Temozolomide and irinotecan (TEMIRI regimen) as salvage treatment of irinotecan-sensitive advanced colorectal cancer patients bearing MGMT methylation

Original Research Article

Temozolomide and irinotecan (TEMIRI regimen) as salvage treatment of irinotecan-sensitive advanced colorectal cancer patients bearing *MGMT* methylation

Running title: TEMIRI for advanced *MGMT* methylated colorectal cancer

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Abstract

Background: Non-randomized studies showed that temozolomide (TMZ) achieves an average 10% response rate in heavily pretreated metastatic colorectal cancer (mCRC) patients with promoter methylation of the DNA repair gene O6-methylguanine-DNA methyltransferase (*MGMT*). In this phase II trial, irinotecan and temozolomide (TEMIRI) combination regimen was assessed in irinotecan-sensitive, *MGMT* methylated/microsatellite stable (MSS) pretreated mCRC patients.

Patients and methods: Key inclusion criteria were: centrally confirmed *MGMT* methylation by methylation-specific PCR, MSS mCRC, progression after at least two prior chemotherapy regimens for advanced disease and irinotecan-free interval (IFI) >3 months. TEMIRI (TMZ 150mg/sqm on days 1-5 plus irinotecan 100mg/sqm on days 1,15 q28 days) was administered for six cycles, followed by maintenance with TMZ. The primary endpoint was overall response rate (ORR). Exploratory translational analyses included MGMT immunoistochemistry (IHC) and methylBEAMing (MB).

Results: Between December 2014 and June 2017, 25 patients were enrolled. The primary endpoint was met, since 6 patients achieved a partial response (ORR 24%, 95% CI, 11%-43%). At a median follow-up of 15.6 months, median progression-free survival (mPFS) and overall survival (mOS) were 4.4 and 13.8 months, respectively. Only 4 (16%) patients had ≥ grade 3 adverse events. All patients whose cancer was MGMT-positive IHC were non-responders. Consistently, patients with MGMT-negative/low tumors had a significantly longer mPFS than others (6.9 versus 2.0 months; HR=0.29, 95%CI, 0.02-0.41; p=0.003) and a non-significant trend for longer mOS. MB testing showed similar accuracy.

Conclusions: TEMIRI regimen is a safe and active option in pre-treated, irinotecan- sensitive mCRC patients with *MGMT* methylation.

Keywords: *MGMT*; colorectal cancer; temozolomide; irinotecan; TEMIRI.

Key message:

We combined temozolomide with irinotecan (TEMIRI phase II study) in metastatic colorectal cancer patients selected for MGMT methylation by methylation-specific PCR/microsatellite stability plus benefit from previous irinotecan-based treatment/irinotecan-free interval >3 months. Response rate was 24%, providing a promising option for patients failing standard regimens. Negative MGMT expression by IHC further refines patients' selection.

Introduction

MGMT is responsible of the elimination of alkyl groups from the O6-position of guanine; its promoter methylation results in diminished DNA-repair of O6-alkylguanine adducts and enhanced sensitivity to alkylating agents [1-4]. After failure of initial studies with dacarbazine or temozolomide (TMZ) in all-comers with metastatic colorectal cancer (mCRC) [5], recent phase II studies in the *MGMT* methylated subgroup showed an average response rate of 10% in chemorefractory disease [6-10] (Table S1). Subsequent studies have been focused at improving such results by: 1) restricting the molecular selection to homogeneously *MGMT* hyper-methylated/MGMT immunohistochemistry (IHC) negative tumors, 2) bringing forward the use of TMZ in second-line treatment prior to the refractory setting (NCT02414009) and/or by adding TMZ to other active agents used in mCRC.

The role of chemotherapy reintroduction in mCRC patients with potential retained chemosensitivity to a specific agent has been mainly studied for oxaliplatin-based chemotherapy [11]. Conventionally, a chemotherapy-free interval of more than 3 months is adopted to assume potential disease sensitivity to specific agents used in previous treatment lines. The rationale for adding TMZ to irinotecan reintroduction (TEMIRI regimen) as salvage treatment of irinotecan-sensitive mCRC patients bearing *MGMT* methylation also relies on the synergy between topoisomerase II inhibitors and alkylating agents [12] and the need to improve the efficacy of each of these agents when used as monotherapy in later lines. In particular, successful treatment with TEMIRI plus bevacizumab had been reported in a refractory mCRC patient [13].

Here we hypothesized that TEMIRI salvage regimen may be a novel treatment option for pretreated irinotecan-sensitive mCRC patients with *MGMT* methylated and microsatellite stable (MSS) tumors.

