Received: 2 September 2020 | Revised: 17 November 2020 | Accepted: 22 November 2020

DOI: 10.1111/cts.12960

ARTICLE

A phase 2 trial of gemcitabine and docetaxel in patients with metastatic colorectal adenocarcinoma with methylated checkpoint with forkhead and ring finger domain promoter and/or microsatellite instability phenotype

Marina Baretti[1] | Enusha Karunasena[1] | Marianna Zahurak[2] | Rosalind Walker[1] | Yang Zhao[3] | Thomas R. Pisanic 2nd[3] | Tza-Huei Wang[3] | Tim F. Greten[4] | Austin G. Duffy[4] | Elske Gootjes[5] | Gerrit Meijer[5] | Henk M.W. Verheul[5] | Nita Ahuja[6] | James G. Herman[7] | Nilofer S. Azad[1]

[1]Johns Hopkins Medicine Sidney Kimmel Comprehensive Cancer Center, Baltimore, Maryland, USA

[2]Department of Oncology, Biostatistics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[3]Johns Hopkins Institute for NanoBioTechnology, Baltimore, Maryland, USA

[4]Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

[5]Amsterdam University Medical Center, location VUMC, Amsterdam and Radboud UMC, Nijmegen, The Netherlands

[6]Oncology and Pathology, Smilow Cancer Hospital, Yale University School of Medicine, New Haven, Connecticut, USA

[7]Department of Medicine, UPMC Hillman Cancer Center, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Correspondence
Nilofer S. Azad, Johns Hopkins Medicine Sidney Kimmel Comprehensive Cancer Center, 1650 Orleans Street, Baltimore, MD 21287, USA.
Email: nazad2@jhmi.edu

Funding information
American Cancer Society Research Scholar Award 127343-RSG-15-068-01-TBG.

Abstract

We previously reported *CHFR* methylation in a subset of colorectal cancer (CRC; ~30%) with high concordance with microsatellite instability (MSI). We also showed that *CHF*R methylation predicted for sensitivity to docetaxel, whereas the MSI-high phenotypes were sensitive to gemcitabine. We hypothesized that this subset of patients with CRC would be selectively sensitive to gemcitabine and docetaxel. We enrolled a Phase 2 trial of gemcitabine and docetaxel in patients with MSI-high and/or *CHFR* methylated CRC. The primary objective was Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 response rate. Enrolled patients were treated with gemcitabine 800 mg/m^2 on days 1 and 8 and docetaxel 70 mg/m^2 on day 8 of each 21-day cycle. A total of 6 patients with *CHFR*-methylated, MSI-high CRC were enrolled from September 2012 to August 2016. The study was closed in September of 2017 due to poor accrual prior to reaching the first interim assessment of response rate, which would have occurred at 10 patients. No RECIST criteria tumor responses were observed, with 3 patients (50%) having stable disease as best response, 1 lasting more than 9 months. Median progression-free survival (PFS) was 1.79 months (95%

ClinicalTrials.gov Identifier: NCT01639131. Date of registration: July 12, 2012. https://clinicaltrials.gov/ct2/show/NCT01639131.

confidence interval [CI] = 1.28, not available [NA]) and median overall survival (OS) was 15.67 months (95% CI = 4.24, NA). Common grade 3 toxicities were lymphopenia (67%), leukopenia (33%), and anemia (33%). Although negative, this study establishes a proof-of-concept for the implementation of epigenetic biomarkers (*CHFR* methylation/MSI) as inclusion criteria in a prospective clinical trial to optimize combinatorial strategies in the era of personalized medicine.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

CHFR silencing via DNA methylation has been suggested to be predictive of taxane sensitivity in diverse tumors. The frequent association of CHFR methylation with microsatellite instability (MSI) suggested a possible combination therapy with gemcitabine, because the MSI phenotype may result in sensitivity to nucleoside analogues.

WHAT QUESTION DID THIS STUDY ADDRESS?

We hypothesized that metastatic colorectal cancer (mCRC), which have CHFR methylation and MSI phenotype were sensitive to gemcitabine and docetaxel, and have designed this Phase 2 trial in biomarker-selected mCRC to test this prediction.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

The study enrolled a molecularly defined subgroup of patients with colorectal cancer (CRC) and showed that the combination is safe in this population. Nevertheless, due to poor enrollment and early termination, no conclusions on the primary and secondary end points could be made.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

This study supports the feasibility of implementing DNA methylation markers in a prospective clinical trial and further efforts toward their application as predictive biomarkers for therapeutic agents in defined subsets of patients are warranted.

