Prognostic value of *MGMT* promoter methylation in glioblastoma patients treated with temozolomide-based chemoradiation: A Portuguese multicentre study

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Abstract. Glioblastoma (GBM) is the most common and aggressive primary brain tumor. The identification of novel molecular prognostic markers of GBM has recently been an area of great interest in neuro-oncology. The methylation status of the *MGMT* gene promoter is currently a promising molecular prognostic marker, but some controversial data have precluded its clinical use. We analyzed *MGMT* methylation by methylation-specific PCR in 90 GBM patients from four Portuguese hospitals, uniformly treated with radiotherapy combined with concomitant and adjuvant temozolomide (Stupp protocol). The Kaplan-Meier method was used to construct survival curves, and the log-rank test and a Cox-regression model were used to analyze patient survival. The methylation status of *MGMT* was successfully determined in 89% (80/90) of the tumors. The frequency of tumoral *MGMT* promoter methylation was 47.5%. The median overall survivals (OSs) were 16 months (95% CI 12.2-19.8) and 13 months (95% CI 13.3-18.7) for patients whose tumors had a methylated or unmethylated *MGMT*, respectively. Univariate and multivariate analyses did not show any statistically significant association between *MGMT* methylation status and patient OS (P=0.583 by the log-rank test; P=0.617 by the Cox-regression test) or progression-free survival (P=0.775 by the log-rank test; P=0.691 by the Cox-regression test). None of the patient clinical features were significantly correlated with survival. This is the first study to report the frequency of *MGMT* methylation among Portuguese GBM patients. Our data did not show statistically significant associations between *MGMT* promoter methylation and the outcome of GBM patients treated with temozolomide. Additional robust prospective studies are warranted to clarify whether the *MGMT* status should be used in clinical decisions.

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Abbreviations: GBM, glioblastoma multiforme; TMZ, temozolomide; OS, overall survival; PFS, progression-free survival; KPS, Karnofsky performance status; EORTC, European Organisation for Research and Treatment of Cancer; NCIC, National Cancer Institute of Canada

Key words: glioblastoma, prognosis, *MGMT* methylation, temozolomide, chemoradiation

Introduction

Tumors of the central nervous system account for a small percentage of all types of human tumors (1), but encompass a wide variety of distinct disease entities (2). Data from the GLOBOCAN 2002 database report an incidence of brain and central nervous system tumors in Portugal of ~422 cases per year in males and ~362 cases per year in females (3). Gliomas are the most common primary brain tumors (2,4) and encompass a wide variety of tumors thought to originate either from stem cells, glial precursor cells or glial differentiated cells. Astrocytomas are the major histological subtype, accounting for ~75% of all gliomas (4), of which GBM

(WHO grade IV) is by far the most common and malignant. The highly infiltrative nature coupled with the high proliferative potential causes this tumor to be particularly devastating, for which no curative therapies are currently available (5,6). Two main subtypes of GBM can be distinguished based on their clinical presentation: the most common subtype, primary (or *de novo*) GBM, develops without the presence of any precursor neoplastic lesion and manifests after a short clinical history (usually less than 3 months); secondary GBMs are much rarer and develop from lower grade tumors (2,7).

Despite recent improvements in therapeutic approaches, treatment still remains mostly palliative, and GBM patients usually present an extremely poor prognosis. The median survival is typically reported to be ~12 months in clinical trials (2), but a large population-based study in 2004 reported a median survival for these patients of ~5 months, with only ~18% and ~3% of the patients surviving over 1 and 2 years, respectively (8). The current paradigm of therapy for patients with newly diagnosed GBM includes surgical resection (when feasible), radiotherapy and chemotherapy. A phase III trial by Stupp *et al* clearly showed that GBM patients treated with radiotherapy combined with concomitant and adjuvant temozolomide (TMZ, an orally administered alkylating agent), also known as TMZ-based chemoradiation, had an increased overall survival (OS) compared with patients treated with radiotherapy alone (9), establishing a new standard in the management of these tumors. Despite a significant improvement, it is important to note that this treatment resulted in a small difference in overall survival. In order to improve the clinical outcome of these patients, it is widely acknowledged that the ideal treatment of GBMs must be individualized, based on the particular features of the tumor. Indeed, the prognosis of these patients is quite variable and unpredictable. Some of the most well-established prognostic markers in GBM include patient clinical features, such as age, Karnofsky performance status (KPS) (10) and extent of tumor resection; however, these markers do not satisfactorily predict patient outcome (11). One of the major goals of current neuro-oncology research is to identify robust and clinically relevant molecular markers that can add value to those more classic clinical prognostic factors.

