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Epidermal growth factor receptor mRNA expression: A potential molecular escape mechanism from regorafenib

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Abstract

Regorafenib has improved the survival of patients with refractory metastatic colorectal cancer (mCRC), yet the mechanisms of inherited or acquired resistance are not well understood. A total of 50 patients with refractory mCRC were enrolled. Circulating tumor cell (CTC) enumeration was carried out at baseline, day 21 after initiation of regorafenib, and at the time of progression of disease (PD) using the CellSearch System (Veridex LLC, NJ, USA). Poly(A) mRNA was extracted from CTCs, and gene expression of epithelial and epithelial-mesenchymal transition markers was analyzed by a multiplex-PCR based DNA Chip. Patients with fewer than 3 CTCs at baseline and day 21 had a longer progression-free survival than those with 3 or more CTCs (3.3 vs 2.0 months, $P = .008$ and 3.3 vs 2.0 months, $P = .004$, respectively). Patients with fewer than 3 CTCs at baseline and day 21 had a longer overall survival (OS) than those with 3 or more CTCs (10.0 vs 4.6 months, $P < .001$ and 8.7 vs 3.8 months, $P = .003$, respectively). In multivariable analysis, CTC counts remained significantly associated with OS at baseline and day 21 ($P = .019$ and $P = .028$). Circulating tumor cell *EGFR* gene expression was upregulated at day 21 and/or PD in 64% of patients. Patients had significantly increased *EGFR* expression at PD compared to baseline ($P = .041$) and at day 21 and/or PD compared to baseline ($P = .004$). Our findings suggest that CTC count and *EGFR* expression could be useful markers of regorafenib efficacy and outcomes. Upregulation of CTC *EGFR* expression might be a molecular escape mechanism under regorafenib therapy.

KEYWORDS

circulating tumor cell, colorectal cancer, epidermal growth factor receptor, epithelial marker and EMT, regorafenib

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1 | INTRODUCTION

Regorafenib, an oral multikinase inhibitor, blocks the activity of several protein kinases, including v-raf murine sarcoma viral oncogene homolog B1 (BRAF),[1] and improves the progression-free survival (PFS) and overall survival (OS) of chemorefractory metastatic colorectal cancer (mCRC) patients.[1,2] A retrospective exploratory analysis of the pivotal phase III CORRECT trial proposed BEAMing analysis of circulating DNA as a potential biomarker and viable approach to obtain real-time tumor-associated genotypic information in mCRC patients treated with regorafenib. Nonetheless, there are currently no validated predictive or prognostic biomarkers of regorafenib efficacy.

Circulating tumor cells (CTCs) are shed from the primary tumor, migrate to sites of metastases, and serve as a noninvasive means of monitoring the dynamic alterations driving treatment efficacy and disease progression. The most widely studied CTC detection methods are based on immunomagnetic enrichment with antiepithelial cell adhesion molecule (EpCAM) Abs and subsequent immunological identification with anticytokeratin (anti-CK). The CellSearch system is one such method and is the only FDA-approved assay for the enumeration of CTCs in peripheral blood.[3-5] In a study by Cohen et al using CellSearch, the presence of 3 or more CTCs at baseline and follow-up was an independent prognostic marker of inferior survival in mCRC patients.[5-7] However, it is unclear whether CTC enumeration at baseline and over time is predictive or prognostic in mCRC patients specifically treated with regorafenib.

In addition to enumeration, protein expression and molecular profiling of CTCs might serve as more refined biomarkers and help inform a more personalized treatment approach. Under the pressure imposed by chemotherapy and mAbs, clonal selection and genomic instability emerge and provoke eventual treatment resistance. The ability to characterize such intratumoral heterogeneity could help to identify novel predictive and prognostic biomarkers and improve treatment decision-making. To this end, our group has previously examined the prognostic role of epithelial-mesenchymal transition (EMT) gene (*PI3K*, *Akt-2*, and *Twist1*) expression within CTCs of patients with mCRC (Ning Y et al).[8]

In this study, we examined the ability of CTC enumeration and molecular characterization, using mRNA levels of epithelial (*EGFR*, *EpCAM*, and *CEA*) and EMT (*PI3K*, *Akt-2*, and *Twist1*) markers, to predict outcomes and identify potential molecular escape mechanisms in mCRC patients undergoing regorafenib therapy.

2 | MATERIALS AND METHODS

2.1 | Study design and patient population

This was a retrospective analysis of a prospective single-arm study examining the efficacy and toxicity of regorafenib in patients with refractory mCRC undertaken at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research. Eligible patients were 18 years or older with histologically confirmed mCRC, who had progressed or were intolerant to standard therapy (including a fluoropyrimidine, irinotecan, oxaliplatin, bevacizumab, and an antiepidermal growth factor receptor [EGFR] agent if indicated), and were subsequently treated with regorafenib at a dose of 160 mg orally daily for 21 days of every 28-day cycle. Treatment was continued until the development of intolerable toxicities or disease progression. Standard inclusion criteria applied, including ECOG performance status of 0 or 1, presence of measurable disease, life expectancy of at least 3 months, and adequate organ function. Between March 2013 and December 2014, a total of 62 patients were enrolled, of which 50 met inclusion/exclusion criteria and had sufficient blood samples for analysis. Study protocols were approved by the Institutional Review Board at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research. All patients provided written informed consent for the evaluation of molecular correlates.