INTRODUCTION

Colorectal cancer (CRC) is the fourth most diagnosed cancer and the second leading cause of cancer-related deaths in the United States.[1] Despite significant improvements in CRC treatment, the long-term prognosis of patients with metastatic CRC (mCRC) disease remains poor, with a median overall survival (OS) of ~30 months.[2]

CRC has distinct phenotypes and can be divided into groups depending on the type of genomic instability: those with chromosomal instability (CIN), characterized by aneuploidy, amplification, chromosomal gains, and losses,[3] and those with microsatellite instability (MSI).[4] The MSI appears in tumors with deficient mismatch repair (dMMR) due to the inactivation of the four DNA MMR genes: MSH2, MLH1, MSH6, and PMS2, either by mutation or by epigenetic silencing via promoter methylation. An association between MSI and a hypermethylator phenotype (CpG island methylator phenotype [CIMP]-high) has been well described.[5,6] Preclinical and clinical studies assessing the relative chemosensitivity of MSI versus microsatellite stability (MSS) CRC have yielded mixed results, especially in regard to 5FU-based treatments.[7–10] Previous studies have reported that the MSI-H phenotype is associated with increased sensitivity to nucleoside analogs, such as gemcitabine, due to the cells' inability to tolerate DNA damage caused by this class of agents.[11]

Many genes are silenced by promoter region hypermethylation in colon cancer compared with normal colonic epithelium, including *CHFR*. The *CHFR* (checkpoint with forkhead and RING finger domains) encodes a protein that inhibits polo-like kinase-1, which controls the G2/M checkpoint by delaying entry into metaphase when alterations of the mitotic spindle occur, thus delaying G2 to M transition.[12,13] When cells are treated with microtubule inhibitors, loss of CHFR, including through hypermethylation, leads to mitotic catastrophe and apoptosis.[12] *CHFR* is frequently inactivated by promoter CpG island methylation in CRC,[14,15] is associated with reduced survival in stages II and III CRC,[16] and is an independent predictor for recurrence for patients with locally advanced CRC.[17] We, and others, have demonstrated that MSI and *CHFR* methylation are frequently found together in CRC cell lines and primary tumors.[14,15]

Epigenetic silencing of *CHFR* expression via CpG promoter methylation has been shown to increase sensitivity to microtubule inhibitors, including taxanes, and previous

preclinical and clinical studies in other cancer types, including gastric, endometrial, and cervical cancer, have suggested this correlation.[18–21] Our laboratory investigations have confirmed taxane sensitivity in CRC cell lines that have completely or partially methylated *CHFR* promoter. We have reported a significant activity of the combination of gemcitabine and docetaxel in CRC cell lines and xenograft models with this *CHFR* methylation/MSI-H phenotype.[16]

Gemcitabine and docetaxel have been studied in the treatment of patients with unselected CRC as monotherapy, but did not show significant efficacy.[17,22–24] However, based on our preclinical studies, we hypothesized that the combination of these cytotoxic chemotherapy agents would have activity in patients with biomarker-selected CRC with the MSI phenotype and/or *CHFR* promoter methylation. To test this hypothesis, we conducted a Phase 2, biomarker-driven trial, evaluating gemcitabine with docetaxel in *CHFR* promoter methylated or MSI-H pretreated, patients with mCRC.

METHODS

Study design

This was an open-label, multicenter, trial conducted at the Sidney Kimmel Comprehensive Cancer Center (SKCCC) at Johns Hopkins University (JHU), the National Cancer Institute, and the Amsterdam University Medical Center location VUMCmc Cancer Center (Vu). The primary objective was to evaluate the efficacy of combination gemcitabine and docetaxel chemotherapy in the treatment of mCRC with *CHFR* and/or MSI phenotype. The primary end point was overall tumor response rate (ORR), defined as the percentage of patients who show a complete response (CR) or partial response (PR). Secondary end points included time to progression-free survival (PFS), overall survival (OS), safety and toxicity assessments, and correlative science studies.

Enrolled patients were treated with gemcitabine 800 mg/m^2 on days 1 and 8 and docetaxel 70 mg/m^2 on day 8 of each 21-day cycle.[25–27] Patients received filgrastim (granulocyte colony-stimulating factor [G-CSF]) on days 9 through 15 or pegfilgrastim 6 mg on day 9 or 10 of each cycle. Patients were treated until disease progression or unacceptable adverse events (AEs), or withdrawn of the consent.

Dose reductions were mandated for grade 3 or higher toxicities related to the study drug. The study drug could be resumed at a lower dose once the toxicity resolved to grade 1 or baseline prior to the next scheduled dose. If toxicity did not resolve to grade 1 or baseline parameters within 21 days, treatment was discontinued. Dose re-escalation was not allowed.

The protocol was approved by the institutional review boards (IRBs) at all study sites, and complied with the International Ethical Guidelines for Biomedical Research Involving Human Subjects and the Declaration of Helsinki. Eligible patients were enrolled centrally at the SKCCC at JHU. All patients provided written informed consent for this study. The trial was registered under ClinicalTrials.gov as (NCT01639131).

Patients

Patients were eligible for the trial if they were 18 years or older with histologically confirmed mCRC, with either methylated *CHFR* promoter and/or MSI-high phenotype. MSI had to be assessed by a Clinical Laboratory Improvement Amendment (CLIA) certified laboratory using either polymerase chain reaction (PCR)-based microsatellite testing or immunohistochemistry for MMR proteins. Eastern Cooperative Oncology Group (ECOG) performance status ≤1, and adequate organ function as defined by absolute neutrophil count greater than or equal to 1500 cells/μL, platelet count greater than or equal to 100,000 cells/μL, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) less than or equal to 2.5 times the upper limit of normal (or ≤5× upper limit of normal in patients with liver metastases) total bilirubin less than or equal to 1.5 times the upper limit of normal, and serum creatinine within normal institutional limits or creatinine clearance greater than or equal to 60 mL/min. There was no limit on prior therapies. Measurable disease according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria was also required.

