Promoter CpG Island Hypermethylation of the DNA Repair Enzyme MGMT Predicts Clinical Response to Dacarbazine in a Phase II Study for Metastatic Colorectal Cancer

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Abstract

Purpose: O6-methylguanine-DNA-methyltransferase (MGMT) is a DNA repair protein removing mutagenic and cytotoxic adducts from O6-guanine in DNA. Approximately 40% of colorectal cancers (CRC) display MGMT deficiency due to the promoter hypermethylation leading to silencing of the gene. Alkylating agents, such as dacarbazine, exert their antitumor activity by DNA methylation at the O6-guanine site, inducing base pair mismatch; therefore, activity of dacarbazine could be enhanced in CRCs lacking MGMT. We conducted a phase II study with dacarbazine in CRCs who had failed standard therapies (oxaliplatin, irinotecan, fluoropyrimidines, and cetuximab or panitumumab if KRAS wild-type).

Experimental Design: All patients had tumor tissue assessed for MGMT as promoter hypermethylation in double-blind for treatment outcome. Patients received dacarbazine 250 mg/m2 intravenously every four consecutive days, every 21 days, until progressive disease or intolerable toxicity. We used a Simon two-stage design to determine whether the overall response rate would be 10% or more. Secondary endpoints included association of response, progression-free survival, and disease control rate with MGMT status.

Results: Sixty-eight patients were enrolled from May 2011 to March 2012. Patients received a median of three cycles of dacarbazine (range 1–12). Grades 3 and 4 toxicities included: fatigue (41%), nausea/vomiting (29%), constipation (25%), platelet count decrease (19%), and anemia (18%). Overall, two patients (3%) achieved partial response and eight patients (12%) had stable disease. Disease control rate (partial response + stable disease) was significantly associated with MGMT promoter hypermethylation in the corresponding tumors.

Conclusion: Objective clinical responses to dacarbazine in patients with metastatic CRC are confined to those tumors harboring epigenetic inactivation of the DNA repair enzyme MGMT. Clin Cancer Res; 19(8); 2265–72. ©2013 AACR.
Translational Relevance

O6-methylguanine-DNA-methyltransferase (MGMT) is a DNA repair protein removing mutagenic and cytotoxic adducts from O6-guanine in DNA. Approximately 40% of colorectal cancers (CRC) display MGMT deficiency due to promoter hypermethylation leading to silencing of the gene. Alkylating agents, such as dacarbazine, exert their antitumor activity by DNA methylation at the O6-guanine site, inducing base pair mismatch; therefore, activity of dacarbazine could be enhanced in CRCs lacking MGMT. Although several reports have shown anecdotal efficacy of dacarbazine in metastatic CRC, there is a lack of translational evidence of CRC sensitivity to this drug based on MGMT status. We report here a phase II clinical study showing for the first time that dacarbazine activity is confined to CRC harboring promoter CpG hypermethylation of MGMT. These data therefore highlight a previously unidentified subgroup of the patients with CRC who benefit from treatment with alkylating agents based on a specific epigenetic alteration in individual tumors.

options, based on specific molecular alterations that could prove their effectiveness also in the wide KRAS-mutated subgroup of CRCs.

O6-methylguanine-DNA-methyltransferase (MGMT) is a DNA repair protein that removes mutagenic and cytotoxic adducts from O6-guanine in DNA. MGMT protects cells against these lesions, transferring the alkyl group from the O6-guanine in DNA to an active cysteine within its own sequence. Such reaction inactivates one MGMT molecule for each lesion repaired (7). The inactivation of tumor suppressor genes by the presence of cytosine methylation encompassing the corresponding transcription start site located in a CpG island is gaining “momentum” in the management of oncology patients (8) and, in this regard, promoter CpG island hypermethylation leads to the transcriptional silencing of MGMT (9). The subsequent lack of repair of O6-methylguanine adducts can result in a higher frequency of G:C > A:T transitions (10, 11). It is known that approximately 40% of CRCs have silencing of MGMT. Interestingly, in a retrospective analysis on 244 CRCs samples, it has been found that 71% of tumors with G to A mutation in KRAS showed MGMT epigenetic inactivation, showing a strong association between the MGMT inactivation by promoter hypermethylation and the appearance of G to A mutations at KRAS (10). Furthermore, MGMT hypermethylation was also found in 35% of wild-type KRAS mCRCs. de Vogel and colleagues (12) found that MGMT hypermethylation is associated with G:C > A:T mutations in KRAS, but not in adenomatous polyposis coli (APC), suggesting that MGMT hypermethylation may succeed APC mutations but it precedes KRAS mutations in colorectal carcinogenesis.

