Clinical Trial Substantiates the Predictive Value of O-6-Methylguanine-DNA Methyltransferase Promoter Methylation in Glioblastoma Patients Treated with Temozolomide

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Abstract

**Purpose:** In the setting of a prospective clinical trial, we determined the predictive value of the methylation status of the O-6-methylguanine-DNA methyltransferase (MGMT) promoter for outcome in glioblastoma patients treated with the alkylating agent temozolomide. Expression of this excision repair enzyme has been associated with resistance to alkylating chemotherapy.

**Experimental Design:** The methylation status of MGMT in the tumor biopsies was evaluated in 38 patients undergoing resection for newly diagnosed glioblastoma and enrolled in a Phase II trial testing concomitant and adjuvant temozolomide and radiation. The epigenetic silencing of the MGMT gene was determined using methylation-specific PCR.

**Results:** Inactivation of the MGMT gene by promoter methylation was associated with longer survival ($P = 0.0051$; Log-rank test). At 18 months, survival was 62% (16 of 26) for patients testing positive for a methylated MGMT promoter but reached only 8% (1 of 12) in absence of methylation ($P = 0.002$; Fisher’s exact test). In the presence of other clinically relevant factors, methylation of the MGMT promoter remains the only significant predictor ($P = 0.017$; Cox regression).

**Conclusions:** This prospective clinical trial identifies MGMT-methylation status as an independent predictor for glioblastoma patients treated with a methylating agent. The association of the epigenetic inactivation of the DNA repair gene MGMT with better outcome in this homogenous cohort may have important implications for the design of future trials and supports efforts to deplete MGMT by O-6-benzylguanine, a noncytotoxic substrate of this enzyme.

Introduction

A key role of the O-6-methylguanine-DNA methyltransferase (MGMT) as a mechanism of resistance to alkylating agents in tumors has been repeatedly suggested (1–3). This enzyme efficiently removes methyl adducts at the O-6-position of guanine, one of the most prominent and biologically important targets of alkylating agents. High repair activity of MGMT is expected to protect tumors against therapeutic agents, such as carmustine and temozolomide (4). Thus, MGMT may be considered as a therapeutic target.

MGMT function is frequently lost in the presence of CpG island hypermethylation in the promoter region of certain types of human primary neoplasms, including brain tumors (5). Inactivation of genes by methylation is a common epigenetic mechanism during malignant progression of tumors (6). A recent study suggested that methylation of the MGMT promoter is predictive for good outcome in patients with malignant gliomas treated with alkylating agents (7). That study has been criticized because the data were obtained from a heterogeneous group of patients with either anaplastic astrocytoma (WHO grade III) or glioblastoma multiforme (WHO grade IV), and who were treated with varying carmustine-containing chemotherapy regimens, including some patients undergoing subsequent high-dose chemotherapy with autologous bone marrow support (8). Similarly, Friedman et al. (9) found a correlation between MGMT expression as assessed by immunohistochemistry and response to temozolomide therapy.

Here, we tested MGMT silencing by hypermethylation in a prospective phase II trial of a homogenous cohort of patients with newly diagnosed glioblastoma treated with the novel alkylating agent temozolomide. In contrast to previous reports, our patient population and treatment are homogeneous. The end point of the analysis is survival.

Patients and Methods

**Clinical Trial.** All patients had newly diagnosed and histologically confirmed glioblastoma (10). The alkylating...
agent temozolomide (75 mg/m²/day × 5 days/week for 6 weeks) was administered orally concomitant to standard fractionated radiotherapy (60 Gy total dose; 2 Gy × 5 days/week for 6 weeks) followed by up to six cycles of adjuvant temozolomide (200 mg/m²/day × 5 days, every 28 days). The results of this trial were reported previously and a recent update showed a promising 2-year survival of 28% (10, 11).

