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Genomic profiling of blood-derived circulating tumor DNA from patients with colorectal cancer: Implications for response and resistance to targeted therapeutics

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Circulating tumor DNA from colorectal cancer

Keywords: circulating tumor DNA; liquid biopsy; colorectal cancer; genomic

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ABSTRACT

Molecular profiling of circulating tumor DNA (ctDNA) is a promising non-invasive tool. Here, next-generation sequencing (NGS) of blood-derived ctDNA was performed in patients with advanced colorectal cancer (CRC). We investigated ctDNA-derived genomic alterations, including potential actionability, concordance with tissue NGS, and serial dynamics in 78 patients with CRC using a clinical-grade NGS assay that detects single nucleotide variants (54-73 genes) and selected copy number variants, fusions, and indels. Overall, 63 patients (80.8% [63/78]) harbored ctDNA alterations; 59 (75.6% [59/78]), ≥1 characterized alteration (variants of unknown significance excluded). All 59 patients had actionable alterations potentially targetable with Food and Drug Administration-approved drugs (on-label and/or off-label (N=54) or with experimental drugs in clinical trials (additional five patients)) (University of California San Diego Molecular Tumor Board assessment): 45, by OncoKB (http://oncokb.org/#/. The tissue and blood concordance rates for common specific alterations ranged from 62.3% to 86.9% (median=5 months between tests). In serial samples from patients on anti-EGFR therapy, multiple emerging alterations in genes known to be involved in therapeutic resistance, including *KRAS*, *NRAS*, *BRAF, EGFR*, *ERBB2,* and *MET* were detected. In conclusion, over 80% of patients with stage IV colorectal cancer had detectable ctDNA, and the majority had potentially actionable alterations. Concordance between tissue and blood was between 62% and 87%, despite a median of five months between tests. Resistance alterations emerged on anti-EGFR therapy. Therefore, biopsy-free, non-invasive ctDNA analysis provides data relevant to the clinical setting. Importantly,

sequential ctDNA analysis detects patterns of emerging resistance allowing for precision planning of future therapy.

INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous disease that develops as a consequence of different combinations of epigenetic and genetic alterations, with significant variability observed in individual patient prognosis and therapy response, perhaps due to molecular heterogeneity. Advances in the treatment of patients with metastatic CRC have led to an improvement in patient survival partly due to the use of biologic agents targeting the EGFR signaling pathway or tumor angiogenesis (1-5). Additionally, certain biomarkers with prognostic or predictive value have been identified. For example, activating mutations of *KRAS* and *NRAS* can predict a lack of response to anti-EGFR therapy. These mutations frequently co-exist with alterations in the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway encoding genes (6). Hyper-mutated CRC tumors have been found to be excellent targets for programmed death 1 (PD-1) inhibitors (7). Finally, *BRAF* mutation is a known poor prognostic feature (1, 8, 9).

Further understanding of underlying genomic alterations in CRC has been made possible by recent improvements in DNA sequencing technology. The Cancer Genome Atlas network conducted a comprehensive genome-wide analysis of somatic mutations in colorectal tumors. The most commonly altered genes were as follows in non-hyper-mutated CRCs: *APC* (81%), *TP53* (60%), *KRAS* (43%), and *PIK3CA* (18%). *BRAF* mutations were frequently associated with hyper-mutated CRCs (10). In addition, understanding of heterogenous genetic makeup of CRC has led to a consensus on molecular subtypes used to classify CRC (11).

The development of next-generation sequencing (NGS) platforms and their implementation into clinical use allows for rapid clinical-grade genomic analysis to identify actionable genomic alterations, and thus has led to the use of targeted therapies matched to patients' specific alterations (12-15). Until recently, genomic sequencing was typically performed on archival tumor tissue. However, acquisition of tumor tissue is not always feasible, and a tissue biopsy at a single time point may not, due to tumor heterogeneity, provide a recent or complete picture of the molecular background of tumor evolution, response, and resistance (16, 17). One strategy to overcome these challenges is to investigate circulating biomarkers. Circulating tumor DNA (ctDNA) is shed into bloodstream from cancer cells and can be isolated from blood (also known as a "liquid biopsy") (18-20). More recently, the technology has rapidly advanced, and evaluating multiple genes by performing NGS on ctDNA has proven useful when applied in the clinic (21-26).

