CHFR-Promoter-Methylation Status Is Predictive of Response to Irinotecan-based Systemic Chemotherapy in Advanced Colorectal Cancer

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Abstract. *Background/Aim:* We investigated whether promoter methylation of the checkpoint-with-forkhead-and-ring-finger-domains (CHFR) gene is a predictor of the efficacy of irinotecan-based systemic chemotherapy for advanced colorectal cancer (CRC) patients. *Materials and Methods:* CHFR-promoter methylation was measured by quantitative methylation-specific PCR (qMSP). The histoculture drug response assay (HDRA) was used in vitro to analyze the correlation between CHFR-promoter methylation and the efficacy of the irinotecan-active-metabolite SN38 in colorectal-cancer tissues from 44 CRC patients. CHFR promoter-methylation was also analyzed for its correlation with clinical response to irinotecan-based systemic chemotherapy of 49 CRC patients. *Results:* CHFR-promoter methylation significantly-positively correlated with inhibition of colon cancer by SN38 in the HDRA ($p=0.002$). CHFR-promoter methylation also significantly-positively correlated with clinical response to irinotecan-based systemic chemotherapy ($p=0.04$ for disease control). CHFR-promoter methylation also significantly-positively correlated ($p=0.01$) with

increased progression-free survival for patients treated with irinotecan-containing FLOFIRI in combination with bevacizumab, the most-frequent regimen in the cohort. *Conclusion:* Sensitivity of advanced CRC patients to irinotecan-based systemic chemotherapy can be predicted by the extent of CHFR-promoter methylation.

Colorectal cancer (CRC) is the third most-common malignancy and the second-most-frequent cause of cancer-related mortality worldwide (1). Recent advances in systemic chemotherapy have improved post-operative outcomes in advanced or metastatic CRC (2, 3). The current first-line therapies for unresectable CRC are oxaliplatin-based and irinotecan-based regimens. The response rates (RR) are approximately 40-60% for these regimens (4, 5), with time to progression (TTP) of 7-8 months and median overall survival (OS) of 14-21 months (6). The two drugs are equivalent in efficacy in this clinical setting (7). Oxaliplatin causes severe chronic neurotoxicity, which limits the duration of treatment (8). Therefore, irinotecan-based therapy is often preferred for advanced or metastatic CRC. However, there is currently no established biomarker that is predictive of the clinical benefit of irinotecan-based systemic chemotherapy (9).

DNA methylation is altered in human cancer as originally discovered by Hoffman *et al.* (10). DNA methylation is involved in the development and progression of CRC (11, 12). DNA methylation of tumor-suppressor genes is useful as a prognostic and susceptibility factor to anti-cancer drugs (13, 14). Recently, checkpoint-with-fork-head-and-ring-finger-domains (CHFR)-promoter methylation has been suggested to be associated with favorable treatment

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outcomes of irinotecan-based chemotherapy in patients with advanced or metastatic CRC (9). However, the clinical implications of the methylation status of CHFR in advanced or metastatic CRC cases are largely unexplored. In the present report, we correlated CHFR-promoter methylation with the efficacy of irinotecan-based chemotherapy for advanced or metastatic CRC.

Materials and Methods

Study populations. Methylation analyses were performed on a training cohort of 44 frozen colorectal tumor tissues from patients with CRC who underwent surgery at Juntendo University Hospital between 2017 and 2020. We also established a test cohort, to measure CHFR-promoter methylation data using frozen colorectal-cancer tissues from 49 patients with CRC who were treated with at least six cycles of irinotecan-based systemic chemotherapy for advanced or metastatic CRC at Juntendo University Hospital between 2011 and 2019. Clinical data from each patient were obtained from institutional databases and medical records. All procedures followed were in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent to be included in the study, or the equivalent, was obtained from all patients. The study was approved by the Institutional Review Board of Juntendo University Hospital (training cohort: IRB No. 17-036; test cohort: IRB NO. 20-097).

