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Activity of temozolomide in patients with advanced chemorefractory colorectal cancer and *MGMT* promoter methylation

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Background: No evidence-based treatment options are available for patients with advanced colorectal cancer (CRC) progressing after standard therapies. *MGMT* is involved in repair of DNA damage and *MGMT* promoter methylation may predict benefit from alkylating agents such as temozolomide. The aim of our study was to evaluate the activity of temozolomide in terms of response rate in patients with metastatic CRC and *MGMT* methylation, after failure of approved treatments.

Patients and methods: Patients were enrolled in a monocentre, open-label, phase II study and treated with temozolomide at a dose of 150 mg/m^2/day for 5 consecutive days in 4-weekly cycles. The treatment was continued for at least six cycles or until progressive disease.

Results: Thirty-two patients were enrolled from August 2012 to July 2013. Treatment was well tolerated with one grade 4 thrombocytopenia and no other grade ≥3 toxicities. No complete response occurred. The objective response rate was 12%, reaching the pre-specified level for promising activity. Median progression-free survival and overall survival were 1.8 and 8.4 months, respectively. Patients with KRAS, BRAF and NRAS wild-type CRC showed significantly higher response when compared with those with any RAS or BRAF mutation (44% versus 0%; $P = 0.004$). TP53 status had no influence on the primary end point.

Conclusions: Temozolomide is tolerable and active in heavily pre-treated patients with advanced CRC and MGMT promoter methylation. Further studies in biomolecularly enriched populations or in a randomized setting are necessary to demonstrate the efficacy of temozolomide after failure of standard treatments.

Key words: colorectal cancer, temozolomide, MGMT, clinical trial

introduction

Treatment strategies for colorectal cancer (CRC) have changed in the past 10 years and resulted in significant improvement of survival. When deemed not suitable for surgical resection, patients with metastatic CRC are still not curable with available treatments. Several drugs including cytotoxics (fluoropyrimidines, oxaliplatin, irinotecan), the antiangiogenic agents bevacizumab and aflibercept and the anti-EGFR monoclonal antibodies cetuximab and panitumumab—either given in combination or as monotherapy in KRAS wild-type CRC—demonstrated to improve outcomes [1]. Recently, a randomized, double-blind, placebo-controlled, phase III trial met its primary end point of significant improvement of overall survival (OS) in patients receiving regorafenib—a multi-targeted tyrosine kinase inhibitor—when compared with placebo after failure of standard treatments [2]. As a matter of fact, there are no effective drugs currently available beyond the approved treatments.

The DNA repair gene O^6-methylguanine-DNA methyltransferase (*MGMT*) is responsible of the elimination of alkyl groups from the O^6-position of guanine. If inactive, it may be involved in early steps of colorectal tumorigenesis through an increase of the mutational rate—particularly, G-to-A point mutations of *KRAS* gene [3, 4]. In several tumour types, the protein encoded by the *MGMT* repairs DNA damages induced by alkylating agents [5, 6]. Epigenetic silencing of *MGMT* during colorectal tumorigenesis is associated with hypermethylation of the CpG island in its promoter [7]. This transcriptional gene silencing is

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responsible for diminished DNA-repair of O^6-alkylguanine adducts, with the consequence of enhancing chemosensitivity to alkylating agents—in particular dacarbazine and its oral prodrug temozolomide [8].

In malignant glioblastoma, *MGMT* promoter methylation was validated as predictive factor for benefit from alkylating agents such as temozolomide [9]. In chemorefractory tumours, the rationale for the so-called New Target Identification relies in a molecular profiling assay, with the aim to identify predictive biomarkers of tumour response to selected cytotoxics or target therapies [10]. A recently published case report described 2 patients with metastatic CRC, low immuno-histochemical MGMT expression and clinical response to temozolomide [11].

