Open access • Journal Article • DOI:10.1038/NRNEUROL.2009.197

MGMT promoter methylation in malignant gliomas: ready for personalized medicine? — Source link

Michael Weller, Roger Stupp, Guido Reifenberger, Alba A. Brandes ...+3 more authors

Institutions: University of Zurich, University of Lausanne, University of Düsseldorf, Erasmus University Rotterdam ...+1 more institutions

Published on: 01 Jan 2010 - Nature Reviews Neurology (Nature Publishing Group)

Topics: Glioma

MGMT promoter methylation in malignant gliomas: ready for personalized medicine?

Weller, M; Stupp, R; Reifenberger, G; Brandes, A A; van den Bent, M J; Wick, W; Hegi, M E

MGMT promoter methylation in malignant gliomas: ready for personalized medicine?

Michael Weller, Roger Stupp, Guido Reifenberger, Alba A. Brandes, Martin J. van den Bent, Wolfgang Wick and Monika E. Hegi

ABSTRACT

The DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) antagonizes the genotoxic effects of alkylating agents. *MGMT* promoter methylation is the key mechanism of *MGMT* gene silencing and predicts a favorable outcome in patients with glioblastoma who are exposed to alkylating agent chemotherapy. This biomarker is on the verge of entering clinical decision-making and is currently used to stratify or even select glioblastoma patients for clinical trials. In other subtypes of glioma, such as anaplastic gliomas, the relevance of *MGMT* promoter methylation might extend beyond the prediction of chemosensitivity, and could reflect a distinct molecular profile. Here, we review the most commonly used assays for evaluation of *MGMT* status, outline the prerequisites for standardized tests, and evaluate reasons for difficulties in reproducibility. We critically discuss the prognostic and predictive value of *MGMT* silencing, reviewing trials in which patients with different types of glioma were treated with various chemotherapy schedules, either upfront or at recurrence. Standardization of *MGMT* testing requires comparison of different technologies across laboratories, and prospectively validated cut-off values for prognostic or predictive effects. Moreover, future clinical trials will need to determine, for each subtype of glioma, the degree to which *MGMT* promoter methylation is predictive or prognostic, and whether testing should become routine clinical practice.

Weller, M. *et al. Nat. Rev. Neurol.* advance online publication XX Month 2009; doi:10.1038/

Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, CH-8091 Zurich, Switzerland (**M. Weller**). Department of Neurosurgery, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Rue du Bugnon 46, CH-1011 Lausanne, Switzerland (**R. Stupp, M . E. Hegi**). Department of Neuropathology, Heinrich-Heine-University, Moorenstrasse 5, D-40225 Düsseldorf, Germany (**G. Reifenberger**). Department of Medical Oncology, Bellaria Hospital, Via Altura 3, 40139 Bologna, Italy (**A. A. Brandes**). Neuro-Oncology Unit, Daniel de Hoed Cancer Centre/Erasmus, University Hospital Rotterdam, 3008AE Rotterdam, The Netherlands (**M. J. van den Bent**).Department of Neurooncology, University of Heidelberg, Im Neuenheimer Feld 400, D-69120 Heidelberg, Germany (**W. Wick**).

Correspondence to: M. Weller, michael.weller@usz.ch

Competing interests

M. Weller, R. Stupp and M. E. Hegi declare associations with the following companies: Merck Serono, OncoMethylome Sciences, Schering-Plough. G. Reifenberger declares associations with the following companies: Essex Pharma, OncoMethylome Sciences, Miltenyi Biotec. A. A. Brandes declares associations with the following companies: OncoMethylome Sciences, Schering-Plough. M. van den Bent declares associations with the following companies: Merck AG, Oncomethylome Sciences, Schering-Plough.

W. Wick declares associations with the following companies: Merck Serono, Schering-Plough. See the article online for full details of the relationships.

Key points

- *MGMT* (O^6-methylguanine-DNA methyltransferase) promoter methylation has become the most powerful molecular prognosticator in malignant gliomas
- *MGMT* promoter methylation is predictive for response to alkylating agent chemotherapy in glioblastoma
- Methylation-specific PCR is the only validated technique to derive prognostic information from determination of the *MGMT* status
- The *MGMT* status has become a parameter for stratification of patients with glioma within clinical trials

INTRODUCTION

O^6-methylguanine-DNA methyltransferase (MGMT) is a ubiquitous DNA repair enzyme that has been highly conserved throughout evolution. MGMT is associated with resistance to alkylating agent cancer therapy, and modulation of this enzyme as a treatment target has been under investigation for over 2 decades.[1,2] MGMT rapidly reverses alkylation, including methylation, at the O^6 position of guanine by transferring the alkyl group to the active site of the enzyme.[3] Although O^6-alkylguanine is not the main lesion induced by alkylating agents, it seems to be the most cytotoxic one. Lack of MGMT in the cell allows accumulation of O^6-alkylguanine in the DNA, which, subsequent to incorrect pairing with thymidine, triggers mismatch repair, thereby inducing DNA damage signaling and, eventually, cell death.[4,5] In accordance with this postulated mechanism, mismatch repair-deficient cells are highly resistant to alkylating agents, even in the absence of MGMT.

In this article, we critically review the prognostic and predictive value of *MGMT* silencing in gliomas, drawing on the results of trials in which various chemotherapy schedules were used to treat patients with these tumors. We discuss the assays that are most commonly used to evaluate *MGMT* status, outline the prerequisites for standardized tests, and consider possible reasons for difficulties in reproducibility.

THE *MGMT* GENE AND ITS PROMOTER

The *MGMT* gene is located on chromosome 10q26. Its promoter lacks the constitutive regulatory elements known as the TATA box and the CAT box, similar to many housekeeping genes, and contains a CpG island. CpG islands are genomic regions, typically of 300–3000 bp, that contain a high frequency of CG dinucleotides (CpG sites),

and are often located in the vicinity of the transcription initiation site. The region required for maximal promoter activity lies at the 5' end of the gene (from bps –953 to +202; transcription initiation site +1 bp) and comprises a minimal promoter, an enhancer region to which the *MGMT* enhancer-binding protein (MEBP) binds, and a number of transcription factor binding sites, such as those for Sp1 and AP1 (Figure 1). Expression levels of *MGMT* vary considerably between organs, with relatively low levels in the brain and the highest levels in the liver. Tumors frequently exhibit higher levels of expression than do their tissue of origin.[1]

The CpG island is located in the 5' region of *MGMT* (bps –552 to +289) and includes 97 CpGs (Figure 1), which are usually unmethylated in normal tissues. Methyl-CpG-binding proteins, such as methyl-CpG-binding protein 2 (MeCP2) and methyl-CpG-binding domain protein 2 (MBD2), bind to aberrantly methylated sequences, leading to alterations of chromatin structure and preventing binding of transcription factors, thereby silencing the gene (Figures 1 and 2a).[16] Some studies have provided insight into the relationship between gene expression and the patterns and localization of dense CpG methylation in the *MGMT* promoter.[16,17] Two regions that are prone to high levels of methylation have been identified, of which the region comprising the enhancer element seems to be more critical for the loss of *MGMT* gene expression upon methylation, on the basis of luciferase reporter assays interrogating different regions of the methylated promoter.[16,17] Hence, most methylation-specific tests are designed to interrogate this region (Figure 2b).

MGMT PROMOTOR METHYLATION IN GLIOMAS

The first striking observations on a potential predictive value of MGMT protein levels, as determined by immunofluorescence microscopy, in patients with malignant glioma were made more than 10 years ago.[6,7] Patients with low levels of MGMT seemed to derive considerably more benefit from carmustine (BCNU) than those with high levels. Similarly, low levels of MGMT protein, as detected by immunohistochemistry, predicted prolonged progression-free survival (PFS) in patients with glioma treated upfront with temozolomide,[8] or prolonged overall survival in patients with newly diagnosed, inoperable glioblastoma treated with neoadjuvant temozolomide.[9]

Decreased levels of MGMT protein can be attributed to epigenetic silencing mediated by *MGMT* gene promoter methylation, which can be assessed by a simple methylation-specific PCR (MSP). A correlation with survival was demonstrated when glioma patients were treated with nitrosoureas[10] or temozolomide,[11] strongly suggesting that *MGMT* promoter methylation assessment could provide a prognostic or predictive biomarker for benefit from alkylator-based chemotherapy added to radiotherapy. Subsequently, in the randomized European Organisation for Research and Treatment of Cancer (EORTC) 26981-22981–National Cancer Institute of Canada (NCIC) CE.3 trial,[12,13] *MGMT* promoter methylation was shown to predict prolonged PFS specifically in patients treated with temozolomide and radiotherapy, consistent with the idea that methylation predicts benefit from alkylating agent chemotherapy rather than simply being yet another prognostic marker. By contrast, only a slight trend was observed towards longer PFS in patients with methylated versus unmethylated tumors who were treated with radiotherapy alone.[13] This study used a nonquantitative gel-based MSP assay and dichotomized patients into methylated and unmethylated groups (45% versus 55%). Quantitative assays, such as real-time, quantitative PCR (qMSP), suggest that a

subgroup of patients with intermediate methylation exists, representing a 'gray zone' in the test results. This observation could account for the fact that some patients with *MGMT*-methylated tumors seemed to derive no benefit from temozolomide, whereas some patients in the unmethylated group did benefit from the treatment. The modest effect of temozolomide in patients lacking *MGMT* promoter methylation has provoked an ongoing discussion as to whether *MGMT* testing should be made mandatory, and whether temozolomide should be withheld from patients with tumors that lack *MGMT* promoter methylation.

