O6-methyl-guanine-DNA methyltransferase Methylation in Serum and Tumor DNA Predicts Response to 1,3-Bis(2-Chloroethyl)-1-Nitrosourea but not to Temozolamide Plus Cisplatin in Glioblastoma Multiforme

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ABSTRACT

Purpose: In glioblastoma multiforme (GBM), the cytotoxic effect of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolamide is dependent on O6 alkylation, which correlates inversely with expression of the DNA repair enzyme O6-methyl-guanine-DNA methyltransferase (MGMT). Thus, MGMT assessment can be useful in predicting response in GBM, but the scarcity of neoplastic cells limits the practicality of MGMT assessment in these tumors. Although GBM grows within the skull, we investigated the concordance of methylation in glioma tissue, and paired serum DNA and the potential correlation with response and time to progression.

Experimental Design: Using MSP assay, we assessed the methylation of MGMT, p16, DAPK, and RASSFIA in tumor and serum DNA from 28 GBM patients treated with BCNU or with temozolamide plus cisplatin.

Results: The concordance between methylation in tumor and serum was highly significant. Overall, response plus stable disease was noted in 10 of 11 (90.9%) patients with MGMT methylation and in 5 of 14 (35.7%) patients without (P = 0.01). In the 16 patients treated with temozolamide plus cisplatin, no significant correlation between MGMT methylation status and response was observed, whereas in BCNU-treated patients, a significant difference was observed in favor of those with methylated MGMT. Time to progression was 29.9 weeks in 12 patients with MGMT methylation and 15.7 weeks in 10 patients without (P = 0.006). No correlation was observed between response or time to progression and p16, DAPK, or RASSFIA methylation.

Conclusions: Methylated MGMT, p16, DAPK, and RASSFIA were found in serum DNA of GBM patients, with a good correlation between serum and primary tumor tissue. Serum MGMT methylation predicted response and time to progression in BCNU-treated GBM patients. The methylation-specific PCR assay in serum DNA could be a good predictive tool for selecting GBM patients to be treated with BCNU or alternatively with the combination of temozolamide plus cisplatin.

INTRODUCTION

GBM1 is the highest grade glioma; complete resection is difficult, and even when possible, it is associated with severe neurological damage. Treatment consists of cytoreductive surgery followed by radiotherapy. Median survival time is only 9 months, and 5–10% of patients survive up to 2 years. Meta-analyses have shown that chemotherapy could improve survival with an absolute increase in 1-year survival of 6% (1). Pharmacogenetic research to predict response to chemotherapy has not been fully explored. Until recently, prognostic groups were based on age, Karnofsky performance status, biopsy only, or radiotherapy dose (2). Many genetic abnormalities are involved in gliomagenesis, and relevant mechanisms of resistance to common drugs used in the treatment of GBM have been identified (3). One-step repair (the direct reversal of DNA damage) targets 2-chloroethylnitrosoureas, such as BCNU (carmustine) and temozolamide, both of which involve alkylation of the O6 site of guanine. It is performed by the repair protein O6-alkylguanine-DNA alkyltransferase or MGMT through direct removal of an alkyl group from the O6-atom of guanine in the DNA of cells exposed to alkylating agents. With increasing size of the alkyl group (≥ethyl), the relative contribution of MGMT to the repair of O6-alkylguanines in DNA decreases and excision repair steps in as a backup modality (4). This O6-alkylguanine DNA lesion is highly cytotoxic and correlates inversely with the activity of the DNA repair enzyme MGMT, which removes the monofunctional O6 adduct from the DNA, limiting response to these cytotoxic drugs. MGMT activity in gliomas is

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3 The abbreviations used are: GBM, glioblastoma multiforme; MGMT, O6-methyl-guanine-DNA methyltransferase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MSP, methylation-specific PCR; MRI, magnetic resonance imaging; CI, confidence interval; NSCLC, non-small cell lung cancer; GSTP1, glutathione S-transferase 1.

