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Translational Cancer Mechanisms and Therapy

Clinical Cancer Research

Response to Anti-EGFR Therapy in Patients with BRAF non-V600–Mutant Metastatic Colorectal Cancer

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Abstract

Purpose: While mutations in BRAF in metastatic colorectal cancer (mCRC) most commonly occur at the V600 amino acid, with the advent of next-generation sequencing, non-V600 BRAF mutations are increasingly identified in clinical practice. It is unclear whether these mutants, like BRAF V600E, confer resistance to anti-EGFR therapy.

Experimental Design: We conducted a multicenter pooled analysis of consecutive patients with non-V600 BRAF-mutated mCRCs identified between 2010 and 2017. Non-V600 BRAF mutations were divided into functional classes based on signaling mechanism and kinase activity: activating and RAS-independent (class 2) or kinase-impaired and RAS-dependent (class 3).

Results: Forty patients with oncogenic non-V600 BRAF–mutant mCRC received anti-EGFR antibody treatment [$n = 12$ (30%) class 2 and $n = 28$ (70%) class 3]. No significant differences in clinical characteristics were observed by mutation class. In contrast, while only 1 of 12 patients with class 2 BRAF mCRC responded, 14 of 28 patients with class 3 BRAF responded to anti-EGFR therapy (response rate, 8% and 50%, respectively, $P = 0.02$). Specifically, in first- or second-line, 1 of 6 (17%) patients with class 2 and 7 of 9 (78%) patients with class 3 BRAF mutants responded ($P = 0.04$). In third- or later-line, none of 6 patients with class 2 and 7 of 19 (37%) patients with class 3 BRAF mutants responded ($P = 0.14$).

Conclusions: Response to EGFR antibody treatment in mCRCs with class 2 BRAF mutants is rare, while a large portion of CRCs with class 3 BRAF mutants respond. Patients with colorectal cancer with class 3 BRAF mutations should be considered for anti-EGFR antibody treatment.

See related commentary by Fontana and Valeri, p. 6896

Introduction

BRAF is a member of the RAF family of serine/threonine kinases and transduces signals in the MAPK pathway (1). It is directly activated by RAS, which induces the formation of RAF dimers that carry the MAPK signal downstream. Between 8% and 12% of metastatic colorectal cancer (mCRC) cases harbor a *BRAF* mutation (2–4). While mutations in *BRAF* most commonly occur at the V600 amino acid, up to a quarter of mutations in *BRAF* do not involve this residue, and, with next-generation sequencing, these mutants are increasingly identified in clinical practice (4–6).

BRAF mutations can be classified into three groups based on their biochemical and signaling mechanisms (7–9). Class 1 consists of BRAF V600 mutants, which exhibit high kinase activity and are RAS independent because they can signal as monomers. Mutations outside of the V600 amino acid in *BRAF* are divided into class 2 and class 3. BRAF class 2 mutants are activating and RAS independent; they dimerize and signal without RAS activation. Class 3 BRAF mutants exhibit low kinase activity or are kinase-dead but activate the MAPK pathway through enhanced RAS binding and subsequent RAS-dependent CRAF activation (8–10). These distinct mechanisms of MAPK activation may affect clinical characteristics of *BRAF*-mutant colorectal cancers. While *BRAF* V600–mutant tumors are often right-sided, high grade, microsatellite instability (MSI)-high, and associated with a worse prognosis compared with *BRAF* wild-type (WT) tumors, *BRAF* non-V600 tumors are observed in younger patients with left-sided primaries and tend to have a favorable prognosis similar to *BRAF* WT tumors (5, 11).

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Translational Relevance

BRAF mutations can be classified into three groups based on their biochemical properties: class 1 mutants (i.e., V600) exhibit high kinase activity and signal as RAS-independent monomers; class 2 mutants are activating and signal as RAS-independent dimers; and class 3 mutants have impaired kinase activity and depend on RAS to activate signaling. In metastatic colorectal cancer (mCRC), BRAF mutations consist of 2/3 class 1 and 1/3 class 2 and 3 alterations. The class 2 and 3 BRAF mutants do not respond to approved RAF inhibitors. Class 3 mutants, however, may be sensitive to inhibition of receptors activating RAS, such as EGFR in colorectal cancer. Here, we have assembled a large series of non-V600 BRAF–mutant mCRC treated with EGFR-targeting antibodies to test this hypothesis. We show that patients with class 3 BRAF mutations commonly respond to anti-EGFR treatment, while patients with mCRC with class 2 BRAF mutations are unlikely to respond.

RAF inhibitors currently in use potently inhibit BRAF monomers but not dimers. They therefore are predicted to be useful for the treatment of *BRAF* V600–mutant tumors but not those driven by class 2 or class 3 mutations. This is the case in preclinical models and has, for the most part, been born out in clinical trials. Class 2 and 3 mutant-driven tumors are ERK driven and predicted to be sensitive to MEK inhibition, but the effectiveness of the latter is limited by the narrow therapeutic index of these drugs. The data do offer the possibility that tumors driven by class 3 BRAF mutants in which RAS activation is dependent on receptor tyrosine kinase (RTK) signaling will be sensitive to inhibition of that RTK.

The obstacles to test this idea are the difficulty in identifying the dominant kinase driving the pathway in a particular tumor and in determining whether there is a single dominant driver at all. The epidermal growth factor receptor (EGFR) has been identified as important in the normal development and physiology of the colon and an important driver of a subset of colorectal cancers. We therefore undertook an international collaboration to assemble a large number of consecutive non-V600 *BRAF*–mutant mCRC to determine whether colorectal cancers with non-V600 *BRAF*–mutants would be sensitive to EGFR inhibition.