Herein, we describe the results of ctDNA testing in 78 patients with CRC whose blood-derived ctDNA was interrogated by a targeted NGS panel. Type, distribution, and frequency of genomic alterations, potential actionability, concordance with tissue testing, and emerging resistance alterations detected in ctDNA after anti-EGFR-based therapy (including an illustrative case) are described.

MATERIALS AND METHODS

Patients

We investigated genomic alterations and clinic-pathologic data from electronic medical records in 78 consecutive patients with CRC followed at the University of California San Diego (UCSD) Moores Cancer Center, for whom ctDNA testing was performed on their blood samples between December 2014 and November 2016. This was a retrospective analysis of all eligible patients with available data during the time period. A total of 1005 eligible patients with various cancers had ctDNA analysis during this time period.

Next generation sequencing

ctDNA analyses: Digital sequencing was performed by clinical laboratory improvement amendments (CLIA)-licensed and College of American Pathologist (CAP)-accredited laboratory (Guardant Health, Inc., http://www.guardanthealth.com/). ctDNA was extracted from whole blood collected in 10mL Streck tubes, and 5ng-30ng of ctDNA was prepared for sequencing as previously described (23). The fractional concentration or variant allele fraction for a given somatic mutation is calculated as the fraction of ctDNA harboring that mutation in a background of wild-type ctDNA fragments at the same nucleotide position. Germline alterations are filtered out and not reported. This ctDNA assay has high sensitivity (detects 85%+ of the single nucleotide variants (SNVs) detected in tissue in advanced cancer patients) and specificity (> 99.9999%) (23).

Throughout the timeframe of this study, the ctDNA assay expanded gene panels from 54 to 73 genes (**Supplemental Table 1**). There were 104 blood samples collected from a total of 78 patients in this study (17 patients had multiple blood samples assayed): four samples were tested with the 54 gene panel; 44 with the 68 gene panel; 55 with the 70 gene panel; and one with the 73 gene panel. The assay reports SNVs in all genes and selects fusions, copy number variants (CNVs), and indels (**Supplemental Table 1**). Degree of CNVs were reported as follows: 1+, 2.13-2.40, which is the 10^{th} to 50^{th} percentile; 2+, 2.41-4.00, which is >50^{th} to 90^{th} percentile; and 3+, greater than 4.0 copy numbers, which is >90^{th} percentile. For ctDNA, the following information was evaluated; the number of total alterations and the number of characterized alterations. We counted both variants of unknown significance (VUSs) and characterized alterations when we refer to total alterations. Synonymous alterations were not included in any analysis.

Tumor tissue analyses: Tissue testing was performed on formalin-fixed, paraffin-embedded tissue by Foundation Medicine (FoundationOne, http://www.foundationone.com), which is a clinical-grade NGS test (315 genes) (27). For tissue NGS, we included only characterized alterations in our analyses.

Concordance

We investigated the concordance for the 61 patients who had both tests performed (tissue and blood). Concordance between tissue and blood was calculated using a kappa coefficient. We examined specific

concordance rates for the most frequent alterations including *TP53, KRAS, APC, PIK3CA, BRAF and MYC*, and all the genes examined were present in the tissue and ctDNA panels.

Definition of actionability

An actionable alteration was defined by UCSD Molecular Tumor Board as a genomic alteration that produces a protein product serving as either the direct target or as part of a signaling pathway that could be impacted by drugs. Drug impact could be impact or via differential expression on tumor versus normal cells. Drugs might be available for on-label (Food and Drug Administration (FDA)-approved for CRC) and/or off-label use (FDA-approved for an indication other than CRC) or in experimental clinical trials. Protein products of genes were considered actionable by small molecule inhibitors if the compound impacted the protein at low 50% inhibitory concentrations. Genes that produced proteins that were recognized as the main target of an antibody were also considered actionable. We also utilized OncoKB (28) as an additional source to define actionability.

Statistical Design

The study was performed under an IRB-approved registry type study (PREDICT). PREDICT has both prospective and retrospective components but, in this case, data was gathered retrospectively. This study was performed in accordance with the UCSD Moores Cancer Center Institutional Review Board guidelines for NCT02478931 and for any

investigational therapies to which the patients consented.

