CpG Island Hypermethylation of the DNA Repair Enzyme Methyltransferase Predicts Response to Temozolomide in Primary Gliomas

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ABSTRACT

Purpose: The DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) inhibits the killing of tumor cells by alkylating agents, and its loss in cancer cells is associated with hypermethylation of the MGMT CpG island. Thus, methylation of MGMT has been correlated with the clinical response to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in primary gliomas. Here, we investigate whether the presence of MGMT methylation in gliomas is also a good predictor of response to another emergent alkylating agent, temozolomide.

Experimental Design: Using a methylation-specific PCR approach, we assessed the methylation status of the CpG island of MGMT in 92 glioma patients who received temozolomide as first-line chemotherapy or as treatment for relapses.

Results: Methylation of the MGMT promoter positively correlated with the clinical response in the glioma patients receiving temozolomide as first-line chemotherapy (n = 40). Eight of 12 patients with MGMT-methylated tumors (66.7%) had a partial or complete response, compared with 7 of 28 patients with unmethylated tumors (25.0%; P = 0.030). We also found a positive association between MGMT methylation and clinical response in those patients receiving BCNU (n = 35, P = 0.041) or procarbazine/1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (n = 17, P = 0.043) as first-line chemotherapy. Overall, if we analyze the clinical response of all of the first-line chemotherapy treatments with temozolomide, BCNU, and procarbazine/1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea as a group in relation to the MGMT methylation status, MGMT hypermethylation was strongly associated with the presence of partial or complete clinical response (P < 0.001). Finally, the MGMT methylation status determined in the initial glioma tumor did not correlate with the clinical response to temozolomide when this drug was administered as treatment for relapses (P = 0.729).

Conclusions: MGMT methylation predicts the clinical response of primary gliomas to first-line chemotherapy with the alkylating agent temozolomide. These results may open up possibilities for more customized treatments of human brain tumors.

INTRODUCTION

Alkylating agents are among the most widely used chemotherapeutic drugs in the treatment of human cancer (1). Several alkylation sites in DNA have been described as being targets of the action of these compounds; the most frequent site is the O-6 position of guanine. This modification can produce DNA interstrand cross-links (1), and this base is the preferred point of attack in the DNA of numerous alkylating chemotherapeutic drugs such as BCNU [carmustine, 1,3-bis(2-chloroethyl)-1-nitrosourea], ACNU [nimustine, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea], CCNU [lomustine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea], procarbazine, streptozotocin, and temozolomide. However, the toxicity of alkylating agents is reduced in the presence of O6-methylguanine DNA methyltransferase (MGMT) by rapidly reversing the formation of adducts at the O6 position of guanine (2, 3), thereby averting the formation of lethal cross-links. Thus, MGMT activity is a major mechanism of resistance to alkylating drugs (2–4).

In gliomas, enhanced sensitivity to the action of BCNU was initially suggested in the subgroup of patients with reduced...
MGMT activity (4–10). In human cancer, the MGMT gene is not commonly mutated or deleted, and loss of MGMT function is most frequently due to epigenetic changes, specifically promoter-region methylation (4, 10). Hypermethylation of the MGMT CpG island as the cause of MGMT transcriptional silencing in cell lines defective in O\textsuperscript{6}-methylguanine repair has been demonstrated (11–13); in vitro treatment of cancer cells with demethylating drugs restores MGMT expression (11, 14); and the presence of MGMT methylation has been correlated with loss of mRNA expression (14), lack of MGMT protein (13, 15), and loss of enzymatic activity (15) in primary tumors, among them gliomas.

Following the demonstration that CpG island hypermethylation of MGMT was the main cause of its loss in gliomas (13), we hypothesized that gliomas hypermethylated at MGMT would be more sensitive to the action of these alkylating agents because their DNA lesions could not be repaired in the cancer cell, thereby leading to cell death. We gave proof of principle for this hypothesis and MGMT promoter hypermethylation predicted a good response to chemotherapy, greater overall survival, and longer time to progression in gloma patients treated with BCNU (16). The methylation status of the MGMT promoter was a better predictor of the outcome of BCNU treatment than classical prognostic factors such as grade of tumor, Karnofsky performance status, or patient age (16). Furthermore, the association between MGMT hypermethylation and good clinical response in gliomas was also recently observed by an independent group (17).