Assessments

Patients were evaluated every cycle for trial therapy compliance and monitoring of AEs. The National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 was implemented for AE monitoring. The treatment protocol allowed dose delays or reduction if patients experienced unacceptable side effects and adverse reactions related to study drug(s). Disease assessments (computed tomography or magnetic resonance imaging) were performed at baseline and then every 6 weeks. Response was evaluated according to the RECIST version 1.1.[28] In the event that the patient was deemed to be receiving continued clinical benefit in the face of progressive disease by RECIST criteria, the patient may have continued on therapy with agreement of the Principal Investigator. If progressive disease was confirmed on successive imaging or clinical examination, the date of progression was marked as the first timepoint when progression was noted. Upon progression of disease, patients were monitored for long-term AEs and survival.

Genomic DNA extraction, sodium bisulfite conversion, and quality assurance

DNA extraction and quality assurance were carried out as described previously.[29,30] Tissue DNAs were treated with sodium bisulfite and analyzed by methylation-specific PCR (MSP) as described by Herman et al.[31] This process converts nonmethylated cytosine residues to uracil, whereas methylated cytosines remain unchanged. Bisulfite-modified samples were aliquoted and stored at −80°C.

Microsatellite instability and methylation of *CHFR* gene promoter in archival tissue biopsy

For study enrollment, mismatch repair deficiency was determined at each participating institution by immunohistochemistry (IHC) for MMR proteins MLH1, MSH2, MSH6, or PMS2, or by PCR-based tests for microsatellite instability. For the latter, six slides of tumor and normal (uninvolved lymph node or margin of resection) were cut (5 microns each), deparaffinized (xylene), and one stained with hematoxylin and eosin (H&E). A tumor area containing at least 20% neoplastic cells, designated by a board-certified Anatomic Pathologist was macrodissected using the Pinpoint DNA isolation system (Zymo Research), digested in proteinase K for 8 h and DNA was isolated using a QIAamp DNA Mini Kit (Qiagen). MSI was assessed using the MSI Analysis System (Promega), composed of five pseudomonomorphic mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) to detect MSI and 2-pentanucleotide repeat loci (PentaC and PentaD) to confirm identity between normal and tumor samples, per manufacturer's instructions. Following amplification of 50–100 ng DNA, the fluorescent PCR products were sized on an Applied Biosystems 3130xl capillary electrophoresis instrument (Invitrogen). Pentanucleotide loci confirmed identity in all cases. Controls included water as a negative control and a mixture of 80% germline DNA with 20% MSI cancer DNA as a positive control. The size in bases was determined for each microsatellite locus and tumors were designated as MSI if two or more mononucleotide loci varied in length compared with the germline DNA.

CHFR methylation was assessed by standard methylation-specific PCR and DREAMing (Discrimination of Rare EpiAlleles by Melt; Supplementary Methods).[32,33] Patients were assessed for *CHFR* methylation if he/she had methylation specific amplification using MSP for the *CHFR* gene or lack of expression by IHCs; MSP primers are previously reported.[14] Patients with MSI and a family history supportive for a possible diagnosis of HNPCC were referred to a genetics counselor for further evaluation and recommendations.

Statistical methods

The primary objective of this study was ORR, PR, plus CR. The study was designed to have the goal of improving a 20% historical response rate to a rate of 30% with the combined therapy. Proportions were reported with exact 95% binomial confidence intervals (CIs). Event time distributions for OS and PFS were estimated with the method of Kaplan–Meier (KM).[34] Follow-up was reported using the reverse KM method. The median follow-up was calculated as the 50% point of this curve. Safety analyses included all patients who received at least one dose of study drug.

RESULTS

Patient characteristics

From September 2012 to August 2016, 17 patients were screened. Ten patients were deemed not eligible because they had MSS mCRC and absent *CHFR* promoter methylation. During the time this protocol was open, six patients were treated (accrual rate 1.5 patients per year). The study was closed in September of 2017 due to poor accrual prior to reaching the first interim assessment of response rate, which was planned at 10 patients.

The demographic characteristics of enrolled patients are presented in Table 1. All six patients were evaluable for primary end point evaluation. All patients were pretreated, with a median of 2 (range 2–4) prior therapies. All patients discontinued therapy due to disease progression. One patient tested positive for MSI (MSI low), the remaining five patients were classified as MSI-H (Table 2). *CHFR* promoter methylation was found in four of the enrolled patients, and was negative and undetermined for one patient, respectively (Table 2). All patients were started on the planned doses of gemcitabine and docetaxel and were evaluable for toxicity end points. Median dose administered per treated cycle of gemcitabine and docetaxel was 90% for gemcitabine and 89% for docetaxel (range 66%–100% and 66%–100%, respectively). Patients were treated for a median of two cycles (range 1–14).

