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Clinical Cancer Research

Evaluation of Emergent Mutations in Circulating Cell-Free DNA and Clinical Outcomes in Patients with Metastatic Colorectal Cancer Treated with Panitumumab in the ASPECCT Study

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Abstract

Background: Mutations in *EGFR* pathway genes are poor prognostic indicators in patients with metastatic colorectal cancer. Plasma analysis of cell-free DNA is a minimally invasive and highly sensitive method to detect somatic mutations in tumors.

Methods: Plasma samples collected from panitumumab-treated patients in the ASPECCT study at baseline and safety follow-up (SFU) were analyzed by a next-generation sequencing–based approach for extended *RAS* mutant allele frequency as a continuous variable and their association with clinical outcomes and the mutational prevalence of 63 cancer-related genes. The correlation between patient outcome and baseline mutational status of *EGFR* pathway genes was also examined.

Results: Overall, 261 patients in the panitumumab arm had evaluable plasma samples. Patients with a higher *RAS* mutant allele frequency at baseline had worse clinical outcomes than those with a lower frequency ($P < 0.001$, Cox PH model); however, *RAS* mutations did not necessarily preclude patients from deriving benefits. The objective response rate (complete or partial response) was 10.8% for patients with baseline *RAS* mutations and 21.7% for those with *BRAF* mutations. The 63-gene panel analysis revealed an increase in tumor mutational burden from baseline to SFU ($P < 0.001$, Wilcoxon signed rank test). Baseline mutations in *EGFR* pathway genes, when analyzed both categorically and continuously, were associated with shorter survival.

Conclusions: When mutations in *EGFR* pathway genes were analyzed continuously, higher mutant allele frequency correlated with poorer outcomes. However, extended *RAS* mutation, by itself, did not preclude clinical responses to panitumumab in a monotherapy setting.

Introduction

Colorectal cancer, the third most commonly diagnosed cancer worldwide, is associated with high mortality (1, 2). Activation of the *EGFR* signaling is frequently implicated in a number of human malignancies including colorectal cancer, and the *EGFR* pathway has become an important therapeutic target (3). EGFR is a transmembrane receptor tyrosine kinase that, upon ligand binding, can activate two main downstream signaling cascades including the *RAS–RAF–MEK–ERK* axis, which regulates cell-cycle events and proliferation (4), and the *PI3K–PTEN–AKT* axis, which is mainly involved in cell survival, motility, and invasion (5). Cetuximab and panitumumab are chimeric and fully human anti-EGFR monoclonal antibodies, respectively, which have demonstrated clinical efficacy as single agents or in combination with chemotherapy in patients with metastatic colorectal cancer (mCRC; refs. 6–9).

ASPECCT was a randomized, open-label, phase III, noninferiority study in patients with chemo-refractory wild-type *KRAS* exon 2 mCRC. This head-to-head comparison showed that panitumumab was noninferior to cetuximab for OS [median OS, 10.4 months vs. 10.0 months; *Z*-score = –3.19; $P = 0.0007$; HR = 0.97; 95% confidence interval (CI), 0.84–1.11] and progression-free survival (PFS), with no substantial differences in safety (10).

Activating mutations in the *RAS* family genes predict a lack of benefit from EGFR blockade in patients with mCRC who received anti-EGFR antibodies as monotherapy or in combination with chemotherapy (11–13). Extended genetic testing for *RAS* mutations [*KRAS* and *NRAS* codons 12 and 13 (exon 2), 59 and 61 (exon 3), and 117 and 146 (exon 4)] is required prior to anti-EGFR therapy in order to select patients who are likely to derive clinical benefit and to avoid unnecessary treatment-related toxicities and costs (14). Despite the therapeutic advances and implementation of biomarker testing, patients with wild-type *RAS* mCRC invariably experience tumor progression and relapse, which may be attributable to the clonal evolution that confers

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Translational Relevance

We extensively investigated the mutant allele frequencies in plasma cfDNA in the extended *RAS* as well as additional *EGFR* pathway genes in a large panitumumab-treated population with mCRC. Despite an observed trend of higher *RAS* mutant allele frequency correlating with worse outcomes, baseline extended *RAS* mutations did not preclude clinical response to panitumumab. Even though baseline mutations in *EGFR* pathway genes were associated with shorter overall survival, our prognostic model analyzing mutations as a continuous variable showed that patients who were mutant for *EGFR* pathway genes but with a low mutant allele frequency may still derive clinical benefit from panitumumab. Our work also highlights the clinical utility of cfDNA-based "liquid biopsy" in the assessment of tumor molecular heterogeneity.

growth advantage under the selective pressure of EGFR inhibition (11, 15). The emergence of *RAS* mutant clones during treatment is a potential mechanism for acquired resistance to EGFR blockade (16). In addition, genetic alterations in other downstream effectors of the EGFR signaling pathway such as *BRAF*, *PIK3CA*, and *PTEN* have been shown to be potentially associated with a low response rate and poor prognosis (12, 17, 18) and therefore are being explored as putative alternative resistance mechanisms. The ASPECCT study provides a unique opportunity to investigate the mutational profiles and genetic changes in a large global population with chemo-refractory colorectal tumors with no previous exposure to EGFR antibodies.

In the ASPECCT study, eligible patients with wild-type *KRAS* exon 2 mCRC were identified by analyzing their tumor biopsies, which was the standard of care at the time when ASPECCT was conducted. However, this technique has inherent limitations. A tissue biopsy captures the genomic profile of only a tumor fraction instead of the overall heterogeneity, and the primary tumor biopsy may not always fully reflect the genetic characterization of the metastatic disease (19). The genomic profile from a tissue biopsy is a snapshot of the unstable cancer genome and may significantly alter over time, particularly under the selective pressure of treatment with targeted therapies. Moreover, the invasive nature and potential complications of tissue biopsy–related procedures prevent the repeated and extensive use of tissue biopsies for continuous genetic profiling. Recently, plasma testing of isolated cell-free DNA (cfDNA) has been proposed as a less-invasive surrogate for tissue biopsy (20). Such procedure, known as "liquid biopsy," may overcome some of the challenges posed by tissue genotyping and allow for a comprehensive assessment of real-time global mutational status in patients with cancer, but its clinical utility remains limited as of now due to the lack of relevant research and knowledge, as well as standardization across multiple analysis platforms.