The possible predictive value of *MGMT* promoter methylation specifically for the benefit derived from alkylating chemotherapy in glioblastoma patients has recently been challenged in patients with anaplastic glioma. A trial by the German Cancer Society's Neuro-Oncology Working Group (NOA-04) showed prolonged PFS and overall survival in WHO grade III anaplastic glioma patients with *MGMT* promoter methylation, irrespective of initial treatment with radiotherapy or alkylating agent chemotherapy, temozolomide or procarbacin, CCNU and vincristin (PCV).[14] Similarly, in the EORTC trial 26951 on adjuvant PCV chemotherapy, PFS was prolonged in patients whose tumors showed *MGMT* promoter methylation independent of the administration of alkylating agent chemotherapy.[15] Since no evidence exists that MGMT is involved in the repair of radiation-induced DNA damage, other, as yet unknown, genetic alterations associated with *MGMT* promoter methylation and predictive for sensitivity to irradiation could be operating in *MGMT*-methylated anaplastic gliomas. These findings raise the question of whether *MGMT* promoter methylation is merely an epiphenomenon of other important predictive and prognostic markers, and they also underline the idea that grade III and grade IV gliomas need to be studied as separate entities.

Assessing *MGMT* status in tumor tissue

The potential clinical utility of *MGMT* status as a biomarker in gliomas has led to an ongoing debate regarding how this status should be assessed—by promoter methylation analysis, at the level of mRNA or protein expression, or by enzyme activity—and which specific procedure is best suited for routine clinical applications.[18,19] A biomarker test for *MGMT* status needs to be standardized, suitable for high-throughput analyses and reproducible in independent laboratories, and must have a clinically relevant cut-off point. The test should allow prospective patient selection and individualized therapy, thereby pursuing the strategy of personalized medicine for patients with brain tumors.

Enzyme activity

The enzymatic activity of MGMT can be assessed in cell lysates from freshly resected or frozen tumor tissue. The main drawbacks to this approach are the potential contamination by non-neoplastic cells,[20] and the requirement for rapid and standardized processing of the samples. By means of an assay that measured the transfer of ^{3}H-labeled methyl groups from the O^6 position of guanine to protein in the cell extract, MGMT activity was shown to be increased in recurrent tumors specifically in patients who had received alkylating agent chemotherapy.[21,22] However, such data have not been generated in a controlled, prospective manner and with parallel assessment of *MGMT* promoter methylation.

Immunohistochemistry

The early studies that used immunofluorescence detection in malignant gliomas,[6,7] as well as more-recent studies in progressive low-grade oligodendroglial tumors[23] or newly diagnosed glioblastoma,[9] reported that low MGMT protein levels had predictive value for the response to alkylating agents. The clinical value of immunohistochemical detection of MGMT protein in human gliomas, however, remains controversial for several reasons. First, MGMT assessment by immunochemical techniques has failed to correlate consistently with outcome.[18,24] Second, a high interobserver variablility, even among expert neuropathologists, casts doubt on the reproducibility of this method of assessment.[18] Third, many authors have failed to identify a correlation between *MGMT* promoter methylation assessed by MSP and protein levels in glioma tissue assessed by immunochemistry.[18,24-26] One explanation for this lack of correlation is the considerable and highly variable contamination of glioma tissue sections with non-neoplastic cells expressing MGMT that are not always easy to distinguish from tumor cells (Figure 3). Furthermore, published cut-off levels employed to define low versus high MGMT expression are highly variable (ranging from >10% to >50% positive cells).[18] The extent to which such variation accounts for the conflicting results remains unknown.

mRNA expression

MGMT mRNA levels can be determined in fresh surgical specimens, although contamination by mRNA from non-neoplastic cells makes the results difficult to interpret. *In situ* hybridization might circumvent this problem, but specific delineation of tumor cells, as well as careful and uniform handling of the samples to prevent degradation, remains a challenge, as does any effort at quantification.

MGMT promoter methylation

The widespread recognition of the value of the *MGMT* promoter methylation status derives from its identification as a predictive marker for prolonged PFS and overall survival in temozolomide-treated patients in the EORTC–NCIC study. This study used a gel-based MSP assay that is now widely employed.[13,27,28]

Diagnostic methylation-specific assays aim at predicting the activity of the whole *MGMT* promoter by interrogating only a fraction of the CpGs for their methylation status. In other words, the test needs to predict overall dense promoter methylation that is associated with silencing of the gene (Figure 2a). The CpGs interrogated by different methylation-specific assays are depicted in Figure 2b. The principle for the discrimination of unmethylated from methylated sequences that is used by most methylation-specific assays is based on a bisulfite treatment step that converts unmethylated cytidine—but not 5-methylcytidine—in the DNA to uracil (Figure 4). Subsequent detection and quantification of the methylated and unmethylated sequences can be performed by various technologies to create semiquantitative or quantitative assays (Table 1).

MSP is the most commonly used technology at present (Tables 2 and 3). This technique uses methylation-specific primers, each of which is designed to bind only to completely methylated or unmethylated sequences. Each primer typically interrogates a series of three to five CpGs.[27–29] MSP can be performed using real-time PCR platforms that allow standardization, high-throughput analysis, and definition of cut-off points.[30,31]

Other quantitative or semiquanitative methods include methylation-specific pyrosequencing, which interrogates between 4 and 12 CpGs,[32] and methylation-specific

clone sequencing. Restriction enzymes that differentiate between methylated and unmethylated sequences are used in combined bisulfite restriction analysis (COBRA)[32] and methylation-specific multiplex ligation-dependent probe amplification (MS-MPLA).[33] The latter technique does not depend on bisulfite conversion. Recently developed technologies that analyze bisulfite-converted DNA include methylation-sensitive high-resolution melting (MS-HRM; a PCR-based method that differentiates the melting behavior of the amplicons derived from methylated and unmethylated sequences),[34] bead array-based technologies,[35] mass spectroscopy,[36] and denaturing high-performance liquid chromatography.[37]

Each technology must define a cut-off point for the prognostic effect of the *MGMT* methylation status, which needs to be validated prospectively. Quantitative assays are more amenable to definition of technical cut-off points and quality control than are qualitative assays, as illustrated by qMSP.[29,31] Completely quantitative or semiquantitative assays that normalize to a control gene or the copy number of the unmethylated *MGMT* promoter sequence might underestimate *MGMT* methylation, because contaminating nontumoral tissue will contribute to the signal of the normalizing gene. The tissue used for DNA isolation must, therefore, be macrodissected by the neuropathologist, so as to avoid infiltration zones, lymphocyte infiltrates, and regions dominated by vascular proliferation. Samples consisting of compact tumor tissue of sufficient size; for example, four paraffin sections with a compact tumor surface of 0.5x1 cm, generally provide good results. Stereotactic biopsies should be controlled for tumor content, and usually only yield sufficient DNA when obtained frozen.[25] Tissues should be fixed in buffered formalin. Overfixation decreases the quality of the DNA owing to formation of cross-links, and can impede successful testing.

Bisulfite conversion is the most critical step, since incomplete conversion yields an apparently 'methylated' CpG. The procedure should, therefore, be controlled for completion of the reaction.[29] A number of commercial kits work reliably. Bisulfite-treated DNA is unstable and should be used rapidly, although storage at –20°C in aliquots will slow down the decay.