In recent years, temozolomide has emerged as another potent alkylating agent for the treatment of human tumors, especially gliomas (18–21), either as the first-line of chemotherapy or as treatment for the relapses. The type of DNA lesion induced by temozolomide also involves alkylation at the O\textsuperscript{6} position of guanine and is repaired by MGMT. Furthermore, temozolomide depletes MGMT activity (22, 23), and antitumor activity occurs when sufficient adducts are formed by temozolomide to inactivate MGMT, whereas administration of MGMT inhibitors before treatment with temozolomide is of benefit in glioma cells expressing high levels of the protein (24).

These data have prompted us to examine whether the presence of MGMT methylation in a large collection of human gliomas predicts the clinical response to temozolomide of patients receiving the drug as first-line chemotherapy or as treatment for relapses.

MATERIALS AND METHODS

Patients. We studied specimens of glioma tumors from 92 patients who had been referred to six hospitals in Spain and France between April 1993 and November 1998. Informed written consent was obtained from all patients, and tissue collection was approved by each Institutional Review Board. Sixteen patients had anaplastic astrocytomas, 51 had glioblastoma multiforme, 20 had anaplastic oligodendroglioma, and 5 had anaplastic oligoastrocytoma. From these cases, the histology of the relapsed tumors were 15 glioblastoma multiforme, 16 anaplastic astrocytoma, 17 anaplastic oligodendroglioma, and 4 anaplastic oligoastrocytoma. The age of patients ranged from 29 to 79 years (mean age at surgery, 52 years); 58 were men and 34 were women. Forty patients received temozolomide as first-line chemotherapy, whereas in 52 cases, temozolomide was administered as treatment for glioma relapse. These latter 52 patients either received BCNU (n = 35) or procarbazine/CCNU (n = 17) as first-line chemotherapy. A complete response was defined as the absence of any evidence of the tumor on computed tomography and magnetic resonance imaging scans, no need for steroid treatment, and an improvement in the patient’s general condition. Patients with persistent computed tomographic abnormalities but with more than a 50% reduction in the diameter and volume of the tumor, a reduced need for steroid treatment, and a stabilized neurological condition were considered to have a partial response. The disease was considered to have progressed if both the diameter and volume of the tumor increased by ≥25% of the initial measurements, if a new lesion was evident from computed tomography or magnetic resonance imaging scans, or if the patient’s neurological condition worsened and required an increased dose of steroids.

Analysis of the CpG Island Methylation Status of the MGMT Gene. DNA was extracted according to standard protocols. Methylation patterns in the CpG island of MGMT were determined by chemical modification of unmethylated, but not methylated, cytosines to uracil. Methylation-specific PCR (MSP) was performed with primers specific for either modified methylated or unmethylated DNA, as described previously (13, 16, 25). DNA (1 μg) was denatured with sodium hydroxide and modified with sodium bisulfite. DNA samples were then purified with the Wizard DNA purification resin (Promega, Madison, WI), treated again with sodium hydroxide, precipitated with ethanol, and resuspended in water. Primer sequences for the unmethylated reaction were 5'-TTTGTGTGGTTTGAGTTQGTTTTGTT-3' (forward primer) and 5'-AATCTCCTACTTCCTCCAAAAAACA-3' (reverse primer), and for the methylated reaction, they were 5'-TTTGGCAAGTTCTAGTGGTTTGCG-3' (forward primer) and 5'-GCACCTCTCCGAAAAGAAGACG-3' (reverse primer). Methylation at these sites has been correlated with gene silencing in cancer cell lines and primary tumors (13, 14, 26). A second set of MSP primers adjacent to those described above was also used in the same samples and gave identical results. We carried out this independent second MSP reaction just to double-check the methylation results. These second MGMT MSP primers for the unmethylated reaction were 5'-ATTTGGTG-GAGTTGGTTGGTTTT-3' (sense) and 5'-AAAAACACAC-CTAAAAACTCACCCC-3' (antisense) and, for the methylated reaction, 5'-ATTGGTGAGTTGGTTGGTT-3' (sense) and 5'-AAAAACACCTAAAACCTGCC-3' (antisense). The annealing temperature for both sets of MSP primers was 59°C. Plasmid DNA treated in vitro with SsII methyltransferase (New England Biolabs, Beverly, MA) was used as a positive control for methylated alleles of MGMT, and DNA from normal lymphocytes was used as a negative control. Controls without DNA were used for each set of MSP assays. Ten μl of each 50-μl MSP product were loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and examined under UV illumination.