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variable; 25% of gliomas have no detectable MGMT activity (<0.25 fmol/10^6 cells or 150 molecules/cell), and MGMT activity correlates with aneuploidy (5). Gliomas without MGMT activity responded better to surgery plus alkylating agent-based chemotherapy than to surgery alone (6). MGMT activity is commonly elevated in pediatric brain tumors, raising the hypothesis that elevated MGMT activity could enhance resistance to alkylating agent-based chemotherapy (7). MGMT-predictive chemoresponsiveness has been reported in a mixed group of BCNU-treated GBM and anaplastic astrocytoma patients by a quantitative indirect immunofluorescence assay. MGMT levels were scored high or low both according to the threshold of 60,000 molecules/nucleus.

Time to progression was significantly longer in GBM patients with low MGMT levels (6 months) than in those with high MGMT levels (3 months; \( P = 0.008 \)). Median survival was 12 versus 7 months, respectively (8). The role of MGMT was also examined in temozolomide-treated GBM patients. Responses were observed more frequently in patients with low immunostaining for MGMT (<20% of cells; Ref. 9). To overcome the one-step repair mechanism of chemoresistance, MGMT inhibitors have been developed, such as \( O^6 \)-benzyl-2'-deoxyguanosine and \( O^6 \)-benzylguanine. Administration of these non-specific MGMT inhibitors before treatment with either BCNU or temozolomide has shown a benefit in tumors having MGMT activity >45 fmol/mg protein (10). To make matters more complex, it seems that cisplatin can abrogate MGMT activity, which makes novel chemotherapy approaches like temozolomide plus cisplatin particularly attractive in the treatment of GBM (11). With MSP, 40% of brain tumors showed no methylated MGMT, which correlated with better response and survival in BCNU-treated patients. Anaplastic astrocytomas were also included in this study, and striking differences in median time to progression were observed (21 months for methylated versus 8 months for unmethylated gliomas; \( P < 0.001 \); Ref. 12).

However, whereas MGMT transcript is a good predictor of response and survival, GBM is characterized by necrosis, with cells arranged around the edge of the necrotic tissue (pseudo-palisading cells), and tumor tissue is often scarce, because in a number of patients only stereotactic tumor biopsy is performed. For this reason, analysis of serum DNA may be a useful method for assessing MGMT methylation status in GBM patients, because the level of free serum DNA was observed to be high in brain tumors. More than 3 decades ago, significant amounts of serum DNA were identified in brain tumors by a radioimmunoassay (13). More recently, several reports indicate that genetic microsatellite alterations in serum DNA mirror those observed in paired tumor samples in head and neck (14), and small-cell lung cancer (15). Similar methylation patterns of \( p16 \), \( DAPK \), \( GSTP1 \), and MGMT in paired tumor and serum DNA were observed in resected NSCLCs (16). \( p16 \), \( DAPK \), and MGMT hypermethylation has also been observed in paired tumor and serum DNA of head and neck cancer patients (17). Interestingly, \( p16 \) methylation has been observed in GBM (18). Loss of \( DAPK \) expression has been associated significantly with more biologically aggressive pituitary tumors (19). \( RASSF1A \) has not yet been explored in adult GBM, although \( RASSF1A \) methylation is observed frequently in pediatric tumors, including neuroblastoma (20). \( RASSF1A \) contains a region for the DNA-dependent ataxia-telangiectasia-mutated and ataxia-telangiectasia-related kinases.

In the present study, we have collected tumor and serum from 28 GBM patients treated prospectively with surgery, radiotherapy, and chemotherapy with BCNU or temozolomide plus cisplatin. Our objectives were to confirm the presence of circulating serum DNA in these patients, to examine the concordance between methylation of \( MGMT \), \( p16 \), \( DAPK \), and \( RASSF1A \) in tumor and in serum, and to correlate the methylation of these genes with response and time to progression.

