A Phase II Study of Temozolomide in Patients with Advanced Aerodigestive Tract and Colorectal Cancers and Methylation of the O^6^-Methylguanine-DNA Methyltransferase Promoter

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Abstract

Responses of patients with gliomas to temozolomide are determined by O^6^-methylguanine-DNA methyltransferase (MGMT) and mismatch repair (MMR) pathways. This phase II study (NCT00423150) investigated whether MGMT promoter methylation predicts response in patients with advanced aerodigestive tract and colorectal cancers (CRC). Tumor and serum samples were screened for MGMT promoter methylation. In methylation-positive patients, 150 mg/m^2 temozolomide was administered daily on a seven-day-on, seven-day-off schedule for each 28-day cycle. The primary efficacy endpoint was response rate (RR). MMR status was determined by a microsatellite instability assay. Among 740 patients screened, 86 were positive for MGMT promoter methylation and enrolled. Nineteen percent of the screened population (137/740) had confirmed tissue and/or serum MGMT promoter methylation, including 25% (57 of 229) for CRC, 36% (55 of 154) for esophageal cancer, 11% (12 of 113) for head and neck cancer, and 5% (13 of 242) for non–small cell lung carcinoma. Among patients with valid methylation results in both tissue and serum samples, concordance was 81% (339 of 419). The majority of enrolled patients (69 of 86; 80%) had microsatellite stable cancer. Overall RR was 6% (5 of 86 partial responses); all responders had microsatellite stable cancer. Temozolomide resulted in 81% (339 of 419). The majority of enrolled patients (69 of 86; 80%) had microsatellite stable cancer. Overall RR was 6% (5 of 86 partial responses); all responders had microsatellite stable cancer. Temozolomide resulted in low RRs in patients enriched for MGMT methylation. MGMT methylation status varied considerably in the patient population. Although serum methylation assay is an option for promoter methylation detection, tissue assay remains the standard for methylation detection. The low RR of this cohort of patients indicates that MGMT methylation as a biomarker is not applicable to heterogeneous tumor types, and tumor-specific factors may override validated biomarkers. Mol Cancer Ther; 12(5); 809–18. ©2013 AACR.

Introduction

Despite advances in targeted therapy, chemotherapy drugs remain the mainstay of treatment for most patients with solid tumors. However, few studies have allowed personalized choice of specific chemotherapy regimens for individual cancers. Determining predictors of sensitivity to chemotherapeutic agents would allow increased response rates (RR) with restriction of potential toxicity to populations of patients more likely to benefit.

Temozolomide, a methylating agent of the imidotrazine class, is commonly used in the treatment of glioblastoma and metastatic melanoma (Fig. 1; ref. 1). Its primary cytotoxic effect is attributable to alkylation at the O^6^ position of guanine, where it primarily forms O^6^-methylguanine-DNA adducts (1, 2). If the methyl group is not removed by O^6^-methylguanine-DNA methyltransferase (MGMT) before cell division, the DNA mismatch repair (MMR) pathway is triggered, resulting in a futile recycling that ultimately leads to DNA strand breaks and apoptotic cell death in MMR-proficient (microsatellite stable) cells (2, 3). MGMT rapidly reverses methylation via suicide inactivation by efficiently removing mutagenic and cytotoxic adducts from O^6^-guanine in DNA, thus restoring normal base pairing. The MMR system recognizes base mismatches and insertion–deletion loops, cuts the nucleotide sequence containing the lesion, and restores the correct base sequence. Both MGMT and MMR are potential determinants of tumor response to alkylating drugs such as temozolomide (3–7).

High levels of MGMT expression in normal and tumor cells are associated with resistance to alkylating chemotherapy (2). In contrast, tumors with a functional MMR...
system would likely be susceptible to temozolomide in the absence of MGMT (8, 9).

Molecular studies, primarily in patients with gliomas, suggest that the benefit of temozolomide is greatest in patients whose tumors have a methylated MGMT gene promoter and are thus unable to repair chemotherapy-induced DNA damage (7, 10). Although the value of promoter methylation status has been most studied in patients with gliomas, the response of nonglioma tumors with MGMT deficiency to temozolomide is not well described. A recent case report described 2 patients with metastatic colorectal cancer (CRC) and decreased expression of MGMT who responded positively to temozolomide (11). Promoter hypermethylation of tumor suppressor genes has been shown in tissue and serum DNA of patients with malignancies besides gliomas, particularly CRC and cancers of the aerodigestive tract (incidence 18%–73%; refs. 12–22).

