

Review

MGMT analysis at DNA, RNA and protein levels in glioblastoma tissue

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Summary. Evidence from several studies supports that the epigenetic, transcriptional and translational regulation and expression of O6-methylguanine-methyltransferase (MGMT) is relevant for prognostic and predictive considerations in glioblastoma patients. MGMT status is being used as a stratifying factor or eligibility criterion in ongoing and accruing clinical glioblastoma trials. In some cases, there is also interest in MGMT assessment of glioblastoma tissue in the day-to-day clinical setting. However, a number of different methods and protocols have been used for MGMT analysis and it is unclear which methods harbour the greatest potential for translation into routine clinical use. This article reviews methods that have been used for MGMT assessment at DNA-, RNA- and protein-level in glioblastoma with a focus on their potential clinical utility. **Conclusions.** (1) DNA-based methods for MGMT analysis seem more promising for translation into the clinical setting than RNA- or protein-based methods. However, at present there is lack of data to base recommendations for a specific method or protocol for MGMT testing on. There is a strong need for systematic comparisons and validation of intra- and interlaboratory reproducibility and clinical performance of different methods for MGMT assessment to identify the best method for clinical MGMT testing. (2) The current practice of formalin-fixation of neurosurgical specimens considerably limits the spectrum of methods that can be applied for molecular diagnosis in clinical neuro-oncology. Further studies may be helpful to establish more appropriate protocols for tumour tissue preservation (e.g. identification of alternative fixatives that do not deteriorate DNA and RNA quality).

Key words: Glioblastoma, MGMT, Molecular diagnosis

Introduction

Glioblastoma multiforme (GBM) is the most common malignant type of primary brain tumor (Kleihues et al., 2007). For decades, surgery and radiation have been the sole therapy modalities with proven efficacy in GBM patients, while the benefit of systemic antineoplastic therapy was unclear (Medical Research Council Brain Tumour Working Party, 2001). However, a recent prospective multicenter study showed that the addition of the alkylans temozolomide to radiotherapy for newly diagnosed GBM results in a clinically meaningful and statistically significant survival benefit with minimal toxicity (Stupp et al., 2005). As a consequence, postoperative combined radio-chemotherapy followed by adjuvant chemotherapy with temozolomide has been established as standard adjuvant therapy for GBM patients. A translational study performed in parallel to the clinical trial by Stupp et al. showed a strong association of the methylation status of the O6-methylguanine-methyltransferase gene (MGMT) promoter with patient survival (Hegi et al., 2005). The MGMT gene is located on chromosome 10 and encodes MGMT, a DNA repair protein that is considered to counteract the effect of alkylating chemotherapy by removing methyl groups from the O6-position of guanine (Jacinto and Esteller, 2007). Epigenetic MGMT promoter methylation at cytosine guanine dinucleotide clusters ("CpG islands") results in transcriptional silencing and, therefore, inhibition of expression of MGMT protein. The relevance of MGMT as potential prognostic or predictive factor in malignant glioma patients is supported by a number of independent studies. Therefore, there is strong interest in incorporating testing of MGMT promoter methylation status into clinical GBM trials (Friedman et al., 1998; Esteller et al., 2000; Paz et al., 2004; Brell et al., 2005; Hegi et al., 2005; Pollack et al., 2006; Chinot et al., 2007; Criniere et al., 2007; Donson et al., 2007; Krex et al., 2007; Martinez et al., 2007). In fact, MGMT status is being used as a stratifying factor or eligibility criterion

in ongoing and upcoming clinical glioblastoma trials (Idbaih et al., 2007; Jacinto and Esteller, 2007; Stupp and Hegi, 2007; Yip et al., 2008). Concerning the day-to-day clinical practice, MGMT assessment is not yet part of the routine diagnostic work-up of GBM specimens, because the current standard therapy strategy for newly diagnosed GBM is regardless of MGMT status. Still, in individual cases patients or oncologists request testing of the MGMT status for prognostic considerations or adaptations of alkylating chemotherapy. Therefore, more and more neuropathological and neurooncological laboratories are considering establishing MGMT analysis. However, a large number of different methods and protocols have been applied for MGMT analysis in GBM, and there is no consensus on which method should be primarily used. Most studies focus on MGMT gene promoter methylation status using a variety of methods, but there are also several studies that evaluated expression of MGMT at protein- or RNA-level (Table 1). This article aims at providing an overview of methods that have been applied to MGMT testing with a focus on their potential clinical utility.