MGMT (O-6-methylguanine-DNA methyltransferase), a gene located on chromosome 10q26, encodes a DNA-repair enzyme that has been shown to contribute to the chemoresistance of GBM cells to alkylating agents (12). Specifically, a landmark study by Hegi *et al* showed that GBM patients whose tumors had a methylated *MGMT* promoter presented a significantly longer median OS (21.7 months) and 2-year survival rate (46%) when treated with TMZ-based chemoradiation compared with patients without *MGMT* promoter methylation who were treated similarly (median survival of 12.7 months and 2-year survival rate of 13.8%) (13). Additionally, in the data set of Hegi *et al*, *MGMT* promoter methylation was an independent favorable prognostic factor, irrespective of treatment. Due to its potential prognostic value, the assessment of *MGMT* methylation status is currently a common practice in clinical trials involving GBM patients (14). Despite these striking findings, there is a significant body of controversial data surrounding the reproducibility of these results, questioning the true implication of *MGMT* as a GBM prognostic marker and/or a specific predictor of TMZ-based chemotherapy (15-17). In this context, we aimed to assess the frequency and clarify the prognostic capacity of *MGMT* promoter methylation in a set of Portuguese GBM patients uniformly treated with TMZ-based chemoradiation.

Table I. Clinicopathological features of glioblastoma patients treated with temozolomide-based chemoradiation (n=90).

Male/female ratio	1.9
Age, years (median ± SD)	56±11
Karnofsky performance score	
≥80 (n)	48
<80 (n)	42
Extent of resection	
Total or subtotal (n)	81
Biopsy (n)	9

n, number of patients.

Materials and methods

Human tumor samples. Human tumor samples were obtained from primary GBM patients newly diagnosed according to the WHO criteria (2) and surgically resected between 2004 and 2007 at 4 hospitals in northern Portugal: Hospital São João (n=36), Hospital Pedro Hispano (n=33), Hospital Santo António (n=11) and Hospital São Marcos (n=10). All patients underwent radiotherapy plus continuous concomitant TMZ after surgery, followed by maintenance cycles of TMZ, according to the Stupp protocol (9). Extension of tumor resection was assessed by the neurosurgeon and by post-operative magnetic resonance imaging (MRI) and were classified as gross resection (total or subtotal) or biopsy. The clinicopathological features are summarized in Table I. All procedures followed in this study were in accordance with institutional ethical standards, and the biological samples were unlinked and unidentified from their donors. Follow-up data were available for all patients as of July 2008, and were collected through direct interview with patients or their relatives and by review of in-hospital patient files.

DNA isolation. DNA was isolated by macrodissection from 10-μm sections of formalin-fixed paraffin-embedded tumor tissue samples, avoiding the harvesting of surrounding normal brain tissue by comparing each slide with the corresponding hematoxylin and eosin (H&E)-stained slide (marked for the area of tumoral tissue) (18). The recovered tissues were processed with the QIAamp® DNA Micro Kit (Qiagen) following the manufacturer's instructions. DNA quality control and yield were assessed by spectrophotometry using Nanodrop™ .

DNA bisulfite treatment and MGMT methylation-specific PCR analysis. Genomic DNA was subjected to bisulfite treatment using the EZ DNA Methylation-Gold™ Kit (Zymo

Table II. Associations between clinical features or status of tumoral *MGMT* promoter methylation and prognosis of GBM patients, assessed by univariate (log-rank test) and multivariate (Cox-regression) analyses.