EGFR inhibitors represent an important regimen for mCRC associated with an overall survival (OS) benefit (12). Molecular markers have refined the population of patients treated with these agents. Colorectal cancers with activating *RAS* mutations, which constitutively signal downstream of EGFR, do not benefit from anti-EGFR mAbs. Similarly, *BRAF* mutation may confer resistance to anti-EGFR therapy. Several retrospective studies and meta-analyses suggest that the presence of *BRAF* V600E is a negative predictor for response to these treatments (3, 13–16). However, the effect of *BRAF* non-V600 mutations on response to targeted EGFR inhibition is largely unknown. Small studies and published case reports describe variable responses (17–19). We have now functionally characterized unknown BRAF mutants found in clinical practice and present an analysis of response of patients with mCRC with non-V600 *BRAF* alterations to EGFR antibody therapy based on *BRAF* mutation functional class.

Materials and Methods

Study design

To identify all mCRCs with non-V600 *BRAF* alterations sequenced between 2010 and 2017, we queried databases or searched the electronic records from prospective institutional sequencing of patients with mCRC from the Japanese nationwide cancer genome screening project for gastrointestinal cancers (SCRUM-Japan GI-SCREEN), Memorial Sloan Kettering Cancer Center (MSK), the Biomarker Research for anti-EGFR monoclonal Antibodies by Comprehensive Cancer genomics (BREAC) study (18), Aichi Cancer Network, West Japan Oncology Group, and Massachusetts General Hospital Cancer Center. Data from consecutive patients with non-V600 *BRAF* mutant mCRC collected included: age at diagnosis, sex, primary tumor site, histology, stage, adjuvant chemotherapy status, *RAS* mutation status, MSI/mismatch repair protein status, last date of follow-up or date of death, and vital status. Primary site was designated as right-sided from the cecum to the distal transverse colon and left-sided from the distal transverse colon/splenic flexure (inclusive) to the rectum. Patients who received any systemic chemotherapy for metastatic disease were analyzed for OS, defined as the time from start of systemic chemotherapy for metastatic disease to death from any cause.

Efficacy of anti-EGFR antibody was assessed in patients with *RAS* WT tumors who received anti-EGFR antibody with or without chemotherapy. For these patients, information on prior treatments and clinical outcome of anti-EGFR antibody treatment were also collected. The efficacy endpoints were progression-free survival (PFS), defined as the time from the start of anti-EGFR antibody treatment to disease progression or death from any cause; OS; and response rate, defined as the proportion of patients who had a complete or partial response to anti-EGFR–containing regimen. Response was assessed on the basis of review of radiology reports and categorized according to the RECIST version 1.1.

This study was approved by the respective institutional review boards in each participating center and complied with the Declaration of Helsinki. Each patient provided written informed consent.

Tumor genotyping

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue obtained from biopsies or resections and sequenced with a multi-gene assay in a Clinical Laboratory Improvement Amendments (CLIA)-certified setting. For patients treated in Japan, sequencing was performed with either Oncomine Cancer Research Panel (Life Technologies), Oncomine Comprehensive Assay (Life Technologies), or GENOSEARCH BRAF or MEBGEN RASKET (MBL). For the BREAC study, targeted capture sequencing was done with the Illumina HiSeq 2000 system (18). Tumor genomic analysis for MSK was performed with the mass spectrometry–based Sequenom assay (20) before 2015 and with MSK-IMPACT next-generation sequencing assay afterwards (21). Patients identified at Massachusetts General Hospital had tumor genomic analysis with SNaPshot, which utilizes next-generation sequencing and interrogates BRAF exons 11 and 15.

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Circulating free DNA analysis

Circulating free DNA (cfDNA) analysis was performed with the Guardant360 Commercial Assay (Guardant Health; ref. 22). This is a CLIA-certified targeted digital sequencing panel designed to detect single-nucleotide variants and select insertions/deletions, amplifications, and fusions in 73 cancer genes.

Patient-derived xenograft models

Patient-derived tumor models were obtained from Crown Bioscience or generated as described previously (23). The patient-derived tumor was implanted as subcutaneous xenografts into 6 weeks old NSG mice (Jackson Laboratories), and treatments started when tumors reached 100 mm^3 volumes. Mice (at least 5/group) were randomized and dosed with vehicle or cetuximab (50 mg/kg twice weekly intraperitoneal). Mice were observed daily throughout the treatment period for signs of morbidity/mortality, and body weights were recorded twice weekly. Tumors were measured twice weekly using calipers, and volume was calculated using the formula length $\times$ width^2 $\times$ 0.52 (24). Cetuximab for animal experiments was purchased from the hospital pharmacy. All studies were performed in compliance with institutional guidelines under an Institutional Animal Care and Use Committee–approved protocol. Investigators were not blinded when assessing the outcome of *in vivo* experiments.

Classification of BRAF mutants in mCRC cases

The conditional RAS-knockout ("RAS-less") cells, kindly provided by Mariano Barbacid (Centro Nacional de Investigaciones Oncologicas) (25), were used to classify unknown BRAF mutants. These HRAS$^{-/-}$, NRAS$^{-/-}$, KRASlox/lox; RERTert/ert mouse embryonic fibroblasts (MEF) cells were transfected to inducibly express WT BRAF or the BRAF mutants of interest (A33T, F24L, R558Q, L485F, and L525R) as described previously (9). Briefly, cells were grown in medium without 4-OHT (4-hydroxytamoxifen, control cells expressing KRAS) or with 1 μmol/L 4-OHT (cells with no RAS isoform expression) for 1 week. Expression of the BRAF proteins was induced by 10 ng/mL doxycycline treatment for 24 hours. Cells grown without 4-OHT were used to assay ERK activation and dimerization dependence of the expressed BRAF mutants of interest; cells grown with 4-OHT to knockout the last *RAS* allele were used to investigate RAS dependence of the expressed BRAF mutants. Cells were collected, and whole-cell lysates were prepared and examined by Western blot analysis. RAS-GTP levels were determined using the active RAS pull-down assay (Thermo Fischer Scientific).