**MATERIALS AND METHODS**

**Patients.** We collected freshly resected surgical specimens and biopsies, and paired peripheral venous blood drawn in the operating theater from 28 GBMs. The study protocol was approved by the Clinical Research Ethics Committee, and all of the patients gave informed consent for obtaining the blood and tumor samples, and for the gene methylation assays. After central pathologic review, 27 patients had confirmed GBM, and 1 had gliosarcoma. After partial resection or biopsy, 26 of the patients had measurable tumor by MRI. Only 21 of 28 patients had glioma tissue available. Sixteen patients were treated with temozolomide 200 mg/m^2/day in a fasting state for 5 consecutive days plus cisplatin 100 mg/m^2 every 28 days for three cycles before radiotherapy 60 Gy over the primary tumor; 8 patients were treated with BCNU 200–250 mg/m^2 every 6 weeks followed by radiotherapy. One patient received only temozolomide plus radiotherapy, and 3 patients received no chemotherapy because of early progression (Fig. 1).

Complete response was defined as the disappearance of all of the enhancing tumor on consecutive MRI scans at least 1 month apart and no corticosteroid use, with stable or improved neurological condition. Partial response was a ≥50% reduction in contrast enhancement, steroids stable or reduced, and neurologically stable or improved. Progressive disease was defined as ≥25% increase in tumor volume, or neurologically worse, and steroids stable or increased. All of the other conditions were considered to be stable disease (21). Time to progression was calculated as the time from surgery to radiologically confirmed progression.

**DNA Extraction from Glioma and Serum Samples.** Tumor samples (\( n = 21 \)) were collected at resection time, immediately snap-frozen in liquid nitrogen, and stored at −80°C until DNA extraction was performed. Serum samples (\( n = 28 \)) were collected before surgery or at the time of surgery. Five to 10 ml of venous blood from each patient was withdrawn into vacutainer tubes containing SST gel and clot activator (Becton Dickinson, Plymouth, United Kingdom). Serum was isolated after centrifugation at 2500 rpm for 10 min and stored at −20°C until use.

Genomic DNA was obtained from 25 mg of each tissue sample, using DNAeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Serum DNA was extracted using the QIAamp Blood Mini kit (Qiagen) according to the manufacturer’s instructions.

**MSP.** DNA methylation patterns in the CpG island of \( MGMT \) (GenBank accession no. X_61657), \( p16 \) (GenBank...
accession no. X_94154), RASSF1A (GenBank accession no. XM_011780), and DAPK (GenBank accession no. NM_004938) were determined by MSP. Primers were designed for either methylated or unmethylated versions for the different genes: MGMT sense 5′-TTTCGACGTTCGTTTTCGC-3′ and MGMT antisense 5′-GCACCTTTCCGAAAAACGAAACG-3′ for methylated sequences; MGMT sense 5′-TTT GTGTITTTGAGTTTTTGT-3′, and MGMT antisense 5′-AACCTCCACATCTTC CAAAACAAAAACA-3′ for unmethylated sequences; p16 sense 5′-TTATTAGAGGTGGG GGCAGATCGCAGGC-3′, and p16 antisense 5′-ACCCGACCCCAGACCGACCGTAA-3′ for methylated sequences; p16 sense 5′-TTATTAGAGGTGGGTGTTTGTGTTTGT-3′, and p16 antisense 5′-CAACCCCAAACCCTCACTG-3′. MSP distinguished unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated (but not methylated) cytosines to uracil. Briefly, 1–2 μg of genomic DNA was denatured with 0.2 mM NaOH. Ten μM hydroquinone (Sigma) and 3 μM sodium bisulfite (Sigma) were added to each sample and incubated at 55°C for 16 h. After purification with Wizard DNA Purification System (Promega), the DNA was treated with 3 μl of NaOH and precipitated with 3 volumes of 100% ethanol, 0.3 volumes of 10 μM sodium acetate, and 1 μl of glycerol (Roche Molecular Biochemicals) at −20°C. Samples were centrifuged for 30 min at 12,000 rpm, and DNA was washed with 70% ethanol and dissolved in distilled water. The PCR mixture contained 1× PCR buffer [16.6 mM [NH₄]₂SO₄, 67 mM TRIS-HCl (pH 8.8), and 0.01% Tween 20], 6 μM MgCl₂, dNTPs (each at 1.25 mM; Roche Diagnostics, Germany), 0.5 μM of each primer, 0.5 units of TaqDNA Polymerase, and bisulfite-modified DNA in a final volume of 50 μl. Each PCR product (15 μl) was loaded onto a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. DNA from peripheral blood lymphocytes and placenta treated with SssI methyltransferase (New England Biolabs) was used as a positive control for methylated reaction. In addition, all of the amplification assays included the MOLT-4 cell line as a positive control for RASSF1A methylation and bisulfite-treated DNA from human colorectal cell line SW-48 as a positive control for MGMT methylation. All of the experiments were repeated at least twice to confirm the results.

