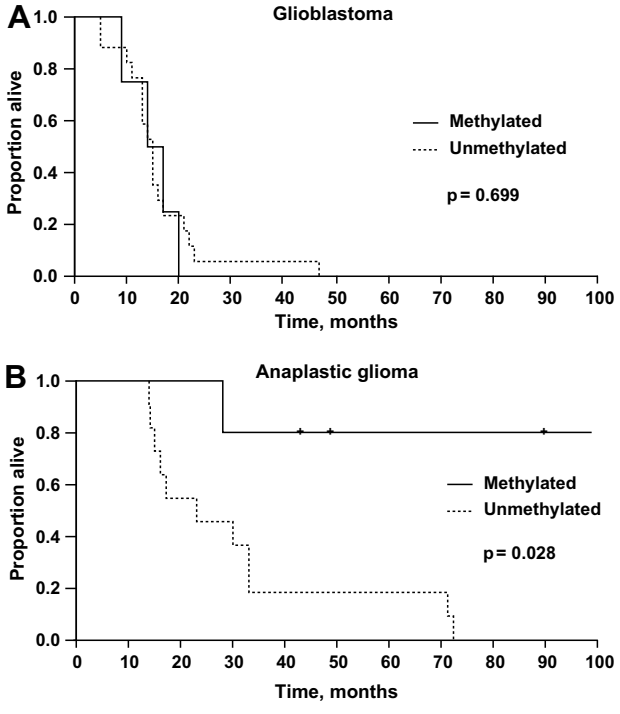

Fig. 3 – Kaplan–Meier estimate of overall survival from diagnosis of high-grade glioma based on O^6-methylguanine-DNA-methyltransferase promoter methylation status and by tumour histology (glioblastoma, n = 32 [panel A]; anaplastic astrocytoma/anaplastic oligoastrocytoma, n = 18; [panel B]).

The results of this present study are consistent with the hypothesis that MGMT promoter methylation determines the sensitivity of HGG to alkylating agents. We found no correlation between MGMT methylation status and time from initial diagnosis of HGG to the first recurrence (data not shown), during which time none of the patients included in this analysis were treated with TMZ. Similarly, MGMT promoter methylation did not influence TTP from diagnosis in glioblastoma patients treated with RT only in the EORTC/NCIC trial.[11]

The observation that MGMT promoter methylation status was not correlated with response or survival in the small subgroup of 1p/19q intact AA/AOA patients who were treated with the 21/28-d extended dosing schedule suggests that resistance in unmethylated gliomas might be overcome. This dose-dense regimen delivers a 2-fold higher dose per cycle, and has been shown to deplete MGMT in peripheral blood monocytes.[22] However, numerically the outcome of MGMT methylated patients was superior in our series. This was also the case in 2 other correlative studies in which patients with recurrent glioblastoma were treated with either a 7 of 14 d or the 21/28-d regimen (Table 4).[23,25] Therefore, there are as yet limited data to suggest that resistance to TMZ can be overcome by depletion of MGMT in tumour tissue using a dose-dense regimen.

Finally, this present study highlights an important limitation with regard to the clinical implementation of the QMSP assay based on archival tumour tissue. Analysis of approximately one third of the tumour blocks failed to provide useful data because of insufficient material, extensive tumour necrosis, fixation under conditions that adversely affected

Table 4 – Correlative studies between MGMT promoter methylation and survival following dose-dense TMZ in recurrent HGG

	TMZ regimen	Histopathology	Methylation status	N	Median PFS, weeks	Log-rank *p* value
This study	21/28-d 100 mg/m^2/d	AA/AOA	M	4	36	$p = 0.52$
			UM	7	20	
Wick et al.[25]	7/14-d 150 mg/m^2/d	Glioblastoma	M	17	27	$p = 0.22$
			UM	19	19	
Brandes et al.[23]	21/28-d 75 mg/m^2/d	Glioblastoma	M	10	16	$p = 0.86$
			UM	12	12	

MGMT = O^6-methylguanine-DNA-methyltransferase; TMZ = temozolomide; HGG = high-grade glioma; AA = anaplastic astrocytoma; AOA = anaplastic oligoastrocytoma; PFS = progression-free survival; M = methylated; UM = unmethylated.

the quality of the DNA or because the assay result was indeterminant (i.e. the ratio was in the grey zone). Prospective freezing of tumour tissue at surgery could greatly improve the success of DNA extraction and MGMT promoter methylation testing. The ongoing CATNON and RTOG0525/EORTC26052-22053 prospective phase III studies that incorporate MGMT promoter methylation testing for stratification purposes and that investigate dose-dense regimens will provide important answers regarding the potential of MGMT promoter methylation testing as a useful clinical tool to predict benefit from TMZ treatment at diagnosis. Use of MGMT promoter methylation status as a stratification factor in future clinical trials involving alkylating agents for the treatment of patients with recurrent glioma seems appropriate.

Conflict of interest statement

J. Menten and B. Neyns have served as advisors to Schering-Plough, and B. Neyns has received honoraria or speaker fees from Schering-Plough in the amount of <$5000 over the past 3 years. No other authors have any disclosures.

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