In cells, loss of MGMT expression leads to compromised DNA repair and may play a significant role in cancer progression and response to chemotherapy as it occurs in glioma (13–16). The mechanism of action of dacarbazine and temozolomide is DNA methylation at the O6-guanine site, inducing base pair mismatch. The methyl group at O6-site is removed by MGMT in a one-step methyl transfer reaction. Therefore, we hypothesized that MGMT inactivation by hypermethylation may confer sensitivity to these agents (17). However, discrepant data about the clinical activity of these drugs in mCRC are reported in the literature (18–21). A response rate of 19%, including one complete response, was reported in 26 fluoropyrimidine-resistant patients receiving cisplatin and dacarbazine (19). In another study, 48 patients refractory to fluoropyrimidine were treated with dacarbazine, irinotecan, and cisplatin obtaining a 33% of response rate (18). Temozolomide is an imidazotetrazine derivative of dacarbazine. The combination of lomeguatrib and temozolomide did not show activity in unselect ed mCRC (20). In a pilot study including patients selected by tumor molecular profiling, temozolomide was effective in 2 patients with mCRC exhibiting loss of MGMT expression (22). The latter finding was confirmed by a recent report by Shacham-Shmueli and colleagues (23) documenting objective responses to temozolomide in 2 patients with MGMT-deficient mCRC.

On the basis of these findings, we designed a phase II trial aimed at assessing the antitumor activity of dacarbazine in patients with mCRC with determined MGMT promoter methylation status and refractory to the standard therapies.

Materials and Methods

Trial design

The study was designed as a phase II trial (DETECT-01 trial, EUiDRACT number 2011-002080-21). Patients were treated with dacarbazine monotherapy until progression or unacceptable toxicity for 18 weeks (6 cycles). In case of partial response with clinical benefit, treatment was allowed until dose-limiting toxicity. Primary endpoint was to assess response rate to dacarbazine according to Response Evaluation Criteria in Solid Tumors (RECIST 1.1) criteria. Secondary endpoints were to assess: disease control rate (DCR), progression-free survival (PFS), identification of KRAS, and O6-methylguanine-DNA-methyltransferase (MGMT) status in individual tumor samples as potential molecular biomarkers of response to dacarbazine. Written informed consent was obtained from each patient. The study followed the Declaration of Helsinki and good clinical practice, being approved by Ethic Committee of Ospedale Niguarda Ca’ Granda (Milan, Italy).

Patients

All patients met the following inclusion criteria: age 18 years or more, Eastern Cooperative Oncology Group performance status of ≤ 1, histologically confirmed metastatic colorectal adenocarcinoma. A paraffin-embedded
block from archival tumor tissue of primary and/or metastases for MGMT status analysis was requested. All patients had measurable disease (by RECIST criteria v1.1), and progressed on standard treatment with fluoropyrimidine, oxaliplatin, irinotecan, and cetuximab or panitumumab (the latter 2 drugs if KRAS wild-type). An adequate bone marrow, liver, and renal function was required.