Statistical Analysis. Data were analyzed with the SPSS 10 software package. Categorical data were analyzed by χ² or Fisher’s exact test. Correlation between methylation statuses of different genes in glioma and serum was analyzed by Spearman correlation coefficient. Time to progression and survival data were evaluated according to the Kaplan-Meier method and the log-rank test. Statistical significance was defined as P < 0.05.

RESULTS
Patients and Glioma Characteristics. Median age was 59 years (range, 30–76 years), 16 men and 12 women, with Karnofsky performance status 80–100 in 16 patients and 70 in 12 patients. Twenty-one patients underwent partial resection, and only 2 had radical surgery, with no residual disease observed in postoperative brain MRI. In the remaining 5 patients, stereotactic biopsy was performed. Three patients received no treatment after surgery, 2 because of early postoperative death and 1 because of multicentric glioma that precluded optimal radiotherapy (Table 1).

Gene Promoter Hypermethylation in Glioma and Serum. The prevalence of MGMT, p16, DAPK, and RASSF1A promoter methylation was 8 of 21 (38.1%), 14 of 21 (66.7%), 11 of 21 (52.4%), and 12 of 21 (57.1%), respectively, in glioma tissue, and 11 of 28 (39.3%), 15 of 27 (53.6%), 9 of 26 (34.5%), and 13 of 26 (50%), respectively, in serum (Fig. 2). Table 2 summarizes the concordance between methylation of these genes in tumor and serum. All of the correlation coefficients were highly significant.

Methylation Status and Response. MGMT status was studied in DNA extracted from 28 serum samples and 21 avail-
able paired glioma samples. Methylation status did not differ according to age, gender, or treatment approach (Table 1).

Overall, complete or partial response, or stable disease was observed in 15 patients (60%), whereas 10 patients (40%) had progressive disease. When patients were divided into those with \textit{MGMT} methylation in either tumor or serum or both and those

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics according to \textit{MGMT} methylation pattern in glioma and serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients no. (%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16 (57.1)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (42.9)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>(\leq 50)</td>
<td>59</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>22 (78.6)</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
</tr>
<tr>
<td>Stereotactic biopsy</td>
<td>5 (17.9)</td>
</tr>
<tr>
<td>Partial resection</td>
<td>21 (75)</td>
</tr>
<tr>
<td>Complete resection</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>3 (10.7)</td>
</tr>
<tr>
<td>BCNU</td>
<td>8 (28.6)</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>Temozolomide/cisplatin</td>
<td>16 (57.1)</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>6 (21.4)</td>
</tr>
<tr>
<td>Yes</td>
<td>22 (78.6)</td>
</tr>
</tbody>
</table>

* NS, not significant.

**Negative postoperative MRI.

MSP of \textit{MGMT}, \textit{p16}, \textit{DAPK}, and \textit{RASSF1A}. Genomic DNA was obtained from tissue samples using DNAeasy tissue kit (Qiagen). Serum DNA was extracted using the QIAmp Blood Mini kit (Qiagen). DNA methylation patterns in the CpG dinucleotides of \textit{MGMT} (GenBank accession no. U95038), \textit{p16} (GenBank accession no. X94154), \textit{DAPK} (GenBank accession no. NM_004938), and \textit{RASSF1A} (GenBank accession no. XM_004938) were determined by MSP assay. Bisulfite-treated DNA was purified using the Wizard DNA clean-up system (Promega Co., Madison, WI). Five \(\mu\)l of bisulfite-modified DNA was amplified by PCR using primers that were specific for methylated or unmethylated sequences of each gene. T, tumor DNA; S, serum DNA; L, lymphocyte DNA used as negative and positive control for methylated and unmethylated PCR reaction, respectively; SW-48, HT-29, MOLT-4 cell lines, and MP (\textit{in vitro SssI} modified placental DNA) used as positive and negative control for methylated and unmethylated PCR reaction, respectively.