It is now recognized that cell-free tumor DNA shed into the bloodstream may be detectable (15, 23–32). Recently, promoter methylation status determined in serum DNA isolates from patients with cancer has been shown to highly correlate with methylation status in those patients’ tumors (24–27, 30, 32). Accordingly, determination of promoter methylation from plasma/serum samples potentially provides a minimally invasive method for large-scale screening of patients for a specific promoter methylation in tumor DNA.

To evaluate the efficacy and safety of temozolomide in patients with nonglioma cancers, we conducted a nonrandomized, open-label, single-agent phase II study in patients with advanced CRC, non–small-cell lung carcinoma (NSCLC), head and neck cancer (H&N), or esophageal cancer who had confirmed MGMT promoter methylation. We also explored the concordance of tissue and serum for MGMT promoter methylation, their positive and negative predictive values, and the impact of MMR status on response to temozolomide.

Materials and Methods

Patients and treatment

Eligible patients were 18 years or older with histologically or cytologically confirmed metastatic CRC, locally advanced/inoperable/metastatic NSCLC, recurrent/metastatic H&N (including squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx), recurrent/metastatic esophageal cancer (including squamous cell carcinoma and adenocarcinoma), or cancers of the gastrointestinal junction. Detection of tissue and/or serum methylated MGMT promoter was a necessary entry criterion. In addition, each enrolled patient had at least 1 measurable lesion, as defined by Response Evaluation Criteria in Solid Tumors (RECIST), and an Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2. Prior therapy for advanced/metastatic disease was limited to 2 or less prior regimens for H&N and esophageal cancer and 3 or less prior regimens for CRC and NSCLC.

Patients received temozolomide (Temodal/Temodar; Merck Sharp & Dohme Corp.) orally at a dose of 150 mg/m² daily on a 7-day-on/7-day-off schedule (i.e., on days 1–7 and 15–21) for each 28-day cycle. Treatment was to be continued until occurrence of disease progression, intolerable toxicity, or death. Each patient provided written informed consent before obtaining molecular studies of their tumor and serum samples and before receiving study drug. The protocol was reviewed and approved by the Institutional Review Board/ethics committee at each center.

Evaluation of treatment response

The primary efficacy variables of RR [complete response (CR) and partial response (PR)] and secondary efficacy variables of duration of response and time to disease progression (TTP) were determined according to RECIST 1.0 criteria. Radiologic scans by computed tomography or MRI for evaluation of tumor status were conducted at baseline and every 8 weeks from day 1, cycle 1 until disease progression or death, and were intended to include patients who discontinued treatment prematurely. Overall survival (OS) was a secondary endpoint, determined from clinic visit or telephone contact. Exploratory objectives examined the concordance of MGMT status of tumor tissue and serum samples, and assessed the impact of MMR status on response.

The status of MGMT (methylated/unmethylated/unknown) and MMR [proficient (microsatellite stable)/deficient (microsatellite instability (MSI)-low or MSI-high)/unknown] was determined from tumor tissue blocks or slides (from initial diagnosis or later) and from serum samples collected at screening (details later). Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, version 3.0 (NCI CTCAE v3.0).

DNA extraction and methylation-specific PCR assay

One central laboratory conducted all MGMT analyses (OncoMethylome Sciences Testing Laboratory) in all screened patients using a direct, real-time methylation-specific PCR assay (MSP) to determine methylation status of the MGMT gene promoter (33). This assay detects CpG island methylation in the same MGMT promoter region as...
the nested, gel-based assay used in the phase III glioblastoma trial (7). When compared with the clinically validated nested, gel-based assay, the real-time MSP assay showed good concordance [Cohen’s κ coefficient of 0.80; 95% confidence interval (CI), 0.67–0.92; positive and negative agreement of 0.80 and 0.98, respectively]. The lack of a gold standard or other source of "true values" made discussion of sensitivity and specificity of the real-time assay difficult (34).

Mismatch repair assay
One centralized laboratory conducted all MMR analyses for all enrolled patients (Functional Genomics, Discovery Technologies, Schering-Plough Research Institute). The MSI Analysis System version 1.1, a fluorescent PCR-based assay (Promega), was used to determine MSI. The assay included fluorescently labeled primers for coamplification of 7 markers consisting of 5 mononucleotide repeated markers (BAT-25, BAT-26, NR-21, NR-24, and MNGO-N-D) and 2 pentanucleotide repeat markers (Penta C and Penta D; ref. 34). MSI classification is based on 2002 guidelines suggested by the NCI workshop on hereditary nonpolyposis CRC (Lynch syndrome) and MSI. Samples with instability in 2 or more of these markers are defined as MSI-high, whereas those with 1 unstable marker are designated as MSI-low. Samples with no detectable alterations are microsatellite stable.