To date, MSP is the only test that has repeatedly been shown to be of predictive or prognostic value in clinical trials (Tables 2 and 3).[11,38] At present, qMSP[31] is being used for patient selection in the CENTRIC trial (http://clinicaltrials.gov, NCT00689221). This trial is assessing a role for cilengitide in newly diagnosed glioblastoma in light of promising phase II data indicating that the activity of this drug is restricted to patients with *MGMT* promoter methylation (Stupp, R. *et al.*, unpublished work). Furthermore, on the basis of a strong preclinical rationale, the activity of enzastaurin and radiotherapy in newly diagnosed glioblastoma is being assessed only in patients with an unmethylated *MGMT* promoter.[39] The results of prospective validation of a qMSP-based test,[31] in a randomized phase III trial of glioblastoma patients treated with temozolomide in the Radiotherapy Oncology Group (RTOG) 0525 EORTC Intergroup trial (http://clinicaltrials.gov, NCT00304031), are expected by early 2010. The quantitative evaluation of *MGMT* methylation in this trial is expected to provide a clinically relevant cut-off point, as opposed to the technical cut-off already defined.[31] Validation of other technologies is awaited. Given the large variation of 30–60% *MGMT* methylation reported in the literature for glioblastoma, clinical validation of cut-offs for individual tests is crucial. Methylation testing performed outside the academic trial context should also follow a specified protocol that lends itself to independent reproduction. In the future, the use of prospectively validated tests should become standard.

THE ROLE OF MGMT IN GLIOMA SUBTYPES

Glioblastoma

The *MGMT* promoter methylation status, as determined by MSP, is the strongest prognostic factor for outcome in patients with newly diagnosed glioblastoma, and is a powerful predictor of response to alkylating chemotherapy (Table 2).[10,11,13,38,40,41] The 2 year and 5 year survival rates in patients with a methylated *MGMT* promoter treated with concomitant and adjuvant temozolomide were 49% and 14%, respectively, while the corresponding figures for patients initially treated with radiotherapy only were 24% and 5%. Of patients with an unmethylated *MGMT* promoter, 15% and 8% were alive at 2 years and 5 years, respectively, after treatment with combined chemoradiotherapy, compared with 2% and 0% in those initially treated with radiotherapy alone.[42] The small improvement in outcome even in the patients with an unmethylated *MGMT* promoter could be attributable to the above-mentioned gray zone separating methylated and unmethylated tumors, and the consequent somewhat arbitrary separation into two groups. Incorrect test results, misdiagnosis of some lower grade gliomas, differences in post-progression therapy, and individual variability due to other, as yet unrecognized, prognostic factors could also explain the marginally improved outcome of combined-modality treatment in patients with an unmethylated *MGMT* promoter.

The prognostic relevance of *MGMT* promoter status has been confirmed in elderly glioblastoma patients treated with concomitant and adjuvant temozolomide.[43] In addition, the observation that 74% of patients with glioblastoma who survive for >5 years have *MGMT* promoter methylation—as opposed to <50% in an unselected population of glioblastoma patients—also underlines the prognostic value of *MGMT* status.[44]

Nevertheless, long-term survival can be observed even in the absence of *MGMT* promoter methylation, indicating that *MGMT* promoter methylation is only one aspect of a very complex biological system.[44,45]

A study published this year proposed that the extent of *MGMT* methylation, as measured by pyrosequencing, is a prognostic factor in glioblastoma patients treated with temozolomide and radiotherapy.[46] Patients with >29% *MGMT* methylation over the 12 CpG sites measured had a significantly better outcome than patients with >9% but ≤ 29% methylation, a clinically interesting finding that will need prospective validation. The authors defined 9% methylation as the cut-off point for determining outcomes between methylated and unmethylated tumors **[Au: OK?; yes]**. This cut-off for methylation was considered to be statistically different from the background determined in non-neoplastic brain. Leaving aside the possibility that methylation detected at a low level could reflect a false-positive result, a low methylation average could, in some cases, signify the presence of only a few methylated CpGs, as suggested by the unsupervised analysis of the *MGMT* methylation pattern used in this study. Such low positive scores might not necessarily reflect the dense methylation that is required for silencing of the gene. In comparison, MSP would only recognize dense methylation and would not detect small numbers of methylated CpGs, highlighting the different information content of the results rendered by these two technologies.

Gelöscht: , a clinically interesting finding that will need prospective validation

In contrast to newly diagnosed glioblastoma, the predictive value of *MGMT* promoter methylation has remained controversial in recurrent glioblastoma. The absence of a strong predictive effect on tumor response or outcome with various temozolomide administration schedules[47–49] suggests that MGMT-independent mechanisms of resistance have a predominant role in the setting of recurrent

glioblastoma. Nevertheless, patients with *MGMT*-methylated tumors still showed improved survival in these series, although no such effect was seen in a recent study from Belgium.[50] Selection for loss of mismatch repair proteins, such as MSH6, could be involved in a minority of patients.[51–53] *In vivo* evidence for the direct involvement of MGMT in the response of glioblastoma to alkylating agents has been provided by The Cancer Genome Atlas.[54] Mutation analyses of 601 genes in 91 matched tumor and normal samples identified a hypermutator phenotype in the recurrent glioblastoma of a subset of 7 of 19 patients pretreated with alkylating agents. This phenotype was much more common in tumors with a methylated *MGMT* promoter (6 of 6 *MGMT*-methylated cases) than those with an unmethylated promoter. Moreover, in all 6 treated and *MGMT*-methylated glioblastomas that were hypermutated, at least one of the mismatch repair genes *MLH1*, *MSH2*, *MSH6* and *PMS2* was mutated, compared with only 1 of 84 non-hypermutated, untreated glioblastomas. The gene mutation pattern, including mutations of the mismatch repair genes, was different in the six patients whose glioblastoma carried a methylated *MGMT* promoter from the pattern seen in unmethylated and treated tumors (n=13). This discovery was compatible with a deficiency in repair of alkylated guanine residues, as reflected in a strong predominance of G:C to A:T transitions at non-CpG sites (146/181 [81%] mutations in the 6 treated, *MGMT*-methylated patients versus 29/99 [29%] in the 13 treated, *MGMT*-unmethylated patients). These findings are consistent with escape from *MGMT* methylation-mediated sensitivity to the alkylating drug by selection for mismatch repair deficiency.

The value of temozolomide in the setting of recurrent glioblastoma, including its relationship with *MGMT* promoter methylation status, must now to be determined in

patients who have already been exposed to temozolomide in the first-line setting,[55,56] as is being pursued in the DIRECTOR trial (http://clinicaltrials.gov, NCT00941460).

Anaplastic glioma

The NOA-04 trial showed no difference in PFS or overall survival between patients with anaplastic glioma started on radiotherapy alone and patients started on temozolomide or PCV alone. Interestingly, *MGMT* promoter methylation predicted prolonged PFS irrespective of the initial treatment.[14] Similar results were obtained in the EORTC trial 26951, in that *MGMT* promoter methylation was prognostic for PFS in both arms—radiotherapy alone and radiotherapy followed by PCV.[15] The high correlation of *MGMT* promoter methylation with the 1p19q co-deletion[15,57,58] and isocitrate dehydrogenase (*IDH*) gene mutations,[59] which are known to be favorable prognostic factors in anaplastic glioma,[14,60,61] might indicate that epigenetic deregulation of *MGMT* occurs in a specific pathogenetic context in anaplastic gliomas. Since *MGMT* promoter methylation is prognostic and not predictive for chemotherapy response in anaplastic gliomas, a methylated *MGMT* promoter should not be used to justify the upfront treatment of these tumors with temozolomide-based radiochemotherapy in the absence of appropriate data from studies such as CATNON (http://clinicaltrials.gov, NCT00626990).

Low-grade glioma

An initial study on 49 patients reported that *MGMT* promoter methylation is a negative prognostic factor for PFS in patients with low-grade astrocytomas.[62] The population was, however, mixed, in that approximately one-quarter of the patients were untreated after surgery, one-quarter received radiotherapy, and half received interferon only. By

contrast, protracted treatment with temozolomide in a phase II study in low-grade glioma showed improved outcome in patients with *MGMT* promoter methylation.[63] One might speculate that the differences in outcome attributed to the *MGMT* status were, in fact, due to the alkylating agent therapy that was present in the latter study. Both *MGMT* promoter methylation[64] and low MGMT protein levels[23] were reported to predict a favorable response to temozolomide in low-grade oligodendrogliomas. In addition, a correlation exists between *MGMT* promoter methylation and the 1p19q co-deletion and mutations of the *IDH1* gene in these tumors,[57,59] as well as in anaplastic gliomas[14,59] (see above). Which of these changes, or other aberrations yet to be identified, contribute most to the chemosensitivity of these tumors remains to be elucidated.