Irinotecan-based systemic chemotherapy. The regimen in each case was determined based on a discussion between the physician and patient. Patients were treated with one of the following irinotecan-containing chemotherapy regimens: FOLFIRI [irinotecan (180 mg/m² *i.v.*), leucovorin (200 mg/m² *i.v.* on day 1), and 5-fluorouracil (5-FU) (400 mg/m² *i.v.* bolus followed by 2,400 mg/m² continuous *i.v.* over 46 h on day 1) every 2 weeks]; XELIRI [irinotecan (200 mg/m² *i.v.* on day 1) and capecitabine (800 mg/m² *p.o.* twice a day for 2 weeks) every 3 weeks]; IRIS [irinotecan (125 mg/m² *i.v.* on day 1 and 15) and S-1 (40-60 mg *p.o.* twice a day for 2 weeks) every 4 weeks]; SIR [irinotecan (150 mg/m² *i.v.* on day 1) and S-1 (40-60 mg *p.o.* twice a day for 2 weeks) every 3 weeks]; and irinotecan alone [irinotecan (150 mg/m² *i.v.* on day 1) every 2 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 5 mg/kg *i.v.* on day 1 and day 15 of each cycle of IRIS or 7.5 mg/kg *i.v.* on day 1 of each cycle of SIR), or ramucirumab (8 mg/kg *i.v.* on day 1 of each cycle of FOLFIRI) or cetuximab (weekly dosage: initial dose: 400 mg/m² *i.v.*, subsequent doses: 250 mg/m²/week; biweekly dosage: initial and subsequent doses: 500 mg/m² *i.v.* on day 1 of each cycle of FOLFIRI and day 8, cetuximab only) or panitumumab (6 mg/kg *i.v.* on day 1 of each cycle of FOLFIRI or irinotecan) were administered. Changes in tumor burden were assessed using the revised RECIST guidelines (version 1.1) (15).

Clinico-pathological analyses. All specimens were examined in the following manner (16): After resection of the colon and rectum, the excised specimen was cut open by the surgeon. Following formalin fixation, the specimen, lymph nodes and metastatic lesions were examined by a pathologist and clinico-pathological factors [*i.e.*, age, sex (male/female), location of primary tumor (colon/rectum or

right side: cecum, ascending colon, transverse colon; left side: descending colon), sigmoid colon, rectosigmoid colon, rectum, pre-operative serum carcinoembryonic antigen (CEA), maximum diameter of primary tumor, differentiation of primary tumor, T-classification, N-classification, metastatic sites, TNM stage (17)] were recorded.

Histoculture drug response assay (HDRA). The HDRA was invented by Hoffman *et al.* (18). The HDRA chemosensitivity results have been previously correlated to clinical outcome, including CRC (19, 20). The HDRA was performed as described previously (20, 21). Cancer tissues from excised specimens were immediately washed with Hank's balanced salt solution (HBSS), and divided into pieces of about 10 mg. These tissue fragments were placed on Gelfoam® (Health Design, Rochester, NY, USA) in 24-well plates and incubated for 7 days in RPMI 1640 medium (GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 20% fetal calf serum (GIBCO; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 95% air/5% CO₂. Preliminary determination of IC₅₀ values (the concentration required for 50% growth inhibition) indicated 300 µg/ml for 5-FU and 0.4 µg/ml for SN38 (a topoisomerase I inhibitor that is the active metabolite of irinotecan). Following histoculture, HBSS (100 µl) containing 0.1 mg/ml type I collagenase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and MTT solution (2 mg/ml, 100 µl) were added to each well, and the mixture was incubated at 37°C for another 16 h. Following extraction with dimethyl sulfoxide (DMSO), the absorbance in each well was measured with a microplate reader at 540 nm. The mean absorbance from four parallel culture wells was used to calculate the absorbance per gram of cultured tumor tissue. The tumor-tissue weight was determined prior to culture. The inhibition rate (IR) was calculated as: IR=(1-T/C)×100%, where T and C are the mean absorbances of the treated tumor/weight and control tumor/weight, respectively.

Genomic DNA extraction and bisulfite treatment. Genomic DNA was purified from fresh-frozen samples with an AllPrep DNA/RNA Mini Kit (Qiagen, Germantown, MD, USA). A sample (500 ng) of this DNA was bisulfite-converted using reagents contained in the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) and samples were processed according to the manufacturer's instructions.