2.2 | Isolation and enumeration of CTCs

Circulating tumor cell enumeration was carried out at baseline prior to initiation of regorafenib, day 21 after initiation of therapy, and at the time of treatment intolerance or progression of disease (PD) using the CellSearch system. A total of 7.5 mL whole blood was collected on a CellSave Preservative Tube, and the CellSearch Circulating Tumor Cell Kit (Veridex) was applied for CTC enrichment and enumeration. Immunomagnetic enrichment was undertaken using anti-EpCAM ferrofluid.

Enriched cells were then fluorescently stained with the nucleic acid dye DAPI and labeled with mAbs specific for epithelial cells (anti-CK8, 18, and 19) and leukocytes (anti-CD45). A CTC was defined as an EpCAM, isolated, intact, round to oval cell, with a visible nucleus (DAPI-positive), positive staining for CK, and negative staining for CD45. Results of CTC enumeration were then expressed as the number of cells per 7.5 mL blood.[9]

2.3 | Gene expression analysis using quantitative RT-PCR and multiplex PCR

The mRNA expression of epithelial (*EGFR*, *EpCAM*, and *CEA*) and EMT (*PI3K-α*, *Akt-2* and, *TWIST1*) markers in the enriched CTC cartridges after counting the CTC number using the CellSearch system was measured using the HotStarTaq Master Mix (#203443; Qiagen), a thermocycler, and the Agilent 2100 Bioanalyzer on a DNA LabChip (Agilent Technologies).

Primers for epithelial (*EGFR*, *EpCAM*, and *CEA*) and EMT (*PI3K-α*, *Akt-2*, and *TWIST1*) markers were provided by AdnaGen. The analysis of tumor cell-derived mRNA was carried out by quantitative RT-PCR for the following transcripts: *EGFR*, *EpCAM*, *CEA*, *PI3K-α*, *Akt-2*, and *TWIST1*. AdnaTest ColonCancerDetect and AdnaTest EMT-2/StemCell Detect kits (AdnaGen), containing oligo$(dT)_{25}$-coated beads, were used to isolate mRNA from tumor cells in the enriched CTC cartridges of CellSearch system. PrimerMix ColonDetect was first used

to amplify 3 genes (*EGFR*, *EpCAM*, and *CEA*) and 1 control gene (*Actin*). PrimerMix EMT-2/StemCell Detect was used to amplify 3 EMT-related genes (*PI3K-α*, *Akt-2*, and *TWIST1*) and 1 control gene (*Actin*). Visualization of PCR fragments was carried out with the Agilent 2100 Bioanalyzer, with a limit of detection of 0.01 ng/μL or more.

2.4 | Statistical analysis

The primary outcome measure was PFS, defined as the date of randomization to the date of first documented disease progression or death from any cause. If disease progression or death was not observed, PFS was censored on the day of the last computed tomography scan. Secondary end-points were response rate and OS. Overall survival was defined as the period from randomization to the date of death or censored on the date of last contact if alive. Patients were dichotomized into responders (including complete or partial response) and nonresponders (including stable or progressive disease) as defined by RECIST 1.1 criteria.

Circulating tumor cell levels were dichotomized into low and high values based on the widely accepted cut-off value of 3.[5] The mRNA levels of CTC *EGFR*, *EpCAM*, *CEA*, *PI3K-α*, *Akt-2*, and *TWIST1* were categorized into positive and negative groups. The cut-off value of 0.05 for *EGFR* expression at baseline was chosen based on the maximum χ^2 approach. Associations between CTC and gene expression levels, and PFS and OS were analyzed by Kaplan-Meier curves and log-rank test in univariable analysis, and the Cox regression model in a multivariable model, adjusting for baseline patient and tumor characteristics.

SAS 9.4 (SAS Institute) was used to perform all analyses. All tests were 2-sided at a significance level of .05.

3 | RESULTS

3.1 | Patient and tumor characteristics

Clinicopathologic characteristics are presented in Table 1. The median follow-up time was 180 days. The median PFS and OS were 69 days and 192 days, respectively. Six patients were deemed not evaluable; 2 patients had rapid disease progression, and 4 patients had adverse events. Associations between baseline characteristics and clinical outcomes were examined using the log-rank test in univariate analysis. Using the Cox regression model in a multivariable model, the presence of liver metastases, metastases to other organs, and *KRAS* mutation status were significantly associated with PFS and OS.

