Clinical Cancer Research

Impact of Emergent Circulating Tumor DNA *RAS* Mutation in Panitumumab-Treated Chemoresistant Metastatic Colorectal Cancer

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Abstract

Purpose: The accumulation of emergent *RAS* mutations during anti-EGFR therapy is of interest as a mechanism for acquired resistance to anti-EGFR treatment. Plasma analysis of circulating tumor (ct) DNA is a minimally invasive and highly sensitive method to determine *RAS* mutational status.

Experimental Design: This biomarker analysis of the global phase III ASPECCT study used next-generation sequencing to detect expanded *RAS* ctDNA mutations in panitumumab-treated patients. Plasma samples collected at baseline and posttreatment were analyzed categorically for the presence of *RAS* mutations by the Plasma*Select*-R 64-gene panel at 0.1% sensitivity.

Results: Among panitumumab-treated patients with evaluable plasma samples at baseline ($n = 238$), 188 (79%) were wild-type (WT) *RAS*, and 50 (21%) were mutant *RAS*. Of the 188 patients with baseline ctDNA WT *RAS* status, 164 had evaluable posttreatment results with a 32% rate of emergent *RAS* mutations. The median overall survival for WT and *RAS* mutant status by ctDNA at baseline was 13.7 (95% confidence interval, 11.5–15.4) and 7.9 months (6.4–9.6), respectively ($P < 0.0001$). Clinical outcomes were not significantly different between patients with and without emergent ctDNA *RAS* mutations.

Conclusions: Although patients with baseline ctDNA *RAS* mutations had worse outcomes than patients who were WT *RAS* before initiating treatment, emergent ctDNA *RAS* mutations were not associated with less favorable patient outcomes in panitumumab-treated patients. Further research is needed to determine a clinically relevant threshold for baseline and emergent ctDNA *RAS* mutations. *Clin Cancer Res; 24(22); 5602–9. ©2018 AACR.*

Introduction

The development of resistance to molecularly targeted therapies is of intense clinical interest in oncology. This study examined the impact of baseline-extended *RAS* and emergent *RAS* mutations, detected by using a highly sensitive assay, on tumor response to targeted therapy in patients with metastatic colorectal cancer (mCRC). Colorectal cancer is the fourth-leading cause of cancer-related deaths worldwide (1). For patients with mCRC, treatment with irinotecan-based and oxaliplatin-based chemotherapy regimens in combination with targeted therapy can improve overall survival (OS; refs. 2, 3). Advances in chemotherapy provision have resulted in a group of patients with chemorefractory disease who remain fit to receive third-line treatment. The anti-EGFR monoclonal antibodies panitumumab and cetuximab have shown clinical benefit in patients with treatment-naïve and chemorefractory wild-type (WT) *RAS* mCRC (4–10).

The phase III ASPECCT 20080763 study was the first prospective comparison of efficacy and safety for panitumumab versus cetuximab monotherapy in the treatment of chemorefractory mCRC. The primary analysis demonstrated that panitumumab is non-inferior to cetuximab for OS in chemorefractory WT *KRAS* exon 2 mCRC [median, 10.4 vs. 10.0 months; Z-score $= -3.19$; $P = 0.0007$; HR $= 0.97$; 95% confidence interval (CI) $= 0.84–1.11$] and showed similar safety profiles between the two groups (11). As the canonical testing paradigm for patients with mCRC is to test for DNA mutations present in the initial tumor resection specimen prior to chemotherapy, the ASPECCT trial provides a unique opportunity to rigorously interrogate the effect of late-line EGFR selection in tumors that have become resistant to both platins and topoisomerase inhibitors. Next-generation sequencing (NGS) technology on plasma samples allows for posttreatment sampling and analysis of circulating tumor DNA (ctDNA). This liquid biopsy format also allows for interrogation of extended *RAS* mutations from baseline plasma samples.

At the time the ASPECCT study was conducted, assessment for *KRAS* exon 2 WT status by tumor tissue was the standard of care before initiating treatment with anti-EGFR therapy. Since the inception of the ASPECCT trial, the value of expanded *RAS* testing has been demonstrated (12–14), and high-sensitivity technology has become available for the detection of ctDNA mutations in plasma (although it has not yet been clinically substantiated;

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Baseline mutations in *RAS* predict a lack of response to EGFR blockade in patients with colorectal cancer, and *RAS* testing is broadly implemented to select patients with wild-type tumors. Utilizing the next-generation sequencing technology to detect plasma ctDNA mutations in *KRAS* and *NRAS* in patients both before and after treatment with panitumumab, this study investigated the predictive value of emergent *RAS* mutation status as a potential driver of developing acquired resistance. Patients with baseline mutant *RAS* had worse outcomes than patients with wild-type *RAS*. However, emergent ctDNA *RAS* mutation status lacks significant association with patient outcomes. Therefore, although baseline *RAS* mutations predict a poor prognosis, emergent *RAS* mutation status should not be used to inform clinical decisions or changes to current therapy. Our study, however, does demonstrate that ctDNA-based liquid biopsy is a sensitive and minimally invasive approach that can be used to dynamically monitor the clonal evolution of the tumor.