Statistical analysis

Fisher exact test was used to compare clinicopathologic features between *BRAF* mutational classes. For PFS and OS, survival curves according to *BRAF* mutational classification were estimated by the Kaplan–Meier method and were compared using log-rank test. A two-sided $P < 0.05$ was considered significant. All statistical analyses were performed using IBM SPSS statistics version 22.0 (IBM Corp).

Results

Response to EGFR inhibitors in preclinical patient-derived models

The effect of non-V600 BRAF mutants on clinical response to EGFR targeting antibodies in colorectal cancer is unknown. Given that mutant BRAF activates MAPK signaling downstream of EGFR, we hypothesized that CRCs with class 2 BRAF mutants may be refractory to anti-EGFR antibody treatment similar to those with class 1 BRAF V600 mutants. In contrast, class 3 BRAF mutants may amplify RAS signaling downstream of EGFR, and it has been proposed that these tumors may depend on EGFR activation for MAPK signaling (9). Therefore, we first analyzed patient-derived colorectal cancer models to evaluate response to EGFR antibodies. Five patient-derived xenografts (PDX) were generated from clinical samples of colorectal cancer, expanded in mice, and then treated with vehicle or the EGFR antibody cetuximab. The effect of cetuximab treatment in one PDX model was reported previously (9) and the other four PDX models are shown in Fig. 1. Two of the PDXs were extremely sensitive to cetuximab treatment (Fig. 1; ref. 9), while the three others continued to grow despite cetuximab exposure, with no significant difference from vehicle treatment. Analysis by BRAF mutation class indicated that the two PDXs harboring class 2 BRAF mutants (K601E and T599dup) were resistant to cetuximab treatment, while the three PDXs harboring class 3 BRAF mutants (D594N and G466V) included two EGFR antibody–sensitive tumors and a resistant tumor. Our preclinical models suggest that CRCs with *BRAF* mutations may respond differently to EGFR antibody treatment and response may vary by *BRAF* mutation class.

Classification of BRAF mutants in mCRC cases

On the basis of our preclinical observations, we sought to evaluate the effect of non-V600 BRAF mutants on clinical response to EGFR antibodies in patients. We assembled an international collaboration to identify consecutive patients with colorectal cancer with non-V600 *BRAF* alterations seen in the clinic. A total of 153 patients with non-V600 *BRAF*–mutant mCRC were identified (Supplementary Fig. S1). Four patients with concurrent class 1 *BRAF* mutations and 31 patients without chemotherapy treatment for metastatic disease were excluded from further analysis. In the total population of 118 patients, 41 received anti-EGFR antibody treatment.

We first classified the BRAF mutants in the 118 cases into class 2 or class 3 on the basis of published studies of the biochemical effects of the BRAF alterations (9, 18, 26–29). We sought to evaluate the effect of EGFR antibodies in all patients exposed to these agents by BRAF mutation class. Three patients had non-V600 *BRAF* mutations that had not been previously characterized (A33T, F247L, and R558Q), and two cases had *BRAF* mutations (L485F and L525R) that had not been fully characterized. To classify these mutants, we first expressed each of these BRAF mutants in MEFs to assess their ability to activate downstream proteins phospho-ERK and phospho-MEK (Fig. 2A). A change in ERK signaling was not detectable in cells when WT BRAF was overexpressed. Expression of BRAF A33T also had no effect on ERK signaling. BRAF F247L and R558Q led to modest activation of ERK signaling, and BRAF L485F and L525R both strongly activated ERK signaling, compared with WT BRAF. We next evaluated whether dimerization is required for these mutants to activate the MEK/ERK cascade by introducing the R509H mutation into BRAF. It has been reported that this mutation impairs BRAF dimerization (30). The activation of ERK signaling seen with the BRAF mutants F247L, R558Q, L485F, and L525R was abrogated by the R509H mutation, indicating that all these mutants require dimerization to signal. Furthermore, we determined RAS independence by expressing each of these BRAF

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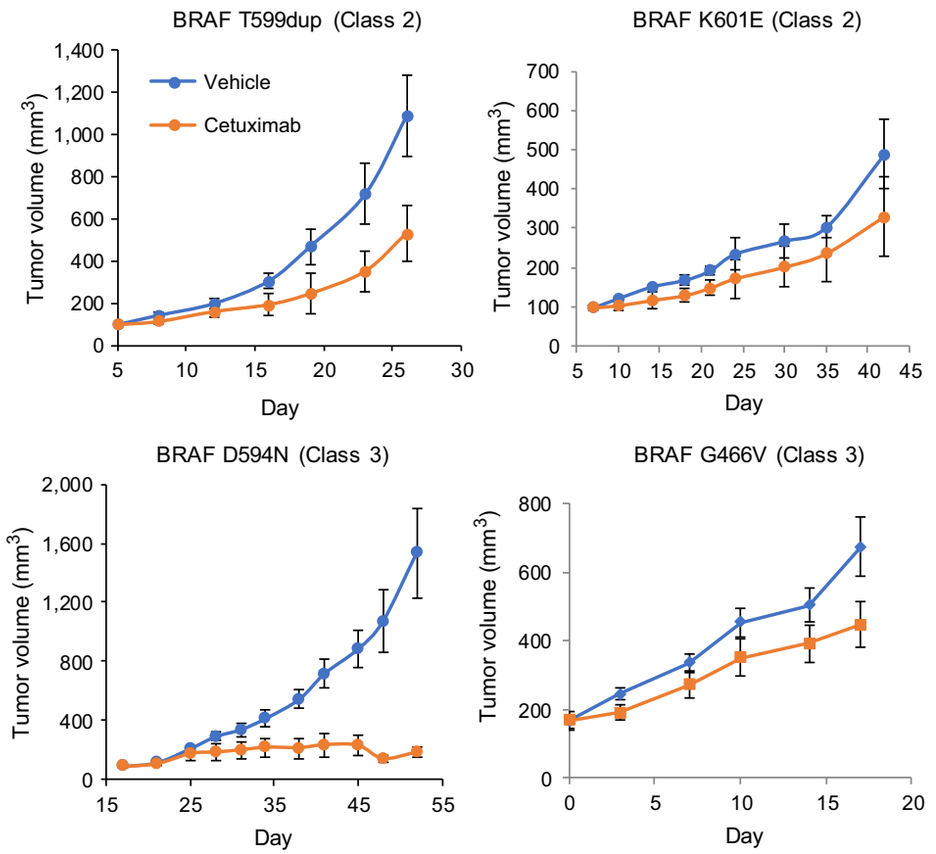