Patients and Methods

Study population

Key inclusion criteria were: ECOG PS 0-1; at least two previous treatment lines for advanced disease (oxaliplatin-based adjuvant treatment was acceptable as a treatment line in case of disease relapse within 6 months); previous treatment with an anti-EGFR monoclonal antibody if *RAS (BRAF)* wild-type disease and at least one antiangiogenic agent including bevacizumab, aflibercept and/or regorafenib; documented benefit (complete response, partial response or stable disease) from the last irinotecan-based regimen; irinotecan-free interval (IFI) defined as the time elapsed from the last administration of an irinotecan-based regimen to progressive disease (PD) >3 months, i.e. interruption of irinotecan-based therapy for reasons other than PD (>1 previous irinotecan- based line eligible); central confirmation on archival tumor tissue samples of *MGMT* methylation and MSS status by means of methylation-specific PCR (MSP) and multiplex PCR, respectively [8]. Key exclusion criteria were: life expectancy ≤12 weeks; inadequate liver, kidney and/or hematologic function; serious illness or medical conditions that contraindicated the treatment according to the investigators. All patients signed an IRB approved consent form (INT 20/13) prior to any study procedure.

Treatment schedule and study procedures

As shown in Figure S1, patients received a maximum of 6 cycles of TEMIRI regimen (TMZ 150 mg/sqm on days 1-5 plus irinotecan 100 mg/sqm on days 1 and 15, every 28 days), followed by maintenance with single-agent TMZ at the same dose and schedule used in the combination phase, until PD, unacceptable toxicity or informed consent withdrawal. Radiological disease assessments were performed at baseline and every 8 weeks until PD. Adverse events (AEs) and laboratory changes were recorded and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.0).

Study endpoints

The primary endpoint of the study was overall response rate (ORR) according to RECIST v1.1 criteria. Secondary endpoints included clinical benefit rate (CBR), duration of response (DoR), progression-free survival (PFS), overall survival (OS) and safety. Pre- specified exploratory endpoints included the correlation of activity/efficacy parameters with biomarkers, including MGMT IHC and Methyl-BEAMing (MB). IHC and MB analyses were performed as previously described [9,14] and patients were divided into IHC negative/low versus positive or according the MB cut-off of 63% identified previously [14,15], respectively.

Statistical design and analysis

TEMIRI treatment would have been judged promising for a hypothetical increase of ORR from 10% with TMZ alone [8-10] to 35% with TEMIRI regimen. According to the Fleming single-stage design and setting p0 (ORR in the null hypothesis) 0.10, and p1 (ORR in the alternative hypothesis) 0.35, α and β errors of 0.05 and 0.20 respectively, a total of 25 patients were required. Null hypothesis would have been rejected if RECIST response had been observed in at least 6 patients. Using the Kaplan-Meier method, PFS was calculated from the date of enrollment to the first event (i.e., disease progression or death from any cause) and OS from the date of enrollment to death or last follow-up. The Fisher's exact test was used to compare subgroups and variables.

Results

Patients' population

Between December 2014 and June 2017, 85 patients with irinotecan-sensitive mCRC were screened at two Italian institutions. Thirty-three patients (38%) had *MGMT* methylation by qualitative MSP assay. One patient died before the enrollment and 7 were deemed not eligible for the trial (Figure S2), until the target accrual of 25 patients was reached.

Patients demographics and disease characteristics are shown in Table 1. Notably, even if 17 (68%) patients had received ≥3 treatment lines, the median IFI was 6.8 (IQR, 4.4-12.8) months.

Treatment activity and efficacy

The primary endpoint of the study was met, since 6 patients achieved a confirmed partial response (ORR 24%, 95% CI, 11%-43%). CBR was 68% (95% CI, 48%-83%). Median DoR was 5.4 months (95% CI, 0.7-8.3). One patient experienced rapid clinical deterioration and was not assessable for response. The waterfall and spider plots describing RECIST best responses and their dynamics are depicted in Figure 1A and 1B, respectively.

At a median follow-up of 15.6 (95% CI, 10.2-21.3) months, 22 (88%) patients progressed and 15 (60%) died. Median PFS and OS were 4.4 (95% CI 2.9-8.3) and 13.8 (95% CI, 8.8-17.3) months, respectively (Figure 2A and 2B).

No significant differences in outcomes were observed according to best response (SD versus PR) to the last irinotecan-based regimen: in particular, ORR was 22% versus 20%, respectively (p=1.00).

Treatment safety

All patients that received at least one dose of treatment were evaluated for safety. Overall, the TEMIRI regimen was relatively well tolerated (Table 2): only 4 (16%) patients had grade 3 or higher AEs and there were no treatment-related deaths. Three patients (12%) required a 25% reduction of irinotecan due to G3 pneumonia, protracted G1 hematologic toxicities and G2 gastrointestinal toxicities, respectively. One of them (4%) had also a 50% reduction of TMZ due to G3 diarrhea. Two patients (8%) started with a reduced irinotecan dose as per investigator decision, due to concomitant medical conditions. There were two treatment delays of more than 15 days due to a G3 pneumonia and G2 thrombocytopenia, respectively. Treatment was permanently interrupted in one case because of persistent G2 nausea and

diarrhea.