Fig. 2  MSP of \textit{MGMT}, \textit{p16}, \textit{DAPK}, and \textit{RASSF1A}. DNA methylation status did not differ according to age, gender, or treatment approach (Table 1).

Overall, complete or partial response, or stable disease was observed in 15 patients (60%), whereas 10 patients (40%) had progressive disease. When patients were divided into those with \textit{MGMT} methylation in either tumor or serum or both and those
without MGMT methylation, response plus stable disease was noted in 10 of 11 (90.9%) with methylation as opposed to 5 of 14 (35.7%) without (Fisher’s exact test, \( P < 0.01 \)). When response was broken down by methylation status in serum only, response plus stable disease was seen in 8 of 8 (100%) patients with methylated bands and in 7 of 17 (41.2%) with unmethylated bands (Fisher’s exact test, \( P < 0.008 \)). Differences in response were not significant when methylation status only in tumor tissue was considered, possibly because of the few samples available (Table 3). Fig. 3 illustrates a partial response in a GBM patient with methylated MGMT in both tumor and serum.

When patients were broken down by treatment group, in the 16 patients treated with temozolamide plus cisplatin (Table 4), no significant correlation between MGMT methylation status and response was observed, whereas in BCNU-treated patients, a significant difference was observed in favor of those with methylated MGMT.

No correlation was observed between response and p16, DAPK, or RASSF1A methylation in tumor or serum (data not shown).

**Methylation Status and Survival.** Overall, median time to progression was 23.9 weeks (95% CI, 14.2–33.5). Time to progression was 29.9 weeks (95% CI, 24.3–35.4) in 12 patients with MGMT methylation in either tumor or serum, and 15.7 weeks (95% CI, 14.3–17.2) in 10 patients without MGMT methylation (log-rank test, \( P = 0.006 \); Fig. 4).

Neither p16, DAPK, nor RASSF1A methylation correlated with time to progression. Time to progression for 17 patients with methylated p16 was 20.5 weeks, whereas for 7 patients with unmethylated p16, it was 29.8 weeks (\( P = 0.75 \)). For 10 patients with methylated DAPK, it was 44.4 weeks, and for 13 patients with unmethylated DAPK, it was 45.1 weeks (\( P = 0.28 \)). For 13 patients with methylated RASSF1A, it was 19.4 weeks and for 10 patients with unmethylated RASSF1A, it was 30.5 weeks (\( P = 0.72 \)).

![Fig 3](https://example.com/docs/fig3.jpg) **Fig. 3** Response to temozolomide plus cisplatin in a patient with methylated MGMT in serum and tissue. Two different brain computed tomography layers taken immediately after biopsy (a and b) show a glioblastoma with edema, and midline shift with an enhanced ring and doughnut shape. In the same computed tomography layers taken after three cycles of chemotherapy (c and d), a small residual enhancing lesion can be seen, and the midline shift has already disappeared. This patient was classified as a partial response.

