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Association of *CHFR* Promoter Methylation with Treatment Outcomes of Irinotecan-Based Chemotherapy in Metastatic Colorectal Cancer

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Abstract

Aberrant promoter methylation plays a vital role in colorectal carcinogenesis. However, its role in treatment responses is unclear, especially for metastatic disease. Here, we investigated the association between promoter methylation and treatment outcomes of irinotecan-based chemotherapy in 102 patients with metastatic colorectal cancer. Promoter methylation was examined by methylation-specific polymerase chain reaction for three loci (*CHFR*, *WRN*, and *SULF2*) associated with chemotherapy response and five CpG island methylator phenotype (CIMP)–specific markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*). Association between *CHFR* methylation and *in vitro* sensitivity to irinotecan was also evaluated. Promoter methylation of *CHFR*, *WRN*, and *SULF2* was identified in 16 (15.7%), 24 (23.5%), and 33 (32.4%) patients, respectively. CIMP status was positive in 22 (21.6%) patients. *CHFR* methylation was associated with a significantly longer time to progression (TTP) (median: 8.77 vs. 4.43 months, $P = .019$), with trends favoring higher overall survival (OS) (median: 22.83 vs. 20.17 months, $P = .300$) and response rates (31.3% vs. 17.4%, $P = .300$). For patients with unmethylated *CHFR*, TTP (median: 5.60 vs. 3.53, $P = .020$) and OS (median: 20.57 vs. 9.23, $P = .006$) were significantly different according to CIMP status. Colorectal cancer cell lines with *CHFR* methylation demonstrated increased sensitivity to irinotecan. Both *CHFR* overexpression and combination with 5-aza-2′-deoxycytidine reversed irinotecan sensitivity in *CHFR*-methylated cell lines, whereas *CHFR* knockdown in unmethylated cells restored sensitivity to irinotecan. These data suggest that *CHFR* methylation may be associated with favorable treatment outcomes of irinotecan-based chemotherapy in patients with metastatic colorectal cancer.

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide [1]. Since the adoption of anti-EGFR and anti-VEGF(R) antibodies in 2004, the median survival time of patients with metastatic CRC has reached 30 months [2]. However, despite the recent advances in molecular targeted therapy as well as

Abbreviations: 5-Aza-CdR, 5-Aza-2′-deoxycytidine; CCLE, the Cancer Cell Line Encyclopedia; CIMP, CpG island methylator phenotype; EGFR, epidermal growth factor receptor; MSP, methylation-specific polymerase chain reaction; OS, overall survival; TCGA, The Cancer Genome Atlas; TTP, time to progression; VEGF, vascular endothelial growth factor

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immunotherapy, cytotoxic chemotherapy with fluoropyrimidines and oxaliplatin and/or irinotecan remains the mainstay of therapy and is responsible for the majority of survival gain in metastatic CRC. Therefore, prediction of treatment response or resistance to cytotoxic chemotherapy is a highly significant and clinically relevant issue to further improve the treatment outcomes of patients with metastatic CRC.

Irinotecan, a topoisomerase I inhibitor, is one of the major chemotherapeutic agents for metastatic CRC along with fluoropyrimidines and oxaliplatin. However, only 30%-40% of the patients show an objective response to irinotecan, and there is currently no established biomarker predictive of clinical benefit from irinotecan chemotherapy.

CRC is known to have an abundance of aberrant promoter methylations [3], and methylation status has been studied for potential correlations with treatment outcomes of CRC in this regard [4–15]. Patients with the CpG island methylator phenotype (CIMP), reflecting extensive promoter methylation, were found to benefit from irinotecan chemotherapy in the adjuvant setting. A recent study also demonstrated that the treatment outcomes of metastatic CRC differed significantly according to CIMP status, adding a further prognostic role for CIMP status besides stage II and III CRC [8]. In this study, while CIMP-high metastatic CRC was associated with poorer progression-free survival and overall survival (OS) for oxaliplatin-based first-line chemotherapy, the correlation between CIMP status and treatment outcomes was less prominent for irinotecan-based second-line treatment [8]. As CIMP is one of the key molecular pathways in CRC carcinogenesis and the association between CIMP status and irinotecan response remains unclear, we aimed to further investigate the implications of CIMP in patients with metastatic CRC treated with irinotecan [16].

In addition to CIMP status, silencing by promoter methylation in individual genes has also been suggested to induce resistance or response to irinotecan [15,17]. Checkpoint with forkhead and ring finger domains (*CHFR*) encodes the E3 ubiquitin-protein ligase CHFR and has been identified as a mitotic stress checkpoint and tumor suppressor gene. *CHFR* is frequently inactivated by promoter CpG island methylation in CRC [18–20]. *CHFR* methylation was associated with reduced survival in stage II and III CRC [21,22] and was suggested to be associated with enhanced sensitivity to taxanes in CRC, non–small cell lung cancer, and gastric cancer [23–27]. Although *CHFR* methylation has not been directly evaluated in conjunction with irinotecan therapy, recent studies have found that CHFR plays an important role in the early stage of the DNA damage response [28,29]. As *CHFR* is highly methylated in CRC and a well-coordinated DNA damage response pathway is required for the repair of irinotecan-induced cellular damage, we postulated that *CHFR* may be involved in the therapeutic response to irinotecan in addition to taxanes.

Werner syndrome RecQ-like helicase (*WRN*), known as a tumor suppressor gene with exonuclease function, was reported to be frequently inactivated epigenetically and to correlate with mucinous differentiation in CRC [12,13,30]. *WRN* methylation has been predicted to enhance topoisomerase inhibitor activity by abrogation of its exonuclease function, and the association of *WRN* methylation and irinotecan response was suggested in a few studies with a relatively small number of patients with colorectal, gastric, and cervical cancers [13,31,32].

Heparan sulfate 6-O-endosulfatase gene (*SULF2*) encodes an oncoprotein with heparin-degrading endosulfatase activity, which activates receptor tyrosine kinases and downstream pathways including MAPK, AKT, and WNT [33]. While the role of *SULF2* methylation has been mostly unknown in CRC, *SULF2* methylation was associated with irinotecan sensitivity in patients with gastric cancer [32]. In addition, *SULF2* silencing increased sensitivity to topoisomerase 1 inhibitors via increased expression of interferon-inducible genes, including *ISG15,* in non–small cell lung cancer [34].

In this study, we aimed to investigate the association of treatment outcomes for irinotecan-based systemic chemotherapy with methylation in *CHFR*, *WRN*, and *SULF2* as well as CIMP status in patients with metastatic CRC.

