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O^6-methylguanine-DNA methyltransferase in glioma therapy: Promise and problems

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Abstract

Gliomas are the most frequent adult primary brain tumor, and are invariably fatal. The most common diagnosis glioblastoma (GBM) afflicts 12,500 new patents in the U.S. annually, and has a median survival of approximately one year when treated with the current standard of care. Alkylating agents have long been central in the chemotherapy of GBM and other gliomas. The DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT), the principal human activity that removes cytotoxic O^6-alkylguanine adducts from DNA, promotes resistance to anti-glioma alkylators, including temozolomide and BCNU, in GBM cell lines and xenografts. Moreover, MGMT expression assessed by immunohistochemistry, biochemical activity or promoter CpG methylation status is associated with the response of GBM to alkylator-based therapies, providing evidence that MGMT promotes clinical resistance to alkylating agents. These observations suggest a role for MGMT in directing adjuvant therapy of GBM and other gliomas. Promoter methylation status is the most clinically tractable measure of MGMT, and there is considerable enthusiasm for exploring its utility as a marker to assign therapy to individual patients. Here, we provide an overview of the biochemical, genetic and biological characteristics of MGMT as they relate to glioma therapy. We consider current methods to assess MGMT expression and discuss their utility as predictors of treatment response. Particular emphasis is given to promoter methylation status and the methodological and conceptual impediments that limit its use to direct treatment. We conclude by considering approaches that may improve the utility of *MGMT* methylation status in planning optimal therapies tailored to individual patients.

Keywords

alkylating agents; biomarker; glioblastoma; glioma; MGMT; chemotherapy resistance

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1. Introduction

The protein O^6-methylguanine-DNA methyltransferase is one of the most extensively studied DNA repair activities [1,2]. First discovered in bacteria in 1977 [3], the activity was described shortly thereafter in rodent and human cells [1]. Subsequent work revealed MGMT[1] to have biochemical and biological properties of translational significance (Table 1), including silencing of expression mediated by methylation of deoxycytidine in CpG dinucleotides in the *MGMT* promoter [4,5]. Promoter methylation is associated with prolonged progression-free and overall survival in newly diagnosed glioblastomas (WHO grade IV) treated with temozolomide during and after the completion of radiotherapy [6], the present standard of care; hence MGMT is the first DNA repair protein found to be predictive of treatment response in GBM. MGMT promoter methylation status is also predictive in low-grade and anaplastic gliomas treated with other alkylating agent regimens [*e.g.*,7,8]. While these findings have suggested that MGMT promoter status can be used for treatment stratification of gliomas, important questions about the clinical utility and biological significance of MGMT promoter status remain to be resolved. We will address these questions in this review. We will first describe the biochemical properties and biological activities that suggest MGMT as a biomarker for alkylating agent response in gliomas. We will then summarize the major studies that have sought association of MGMT protein expression, biochemical activity or promoter methylation status with response to alkylator therapy. The discussion will include an examination of the uncertainties that currently limit use of MGMT as a predictive and prognostic marker to guide treatment.

2. Biochemical, genetic and biological characteristics

2.1. Overview

The rational development of individualized treatments for GBM and other gliomas requires identifying tumor-specific biomarkers that predict clinical outcome. As discussed in this section, MGMT possesses biochemical, genetic and biological features that recommend it for such a role.

2.2. Biochemical function

Human MGMT is a monomeric 22 kD protein, and is apparently the sole human repair activity that excises methyl adducts from the O6 position of guanine in DNA [1,2,9]. The preferred substrate is O^6-methylguanine in double-stranded DNA, but the protein also removes larger aliphatic and chloroethyl adducts. Structure-function studies indicate that MGMT binds to the minor groove of DNA and detects O^6-alkylguanine adducts by a base flipping mechanism that rotates the alkylated base out of the DNA helix and into the active site of the protein. The alkyl adduct is then covalently transferred to an active site cysteine residue (Fig. 1), yielding S-alkylcysteine and guanine [2]. This mechanism differs from the pathways that excise most other DNA adducts in that repair is carried out by a single protein without cleavage of the phosphodiester backbone. MGMT is unique among DNA repair activities in that the active site cannot be regenerated, and alkylation of the protein targets it for ubiquitination and subsequent degradation. This “suicide” mechanism implies that the number of O^6-alkylguanine adducts that can be removed from DNA *in vivo* is limited by the number of MGMT molecules in cells and the rate of *de novo* synthesis of the protein. This distinctive property has stimulated a search for pseudosubstrate analog inhibitors that could be used to deplete tumor cells of MGMT during alkylator therapy. While a large number of

[1]**Abbreviations:** BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea, carmustine; CCNU, 3-(2-chloroethyl)-3-cyclohexyl-1-nitrosouea, lomustine; GBM, glioblastoma multiforme; IHC, immunohistochemistry; MGMT, O^6-methylguanine-DNA methyltransferase; MSP, methylation-specific PCR; PCV, procabazine, CCNU, vincristine; TMZ, temozolomide

potential inhibitors has been described [2,10], to date none has proved to be unequivocally suitable for clinical use [11].

2.3. Physiological function

MGMT prevents the genotoxic effects of O^6-alkylguanine adducts produced by exogenous and endogenous alkylators in mammalian and human cells [2,9,12]. MGMT activity varies widely among normal tissues, with brain usually having low levels of expression, and also varies widely among individuals. Notably, MGMT is not necessary for mammalian development as evidenced by the viability of MGMT knockout mice [13]. Knockout animals, however, display heightened sensitivity to the deleterious effects of alkylating agents, including therapeutic alkylators: These findings are immediately relevant to neuro-oncology, demonstrating that MGMT promotes resistance to O^6-alkylguanine-induced cytotoxicity in mammalian cells *in vivo* [*e.g.*, 10,14]. Also relevant is the role demonstrated for MGMT in preventing alkylator-induced carcinogenesis. O^6-alkylguanines are potent mutagenic lesions that characteristically produce point mutations, as well as gross chromosomal deletions and rearrangements that are associated with tumor formation and malignant progression [9]. It has long been recognized that O^6-methyl and O^6-ethylguanine are strong neurocarcinogens in rodents and primates [15,16]. Human exposure to endogenous and exogenous alkylators is believed to be continuous and life-long and there is epidemiologic evidence implicating environmental alkylator exposure with increased risk for human primary brain tumors, including adult gliomas [17 and refs therein]. As discussed below, histologically normal brain of glioma patients is more likely to lack detectable MGMT activity than brain from tumor-free individuals [18,19], suggesting that MGMT acts as a tumor suppressor in the central nervous system.