RESULTS

Patient characteristics

The median age at first blood draw was 52 years (range, 27 to 82 years); 40 patients (51.3%) were men (**Table 1**). All patients had a diagnosis of colorectal adenocarcinoma. Overall, 53 individuals (67.9%) had low-grade tumor; 11 (14.1%), high-grade tumor; and six had mucinous adenocarcinoma. Regarding location, 60 patients had tumors involving the colon (21 right colon; 36 left colon; 3 transverse colon), and 18 involved the rectum. Seventy-seven patients had stage IV disease at the time of first blood draw; one, stage III disease.

Genomic alterations among colorectal cancer; ctDNA results and percentage

The total number of genomic alterations were 310 and, of these, 214 (69.0%) were characterized alterations, including substitutions (N=148), amplifications (N=64), and indels (N=2). No fusions were observed. Ninety-six alterations (31.0%) were VUSs (**Table 1**). Of the 214 characterized alterations, 34 were genomically distinct, and 119 were molecularly distinct alterations (e.g. *TP53* R248W and *TP53* R248Q were considered genomically identical but molecularly distinct) (**Supplemental Tables 2 and 3**).

Among 78 patients, 63 patients (80.8%) had ctDNA alterations detected with 59 (75.6%) having ≥1 characterized alteration and 48 (61.5%) having ≥2 characterized alterations. The median number of alterations per patient was 3 (range, 0-26); median number of characterized alterations, 2 (range, 0-13) (**Table 1**).

Focusing on characterized alterations, the most frequently altered gene was *TP53* (52.6% [41/78]) followed by *KRAS* (35.9% [28/78]), *APC* (28.2% [22/78]), *EGFR* (16.7% [13/78]), *BRAF* (15.4% [12/78]), and *PIK3CA* (15.4% [12/78]) (**Figure 1, Panel A and Supplemental Table 2**).

When alterations were grouped depending on the oncogenic pathways, genes involved in the mitogen-activated protein kinase (MAPK) signaling pathway were altered in 59.0% (46/78) of patients; *TP53*-associated genes were altered in 53.8% (42/78); tyrosine kinase families were altered in 37.2% (29/78); the Wnt signaling was altered in 33.3% (26/78); the PI3K signaling was altered in 17.9% (14/78); and cell-cycle associated genes were altered in 14.1% (11/78) (**Table 2).**

Among frequently altered genes, *APC* had the highest median mutant allele ctDNA fraction of 6.6% (range, 0.1–55.5). Most of the other characterized mutations had a median ctDNA fraction of less than 5%. There was no clear association between the frequencies of gene alterations and the fraction of ctDNA detected in the blood (**Figure 1, Panel B**).

Actionable genomic alterations among patients with CRC

We determined actionability based on our Molecular Tumor Board assessments (**Supplemental Table 4)** and by OncoKB (28).

Per our Molecular Tumor Board (UCSD) assessment, of the 214

characterized alterations, 70.1% (150/214) were potentially targetable with FDA-approved drugs as on-label and/or off-label use, and an additional 26.2% (56/214) were theoretically targetable with drugs that are currently in clinical trials. Altogether, 96.3% (206/214) were actionable alterations that were targetable either with FDA-approved drugs or with experimental drugs in clinical trials (**Supplemental Tables 3 and 4**).

Of the 59 patients who harbored ≥1 characterized alteration, all had ≥1 potentially actionable alteration. FDA-approved, on-label therapies were available for 20.5% (16/78) of patients and another 48.7% (38/78) had alterations potentially targetable with FDA-approved drugs in a different indication (off-label use). An additional 6.4% (5/78) of patients had alterations theoretically targetable with experimental drugs in clinical trials (**Supplemental Figure 1, Supplemental Tables 3 and 4)**.

Per OncoKB assessment (http://oncokb.org/#/), of the 214 characterized alterations, 5.1% (11/214) were potentially targetable alterations for colorectal cancer and an additional 34.1% (73/214) were theoretically targetable for cancer types other than colorectal cancer. Altogether, 39.2% (84/214) were actionable alterations that were considered to be targetable alterations (**Supplemental Tables 3**). Of the 59 patients who harbored ≥1 characterized alteration, 45 had ≥1 potentially actionable alterations that were targetable per OncoKB assessment. At least one targetable alteration indicated for colorectal cancers was found in 10.3% (8/78) of patients and another 47.4% (37/78) had alterations that were theoretically targetable for cancer types other than colorectal cancer (**Supplemental Tables 3**).