Patients and Methods

Patients and Irinotecan-Based Systemic Chemotherapy

Patients who underwent surgical resection of CRC at the National Cancer Center (NCC), Korea, from 2001 to 2004 were eligible for this retrospective biomarker study if the following criteria were met: pathologically confirmed diagnosis of colorectal adenocarcinoma, age ≥19 years, synchronous or metachronous metastasis, systemic chemotherapy with one of the irinotecan-containing regimens, tumor tissues available at NCC Tumor Bank/Pathology Department, and presence of evaluable lesion(s) before initiation of irinotecan-containing chemotherapy. This study protocol was reviewed and approved by the Institutional Review Board of NCC (IRB No: NCC2014-0075). The study was conducted in accordance with the recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

Patients were treated with one of the following irinotecan-containing chemotherapy regimens: FOLFIRI [irinotecan (180 mg/m^2 i.v.), leucovorin (200 mg/m^2 i.v. on day 1), and 5-fluorouracil (400 mg/m^2 i.v. bolus followed by 2400 mg/m^2 continuous i.v. over 46 hours on day 1) every 2 weeks]; XELIRI [irinotecan (250 mg/m^2 i.v. on day 1) and capecitabine (1000 mg/m^2 p.o. twice a day for 2 weeks) every 3 weeks]; IFL [irinotecan (125 mg/m^2 i.v.), leucovorin (20 mg/m^2 i.v. on day 1), and 5-fluorouracil (500 mg/m^2 i.v. on day 1) weekly for 4 weeks, every 6 weeks]; and irinotecan alone [irinotecan (350 mg/m^2 i.v. on day 1) every 3 weeks]. At the physician's discretion, bevacizumab (5 mg/kg i.v. on day 1 of each cycle of FOLFIRI or 7.5 mg/kg i.v. on day 1 of each cycle of XELIRI) or cetuximab (400 mg/m^2 i.v. on day 1 and 250 mg/m^2 on day 8 and weekly thereafter) was combined with the cytotoxic chemotherapy. Computed tomography was performed after every four cycles for biweekly regimens and after three cycles for three-weekly regimens during the chemotherapy period or earlier if disease progression was suspected. Disease progression was defined based on the computed tomographic findings.

Methylation Analyses

Analysis of DNA methylation was performed as described previously [35]. Genomic DNA samples from the tumor and adjacent normal tissue were bisulfite-modified using the EZ DNA methylation kit (Zymo Research, Orange, CA) and analyzed for methylation in five CIMP-specific CpG island loci (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, *SOCS1*), as well as *CHFR*, *WRN*, and *SULF2* using a methylation-specific polymerase chain reaction (MSP) method. Briefly, 1 μg of DNA was denatured using sodium hydroxide, modified by sodium bisulfite, treated again with sodium hydroxide, precipitated with ethanol, and resuspended in water. For each locus, two primer pairs were used for the MSP analysis; the first recognizes and anneals to methylated sequences only, whereas the second set anneals to and amplifies unmethylated alleles. The detailed primer information is provided in Supplementary material 1. The

PCR products were then purified using the Wizard DNA purification resin (Promega, Madison, WI). Each PCR product was directly loaded on an 8% acrylamide gel, stained with ethidium bromide, and visualized under UV illumination. CIMP status was considered positive when at least three methylated promoters were identified and as negative when zero to two methylated promoters were identified.

The Cancer Genome Atlas (TCGA) Data Analysis

CHFR DNA methylation (Illumina Infinium HM27 bead array; HM27) and mRNA expression microarray) data from 223 colorectal adenocarcinoma samples from TCGA project were downloaded through cBioPortal (http://www.cbioportal.org; accessed on Jun. 12, 2018).

Cell Lines and Cell Culture

Human CRC cell lines (RKO, HT-29, HCT-116, SNU-81, SW480, DLD-1, SNU-407, CaCo-2, LoVo, SW620, SNU-C4, and SNU-C5) were obtained from the Korea Cell Line Bank and the American Type Culture Collection. Cell lines were grown in DMEM or RPMI-1640 with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO_2-humidified atmosphere. All cell lines were certified using the GenePrint 10 System (Promega, Madison, WI) by the Omics Core Lab of NCC.

Reagents

Irinotecan HCl trihydrate was purchased from Selleckchem (Houston, TX). The DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-Aza-CdR) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in dimethyl sulfoxide and stored at −20°C.

Growth Inhibition Assays

A colorimetric assay using the tetrazolium salt MTS was used to assess cell proliferation after treatment with irinotecan. Equivalent numbers of cells (5×10^3 cells/well) were incubated in 0.2 ml culture medium in each well. After 1, 2, and 3 days of culture, 0.1 mg MTS solution (Promega, Madison, WI) was added to each well followed by incubation at 37°C for a further 4 hours. Plates were centrifuged at 450×*g* for 5 minutes at room temperature, and the medium was removed. Dimethyl sulfoxide (0.15 ml) was added to each well to solubilize the crystals, and the plates were immediately read at 540 nm using a scanning multiwell spectrometer (Bio-Tek instruments Inc., Winooski, VT). The cell proliferation rate was obtained from three biological replicates, and all experiments were performed three times.

Cancer Cell Line Encyclopedia (CCLE) Data Analysis

Pharmacological profiling data for irinotecan was downloaded for 12 colorectal adenocarcinoma cell lines along with the DNA methylation data from the CCLE (http://portal.broadinstitute.org/ccle; accessed on Jun. 13, 2018)

Establishment of CHFR-Overexpressing Cells Using Plasmid DNA Vector

The recombinant plasmid DNA human *CHFR* (target sequence: 5′-GCGATCGCACGCGT-3′) (RC228526) was purchased from OriGene (Rockville, MD). The recombinant plasmid was then transformed into competent *Escherichia coli* cells. The bacteria were cultured, and the recombinant plasmids were extracted and purified using PureLink HiPure Plasmid DNA Purification kits (Invitrogen, Carlsbad, CA). HCT-116 and SNU-C5 cells were plated in six-well plates at a density of 3×10^5 cells per well and incubated overnight. Cells were then transfected with the human *CHFR* vector or a blank control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection was verified by Western blot analysis.