Figure 1.
Response to EGFR inhibitors in preclinical patient-derived models. PDXs established from class 2 (T599dup and K601E) or class 3 (D594N and G466V) mutant BRAF were treated with either vehicle or cetuximab. Each treatment group consisted of 5 mice. Error bars, SD.

mutants in the RAS-less cells (9). In these cells, the only RAS allele (*kras)* was removed by 4-OHT–induced Cre expression. Then, the dependence of ERK signaling of the mutants on RAS signaling was assayed (Fig. 2B). Absence of RAS expression abrogated ERK activation by BRAF F247L and R558Q but had no effect on ERK activation by BRAF L485F and L525R. On the basis of these data, BRAF A33T was considered to have no effect on ERK signaling,

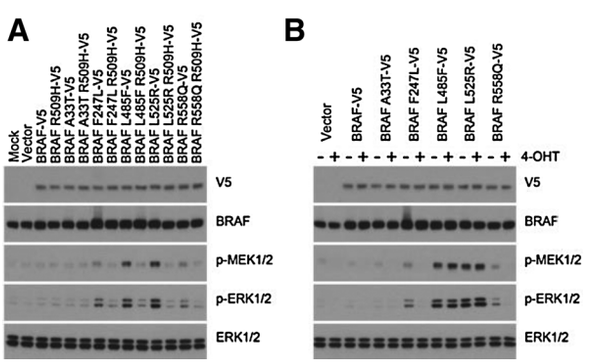

Figure 2.
Classification of BRAF mutants. **A,** MEFs were transfected to express the V5-tagged, indicated BRAF proteins or the same BRAF proteins together with R509H mutation. Cells were collected to assay expression of activated pathway intermediates. **B,** Expression of the indicated BRAF proteins was induced in the conditional "RAS-less" MEFs after pretreatment with 4-OHT to knockout the last *RAS* allele. Cells were collected to assay expression of activated pathway intermediates.

BRAF F247L and R558Q were classified as class 3 mutants, and BRAF L525R and L485F were classified as class 2 mutants.

Concurrent MAPK pathway alterations

The 118 cases in our series thus consisted of 32 class 2 BRAF-mutant tumors (27%), 72 class 3 BRAF-mutant cases (61%), 13 uncharacterized BRAF mutants, and a mutant with no effect on ERK signaling (Supplementary Table S1). Elevated induction of ERK output by class 3 BRAF mutants requires adequate RAS activity, which could result from RTK activation or mutations upstream in the pathway (e.g., RAS). In class 3 BRAF-mutant epithelial tumors such as non–small cell lung cancer (NSCLC) and colorectal cancer, we have shown that RAS activation often relies on RTK signaling. In contrast, in melanoma, class 3 BRAF mutants almost always coexist with mutants that activate RAS signaling, usually inactivating mutations of *NF1* (9). In our larger dataset of non-V600 BRAF–mutant colorectal cancers, concurrent *RAS* hotspot mutations were detected in 31 patients and were enriched in tumors with class 3 BRAF mutants versus those with class 2 BRAF mutants (25% vs. 6.3%, respectively; $P = 0.03$; Supplementary Table S2). Furthermore, 20 of the 53 class 3 *BRAF*-mutant colorectal cancers co-occurred with another MAPK pathway alteration, including *RAS, NF1*, and *MAP2K1* alterations, and concurrent MAPK pathway alterations were significantly more common in the class 3 *BRAF*-mutant colorectal cancers compared with class 2 *BRAF*-mutant ones (20/53 vs. 4/29; $P = 0.025$; Supplementary Fig. S2). These results indicate that about a third of class 3 *BRAF*-mutant colorectal cancers coexist with a MAPK alteration expected to confer resistance to EGFR inhibitors and

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that the remaining about two thirds may be sensitive to RTK inhibition.

Clinical characteristics and survival

Clinical characteristics of the 118 patients are summarized in Supplementary Table S2. There was a preponderance of male patients in the entire cohort and also within the class 2 and class 3 *BRAF*-mutant colorectal cancer cases. A larger portion of class 3 *BRAF*-mutant colorectal cancers originated in the rectum, but this trend did not reach statistical significance. The large majority of cases exhibited conventional histology, not mucinous. In addition, most of the non-V600 *BRAF* mutations occurred in microsatellite stable tumors; one class 2 *BRAF* mutation occurred in a MSI tumor and one frameshift alteration of unknown significance occurred in a MSI tumor. The novel *BRAF* alteration A33T that did not affect ERK signaling occurred in a MSI tumor.

Median OS for the patients with non-V600 *BRAF*–mutant mCRC was 40.2 months [95% confidence interval (CI), 30.4–50.0 months; Supplementary Fig. S3A). Patients with concurrent *RAS* tumor mutation appeared to have a poorer prognosis compared with patients with *RAS* WT tumors, but this difference did not reach statistical significance (median OS 29.0 months vs. 44.2 months, respectively; $P = 0.07$; Supplementary Fig. S3B). Among patients with *RAS* WT tumors, those with class 2 *BRAF*-mutant mCRC had a trend toward shorter survival compared with those with class 3 *BRAF*-mutant tumors (median OS 26.0 vs. 44.8 months, respectively; $P = 0.21$; Supplementary Fig. S3C).