ref. 15). In addition, somatic mutations in the *RAS* family of genes [as detected in formalin-fixed paraffin-embedded (FFPE) tumor samples] have been established as a negative predictor of response to anti-EGFR therapy (13). Mutations in *RAS* acquired while on anti-EGFR therapy are of tremendous interest as a potential explanation for acquired resistance to anti-EGFR therapeutics. Analysis of ctDNA isolated from plasma is a less invasive approach for tumor mutation assessment that may also allow for the determination of global mutation status and can, in parallel, provide insight into tumor heterogeneity and intertumor clonal dynamics under target therapy selection (16, 17). In the context of early stage disease, ctDNA is also a promising marker of minimal residual disease (18). Although assessing ctDNA *RAS* mutations in plasma appears to represent a potential useful source of tumor DNA for *RAS* mutational profiling, little has been established regarding its reliability and correlative association or predictive utility in large global clinical trial cohorts of colorectal cancer. The clinical implications of evolving plasma *RAS* mutations are therefore an area of substantial clinical interest.

Current advances in ctDNA isolation and sequencing technology allow for the detection of mutations in plasma ctDNA at exceptionally high levels of sensitivity when compared with traditional Sanger sequencing. Currently, there is no consensus across assays or platforms for clinically validated ctDNA threshold values that warrant changes in clinical decisions. This exploratory biomarker analysis of the ASPECCT trial utilized a highly sensitive NGS assay to detect plasma ctDNA mutations in full coding regions of *KRAS* and *NRAS* at two study timepoints—baseline (prior to initiation of therapy) and posttreatment [at safety follow-up (SFU)]. The primary objective of this study was to evaluate the impact of emergence of ctDNA *RAS* mutations in panitumumab-treated chemorefractory patients by comparing clinical outcomes of patients with and without detectable emergent mutations using a plasma-based platform that allowed for analysis of expanded *RAS* status. The secondary aim of this study was to assess outcomes for patients found to be *RAS* mutant by plasma at baseline.

Materials and Methods

Patients

ASPECCT was an open-label, phase III, non-inferiority study of panitumumab versus cetuximab monotherapy for chemorefractory WT *KRAS* exon 2 mCRC (ClinicalTrials.gov, number NCT01001377; ref. 11). The study included 1,010 patients (aged $\geq$ 18 years) who were screened prospectively for metastatic adenocarcinoma of the colon or rectum with confirmed *KRAS* exon 2 WT status prior to enrollment. *KRAS* mutational status was evaluated using the FDA-approved *therascreen*® *KRAS* assay in central lab testing, which detects mutations at 1% to 6% sensitivity. Specifically, *KRAS* tumor status was assessed in FFPE tissues prior to randomization in one of three central labs for the presence or absence of the seven most common *KRAS* exon 2 mutations. Expanded *RAS* testing was not performed on tissue, neither at the time ASPECCT was conducted nor during this exploratory analysis.

Eligibility criteria included measurable disease per RECIST version 1.1, an Eastern Cooperative Oncology Group (ECOG) performance status of $\leq$2, intolerance to or disease progression with irinotecan and oxaliplatin-containing regimens, and previous treatment with a thymidylate synthase inhibitor for colorectal cancer. Patients were excluded for prior anti-EGFR therapy, antitumor therapy within 30 days, serum magnesium below lower limit of normal, major surgery within 28 days, and inadequate hematologic, renal, or hepatic function. The protocol received institutional/ethical approval at each trial site. Patients provided written-informed consent.

ASPECCT study design and treatment

Participants were randomized 1:1 and treated with either panitumumab (6.0 mg/kg biweekly; $n = 499$) or cetuximab (400 mg/m^2 loading dose, followed by 250 mg/m^2 weekly; $n = 500$) until disease progression, intolerability, or withdrawal of consent. The primary endpoint of ASPECCT was OS; secondary endpoints were progression-free survival (PFS) and objective response rate (ORR).

Exploratory biomarker analysis

A subset of ASPECCT study patients provided written informed consent for participation in a plasma biomarker study. The focus of this Amgen-sponsored analysis was confined to the panitumumab-treated population. Paired plasma samples were collected at baseline and at SFU 30 to 33 days after last dose of panitumumab and were subsequently analyzed for the presence of *RAS* mutations by deep sequencing via the Illumina NGS platform. Analysis was performed by staff blinded to patient outcome and treatment. All consented patients who received $\geq$1 dose of panitumumab were included in the analysis set. The studies were conducted under ICH (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) guidelines for Good Clinical Practice, which follows the principles of the Declaration of Helsinki and CIOMS (International Ethical Guidelines for Biomedical Research Involving Human Subjects).