Pre-specified exploratory biomarkers

Eighteen (72%) samples were classified by IHC as MGMT-low/negative, whereas 7 (28%) as MGMT-positive; 15 (60%) and 10 (40%) had a MB value ≥ or <63%, respectively.

The association of MGMT IHC assessment and/or MB with ORR is described in Table S2. A positive MGMT IHC was associated with a negative predictive value of 100% (7 out of 7 MGMT-positive patients were non-responders), while the positive predictive value of MGMT IHC was 33% (only 6 out of 18 patients with MGMT-negative/low achieved a response). The predictive accuracy of MGMT IHC was 52%. Regarding MB analysis, its negative and positive predictive values were 89% and 33%, respectively, with a predictive accuracy of 54%.

Regarding the impact of MGMT IHC on survival endpoints, patients with MGMT-negative/low tumors had a significantly longer median PFS (mPFS) than MGMT-positive ones (6.9 versus 2.0 months; HR=0.29, 95% CI, 0.02-0.41; p=0.003; Figure 2C), while no significant difference in terms of OS was observed (17.3 versus 13.8 months; HR=0.56, 95%CI: 0.13-1.85; p=0.303; Figure 2D). Similarly, patients with methylation percentage ≥63% by MB had a significantly longer mPFS than others (6.6 versus 3.8 months; HR=0.46, 95% CI, 0.13-0.95; p=0.049; Figure 2E), with no OS differences (15.5 versus 12.7 months; HR=0.75, 95% CI 0.24-2.11; p=0.327; Figure 2F).

Neoantigen evolution during treatment

Interestingly, one MGMT IHC-negative, MSS, *KRAS* mutant female patient with initial RECIST response to TEMIRI underwent tumor re-biopsy at acquired resistance and Foundation One™ test was ordered on both archival and re-biopsy matching tumor samples. Mutational burden was estimated as high as 68 Muts/Mb only in the post- progression sample, whereas the expected non-hypermutated status (4 Muts/Mb) was confirmed in the archival sample. A *BRCA2 E2198** somatic mutation emerged at acquired resistance, whereas mutations in

mismatch repair genes did not even emerge at subclonal level (MSS status retained on both IHC and multiplex PCR).

Discussion

Life expectancy of mCRC patients following the failure of second-line treatment is usually poor, particularly for *RAS* or *BRAF* mutated subgroups. Regarding third-line treatment and beyond, two options are nowadays approved for chemorefractory disease: regorafenib and TAS-102. Such agents, however, confer a modest median OS gain of 1.4 and 1.8 months, respectively [16,17], with significant toxicities and financial burden. Even if breakthrough treatments are emerging for selected molecular subgroups, such as immunotherapy for MSI-high [18], dual HER2 blockade for HER2-positive [19], or entrectinib for ALK, ROS1 and NTRK1-3 fusion positive [20] mCRC patients, the individual prevalence of such predictive biomarkers is below 5%. Therefore, the majority of patients with mCRC are currently excluded from such promising options, highlighting the urgent need of active agents or regimens for pretreated patients.

MGMT methylation has emerged as a potential biomarker of response to alkylating agents in mCRC and several non-randomized clinical trials showed that the average ORR to TMZ is 10% in heavily pretreated patients [6, 8-10]. Additionally, given the evidence supporting chemo-holidays or maintenance strategies in the first-line setting, reintroduction of oxaliplatin- or irinotecan-based treatment – even if supported by retrospective data with low levels of evidence [11] - may be a reasonable option in individual patients carefully selected based on initial benefit, tolerability and chemotherapy-free intervals.

The rationale for combining TMZ and irinotecan as salvage treatment of irinotecan- sensitive, *MGMT* methylated mCRC patients relies on 2 major points: 1) the unsatisfactory activity of single-agent TMZ in refractory patients with no proper molecular selection and 2) the safety of

the TEMIRI regimen, proven by several studies, even if with different schedules and dosages [21,22].

In our study, TEMIRI regimen achieved an ORR of 24%, mPFS and OS of 4.4 and 13.8 months, respectively, in heavily pretreated, carefully selected patients. Even if the activity of irinotecan/FOLFIRI was modest in second-line trials (4-16%) [23-25], it must be pointed out that our patients were selected among those with clinically demonstrated chemosensitivity. However, our results suggest how TMZ and irinotecan may achieve synergistic rather than additive effects. In fact, based on the specific mechanisms of action of the two DNA-damaging agents, preclinical evidences showed how the inhibition of topoisomerase II may enhance the cytotoxicity of alkylating agents [12].

Even if our study was non-randomized and therefore TEMIRI regimen cannot be properly compared with other evidence-based options such as regorafenib or TAS-102, it must be pointed out that both agents achieved an ORR close to 0%, as well as mPFS and OS of 1.9 and 6.4 months for regorafenib, and 2.0 and 7.1 months for TAS-102, respectively.