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**Table 2** Concordance between methylation of MGMT, p16, DAPK, and RASSF1A in tumor and serum DNA from glioblastoma patients

<table>
<thead>
<tr>
<th>Markers</th>
<th>Assessment in tissue</th>
<th>Assessment in serum</th>
<th>Spearman correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unmethylated no. (%)</td>
<td>Methylated no. (%)</td>
<td></td>
</tr>
<tr>
<td>MGMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 (57.1)</td>
<td>3 (14.3)</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>1 (4.8)</td>
<td>5 (23.8)</td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (28.6)</td>
<td>2 (9.5)</td>
<td>0.00001</td>
</tr>
<tr>
<td></td>
<td>4 (4.8)</td>
<td>12 (57.1)</td>
<td></td>
</tr>
<tr>
<td>DAPK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (47.6)</td>
<td>3 (14.3)</td>
<td>0.00001</td>
</tr>
<tr>
<td></td>
<td>1 (4.8)</td>
<td>8 (38.1)</td>
<td></td>
</tr>
<tr>
<td>RASSF1A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 (38.1)</td>
<td>2 (9.5)</td>
<td>0.00001</td>
</tr>
<tr>
<td></td>
<td>1 (4.8)</td>
<td>10 (47.6)</td>
<td></td>
</tr>
</tbody>
</table>

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**Table 3** Response to treatment according to MGMT methylation pattern in serum, glioma, or both

<table>
<thead>
<tr>
<th>MGMT status</th>
<th>Progressive disease no. (%)</th>
<th>Objective response and stable disease no. (%)</th>
<th>( P ) (Fisher’s exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue and serum</td>
<td>Unmethylated</td>
<td>9 (64.3)</td>
<td>5 (35.7)</td>
</tr>
<tr>
<td>Serum</td>
<td>Methylated</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
</tr>
<tr>
<td>Tissue</td>
<td>Unmethylated</td>
<td>10 (58.8)</td>
<td>7 (41.2)</td>
</tr>
<tr>
<td>Tissue</td>
<td>Methylated</td>
<td>1 (16.7)</td>
<td>5 (83.3)</td>
</tr>
<tr>
<td>Tissue</td>
<td>Methylated</td>
<td>1 (16.7)</td>
<td>5 (83.3)</td>
</tr>
</tbody>
</table>
Serum DNA MGMT Methylation Predicts GBM BCNU Response

average number of CpG islands aberrantly methylated in a methylation of CpG islands is a common trait of cancer, and theing of tumor suppressor or other cancer-associated genes by methyl group to cytosine residues at CpG dinucleotides. Silenc-
dNA methyltransferase enzymes that catalyze the addition of a late with resistance to BCNU (6, 8) and temozolamide (9).

Fig. 4 Time to progression according to MGMT methylation pattern in serum or tissue.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Response to chemotherapy for patients treated with temozolomide plus cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGMT status in serum/tissue</td>
<td>No.</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>8</td>
</tr>
<tr>
<td>Methylated</td>
<td>8</td>
</tr>
</tbody>
</table>

* PR, partial response; CR, complete response.

DISCUSSION

Multiple genetic alterations commonly outlined in GBM include PTEN and p53 mutations (22), amplification of the epidermal growth factor receptor oncogene, loss of the q arm of chromosomes 7 and 10, and overexpression of human telomerase reverse transcriptase (23, 24). High levels of MGMT correlate with resistance to BCNU (6, 8) and temozolamide (9).

DNA methylation is the result of the activity of a family of DNA methyltransferase enzymes that catalyze the addition of a methyl group to cytosine residues at CpG dinucleotides. Silencing of tumor suppressor or other cancer-associated genes by methylation of CpG islands is a common trait of cancer, and the average number of CpG islands aberrantly methylated in a single tumor is calculated to be ~400 (25). Therefore, GBM is not an exception and may be characterized by the simultaneous methylation of multiple CpG islands, as occurs in other cancers (25) and in adult acute lymphocytic leukemia (26). Increased CpG island methylation can lead to the inactivation of many tumor suppressor genes and to the inactivation of DNA repair genes like MGMT, resulting in increased levels of genetic damage. In addition, extensive demethylation of DNA outside CpG islands is also a common feature of cancer, involving reduced chromosome stability, activation of retrotransposon, or activation of oncogenes. To date, there are few studies correlating MGMT hypermethylation with longer progression-free survival in brain tumors. However, the Esteller et al. (12) study found an increased responsiveness to BCNU and cisplatin in MGMT-deficient anaplastic astrocytomas and GBM, and Tang et al. (27) reported that methylation of the DAPK gene was associated with decreased survival in chemonaive resected NSCLC patients.