2.4. Gene structure

The 170 kb human *MGMT* gene is located on the distal end of the long arm of chromosome 10 at 10q26 and contains 5 exons, the first of which is untranslated [12]. The maximal MGMT promoter extends from position −953 to +202 and contains a minimal promoter (−69 to +19) as well as an enhancer (+143 to +202) that binds the MGMT enhancer binding protein (MEBP). The protein is sequestered in the cytoplasm of MGMT-deficient but not MGMT-proficient cells, suggesting a role in regulating MGMT expression [20].

The *MGMT* promoter has a CpG island containing 97 CpG sites, the majority of which are in a region extending from ~300 nt 5' to the transcription start site through the first exon [21]. Promoter CpG islands, defined as regions 0.3 to 3 kb in length with CpG content greater than 60%, are hallmarks of genes subject to epigenetic silencing of expression that is mediated by methylation at the 5 position of cytosine in CpG pairs [22]. Ten binding sites for the transcription promoting protein Sp1 are found in the promoter. Binding of Sp1, which is subject to regulation by p53 [23], stimulates transcription, suggesting a mechanism by which MGMT expression may be regulated in response to DNA damage. In addition, the promoter contains two stress-responsive transcription factor (*e.g.*, *Jun*, *Fos*) binding AP-1 sites and two glucocorticoid response elements. While these features suggest that MGMT expression is responsive to genomic insult and steroids, only modest (2- to 4-fold), transient elevations of MGMT mRNA content have been reported in rat hepatoma cells exposed to DNA damaging agents, including X-rays and alkylators, and to dexamethasone [24]. Moreover, we believe there is no convincing evidence to date that MGMT is inducible by DNA damaging agents or steroids in human cells.

2.5. Regulation of MGMT expression in human tumor-derived cell lines

Approximately 20% to 30% of cell lines derived from diverse human tumors and 50% of virally transformed cell lines do not have detectable MGMT activity [12]. These cell lines

possess an intact MGMT gene as well as the trans-acting factors requisite for transcription, but contain neither MGMT mRNA nor protein, indicating that transcriptional silencing underlies the absence of MGMT activity. Gene silencing in tumor cell lines is commonly accompanied by hypermethylation of promoter CpG islands, suggesting that methylation mediates *MGMT* silencing in human tumors [25,26]. In accord, an early study found that the level of methylation of 21 of 25 CpG sites in the *MGMT* promoter was inversely associated with activity in a panel of human-derived cell lines [27]. Additional experiments using methylation-sensitive restriction endonucleases revealed methylation of 8 promoter CpG dinucleotides in MGMT-deficient but not MGMT-proficient glioma cell lines [21,28,29]. Sequence analysis of bisulfite-treated DNA -- bisulfate converts cytosine, but not 5-methylcytosine, to uracil (Fig. 2) -- from MGMT-deficient and -proficient colorectal tumor [21] and myeloma cell lines [28], revealed 3 clusters of CpG sites, one in the first exon and two 5' to the transcription start site, that were heavily methylated in MGMT-deficient lines and essentially unmethylated in MGMT-proficient lines. In agreement, sequencing all *MGMT* promoter CpGs following bisulfite treatment in 19 MGMT-deficient human tumor cell lines, including 3 from primary brain tumors, showed two heavily methylated CpG clusters, one in the minimal promoter region of exon 1 and the other encompassing the enhancer region 5' to the transcription start site [30]. Subsequent work using methylation-specific PCR of bisulfite-treated DNA (Fig. 3) from glioma cell lines and xenografts substantiated these earlier results [*e.g.*, 31,32]. That promoter methylation has a mechanistic rather than merely correlative association with the MGMT-deficient phenotype is supported by two findings. First, demethylation by long-term exposure to 5-azacytidine induced MGMT expression in one of three MGMT-deficient cell lines [21]. Second, CpG methylation of the *MGMT* promoter in transfected reporter gene constructs inhibited promoter activity [33]. Additional studies have suggested that methylation-related chromatin structure affects MGMT expression by influencing access of transcription factors to the promoter [34]. More recently, CpG methylation has been shown to promote binding of transcription-repressing methyl-CpG binding proteins in MGMT-deficient human tumor cell lines [30,35]. Binding of these proteins condenses chromatin structure, suppressing gene expression by excluding transcription factors [*e.g.*, 30].

2.6. MGMT content of human gliomas and normal brain

Most human tumor specimens express MGMT activity [12]. However, early studies revealed that an appreciable fraction of gliomas lack detectable biochemical activity [19,36 and refs therein]. For example, a survey of 174 adult gliomas revealed a 300-fold range of MGMT activity in 76% of samples, with 24% exhibiting no detectable activity, defined as < 0.25 fmol/10^6 cells or 151 molecules/cell [36]. Another study reported that 7 of 40 (18%) newly operated GBMs had undetectable MGMT activity [37]. In a more recent examination of 149 GBM and anaplastic (*i.e.*, grade 3) gliomas, MGMT activity varied ~220-fold (0.26–57 fmol/10^6 cells; mean ± SD = 8.4 ± 10 fmol/10^6 cells or ~5,000 MGMT molecules/cell); 15 tumors (10%) had no detectable activity (Bobola & Silber, in preparation). These data indicate that the MGMT-deficient phenotype is relatively frequent in GBM and may identify a sub-set of tumors that are more sensitive to alkylating agent-based chemotherapy. In accord with this conclusion is the finding that the frequency of MGMT-deficient gliomas was 7-fold lower (4% vs 29%) among tumors recurring after alkylator therapy compared to newly operated tumors. In contrast, prior radiotherapy, which does not cause alkylation, had no effect on the fraction of MGMT-deficient gliomas [19]. More recently, Kaina and colleagues examined the effect of adjuvant therapy on MGMT activity in a sample of 40 GBM [37]. They found that a statistically significant 2-fold elevation of mean MGMT activity accompanied initial recurrence after radiotherapy and alkylating agent chemotherapy compared to newly operated tumors. No statistically significant change in activity accompanied recurrence after treatment with radiation alone. These results strongly

suggest that selection for cells expressing higher levels of MGMT accompanies alkylator therapy, and support the hypothesis that MGMT promotes alkylating agent resistance *in vivo*.