3.2 | Clinical outcomes by CTC count and gene expression in patients receiving regorafenib

The distribution of CTC count and CTC gene expression at baseline and day 21 is outlined in Table 2. At baseline, 64% of patients had detectable CTCs, all of whom had measurable CTC *EGFR* expression. Among patients without detectable CTCs, all had measurable mRNA expression of at least one of the tested genes at baseline.

TABLE 1 Patients with metastatic colorectal cancer and tumor characteristics (N = 50)

Characteristic	N	%
Age, y		
Median (range)	65	34-78
Sex		
M	22	44
F	28	56
Performance status		
0	27	54
1	23	46
Primary tumor site		
Right	14	28
Left	36	72
Primary tumor resection		
No	42	84
Yes	8	16
KRAS mutation status		
Wild type	34	69
Mutant	15	31
Metastases		
Liver	32	64
Lung	26	52
Lymph nodes	28	56
Peritoneal	12	24
Other organs	13	26
Line of chemotherapy		
2	1	2
3	11	22
4	35	70
5	3	6
Response		
Complete response	0	0
Partial response	1	2
Stable disease	17	34
Progressive disease	26	52
NE	6	12
Histological type		
tub1	11	22
tub2	33	66
por	5	10
Unknown	1	2

[a]A patient could have multiple sites of metastases.

NE, not evaluable; *por*, poorly differentiated adenocarcinoma; *tub1*, well-differentiated adenocarcinoma; *tub2*, moderately differentiated adenocarcinoma;

TABLE 2 Circulating tumor cell (CTC) count and gene expression distribution

Marker	N	No detectable expression	Median	Range	Interquartile range
Baseline					
CTC count	50	18	2.00	0-105	0-6
EGFR	50	17	0.01	0.00-0.83	0.00-0.02
CEA	50	24	0.01	0.00-0.51	0.00-0.01
EpCAM	50	39	0.00	0.00-2.11	0.00-0.00
TWIST1	50	36	0.00	0.00-0.06	0.00-0.01
Akt2	49	38	0.00	0.00-0.48	0.00-0.00
PI3Kα	50	42	0.00	0.00-0.06	0.00-0.00
Day 21					
CTC Count	50	20	1.00	0-346	0-4
EGFR	50	25	0.01	0.00-1.48	0.00-0.01
CEA	50	37	0.00	0.00-0.02	0.00-0.01
EpCAM	50	40	0.00	0.00-0.02	0.00-0.00
TWIST1	49	39	0.00	0.00-0.21	0.00-0.00
Akt2	50	39	0.00	0.00-0.02	0.00-0.00
PI3Kα	50	43	0.00	0.00-0.02	0.00-0.00

Associations between CTC count and outcomes are analyzed (Table 3). The CTC count at baseline and at day 21 was significantly associated with PFS and OS. Specifically, patients with fewer than 3 CTCs at baseline and day 21 had a longer median PFS of 3.3 and 3.3 months, respectively, compared to those with 3 or more CTCs, who had a median PFS of 2.0 and 2.0 months, respectively (hazard ratio [HR], 2.1 and 2.4; 95% confidence interval [CI], 1.1-4.2 and 1.0-5.6; P = .008 and P = .004, respectively) (Table 3, Figure 1A,1). Patients with less than 3 CTCs at baseline and day 21 had a longer median OS of 10.0 and 8.7 months, respectively, compared with those with 3 or more CTCs, who had a median OS of 4.6 and 3.8 months, respectively (HR, 3.0 and 2.5; 95% CI, 1.5-5.8 and 1.3-4.8; P < .001 and P = .003, respectively) (Table 3, Figure 1C,1). In multivariable analysis, CTC counts remained significantly associated with OS at baseline and day 21 (HR, 2.7 and 2.3, adjusted P = .019 and P = .028) (Table 3). Additionally, patients with less than 3 CTCs at baseline and day 21 had a significantly higher tumor response rate compared to those with 3 or more CTCs (Fisher's exact test, P = .007 and P = .03, respectively) (Table 3).

We then examined associations between CTC gene expression levels and outcomes. We used the optimal cut-off value method as previously described.[10-12] The cut-off value of 0.05 for *EGFR* expression at baseline was chosen based on the maximum χ^2 approach. Patients with baseline CTC *EGFR* levels 0.05 or higher had a shorter PFS, compared to those with lower levels, both in univariable (HR 3.20, 95% CI, 1.16-8.80, P = .007) and multivariable (HR 4.26, 95% CI, 1.41-11.24, P = .007) analyses. All 5 patients with increased baseline CTC *EGFR* expression had disease progression as their best response. No significant associations were seen with the other markers that were analyzed (Table 3).