	Overall survival			Progression-free survival		
	Median (95% CI)[a]	P-value (Log-rank)	P-value (Cox)	Median (95% CI)[a]	P-value (Log-rank)	P-value (Cox)
Gender						
Males	14 (10.6-17.4)			9 (6.9-11.1)		
Females	14 (11.6-16.4)	0.436	0.807	9 (5.9-12.1)	0.864	0.990
Age[b]						
>56	13 (9.5-16.5)			9 (6.9-11.1)		
≤56	14 (11.0-17.0)	0.978	0.951	10 (8.0-12.0)	0.754	0.431
KPS						
≥80	16 (14.2-17.8)			10 (7.2-12.8)		
<80	12 (9.8-14.2)	0.165	0.311	8 (5.7-10.3)	0.096	0.125
Extent of resection						
Total or subtotal	13 (10.4-15.6)			9 (7.5-10.5)		
Biopsy	16 (13.3-18.7)	0.458	0.481	6 (1.1-10.9)	0.942	0.930
MGMT status						
Methylated	16 (12.2-19.8)			9 (5.0-13.0)		
Unmethylated	13 (11.1-14.9)	0.583	0.617	10 (8.1-12.0)	0.775	0.691

[a]Median survival and 95% confidence intervals, in months. [b]Patient age was used as a continuous variable for the Cox-regression model. KPS, Karnofsky performance status.

Research) following the manufacturer's instructions. The promoter *MGMT* methylation-specific polymerase chain reactions (MSP) were performed using a two-step nested approach, and the results were confirmed by one-step MSP in a subset of tumors as previously described (13,19). Peripheral blood DNA from tumor-free controls and CpGenome™ Universal Methylated DNA (Chemicon International) were used as *MGMT* unmethylated and methylated controls, respectively. The PCR products were resolved on 4% low-melting point agarose gels. Analysis of MSP data was performed by investigators who were blind to the clinical data.

Statistical analyses. The Kaplan-Meier method was used to estimate OS and progression-free survival (PFS), and the log-rank test was used to assess the differences. OS was measured from the time of surgical resection to patient death, or the last date when the patient was known to be alive. PFS time was defined as the time from surgical resection to the time of demonstrated tumor growth on follow-up imaging, or evidence of neurological decline. Multivariate survival analyses by use of Cox proportional hazards models (backward selection) were performed to adjust for the effects of potential confounding factors, including patient age (used as a continuous variable), gender, KPS and extent of tumor resection. All statistical tests were two-sided, and significance was considered at values of $P<0.05$. Data analysis was performed using SPSS 16.0 software (SPSS, Inc.).

Results

Patient clinical features which may have an effect on prognosis, such as gender, age at diagnosis, KPS and extent of tumor resection, are summarized in Table I. The tumoral *MGMT* methylation status was successfully determined by MSP in 80 tumor samples (89%), of which 38 GBMs (47.5%) had a methylated *MGMT* promoter. Fig. 1 presents a typical MSP analysis of the methylation status of the *MGMT* promoter.

Considering the whole tumor set, independently of clinical features or *MGMT* methylation status, the median OS and PFS were 14 months (95% CI 11.5-16.5) and 9 months (95% CI 7.5-10.5), respectively. The overall 2-year survival rate was 16.7%. Table II summarizes the median OS and median PFS of patients based on clinicopathological features (gender, age, KPS and extent of tumor resection) and *MGMT* methylation status. By using the log-rank test, no statistically significant associations were detected between each individual variable and GBM patient OS or PFS ($P>0.05$) (Table II). Similarly, a multivariate Cox proportional model did not show any statistically significant correlation between the studied variables and GBM patient outcome ($P>0.05$) (Table II). Nevertheless, patients whose tumors had a methylated *MGMT* promoter showed a slightly improved median OS of 16 months (95% CI 12.2-19.8) as compared to patients with an unmethylated *MGMT* promoter (13 months, 95% CI 11.1-14.9; Fig. 2), but the differences were not statistically significant ($P=0.583$ by the univariate test; $P=0.617$ by the multivariate