Plasma sample collection. The collection of plasma samples followed a standard protocol: (1) Fill a 5 mL K2-EDTA drawing tube until the vacuum is exhausted and blood flow ceases; (2) Gently invert 8–10 times; (3) Centrifuge at 1,500 $\times$ *g* for 15 minutes at 4°C within 30 minutes of collection (if a refrigerated centrifuge is

not available, place samples on wet ice bath for 5–10 minutes and centrifuge as normal); (4) Use a pipette to remove plasma from the top of the tube without disturbing the blood cells and transfer an equal volume (ideally 0.5 mL each) into each of the two 2 mL cryovials (SARSTEDT Microtube, 2 mL, pp No./REF 72.694.005). If there is an inadequate amount of plasma for 1 mL per cryovial, then split the available plasma volume equally into all cryovials; (5) Complete the preprinted labels provided in the Sample Collection/Shipment Notebook with subject identification number, randomization number, date, and time of collection. Verify that the label corresponds to the appropriate assay type and time point; (6) Attach one label to each cryovial and K2-EDTA tube containing the cell pellet and ensure the bar code is not obscured (refer to tube labeling instructions in the Sample Collection/Shipment Notebook); (7) Immediately place the 2 mL cryovials containing the plasma sample and the K2-EDTA tube containing the cell pellet in a −70°C or colder freezer (if no −70°C freezer is available, freeze on dry ice and ship frozen to BST on the day of collection; the plasma sample and the cell pellet must be frozen within 60 minutes of blood collection).

Next-generation sequencing. Plasma samples were analyzed using the Plasma*Select*-R 64-gene panel assay, which includes *RAS* mutations [*KRAS* and *NRAS*, exons 2 (codons 12/13), 3 (codons 59/61), or 4 (codons 117/146)]. Briefly, ctDNA fragments were isolated from plasma, followed by molecular barcoding of individual DNA molecules and amplification of full coding regions of *RAS*. Redundant sequencing of each barcoded DNA molecule allowed for the discrimination of true mutations from artifacts. Sequenced DNA was aligned to the *RAS* sequence within the reference human genome to report mutations with a sensitivity of 0.1% mutant DNA, which is the limit of detection (LOD) for the assay (19). The human genome assembly, GRCh37/hg19 (GCA_000001405.1), was used as the reference genome.

Identification of RAS mutation status. *RAS* mutation status was defined categorically by the detection of any mutant result in the patient's plasma samples. Emergent *RAS* was defined as a mutation in the previously specified exons of *KRAS* or *NRAS* at posttreatment in patients who were *RAS* WT by plasma ctDNA testing at baseline.

Statistical analysis. In this exploratory analysis, the emergence of *RAS* mutation rate at SFU was evaluated. This study was hypothesis generating, and no formal exploratory hypothesis was prospectively tested. The evaluable *RAS* analysis set was defined as the subset of patients in the primary analysis set with known *RAS* mutation status for the baseline plasma sample. The incidence of emergence of mutant *RAS* was evaluated with an exact 95% confidence interval (CI) for the incidence rate. Mutation findings were analyzed and correlated with treatment outcomes from the primary analysis of ASPECCT.

To assess the association between outcomes and emergence of mutant *RAS*, OS and PFS were analyzed by mutation status using a univariate Cox proportional-hazards (PH) model. In addition, outcomes were analyzed by baseline *RAS* mutation status. ORR was calculated by *RAS* mutation status in the baseline plasma sample for patients with evaluable *RAS*. An exact 95% CI was calculated for the common OR for ORR across strata for WT relative to mutant. Wilson's score method with continuity correction was used to calculate a 95% CI for the difference in rates for each mutation type.

Results

Patients

The ASPECCT primary analysis demonstrated that panitumumab was non-inferior to cetuximab for OS in chemorefractory WT *KRAS* exon 2 mCRC (11). Of the 1,010 participants enrolled in the ASPECCT study, 499 patients received panitumumab treatment. Of those patients treated with panitumumab, 238 (48%) had evaluable plasma samples at baseline that had paired posttreatment plasma samples (Fig. 1). Although baseline patient demographics and tumor characteristics were similar between the plasma analysis patients and the larger ASPECCT intent-to-treat (ITT) population, clinical outcomes for the plasma analysis set were numerically higher than those for the ITT population (Supplementary Table S1).