Therefore, we conducted a mono-institutional, open-label, single-arm, phase II study of treatment with temozolomide in patients with metastatic CRC and tumour *MGMT* promoter methylation, who progressed after all approved standard therapies including fluoropyrimidines, oxaliplatin, irinotecan, bevacizumab and cetuximab or panitumumab (if *KRAS* wild-type).

patients and methods

study population

Between August 2012 and July 2013, 32 patients with advanced, chemorefractory CRC were included in this study at the Department of Medical Oncology of the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan. Patients with histologically confirmed MGMT-methylated metastatic CRC and measurable disease were eligible if they met the following criteria: age ≥18 years, life expectancy of at least 3 months, adequate organ function (defined as absolute neutrophils ≥1500/μl, platelets ≥100 000/μl, haemoglobin ≥9 g/dl; creatinine ≤2.0 mg/dl and ≤1.5 × the upper normal level [ULN]; bilirubin ≤1.5 mg/dl; alanine aminotransferase, aspartate aminotransferase and alkaline phosphatise ≤2.5 × ULN, or ≤5 × ULN for subjects with liver metastases) and ECOG performance status ≤2. Radiologically documented progressive disease (PD) during or within 3 months following the most recent dose of treatment including all of the following: fluoropyrimidines, oxaliplatin, irinotecan, bevacizumab and cetuximab or panitumumab—the latter only in *KRAS* wild-type CRC. Subjects treated with oxaliplatin in an adjuvant setting should have progressed during or within 6 months of treatment completion. Subjects withdrawn from standard treatment due to unacceptable toxicity were also eligible. Patients had completed any previous chemotherapy, radiotherapy and/or major surgery at least 4 weeks before enrolment. Patients with history of malignancy in the previous 5 years were excluded. Women of childbearing potential and men must agree to use adequate contraception since enrolment until at least 3 months after the last study drug administration. The study was conducted according to Good Clinical Practices and was approved by the local ethics committee. All subjects provided written informed consent.

treatment regimen

Temozolomide was administered orally under fasting conditions once a day for 5 consecutive days at the dose of 150 mg/m^2/day every 28 days. Treatment was continued until PD, unacceptable toxicity or consent withdrawal. The dose was reduced by 25% of the starting dose when grade 3 or 4 haematologic toxicity occurred or if retreatment was delayed for 2 weeks or more. A 50% dose reduction was required in cases of grade 3 or 4 non-haematologic toxicity. Patients requiring more than two dose reductions were discontinued from treatment. Treatment was allowed once the absolute neutrophils were ≥1500/mm^3 and platelets were ≥100 000/mm^3, for up to six cycles.

study end points and evaluations

The primary end point of the study was response rate, while secondary end points were progression-free survival (PFS), OS, duration of response and safety. Pre-treatment evaluations included the following: medical history and physical examination; complete blood count and biochemical profile; electrocardiogram; chest x-ray, computed tomography (CT) scan of the chest, abdomen and pelvis, with documentation of tumour measurements. During treatment, complete blood cell counts and biochemical profiles, physical examinations and assessment of toxicities were done before each treatment cycle. CT scans were repeated every two cycles during treatment phase (and every 8 weeks thereafter) according to RECIST 1.1 criteria to define complete response (CR), partial response (PR), stable disease (SD) and PD. At the discretion of the investigators, CT scans could be carried out earlier than required by protocol if appropriate. Treatment toxicities were evaluated according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 3.0.

statistical analysis

The study was planned and design according to Simon's Minimax two-stage design. The error rates used are 10% for accepting the null hypothesis of a 5% response rate and 10% for rejecting the alternative hypothesis of a promising 20% response rate. Eighteen patients were to be treated in the first stage, and if at least one response had not been observed, the study would have been stopped and the regimen declared ineffective. If one or more responses were seen, accrual of an additional 14 patients (for a total of 32) was planned. The regimen was to be declared promising if ≥4 responses were seen. Associations between pre-specified biomarkers and RECIST response was assessed by two-tailed Fisher's exact test.