Table 1. Response details for patients treated with anti-EGFR antibody therapy

Class	BRAF	Line	Regimen	Response	PFS
2	G469V	1	Folfox+Pmab	PR	18.9
2	T599_V600TinsT	1	Folfox+Pmab	PD	1.2
2	G469A	2	Irinotecan+Pmab	SD	4.0[a]
2	G469A	2	Irinotecan+Cmab	SD	3.5[a]
2	L597R	2	Irinotecan+Pmab	PD	3.3
2	L597R	2	FOLFIRI+Pmab	SD	16.5
2	G469A	3	FOLFIRI[b]+Cmab	SD	4.4
2	G469A	3	Cmab	SD	2.8
2	G469V	3	Irinotecan[b]+Cmab	SD	10.9
2	L485F	3	Irinotecan[b]+Cmab	SD	6.3
2	L525R	3	Cmab	SD	4
2	K601E	3	Pmab	PD	1.4
3	N581I	1	Irinotecan+Cmab	SD	10.3[a]
3	N581S	1	FOLFIRI+Cmab	PR	16
3	N581T	1	FOLFOX+Pmab	PR	1.4[a,d]
3	D594G	1	FOLFOX+Pmab	SD	7.0[a]
3	D594G	1	FOLFOX+Pmab	PR	2.8[a,d]
3	D594G	1	FOLFOX+Pmab	PR	8.3[a]
3	F595L	1	FOLFOX+Pmab	PR	10.2[a]
3	G466E	2	Irinotecan[b]+Pmab	PR	8.3[a]
3	D594G	2	FOLFIRI+Cmab	PR	3.2
3	G466E	3	Irinotecan[b]+Pmab	PR	8.3
3	G466V	3	Irinotecan[b]+Pmab	PR	6.1
3	Q524L	3	Pmab	PD	2.3
3	D594G	3	Irinotecan[b]+Pmab	NE	1.8[a]
3	D594G	3	Irinotecan[b]+Pmab	SD	1.8[a]
3	D594G	3	FOLFIRI[b]+Cmab	SD	3.6
3	D594G	3	Irinotecan[b]+Pmab	PD	2.8
3	D594G	3	FOLFIRI[b]+Cmab	PD	2.4
3	D594G	3	FOLFIRI[b]+Pmab	SD	2.6[a]
3	D594G	3	FOLFIRI[b]+Cmab	SD	3.7
3	D594G	3	Irinotecan[b]+Cmab	SD	6.6
3	D594G	3	Irinotecan[b]+Pmab	SD	2.3
3	D594G	3	Irinotecan[b]+Cmab	SD	2.2[a]
3	D594G	3	Irinotecan[b]+Pmab	PR	6.7[a]
3	D594N	3	Irinotecan[b]+Pmab	PR	14.9
3	D594N	3	5-FU[c]+Cmab	PD	1.6
3	D594N	5	Irinotecan[b]+Pmab	PR	14.7[a]
3	F247L	3	FOLFIRI[b]+Cmab	CR	12.6
3	R558Q	4	Irinotecan[b]+Cmab	CR	37.7[a]

Abbreviations: CR, complete response; Cmab, cetuximab; NE, not evaluable; PD, progressive disease; PR, partial response; SD, stable disease; Pmab, panitumumab.
[a]Censored.
[b]Irinotecan refractory.
[c]5-FU refractory.
[d]Received surgical resection.

Table 2. Response to anti-EGFR antibody in patients based on BRAF classification

	Class 2		Class 3		
	No. of patients	No. Responded (%)	No. of patients	No. Responded (%)	*P*
First- or second-line	6	1 (17)	9	7 (78)	0.04
Third- or later-line	6	0	19	7 (37)	0.14
Total	12	1 (8)	28	14 (50)	0.02

Efficacy of anti-EGFR antibody treatment

Clinical characteristics for the 40 patients with class 2 or class 3 *BRAF*-mutant mCRC treated with anti-EGFR antibody are summarized in Supplementary Table S3; no significant differences were observed by *BRAF* mutation class. The regimen received, tumor *BRAF* mutation, and best response are listed in Table 1. We found clear differences in response to anti-EGFR therapy by mutational class. The response rate to anti-EGFR containing regimen in class 2 and 3 *BRAF* mutants was 8% and 50%, respectively ($P = 0.02$; Table 2). In the subgroup analysis according to line of therapy and sidedness, there was also trend toward higher response in class 3 patients. In the first-line or second-line setting, about half of patients had a response to treatment containing an EGFR antibody, including 7 of 9 patients with class 3 *BRAF*-mutant mCRC and 1 of 6 patients with class 2 *BRAF*-mutant mCRC (response rate, 78% and 17%, respectively; $P = 0.04$). Notably, 2 patients with class 3 *BRAF* mutation were able to undergo resection after response to chemotherapy. In third- or later-line, no patients with class 2 *BRAF*-mutant mCRC responded to EGFR inhibitors, while 7 of 19 (37%) patients with class 3 *BRAF*-mutant mCRC responded ($P = 0.14$). More than half of the left-sided class 3 *BRAF*-mutant tumors responded to anti-EGFR therapy whereas only 2 of 7 right-sided class 3 tumors achieved response (Supplementary Table S4).

PFS for patients who received EGFR inhibitor containing therapy at third- or later-line was 4.4 months (95% CI, 0.9–7.9 months; Fig. 3A). In the subgroup analysis, the median duration of response to EGFR antibody therapy was 4.0 months (95% CI, 2.1–5.9 months) for class 2 *BRAF*-mutant mCRC and 6.1 months (95% CI, 0.8–11.4 months) for class 3 patients with *BRAF*-mutant mCRC ($P = 0.25$; Fig. 3B).