Is there a way to improve the activity and efficacy of TMZ-based therapy in mCRC patients bearing *MGMT* methylation? The extremely low positive predictive value of MSP has clearly shown that qualitative assessment of *MGMT* methylation is necessary but not sufficient to predict response to TMZ [8-10]. Besides the exclusion of the small fraction of patients with concomitant MSI-high status, which is putative of intrinsic resistance [26], previous studies showed that absent or low MGMT IHC expression [9], or high/homogeneous *MGMT* hyper-methylation [6,14] may be associated with improved response to TMZ. Here, the easily available IHC analysis achieved the maximal negative predictive value since all patients with MGMT-positive tumors were non-responders. The lack of a formal statistical significance of our pre-planned exploratory biomarkers analysis for predicting treatment response may be due to both the small sample size and the relatively low positive predictive value. In fact,

even if both IHC and MB (second-level analyses) outperformed MSP, they still had insufficient sensitivity to accurately predict TMZ response.

Finally, TMZ has gained a renewed attention for potentially novel therapeutic applications. In fact, while microsatellite instability (MSI)-high glioblastoma is primarily resistant to TMZ, acquired resistance may also emerge through the induction of a microsatellite instability (MSI)-like status [27,28]. TMZ may induce an exponential increase of tumor mutational burden in MGMT deficient melanoma or glioblastoma [29-31]. Based on such evidences, we recently showed that inactivation of DNA mismatch repair (MMR), driven by acquired resistance to TMZ, increased mutational load and promoted continuous renewal of neoantigens in human colorectal cancers while triggering immune surveillance in mouse models [32]. From this point of view, refining the prediction of TMZ response may be helpful in the next future to exploit treatment-induced hyper-mutational status as a potential target for immunotherapy strategies in selected patients. Namely, the emergence of high mutational-burden (and, as new report, an acquired *BRCA2* mutation) in one patient confirms our previous report [32] and suggests that TMZ may be exploited as a priming agent to pharmacologically reshape the genetic and immunological landscape of MSS cancers, as will be tested by upcoming proof-of-concept clinical trials.

The biomarkers investigated here should be however considered as hypothesis- generating and, even if their effect on ORR and PFS suggest a predictive rather than prognostic value, the absence of a control group untreated with TMZ prevents from drawing conclusive results. Moreover, even the assessment of *MGMT* methylation itself should be considered investigational in the absence of randomized studies definitively demonstrating TMZ clinical effectiveness in molecularly selected mCRC patients.

In conclusion, TEMIRI is worth of investigation in randomized studies assessing the role of this regimen as compared with standard of care (such as regorafenib or TAS-102) in pretreated

irinotecan-sensitive mCRC patients with *MGMT* methylation and absent/low MGMT immunohistochemistry protein expression.

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Disclosure: The authors have declared no conflicts of interest.

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Figure 1

RECIST best tumor response and dynamics for patients who were treated with TEMIRI regimen.

Panel A shows the waterfall plot of the best percentage change in the tumor burden from baseline evaluation. One patient rapidly progressed after one cycle, so RECIST tumor response was not assessed. Heat map shows results of *MGMT* immunohistochemistry analysis and methylation % by methylBEAMing. Patients were divided into IHC negative/low versus IHC positive and methylation values were corrected for tumor content and categorized according the previously validated cut-off of 63%.

Panel B shows the dynamic response for each patient. In particular, each line represents the response trend form baseline (day 0) up to progression or latest scan. Red, green and blue histograms (panel A) or lines (panel B) represent patients with progressive disease, stable disease and partial response as RECIST v1.1-defined best response, respectively. Dotted

lines show a 20% increase (red) and 30% reduction (green) from baseline. The plus signs represent ongoing treatments.