Response duration was calculated as the time from first documented response to PD or death due to underlying cancer. PFS was calculated from date of enrolment to the date of the first documented PD or death for any cause. OS was calculated from date of enrolment to the date of death due to any cause, or censored at the date of last follow-up for living patients. PFS and OS were determined by Kaplan–Meier methodology. Median value were estimated and presented with 95% confidence interval (CI). Data analysed were using SPSS version 16.0 for Windows (SPSS, Chicago, IL).

analysis of MGMT gene methylation

DNA was extracted from formaline-fixed paraffin-embedded selected tumours using the QIAmp DNA Mini Kit (Qiagene). MGMT promoter methylation was assessed by methylation-specific PCR. One microgram of DNA was bisulphate treated using Methylation KIT-Zymo Research. The bisulphate-modified template was amplified by using primers specific for methylated (Met) and unmethylated (UnMet) template: MGMT Met Fw: 5′-cgaatatactaaaacaacccgcg-3′; MGMT Met Rev: 5′-gtatttttttcgggagcgaggc-3′. MGMT UnMet Fw: 5′-ccaaatatactaaaacaacccaca-3′; MGMT UnMet Rev: 5′-tgtatttttttgggagtgaggt-3′ following the methodology previously described [12].

Two templates provided by the Methylation KIT were used as positive controls for methylation and unmethylation reactions. The products of PCR-specific amplications were separated by means of 2% agarose gel electrophoresis and visualized using ethidium bromide staining. A sample was classified as methylated when a band of the expected molecular weight using primers specific for Met template was detected; a sample was classified as unmethylated when a band of the expected molecular weight using primers specific for UnMet template was detected only.

predictive biomarkers assessment

Mutational analysis of *KRAS* exons 2 and 3 was carried out as previously described [13]. *BRAF* (exon 15), *NRAS* (exon 2 and 3) and *TP53* (exons 5–8) mutational analysis was carried out by means of PCR using specific primers previously described [13, 14]. The PCR products were subjected to direct sequencing using an ABI Prism 3500 DX Genetic Analyzer (Applied Biosystems, Foster City, CA) and then evaluated by means of the ChromasPro software. For the detection of microsatellite instability (MSI), we used a single fluorescent multiplex PCR system of five quasi-monomorphic mononucleotide repeats including BAT-26, BAT-25, NR-21, NR-22 and NR-24, as previously described [15].

results

patients characteristics and outcome

Thirty-two patients were enrolled in the study, and all received at least one cycle of chemotherapy. Patient demographics and disease characteristics are shown in Table 1.

All patients had serial measurements adequate to determine their response. No CR occurred, while 4 (12%) patients demonstrated a PR, 6 (19%) had SD and 22 (69%) had PD as best response. Overall objective response rate was therefore 12%, reaching the pre-specified level for promising activity. The median duration of response was 7 months (range, 3.7–9.2 months). The disease control rate (CR + PR + SD ≥4 months) was 31%.

At a median follow-up time of 8 months, 28 (88%) of patients experienced PD and 15 (47%) died. Kaplan–Meier curves for PFS and OS of the 32 patients are displayed in Figures 1 and 2, respectively. The median PFS was 1.8 months (95% CI 1.7–3.9 months) and the median OS was 8.4 months (95% CI 5–14.1 months). Six- and 12-month OS rates were 52% and 38%, respectively. The median PFS was significantly improved for patients achieving clinical benefit when compared with patients with PD (1.6 versus 6.2 months; $P < 0.0001$). A similar outcome was observed for median OS (5.3 months versus not reached; $P = 0.0018$).

Post-study treatment was conducted in 8 patients (25%)—including regorafenib in 3, chemotherapy rechallenge in 2 and investigational drugs in 3 subjects. No patient had clinical benefit from post-progression treatment.

Table 1. Main patient and disease characteristics

	Overall *N* (%)
Total	32
Patient's age (years)	
Median (range)	60 (41–75)
Gender	
Male	12 (36)
Female	20 (64)
Primary tumour location	
Right colon	12 (38)
Left colon	9 (28)
Rectum	11 (34)
Metastases presentation	
Synchronous	20 (64)
Metachronous	12 (36)
Prior adjuvant chemotherapy	
Yes	5 (16)
No	27 (84)
Number of metastatic sites	
1	6 (19)
2	18 (56)
>2	8 (25)
Number of treatment lines for advanced disease	
2	14 (44)
3	6 (19)
4	10 (31)
5	2 (6)
Performance status (ECOG)	
0	17 (54%)
1	11 (34%)
2	4 (12%)

predictive biomarkers

Tissue blocks were available for 31 patients who provided written informed consent for a biological ancillary study. MSI, *KRAS*, *BRAF*, *NRAS* and *TP53* status was fully evaluable for all 31 patients and the results are shown in Supplementary Table 1 and 2, available at *Annals of Oncology* online. No MSI-high CRC was detected. *KRAS*, *BRAF* and *NRAS* mutations were always mutually exclusive. The majority of cases—22 of 31 (71%)—were *KRAS* or *BRAF* or *NRAS* mutated, while 9 (29%) were all genes wild type. *TP53* mutations were all considered as non-functional according to Kato et al. [16] and were detected in 15 of 31 (48%) samples.