Statistical Analysis. Data were analyzed with the SPSS 10 program. Categorical data were analyzed by χ² contin-
MGMT Methylation Status and Clinical Response to BCNU or Procarbazine/CCNU as First-Line Chemotherapy. We also found a positive association between MGMT methylation and clinical response in glioma patients receiving BCNU as first-line chemotherapy \((n = 35)\), in agreement with previous data \((16, 17)\). Six of 11 patients with methylated tumors \((54.5\%)\) had a partial or complete response, compared with 4 of 24 patients with unmethylated tumors \((16.7\%)\) \((Fisher’s exact test, \(P = 0.041\); Table 2)\. Most interestingly, we also found that MGMT methylation was associated with the clinical response of glioma patients to procarbazine/CCNU \((n = 17)\) as first-line chemotherapy \((Fisher’s exact test, \(P = 0.043\); Table 2)\. Overall, if we analyze the clinical response of all of the first line chemotherapy treatments with temozolomide, BCNU and procarbazine/CCNU as a group in relation to the MGMT methylation status, MGMT hypermethylation was strongly associated with the presence of positive clinical response: seventeen of 28 patients with methylated tumors \((60.7\%)\) had a partial or complete response, compared with 12 of 64 patients with unmethylated tumors \((18.7\%)\) \((Fisher’s exact test, \(P < 0.001\); Table 2)\. 

MGMT Methylation Status and Survival. No statistically significant association between MGMT methylation and time-to-progression or overall survival was found among the 92 patients who received temozolomide or when considering separately those who received temozolomide as first-line chemotherapy \((n = 40)\) and those who were received it for the treatment of relapses \((n = 52)\) \((Fisher’s exact test, \(P > 0.05\); Kaplan-Meier, \(P > 0.05)\). However, live patients were better represented in the group with methylated MGMT than in that with nonmethylated MGMT \((53.85\% versus 50.85\%)\). This trend toward a better outcome in those gliomas with methylated MGMT was also more apparent when the tumors were subdivided by histology. In anaplastic oligodendroglioma, 66.6% of the patients were alive when MGMT was methylated \textit{versus} 50% when it was unmethylated; and in glioblastoma multiforme, 55.5% were alive when MGMT was methylated \textit{versus} 44.4% when it was not methylated. None of these differences was statistically significant. No differences were observed for anaplastic astrocytoma or anaplastic oligoastrocytoma.

### Table 1: Characteristics of patients with glioma in relation to the methylation status of the MGMT promoter

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>MGMT methylated</th>
<th>MGMT unmethylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;45)</td>
<td>10 (35.7%)</td>
<td>22 (34.4%)</td>
</tr>
<tr>
<td>(45–59)</td>
<td>5 (17.9%)</td>
<td>21 (32.8%)</td>
</tr>
<tr>
<td>(\geq 60)</td>
<td>13 (46.4%)</td>
<td>21 (32.8%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18 (64.3%)</td>
<td>42 (65.6%)</td>
</tr>
<tr>
<td>Female</td>
<td>1 (35.7%)</td>
<td>22 (34.4%)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBM</td>
<td>20 (71.4%)</td>
<td>32 (50%)</td>
</tr>
<tr>
<td>AA</td>
<td>2 (7.1%)</td>
<td>14 (21.9%)</td>
</tr>
<tr>
<td>AOD</td>
<td>6 (21.5%)</td>
<td>14 (21.9%)</td>
</tr>
<tr>
<td>AOA</td>
<td>0 (0%)</td>
<td>4 (6.2%)</td>
</tr>
</tbody>
</table>

Abbreviations: GBM, glioblastoma multiforme; AA, anaplastic astrocytoma; AOD, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma.
Table 2 Clinical response of glioma patients according to the methylation status of the MGMT promoter

<table>
<thead>
<tr>
<th>First-line chemotherapy</th>
<th>MGMT unmethylated</th>
<th>MGMT methylated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of patients</td>
<td>Number</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>40</td>
<td>28</td>
</tr>
<tr>
<td>BCNU</td>
<td>35</td>
<td>24</td>
</tr>
<tr>
<td>Procarbazine/CCNU</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>All drugs</td>
<td>92</td>
<td>64</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Tailoring the correct anticancer drug to the right cancer patient should be one of the greatest goals of oncologic treatment. The young discipline of pharmacogenomics has addressed this long-mooted aim by searching for mutations and germ-line variants of transforming genes or genes involved in the drug metabolism, and expression microarray profiles have recently been added for this purpose (28). Aberrant DNA methylation is a common hallmark of human cancer (29–31), and so the methylation-associated silencing of certain genes is another good candidate in the pharmacogenomics field (32). A good example in human gliomas is the association between promoter hypermethylation of the DNA repair gene MGMT and good clinical response and survival to the chemotherapy with BCNU (16, 17), which we also found in our current study.