Small Interfering RNA (siRNA)–Mediated Knockdown of CHFR

siRNA against *CHFR* and the control sequence were purchased from Qiagen (Chatsworth, CA). The sequence of the *CHFR*-specific siRNA was 5′-AACCAGAGGTTTGACATGGAA-3′, and AllStars Negative Control siRNA (catalog no. 1027281) was used as the control (nonspecific). siRNA transfection was performed using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 12 μl of 20 nM siRNA solution and 20 nM HiPerFect Transfection Reagent was incubated in 100 ml of serum-free RPMI 1640 medium for 10 minutes to facilitate complex formation. The resulting mixture (final concentration 5 nM) was added to SNU-81 and CaCo-2 cells (1×10^6) and incubated in a 60-mm tissue culture dish with 4 ml of RPMI 1640. The cells were then washed at 0, 24, 48, and 72 hours after transfection.

Western Blotting

Briefly, cell homogenates containing equivalent amounts of protein were centrifuged at 4000×*g*, and the supernatant fractions were subjected to SDS-PAGE. Following electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA) and blocked by incubation for 2 hours at 4°C in 1% Tween 20-TBS buffer containing 1.5% nonfat dry milk (Bio-Rad, Hercules, CA) and 1 mM $MgCl_2$. Membranes were then incubated for 2 hours at room temperature with primary antibodies against CHFR (Santa Cruz bio Technology, Santa Cruz, CA) or β-actin (Cell Signaling Technology, Beverly, MA). Next, the membranes were washed thrice for 15 minutes with blocking solution and incubated with diluted HRP-conjugated secondary antibody (SouthernBiotech, Birmingham, AL) for 1 hour at room temperature. This was followed by washing with blocking solution (thrice for 15 minutes), incubation with WEST-ZOL plus chemiluminescence reagent (iNtRON Biotechnology, Seoul, Korea) for 1 minute, and exposure to film (Kodak Blue XB-1).

Statistical Analyses

The primary aim of the present study was to determine the association of methylation in *CHFR*, *WRN*, and *SULF2*, as well as CIMP status, with time to progression (TTP) of irinotecan-based systemic chemotherapy in patients with metastatic CRC. In addition to TTP, OS and response were analyzed as indicators of treatment outcomes with irinotecan treatment. TTP was defined as the time interval from the date of treatment initiation to the date of disease progression. OS was calculated from the date of treatment initiation to the date of death from any cause. Categorical variables were compared using Pearson's χ^2 test or Fisher's exact test, and continuous variables were compared using Mann-Whitney test. The Kaplan-Meier method was used for estimating TTP and OS, and comparisons were made using log-rank tests. To adjust for baseline characteristics, we performed multivariate analyses with a Cox proportional hazard model using a forward conditional variable selection method. Age (continuous variable), sex, differentiation (well-differentiated to moderately differentiated vs. poorly differentiated), tumor location (proximal vs. distal), number of metastatic

organs (1-2 vs. ≥3), serum carcinoembryonic antigen (CEA) levels (≤5 vs. >5 ng/ml), *BRAF* mutation status, *hMLH1*/*hMSH2* (proficient vs. deficient), and number of prior systemic chemotherapy lines in the metastatic setting were included as covariates based on previous studies on metastatic CRC [8]. Two-sided *P* values < .05 were considered statistically significant. Statistical analyses for the clinical study were conducted using R-3.3.4 software, and analyses for *in vitro* study were performed with GraphPad Prism version 7.0.0 for Windows (GraphPad Software, La Jolla, CA). This study was analyzed and reported according to the Reporting Recommendations for Tumor Marker Prognostic Studies [36].

Results

Patient Characteristics and Irinotecan-Based Chemotherapy

In total, 102 patients were included in this study (Supplementary material 2). The patient characteristics are described in Table 1. According to the inclusion criteria, all patients had undergone resection of the primary tumor. All surgical specimens used for this study were collected from the primary tumors before the initiation of irinotecan treatment and therefore do not reflect the potential effects from subsequent treatments. Twenty-one patients (20.6%) exhibited proximal (cecum to transverse colon) lesions, and 81 (79.4%) exhibited distal lesions. In total, 73 patients (71.6%) presented with synchronous metastatic disease and 29 (28.4%) with metachronous metastasis. Irinotecan-based chemotherapy was administered as first-line therapy in 51 patients (50.0%), second-line in 38 (37.3%), and ≥third-line in 13 patients (12.7%). The irinotecan-containing chemotherapy regimens include FOLFIRI in 44 patients (43.1%), IFL in 15 (14.7%), XELIRI in 21 (20.6%), and irinotecan as a single agent in 22 patients (21.6%). Bevacizumab or cetuximab was added for five and four patients, respectively. The TTP following irinotecan therapy was 5.57 months (95% CI, 3.61-7.52) in the entire population. At the time of last follow-up (March 2018), all 102 patients had died. The median OS after the initiation of irinotecan-based chemotherapy was 20.4 months (95% CI, 18.0-22.8).

CHFR Methylation and Irinotecan Treatment Outcomes

Promoter methylation in *CHFR*, *WRN*, and *SULF2* was observed in 16 (15.7%), 24 (23.5%), and 33 (32.4%) patients, respectively. Twenty-two (21.6%) patients exhibited at least three methylated CIMP-specific loci and were defined as CIMP-positive. Among the five CIMP-specific loci, *NEUROG1* was the most frequently methylated locus (63 patients; 61.8%), followed by *CACNA1G* (41; 40.2%), *IGF2* (28; 27.5%), *SOCS1* (16; 15.7%), and *RUNX3* (16; 15.7%). In our analysis, *CHFR* and *WRN* methylation was significantly associated with CIMP positivity, whereas *SULF2* methylation was not (Supplementary Material 3). *CHFR* methylation was closely associated with the *WRN* methylation as well. We confirmed a significant increase in *CHFR* methylation level according to CIMP subtypes using the TCGA dataset (Supplementary material 3). The *CHFR*-methylated group showed more frequent mutations in *KRAS*, *NRAS*, or *BRAF* compared to the unmethylated group (62.5% vs. 31.2%; *P* = .024). However, other clinicopathological characteristics including tumor location, *BRAF* mutation, and MMR status were not associated with methylation of *CHFR*, *WRN*, *SULF2,* or the CIMP status in our analysis (Table 1).

TTP after the initiation of irinotecan-containing chemotherapy was significantly different according to *CHFR* methylation status (*P* = .019; Figure 1*A*). The median TTP was 8.77 months (95% CI, 6.81-10.73) for the *CHFR*-methylated group and 4.43 months (95% CI, 3.09-5.78) for the unmethylated group. OS showed a trend towards the *CHFR*-methylated group, but the difference was not significant (*P* = .300; Figure 1*B*). The median OS was 22.83 months (95% CI, 13.03-32.63) for the *CHFR*-methylated group and 20.17 months (95% CI, 17.65-22.68) for the unmethylated group. The response rate was 31.3% vs. 17.4% for patients with methylated and unmethylated *CHFR*, respectively (*P* = .278).