3.3 | Changes in CTC count and CTC gene expression at day 21

Next, we assessed CTC count and CTC gene expression levels at day 21 and at the time of disease progression as compared to baseline. Circulating tumor cell *EGFR* expression was significantly increased at day 21 and/or PD (P = .004) in 64% of patients (Table 4). More specifically, CTC *EGFR* expression was significantly increased at PD compared to baseline (P = .041) in 48% of patients. No significant associations were seen with the other genes that were analyzed.

3.4 | Associations between changes in CTC *EGFR* and EMT marker expression

We then hypothesized that increased CTC *EGFR* expression could result in an increase in the expression of CTC EMT markers.[13] However, there were no significant associations between changes in CTC *EGFR* and EMT marker gene expression.

4 | DISCUSSION

Our study is the first to show that CTC enumeration and mRNA gene expression levels could be useful markers of regorafenib efficacy in mCRC patients. Specifically, we found the presence of 3 or more CTCs at baseline and day 21 to be an independent prognostic factor for inferior survival. Moreover, upregulation of CTC *EGFR* expression induced by regorafenib was associated with disease progression.

Regorafenib blocks both WT and mutant *BRAF* (V600E) signaling. Preclinical studies have previously indicated that *BRAF*

TABLE 3 Associations between circulating tumor cell (CTC) count and gene expression and outcomes in patients with metastatic colorectal cancer treated with regorafenib

		Tumor response			Progression-free survival[a]					Overall survival				
Marker	N	PR + SD	PD	P^b	Median (95%CI)	HR (95% CI)	P^b	HR (95% CI)[c]	$P^{*‡}$	Median (95% CI)	HR (95% CI)	P^b	HR (95% CI)[c]	$P^{b,c}$
Baseline														
CTC count														
<3	27	14 (61%)	9 (39%)	.007	3.3 (1.8,4.7)	1 (ref.)	.008	1 (ref.)	.96	10.0 (6.5,13.9)	1 (ref.)	<.001	1 (ref.)	.019
≥3	23	4 (19%)	17 (81%)		2.0 (1.7,2.5)	2.12 (1.06,4.23)		0.98 (0.41,2.50)		4.6 (3.4,6.3)	2.96 (1.51,5.83)		2.68 (1.19,6.14)	
EGFR														
Negative	17	7 (50%)	7 (50%)	.51	2.7 (1.8,3.7)	1 (ref.)	.77	1 (ref.)	.44	6.3 (3.3,7.9)	1 (ref.)	.23	1 (ref.)	.90
Positive	33	11 (37%)	19 (63%)		2.0 (1.8,3.3)	1.10 (0.56,2.14)		1.32 (0.67,2.73)		8.0 (4.1,10.8)	0.68 (0.36,1.29)		0.95 (0.48,1.96)	
EGFR														
<0.05	45	18 (46%)	21 (54%)	.069	2.3 (1.8,3.3)	1 (ref.)	.007	1 (ref.)	.007	7.8 (4.8,10.0)	1 (ref.)	.25	1 (ref.)	.030
≥0.05	5	0 (0%)	5 (100%)		1.7 (0.6,2.5)	3.20 (1.16,8.80)		4.26 (1.41,11.24)		5.2 (1.2,12.9)	1.72 (0.67,4.46)		3.14 (1.05,8.19)	
CEA														
Negative	24	4 (20%)	16 (80%)	.015	2.0 (1.7,2.7)	1 (ref.)	.16	1 (ref.)	.27	5.9 (4.1,8.0)	1 (ref.)	.23	1 (ref.)	.56
Positive	26	14 (58%)	10 (42%)		2.7 (1.8,4.5)	0.66 (0.34,1.28)		0.68 (0.35,1.34)		7.9 (4.0,11.8)	0.69 (0.37,1.29)		0.82 (0.43,1.58)	
EpCAM														
Negative	39	13 (37%)	22 (63%)	.45	2.3 (1.8,3.2)	1 (ref.)	.29	1 (ref.)	.66	6.7 (4.0,9.6)	1 (ref.)	.71	1 (ref.)	.29
Positive	11	5 (56%)	4 (44%)		2.0 (1.7,7.6)	0.69 (0.32,1.51)		1.21 (0.49,2.70)		7.6 (3.6,12.0)	0.87 (0.41,1.83)		1.53 (0.67,3.24)	
TWIST1														
Negative	36	12 (35%)	22 (65%)	.27	2.0 (1.8,2.7)	1 (ref.)	.11	1 (ref.)	.61	6.7 (5.0,10.3)	1 (ref.)	.60	1 (ref.)	.36
Positive	14	6 (60%)	4 (40%)		4.2 (1.1,7.6)	0.56 (0.27,1.19)		0.81 (0.35,1.73)		5.8 (3.3,11.8)	1.19 (0.61,2.34)		1.40 (0.67,2.80)	
Akt2														
Negative	38	15 (43%)	20 (57%)	1.00	2.5 (1.8,3.3)	1 (ref.)	.62	1 (ref.)	.20	6.5 (4.6,9.6)	1 (ref.)	.58	1 (ref.)	.30
Positive	11	3 (38%)	5 (63%)		2.2 (1.6,3.7)	1.20 (0.54,2.68)		1.76 (0.70,3.99)		7.6 (3.3,11.8)	1.24 (0.56,2.75)		1.57 (0.63,3.51)	
PI3Kα														
Negative	42	13 (36%)	23 (64%)	.24	2.2 (1.8,2.7)	1 (ref.)	.51	1 (ref.)	.61	6.3 (4.6,9.6)	1 (ref.)	.18	1 (ref.)	.15
Positive	8	5 (63%)	3 (38%)		3.3 (0.6,4.7)	0.77 (0.34,1.75)		0.80 (0.32,1.78)		8.7 (1.2,24.9)	0.54 (0.21,1.41)		0.47 (0.15,1.19)	
Any EMT marker														
Negative	27	10 (38%)	16 (62%)	.75	2.3 (1.8,3.2)	1 (ref.)	.55	1 (ref.)	.82	6.3 (4.1,9.6)	1 (ref.)	.74	1 (ref.)	.93
Any positive	22	8 (47%)	9 (53%)		2.3 (1.8,4.2)	0.83 (0.44,1.57)		1.08 (0.55,2.10)		7.8 (3.6,11.8)	0.90 (0.47,1.71)		1.03 (0.52,2.00)	

