

# Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment

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Received 22 September 2014; revised 16 December 2014; accepted 17 December 2014

**Introduction:** *KRAS* and *EGFR* ectodomain-acquired mutations in patients with metastatic colorectal cancer (mCRC) have been correlated with acquired resistance to anti-EGFR monoclonal antibodies (mAbs). We investigated the frequency, co-occurrence, and distribution of acquired *KRAS* and *EGFR* mutations in patients with mCRC refractory to anti-EGFR mAbs using circulating tumor DNA (ctDNA).

**Patients and methods:** Sixty-two post-treatment plasma and 20 matching pretreatment archival tissue samples from *KRAS*<sup>wt</sup> mCRC patients refractory to anti-EGFR mAbs were evaluated by high-sensitivity emulsion polymerase chain reaction for *KRAS* codon 12, 13, 61, and 146 and *EGFR* 492 mutations.

**Results:** Plasma analyses showed newly detectable *EGFR* and *KRAS* mutations in 5/62 [8%; 95% confidence interval (CI) 0.02–0.18] and 27/62 (44%; 95% CI 0.3–0.56) samples, respectively. *KRAS* codon 61 and 146 mutations were predominant (33% and 11%, respectively), and multiple *EGFR* and/or *KRAS* mutations were detected in 11/27 (41%) cases. The percentage of mutant allele reads was inversely correlated with time since last treatment with EGFR mAbs ( $P = 0.038$ ). In the matching archival tissue, these mutations were detectable as low-allele-frequency clones in 35% of patients with plasma mutations after treatment with anti-EGFR mAbs and correlated with shorter progression-free survival (PFS) compared with the cases with no new mutations (3.0 versus 8.0 months,  $P = 0.0004$ ).

**Conclusion:** Newly detected *KRAS* and/or *EGFR* mutations in plasma ctDNA from patients refractory to anti-EGFR treatment appear to derive from rare, pre-existing clones in the primary tumors. These rare clones were associated with shorter PFS in patients receiving anti-EGFR treatment. Multiple simultaneous mutations in *KRAS* and *EGFR* in the ctDNA and the decline in allele frequency after discontinuation of anti-EGFR therapy in a subset of patients suggest that several resistance mechanisms can co-exist and that relative clonal burdens may change over time. Monitoring treatment-induced genetic alterations by sequencing ctDNA could identify biomarkers for treatment screening in anti-EGFR-refractory patients.

**Key words:** ctDNA, *KRAS* acquired mutation, *EGFR* acquired mutation, anti-EGFR mAbs

## Introduction

The anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies (mAbs) cetuximab and panitumumab have improved clinical outcomes for patients with metastatic

colorectal cancer (mCRC) who have tumors expressing wild-type *KRAS* [1].

Researchers have found a relationship between the occurrence of novel genetic events in key downstream effectors of the EGFR pathway and treatment-acquired resistance. In one study, an *EGFR* extracellular domain (S492R) mutation was newly detected in 2/20 patients whose disease initially responded but later progressed on cetuximab [2]. Other studies have reported the presence of acquired *KRAS* mutations in patients in whom resistance to anti-EGFR mAbs developed after an initial clinical benefit [3–5].

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Retrospective studies had identified pre-existing low-frequency *KRAS* mutations using high-sensitivity sequencing techniques, despite the fact that standard-of-care *KRAS* testing had failed to detect these mutations. These low-frequency *KRAS* mutations seemed to correlate with a poor response to anti-EGFR treatment [6, 7].

In this present retrospective study, we evaluated the frequency and distribution of newly detectable *KRAS* and *EGFR* mutations after progression on anti-EGFR mAb by sequencing cell-free circulating tumor DNA (ctDNA), and correlated these findings with high-sensitivity testing of the primary tumor to characterize the presence of pre-existing rare *KRAS* and *EGFR* mutant clones.

Additional goals were to understand whether multiple genetic changes may develop under treatment pressure and to track the temporal dynamics of these mutations upon completion of anti-EGFR therapy.

