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Original Article

Arginine methylation of EGFR: a new biomarker for predicting resistance to anti-EGFR treatment

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Abstract: Arginine methylation of the epidermal growth factor receptor (meEGFR) increases the binding affinity of EGFR ligands and is reported to have a role in predicting response to anti-EGFR agents. This study investigated the predictive impact of meEGFR in metastatic colorectal cancer (mCRC) patients treated with anti-EGFR agents. Two patient cohorts were evaluated. Cohort 1 consisted of mCRC patients with documented disease progression following anti-EGFR treatment. Circulating tumor cells (CTCs) were isolated and distinguished based on $CD45^-$ and $Epcam^+$. Cohort 2 consisted of formalin fixed paraffin-embedded (FFPE) blocks from a prospective cohort. meEGFR in both cohorts was identified by positive staining for me-R198/200 EGFR signal. CTCs were identified in 30 out of 47 cases in cohort 1. Of those 30, meEGFR-CTCs were identified in 19 cases. Mean total meEGFR-CTCs counts was 2.3 (range 0-30) cells per 7.5 ml. There was no association between meEGFR-CTCs and clinic-pathological-molecular features. In RAS^{wt}/$BRAF^{wt}$ patients with high levels of meEGFR-CTCs ratio (≥ 0.23) had significantly inferior PFS with anti-EGFR treatment (HR = 3.4, 95% CI 1.5-7.9, P = 0.004). By contrast, high levels of meEGFR in the untreated tumor tissues had no correlation with anti-EGFR treatment duration in cohort 2. Therefore, meEGFR-CTCs may have the potential to serve as a "liquid biopsy" biomarker to predict anti-EGFR treatment efficacy.

Keywords: Liquid biopsy, circulating tumor cells, EGFR, arginine methylation, colorectal cancer, predictive marker

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide. In the Unit-ed States, it is estimated that more than 130,000 new cases will be diagnosed with nearly 50,000 deaths from CRC in 2016. Despite the recent increase in cases with molecular descriptions, treatment advances have not kept pace with the new information, and the 5-year survival rate of advanced-stage CRC is only 15% [1]. Monoclonal antibodies against epidermal growth factor receptor (anti-EGFRab), including cetuximab and panitumumab, are currently the standard treatment for metastatic colorectal cancer (mCRC). The U.S. Food and Drug Administration has recommended the use of anti-EGFRab treatment in colorectal cancers with wild type (WT) *RAS* (both *KRAS/NRAS)* as mutant *RAS* is associated with poor response to cetuximab [2-4]. However, only 40-60% of the *RAS* WT patient population respond to anti-EGFRab [5], and not all patients harboring mutant *KRAS* show resistance to anti-EGFRab treatment [6]. Therefore, these outcomes suggested there exists some heterogeneity in EGFR signaling and dependency even among *RAS* WT patients. Similarly those patients who initially respond to anti-EGFRab treatment often develop resistance within a year.

Resistance mechanisms to anti-EGFRab have been wildly studied. Primary resistance mechanisms have been reported, including: 1) Alter-

ation in EGFR and EGFR ligand [7, 8]; 2) RAS mutation [9]; 3) Mutation of V-raf murine sarcoma viral oncogene homolog B (BRAF) [10]; 4) Activation of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*)/phosphatase and tensin homolog (PTEN) pathway [11, 12]. Mechanisms underlying acquired resistance to EGFRab have also been proposed: 1) Acquired mutation of RAS and EGFR [13]; 2) Acquired mutation of BRAF [14, 15]; 3) Amplification of human epidermal growth factor receptor 2 (HER2) [16, 17] or MET [18] signaling; 4) Mutation of *PIK3CA* [11, 12]; 5) Loss of expression of PTEN [11, 19, 20]. However, accumulating data on these signal transduction pathways currently shows that these mutations serve as prognostic markers only and not predictive markers [21]. Hence, further investigation into the underlying mechanism of both primary and acquired anti-EGFRab resistance and identification of better predictors for anti-EGFRab response are needed.

Aberrant EGFR activation caused by *EGFR* gene mutation, amplification and/or ligand overexpression is involved in the pathogenesis of multiple cancers [22]. Although EGFR mutations are common in many cancer types, very few occur in CRC. When they do occur, the EGFR mutations are generally localized to the intracellular catalytic domain, resulting in oncogenic activation. There is emerging evidence to suggest that alterations affecting the extracellular domain of EGFR also drive oncogenic activities [23]. Recently, our group reported a post-translational arginine methylation on the extracellular domain of EGFR by protein arginine methyltransferase (PRMT) 1 at R198 and R200 that resulted in increased ligand binding to promote EGFR receptor dimerization and activation, and alters EGFR signaling. Additionally, patients with high methylated EGFR expression in the tumor tissues correlated with shorter duration of cetuximab response [24]. Overall, these results suggested that EGFR R198/200 has the potential to serve as a predictive biomarker for anti-EGFRab treatment response.

Liquid biopsies are innovative types of molecular tumor sampling methods because they can serve as a non-invasive and an easy technique to obtain the gene mutation profiles from either circulating tumor cells (CTCs) or cell-free circulating tumor DNA (cfDNA). Mutation detection in blood can produce results highly similar to those of traditional biopsies [25]. Moreover, liquid biopsies can also identify mutations that are associated with treatment resistance that is not possible to detect in the original tissue biopsy [26]. Several studies reported the unfavorable prognostic impact of high CTCs number on patient survival in CRC [27-30]; however, none of those studies demonstrated the predictive impact on CRC treatment.