Previously, next-generation sequencing (NGS) was performed on cfDNA for extended *RAS* in patients enrolled in the panitumumab arm of the ASPECCT study. The categorical results of cfDNA *RAS* mutations were analyzed at 2 study time-points—baseline (prior to treatment) and safety follow-up (SFU; post-treatment). Patients were segregated into non-emergent (*RAS* wild-type at both baseline and SFU) or emergent group (*RAS* wild-type at baseline and then mutant at SFU). We reported that patients with emergent *RAS* had similar OS to those who were non-emergent [13.1 months (95% CI, 10.5–16.0) for emergent *RAS* group; 13.8 months (95% CI, 10.8–16.4) for non-emergent *RAS* group; HR = 1.163 (95% CI, 0.807–1.675); $P = 0.4185$; ref. 21]. In this updated analysis of the same patient population, the fraction of cfDNA *RAS* mutations was analyzed as a continuous variable in each patient, indicative of plasma *RAS* mutant allele frequency.

This exploratory biomarker study employed a highly sensitive NGS technology with the limit of detection (LOD) of 0.1% to analyze plasma samples from panitumumab-treated patients in the ASPECCT study. We sought to explore the association between the mutant allele frequency of extended *RAS* and clinical outcomes and investigate the mutational prevalence and net genetic changes from baseline to SFU by a 63-gene panel assay. Given the potential clinical relevance of *EGFR* downstream effectors in the resistance to anti-EGFR therapy, we have also expanded the biomarker outcomes analysis to include 6 of the *EGFR* pathway genes—*KRAS and NRAS (*extended *RAS)*, *BRAF*, *MAP2K1*, *PIK3CA*, and *PTEN*—and analyzed the prognostic value of mutations in these genes at baseline as both categorical and continuous measures.

Materials and Methods

Patients

The ASPECCT study enrolled 1010 patients with chemo-refractory wild-type *KRAS* exon 2 mCRC (ClinicalTrials.gov identifier: NCT01001377). Eligible patients (aged ≥18 years) had histologically or cytologically confirmed metastatic adenocarcinoma of the colon or rectum, an Eastern Cooperative Oncology Group performance status of 2 or less, progressive disease (PD) on or intolerance to irinotecan-based and oxaliplatin-based therapies, and previously received a thymidylate synthase inhibitor (including 5-fluorouracil, capecitabine, raltitrexed, or fluorouracil-uracil) for colorectal cancer. Prior to randomization, *KRAS* exon 2 status was prospectively evaluated in formalin-fixed, paraffin-embedded tumor tissue sections by the Food and Drug Administration–approved *Therascreen KRAS* assay in central lab testing. Extended *RAS* testing was not performed on the tissue samples. The protocol received institutional and ethical approval at each study site; all patients provided written informed consent.

Study design and treatment

Eligible patients were randomized 1:1 to receive either panitumumab or cetuximab. Panitumumab was administered intravenously at 6 mg/kg on day 1 of each 14-day cycle, and cetuximab was administered intravenously at an initial dose of 400 mg/m^2 followed by 250 mg/m^2 on day 1 of each 7-day cycle. Treatment continued until PD, intolerability, or withdrawal of consent. The primary objective was OS. Secondary objectives included PFS, objective response rate, time to treatment failure, time to response, duration of response, and safety (Fig. 1).

Exploratory biomarker analysis

The objectives of this exploratory biomarker study were to investigate the association of circulating cfDNA *RAS* mutant allele frequency with patient outcomes, investigate the mutational landscape at baseline or SFU and net changes in key cancer-driving genes before and after therapy, and analyze the effect of baseline mutations in *EGFR* pathway genes on OS.

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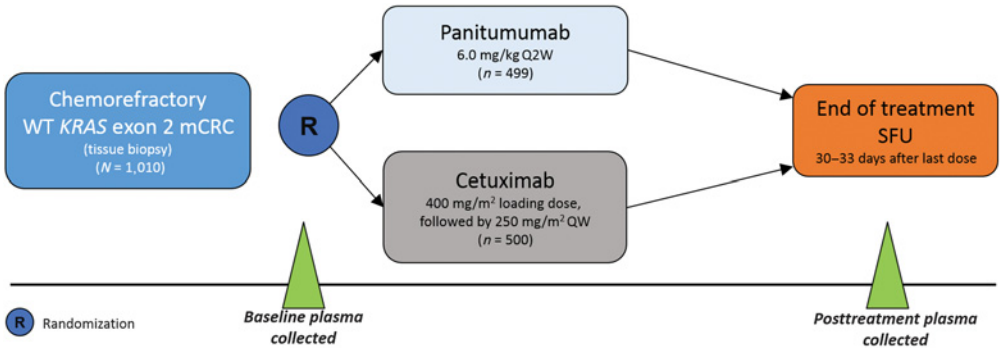

Figure 1.
Study schema of the ASPECCT phase III clinical study. WT, wild-type.

A subset of ASPECCT patients had provided written informed consent for participating in the plasma biomarker study. Patients involved in this current biomarker analysis were from the panitumumab arm, had received at least 1 dose of panitumumab, and had plasma samples available at both baseline and SFU. Plasma samples were collected at baseline (prior to randomization) and SFU (30–33 days after the last dose of panitumumab). Plasma samples collected were analyzed for extended *RAS* mutations (*KRAS* and *NRAS*, exon 2 [codons 12/13], 3 [codons 59/61], and 4 [codons 117/146]) as well as mutations in other *EGFR* pathway genes, including *BRAF*, *MAP2K1*, *PIK3CA*, and *PTEN*. Mutations in extended *RAS* were analyzed as a continuous variable and further correlated with clinical outcomes from the primary analysis of ASPECCT. The plasma levels of cfDNA *RAS* mutations were analyzed as a continuous variable for the association with clinical outcomes. Patients were classified into 4 groups by their respective best responses—progressive disease (PD), stable disease (SD), partial response (PR), and complete response (CR) or their survival outcomes. The results of extended *RAS* mutant allele frequencies were reported as percent mutant reads, indicating the percentage ratio of mutant DNA alleles to the total alleles (wild-type plus mutant), with 0 indicating undetectable mutant *RAS* allele and 100 indicating that all *RAS* alleles were mutant. Mutational prevalence of a panel of 63 cancer-related genes and net change in mutations of these genes from baseline to SFU in all patients were analyzed on a per-patient basis. For biomarker analysis of *EGFR* pathway genes, patient outcomes were analyzed according to the baseline mutation as both categorical and continuous variables. This biomarker analysis was blinded to both treatment and patient outcome and included all evaluable samples from panitumumab-treated patients for whom clinical data were available. Procedures of plasma sample collection were previously described (21). 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 follow the principles of the Declaration of Helsinki and CIOMS (International Ethical Guidelines for Biomedical Research Involving Human Subjects).