## materials and methods

### study population

We retrospectively studied 70 patients with mCRC or unresectable locally advanced colorectal cancer who had been treated with systemic chemotherapy and anti-EGFR mAbs. Patients had been enrolled in the Assessment of Targeted Therapies in Colorectal Cancer (ATTACC) biomarker screening program, undergoing to evaluation for biomarker-matched clinical trials at The University of Texas MD Anderson Cancer Center. All patients had *KRAS* and *BRAF* sequenced as part of standard-of-care testing in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. Tumor measurements by RECIST1.1 were calculated from computed tomography (CT) scans obtained in proximity to plasma sample collection. Tumor burden assessment (Figure 2B) was calculated from CT scans using the commercially available volumetric-dedicated software MyrianXL-Onco (Intrasense, Montpellier, France) [8]. This study was carried out under an Institutional Review Board-approved protocol, and informed consent was collected from all patients enrolled.

### sample collection, ctDNA isolation, and *KRAS* and *EGFR* mutation analysis

Baseline *KRAS* mutation analysis carried out in a CLIA-compliant laboratory on patient formalin-fixed paraffin-embedded (FFPE) tissue samples using either DNA sequencing (IonTorrent PGM, Life Technologies, Gray Island, NY) or allele-specific polymerase chain reaction (PCR) combined with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MassARRAY, Sequenom, San Diego, CA) for mutations in the four most common hot spots (codons 12, 13, 61, and 146) of *KRAS*. After resistance on EGFR-inhibitors blood samples for ctDNA were collected and processed within 2 h of collection in cell preparation tubes (BD, Franklin Lakes, NJ). Plasma was separated after a single centrifugation step and stored in 2-ml aliquots at  $-80^{\circ}\text{C}$  until analysis. ctDNA was purified from the plasma aliquots at Sysmex Inostics Laboratory (Hamburg, Germany). A subsequent *KRAS* and *EGFR* analysis was carried out using high-sensitivity emulsion PCR on DNA extracted from FFPE samples of primary tumor tissues from patients who had newly detected *KRAS* and *EGFR* mutations identified in post-progression plasma samples. DNA was extracted from five 10- $\mu\text{m}$ -thick slides made from the FFPE sample [9]. Sequencing was carried out using Bead Emulsion Amplification Magnetic (BEAMing) technology, and samples with a detectable mutation rate above 0.02% were considered positive [9]. Specific *KRAS* and *EGFR* mutations were analyzed (supplementary Table S1).

### statistical analysis

Differences in progression-free survival (PFS) for initial treatment with EGFR inhibitors were evaluated using the log-rank test (Mantel-Cox test). The relationship between pre- and post-treatment *KRAS* mutation reads was assessed by a Mann-Whitney non-parametric test. The correlation between *KRAS* mutation reads as a percentage of total reads and months from last anti-EGFR treatment was analyzed by the non-parametric Kruskal-Wallis test. Comparisons were considered significant if the *P* value was  $\leq 0.05$ . Statistical analyses were carried out using GraphPad Prism software, version 6.1 (GraphPad Software, Inc., La Jolla, CA).

## results

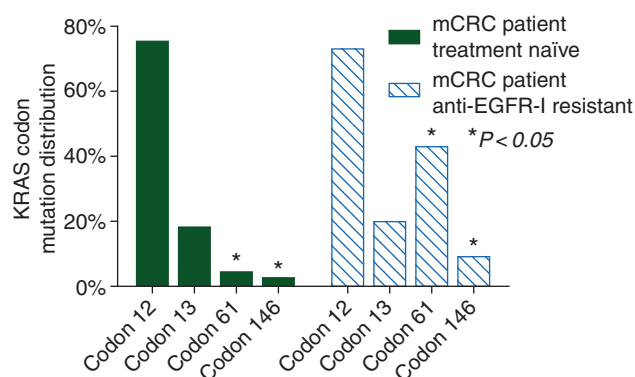
Of the 70 patients studied, 62 had acquired resistance to anti-EGFR mAbs after an initial response, 8 had tumors known to carry *KRAS*<sup>mut</sup> and were used as positive controls for *KRAS* mutation detection in the plasma. Among the 62 patients with anti-EGFR mAb resistance, 55 were treated with a cetuximab-based regimen, 4 with panitumumab-based regimen, and 3 received both. Median patient age was 60 years, and 74% of the patients had primary colon cancer, whereas 26% had tumors that arose in the rectum. Anti-EGFR mAbs were administered as first-line treatment in 4/62 (6%) patients, as second-line treatment in 30/62 (49%), as third-line treatment in 24/62 (39%), and as fourth-line treatment in 4/62 (6%). Analysis of specific *EGFR* and *KRAS* mutations was possible in all samples. *KRAS* mutational analysis carried out on ctDNA extracted from post-treatment plasma samples from the eight control patients known to harbor a mutation in *KRAS* confirmed the presence of such mutations detected at the time of diagnosis. No *EGFR* mutations were detected in any of these samples.

