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Tumor *MGMT* promoter hypermethylation changes over time limit temozolomide efficacy in a phase II trial for metastatic colorectal cancer

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Background: Objective response to dacarbazine, the intravenous form of temozolomide (TMZ), in metastatic colorectal cancer (mCRC) is confined to tumors harboring *O*(6)-methylguanine-DNA-methyltransferase (MGMT) promoter hypermethylation. We conducted a phase II study of TMZ enriched by MGMT hypermethylation in archival tumor (AT), exploring dynamic of this biomarker in baseline tumor (BT) biopsy and plasma (liquid biopsy).

Patients and methods: We screened 150 mCRC patients for MGMT hypermethylation with methylation-specific PCR on AT from FFPE specimens. Eligible patients ($n = 29$) underwent BT biopsy and then received TMZ 200 mg/m^2 days 1–5 q28 until progression. A Fleming single-stage design was used to determine whether progression-free survival (PFS) rate at 12 weeks would be ≥35% [H0 ≤ 15%, type I error = 0.059 (one-sided), power = 0.849]. Exploratory analyses included comparison between MGMT hypermethylation in AT and BT, and MGMT methylation testing by MethylBEAMing in solid (AT, BT) and LB with regard to tumor response.

Results: The PFS rate at 12 weeks was 10.3% [90% confidence interval (CI) 2.9–24.6]. Objective response rate was 3.4% (90% CI 0.2–15.3), disease control rate 48.3% (90% CI 32.0–64.8), median OS 6.2 months (95% CI 3.8–7.6), and median PFS 2.6 months (95% CI 1.4–2.7). We observed the absence of MGMT hypermethylation in BT in 62.7% of tumors.

Conclusion: Treatment of mCRC with TMZ driven by MGMT promoter hypermethylation in AT samples did not provide meaningful PFS rate at 12 weeks. This biomarker changed from AT to BT, indicating that testing BT biopsy or plasma is needed for refined target selection.

Key words: colorectal cancer, temozolomide, MGMT, MethylBEAMing, liquid biopsy

introduction

Colorectal cancer (CRC) is the third most common type of cancer in men and the second in women worldwide [1]. CRC is also the fourth leading cause of cancer deaths worldwide, accounting for 9.7% of global new cancers, with 1.4 million cases and 694 000 deaths [2]. Approximately 25% of patients have metastatic disease at diagnosis, and almost 50% of resected patients with early-stage disease will eventually develop metastases, accounting for the relevant mortality rates [2]. In the last 20 years, research efforts in metastatic colorectal cancer (mCRC) have led to the approval of several targeted agents in addition to standard chemotherapy, including bevacizumab [3], cetuximab [4], panitumumab [5], aflibercept [6], and regorafenib [7]. Apart from the anti-EGFR monoclonal antibodies cetuximab and panitumumab, for which *RAS* gene mutations have been shown to play a negative predictive role [8], in CRC there is a lack of clinically validated biomarkers effectively directing therapy.

MGMT is a repair protein which removes alkylating groups from the O^6-guanine in DNA. MGMT protects normal and tumor cells from this type of DNA damage, moving the

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alkylating group to a cysteine residual within its own protein [9, 10]. Approximately 40% of mCRC show silencing of the *MGMT* gene leading to the absence of the corresponding protein [11]. Due to this deficiency, the tumor cell is not able to effectively repair O^6-methylguanine adducts, thus determining a higher frequency of G:C > A:T transitions and potentially enhancing the cytotoxic effect of alkylating agents such as temozolomide (TMZ) or dacarbazine [10, 12].

We previously reported that objective response to dacarbazine, the intravenous form of TMZ, is confined to tumors harboring *MGMT* promoter hypermethylation [13]. Initial reports indicated that mCRC selected by MGMT deficiency achieved remarkable responses to TMZ [14]. Subsequent phase II studies enriched for *MGMT* methylation showed objective responses up to 12% [15, 16].