Statistical methods
An adaptive design was intended to assess activity of treatment in terms of RR within each cancer type and across cancer types. The focus of the statistical analysis was the probability that RR to temozolomide would be at least modest (10%). The trial design adapted to the data that accumulated in such a way as to accommodate 2 possibilities: (i) the response to temozolomide is specific to MGMT methylation, independent of tumor site; and (ii) various cancer types have distinct RRs to temozolomide. After each cohort of 20 subjects (across all cancer types) became evaluable (assessed for response up to at least cycle 4 or had discontinued prior), an interim analysis using a Bayesian hierarchical logistic regression was conducted, with protocol-specified stopping rules for futility (unlikely chance that RR was at least 10%) and success (very high chance that RR was >10%). A maximum of 40 subjects of each cancer type were to be enrolled using the adaptive design. However, the adaptive process was discontinued after the third interim analysis because enrollment to NSCLC and H&N cohorts was too slow. The CRC cohort had met its enrollment target, and the esophageal cancer cohort, which did not meet either the futility or success criterion, was to be analyzed when approximately 25 subjects were evaluable.

Final analysis of tumor response was conducted on all enrolled patients [intent-to-treat (ITT) population] and the evaluable population (i.e., treated patients assessed for response up to at least cycle 4 or had progressive disease before cycle 4), overall and by cancer type, with corresponding 95% CIs. The Kaplan–Meier method was used to estimate TTP and OS for each cancer type. The concordance of MGMT promoter methylation status between tissue and serum samples was summarized on the basis of data from all screened patients and all enrolled patients in each disease category. For comparisons of methylation results for serum and tumor samples, sensitivity, specificity, and positive and negative predictive values were calculated, along with 95% CIs. The association between MMR status and response to treatment was summarized descriptively. Descriptive statistics were provided for safety data [NCT00423150; ClinicalTrials.gov identifier; study protocol number: P04273 (Merck)].

Results
From January 2007 until June 2009, 740 patients from 64 institutions in 11 countries were screened for eligibility. A total of 86 patients who were positive for MGMT promoter methylation were enrolled in the study and received treatment (ITT population), 82 of whom met the criteria for evaluable. Among the 4 patients excluded from the evaluable population, 3 discontinued before having any tumor response assessments and 1 discontinued after 2 cycles of temozolomide with stable disease. All 86 patients eventually discontinued temozolomide therapy; the primary reason for discontinuation was disease progression (69 patients; 80%). Study enrollment was terminated at 86 patients because of slow enrollment in H&N and NSCLC, and the observed low RRs in the other 2 cohorts after the adaptive process was discontinued.

Baseline characteristics of the ITT population are summarized in Table 1. The 86 treated patients received a median total dose of temozolomide of 4,322 mg/m² (range, 445.0–22,158.0) for a median 10 weeks (range, 1.4–50.9). The median number of cycles was 2 (range, 1–12). By cancer type, the median number of cycles (range) was 2 (1–7) for CRC, 4 (1–12) for esophageal cancer, 3 (1–7) for H&N, and 2 (1–6) for NSCLC. Only 12 patients (14%) had dose reductions, most of whom (n = 10) had a single dose reduction. The most common reason for dose reduction was adverse events.

MGMT promoter methylation status
A total of 19% of the screened population (137 of 740) had confirmed MGMT promoter methylation, as determined by either tissue or serum samples, including 25% (57 of 229) for CRC, 36% (55 of 154) for esophageal cancer, 11% (12 of 113) for H&N, and 5% (13 of 242) for NSCLC.