For the 62 patients initially characterized as having *KRAS*<sup>wt</sup> tumors, acquired mutations in *KRAS* and *EGFR* following resistance to anti-EGFR therapies were seen in 27/62 cases [44%, 95% confidence interval (CI) of 30% to 56%] and 5/62 cases (8%, 95% CI of 2% to 19%), respectively. Although *KRAS* codon 12 and 13 mutations represented, as expected, the majority of the acquired mutations, *KRAS* mutations in codons 61 and 146 occurred more frequently in patients with acquired *KRAS* mutations after anti-EGFR mAb treatment than in treatment-naïve patients with *KRAS* mutations. Codon 61 and 146 mutations were identified in 9/27 (33%) and 3/27 (11%) samples compared with 9/240 (4%) and 5/240 (2%) samples, respectively, of untreated colorectal tumors classified as *KRAS*-mutant at baseline and analyzed by standard-of-care sequencing ( $P < 0.01$  for comparison with acquired mutation frequencies, Figure 1). Newly detected *EGFR* S492R mutations were seen in five of the plasma samples from patients treated with cetuximab but in none of those treated with panitumumab. Interestingly, 4/5 patients with acquired *EGFR* mutations also had newly detected *KRAS* mutations suggesting that these mutations are not mutually exclusive. Multiple acquired *KRAS* codon mutations were simultaneously present in the ctDNA of 11/27 (41%) patients (supplementary Table S3).

The presence of multiple clones carrying different genetic alterations and the dynamic clonal selection induced by anti-EGFR treatment pressure was confirmed by an analysis of serial samples from one of the patients (supplementary Figure S1). Analysis of *KRAS* and *EGFR* mutations in the patient's primary

tumor archival sample using BEAMing confirmed *KRAS*<sup>wt</sup> and *EGFR*<sup>wt</sup> status, which correlated with the patient's response to the initial anti-EGFR treatment. The blood samples collected after progression and before the time of anti-EGFR re-challenge within a clinical trial showed multiple newly detected *KRAS* and *EGFR* mutations. After retreatment with cetuximab, a sample demonstrated an increased percentage of *KRAS* mutant allele reads and continued acquisition of new *KRAS* and *EGFR* mutations.

The quantitative analysis of the plasma sample data showed that the acquired *KRAS* mutations occurring after progression on anti-EGFR therapy had a lower concentration of detectable mutant alleles than the *KRAS* mutants identified at diagnosis (Figure 2A,  $P < 0.001$ ). These differences remained after controlling for metastatic tumor volume (Figure 2B).



**Figure 1.** *KRAS* codon mutation distribution in treatment-naïve patients versus patients with acquired EGFR-inhibitor resistance (EGFR-I resistant). Atypical *KRAS* codon 61 and codon 146 mutations were more frequent in metastatic colorectal cancer patients with acquired *KRAS* mutations after anti-EGFR mAb treatment than in treatment-naïve patients with *KRAS* mutations ( $*P < 0.05$ ).

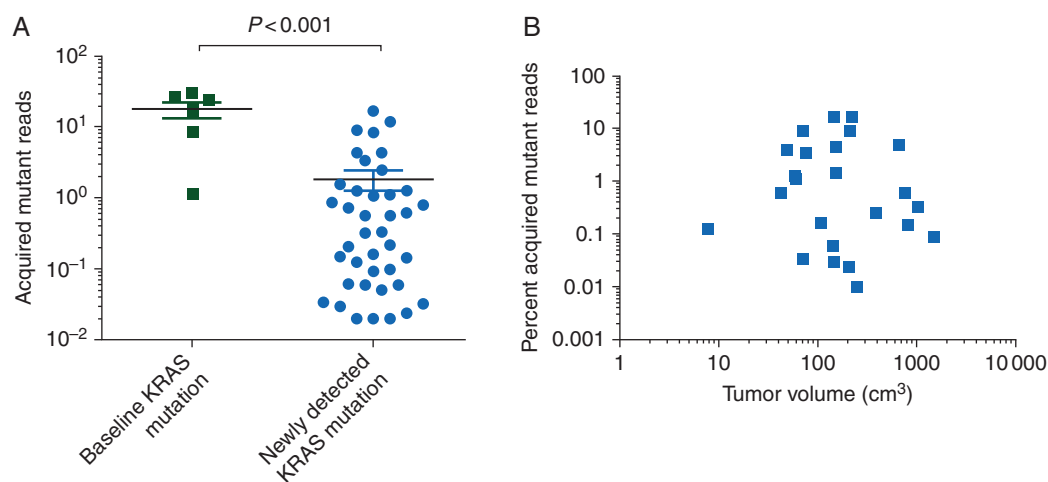
Because it is possible that clones with acquired *KRAS* and *EGFR* mutations no longer have a growth advantage relative to other clones after discontinuation of the selective pressure, we evaluated the impact of time since the last cycle of anti-EGFR mAb treatment on the mutant/wild-type allele ratio for the most prevalent acquired allele in each patient. A significant inverse relationship between mutant allele levels and time since last treatment was observed ( $P = 0.038$ , Figure 3).