Pseudoprogression, relapse patterns and *MGMT*

Gelöscht: status [Au: OK? Level 2 subheadings can be no longer than 45 characters, including spaces]

The idea that *MGMT* methylation status might have clinical relevance was supported by the analysis of peculiar false-positive neuroradiological patterns mimicking early disease progression in patients after radiochemotherapy involving temozolomide. These patterns, termed 'pseudoprogression', are usually seen in the first 3 months after completion of radiotherapy with concomitant chemotherapy, and may be more common in patients with *MGMT*-methylated tumors, possibly signifying the extent of cytotoxic effects of treatment.[65]

Some have speculated that the increase in overall survival observed in patients with glioma treated with temozolomide concomitant with radiotherapy could be associated with altered patterns of relapses. For many years, relapse inside the radiotherapy field in 90% of all cases has been the rule for patients with glioblastoma. A recent analysis of the EORTC 26981–22981-NCIC CE.3 trial showed a frequency for

distant recurrences of 20%.[66] This analysis, which was carried out using a novel observer-independent tool, demonstrated no differences in the recurrence pattern (distant versus local) according to either therapy or *MGMT* status. In another series, *MGMT* promoter methylation was associated with an increased frequency of distant recurrences, defined as recurrences with ≤20% enhancing tumor residing inside the 95% isodose of the radiation field.[67] The best methodology to determine this clinically relevant point needs to be confirmed prospectively.

A ROLE FOR ROUTINE *MGMT* TESTING?

MGMT promoter methylation is now recognized to be a relatively early molecular lesion in the pathogenesis of gliomas. The frequencies of methylation seem to be specific to the glioma subtype and malignancy grade, as depicted in Figure 5. Whether a methylated *MGMT* promoter has the same relevance in all types of gliomas and for all grades of malignancy, however, remains a matter for debate. In glioblastoma, for example, in contrast to all other glioma subtypes, one *MGMT* allele is frequently lost by deletion of one copy of chromosome 10. Loss of one allele plus methylation is likely to have more profound effects on MGMT expression than methylation alone.

The true value of *MGMT* promoter methylation as a diagnostic and prognostic marker suitable for treatment decisions depends in part on the answers to several crucial questions. First, which area of the gene is most relevant for silencing through methylation? Second, how do we set the clinically relevant cut-off point in quantitative assays? Third, how homogeneous is the *MGMT* promoter methylation pattern within a given tumor? Last, how stable is the methylation pattern throughout the course of disease and on disease progression? Importantly, at present we have no appropriate

alternative treatments for patients whose *MGMT* promoter methylation profile does not suggest a substantial benefit from alkylating agent chemotherapy. Withholding such treatment on the basis of this profile, therefore, would seem to be inappropriate at this stage.

A thorough analysis of two to four biopsy specimens from each of 25 WHO grade III or IV gliomas revealed that *MGMT* promoter methylation is a highly homogeneous marker in malignant gliomas. MSP and sodium bisulfate sequencing showed identical results, and only one tumor showed inconsistent results between biopsies.[25]

Little is known regarding therapy-induced changes in *MGMT* promoter methylation or MGMT expression levels in the tumor. Neither dexamethsone nor irradiation induced *MGMT* gene transcription in glioma cells *in vitro*.[68] In tissue culture and animal studies, however, temozolomide strongly induced MGMT protein expression in temozolomide-resistant glioma cells lacking *MGMT* promoter methylation.[69]

Loss of *MGMT* promoter methylation might represent a key mechanism by which patients with initially methylated tumors eventually acquire resistance to temozolomide, leading to progression or relapse. Data on changes in methylation status, however, remain scarce. A study of 14 patients with initial low-grade astrocytoma histology showed that three patients acquired methylation at recurrence, but no initially methylated tumor lost its methylation.[62] Another small study reported changes in methylation status in three of ten patients, but a possible relationship with treatment was not explored.[70] Of ten patients treated with temozolomide chemoradiation in phase II or III trials,[11,13] eight tumors remained unchanged (one unmethylated and seven methylated), while one gained and another lost *MGMT* promoter methylation (Hegi, M. E. unpublished work). Similarly, an analysis of paired primary and recurrent glioblastoma tissue samples from

patients initially treated with radiotherapy and temozolomide by the German Glioma Network indicated that the *MGMT* promoter methylation status remained stable in the vast majority of patients (Reifenberger, G. and Weller, M., unpublished work). Hence, treatment resistance seems not to be associated with changes in the *MGMT* methylation status.

Taken together, the data summarized so far indicate that the determination of *MGMT* promoter methylation undoubtedly yields prognostic information, but is rarely useful for clinical decision-making in individual patients. Could *MGMT* testing have a role in clinical trials for patients with glioma? Several current trial concepts use the *MGMT* status, as determined by MSP, for stratification or as an inclusion criterion, limiting enrollment to patients either with methylated or unmethylated tumors. Moreover, in Europe at least, withholding temozolomide from patients with newly diagnosed glioblastoma without *MGMT* promoter methylation is considered to be justified in the context of clinical trials to test the effect of a new compound. Indeed, such an approach is being used in a current phase II study led by the EORTC, comparing radiotherapy plus temsirolimus versus radiotherapy plus temozolomide. This is view is not, however, shared by most neuro-oncologists in the US.

MGMT-DEPLETING STRATEGIES

The presence of MGMT as a key DNA repair protein is an undisputed mechanism of resistance to chemotherapy with alkylating agents. Strategies to overcome MGMT-mediated resistance have been and are being pursued. MGMT-depleting agents, such as O^6-benzylguanine (O^6-BG), act as a pseudosubstrate for MGMT, which in turn is consumed and subsequently targeted for proteasomal degradation. Thus, *de novo*

synthesis of MGMT is required to maintain DNA repair. Systemic application of O^6-BG was shown to decrease MGMT activity in glioma tissue when assessed 6 hours later, but was no longer effective at 18 hours,[71] suggesting that MGMT is rapidly resynthesized *in vivo*. Accordingly, multiple daily dosing would be necessary to maintain low MGMT activity. Furthermore, O^6-BG depletes MGMT nonselectively, resulting in substantial systemic toxicity, most notably dose-limiting myelosuppression, which necessitates substantial dose reductions for nitrosoureas or temozolomide. The clinical experience has, therefore, been disappointing overall in recurrent glioblastoma, although some responses (16% [5/32]) were reported in patients with anaplastic glioma.[72] The feasibility of administering O^6-BG locally into the tumor cavity via an Ommaya reservoir, in combination with systemic temozolomide, has been explored in a single patient.[73]

With the aim of increasing the dose-limiting tolerance of the bone marrow towards alkylating chemotherapy or inducing long-term selection of genetically modified hematopoietic stem and precursor cells (HSCs), HSCs were transduced with a retroviral vector that expresses the Pro140Lys mutant of MGMT (MGMT*), which confers resistance to inhibition by O^6-BG. Rhesus macaque experiments with $CD34^+$ HSCs demonstrated the feasibility of generating long-term repopulating MGMT* HSCs. O^6-BG plus temozolomide or BCNU treatment provided chemoprotection of progenitor cells but no selection of long-term repopulating HSCs.[74] A phase I clinical trial of this approach for patients with newly diagnosed glioblastoma or grade III astrocytoma was opened in 2006 (http://clinicaltrials.gov, NCT00272870), but was suspended owing to low recruitment levels and drug availability.

Since temozolomide is itself a substrate for MGMT, alternative, more-protracted dosing regimes of temozolomide have been explored in first-line and recurrent

glioblastoma settings. The RTOG–0525–EORTC Intergroup trial has set out to determine whether alternative dosing of temozolomide in the adjuvant phase after completion of the concomitant treatment phase, using a 3 weeks on–1 week off schedule, could overcome resistance to chemotherapy in the nonmethylated population in the first-line setting. Even if this trial proves to be positive for patients without *MGMT* methylation, the question of whether the success depended on a dose-intense temozolomide-dependent depletion of MGMT levels in the tumor cells still remains open. Two small phase II trials aiming at improving PFS and overall survival by intensifying alkylating agent treatments have resulted in clinical benefits only for patients with *MGMT* promoter methylation.[38,75,76] The use of dose-intense temozolomide in patients with recurrent disease resulted in PFS rates at 6 months of 30–45%,[47,48,55] suggesting superiority over conventional dosing using the 5 out of 28 days schedule (21%).[77] Notably, none of these studies have been able to demonstrate that the presumed superiority of dose-intense temozolomide regimens is truly mediated by MGMT depletion, because serial biopsies of brain tumors are ethically not feasible. Moreover, preliminary results of the UK Medical Research Council BR12 trial, which allocated patients with recurrent anaplastic astrocytoma or glioblastoma randomly, in a noncomparative design, to temozolomide for 5 out of 28 days or 21 out of 28 days, indicated an inferior outcome with the continuous adminstration schedule.

