# Low Leukocyte MGMT Accompanies Temozolomide-Induced Myelotoxicity in Brain Tumor Patients

Julia E. Stokes<sup>#</sup>, Michael S. Bobola<sup>#</sup>, Marc C. Chamberlain<sup>#,\*</sup> and John R. Silber<sup>#</sup>

Departments of Neurological Surgery (JES, MSB, MCC, JRS), and Neurology (MCC), University of Washington, USA

**Abstract:** Objective: The methylating agent temozolomide (TMZ) has markedly improved clinical outcome for patients with glioblastoma and other gliomas. While TMZ has comparatively low systemic toxicity, a minority of patients experience severe myelotoxicity that compromises TMZ treatment, necessitating dose reductions and treatment delays. These limitations emphasize the need to develop markers to identify individuals susceptible to TMZ-induced myelosuppression. The purpose of this small pilot study is to examine the association between treatment-limiting myelosuppression in primary brain tumor patients receiving TMZ and expression of O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) in peripheral blood leukocytes (PBL). MGMT is the sole human activity that removes TMZ-induced, cytotoxic O<sup>6</sup>-methylguanine adducts from DNA.

*Methods*: MGMT biochemical activity and *MGMT* promoter methylation status, a surrogate measure of MGMT expression, were assayed in PBL from 10 patients who experienced treatment-limiting myelotoxicity during TMZ therapy, 8 patients who experienced no myelotoxicity during TMZ treatment, and 10 disease-free, untreated controls.

Results: MGMT activity was detectable in all 28 PBL samples, and all displayed an unmethylated promoter indicative of MGMT expression. Mean PBL MGMT activity was 2-fold lower in patients who experienced myelotoxicity compared to patients without myelotoxicity (8.9  $\pm$  3.9 vs. 18  $\pm$  8.1 fmol/10<sup>6</sup> cells;  $P \le 0.015$ ) and to untreated controls (8.9  $\pm$  3.9 vs. 16  $\pm$  6.8 fmol/10<sup>6</sup> cells;  $P \le 0.015$ ).

Conclusions: These preliminary data indicate that low MGMT activity in PBL is associated with myelotoxicity in primary brain tumor patients receiving TMZ, and may have value if confirmed in a larger study as a marker to identify patients at greater risk of treatment-limiting myelosuppression.

**Keywords:** Primary brain tumor, temozolomide (TMZ), glioblastoma, myelosuppression, thrombocytopenia, O6-methylguanine-DNA methyltransferase, promoter methylation, MGMT enzymatic activity, peripheral blood leukocytes, biomarkers.

## INTRODUCTION

Inclusion of temozolomide (TMZ) in post-surgical therapy has produced clinically relevant improvement in survival for patients with glioblastoma [1]. In part this benefit derives from the low cumulative toxicity associated with TMZ, as contrasted with other alkylating chemotherapies. However, a small fraction of (approximately 7%) develop patients myelosuppression (defined as clinically relevant > grade III cytopenias), which limits or halts treatment [2,3]. Even when TMZ is discontinued, persistence of cytopenia can delay initiation of alternative cytotoxic therapies compromising further treatment. Such adverse consequences highlight the need for a clinically tractable biomarker of susceptibility to TMZinduced myelotoxicity.

The for molecular search markers myelosensitivity has focused primarily on the repair O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT), the sole human activity that removes TMZinduced cytotoxic O<sup>6</sup>-methylguanine adducts from DNA [4]. A role for MGMT in myelosensitivity to TMZ is suggested by reports that activity in peripheral blood mononuclear cells varies widely in normal individuals [5], and that low levels of MGMT in peripheral blood mononuclear cells more frequently accompanied higher-grade leukopenia and thrombocytopenia in melanoma patients treated with TMZ or its analog dacarbazine [6]. In this preliminary work, we assayed MGMT activity in peripheral blood leukocytes (PBL) from primary brain tumor patients treated with TMZ. We found significantly lower MGMT accompanies treatment-limiting myelosuppression in TMZ-treated patients. Our results indicate that TMZinduced myelotoxicity in primary brain tumor patients reflects, at least in part, a reduced capacity to remove O<sup>6</sup>-methylguanine adducts from DNA, and suggest that PBL MGMT activity may serve as a marker for TMZ myelosensitivity in this patient population.

