

## TOPIC OF THE ISSUE: REVIEW ARTICLE

Year : 2011 | Volume : 59 | Issue : 2 | Page : 229--235

## O<sup>6</sup>-methylguanine DNA methyltransferase gene promoter methylation in high-grade gliomas: A review of current status

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## Abstract

Assessment of promoter methylation of the O<sup>6</sup>-methylguanine DNA methyltransferase (MGMT) gene has recently gained importance in molecular profiling of high-grade gliomas. It has emerged not only as an important prognostic marker but also as a predictive marker for response to temozolomide in patients with newly diagnosed glioblastoma. Further, recent studies indicate that MGMT promoter methylation has strong prognostic relevance even in anaplastic (grade III) gliomas, irrespective of therapy (chemotherapy or radiotherapy). This article provides an overview of its use as a predictive and prognostic biomarker, as well as the methods employed for its assessment and use in therapeutic decision making.

### How to cite this article:

Suri V, Jha P, Sharma MC, Sarkar C. O<sup>6</sup>-methylguanine DNA methyltransferase gene promoter methylation in high-grade gliomas: A review of current status. *Neurol India* 2011;59:229-235

### How to cite this URL:

Suri V, Jha P, Sharma MC, Sarkar C. O<sup>6</sup>-methylguanine DNA methyltransferase gene promoter methylation in high-grade gliomas: A review of current status. *Neurol India* [serial online] 2011 [cited 2011 Apr 7 ];59:229-235

Available from: <http://www.neurologyindia.com/text.asp?2011/59/2/229/79128>

## Full Text

### Introduction

The unique pharmacological profile of temozolomide (TMZ), its availability as an oral agent, and its safety and efficacy documented in several clinical trials supports its potential in the treatment of malignant gliomas. [1],[2],[3] The role of TMZ in the treatment of glioblastomas (GBMs) was established by the randomized multicentric phase 3 trial of the European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups (EORTC 26981) and the National Cancer Institute of Canada Clinical Trials Group (NCIC trial CE.3). [4] This trial which compared the efficacy of TMZ and radiotherapy (RT) followed by adjuvant TMZ with RT alone showed a statistically significant survival benefit in the TMZ + RT treated GBM group. At a median follow-up of 28 months, the median survival was 14.6 months with TMZ + RT and 12.1 months with RT alone. [4] The final results of EORTC 26981 with a median follow-up of more than 5 years showed an overall survival (OS) of 27.2% at 2 years, 16.0% at 3 years, 12.1% at 4 years, and 9.8% at 5 years with TMZ versus 10.9%, 4.4%, 3.0%, and 1.9% at 2, 3, 4 and 5 years, respectively, with RT alone. A benefit of combined

therapy was recorded in all clinical prognostic subgroups. [5]

The therapeutic benefit of TMZ depends on its ability to damage DNA by introducing alkyl groups at multiple sites along the DNA backbone, thus impairing DNA replication and triggering cell death. The most cytotoxic lesion is alkylation of O 6 position of guanine. However, normal cells contain DNA repair proteins which correct the damage and allow normal DNA replication. One important DNA repair protein is O 6 -alkylguanine-DNA alkyltransferase (AGT) which is encoded by the gene O 6 -methylguanine DNA methyltransferase (MGMT) located on chromosome 10q26. [6] MGMT prevents TMZ induced cell death by removing alkyl adducts from the O 6 position of guanine and O 4 position of thymine. Thus, tumor cells expressing MGMT are resistant to alkylating agents, while those that lack the enzyme appear to be chemosensitive. The most important mechanism of silencing of the MGMT gene is by methylation of its promoter. This results in loss of MGMT expression and diminished repair activity. In recent years, MGMT promoter methylation status, an indicator of gene silencing, has emerged as a potentially important predictor of response to TMZ therapy as well as an important prognostic marker in GBMs and anaplastic gliomas [6],[7],[8] [Table 1].{Table 1}