This plasma analysis focused on patients who were *RAS* WT by plasma at baseline. Fifty (21%) patients had mutant *RAS* plasma status at baseline and were excluded from the emergent mutation analysis. These findings are similar to those in the PEAK clinical trial, which found that 23% of patients previously identified as *KRAS* exon 2 WT by tissue were mutant in other *RAS* exons (20). There were 188 patients with WT *RAS* at baseline who were also evaluable at posttreatment for emergent mutations. Baseline demographics and disease characteristics were similar between the two arms (Table 1). The median age was 60.5 years for both WT (Min–Max: 19–84) and mutant *RAS* (33–83). For WT and mutant *RAS*, 63% and 52% patients had a primary tumor diagnosis for the colon; 12% and 14% had liver-only metastatic disease; and 27% and 22% had received prior bevacizumab treatment, respectively (Table 1). At posttreatment, of the 188 patients with WT samples at baseline, 164 were evaluable, and 24 were unevaluable due to insufficient quantity of captured DNA. Of 164 patients with evaluable samples, 111 remained WT for *RAS* in posttreatment plasma (non-emergent), whereas 53 had plasma-detected *RAS* mutation and were considered to have emergent *RAS* mutations (Fig. 1).

Description of baseline and emergent *RAS* mutations

In this study, the rate of emergent mutant *RAS* was 32.3% (95% CI, 25.23%–40.05%; $n = 164$). Mutations were observed in multiple exons for *RAS* alleles at baseline and posttreatment (Table 2; Supplementary Table S2). For baseline *RAS* mutants, the dominant mutation locations reported were *KRAS* exons 2 (12%), 3 (34%), and 4 (12%), as well as *NRAS* exons 2 (20%), 3 (18%), and 4 (4%). For emergent mutants, the dominant mutation locations reported were *KRAS* exons 2 (25%), 3 (38%), and 4 (9%), as well as *NRAS* exons 2 (9%) and 3 (19%). There were 2 patients at baseline and 12 patients at posttreatment who had mutations in multiple exons suggesting multiple coexisting mutant clones in these patients. Patients with multiple concurrent *KRAS*/*NRAS* ctDNA mutations at SFU were listed in Supplementary Table S3.

Emergent ctDNA *RAS* mutation status and efficacy

OS. There was no significant difference in OS between patients with emergent ctDNA *RAS* mutation and those without emergent mutations. For emergent *RAS* and non-emergent *RAS*, median

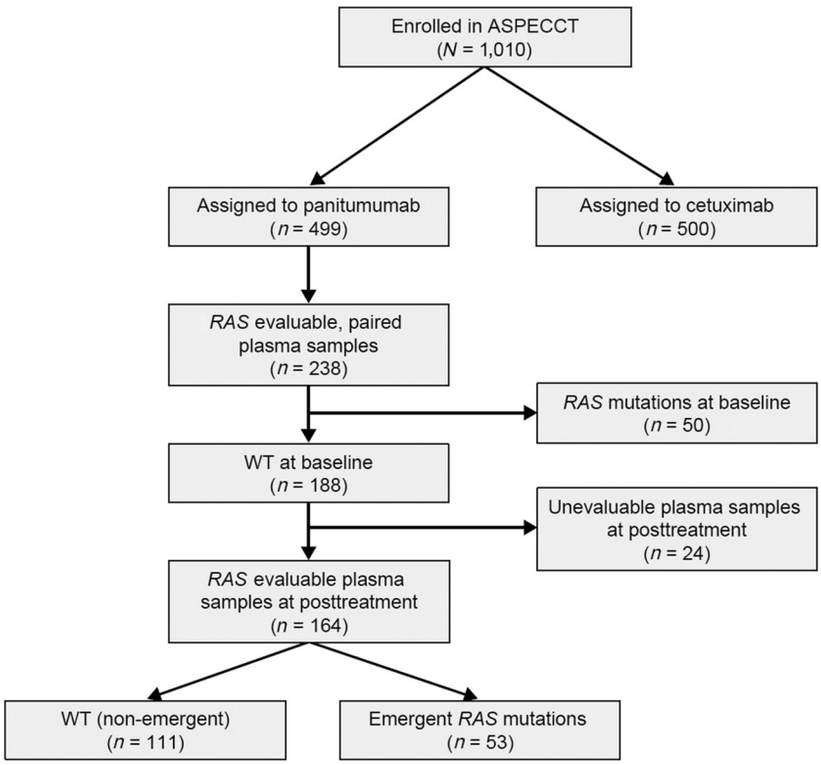

Figure 1.
Study schema for this exploratory biomarker analysis of the ASPECCT phase III study. *RAS,* rat sarcoma.

OS was 13.1 (95% CI, 10.5–16.0) and 13.8 months (95% CI, 10.8–16.4), respectively [HR = 1.16 (95% CI, 0.81–1.68); $P = 0.42$; Fig. 2A].