It has been also reported that histologically normal brain adjacent to gliomas displayed MGMT-deficient phenotype in 55%–65% of cases [18,19]. In contrast, normal brain from individuals without glioma had undetectable MGMT activity in 12% of cases, a finding suggesting that absence of MGMT may be a risk factor for human gliomagenesis. Subsequent work indicated that MGMT-deficiency in brain may arise during development [38]. In this study, 76% of 6- to 8-week old embryonic brain specimens lacked detectable MGMT activity. This fraction decreased with developmental age such that 13% of 16–18 week fetal brain specimens were MGMT-deficient, essentially the same fraction as observed in adult normal brain from patients without glioma. These results suggest that lack of MGMT in the normal brain of a minority of individuals represents the persistence of a pre-natal phenotype, and that absent MGMT may be an epigenetically determined susceptibility factor for gliomagenesis. Whether the absence of MGMT activity in normal brain adjacent to gliomas reflects silencing by promoter methylation or atypical regulation by transcription factors during development, and/or other processes, remains to be elucidated.

2.7. MGMT promotes resistance to clinical alkylating agents in glioma cells

It is well established that MGMT promotes resistance to the methylating imidazole derivative TMZ, and to nitrosourea-derived methylating (*e.g.*, procarbazine) and chloroethylating (*e.g.*, BCNU, CCNU) agents in glioma cell lines [*e.g.*, 39–41] and xenograft models of human gliomas [*e.g.*, 29,42]. This conclusion is based on extensive results with cells that either lack MGMT expression, or in which MGMT activity was ablated by treatment with substrate analog inhibitors (*e.g.*, O^6-benzylguanine, lomeguatrib) [10,11,32]; in all cases the absence of MGMT was accompanied by greater sensitivity to methylator and chloroethylator cytotoxicity. The extensive findings in glioma cells are concordant with a larger body of evidence obtained in other human and mammalian cells (*e.g.*, [9], and references therein, from Kaina's group). The lethality of O^6-methylguanine and O^6-chloroethylguanine is emphasized by the fact that both constitute only a small fraction (~7% and 3.5%, respectively) of the base adducts produced by their respective alkylators [43–45]. The repair of these minority lesions by MGMT prevents interruption of DNA replication, thus avoiding the genesis of double-strand breaks, potently cytotoxic lesions that induce apoptosis [9].

The mechanism by which O^6-methylguanine induces cytotoxicity has been the subject of intensive investigation [9,46]. The current consensus reflects the observations that replicative DNA polymerases do not stall at O^6-methylguanine but incorporate deoxycytidine or thymidine opposite the lesion with approximately equal frequency. In either case, O^6-meG does not correctly base pair with the newly incorporated nucleotide, producing a single nucleotide mispair substrate for mismatch repair. Mismatch repair excises a long strand of newly synthesized DNA that encompasses the mispair. Subsequent DNA repair synthesis to fill the gap produces yet another mispair at the adduct remaining in the template strand, thus again eliciting the action of mismatch repair. This futile cycle of abortive attempts to resolve the mispair has the effect of producing a persistent single-strand gap that is converted into an apoptosis-inducing double-strand break during DNA replication in the next S-phase. This model provides a mechanism to account for the resistance to methylating agents that accompanies loss of mismatch repair [46]. Single-strand gap formation can also account for the radiosensitizing effect of O^6-methylguanine in DNA, as these gaps are easily converted to DNA double-strand breaks by attack of radiation-induced oxidative free radicals [41].

O^6-chloroethylguanine rapidly undergoes rearrangement to form three lesions: O^6-hydroxyethylguanine, O^6-aminoethylguanine and the exocyclic ethano adduct 1-O^6-ethanoguanine [47,48]. The latter adduct slowly undergoes an intramolecular rearrangement to form the 1-(1-guanyl)-2-(3-cytosinyl)ethane inter-strand crosslink, a physical barrier to DNA replication. Stalled DNA replication forks are sites of double-strand break formation during the subsequent round of DNA replication [9]. It is well established that MGMT-mediated resistance to chloroethylating agents is accompanied by reduced crosslink formation. Less certain is whether this is accomplished by the interaction of MGMT with O^6-chloroethylguanine or with 1,O^6-ethanoguanine. Data from Brent's group indicates that MGMT reacts with the ethano intermediate to produce a DNA-protein crosslink that is excised by nucleotide excision repair [49].

3. MGMT as a predictive and prognostic marker

3.1. Overview

While the contribution of MGMT activity to glioma cell alkylating agent resistance in cell lines and xenografts is firmly documented, evidence that MGMT is a clinical resistance mechanism requires establishment that MGMT expression is inversely associated with progression-free and overall survival following alkylator therapy. At present, this relationship has been established retrospectively for GBM [*e.g.*, [50], and prospective analysis is ongoing (RTOG 0525 trial) [51]. Numerous studies have examined MGMT protein expression visualized by immunohistochemistry, activity measured by biochemical assay, and promoter CpG methylation status determined by a variety of methods that exploit the bisulfite-induced deamination of cytosine, but not 5-methylcytosine, to uracil (Table 2; Fig. 2). However, whether any of these measures of MGMT expression can be used to individualize treatment remains an open question. As discussed below, they have limitations that potentially confound biological interpretation and compromise clinical utility.