Here, we evaluated the possibility of using protein arginine methylation of the EGFR (meEGFR) to predict response to anti-EGFR agents by systemically analyzing two different CRC patient sample cohorts. We analyzed the expression of meEGFR on CTCs in blood samples from patients from first cohort who were previously treated with EGFRab using the Parsortix™ system. We evaluated meEGFR expression in formalin fixed paraffin embedded (FFPE) tumor tissues from patients in the second cohort [tissues]. The association between meEGFR expression and progression-free survival (PFS) were evaluated for both cohorts.

Material and methods

All studies performed were approved by the Institutional Review Board at The MD Anderson Cancer Center.

The CTC sample cohort involved a prospective study. The inclusion criteria were mCRC patients whose histology confirmed colorectal adenocarcinoma, document disease progression after anti-EGFR agents, and age ≥ 18 years and an Eastern Cooperative Oncology group performance status ≤ 2. Patients' blood was obtained between September 2015 and July 2016. Circulating tumor cell (CTC) isolations involved collecting a maximum 15 mL of blood in Vacutainer tubes containing EDTA (BD Biosciences).

The FFPE cohort involved mCRC patient medical record review and sample identification as part of the Assessment of Targeted Therapies Against Colorectal Cancer (ATTAAC) program. These ATTACC patients were enrolled between February 13, 2009, and November 18, 2015. Last follow up date was January 31, 2017. All patients were provided with a written informed consent for blood collection under IRB protocols 2009-0091 or LAB 10-0963 protocol. The

primary objective was to investigate the association of meEGFR expression with progression free survival (PFS) in patients receiving anti-EGFR treatment; the secondary objective was to examine the associations between meEGFR expression and with various clinico-pathological-molecular variables.

Clinical characteristics

Demographic information was collected from a medical record review, including age, gender, primary tumor site, dates of anti-EGFR treatment, lines of anti-EGFR agents used, tumor metastatic sites, previous treatment with irinotecan, date of last follow-up, and date of death. Right-sided colon cancer was defined as cancer in the region from the cecum to the transverse colon, whereas left-sided colon cancer was defined as cancer in the region from the splenic flexor through the rectum. The staging was done per the American Joint Committee on Cancer/Union for International Cancer Control TMN staging system (version 7, 2010) [31]. Progression free survival (PFS) is defined as the interval between the start date for anti-EGFR agents and the stop date of anti-EGFR agents due to disease progression.

Isolation of circulating tumor cells

Tumor cells were isolated from patient blood using the ANGLE Parsorter PR1 system (Parsortix™). This system uses a microfluidic cassette, which separates CTCs by size differences of blood cells in a micro-flow environment. No antibodies are used in this system. Detailed methods associated with this assay have been previously published [32]. In brief, a CTC separation cassette narrows stepwise to a 10-μm gap and traps larger cells (> 10 μm in diameter). After rinsing the microfluidics cassette with 70% Ethanol and PBS, whole blood containing EDTA is loaded on the Parsorter™ system (Angel, Inc.) and then washed with buffer. Each blood sample was then separated by size, and CTCs were isolated over the course of approximately two hours. CTCs were then spread onto two glass slide by Cytospin 2™ (Shandon Inc.) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min at room temperature, then washed with PBS three times. Sample slides were then stored in -80°C for further analysis.

Identification CTCs and meEGFR-CTCs

CTCs were identified based on the combination of positive Epcam signal and lack of CD45 biomarker expression. In brief, sample slides were first blocked with goat serum at room temperature for 60 min. After blocking, Alexa647 conjugated anti-Epcam antibody (Cell Signaling) and Alexa488 conjugated CD45 antibody (abcam) were applied to the sample with 1:100 and 1:500 dilution, respectively, in antibody dilution buffer (1% BSA, 0.3% Triton X-100). After overnight incubation at 4°C, unbound antibodies were removed by phosphate-buffered saline (PBS) wash and coverslip slides with Prolong Gold Antifade Reagent with DAPI (Cell Signaling). Stained slides were scanned using a high-content imaging system (Molecular Devices), and total CTCs numbers were determined by counting $Epcam^+$ and $CD45^-$ cells staining across the entirety of each slide image. A high CTC count was defined as ≥ 3 CTC per 7.5 ml of blood based on the data from a previous study [27]. The number of meEGFR positive cells were determined by immunohistochemistry staining (IHC) using me-R198/200 antibody generated by our lab as previously described [24]. Interpretation of immunohistochemical analysis for meEGFR-CTCs was shown in Supplementary Figure 1.