We designed the present study to evaluate the antitumor activity of TMZ in mCRC after failure of standard therapies selecting patients based on *MGMT* promoter hypermethylation assessed in individual archival tumor (AT) samples. In addition, we explored baseline tumor (BT) biopsy and blood (liquid biopsy) as biomarkers of the actual epigenetic status of the tumor before and during therapy.

materials and methods

study design

The study was designed as a single-institution, open-label, single-arm phase II trial (TEMECT—TEMozolomide Evaluation in ColorecTal cancer, EUDRACT number 2012-003338-17). The aim of the study was to evaluate the efficacy of TMZ treatment in a molecularly selected population of mCRC patients by assessing its ability to achieve a clinically meaningful prolongation of progression-free survival (PFS) when compared with the expected outcome in this setting [7]. Patients were treated with TMZ until progression or unacceptable toxicity. Primary end point was PFS rate at 12 weeks, i.e. the proportion of patients known to be alive and progression free at 12 weeks or later since TMZ treatment start. Secondary end points included objective response rate [ORR = CR + partial response (PR)] according to RECIST 1.1 [17], disease control rate (DCR) [confirmed ORR + stable disease (SD) rate], overall survival (OS), and overall safety profile evaluated by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) Version 3.0. Computed tomography or magnetic resonance imaging scan for tumor evaluation were carried out every 6 ± 1 weeks until tumor progression. Preplanned exploratory analyses included quantitative MGMT methylation assessed by new-generation PCR (MethylBEAMing) and its relationship with primary clinical efficacy, both in tumor tissue from AT samples as well as blood and BT collected at baseline within 28 days before initiation of study treatment (see supplementary Materials and Methods, available at *Annals of Oncology* online). *KRAS* mutations in exons 2, 3, and 4 have been also evaluated in AT specimens and tested for association with clinical outcome. As for the exploratory end points, tumor shrinkage/increase was also computed as absolute difference (mm) between sum of target lesions at baseline and at best response. Patients provided written informed consent and the study followed the Declaration of Helsinki and Good Clinical Practice, being approved by Ethic Committee of Ospedale Niguarda Ca' Granda (Milan, Italy). Details about the analysis of MGMT with methylation-specific PCR (MSP) and MethylBEAMing, patients population, treatment, and statistical design are available in supplementary Materials and Methods, available at *Annals of Oncology* online.

results

patient's characteristics

From December 2012 to May 2014, we screened 150 patients for *MGMT* promoter hypermethylation by MSP on AT FFPE tissue samples from primary tumor or metastases. Twenty-nine patients were enrolled (supplementary Figure S1, available at *Annals of Oncology* online, CONSORT diagram). AT consisted of primary tumors in 21/29 (72.4%) and metastases in 8/29 (27.6%) (6 lung, 2 liver metastases); median time from histological diagnosis on primary or metastases and enrollment was 3.2 (range 1.1–8.8) and 1.9 years (range 0.3–6.7), respectively.

Ninety-three percent of patients had received prior bevacizumab, 28% regorafenib (Table 1) and 69% more than four lines of treatment.

MGMT molecular evaluation in archival tissue and in pre-TMZ treatment tumor biopsies

At prescreening, we detected 95/149 (63.8%) unmethylated and 54/149 (36.2%) methylated CRCs, among the latter 29/54 (53.7%) were enrolled. All patients enrolled in the study underwent BT of a metastatic site before the start of treatment with TMZ (median time 2 days, range 0–18 days). Twenty-two of 29 matched pairs of AT and BT in individual patients were evaluable by the MSP assay (supplementary Figure S1, available at *Annals of Oncology* online). Concordance in *MGMT* promoter methylation status was found in 6 of 22 evaluable (27.3%) sample pairs. There was no difference in concordance rates between the matched pairs of metastases/metastases (0/6) or primary/metastases (6/16) (Fisher's exact test $P = 0.1328$). Further molecular characterization was performed on AT and included status of *KRAS* and *BRAF* (Table 1).

treatment efficacy and toxicity

The primary end point of the study was not met, since PFS rate at 12 weeks was 10.3% [confidence interval (CI) 2.9% to 24.6%].

