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Correlation of O6-Methylguanine Methyltransferase (MGMT) Promoter Methylation With Clinical Outcomes in Glioblastoma and Clinical Strategies to Modulate MGMT Activity — Source link

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Abstract

Resistance to alkylating agents via direct DNA repair by O(6)-methylguanine methyltransferase (MGMT) remains a significant barrier to the successful treatment of patients with malignant glioma. The relative expression of MGMT in the tumor may determine response to alkylating agents, and epigenetic silencing of the MGMT gene by promoter methylation plays an important role in regulating MGMT expression in gliomas. MGMT promoter methylation is correlated with improved progression-free and overall survival in patients treated with alkylating agents. Strategies to overcome MGMT-mediated chemoresistance are being actively investigated. These include treatment with nontoxic pseudosubstrate inhibitors of MGMT, such as O(6)-benzylguanine, or RNA interference-mediated gene silencing of MGMT. However, systemic application of MGMT inhibitors is limited by an increase in hematologic toxicity. Another strategy is to deplete MGMT activity in tumor tissue using a dose-dense temozolomide schedule. These alternative schedules are well tolerated; however, it remains unclear whether they are more effective than the standard dosing regimen or whether they effectively deplete MGMT activity in tumor tissue. Of note, not all patients with glioblastoma having MGMT promoter methylation respond to alkylating agents, and even those who respond will inevitably experience relapse. Herein we review the data supporting MGMT as a major mechanism of chemotherapy resistance in malignant gliomas and describe ongoing studies that are testing resistance-modulating strategies.

Correlation of MGMT Promoter Methylation With Clinical Outcomes in Glioblastoma and Clinical Strategies to Modulate MGMT Activity

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ABSTRACT

Resistance to alkylating agents via direct DNA repair by O^6-methylguanine methyltransferase (MGMT) remains a significant barrier to the successful treatment of patients with malignant glioma. The relative expression of MGMT in the tumor may determine response to alkylating agents, and epigenetic silencing of the *MGMT* gene by promoter methylation plays an important role in regulating MGMT expression in gliomas. *MGMT* promoter methylation correlates with improved progression-free and overall survival in patients treated with alkylating agents. Strategies to overcome MGMT-mediated chemoresistance are being actively investigated. These include treatment with nontoxic pseudo-substrate inhibitors of MGMT, such as O^6-benzylguanine, or RNA interference-mediated gene silencing of *MGMT*. However, systemic application of MGMT inhibitors is limited by an increase in hematologic toxicity. Another strategy is to deplete MGMT activity in tumor tissue using a dose-dense temozolomide schedule. These alternative schedules are well tolerated; however, it remains unclear whether they are more effective than the standard dosing regimen or whether they effectively deplete MGMT activity in tumor tissue. Of note, not all glioblastoma patients with *MGMT* promoter methylation respond to alkylating agents, and even those who respond will inevitably relapse. Herein we review the data supporting MGMT as a major mechanism of chemotherapy resistance in malignant gliomas and describe ongoing studies that are testing resistance-modulating strategies.

Key words: O^6-methylguanine methyltransferase, temozolomide, tumor resistance, glioma, alkylating agents, glioblastoma

INTRODUCTION

Alkylating agents, including the chloroethylnitrosoureas (carmustine [BCNU], lomustine [CCNU], and fotemustine), procarbazine, and temozolomide, are commonly used to treat malignant brain tumors. These agents cause DNA damage by adding alkyl groups to DNA, which triggers DNA repair, thereby inducing apoptosis. Temozolomide is a methylating agent that modifies the DNA at several sites, most commonly N^7-methylguanine and N^3-methyladenine, which constitute nearly 90% of the total methylation events.[1] However, these adducts are efficiently repaired by the base excision repair pathway and have a low cytotoxic potential. Only 5% to 10% of the methylation events mediated by temozolomide yield O^6-methylguanine, but if the methyl group is not removed before cell division, these adducts trigger the DNA mismatch repair (MMR) pathway and are highly cytotoxic.[2]

O^6-methylguanine methyltransferase (MGMT) is a cellular DNA repair protein that rapidly reverses alkylation (including methylation) at the O^6 position of guanine,[3] thereby neutralizing the cytotoxic effects of alkylating agents such as temozolomide. The protective effect of MGMT activity against the cytotoxic effects of alkylating agents has been demonstrated in human cell lines, xenograft models, and *MGMT* transgenic mice.[4-9] High levels of MGMT activity in tumor tissue are associated with resistance to alkylating agents.[7] In contrast, epigenetic silencing of the *MGMT* gene by promoter methylation results in decreased MGMT expression in tumor cells.[10,11] Methylation of the *MGMT* promoter has been observed in a variety of tumor types,[12] which is consistent with the observation that epigenetic “silencing” of genes is a common mechanism for inactivating tumor suppressor genes during malignant progression.[13,14] Herein, we review the role of MGMT in conferring resistance to alkylating agents and strategies to modulate MGMT activity in malignant gliomas.

THE ROLE OF MGMT IN RESISTANCE TO ALKYLATING CHEMOTHERAPY

O^6-methylguanine methyltransferase is ubiquitously expressed in normal human tissues.[15] Most of our knowledge about MGMT as a DNA repair protein is based on observations following exposure to alkylating agents.[5] In several ways, the MGMT-mediated repair process (Fig 1) is unique and differs from other DNA repair pathways; MGMT is not part of a repair complex but acts alone. It specifically removes the methyl group from the O^6 position of guanine, thereby restoring the nucleotide to its native form without causing any DNA strand breaks, and it is a "suicide enzyme." On transfer of the alkyl group to an internal cysteine residue in the active site of MGMT, the enzyme is irreversibly inactivated, thus requiring *de novo* protein synthesis to maintain enzyme activity. In fact, the process is saturable; an excess of O^6-methylguanine in the DNA can deplete MGMT. Although the O^6 position of guanine is not the most common target of alkylating agents, the resulting promutagenic lesions act as an important trigger for cytotoxicity and apoptosis.[16] Left unrepaired, this modified guanine preferentially pairs with thymine during DNA replication, which activates the MMR pathway. However, MMR only targets and corrects the newly synthesized daughter strand, leaving behind the O^6-methylguanine in the template strand. It has been hypothesized that the MMR pathway, in an attempt to repair this mismatch, undergoes reiterative cycles of resynthesis and attempted repair (ie, futile cycling). This results in DNA double-strand breaks, thereby activating apoptotic pathways leading to cell death.[17,18] The essential role of the MMR pathway is illustrated by studies showing that cells deficient in both MGMT and the MMR pathway are 100 times more resistant to methylating agents.[19] It has also recently been shown that treatment of glioblastoma (GBM) with temozolomide seems to select for inactivation of MMR as a result of mutation or loss of expression of the MMR-associated protein MSH6.[20] Consequently, the highest therapeutic activity of alkylating agents is expected in tumor cells with low levels of MGMT and an intact MMR system.[5] Given the central

role of MGMT in resistance to alkylating agents and its unique properties, MGMT is an ideal potential target for biochemical modulation of drug resistance.[21-23]

Analytic Determination of MGMT

Several methods for measuring MGMT levels within tumors have been described. The MGMT protein can be detected in tissue samples by immunohistochemistry,[24] enzyme activity can be measured by high-performance liquid chromatography (HPLC),[25,26] and epigenetic silencing of the *MGMT* gene by promoter methylation can be assessed using a methylation-specific polymerase chain reaction assay (MSP).[27,28] Determination of MGMT activity by HPLC requires fresh tissue and is most suitable for quantitative studies in pure populations of cells.[25,26] Immunohistochemical analysis has revealed heterogeneous MGMT expression patterns within tumors, often with regions of intense staining bounded by adjacent cells that lack expression.[24,29,30] Tumor-infiltrating lymphocytes, microglia, and blood vessels may also express high levels of MGMT protein (Fig 2). Both HPLC and immunohistochemistry have been used to study the correlation between MGMT activity and drug resistance in cell lines and xenografts derived from a variety of human tumors.[31] These studies have shown that MGMT expression varies widely in different tumor types and cell lines.