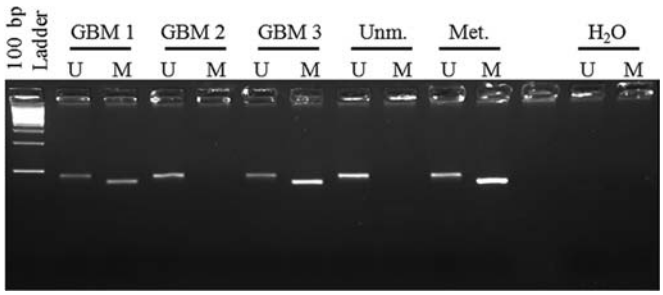

Figure 1. Methylation-specific PCR (MSP) analyses of the *MGMT* promoter in glioblastoma tumor tissue from three patients (GBM 1, GBM 2 and GBM 3). MSP control reactions consisted of blood-extracted DNA from a cancer-free individual for use as the umethylated DNA control (Unm.), and a CpGenome Universal Methylated DNA as the methylated DNA control (Met.). Note the presence of bands in both the unmethylated (U, 93 bp) and methylated (M, 81 bp) lanes for glioblastoma samples 1 and 3, reflecting a methylated *MGMT* promoter. The lack of a band in the lane corresponding to methylation-specific primers for glioblastoma sample 2 reflects the absence of *MGMT* promoter methylation. PCR reactions in the absence of DNA (H_2O) were performed as negative controls for both the unmethylated and methylated reactions.

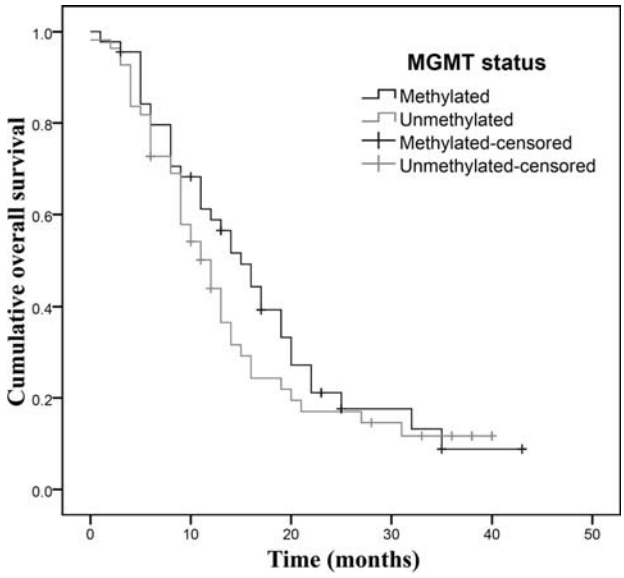

Figure 2. Kaplan-Meier analyses of the influence of *MGMT* promoter methylation on overall survival of glioblastoma patients treated with temozolomide-based chemoradiation. Patients whose tumors present a methylated *MGMT* promoter (black curve) show a trend of improved overall survival than patients whose tumors have an unmethylated *MGMT* promoter (grey curve), but the differences did not reach statistical significance (P=0.583 by the log-rank test; see text for details).

model; Table II). The median PFS was closely similar between patients whose tumors had methylated (9 months, 95% CI 5.0-13.0) and unmethylated (10 months, 95% CI 8.1-12.0) *MGMT* promoter (P=0.775 by the log-rank test; P=0.691 by the Cox model; Table II). The 2-year survival rate was 15.8% and 16.7% in patients with methylated and unmethylated *MGMT*, respectively (P=0.915).

Discussion

GBM is a particularly devastating disease as no curative therapies are available, and very few well-established prognostic factors have been identified. Many recent efforts in the field of neuro-oncology are directed to developing more efficient therapies, but only a small fraction of patients experience significant clinical benefit and prolonged survival. Despite the variability of the clinical responses, the majority of patients with GBM are presently treated in a uniform standardized way, following a 'one fits all' therapeutic approach, regardless of the individual molecular characteristics of each tumor that most likely affect patient prognosis. Consequently, many patients display minor responses and major therapy-related toxicities.