In a patient with *BRAF* D594G–mutant (class 3) mCRC who responded to panitumumab and irinotecan after prior irinotecan, we were able to collect plasma for cfDNA analysis before treatment and at 8 weeks. cfDNA analysis indicated that the *BRAF* mutation had the highest variant allelic frequency in blood at the start of treatment of 36.9%, suggesting it is a truncal mutation and not a subclonal event (Fig. 3C). After 8 weeks of treatment, the frequency of *BRAF* D594G decreased to 0.8%, indicating that

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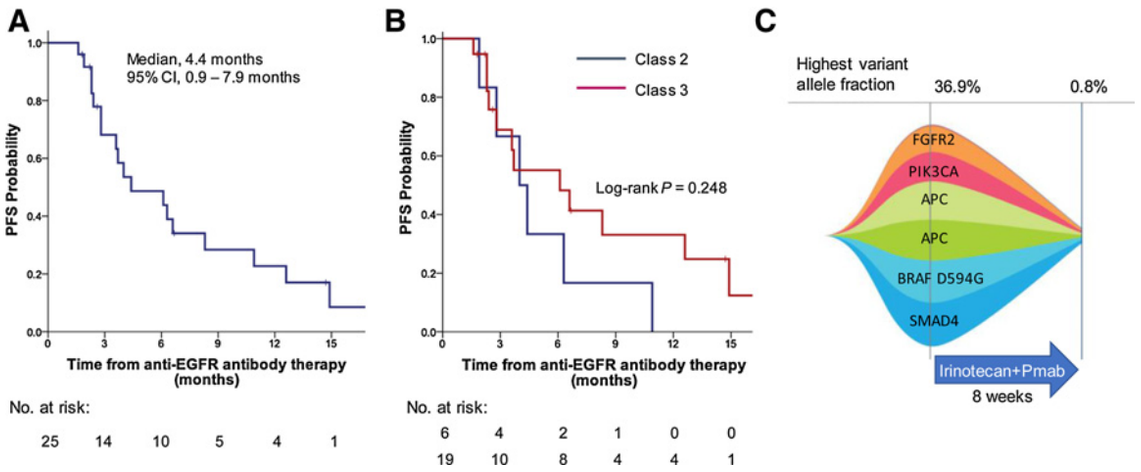

Figure 3.

Survival of patients with non-V600 BRAF alterations. PFS in a total of 25 patients treated with anti-EGFR antibody at the third- or later-line (**A**), or stratified by BRAF mutation class (**B**). **C,** Measurements of cell tumor DNA before and during treatment with panitumumab and irinotecan. Numbers for highest allelic fraction correspond to BRAF D594G–mutant allelic fraction detected in blood.

the *BRAF*-mutant clone had decreased with EGFR inhibitor treatment, rather than a separate *BRAF* WT clone responding. The variant allelic frequency of all the circulating tumor DNA detectable in blood in this patient decreased with the panitumumab/irinotecan treatment, supporting the benefit of EGFR inhibitor treatment in this patient with a non-V600 *BRAF* mutation.

Class 2 mutations can cause secondary resistance to EGFR inhibitors

The patient with *BRAF* F247L mCRC achieved a long-lasting response to EGFR antibody–containing therapy of over a year. At resistance, a new, additional class 2 *BRAF* mutation was detected. Specifically, the patient received third-line FOLFIRI and cetuximab for biopsy proven metastatic thoracic lymphadenopathy (clinical course summarized in Fig. 4A). Treatment led to complete regression of the hypermetabolic thoracic nodes (Fig. 4B), but the patient developed a brain metastasis after a year of treatment (Fig. 4C). The brain metastasis was removed and sequencing identified a new *MKRN1-BRAF* fusion (Fig. 4D), a class 2 activating alteration that no longer includes the RAS binding domain of BRAF. This fusion does not require RAS binding to dimerize. Sequencing of both the primary colon cancer and the thoracic lymph node biopsy specimen did not identify this fusion confirming that this alteration was acquired in the resistant brain metastasis. We were able to generate a PDX from the cerebellar metastasis. Treatment of the PDX with vehicle or cetuximab (5 mice per group) indicated that cetuximab did not inhibit tumor growth (Fig. 4E). These data suggest that the acquired class 2 BRAF alteration caused resistance to cetuximab treatment and further supports the limited efficacy of EGFR inhibitors against class 2 BRAF mutants.

Discussion

In this study, we find in both preclinical models and in our large clinical series that response to EGFR antibody treatment in colorectal cancers with class 2 *BRAF* mutants is rare, while a large portion of colorectal cancers with class 3 *BRAF* mutants respond to EGFR antibody treatment. This is the first demonstration of the sensitivity of tumors driven by RAS- or RAF-dependent activation of ERK signaling to RTK inhibition. Moreover, we have defined a biomarker (class 3 *BRAF* mutation) that allows identification of patients who are candidates for this therapy.

Our data indicate that patients with colorectal cancer with class 3 *BRAF* mutations should not be excluded from EGFR antibody treatment. Anti-EGFR antibody containing treatment decreased the frequency of *BRAF* D594G circulating tumor DNA, indicating that the *BRAF*-mutant clone responded to the treatment, rather than a separate *BRAF* WT clone responding. When anti-EGFR antibody treatment was used with first-line chemotherapy, 7 of 9 patients treated achieved responses, and 2 of them were able to undergo surgical resection. Consistent with our data, Cremolini and colleagues found that 3 of 4 patients with either *BRAF* 594 or 596 mutation achieved partial response with upfront chemotherapy plus cetuximab (31). Together these data indicate that RAS activation in many class 3 BRAF-mutant mCRC is driven by EGFR and targeting this RTK leads to regression of these tumors.