The MSP assay has been developed for determining the *MGMT* methylation status of CpG islands in the gene promoter (Fig 3).[28,32] The MSP assay detects CpG island methylation with high sensitivity and specificity. This assay requires only small quantities of DNA and can be performed on DNA extracted from paraffin-embedded tissue samples; however, fixation may cause deterioration of the DNA. Therefore, best results are obtained with cryopreserved tumor specimens.

For diagnostic purposes in patients with glioma, one advantage of the MSP assay lies in the fact that detection of the methylated *MGMT* allele can be attributed solely to neoplastic cells.[33] Therefore, nontumor tissue contamination of the surgical specimen does not interfere

with the result. Furthermore, *MGMT* gene expression may be induced in tumor cells in response to DNA damage from alkylating agents or radiotherapy (RT) and also by corticosteroids,[6,34] although this remains to be demonstrated in primary gliomas. Aberrant methylation of CpGs is a heritable change in the DNA that may be less susceptible to treatment-induced alterations. Recent reports have reviewed and discussed the different techniques for assessing MGMT and whether they can predict clinical outcome in glioma patients.[35-37] The correlation with outcome was variable throughout the studies analyzed, regardless of the techniques employed; however, the overall interpretation of these results is limited because much of the data come from small series or cohorts of mixed glioma subtypes, use of different cut-offs (eg, for immunohistochemistry), and heterogeneity of treatment or complete lack of treatment information. The latter is highly relevant, since MGMT status is thought to predict benefit primarily from treatment with alkylating agents.[32,38] Clearly, the methods useful for diagnostic purposes will have to be standardized and validated in prospective studies.[39,40]

MGMT Expression/Methylation and Clinical Outcome in Malignant Glioma

Clinical studies have correlated MGMT expression or *MGMT* promoter methylation with response to chloroethylnitrosoureas and methylating agents and with survival.[41-46] A Southwest Oncology Group study found that MGMT expression levels in newly diagnosed malignant astrocytoma assessed by quantitative immunofluorescence were inversely correlated with tumor response and survival in patients treated with BCNU.[43] The difference in median survival among patients with tumors showing high (n = 41) versus low (n = 23) MGMT expression levels was significant (8 versus 29 months, respectively; $P = .0002$; Fig 4).[43] In a retrospective analysis of 47 glioma patients treated with whole-brain RT and various alkylating agents, Esteller et al[42] demonstrated an improved clinical outcome in the presence of a methylated *MGMT* promoter. Similarly, in a phase II trial, we demonstrated significantly improved clinical outcome in patients with newly diagnosed GBM who had a methylated *MGMT* promoter and were treated with

temozolomide and RT.[47] The 18-month survival rate was 62% among patients with a methylated *MGMT* promoter compared with only 8% in the absence of promoter methylation ($P = .002$). These studies identified *MGMT* promoter methylation status as a potential independent prognostic factor for survival in GBM patients treated with alkylating agents. Furthermore, Paz et al[44] showed that *MGMT* promoter methylation was associated with a higher rate of clinical response in patients with primary glioma treated with temozolomide or other alkylating agents (Table 1). However, this association was not observed when patients were treated with alkylating agents at relapse, suggesting that selection for treatment resistance had occurred.[44]

More recently, *MGMT* promoter methylation was analyzed in tissue samples from patients with newly diagnosed GBM treated within an international, randomized phase III trial (European Organization for Research and Treatment of Cancer [EORTC] trial 26981-22981 and National Cancer Institute of Canada [NCIC] trial CE.3) comparing RT alone with RT plus concomitant and adjuvant temozolomide.[32] Paraffin-embedded tissue samples were available for 307 (54%) patients. High-quality DNA, allowing for analysis by MSP assay, could be extracted from two thirds of the available tissue, and 45% of this group were shown to have a methylated *MGMT* promoter. The MSP assay consisted of a 2-stage polymerase chain reaction using nested primers.[48] The results showed that *MGMT* promoter methylation was associated with improved overall survival in patients treated with RT plus temozolomide but not in patients initially treated with RT alone (Fig 5).[32] Among patients whose tumors contained a methylated *MGMT* promoter, patients treated with RT plus temozolomide had a median survival of 21.7 months compared with 15.3 months for patients assigned to RT alone. In patients treated with initial RT alone, *MGMT* promoter methylation was also associated with a slight improvement in survival among patients surviving beyond 9 months. This was expected because more than 70% of the patients in the RT arm received chemotherapy (most likely with an alkylating agent) at progression, and 60% of these patients received temozolomide at progression. When progression-free survival (PFS) was analyzed, thereby eliminating second-line therapy as a

confounding factor, PFS was only prolonged in patients with a methylated *MGMT* promoter who were treated with RT plus temozolomide (Fig 6).[32] In patients with a methylated *MGMT* promoter, median PFS was 10.3 months for patients treated with RT plus temozolomide compared with 5.9 months in the RT-only group ($P = .001$).

CLINICAL STUDIES OF TEMOZOLOMIDE AND MGMT INHIBITORS

Given the important role of MGMT in tumor resistance to alkylating agents, a variety of MGMT inhibitors have been investigated with the goal of modulating chemoresistance (reviewed by McMurry[49]). O^6-benzylguanine (O^6-BG) is a potent MGMT-inactivating agent that has been studied in combination with alkylating agents. It inactivates MGMT stoichiometrically.[50] However, O^6-BG lowers MGMT levels in normal cells as well, increasing toxicity from chemotherapy. A dose-escalation study was conducted to determine the dose of O^6-BG that would effectively suppress MGMT activity in anaplastic gliomas.[51] Escalating doses of O^6-BG were administered 6 or 18 hours before tumor resection, and MGMT activity was measured in snap-frozen tumor tissue. A dose of 120 mg/m^2 given 6 hours before surgery inhibited MGMT activity in > 90% of patients, whereas MGMT activity was inhibited in only 45% of patients who received O^6-BG 18 hours before surgery (Table 2). Although this analysis did not take into account that MGMT activity may have been constitutively low due to promoter methylation in 30% to 50% of patients, these data suggest either that O^6-BG is only able to transiently inhibit the enzyme or that resynthesis of MGMT can occur rapidly in tumor tissue.[51] No toxic side effects were observed in this study. Importantly, this study illustrated how transient the reduction in MGMT activity may be with such a treatment approach.