To determine whether the newly detected *KRAS* and *EGFR* mutations were already present in the treatment-naïve primary tumors as undetectable low-frequency clones or were actual acquired mutations, we used BEAMing to re-analyze the archival samples from 20 of the 27 patients positive for newly detected *KRAS* mutations. *KRAS* mutations were detectable in 7 (35%) of the 20 primary tumors analyzed (95% CI of 0.15–0.69), whereas *EGFR* extracellular domain mutations were not detected in any samples [10] (supplementary Table S2). Although the sample size was small, patients with low-frequency mutations in their primary tumor samples had significantly worse PFS than patients with *KRAS*<sup>wt</sup> primary tumors after BEAMing reanalysis of their archival tissue (median PFS: 3.0 versus 8.0 months,  $P = 0.0004$ , Figure 4).

## discussion

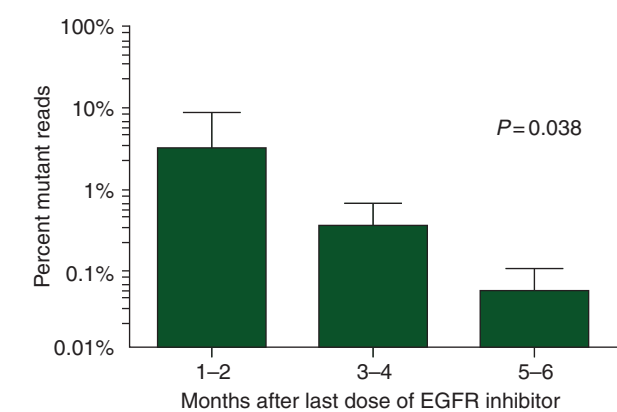
Our findings demonstrate that *KRAS* mutations newly detected in the plasma of patients with colorectal cancer who had disease progression following treatment with anti-EGFR mAbs were already present as minor clones in more than a third (7/20) of the archival tissue collected at the time of diagnosis and that the pattern of genetic alterations in plasma and tissue samples was consistent.

In agreement with our findings, Diaz et al. [3] presented modeling data suggesting that acquired *KRAS* mutations were pre-



**Figure 2.** Concentration of ctDNA-detectable alleles by timing of mutation and tumor size. (A) Patients with tumors carrying *de novo* *KRAS* mutations had a significantly higher percentage of *KRAS* mutant alleles (defined as the number of mutant *KRAS* allele reads divided by the total number of wild-type or mutant *KRAS* allele reads) detected in plasma ctDNA than patients characterized as having wild-type *KRAS* initially but who had newly detected *KRAS* mutations after progression on EGFR mAb treatment ( $P < 0.001$ , Mann–Whitney *U*-test). (B) Tumor volumes were calculated by three independent readers by manually tracing the outer edge of the lesions on each image, and the whole-tumor burden was calculated by summing each lesion volumes. No correlation was found between percentage of acquired mutant alleles and tumor burden.

existent in minor clones and expanded under selective pressure. Our data confirm this in a subset of patients by demonstrating the presence of clones missed by standard-of-care sequencing.