None of the patients with *RAS*- or *BRAF* -mutated tumours responded to treatment, while four of nine patients with *RAS* and *BRAF* wild-type had an objective response (0% versus 44%, respectively; $P = 0.004$). On the other hand, there was no significant difference in terms of response rate between *TP53* mutated and *TP53* wild-type tumours (20% versus 3%; $P = 0.33$).

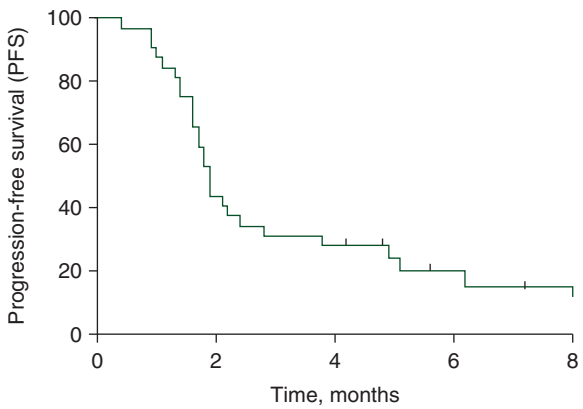

Figure 1. Kaplan–Meier curves for progression-free survival in the intent-to-treat population.

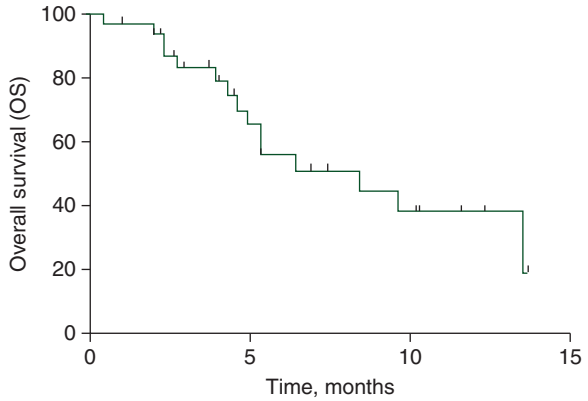

Figure 2. Kaplan–Meier curve for overall survival in the intent-to-treat population.

Table 2. Treatment-related toxicity

Side-effects	No. of patients (%) grade NCI CTC			
	G1	G2	G3	G4
Nausea	1 (3%)	–	–	
Vomiting	1 (3%)	1 (3%)	–	–
Asthenia	3 (9%)	–	–	–
Anaemia	21 (66%)	0%	0%	0%
Neutropenia	0%	0%	0%	0%
Thrombocytopenia	1 (3%)	–	–	1 (3%)

safety

Ninety-two chemotherapy cycles were administered, with a median number of two cycles per patient (range 1–6 cycles). Overall, any-grade adverse events were reported in 14 (44%) of patients. Treatment-related side-effects are presented in Table 2. No toxic death occurred. Severe thrombocytopenia (grade 4) was experienced from one patient (3%), while no other grade 3–4 haematological toxicity was observed. All non-haematological side-effects were considered as mild or moderate. Dose reduction was seen in three patients (9%). Trial discontinuation was carried out before treatment completion in two patients (due to cholangitis and patient decision, respectively).

discussion

We showed that temozolomide induced an objective response rate by RECIST criteria in 12% of heavily pre-treated patients with advanced CRC and *MGMT* promoter methylation. Treatment was well tolerated, and the only grade 4 toxicity was one thrombocytopenia episode (3%), with no other grade ≥3 toxicities. The trial met its primary end point of acceptable response rate, with a disease control rate of 31%, a median PFS and OS of 1.8 and 8.4 months, respectively.