Treatment safety

Hematological and nonhematological toxicities are shown in Table S1. Toxicity was evaluable for all six patients that started treatment. There was one grade 4 AE at least possibly related to study treatment (sepsis). The most frequently reported grade 3 drug-related toxicities were lymphopenia (67%), leukopenia (33%), and anemia (33%); grade 3 neutropenia, abdominal pain, deep vein thrombosis, and albumin

TABLE 1 Baseline characteristics

Characteristics	*N* (%)
Age, mean (SD)	56.3 (11.1)
Sex	
Female	2 (33.3)
Male	4 (66.7)
Race	
White	3 (50.0)
African American	2 (33.3)
Asian	1 (16.7)
ECOG PS	
0	2 (33.3)
1	4 (66.7)
Family history of CRC	
Yes	3 (50.0)
No	3 (50.0)
Synchronous metastases	
Yes	3 (50.0)
No	3 (50.0)
Primary tumor site	
Right	4 (66.7)
Left	1 (16.7)
Rectum	1 (16.7)
Number of metastatic sites	
1	3 (50.0)
>1	3 (50.0)
Prior number of therapies	
1–2	5 (83.3)
3	0 (0.0)
≥4	1 (16.7)
RAS mutation[a]	
Yes	2 (33.3)
No	4 (66.7)
BRAF mutation[a]	
Yes	0 (0.0)
No	3 (50.0)
Unknown	3 (50.0)

Abbreviations: CRC, colorectal cancer; ECOG, Eastern Cooperative Oncology Group; RAS, renin angiotensin system.

[a]RAS and BRAF mutation status was based on historical patient record.

reduction occurred in one patient each. Overall, hematological AEs were frequent, with the most common hematologic AEs being lymphopenia (83%). Three patients (50%) required dose modifications (2 patients at dose level 1 (75% of original dose), and 1 patient at dose level 2 (66% of original dose) to due to grade 3 grade or higher hematological toxicities related to the study drug. The most frequent nonhematologic AEs were alopecia (67%) and neuropathy (67%). No patients discontinued treatment due to drug-related AEs.

TABLE 2 Microsatellite stability status, *CHFR* gene methylation status of treated patients

Patient ID	MSI status	CHFR promoter methylation
001	MSI-low	Unmethylated
002	MSI-high	Methylated
003	MSI-high	Methylated
004	MSI-high	Methylated
009	MSI-high	Methylated
0013	MSI-high	Unknown

Abbreviation: MSI, microsatellite instability.

Treatment efficacy

Among six evaluable patients, median follow-up, calculated as the 50% point of the censoring function, was 60.9 months (range 2.3–61.3 months). Two of the six patients (33%) were censored for OS. Median PFS was 1.79 months (95% CI = 1.28, NA; Figure 1a) and median OS was 15.67 months (95% CI = 4.24, NA; Figure 1b). No RECIST criteria tumor responses were observed. Three patients (50%, patients 002, 009, and 013) had stable disease as best response, for 9.3, 3.5, and 1.8 months each (Table 3). All three patients had MSI-H tumor. *CHFR* was methylated for one patient, not methylated for another patient, and unknown status for the third patient's status. Best responses, number of cycles and off study reasons, and post-trial treatment are summarized in Table 4. DREAMing[32] analysis of baseline plasma *CHFR* methylation status was conducted in one patient achieving long lasting stable disease, showing all detected methylated epialleles were heavily methylated, Figure 2.

DISCUSSION

Epigenetic abnormalities are widespread in malignant tissue and have been shown to play key roles in the development, progression, and outcome of many cancers, including CRC.[35,36] As aberrant DNA methylation is an early and frequent event during carcinogenesis, clinical investigations into associations among abnormal methylation and cancer diagnosis, prognosis, and response to therapy have been conducted in various cancers.[37–39] However, epigenetic biomarkers are not routinely utilized in gastrointestinal malignancies to prospectively choose therapy. Examples, such as the use of O6-Methylguanine-DNA Methyltransferase (MGMT) methylation to predict a lack of benefit of alkylating agents in the treatment of glioblastoma multiforme[40] and homozygous

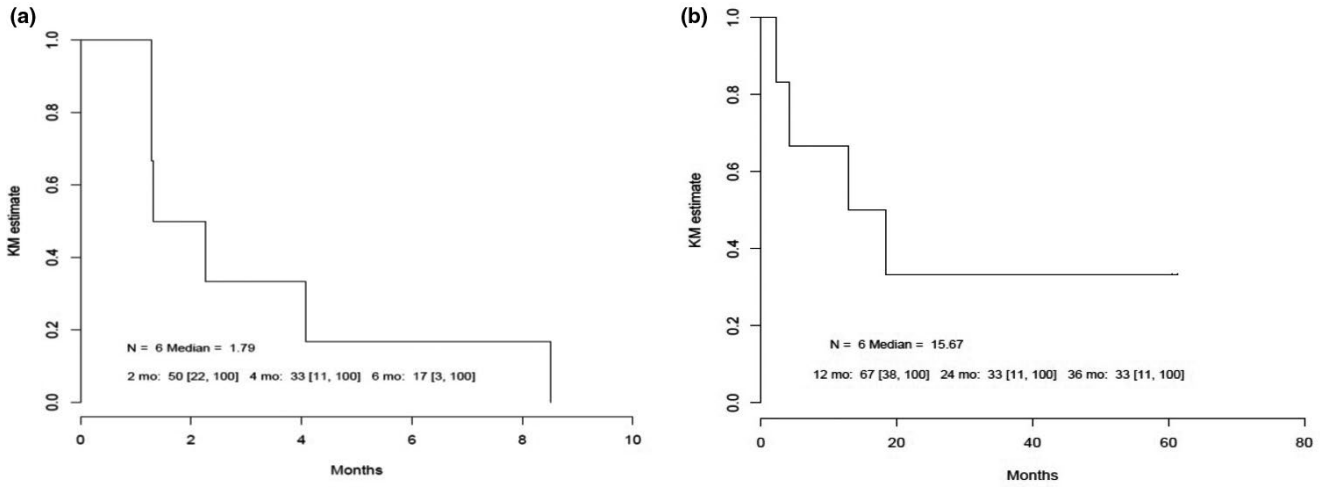

FIGURE 1 Kaplan-Meier (KM) estimates of progression-free survival (a) and overall survival (b)