In this article, we show that the potential of MGMT methylation to predict the clinical response of human tumors to alkylating agents is not limited to BCNU, but it also extends to the treatment of human gliomas with temozolomide (and procarbazine/CCNU) as first-line chemotherapy. Data from cancer cell lines had previously suggested that the sensitivity to temozolomide was associated with MGMT expression levels (33, 34). For primary gliomas, an early study with a small number of cases found increased clinical responses in temozolomide-treated patients with MGMT methylation, although without reaching statistical significance (17). However, a very recent study, focused only in glioblastomas, found that MGMT was a marker of good response to temozolomide (35). Similar studies to ours with primary tumors have also recently shown that MGMT promoter hypermethylation and low expression also predict good response and longer survival in human gliomas for a similar alkylating agent, ACNU (36, 37). Future studies should examine the use of MGMT methylation as a marker of response to temozolomide in such conditions as non-small cell lung cancer or melanoma in which this drug has begun to be tested (38–40).

The potential of MGMT methylation to pinpoint tumors that are more sensitive to alkylating agents may extend to other non-BCNU-like alkylating drugs such as cyclophosphamide (41, 42). This has been demonstrated in vivo in diffuse large cell lymphomas treated with chemotherapeutic regimens, including the alkylating agent cyclophosphamide (26), where MGMT hypermethylation was the strongest predictor of overall survival and time-to-progression and was far superior to classical clinical factors such as the International Prognostic Index (26). MGMT methylation is present in other tumor types where chemotherapy regimens with alkylating agents are not commonly used such as head and neck tumors and colorectal carcinomas, and so these findings may make it worthwhile reexamining the use of these agents in a particular subset of these patients.

One might also try increasing the sensitivity of the resistant tumors, those without MGMT inactivation, to alkylating agents (10). The development of the MGMT inhibitor O6-benzylguanine (43) is being investigated for this purpose. O6-Benzylguanine is a MGMT substrate that inactivates MGMT by binding to the protein in a suicide reaction. Although this inhibitor has been found primarily to enhance the response to alkyl-nitrosourea both in vitro and in vivo (43), O6-benzylguanine has also been shown to increase sensitivity to cyclophosphamide metabolites (44). In the particular case of temozolomide, the depletion of MGMT by O6-benzylguanine causes sensitization to this drug in cancer cell lines and tumor xenografts (24, 34, 44). It should be also noticed that there is a broad spectrum of responses to the alkylating agents in these MGMT unmethylated tumors, suggesting the existence of additional chemoresistance factors.

It is important to note that MGMT hypermethylation alone, without treatment with an alkylating agent, is, in fact, a poor prognostic factor (45–47). This is also the case for brain tumors, where MGMT hypermethylation has been associated with unfavorable clinical course in astrocytoma patients that were not treated with alkylating agents (48). One reason may be because patients with epigenetic silencing of MGMT accumulate more mutations in oncogenes and tumor suppressor genes such as p53 and K-ras (4). This phenomenon may explain why MGMT methylation did not predict response to temozolomide when used as treatment for the relapses, in addition to the selection by temozolomide of the resistant unmethylated clones, or why it did not affect the overall survival of these patients, despite their better objective clinical responses. This is an example of the difference between predictive and prognostic markers. Prognos-
tic markers suggest a difference in outcome that is independent of the treatment received, including the possibility of no treatment. An example of a prognostic marker is the aforementioned International Prognostic Index for lymphomas, which summarizes variables related to tumor stage (stage itself, extra-nodal sites, and lactate dehydrogenase level) and host factors (age and performance status). Predictive markers, on the other hand, predict a response and, thereby often but not always, survival differences related to a specific form of therapy. Thus, although both types of markers can predict differences in survival, predictive markers potentially provide information that lead to treatment decisions. MGMT methylation is probably a negative prognostic marker but a positive predictive marker.

To summarize, our study suggests the potential use of MGMT methylation as a predictive marker for the clinical response of gliomas to temozolomide as first-line chemotherapy. Furthermore, the methylation of the MGMT promoter was also associated with the response to procarbazine/CCNU. These findings may also extend to other types of neoplasms treated with nitrosoureas or other alkylating agents. Our results may stimulate the additional development of inhibitors of MGMT for those tumors unmethylated at the MGMT gene and provide an experimental foundation for future personalized treatment based on pharmacogenomic strategies.

REFERENCES
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