TTP was marginally significant favoring the *WRN*-methylated group (*P* = .050; Supplementary material 4). The median TTP was 7.03 months (95% CI, 1.89-12.2) vs. 5.43 (95% CI, 3.32-7.55) for the *WRN*-methylated and unmethylated groups. There was no difference in OS based on *WRN* methylation. The treatment outcomes in terms of TTP and OS following irinotecan treatment were not different based on *SULF2* methylation or CIMP status (Supplementary Material 4).

Table 1. Patient Characteristics and *CHFR* Methylation

	Total (%)	*CHFR* Methylated (%)	*CHFR* Unmethylated (%)	*P*
Total	102 (100)	16 (100)	86 (100)	
Age				.550
Median (range)	56 (27-78)			
<65 years	75 (73.5)	13 (81.2)	62 (72.1)	
≥65 years	27 (26.5)	3 (18.8)	24 (27.9)	
Sex				>.999
Male	68 (66.7)	11 (68.8)	57 (66.3)	
Female	34 (33.3)	5 (31.2)	29 (33.7)	
Location				.737
Right-sided	21 (20.6)	4 (25.0)	17 (19.8)	
Left-sided	81 (79.4)	12 (75.0)	69 (80.2)	
Histology				.950
Nonmucinous	92 (90.2)	15 (93.8)	77 (89.5)	
Mucinous	10 (9.8)	1 (6.2)	9 (10.5)	
Differentiation				.732
Well to moderate	82 (80.4)	14 (87.5)	68 (79.1)	
Poor	20 (19.6)	2 (12.5)	18 (20.9)	
KRAS/*NRAS*				.138
Wild type	65 (68.5)	8 (50.0)	57 (66.3)	
Mutant	30 (31.6)	8 (50.0)	22 (25.6)	
BRAF status				.073
Wild type	92 (96.8)	14 (87.5)	78 (98.7)	
Mutant	3 (3.2)	2 (12.5)	1 (1.3)	
hMLH1/*hMSH2*				>.999
Proficient	98 (96.1)	16 (100.0)	82 (95.3)	
Deficient	4 (3.9)	0 (0.0)	4 (4.7)	
Presentation				>.999
Synchronous	73 (71.6)	12 (75.0)	61 (70.9)	
Metachronous	29 (28.4)	4 (25.0)	25 (29.1)	
No. metastatic organs				.710
1	50 (49.0)	7 (43.8)	43 (48.8)	
2	39 (38.2)	6 (37.5)	33 (38.4)	
≥3	14 (13.7)	3 (18.8)	11 (12.8)	
CEA				.891
<5.0 ng/ml	27 (26.5)	5 (31.2)	22 (25.9)	
≥5.0 ng/ml	74 (72.5)	11 (68.8)	63 (74.1)	
No. prior treatment(s)				.710
0	51 (50.0)	7 (43.8)	44 (51.2)	
1	38 (37.3)	6 (37.5)	32 (37.2)	
≥2	13 (12.7)	3 (18.8)	10 (11.6)	
Irinotecan regimen				.658
FOLFIRI	44 (43.1)	6 (37.5)	38 (44.2)	
IFL	15 (14.7)	4 (25.0)	11 (12.8)	
XELIRI	21 (20.6)	3 (18.8)	18 (20.9)	
Irinotecan alone	22 (21.6)	3 (18.8)	19 (22.1)	

P = .024 for *KRAS*/*NRAS*/*BRAF* mutation frequency and *CHFR* methylation (62.5% vs. 31.2% for *CHFR* methylated and unmethylated groups, respectively)