Table 1. Baseline demographics and disease characteristics

Baseline demographics and disease characteristics	Wild-type ($n = 188$)	Mutant ($n = 50$)	*P* value[a]
Age, years, median (range)	60.5 (19–84)	60.5 (33–83)	0.1610
Men, *n* (%)	118 (62.8)	27 (54.0)	0.2589
Race, *n* (%)			0.2327
Asian	99 (52.7)	21 (42.0)	
White/Caucasian	86 (45.7)	29 (58.0)	
Other	3 (1.6)	0 (0.0)	
Geographic region, *n* (%)			0.3801
North American, Western Europe, and Australia	45 (23.9)	15 (30.0)	
Rest of the world	143 (76.1)	35 (70.0)	
ECOG performance status, *n* (%)			0.4765
0	59 (31.4)	16 (32.0)	
1	117 (62.2)	33 (66.0)	
2	12 (6.4)	1 (2.0)	
Primary tumor diagnosis, *n* (%)			0.1663
Colon	118 (62.8)	26 (52.0)	
Rectum	70 (37.2)	24 (48.0)	
Number of metastatic sites, *n* (%)			0.9727
1	36 (19.1)	10 (20.0)	
2	71 (37.8)	18 (36.0)	
≥3	81 (43.1)	22 (44.0)	
Liver-only metastatic disease, *n* (%)	22 (11.7)	7 (14.0)	0.6589
Prior bevacizumab treatment, *n* (%)			0.4628
Yes	51 (27.1)	11 (22.0)	
No	137 (72.9)	39 (78.0)	

[a]*P* value was calculated from independent sample *t* test for age and from χ^2 test for all other variables.

PFS. There was no significant difference in PFS between patients with emergent ctDNA *RAS* mutations and those without emergent mutations. For emergent *RAS* and non-emergent *RAS*, median PFS was 6.4 (95% CI, 5.0–6.7) and 4.9 months (95% CI, 4.5–5.0), respectively [HR = 0.91 (95% CI, 0.65–1.26); $P = 0.56$; Fig. 2B].

ORR. There was no significant difference in ORR between patients with emergent ctDNA *RAS* mutation and those without emergent mutations [35% (95% CI, 22.0–49.1) vs. 32% (95% CI, 23.3–41.8); Table 3]. Partial response rates were nearly identical in patients with and without emergent *RAS* mutations (35% vs. 32%; Table 3). Similarly, rates of stable disease (SD) and progressive disease (PD) were comparable in patients with and without emergent *RAS* mutations (SD: 52% vs. 48%; PD: 14% vs. 20%; Table 3). The mutation

Table 2. Description of ctDNA plasma *RAS* mutations

Mutation location,[a] *n* (%)	Baseline mutants[b] ($n = 50/238$)	Emergent mutants[c] ($n = 53/164$)	*P* value[d]
			0.2563
KRAS exon 2	6 (12.0)	13 (24.5)	
KRAS exon 3	17 (34.0)	20 (37.7)	
KRAS exon 4	6 (12.0)	5 (9.4)	
NRAS exon 2	10 (20.0)	5 (9.4)	
NRAS exon 3	9 (18.0)	10 (18.9)	
NRAS exon 4	2 (4.0)	0 (0.0)	

Abbreviations: *KRAS*, Kirsten *RAS*; *NRAS*, neuroblastoma *RAS*; *RAS*, rat sarcoma.
[a]Dominant mutation reported for each patient.
[b]Two patients at baseline had mutations in multiple exons.
[c]Twelve patients at safety follow-up had emergent mutations in multiple exons.
[d]*P* value was calculated from the χ^2 test.