Next-generation sequencing

Plasma samples were analyzed by the *PlasmaSelect* 63-gene panel assay (Personal Genome Diagnostics; Supplementary Table S1). After plasma sample collection, individual DNA molecules in the plasma were bar-coded, and full coding regions of extended *RAS* and *EGFR* pathway genes were amplified, followed by redundant sequencing that allows for the discrimination of true mutations from sequencing artifacts. Sequenced DNA was aligned to the sequence in the reference human genome to report identified mutations. The LOD of the assay was 0.1% (22). The human genome assembly, GRCh37/hg19 (GCA_000001405.1), was used as the reference genome.

Model building process to characterize hazard based on mutant allele fraction of EGFR pathway genes

A disease severity index was created based on the weighted sum of allele frequencies for candidate mutant genes. The weight was based on the Cox proportional hazards model parameter estimate. A Gaussian-Process Cox proportional hazards (Cox PH) model of the disease index was fit to the data in order to estimate a non-linear relative hazard, and the mutant allele frequency with a hazard of 1 was selected to be the cut-point for defining high- and low-risk patients (23). The model allows curvature to the log-linear Cox PH model and is more appropriate when the hazard may not change monotonically over the domain of interest—in this case, from 0% to 100% mutant allele reads. The cut-point is arbitrarily chosen at a mutant allele frequency where the coefficient is 0 (the hazard is 1) because at this level the patient has an "average" risk. It is possible that the curve could cross 1 more than once due to the non-linear nature of the function. If this occurs, the first one was selected as the cut-point.

Results

Patients

Of the 499 patients randomized to the panitumumab arm of the ASPECCT study, 261 had plasma samples available for evaluation of extended *RAS* mutations. Of these, 235 and 234 patients had mutation results available at baseline and SFU, respectively; and 208 had paired results. A total of 469 patient samples were sequenced (Supplementary Table S2; Fig. 1).

Circulating *RAS* mutant allele frequencies and clinical responses

All patients with CR were *RAS* wild-type at baseline or SFU. The percentage of patients with mutant *RAS* status was the highest in PD group followed by SD and PR at both time points, and an increase from baseline to SFU was observed in all 3 groups (percentage of *RAS* mutant at baseline vs. SFU: PR, 6% vs.

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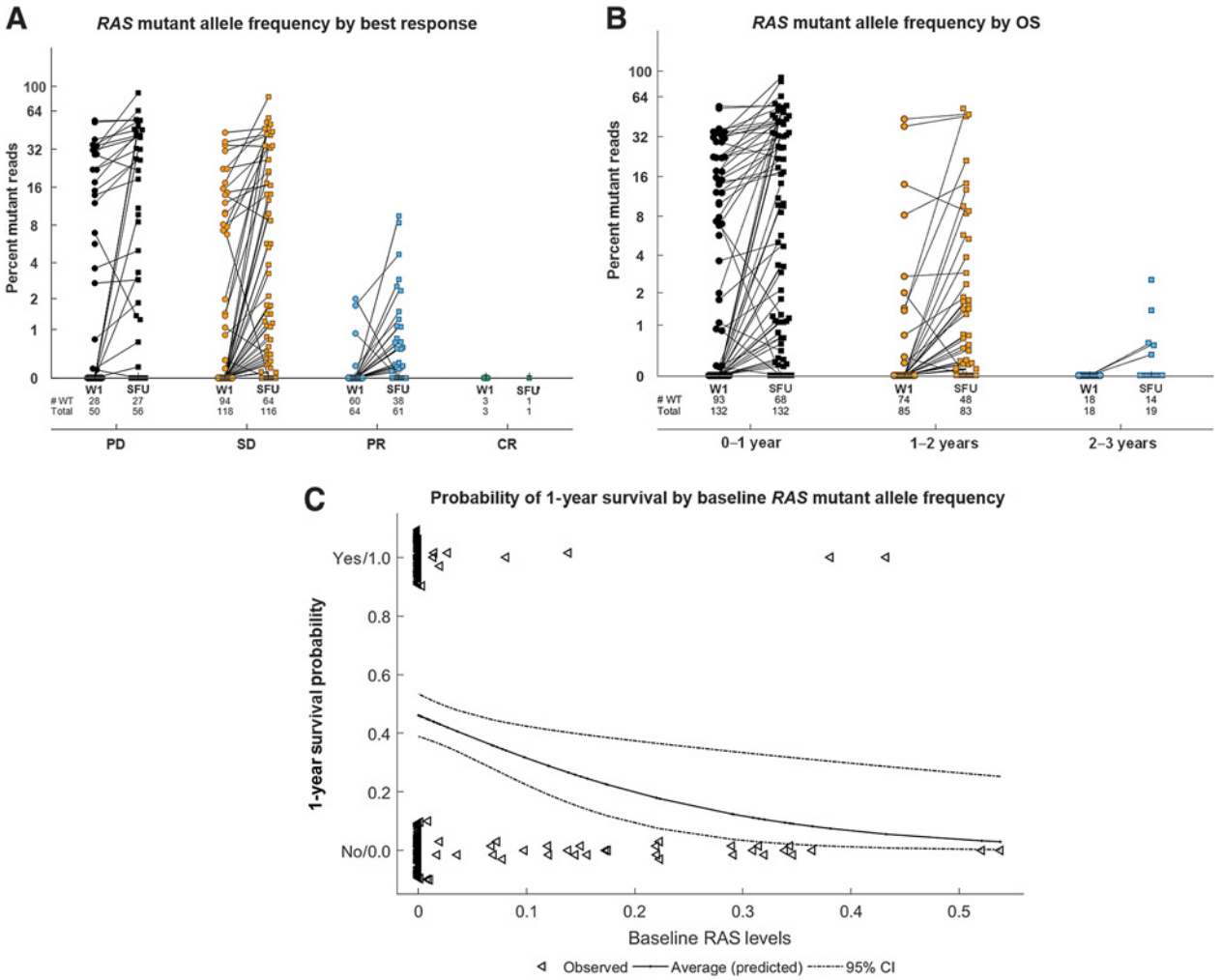

Figure 2.