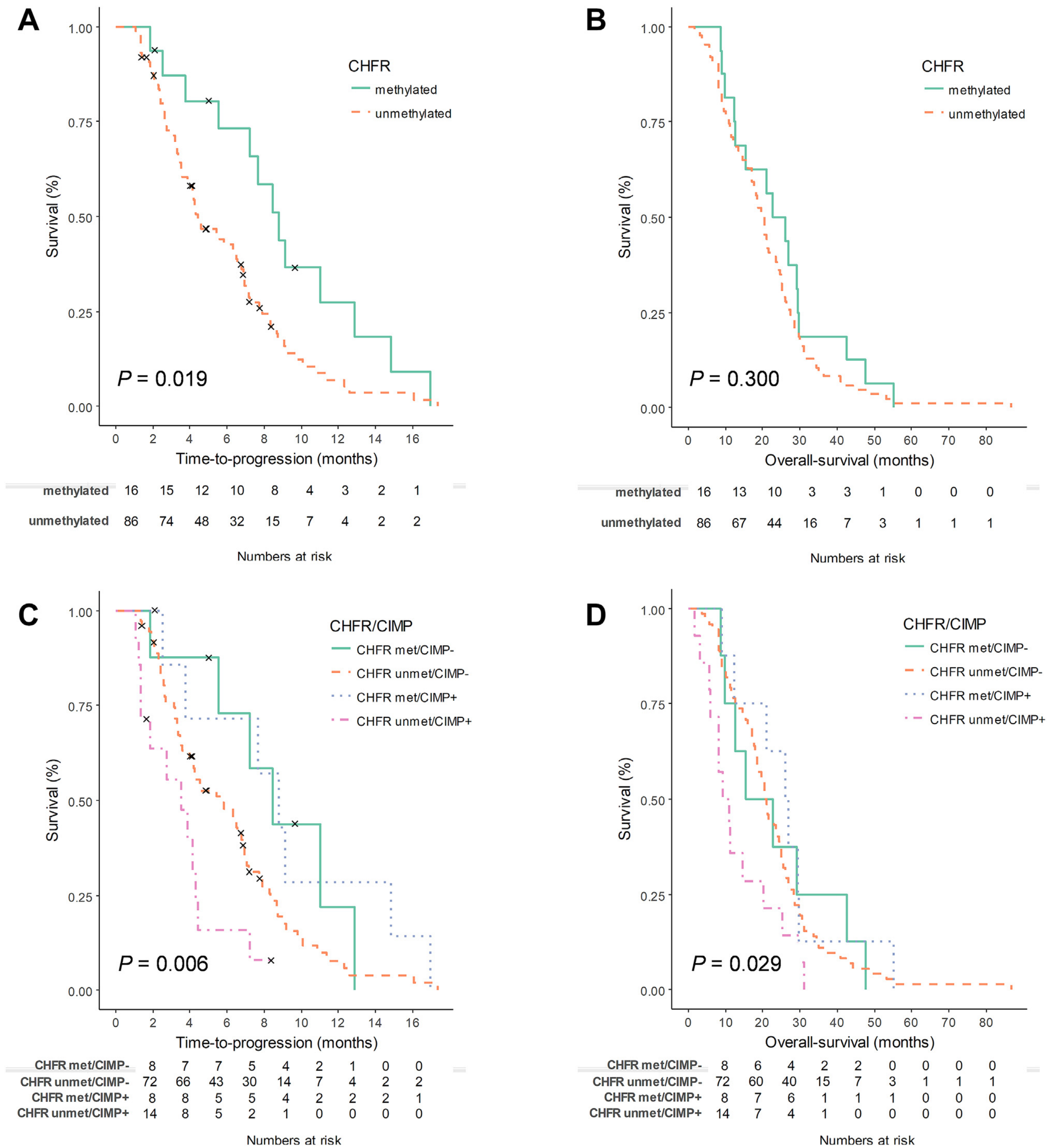

Figure 1. (A-B) Kaplan-Meier curves for TTP and OS of irinotecan treatment according to *CHFR* methylation. (C-D) Kaplan-Meier curves for TTP and OS of irinotecan treatment according to *CHFR* methylation and CIMP status.

Table 2. Multivariate Analyses for TTP and OS According to *CHFR* Status

TTP			OS		
Covariates in the Final Model	Adjusted HR (95% CI)	*P*	Covariates in the Final Model	Adjusted HR (95% CI)	*P*
CHFR unmethylated	2.88 (1.50-5.52)	.001	CEA >5.0 ng/ml	2.33 (1.42-3.80)	.01
No. prior treatments		<.001	No. prior treatments		.002
1	2.78 (1.60-4.86)	<.001	1	2.78 (1.60-4.86)	<.001
≥2	3.43 (1.69-6.97)	<.001	≥2	3.43 (1.69-6.97)	<.001
			hMLH/*hMSH2* deficient	4.49 (1.02-19.66)	.046
			Metastatic organs ≥3	2.28 (1.19-4.37)	.013

Multivariate analyses were performed by Cox regression analysis with forward conditional selection method adjusted for age (continuous), sex, differentiation (well to moderately differentiated vs. poorly differentiated), tumor location (right vs. left), metastatic organs (1-2 vs. ≥3), CEA level (≤5 vs. >5.0 ng/ml), *BRAF* mutation, *hMLH1*/*hMSH2*, number of prior treatment(s), promoter methylation, and CIMP status.

Table 3. Multivariate Analyses for TTP and OS According to *CHFR* and CIMP Status

TTP			OS		
Covariates in the Final Model	Adjusted HR (95% CI)	*P*	Covariates in the Final Model	Adjusted HR (95% CI)	*P*
CHFR/CIMP status		<.001	*CHFR*/CIMP status		<.001
CHFR unmethylated/CIMP negative	2.43 (1.23-4.81)	.011	*CHFR* unmethylated/CIMP negative	3.94 (2.02-7.69)	<.001
CHFR unmethylated/CIMP positive	6.45 (2.66-15.63)	<.001	*CHFR* unmethylated/CIMP positive	4.72 (2.10-10.64)	<.001
No. prior treatment(s)		<.001	No. prior treatment(s)		.001
1	2.77 (1.58-4.84)	<.001	1	0.98 (0.59-1.63)	.935
≥2	3.84 (1.87-7.88)	<.001	≥2	3.42 (1.78-6.60)	<.001
			CEA >5.0 ng/ml	2.80 (1.66-4.72)	<.001

Multivariate analyses were performed by Cox regression analysis with forward conditional selection method adjusted for age (continuous), sex, differentiation (well to moderately differentiated vs. poorly differentiated), tumor location (right vs. left), metastatic organs (1-2 vs. ≥3), CEA level (≤5 vs. >5.0 ng/ml), *BRAF* mutation, *hMLH1*/*hMSH2*, number of prior treatment(s), and *CHFR*/CIMP status.

In the multivariate analyses for TTP and OS of irinotecan treatment, *CHFR* methylation was confirmed to be significantly associated with TTP after adjustment for potential confounding factors [adjusted HR, 2.88 (95% CI, 1.50-5.52), *P* = .001; Table 2], whereas methylation of *WRN* and *SULF2*, and the CIMP status were not. Methylation of *CHFR*, *WRN*, *SULF2*, and the CIMP status were not significant factors in the multivariate analysis for OS.

As *CHFR* methylation was associated with CIMP-positive status, the association of *CHFR*/CIMP status was also analyzed for TTP and OS. TTP and OS were significantly different according to the *CHFR*/