Concordance of the ctDNA test with tissue NGS test

Of the 78 patients who had ctDNA test, 61 patients also had a tissue genomic test. The time between biopsy and blood draw ranged from 1 day to 6.6 years, with a median of 5.0 months. We examined concordance for the most frequent alterations. The concordance rates were 70.5% for *TP53*, 77.0% for *KRAS*, 62.3% for *APC*, 80.3% for *PIK3CA*, 86.9% for *BRAF*, 83.6% for *MYC* (**Table 3)**. There was no statistically significant difference in concordance rates in patients for whom the time interval between biopsy and blood draw used for testing was ≤6 months (N = 35) versus those for whom the time interval was >6 months (N=26).

Emerging resistance alterations in ctDNA along with therapeutic intervention

We documented that emerging resistance alterations could be detected in ctDNA from patients treated with anti-EGFR antibodies. There were nine patients treated with anti-EGFR-based therapy who also had tissue NGS prior to or soon after the therapy. Tissue NGS test was performed at various time points (range, 19 days to 26.9 months) before the initiation of anti-EGFR-based therapy; one patient had it performed one month after the initiation of the therapy (#12). Pretreatment ctDNA was also available in two patients, and both showed no detectable alterations (#1, #30) (**Table 4**).

In total, we observed 31 alterations that were not detected in the tumor and only appeared in the ctDNA after the therapy was initiated. Of these, we found 15 alterations in genes known to be involved in

therapeutic resistance to anti-EGFR therapy (**Table 4**, #2, #12, #38, #68). The progression-free survival of these four patients were 4.6 months, 12.4 months, 6.7 months, and 2.5+ months, respectively; three patients (#2, #12, #38) experienced disease progression at the time of blood draw after a prior response/stable disease, and one patient (#68) was assessed as having stable disease at the time of blood draw, but lost to follow up thereafter. *KRAS* alterations were observed in two patients (#12, #38); one patient had five molecularly distinct *KRAS* alterations (*KRAS* G12A, *KRAS* G12C, *KRAS* G13D, *KRAS* Q61H, and *KRAS* amplification) and the other had *KRAS* amplification. Two *NRAS* mutations in one patient (#12) were observed; *NRAS* Q61H, *NRAS* Q61K. *BRAF* G469A mutation was observed in one patient (#12). *EGFR* S492R mutation was observed in two patients (#12, #68). Additionally, amplifications in *ERBB2* (N = 3) (#2, #12, #38) and *MET* (N = 1) (#68) were observed.

A representative case (#38) with emerging *KRAS* and *ERBB2* alterations upon progression on anti-EGFR-based therapy is presented (**Figure 2**).

DISCUSSION

We investigated genomic alterations in 78 patients with CRC (77, stage IV) using a targeted NGS assay that analyzed blood-derived ctDNA. Of the total, 63 patients (80.8%) had ≥1 detectable alteration(s), which is consistent with previous reports (23-25, 29). Of 59 patients (75.6%) who harbored at least one characterized alteration, the most frequent alterations were *TP53* mutations (52.6%), followed by alterations in *KRAS* (35.9%), *APC* (28.2%), *EGFR* (16.7%), *BRAF* (15.4%), and *PIK3CA* (15.4%). The mutation frequencies for *KRAS* and *BRAF* are similar to those reported in tissue, which generally show that about 35% to 45% of patients have a *KRAS* mutation (30, 31) and about 10% of patients with CRC have a *BRAF* alteration (32). Examining ctDNA, Thierry *et al* (33) noted a higher rate of *KRAS* mutations in ctDNA (~59%), while our rate of 35.9% was more consistent with the lower range found in tissue studies. Differences between studies can be attributable to different sample sources (tissue versus ctDNA in blood), relatively small sample size, technical differences such as those in depth of sequencing, and other factors relating to the phenotype of patients tested.