A novel tool for the assessment of tumor-specific DNA abnormalities is circulating DNA extracted from the blood of the patients. Double-strand DNA fragments appear frequently in considerable quantities in the serum of cancer patients, even in GBM (13). A recent study indicated that the concentration of free DNA varies widely, with peaks up to 219 ng/ml in the plasma of cancer patients (28). A pioneering study of p16, MGMT, GSTP1, and DAPK demonstrated that aberrant methylation of at least one of these genes was present in 68% of resected NSCLC tumor tissue, and the assay revealed identical methylation patterns in the serum of 73% of patients positive for methylated DNA in the primary NSCLC tumors (16). Recently, methylation of adenomatous polyposis coli promoter DNA was observed in almost all of the lung cancer tissues examined, and 47% of paired serum samples also exhibited detectable amounts of methylated adenomatous polyposis coli promoter DNA. p16 methylation in tumor tissue and matching serum DNA has also been documented in colorectal cancer (29), and methylation of p16, DAPK, GSTP1, and other genes has been seen in tumor and serum of gastric cancer patients (30).

We have assessed methylation of MGMT, p16, DAPK, and RASSF1A in tumor and matching serum of GBM patients treated with either BCNU or temozolomide plus cisplatin. Brain tumors are expanding lesions that produce symptoms primarily by increasing the mass of tissue residing within the rigid skull. In fact, when malignant gliomas are transplanted into s.c. tissues, the glioma cells grow locally but do not metastasize, indicating that primary brain tumors are fundamentally different from tumors originating elsewhere (31). For this reason, it was particularly intriguing to screen methylation in serum of GBM patients. The only previous research along these lines was the seminal work by Leon et al. (13) examining the concentration of free serum DNA in several tumors including four gliomas.

Whereas all of the normal human tissues express MGMT, a subset of tumor cell lines appears to be totally MGMT-deficient. In MGMT-proficient cells, MGMT mRNA levels generally correlate well with protein levels (32). However, translational and post-translational processes could alter MGMT levels (32, 33). In the present study, MGMT methylation, especially in serum DNA, was significantly associated with response and survival in all of the patients, although we found no association between older age and unmethylated MGMT, as had been suggested previously (34, 35). However, when patients were broken down by treatment group, no significant correlation between MGMT methylation and response was observed in the 16 patients treated with temozolomide plus cisplatin. The cisplatin-induced inactivation of MGMT in HeLa CCL2 cells (11) sheds light on how cisplatin can protect temozolomide from...
the MGMT direct reversal of temozolomide-induced DNA damage. Our results support previous in vitro experiences, which suggested that cisplatin could enhance the activity of temozolomide by the inhibition of MGMT activity (36). We also observed a good correlation between methylation of p16, DAPK, and RASSF1A in tumor and serum but with no impact on response or time to progression. In the future, assessment of other target genes could contribute to our understanding of the different pieces in the puzzle of cancer resistance. For instance, the death-associated protein 3, also reported as human ionizing resistance conferring protein, has been found to be overexpressed in GBM cells (37). Other DNA repair pathways are also important in chemoresistance, but the examination of the factors involved in these pathways requires tumor tissue, whereas the MSP assay of MGMT methylation can be easily performed in serum DNA template.

In summary, this is the first report demonstrating the presence of methylated MGMT, p16, DAPK, and RASSF1A in serum DNA of GBM patients, with a good correlation between methylation in serum and in primary tumor tissue. Moreover, serum MGMT methylation predicted response and time to progression in BCNU-treated GBM patients. The MSP assay in serum DNA could be a good predictive tool for selecting GBM patients to be treated with BCNU or alternatively with the combination of temozolomide plus cisplatin.

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O6-methyl-guanine-DNA methyltransferase Methylation in Serum and Tumor DNA Predicts Response to 1,3-Bis(2-Chloroethyl)-1-Nitrosourea but not to Temozolamide Plus Cisplatin in Glioblastoma Multiforme

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