3.2. Immunohistochemistry

Examination of paraffin-embedded, formalin-fixed tumor sections by IHC offers a clinically convenient approach to determining MGMT expression in human gliomas. To date, the utility of MGMT expression, assessed by IHC, as a marker of progression-free and overall survival remains to be unequivocally demonstrated. Some studies have found an inverse association between immunopositivity for MGMT and outcome. The earliest of these studies used quantitative immunofluorescence microscopy to evaluate MGMT content in 99 GBM and 47 anaplastic astrocytomas that received BCNU during adjuvant treatment [52]. This method is notable in that it quantifies the number of MGMT molecules per nucleus rather than the fraction of immunopositive cells. Tumors with MGMT expression less than 60,000 MGMT molecules/nucleus (*ie.*, ~100 fmol MGMT /10^6cells or ~33 fmol MGMT/mg protein, assuming one cell contains 0.3 ng protein) had significantly longer progression-free and overall survival. Comparable results were observed in another study of 64 GBM and anaplastic astrocytomas treated with radiation and BCNU [53]. Several subsequent studies using standard immunostaining techniques found that low MGMT expression (*i.e.*, low fraction of immunopositive cells) was prognostic of longer overall [*e.g.*, 54–57] or progression-free [58] survival following alkylator therapy. The cut-off points for low expression ranged from 5% to 50% and were chosen to reveal the most significant between-group differences. Other studies have not found an association between MGMT immunopositivity and outcome [*e.g.*, 59–62]. The lack of accord among all these reports highlights the intrinsic limitations of IHC [*e.g.*, variability caused by section thickness, fixation and embedding procedures, and antibody dilution as well as poor reproducibility imposed by observer variability (detailed in [61]). Importantly, the IHC studies revealed highly variable intra-tumoral intensity of immunostaining, suggesting heterogeneous

MGMT expression. However, unlike the earlier quantitative immunofluorescence microscopy studies, standard IHC cannot quantify this variability. It should be noted that compared to other MGMT assays, IHC is relatively insensitive, with a lower limit of detection ranging from 3,000 to 12,000 molecules/cell [52,63]. By comparison, biochemical assay can detect as few as ~150 molecules/cell [19] and is at least 20-fold more sensitive. As discussed below, median MGMT activity in GBM is ~ 3,000 molecules/cell, suggesting the potential for a high frequency of false negatives using IHC.

3.3. Biochemical activity

The unique reaction mechanism of MGMT, in which an alkyl group is irreversibly bound to the protein (Fig. 1), is the basis for the most commonly used assay to determine activity in tissue samples. Briefly, cleared supernatants of intact tissue homogenates are incubated with DNA containing [^{3}H]-labeled O^6-methylguanine. After acid hydrolysis to release the remaining O^6-methylguanine from the DNA substrate, [^{3}H]-labeled protein is recovered by filtration. Because only one methyl group is bound per MGMT molecule, the assay determines the number of MGMT molecules in the volume of extract assayed. As noted above, the assay is very sensitive, capable of detecting as few as 150 MGMT molecules/cell [19,36]. Biochemical assay has the advantage of providing an objective, continuous variable, *i.e.*, MGMT activity, which facilitates analysis of the association of activity with clinical outcome. However, in its current form the biochemical assay is not practical for routine clinical laboratory analysis because of the requirement for extract preparation from intact, fresh tissue and the use of a radioactive DNA substrate.

There are only a few studies of the association of glioma MGMT activity with response to alkylating agent-based chemotherapy. In an examination of 62 high-grade gliomas, no difference was reported in progression-free survival following treatment either with BCNU or PCV between 20 MGMT-deficient and 42 MGMT-proficient tumors [19]. A major drawback of this study was the inclusion of diverse diagnoses that vary widely in intrinsic response to adjuvant treatment. In a recent study of 77 *de novo* GBM, tumors with MGMT activity less than the median value (~3,000 molecules/cell) were significantly less likely to progress than higher activity tumors (hazard ratio = 2.06; $P \leq 0.004$); this difference was observed for tumors treated with radiation followed by alkylators and for tumors treated with concurrent radiation and TMZ (Bobola and Silber, in preparation). When entered in Cox regression analyses as a continuous variable, MGMT activity was inversely associated with progression-free survival and showed a greater than 100-fold difference in risk for progression between GBM with the highest and lowest activities. Comparable results were observed for 72 anaplastic gliomas. These findings are in accord with a recent study of 40 GBM showing significantly greater progression-free survival for tumors with MGMT less than the median value of 30 fmol/mg extract protein [37]. It must be kept in mind that MGMT activity measured in tumor tissue represents an average activity of all cells, both neoplastic and normal. For example, vascular endothelial cells in gliomas have been reported to be immunopositive for MGMT (*e.g.*, [64]) and the majority of peripheral blood leukocytes, which are likely to be present in tumor specimens, either express MGMT activity ([18]; Chamberlain and Silber, submitted) or display unmethylated promoters in glioma patients (*e.g.*, [65]). However, the contribution of contaminating nonneoplastic neural cells to tumor specimen activity may be negligible in many instances, as data suggest that brain adjacent to glioma lacks detectable activity in the majority of cases [18,19].

3.4. Promoter CpG methylation status

Numerous studies have shown that methylation of the *MGMT* promoter CpG island is associated with prolonged progression-free survival and overall survival after alkylator therapy in GBM and other gliomas [4,5]. In the landmark EORTC-NCIC trial, promoter

methylation was associated with prolonged progression-free and overall survival in newly diagnosed GBM treated with concurrent TMZ and radiotherapy followed by adjuvant TMZ [6,50]. Patients with methylated tumors had 2-year and 5-year survival rates of 49% and 14%, respectively, compared to 15% and 8% for unmethylated tumors. Additional clinical trials have documented that GBM harboring methylated MGMT promoters show longer survival following concurrent therapy [*e.g.*, 58,62,66–70], adjuvant TMZ together with CCNU [*e.g.*, 71]) and adjuvant BCNU [72]. In addition, the majority of GBM from patients surviving more than 3 years following radiotherapy and various alkylating regimens displayed *MGMT* promoter methylation [73,74]. Other studies have shown that promoter methylation is associated with better outcome for anaplastic gliomas and grade II gliomas treated with a variety of alkylator-based regimens [*e.g.*, 7,8,75–77]. These findings convincingly demonstrate that promoter methylation status identifies alkylation-sensitive GBM and other gliomas.

4. Determination of methylation status

4.1. Overview

In most assays, the methylation status of CpG residues is determined by the bisulfite-mediated deamination of cytosine, but not 5-methylcytosine, to uracil (Fig. 2; [78]). Considerable effort has been expended developing simple, reliable, and, more recently, quantitative techniques to distinguish uracil from 5-methylcytosine, as well as to identify CpG residues whose methylation status is most tightly associated with treatment outcome. Single molecule sequencing of bisulfite-treated promoter DNA is the gold standard for assaying the frequency of methylation at any given CpG site. While this approach permits determination of the methylation frequency at all 97 CpG residues in the *MGMT* promoter [*e.g.*, 58], the technique is not clinically tractable, although it has been used to validate other methylation assays [*e.g.*, 79]. Methylation-specific PCR (MSP) assays that discriminate between uracil and 5-methylcytosine at a limited number of promoter CpG residues, as illustrated in Fig. 3, have proved to be more tractable, permitting the analyses of large numbers of tumors that have revealed the association of clinical outcome with promoter methylation status.