Requirements for use of a method in the diagnostic setting

The optimal method for diagnostic assessment of MGMT assessment should be widely available, easy to establish, cost-effective, reproducible both within a given laboratory and in between different laboratories and its results should show a consistent association with patient outcome (Gutman and Kessler, 2006; Yip et al., 2008).

Tumor tissue quantity and quality as limiting factor

The spectrum of potentially useful methods for MGMT assessment in the clinical setting is considerably

limited by the quality and quantity of neurosurgical tumor tissue specimens. Tissue preservation protocols used for histopathological diagnostic work-up and tissue archiving usually involve formalin-fixation and paraffin embedding (FFPE). However, formalin-fixation deteriorates the DNA and RNA quality and thus makes application of some methods for molecular diagnostics difficult (Srinivasan et al., 2002; Ferrer et al., 2007; Cox et al., 2008). Furthermore, in some cases tumor localization (e.g. localization in eloquent brain areas) or extensive tumor necrosis may allow extraction of only small fragments of viable and informative tumor tissue. Also, the infiltrative growth pattern of gliomas leads to a high content of non-neoplastic cells (e.g. astrocytes, oligodendrocytes, microglial cells, hematogenous cells) in some biopsy specimens, thus complicating molecular analysis.

MGMT assessment at DNA-level

Bisulfite sequencing

Bisulfite sequencing is currently regarded as the gold standard for the analysis of DNA methylation profiles because it provides single base pair resolution and quantitative methylation information. This method is widely used in biomedical basic research, but it is too expensive and complex for routine clinical application.

Methylation-specific PCR (MSP)

Several studies, including the study by Hegi et al. have used methylation-specific polymerase-chain reaction (MSP) for detection of MGMT promoter methylation status (Esteller et al., 2000; Paz et al., 2004; Hegi et al., 2005; Cankovic et al., 2007; Wick et al., 2007). A pivotal step of MSP is sodium bisulfite modification of DNA, by which unmethylated cytosines

Table 1. Summary of methods that have been used for O6-methylguanine methyltransferase (MGMT) assessment at DNA, RNA and protein levels in glioma.

Analysis level	Method
DNA level	Bisulfite sequencing
	Combined bisulfite restriction analysis (COBRA)
	Melting curve analysis-based semiquantitative real time polymerase-chain reaction
	Methylation-sensitive high resolution melting
	Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)
	Methylation-specific polymerase chain reaction (MSP)
	Methylation-specific SYBR-green-based quantitative polymerase-chain reaction
	Pyrosequencing
	Real-time quantitative MSP (rt-MSP)
	Regional methylation elongation assay
RNA level	Nested in situ rt-RT-PCR
	Real-time reverse transcription-polymerase chain reaction (rt-RT-PCR)
Protein level	Activity assay
	Immunohistochemistry
	Western blot

are converted to uracil, while methylated cytosines are left unchanged (Derks et al., 2004). This modification results in two DNA strands of different sequences that can be separated by polymerase-chain reaction (PCR) using specific primer sets for both unmethylated and methylated DNA. MSP has been described as a sensitive method to detect DNA methylation. However, there are several problems that limit the usefulness of MSP for routine MGMT testing.