TABLE 3 Efficacy outcomes in evaluable patients and poststudy treatment

	N = 6
Type of response, *N* (%)	
Complete response	0 (0.0)
Partial response	0 (0.0)
Stable disease	3 (50.0)
Progressive diseases	3 (50.0)
Objective response rate (95% CI)	0 (0.0, 39.3%)
Median progression-free survival (95% CI, months)	1.79 (1.28, NA)
Median overall survival (95% CI, months)	15.67 (4.24, NA)

Abbreviations: CI, confidence interval; NA, not available.

BRCA1 promoter methylation to predict sensitivity to ovarian tumor's susceptibility to PARP inhibitors are instructive[41]: when the appropriate population of patients is selected based on the synthetic lethality produced by this epigenetic change, there is the possibility of increased clinical benefit.

The main goal of this study was to evaluate new therapeutic options through application of novel predictive biomarkers of chemosensitivity for refractory, patients with mCRC with distinct epigenetic features, exploring the activity of drugs not traditionally used in this disease.

Our hypothesis was that epigenetic biomarkers might be used to identify patients more likely to respond to a specific treatment targeting molecular vulnerabilities associated with epigenetic silencing of the biomarker-associated genes. In the present study, we sought to evaluate whether taxane therapy might prove more efficacious in patients exhibiting epigenetic silencing of *CHFR* with corresponding sensitivities due to cell-cycle checkpoint dysregulation.

In the initial Phase 1 study of gemcitabine, two partial responses were observed, one being in a patient with advanced CRC.[42] However, Phase 2 studies of monotherapy gemcitabine showed minimal activity in an unselected population in both treatment naïve and patients with refractory CRC.[43] Similarly, docetaxel monotherapy showed minimal benefit in unselected patients with CRC, with 3 Phase 2 studies, including a combined 76 patients reported only 3 patients with objective responses (1 CR and 2 PRs). An additional two patients experienced a minor response and nine patients demonstrated stable disease.[22–24] These patients were not molecularly characterized.

In our trial, patients received intravenous gemcitabine 800 mg/m^2 on days 1 and 8 and docetaxel 70 mg/m^2 on day 8 of each 21-day cycle.

Gemcitabine and docetaxel combination have a broad range of activity against human solid tumors. Preclinical data, however, initially suggested that they may have an antagonistic effect on cytotoxicity when used concurrently. Nevertheless, retrospective reviews of gemcitabine and docetaxel in patients with locally advanced or metastatic disease included an in vitro study to investigate the dosing sequence of gemcitabine and docetaxel, finding that gemcitabine followed by docetaxel was synergistic[44] and the sequence of gemcitabine followed by docetaxel showed objective responses[45] in prior study in patients with advanced solid tumors. Hence, the currently used schedule of gemcitabine and docetaxel in this trial was established. Preclinical studies demonstrated that epigenetic silencing of *CHFR* gene through promoter methylation could make cancer cells more sensitive to taxane chemotherapy.[18,20,21]Initial reports suggested that 30% of CRC cases exhibit *CHFR* methylation, and this was particularly in MSI-high phenotypes, which have also been suggested to confer increased sensitivity to gemcitabine.[11,42] Salient to this study, we have previously reported that the biomarkers of *CHFR* methylation and MSI characterize a population of mCRC that would be potentially characterized by differential sensitivity to taxanes and gemcitabine, respectively.[16]

TABLE 4 Summary of off study reasons, post-trial treatment and causes of death

Patient ID	Number of cycles received within the clinical trials	Best response according to RECIST 1.1	Off study reasons	Post-trial treatment	Outcome
001	2	Disease progression	Disease progression	—	Death due to cancer progression
002	14	Stable disease	Disease progression	Pembrolizumab	Death due to cancer progression
003	2	Disease progression	Disease progression	Pembrolizumab	Censored for OS[a]
004	2	Disease progression	Disease progression	Pembrolizumab	Censored for OS[a]
009	5	Stable disease	Disease progression	Pembrolizumab	Death due to cancer progression
013	2	Stable disease	Clinical progression	—	Death due to cancer progression

Abbreviations: OS, overall survival; RECIST, Response Evaluation Criteria in Solid Tumors.

[a]At the time of the last follow-up, two patients were alive and were censored for OS.