4.2. MSP-based techniques

For most major clinical studies, MSP has been used to examine the status of 4–12 CpG sites in the *MGMT* promoter [4,5]. MSP techniques use two sets of primers to interrogate CpG dinucleotides that are frequently methylated in MGMT-deficient cell lines [*e.g.*, 78,80]; one set contains guanine complementary to 5-methylcytosine and the other contains adenine complementary to uracil (Fig. 3). In its original form, also referred to as nested MSP, the assay examines 9 CpG sites and is designed to produce different-sized PCR products from methylated and unmethylated primers that are resolved by agarose gel electrophoresis. Thus, the result is dichotomous, assigning tumors to one of two categories. The assay was validated by early work that yielded a single, expected band for MGMT-proficient and MGMT-deficient human cell lines [72,80]. However, results with tumor tissue were not as definitive. While MGMT-expressing tumors, evidenced by IHC showing frequent, unambiguous nuclear staining, displayed the unmethylated promoter band, MGMT-non-expressing tumors almost invariably displayed both the methylated and unmethylated products. Appearance of the unmethylated signal has been attributed to the presence of normal, MGMT-expressing cells [*e.g.*, 50,80], and GBM and other gliomas displaying both PCR products are assigned methylated status. The appearance of both PCR products may also reflect the presence of MGMT-deficient and -proficient glioma cells in a tumor, or partially methylated sequences, or even possibly the presence of promoter hemimethylation

(*i.e.*, only one MGMT allele is methylated) as recently reported for MGMT-proficient cell lines [32].

Quantitative-MSP (Q-MSP), a variant of MSP utilizing real time PCR techniques [81], attempts to standardize the assay, better differentiate methylated and unmethylated tumors and provide a quantitative estimate of the frequency of methylation. This technique utilizes specific primer sets to simultaneously amplify an *MGMT* promoter sequence encompassing 8 CpG sites and an unmethylated reference gene, typically *Actin B*. Amplification of promoter and reference sequences is quantified by generation of fluorescent signals, *e.g.*, by using Taqman- or SYBR-green-based assays. Fluorescence intensity, expressed as C_T, the cycle number at which signal is significantly higher than background, is converted to sequence copy number by using standard curves generated with bisulfite-treated plasmids containing the methylated promoter or reference sequence. Promoter copy number is normalized to the reference sequence to allow comparison between tumor samples. These techniques quantify methylation status as either copy number of fully methylated promoters [*e.g.*, 82] or as the percentage of total promoters that are fully methylated [*e.g.*, 62].

Hegi and colleagues used Q-MSP with *Actin B* as a reference to determine the *MGMT* promoter methylation status of 134 gliomas [82]. A number of stringent criteria, including *Actin B* copy number greater than 1,000, had to be satisfied to validate each assay. Interestingly, the normalized copy number of methylated *MGMT* promoters could be fitted to a bimodal distribution, suggesting that the technique revealed an objective cut-point for distinguishing methylated and unmethylated tumors. In agreement with this hypothesis, the methylation status determined by Q-MSP and MSP in 91 gliomas was concordant in 90% of tumors. While this work suggests that Q-MSP affords an objective basis for assigning methylation status, there is no *a priori* reason to expect a bimodal distribution of methylated promoters, and the diagnostic heterogeneity of the tumor sample precluded examination of the association of methylated promoter copy number with clinical response. There was also no examination of the relationship of methylated promoter copy number with MGMT expression, limiting insights into the possible biological and biochemical significance of the putative bimodal distribution. A recent study illustrated the potential clinical utility of a variant Q-MSP technique that measured both methylated and unmethylated promoter copy number (semi-quantitative- or SQ-MSP). In an analysis of 81 GBM treated with concurrent TMZ and radiotherapy, overall survival was significantly longer in tumors with a greater than median fraction (35%) of methylated promoters [62]. Fitting the fraction of methylated promoters to a bimodal distribution was reported to yield a cut-point for prolonged survival that was essentially the same as the median (30–35%). Notably, the prognostic value of SQ-MSP was greater than that of gel-based MSP, suggesting that determination of methylation status by real time PCR-based techniques has clinical utility. The methods employed, however, are technically demanding and better suited for dedicated laboratories. In this regard, we note that analysis of methylation status by Q-PCR is commercially available [*e.g.*, 82].

4.3. MSP: Limitations and caveats

Current MSP techniques have shortcomings that limit detection and quantification of CpG methylation. For example, techniques are optimized for primer binding to either *completely* methylated or unmethylated template sequences. Heterogeneous methylation, either inherent or the result of incomplete bisulfite-mediated deamination, can prevent primer annealing and extension, or foster indiscriminate annealing of primers, producing both methylated and unmethylated products from the same promoter sequence. Methylation-specific pyrosequencing addresses the problem of heterogeneous methylation by providing an average frequency of methylation at all CpG sites assayed [79,81]. Other approaches include digestion with restriction endonucleases to determine CpG methylation status. Combined

bisulfite restriction analysis (COBRA; [79]) employs digestion of bisulfite-treated, PCR-amplified DNA with *BstU*I or *Taq*I to eliminate unmethylated CpGs. In the case of methylation-specific multiplex ligation-dependent probe amplification (MS-MPLA; [83]), digestion of native DNA with methylation-sensitive *HhaI* eliminates unmethylated CpG sites from the analysis. The complexity of the latter two methods will likely limit their widespread clinical application.