Table 1. Patient's demographic and characteristics

Clinical characteristics	All patients ($N = 29$) (%)
Age	
Median age (range)	60 (38–77)
≥65 years	7 (24%)
Gender, *n* (%)	
Female	9 (31%)
Male	20 (69%)
Performance status ECOG	
0	22 (76%)
1	7 (24%)
Number of previous treatments	
Median (range)	5 (3–9)
Previous treatment with bevacizumab	27 (93%)
Previous treatment with regorafenib	8 (28%)
Molecular status (*n* = assessable patients)	
KRAS mut (*n* = 29)	18 (62%)
BRAF mut[a] (*n* = 22)	1[a] (5%)

[a]BRAF p.S602S, c.1806T > A.

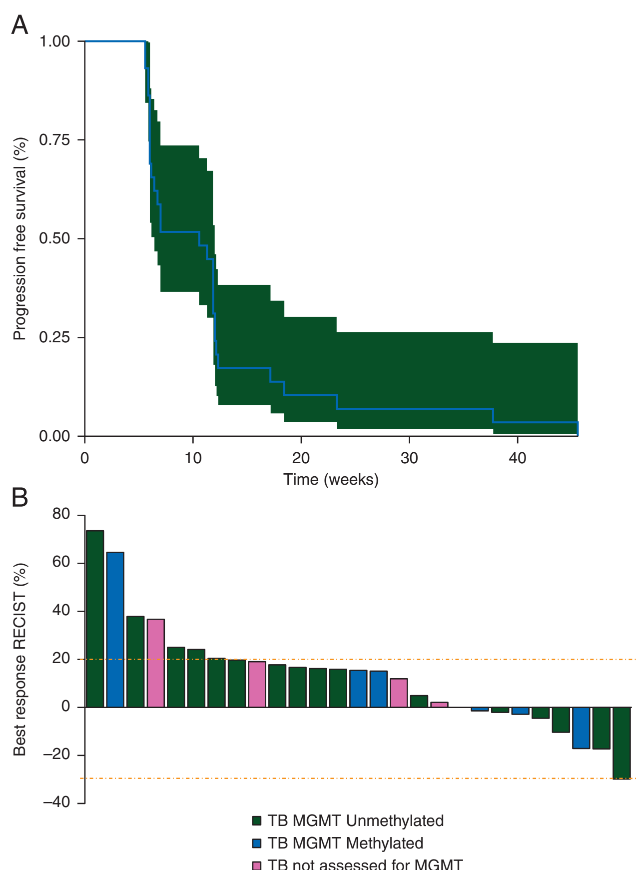

Figure 1. (A) Kaplan–Meier analysis of PFS for ITT population. (B) Waterfall plot showing changes in sum of target lesions at best response in assessable patients. Dotted line shows +20% increases and −30% of tumor shrinkage. Different patterns in bars show the MGMT status in baseline tumor biopsy before starting TMZ treatment.

Median follow-up was 9.8 months (CI 9.46–not reached). Median PFS was 2.6 months (CI 1.4–2.7) (Figure 1A). We observed one partial response (PR, 3.4%, CI 0.2% to 15.3%) and 13 SD, accounting for a DCR (CRs/PRs and SDs) of 48% (CI 32.0%–64.8%) (Figure 1B). The OS was 6.2 months (CI 3.8–7.6). The median number of treatment cycles was 2 (range 1–5), with a median dose intensity of 208.3 (range 102.6–242.4) mg/m^2/week. Treatment modifications, i.e. dose delays or reductions, occurred in 11 (37.9%) patients [6 patients (20.7%) required ≥1 dose reduction; 11 (37.9%) required ≥1 dose delay]. Hematological adverse events were the most common reason for dose modification (7 patients, 24%). Common adverse events (occurring in >10% of patients) are reported in supplementary Table S1, available at *Annals of Oncology* online.

exploratory biomarkers analysis

Since despite *MGMT* silenced status selection by the qualitative MSP assay (yielding either positive or negative), TMZ showed poor efficacy in this setting, a quantitative measurement of MGMT, i.e. the PCR-based method MethylBEAMing, was then applied in individual AT and BT tissue samples as well as in plasma (liquid biopsy) to evaluate whether the percentage of tumor MGMT promoter hypermethylation in individual patients could explain the lack of efficacy.