Firstly, mosaic methylation patterns with variable degrees of methylation at the primer site may lead to false-positive or false-negative results (Mikeska et al., 2007). Furthermore, there is no control of efficacy of bisulfite modification, thus there is the possibility of false positive results due to inadequate conversion of unmethylated cytosines (Derks et al., 2004; Shaw et al., 2006; Mikeska et al., 2007). It has been noted that inadequate conversion may occur in up to 10% of MSP analyses and is particularly common when DNA quality or quantity is poor and high numbers of PCR cycles or nested primers are used (Shaw et al., 2006). Such conditions are common in the clinical setting, because samples are typically obtained from FFPE tumor tissue specimens. A relatively high percentage of FFPE tissue specimens do not yield DNA of sufficiently high quality to successfully perform MSP. In the study by Hegi et al the MGMT methylation status could be determined by MSP in only 67% of FFPE samples (Hegi et al., 2005). In this multi-centre study, the success rate of MSP was found to be centre-dependent, thus supporting the notion that MSP is highly dependent on tissue handling (e.g. type and concentration of tissue fixative, storage conditions). Also, several groups have reported problems with establishing a reliable MGMT MSP protocol using FFPE tissues (personal communications). However, Cankovic et al reported a simplified MSP protocol involving the use of thick tissue sections and purification of bisulfite treated DNA with the help of commercial methylation kits (Cankovic et al., 2007). This modified MSP protocol allowed them to assess MGMT promoter methylation status of 164 out of 167 FFPE brain tumor resection specimens submitted for MGMT MSP testing. So, the reliability of MGMT MSP in FFPE tissue seems to differ between research groups. Possible explanations could be laboratory-specific differences in tissue preservation (e.g. use of different pH, temperature, or concentration of formalin) or differences in the MSP protocols. MGMT MSP seems to be more reliable in fresh frozen tumour tissue samples (Yachi et al., 2008). Further interlaboratory studies are needed to establish reliable protocols for appropriate tissue handling and MGMT MSP.

Real-time quantitative MSP (rt-MSP)

Real-time quantitative MSP (rt-MSP) has been proposed as a potentially feasible alternative to conventional gel-based MSP (Vlassenbroeck et al., 2008). In a recent study, quantitative rt-MSP results

showed a bimodal distribution, thus potentially allowing definition of a diagnostic cut-off for reliable separation of cases with unmethylated and methylated MGMT promoter. Rt-MSP is suitable for high-throughput analysis and is claimed to work reliably in FFPE. A high rate of agreement of results obtained by conventional gel-based MSP and by rt-MSP has been reported (Vlassenbroeck et al., 2008). Furthermore, rt-MSP yielded interpretable results in significantly more cases than conventional MSP. However, reproducibility of rt-MSP should be validated in independent studies in the academic setting. rt-MSP is currently being applied for randomizing 1153 patients according to MGMT promoter methylation status in an ongoing clinical phase III trial (RTOG 0525/ EORTC 26052-22053) testing standard versus dose intense adjuvant temozolomide in patients with newly diagnosed GBM (Stupp et al., 2007) and in the CENTRIC trial.

Multiplex ligation-dependent probe amplification (MLPA)

Methylation-specific (MS) multiplex ligation-dependent probe amplification (MLPA) has been described as a method to evaluate the methylation status of multiple CpG dinucleotides (Nygren et al., 2005). A recent study has applied MS-MLPA for analysis of MGMT promoter methylation status in glioma and found that it is a robust and reliable method (Jeuken et al., 2007). Of particular relevance is that, in contrast to MSP, MS-MLPA does not require the troublesome step of bisulfite conversion. In the study by Jeuken et al, MS-MSPA provided methylation status of all analysed samples, even in FFPE tumor material. There was a high agreement of results obtained by MSP and MLPA. Therefore, MLPA is a promising candidate method for MGMT promoter methylation testing in gliomas. However, analysis of interlaboratory reproducibility and validation within prospective trials are yet to be performed.

Pyrosequencing

Pyrosequencing is a sequencing-based method that allows analysis of several CpG positions simultaneously. Mikeska et al. compared the accuracy of detecting MGMT promoter methylation of pyrosequencing with two other methods: combined bisulfite restriction analysis (COBRA) and a primer extension- and denaturing high-performance liquid chromatography-based method (SIRPH) (Mikeska et al., 2007). Of these three methods, the pyrosequencing assay had the best reliability and reproducibility on both snap-frozen and FFPE tumour tissue specimens. The authors identified a specific pyrosequencing marker (Py15) as particularly accurate and robust and recommended its use for MGMT testing in the clinical setting. However, the authors noted that the fact that pyrosequencers are not widely available may limit the use of pyrosequencing in the clinical setting.