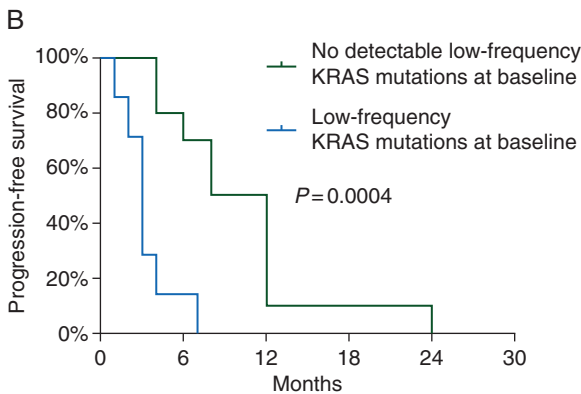
**Figure 3.** Time from last treatment with EGFR mAbs correlates with quantification of *KRAS* mutations detected in ctDNA. Time from last dose of EGFR mAbs correlated with the percentage of *KRAS* mutation reads detected in ctDNA from the plasma of metastatic colorectal cancer patients who developed resistance to treatment ( $P = 0.038$ ).

The pattern of genetic alterations in cancer patients is a dynamic model affected by several intrinsic and extrinsic factors including tumor genetic instability and treatment pressure. The presence of low-frequency *KRAS* mutations in the primary tumor correlated with a worse response to anti-EGFR treatment. Our findings confirm the data presented by Tougeron et al. [6] who demonstrated that low-frequency *KRAS* mutations, detected by pyrosequencing, were present in ~20% of tumors previously characterized as *KRAS*<sup>wt</sup> and that these mutations were associated with a shorter PFS. Our data extend this finding by demonstrating that these low-frequency mutations are indeed the dominant clone by allele frequency in ctDNA at the time of progression on anti-EGFR therapy.

Overall, these data support the hypothesis that utilizing a high-sensitivity mutation detection technique that provides quantitative information about the presence of mutant alleles in the gene of interest would allow for the selection of patients most likely to benefit from anti-EGFR treatment. However, it remains to be seen whether low-frequency *KRAS*-mutant clones in the primary tumor provide relative or absolute resistance to anti-EGFR mAb therapy; this question will require evaluation in randomized clinical trials. An additional limitation is that standard-of-care sequencing rarely utilizes microdissection, and it is

A

KRAS mutation detected			
Patients	Archival tissue (standard care)	Archival tissue (BEAMing, micro-dissected)	Post-treatment plasma (BEAMing)
Baseline low-frequency mutations	WT	G12V (12.7%)	G12V (17%) Q61H (0.1%)
	WT	G12D (18%)	G12D (9%)
	WT	G12C (5.37%) G12D (9.6%)	G12C (0.09%)
	WT	G12C (5.6%)	G12C (11%)
	WT	G128 (16%)	G128 (8.8%)
	WT	A146T (6%) G12V (26%)	A146T (5.7%)
No low-frequency mutations detectable at baseline	WT	WT	G128 (0.3%) G13D (0.3%) Q61H (0.4%)
	WT	WT	G12C (0.3%)
	WT	WT	G12C (1.3%)
	WT	WT	G12V (0.1%)
	WT	WT	G12C (3%)
	WT	WT	Q61H (1.1%)
	WT	WT	G12D (0.2%)
	WT	WT	Q61H (0.4%) G128 (2.2%) G13D (0.3%)



**Figure 4.** Low-frequency *KRAS* mutation expression in patient archival tissue and patient plasma samples correlate with shorter PFS with anti-EGFR treatment. (A) In 7 of 20 patients with newly detected *KRAS* mutations in the plasma and wild-type *KRAS* by standard-of-care testing in the primary tumor, there were detectable low-frequency mutations present in microdissected tumor analyzed by BEAMing assay. (B) Patients with low-frequency *KRAS* mutations detected in archival primary tumor tissues had shorter PFS than patients with no detectable mutations by BEAMing ( $P = 0.0004$ ).



possible that higher prevalence *KRAS*-mutant clones may not be detected in a stromal-rich primary tumor. This was seen in 4 of our 14 primary tumors, where microdissected tumor resulted in allele frequencies >10% that were missed by routine standard-of-care testing. Our study did not include other mutations that have subsequently been shown to be associated with EGFR resistance, including *NRAS* mutations and EGFR codon 491 [11]. Further efforts should utilize a larger panel of mutations to more fully characterize the spectrum molecular changes with acquired resistance.