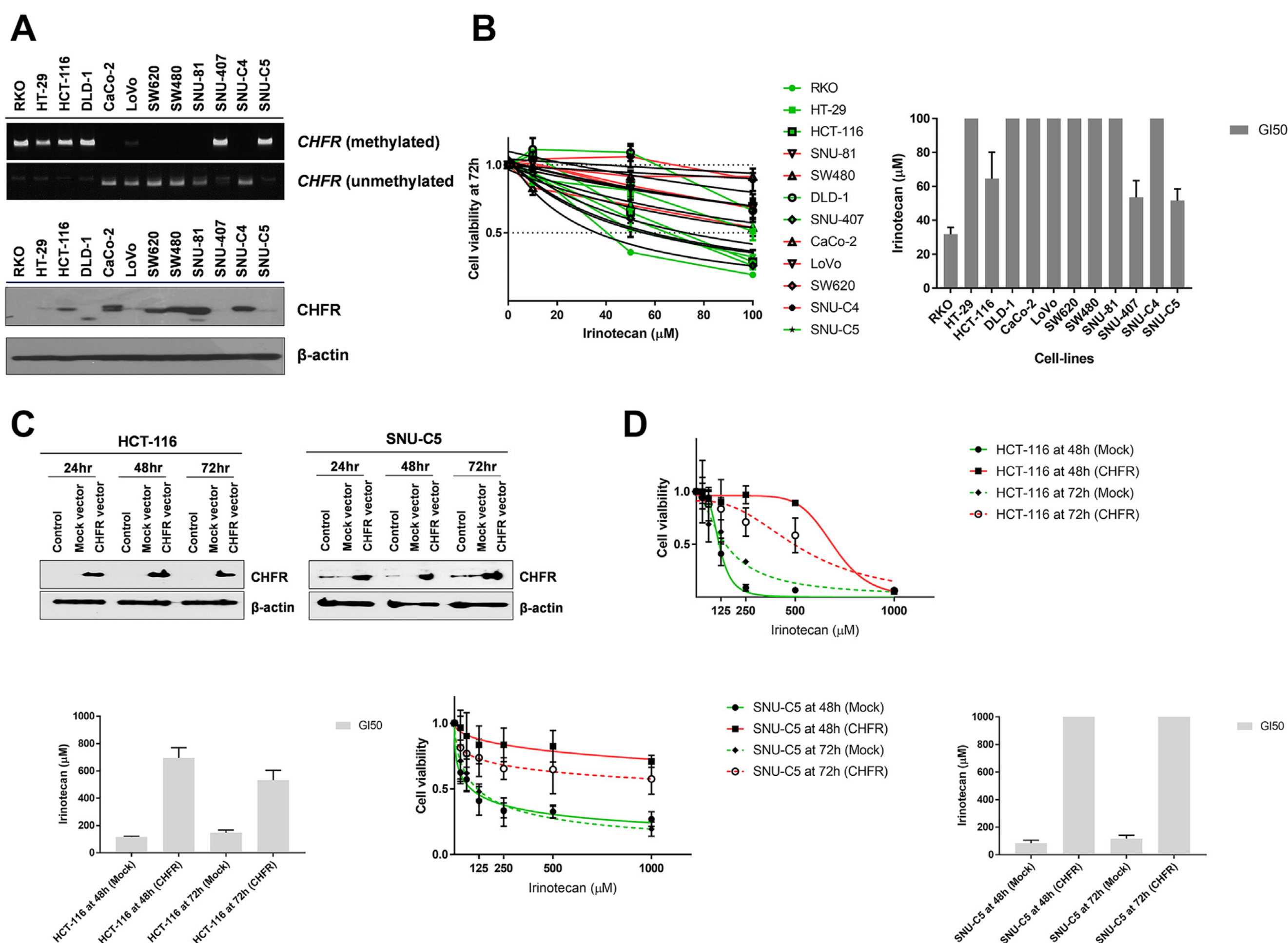

Figure 2. (A) *CHFR* promoter methylation in 12 colorectal cancer cell lines as determined by methylation-specific PCR assays (upper panel). CHFR protein expression as determined by Western blotting (lower panel). (B) *In vitro* sensitivity of 12 colorectal cancer cell lines to increasing concentrations of irinotecan as determined by MTS cell proliferation assays. Cell growth curves (green, *CHFR*-methylated; red, unmethylated) are based on three independent experiments performed in triplicate (left panel). Nonlinear fit curves for each cell line are shown in black. GI50 values for each cell line are shown as bars representing the mean ± SEM (right panel). (C) Overexpression of *CHFR* in *CHFR*-methylated cell lines (HCT-116 and SNU-C5) was confirmed by Western blotting. (D) Irinotecan sensitivity after *CHFR* overexpression in HCT-116 and SNU-C5. Cell growth curves and GI50 values were determined at 48 and 72 hours after *CHFR* overexpression following treatment with increasing concentrations of irinotecan. Values are based on three independent experiments performed in triplicate.

CIMP status (P = .006 for TTP, Figure 1*C*; P = .029 for OS, Figure 1*D*). For patients with unmethylated *CHFR*, TTP and OS were significantly different according to the CIMP status. The median TTP was 5.60 months (95% CI, 3.40-7.81) for the *CHFR*-unmethylated/CIMP-negative group and 3.53 months (95% CI, 1.60-5.47) for the *CHFR*-unmethylated/CIMP-positive group (P = .020). The median OS was 20.57 months (95% CI, 18.52-22.61) for the *CHFR*-unmethylated/CIMP-negative group and 9.23 months (95% CI, 4.28-14.18) for the *CHFR*-unmethylated/CIMP-positive group (P = .060). In the multivariate analyses for TTP and OS, *CHFR*/CIMP status was found to be significantly associated with TTP and OS after adjustment for potential confounding factors (Table 3).

CHFR Methylation and In Vitro Sensitivity to Irinotecan

Irinotecan sensitivity *in vitro* based on *CHFR* methylation status was evaluated in 12 CRC cell lines. When *CHFR* promoter methylation status was assessed with MSP, six cell lines (RKO, HT-29, HCT-116, DLD-1, SNU-407, and SNU-C5) showed *CHFR* methylation, whereas five cell lines (CaCo-2, SW-620, SW-480, SNU-81, SNU-C4) had an unmethylated promoter (Figure 2*A*). *CHFR* was partially methylated in LoVo cells. Western blot analysis revealed that protein expression levels of CHFR were inversely correlated with *CHFR* methylation status (Figure 2*A*). We confirmed the inverse correlation between *CHFR* methylation and mRNA expression in clinical samples as well using the TCGA dataset (Pearson correlation coefficient = −0.738, P < 0.001; Supplementary Material 5).

Cell proliferation assay demonstrated that CRC cell lines with *CHFR* methylation were more sensitive to irinotecan treatment compared to those with unmethylated *CHFR* (Figure 2*B*). Notably, all four cell lines with GI50 values <100 μM for irinotecan had methylated *CHFR* (RKO, SNU-C5, SNU-407, and HCT-116). We also compared the IC50 of 12 CRC cell lines according to *CHFR* methylation status using CCLE data (Supplementary material 6). Although the mean IC50 was lower in *CHFR*-methylated cells (n = 6) than in unmethylated cells (n = 6), the difference between groups was not significant (P = .441).