(Continues)

TABLE 3 (Continued)

Marker	N	Tumor response			Progression-free survival[a]					Overall survival				
		PR + SD	PD	P^b	Median (95%CI)	HR (95% CI)	P^b	HR (95% CI)[c]	$P^{*‡}$	Median (95% CI)	HR (95% CI)	P^b	HR (95% CI)[c]	$P^{b,c}$
Day 21														
CTC count														
<3	32	15 (54%)	13 (46%)	.030	3.3 (2.0,4.5)	1 (ref.)	.004	1 (ref.)	.060	8.7 (6.5,10.8)	1 (ref.)	.003	1 (ref.)	.028
≥3	18	3 (19%)	13 (81%)		2.0 (1.8,2.3)	2.42 (1.04,5.63)		2.39 (0.99,5.94)		3.8 (2.8,5.1)	2.49 (1.28,4.83)		2.27 (1.10,4.66)	
EGFR														
Negative	25	8 (36%)	14 (64%)	.76	2.5 (1.8,4.5)	1 (ref.)	.37	1 (ref.)	.56	7.8 (4.6,12.0)	1 (ref.)	.37	1 (ref.)	.63
Positive	25	10 (45%)	12 (55%)		2.3 (1.8,4.1)	1.32 (0.65,2.65)		1.25 (0.59,2.64)		6.7 (4.0,10.0)	1.32 (0.71,2.48)		1.17 (0.62,2.25)	
EGFR														
<0.05	45	16 (40%)	24 (60%)	1.00	2.3 (1.8,3.3)	1 (ref.)	.69	1 (ref.)	.84	7.6 (5.0,9.6)	1 (ref.)	.40	1 (ref.)	.25
≥0.05	5	2 (50%)	2 (50%)		2.7 (1.8,4.1)	1.26 (0.38,4.18)		1.14 (0.27,3.80)		4.1 (1.6,12.9)	1.55 (0.55,4.42)		1.93 (0.57,5.44)	
CEA														
Negative	37	12 (38%)	20 (63%)	.51	2.3 (1.8,3.7)	1 (ref.)	.64	1 (ref.)	.91	6.7 (5.0,8.0)	1 (ref.)	.42	1 (ref.)	.88
Positive	13	6 (50%)	6 (50%)		2.7 (1.8,4.2)	0.85 (0.40,1.81)		1.05 (0.45,2.29)		8.7 (2.8,25.7)	0.74 (0.35,1.58)		0.94 (0.41,2.00)	
EpCAM														
Negative	40	13 (37%)	22 (63%)	.45	2.3 (1.8,3.7)	1 (ref.)	.98	1 (ref.)	.57	7.8 (5.0,10.8)	1 (ref.)	.086	1 (ref.)	.25
Positive	10	5 (56%)	4 (44%)		3.0 (1.1,4.2)	1.01 (0.43,2.38)		0.78 (0.31,1.75)		5.5 (1.7,8.0)	1.86 (0.84,4.10)		1.60 (0.70,3.41)	
TWIST1														
Negative	39	13 (38%)	21 (62%)	.46	2.0 (1.8,3.3)	1 (ref.)	.50	1 (ref.)	.49	8.0 (4.6,10.3)	1 (ref.)	.29	1 (ref.)	.11
Positive	10	5 (56%)	4 (44%)		3.7 (1.8,4.8)	0.77 (0.35,1.72)		0.73 (0.28,1.69)		5.5 (2.8,7.9)	1.46 (0.71,3.04)		1.99 (0.83,4.51)	
Akt2														
Negative	39	14 (40%)	21 (60%)	1.00	2.3 (1.8,3.2)	1 (ref.)	.51	1 (ref.)	.39	6.7 (4.6,9.6)	1 (ref.)	.93	1 (ref.)	.81
Positive	11	4 (44%)	5 (56%)		3.3 (1.7,6.6)	0.77 (0.33,1.76)		0.68 (0.27,1.55)		8.7 (3.5,13.9)	1.03 (0.51,2.09)		0.91 (0.41,1.91)	
PI3Kα														
Negative	43	15 (41%)	22 (59%)	1.00	2.3 (2.0,3.3)	1 (ref.)	.71	1 (ref.)	.67	7.8 (4.8,10.8)	1 (ref.)	.078	1 (ref.)	.039
Positive	7	3 (43%)	4 (57%)		3.0 (1.7,4.7)	1.17 (0.48,2.88)		1.23 (0.44,3.01)		5.9 (3.5,7.9)	2.02 (0.85,4.78)		2.61 (1.01,6.15)	
Change at day 21														
CTC count														
Stable/ decreased	40	15 (44%)	19 (56%)	.49	2.7 (1.8,4.1)	1 (ref.)	.31	1 (ref.)	.25	6.7 (5.0,9.6)	1 (ref.)	1.00	1 (ref.)	.85
Increased	10	3 (30%)	7 (70%)		2.1 (1.8,2.7)	1.47 (0.65,3.29)		1.68 (0.67,3.92)		8.3 (1.2,15.3)	1.00 (0.45,2.20)		1.08 (0.46,2.33)	