EGFR mutations were detected in three patients (3.8%) (two, S492R and one, G465R), and *BRAF* mutations in four patients (5.1%) (three, V600E and one, G469A). Several previous studies have shown that resistant *EGFR* ectodomain mutations (S492R, G465R) emerge in patients treated with anti-EGFR therapy (34-36). In our study, all three patients who harbored *EGFR* mutations in ctDNA were previously treated with cetuximab-based regimens, and available pretreatment tumor specimens from two patients confirmed that the *EGFR* S492R mutation was not

present before cetuximab treatment (**Table 4**). Finding this mutation in ctDNA has particular clinical relevance because patients with EGFR S492R mutation appearing under the pressure of cetuximab therapy may still respond to panitumumab (35).

We also examined the concordance in the patients who had both a tissue and ctDNA test. Of interest in this regard, Khan *et al*, (37) have shown that a significant proportion of patients with colorectal cancers defined as *RAS* wild-type based on tissue evaluation harbor alterations in the RAS pathway in pretreatment cfDNA and do not benefit from EGFR inhibitors. In our study, the concordance rates for common specific alterations were in the range of 62.3-86.9% with a median of five months between tests (**Table 3**). Testing of ctDNA therefore demonstrated acceptable concordance with NGS testing of tumor tissue obtained during clinical practice in this study. Previous studies have shown that concordance decreases with the temporal separation between tissue and ctDNA tests (25), but we did not observe such a difference, perhaps because of the limited number of patients in the study. It is also unclear why our results showed a lower concordance for *APC* compared to other genes (62.3% versus 70.5%–86.9%); this may reflect a lower rate of *APC* mutation (28.2%) in our ctDNA versus 67.2% (41/61) in tissue, perhaps suggesting that *APC*-bearing ctDNA is less likely to shed into the circulation. In one study of mutation analysis in ctDNA using a targeted NGS panel in a variety of metastatic solid tumor patients including CRC, the concordance rate for all mutations found across 54 genes was 85.9% between tissue and ctDNA (38). In another study of 42 patients with advanced-stage non-small cell lung cancer, concordance between tissue

and plasma was 76% when concordance for mutations in *EGFR, KRAS, PIK3CA,* and *TP53* was measured using a targeted sequencing method (39). In these studies, tissue and blood sampling were performed concurrently, while in our study, the time between biopsy and blood draw ranged from 1 day to 6.6 years (median = 5.0 months). Some discordance between genomic profiles from tissue and ctDNA is generally observed. On one hand, ctDNA tests can theoretically detect shed DNA from multiple tumor sites, while tissue biopsy demonstrates only the alterations found at the specific site of sampling. Recent reports focusing on discordance of the *KRAS* mutational status between primary tumor and paired metastasis showed discordance rates of 3.6%–17.5% (40). On the other hand, in cancers with small tumor burden, mutations identified in tissue may not be detected in ctDNA test due to the low content of ctDNA. Additionally, emergence of resistance alterations can occur under the pressure of therapy during the elapsed interval between each test (41-43); at the same time, targeted therapies may suppress ctDNA (44). Of note, we observed that ctDNA samples harbor unique CNVs that were absent from tissues. For instance, amplifications in *BRAF* (N=6), *KRAS* (N=3), *PIK3CA* (N=3), *MET* (N=2), *FGFR1* (N=2), and *FGFR2* (N=2) were only detected in ctDNA (not in tissue in the 61 patients who had both tested), which, at least theoretically, may be attributed to the inadequate representation of tumor heterogeneity by tissue testing. In contrast, *APC* mutations were more frequently detected in tissue than in ctDNA (67.2% versus 28.2%). Thus, complementary assessment of both tissue and ctDNA appears advantageous to assess dynamic tumor profiles.

Regarding actionability to guide therapeutic decisions, per the UCSD

Molecular Tumor Board assessment, 69.2% of patients had at least one actionable alteration that could be impacted by an FDA-approved drug, and an additional 6.4% had alterations targetable with experimental drugs in clinical trials. Genes involved in the MAPK pathway, *TP53*-associated genes, tyrosine kinase families and Wnt signaling were frequently altered (33.3-59.0%). Although less frequent, alterations in PI3K and cell cycle machinery were also observed (14.1-17.9%). Per OncoKB (28), the percent of patients with at least one actionable alteration was 57.7% (45/78). Recent actionability studies performed on ctDNA in various solid tumor patients reported that 71-81.5% of patients had at least one clinically actionable alteration (24, 25), which is consistent with our results. Accumulating evidence suggests that biomarker-based treatment approach may be able to improve clinical outcome (12-15). Clinical utility of this approach in genomically-matched patient populations continues to be investigated in large prospective clinical trials.