The recent introduction of radiotherapy plus concomitant and adjuvant TMZ treatment for GBM has led to a small but significant improvement in patient outcome (9); however, the responses are still very poor and unpredictable. While the EORTC-NCIC trial by Hegi *et al* (13) implicated the status of *MGMT* promoter methylation as a biomarker of GBM patient response to TMZ, all GBM patients continue to be treated with TMZ, regardless of their *MGMT* status (13). To note, the conclusions of Hegi *et al* were somewhat confounded and questioned due to several limitations of the study. First, the studies were performed retrospectively on a subset of patients from whom adequate tumor tissue was available. Second, although the difference in survival based on *MGMT* methylation status was highly significant, more than half of the methylated *MGMT* patients (with a predictably favorable outcome) did not survive for 2 years, while ~15% of the patients with unfavorable unmethylated *MGMT* did survive over 2 years. Third, *MGMT* methylation was associated with improved outcomes both in the temozolomide-based chemoradiation and radiation-only arms, suggesting that it may partly be a prognostic marker associated with the nature of the tumor, rather than a truly therapy-specific predictive marker. Lastly, many of the patients in the radiation-only arm also received TMZ at recurrence. Regardless, this was a landmark study suggesting that methylation of *MGMT* is both a prognostic marker and a specific marker of response to TMZ in GBM patients. Indeed, the conclusions of Hegi *et al* were recently corroborated by a 5-year analysis of the EORTC-NCIC trial, where Stupp *et al* (20) confirmed that the methylation of the *MGMT* promoter is associated with better outcome and benefit from TMZ chemotherapy. The first study implicating a correlation between *MGMT* levels and survival of malignant glioma patients dates back to 1998, when Jaeckle *et al* showed that patients treated with BCNU (a nitrosourea used as a chemotherapeutic agent) whose tumors had low or undetectable levels of MGMT had an improved survival as compared to those whose tumors had detectable MGMT activity (21). Soon after, Esteller *et al* reported for the first time that methylation of the *MGMT* gene promoter led to loss of MGMT expression in human tumors *in vivo* (22). In 2000, Esteller *et al* found that a methylated *MGMT* promoter in malignant glioma was associated with improved overall and PFS, independently of patient age and KPS (12). Mechanistically, the ability of functional MGMT protein to remove the cytotoxic chemotherapy-induced alkyl groups from the O^6-guanine of DNA, and thus prevent killing of tumor cells, supported the clinical

Table III. Prognostic significance of *MGMT* methylation in glioblastoma reported in the literature.