We then investigated the effect of *CHFR* overexpression on sensitivity to irinotecan in *CHFR*-methylated HCT-116 and SNU-C5 cells. After *CHFR* overexpression (Figure 2*C*), sensitivity to irinotecan measured at 48 and 72 hours was significantly diminished

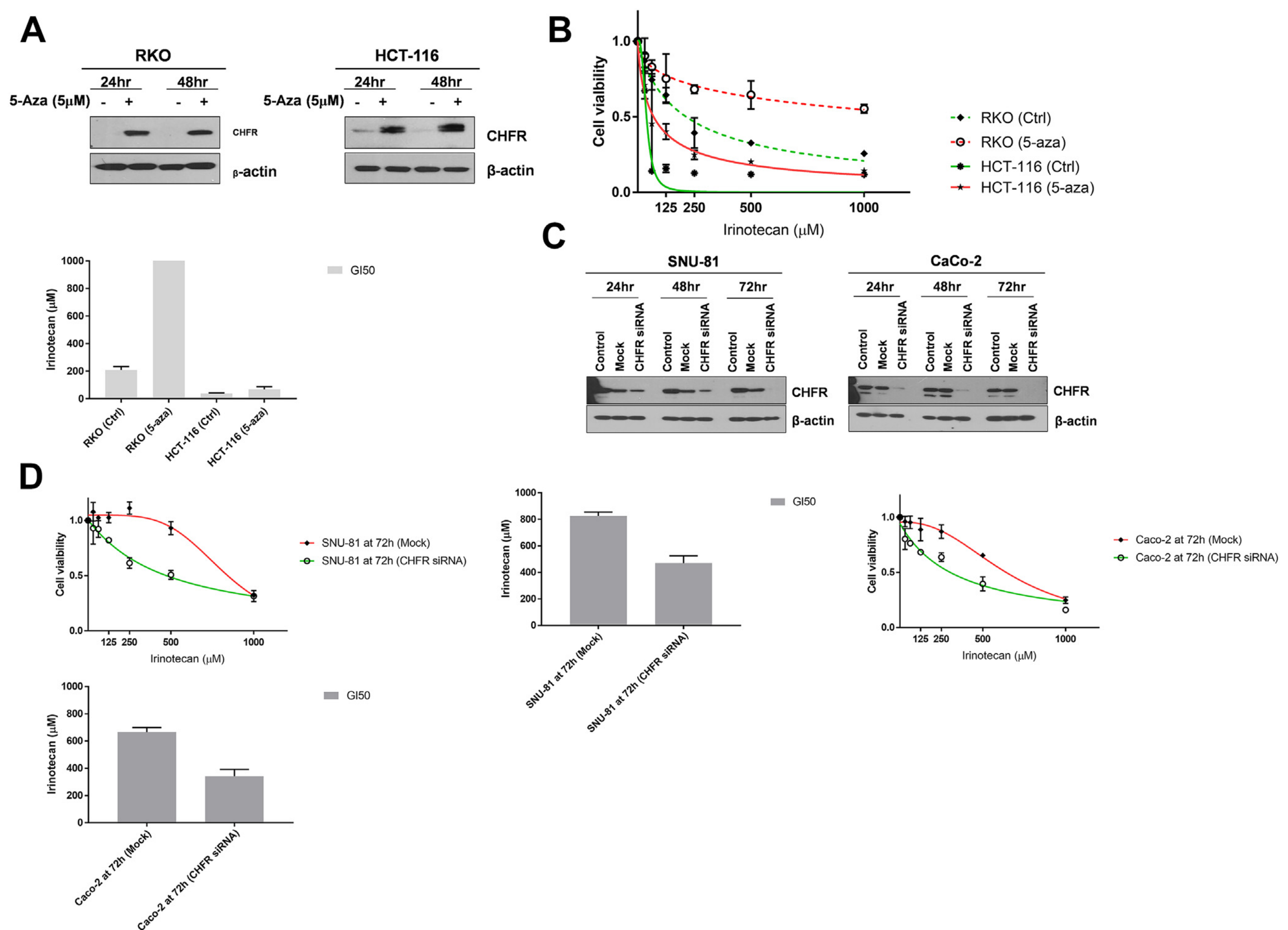

Figure 3. (A) Western blotting for CHFR in *CHFR*-methylated cell lines (RKO and HCT-116) at 24 and 48 hours after 5-Aza-CdR treatment at 5 μM. (B) Irinotecan sensitivity in RKO and HCT-116 cells after 5-Aza-CdR treatment. Cell growth curves and GI50 values were plotted at 72 hours after 5-Aza-CdR treatment following treatment with increasing concentrations of irinotecan. (C) Western blotting for CHFR in *CHFR*-unmethylated cell lines (SNU-81 and CaCo-2) after *CHFR* knockdown with siRNA at 24, 48, and 72 hours. (D) Irinotecan sensitivity in SNU-81 and CaCo-2 cells after *CHFR* knockdown. Cell growth curves and GI50 values were plotted at 72 hours after *CHFR* knockdown following treatment with increasing concentrations of irinotecan. All values are based on three independent experiments performed in triplicate.

in both cell lines compared to their parental cells (Figure 2*D*). We also compared the viability of *CHFR*-methylated cell lines (RKO and HCT-116) with increasing concentrations of irinotecan along with or without 5-Aza-CdR (5 μM), a DNA-demethylating agent (Figure 3, *A* and *B*). Both RKO and HCT-116 cells showed *CHFR* upregulation after 5-Aza-CdR treatment (Figure 3*A*), and combination with 5-Aza-CdR decreased the growth inhibitory potential of irinotecan in all three cell lines (Figure 3*B*).

We then tested the effect of *CHFR* knockdown on irinotecan sensitivity in *CHFR*-unmethylated cell lines (Figure 3, *C* and *D*). Both SNU-81 and CaCo-2 cell lines demonstrated loss of *CHFR* expression at 72 h after siRNA treatment (Figure 3*C*), and GI50 values were significantly lower in cell lines subjected to *CHFR* knockdown compared to their parental cells (Figure 3*D*).

Discussion

In this study, we found that *CHFR* methylation was predictive of favorable treatment outcomes in terms of TTP in patients with metastatic CRC treated with irinotecan-based systemic chemotherapy. We further expanded our study *in vitro* and confirmed that CRC cell lines with *CHFR* methylation were more susceptible to irinotecan compared to those without methylation and that sensitivity to irinotecan could be modulated negatively by *CHFR* upregulation or positively by downregulating *CHFR*. In addition, irinotecan treatment outcomes differed according to CIMP status in patients with unmethylated *CHFR*. To the best of our knowledge, this is the first report on the clinical impact of *CHFR* solely focused on metastatic CRC and suggests *CHFR* methylation as a biomarker for irinotecan-based chemotherapy in patients with CRC.