Current promoter methylation assays are also limited by inability to distinguish signal specific to tumor from that contributed by accompanying normal cells. Most clinical studies circumvent this problem by extracting DNA from tumor cells that have been micro-dissected from formalin fixed, paraffin embedded tissue [*e.g.*, 84]. This approach is attractive in that it allows analysis of archived specimens as well as minimizing contamination by normal cells. However, several caveats should be kept in mind concerning archived material. Very small amounts of DNA, typically no more than several hundred nanograms, are usually recovered, and as much as 90% of DNA can be lost because of strand breaks produced during bisulfite treatment. In addition, fixation promotes DNA-protein cross-links that can compromise template function during PCR [85,86]. As a result of these limitations, MSP can be uninformative in as many as 25% of cases [*e.g.*, 82]. Low yield of poor quality DNA necessitates increasing the number of PCR cycles required to produce an interpretable result. This can introduce uncertainty in the assay by heightening the risk of extending primers indiscriminately bound to heterogeneously methylated templates. Increasing cycle number of PCR assays that can detect fewer than one sequence in a thousand also elevates the risk of detecting signal from contaminating normal tissue. We note that it is commonly assumed that contaminating normal cells invariably express MGMT, as has been reported for endothelial cells in gliomas [64] and therefore contribute only unmethylated promoters that can be discounted in the presence of methylated signal [*e.g.*, 80,87]. However, this is not necessarily the case. Contamination of gliomas with normal cells also results from tumor infiltration of surrounding non-neoplastic brain that frequently lacks detectable MGMT activity [18, 36], raising the possibility that contaminating normal brain cells may also contribute to a methylated signal. Moreover, an appreciable fraction (18% to 25%) of GBM and other gliomas lack detectable MGMT activity [19,32,36], implying that neither tumor nor normal cells express MGMT.

An alternative approach to limiting the contribution of accompanying normal cells would be to isolate cells from fresh tissue prior to analysis. In a recent report [88], Hegi and co-workers compared MGMT mRNA expression, activity and promoter methylation in 10 GBM and paired cultures established from $CD133^+$ cells isolated from the same tumors. All tumors had detectable MGMT activity although seven were methylated by MSP as evidenced by the presence of PCR product for both methylated and unmethylated promoters. Single clone sequencing of bisulfite-treated DNA revealed heavy methylation at 28 CpGs within the minimal promoter region in 25% to 90% of clones of the seven methylated GBM. The $CD133^+$ cultures established from the methylated tumors had little or no MGMT activity, and 100% of bisulfite-sequenced clones showed extensive methylation comparable to that of the tumor of origin. In contrast, the three cultures derived from unmethylated tumors had MGMT activity and displayed little or no promoter methylation. As CD133 expression is a marker of a sub-set of GBM stem cells that are believed to determine the biological and clinical course of GBM [89], these findings suggest that methylation is indicative of low or absent MGMT expression in tumor. The presence of MGMT activity and unmethylated promoters in the seven GBM was attributed to contaminating normal cells. This study represents the most comprehensive effort to date to compare MGMT activity and promoter methylation in GBM stem cells with activity and methylation in the tumor of origin. The authors stated that $CD133^+$ cultures could be established from only about 50% of tumors, raising the possibility that methylation of the MGMT promoter

accompanies other traits that promote *in vitro* proliferation. It should be noted that growth in culture is frequently accompanied by extensive changes in CpG methylation, and the $CD133^+$ cells were cultured for 2 to 12 months prior to analysis. Also, culture conditions can affect MGMT expression, as illustrated by a lymphoblastoid line that expressed MGMT when grown as adherent cells, but lost MGMT expression when grown in suspension [90]. Thus, how representative the methylation pattern of a subset of cultured GBM cells is of tumor cells *in vivo* remains uncertain.

The fraction of GBM found to have methylated *MGMT* promoters ranges from 35% to 73%, with similar variability observed for anaplastic and grade II gliomas [4]. This frequency is much greater than the fraction of GBM and other gliomas that lack biochemically detectable activity (~18%–25%; [19,37]). While the discrepancy between the results of promoter methylation and activity assays likely reflects the convention of assigning methylated status to tumors exhibiting both methylated and unmethylated products, the varying estimates of methylation frequency may also reflect, in part, the technical difficulties inherent in all CpG methylation measurements and lack of objective criteria for determining a cutoff point for the fraction of methylated promoters that define the methylated phenotype. The wide range in promoter methylation may also reflect the assumption that contaminating normal cells invariably display unmethylated promoters, an assumption that, as discussed above, may not be valid in all cases.

5. Utilization of methylation status to direct treatment

Identification of biomarkers that predict GBM response to therapy is a critical step in the rational development of treatments tailored to individual patients that produce optimal clinical outcome. Currently, only MGMT promoter methylation assays have been shown to consistently identify GBM that are more likely to respond favorably to alkylating agent-based therapies in multiple independent studies [4,5,50]. The question of whether or not methylation status should be used to allocate treatment, particularly for unmethylated GBM, has been asked repeatedly [*e.g.*, 4,89]. Several considerations and outstanding questions indicate that the answer remains no, and the clinical utility of promoter methylation status is currently limited to providing an estimate of overall prognosis.

Perhaps the greatest impediment to clinical utilization is the lack of a validated, standardized promoter methylation assay. Standardization would permit inter-group comparison of results, facilitating the inclusion of methylation data in the design of clinical trials. Standardization is currently impeded by lack of consensus about what technique is most tractable in the clinical setting, which promoter CpG sites are most informative and what criteria permit an objective assignment of methylation status. Conceivably these impediments can be circumvented by emerging DNA sequencing methods that can detect methylation at all promoter CpG sites without the use of bisulfite-mediated deamination [*e.g.*, 92]. Assay standardization and rigorous criteria for methylation status may also help overcome another impediment, the high backgrounds of false negative and positive results produced by current methylation assays. This lack of specificity is exemplified by the observation that 15% of patients with unmethylated GBM treated with concurrent TMZ-radiotherapy survive 2 years, a 7-fold increase in frequency compared to tumors receiving radiation alone [6]. It is important to keep in mind that this uncertainty, while likely reflecting to some extent the inherent limitations of current assays, may also be indicative of tumor sub-groups in which MGMT is not the primary determinant of treatment outcome.

Another limitation of methylation status as a biomarker is that it does not aid in choosing a particular alternative therapy for unmethylated tumors, other than that the regimen not include alkylating agents. It has been proposed that unmethylated GBM are candidates for

MGMT ablative therapy, either using substrate analog inhibitors or TMZ dose intensification [4,11]. However, there is no compelling evidence that methylated GBM would not also benefit from MGMT ablation since assay of these tumors almost invariably reveals evidence of unmethylated promoters, conceivably from MGMT-expressing tumor cells. While, on average, promoter methylation may be associated with no/low MGMT activity in GBM tissue, the correspondence is not absolute, and thus the status of expression in any individual tumor is not unequivocal. For example, methylation status does not consistently correlate with MGMT expression assessed by IHC [*e.g.*, 61,62]. Likewise, MGMT activity of methylated GBM was found to be lower than that of unmethylated tumors in one study [32], but not in another [93]. More recently, we have observed that MGMT activity in 39 unmethylated GBM was significantly higher than that in 15 methylated tumors (10 ± 8.9 *vs* 4.8 ± 2.6 fmol/10^6 cells; $P \leq 0.002$; Silber unpublished). However, there is considerable overlap of activities between the two groups, reflecting the presence of low activity unmethylated and high activity methylated GBM. This finding reflects a major limitation imposed by the inability of current CpG methylation and biochemical assays to distinguish not only tumor from normal cells but methylated from unmethylated GBM cells within the same tumor.