### **O 6 -methylguanine DNA Methyltransferase Promoter Methylation in Adult Glioblastomas**

Interest in the role of MGMT promoter methylation in determining responsiveness to alkylating drugs was generated by a study conducted by Esteller et al., which showed that this alteration was associated with responsiveness to carmustine and an increase in OS and the time to progression of disease. [9] MGMT promoter methylation was observed in 40% of their cases. Later, Hegi et al. tested 206 GBM patients enrolled in the EORTC 26981/NICC trial and noted MGMT promoter methylation in 45% cases. [6] This landmark study showed that irrespective of treatment, MGMT promoter methylation was an independent favorable prognostic factor. The study also highlighted that MGMT promoter methylation was a clinically relevant predictor of benefit from TMZ CT. [6] Thus, among the patients whose tumor contained a methylated MGMT promoter, median survival was 21.7 months in patients treated with TMZ and RT as compared with 15.3 months among those who were assigned to only RT (P = 0.007). In the absence of methylation of the MGMT promoter, there was a smaller and statistically insignificant difference in survival between the treatment groups. The 5-year survival analysis of EORTC/NICC trial showed that methylation of the MGMT promoter was the strongest predictor for outcome and benefit from TMZ CT. [5] The 2-year and 5-year survival in patients with methylated and unmethylated MGMT receiving TMZ + RT was 49% versus 15% and 14% versus 8%, respectively. Only slight improvement was observed in RT arm, the 2-year and 5-year survival being 24% versus 2% and 5% versus 0%, respectively.

A study to evaluate prognostic significance of molecular markers in newly diagnosed GBMs recruited into German Glioma Network (GGN) also showed that MGMT promoter methylation was associated with prolonged progression-free survival (PFS) and OS in patients receiving TMZ. [21] Several other studies have also shown predictive and prognostic significance of MGMT promoter methylation in GBMs [Table 1]. In our study on adult GBMs with long-term survival (median survival 3.88 years) treated with adjuvant TMZ, 83.3% (5/6) patients had methylated promoter. [23]

### **O 6 -methylguanine DNA Methyltransferase Promoter Methylation in Adult Recurrent Glioblastomas**

The prognostic and predictive value of MGMT promoter methylation in recurrent GBMs is not very definitive. Brandes et al. in their study of 33 recurrent GBMs, did not observe significant difference between MGMT promoter methylated versus unmethylated patients for median PFS and PFS at 6 months (PFS-6) (15.6 weeks and 20% vs. 11.9 weeks and 21.4%). [11]

In a study by Wick et al., 17 of the 36 recurrent GBMs had a methylated MGMT promoter. However, PFS did not significantly differ with regard to the methylation of the MGMT promoter. The median PFS and PFS-6 were 19

weeks and 34% with an unmethylated promoter versus 27 weeks and 52% with a methylated MGMT promoter. [15] Brandes et al. evaluated MGMT status at first and second surgery in 44 recurrent GBMs and noted that the methylation status determined at first surgery was of prognostic value. [19] However, it was not predictive of outcome following second surgery. Sadones et al., in their study of 38 recurrent gliomas, documented that promoter methylation correlated with superior OS in anaplastic gliomas but not in GBMs. [20]

### **O 6 -methylguanine DNA Methyltransferase Promoter Methylation in Adult Anaplastic (grade III) Gliomas**

Two major independent studies on grade III gliomas have shown prognostic value of MGMT promoter methylation even in patients treated with RT. A trial by the German Cancer Society's Neuro-Oncology Working Group (NOA-04) in patients with newly diagnosed, supratentorial anaplastic gliomas (WHO grade III) showed that methylation of the MGMT promoter was associated with prolonged PFS in both the CT and RT arms. [8] In the EORTC 26951 study, 368 patients with anaplastic oligodendroglial tumors were randomly assigned to either RT alone or to RT followed by adjuvant PCV. The frequency of MGMT promoter methylation was 80% in 152 assessable cases. [7] The prognostic significance of MGMT promoter methylation was equally strong in the RT arm and the RT/PCV arm for both PFS and OS. Analysis was done with MGMT average (MGMT av) as binary variable (a cutoff of MGMT av > 0.25 was considered as indicative of methylation). In the RT arm, the 2-year PFS was 12.5% versus 48.1% and OS was 31.3% versus 65.4% in patients with MGMT av ≤ 0.25 and MGMT av > 0.25, respectively. Similarly, for the patients in RT/PCV arm, the 2-year PFS and OS was 20.0% versus 56.8% and 46.7% versus 71.7%, respectively, for the unmethylated versus methylated groups. The study highlighted that MGMT promoter methylation has prognostic significance and is not predictive for outcome to adjuvant PCV chemotherapy. [7] Thus, in anaplastic gliomas, MGMT promoter methylation is a prognostic marker of good outcome irrespective of the treatment arm.