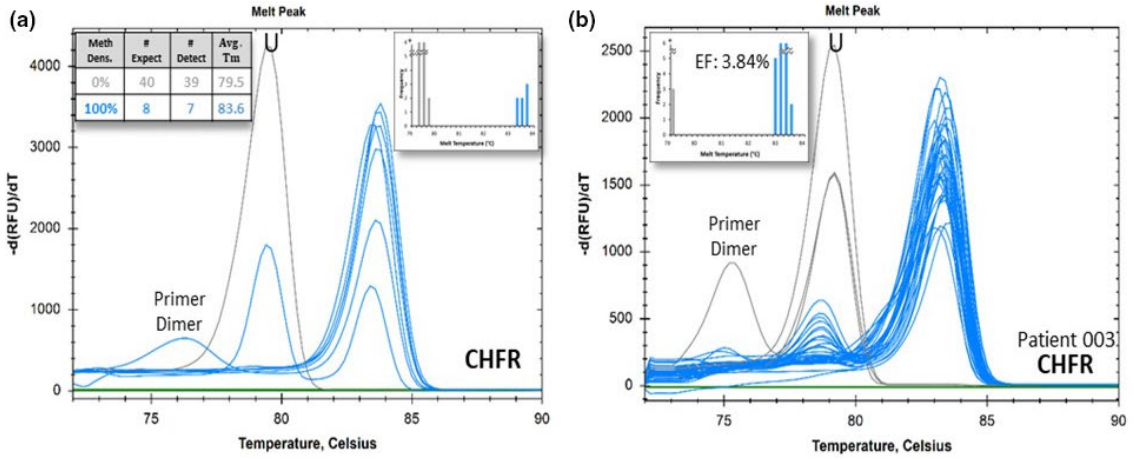

FIGURE 2 Assessment of *CHFR* methylation by DREAMing. (a) Validation of the single-copy sensitive CHFR DREAMing assay, showing detection of 7 of 8 synthetic BST fully methylated epialleles (blue) in a background of 4800 healthy human haploid cfDNA genomic equivalents (grey representative trace). Average melt temperatures and "DREAM analysis" are shown in the upper left and right insets, respectively. (b) CHFR DREAMing assessment of cfDNA obtained from liquid biopsy of patient 003. The calculated overall epiallelic fraction (EF) of methylated *CHFR* is 3.84%, as estimated by Poissonian distribution. All detected methylated epialleles were heavily methylated, exhibiting T_m >83.0°C

Accordingly, we designed a Phase 2 clinical trial of biomarker-driven therapy in which we used epigenetic alterations to prospectively select patients with mCRC for combined docetaxel and gemcitabine treatment. The main effect of gemcitabine/docetaxel in the population of mCRC with MSI-H or methylated *CHFR* promoter was disease stabilization, rather than tumor shrinkage. Three patients had stable disease as best response, one of which lasting for 23 months, resulting in a disease control rate (DCR) of 50%. The safety profile of the combination treatment was consistent with clinical experience in other settings and histologies.[46,47]

Unfortunately, despite the strong preclinical rationale and results, and the highly innovative approach to repurpose already approved anticancer agents, our study was terminated early due to failure to accrual. Multiple reasons have contributed to this failure. The strong concordance of the MSI-high phenotype with the *CHFR* methylated phenotype led to an overestimate of the prevalence of *CHFR* methylation in an advanced CRC setting. Although dMMR/MSI-H CRCs are found in 15–20% of stage II and III CRCs, this prevalence is drastically lower in the metastatic setting, with only 5% of stage IV CRCs being MSI-H. Accordingly, advanced CRCs also have a much lower percentage of *CHFR* methylation than predicted within The Cancer Genome Atlas (TCGA), which is predominantly earlier stage disease.[15,48] However, the primary reason for poor accrual was another study at our institution, which examined MMR deficiency as a predictive marker for anti-PD1 immune checkpoint treatment. An investigator-initiated, Phase 2 study (KEYNOTE-016) was concurrently opened at our institution, demonstrating the

unprecedented benefit of PD-1 blockade therapy with pembrolizumab in MSI-H or dMMR unresectable or metastatic solid tumors, including CRC, leading to US Food and Drug Administration (FDA) approval of pembrolizumab in MSI-high patients.[49] The prolonged OS observed in our patients is likely explained by the fact that four of the six patients, all MSI-H, received post-study treatment with pembrolizumab within the KEYNOTE-016 clinical trial.

Our trial was based on strong preclinical data and aimed to prove that epigenetic biomarkers, such as *CHFR* methylation/MSI, could can guide therapy and enhance the utility of established chemotherapeutic agents, such as taxanes and gemcitabine for patients with mCRC. Unfortunately, the low percentage of MSI-H/*CHFR*-methylated mCRC, and confounding issues associated with a competing trial, resulted in premature closure of the study. Nonetheless, there is evidence that the proposed strategy is worthy of further exploration. Although a small sample size, three of the six patients did maintain stable disease and the patient with the longest response (9.3 months) was both *CHFR*-methylated and MSI-high. This trial enriches the list of studies supporting the feasibility of implementing DNA methylation markers in a prospective clinical trial. Although none have yet achieved regulatory approval for clinical use, a small number of examples are established in the literature. Among the best known is DNA methylation of the MGMT promoter encoding a DNA repair enzyme, which is associated with better response to alkylating neoplastic agents like temozolomide, as first shown in glioblastoma by Esteller et al.[40] and later by Hegi et al.[50] Other published predictive epigenetic biomarker examples include BRCA1. The BRCA1 gene plays a role in DNA damage response and hypermethylation of its promoter region may be predictive of enhanced sensitivity to PARP inhibitors and platinum-derived drugs in patients with ovarian cancer.[41,51]

Finally, we used a novel a novel, quasi-digital high resolution melt platform assay, named DREAM-ing, to quantitatively analyze changes in DNA methylation in circulating free DNA, confirming its applicability for clinical trial samples.[32,52]

Considering the concordance of *CHFR* methylation and mismatch repair deficiency, the latter of which predicts for durable disease response in patients with anti-PD-1 therapy, it is unlikely that a properly powered study testing our hypothesis will ever be accrued in this setting. As epigenetic changes are key components in CRC carcinogenesis and progression, further efforts toward the application of aberrant DNA methylation as feasible biomarkers for CRC to predict clinical benefit for therapeutic agents in defined subsets of patients with CRC are warranted.