Other studies have attempted to determine the maximum tolerated dose of chemotherapy that can be administered in combination with O^6-BG. One study showed that 120 mg/m^2 of O^6-BG inhibited MGMT activity to undetectable levels for up to 18 hours in the tumor, and O^6-BG either alone or in combination with 13 mg/m^2 BCNU resulted in no serious side effects.[26] However, in a subsequent phase I study, 100 mg/m^2 of O^6-BG increased hematologic toxicity when used in conjunction with escalating doses of BCNU (13.5, 27, 40, and 55 mg/m^2).[52] At the highest dose of BCNU (55 mg/m^2), grade 3/4 thrombocytopenia and

neutropenia became dose limiting. The maximum tolerated dose of BCNU in combination with 100 mg/m^2 O^6-BG was 40 mg/m^2 administered at 6-week intervals. In the following phase II trial, 18 patients with recurrent or progressive nitrosourea-resistant malignant gliomas were treated with O^6-BG and BCNU.[53] No objective tumor responses were observed, but 5 patients had stable disease for 6 to 18 weeks. Again, 66% of the patients experienced grade 3/4 thrombocytopenia and neutropenia. These studies did not stratify patients based on the *MGMT* methylation status of their tumors.

O^6-(4-bromothenyl)guanine (lomeguatrib, PaTrin-2; KuDOS Pharmaceuticals, Cambridge, UK) is similar to O^6-BG. Preclinical data demonstrated that lomeguatrib sensitized an ovarian tumor cell line to temozolomide,[54] and the combination of lomeguatrib and temozolomide significantly improved inhibition of tumor growth in human melanoma xenografts.[55] Lomeguatrib is also an oral agent and could be conveniently combined with temozolomide. No data on inhibition of MGMT in the tumor or any safety data on the combination of lomeguatrib and temozolomide are yet available.

Furthermore, 2-amino-O^4-benzylpteridine derivatives are in preclinical studies and have greater tumor specificity,[56,57] which could counter the problem of hematologic toxicity associated with systemic application of other MGMT inhibitors. The cytotoxicity of BCNU in combination with O^4-benzylfolates appears to be a function of the R-folate receptor, which is overexpressed in many tumor types.[57] The compound O^4-benzylfolic acid, which has the added advantage of greater water solubility, was shown to be 30 times more effective than O^6-BG and was effective against the mutant P140K-MGMT that is resistant to O^6-BG. Thus, this class of compounds may have greater clinical potential.

Temozolomide and MGMT Depletion: Alternative Dosing Schedules

It is important to note that alkylating agents themselves are also powerful MGMT-depleting agents because of their capacity to induce DNA damage. MGMT depletion in the tumor could potentially be achieved with alternative dosing schedules of temozolomide that deliver more prolonged exposure and higher cumulative doses than the standard 5-day regimen (150 to 200 mg/m^2/day for 5 days every 28-day cycle).[58] Both compressed and extended temozolomide schedules have been tested preclinically and clinically. Early results suggested that continuous daily administration was more effective than a single dose,[59] and more frequent administration (ie, twice daily or every 4 hours) yielded the most effective depletion of MGMT activity and the highest levels of O^6-methylguanine DNA adducts in tumor tissue.[60,61] These studies suggested that tumor cells become sensitized to temozolomide as MGMT is depleted, resulting in greater cytotoxicity with subsequent doses. However, normal hematopoietic cells can also become sensitized. For example, in a phase II trial in 30 patients with metastatic melanoma who received 5 doses of temozolomide (200 mg/m^2 every 4 hours) within 16 hours (total dose = 1,000 mg/m^2), 68% of patients developed grade 3/4 thrombocytopenia and 54% developed grade 3/4 leukopenia, but the compressed dosing schedule did not appear to significantly enhance antitumor activity.[62] Notably, pretreatment MGMT levels in peripheral blood mononuclear cells (PBMCs) correlated with the dose intensity that patients were able to tolerate. Profound lymphopenia has also been reported in patients receiving extended daily treatment with temozolomide at a dose of 75 mg/m^2, which can lead to opportunistic infections such as *Pneumocystis carinii* pneumonia.[63-65]

Subsequent clinical trials with temozolomide have explored a variety of dosing schedules (Table 3) with the intention of maximizing MGMT depletion in tumor cells. The first alternative regimen tested was the extended daily regimen,[58] which was subsequently used in combination with RT in the trials conducted by the EORTC. These regimens increase exposure to temozolomide over a 28-day cycle by approximately 1.5- to 2-fold compared with the

standard 5-day regimen, and the hope is that these alternative regimens will increase antitumor activity compared with the 5-day regimen without dramatically increasing hematologic toxicity. However, to date, there is no empiric evidence that this can be achieved in clinical practice.

This concept of enhanced MGMT depletion with alternative temozolomide dosing regimens has been rigorously tested by Tolcher et al.[66] Patients received temozolomide at various doses and on 2 alternative dosing schedules: either 7 consecutive days of every 14 days (7 days on/7 days off) or 21 consecutive days of every 28 days (21/28-day schedule). Serial blood samples were taken to assess MGMT enzyme activity in PBMCs. This study demonstrated a time- and dose-dependent decrease in MGMT activity with both regimens. Continuous dosing for 7 days at a dose of 75 to 175 mg/m^2 produced a rapid reduction from mean baseline MGMT activity (72% on day 8), which appeared to be fairly dose dependent. However, during the 7-day rest period, there was recovery of MGMT activity to approximately 55% of baseline (Fig 7).[66] The 21-day continuous schedule at a lower daily dose (85 to 125 mg/m^2) resulted in a similar reduction (~70%) in mean MGMT activity by day 15, which was sustained through day 21.

This study established a benchmark against which to evaluate alternative dosing regimens and illustrated several important concepts with regard to MGMT depletion with temozolomide. The depletion of MGMT activity was a function of both the total cumulative dose and the area under the concentration time curve.[66] The daily and cumulative doses administered using a variety of dose-dense regimens are shown in Table 3. Higher doses of temozolomide appear to deplete MGMT levels in PBMCs more rapidly than lower doses. Administration of temozolomide for 21 consecutive days at a daily dose that resulted in a comparable cumulative dose per cycle as the 7-days-on/7-days-off regimen resulted in comparable depletion of MGMT activity at lower daily doses. However, this regimen appears to achieve more protracted MGMT depletion at least in PBMCs. On the other hand, the 7-days-

on/7-days-off regimen allows for better recovery of MGMT in PBMCs, which may result in less hematologic toxicity.

Currently, it is not known which of these schedules is more effective at depleting MGMT in tumor cells or which will strike a better balance between antitumor activity and hematologic toxicity. Although the Tolcher study provides the best rationale for protracted temozolomide schedules, there remain a number of important unanswered questions. In particular, it remains unknown how these schedules affect MGMT activity in brain tumor tissue. Studies reported by Spiro et al[26,61] suggest that depletion of MGMT activity in PBMCs occurs at lower doses of O^6-BG or temozolomide and is not a reliable predictor of MGMT depletion in tumor tissue.