Quantitative methylation-specific PCR (qMSP). The methylation status of the CHFR-promoter region was subjected to qMSP analysis, as follows. Bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR. qMSP was performed using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). CHFR-promoter methylation was examined using a 200 nM forward primer, 200 nM reverse primer and 80 nM probes, with cycling conditions of 95°C for 5 min, followed by 55 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. The master mix contained 16.6 mM (NH₄)₂SO₄, 67 mM Tris pH 8.8, 10 mM β-mercaptoethanol, 10 nM fluorescein, 0.166 mM of each deoxynucleotide triphosphate (dNTP) and 0.04 U/µl of Platinum Taq polymerase (ThermoFisher Scientific). Each assay had a final reaction volume of 25 µl. Human-female Jurkat genomic DNA treated with CpG Methylase (M.SssI) (New England Bio Labs, Ipswich, MA, USA) was used as a positive methylation-control. Due to low levels of DNA methylation, replicates for some samples

Figure 1. Correlation of efficacy in the HDRA and CHFR-RMV in the training cohort. (A) CHFR-RMV did not show significant correlation with 5-FU-induced inhibition ($R_s=0.21, p=0.17$). (B) CHFR-RMV was significantly correlated with inhibition by SN38 ($R_s=0.37, p=0.01$). CHFR: Checkpoint-with-forkhead-and-ring-finger domains; HDRA: histoculture drug response assay; RMV: relative methylation value.

had no detectable methylation, as anticipated. Methylation was quantified using the Relative Methylation Value (RMV), based on calculation of $2^{-\Delta\Delta C_t}$ for each detectable replicate compared to the mean C_t for β -actin (ACTB) (22, 23). The bisulfite-modified DNA was assessed for CHFR-promoter methylation with 200 nM forward primer, 5'-GTT ATT TTC GTG ATT CGT AGG CGA C-3', 200 nM reverse primer, 5'-CGA AAC CGA AAA TAA CCC GCG-3', and 80 nM probe, 5'-56-FAM\ CGC TCG ACC \ZEN\ ATC TTT AAT CCT AAC CAA ACG ACT TC \3IABkFQ\ -3'. The sequences for beta-actin (ACTB) recognizing both methylated and unmethylated templates were: 200 nM forward primer, 5'-TAG GGA GTA TAT AGG TTG GGG AAG TT-3', 200 nM reverse primer, 5'-AAC ACA CAA TAA CAA ACA CAA ATT CAC-3', and 80 nM probe, 5'-56-FAM\ TGT GGG GTG \ZEN\ GTG ATG GAG GAG GTT TAG \3IABkFQ\ -3'. For non-detectable replicates, a C_t of 100 was used to give a near zero value for $2^{-\Delta\Delta C_t}$. The mean $2^{-\Delta\Delta C_t}$ value (RMV) was calculated as: $\text{mean } 2^{-\Delta\Delta C_t} (\text{RMV}) = (2^{-\Delta\Delta C_{t\_replicate\_1}} + 2^{-\Delta\Delta C_{t\_replicate\_2}} + 2^{-\Delta\Delta C_{t\_replicate\_3}}) / 3$ (22, 23).

RNA extraction and real-time quantitative PCR (RT-PCR). RNA expression was investigated in 90 patients (training cohort: 44 patients; test cohort: 46 patients) with cancer tissue of sufficient quality to analyze RNA expression. RNA was extracted using an AllPrep DNA/RNA Mini Kit (Qiagen). RNA samples (1,000 ng) were reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ThermoFisher Scientific). cDNA was suspended in TE buffer. RT-PCR was

performed on an ABI StepOnePlus Real-Time PCR System (Applied BioSystems). Each PCR contained 4 μ l of cDNA product, 10 μ l of PowerUp SYBR Green Master Mix (ThermoFisher Scientific), 1 μ l of forward and reverse primers combined, made up to a total volume of 20 μ l with sterile water. Following initial incubation at 95°C for 5 min, quantitative RT-PCR amplification was performed using 40 denaturing cycles of 30 s at 95°C, 30 s annealing at 55°C, and 30 s extension at 72°C. These experiments were performed in triplicate and the mean value was calculated. The sample value was determined as the CHFR gene/ACTB values. The forward primer for the CHFR gene was GGCAACCAG AGGTTTGACAT and reverse primer was AGTCAGGACGG GATGTTACG. Primers for ACTB were: forward: TTCTA-CAATGA GCTGCGTGTG and reverse: GGGGTGTTGAAGTCTCAAA.