Relationship between circulating *RAS* mutant allele frequencies and patient outcomes. Figure shows the correlation between circulating *RAS* mutant allele frequencies and best responses (**A**) or OS (**B**). *RAS* mutations were analyzed as a continuous variable indicated as "Percent Mutant Reads" shown on the y-axis. Samples were segregated into different groups by best response or duration of OS and plasma sample collection time points (baseline or SFU) as indicated on the x-axis. For patients with matched samples, a line connects the baseline and SFU results. **C,** Logistic regression between 1-year survival and baseline *RAS* mutant allele frequencies. Baseline *RAS* mutant allele frequencies are shown on the x-axis; the probability of 1-year survival is shown on the y-axis as either 1, indicating survival of 1 year or longer, or 0, indicating zero probability of 1-year OS. Each triangle represents an individual patient. Data were jittered around 1 or 0 on the y-axis to avoid overlap. The logistic regression model analysis shows that the probability of surviving past 1 year decreased as *RAS* mutant allele frequencies rose. MT, mutant; W1, week 1/baseline; WT, wild-type; MT, mutant.

38%, $P < 0.001$; SD, 20% vs. 45%, $P < 0.001$; and PD, 44% vs. 52%, $P = 0.42$; Fig. 2A). There was an association between low or absence of mutant *RAS* clones at baseline and better outcomes. Most of the patients with PR (94%) had no detectable *RAS* mutations at baseline. Interestingly, 6% of the patients with PR and 20% with SD were *RAS* mutant at baseline, indicating that baseline cfDNA *RAS* mutations did not completely preclude SD or PR. However, while PR and CR were attributable to treatment with panitumumab, patients with an indolent disease biology might achieve SD and long OS without active treatment, independent of anti-EGFR therapy. Of 28 patients who had no detectable *RAS* mutations at baseline but still experienced PD, 9 (32.1%) were *BRAF* mutant at baseline. We analyzed the baseline *RAS*/*BRAF* status and the corresponding best response and survival (Table 1). At baseline, 21.7% (5/23) of patients who were *BRAF* mutant and 10.8% (4/37) of those who were *RAS* mutant achieved objective response (PR). There were 11 patients who were mutant for both *RAS* and *BRAF*, 5 and 6 of whom had PD and SD, respectively, and no patients with mutations in both *RAS* and *BRAF* had an objective response. The 1-year survival rate was 9.1% in patients with mutations in both *BRAF* and *RAS* and 50.4% in wild-type patients. The 1-year survival rate was higher in patients with *BRAF mutations* than in those with *RAS* mutations (30.4% vs. 27.0%). All patients had died after 3 years. In the continuous analysis, patients with PD or SD had higher *RAS* mutant allele frequencies than those with PR (Supplementary Fig. S1, $P < 0.001$ for PD vs. SD; $P = 0.011$ for SD vs. PR). The mean of *RAS* mutant allele frequency was significantly higher among patients in the PD

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Table 1. Best response and survival by baseline *RAS/BRAF* mutation profiles

		Best response, number of patients (%)				Number of death (%)		
RAS	***BRAF***	**PD**	**SD**	**PR**	**CR**	**1-Year**	**2-Year**	**3-Year**
WT	WT	19 (13.9)	70 (51.1)	47 (34.3)	1 (0.7)	68 (49.6)	57 (41.6)	12 (8.8)
WT	MT	9 (39.1)	9 (39.1)	5 (21.7)	0 (0.0)	16 (69.6)	4 (17.4)	3 (13.0)
MT	WT	16 (43.2)	17 (45.9)	4 (10.8)	0 (0.0)	27 (73.0)	10 (27.0)	0 (0.0)
MT	MT	5 (45.5)	6 (54.5)	0 (0.0)	0 (0.0)	10 (90.9)	1 (9.1)	0 (0.0)

WT, wild-type; MT, mutant; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response.

group than other groups. In addition, a trend of an overall increase in *RAS* mutant allele frequency from baseline to SFU was observed in most of the patients in PD ($P < 0.001$), SD ($P < 0.001$), and PR ($P < 0.001$) groups. However, it appeared that patients with a low level of *RAS* mutant allele frequency could still derive some clinical benefits. No clear difference in the distribution of baseline *RAS* mutant allele frequencies was found between PD and SD ($P = 0.92$) or between SD and PR ($P = 0.27$), based on the two-sample Kolmogorov–Smirnov test; however, the distribution was significantly different between PR and PD ($P = 0.0012$; Fig. 2A).

Circulating *RAS* mutant allele frequencies and OS

The median OS for panitumumab-treated patients included in this study was 1 year. Mutant *RAS* allele frequencies were then analyzed for association with the duration of OS, according to which patients were classified into 3 groups—0 to 1, 1 to 2, and 2 to 3 years (Fig. 2B). Mutant *RAS* was detected at baseline in 30% (39/132) of the patients with OS of 0 to 1 year and 13% (11/85) of patients with OS of 1 to 2 years. All the patients who survived for 2 to 3 years had no detectable *RAS* mutations at baseline, whereas 26% of them had detectable *RAS* mutations at SFU. Patients who survived longer had lower allele frequencies of mutant *RAS* according to the continuous analysis ($P < 0.001$, Cox PH model). Response and correlation of OS with predominant *RAS* mutants were shown in Supplementary Table S3. The association between OS and baseline *RAS* mutant allele frequency is significant regardless of inclusion of wild-type patients or normalization of cfDNA concentration (Supplementary Table S4).

Next, we analyzed the effect of baseline *RAS* mutant allele frequency on the probability of surviving to 1 year or beyond (Fig. 2C). Baseline *RAS* mutant allele frequencies are shown on the x-axis; the probability of 1-year survival is shown on the y-axis as either 1, indicating survival of 1-year or longer, or 0, indicating zero probability of 1-year OS. The logistic regression model analysis showed that the probability of surviving past 1 year decreased as *RAS* mutant allele frequencies rose. However, patients without detectable *RAS* mutations at baseline (0 on x-axis) had only 46% probability of surviving for $\geq$1 year. Moreover, a few patients still survived to 1 year or beyond despite high *RAS* mutant allele frequency. Taken together, these results indicate that the baseline *RAS* mutation status or the mutant *RAS* allele frequency alone is not a perfect indicator for the probability of 1-year survival especially when detected using highly sensitive NGS platforms that detect mutations below 5% LOD.