Concordance of methylation promoter status between tissue and serum assay for the screened and ITT populations is depicted in Table 2. By tissue assay alone, the overall MGMT promoter methylation rate was 15% (113 of 740) for the screened population, whereas by serum assay alone the rate was 8% (60 of 740). Thirty-six patients (5%) had a methylated MGMT result in both tissue and serum samples and 303 (41%) had an unmethylated MGMT result in both tissue and serum samples. Accordingly, MGMT promoter methylation results between tissue and
serum samples in patients with a valid tissue and serum methylation result \((n = 419)\) were concordant in 81% of patients (339 of 419). However, in the 113 patients with \(\text{MGMT}\) promoter methylation in a tissue sample, only 36 (32%) showed methylation in the serum sample, suggesting that in a large proportion of patients with positive tumor assay, \(\text{MGMT}\) promoter methylation was not detected by the serum assay or that sufficient tumor heterogeneity existed to provide discordant results.

Excluding specimens with unknown results and assuming the tissue assay is indeed the gold standard, the serum assay has a sensitivity (i.e., serum methylated among all tumor methylated) of 34% (95% CI, 0.25–0.43) and a specificity (i.e., serum unmethylated among all tumor unmethylated) of 96% (95% CI, 0.94–0.98). The positive predictive value of the serum assay (i.e., tumor methylated among all serum methylated) was 77% (95% CI, 0.64–0.89) and the negative predictive value (i.e., tumor unmethylated among all serum unmethylated) was 81% (95% CI, 0.78–0.85). Overall, accuracy of the serum assay (i.e., correct prediction/total) was 81% (95% CI, 0.77–0.85).

Following the protocol eligibility criterion, all 86 (100%) patients in the ITT population had confirmed \(\text{MGMT}\) promoter methylation, by either tissue or serum assay. A majority (69 patients; 80%) had a methylated \(\text{MGMT}\) result in a tissue sample and 42 patients (49%) had a methylated \(\text{MGMT}\) result in a serum sample.

### Mismatch repair status

The majority of enrolled patients (69 of 86; 80%) had MMR proficiency (microsatellite stable). A minority (6 of...
MGMT status was unknown in 11 patients (13%).

Efficacy and correlation between tumor response and MGMT and MMR status

Five of 86 (6%) patients had a PR as best response and 39 (45%) patients had stable disease. The overall RR was 5.8% (95% CI, 1.9–13.0), which was highest in the H&N group (14.3%; 95% CI, 0.4–57.9), followed by esophageal cancer (9.4%; 95% CI, 2.0–25.0; Table 3). Similar best overall response findings, overall and by cancer type, were observed in the evaluable population.

The association between MGMT and MMR status and response to treatment is summarized in Table 4, for all cancer types combined. All 5 (100%) patients who had a PR to treatment with temozolomide were MMR-proficient (microsatellite stable). Of the 39 patients with stable disease, 32 (82%) were MMR-proficient, 1 (3%) was MMR-deficient, and 6 (15%) had unknown MMR status. Of the 39 patients with progressive disease, 29 (74%) were MMR-proficient, 5 (13%) were MMR-deficient, and 5 (13%) had unknown MMR status. Because only a minority (6 of 86; 7%) of patients had MMR deficiency, the correlation between MMR status and response cannot be established.

With regard to MGMT status, the rate of PR or stable disease seems to be comparable between patients with methylation detected in serum (20 of 42 patients; 48%) or tissue (38 of 69 patients; 55%), and between patients with methylation detected in serum (20 of 42 patients; 48%) or not detected in serum (24 of 44 patients; 55%).

Sixty-six patients (77%) died during the study period. For the ITT population, all patient cohorts combined and by cancer group, the estimated median OS was 6.7 months (95% CI, 5.8–8.0 months) with an estimated median TTP of 2.8 months (95% CI, 1.9–3.6 months). Kaplan–Meier plots for OS and TTP are provided in Supplementary Figs. S1 and S2.

Safety

Treatment-emergent and treatment-related adverse events reported for 5% or more of patients are presented for all cancer types combined in Table 5. Most adverse events were grade 1 or 2 in severity. Sixty-six (77%) patients died during the study period, primarily due to disease progression (53 patients; 62%) and disease-related complications (8; 9%; Table 6).

Discussion

Although targeted therapies play an increasing role in the treatment of cancers, cytotoxic chemotherapy remains important for treatment of most solid tumors. However, the validation of biomarkers to predict response to chemotherapy has been disappointing. The best example is MGMT methylation status, a documented prognostic factor in patients with gliomas. MGMT methylation status is associated with increased sensitivity of glioblastoma to alkylating agents, correlating with prolonged progression-free survival and OS in glioma patients treated with temozolomide (6, 35). However, use of methylation promoter status to guide temozolomide therapy in other cancers has not been reported. We report the first clinical trial that investigated the efficacy and safety of temozolomide in patients with advanced aerodigestive tract and CRCs with confirmed MGMT promoter methylation. In addition, the concordance of methylation status between tissue and serum samples was evaluated in more than 700 screened patients, which is the largest number of samples studied to date. The study used an adaptive design, which allowed for continuous accrual of patients across all cancer types and stopping the enrollment of any of the 4 cancer types in the event of a positive outcome or futility.