Lastly, analysis of ctDNA in serially collected plasma samples allowed detection of emerging mutant ctDNA, which can be used to monitor patients for disease progression as well as to find mechanisms of resistance. In our patients treated with anti-EGFR agents, we could detect multiple emerging alterations in genes known to be involved in therapeutic resistance to anti-EGFR therapy - *KRAS*, *NRAS*, *BRAF,* and *EGFR* mutations, and amplification of *KRAS*, *ERBB2,* and *MET* - consistent with the implication of many of these genes in both primary as well as acquired resistance to anti-EGFR therapy (29, 34-36, 42, 43, 45-49) (see illustrative case, **Figure 2**). A limitation of our study is that we did not administer therapy that would impact resistant alterations; future studies

should address this issue in order to validate the role of these alterations in resistance.

The study has additional limitations. Importantly, understanding the underlying mechanisms for response and resistance cannot be fully elucidated in the clinic and further bench-side investigations are needed. The median age of patients was 52, which is young for colorectal cancer, and might reflect a selection bias because of the referral pattern to a tertiary care center or because of physician discretion in ordering ctDNA testing. Another limitation relates to whether individual mutations are drivers versus passengers (50). Regarding treatment, some patients who received EGFR inhibitors in the current study were also given chemotherapy. This causes some problems in interpretation of genomic findings, although the presence of *RAS* mutations is highly suggestive of an independent effect of anti-EGFR antibodies and clonal expansion under their selective pressure. Finally, the timing of sample acquisition varied as NGS was performed based on physician discretion. Future studies would benefit from analysis at prospective, pre-defined time-points.

To conclude, over 80% of patients with advanced CRC had detectable ctDNA alterations and more than half (76% [59/78]) per UCSD Molecular Tumor Board and 57.7% (45/78) by OncoKB (28) had potentially actionable alterations. Concordance between tissue and blood for common specific alterations was acceptable. Additionally, we could detect emerging resistance alterations in ctDNA from patients treated with anti-EGFR-based therapy. These data suggest that biopsy-free, non-invasive ctDNA analysis is becoming a valuable option when an invasive tissue

biopsy is not feasible, and ctDNA can yield promising candidate biomarkers for the detection and monitoring of CRC.

Author contributions:
Conception/Design: Choi, Kurzrock
Provision of study material or patients: Choi, Kurzrock
Collection and/or assembly of data: Choi, Kato, Fanta
Data analysis and interpretation: Choi, Kato, Kurzrock
Manuscript writing: All authors
Final approval of manuscript: All authors

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Table 1. Patient characteristics and genomic alterations in patients with colorectal cancer
(N = 78)

Patient characteristic (N=78)	
Age at time of blood draw, median (range), year	52 (27-82)
Women (N (%)).	38 (48.7%)
Men (N (%))	40 (51.3%)
Race (N (%)) Caucacian Hispanic Asian Black Other	 52 (66.7%) 13 (16.7%) 7 (9.0%) 4 (5.1%) 2 (2.6%)
Tumor grade (N (%)) Well/moderately differentiated Poorly differentiated/ undifferentiated Mucinous adenocarcinoma Not available	 53 (67.9%) 11 (14.1%) 6 (7.7%) 8 (10.3%)
Location of tumor Right colon Left colon Transverse colon Rectum	 21 36 3 18
Stage at the time of first blood draw Stage III Stage IV (N, %) Recurrent Initially metastatic	 1 77 31 (40.3%) 46 (59.7%)
Genomic alterations	
Number of alterations*	310
Median number of alterations per patient (range) (includes characterized alterations and VUSs)	3 (0-26)
Number of characterized alterations	214 (69.0%)
Median number of characterized alterations per patient (range)	2 (0-13)
Substitution, No. (%)	148 (47.7%)
Amplification, No. (%)	64 (20.6%)
Fusion, No. (%)	0 (0%)
Indel, No. (%)	2 (0.6%)
Variants of unknown significance, No. (%)	96 (31.0%)