<sup>\*</sup>Address corresponding to this author at the Division of Neuro-Oncology, 825 Eastlake Ave E, PO Box 19023, MS G-4940, Seattle, WA 98109-1023, USA; Tel: 206-288-8280; Fax: 206-288-2000; E-mail: chambemc@uw.edu

<sup>&</sup>lt;sup>#</sup>Julia E. Stokes, data acquisition, data analysis, manuscript preparation.

<sup>\*</sup>Michael S. Bobola, data acquisition, data analysis.

<sup>#</sup>Marc C. Chamberlain, study design, manuscript preparation, data interpretation.

<sup>&</sup>lt;sup>#</sup>John R. Silber, study design, funding, manuscript preparation, study supervision.

#### **MATERIALS AND METHODS**

## **Patient Population and Leukocyte Isolation**

Blood was obtained, with informed consent, from 18 patients with a diagnosis of primary brain tumor who had been treated with TMZ at the University of Washington Medical Center, and from 10 disease-free controls. Thirty mL of blood was collected in 1:1000 sodium heparin and held for no longer than one hour at room temperature prior to isolation of PBL by gradient centrifugation using Histopague (Sigma). Washed cell pellets were flash-frozen and stored as duplicate aliquots at -70 °C. The average interval between the last day of TMZ ingestion and blood draw was 305 ± days (range, 27-849 days) for patients experiencing myelotoxicity and 164 ± 184 days (range, 21-528 patients experiencing days) for myelotoxicity.

## **Extract Preparation and DNA Isolation**

Whole cell extracts were prepared from one PBL aliquot by lysis on ice for 40 minutes in 25 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 10% glycerol and 0.1% Nonidet P40, followed by centrifugation at 10,000 x g for 30 min to pellet insoluble debris. DNA was isolated from a second PBL aliquot by solubilization in sodium dodecylsulfate followed by serial salt and alcohol precipitation using commercially available reagents (PureGene, Gentra Systems).

## **MGMT Assay and CpG Methylation Status**

MGMT activity (fmol  $O^6$ -[ $^3$ H]methylguanine transferred/ $10^6$  cells) was measured by standard biochemical assay that quantifies transfer of [ $^3$ H]methylguanine in DNA to protein [7]. The methylation status of 9 informative CpG residues in the *MGMT* promoter was determined by

Table 1: PBL MGMT, Treatment and Myelotoxicity

Age/Sex	Dx <sup>1</sup>	MGMT <sup>2</sup>	TMZ therapy <sup>3</sup>	Toxicity⁴
Patients with myelosuppression				
39/M	AOA	$3.6 \pm 0.5$	RT + TMZ; TMZ x 1	thrombocytopenia; TMZ discontinued during RT; reduced dose & delays
54/F	GBM	4.1 ± 1.6	RT + TMZ; TMZ x 2	thrombocytopenia; reduced dose & delays
59/F	GBM	7.5 ± 2.6	RT + TMZ; TMZ x 5	thrombocytopenia; TMZ discontinued during RT; reduced dose & delays
44/F	AO	7.9 ± 4.6	RT + TMZ; TMZ x 5	pancytopenia; reduced dose & delays
60/F	GBM	7.9 ± 1.6	RT + TMZ; TMZ x 5	neutropenia; TMZ discontinued during RT; reduced dose & delays
59/M	GBM	8.0 ± 2.3	RT + TMZ; TMZ x 3	thrombocytopenia; reduced dose & delays
64/F	AA	8.7 ± 1.8	RT + TMZ; TMZ x 4	thrombocytopenia; reduced dose & delays
49/F	0	12 ± 1.3	TMZ x 18	neutropenia; reduced dose & delay
28/F	GBM	15 ± 3.8	RT + TMZ; TMZ x 7	thrombocytopenia; reduced dose & delays
28/F	AA	15 ± 4.8	RT + TMZ; TMZ x 6	thrombocytopenia; reduced dose & delays
	II.	<u>I</u>	Patients with r	no myelosuppression
47/F	GBM	7.8 ± 3.5	TMZ x 4	None; continued treatment
34/M	AOA	11 ± 1.9	RT + TMZ; TMZ x 24	None; completed treatment
32/M	PNET	13 ± 4.2	TMZ x 4	None; continued treatment
66/M	GBM	14 ± 2.7	RT + TMZ; TMZ x 5	None; refused cycle 6
40/M	AA	16 ± 4.9	TMZ x 14	None; continued treatment
48/M	AO	22 ± 3.5	RT + TMZ; TMZ x 10	None; continued treatment
64/M	GBM	24 ± 4.1	RT + TMZ; TMZ x 8	None; completed treatment
65/M	0	33 ± 5.4	TMZ x 24	None; completed treatment

<sup>&</sup>lt;sup>1</sup>Diagnosis: AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; AOA, mixed anaplastic oligodendroglioma-astrocytoma; GBM, glioblastoma; O, oligodendroglioma; PNET, primitive neuroectodermal tumor.