(Continues)

TABLE 3 (Continued)

Marker	N	Tumor response			Progression-free survival[a]					Overall survival				
		PR + SD	PD	P^b	Median (95%CI)	HR (95% CI)	P^b	HR (95% CI)[c]	$P^{*‡}$	Median (95% CI)	HR (95% CI)	P^b	HR (95% CI)[c]	$P^{b,c}$
EGFR														
Stable/ decreased	36	11 (33%)	22 (67%)	.09	2.2 (1.8,3.3)	1 (ref.)	.53	1 (ref.)	.42	6.5 (4.8,9.6)	1 (ref.)	.74	1 (ref.)	.76
Increased	14	7 (64%)	4 (36%)		3.2 (1.8,4.5)	0.76 (0.31,1.86)		0.64 (0.20,1.74)		6.7 (2.0,11.8)	1.12 (0.57,2.21)		0.89 (0.42,1.80)	
CEA														
Stable/ decreased	47	17 (41%)	24 (59%)	1.00	2.5 (2.0,3.3)	1 (ref.)	.62	1 (ref.)	.11	6.7 (4.8,8.7)	1 (ref.)	.46	1 (ref.)	.61
Increased	3	1 (33%)	2 (67%)		1.8 (1.8,4.5)	1.33 (0.40,4.42)		3.17 (0.72,12.35)		12.9 (3.5,12.9)	0.59 (0.14,2.46)		1.46 (0.28,5.34)	
EpCAM														
Stable/ decreased	41	14 (39%)	22 (61%)	.70	2.3 (1.8,3.7)	1 (ref.)	.98	1 (ref.)	.68	7.8 (5.0,10.8)	1 (ref.)	.018	1 (ref.)	.085
Increased	9	4 (50%)	4 (50%)		2.7 (1.1,4.2)	1.01 (0.41,2.48)		0.83 (0.31,1.91)		4.8 (1.7,8.0)	2.32 (1.05,5.16)		2.02 (0.88,4.32)	
TWIST1														
Stable/ decreased	40	13 (38%)	21 (62%)	.46	2.0 (1.8,3.3)	1 (ref.)	.65	1 (ref.)	.53	7.6 (4.6,10.3)	1 (ref.)	.44	1 (ref.)	.23
Increased	9	5 (56%)	4 (44%)		3.2 (1.8,4.8)	0.84 (0.38,1.87)		0.75 (0.29,1.77)		6.3 (2.8,13.9)	1.34 (0.63,2.84)		1.72 (0.69,4.03)	
Akt2														
Stable/ decreased	40	14 (40%)	21 (60%)	.70	2.3 (1.8,3.3)	1 (ref.)	.57	1 (ref.)	.48	6.3 (4.6,7.9)	1 (ref.)	.68	1 (ref.)	.51
Increased	9	4 (50%)	4 (50%)		4.2 (1.7,6.6)	0.78 (0.32,1.90)		0.72 (0.27,1.68)		10.0 (3.1,15.3)	0.86 (0.40,1.84)		0.76 (0.32,1.65)	
PI3Kα														
Stable/ decreased	44	16 (42%)	22 (58%)	1.00	2.5 (2.0,3.3)	1 (ref.)	.38	1 (ref.)	.78	7.6 (4.8,10.3)	1 (ref.)	.075	1 (ref.)	.092
Increased	6	2 (33%)	4 (67%)		1.8 (1.7,4.5)	1.50 (0.57,3.97)		1.16 (0.38,2.98)		4.7 (3.5,8.7)	2.13 (0.86,5.29)		2.26 (0.83,5.44)	

Note: Epithelial-mesenchymal transition (EMT) combines *TWIST1*, *Akt2*, and *PI3Kα* at baseline.