ACKNOWLEDGMENTS

The authors would like to acknowledge the patients and their families for participating in this study. In addition, we thank all Research staff for assistance with sample collection and patient-care logistic.

CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHORS CONTRIBUTIONS

M.B., N.A., J.G.H., and N.S.A. wrote the manuscript. M.B., R.W., T.F.G., A.G.D., E.G., G.M., H.M.W.V., N.A., J.G.H., and N.S.A. designed the research. M.B., E.K., M.Z., T.R.P., J.G.H., and N.S.A. analyzed the data. Y.Z., T.R.P., and T.H.W. contributed new analytical tools.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin*. 2018;68(6):394-424.
2. Van Cutsem E, Cervantes A, Adam R, et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. *Ann Oncol*. 2016;27(8):1386-1422.
3. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology*. 2008;135(4):1079-1099.
4. Baretti M, Le DT. DNA mismatch repair in cancer. *Pharmacol Ther*. 2018;189:45-62.
5. Dahlin AM, Palmqvist R, Henriksson ML, et al. The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. *Clin Cancer Res*. 2010;16(6):1845-1855.
6. Ahuja N, Mohan AL, Li Q, et al. Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res*. 1997;57(16):3370-3374.
7. Gavin PG, Colangelo LH, Fumagalli D, et al. Mutation profiling and microsatellite instability in stage II and III colon cancer: an assessment of their prognostic and oxaliplatin predictive value. *Clin Cancer Res*. 2012;18(23):6531-6541.
8. Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med*. 2003;349(3):247-257.
9. Sargent DJ, Marsoni S, Monges G, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol*. 2010;28(20):3219-3226.
10. Tejpar S, Saridaki Z, Delorenzi M, Bosman F, Roth AD. Microsatellite instability, prognosis and drug sensitivity of stage II and III colorectal cancer: more complexity to the puzzle. *J Natl Cancer Inst*. 2011;103(11):841-844.
11. Takahashi T, Min Z, Uchida I, et al. Hypersensitivity in DNA mismatch repair-deficient colon carcinoma cells to DNA polymerase reaction inhibitors. *Cancer Lett*. 2005;220(1):85-93.
12. Scolnick DM, Halazonetis TD. Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature*. 2000;406(6794):430-435.
13. Kang D, Chen J, Wong J, Fang G. The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. *J Cell Biol*. 2002;156(2):249-259.