Distribution and net change of mutational load from the 63-gene panel analysis

The mutation prevalence among patients was analyzed by a 63-gene panel NGS assay at both baseline and SFU (Supplementary Figs. S2, S3, and S4). Overall, 26% and 39% of the patients had multiple mutations in the same gene at baseline and SFU, respectively. *EGFR* and *TP53* had the highest number of mutations in any single patient, followed by *APC*, *ALK*, *KRAS*, *ERBB4*, and *MAP2K1* (Fig. 3A). One patient had 16 different mutations in *EGFR*. The most common *EGFR* mutation was at amino acid position 465, which was only detected at SFU (Supplementary Fig. S5). The best response for patients who were *EGFR* G465 mutant at SFU was either PR or SD but not PD, suggesting that these patients might have derived some clinical benefits from panitumumab despite preclinical evidence showing that this mutation abrogates panitumumab binding to EGFR (24).

The top panels of Fig. 3B show the distribution of mutational load at baseline and SFU, and the distribution of net change in mutational load before and after treatment. A single patient could have up to 50 mutations at baseline and 70 at SFU. A right shift toward higher mutational load was seen from baseline to SFU, indicating an overall increase in tumor mutation burden under the selection pressure of anti-EGFR therapy ($P < 0.001$, Wilcoxon signed rank test; Fig. 3B, top left and middle panels). The lower panels of Fig. 3B show the proportion of patients with mutations in the given gene at baseline and SFU (lower left and middle panels), as well as the net change of the proportion of patients with a net gain of mutation in a given gene minus that with a net loss of mutation in the same gene (lower 95% CI >0%, lower right panel). At baseline, approximately 84% and 75% of the patients had a mutation in *TP53* and *APC*, respectively, and similar results were observed at SFU. We have identified genes with a positive net change, as shown by Fig. 3B lower right panel. As expected, genes with the largest net change were in the *EGFR* pathway such as *KRAS*, *EGFR*, *NRAS*, *MAP2K1*, and *BRAF*. The net change was the highest for *KRAS* (20.1%) and *EGFR* (21.1%).

Overall, 63.5% of the patients had a net gain in the sum of mutations and 26.4% had a net loss. The net changes in mutation burden were further evaluated for clinical outcomes. Compared with patients who had a net gain, those with a net loss had longer OS ($P = 0.02$), implying a potential association between a decreased level of mutational heterogeneity and better outcome.

Biomarker analysis of *EGFR* pathway genes

To explore resistance mechanisms beyond *RAS* and their prognostic value, we expanded the biomarker analysis to include a total of six genes involved in the *EGFR* signaling cascade—*BRAF*, *PIK3CA*, *KRAS*, *NRAS*, *PTEN*, and *MAP2K1*. In total, 208 patients had paired evaluable plasma samples (Fig. 4A). At baseline, 113 patients were wild-type for six *EGFR* pathway genes and 95 patients were mutant. Of 95 mutant patients, 65 had single-gene mutations and 30 had multi-gene mutations. Genes with relatively predominant mutations identified at baseline were *BRAF* (16.3%), *PIK3CA* (14.4%), *KRAS* (13.5%), and *NRAS* (9.6%; ref. Fig. 4B).

Of the 113 patients with no mutations detected at baseline, 54 gained additional mutations during treatment: 29 and 25 patients acquired single-gene and multi-gene mutations, respectively; 59 patients remained wild-type at SFU (Fig. 4A). The genes with

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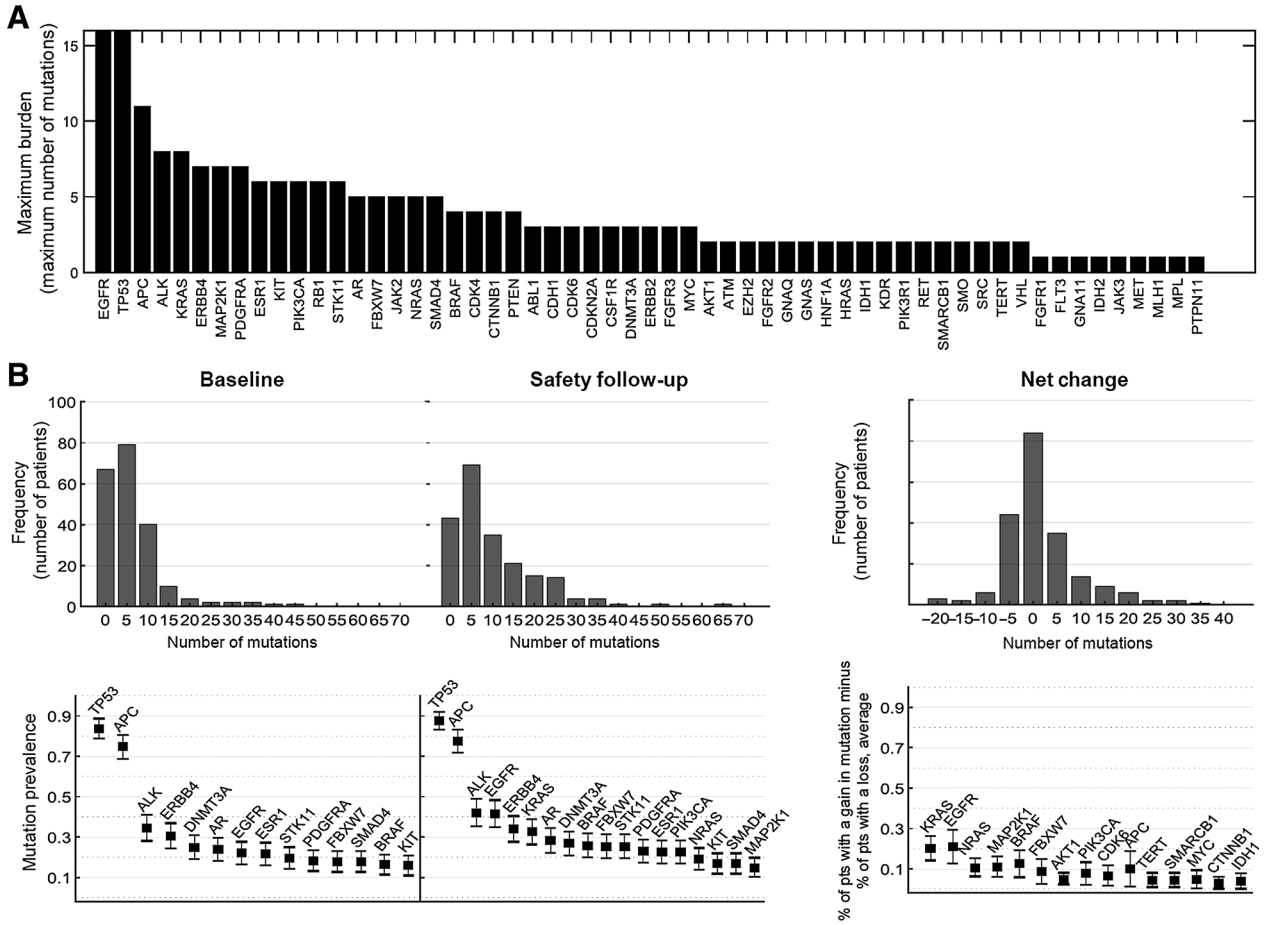

Figure 3.