CI, confidence interval; HR, hazard ratio; ref., reference.

[a]Seven patients with progression-free survival (PFS) shorter than 21 d were excluded from analyses of associations between markers measured at day 21 and PFS.

[b]*P* values based on Fisher's exact test for tumor response, log-rank test for PFS and overall survival (OS) in the univariable analysis, and the Wald test in the multivariable Cox regression model. *P* values were not adjusted for multiple testing.

[c]Based on the multivariable Cox regression model adjusting for liver metastases, metastases of other organs, and *KRAS* mutation status for PFS and OS.

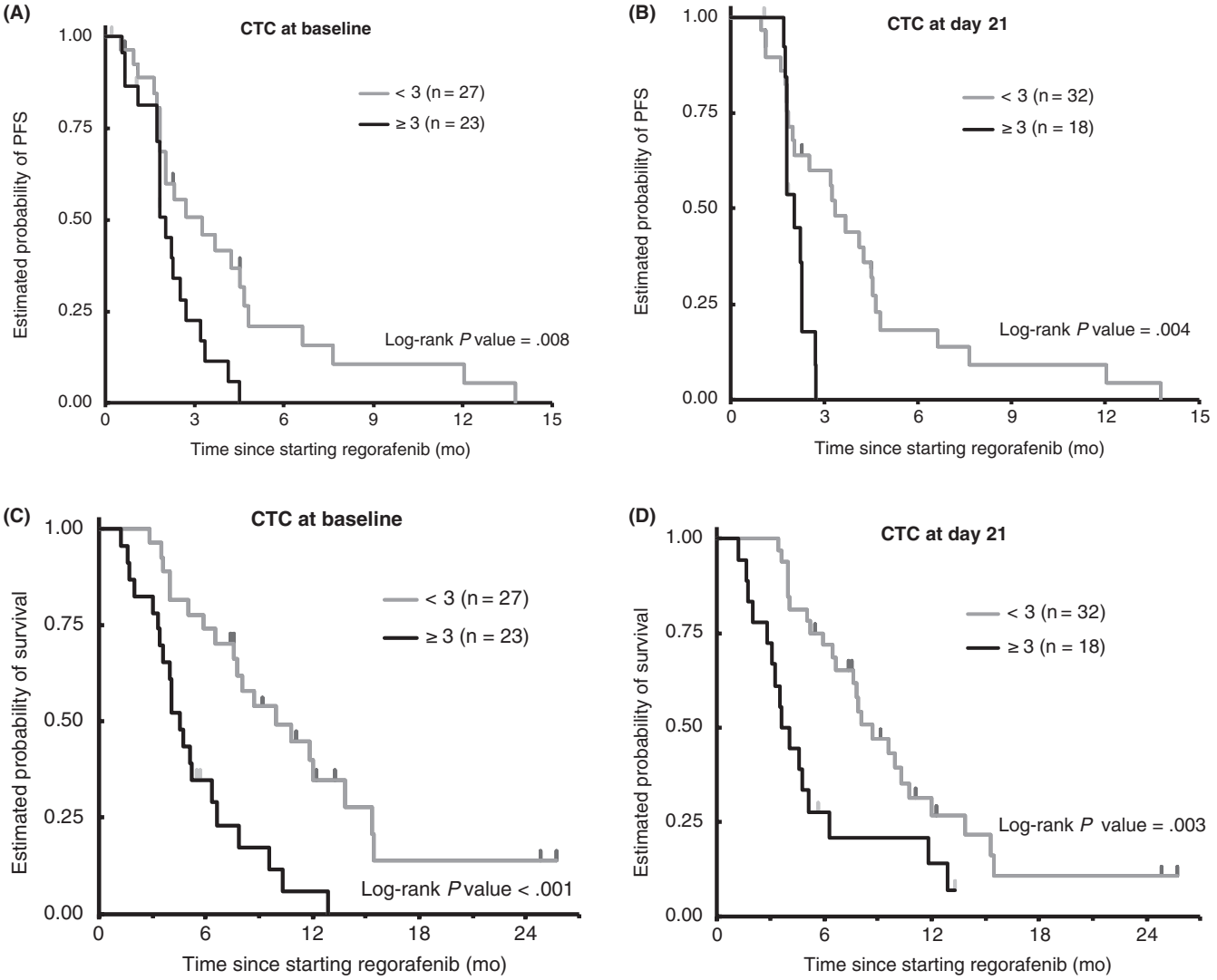

FIGURE 1 Comparison of clinical outcomes by circulating tumor cell (CTC) count at baseline and day 21 of treatment with regorafenib in patients with metastatic colorectal cancer. A,B, Progression-free survival probability at (A) baseline and (B) day 21. C,D, Overall survival probability at (C) baseline and (D) day 21