CHFR was methylated in 15.7% of patients with metastatic CRC in our study, and this was lower than in previous studies reporting *CHFR* methylation in 31%-63% of patients, mostly with stage I-III CRC [18,19,21,22,37]. When we analyzed the TCGA dataset, *CHFR* methylation levels did not differ by tumor stage in patients with CRC (P = .511 by ANOVA). Therefore, the lower prevalence of *CHFR* methylation in this study may be explained by differences in methylation assay or patient selection criteria. The correlation of *CHFR* with chemotherapy response or resistance has been mostly conducted for taxanes such as paclitaxel or docetaxel [23,24,27,38]. This is because *CHFR* is known to encode a checkpoint protein that delays entry into metaphase [39]. Hence, the antitumor activity of microtubule inhibitors like taxanes could be boosted in cells with *CHFR* deficiency. In a previous study, the association of *CHFR* and chemotherapy response was specific to microtubule inhibitors but not to etoposide, a topoisomerase II inhibitor, or to cisplatin [40]. In a study including 20 patients with CIMP-positive metastatic CRC who received nab-paclitaxel, however, treatment outcomes did not differ according to plasma *CHFR* methylation status, suggesting that *CHFR* methylation might not be predictive of taxane sensitivity, at least for patients with CRC. One potential explanation for the association between *CHFR* methylation and irinotecan response demonstrated in our study comes from the fact that *CHFR* was first described to function as an E3 ubiquitin ligase, which ubiquitinates and targets proteins for degradation by the S26 proteasome [41]. One of the cellular mechanisms of irinotecan resistance is the repair of irinotecan-induced DNA damage [17]. When the reversible Topo-I-irinotecan-DNA cleavable complex formed by irinotecan treatment collides with the advancing replication fork, Topo-I, the cellular target of irinotecan, is degraded through an ubiquitin/26S proteasome-dependent system, and this facilitates the repair of single-strand breaks, thereby evading irinotecan-induced cellular damage. If the level of CHFR protein, a ubiquitin ligase, is decreased by DNA methylation, ubiquitination of Topo-I by 26S proteasome is impaired, and the subsequent upregulation of Topo-I could again render cancer cells more sensitive to irinotecan-induced cellular damage. This hypothesis is difficult to validate using clinical samples collected at a single time point and is also difficult to demonstrate using public data such as TCGA dataset. In fact, when we correlated *CHFR* methylation and *TOP1* mRNA expression levels using the TCGA dataset, there was no apparent correlation between them (data not shown).

We demonstrated that *CHFR* methylation was closely associated with CIMP status in our clinical samples as well as in TCGA dataset. Although OS showed a trend favoring the CIMP-negative group (P = .268 for OS after irinotecan treatment) in our cohort, the difference in TTP or OS based on CIMP status was not apparent in the overall population treated with irinotecan. We speculate that the different methodologies used in this study (MSP) and the previous one (MethyLight assay) or differences in patient selection criteria (irinotecan-based chemotherapy) may at least partially contribute to these results. However, in patients with unmethylated *CHFR*, TTP was poorer in the CIMP-positive group than in the CIMP-negative group, and multivariate analysis confirmed that the combination of *CHFR*/CIMP was a significant factor for both TTP and OS. Therefore, the significance of *CHFR* methylation in the treatment response to irinotecan may have weakened the prognostic impact of CIMP in our study cohort.

In our study cohort, *WRN* was found to be hypermethylated in 23.5% of patients with metastatic CRC, and this rate was lower than the ~40% reported previously [5,13]. Although DNA hypermethylation in a specific gene has long been regarded to reflect decreased mRNA and protein expression in the corresponding gene, it may not be correlated with repressive gene expression in some cases because of the complex regulation of gene expression besides DNA methylation [42]. Although we included *WRN* methylation as one of the potential biomarkers in this study, *WRN* methylation was marginally correlated with TTP, and the correlation was not maintained in a multivariate analysis. Recently, it was reported that *WRN* methylation was not predictive of good clinical outcomes in 93 patients with metastatic CRC treated with irinotecan and capecitabine [5]. Remarkably, *WRN* mRNA and protein expression levels were independent of *WRN* methylation status in the study, which was also confirmed with TCGA data. We suspect that this absence of correlation between *WRN* methylation and mRNA or protein levels might be the main reason for the lack of correlation in *WRN* methylation with treatment outcome in our study.

Currently, most of the irinotecan-based systemic chemotherapy is given in combination with anti-EGFR antibodies or anti-VEGF/VEGFR antibodies because combination chemotherapy in both forms have been proven to provide survival benefit in the first-line (cetuximab, panitumumab, bevacizumab) as well as second-line (bevacizumab, ziv-aflibercept, ramucirumab) and third-line settings (cetuximab) when compared to irinotecan-based cytotoxic chemotherapy alone. In our study, we selected patients that had been treated in the era when there was limited access to combination with molecular targeted agents. In fact, most (93/102 patients, 91.2%) of the patients in our study cohort were treated with irinotecan-based systemic chemotherapy alone. Although this selection was not intended and was mainly because there were limitations to the archival tumor tissues collected after 2005 made by

domestic legal provisions when this study was planned, we believe that this selection allowed us to better define the pure impact of irinotecan on patient outcomes.

Our study has the following limitations. First, we did not validate our findings in a separate cohort. However, the preclinical observation of our study is fully in agreement with the clinical findings, and the association of *CHFR* and irinotecan could be mechanistically explainable. Although our findings are not confirmative and are more likely to be hypothesis generating, we believe that these points make our study more generalizable and worthy of further investigation in a larger study cohort. Second, in the absence of another treatment arm, we could not verify that *CHFR* methylation was predictive or prognostic in nature. However, the changes shown after overexpression and knockdown of *CHFR* in the preclinical study suggest that *CHFR* methylation is more likely to be predictive of irinotecan treatment. Third, mainly because of tissue availability, a limited number of patients heterogeneous in their clinical characteristics, including treatment lines, were included. In our study, number of treatment lines was one of the most significant factors in univariate analyses for both TTP and OS, and the association of *CHFR* methylation or CIMP status with treatment outcomes was more prominent when stratified by number of prior treatment(s). Therefore, the correlation between *CHFR* methylation and irinotecan sensitivity might have been more evident if we had evaluated a more homogeneous population.

In summary, we report that *CHFR* is recurrently hypermethylated in metastatic CRC and that methylated *CHFR* is associated with better treatment outcomes in terms of TTP in patients with metastatic CRC treated with irinotecan-based systemic chemotherapy. Interestingly, in patients with unmethylated *CHFR*, the CIMP status could further discriminate patient outcomes. The association of *CHFR* and irinotecan treatment outcomes was confirmed in a preclinical study by overexpressing or knocking down *CHFR* and investigating the changes in irinotecan sensitivity accordingly. Considering the major importance of irinotecan-based chemotherapy in patients with metastatic CRC and the absence of a biomarker to guide treatment in these patients, we believe that our findings deserve confirmation in a larger patient cohort and could facilitate patient selection for irinotecan chemotherapy.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.11.010.

Declarations of Interest

None.

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