### **O 6 -methylguanine DNA Methyltransferase Promoter Methylation in Pediatric Glioblastomas and Anaplastic (Grade III) Gliomas**

Few studies on pediatric GBMs have also shown MGMT promoter methylation to be associated with longer survival. Donson et al., in a study on 10 pediatric GBMs, demonstrated average survival time for TMZ treated children with methylated MGMT to be 13.7 months as compared to 2.5 months for patients with an unmethylated MGMT promoter. Based on a strong correlation of MGMT promoter methylation with OS, the authors suggested that MGMT methylation may be a prognostic factor for survival in pediatric GBM as well as a marker for TMZ sensitivity. [13]

Schlosser et al. evaluated 24 children with high-grade glioma (HGG) registered into the German HIT-GBM database, receiving TMZ. DNA methylation, but not protein expression of MGMT, was associated with an increased median event free survival (EFS) and OS of children with relapsed HGG. [24]

Pollack et al. evaluated a large cohort of pediatric HGGs (109 cases of GBM, anaplastic astrocytomas (AA) or anaplastic mixed gliomas) from the Children's Cancer Group 945 study. All the patients received alkylator-based chemotherapy as a component of adjuvant therapy. The association between MGMT status and outcome was not significant in the patients with AA but was significant in patients with GBMs (5-year PFS of 29 ± 9% in those without expression vs. 0% in those with overexpression). [17]

In Children's Oncology Group study ACNS0126, 107 children with a diagnosis of AA, GBM and gliosarcoma were enrolled. The 2-year EFS rate was 17 ± 5% among patients without MGMT overexpression and 5 ± 4% among those with overexpression. [22]

### **Pseudoprogression and O 6 -methylguanine DNA Methyltransferase Promoter Methylation Status**

Pseudoprogression (PsPD) is a peculiar neuroradiological pattern mimicking early disease progression in patients after radiochemotherapy involving TMZ. This is believed to be a false-positive sign usually seen in the first 3 months after completion of RT and concomitant CT and may be more common in patients with MGMT methylated tumors. In a study conducted by Brandes et al. in 2008 on 103 GBMs, PsPD was observed in 38 patients: 21 (91%) of 23 patients with methylated MGMT promoter and 11 (41%) of 27 with unmethylated MGMT promoter showed PsPD. [16]

### **Correlation of O<sup>6</sup>-methylguanine DNA Methyltransferase Promoter Methylation with Other Genetic Alterations**

Some studies have shown a strong correlation of MGMT promoter methylation with combined loss of heterozygosity (LOH) of 1p and 19q in anaplastic oligodendrogliomas. [7],[11],[25] However, Everhard et al., Wantanabe et al., and Huang et al. did not find any significant association. [26],[27],[28]

van den Bent et al. (EORTC brain tumor group) reported a strong correlation of MGMT promoter methylation with IDH1 mutations and 1p/19q deletion in anaplastic oligodendroglial tumors. [7] Similar results were documented by Sanson et al. (58% IDH1 mutations in methylated vs. 26% IDH1 mutations in unmethylated tumors). [29] However, Weller et al. and Felsberg et al. did not find any correlation between these two alterations. [21],[30]

Reports on association of MGMT promoter methylation with epidermal growth factor receptor (EGFR) amplification and TP53 mutation are highly contradicting. Shamsara et al. found a significant association of p53 immunopositivity with methylated GBMs. [31] Watanabe et al. also reported a significant association of MGMT methylation with TP53 mutation in diffuse astrocytomas. [27] However, Everhard et al. and Jesien-Lewandowicz et al. did not find any significant correlation. [26],[32] Eoli et al. found EGFR amplification to be more frequent in unmethylated than in methylated GBMs (40% vs. 28%) though the difference was not statistically significant. [14]

In our study of 102 adult gliomas, there was an inverse correlation of MGMT promoter methylation frequency with tumor grade, observed in 80%, 71% and 57% of grade II, grade III gliomas and GBMs, respectively. Majority of cases with 1p/19q LOH also showed MGMT methylation, although the association was not significant. There was no significant correlation of MGMT status with IDH1 mutation. In astrocytic tumors, there was no correlation of EGFR amplification and p53 immunopositivity with MGMT methylation. [33]

### **Methods of Assessment of O<sup>6</sup>-methylguanine DNA Methyltransferase Promoter Methylation Status: Challenges and Pitfalls**

Several methods have been used for the assessment of MGMT methylation at DNA, RNA and protein levels. However, all of them have their own advantages and disadvantages.