**Treatment schedules**

Dacarbazine 250 mg/m² intravenously everyday for 4 consecutive days, every 21 days, was administered until progression, death, unacceptable toxicity, or patient withdrawal of consent. Antiemetic agents and supportive care were provided by treating physician as per standard clinical practice. In case of G3 hematologic toxicity (absolute neutrophil count $<1.5 \times 10^9$/L and platelet count $<100 \times 10^9$/L) dacarbazine was delayed by 1-week interval until recovery. Prophylactic use of colony-stimulating factors was allowed as per standard clinical practice.

**Evaluation criteria**

Patients were evaluated for primary overall response rate (ORR) and secondary endpoint (DCR and PFS) according to RECIST criteria v1.1. Tumors were measured every 8 ± 1 weeks through week 18 and then every 8 ± 1 weeks until the tumor progressed. Complete response was defined as disappearance of all target lesions. Any pathologic lymph nodes (whether target or nontarget) must have reduction in short axis to 10 mm or less. An objective response (partial response) was defined as a reduction of at least 30 percent in the sum of diameters of target lesions, while on study. Clinical investigators and radiologists were blinded as for MGMT status of the tumors.

**Safety assessment**

Safety assessments and blood biochemistry including complete blood counts were carried out at baseline and at the beginning of each treatment cycle. Any toxicity was assessed using the National Cancer Institute (NCI)-CTCAE version 4.0 and recorded at every visit until resolved.

**Analysis of MGMT promoter methylation status**

Loss of expression of MGMT was defined as promoter hypermethylation 25% or more as previously described (9). Tumor samples from patients’ primary tumor were obtained from Pathology Department of the Ospedale Niguarda Ca’ Granda or others Pathology Departments as referral. Formalin-fixed paraffin-embedded tumor blocks were reviewed for quality and tumor content. A single representative block, from either the primary tumor or metastasis, depending on availability, was selected for each case. White slides (2 cut of 10 μm, if from a tumor tissue paraffin block, or 3 cuts of 10 μm if from a biopsy) were sent to Bellvitge Biomedical Research Institute (IDIBELL; Barcelona, Spain) for DNA extraction and evaluation of MGMT promoter methylation status in blind as for clinical outcome. Genomic DNA was extracted from paraffin tissue samples following manufacturer's instructions (QiAamp DNA FFPE Tissue Kit). DNA was then subjected to bisulfate treatment using EZ DNA methylation kit (Zymo Research). Briefly, 1 μg of genomic DNA was denaturated by incubating with 0.2 mol/L NaOH. Aliquots of 10 mmol/L hydroquinone and 3 mol/L sodium bisulfate (pH 5.0) were added, and the solution was incubated at 50°C for 16 hours. Treated DNA was purified, desulfonated with 0.3 mol/L NaOH, repurified on Zymo-Spin columns, and eluted with 25 μL water. MGMT promoter methylation status was analyzed by methyl-specific polymerase chain reaction (MSP). It was carried out in a 15 μL volume containing 1 μL of the sodium bisulfite-modified DNA. The characteristics of the MSP reactions and the primer sequence have been previously described (14). SW620 cell line was used as a positive control for hypermethylated alleles of MGMT and DNA from RKO cell line used as a negative control (Fig. 1).

![Figure 1. Methyl-specific PCR for MGMT promoter. Example of results obtained for 6 metastatic colorectal cancer primary tumors from the study cohort. Tumors T2 and T7 were methylated and all the others unmethylated. U indicates unmethylated tumors and M methylated tumors. RKO was the human colorectal cancer cell line used as negative control for methylation and SW620 the human colorectal cancer cell line used as positive one. H₂O is the experiment negative control.](image-url)
According to clinical considerations and on the basis of the available literature, the efficacy of a treatment in this setting of mCRC chemorefractory patients would be considered poor if the ORR is 3% or less, whereas it could be considered of clinical usefulness if the ORR is 10% or more. Assuming $\alpha = 0.05$ and $\beta = 0.20$, a Simon Optimal 2-stage design has been then chosen to test the null hypothesis that $P \leq 0.03$ versus the alternative that $P \geq 0.10$. According to this design, if at least 2 of the first 40 patients would have achieved an objective response, enrollment would have been extended by 28 patients. Overall, objective response rate of dacarbazine monotherapy would have been deemed unacceptable if objective response was 4 or less. The association between MGMT promoter methylation status and ORR and DCR was determined by 2-sided Student $t$-test or Fisher exact test. PFS was estimated by Kaplan–Meier product-limit method followed by log-rank test.