CONCLUSIONS

MGMT promoter methylation has emerged as an important molecular marker in patients with gliomas. Furthermore, the EORTC–NCIC trial has suggested that *MGMT* promoter methylation is not only a prognostic marker, but is also a predictive marker for response

to temozolomide in patients with newly diagnosed glioblastoma.[13] Emerging data indicate that *MGMT* promoter methylation has strong prognostic relevance following therapy with both radiation therapy and alkylating chemotherapy in patients with anaplastic glioma.[14,42] In this setting, *MGMT* promoter methylation is likely to be indicative of a broader molecular phenotype with prognostic significance. Correlative analysis with other prognostic molecular markers, such as the 1p19q co-deletion or *IDH1* and *IDH2* mutations, should further clarify the importance of *MGMT* promoter methylation in this patient population. In anticipation of the results of ongoing prospective phase III trials incorporating *MGMT* promoter methylation status, and validation of a diagnostic assay, treatment decisions should not yet be based on *MGMT* promoter methylation status outside clinical trials. However, all investigators conducting ongoing and future clinical trials in patients with glioma should consider assessing *MGMT* promoter methylation status, and probably including this factor as a stratification parameter.

Review criteria

The reference database MEDLINE served as the basis for the present Review. A literature search was performed for papers published in the English language up to August 2009. The keywords used were as follows: “glioma”, “MGMT”, “methylation”, “trial”, “alkylating agent”, “DNA repair”, and combinations thereof. The papers identified by this search were reviewed, as were references cited therein. The database available at http://clinicaltrials.gov—a registry of federally and privately supported clinical trials conducted in the US and around the world—was consulted for information on clinical

trials using the following keywords: “glioma”, “MGMT”, “methylation”, “temozolomide” and “alkylating agent”.

1. Gerson, S. L. *MGMT*: its role in cancer aetiology and cancer therapeutics. *Nat. Rev. Cancer*. **4**, 296–307 (2004).
2. Kaina, B., Christmann, M., Naumann, S. & Roos, W. P. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair (Amst.)* **6**, 1079–1099 (2007).
3. Pegg, A. E. Repair of O^6-alkylguanine by alkyltransferases. *Mutat. Res.* **462**, 83–100 (2000).
4. Ochs, K. & Kaina, B. Apoptosis induced by DNA damage O^6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. *Cancer Res.* **60**, 5815–5824 (2000).
5. Stojic, L. *et al.* Mismatch repair-dependent G_2 checkpoint induced by low doses of S_N1 type methylating agents requires the ATR kinase. *Genes Dev.* **18**, 1331–1344 (2004).
6. Belanich, M. *et al.* Retrospective study of the correlation between the DNA repair protein alkyltransferase and survival of brain tumor patients treated with carmustine. *Cancer Res.* **56**, 783–788 (1996).
7. Jaeckle, K. A. *et al.* Correlation of tumor O6 methylguanine-DNA methyltransferase levels with survival of malignant astrocytoma patients treated with bis-chloroethylnitrosourea: a Southwest Oncology Group study. *J. Clin. Oncol.* **16**, 3310–3315 (1998).
8. Friedman, H. S. *et al.* DNA mismatch repair and O6-alkylguanine-DNA alkyltransferase analysis and response to Temodal in newly diagnosed malignant glioma. *J. Clin. Oncol.* **16**, 3851–3857 (1998).
9. Chinot, O. L. *et al.* Correlation between O^6-methylguanine-DNA methyltransferase and survival in inoperable newly diagnosed glioblastoma patients treated with neoadjuvant temozolomide. *J. Clin. Oncol.* **25**, 1470–1475 (2007).
10. Esteller, M. *et al.* Inactivation of the DNA-repair gene *MGMT* and the clinical response of gliomas to alkylating agents. *N. Engl. J. Med.* **343**, 1350–1354 (2000).
11. Hegi, M. E. *et al.* Clinical trial substantiates the predictive value of *O-6-methylguanine-DNA methyltransferase* promoter methylation in glioblastoma patients treated with temozolomide. *Clin. Cancer Res.* **10**, 1871–1874 (2004).
12. Stupp, R. *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N. Engl. J. Med.* **352**, 987–996 (2005).
13. Hegi, M. E. *et al. MGMT* gene silencing and benefit from temozolomide in glioblastoma. *N. Engl. J. Med.* **352**, 997–1003 (2005).
14. Wick, W. *et al.* NOA-04 randomized phase III trial of sequential radiochemotherapy of anaplastic glioma with PCV or temozolomide. *J. Clin. Oncol.* (in press).
15. van den Bent, M. J. *et al. MGMT* promoter methylation is prognostic but not predictive for outcome to adjuvant PCV chemotherapy in anaplastic

oligodendroglial tumors: a report from EORTC Brain Tumor Group study 26951. *J. Clin. Oncol*. (in press).
16. Nakagawachi, T. *et al.* Silencing effect of CpG island hypermethylation and histone modifications on O6-methylguanine-DNA methyltransferase (*MGMT*) gene expression in human cancer. *Oncogene* **22**, 8835–8844 (2003).
17. Everhard, S. *et al.* Identification of regions correlating MGMT promoter methylation and gene expression in glioblastomas. *Neuro Oncol.* **11**, 348–356 (2009).
18. Preusser, M. *et al.* Anti-O6-methylguanine-methyltransferase (MGMT) immunohistochemistry in glioblastoma multiforme: observer variability and lack of association with patient survival impede its use as clinical biomarker. *Brain Pathol.* **18**, 520–532 (2008).
19. Hegi, M. E. *et al.* Correlation of O^6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. *J. Clin. Oncol.* **26**, 4189–4199 (2008).
20. Voelter, V. *et al.* Infrequent promoter methylation of the *MGMT* gene in liver metastases from uveal melanoma. *Int. J. Cancer* **123**, 1215–1218 (2008).
21. Silber, J. R. *et al.* O^6-methylguanine-DNA methyltransferase-deficient phenotype in human gliomas: frequency and time to tumor progression after alkylating agent-based chemotherapy. *Clin. Cancer Res.* **5**, 807-814 (1999).
22. Wiewrodt, D. *et al.* MGMT in primary and recurrent human glioblastomas after radiation and chemotherapy and comparison with p53 status and clinical outcome. *Int. J. Cancer* **122**, 1391–1399 (2008).
23. Levin, N. *et al.* Progressive low-grade oligodendrogliomas: response to temozolomide and correlation between genetic profile and O^6-methylguanine DNA methyltransferase protein expression. *Cancer* **106**, 1759–1765 (2006).
24. Rodriguez, F. J. *et al.* MGMT immunohistochemical expression and promoter methylation in human glioblastoma. *Appl. Immunohistochem. Mol. Morphol.* **16**, 59–65 (2008).
25. Grasbon-Frodl, E. M. *et al.* Intratumoral homogeneity of MGMT promoter hypermethylation as demonstrated in serial stereotactic specimens from anaplastic astrocytomas and glioblastomas. *Int. J. Cancer* **121**, 2458–2464 (2007).
26. Lavon, I. *et al.* Longitudinal assessment of genetic and epigenetic markers in oligodendrogliomas. *Clin. Cancer Res.* **13**, 1429–1437 (2007).
27. Herman, J. G., Graff, J. R., Myöhänen, S., Nelkin, B. D. & Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA* **93**, 9821–9826 (1996).
28. Esteller, M., Hamilton, S. R., Burger, P. C., Baylin, S. B. & Herman, J. G. Inactivation of the DNA repair gene *O^6-methylguanine-DNA methyltransferase* by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res.* **59**, 793–797 (1999).
29. Ogino, S. *et al.* Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. *J. Mol. Diagn.* **8**, 209–217 (2006).
30. Eads, C. A. *et al.* MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* **28**, E32 (2000).