In this trial, efficacy of temozolomide for aerodigestive tract and CRCs in patients with confirmed methylation MGMT promoter status was inconclusive. Low RRs in

<table>
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<th>Table 3. Best overall RR by cancer type</th>
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<tr>
<td><strong>ITT population</strong></td>
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<tr>
<td>CR</td>
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<tr>
<td>n (%)</td>
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<tr>
<td>CR</td>
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<tr>
<td>PR</td>
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<td>Stable disease</td>
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<td>Progressive disease</td>
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<tr>
<td>No response data</td>
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<td>RR, % (95% CI)</td>
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| **Evaluable population**               |
| n (%)                                  | n = 36 | n = 31 | n = 6 | n = 9 | n = 82 |
| CR                                     | 0 | 0 | 0 | 0 | 0 |
| PR                                     | 1 (3) | 3 (10) | 1 (17) | 0 | 5 (6) |
| Stable disease                         | 15 (42) | 16 (52) | 3 (50) | 4 (44) | 38 (46) |
| Progressive disease                    | 20 (56) | 12 (39) | 2 (33) | 5 (56) | 39 (48) |
| RR, % (95% CI)                         | 2.8 (0.1–14.5) | 9.7 (2.0–25.8) | 16.7 (0.4–64.1) | 0 (0–33.6) | 6.1 (2.0–13.7) |

*RR = complete + partial responses.*
patients with CRC and esophageal carcinoma led to early termination of the study, and unexpectedly low methylation rates in patients with H&N and NSCLC resulted in low enrollment and, therefore, uninterpretable results. For the 86 enrolled patients (all cancer groups combined), the estimated median TTP was 2.8 months (95% CI, 1.9–3.6 months) and estimated median OS was 6.7 months (95% CI, 5.8–8.0 months). Only 5 (6%) patients overall had a PR (1 CRC, 3 esophageal, and 1 H&N) and no patient achieved a CR. Stable disease was the best response for 45% of temozolomide-treated patients. The standard of care for patients with advanced aerodigestive tract and CRCs is tenuous at best; thus, it is disappointing that temozolomide as a single agent offered few apparent benefits. For CRC, RRs in our patient population were lower (2.7%) compared with other third- or fourth-line investigational agents such as cetuximab monotherapy (10.8%) or the combination of cetuximab and irinotecan (22.9%; ref. 36). However, for esophageal cancer, our RR (9%) was similar to gefitinib (37), but lower than other investigational agents such as irinotecan combined with capecitabine, 5-fluorouracil, or cisplatin plus cetuximab, and thoracic radiotherapy (17%–20%; refs. 38–40) or second-line paclitaxel and capecitabine (45%; ref. 41). As such, the role of temozolomide monotherapy for CRC and esophageal cancer seems limited. Although we did not formally assess the contribution of prior therapies to resistance, potential reasons for the lack of efficacy could include insensitivity to temozolomide after multiple prior therapies (e.g., the majority of CRC patients had multiple previous treatments, and most patients likely received prior alkylating agent therapy) and/or other mechanisms of resistance. Results from trials of combination therapy with temozolomide will be interesting.

It is clear that methylation of the MGMT promoter is not the only factor determining response to temozolomide. A recent study showed that the base excision repair enzyme alkylpurine-DNA N-glycosylase (APNG), which repairs the cytotoxic lesions N3-methyladenine and N7-methylguanine, may contribute to temozolomide resistance. Reduction of APNG expression in a variety of cellular models attenuated repair of temozolomide-induced DNA damage. Patients with tumors expressing high levels of nuclear APNG showed worse survival compared with those whose tumors lacked APNG (42).

Despite the low RR in this trial, temozolomide was well tolerated and showed a safety profile similar to that seen in other malignancies (43). Most adverse events were of grade 1 or 2 severity. Similar to patients with gliomas, gastrointestinal adverse events (nausea and vomiting) and myelosuppression (thrombocytopenia) were the most commonly reported drug-related events, and all are known to be associated with temozolomide.