IHC analysis of meEGFR expression in FFPE

To detect meEGFR in FFPE tumor samples, slides were deparaffinized and rehydrated, and antigen retrieval was performed by the Lab VisionTM PT Module (ThermoFisher Scientific) The sections were treated with 1% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. After 1 hour of serum blocking, the samples were incubated with primary antibodies at 4°C overnight. The sections were then treated with biotinylated secondary antibody, followed by incubations with avidinbiotin peroxidase complex solution for 1 hour at room temperature. Color was developed using 3-amino-9-ethylcarbazole solution. Counterstaining was done using Mayer's hematoxylin. The total protein expression score was calculated as a function of the percentage of immunopositive cells and immunostaining intensity. High meEGFR expression was defined as more than 50% of the immune score activity which was greater than 150. Interpretation of immunohistochemical (IHC) analysis for meEG-

Table 1. Clinical-pathological and molecular characteristic of study populations, n (%)

A. CTC cohort		
Variable	Value	%
No. of patients	47	100
Median age (yr, range)	52, 25-71	
Age		
< 50 years	19	40.4
≥ 50 years	28	59.6
Sex		
Female	22	46.8
Male	25	53.2
Primary tumor site		
Ascending	12	25.5
Transverse	1	2.1
Descending	7	14.9
Sigmoid	22	46.8
Rectum	5	10.6
Line of anti-EGFR Rx		
1st line	6	12.8
2nd line	26	55.3
3rd line	15	31.9
Previous treatment		
Irinotecan	20	42.6
Oxaliplatin	40	85.1
Bevacizumab	36	76.6
Chemotherapy regimen		
Anti-EGFRab monotherapy	8	17.0
Irinotecan-based+anti-EGFRab	35	74.5
Oxaliplatin-based+anti-EGFRab	2	4.3
Vemurafenib+Cetuximab+irinitocan	2	4.2
Liver metastasis		
No	11	23.4
Yes	36	76.6
Lung metastasis		
No	21	44.7
Yes	26	55.3
Differentiated		
Moderate	40	85.1
Poorly	7	14.9
NRAS		
wt	41	87.2
mt	2	4.3
No data	4	8.5
BRAF		
wt	39	83
mt	6	12.8
No data	2	4.3
PIK3CA		
wt	33	70.2
mt	4	8.5
Variant	2	4.3
No data	8	17
MSI		

FR on tissues was shown in Supplementary Figure 2.

Gene mutational analysis

DNA was extracted from FFPE tumor tissue. Samples were evaluated for somatic mutation using a next-generation sequencing platform with 46- or 50-gene panels. Alternately, samples were analyzed for targeted gene mutation of frequently reported point mutations found in human malignancies. Targeted mutation analysis was conducted in a Clinical Laboratory Improvement Amendments (CLIA)-certified molecular diagnostics laboratory. This testing determined the effective lower limit of detection (analytical sensitivity) for single nucleotide variations to be in the range of 5% (one mutant allele in the background of nineteen wild type alleles) to 10% (one mutant allele in the background of nine wild type alleles).

Determination of mismatch repair (MMR) status

MMR status was determined by IHC analysis of MMR protein expression or by polymerase chain reaction (PCR) in the clinical lab. Detailed methods associated with both assays have been previously published [33]. dMMR was defined as the presence of high-level microsatellite instability on PCR and/or the loss of MMR protein expression in IHC. pMMR was defined as the presence of microsatellite stability or low-level microsatellite instability on PCR and/or no loss of MMR protein expression in IHH.

Statistical analysis

Patient characteristics are reported as categorical frequency and percent for each cohort. Correlations between clinical-pathological-molecular variables and meEGFR-CTCs status or meEGFR expression status on tissues were initially tested using Pearson's χ^2 or Fisher exact test. The association between patient and molecular characteristics with PFS was further explored using Kaplan-Meier curves. Cox proportional hazards regression was used to adjust for potential confounders and significant differences were assessed us-

MSS/MSI-L	35	74.5
MSI-H	3	6.4
No data	9	19.1

B. Tissues cohort

Variable	Value	%
No. of patients	176	100
Median age (yr, range)	55, 20-79	
Age		
< 50 years	51	29
≥ 50 years	125	71
Sex		
Female	79	44.9
Male	97	55.1
Primary tumor site		
Ascending	50	28.4
Transverse	14	8
Descending	10	5.7
Sigmoid	66	37.5
Rectum	36	20.5
Type of tissue tested		
Primary CRC tissues	156	88.6
Metastatic tissues	20	11.4
Line of anti-EGFR Rx* (n = 74)		
1st line	8	10.8
2nd line	34	45.9
3rd line	32	42.3
Previous treatment* (n = 74)		
Irinotecan	42	56.8
Differentiated		
Moderate	109	61.9
Poorly	64	36.4
Unknown	3	1.7
KRAS		
wt	127	72.2
mt	48	27.3
No data	1	0.6
NRAS		
wt	140	79.5
mt	4	2.3
No data	32	18.2
BRAF		
wt	136	77.3
mt	24	13.6
No data	16	9.1
PIK3CA		
wt	132	75
mt	19	10.8
No data	25	14.2
MSI		
MSS/MSI-L	98	55.7
MSI-H	7	4
No data	71	40.3

*only the patients that confirmed RAS^{wt} and treated with anti-EGFR agents. wt: wild type, mt: mutation.

ing the log-rank test. Calculations were performed with SPSS-version 23.0 software (IBM Corp., Armonk, NY). *P* values of less than 0.05 were considered statistically significant.

Results

In CTC cohort

A total of 47 mCRC patients were included in this cohort between September 2015 and July, 2016. The median age of the cohort was 52 years (range 25-71 years), and the ratio of males to females was 1.1. The majority of primary tumors were left-sided colon tumors (29 patients), followed by right-sided colon tumors (13 patients), then rectal tumors (5 patients). Anti-EGFRab were most commonly used in the second line of treatment in 26 patients, followed by third line in 15 patients and first line in 6 patients. Previous irinotecan used in 42.6% of all patients. Patient and tumor characteristics are shown in **Table 1A**.