Methylation status of *MGMT* by methylBEAMing was retrospectively assessed in 28 AT and 25 BT (supplementary Table S2, available at *Annals of Oncology* online). Median methylation value by this methodology was 26.17% (range 1.9–100) and 10.9% (range 0–100), and this difference was statistically significant (Wilcoxon rank sum test, $P = 0.01$). We did not find a linear correlation between tumor shrinkage and the percentage of *MGMT* promoter hypermethylation by methylBEAMing in AT ($P = 0.6$) (Figure 2A). However, a significant correlation was retrieved when the same analysis was carried out in BT ($P = 0.03$) (Figure 2B). In particular, all patients displaying tumor increase segregated below a cutoff value of 17.4% of MGMT methylation in BT.

In plasma, we found that median MGMT methylation by methylBEAMing at baseline was 14.6% (range 0.0%–81.0%). This value is significantly different when compared with the same method applied to AT ($P = 0.025$ by Wilcoxon rank sum test), but not to BT ($P = 0.627$). Interestingly, seven patients displayed no methylation at all in plasma (0%), mirroring the results by MSP in baseline biopsy. After one cycle of TMZ treatment, we found a correlation between methylation variation in plasma and tumor shrinkage ($P = 0.008$) (supplementary Figure S2, available at *Annals of Oncology* online). Finally, we found no association between *KRAS* mutations assessed in AT and DCR ($P = 0.48$).

discussion

Three phase II clinical trials have previously assessed the clinical efficacy of alkylating agents in mCRC. All of them have assessed the role of MGMT promoter hypermethylation as a predictive biomarker of response by the analysis of archival tumor tissues [13, 15, 16]. Despite some evidence of improved DCR in patients with MGMT hypermethylated tumors, the role of this biomarker in mCRC remains unclear.

While the validation of MGMT as a predictive biomarker is ascertained in melanoma and glioblastoma, a subset of cancers where alkylating agents have been the backbone of systemic treatment for years [18, 19], this is not the case for CRC where these drugs are of very limited use. Indeed, only few data are available regarding treatment of CRC with alkylating agents based on MGMT methylation [13, 15, 16]. However, MSP is a well-standardized assay [9] and, despite issues of reproducibility, it has been the gold standard for the development of methylation biomarkers [20, 21].

In the present study, we report that, in a cohort of 29 patients, all selected for *MGMT* promoter hypermethylation by MSP in individual archival tumor, TMZ treatment did not overcome the threshold of a meaningful PFS rate at 12 weeks of 35%, with ORR of 3.4% and DCR of 48.3%. The innovative study design included MGMT assessment in archival tumor specimens, in biopsy of tumor at baseline and in plasma (liquid biopsy), leading to the following novel findings about the dynamic of this epigenetic biomarker.

First, we observed a previously unreported change in *MGMT* status occurring over time, i.e. MGMT methylation declined significantly from archival tumor samples compared with a biopsy taken at the actual moment of starting treatment with TMZ. It has been previously reported that in glioblastoma *MGMT*