It has recently been appreciated that within *RAS* WT tumors, patients with a left-sided colon primary benefit from anti-EGFR antibodies, and those with right-sided primaries obtain limited benefit (32–34). Consistent with this observation, we found that left-sided tumors were significantly more sensitive to EGFR antibody treatment. Given that class 3 BRAF mutants require RAS activation to effectively amplify MAPK signaling, our data suggest that EGFR, which is primarily activated in the left-colon, often provides the activation of RAS for MAPK pathway activation and tumorigenesis in class 3 *BRAF*-mutant tumors.

Not all the patients with class 3 *BRAF* colorectal cancer responded to anti-EGFR therapy. In addition to *RAS* mutations, activation of other RTKs leads to resistance to anti-EGFR therapy (35, 36). By identifying RTKs playing dominant roles in RAS activation, class 3 BRAF-mutant tumors may be better targeted by treating with the matched RTK inhibitor with or without a MEK inhibitor. Despite this, our data suggest that in a large portion of class 3 *BRAF* mutants, the single dominant driver is EGFR, which can be effectively targeted in the clinic.

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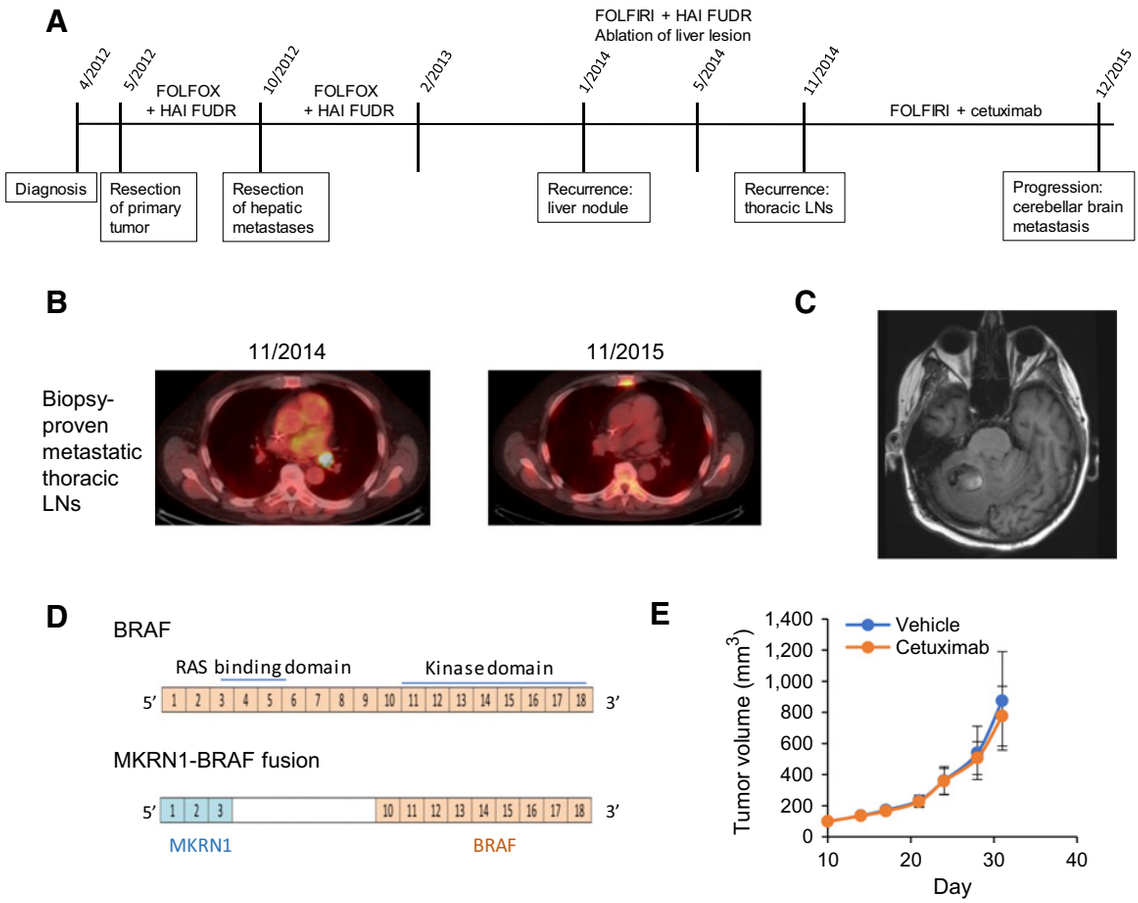

Figure 4.

Class 2 mutations can cause secondary resistance to EGFR inhibitors. **A,** Timeline of patient's treatment history. **B,** Representative images from PET/CT showing biopsy-proven, hypermetabolic metastatic thoracic lymphadenopathy at the start of FOLFIRI/cetuximab treatment and after 1 year of treatment. **C,** Representative MRI image showing new cerebellar metastasis. **D,** Schema of the MKRN1-BRAF fusion identified on sequencing the cerebellar metastasis. **E,** Growth curve of mice bearing the PDX with MKRN1-BRAF fusion treated with vehicle or cetuximab. Five mice were treated in each group and SDs are indicated with error bars. LN, lymph node.