Detection of CTCs and meEGFR-CTCs

In this cohort, CTCs were identified in 30 out of 47 cases (63.8%). Of these 30 cases, meEGFR-CTCs were identified in 19 cases (63.3%) (**Figure 1**). Mean total CTCs and cell counts of CTCs positive for meEGFR were 3.6 cells (range 0-52) and 2.3 cells (range 0-30) per 7.5 ml, respectively. The ratio of meEGFR CTCs per total CTCs is shown in **Figure 2**. The mean ratio of mEGFR CTCs to total CTCs was 0.23 with the range from 0 to 1. Therefore, we considered cases with a ratio ≥ 0.23 as high meEGFR-CTC cases.

Association between total CTCs or meEGFR-CTCs and clinic-pathologic-molecular characteristic

We compared the clinic-pathological and molecular variables of patients, including age, sex, site of the primary tumor, histologic grade, previous irinotecan used, line of anti-EGFR treatment, and, *NRAS, BRAF, PIK3CA,* and MSI status, by the status of CTCs and meEGFR-CTCs. No clinic-pathological-molecular features were associat-

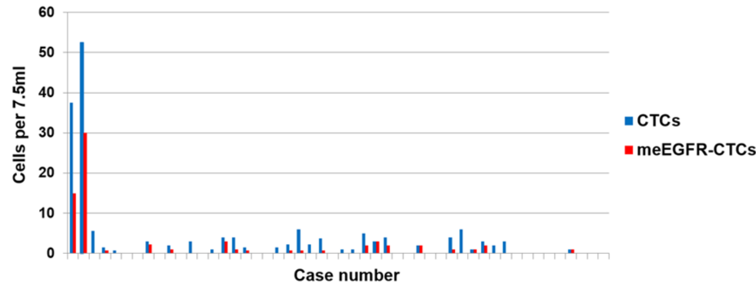

Figure 1. Total CTCs and meEGFR-CTCs detected. CTCs were identified in 30 out of 47 cases (63.8%). Of these 30 cases, meEGFR-CTCs were identified in 19 cases (63.3%).

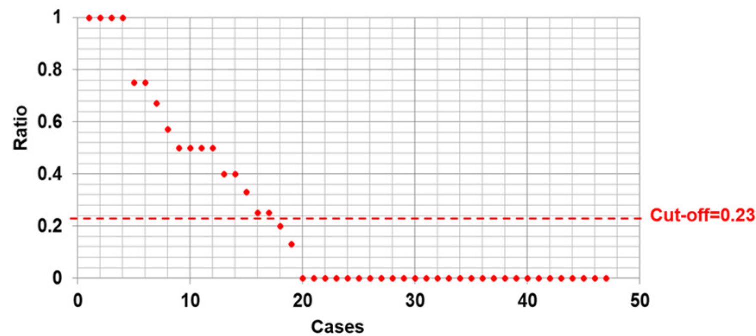

Figure 2. Ratio of meEGFR per total CTCs. The mean ratio of mEGFR CTCs to total CTCs was 0.23 with the range from 0 to 1.

ed with either detectable/non-detectable meEGFR-CTCs or total CTCs less than/at least 3 cells per 7.5 ml (Supplementary Table 1). Further, there was no significant difference between patients with high *vs*. low meEGFR-CTCs ratio (**Table 2**). This suggests that the meEGFR-CTCs are not a surrogate for existing prognosis or predictive features but represents unique molecular feature.

Progression free survival analysis

To test the potential associations between meEGFR-CTCs and progression free survival (PFS), we first performed univariate analyses of PFS by meEGFR-CTC ratio and previously established prognostic factors: sidedness, line of anti-EGFR treatment, and *PIK3CA* status. The only factor that was significantly associated with worse PFS in this cohort was meEGFR ratio ≥ 0.23. In *RAS*^{wt} *BRAF*^{wt} mCRC patients with meEGFR ratio ≥ 0.23 had significantly worse PFS for anti-EGFR treatment compared with patients with the ratio < 0.23 (HR 3.4, 95% CI 1.47-7.90. P = 0.004) and remain statistically significant in multivariate analysis (HR = 3.0, 95% CI 1.03-8.5, P = 0.04) (**Table 4A**; **Figure 3A**). This ratio had 100% sensitivity and 59% specificity to detect the difference if we used the cut point of 3 months to define the patients as responder and non-responder group.

In tissues cohort

A total of 176 mCRC patients were included in the tissue analysis cohort. Of these, we had tumor samples from primary CRC in 156 cases, and metastatic tumor samples from 20 cases. Tissues were collected prior to anti-EGFR treatment. The median age was 55 years (range 20-79 years), and the ratio of males to females was 1.24. Patient and tumor characteristics are shown in **Table 1B**.

Association between meEGFR expression and clinic-pathologic-molecular characteristic

Out of 176 samples, 164 had data on meEGFR expression. A total of 76 cases (46.3%) exhibited high expression of meEGFR. Further, 63 cases exhibited low expression of EGFR (score > 0-150) and 25 cases showed no expression (score = 0). Comparing the clinical-pathological and molecular variables by meEGFR expression revealed that only *KRAS*^{mt} *and NRAS*^{mt} were significantly associated with meEGFR high expression (P = 0.03 and P = 0.02, respectively) (**Table 3**).