BRAIN TUMOR TRIALS WITH NOVEL SCHEDULES OF TEMOZOLOMIDE

A number of studies in patients with high-grade glioma have investigated alternative temozolomide dosing schedules and have begun to look at important clinical questions with regard to the best treatment strategy in both the first-line and recurrent settings (Table 4).[67-74]

One particularly relevant question is whether concomitant RT plus temozolomide is sufficient to confer a survival benefit in patients with newly diagnosed GBM. This question is the object of an ongoing international Intergroup trial designed to establish the optimal sequence and relative contribution of the concomitant and adjuvant chemotherapy compared with RT alone in patients with anaplastic astrocytoma. Over 700 patients will be randomized in the CATNON trial (Concurrent and Adjuvant Temozolomide in NON-deleted anaplastic astrocytoma).

One study in Greece has examined whether dose intensification of the maintenance regimen affects clinical outcome. In a randomized multicenter phase II trial patients with newly diagnosed GBM received the standard regimen of concomitant RT plus temozolomide followed by temozolomide at a dose of 150 mg/m^2 x 5 days every other week compared with RT alone.[67] The results suggested that this dose-intensified maintenance regimen may improve PFS in the absence of significant hematologic toxicity.[67] Patients treated with RT plus temozolomide had a median PFS of nearly 11 months, and 37% of patients had not yet progressed at 1 year. This was substantially better than the PFS achieved with RT plus temozolomide in the EORTC/NCIC trial (median of 7 months, and 1-year PFS of 27%).[75] However, this improvement in PFS did not appear to translate into a substantial improvement in overall survival; median overall survival was 13.4 months with RT plus temozolomide (compared with 14.6 months in the EORTC/NCIC trial[75]) and only 7.7 months in the RT-only arm.[67] Although patient selection cannot be ruled out, the poor survival outcome, particularly in the control arm, may have been due to the small

number of patients who received chemotherapy at recurrence. Nevertheless, this trial provides evidence of the safety and feasibility of a dose-intensified temozolomide maintenance regimen.

A French phase II study also examined the effect of the 7-days-on/7-days-off regimen (150 mg/m^2/day) as both neoadjuvant therapy before RT for up to 4 cycles (8 weeks) and adjuvant (ie, maintenance) therapy after RT until progression (up to 8 cycles) in patients with inoperable tumors.[76] This study provided information about antitumor activity in the neoadjuvant setting, although the median number of cycles was only 3 (range, 1 to 8). Consistent with the previous attempts at neoadjuvant therapy for GBM, 25% of patients had a partial response, and 31% had stable disease. Median PFS in this group of patients with poor prognosis was 3.8 months, and median overall survival was 6 months. Hematologic toxicity requires careful monitoring; 24% of patients developed grade 3 or 4 thrombocytopenia, 14% had grade 4 granulocytopenia, and 14% had grade 4 lymphopenia. In addition, 5 patients developed interstitial pneumopathy, and 6 patients required dose reductions. This study also provided evidence that MGMT expression correlated with response to temozolomide; despite dose intensification, patients with high levels of MGMT expression in their tumor tissue were unlikely to respond and more likely to progress early.

To date, the largest clinical experience with an alternative temozolomide regimen in the first-line setting is from the joint German-Swiss randomized phase III trial of the Neuro-oncology Working Group within the German Cancer Society (NOA-08).[71] This trial is currently investigating the benefit of temozolomide (100 mg/m^2 7 days on/7 days off) versus RT alone as a primary therapy for high-grade glioma until treatment failure and shall enroll 340 patients over the age of 65 years.

In the recurrent setting, several studies have investigated dose-dense regimens. Italian investigators studying temozolomide at 75 mg/m^2 on a 21/28-day schedule reported grade 3 lymphocytopenia in one fourth of patients and an increased incidence of infections.[72,77] In our previous experience with temozolomide administered at 75 mg/m^2 for 6-7 weeks concurrent with

RT, up to 80% of patients developed profound lymphocytopenia, further promoted by the frequent administration of corticosteroids at diagnosis.[63] A small Belgian phase II trial has also examined the 21/28-day schedule at a dose of 100 mg/m^2 in 19 patients with recurrent anaplastic astrocytoma and anaplastic oligoastrocytoma.[73] A report on the safety profile of this regimen indicated a high incidence of grade 3 and 4 lymphopenia in 10 and 9 patients, respectively. In addition, there were 2 suspected opportunistic infections: 1 herpes zoster reactivation during lymphopenia and 1 case of *Pneumocystis carinii* pneumonia after the patient had discontinued study treatment. Therefore, it appears that regular lymphocyte counts and prophylaxis against opportunistic infections may be required when using this regimen, particularly in the recurrent setting. Similar observations have been made in melanoma patients who were treated with the daily schedule (75 mg/m^2 $\times$ 6 to 7 weeks).[64] The high frequency of lymphopenia in patients receiving prolonged daily schedules of temozolomide raises the question whether more intermittent dosing might be better tolerated. The most compelling data come from a German study in 39 patients with recurrent GBM who were treated with 150 mg/m^2 on the 7-days-on/7-days-off regimen, which was associated with a relatively low incidence of lymphopenia.[69,70] This regimen produced an overall response rate of 9.5%, a 6-months PFS rate of 48%, and a median PFS of 21 weeks (~5 months),[69] which is superior to the data reported on the standard 5-day dosing regimen.[78] These data have since been confirmed and extended.[74] Importantly, in the more recent trial involving 90 patients, there was no significant difference in median PFS between patients with a methylated or unmethylated *MGMT* promoter at initial diagnosis.[74] This suggests either that patients with a methylated *MGMT* promoter at diagnosis acquired resistance to temozolomide during progression[20,44] or that this regimen may indeed overcome MGMT-mediated resistance, thereby extending time to progression in patients with an unmethylated promoter.

CONCLUSIONS AND FUTURE DIRECTIONS

A variety of molecular markers may have prognostic value in patients with malignant glioma. These markers include high expression of MGMT, overexpression of the epidermal growth factor receptor (EGFR), presence of the EGFRvIII mutation, expression of the *YKL-40* gene, tenascin expression, loss or mutation of the *PTEN* gene, loss of chromosome 10, and mutation or loss of the *p53* gene.[79-82] Although MGMT expression appears to have a strong influence on response to alkylating agents and clinical outcome in patients with GBM, to date none of these markers, including MGMT, has been definitively confirmed.

An international group of investigators from the United States, Canada, and Europe is collaborating on a successor clinical trial to the EORTC/NCIC study. This trial, RTOG 0525, led by the Radiation Therapy Oncology Group Intergroup (RTOG) along with the EORTC and the North Central Cancer Treatment Group, will test whether increasing the dose intensity of adjuvant temozolomide will improve survival compared with the standard regimen established by the EORTC/NCIC study.[83,84] The study design is shown in Fig 8.[83,84] The underlying hypothesis for this phase III trial is based on the laboratory finding that prolonged exposure of tumor cells to temozolomide results in reduced MGMT activity.[85] An accrual of 1,153 patients is expected, and both clinical and molecular markers are being used to assign patients to the two treatment arms. Clinical factors include the validated subgroups identified by the recursive partitioning analysis of the RTOG, and patients in classes III, IV, and V are eligible for study entry. In addition, immediate tumor tissue sample submission is mandatory to prospectively assess *MGMT* promoter methylation status (using a quantitative MSP assay), and patients are then stratified by *MGMT* promoter methylation status before randomization. This will ensure a balanced number of patients with *MGMT*-methylated tumors assigned to the control and experimental arms, which will be critical for validation of *MGMT* promoter methylation as a molecular marker of response and/or prognosis.