### Results

#### Patients' characteristics

Sixty-eight patients were enrolled in our institution from May 2011 until March 2012. All patients had progressed on fluoropyrimidines, oxaliplatin, irinotecan, and cetuximab or panitumumab (the latter 2 drugs if KRAS wild-type). 87% of patients had received prior bevacizumab and 19% patient had received more than 4 lines of treatment. Twenty percent of patients received mitomycin C, 4% raltitrexed, and 12% previous experimental agents within clinical trials. Clinical characteristics of patients in this trial are reported in Table 1. Reasons for discontinuation of dacarbazine treatment included hematologic toxicity (1 patient), progression (61 patients), death (4 patients), and withdrawal of consent (2 patients). Cause of death was recorded as mCRC in all deceased patients.

#### Toxicity

Adverse events were listed in Supplementary Table S1. Hematologic toxicity was the most frequent adverse event reported and general toxicity was consistent with the known toxicity profile of dacarbazine. We observed 3 hematologic G4 adverse events (2 platelet count decreased and one neutrophil count decreased). Hepatic failure with increased bilirubin due to progression of disease was observed in 3 patients with extensive metastatic liver involvement.

#### Analysis of MGMT promoter hypermethylation

Sixty-five of 68 patients were tested for MGMT promoter CpG island methylation, as showed in Table 1. Overall, MGMT hypermethylation was found in 40% (26/65) of the colorectal neoplasms DNAs analyzed, a similar frequency to the previously reported for this tumor type (9). According to the location of the tumor, MGMT promoter status was assessed in 69% (45/65) in primary tumor, in 14% (9/65) in metastatic site, and in 17% (11/65) in both primary
and metastatic site from the same patient. In the latter case, we observed concordance in 10 of 11 pairs, with only one case showing a hypermethylated primary with unmethylated liver metastasis, and the result from liver metastasis was considered for the purpose of analysis. Sites of metastases were: liver 75% (15/20), 5% (1/20) ovary, 10% (2/20) lung, 5% (1/20) spleen, and 5% (1/20) cutaneous.

**MGMT hypermethylation** was more frequent (61% and 31%, respectively) in tumors carrying **KRAS** mutation with G > A transition (G12D, G12V, or G13D), as previously described (10, 11), although the difference was not statistically significant due to the small size (only 26 patients were evaluable for both analysis; \( P = 0.238 \)).

**Antitumor activity of dacarbazine**

ORR was 3%, with 2 partial responses. Stable disease was achieved in 8 of 68 patients (12%), accounting for a DCR (partial response + stable disease) of 15%. Median PFS was 57 days. Preplanned analysis of secondary endpoints based on assessments of MGMT methylation and KRAS mutation status in individual tumors showed that objective responses occurred only in patients displaying MGMT hypermethylation (Fig. 2A and Fig. 3). In addition, we observed a significantly higher DCR (44.0% vs. 6%, \( P = 0.012 \)) in the MGMT-hypermethylated group (Fig. 2). A trend toward better PFS [HR = 0.66; 95% confidence interval (CI) 0.40–1.10; \( P = 0.0982 \)] was also found in the MGMT-hypermethylated cases (Fig. 4A). A similar tendency was found between reduction of tumor volume following dacarbazine treatment and MGMT methylation status: tumor shrinkage of any size occurred more frequently in patients displaying MGMT hypermethylation (Fisher exact test, \( P = 0.093 \)). In contrast, KRAS status was not associated with PFS, DCR, and ORR (KRAS mutant vs. KRAS wild-type, \( P = 0.735, 0.999, \) and 0.492, respectively; Fig. 4B).