Our study in patients with advanced aerodigestive tract and CRCs screened 740 patients for MGMT promoter methylation status—the largest single database reported to date. The frequency of MGMT promoter methylation in our patient population was within the expected range for CRC and esophageal cancer (25% and 36%, respectively). However, among more than 350 patients screened, unexpectedly low rates were found for H&N and NSCLC (11% and 5%, respectively). Previous studies, including more than 600 samples from patients with CRC, reported that 24% to 41% tested positive for MGMT promoter methylation (12, 21, 22). In esophageal cancer, 20% to 73% of approximately 200 samples displayed promoter MGMT methylation (12, 18–20). In more than 400 NSCLC samples, 15% to 51% had methylated MGMT promoters (12–14, 44). The

<table>
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<th>Overall best response, n (%)</th>
<th>MGMT status</th>
<th>MMR status</th>
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<tr>
<td></td>
<td>Proficiency (microsatellite stable)</td>
<td>Deficiency (MSI-low)</td>
</tr>
<tr>
<td>PR</td>
<td>Methylated</td>
<td>5 (6)</td>
</tr>
<tr>
<td></td>
<td>Both tissue and serum</td>
<td>3 (3)</td>
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<tr>
<td></td>
<td>Tissue only</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>Methylated</td>
<td>32 (37)</td>
</tr>
<tr>
<td></td>
<td>Both tissue and serum</td>
<td>10 (12)</td>
</tr>
<tr>
<td></td>
<td>Tissue only</td>
<td>18 (21)</td>
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<tr>
<td></td>
<td>Serum only</td>
<td>4 (5)</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>Methylated</td>
<td>29 (34)</td>
</tr>
<tr>
<td></td>
<td>Both tissue and serum</td>
<td>7 (8)</td>
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<tr>
<td></td>
<td>Tissue only</td>
<td>15 (17)</td>
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<td></td>
<td>Serum only</td>
<td>7 (8)</td>
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<tr>
<td>No response data</td>
<td>Methylated</td>
<td>3 (3)</td>
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<tr>
<td></td>
<td>Both tissue and serum</td>
<td>1 (1)</td>
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<td></td>
<td>Tissue only</td>
<td>1 (1)</td>
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<td></td>
<td>Serum only</td>
<td>1 (1)</td>
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high upper-end range for NSCLC is partially explained by the inclusion of patients who had never smoked and who had higher expression of MGMT promoter methylation compared with smokers (14). Patients with H&N had 18% to 41% of MGMT promoter methylation in approximately 400 samples (12, 15–17, 45, 46). In our study and most others, MGMT promoter methylation status was determined by a methylation-specific PCR assay conducted on tumor DNA. The aberrant methylation rates for H&N and NSCLC are likely explained by the inclusion of patients with a range of disease stages and variable smoking histories as well as specific assay and institutional differences.

The discovery of circulating cell-free DNA in serum or plasma (15, 23–32) presents a new opportunity for identifying MGMT promoter methylation in tumor cells. There have been limited reports on the concordance between serum, plasma, or sputum and tumor methylation biomarkers in patients, such as APC promoter methylation in lung cancer (29); hMLH1 promoter methylation in CRC (23); p16 methylation in NSCLC, H&N, esophageal, breast, and hepatocellular cancers (15, 24, 28, 30, 32); and MGMT methylation in lung cancer (44). Although these reports used similar MSP analysis, the concordance between assays of tumor and surrogate tissue varied across different cancer types and biomarkers. In our large series of 740 tumor and serum samples, the concordance between tissue and serum MGMT promoter methylation in patients with known tissue and serum MGMT methylation status was 81%, and positive and negative predictive values were relatively high (77% and 81%, respectively). Yet, it is important to highlight that the serum assay detected MGMT promoter methylation in only 32% of subjects (36 of 113 patients) who showed MGMT promoter methylation in their tissue samples. These findings suggest that serum assay alone may underreport patients with tumors that have MGMT promoter methylation. Several explanations are possible for the low sensitivity in serum, including variations in the amounts of circulating free DNA, size of free DNA, and degree of tumor-shedding DNA. In addition, the high-throughput direct real-time PCR (MSP) assay (33) includes a step of chemical treatment of DNA (i.e., sodium bisulfite conversion), which may damage DNA, ultimately leading to a reduced amount of DNA available for testing.