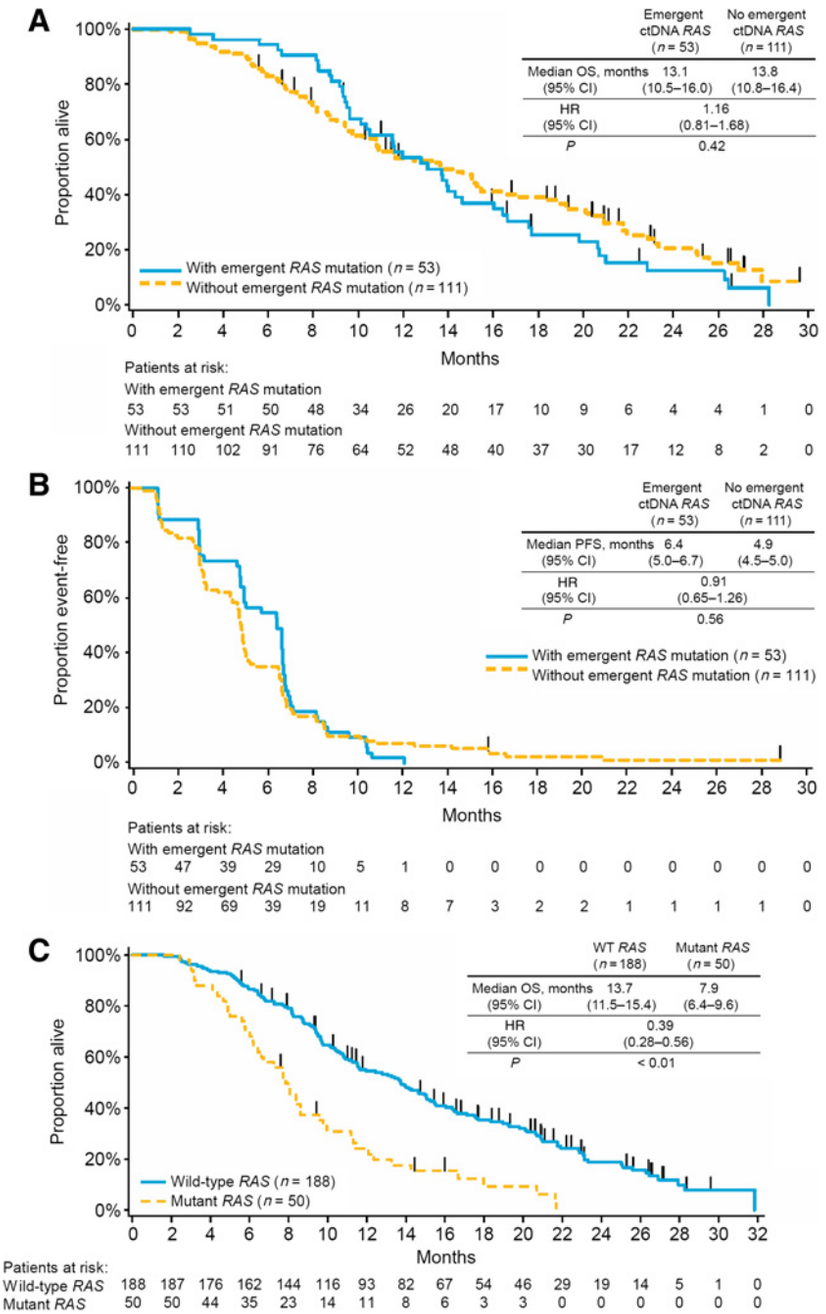

Figure 2.
Analyses for the emergence of ctDNA and baseline *RAS* mutations, and clinical outcomes in panitumumab-treated patients. Panels show Kaplan–Meier estimates for the probability of (**A**) OS (**B**) PFS by emergent ctDNA *RAS* mutation status, and (**C**) OS by baseline *RAS* mutation status. *RAS*, rat sarcoma.

odds ratio, which measures the odds of objective response in the event of emergent ctDNA *RAS* mutation versus the odds in the absence of mutation, was 1.12 (95% CI, 0.52–2.38; $P = 0.86$; Table 3).

RAS mutation analysis

Baseline RAS mutants. There were 50 patients who were *RAS* mutant by plasma at baseline, with a range of 0.15% to 3.8% mutant ctDNA detected. For WT and *RAS* mutant status at baseline plasma, median OS was 13.7 (95% CI, 11.5–15.4) and 7.9 months (95% CI, 6.4–9.6), respectively [HR = 0.39 (95% CI, 0.28–0.56); $P < 0.01$; Fig. 2C]. Patients who were WT at baseline plasma showed a greater ORR compared with patients who were *RAS* mutant at baseline plasma [34% (95% CI, 27.4–41.7) vs. 8% (95% CI, 2.2–19.2)]. Rate of SD was similar between patients who were WT and *RAS* mutant at baseline plasma (50% vs. 48%); however, fewer patients with WT status compared with *RAS* mutant status at baseline plasma went on to have PD (16% vs. 44%).

Revertant mutant (to WT). There were 5 patients with *RAS* mutant status at baseline, who reverted to WT at posttreatment (Supplementary Table S4). Three of these patients had a best response of SD, and 2 had partial response (PR).

Table 3. Emergent ctDNA *RAS* mutation status and ORR in panitumumab-treated patients

ORR	Emergent ctDNA *RAS* mutation ($n = 52$)	Non-emergent ctDNA *RAS* mutation ($n = 106$)
Response over the study, *n* (%)		
Partial response	18 (34.6)	34 (32.1)
Stable disease	27 (51.9)	51 (48.1)
Progressive disease	7 (13.5)	21 (19.8)
Patients with objective response		
Percentage of patients	35	32
95% CI	21.97–49.09	23.34–41.84
Mutation odds ratio	1.12	
Exact 95% CI	0.52–2.38	
P value	0.86	

Abbreviation: *RAS*, rat sarcoma.

Range of positivity/cumulative distribution frequency of allele fraction

The Plasma*Select*-R assay (Supplementary Table S5) is able to detect mutant ctDNA at a high level of sensitivity, with an LOD of 0.1% mutant DNA. Figure 3 provides a cumulative frequency distribution for the percentage of emergent mutant *RAS* ctDNA detected. A large subset of the patients in this analysis had detectable mutant *RAS* DNA only slightly above the assay LOD: 25% of patients with emergent ctDNA *RAS* mutations had 0.32% or less mutant DNA detected. The upper quartile of patients had 2.72% or more mutant *RAS* ctDNA detected. Very few patients had 5% or more mutant ctDNA detected, which is approximately the LOD for *RAS* mutations in tumor using other technologies (including PCR, the current standard for *RAS* status determination). At baseline, 25% of patients with ctDNA *RAS* mutations had 1.97% or less mutant DNA detected, whereas the upper quartile of patients had 22.3% or more mutant DNA detected.