6. Future considerations and outstanding questions

As set out in this review, current promoter methylation assays suffer from a number of methodological and conceptual limitations that confound assignment of methylation status. Current assays are poor surrogates for MGMT activity and do not reflect intra-tumoral heterogeneity in MGMT expression that is evident by IHC [57]. These factors are problematic if MGMT activity is the exclusive or predominant determinant underlying the predictive power of promoter methylation (See discussion below). Moreover, unlike genetic aberrations that can be unambiguously assigned to tumor cells, promoter methylation assays do not distinguish signal specific to tumor from that contributed by accompanying normal cells. These limitations, together with a number of outstanding questions pertinent to both biological significance and clinical utility (Table 3), need be addressed in order to increase specificity and predictive power such that the assays can identify treatment-responsive individuals.

The clinical utility of *MGMT* promoter methylation assays would be greatly increased if there were methylation patterns that distinguished tumor from surrounding normal tissue, a possibility yet to be rigorously investigated. Tumorigenesis is frequently accompanied by altered CpG methylation that may change MGMT expression and that may differ from distinctive patterns that are associated with MGMT expression in normal progenitor tissue [26,94]. Moreover, the methylation patterns currently believed to be indicative of *MGMT* silencing in tumor tissue were derived from GBM cell lines that had been in continuous culture for years, raising the possibility that physiologic patterns of methylation were modified or lost in response to selective pressures exerted by long-term growth *in vitro*. These considerations suggest that single-molecule bisulfite-sequencing of the promoter and gene body in GBM and normal brain specimens with known MGMT activity may reveal distinctive methylation patterns that distinguish tumor and normal cells, and also allow a quantitative estimation of the fraction of MGMT-non-expressing tumor cells and the distribution of MGMT activity among tumor cells. This information may provide the means to improve stratification by risk for progression among patients, especially if expressed as a continuous variable.

Also complicating interpretation of the significance of promoter methylation status is evidence that it may not be specific for alkylating agent response. Some [*e.g.*, 7,8,95,96], but not all [6], studies have found that methylation is associated with better outcome in gliomas

treated with radiation only. In accord are recent reports that radiotherapy as well as alkylator treatment selects strongly against promoter methylation in recurrent tumors [*e.g.*, 32,97]. As radiation sensitivity is not mediated by MGMT activity, these findings suggest that MGMT promoter methylation status may reflect a broader DNA damage sensitivity phenotype. In agreement with this hypothesis, it has been demonstrated that MGMT-non-expressing GBM cell lines display not only greater sensitivity than MGMT-expressing lines to O^6-methylguanine, but also to 3-methyladenine, a TMZ-induced cytotoxic adduct that is removed by base excision repair [98]. In addition, other DNA repair proteins are epigenetically regulated [99], including the Werner syndrome helicase (*WRN*) that promotes TMZ resistance in human GBM cells [100]. Suppression of MGMT together with changes in expression of additional DNA repair proteins could result in sensitivity to alkylator-induced adducts other than O^6-methylguanine and to DNA damaging agents in addition to alkylators. Definitive demonstration that MGMT promoter methylation status reflects a global response to therapeutic agents that act by damaging DNA could have an immediate impact on choosing alternative therapies for unmethylated GBM.

Inclusion of additional informative markers together with *MGMT* promoter methylation status in multivariate regression models may increase the ability to stratify treatment by projected response in GBM. While MGMT is the sole DNA repair protein that removes O^6-methylguanine from DNA, additional repair mechanisms promote resistance to the consequences of failure to excise this and other TMZ-induced adducts, *i.e.*, interrupted DNA replication leading to single-strand gaps and double-strand breaks. Human cells possess numerous pathways that promote replication re-start at stalled replication forks [101], gap filling by error-prone DNA polymerases [102] and rejoining of double-strand breaks by homologous and non-homologous recombination [103]. TMZ produces at least two additional cytotoxic lesions, 3-methyladenine and abasic sites, which are not substrates for MGMT. Notably, the abasic site endonuclease activity of Ape1/Ref-1, the DNA repair enzyme primarily responsible for initiating the excision of abasic sites in human cells [104], is inversely associated with progression-free survival in anaplastic gliomas treated with radiotherapy or radiotherapy followed by alkylating agent-based chemotherapy [105]. In addition to DNA repair proteins, a host of genetic and epigenetic molecular markers have been identified in GBM that govern proliferation, response to treatment and survival [*e.g.*, 5,106,107].

As alluded to above, an unresolved question is whether the longer survival following alkylating agent treatment of GBM displaying promoter methylation is solely due to reduced MGMT expression. The genesis of GBM is characterized by changes in gene expression mediated by global DNA hypomethylation accompanied by gene-specific methylation of promoter CpG islands. Ongoing work indicates that GBM can be classified into clinically informative sub-types based on distinctive methylation patterns [*e*.g., 108,109,110], including one having the hallmarks of a glioma CpG island methylator phenotype (G-CIMP; [106,110]). The observation that the G-CIMP sub-group of GBM has prolonged survival emphasizes the question of whether MGMT promoter methylation accompanies other epigenetic changes associated with better outcome. A recent examination by Etcheverry *et al.* of 50 adult GBM patients treated with concurrent radiation and TMZ suggests that this may be the case [109]. This study found that depending on the gene, promoter hypermethylation or hypomethylation was associated with better clinical response independent of *MGMT* methylation status. The promoter methylation of some genes strengthened the prognostic power of *MGMT* methylation status, suggesting that epigenetic silencing of both contributed to better clinical outcome. However, for other genes, promoter methylation negated the better outcome usually associated with *MGMT* methylation, suggesting that silencing of these genes promoted resistance to the consequences of not removing O^6-methylguanine or to some other, unrelated resistance mechanism(s). While the

work of Etcheverry *et al.* was a small study that requires independent confirmation with a larger sample of GBM, these findings strongly suggest that clinical response to concurrent therapy is multifactorial and that MGMT is not necessarily the predominant determinate of response. They also suggest a mechanistic explanation for the failure of some tumors to respond to therapy as predicted by *MGMT* promoter methylation status and may identify future targets for therapeutic intervention.