**Discussion**

In this study, we document that dacarbazine is active after failure of standard therapies only in those patients with mCRC whose tumor is harboring epigenetic inactivation of the DNA repair enzyme MGMT. Overall, we observed 2 objective responses, accounting for 3% of ORR, and 8 stable diseases, accounting for 12% of the cases. The observation of a significant association between MGMT promoter hypermethylation and these clinical endpoints supports the

**Figure 2.** A, waterfall plot showing best change in tumor size (%) along with MGMT promoter methylation status (hypermethylated/unmethylated) and KRAS mutation status, if available. WT, KRAS wild-type; mutated, type of KRAS mutation not available. B, pie-charts showing disease control rate (DCR = partial response (PR) + stable disease (SD)) according to MGMT promoter methylation in individual CRC tumors.
hypothesis that DNA repair-defective mCRC tumors are more susceptible to this chemotherapeutic agent. However, even in the case of MGMT hypermethylation, we observed that a fraction of 44% of patients achieved control of disease (stable disease + partial response), thus suggesting that a multiparametric signature including the DNA methylation-associated silencing of MGMT together with other molecular traits would improve the identification of CRC tumors with defects in DNA repair, susceptible to the action of dacarbazine.

The low response rate observed in the present cohort could be linked to the inclusion of heavily pretreated patients (median 4 lines of previous treatments). To interpret this clinical result in the context of therapy-resistant mCRC, one should consider that second-line treatment with FOLFIRI or FOLFOX combination regimens induces ORR of 10% to 12% (24–26) and dramatically decreases in subsequent lines (6). It is also known that dacarbazine is activated in liver by CYP450 microsomal N-demethylation with formation of 5-[3-hydroxymethyl-3-methyl-triazen-1-yl]-imidazole-4-carboxamide and 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide (MTIC). Rapid decomposition of MTIC produces the major plasma and urine metabolite 5-amino-imidazole-4-carboxamide and the reactive species methane diazohydroxide, which produces molecular nitrogen and a methyl cation supposed to be the methylating species (27). It is therefore conceivable that the multiple (median 4) previous lines of cancer treatment as well as the high (79%) rate of liver involvement in the present study population may have exhausted the liver function capacity to activate dacarbazine.

It was our hypothesis that anticancer activity of dacarbazine could be enhanced by a specific defect in DNA repair, possibly MGMT hypermethylation.
repair system as evaluated by MGMT promoter hypermethylation in individual tumors. This epigenetic defect occurs in about 35% to 40% of mCRCs (9) and it is detected in more than 70% of KRAS-mutated tumors carrying the G > A transitions subtypes of mutation (10, 11), a subgroup of mCRCs with limited therapeutic options. Although the present trial was not designed, and thus, powered to assess a significant difference in PFS between MGMT-hypermethylated/unmethylated groups, we observed a trend toward better PFS in the MGMT-hypermethylated group, together with a better DCR. The 2 patients displaying objective response were indeed carrying MGMT-hypermethylated tumors (Fig. 2A) and one of them showed a long-lasting maintenance of response of 6 months, which is uncommon in the advanced setting of mCRC.

In conclusion, present data document that specific DNA repair defects can be associated with susceptibility to dacarbazine. The use of an alkylating agent that does not require hepatic activation may be preferable in heavily pretreated patients with metastatic liver disease. In this regard, temozolomide is an alkylating agent whose activity is also enhanced in tumors with MGMT loss (17) that is hydrolyzed in cells producing the active compound MTIC without requiring liver passage. A phase II trial with temozolomide has been designed and it is ongoing at our institution to assess the efficacy in patients with MGMT hypermethylated mCRCs after failure of standard therapies.

Disclosure of Potential Conflicts of Interest
Andrea Sartore-Bianchi has received honoraria from speakers’ bureau from Bayer, Roche, and Amgen and is a consultant/advisory board member of Amgen. No potential conflicts of interest were disclosed by the other authors.

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