Discussion

Mutant *RAS* status is an established negative predictor of response to panitumumab therapy, and the emergence of *RAS* mutations is therefore of considerable interest as a potential explanation for resistance to treatment. This study sought to understand the rate of emergent *RAS* mutations following panitumumab treatment in the third-line, chemorefractory, monotherapy setting and to characterize the distribution of specific *RAS* mutations that emerge while on panitumumab monotherapy. This study also sought to explore overall expanded *RAS* mutation status using a plasma-based platform on a robust sample set in a monotherapy, third-line setting. Although posttreatment samples in this analysis were not collected immediately upon radiologic progression, all plasma samples were collected within 30 to 33 days of end-of-treatment SFU, allowing for characterization of plasma mutation status after therapy cessation. Discontinuation of therapy was mainly due to progression or toxicity; in the plasma analysis set, 220 patients discontinued treatment due to PD and 13 due to toxicity. Although this study did not analyze expanded *RAS* status from tissues, the results from baseline plasma samples of an additional 20% mutant *RAS* identified after the initial *KRAS* exon 2 screening are similar to other panitumumab studies (20, 21). Furthermore, greater OS in the panitumumab arm is also consistent with previous findings (2, 20). In this plasma-based analysis, 32% of patients treated with panitumumab developed ctDNA-detectable emergent *RAS* mutations. This is consistent with findings from Siena and colleagues, another panitumumab study that interrogated emergent *RAS* mutations using a different technology to assess ctDNA (22). This current study highlights the response of *RAS*-dependent tumors to the selective pressure of EGFR blockade. Clonal evolution and dynamic *RAS* mutation status are indicators of intratumoral competition; however, in contrast to baseline *RAS* mutant status, the lack of association between the emergence rate and OS suggests that mutation emergence itself may not be the sole driver of resistance as measured by clinical tumor progression. Moreover, Siena and colleagues showed that, in serial plasma collections, *RAS* mutation status and the emergence of mutations did not correlate with immediate clinical changes. We have observed similar results in patients from the cetuximab arm of the ASPECCT trial. The emergence rate of *RAS* mutations in patients treated with cetuximab was 34.04% (95% CI, 20.86–49.31). Baseline *RAS* mutant status was significantly associated with shorter OS [13.3 months (95% CI, 11.7–16.2) for baseline *RAS* WT group; 8.2 months (95% CI, 4.8–13.9) for baseline *RAS* mutant group, HR = 0.393, $P < 0.01$]. Similarly, we did not observe significant association between emergent *RAS* mutant status and OS [11.9 months (95% CI, 9.9–16.2) for *RAS* emergent group; 13.3 months (95% CI, 11.7–17.1) for *RAS* non-emergent group, HR = 0.993, $P = 0.98$].

Given the lack of correlation between emergent *RAS* mutations and clinical outcomes, as well as the intrinsic molecular heterogeneity of colorectal tumors, ctDNA mutations in non-*RAS* genes are worth being taken into consideration in the exploration of mechanisms for acquired resistance. Several other resistance

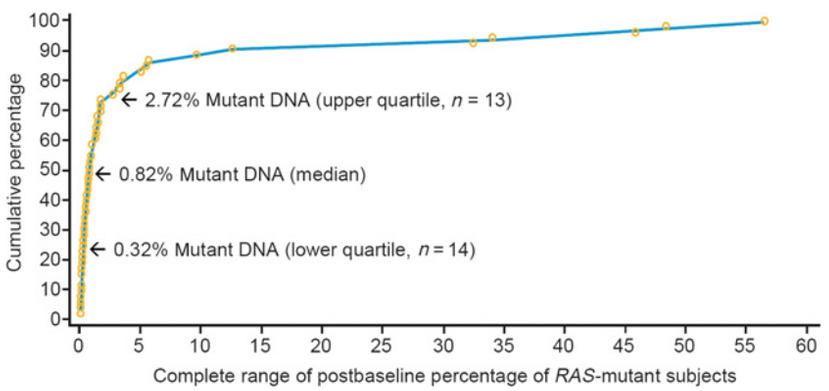

Figure 3.
Cumulative frequency distribution for mutant *RAS* ctDNA upon emergence. *RAS*, rat sarcoma.

mechanisms have been described previously in patients with mCRC resistant to EGFR blockade, including *EGFR* extracellular domain (ECD) mutations, *MET* amplifications, *BRAF* mutations, and *HER2* amplifications (23, 24). It was shown in a retrospective analysis that patients with longer responses to anti-EGFR therapy preferentially developed *EGFR* ECD mutations, whereas *RAS* mutations frequently emerged in patients with limited response and shorter PFS (24). In addition, patients with acquired *MET* amplification seemed to have a shorter PFS during anti-EGFR therapy as compared with those without (23). In the current study, we have analyzed emergent mutations in *BRAF* and *EGFR*, and their correlations with patient outcomes (Supplementary Table S6). The rates of emergent mutations in *BRAF* and *EGFR* were 19.66% (95% CI, 14.09–26.27) and 34.94% (95% CI, 27.71–42.71), respectively. Emergence of *BRAF* mutations during treatment was found to be associated with shorter OS (HR = 1.680; 95% CI, 1.123–2.513; $P = 0.01$); the PFS in patients who developed emergent mutations in *BRAF* was comparable with that in patients who remained *BRAF* WT (HR = 0.928; 95% CI, 0.639–1.346; $P = 0.69$).