While differences in *MGMT* promoter methylation may determine the clinical course in GBM patients treated with temozolomide, it is presently not recommended to use the *MGMT* promoter methylation assay to determine who should receive temozolomide and who should not.[37] First, an independent confirmation of the retrospective analysis from the EORTC/NCIC trial is necessary. Second, there is reason to believe that alternative dose-intensified schedules may overcome resistance in patients with an unmethylated *MGMT* promoter.[86] Third, allocating GBM patients to specific treatments on the basis of *MGMT* promoter methylation status will only assume clinical relevance when effective alternative treatments become available. At present, the only established alternative is nitrosourea-based chemotherapy, which is also subject to resistance mediated by MGMT.

A promising strategy to overcome resistance mediated by MGMT appears to be depletion of MGMT by prolonged exposure to low doses of alkylating agents. For these agents, the feasibility of intensified dosing schedules has been demonstrated. Optimizing this approach in conjunction with modulation of dosing schedules is of paramount importance to maximize the clinical effectiveness of temozolomide. However, myelosuppression continues to be dose-limiting when MGMT depletion is maximized by dose intensification or the addition of MGMT inhibitors such as O^6-BG. The development of tumor-specific MGMT inhibitors may overcome this limitation. This is an area of intense ongoing research, which will hopefully result in further improvements in clinical outcomes.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Final approval of manuscript: Monika E. Hegi, Lili Liu, James Herman, Roger Stupp, Wolfgang Wick, Michael Weller, Minesh P. Mehta, Mark R. Gilbert

TABLES

Table 1. Clinical Response to First-Line Alkylating Agent Chemotherapy in Glioma Patients According to Methylation Status

		MGMT Unmethylated		MGMT Methylated		
First-Line Chemotherapy	Total. Patients, n	Patients, n	Positive Response, n (%)	Patients, n	Positive Response, n (%)	*P*
Temozolomide	40	28	7 (25)	12	8 (68)	.030
BCNU	35	24	4 (17)	11	6 (55)	.041
Procarbazine/CCNU	17	12	1 (8)	5	3 (60)	.043
All drugs	92	64	12 (19)	28	17 (61)	.001

Abbreviations: MGMT, O^6-methylguanine-methyltransferase; BCNU, carmustine; CCNU, lomustine.
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Table 2. Rapid Reconstitution of MGMT Activity in Brain Tumor Tissue After Effective Depletion by O^6-Benzylguanine

O^6-BG dose, mg/m^2	No. of Evaluable Patients	Hours From O^6-BG Dose to Tumor Resection	No. of Patients With Undetectable MGMT Activity* (%)
40	5	6	2 (40)
60	8	6	4 (50)
80	15	6	9 (60)
100	8	6	5 (63)
120	18	6	17 (94)
120	11	18	5 (45)

Abbreviations: MGMT, O^6-methylguanine-methyltransferase; O^6-BG, O^6-benzylguanine.
*< 10 fmol/mg protein.
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Table 3. Alternative Temozolomide Dosing Schedules

Regimen	Daily Dose, mg/m^2	Schedule	Dose Intensity, mg/m^2 Per 28-Day Cycle	Relative Dose Intensity
5/28 days	200	5 days every 28 days	1,000	1
Daily	75	Daily × 6 to 7 weeks, every 10 weeks	1,470	1.5
Daily	50	Daily continuously	1,400	1.4
10/28 days	150	Day 1 to 5 and 15 to 19 every 28 days	1,500	1.5
14/28 days	150 - 175	7 days on/7 days off	2,100 - 2,450	2.1 - 2.5
21/28 days	100	21 days on/7 days off	2,100	2.1

Table 4. Clinical Studies of Alternative Temozolomide Dosing Regimens in Primary Brain Tumors

Study	Patient Population	Schedule	Median PFS, Months	Median OS, Months
Athanassiou[67] (N = 110)	Newly dx GBM	RT + 75 mg/m^2 TMZ concomitant 150 mg/m^2 days 1 to 5, 15 to 19 q28 days	10.8	13.4
		v RT alone (60 Gy)	5.2	7.7
Chinot[68] (N = 30)	Inoperable GBM	150 mg/m^2 7 days on/7 days off as neoadjuvant tx before RT and maintenance after RT	3.8 (6-month PFS = 21%)	6
Wick[69,70] (N = 39)	Recurrent GBM	150 mg/m^2 7 days on/7 days off	5 (6-month PFS = 48%)	
NOA-08*[71]	Newly dx AA or GBM > 65 years old	100 mg/m^2 7 days on/7 days off *v* RT alone	NA	NA
Tosoni[72] (N = 51)	Glioma	75 mg/m^2 21/28 days	NA	NA
Neyns[73] (N = 19)	Recurrent AA & AO	100 mg/m^2 21/28 days	6-month PFS = 56%	12.9
Wick[74] (n = 90)	Recurrent glioma	150 mg/m^{2†} 7 days on/7 days off	5.5 (6-month PFS = 44%)	8.8

Abbreviations: PFS, progression-free survival; OS, overall survival; dx, diagnosed; GBM, glioblastoma; RT, radiotherapy; TMZ, temozolomide; tx, treatment; NOA, Neuro-Oncology Working Group of the German Cancer Society; AA, anaplastic astrocytoma; NA, not available; AO, anaplastic oligoastrocytoma.

*This phase III trial expects to enroll 340 patients in up to 30 German and Swiss centers.

†with individual dose adjustments according to hematologic toxicity

FIGURE LEGENDS

Fig 1. The DNA repair process mediated by O^6-methylguanine DNA methyltransferase (MGMT). The MGMT enzyme transfers the methyl group from the O^6-methylguanine DNA adduct to a cysteine residue in the enzyme and becomes irreversibly inactivated.