Statistical analysis. A Fisher Exact probability test was used to compare discrete variables. Continuous variables were compared by the Mann-Whitney *U*-test for individual comparisons and the Wilcoxon signed rank test for paired comparisons. The cumulative-survival rate was calculated using the Kaplan-Meier method and univariate analyses were performed by the log-rank test. A Cox proportional-hazard regression model was used with the hazard ratio (HR). The cut-off value for RMV was determined using a receiver operating characteristic (ROC) curve, as the value that gave the largest area under the curve (AUC). Data were analyzed using JMP 14 (SAS Institute Inc., Cary, NC, USA). Differences were considered significant at $p \leq 0.05$. Values are expressed as median (range).

Figure 2. Comparison of efficacy of SN38 in the HDRA between CHFR-RMV high- and low- groups. The cohort tested with the HDRA was divided into two groups: CHFR-RMV high- and low-, based on a cut-off of 0.0001. The median inhibition rates of SN38 were 47.0% (2.3%-80.9%) in the CHFR-RMV high-group (n=26) and 10.4% (0.0%-66.9%) in the CHFR-RMV low-group (n=18). There was significantly greater inhibition by SN38 on patient tumors in the HDRA in the CHFR-RMV high-group ($p=0.002$). CHFR: Checkpoint-with-forkhead-and-ring-finger domains; HDRA: histoculture drug response assay; RMV: relative methylation value.

Results

Correlation of the efficacy of the irinotecan active- metabolite SN38 in the HDRA with CHFR-promoter methylation of CRC clinical specimens. CHFR-RMV was significantly positively correlated with inhibition by SN38 in the HDRA ($R_s=0.37$, $p=0.01$) (Figure 1). In contrast, CHFR-RMV was not correlated with inhibition by 5-FU in the HDRA ($R_s=0.21$, $p=0.17$). The median inhibition rate by SN38 was 30.4% (0.0%-80.9%). The chemosensitivity of tumors in the HDRA was defined as positive at an inhibition rate $\geq 50\%$ (24, 25), and the optimal cut-off for CHFR-RMV corresponding to an inhibition rate $\geq 50\%$ was determined. A cut-off value of CHFR-RMV of 0.0001 gave the largest area under the curve (AUC) (AUC=0.703, $p=0.01$). The training cohort tested in the HDRA was then divided into two groups: CHFR-RMV high- and low-groups, based on the cut-off of 0.0001. The median inhibition rates for SN38 were 47.0% (2.3%-80.9%) in the CHFR-RMV high-group (n=26) and 10.4% (0.0%-66.9%) in the CHFR-RMV low-group (n=18). There was a significantly higher inhibition by SN38 on tumors from patients in the CHFR-RMV high-group compared to the

Figure 3. Correlation of CHFR-RMV and CHFR RNA expression in the entire cohort. There was a significantly negative correlation between CHFR-RMV and CHFR RNA expression in the entire cohort (n=90) ($R_s=-0.51$, $p<0.001$).

CHFR-RMV low-group in the HDRA ($p=0.002$) (Figure 2). Upon comparison of clinico-pathological factors between the two groups (Table I), univariate analysis indicated that the location of the primary tumor differed significantly ($p=0.0001$), with patients in the CHFR-RMV high-group more likely to have right-sided CRC. There were no significant differences in other clinico-pathological factors between the groups (Table I).

Clinico-pathological characteristics in the low- and high-CHFR-RMV patient training and test cohorts. The demographics of the patients in the test and training cohorts are shown in Table I. To examine the correlation of the CHFR-promoter methylation and the response to irinotecan-based systemic chemotherapy, the test cohort was divided into two groups: CHFR-RMV high- (n=28) and low- (n=21). In univariate analysis, there were no significant differences between the groups.

Comparison of chemotherapy regimens in the low- and high-CHFR-RMV patient cohorts. The details of the irinotecan-based systemic chemotherapy regimens are shown in Table II. These regimens included FOLFIRI (n=13 patients), IRIS (n=6), SIR (n=1), and irinotecan as a single agent (n=1) in the CHFR-RMV low-group; and FOLFIRI (n=21), XELIRI (n=1), IRIS (n=5), and irinotecan as a single agent (n=1) in the CHFR-RMV high-group. Anti-VEGF antibody was

Table I. Patient characteristics.