Several factors must be considered in the interpretation and applicability of plasma mutation results. Even though the baseline plasma mutation status described in this study was consistent with results in the literature reporting on tissue mutation status, it cannot be assumed that the mutation status in baseline plasma samples represents tissue mutation status. Baseline tumor tissues were not analyzed for extended *RAS* mutations; therefore, a direct correlation of *RAS* mutations between tumor tissue and plasma-derived mutations is unknown. Hence, it is unclear whether those with WT *RAS* by tissue but mutant *RAS* by plasma may still benefit from panitumumab therapy and have similar OS.

Furthermore, detection of ctDNA may be difficult to accurately quantify, as it is often present in very small amounts (potentially < 1.0% of total circulating free DNA; ref. 25). Metastatic colorectal cancer is among the advanced malignancies that are more likely to be associated with detectable ctDNA, but the amount of detectable DNA and the proportion of mutated ctDNA fragments vary widely (26, 27). The detectability of ctDNA may be affected by the total body tumor burden, apoptotic or necrotic foci within the tumor, and the clearance rate of ctDNA (28, 29).

Even when tumor burden is substantial, tumor cell heterogeneity may affect the interpretation of plasma *RAS* mutations. Some authors describe the pool of ctDNA as representing an average of the whole tumor genome (30), whereas others have claimed varying heterogeneity in the representation of mutations detected by ctDNA (29). Multiple exon mutations in *RAS*, as seen in a limited number of patients in this study, suggest that only a fraction of the entire population of neoplastic cells may harbor a given mutation and that detected mutations may or may not play an active role in overall tumor growth even when they are detectable. Clinical utility and appropriate interpretation remain undefined at this time.

A strength of this study is that the analysis stemmed from a global trial, which is a highly informative population for addressing the emergence of mutations in response to treatment selection. Limitations of this study include the lack of a non-treatment control arm, lack of paired samples for all patients from the original ASPECCT ITT population, and variability in clinical outcomes for the plasma analysis set and the ITT population. This variability may be due to the inevitable selection of survivors in the plasma analysis set, which may have comprised healthier patients who were able to provide SFU blood samples compared with patients with PD or those who did not survive. Another limitation is the lack of testing for tissue *RAS* status, as discussed above. In addition, the exact timing of mutation emergence is unknown, albeit of uncertain significance given that posttreatment samples were collected at SFU rather than serially at defined intervals over the course of therapy and immediately upon progression. Furthermore, this analysis used the assay's LOD to classify the presence or absence of emergent *RAS* mutation status, which does not represent a clinically relevant threshold. Further research is needed to better define a clinically relevant *RAS* mutation threshold and demonstrate its clinical utility. Work is ongoing to explore the relevance of *RAS* mutation levels as opposed to mutation status in association with outcomes.

This exploratory study of the global phase III ASPECCT trial provides a robust analysis of baseline and emergent ctDNA *RAS* mutations using a sophisticated platform with a very sensitive level of detection. Emergent ctDNA *RAS* mutations were not associated with less favorable patient outcomes in panitumumab-treated patients from the ASPECCT study. Plasma mutation analysis presents a compelling potential alternative to tissue-based assessment of mutations, because it is minimally invasive and, therefore, an attractive option for both baseline and intermittent mutation assessment. However, the lack of significant association between emerging *RAS* mutations and clinical response or survival in this patient cohort strongly suggests that using emergent ctDNA *RAS* mutation status to make clinical decisions may be premature. The role of plasma mutation testing at baseline is also yet to be conclusively proven, and tumor tissue testing remains the gold standard. Additional studies are warranted when a validated threshold has been established and confirmed using prospective studies.

Disclosure of Potential Conflicts of Interest

M. Peters reports receiving speakers bureau honoraria from Amgen, Bayer, Roche, Sanofi, Servier, and Terumo, is a consultant/advisory board member for Bayer, Merck and Lilly, and reports receiving commercial research grants from Amgen and Roche. A. Thomas reports receiving speakers bureau honoraria from Amgen and Roche, and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb and Servier. P. Gibbs is a consultant/advisory board member for Amgen, Merck, and Roche. A.L. Ang has ownership interests (including patents) at Amgen. B.A. Bach has ownership interests (including patents) at AbbVie and Amgen. No potential conflicts of interest were disclosed by the other authors.

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