#### **i) Promoter methylation based techniques**

Methylation-specific PCR (MSP) is a highly sensitive and most commonly employed bisulfite-based method for analyzing the MGMT promoter methylation status. It has been used in majority of the clinical trials till date [Table 1]. However, the frequency reported in GBMs varies from as low as 35% to as high as 68%. [10],[16] We observed a frequency of 57% and 50% in adult and pediatric GBMs, respectively. [33],[34]

The drawbacks of this assay are non-quantitative information, a lower number of assessable CpG dinucleotides and a misleading risk of false negativity largely due to low quality or quantity of amplifiable DNA. [35],[36],[37]

Several groups have reported problems with establishing a reliable MGMT MSP protocol using formalin-fixed, paraffin-embedded tissues. [38] Mosaic methylation patterns with variable degrees of methylation at the primer site as well as problems with bisulfite modification may lead to false-positive or false-negative results. [37],[39],[40]

A sensitive and specific quantitative MSP (qMSP) assay has been developed which allows a higher level of standardization, and definition of cutoffs for methylation. [41] MGMT methylation assessed by this technique has been consistently shown to predict response to the alkylating agent TMZ, measured by either PFS or OS, in GBM patients. [5],[14],[19],[30],[42],[43] However, definitive confirmation of the predictive value of commercially available methylation-specific quantitative PCR (qMSP) test (Labcorp, San Diego, CA, USA) awaits the results of a randomized prospective trial. [41],[44]

Pyrosequencing and bisulfite sequencing are currently regarded as the gold standard for the analysis of DNA methylation profiles because they provide single base pair resolution and quantitative methylation information. These methods are widely used in biomedical basic research. [37],[45] Unfortunately, these methods are cost and labor intensive.

Several other semiquantitative techniques with unique advantages have been tried. Combined bisulfite restriction analysis (COBRA) utilizes restriction endonucleases that recognize DNA methylation at specific sites (Hassel et al.). [46] Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is a semiquantitative assay in which troublesome bisulfite conversion of unmethylated cytosines can be omitted. [36] Methylation-sensitive high-resolution melting (MS-HRM) allows for estimation of the methylation level by comparing the melting profiles of unknown PCR products to the melting profiles of PCR products derived from standards with a known unmethylated to methylated template ratio. [47] Denaturing high-performance liquid chromatography (HPLC) circumvents the cloning and sequencing steps required for methylation detection. [48]

#### ii) mRNA expression

DNA methylation at CpG sites in the promoter region of a gene can alter mRNA expression which can be determined in fresh tumor specimens. However, this technique is difficult to accept for routine patient care. [49]

#### iii) MGMT expression by immunohistochemistry

MGMT protein can be visualized immunohistochemically and commercial anti-MGMT antibodies are available. All of the large-scale studies to date have failed to find any concordance between MGMT immunohistochemistry (IHC) results and promoter methylation as measured by the MSP test and patient survival. [42],[50],[51],[52] The unreliability of IHC seems to be due to tumor heterogeneity, interobserver variability, lack of cut-off values and admixture with non-neoplastic MGMT expressing cells. [53]

### **How Far have we Reached from Bench to Bedside?**

It is apparent that MGMT promoter methylation has taken hold as a prognostic and predictive biomarker and clinicians expect its routine implementation for therapeutic monitoring and prognostication. The biggest challenge however remains to decide which method should be employed for routine assessment. Although various techniques are available, a definite relationship between MGMT promoter methylation, mRNA expression and immunohistochemical assay has not been established.

A major issue which needs to be determined is which of the MGMT promoter regions are most predictive of patient's response to therapy. The MGMT promoter consists of 97 CpG sites which are not uniformly methylated in individual patients. In order to optimize probe design for PCR based techniques, the heterogeneity of methylation patterns across the promoter region needs to be identified. Further, there is little information on relative cut-off values. Thus, the need of the hour is to develop a consensus high throughput, robust, relatively

affordable, standardized and clinically validated test to assess MGMT promoter methylation for treatment planning in this era of genetically directed rational cancer therapy.

Secondly, it is important to note that the absolute clinical necessity of MGMT testing is also not clear at this juncture. Till date, TMZ is the most effective and best tolerated chemotherapeutic agent for high-grade glioma patients and we have no appropriate alternatives for patients with unmethylated MGMT promoter. So, whether it is justifiable to deny a patient of this treatment based on a single prognostic/predictive parameter available still remains a big question.

To conclude, it appears that only when alternative treatment regimens become available and consensus test method is developed, will the assessment of MGMT promoter methylation status become a routine in patient management. Till then, perhaps it will remain confined to stratifying patients in clinical trials.

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Thursday, April 07, 2011

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