Combined bisulfite restriction analysis (COBRA)

Combined bisulfite restriction analysis (COBRA) uses a restriction enzyme analysis of PCR-amplified and bisulfite treated DNA and has been applied to testing of MGMT promoter methylation status (Brena et al., 2006). However, a comparative study showed that the pyrosequencing assay is more reliable for assessment of MGMT promoter methylation status on both snap-frozen and FFPE tumour tissue specimens (Mikeska et al., 2007).

Other DNA-based methods

Other DNA-based methods that have been used for MGMT analysis include methylation-sensitive high resolution melting (MSHRM) (Wojdacz and Dobrovic, 2007), regional methylation elongation assay (Zhang et al., 2008), methylation-specific SYBR-green-based quantitative PCR (Hattermann et al., 2008), melting curve analysis-based semiquantitative real time PCR (Lorente et al., 2008). So far, there are limited data on these methods and further studies exploring their feasibility for use in the clinical setting are needed.

MGMT assessment at RNA-level

Real-time reverse transcription-polymerase chain reaction (rt-RT-PCR)

Few studies have investigated MGMT RNA expression and its potential clinical usefulness in malignant glioma. In 1996, Mineura et al. described a significantly longer time to tumor progression in malignant glioma patients treated with alkylating chemotherapy (nitrosourea) with low intratumoral levels of MGMT mRNA than for patients with high levels of MGMT mRNA (Mineura et al., 1996). While Rolhion et al. failed to confirm a significant association of MGMT mRNA expression with patient survival (Rolhion et al., 1999), two more recent studies support the findings of Mineura et al. (Tanaka et al., 2003, 2005). However, in these studies frozen tumor tissue specimens were used that are usually not available in the routine diagnostic setting. Ohe et al used nested in situ rt-RT-PCR for detection of O6-methylguanine-DNA methyltransferase messenger RNA in FFPE human astrocytic tumor tissues and found that this method may be useful for predicting chemotherapy-resistance of tumors (Ohe et al., 2003). In a study on 55 patients with high-grade gliomas, Tanaka et al individualized the adjuvant therapy regimen according to MGMT mRNA expression as assessed by rt-RT-PCR (Tanaka et al., 2008). Patients with low expression of MGMT mRNA were treated with 1-(4-amino-2-methyl-5-pyrimidynyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU)-based chemotherapies. Patients with high MGMT mRNA expression received platinum-based chemotherapy (cisplatin or carboplatin). The authors reported a median

survival period of 21.7 months and a 2-year survival rate of 70.9% for patients with glioblastoma and concluded that their “individualized” adjuvant therapy approach based on the results of rt-RT-PCR may lead to a beneficial glioma therapy. These results await validation by independent studies. However, the high dependency of RNA-based methods on trained personnel, laboratory equipment and high quality tumor tissue may limit their feasibility for widespread use in the routine clinical setting.

MGMT assessment at protein-level

It has been suggested that the favourable association of MGMT promoter hypermethylation with patient survival in patients treated with alkylating chemotherapy is due to transcriptional silencing and therefore inhibition of expression of MGMT protein. It is thought that alkylating chemotherapy can do more harm to tumor cells in the absence of MGMT protein expression, because its cytotoxic effect is not counteracted by MGMT protein. Therefore, it seems in principle conceivable that MGMT assessment at protein level may be more appropriate for prognostic/predictive considerations than MGMT assessment at DNA- or RNA-level. On the other hand, it has been suggested that the favourable prognostic impact of MGMT promoter methylation status may not be due to specific MGMT protein activity at all. Instead, MGMT promoter hypermethylation could simply correlate to general hypermethylation in the tumour DNA (“CpG island methylator phenotype” = CIMP) (Teodoridis et al., 2008). In such a case, the suppression of another gene or a set of genes could be more important for modification of therapy response.