Progression free survival analysis

Univariate analysis of PFS was performed using previously established prognostic factors: sidedness, line of anti-EGFR treatment, and *PIK3CA* status. In 176 cases, there were 107 (60.7%) *RAS*^{wt} mCRC patients. Of these 107, 67 cases were *RAS*^{wt} *BRAF*^{wt} mCRC and had available data on outcome with anti-EGFR treatment. Median PFS were 6, 8, 11 mo in 3rd, 2nd, and 1st line, respectively (P = 0.02). There was no correlation between high meEGFR expression in the tumor tissues and PFS in *RAS*^{wt}/

Table 2. Association between meEGFR-CTCs ratio and clinical-pathological and molecular factors

Variable	meEGFR-CTCs ratio		*P* value
	< 0.23	≥ 0.23	
Age			
< 50 years	15 (50)	4 (23.5)	0.08
≥ 50 years	15 (50)	13 (76.5)	
Sex			
Female	14 (46.7)	8 (47.1)	0.99
Male	16 (53.3)	9 (52.9)	
Site			
Right-sided	8 (26.7)	5 (29.4)	0.84
Left-sided	22 (73.3)	12 (70.6)	
Line of anti-EGFR Rx			
1st line	4 (13.3)	1 (5.9)	0.38
2nd line	15 (50)	12 (70.6)	
3rd line	11 (36.7)	4 (23.5)	
Previous irinotecan			
No	18 (60)	9 (52.9)	0.64
Yes	12 (40)	8 (47.1)	
Liver metastasis			
No	5 (16.7)	6 (35.3)	0.15
Yes	25 (83.3)	11 (64.7)	
Lung metastasis			
No	13 (43.3)	8 (47.1)	0.81
Yes	17 (56.7)	9 (52.9)	
Differentiated			
Moderate	25 (83.3)	15 (88.2)	0.65
Poorly	5 (16.7)	2 (11.8)	
NRAS (n = 43)			
wt	26 (96.3)	14 (93.8)	0.70
mt	1 (3.7)	1 (6.3)	
BRAF (n = 45)			
wt	24 (85.7)	15 (18.2)	0.81
mt	4 (14.3)	2 (11.8)	
PIK3CA (n = 37)			
wt	18 (85.7)	15 (93.8)	0.44
mt	3 (14.3)	1 (6.3)	
MSI (n = 38)			
MSS/MSI-L	21 (91.3)	14 (93.3)	0.82
MSI-H	2 (8.7)	1 (6.7)	

wt: wild type, mt: mutation.

RAF^{wt} mCRC in this cohort (HR 0.8, 95% CI 0.45-1.44, P = 0.46) (**Table 4B**; **Figure 3B**).

meEGFR expression in CTCs vs. tissues

In this study, there were 10 cases that had data on both meEGFR-CTCs and meEGFR expression from tissues. There was no association between meEGFR-CTC ratio and tumor meEGFR expression status (P = 0.67). Detail of these cases was shown in Supplementary Table 2.

Discussion

In this study, we successfully isolated CTCs from CRC patients' blood and were able to assess arginine methylated EGFR in the isolated CTCs. We showed for the first time that elevated levels meEGFR-CTCs were associated with a shorter duration of anti-EGFR-based treatment. EGFR arginine-methylation in CTC may serve as a biomarker to stratify the patients that response to anti-EGFR therapy.

EGFR methylation in CRC has been reported at the level of pre-transcriptional and post-translational modification. Arginine methylation represents a common post-translational modification of EGFR [34]. Protein arginine methylatransferases (PRMTs) mediate the methylation of protein substrates of arginine residue and can play an important function in cancer development [35]. The activity of PRMT1, a member of the PRMT family, accounts for more than 90% of the methylarginine residues in mammalian cells [36]. PRMT1 is the major asymmetric arginine methyltransferase and is deregulated in multiple cancers, including breast, prostate, lung, bladder, leukemia, and colon cancer [37-41]. More recently, our group demonstrated that patients with high levels of PRMT1-mediated EGFR methylation had worse PFS with cetuximab treatment and poor OS [24] compared with patients with low levels of PRMT1-mediated EGFR methylation. Although EGFR expression does not appear to be a predictive marker for anti-EGFR treatment [42], methylated EGFR expression may serve as a potential predictive marker in anti-EGFR therapy. However, further validation of this result is needed.

Compared to standard tissue biopsy, liquid biopsy has several unique advantages. First, it is minimally invasive, avoiding the potential complications of biopsies. Second, it provides an opportunity to obtain tumor information when tissue biopsy is difficult or contraindicated. Additionally, the safety and simplicity of such an option allows for serial sampling, which are important for assessing treatment response [43]. Previous studies demonstrated that high CTC numbers in blood correlate with poor

Table 3. Association between meEGFR expression and clinical-pathological and molecular factors (N = 164, exclude 12 cases that had no data on meEGFR expression)

Variable	meEGFR		*P* value
	Low/No expression	High expression	
Age (n = 164)			
< 50 years	25 (28.4%)	21 (27.6%)	0.91
≥ 50 years	63 (71.6%)	55 (72.4%)	
Sex (n = 164)			
Female	36 (40.9%)	36 (47.4%)	0.41
Male	52 (59.1%)	40 (52.6%)	
Site (n = 164)			
Right-sided	34 (38.6%)	27 (35.5%)	0.68
Left-sided	54 (61.4%)	49 (64.5%)	
Differentiated (n = 161)			
Moderate	45 (56.3%)	52 (64.2%)	0.30
Poorly	35 (43.8%)	29 (35.8%)	
KRAS (n = 163)			
wt	73 (83%)	51 (68%)	0.03
mt	15 (17%)	24 (32%)	
NRAS (n = 136)			
wt	76 (100%)	56 (93.3%)	0.02
mt	0 (0%)	4 (6.7%)	
BRAF (n = 150)			
wt	68 (84%)	58 (84.1%)	0.97
mt	13 (16%)	11 (15.9%)	
PIK3CA (n = 142)			
wt	73 (90.1%)	51 (83.6%)	0.25
mt	8 (9.9%)	10 (16.4%)	
MSI (n = 99)			
MSS/MSI-L	41 (91.1%)	51 (94.4%)	0.52
MSI-H	4 (8.9%)	3 (5.6%)	

wt: wild type, mt: mutation.