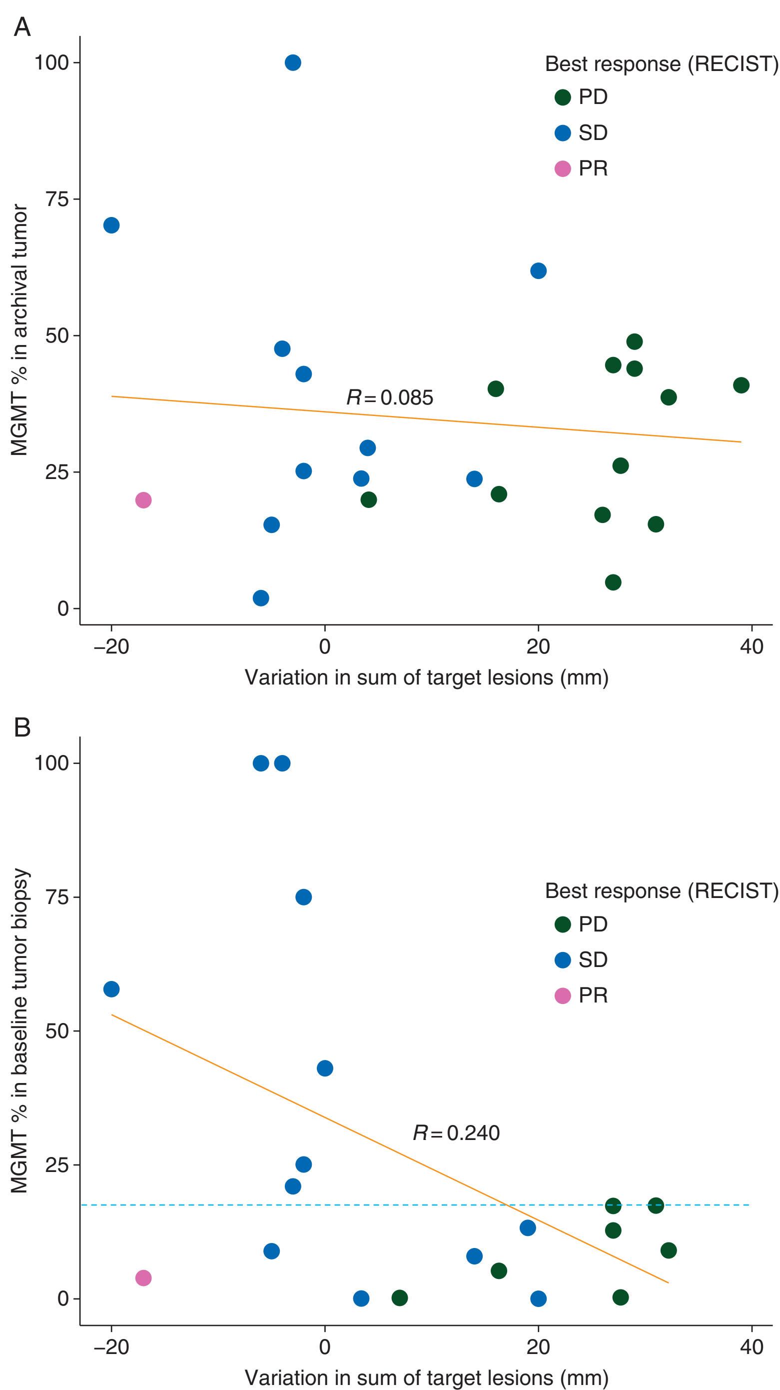

Figure 2. Scatterplot showing relationship between variations in sum of target lesions (mm) and MGMT promoter hypermethylation values (%) in archival tumor (A) or baseline tumor biopsy by MethylBEAMing before starting TMZ treatment (B). Dotted line shows a threshold of 17.4% of MGMT in baseline tumor biopsy segregating patients with an increased sum of target lesion as best response. PD, progression; SD, stable disease; PR, partial response, according RECIST criteria 1.1 .

promoter methylation status can change after chemotherapy [22], but very limited data are available for CRC [12, 23, 24]. It should be acknowledged that, in our study, the time between sample collection and analysis of *MGMT* methylation in archival tumor versus baseline TB was remarkably different (median 2.8 years versus 2 days); therefore, the observed reduction in *MGMT* methylation of the tumors may be due also to long-term storage [25]. Moreover, it has been reported in limited series that MGMT methylation may change between primary tumor and metastases [24], as it occurs with other biomarkers in CRC [26]. Since in our study most of the pairs were primary/metastasis, we cannot exclude that this was a major factor impacting on observed discordance between AT and BT; however, among the six pairs of metastasis/metastasis, the concordance was 0%, thus suggesting that the change in MGMT methylation status may occur independently of the site of tumor sampling. Further,