The role of circulating cell-free DNA as a diagnostic/prognostic/therapeutic biomarker continues to evolve. It is important to recognize that detection of circulating free DNA may also reflect physiologic and pathologic processes that are not tumor-specific (47). On the basis of the

### Table 5. Treatment-related adverse events reported for ≥5% of patients, all cancer types combined (ITT population)

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>All grades</th>
<th>Grade 3/4</th>
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<tbody>
<tr>
<td>Blood and lymphatic system disorders</td>
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<td></td>
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<tr>
<td>Anemia</td>
<td>13 (15)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>8 (9)</td>
<td>3 (3)</td>
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<tr>
<td>Thrombocytopenia</td>
<td>19 (22)</td>
<td>7 (8)</td>
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<tr>
<td>Gastrointestinal disorders</td>
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<td></td>
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<tr>
<td>Constipation</td>
<td>6 (7)</td>
<td>0</td>
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<tr>
<td>Diarrhea</td>
<td>11 (13)</td>
<td>0</td>
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<tr>
<td>Nausea</td>
<td>37 (43)</td>
<td>1 (1)</td>
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<tr>
<td>Vomiting</td>
<td>28 (33)</td>
<td>1 (1)</td>
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<tr>
<td>General disorders and administration site conditions</td>
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<td></td>
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<tr>
<td>Asthenia</td>
<td>5 (6)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>29 (34)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Investigation</td>
<td></td>
<td></td>
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<tr>
<td>Platelet count decreased</td>
<td>5 (6)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Metabolism and nutrition disorders</td>
<td></td>
<td></td>
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<tr>
<td>Anorexia</td>
<td>15 (17)</td>
<td>2 (2)</td>
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<tr>
<td>Nervous system disorders</td>
<td></td>
<td></td>
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<tr>
<td>Dizziness</td>
<td>5 (6)</td>
<td>0</td>
</tr>
<tr>
<td>Headache</td>
<td>6 (7)</td>
<td>0</td>
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<tr>
<td>Skin and subcutaneous tissue disorders</td>
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</tr>
<tr>
<td>Rash</td>
<td>8 (9)</td>
<td>0</td>
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### Table 6. Summary of cause of death by cancer type (ITT population)

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<tr>
<th></th>
<th>CRC patients, n (%) (n = 37)</th>
<th>Esophageal cancer patients, n (%) (n = 32)</th>
<th>H&amp;N patients, n (%) (n = 7)</th>
<th>NSCLC patients, n (%) (n = 10)</th>
<th>All combined patients, n (%) (n = 86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All causes</td>
<td>33 (89)</td>
<td>19 (60)</td>
<td>5 (71)</td>
<td>9 (90)</td>
<td>66 (77)</td>
</tr>
<tr>
<td>Adverse event</td>
<td>1 (3)</td>
<td>1 (3)</td>
<td>1 (14)</td>
<td>1 (1)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>Disease progression</td>
<td>26 (70)</td>
<td>18 (56)</td>
<td>2 (29)</td>
<td>7 (70)</td>
<td>53 (62)</td>
</tr>
<tr>
<td>Disease-related complications</td>
<td>5 (14)</td>
<td>0 (0)</td>
<td>2 (20)</td>
<td>1 (10)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>Other*</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

*Patient had discontinued treatment after 2 cycles because of disease progression and died 172 days later; no further information is available.
findings from our study, tumor tissue still represents the gold standard for methylation detection when samples are available. Studies evaluating MGMT promoter methylation within glioblastoma lesions as well as in paired primary and recurrent glioblastoma samples suggested that MGMT promoter methylation could be a homogeneous marker throughout malignant gliomas (48) and that most recurrent lesions retained the methylation profile from the primary lesions (49). However, considering the increasing awareness of tumor heterogeneity (50) and the positive predictive value (77%) for serum assay observed in our study, the serum methylation assay may provide a possible alternative for patients with no available tumor samples. It should be noted that we observed MGMT methylation in serum but not in the corresponding tumor in 1% of screened patients and in 8% of enrolled patients. The reasons for the lack of concordance between serum and tumor methylation pattern in such cases are unknown but could include other sources of DNA detected in plasma or tumor heterogeneity. Although this is a relevant and important consideration in the development of serum-based biomarker assays, it is beyond the scope of our study and warrants further investigation.