In advanced CRC, the occurrence of chemorefractory disease poses a major therapeutic challenge—for presence of an adequate performance status to potentially receive further treatments, but absence of effective drugs which may be offered to patients with an evidence-based algorithm. Recently, regorafenib significantly improved OS when compared with placebo in patients with heavily treated CRC. However, median PFS and OS were 1.9 and 6.4 months in the study drug arm when compared with 1.7 and 5 months in the placebo arm ($P < 0.0001$ and $P = 0.0052$, respectively) [2], highlighting the unmet need of effective treatments for chemorefractory disease. Patients who progress after all approved treatments may be generally considered suitable for new investigational drugs or strategies. Thus, in the era of personalized medicine, tumour molecular profiling may lead to the identification of therapeutic targets or predictive biomarkers for pharmacological intervention [10].

MGMT methylation is a biomarker linked to sensitivity to alkylating agents such as dacarbazine and temozolomide [8]. The association between the *MGMT* status and responsiveness to temozolomide was extensively studied in glioblastoma patients. Thus, we selected the presence of *MGMT* methylation as inclusion criteria, since immuno-histochemistry may be less reproducible and was not sufficiently studied in CRC. In the landmark study, *MGMT* promoter methylation was validated as predictive factor of benefit from temozolomide-based chemoradiation, but also as independent prognostic biomarker—regardless of treatment [9]. Some data on *MGMT* methylation as potential target for alkylating agents in advanced CRC were recently published, ranging from case reports [11, 17] to prospective non-randomized studies [18–20]. Hochhauser et al. [20] recently reported a phase II study of temozolomide in patients with advanced aerodigestive tract—including oesophageal, head and neck and non-small-cell lung cancers—and CRC with *MGMT* promoter methylation. Despite a 6% response rate in the overall patients population, only one response (3%) was observed in the subgroup of 37 CRC patients [20]. A phase II study of dacarbazine in 68 patients with advanced, chemorefractory CRC, showed a response rate of 3% [19]. All two patients with objective response had *MGMT* promoter methylation—which was associated with higher disease control rate when compared with non-methylation (44% versus 6%; $P = 0.012$) [19].

In our study, significantly more women had MGMT promoter methylation (Table 1), as reported in the literature [4]; it was also previously shown that *MGMT* promoter methylation is more frequent in MSI-high CRC [21]. However, none of the patients included in this study displayed deficient mismatch repair, probably due to its association with better prognosis and non-metastatic disease [22]. For glioblastoma, it was hypothesized that an intact mismatch repair pathway may be necessary for apoptotic response to alkylating agents, since the O^6-methylguanine:cytosine pairs induced by temozolomide are not repaired by *MGMT* [23].

Not surprisingly, *KRAS*, *BRAF* and *NRAS* mutations were highly represented (overall, 71%) in this dataset of patients with MGMT methylated CRC, as shown for CRC developing through the 'serrated' pathway [4, 24]. The presence of mutation in any of these components of the mitogen-activated protein kinases (*MAPK*)—either *RAS* or *BRAF*—was associated with clinical resistance to temozolomide. As already shown for glioblastoma, *MAPK* signalling may enhance MGMT activity and drive cellular resistance to temozolomide [25]. Finally, even if p53 is involved in apoptosis and DNA repair, no significant impact of *TP53* gene status on tumour response was observed.

In conclusion, this is the first study to investigate the activity of temozolomide in patients with advanced, chemorefractory CRC and *MGMT* promoter methylation. Even if our results may be

promising, the efficacy of temozolomide in this setting warrants further confirmation through adequately powered and randomized studies. Moreover, the identification of predictive biomarkers of response is a fundamental issue in order to identify a biomolecular subset of patients who may derive a consistent benefit from temozolomide-based treatment. In this regard, the investigation of temozolomide in combination with *MAPK* inhibitors, as well as further studies in the molecularly enriched population of patients with *RAS* and *BRAF* wild-type status, may be advocated.

funding

This is an investigator-driven study. Financial support was granted by institutional funds.

disclosure

The authors have declared no conflicts of interest.

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