Clinico-pathological factors	Variables	Training cohort (n=44)			Test cohort (n=49)		
		CHFR-RMV Low (n=18)	CHFR-RMV High (n=26)	p-Value	CHFR-RMV Low (n=21)	CHFR-RMV High (n=28)	p-Value
Number of patients		18	26	-	21	28	-
Age	Years ^a	69.5 (45-88)	70 (43-84)	0.77	61 (25-80)	65 (40-83)	0.24
Gender	Male	9 (50.0%)	10 (38.5%)	0.54	12 (57.1%)	16 (57.1%)	1.00
	Female	9 (50.0%)	16 (61.5%)		9 (42.9%)	12 (42.9%)	
Location	Colon	16 (88.9%)	25 (96.2%)	0.56	14 (66.7%)	21 (75.0%)	0.54
	Rectum	2 (11.1%)	1 (3.9%)		7 (33.3%)	7 (25.0%)	
Location	Right-sided	1 (5.6%)	17 (65.4%)	0.0001	5 (23.8%)	8 (28.6%)	0.76
	Left-sided	17 (94.4%)	9 (34.6%)		16 (76.2%)	20 (71.4%)	
Serum CEA level at time of resection of primary tumor	ng/ml ^a	4.4 (0.8-59.7)	5.0 (0.6-368)	0.73	12.7 (1.5-6,796)	7.2 (0.3-1,403)	0.25
Maximum diameter of primary tumor	mm ^a	45 (30-70)	50 (23-180)	0.39	50 (30-200)	49 (27-90)	0.70
Predominant histological type of primary tumor	Differentiated	17 (94.4%)	20 (76.9%)	0.21	17 (81.0%)	25 (89.3%)	0.44
	Undifferentiated	1 (5.6%)	6 (23.1%)		4 (19.1%)	3 (10.7%)	
Undifferentiated component in primary tumor	Present	4 (22.2%)	11 (42.3%)	0.21	6 (28.6%)	9 (32.1%)	1.00
	Absent	14 (77.8%)	15 (57.7%)		15 (71.4%)	19 (67.9%)	
T-classification	T1-3	17 (94.4%)	23 (88.5%)	0.63	13 (61.9%)	22 (78.6%)	0.22
	T4	1 (5.6%)	3 (11.5%)		8 (38.1%)	6 (21.4%)	
N-classification	N0	10 (55.6%)	16 (61.5%)	0.76	5 (23.8%)	3 (10.7%)	0.26
	N1, 2	8 (44.4%)	10 (38.5%)		16 (76.2%)	25 (89.3%)	
Metastatic site ^b	Liver	3 (16.7%)	3 (11.5%)	-	10 (47.6%)	18 (64.3%)	-
	Lung	0 (0%)	1 (3.8%)		11 (52.4%)	12 (42.9%)	
	Peritoneum	0 (0%)	1 (3.8%)		2 (9.5%)	2 (7.1%)	
	Local	0 (0%)	0 (0%)		1 (4.8%)	1 (3.6%)	
	Para-aortic LN	0 (0%)	0 (0%)		2 (9.5%)	1 (3.6%)	
	Others	0 (0%)	0 (0%)		3 (14.3%)	5 (17.9%)	
Stage at resection of primary tumor	I-III	15 (83.3%)	22 (84.6%)	1.00	8 (38.1%)	10 (35.7%)	1.00
	IV	3 (16.7%)	4 (15.4%)		13 (61.9%)	18 (64.3%)	
	Wild	1 (5.6%)	4 (15.4%)	0.42	10 (47.6%)	16 (57.1%)	0.57
RAS	Mutant	2 (11.1%)	1 (3.8%)		10 (47.6%)	11 (39.3%)	
	Unknown	15 (83.3%)	21 (80.8%)	0.26	1 (4.8%)	1 (3.6%)	0.71
	Wild	0 (0%)	3 (11.5%)		2 (13.3%)	5 (17.9%)	
UGT1A1 gene ^c	Variant	0 (0%)	0 (0%)		6 (30.0%)	7 (25.0%)	
	Unknown	18 (100%)	23 (88.5%)		13 (56.7%)	16 (57.1%)	

^aMedian (minimum-maximum); ^bduplication involved. ^cUGT1A1 protein degrades SN38.

added in 16 patients in the CHFR-RMV low-group and 20 patients in the CHFR-RMV high-group. Anti-EGFR antibody was added in 3 patients and 6 patients, respectively, in the two groups. Use of irinotecan-based systemic chemotherapy regimens did not differ significantly between the two groups. These regimens were administered as first-line therapy in 13 patients in the CHFR-RMV low-group and in 12 patients in the CHFR-RMV high-group. There was also no significant difference in the use of the regimens as first-line systemic chemotherapy between the two groups (Table II).