31. Vlassenbroeck, I. *et al.* Validation of real-time methylation-specific PCR to determine O^6-methylguanine-DNA methyltransferase gene promoter methylation in glioma. *J. Mol. Diagn.* **10**, 332–337 (2008).
32. Mikeska, T. *et al.* Optimization of quantitative *MGMT* promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. *J. Mol. Diagn.* **9**, 368–381 (2007).
33. Jeuken, J. W. *et al.* MS-MLPA: an attractive alternative laboratory assay for robust, reliable, and semiquantitative detection of MGMT promoter hypermethylation in gliomas. *Lab. Invest.* **87**, 1055–1065 (2007).
34. Wojdacz, T. K. & Dobrovic, A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res.* **35**, e41 (2007).
35. Martinez, R. *et al.* A microarray-based DNA methylation study of glioblastoma multiforme. *Epigenetics* **4**, 255–264 (2009).
36. Coolen, M.W., Statham, A.L., Gardiner-Garden, M. & Clark, S.J. Genomic profiling of CpG methylation and allelic specificity using quantitative high-throughput mass spectrometry: critical evaluation and improvements. *Nucleic Acids Res.* **35**, e119 (2007).
37. Ezzeldin, H. H., Lee, A. M., Mattison, L. K. & Diasio, R. B. Methylation of the DPYD promoter: an alternative mechanism for dihydropyrimidine dehydrogenase deficiency in cancer patients. *Clin. Cancer Res* .**11**, 8699–8705 (2005).
38. Herrlinger, U. *et al.* Phase II trial of lomustine plus temozolomide chemotherapy in addition to radiotherapy in newly diagnosed glioblastoma: UKT-03. *J. Clin. Oncol.* **24**, 4412–4417 (2006).
39. Tabatabai, G. *et al.* Synergistic antiglioma activity of radiotherapy and enzastaurin. *Ann. Neurol.* **61**, 153–161 (2007).
40. Weller, M. *et al.* Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. *J. Clin. Oncol.* doi:10.1200/JCO.2009.23.0805
41. Gorlia, T. *et al.* Nomograms for predicting survival of patients with newly diagnosed glioblastoma: prognostic factor analysis of EORTC and NCIC trial 26981-22981/CE.3. *Lancet Oncol.* **9**, 29–38 (2008).
42. Stupp, R. *et al.* Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* **10**, 459–466 (2009).
43. Brandes, A. A. *et al.* Temozolomide concomitant and adjuvant to radiotherapy in elderly patients with glioblastoma: correlation with MGMT promoter methylation status. *Cancer* **115**, 3512–3518 (2009).
44. Krex, D. *et al.* Long-term survival with glioblastoma multiforme. *Brain* **130**, 2596–2606 (2007).
45. Murat, A. *et al.* Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. *J. Clin. Oncol.* **26**, 3015–3024 (2008).
46. Dunn, J. *et al.* Extent of MGMT promoter methylation correlates with outcome in glioblastomas given temozolomide and radiotherapy. *Br. J. Cancer* **101**, 124–131 (2009).

47. Brandes, A. A. *et al.* Temozolomide 3 weeks on and 1 week off as first-line therapy for recurrent glioblastoma: phase II study from Gruppo Italiano Cooperativo di Neuro-Oncologia (GICNO). *Br. J. Cancer* **95**, 1155–1160 (2006).
48. Wick, A. *et al.* Efficacy and tolerability of temozolomide in an alternating weekly regimen in patients with recurrent glioma. *J. Clin. Oncol.* **25**, 3357–3361 (2007).
49. Brandes, A. A. *et al. MGMT* methylation status can change between first surgery for newly diagnosed glioblastoma and second surgery for recurrence: clinical implications. *Neuro Oncol*. (in press).
50. Sadones, J. *et al.* MGMT promoter hypermethylation correlates with a survival benefit from temozolomide in patients with recurrent anaplastic astrocytoma but not glioblastoma. *Eur. J. Cancer* **45**, 146–153 (2009).
51. Cahill, D. P. *et al.* Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. *Clin. Cancer Res.* **13**, 2038–2045 (2007).
52. Maxwell, J. A. *et al.* Mismatch repair deficiency does not mediate clinical resistance to temozolomide in malignant glioma. *Clin. Cancer Res.* **14**, 4859–4868 (2008).
53. Yip, S. *et al. MSH6* mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. *Clin. Cancer Res.* **15**, 4622–4629 (2009).
54. The Cancer Genome Atlas Consortium. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* **455**, 1061–1068 (2008).
55. Perry, J. R., Rizek, P., Cashman, R., Morrison, M. & Morrison, T. Temozolomide rechallenge in recurrent malignant glioma by using a continuous temozolomide schedule: the "rescue" approach. *Cancer* **113**, 2152–2157 (2008).
56. Wick, A. *et al.* Rechallenge with temozolomide in patients with recurrent gliomas. *J. Neurol.* **256**, 734–741 (2009).
57. Möllemann, M., Wolter, M., Felsberg, J., Collins, V.P. & Reifenberger, G. Frequent promoter hypermethylation and low expression of the *MGMT* gene in oligodendroglial tumors. *Int. J. Cancer* **113**, 379–385 (2005).
58. Brandes, A. A. *et al.* Survival following adjuvant PCV or temozolomide for anaplastic astrocytoma. *Neuro Oncol* **8**, 253–260 (2006).
59. Sanson, M. *et al.* Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. *J. Clin. Oncol.* **27**, 4150–4154 (2009).
60. van den Bent, M. J. *et al.* Adjuvant procarbazine, lomustine, and vincristine improves progression-free survival but not overall survival in newly diagnosed anaplastic oligodendrogliomas and oligoastrocytomas: a randomized European Organisation for Research and Treatment of Cancer phase III trial. *J. Clin. Oncol.* **24**, 2715–2722 (2006).
61. Cairncross, G. *et al.* Phase III trial of chemotherapy plus radiotherapy compared with radiotherapy alone for pure and mixed anaplastic oligodendroglioma: intergroup Radiation Therapy Oncology Group Trial 9402. *J. Clin. Oncol.* **24**, 2707–2714 (2006).
62. Komine, C. *et al.* Promoter hypermethylation of the DNA repair gene O6-methylguanine-DNA methyltransferase is an independent predictor of shortened progression free survival in patients with low-grade diffuse astrocytomas. *Brain Pathol.* **13**, 176–184 (2003).

63. Kesari, S. *et al.* Phase II study of protracted daily temozolomide for low-grade gliomas in adults. *Clin. Cancer Res.* **15**, 330–337 (2009).
64. Everhard, S. *et al.* MGMT methylation: a marker of response to temozolomide in low-grade gliomas. *Ann. Neurol.* **60**, 740–743 (2006).
65. Brandes, A. A. *et al. MGMT* promoter methylation status can predict the incidence and outcome of pseudoprogression after concomitant radiochemotherapy in newly diagnosed glioblastoma patients. *J. Clin. Oncol.* **26**, 2192–2197 (2008).
66. Wick, W. *et al.* A novel tool to analyze MRI recurrence patterns in glioblastoma. *Neuro Oncol* **10**, 1019–1024 (2008).
67. Brandes, A. A. *et al.* Recurrence pattern after temozolomide concomitant with and adjuvant to radiotherapy in newly diagnosed patients with glioblastoma: correlation with *MGMT* promoter methylation status. *J. Clin. Oncol.* **27**, 1275–1279 (2009).
68. Hermisson, M. *et al.* O^6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. *J. Neurochem.* **96**, 766–776 (2006).
69. Kitange, G. J. *et al.* Induction of MGMT expression is associated with temozolomide resistance in glioblastoma xenografts. *Neuro Oncol* **11**, 281–291 (2009).
70. Cankovic, M., Mikkelsen, T., Rosenblum, M. L. & Zarbo, R. J. A simplified laboratory validated assay for MGMT promoter hypermethylation analysis of glioma specimens from formalin-fixed paraffin-embedded tissue. *Lab. Invest.* **87**, 392–397 (2007).
71. Schold, S. C. Jr *et al.* O^6-benzylguanine suppression of O^6-alkylguanine-DNA alkyltransferase in anaplastic gliomas. *Neuro Oncol* **6**, 28–32 (2004).
72. Quinn, J. A. *et al.* Phase II trial of temozolomide plus O^6-benzylguanine in adults with recurrent, temozolomide-resistant malignant glioma. *J. Clin. Oncol.* **27**, 1262–1267 (2009).
73. Koch, D., Hundsberger, T., Boor, S. & Kaina, B. Local intracerebral administration of O^6-benzylguanine combined with systemic chemotherapy with temozolomide of a patient suffering from a recurrent glioblastoma. *J. Neurooncol* **82**, 85–89 (2007).
74. Larochelle, A. *et al. In vivo* selection of hematopoietic progenitor cells and temozolomide dose intensification in rhesus macaques through lentiviral transduction with a drug resistance gene. *J. Clin. Invest.* **119**, 1952–1963 (2009).
75. Glas, M. *et al.* Long-term survival of patients with glioblastoma treated with radiotherapy and lomustine plus temozolomide. *J. Clin. Oncol.* **27**, 1257–1261 (2009).
76. Weiler, M. *et al.* Chemoradiotherapy of newly diagnosed glioblastoma with intensified temozolomide. *Int. J. Radiat. Oncol. Biol. Phys.* doi:10.1016/j.ijrobp.2009.05.031
77. Yung, W. K. *et al.* Multicenter phase II trial of temozolomide in patients with anaplastic astrocytoma or anaplastic oligoastrocytoma at first relapse. Temodal Brain Tumor Group. *J. Clin. Oncol.* **17**, 2762–2771 (1999).
78. Yu, J. *et al.* Methylation profiles of thirty four promoter-CpG islands and concordant methylation behaviours of sixteen genes that may contribute to carcinogenesis of astrocytoma. *BMC Cancer* **4**, 65 (2004).