63-gene panel analysis. **A,** Maximum number of mutations in any single patient by gene. Mutations were analyzed as binary variables, and the number of mutations were summarized at the gene level. Data represent any patient with the most mutations in a particular gene. **B,** Distribution of total mutations at baseline (upper left) and safety follow-up (upper middle), and distribution of net changes in mutations (upper right). The histograms are binned as: $0 \leq x < 5$, $5 \leq x < 10$, etc., where x is the number of mutations. Mutation prevalence at baseline (lower left panel) and safety follow-up (lower middle) are shown. Y-axis indicates the proportion of patients with mutations in the given gene. Only genes with prevalence >10% are shown. Lower right panel shows the proportion of patients with a gain of mutation in a gene minus the proportion of patients with a loss of mutation in the same gene.

predominant gain in mutations during treatment were *KRAS* (23.0%), *BRAF* (19.5%), and *MAP2K1* (15.0%; Supplementary Fig. S6). At SFU, 67 patients were wild-type; 141 were mutant, 62 of whom had single-gene mutations and 79 had multi-gene mutations (Fig. 4A). The genes with predominant gain in mutations identified at SFU were *KRAS* (32.7%), *BRAF* (26.0%), and *PIK3CA* (22.6%; Fig. 4C).

We next evaluated the prognostic value of baseline mutations in *EGFR* pathway genes as both categorical and continuous measures (Table 2). According to the categorical analyses, patients with baseline wild-type status for all six genes had longer OS than those with mutant status; the mutation status of *KRAS* and *PTEN* had the strongest association with OS ($P < 0.001$). In the continuous analyses, a cut-point for the mutant allele fraction was defined for all *EGFR* pathway genes except *MAP2K1* (due to insufficient number of samples) to reflect the baseline hazard of 1.0 (average risk; Supplementary Fig. S7). Patients with mutant allele frequency higher or lower than the cut-point were classified as high-risk (above average risk, hazard >1.0) or low-risk (below average risk, hazard <1.0), respectively. Our results showed that patients in the low-risk group had longer OS than those in the high-risk group (Table 2). Furthermore, there were more patients in the low-risk group than the wild-type group (gene, percentage of patients with low-risk vs. wild-type: *BRAF*, 95.7% vs. 83.7%; *KRAS*, 92.3% vs. 86.5%; *NRAS*, 96.6% vs. 90.4%; *PIK3CA*, 96.2% vs. 85.6%; *PTEN*, 94.7% vs. 91.3%); however, both groups frequently had comparable median OS, supporting the notion that patients with low mutant frequencies may still benefit from anti-EGFR treatment despite the mutant status.

Discussion

The highly sensitive NGS platform used in this study enables redundant sequencing to discriminate true mutations from artifacts at a sensitivity of 0.1%. By using this approach to detect plasma cfDNA mutations before and after panitumumab treatment, we extensively analyzed the effect of *RAS* mutant allele frequency on clinical outcomes and the mutation prevalence of 63

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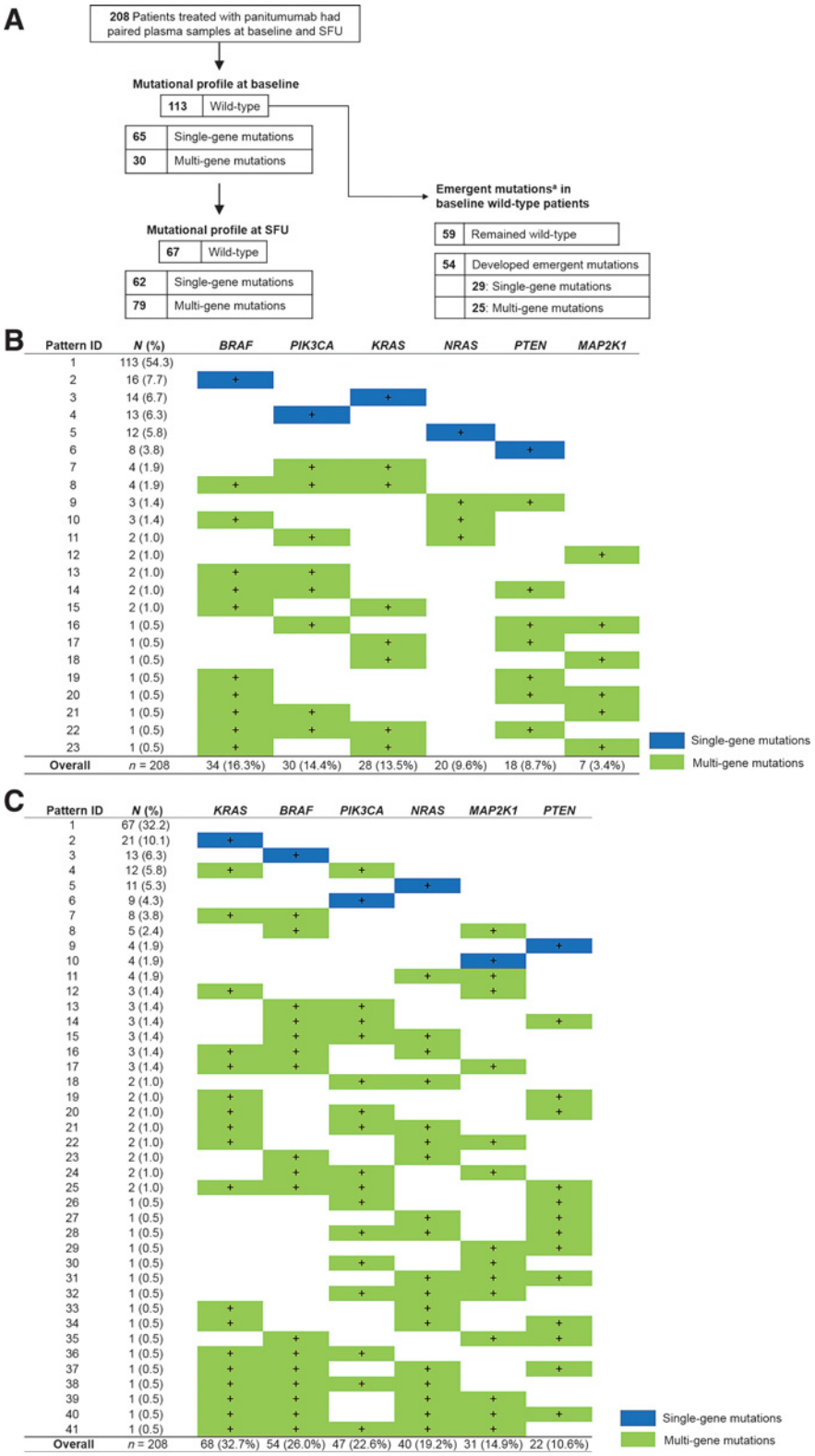