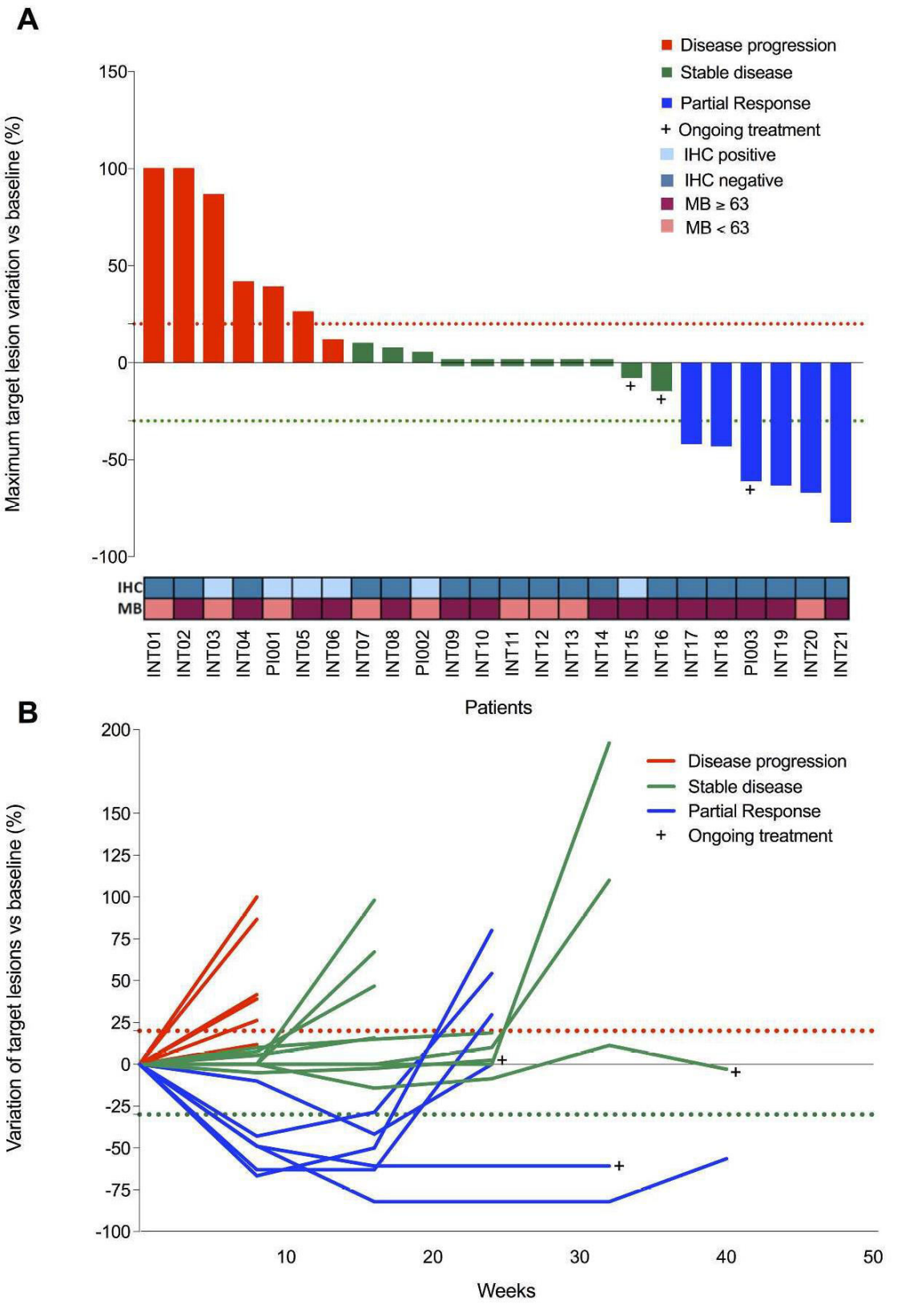
Figure 2.

Panel A and B: Kaplan – Meier curves estimating median Progression Free Survival (PFS) and Overall Survival (OS) respectively, of the study population.
Panel C and D: Kaplan – Meier curves estimating PFS and OS respectively, according to the negativity/low expression (red line) or positivity (blue line) of IHC for MGMT.
Panel E and F: Kaplan – Meier curves estimating PFS and OS respectively, according to the methylation percentage, with corrected methylBEAMing ≥ 63 (red line) or < 63 (blue line).

Legends for Supplementary files:

Table S1. Summary of phase II clinical trials with alkylating agents (single-agent dacarbazine or temozolomide) in refractory metastatic colorectal cancer.
Table S2. Association of pre-specified exploratory biomarkers (MGMT immunohistochemistry and methylBEAMing) with RECIST response in 24 evaluable patients.
Figure S1. Study design.
Figure S2. Study flow diagram.

Figure 1


A
Disease progression
Stable disease
Partial Response
+ Ongoing treatment
IHC positive
IHC negative
MB ≥ 63
MB < 63
Maximum target lesion variation vs baseline (%)
150
100
50
0
-50
-100
IHC
MB
INT01
INT02
INT03
INT04
PI001
INT05
INT06
INT07
INT08
PI002
INT09
INT10
INT11
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INT14
INT15
INT16
INT17
INT18
PI003
INT19
INT20
INT21
Patients
B
Disease progression
Stable disease
Partial Response
+ Ongoing treatment
Variation of target lesions vs baseline (%)
200
150
125
100
75
50
25
0
-25
-50
-75
-100
10
20
30
40
50
Weeks