In our study, newly detected *KRAS* mutations were present in multiple concomitant *KRAS* codons within the same patient in several cases, whereas *EGFR* extracellular domain mutations were confirmed in only a few patients after acquired resistance to cetuximab treatment. Multiple *KRAS* codon mutations are not usually seen in primary tumors because *KRAS* is considered an early mutation in tumor formation and is associated with a low degree of mutational heterogeneity [12]. *KRAS* mutations occurring in codons 61 and 146 are considered rare events in untreated colorectal cancers, and multiple studies have suggested that these biomarkers predict a lack of response to anti-EGFR therapies [13]. In contrast, tumors with codon 13 mutations have been suggested to retain sensitivity to EGFR inhibition in some, but not all, studies [14]. The acquisition of codon 13, 61, and 146 mutations in our study provides strong evidence that these mutations provide a selective growth advantage in the presence of EGFR inhibition; thus, our study provides orthogonal data suggesting that, similar to codon 12 mutations, these mutations are predictive biomarkers of anti-EGFR mAb resistance. It is also of interest that 'atypical' codon 61 and 146 *KRAS* mutations were expressed more frequently in acquired resistance mutations than in the general colorectal cancer population [15]. Although the mechanism for this finding is unknown, prior studies have demonstrated that codon 61 and 146 mutations have weaker RAS-GTPase activity in transforming assay, and therefore may have lower growth advantage compared with exon 2 mutations that may only expand once the tumor is placed under treatment pressure with an EGFR inhibitor [16].

The presence of an *EGFR* S492R mutation has been reported in one study to be a biomarker predictive of cetuximab resistance but panitumumab sensitivity, owing to the different binding sites for each of the drugs [2]. In our study, the *EGFR* S492R mutation was detected in 5/62 (8%) of the EGFR mAb-resistant plasma samples analyzed but in none of the archival tissues [10], and it should be noted that 4/5 patients with newly detectable *EGFR* mutations had concomitant *KRAS* mutations. Although the number of patient treated with panitumumab was very limited, in our study the *EGFR* S492R mutation seemed a more common feature of patients progressing on cetuximab, consistently with the ASPECCT trial data where acquired *EGFR* S492R mutation where detected in 16% of the patient treated with cetuximab and 1% of those treated with panitumumab [17]. Therefore, these *EGFR* mutations need to be better understood in the context of anti-EGFR treatment re-challenge.

It is notable that newly detected *KRAS* and *EGFR* mutations were present at a substantially lower level in ctDNA than pre-existing *KRAS* mutations, which suggests that the acquired *KRAS* mutations may not fully or permanently repopulate the entirety

of the tumor, although direct quantification of the percent of the tumor repopulated with the mutant clone is not possible with the current assay. Indeed, given the appearance of multiple mechanisms of resistance in individual patients, it is possible that these mechanisms may co-exist with other previously reported mechanisms [11, 18, 19]. Additionally, recent reports have noted that paracrine mechanisms arising from resistant clones may propagate resistance through several proposed mechanisms. Hober et al. demonstrated that TGF- $\alpha$  and amphiregulin secreted from resistant cells induced resistance to EGFR inhibition while Troiani et al. identified TGF- $\alpha$  as an activator of the MET and EGFR axis, resulting in cetuximab resistance [20, 21]. In the absence of continued selective pressure from EGFR inhibition, the prevalence of these *KRAS* and *EGFR* mutant clones may decline, as suggested by the trend in mutant read frequency over time that was seen in our population. These data appear to be consistent with a recent report of clinical benefit seen with cetuximab retreatment in previously sensitive patients who were off of EGFR mAb for an extended interval, and suggest future opportunities for ctDNA-guided EGFR mAb rechallenge studies [22].

In conclusion, we confirm that low-frequency *KRAS* mutations detected by high-sensitivity assays are associated with reduced benefit from anti-EGFR mAb therapy and that these rare clones are the dominant clones identified in ctDNA at the time of progression. Analysis of ctDNA in the plasma provides opportunities to interrogate mechanisms of resistance and should be integrated into future prospective studies as a validation measure. Our study shows that, in contrast to the model of a single dominant clone, there are multiple dynamic genetic changes that may develop and lead to resistance in a given patient under treatment pressure [23]. This insight may lead to future efforts to better tailor anti-EGFR re-challenge clinical trials and therapeutic efforts to reverse resistance to EGFR inhibitors [24].

## funding

This work was supported in part by an MD Anderson Cancer Center Institutional Research Grant; by the National Institutes of Health grants CA136980, Cancer Center Support Grant CA16670, and CA172670; by CPRIT RP110584 and 1R01CA172670.

## disclosure

FD and PA have leading position at Sysmex Inostics. No conflict of interest for the other authors.

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