prognosis in many cancer types, including colorectal [27], breast [44], and prostate [45] cancers. Data in a prospective multicenter study demonstrated that mCRC patients with at least three CTCs per 7.5 ml at baseline constitutes a strong independent prognostic factor for inferior PFS and OS [27]. Hence, liquid biopsies are growing in popularity as standard tests and have potential for routine cancer patient care. While the prognostic impact on CTCs in CRC has been established [27, 46, 47], the predictive impact on liquid biopsy in CRC has not been reported.

In this study, CTCs were isolated from patients with Parsortix PR1 system, which isolates CTCs by size and deformation capability. CTCs were identified in 64% of the patients in this cohort, which was higher than the range 28-49% previously reported [27, 28, 48, 49]. However, patients in this cohort were all at stage IV, and when only stage IV disease was considered in other published cohorts, we found similar positive CTCs rate (59.3-60.7%) [29, 48]. meEGFR-CTCs was also identified in 63% of all detected CTCs cases, which indicated that meEGFR occurred in the majority of patients with positive CTC detection. As our study is the first report meEGFR-CTCs in mCRC, further studies are warranted to confirm this finding.

We found no correlation between the occurrences of meEGFR-CTCs and PFS of anti-EGFR treated patients when grouping the patients simply based on the amount of detected meEGFR-CTC (≥ 1 or ≥ 3 per 7.5 ml of blood). However, since meEGFR-CTCs and non-meEGFR-CTCs were both simultaneously detected in most of the cases, simply grouping the patients based on the meEGFR-CTC counts may not accurately reflect anti-EGFRab treatment response. Therefore, we hypothesized that tumor with dominant populations of meEGFR positive tumor cells would have poor response to anti-EGFRab treatment, i.e., the ratio of meEGFR-positive tumor cells in tumor may correlate better with anti-EGFRab treated patients' PFS. Therefore, we used the ratio of meEGFR-CTCs over total CTCs with a cut-off point 0.23 (average ratio = 0.23) to classify the patients into 2 groups. Our study showed that patients with high meEGFR-CTC per total CTCs ratio had significant worse PFS than those who had the ratio < 0.23 (median PFS 5.3 *vs*. 8 months, HR = 3, 95% CI = 1.03-8.5, P = 0.002). This finding confirms our hypothesis that the ratio of meEGFR-CTCs may help predict treatment response and supports the result from our previous paper [24]. No correlation was found between either meEGFR positive or meEGFR ratio with any clinical-pathological and molecular characteristics implying that this is an independent molecular feature not represented by other known factors. Given the small number samples in the current study, these results will need to be confirmed in lager dataset.

In FFPE tissue staining cohort, this study demonstrated meEGFR-positive staining (either low or high expression) in 127/145 (88%) in primary CRC tissues and 12/19 (63%) in metastatic

Table 4. Univariate and multivariate analysis of prognostic factors influencing PFS on *RAS*^{wt} *BRAF*^{wt} with anti EGFR treatment

A. CTC cohort (n = 32)

Variables	N	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Sidedness							
Rt. sided	7	Ref					
Lt. sided	25	0.85	0.4-2.0	0.71			
Line of anti-EGFR Rx							
1st line	3	Ref			Ref		
2nd line	17	2.1	0.6-7.4	0.26	2	0.5-8.0	0.34
3rd line	12	1.6	0.4-5.9	0.47	1.5	0.4-5.7	0.58
PIK3CA							
wt	24	Ref			Ref		
mt	2	0.9	0.2-3.9	0.89	1.3	0.2-7.3	0.77
meEGFR ratio							
< 0.23	20	Ref		0.004	Ref		
≥ 0.23	12	3.4	1.5-7.9		3.0	1.03-8.5	0.04

wt: wild type, mt: mutation, Ref: Reference.

B. *RAS*^{wt}, BRAF^{wt} in tissue cohort

Variables	N	Univariate analysis		
		Median PFS (mo)	95% CI	*P* value
Site				
Rt. sided	16	9.2	4.0-14.4	0.72
Lt. sided	54	7.8	5.3-10.4	
Line of anti-EGFR Rx				
1st line	8	11.0	8.5-13.5	0.02
2nd line	33	8.0	3.5-12.6	
3rd line	29	6.0	4.1-7.9	
PIK3CA				
wt	64	7.8	5.6-10.1	0.37
mt	5	11.1	2.3-20.0	
meEGFR				
Low expression	47	7.4	5.0-9.7	0.46
High expression	20	9.5	2.9-16.0	

wt: wild type, mt: mutation.

tissues. However, there was no correlation between meEGFR levels in CTCs and tumor tissues (Supplementary Table 2). This suggests that arginine methylation of EGFR may be a dynamic process influenced by prior chemotherapy and/or clonal drift in a heterogenous tumors. It is also possible that CTCs do not accurately reflect the protein methylation status of the bulk tumor. However, in contrast to our previous report [24] the meEGFR expression on CRC tissue was not correlated with PFS on anti-EGFR treatment. One potential explanation would be the difference in patients' populations and the difference in the cut-off point to define into high or low meEGFR expression groups. However, our data showed a positive correlation between expression level of meEGFR and PRMT1 (P = 0.03) which confirmed the previous report paper in our group [24] (Supplementary Table 3).