Fig 2. O^6-methylguanine-methyltransferase (MGMT) expression in glioblastoma (GBM). (A) Small cells within the GBM, which may or may not be of tumor origin, express MGMT. (B) Glioblastoma displaying high levels of MGMT expression in most tumor cells. (C and D) Blood vessels expressing MGMT, here shown in vascular proliferations. Higher magnifications are shown below. The GBM tissue microarray is described in by Godard et al.[87]

Fig 3. Methylation-specific polymerase chain reaction (PCR) assay. PCR product indicating a methylated O^6-methylguanine-methyltransferase (MGMT) promoter is shown by arrows. Abbreviations: U, unmethylated MGMT promoter; M, methylated MGMT promoter; L, 100-bp DNA marker ladder; Meth PBL, enzymatically methylated DNA from peripheral blood lymphocytes. Reprinted with permission from Hegi et al.[32]

Fig 4. Kaplan-Meier estimate of survival among patients with tumors showing high versus low O^6-methylguanine-methyltransferase (MGMT) expression levels who were treated with carmustine. Reprinted with permission from the American Society of Clinical Oncology.[43]

Fig 5. Kaplan-Meier analysis of overall survival based on O^6-methylguanine-methyltransferase (MGMT) promoter methylation status (unmethylated [Unmeth] *v* methylated [Meth]) and random assignment to radiotherapy (RT) plus temozolomide (TMZ) or RT alone. Reprinted with permission from Hegi et al.[32]

Fig 6. Kaplan-Meier estimation of progression-free survival according to randomization and O^6-methylguanine-methyltransferase promoter methylation status of the tumors. Abbreviations: Meth, methylated; Unmeth, unmethylated; TMZ, temozolomide; RT, radiotherapy. Reprinted with permission from Hegi et al.[32]

Fig 7. Levels of O^6-methylguanine-methyltransferase (MGMT) enzyme activity in peripheral blood mononuclear cells at baseline and after treatment with the temozolomide 7-days-on/7-days-off schedule at 75 to 175 mg/m^2/day (A) or the 21/28-day schedule at 85 to 125 mg/m^2/day (B). Reprinted with permission from Tolcher et al.[66]

Fig 8. Study schema for the ongoing Radiation Therapy Oncology Group Intergroup trial. MGMT, O^6-methylguanine-methyltransferase; RPA, recursive partitioning analysis; RT, radiotherapy; TMZ, temozolomide. *Dosing will be initiated at 75 mg/m^2 in cycle 1 with dose escalation to 100 mg/m^2 in subsequent cycles if no dose-limiting hematologic toxicity occurs. Data from the National Cancer Institute[83] and the European Organisation for Research and Treatment of Cancer.[84]