<sup>&</sup>lt;sup>2</sup>Mean ± SD of MGMT activity (fmol O<sup>6</sup>-[<sup>3</sup>H]methylguanine/10<sup>6</sup> cells) is the average of 2 or more amounts of extract in at least 2 separate assays.

<sup>&</sup>lt;sup>3</sup>RT + TMZ: TMZ at 75 mg/m<sup>2</sup> was given daily during radiotherapy (RT) and was continued after radiation at 200 mg/m<sup>2</sup> for 5 consecutive days every 28 days for the number of cycles indicated. Five tumors received TMZ following recurrence after prior adjuvant radiotherapy.

methylation-specific PCR [8]. Bisulfite deamination, PCR primers and reaction conditions were as described by Hegi *et al.* [9]. Methylation status was determined by at least two separate bisulfite reactions and a minimum of two amplification reactions for each aliquot of bisulfite-treated DNA. DNA from the MGMT-deficient human glioma line SNB19 and the MGMT-proficient line SF767 served as controls for methylated and unmethylated promoters, respectively.

#### **RESULTS**

#### **Tumor Characteristics and Treatment**

As shown in Table 1, tumors from patients treated with TMZ included 8 WHO grade IV glioblastomas (GBM), 7 WHO grade III anaplastic gliomas, 2 grade II oligodendrogliomas and one supratentorial primitive neuroectodermal tumor (PNET). Thirteen tumors received concurrent TMZ and radiotherapy followed by adjuvant TMZ while the remaining 5 were treated with single agent TMZ. Of the tumors, 10 were from patients that experienced grade III or greater myelosuppression necessitating dose reductions or treatment delays (Table 1). The remaining 8 patients experienced no treatment-limiting count suppression during therapy. The two tumor populations did not differ in age  $(48 \pm 13)$ vs. 50  $\pm$  14 years), although the preponderance (8/10) of patients experiencing myelotoxicity were female in accord with earlier findings [2].

Blood was also obtained from disease-free volunteers (4 females and 6 males), ranging in age

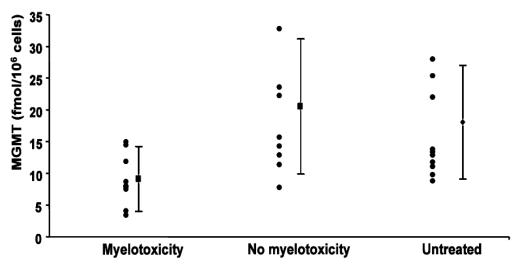
from 24 to 62 years old (mean  $\pm$  SD = 50  $\pm$  14), to serve as untreated controls.

## **MGMT Activity and Promoter Methylation**

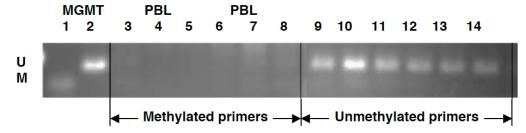
All PBL samples from tumor patients and diseasefree controls had detectable MGMT activity, ranging from 3.6 to 33 fmol/10<sup>6</sup> cells (Figure 1 and Table 1). In addition, all samples displayed only unmethylated MGMT promoters (Figure 2) in accord with previous reports [10]. Activity did not differ between patients who experienced no treatment-limiting myelosuppression and control subjects (18  $\pm$  8.1 vs. 16  $\pm$  6.8 fmol/10<sup>6</sup> cells;  $P \le 0.30$ ), suggesting that prior exposure to TMZ had no lasting effect on MGMT expression. However, as shown in Figure 1, MGMT activity was significantly lower in PBL from patients who experienced treatmentlimiting myelotoxicity compared to patients without myelosuppression (8.9  $\pm$  3.9 vs. 18  $\pm$  8.1 fmol/10<sup>6</sup> cells;  $P \le 0.015$ ) and compared to untreated controls (8.9 ± 3.9 vs. 16  $\pm$  6.8 fmol/10<sup>6</sup> cells;  $P \le 0.015$ ). These data indicate that low MGMT activity is associated with TMZ-induced myelotoxicity and suggest that failure to remove cytotoxic O<sup>6</sup>-methylguanine may increase bone marrow susceptibility to TMZ cytotoxicity.