Our group previously reported that meEGFR is a potential for predicting response to anti-EGFR treatment [24]. This occurred only in CTCs but not in the tumor tissues in the present study. Consequently this finding raises the possibility that meEGFR occur during tumor development and increase over time. Therefore, meEGFR-CTCs maybe better predict response than primary tumor tissues. Additional work based on the current finding could refine cut-off point used to define the correlation between high meEGFR-CTCs ratio and high meEGFR expression in FFPE tissues and could be coupled with serial monitoring of meEGFR-CTCs ratio with treatment response. Furthermore, since PRMT1 mediates meEGFR, PRMT1 inhibitors may reduce meEGFR, potentially sensitizing some tumors to anti-EGFRabs. This represents an exciting area for future study, and the appropriate patient identification could potentially increase those that benefit from anti-EGFR therapy.

We recognize the limitations of the current study. First, it was a small, retrospective study with lack of statistical power. Second, we have no data on longitudinal CTC sampling, so we do not know whether meEFGR can develop as a method of acquired resistance.

In summary, this study is the first to indicate that PRMT1 methylated-EGFR detected in CTCs may serve as a potential liquid biopsy biomark-

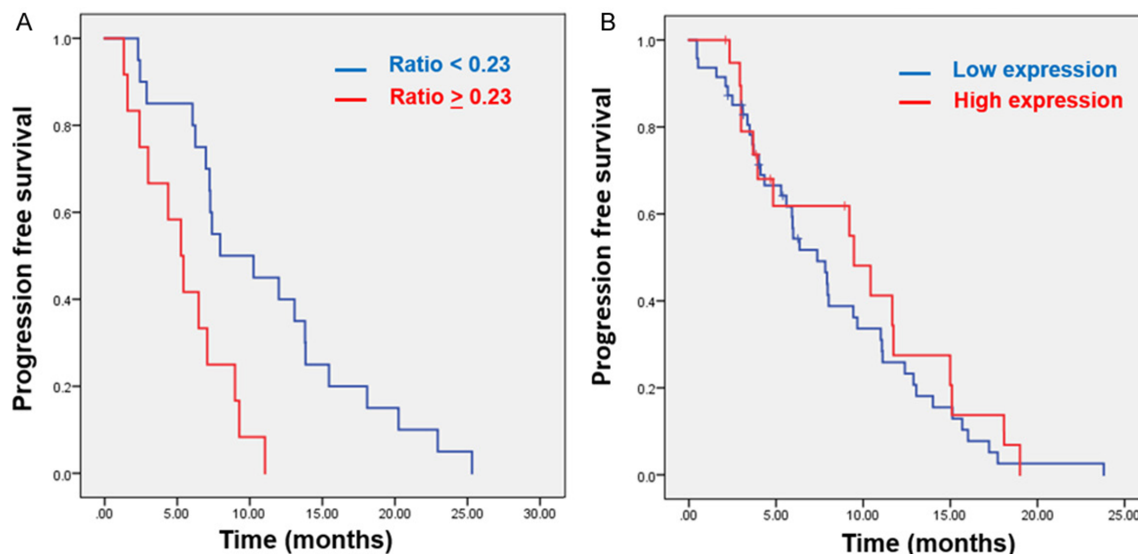

Figure 3. Kaplan-Meier survival curve of *RAS*wt *BRAF*wt mCRC. A. *RAS*wt *BRAF*wt mCRC patients with high meEGFR ratio (≥ 0.23) had significantly worse PFS for anti-EGFR treatment (median PFS 5.3 mo, 95% CI 3.5-7 mo) compared with pts with the ratio < 0.23 (median PFS 8 mo, 95% CI 1.7-14.2 mo, P = 0.002). B. There was no correlation between high or low meEGFR expression in the tumor tissues and PFS for anti-EGFR treatment treatment (median PFS 7.4 mo, 95% CI 5.0-9.7 mo in low expression compared with median PFS 9.5 mo, 95% CI 2.9-16.0 mo in high expression, P = 0.46).

er for predicting anti-EGFR response. Further studies are required to identify the patients most likely to benefit from anti-EGFR treatment. Assessment of meEGFR-CTCs may provide a useful "liquid biopsy" biomarker for identifying patients that may exhibit reduced benefit from anti-EGFR treatment.

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Disclosure of conflict of interest

None.