* Includes both characterized alterations and variants of unknown significance (VUSs)

Abbreviations: NA = not available

Table 2. Selected actionable genomic alterations and examples of possible targeted therapies (N = 78)

Genomic alteration	**No. (%)**	**Example of possible targeted therapies***
Tyrosine kinase families (N= 29, 37.2%)		
EGFR substitutions/amplification	13 (16.7%)	Afatinib, cetuximab, erlotinib
ERBB2 substitutions/amplification	6 (7.7%)	Afatinib, trastuzumab, lapatinib
FGFR1 amplification	3 (3.8%)	Lenvatinib
FGFR2 amplification	3 (3.8%)	
MET amplification	4 (5.1%)	Cabozantinib, crizotinib
MAPK signaling (N= 46, 59.0%)		
KRAS substitution/amplification	28 (35.9%)	MEK inhibitor (e.g. Trametinib or cobimetinib)
NRAS substitution	2 (2.6%)	
NF1 substitution	1 (1.3 %)	
GNAS substitution	2 (2.6%)	
RAF1 amplification	1 (1.3%)	
BRAF substitution/amplification	12 (15.4%)	BRAF inhibitor (e.g. Dabrafenib, vemurafenib), MEK inhibitor (e.g. trametinib or cobimetinib)
PI3K signaling (N= 14, 17.9%)		
PIK3CA substitution/amplification	12 (15.4%)	mTOR inhibitor (e.g. everolimus, temsirolimus)
PTEN substitution	1 (1.3%)	
AKT1 substitution	1 (1.3%)	
Cell cycle associated genes (N=11, 14.1%)		
CDKN2A substitution	2 (2.6%)	Cyclin-dependent kinase inhibitor (e.g. Palbociclib)
CCND1 amplification	1 (1.3%)	
CCND2 amplification	2 (2.6%)	
CDK4 amplification	1 (1.3%)	
CDK6 amplification	4 (5.1%)	
CCNE1 amplification	1 (1.3%)	Proteasome inhibitor (e.g. Bortezomib)
***TP53* associated genes (N= 42, 53.8%)**		
TP53 substitution	41 (52.6%)	Anti-VEGF (e.g. bevacizumab), WEE1 inhibitor (e.g. AZ1775, NCT01748825)
ATM substitution	1 (1.3%)	PARP inhibitor (e.g. olaparib)
Wnt signaling (N= 26, 33.3%)		
APC substitution/indel	22 (28.2%)	COX-2 inhibitor (e.g. celecoxib)
CTNNB1 substitution	1 (1.3%)	Sorafenib, sulindac, and gamma-secretase inhibitor
ARID1A substitution	1 (1.3%)	EZH2 inhibitor (e.g. EPZ-6438 *, NCT01897571)
FBXW7 substitution	2 (2.6%)	mTOR inhibitor (e.g. everolimus, temsirolimus)

*See **Supplemental Table 4** for the rationale of possible targeted therapies.

Table 3. Concordance for specific alterations in patients with colorectal cancer (N=61)

Specific alterations	*TP53* positive in tumor	*TP53* negative in tumor	Total
TP53 positive in ctDNA	32	1	33
TP53 negative in ctDNA	17	11	28
Total	49	12	61
Concordance for *TP53*	43/61 (70.5%); Kappa 0.38 (SE 0.12)		
	KRAS positive in tumor	*KRAS* negative in tumor	Total
KRAS positive in ctDNA	21	2	23
KRAS negative in ctDNA	12	26	38
Total	33	28	61
Concordance for *KRAS*	47/61 (77.0%); Kappa 0.55 (SE 0.11)		
	APC positive in tumor	*APC* negative in tumor	Total
APC positive in ctDNA	18	0	18
APC negative in ctDNA	23	20	43
Total	41	20	61
Concordance for *APC*	38/61 (62.3%); Kappa 0.34 (SE 0.11)		
	PIK3CA positive in tumor	*PIK3CA* negative in tumor	Total
PIK3CA positive in ctDNA	3	4	7
PIK3CA negative in ctDNA	8	46	54
Total	11	50	61
Concordance for *PIK3CA*	49/61 (80.3%); Kappa 0.22 (SE 0.20)		
	BRAF positive in tumor	*BRAF* negative in tumor	Total
BRAF positive in ctDNA	2	6	8
BRAF negative in ctDNA	2	51	53
Total	4	57	61
Concordance for *BRAF*	53/61 (86.9%); Kappa 0.27 (SE 0.24)		
	MYC positive in tumor	*MYC* negative in tumor	Total
MYC positive in ctDNA	2	5	7
MYC negative in ctDNA	5	49	54
Total	7	54	61
Concordance for *MYC*	51/61 (83.6%); Kappa 0.19 (SE 0.23)		
	NRAS positive in tumor	*NRAS* negative in tumor	Total
NRAS positive in ctDNA	1	1	2
NRAS negative in ctDNA	2	57	59
Total	3	58	61
Concordance for *NRAS*	58/61 (95.1%); Kappa 0.38 (SE 0.35)		

