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

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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 CHFR 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/m² on days 1 and 8 and docetaxel 70 mg/m² 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%...
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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 in 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

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.

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.
preclinical and clinical studies in other cancer types, including gastric, endometrial, and cervical cancer, have suggested this correlation. 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.

Gemcitabine and docetaxel have been studied in the treatment of patients with unselected CRC as monotherapy, but did not show significant efficacy. 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/m² on days 1 and 8 and docetaxel 70 mg/m² on day 8 of each 21-day cycle. 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 reductions 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. 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.\(^{20,30}\) 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 macerodissected 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...
reduction occurred in one patient each. Overall, hematologic 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.

### 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 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. 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. 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 and homozygous

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**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<sup>a</sup>         |                |
| Yes                              | 2 (33.3)       |
| No                               | 4 (66.7)       |
| BRAF mutation<sup>a</sup>        |                |
| 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.

<sup>a</sup>RAS and BRAF mutation status was based on historical patient record.

**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.
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BRCA1 promoter methylation to predict sensitivity to ovarian tumor’s susceptibility to PARP inhibitors are instructive: 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 chemo sensitivity 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. However, Phase 2 studies of monotherapy gemcitabine showed minimal activity in an unselected population in both treatment naïve and patients with refractory CRC. 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. These patients were not molecularly characterized.

In our trial, patients received intravenous gemcitabine 800 mg/m² on days 1 and 8 and docetaxel 70 mg/m² 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 and the sequence of gemcitabine followed by docetaxel showed objective responses 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. 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. 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.
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.40 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 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.

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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.

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**SUPPORTING INFORMATION**

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

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