14. Brandes JC, van Engeland M, Wouters KA, Weijenberg MP, Herman JG. CHFR promoter hypermethylation in colon cancer correlates with the microsatellite instability phenotype. *Carcinogenesis*. 2005;26(6):1152-1156.
15. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487(7407):330-337.
16. Pelosof L, Yerram SR, Ahuja N, et al. CHFR silencing or microsatellite instability is associated with increased antitumor activity of docetaxel or gemcitabine in colorectal cancer. *Int J Cancer*. 2014;134(3):596-605.
17. Gusella M, Pasini F, Bolzonella C, et al. Equilibrative nucleoside transporter 1 genotype, cytidine deaminase activity and age predict gemcitabine plasma clearance in patients with solid tumours. *Br J Clin Pharmacol*. 2011;71(3):437-444.
18. Banno K, Yanokura M, Kawaguchi M, et al. Epigenetic inactivation of the CHFR gene in cervical cancer contributes to sensitivity to taxanes. *Int J Oncol*. 2007;31(4):713-720.
19. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*. 2003;349(21):2042-2054.
20. Pillai RN, Brodie SA, Sica GL, et al. CHFR protein expression predicts outcomes to taxane-based first line therapy in metastatic NSCLC. *Clin Cancer Res*. 2013;19(6):1603-1611.
21. Satoh A, Toyota M, Itoh F, et al. Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. *Cancer Res*. 2003;63(24):8606-8613.
22. Pazdur R, Lassere Y, Soh LT, et al. Phase II trial of docetaxel (Taxotere) in metastatic colorectal carcinoma. *Ann Oncol*. 1994;5(5):468-470.
23. Sternberg CN, ten Bokkel Huinink WW, Smyth JF, et al. Docetaxel (Taxotere), a novel taxoid, in the treatment of advanced colorectal carcinoma: an EORTC Early Clinical Trials Group Study. *Br J Cancer*. 1994;70(2):376-379.
24. Taguchi T. An early phase II clinical study of RP56976 (docetaxel) in patients with cancer of the gastrointestinal tract. *Gan To Kagaku Ryoho*. 1994;21(14):2431-2437.
25. Spiridonidis CH, Laufman LR, Jones J, Rhodes VA, Wallace K, Nicol S. Phase I study of docetaxel dose escalation in combination with fixed weekly gemcitabine in patients with advanced malignancies. *J Clin Oncol*. 1998;16(12):3866-3873.
26. Niho S, Kubota K, Goto K, et al. Combination second-line chemotherapy with gemcitabine and docetaxel for recurrent non-small-cell lung cancer after platinum-containing chemotherapy: a phase I/II trial. *Cancer Chemother Pharmacol*. 2003;52(1):19-24.
27. Rizvi NA, Spiridonidis CH, Davis TH, et al. Docetaxel and gemcitabine combinations in non-small cell lung cancer. *Semin Oncol*. 1999;26(5 Suppl 16):27-31; discussion 41-22.
28. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer*. 2009;45(2):228-247.
29. Fackler MJ, McVeigh M, Mehrotra J, et al. Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. *Cancer Res*. 2004;64(13):4442-4452.
30. Swift-Scanlan T, Blackford A, Argani P, Sukumar S, Fackler MJ. Two-color quantitative multiplex methylation-specific PCR. *Biotechniques*. 2006;40(2):210-219.
31. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA*. 1996;93(18):9821-9826.
32. Pisanic TR 2nd, Athamanolap P, Poh W, et al. DREAMing: a simple and ultrasensitive method for assessing intratumor epigenetic heterogeneity directly from liquid biopsies. *Nucleic Acids Res*. 2015;43(22):e154.
33. Overman MJ, McDermott R, Leach JL, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol*. 2017;18(9):1182-1191.
34. Johnson VE, Cook JD. Bayesian design of single-arm phase II clinical trials with continuous monitoring. *Clin Trials*. 2009;6(3):217-226.
35. Baretti M, Azad NS. The role of epigenetic therapies in colorectal cancer. *Curr Probl Cancer*. 2018;42(6):530-547.
36. Azad N, Zahnow CA, Rudin CM, Baylin SB. The future of epigenetic therapy in solid tumours–lessons from the past. *Nat Rev Clin Oncol*. 2013;10(5):256-266.
37. Lee BB, Lee EJ, Jung EH, et al. Aberrant methylation of APC, MGMT, RASSF2A, and Wif-1 genes in plasma as a biomarker for early detection of colorectal cancer. *Clin Cancer Res*. 2009;15(19):6185-6191.
38. Ling ZQ, Lv P, Lu XX, et al. Circulating methylated XAF1 DNA indicates poor prognosis for gastric cancer. *PLoS One*. 2013;8(6):e67195.
39. Zhang Y, Wang R, Song H, et al. Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer. *Cancer Lett*. 2011;303(1):21-28.
40. Esteller M, Garcia-Foncillas J, Andion E, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med*. 2000;343(19):1350-1354.
41. Kondrashova O, Topp M, Nesic K, et al. Methylation of all BRCA1 copies predicts response to the PARP inhibitor rucaparib in ovarian carcinoma. *Nat Commun*. 2018;9(1):3970.
42. Abbruzzese JL, Grunewald R, Weeks EA, et al. A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. *J Clin Oncol*. 1991;9(3):491-498.
43. Correale P, Cerretani D, Clerici M, et al. Gemcitabine (GEM), 5-fluorouracil (5-FU) and folinic acid (FA) in patients with different gastroenteric malignancies. *J Chemother*. 2004;16(2):206-210.
44. Leu KM, Ostruszka LJ, Shewach D, et al. Laboratory and clinical evidence of synergistic cytotoxicity of sequential treatment with gemcitabine followed by docetaxel in the treatment of sarcoma. *J Clin Oncol*. 2004;22(9):1706-1712.
45. Ryan DP, Lynch TJ, Grossbard ML, et al. A phase I study of gemcitabine and docetaxel in patients with metastatic solid tumors. *Cancer*. 2000;88(1):180-185.
46. Passardi A, Cecconetto L, Dall'agata M, et al. Randomized phase II study with two gemcitabine- and docetaxel-based combinations as first-line chemotherapy for metastatic non-small cell lung cancer. *J Transl Med*. 2008;6:65.
47. Novello S, Falcone A, Crino L, et al. Randomised multicenter phase II study of two schedules of docetaxel and gemcitabine or cisplatin/gemcitabine followed by docetaxel as first line

treatment for advanced non-small cell lung cancer. *Lung Cancer*. 2009;66(3):327-332.
48. Toyota M, Sasaki Y, Satoh A, et al. Epigenetic inactivation of CHFR in human tumors. *Proc Natl Acad Sci USA*. 2003;100(13):7818-7823.
49. Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med*. 2015;372(26):2509-2520.
50. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005;352(10):997-1003.
51. Stefansson OA, Villanueva A, Vidal A, Marti L, Esteller M. BRCA1 epigenetic inactivation predicts sensitivity to platinum-based chemotherapy in breast and ovarian cancer. *Epigenetics*. 2012;7(11):1225-1229.
52. Overman MJ, Adam L, Raghav K, et al. Phase II study of nab-paclitaxel in refractory small bowel adenocarcinoma and CpG island methylator phenotype (CIMP)-high colorectal cancer. *Ann Oncol*. 2018;29(1):139-144.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Baretti M, Karunasena E, Zahurak M, et al. A phase 2 trial of gemcitabine and docetaxel in patients with metastatic colorectal adenocarcinoma with methylated checkpoint with forkhead and ring finger domain promoter and/or microsatellite instability phenotype. *Clin Transl Sci*. 2021;14:954–963. https://doi.org/10.1111/cts.12960