For the variety of reasons discussed in this review, the clinical utility of CpG methylation is presently limited to providing an estimate of overall prognosis for populations of tumors. The ultimate goal is to identify with a high degree of certainty treatment-responsive individuals in order to direct patients to the most efficacious therapy. Ongoing research and advances in technology are likely to increase the usefulness of *MGMT* promoter methylation status as a tool for personalizing anti-glioma treatment. However, this goal cannot be realized until alternative therapies for newly diagnosed GBM are developed that produce outcomes comparable to concurrent TMZ and radiation for unmethylated tumors. Only with the advent of new, effective therapies can *MGMT* promoter methylation status truly be used to inform treatment decisions.

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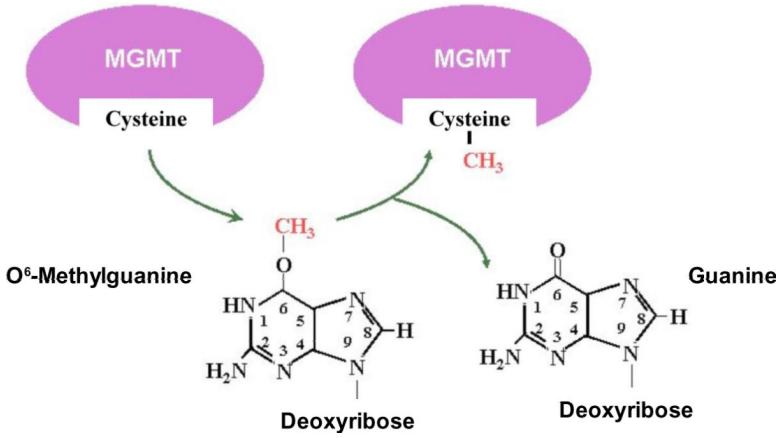

Figure 1. Repair of O^6-alkylguanine adducts by MGMT

MGMT mediates a stoichiometric reaction in which methyl and other alkyl groups bound to the O6 position of guanine in DNA are via a thioester linkage to a cysteine residue in the active site. Alkylation of MGMT leads to rapid degradation of the protein. Thus, the capacity of a cell to remove O^6-alkylguanine from DNA reflects the rate at which adducts are produced by exogenous and endogenous agents and the rate of synthesis of new protein. The "suicide" reaction mechanism of MGMT is unique among human DNA repair enzymes, and suggests the use of substrate analog inhibitors of MGMT in order to increase sensitivity to chemotherapeutic alkylating agents. Image adapted from www.mgmt-agt.net/whatismgmt.htm.

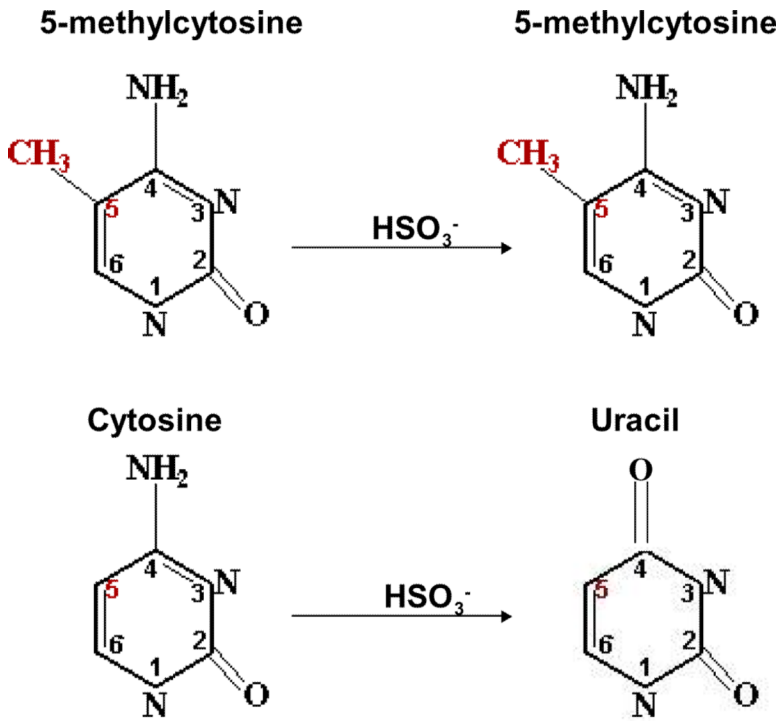

Figure 2. Differential sensitivity of 5-methylcytosine and cytosine to bisulfite-mediated deamination

5-methylcytosine is insensitive to incubation with bisulfite at mildly acidic pH. In contrast, bisulfite treatment promotes the hydrolysis of cytosine, producing uracil and ammonia. This differential sensitivity has been exploited to detect 5-methylcytosine in DNA. Image adapted from www.imb-jena.de/~sweta/renzymes.

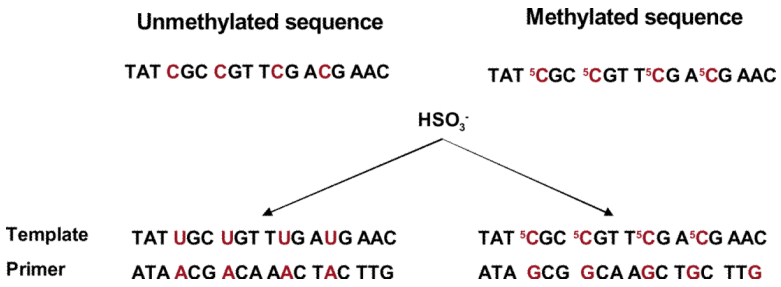

Figure 3. Methylation-specific PCR (MSP)

Bisulfite treatment converts cytosine in DNA to uracil (left) while leaving 5-methylcytosine unaltered (right). The uracil- and 5-methylcytosine-containing template sequences hybridize to unique primers to yield distinct products during PCR amplification.