Author (Reference)	Year	n	Treatment	Effect of *MGMT* methylation on survival[a]	
				PFS	OS
Esteller, *et al* (12)	2000	29	BCNU	Yes	Yes
Balana, *et al* (16)	2003	21	TMZ + cisplatin (n=7)	NS	ND
			BCNU (n=7)	Yes	ND
			No treatment (n=3)	ND	ND
Paz, *et al* (26)	2004	51	BCNU or CCNU or TMZ	NS	NS
Hegi, *et al* (27)	2004	38	Concomitant + adjuvant TMZ	ND	Yes
Blanc, *et al* (28)	2004	44	BCNU or fotemustine (n=2); radiation-only (n=14);		
			Radiation + chemotherapy (BCNU or fomustine) (n=22);		
			No treatment (n=6)	NS	NS
Kamiryo, *et al* (17)	2004	74	ACNU	NS	NS
Watanabe, *et al* (29)	2004	29	IAR regimen	Yes	Yes
Hegi, *et al*[b] (13)	2005	100	Radiotherapy only	ND	Yes
		106	Concomitant + adjuvant TMZ	Yes	Yes
Brandes, *et al* (42)	2006	22 (recurrent GBMs)	TMZ after recurrence	NS	NS
Herrlinger, *et al* (31)	2006	19	CCNU + TMZ	Yes	Yes
Piccirilli, *et al* (41)	2006	22 (≥80 years)	Chemotherapy not specified	ND	Yes
Wick, *et al* (30)	2007	36	Alternating weekly TMZ	NS	NS
Eoli, *et al* (33)	2007	86	1st line cisplatin + BCNU;		
			PCV or TMZ after recurrence	Yes	Yes
Criniere, *et al* (32)	2007	39	Concomitant + adjuvant nitrosureas	ND	Yes
		38	Adjuvant nitrosureas	ND	NS
		85	Radiotherapy only	ND	NS
Donson, *et al* (40)	2007	10 (pediatric GBM)	TMZ (n=7) or etoposide (n=3)	Yes	Yes
Brandes, *et al* (45)	2008	103	Concomitant + adjuvant TMZ	Yes	Yes
Smith, *et al* (48)	2008	27	Gliadel wafers	ND	Yes
Murat, *et al* (39)	2008	42	Concomitant + adjuvant TMZ	Yes	Yes
Sijben, *et al* (50)	2008	29 (≥65 years)	Concomitant + adjuvant TMZ	NS	NS
Gorlia, *et al*[b] (35)	2008	106	Concomitant + adjuvant TMZ	ND	Yes
Dunn, *et al* (44)	2009	109[c]	Concomitant + adjuvant TMZ	Yes	Yes
Brandes, *et al* (23)	2009	37 (≥65 years)	Concomitant + adjuvant TMZ	Yes	Yes
Clarke, *et al* (46)	2009	48	TMZ (metronomic versus dose-dense)	NS	NS
Wemmert, *et al* (36)	2009	27	TMZ after recurrence	ND	NS
Stupp, *et al*[b] (20)	2009	100	Radiotherapy only	ND	Yes
		106	Concomitant + adjuvant TMZ	Yes	Yes
Glas, *et al* (38)	2009	23	CCNU + TMZ	Yes	Yes
Park, *et al* (47)	2009	48	ACNU + cisplatin	ND	NS
Prados, *et al* (37)	2009	44	Erlotinib + TMZ	ND	Yes
Sadones, *et al* (49)	2009	22	TMZ after recurrence	NS	NS
Schaich, *et al* (43)	2009	61	Concomitant + adjuvant TMZ	NS	NS
Sonoda, *et al* (25)	2009	30[d]	ACNU	Yes	Yes
Mellai, *et al* (24)	2009	101	Not specified	ND	Yes/No[e]
Metellus, *et al* (51)	2009	19 (recurrent GBMs)	Carmustine wafers	Yes	Yes
Grossman, *et al* (52)	2009	29	Concomitant + adjuvant TMZ and talampanel	Yes	Yes
Martinez, *et al* (53)	2009	46[f]	TMZ	ND	NS
Piperi, *et al* (54)	2009	17	Not specified	ND	Yes[g]
Weller, *et al* (55)	2009	63	Radiotherapy-only	NS	NS
		183	Concomitant + adjuvant TMZ	Yes	Yes

n, number of patients; PFS, progression-free survival; OS, overall survival; NS, not significant; ND, not determined; ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride (nimustine); BCNU, 1,3-bis-(chloro-ethyl)-1-nitrosourea (carmustine, Gliadel); CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); IAR, IFN-ß, ACNU and radiotherapy; PCV, procarbazine, lomustine and vincristine; TMZ, temozolomide. [a]Only statistically significant results ($P<0.05$) are considered. [b]These studies analyzed the same tumor set from the EORTC-NCIC trial. [c]*MGMT* promoter was considered methylated by pyrosequencing when ≥9% of CpG dinucleotides were methylated. [d]The set of 30 GBM patients in this study compared long-term and short-term survivors only. [e]Statistically significant by univariate analysis, but not significant by multivariate analysis. [f]*MGMT* methylation status was determined by a microarray-based DNA methylation analysis. [g]Patients with a methylated *MGMT* promoter had a significantly worse prognosis than unmethylated cases.

finding that lack of MGMT expression was associated with improved outcomes. Whether this molecular marker should ultimately direct the treatment of newly diagnosed patients is still a fundamental and controversial question. Much of the controversy surrounding the prognostic value of *MGMT* is partly due to studies including very heterogeneous groups of patients with different glioma subtypes and who underwent different treatment regimens. In addition, not all studies analyzed the *MGMT* status at the level of DNA methylation in the promoter region by methylation-specific polymerase chain reaction (MSP); some studies focused on mRNA expression by RT-PCR and protein levels by immunohistochemistry and quantitative immunofluorescence (15). Nevertheless, contradictory results are also reported even in studies that evaluated the influence of *MGMT* promoter methylation on the prognosis of patients with GBM (Table III) (12,13,16,17,20,23-55).