we cannot exclude that sampling procedures along with tumor heterogeneity could have affected the performance of *MGMT* testing on tissue, thus reducing its sensitivity. Indeed, large-scale analyses addressing the issue of epigenomics heterogeneity in colorectal cancer are missing and future studies might shed light on the extent or implications of this phenomenon. As a consequence, regardless of the possible cause or explanation, we emphasize that molecular screening of MGMT hypermethylation by MSP on archival tumor does not properly select an mCRC population for a biomarker-enriched design. Accordingly, we found that only the percentage of *MGMT* methylation in baseline TB, but not that in archival tumor, was correlated with tumor shrinkage after TMZ treatment. However, the observed correlation was weak ($R = 0.24$; Figure 2B), possibly because, in an advanced setting, the heavy pretreatment (in present study 5 median previous lines) gives rise to multiple resistance mechanisms to DNA damaging agents, limiting sensitization by MGMT loss. Moreover, the only patient who achieved partial response did not display *MGMT* methylation in baseline TB, dictating caution in the interpretation of data. Altogether, these findings suggest that MGMT as a biomarker should be evaluated at the time of treatment, and not relying on previous older specimens as it was done in most of published trials for mCRC [13, 15]. Only Hochhauser et al. [16] made a patients selection by using also blood-based MSP assessment at the study entry, but data of matched pairs of tissue/plasma were not provided.

Second, we analyzed *MGMT* methylation status in plasma to test whether liquid biopsy, being performed on a fresh blood sample collected at the time of enrolment, could overcome the previously described spatial and time-dependent variations of the biomarker. This was performed by the quantitative assay methylBEAMing to study fluctuations during treatment. Given its blood-based nature, there might be a role for this test in dynamically assessing epigenetic status of *MGMT* by providing updated results without the need of repeated tumor biopsies. Hochhauser et al. [16] previously reported an assessment of *MGMT* by MSP from blood in a phase II trial with miscellaneous tumor, including aereodigestive, colorectal, and head-and-neck cancers. They show that 61% of patients with positive *MGMT* methylation by MSP in tissue did not confirm this finding in blood, even though distinct data for CRC histology were not provided. In our study, we found that plasma MGMT methylation before treatment was significantly different from that determined, with the same method, in the archival tumor, but not from that assessed on baseline TB, confirming also in plasma the loss of MGMT methylation after time. Interestingly, seven patients displayed no methylation at all (0%) in plasma baseline, which could be explained by the absence of DNA from tumor origin. However, five of the matched biopsies also displayed very low methylation fraction (<1%) suggesting a loss of the methylated status in the tumor. Among the 22 remaining matched pairs of BT biopsies/plasma samples ($n = 22$), we found high sensitivity (100%) and poor specificity (37.5%) for liquid biopsy to detect methylation, thus suggesting its potential use as a prescreening procedure before TB, i.e. excluding those patients with negative results. We did not find an association between pretreatment values of *MGMT* methylation in plasma and tumor response. However, we report a trend between the variation of *MGMT* methylation in plasma after one cycle of TMZ therapy and tumor response, indicating that liquid biopsy provides a surrogate marker of response rather than a predictive marker. Results of a concordance study between tissue and liquid biopsy are clearly warranted before further test it as a biomarker in this setting.

In conclusion, TMZ treatment driven by selection according to *MGMT* promoter hypermethylation in archival tumor samples does not provide meaningful PFS. A possible explanation, implied by this study, resides in the novel finding that *MGMT* hypermethylation in archival tissue is not maintained in paired tumor biopsies assessed at the moment of treatment. The latter, as well as liquid biopsies, might better capture the dynamic epigenetic changes of the tumor. All in all, MGMT as a biomarker for therapeutic intervention in mCRC remains not clinically applicable as in other malignancies such as glioblastoma [27]. Methodological improvements in assessing MGMT in tissue or blood at the actual moment of treatment might overcome some limitations, but clearly further research is needed to identify potential mechanisms of tumor sensitivity acting synergistically with MGMT deficiency.

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disclosure

The authors have declared no conflicts of interest.

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