inhibition causes feedback activation of EGFR signaling and increased *EGFR* expression, allowing for ongoing tumor cell proliferation.[14] In previous studies, tumor tissue *EGFR* expression has been shown to be a negative prognostic marker.[15] Our data further support these findings, suggesting that regorafenib therapy could increase *EGFR* expression and signaling to an extent that leads to molecular escape and treatment resistance. Patients with baseline CTC *EGFR* levels 0.05 or higher had a shorter PFS and had disease progression at the time of their first radiographic evaluation. Although the findings of this study are hypothesis generating, if validated in future studies, CTC *EGFR* expression could serve as a useful marker to identify patients resistant to regorafenib prior to start of therapy.

TABLE 4 Circulating tumor cell *EGFR* upregulation compared to baseline value in patients with metastatic colorectal cancer treated with regorafenib (N = 50)

	Day 21	PD	Day 21 and/or PD
Increased ≥0.01 ng/µL, n (%)	14 (28)	24 (48)	32 (64)
Stable/decreased, n (%)	36 (72)	17 (34)	18 (36)
P^a	.600	.041	.004

Abbreviation: PD, progressive disease.

[a]Based on the sign test for paired data.

Importantly, we investigated CTC gene expression levels in patients with and without detectable CTCs, as defined by the CellSearch assay. A subgroup of patients without detectable CTCs according to CellSearch criteria had expression of epithelial marker genes, suggesting the presence of circulating tumor load, CTCs or cell fragments like exosomes, which were not detected or recognized by the CellSearch system. Similarly, in a study by Mostert et al,[16] CRC patients without detectable CTCs were found to have a circulating

tumor mRNA profile, distinct from both patients with 3 or more detectable CTCs and healthy donors, further suggesting that the blood of patients with no CTCs by CellSearch can contain CTCs or fragments. In patients without detectable CTCs by CellSearch criteria, exosomes could still be present, because CTC isolation is preceded by a Ficoll-based density gradient enrichment.[16] Taken together, our findings highlight a limitation of relying on CTC count alone as a biomarker, and the importance of circulating mRNA expression as a potential prognostic marker in mCRC patients treated with regorafenib.

Furthermore, a considerable proportion of patients in our study did not have detectable CTCs at baseline or subsequent follow-up. Previous reports suggest that CRC cells can lack CK8, 18, or 19 expression, the markers by which a CTC is defined in CellSearch, reflecting the EMT-like phenotype of these cells.[17,18] A lack of CK8, 18, or 19 expression would not affect the isolation of CTCs by immunomagnetic enrichment, which is based on their EpCAM expression. However, the lack of cytokeratin expression could result in these cells not being identified as epithelial cells and thus not counted as CTCs in the subsequent enumeration step. Therefore, in addition to the presence of cell fragments (eg exosomes), another possible explanation for the lack of detectable CTCs according to CellSearch criteria could be the insufficient expression of epithelial markers (ie CK8, 18, and 19).

It has been reported that CTCs often exist in a state of EMT or could coexpress both epithelial and mesenchymal markers. During the EMT, epithelial cells downregulate epithelial-related genes, acquire mesenchymal gene expression, and undergo major changes in their cytoskeleton that result in loss of cell-cell contact and cell polarity, leading to increased motility and invasiveness.[19] Here, we investigated the expression of CTC EMT markers, including *TWIST1*, *Akt2*, and *PI3Kα*, as well as associations between CTC *EGFR* and EMT gene expression, and found a nonsignificant trend towards an inverse association. The lack of a significant association could reflect challenges associated with CTC characterization using the CellSearch technique, including the presence of an excess of leukocytes, which express EMT marker genes, in CTC-enriched blood fractions.

Certain limitations of our study should be acknowledged. Our data are limited by the small sample size and are only exploratory. The lack of a control group of patients not receiving regorafenib preclude any conclusions that can be drawn regarding the use of CTC count or associated *EGFR* expression as predictive markers.

In summary, we identified CTC count as a prognostic marker in mCRC patients treated with regorafenib. Circulating tumor cell *EGFR* expression was significantly increased with regorafenib at the time of disease progression, suggesting this to be a mode of resistance and lending further mechanistic evidence for the synergistic effects seen with regorafenib and anti-EGFR mAbs.[20] Our findings warrant further investigation and, if replicated in future investigations, could inform the selection of mCRC patients best suited for regorafenib in the chemorefractory setting.

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DISCLOSURE

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