Figure 2

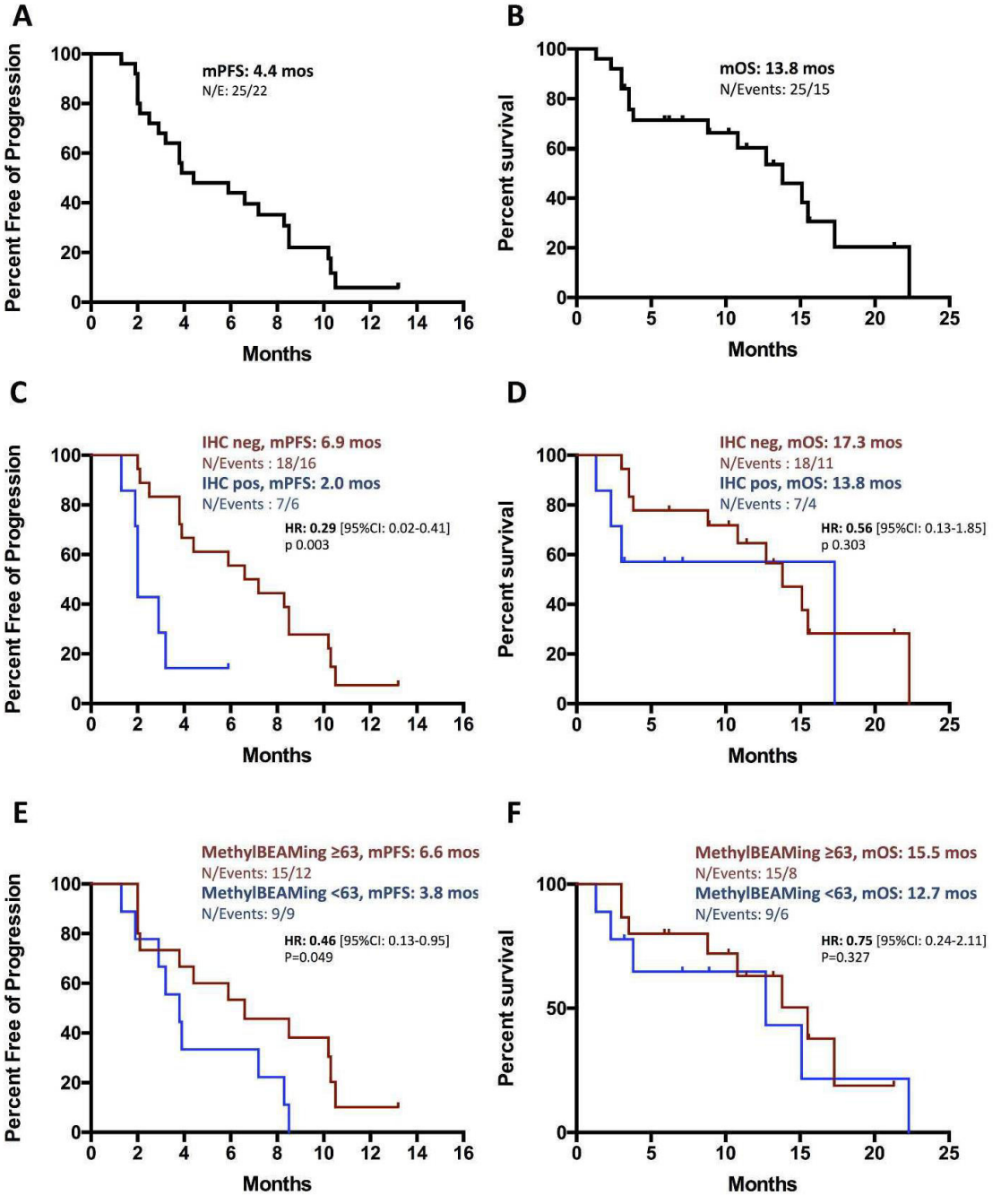

A
Percent Free of Progression
mPFS: 4.4 mos
N/E: 25/22
Months
B
Percent survival
mOS: 13.8 mos
N/Events: 25/15
Months
C
Percent Free of Progression
IHC neg, mPFS: 6.9 mos
N/Events : 18/16
IHC pos, mPFS: 2.0 mos
N/Events : 7/6
HR: 0.29 [95%CI: 0.02-0.41]
p 0.003
Months
D
Percent survival
IHC neg, mOS: 17.3 mos
N/Events : 18/11
IHC pos, mOS: 13.8 mos
N/Events : 7/4
HR: 0.56 [95%CI: 0.13-1.85]
p 0.303
Months
E
Percent Free of Progression
MethylBEAMing ≥63, mPFS: 6.6 mos
N/Events: 15/12
MethylBEAMing <63, mPFS: 3.8 mos
N/Events: 9/9
HR: 0.46 [95%CI: 0.13-0.95]
P=0.049
Months
F
Percent survival
MethylBEAMing ≥63, mOS: 15.5 mos
N/Events: 15/8
MethylBEAMing <63, mOS: 12.7 mos
N/Events: 9/6
HR: 0.75 [95%CI: 0.24-2.11]
P=0.327
Months