Thirty-eight patients of this trial could be evaluated for the methylation status of the MGMT promoter before chemotherapy and radiation (summary of patient profile in Table 1). From a total of 64 patients enrolled, only the 49 patients who underwent surgical debulking could be considered for the analysis, thus excluding 15 patients where the diagnosis was established based on a stereotactic biopsy only. Eleven additional patients were excluded because of insufficient quantity of the tumor material available for analysis or poor quality of the DNA obtained (n = 6), unconfirmed diagnosis on central pathology review (n = 3), or who did not receive the prescribed temozolomide treatment (n = 5). All patients gave written informed consent, and the protocol was approved by the local ethics committees.

DNA Extraction and Methylation-Specific PCR. Genomic DNA was isolated from frozen tumor sections (Qiagen Kit 19060) or from three to four dissected paraffin sections (ExWax paraffin DNA Kit; Intergen S4530) after confirmation of the histology. Areas of normal tissue or necrosis were removed. The methylation-specific PCR was performed basically as reported by Esteller et al. (7). In brief, 2–3 µg of DNA were subjected to bisulfite treatment that modifies unmethylated, but not methylated, cytosines to uracil. After purification (Promega Fig. 1), DNA was amplified in two reactions using specific primers for the methylated (M) and modified unmethylated MGMT promoter (U), respectively. The glioblastoma cell line LN18 is negative for methylation, whereas peripheral blood lymphocyte DNA after enzymatic methylation was used as a positive control for methylation. The PCR products were separated on a 4% agarose gel. Water designates a negative control for PCR (reaction U and M pooled), and the 100-bp marker has been loaded to estimate size. The presence of a PCR product in the U Lane signifies the presence of unmethylated MGMT promoter, whereas a PCR product in the M Lane indicates the presence of methylated promoter. The PCR products were separated on a 4% agarose gel. Water designates a negative control for PCR (reaction U and M pooled), and the 100-bp marker has been loaded to estimate size. The presence of a PCR product in the U Lane signifies the presence of unmethylated MGMT promoter, whereas a PCR product in the M Lane indicates the presence of methylated promoter. The glioblastoma TMZ102 comprises only unmethylated promoter, whereas TMZ242 exhibits only methylated promoter, and TMZ231 and TMZ244 display both. Unmethylated promoter may also be contributed by DNA derived from infiltrating lymphocytes or contaminating normal tissue in the analyzed material.

**Table 1** Characteristics of patients and methylation status of the MGMT promoter

<table>
<thead>
<tr>
<th>Age</th>
<th>Total n = 38</th>
<th>Unmethylated n = 12</th>
<th>Methylated n = 26</th>
<th>Association with methylation status (Fisher’s exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤50</td>
<td>16</td>
<td>42</td>
<td>6</td>
<td>P &gt; 0.7 (2 sided)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>22</td>
<td>58</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Median, 52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range, 26–70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>39</td>
<td>5</td>
<td>P = 1.0 (2 sided)</td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>61</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Karnofsky score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤90</td>
<td>26</td>
<td>68</td>
<td>8</td>
<td>P = 1.0 (2 sided)</td>
</tr>
<tr>
<td>&lt;90</td>
<td>12</td>
<td>32</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Survival</td>
<td></td>
<td></td>
<td></td>
<td>P = 0.002 (1 sided)</td>
</tr>
<tr>
<td>≥18 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;18 months</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a MGMT, O-6-methylguanine-DNA methyltransferase.
A-7280), treated DNA was amplified in two reactions using specific primers for the methylated (M) and modified unmethylated MGMT promoter (U), respectively, as described (7). Results were confirmed in an independent experiment starting at the stage of the bisulfite treatment. Samples with insufficient quality of the DNA that yielded no or no reproducible PCR results were excluded. The glioblastoma cell line LN18 was used as a negative control for methylation, whereas peripheral blood lymphocyte DNA after enzymatic methylation was used as a positive control for methylation. The PCR products were separated on a 4% agarose gel.

Statistical Analysis. Contingency tables were analyzed by Fisher’s exact test. Survival curves were constructed using the Kaplan-Meier method, and multivariate analysis for survival was performed using the Cox proportional hazards model. The proportional hazards assumption was tested using the Schoenfeld residuals and graphical method. The analyses were performed using Stata software (version 8.0; Stata, College Station, TX) and StatView (version 5; SAS Institute, Inc., Cary, NC).