Correlation of CHFR-RMV and CHFR RNA expression in the entire cohort. There was a significantly negative correlation

between CHFR-RMV and CHFR RNA expression in the entire cohort (n=90) ($R_s = -0.51$, $p < 0.001$) (Figure 3).

Correlation of CHFR-RMV status and response to irinotecan-based systemic chemotherapy in the test cohort. First, we compared the best overall responses to irinotecan-based systemic chemotherapy between the CHFR-RMV high- and low-groups (Figure 4). The CHFR-RMV low-group included cases with a partial response (PR) (n=3, 14.3%), stable disease (SD) (n=6, 28.6%) and progressive disease (PD) (n=12, 57.1%). The CHFR-RMV high-group included cases with PR (n=8, 28.6%), SD (n=13, 46.4%) and PD (n=7, 25.0%). There was a trend towards better overall responses

Table II. Details of irinotecan-based systemic chemotherapy regimens.

Regimen	All patients in the test cohort				Patients treated with first-line systemic chemotherapy with irinotecan			
	CHFR-RMV				CHFR-RMV			
	Low group n=21 (%)		High group n=28 (%)		Low group n=13 (%)		High group n=12 (%)	
Regimen								
FOLFIRI	0	(0.0%)	1	(3.6%)	0	(0.0%)	0	(0.0%)
FOLFIRI+bevacizumab	10	(47.6%)	14	(50.0%)	5	(38.5%)	6	(50.0%)
FOLFIRI+ramucirumab	0	(0.0%)	1	(3.6%)	0	(0.0%)	0	(0.0%)
FOLFIRI+cetuximab	1	(4.8%)	2	(7.1%)	1	(7.7%)	1	(8.3%)
FOLFIRI+panitumumab	2	(9.5%)	3	(10.7%)	2	(15.4%)	2	(16.7%)
XELIRI+bevacizumab	0	(0.0%)	1	(3.6%)	0	(0.0%)	0	(0.0%)
IRIS	1	(4.8%)	1	(3.6%)	1	(7.7%)	0	(0.0%)
IRIS+bevacizumab	5	(23.8%)	4	(14.3%)	3	(23.1%)	2	(16.7%)
SIR+bevacizumab	1	(4.8%)	0	(0.0%)	0	(0.0%)	0	(0.0%)
Irinotecan alone	1	(4.8%)	0	(0.0%)	1	(7.7%)	0	(0.0%)
Irinotecan+panitumumab	0	(0.0%)	1	(3.6%)	0	(0.0%)	1	(8.3%)
Total	21	(100%)	28	(100%)	13	(100%)	12	(100%)
Molecular-targeted agents								
Anti-VEGF antibody ^a	16	(76.2%)	20	(71.4%)	8	(61.5%)	8	(66.7%)
Anti-EGFR antibody ^b	3	(14.3%)	6	(21.4%)	3	(23.1%)	4	(33.3%)
None	2	(9.5%)	2	(7.1%)	2	(15.4%)	0	(0.0%)
Total	21	(100%)	28	(100%)	13	(100%)	12	(100%)

^aVEGF: Vascular endothelial growth factor; ^bEGFR: epidermal growth factor receptor.

in the CHFR-RMV high-group ($p=0.07$). The CHFR-RMV high-group had a significantly better disease control (PR+SD) rate (75.0% vs. 42.9%, $p=0.04$) (Figure 4). A similar comparison of best overall-responses in patients who received first-line systemic chemotherapy with irinotecan also showed a trend for a better disease-control rate in the CHFR-RMV high-group (83.3% vs. 46.2%, $p=0.10$) (Figure 5). In patients who also received FOLFIRI+bevacizumab as first-line systemic chemotherapy, which was the most frequent regimen in the cohort, the disease control rate was also higher in the CHFR-RMV high-group (83.3% vs. 40.0%), although the difference was not significant due to the small number of patients ($p=0.24$).