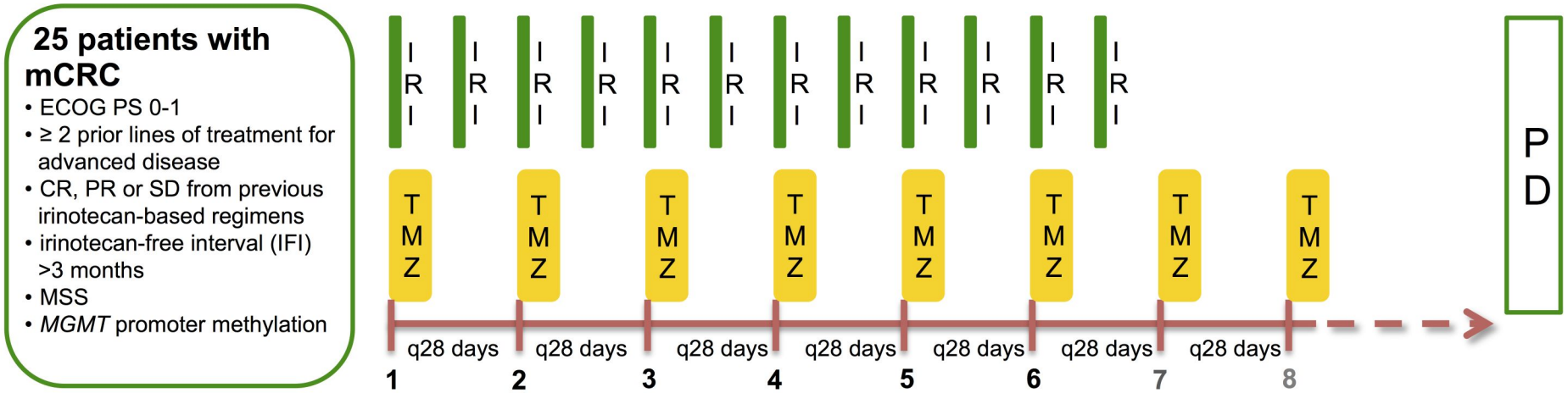
Table 1. Patients demographics and disease characteristics. Abbreviations: IQR: Interquartile Range; CR: Complete Response; PR: Partial Response; SD: Stable Disease.

Variables	***N*=25** ***N* (%)**
Gender	
Male	10 (40)
Female	15 (60)
Age	
Median (IQR)	62 (56-68)
ECOG PS	
0	16 (64)
1	9 (36)
Primary Tumor Location	
Right colon	8 (32)
Left colon	14 (56)
Extraperitoneal Rectum	3 (12)
Prior adjuvant chemotherapy	
Yes	2 (8)
No	23 (92)
***RAS* and *BRAF* mutational status**	
RAS mutated	16 (64)
BRAF mutated	-
RAS and *BRAF* wild-type	9 (36)
Metastases presentation	
Synchronous	17 (68)
Metachronous	8 (32)
No. of metastatic sites	
1	8 (32)
2	9 (36)
>2	8 (32)
No. of previous treatment lines	
2	8 (32)
3	7 (28)
≥4	10 (40)
No. of irinotecan-based treatment lines	
1	19 (76)
2	6 (24)
Irinotecan-Free Interval (IFI)	
Median, months (IQR)	6.8 (4.4-12.8)
Best response to last irinotecan-based regimen	
CR	-
PR	15 (60)
SD	10 (40)

Table 2. Treatment related adverse events according to CTCAE v4.0.

Adverse events	***N*=25** ***N* (%)**		
	G1-G2	**G3**	**G4**
Neutropenia	3 (12)		1 (4)
Anemia	1 (4)		
Thrombocytopenia	3 (12)	1 (4)	
Fever	3 (12)		
Nausea/vomiting	11 (44)		
Diarrhea	9 (36)	1 (4)	
Peripheral neuropathy	1 (4)		
Asthenia	6 (24)		
Anorexia	2 (8)		
Allergic reaction	1 (4)		
Pneumonia		1 (4)	

Figure S1.



TEMIRI regimen

- Temozolomide (TMZ) 150 mg/sqm on days 1-5 q 28 days
- Irinotecan (IRI) 100 mg/sqm on days 1,15 q 28 days

Figure S2.