Cell death after temozolomide treatment also depends upon recognition of O6-guanine/thymine mispairs by the DNA repair pathway (3, 4). Although data are very limited in our study, all 5 patients with a PR were MMR-proficient and most MMR-deficient patients (5 of 6 patients; 83%) had progressive disease. Although the number of responses is too low to permit conclusions about the correlation between MMR status and response to temozolomide, our data suggest that a proficient MMR system seems to be required for the therapeutic effects of temozolomide, consistent with expectations based on the function of the DNA repair system. We did not examine methylation of MMR genes in this study and therefore cannot rule out the possibility of MMR gene methylation and malfunction in the MMR pathway in microsatellite stable tumors.

An unresolved issue is whether the status of MGMT promoter methylation changes over time. Moreover, despite the pivotal findings of Hegi and colleagues (6), it is unclear whether in glioma MGMT status at initial diagnosis is correlated to outcome to temozolomide therapy at progression. Most trials have failed to show such a correlation, which may be due to prior treatments. Apart from the confounding effects of prior chemotherapy on tumor chemosensitivity, methylation changes may be unstable or heterogeneous. We were unable to compare the MGMT methylation status of the primary tumor and metastases because only 1 tumor sample per patient was collected during the study.

The findings of this study must be interpreted on the basis of several limitations. The small number of patients in each cancer cohort does not permit efficacy conclusions; the overall low RR does not permit conclusions to be drawn about influence of MMR status. The type of assay used for promoter methylation status may underestimate the prevalence of methylation detected by the serum assay (33). The source of serum DNA methylation in the absence of tumor methylation, which is an important aspect to be considered in the development of serum-based assays, was not explored. Furthermore, the small number of patients in each cancer cohort and heterogeneity of the study population with regard to number of prior chemotherapies limited the ability to evaluate the effects of multiple lines of chemotherapy on MGMT promoter methylation and MMR status. Although assessment of MGMT methylation status was feasible in this study, the activity of temozolomide was low. Future deep sequencing of human tumors should inform development of biomarkers for responsiveness to conventional chemotherapy such as temozolomide.

A recent study investigated temozolomide in patients with small cell lung cancer without selection for MGMT methylation status (51). The RRs for chemotherapy-sensitive and refractory cases were 23% and 13%, respectively, in this relatively homogeneous population. There was a nonstatistical trend toward increased RRs in the MGMT methylated group. MGMT methylation status was obtained by immunohistochemistry and promoter methylation assay in only 27 patients; the assay was different from that used in our study. In patients for whom MGMT methylation could be determined, the overall promoter methylation rate was 48%, which was higher than in our study, although the sample size was small. A high RR was found in patients with brain metastases, possibly suggesting specific factors that influence response to this agent given the noted sensitivity of gliomas to temozolomide.

In conclusion, temozolomide was safe and well tolerated in a range of advanced cancers studied, with limited efficacy as a single agent. In our large screened population of 740 patients, the frequency of MGMT promoter methylation varied considerably across the cancer spectrum, from 5% for NSCLC to 36% for esophageal cancer. Although concordance of methylation status was observed in nearly half of tested tumor and serum specimens, serum sensitivity was low (34%). Tumor tissue remains the gold standard for methylation detection; however, serum/plasma is a viable option for biomarker detection, especially in patients for whom tumor samples are not available. Although results were negative in relation to response, the implications of our study are of significance for the design of future trials, particularly with temozolomide, and should be considered carefully when designing future studies with DNA-damaging agents.

Disclosure of Potential Conflicts of Interest
R. Glynne-Jones has commercial research grant for drug costs and PET scanning from Roche and Merck Serono for CTAAc approved studies (XERES and BACCHUS), honoraria from Speakers Bureau, fees for lectures, and chairing meetings of Roche/Sanofi/Merck Serono, and is a consultant/advisory board member of Roche/Merck Serono/Sanofi. Q. Zhang is employed as Principal Scientist at Merck. L. Zhang was an employee of Merck and owned Merck stock. E.A. Sausville has commercial research grant from Schering Plough. No potential conflicts of interest were disclosed by the other authors.
Temozolomide in Advanced Cancers: Translational Data

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Hochhauser, R. Glynne-Jones, V. Potter, C. Gevalos, T.J. Doyle, K. Pathiraja, Q. Zhang, L. Zhang, E.A. Sausville
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Molecular Cancer Therapeutics

A Phase II Study of Temozolomide in Patients with Advanced Aerodigestive Tract and Colorectal Cancers and Methylation of the O\textsuperscript{6}-Methylguanine-DNA Methyltransferase Promoter

Daniel Hochhauser, Rob Glynne-Jones, Vanessa Potter, et al.


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