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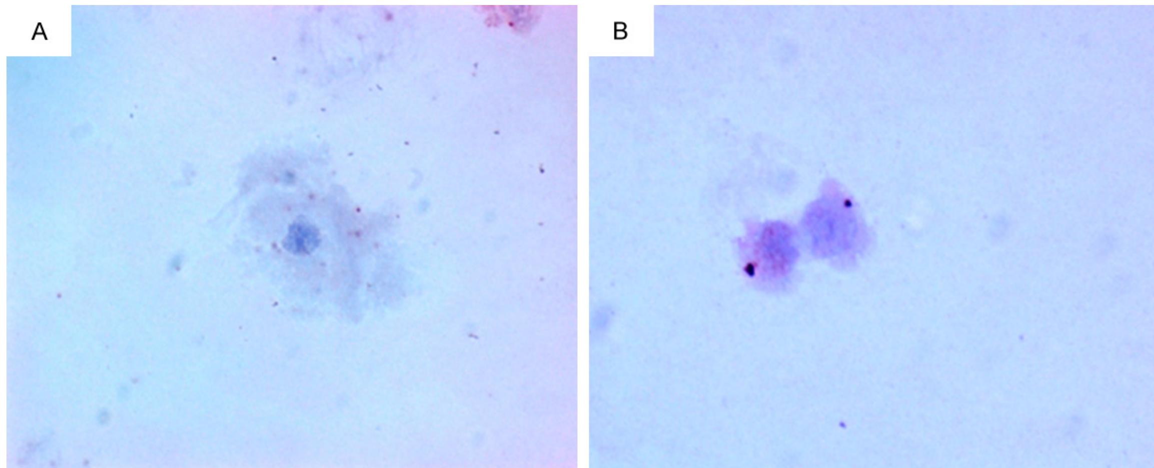
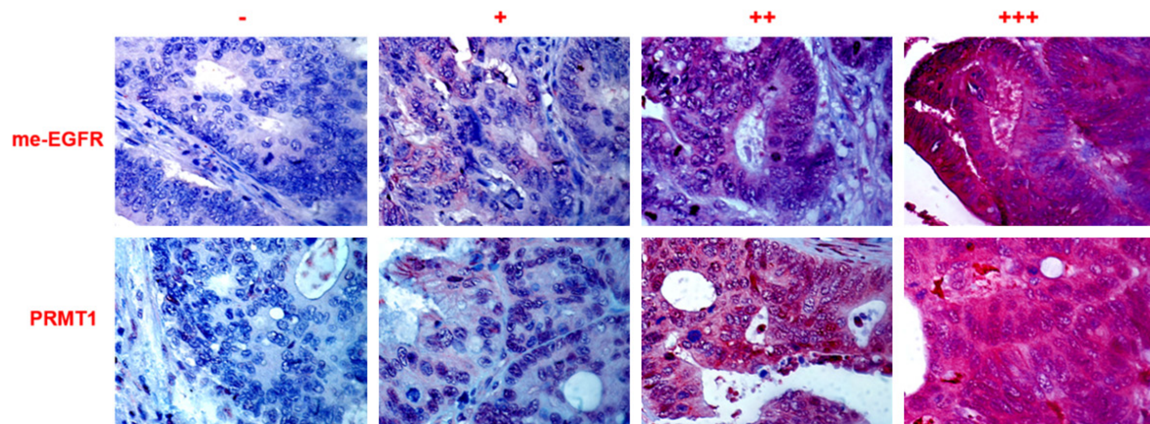


Supplementary Figure 1. Interpretation of immunohistochemical analysis for meEGFR-CTCs. A. meEGFR negative. B. meEGFR positive.



Supplementary Figure 2. Interpretation of immunohistochemical analysis for meEGFR on tumor tissues.

Supplementary Table 1. Association between Total CTCs count/meEGFR-CTCs and clinical-pathological and molecular factors

Variable	Total CTCs count/7.5 ml		P value	meEGFR-CTCs		P value
	< 3	≥ 3		Non-detectable	Detectable	
Age						
< 50 years	15	4	0.12	14	5	0.10
≥ 50 years	16	12		14	14	
Sex						
Female	15	7	0.76	14	8	0.60
Male	16	9		14	11	
Site						
Right-sided	9	4	0.77	8	5	0.87
Left-sided	22	12		20	14	
Line of anti-EGFR Rx						
1st line	3	3	0.59	3	3	0.75
2nd line	17	9		15	11	
3rd line	11	4		10	5	
Previous irinotecan						
No	18	9	0.90	17	10	0.58
Yes	13	7		11	9	
Previous bevacizumab						
No	5	6	0.10	5	6	0.28
Yes	26	10		23	13	
Liver metastasis						
No	4	44	0.85	5	6	0.28
Yes	24	12		23	13	
Lung metastasis						
No	11	10	0.08	12	9	0.76
Yes	20	6		16	10	
Differentiated						
Moderate	25	15	0.23	23	17	0.49
Poorly	6	1		5	2	
NRAS (n = 43)						
wt	27	14	0.65	24	17	0.81
mt	1	1		1	1	
BRAF (n = 45)						
wt	26	13	0.43	22	17	0.64
mt	3	3		4	2	
PIK3CA (n = 37)						
wt	20	13	0.58	16	17	0.32
mt	3	1		3	1	
MSI (n = 38)						
MSS/MSI-L	24	11	0.95	20	15	0.75
MSI-H	2	1		2	1	

wt: wild type, mt: mutation.

Supplementary Table 2. Association between meEGFR ratio from CTCs and meEGFR expression from tissues

		meEGFR tissues		
		High expression	Low/No expression	*P* value
CTCs ratio	< 0.23	3	5	0.67
	≥ 0.23	1	1	

Supplementary Table 3. Association between expression level of meEGFR and PRMT1

		meEGFR		
		Low expression	High expression	*P* value
PRMT1	Low expression	54 (61.4%)	34 (44.7%)	0.03
	High expression	34 (38.6%)	42 (55.3%)	