Results
The methylation-specific PCR (5) identified 26 (68%) glioblastomas in which methylated MGMT promoter was present and 12 (32%) tumors in which only unmethylated MGMT promoter was detectable (Fig. 1; Table 1). In univariate analysis, the presence of methylated promoter was highly correlated with longer overall survival (Fig. 2; P = 0.0051; Log-rank test). The risk of death within 18 months after surgery was increased in absence of MGMT promoter methylation (92 versus 38%; P = 0.002; Fisher’s exact test; Table 1). Methylation of the MGMT promoter was not correlated with other known prognostic factors like patients’ age, gender, or Karnofsky performance score (Fisher’s exact test, two-sided, P > 0.7, all comparisons; Table 1). It is noteworthy that the survival curves of the patients with or without presence of the methylated MGMT promoter start to separate only after 1 year, suggesting that other factors are responsible for outcome in some patients with a particularly poor prognosis. In the multivariate analysis (Cox regression), the above-mentioned prognostic factors actually become non-significant in the presence of the methylation status of MGMT (Table 2).

Discussion
The validation of MGMT methylation as an independent predictive factor in a prospective clinical trial provides the necessary scientific support for individually tailored therapy. Prediction of treatment response will allow selecting patients who are most likely to benefit from alkylating agent chemotherapy and may spare patients with a presumably inferior outcome an expensive and potentially toxic treatment. Alternatively, investigational and possibly more effective treatment approaches could be offered to these patients. Specific inhibitors of the MGMT were recently developed. O6-benzylguanine is currently evaluated in clinical trials. Therapy with O6-benzylguanine will deplete the cells of MGMT and thus make the tumor more sensitive to alkylating agent chemotherapy (12, 13).

Determination of the methylation status of the MGMT promoter, alternatively to analysis of gene or protein expression levels or enzyme activity, has the technical advantage that it is not susceptible to contamination of tumor-infiltrating lymphocytes or normal tissue. In particular, tumor-infiltrating lymphocytes may express considerable amounts of MGMT protein. Thus, on the basis of the fact that the MGMT promoter is unmethylated in normal tissues, the analysis yields a simple readout and will therefore allow rapid integration into routine diagnostics.

With the present study design, we cannot exclude that promoter methylation of the MGMT gene by itself is associated with better prognosis or response to therapy. It is conceivable that a methylated MGMT promoter is a surrogate marker for the presence of an epigenetic activity in the tumor, affecting other unknown genes. Nevertheless, the presence of a methylated MGMT promoter is a marker for response to therapy with temozolomide.

Acknowledgments
We thank our colleagues who made this study possible by participating actively in the clinical trial and providing primary tumor samples, namely Drs. J-G. Villemure, F. Porchet, O. Vernet, A. Reverdin, B. Rilliet, R. O. Mirimanoff, A. Pica, R. C. Janzer, G. Pizzolato, and J. Weis. We also thank M-F. Hamou for excellent technical assistance. Finally, we thank Dr. J. G. Herman for kind technical advice.

References

Table 2 Cox multivariate analysis for survival prediction

<table>
<thead>
<tr>
<th>Hazard ratio</th>
<th>SE</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karnofsky ≥90</td>
<td>0.571</td>
<td>0.225</td>
<td>0.156</td>
</tr>
<tr>
<td>Age ≤ 50</td>
<td>0.661</td>
<td>0.245</td>
<td>0.142</td>
</tr>
<tr>
<td>Male gender</td>
<td>0.574</td>
<td>0.217</td>
<td>0.017</td>
</tr>
<tr>
<td>MGMT-methylation*</td>
<td>0.387</td>
<td>0.154</td>
<td></td>
</tr>
</tbody>
</table>

* CI, confidence interval; MGMT, O-6-methylguanine-DNA methyltransferase.


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