79. Bello, M. J. *et al.* Hypermethylation of the DNA repair gene *MGMT*: association with *TP53* G:C to A:T transitions in a series of 469 nervous system tumors. *Mutat. Res.* **554**, 23–32 (2004).
80. Nakamura, M., Watanabe, T., Yonekawa, Y., Kleihues, P. & Ohgaki, H. Promoter methylation of the DNA repair gene *MGMT* in astrocytomas is frequently associated with G:C --> A:T mutations of the *TP53* tumor suppressor gene. *Carcinogenesis* **22**, 1715–1719 (2001).
81. Watanabe, T. *et al.* Phenotype versus genotype correlation in oligodendrogliomas and low-grade diffuse astrocytomas. *Acta Neuropathol. (Berl.)* **103**, 267–275 (2002).
82. Brell, M. *et al.* Prognostic significance of O^6-methylguanine-DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression in anaplastic gliomas. *Clin. Cancer Res.* **11**, 5167–5174 (2005).
83. Kamiryo, T. *et al.* Correlation between promoter hypermethylation of the O6-methylguanine-deoxyribonucleic acid methyltransferase gene and prognosis in patients with high-grade astrocytic tumors treated with surgery, radiotherapy, and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea-based chemotherapy. *Neurosurgery* **54**, 349–357 (2004).
84. Dong, S. M. *et al.* Concurrent hypermethylation of multiple genes is associated with grade of oligodendroglial tumors. *J. Neuropathol. Exp. Neurol.* **60**, 808–816 (2001).
85. Blanc, J. L. *et al.* Correlation of clinical features and methylation status of MGMT gene promoter in glioblastomas. *J Neurooncol* **68**, 275–283 (2004).
86. Balaña, C. *et al. O^6-methyl-guanine-DNA methyltransferase* methylation in serum and tumor DNA predicts response to 1,3-bis(2-chloroethyl)-1-nitrosourea but not to temozolamide plus cisplatin in glioblastoma multiforme. *Clin. Cancer Res.* **9**, 1461–1468 (2003).
87. Watanabe, T. *et al. O^6-methylguanine-DNA methyltransferase* methylation and *TP53* mutation in malignant astrocytomas and their relationships with clinical course. *Int. J. Cancer* **113**, 581–587 (2005).
88. Crinière, E. *et al.* MGMT prognostic impact on glioblastoma is dependent on therapeutic modalities. *J Neurooncol* **83**, 173–179 (2007).
89. Zawlik, I. *et al.* Promoter methylation and polymorphisms of the *MGMT* gene in glioblastomas: a population-based study. *Neuroepidemiology* **32**, 21–29 (2009).
90. Prados, M. D. *et al.* Phase II study of erlotinib plus temozolomide during and after radiation therapy in patients with newly diagnosed glioblastoma multiforme or gliosarcoma. *J. Clin. Oncol.* **27**, 579–584 (2009).
91. Clarke, J. L. *et al.* Randomized phase II trial of chemoradiotherapy followed by either dose-dense or metronomic temozolomide for newly diagnosed glioblastoma. *J. Clin. Oncol.* **27**, 3861–3867 (2009).
92. Brandes, A. A. *et al.* Correlations between O6-methylguanine DNA methyltransferase promoter methylation status, 1p and 19q deletions, and response to temozolomide in anaplastic and recurrent oligodendroglioma: a prospective GICNO study. *J. Clin. Oncol.* **24**, 4746-4753 (2006).

Figure legends

Figure 1 | Map of the CpG island region of the *MGMT* promoter.[16] **a** | Genomic map of the *MGMT* 5' region. A bent arrow indicates the transcription initiation site. Binding sites for the Sp1 transcription factor are indicated. The CpG island (bps –552 to +289), which includes 97 CpG sites, is numbered from 5' to 3', and an outline of CpG density is shown. Adapted by permission from Macmillan Publishers Ltd: Nakagawachi, T. *et al. Oncogene* **22**, 8835–8844 © 2003.

Figure 2 | Methylation of the CpG island region of the *MGMT* promoter. **a** | Detailed methylation status of the whole CpG island in *MGMT*-expressing and *MGMT*-nonexpressing cell lines. Each circle graph represents the percentage of methylated clones (number of methylated clones/10 analyzed clones x 100) at one of the numbered CpG sites. **b** | Visualization of CpGs interrogated by diverse methylation-specific assays. Alike colors signify CpGs evaluated on the same fragment, except for qMSP^2 where the methylation-specific probe is marked in light blue and recognizes the same molecule. Abbreviations: COBRA, combined bisulfite restriction analysis;[32] HRM, high-resolution melting; *MGMT*, *O*^{6}-methylguanine-DNA methyltransferase;[34] MS-MPLA methylation-specific multiplex ligation-dependent probe amplification;[33] MSP, methylation-specific PCR;[13,28] qMSP^1, quantitative MSP using methylation-specific primers;[31] qMSP^2, MethyLight—including, in addition, a methylation specific probe;[29] PyroSeq, methylation specific pyrosequencing.[32] Part a adapted by permission from Macmillan Publishers Ltd: Nakagawachi, T. *et al. Oncogene* **22**, 8835–8844 © 2003.

Figure 3 | Immunochemical staining for MGMT protein expression in glioblastomas. **a** | Lack of nuclear MGMT expression in tumor cells from a glioblastoma with *MGMT* promoter methylation. Note staining of proliferating microvascular cells as an internal control. **b** | Strong MGMT positivity in tumor and vascular cells in a *MGMT*-unmethylated glioblastoma. **c** | Numerous MGMT-positive cells in a glioblastoma with *MGMT* promoter methylation. **d** | Staining of the same tumor for CD45 reveals prominent contamination of the tumor tissue with $CD45^+$ microglial cells and macrophages, which express MGMT and might be responsible for erroneously positive estimates of MGMT expression and activity levels. The insert in part d shows double staining of perivascular macrophages for CD68 (red) and MGMT (brown) in another case of glioblastoma. All sections are counterstained with hemalum. Original microscopic magnifications were x 400 (parts a and b, and insert in d) or x 100 (parts c and d). Abbreviations: MGMT, O^6-methylguanine-DNA methyltransferase; v, blood vessel.

Figure 4 | Bisulfite conversion of tumor DNA. Treatment of DNA with bisulfite results in the conversion of unmethylated cytidine into uracil, which is replaced by thymidine in the subsequent PCR step. By contrast, 5-methylcytidine (^mC) will not be converted and thus remains as a cytidine. Incomplete bisulfite conversion of unmethylated cytidine will be interpreted as methylation in the subsequent quantification step using any technology and will, therefore, yield a false-positive result.

Figure 5 | Frequency of *MGMT* promoter methylation in glioma subtypes. Frequencies (%) of *MGMT* promoter methylation were taken from 25 publications evaluating 2,994 gliomas (avoiding overlap of studies, not exhaustive). Numbers in bars represent

numbers of cases considered. Data were derived from the studies cited in Tables 2 and 3 and several additional references.[25,57,62,78-87] Most studies used gel-based methylation-specific PCR. Glioblastoma shown as 'GBM' are not listed under PrGBM or ScGBM, with the exception of one population-based study. Abbreviations: AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; GBM, glioblastoma; LA, low-grade astrocytoma; O, oligodendroglioma; OA, oligoastocytoma; PrGBM, primary glioblastoma; ScGBM, secondary glioblastoma.

Table 1 | Commonly used DNA methylation-specific assays

Technology	Pretreatment	Number of CpGs tested	Test properties	Principle	Read out	Advantages	Disadvantages
MS clone sequencing	Bisulfite conversion[a]	Complete sequence	Quantitative	Converted amplified sequences are cloned into vector and amplified in bacteria; each clone is sequenced	Methylation status at all CpGs for each individual allele	Comprehensive	No high-throughput option
MSP, gel-based	Bisulfite conversion	9	Qualitative	Methylation-specific primers: amplification of methylated sequences; amplification of unmethylated sequences	Gel; presence or absence of methylation	Sensitive; associated with prediction in trials	Difficult to standardize
qMSP	Bisulfite conversion	8	Quantitative	MSP using real-time technology	Fully methylated *MGMT* promoter versus β-actin gene	Standardized; high throughput; technical cut off	Recognition only of fully methylated sequence—may make technique too restrictive
MethyLight qMSP	Bisulfite conversion	12	Quantitative	MSP using real-time technology plus methylation-specific probe	Fully methylated *MGMT* promoter versus control gene	Standardized; high throughput; technical cut off	Recognition only of fully methylated sequence—may make technique too restrictive
Pyrosequencing	Bisulfite conversion	4–12	Semiquantitative	Quantification of each added nucleotide during sequencing	Sequence; average (%) methylation over all CpGs measured	Standardized; high throughput; internal control for bisulfite conversion	Cut-off definition
COBRA	Bisulfite conversion; restriction enzymes[b]: *BstU*I, *Taq*I	5	Semiquantitative	Only methylated sequences are cut; fragments are quantified	Percentage of cut sequences; sequencer (gel[c])	Specificity; internal control for bisulfite conversion	Extra step; depends on restriction sites; cut-off definition
MS-MPLA	Restriction enzyme: *Hha*I (methylation sensitive)	4	Semiquantitative	Unmethylated CpG in recognition site results in restriction; only methylated sequences get amplified	Average (ratio methylated versus unmethylated alleles)	No bisulfite conversion; high throughput	Limited to restriction sites; cut-off definition
MS-HRM	Bisulfite conversion	18	Semiquantitative	qPCR, change of melting curve depends on presence of methylated sequences	Percentage of methylated sequences defined by standard curve	Evaluates large regions; standardized; high throughput	Cut-off definition