## **DISCUSSION**

Little is known about the molecular mechanisms responsible for TMZ-induced myelosuppression in primary brain tumor patients. In this initial pilot study, we determined that MGMT activity in PBL is significantly lower in patients who experienced



**Figure 1: MGMT activity in PBL of TMZ-treated and untreated subjects.** Distribution of MGMT activity determined by standard biochemical assay (7) in PBL from TMZ-treated primary brain tumor patients who suffered treatment-limiting myelotoxicity, primary brain tumor patients who experienced no myelotoxicity, and untreated, disease-free volunteers. The mean  $\pm$  SD of MGMT activity (fmol O<sup>6</sup>-[<sup>3</sup>H]methylguanine/10<sup>6</sup> cells) is shown for each group. The activities of three tumors in the first group were essentially identical (Table 1) and appear as a single point in the figure.



**Figure 2: MGMT promoter methylation status in PBL.** Determination of methylation status for 6 representative PBL samples by methylation specific PCR, as described in Materials and Methods, is shown. PCR product was detected only in reactions specific for unmethylated MGMT promoter, indicative of MGMT expression, in the genomic DNA of all PBL samples from TMZ-treated patients and untreated volunteers. The two lanes at the left show the positions of methylated and unmethylated PCR products from SNB19, an MGMT-non-expressing human glioma cell line and SF767, an MGMT-expressing human glioma cell line, respectively; lanes 3-8 show the absence of bands for 5 PBL DNA samples amplified with primers specific for methylated MGMT; lanes 9-14 show bands for the same PBL DNA samples amplified with primers specific for unmethylated MGMT. U, position of unmethylated PCR product; M, position of methylated PCR product.

myelosupression, indicating that TMZ-induced O<sup>6</sup>-meG contributes to myelotoxicity. Our results also suggest that PBL MGMT activity can be used to estimate risk for myelosuppression. Ideally, high-risk patients could be offered TMZ dose reductions or alternative therapies to avoid the development of treatment-related myelotoxicity. Low risk patients could be offered TMZ with little hazard of toxicity, or even dose intensification. However, the overlap of MGMT activities between myelosuppressed and unsuppressed patients (Figure 1 and Table 1) suggests that additional resistance mechanisms contribute to TMZ sensitivity and limit the ability of MGMT alone to predict myelotoxicity. TMZ produces a dozen base adducts, including a number with documented cytotoxicity [4], suggesting that deficits in repair of one or more lesions other than O<sup>6</sup>meG might contribute to toxicity. Also, mechanisms that negate the cytotoxicity of unrepaired O<sup>6</sup>-meG, such as homologous recombination and replicative bypass [4] may greatly reduce TMZ cytotoxicity in cells with relatively low MGMT. Further study is required to resolve these possibilities.

Methylation of CpG islands in the *MGMT* promoter, a presumptive marker of suppressed gene expression, has been associated with decreased MGMT content in brain tumor cells [4]. We therefore hypothesized that methylation-specific PCR analysis of PBL DNA would show a correlation between CpG methylation and low MGMT activity. However, we found no association between methylation status and MGMT activity; all specimens displayed *only* an unmethylated signal. This finding suggests at first glance that promoter methylation status, a clinically tractable assay, has no utility as a marker for risk of TMZ-induced myelotoxicity. Conceivably, MGMT expression in PBL is associated with a different pattern of promoter CpG island methylation than that observed in glial tumors.

Alternatively, MGMT expression in PBL may be mediated by other mechanisms, as evidenced by a recent study that identified MGMT polymorphisms associated with TMZ-induced myelotoxicity [10]. Though these study results are provocative, the small sample size limits generalizing these findings to all patients with gliomas treated with TMZ and suggests confirmation of the study findings is required before this assay is used as a predictive biomarker for TMZ induced myelosuppression.

## **DISCLOSURE**

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## **CONFLICT OF INTEREST**

All authors report no conflict of interest.

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