We also analyzed long-term outcomes in the test cohort through comparison of cancer-specific (CSS) and progression-free (PFS) survival in the CHFR-RMV high- and low- groups (Figure 6). There was no significant difference in CSS from the time of initial diagnosis of advanced or metastatic CRC between the two groups [CHFR-RMV high-group: HR=1.12 (0.55-2.26); $p=0.76$] (Figure 6A). Similarly, there was no significant difference in CSS from the time of resection of the primary tumor [CHFR-RMV high-group: HR=1.30 (0.63-2.66); $p=0.48$] (Figure 6B). Regarding PFS from the time of initiation of systemic chemotherapy with irinotecan, the CHFR-RMV high-group showed a trend towards a better PFS

in the test cohort [HR=0.54 (0.28-1.04); $p=0.07$] (Figure 6C). This trend was maintained in patients treated with first-line systemic chemotherapy with irinotecan in the CHFR-RMV high-group [HR=0.39 (0.13-1.12); $p=0.08$] (Figure 6D). In patients treated with FOLFIRI+bevacizumab as first-line systemic chemotherapy, the most frequently-used regimen, the CHFR-RMV high-group had significantly better PFS ($p=0.01$ by log-rank test; the Cox proportional hazard regression model did not converge) (Figure 6E).

Discussion

Cha *et al.* (9) found that CHFR promoter-methylation was associated with prolonged TTP and OS in patients treated with irinotecan-based systemic chemotherapy for advanced or metastatic CRC, and also showed increased sensitivity to irinotecan in CHFR-methylated cell lines. These *in vivo* and *in vitro* results are consistent with our findings, despite the use of qualitative MSP by Cha *et al.* (9), rather than quantitative MSP as in the present report.

CHFR encodes a checkpoint protein that delays entry into metaphase (26). This suggests that cancer cells that are deficient in CHFR may be more susceptible to microtubule inhibitors such as taxanes. Cha *et al.* (9) suggested that the association of CHFR-promoter methylation with the response

Figure 4. Best overall responses between the CHFR-RMV high- and low- groups. (A) CHFR-RMV low; (B) CHFR-RMV high. The CHFR-RMV low-group included cases with a PR (n=3, 14.3%), SD (n=6, 28.6%) and PD (n=12, 57.1%); whereas the CHFR-RMV high-group had PR (n=8, 28.6%), SD (n=13, 46.4%) and PD (n=7, 25.0%). There was a trend towards better overall responses in the CHFR-RMV high-group ($p=0.07$), with this group having a significantly better disease-control (PR+SD) rate (75.0% vs. 42.9%, $p=0.04$). CHFR: Checkpoint-with-forkhead-and-ring-finger domains; RMV: relative methylation value; PD: progressive disease; PR: partial response; SD: stable disease.

to irinotecan may be based mechanistically on the role of CHFR in protein ubiquitination, as an E3 ubiquitin ligase (27). Irinotecan is a topoisomerase I inhibitor that can induce DNA damage (28). Therefore, repair of irinotecan-induced DNA damage is likely to be a mechanism of irinotecan resistance (29). A decrease in the level of CHFR protein due to DNA methylation will impair ubiquitination of topoisomerase I, and the subsequent upregulation of topoisomerase I may render cancer cells more sensitive to irinotecan-induced damage (9).

To examine this hypothesis, we used SN38 in the HDRA, rather than the more common use of irinotecan (30, 31), since SN38 is the active metabolite of irinotecan and a potent inhibitor of DNA topoisomerase I (32). Irinotecan is converted to SN38 by carboxylesterase enzymes (33). Therefore, the present HDRA study is the first to evaluate the correlation between the efficacy of SN38 and CHFR-promoter methylation directly. The present HDRA study showed no association between response to 5-FU and CHFR-