Immunohistochemistry

MGMT protein can be visualized immunohistochemically and commercial anti-MGMT antibodies are available. There are several potential advantages of immunohistochemistry (IHC) as compared to other methods of MGMT assessment. IHC is a commonly used and reliable method in diagnostic histopathology, is available in most laboratories and usually works reliably on FFPE tissue. Indeed, some retrospective studies on small patient series have reported significant associations of immunohistochemically assessed MGMT expression with patient outcome in glioma (Anda et al., 2003; Nakasu et al., 2004; Brell et al., 2005; Pollack et al., 2006; Chinot et al., 2007; Capper et al., 2008), while such an association could not be found by others (Cahill et al., 2007; Rodriguez et al., 2008a). A recent study investigating MGMT IHC on a large series of tumor tissue specimens from a prospective GBM trial found high observer variability, as well as lack of association with the MGMT promoter methylation status and patient survival (Preusser et al., 2008). Likewise, other groups found no significant correlation between MGMT protein

expression and MGMT promoter methylation or lack of a significant survival difference between MGMT-IHC positive and negative patients (Rodriguez et al., 2008b; Sasai et al., 2008; Yachi et al., 2008). The reason for the unreliability of MGMT IHC seems to be that gliomas contain various types of non-neoplastic MGMT expressing cells including lymphocytes, vascular endothelial cells, and macrophages/microglial cells (Sasai et al., 2008). In summary, evidence prevails that anti-MGMT IHC is not useful as clinical biomarker for diagnostic purposes in glioma.

Western blot

In total, there are only very few studies that used Western blotting for MGMT analysis in brain tumors. Hongeng et al used Western immunoblot assays and reported that medulloblastoma/primitive neuroectodermal tumor and ependymoma had the highest level of MGMT, followed by high-grade glioma and low-grade glioma (Hongeng et al., 1997). Nagane et al analysed intratumoral MGMT expression by Western blot in 19 patients with recurrent GBM treated with temozolomide. They found that patients with low MGMT protein expression had a significantly improved progression-free survival and overall survival compared to those with high expression (Nagane et al., 2007). Currently, a major limiting factor for routine use of Western blotting for MGMT analysis is that the method requires unfixed fresh or frozen tumor material.

MGMT activity assay

MGMT activity can be measured by quantitating the transfer of radioactivity from a DNA substrate containing methylated O6-methylguanine to protein (Domoradzki et al., 1984). Several studies have characterized MGMT activity in brain tumor cell lines (Ostrowski et al., 1991; Bobola et al., 1995; Preuss et al., 1995; Bobola et al., 1996; Hermisson et al., 2006). One study reported that MGMT activity and clonogenic survival after temozolomide exposure are highly correlated in human glioma cell lines (Hermisson et al., 2006), while other studies concluded that MGMT makes only a small contribution to tumor cell resistance to nitrosoureas (Bobola et al., 1995, 1996). However, MGMT activity testing of tumor cell lines in the diagnostic setting seems not to be feasible, as establishing of tumor cell lines is complex and usually not routinely performed.

Only few studies have analysed MGMT activity in human glioma tissues. Maxwell et al showed a highly significant correlation between MGMT protein expression assessed by immunohistochemistry and MGMT activity (Maxwell et al., 2006). However, they found no significant correlation between MGMT protein activity and MGMT promoter methylation status. Mineura et al reported that low intratumoral MGMT activity associates with response to post-operative

adjuvant alkylating chemotherapy with nitrosourea (Mineura et al., 1993). Similarly, another study on extracts of 174 human gliomas found that the frequency of tumors lacking detectable MGMT activity was significantly lower in patients who experience tumor recurrence after surgery, radiation, and alkylating agent-based chemotherapy than in patients treated with surgery alone (Silber et al., 1999). Again, at present the reliance of MGMT activity assay on fresh/frozen tumor tissues limits its translation from the research setting into the routine diagnostic setting.

Summary and conclusions

1. There are a number of different methods that have been used for MGMT assessment in GBM. At present, DNA-based methods for MGMT analysis seem more promising for translation into the clinical setting than RNA- or protein-based methods. However, at present there is lack of data to base recommendations for a specific method or protocol for MGMT testing on. There is a strong need for systematic comparisons and validation of intra- and interlaboratory reproducibility and clinical performance of different methods for MGMT assessment to identify the best method for clinical MGMT testing.

2. The current practice of formalin-fixation and paraffin-embedding of neurosurgical specimens considerably limits the spectrum of methods that can be applied for molecular diagnosis in clinical neuro-oncology. Further studies may be helpful to establish more appropriate protocols for tumour tissue preservation (e.g. identification of alternative fixatives that do not deteriorate DNA and RNA quality) (Srinivasan et al., 2002).

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