Figure 4.

A, Biomarker analysis of *EGFR* pathway genes. Baseline (**B**) and safety follow-up (**C**) mutational profiles are shown. The very left column shows all mutational patterns identified. "+" indicates mutations identified in the corresponding genes. Blue and green represent single-gene and multi-gene mutations, respectively. The bottom row shows the total number of patients (%) with mutations in the given gene. a, Emergent mutations were those defined as wild-type at baseline and then became mutant. Mutational profiles capture emergent mutations and revertants—those that become wild-type during treatment, as well as the gain in additional mutations.

cancer-related genes included in the *PlasmaSelect* assay panel. Baseline cfDNA mutations in *EGFR* pathway genes were found to be prognostic of outcome when analyzed categorically or continuously. For patients included in this analysis, the mutation status of only *KRAS* exon 2 was prospectively analyzed by tissue biopsy because it was the standard of care prior to the initiation of

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Table 2. Baseline mutational status of *EGFR* pathway genes and OS

Gene	Mutant read frequency at baseline hazard (continuous)		Categorical (Y/N) WT	Categorical (Y/N) MT	Continuous Low risk	Continuous High risk
Any/Sum	N/A	*n* (%) median OS, day, (95% CI)	113 (54.3)	95 (45.7)	153 (73.6)	55 (26.4)
		Cox PH *P*-value	458 (379 577)	263 (231 308)	419 (351 464)	221 (174 255)
			<0.001		<0.001	
BRAF	0.17	*n* (%) median OS, day, (95% CI)	174 (83.7)	34 (16.3)	199 (95.7)	9 (4.3)
		Cox PH *P*-value	379 (315 434)	255 (188 344)	351 (303 417)	221 (73 263)
			0.003		0.014	
KRAS	0.35	*n* (%) median OS, day, (95% CI)	180 (86.5)	28 (13.5)	192 (92.3)	16 (7.7)
		Cox PH *P*-value	379 (328 434)	239 (149 262)	364 (320 424)	188 (133 255)
			<0.001		<0.001	
MAP2K1	N/A	*n* (%) median OS, day, (95% CI)	201 (96.6)	7 (3.4)		
		Cox PH *P*-value	350 (296 415)	174 (0 521)	No cut-point found[a]	
			0.025			
NRAS	0.37	*n* (%) median OS, day, (95% CI)	188 (90.4)	20 (9.6)	201 (96.6)	7 (3.4)
		Cox PH *P*-value	350 (296 418)	291 (196 404)	350 (297 415)	183 (0 547)
			0.045		0.002	
PIK3CA	0.37	*n* (%) median OS, day, (95% CI)	178 (85.6)	30 (14.4)	200 (96.2)	8 (3.8)
		Cox PH *P*-value	354 (308 424)	262 (188 353)	350 (297 417)	262 (73 398)
			0.009		0.021	
PTEN	0.04	*n* (%) median OS, day, (95% CI)	190 (91.3)	18 (8.7)	197 (94.7)	11 (5.3)
		Cox PH *P*-value	364 (315 426)	237 (77 287)	351 (303 418)	183 (75 262)
			<0.001		<0.001	

[a]Insufficient numbers above cut-off to perform continuous analysis.

anti-EGFR therapy at the time when ASPECCT was conducted, and therefore, the tissue data of extended *RAS* status beyond *KRAS* exon 2 are unknown.

Activating *RAS* mutations have been shown to predict a lack of response to anti-EGFR therapy and are associated with inferior outcomes in patients with mCRC (11). Our findings revealed that baseline extended *RAS* mutations in cfDNA were detected in 6% of the patients with PR and 20% with SD, suggesting that the presence of *RAS* mutations in plasma prior to treatment does not preclude clinical response to panitumumab. However, patients with indolent disease may still achieve SD and long OS independent of anti-EGFR therapy.

The percentage of patients with mutant *RAS* status increased from 21.3% (50 of 235 patients) at baseline to 44.4% (104 of 234 patients) at SFU. From the continuous analyses, we also observed a patient-level increase in the *RAS* mutant allele frequency from baseline to SFU in most of the patients with detectable mutant *RAS*, regardless of their best response or duration of OS. These results, together with those reported by others (16, 25), indicate that the emergence of activating *RAS* mutations during treatment is a possible mechanism for acquired resistance to EGFR blockade. However, in a previous study, we analyzed the *RAS* mutation status as a categorical variable in the same patient population and reported that emergent cfDNA *RAS* mutations were not associated with less favorable outcomes (21). Similarly, the results from a prospective phase II study of panitumumab in mCRC indicated that patients with emergent *RAS* mutations at progression had similar PFS to those who remained wild-type, and the detection of emergent mutant *RAS* clones did not correlate with immediate clinical changes (26).