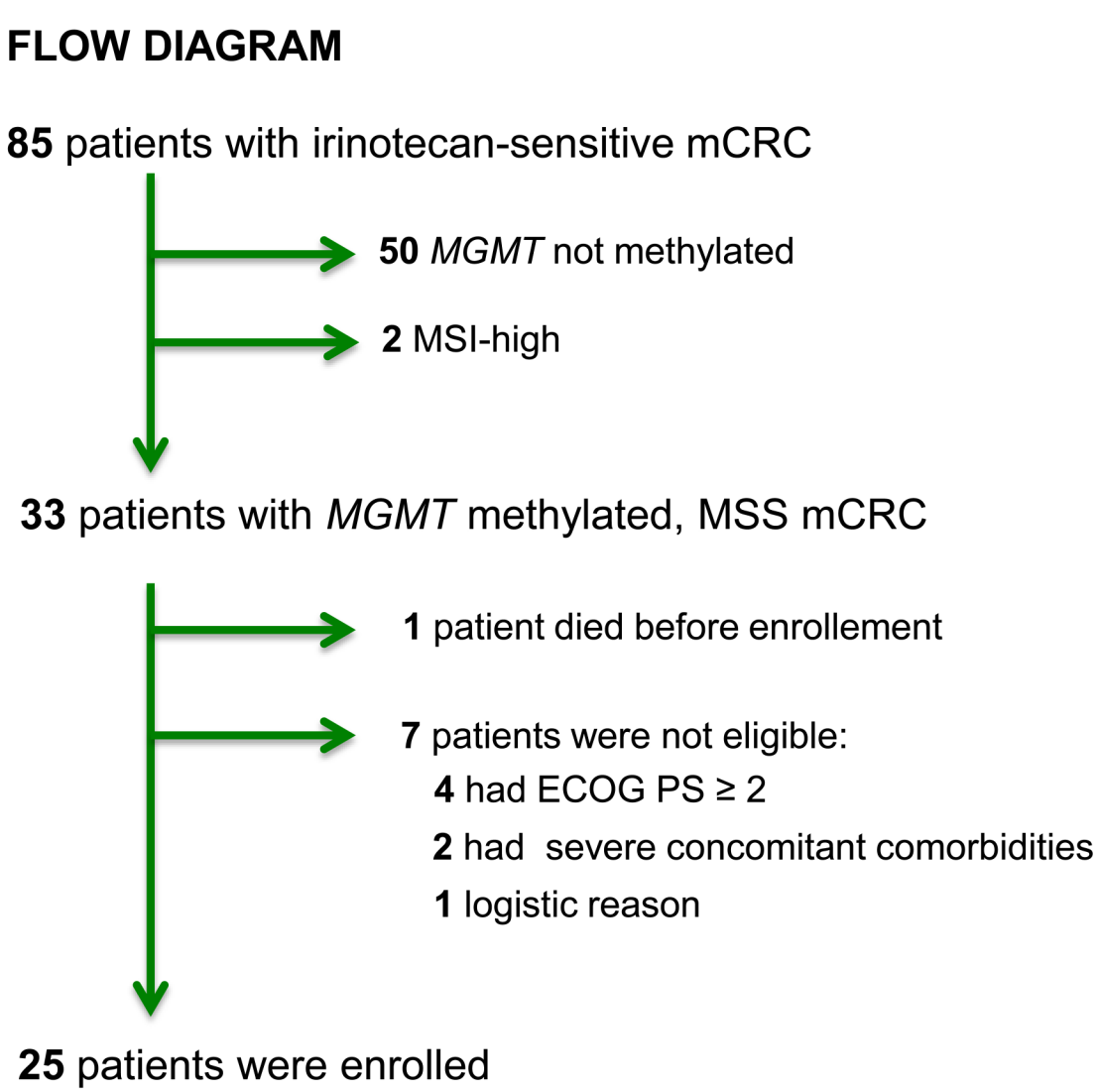

FLOW DIAGRAM
85 patients with irinotecan-sensitive mCRC
50 MGMT not methylated
2 MSI-high
33 patients with MGMT methylated, MSS mCRC
1 patient died before enrollement
7 patients were not eligible:
4 had ECOG PS ≥ 2
2 had severe concomitant comorbidities
1 logistic reason
25 patients were enrolled

Table S1. Summary of phase II clinical trials with alkylating agents (single-agent dacarbazine or temozolomide) in refractory metastatic colorectal cancer.

Ref.	Schedule	*N*	ORR	mPFS
Amatu *et al.* Clin Cancer Res 2013	DTIC 250 mg/sqm d 1-4, q21	68	3%	1.9
Hochauser *et al.* Mol Can Ther 2013	TMZ 150 mg/sqm 7 d on/7 d off, q28	86	5.8%	-
Pietrantonio *et al.* Ann Oncol 2014	TMZ 150 mg/sqm d 1-5, q28	32	12%	1.8
Pietrantonio *et al.* Target Oncol 2016	TMZ 75 mg/sqm d 1-21, q28	32	16%	2.3
Amatu *et al.* Ann Oncol 2016	TMZ 200 mg/sqm d 1-5, q28	29	3.4%	2.6
Calegari *et al.* Br J Cancer 2017	TMZ 150-200 mg/sqm d 1-5, q28	41	10%	1.9

Abbreviations: d: days, ORR: overall response rate, mPFS: median progression-free survival, DTIC: dacarbazine, TMZ: temozolomide.

Table S2. Association of pre-specified exploratory biomarkers (MGMT immunohistochemistry and methylBEAMing) with RECIST response in 24 evaluable patients.

Patients (N=24)	**MGMT IHC**			**MethylBEAMing**		
	Negative	Positive	*p*	Value ≥63	Value <63	*p*
Responders	6 (33%)	0 (0%)	0.277	5 (33%)	1 (11%)	0.350
Non-responders	12 (67%)	6 (100%)		10 (67%)	8 (89%)	

Abbreviations: *p*: Fisher exact test, IHC: immunohistochemistry.