In our study, we attempted to clarify whether *MGMT* methylation is a biomarker of clinical outcome in GBM patients treated with the recently introduced TMZ-based chemoradiation protocol and how it compares to the prognostic value of classic clinicopathological factors. By studying a set of 90 GBM patients from northern Portugal, we were unable to detect any significant correlations between patient clinical features, *MGMT* methylation status and prognosis. Other studies have also failed to show such correlations (Table III). To note, the frequency of *MGMT* promoter methylation in our data set is similar to the previously reported frequencies (13,23,30,33,35,55). Additionally, the median OS and median PFS in our set of GBM patients are in line with other recently published studies (20,23,55). Some possible limitations of our study include the relatively small sample size, which may be a limiting factor in achieving statistical significance, and the fact that we analyzed a multicenter tumor set collected from 4 independent institutions. Nevertheless, our sample size compares favorably with other similar studies published in the literature (Table III); additionally, our population-based study is likely to closely illustrate the difficulties in identifying significant determinants of patient survival, as this type of study is typically less controlled and more heterogeneous than well-designed prospective clinical trials. Obviously, the argument that *MGMT* methylation does not predict GBM patient response to this therapeutic regimen is still equally valid. It is also relevant to stress that the most appropriate method to assess *MGMT* status in gliomas is quite controversial. Validated and commercially available *MGMT* methylation assays have not yet been approved for clinical use and may indeed be technically challenging. In spite of a recent report arguing in favor of the feasibility and reliability of nested, gel-based methylation-specific PCR (MSP) analysis, suggesting it could be routinely implemented in the clinical setting (56), the use of MSP is often considered not to be so straightforward (57-60). Its use has raised some concerns due to inter- and intra-test variability and sensitivity and specificity issues (57,59). One recent report argues that a quantitative MSP test for *MGMT* methylation is more specific than conventional gel-based MSP (61). Furthermore, *MGMT* silencing can occur through methylation of specific cytosines within the CpG island (62); thus, it is important to determine which CpG dinucleotides are the most predictive of potential *MGMT* silencing. A recent study using pyrosequencing methylation analysis argues that a new set of CpG dinucleotides in the *MGMT* promoter CpG island is more robust in predicting gene silencing than those classically tested by the MSP assay (63). Conceptually, while a methylated *MGMT* promoter precludes gene expression, the absence of such methylation does not necessarily translate into activation of gene expression, as specific transcription factors [e.g., Sp1 (64) and TP53 (65)], permissive chromatin states (64,66-69), and absence of negative transcriptional regulators (e.g., IFN-ß) are also required. Additionally, it is reasonable to assume that, given the heterogeneity of *MGMT* expression within individual tumors, a fraction of the cells within the entire tumor load may lack *MGMT* expression, despite an unmethylated *MGMT* status, and may therefore be sensitive to temozolomide. A recent study also suggests that variation in *MGMT* promoter methylation can occur within the same tumor after treatment (70), stressing that clinical decisions based on *MGMT* require caution. Despite these considerations as well as the consensual belief that additional molecular markers of GBM patient outcome are important in determining the tumor response to therapy, if the relevance of *MGMT* promoter methylation status in GBM is corroborated by an ongoing phase III large study by the Radiation Therapy Oncology Group (RTOG 0525), patients with unmethylated *MGMT* promoter may be selected for alternative treatment options in the future. Potential alternative strategies to overcome MGMT-mediated chemoresistance include: (i) use of temozolomide together with MGMT-inactivating drugs (71,72) such as O^6-benzylguanine (73,74) or inhibitors of other DNA repair enzymes; and (ii) use of dose-intensive temozolomide regimens (71) which deplete MGMT levels more rapidly than lower doses (75).

In conclusion, our study is the first to report the frequency of tumoral *MGMT* promoter methylation among Portuguese GBM patients and to analyze the correlations between clinical features, *MGMT* status and outcome in a set of patients uniformly treated with concomitant and adjuvant TMZ chemoradiation. In light of our data, together with the controversies reported in the literature, further studies are warranted to clarify the clinical prognostic relevance of *MGMT* methylation in GBM.

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