In this analysis, 15.1% (28/185) of the patients without detectable plasma *RAS* mutations at baseline still had PD as the best response. As discussed above, a subset of patients with detectable *RAS* mutations at baseline still achieved disease control (SD and PR). In addition, 13% of the patients with detectable *RAS* mutations at baseline survived for 1 to 2 years, longer than the median OS for patients with mCRC treated with anti-EGFR monotherapy (10). Therefore, despite an observed trend that worse clinical outcomes correlated with higher *RAS* mutant allele frequencies, mutant *RAS* detected by liquid biopsy did not preclude clinical responses to panitumumab, and the *de novo* *RAS* mutations acquired during therapy may not be the sole driver for the resistance to anti-EGFR treatment. Genetic alterations in genes other than *RAS* may play a role in the primary or secondary resistance. Of 28 patients who had no detectable *RAS* mutations at baseline but still experienced PD, nine (32.1%) were *BRAF* mutant at baseline, suggesting that *BRAF* mutations might contribute to the disease progression; however, the prognostic value of *BRAF* mutations remains inconclusive based on the results of this study. It should also be noted that in this study, mutant *RAS* status refers to any mutations identified by the extended *RAS* analysis covering mutations in exon 2, 3, and 4 of *KRAS* and *NRAS*. It has been suggested that not all *RAS* mutations are created equal with respect to the impact on clinical response to anti-EGFR therapy, and a particular *RAS* mutation may have a greater or lesser effect on the *RAS* signaling activity, variably affecting tumor dependence on *RAS* mutations as a major oncogenic driver (27–29).

At baseline, 50 (21.3%) of 235 patients who were tested wild-type for *KRAS* exon 2 by tissue biopsy had extended *RAS* mutations according to the liquid biopsy. This group of patients had a shorter median OS compared with those who were wild-type for extended *RAS* at baseline. The prevalence of extended *RAS* mutations at baseline in the population of this study is consistent with the results from a prospective secondary analysis on patient tumor specimens of the PEAK study, which found that 23% of the patients with wild-type *KRAS* exon 2 mCRC had *RAS* mutations beyond *KRAS* exon 2 (30).

High concordance between liquid biopsy and tissue biopsy for molecular characterization of cancer genes has been

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previously demonstrated. A prospective study compared *KRAS* mutation results obtained from plasma cfDNA testing with those obtained from tumor tissue biopsy and showed that plasma cfDNA analysis exhibited 98% specificity and 92% sensitivity for seven *KRAS* point mutations tested, with a concordance rate of 96% (31). It was speculated that the 4% discordance was due to instability of tumor genome and the intratumor and intertumor heterogeneity. Another proof-of-concept study on circulating cfDNA showed that high-depth sequencing of plasma-derived cfDNA captured all mutations detected in the tumor tissue (32). Despite the high concordance, defining a threshold of mutation that is clinically relevant remains challenging with both biopsy approaches. This study was not designed to address the predictive value of mutations and lacks a comparator arm, and therefore, a clinically actionable mutation level could not be determined.

Intrinsic molecular heterogeneity plays a pivotal role in the clonal evolution of colorectal tumors and ensures the selection of subclonal populations capable of growing under the selective pressure of EGFR blockade (16, 18). Our 63-gene panel NGS analysis revealed a high degree of genetic diversification at baseline, which could potentially provide the basis for and prime the development of treatment resistance. There were more patients with multiple mutations in the same gene at SFU than baseline, indicating mutations arising in separate clones in the same gene during anti-EGFR treatment. Most of the patients (63.5%) had a net gain in mutations. As expected, the predominant genes in which additional mutations were gained by most of the patients were in the *EGFR* pathway, including *KRAS*, *EGFR*, *NRAS*, *BRAF*, and *MAP2K1*. Surprisingly, a subset of patients (26.4%) had an overall decrease in the sum of total mutations, indicating that the mutational heterogeneity can decrease as a result of the intraclonal competition under anti-EGFR selective pressure. OS appeared to be inversely associated with the net change in mutational load ($P = 0.02$).

Further analysis on the *EGFR* pathway genes showed that baseline mutant status of six selected *EGFR* pathway genes was associated with shorter median OS, similar to the results from previously studies (33–36). When conducting the continuous analyses, we generated a prognostic model to estimate the cut-point mutant allele frequency used to classify patients into low-risk or high-risk group. Patients with high risk had shorter OS than those with low risk for all selected *EGFR* pathway genes except *MAP2K1*. Given the higher number of patients in the low-risk group versus the categorical wild-type group and the comparable median OS between these two groups across all genes analyzed, our prognostic model offered promise in regard to identifying additional patients who may otherwise be undertreated. However, the prognostic indicators in this exploratory *ad hoc* analysis cannot be interpreted as thresholds to guide clinical treatment decisions for several reasons. In particular, the original ASPECCT study design did not allow us to compare the relative benefit of panitumumab group to a placebo group so that it was not possible to establish a clinical threshold. In addition, the result values are not calibrated across different NGS platforms, and the NGS sequencing has not been shown to be linear in the reported range.

Another limitation of this study is the sparse serial plasma collections. Sequential plasma sample analysis at more frequent intervals may allow for dynamic monitoring of tumor clonal evolution during treatment. Because plasma samples were collected and analyzed at only two time points in this study—prior to and after treatment—the exact timing of emergent mutations and the pattern of mutational changes during the course of treatment were unknown.

In summary, although baseline mutant status for *EGFR* pathway genes as detected by cfDNA analysis was associated with worse outcomes, our analysis, which models mutations as a continuous variable, could potentially identify additional patients with a low mutation frequency who may still derive benefit. Patients with lower allele frequencies of mutant *RAS* tended to have better clinical responses than those with higher *RAS* mutant allele frequency; however, a clinically relevant mutation frequency could not be determined in this exploratory analysis. High mutational heterogeneity was observed by a 63-gene panel analysis at both baseline and SFU, with most patients having a net gain in the sum of mutations while on treatment and a subset of patients having a net loss. Comparator-controlled prospective studies are needed to establish a clinically meaningful threshold for cfDNA mutation frequency and to validate the predictive value of mutations in *EGFR* pathway genes.

Disclosure of Potential Conflicts of Interest

M. Peeters reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Amgen. T. Price is a consultant/advisory board member for Amgen, Merck Serono, and Takeda. M. Boedigheimer is an employee of and holds ownership interest (including patents) in Amgen. P. Ruff reports receiving commercial research grants from Amgen and reports receiving speakers bureau honoraria from Amgen, Merck Serono, and Sanofi. A. Thomas is a consultant/advisory board member for Bristol-Myers Squibb, Amgen, and Servier, and reports receiving other remuneration in the form of an educational award from Bristol-Myers Squibb. G. Demonty holds ownership interest (including patents) in Amgen. K. Hool holds ownership interest (including patents) in Amgen. A. Ang holds ownership interest (including patents) in Amgen. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Price

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Evaluation of Emergent Mutations in Circulating Cell-Free DNA and Clinical Outcomes in Patients with Metastatic Colorectal Cancer Treated with Panitumumab in the ASPECCT Study

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