Figure 5. Best overall responses in patients who received first-line systemic chemotherapy with irinotecan between CHFR-RMV high- and low-groups. (A) CHFR-RMV low; (B) CHFR-RMV high. A comparison of overall responses in patients who received first-line systemic chemotherapy with irinotecan showed a trend for a better disease-control rate in the CHFR-RMV high-group (83.3% vs. 46.2%, $p=0.10$). CHFR: Checkpoint-with-forkhead-and-ring-finger domains; RMV: relative-methylation value.

promoter methylation *in vitro*, suggesting a specific positive correlation of CHFR promoter-methylation and response to SN38. Only one previous report has determined the inhibitory effect of irinotecan itself in colorectal cancer tissues in the HDRA, with the finding of a median inhibition rate of 11.4% in 22 CRC patients (34). The higher inhibition rate found in the present study may be because SN38 is much more potent than irinotecan itself (35).

We also found a significant positive correlation between the clinical response of irinotecan-based systemic chemotherapy

and CHFR-promoter methylation in patients. Especially, it is important that there were clinical benefits in patients in long-term outcomes (*i.e.*, PFS). Thus, evaluation of CHFR-promoter methylation can identify patients who may benefit from irinotecan-based systemic chemotherapy.

There are several limitations in this study. First, only a small number of patients were enrolled due to enrollment being limited to a single center. Second, the regimens of irinotecan-based systemic chemotherapy were not consistent because of the retrospective study design. In particular, the

Figure 6. Long-term outcomes of CRC patients treated with irinotecan-based chemotherapy in the test cohort. Long-term outcome was analyzed in the test cohort by comparison of cancer-specific (CSS) and progression-free (PFS) survival in the CHFR-RMV high- and low-groups. (A) There was no difference in CSS from the time of initial diagnosis of advanced or metastatic CRC between the two groups [CHFR-RMV high-group: $HR=1.12$ (0.55-2.26); $p=0.76$]. (B) Similarly, there was no significant difference in CSS from the time of resection of the primary tumor [CHFR-RMV high-group: $HR=1.30$ (0.63-2.66); $p=0.48$]. (C) For PFS from the time of initiation of systemic chemotherapy with irinotecan, the CHFR-RMV high-group showed a trend towards better PFS in the test cohort [$HR=0.54$ (0.28-1.04); $p=0.07$]. (D) This trend was maintained in patients treated with first-line systemic chemotherapy with irinotecan in the CHFR-RMV high-group [$HR=0.39$ (0.13-1.12); $p=0.08$]. (E) In patients treated with FOLFIRI+bevacizumab as first-line systemic chemotherapy, the CHFR-RMV high-group had significantly better PFS ($p=0.01$ by log-rank test; the Cox proportional hazard regression model did not converge). CHFR: Checkpoint-with-forkhead-and-ring-finger domains; RMV: relative-methylation value; CRC: colorectal cancer; HR: hazard ratio.

influence of molecular-targeted agents on drug response cannot be ignored. However, a previous study gave similar results in a study cohort in which most patients (>90%) were not treated with molecular-targeted agents in combination with irinotecan-based systemic chemotherapy (9). Therefore, it is likely that the mechanisms demonstrated by the present results apply to the effects of irinotecan (SN38) alone.

Since there is no toxicity limit on the cumulative dose of irinotecan, unlike oxaliplatin, it is preferable for patients with advanced or metastatic CRC to continue to receive irinotecan-based systemic chemotherapy. The present results show a positive correlation between increased sensitivity to irinotecan (SN38) and CHFR promoter methylation both *in vitro* and *in vivo*. Our results support the importance of evaluation of CHFR-promoter methylation for patients with advanced or metastatic CRC. Our results also demonstrate the benefits of using the HDRA for patients. A prospective study is warranted to expand our findings.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

TH and Sugimoto K developed the concept of the study and drafted the article. TH and Sugimoto K performed histoculture drug response assay (HDRA) with the assistance of SRL, Inc (Tokyo, Japan). TH, Sugimoto K, HM and TI performed genomic DNA extraction, bisulfite treatment, quantitative methylation-specific PCR (qMSP), RNA extraction and real-time quantitative PCR (RT-PCR). TH, Sugimoto K and TI performed the patient survey and analyzed data. KH, YO, MK, SK, MT, YK, NS, AN and Sakamoto K recruited patients. TH, Sugimoto K and Sakamoto K obtained IRB approval for the protocol of the study. The article was revised by RMH. The article has been approved by all Authors.

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