None of the class 2 *BRAF* mutants responded to anti-EGFR antibodies in treatment beyond the first-line setting. In line with this, cetuximab did not cause regression in our PDX models. In addition, we found that a patient with colorectal cancer acquired a class 2 *BRAF* alteration, the *MKRN1-BRAF* fusion, at resistance to cetuximab, further suggesting that class 2 mutants cause resistance. Similarly, *BRAF* fusions have been reported as a mechanism of acquired resistance to EGFR inhibition in EGFR-mutant NSCLC (37). While class 2 *BRAF* mutants thus appear to rarely respond to EGFR inhibitors, we note that occasional responses have been described (19), although these responses appear short-lived. There may be a range of ERK activation with class 2 BRAF mutants and, in our series, we see a low, but nonzero, rate of concurrent *RAS* mutation in the class 2 *BRAF*-mutant tumors. These data suggest that in some class 2 *BRAF* mutants, while mutant BRAF activates ERK, an additional contribution to ERK activation can come from upstream signaling. Consistent with this understanding, in class 2 *BRAF*-mutant NSCLC lines, EGFR inhibition partially suppressed MAPK signaling and cell growth, but not enough to induce tumor regression (38). Thus, while a subset of class 2 *BRAF*-mutant colorectal cancer may respond to EGFR inhibitors, these cases are rare and EGFR inhibitor treatment may not sufficiently suppress ERK signaling for durable clinical benefit.

Class 2 *BRAF* tumors are resistant to approved BRAF drugs designed to inhibit mutant V600. Newer RAF inhibitors may have activity against these mutants. Type II RAF inhibitors can bind to the Asp-Phe-Gly (DFG)-out inactive conformation of BRAF and induce RAF dimerization but inhibit the kinase activity of the dimer (8, 39). Type II inhibitors demonstrated preclinical efficacy in xenograft models from *BRAF* class 2–mutant NSCLC cell lines (38). MEK inhibitors may also be effective; however, the effects of these drugs are attenuated by reactivation of receptors, such as EGFR. Combined inhibition of BRAF and MEK with or without EGFR could be an effective strategy for class 2 *BRAF* mutants and is currently being investigated in clinical trials (UMIN000031857 and NCT03843775).

While our study is the largest series to interrogate the efficacy of anti-EGFR antibodies in patients with *BRAF* non-V600 mutations, clear limitations of our analysis are the small number of patients

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with *BRAF* class 2 and 3 mutated colorectal cancer, in line with the low frequency of these populations, and the retrospective nature of our cohort. Because non-V600 *BRAF* alterations occur in about 2% of mCRCs, it would be difficult to perform a prospective study; in fact, in *BRAF* V600E mCRC, which is the most frequent *BRAF* alteration in colorectal cancer, the consensus that this genomic alteration is associated with resistance to EGFR antibody treatment comes from meta-analyses. A randomized study of EGFR antibodies has not been conducted for this specific population and the subset analyses of randomized controlled trials that included *BRAF* V600E mCRC did not have sufficient numbers to demonstrate statistically significant differences in response rate (40, 41). Yet despite this, clinically, assessing *BRAF* V600E status is very important in deciding whether to give EGFR antibody treatment alone (42–44). We now show that non-V600 *BRAF* alterations do not clinically represent a uniform group and response to EGFR antibody treatment should be considered by *BRAF* mutation class. Prospective analysis would therefore require very large patient numbers and would not be feasible. We also acknowledge that we could not directly assess the efficacy of anti-EGFR antibodies monotherapy, because most of the patients were treated with chemotherapy. This may contribute to the discordance between the similar PFS intervals for the two *BRAF* mutation classes and the substantially different response rates observed. In addition, many patients underwent mutational analysis after progression through standard chemotherapy to enroll on clinical trials, which could have led to a selection bias resulting in better OS in our cohort.

In conclusion, our results indicate that many class 3, but not class 2, BRAF-mutant–driven colorectal cancers are driven by EGFR and can be sensitive to its inhibition. These data provide clinical guidance on the application of EGFR antibodies in patients with colorectal cancer with non-V600 *BRAF* mutations. Patients with colorectal cancer with class 3 *BRAF* mutations should be considered for anti-EGFR antibody treatment. The lack of efficacy seen in class 2 *BRAF*-mutant tumors suggests that these patients may not benefit from anti-EGFR therapy and highlights the importance to develop new targeted approaches for this patient population.

Disclosure of Potential Conflicts of Interest

R. Yaeger reports receiving commercial research grants from Array Biopharma, GlaxoSmithKline, and Novartis. D. Kotani reports receiving speakers bureau honoraria from Lilly, Takeda, Chugai, and Merck Biopharma. A.R. Parikh reports receiving other commercial research support from Bristol-Myers Squibb, Novartis, Guardant, and Tolero, and is a consultant/advisory board member for Purtech and Foundation Medicine. H. Taniguchi reports receiving other commercial research support from Diichi Sankyo and Takeda, and speakers bureau honoraria from Takeda, Taiho, Merck Biopharma, and Chugai. N. Rosen reports receiving other commercial research support from Chugai, holds ownership interest (including patents) in Zai Laboratory, BeiGene, Kura Oncology, and Araxes, and is a consultant/advisory board member for Zai Laboratory, MapKure, Chugai, BeiGene, AstraZeneca, Array Biopharma, Novartis BioMed, Boehringer Ingelheim, and Revolution Medicines. R.B. Corcoran holds ownership interest (including patents) in Avidity Biosciences, C4 Therapeutics, Fount Therapeutics, nRichDx, and Revolution Medicines, and is a consultant/advisory board member for Amgen, Array Biopharma, Astex Pharmaceuticals, Avidity Biosciences, Bristol-Myers Squibb, FOG Pharma, Fount Therapeutics, Genentech, LOXO, Merrimack, N-of-One, Novartis, nRichDx, Revolution Medicines, Roche, Roivant, Shionogi, Spectrum Pharmaceuticals, Symphogen, Taiho, and Warp Drive Bio. T. Yoshino reports receiving commercial research grants from Novartis Pharma K.K., MSD., K.K., Sumitomo Dainippon Pharma Co., Ltd., Chugai Pharmaceutical Co., LTD., Sanofi K.K., Daiichi Sankyo Company, Limited, Parexel International Inc., and ONO Pharmaceutical CO., LTD. No potential conflicts of interest were disclosed by the other authors.

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