[a]All unmethylated cytidines are converted to uracil, in subsequent PCR uracil is replaced by thymidine; methylated cytidines are resistant to conversion and remain unchanged (Figure 4). [b]Restriction enzymes recognize specific sequences and cut at defined positions. [c]Often used in a qualitative manner (presence versus absence). Abbreviations: COBRA, combined bisulfite restriction analysis; *MGMT*, O^6-methylguanine-DNA methyltransferase; MS, methylation-specific; MS-HRM, MS-high-resolution melting; MS-MPLA, MS-multiplex ligation-dependent probe amplification; MSP, MS PCR; qMSP, quantitative MSP.

Table 2 | *MGMT* promoter methylation in human glioblastoma

Reference	*MGMT* methylation frequency[a]	Clinical significance of *MGMT* promoter methylation
Newly diagnosed glioblastoma		
Esteller *et al.* (2000)[10]	41% (12/29)	Prolonged overall survival in response to radiotherapy plus alkylating agent-containing chemotherapy
Hegi *et al.* (2004)[11]	68% (26/38)	Prolonged overall survival in response to radiotherapy plus concomitant and adjuvant temozolomide; phase II trial
Hegi *et al.* (2005)[13]	45% (92/206)	Prolonged PFS in response to radiotherapy plus concomitant and adjuvant temozolomide as opposed to radiotherapy alone, and prolonged overall survival; randomized phase III trial
Herrlinger *et al.* (2006)[38]	42% (8/19)	Prolonged PFS and overall survival in response to radiotherapy plus temozolomide and lomustin; phase II trial
Criniere *et al.* (2007)[88]	58% (136/219)	Prolonged PFS in response to radiotherapy plus nitrosourea-based chemotherapy, as opposed to radiotherapy alone
Brandes *et al.* (2008)[65]	35% (36/103)	Prolonged overall survival in response to radiotherapy plus concomitant and adjuvant temozolomide; association of pseudoprogression with methylated *MGMT* promoter
Wick *et al.* (2008)[66]	44% (20/45)	Recurrence patterns revealed no difference between groups, treatment arm or *MGMT* methylation status (radiotherapy, *n*=22; radiotherapy plus concomitant and adjuvant temozolomide, *n*=23); randomized phase III trial
Brandes *et al.* (2009)[67]	34% (32/95)	Pattern of and time to recurrence strictly correlated with *MGMT* methylation status in patients treated with radiotherapy plus concomitant and adjuvant temozolomide
Brandes *et al.* (2009)[43]	43% (16/37)	Prolonged PFS in response to radiotherapy plus concomitant and adjuvant temozolomide and prolonged overall survival in elderly patients
Zawlik *et al.* (2009)[89]	44% (165/371)	No prognostic effect in patients treated without chemotherapy (surgery, *n*=105; maximal surgery plus radiotherapy, *n*=208; radiotherapy alone, *n*=10; supportive care only, *n*=29)
Dunn *et al.* (2009)[46]	53% (58/109)	Prolonged PFS and overall survival in response to radiotherapy plus concomitant and adjuvant temozolomide; extent of *MGMT* methylation associated with outcome
Prados *et al.* (2009)[90]	36% (16/44)	Prolonged overall survival in response to radiotherapy plus concomitant and adjuvant temozolomide plus erlotinib; phase II trial
Weller *et al.* (2009)[40]	44% (133/295)	Prolonged PFS and overall survival in response to radiotherapy plus concomitant and adjuvant temozolomide; prospective collection
Weiler *et al.* (2009)[76]	41% (16/39)	Prolonged PFS in response to radiotherapy plus concomitant and adjuvant (1 week on–1 week off) temozolomide plus indomethacin; phase II trial
Clarke *et al.* (2009)[91]	19% (9/48)	No difference of PFS or overall survival in response to radiotherapy plus concomitant and dose-modified adjuvant (1 week on–1 week off versus continuous) temozolomide
Recurrent glioblastoma		
Brandes *et al.* (2006)[47]	46% (10/22)	No prognostic significance in recurrent disease treated with temozolomide (3 weeks on–1 week off)
Wick *et al.* (2007)[48]	47% (17/36)	No prognostic significance in recurrent disease treated with temozolomide (1 week on–1 week off)
Brandes *et al.* (2009)[49]	34% (13/38) at first surgery; 29% (11/38) at second surgery	No prognostic value of *MGMT* promoter methylation at recurrence in patients pretreated with radiotherapy plus adjuvant or concomitant and adjuvant temozolomide

[a] *MGMT* promoter methylation analysis performed by gel-based methylation-specific PCR, except the study by Dunn *et al.* (pyrosequencing).[46] Abbreviations: *MGMT*, O^6-methylguanine-DNA methyltransferase; PFS, progression-free survival.

Table 3 | *MGMT* promoter methylation in various human gliomas

Reference	*MGMT* methylation frequency[a]	Clinical significance of *MGMT* promoter methylation
Anaplastic astrocytoma		
Wick *et al.* (2009)[14]	50% (48/96)	Prolonged PFS and overall survival in response to radiotherapy or chemotherapy with temozolomide (5/28 [Au: OK for all? YES]) or PCV; phase III trial
Anaplastic oligoastrocytoma		
Brandes *et al.* (2006)[92]	69% (37/54)	No prognostic significance in recurrent oligoastrocytoma or oligodendroglioma treated with temozolomide (5/28)
Wick *et al.* (2009)[14]	71% (53/75)	Prolonged PFS and overall survival in response to radiotherapy or chemotherapy with temozolomide (5/28) or PCV; phase III trial
Anaplastic oligodendroglioma		
Wick *et al.* (2009)[14]	71% (22/31)	Prolonged PFS and overall survival in response to radiotherapy or chemotherapy with temozolomide (5/28) or PCV; phase III trial
Anaplastic oligodendroglioma and anaplastic oligoastrocytoma without necrosis		
van den Bent *et al.* (2009)[15]	84% (81/97)	Prolonged PFS and overall survival in response to radiotherapy or chemotherapy with PCV; phase III trial
Anaplastic oligoastrocytoma with necrosis (glioblastoma)		
van den Bent *et al.* (2009)[15]	73% (29/40)	No prolonged PFS and overall survival in response to radiotherapy or chemotherapy with PCV; phase III trial
Recurrent anaplastic astrocytoma or oligoastrocytoma and glioblastoma		
Sadones *et al.* 2009[50]	26% (10/38)	Prolonged overall survival in response to temozolomide (5/28 or 1 week–1 week off) in anaplastic astrocytoma and oligoastrocytoma
Grade II astrocytoma		
Komine *et al.* 2003[62]	43% (21/49)	Decreased PFS with no treatment or radiotherapy or interferon
Grade II oligodendroglioma and oligoastrocytoma		
Everhard *et al.* 2006[64]	93% (63/68)	Prolonged PFS in patients with oligodendroglioma (n=42), oligoastrocytoma (n=18) or astrocytoma (n=8) treated with temozolomide
Kesari *et al.*	60% (12/20)	Prolonged PFS and OS in patients treated with temozolomide (11 weeks on–4 weeks off); phase II trial

[a] *MGMT* promoter methylation analysis performed by gel-based methylation-specific PCR, except studies by Sadones *et al.* (quantitative methylation-specific PCR)[50] and van den Bent *et al.* (methylation-specific multiplex ligation-dependent probe amplification.)[15] Abbreviations: 5/28, 5 out of 28 days; PFS, progression-free survival; PCV, combination of procarbazine, CCNU (lomustine) and vincristine.

Gelöscht: 23
Formatiert: Hervorheben
Formatiert: Hervorheben
Formatiert: Hervorheben
Gelöscht: 23
Gelöscht: 23
Gelöscht: 23
Gelöscht: 23
Gelöscht: 23
Gelöscht: 23