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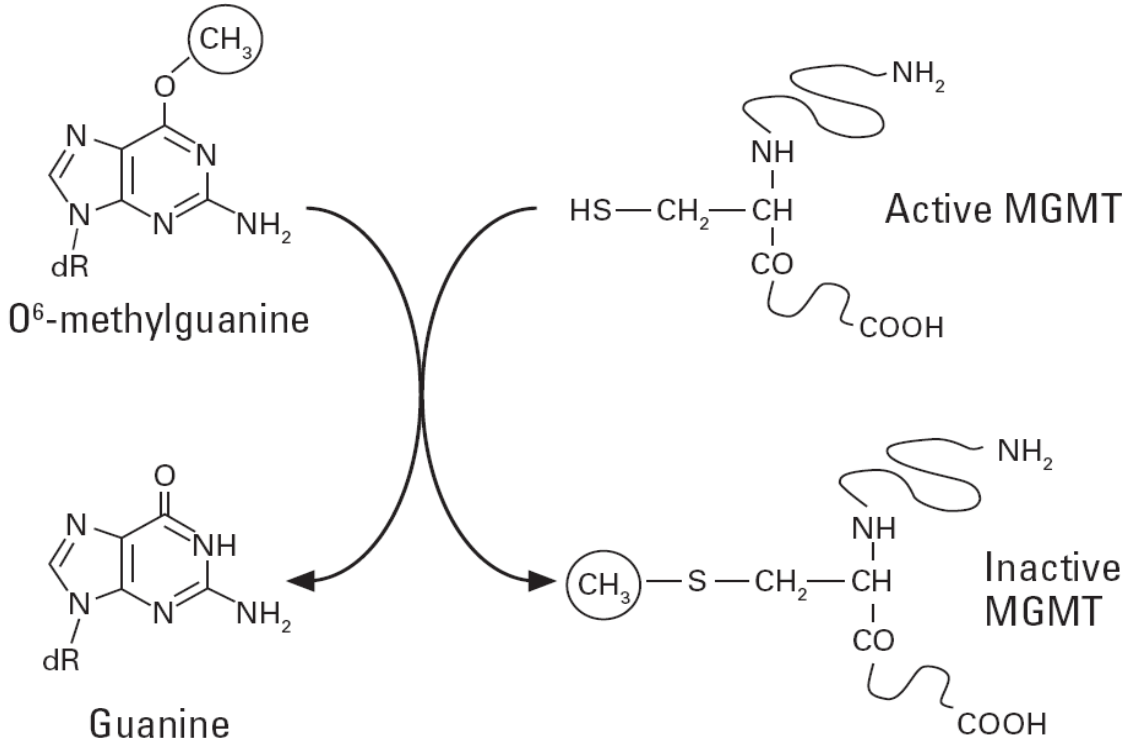
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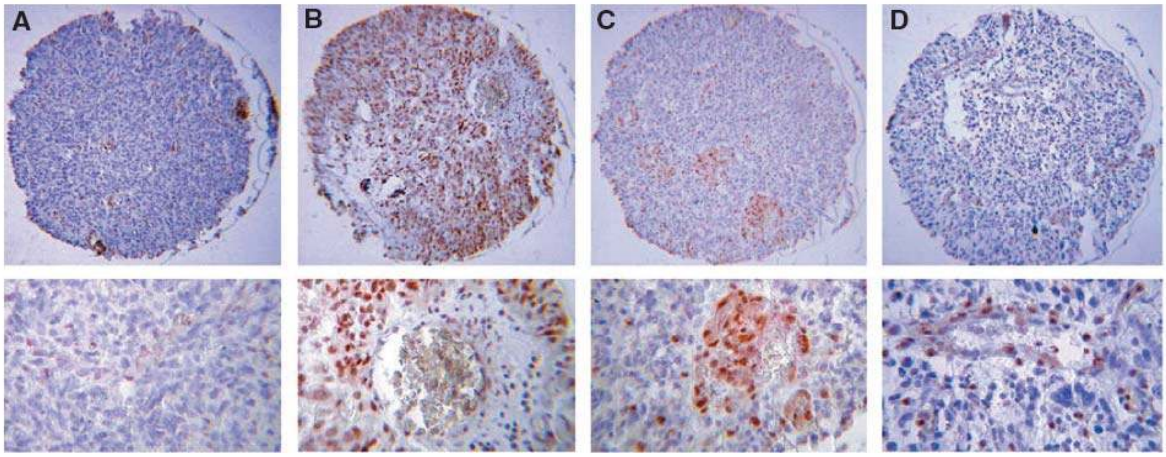
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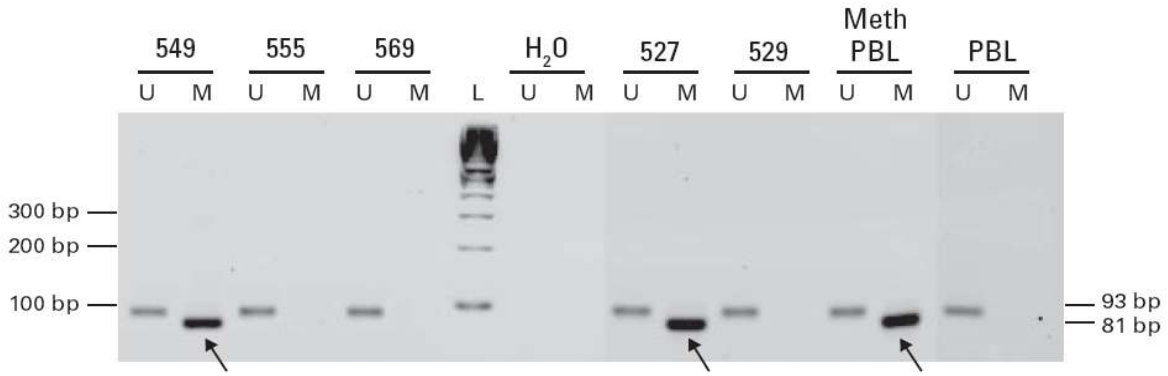
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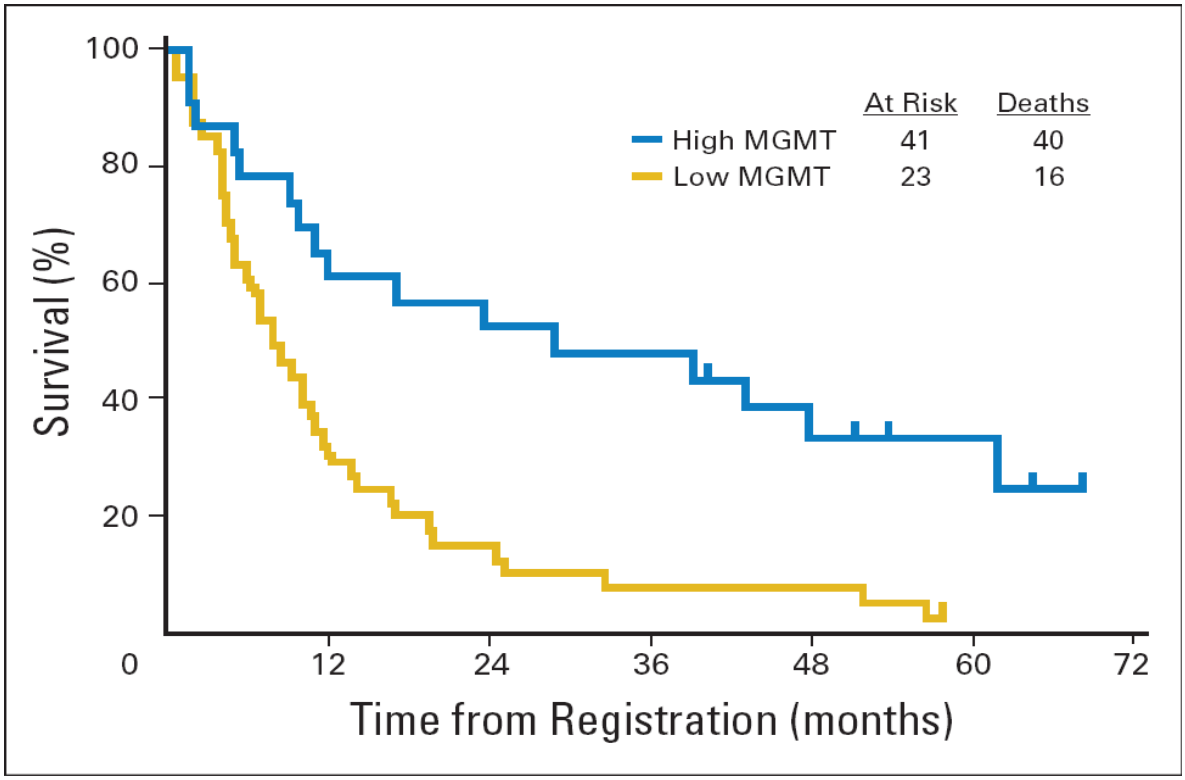
100
80
60
40
20
0
Survival (%)
At Risk
Deaths
High MGMT
41
40
Low MGMT
23
16
12
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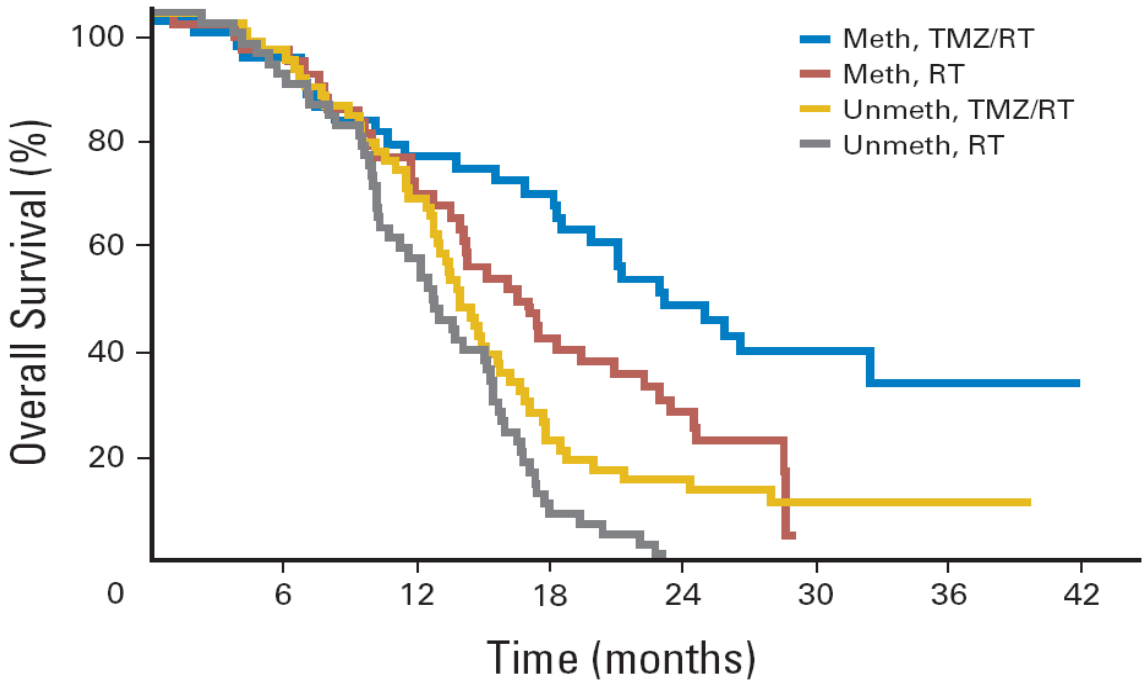
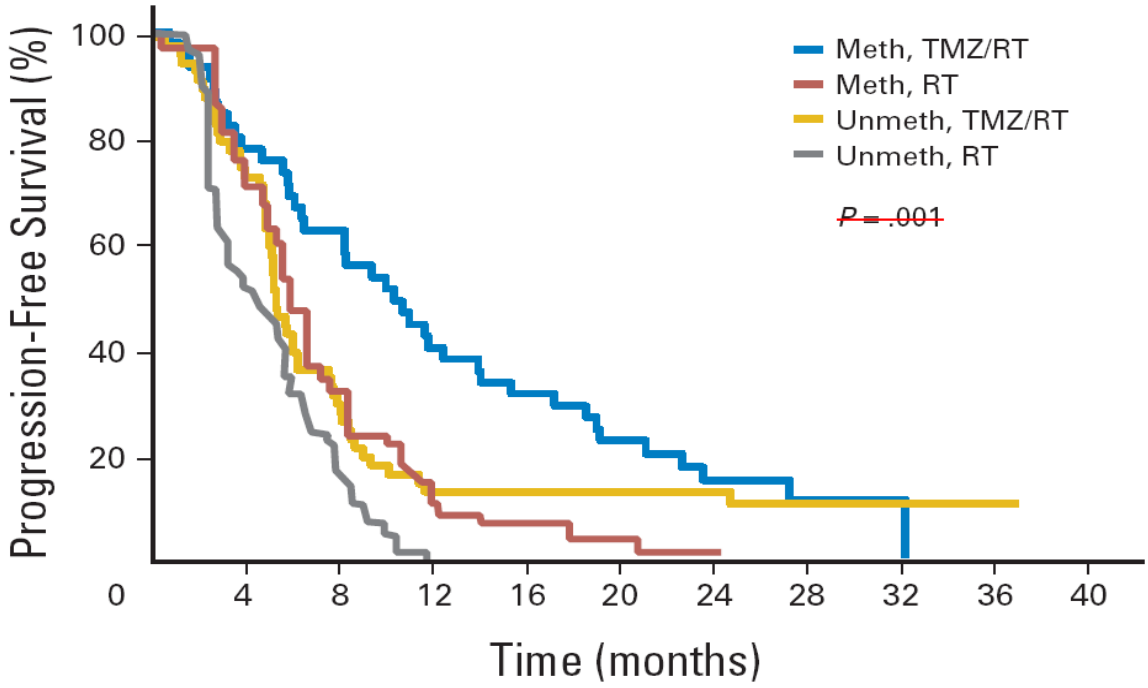
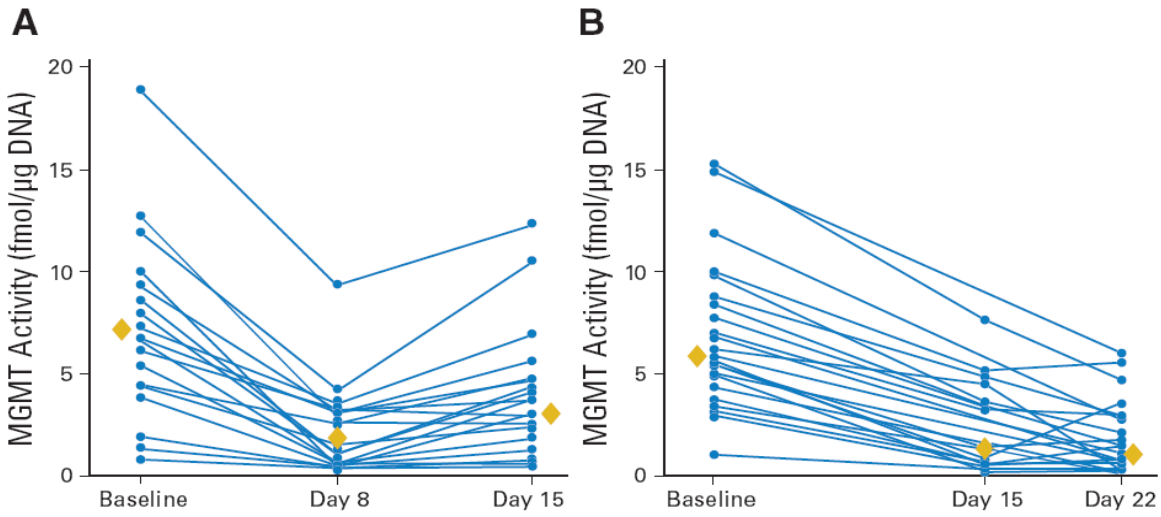
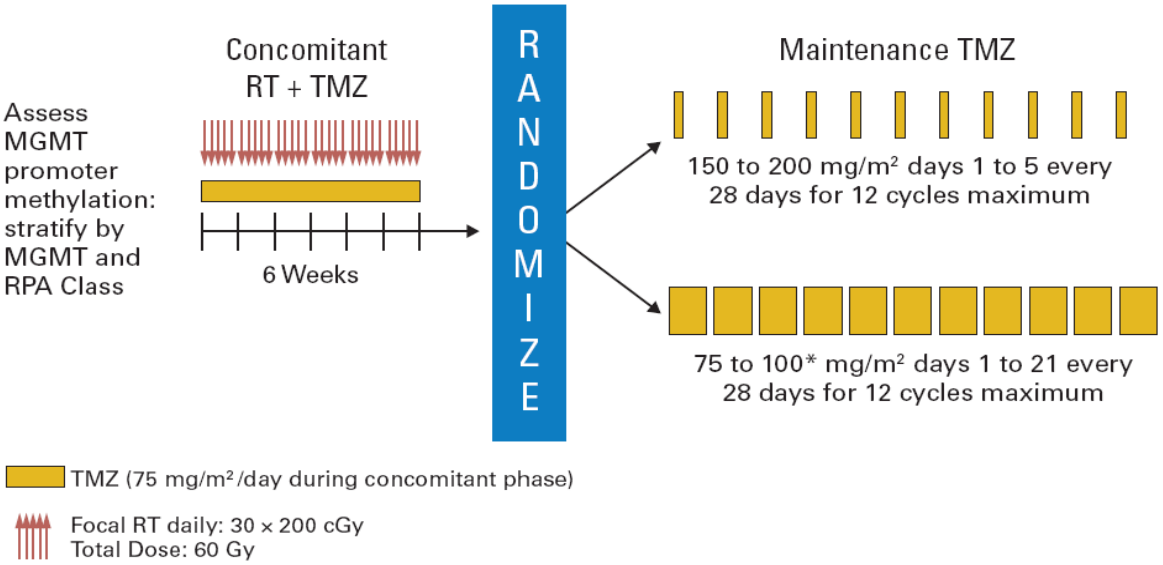
48
60
72
Time from Registration (months)


Meth, TMZ/RT
Meth, RT
Unmeth, TMZ/RT
Unmeth, RT
Overall Survival (%)
100
80
60
40
20
0
6
12
18
24
30
36
42
Time (months)


Progression-Free Survival (%)
100
80
60
40
20
0
4
8
12
16
20
24
28
32
36
40
Time (months)
Meth, TMZ/RT
Meth, RT
Unmeth, TMZ/RT
Unmeth, RT
P = .001


A
B
MGMT Activity (fmol/µg DNA)
20
15
10
5
0
Baseline
Day 8
Day 15
MGMT Activity (fmol/µg DNA)
20
15
10
5
0
Baseline
Day 15
Day 22


Assess MGMT promoter methylation: stratify by MGMT and RPA Class
Concomitant RT + TMZ
6 Weeks
RANDOMIZE
Maintenance TMZ
150 to 200 mg/m² days 1 to 5 every 28 days for 12 cycles maximum
75 to 100* mg/m² days 1 to 21 every 28 days for 12 cycles maximum
TMZ (75 mg/m²/day during concomitant phase)
Focal RT daily: 30 × 200 cGy
Total Dose: 60 Gy