* Only alterations with ≥ 10 patients with the anomaly are represented (except for *NRAS* which was added because of its clinical usefulness for selecting anti-EGFR therapy). All the genes examined (*TP53, KRAS, APC, PIK3CA, BRAF, MYC*

and *NRAS*) were present in the tissue and ctDNA panels.

Table 4: Analysis of resistant markers from anti-EGFR-based therapy with NGS (tissue and/or liquid) (N = 9)

Case ID	Genomic alterations before anti-EGFR -based therapy (days before the initiation of anti-EGFR-based therapy)	Regimen of anti-EGFR-based therapy and clinical outcome	Alterations after anti-EGFR-based therapy (days after the initiation of anti-EGFR-based therapy)		Comment
#1	**ctDNA** (-30 days) None detected **Tissue NGS** (-19 days) *CCND1* amplification *EGFR* amplification *ERBB2* amplification *CDK6* amplification *FGF19* amplification *FGF4* amplification *MYC* amplification *TOP2A* amplification *FGF3* amplification *MUTYH* Y165C *TP53* splice site 672+1G>T	FOLFIRI plus panitumumab PFS: 109 days (3.6 months) Best response = PD with new metastases Taken off due to progression.	**ctDNA** (167 days) *CCND1* ampllification *CDK6* amplification *EGFR* amplification *ERBB2* amplification *MYC* amplification *PIK3CA* amplification		PFS short in patient with baseline *ERBB2* and *EGFR*tissue amplifications. ctDNA obtained after progressing on anti-EGFR-based therapy showed new *PIK3CA* alteration.
#2	**Tissue NGS** (-565 days) TP53 Y163C APC A571fs*18, S1421fs*52	Irinotecan plus cetuximab PFS: 139 days (4.6 months) Best response = SD (+18%) Taken off due to progression.	**Tissue NGS** (144 days) *ERBB2* amplification – equivocal *MYC* amplification – equivocal *TP53* Y163C APC A571fs*18, S1421fs*52 *MYST3* amplification – equivocal	**ctDNA** (194 days) *ERBB2* amplification *CDKN2A* G35W (0.1%) *EGFR* amplification *TP53* Q317K (0.2%) *TP53* Y163C (75.0%)	After progressing on anti-EGFR-based therapy, both tissue and ctDNA revealed emerging *ERBB2* amplification. ctDNA also revealed new *EGFR* amplification.
#12	Not tested	FOLFIRI plus cetuximab PFS: 378 days (12.4 months)	**Tissue NGS** (30 days) *APC* Y935fs*1 *TP53* R282W	**ctDNA** (374 days) *APC* Y935* (3.11%) *PIK3CA* amplification	While patient was responding to the anti-EGFR-based therapy, tissue NGS revealed only

		Best response = PR (-58%) Patient initially had response, but taken off due to progression		*BRAF* G469A (0.18%) *EGFR* amplification *EGFR* S492R (2.68%) *ERBB2* amplification *KRAS* amplification *KRAS* G12A (2.15%) *KRAS* G12C (0.24%) *KRAS* G13D (0.25%) *KRAS* Q61H (8.48%) *NRAS* Q61H (5.02%) *NRAS* Q61K (0.50%)	*TP53* and *APC* alterations. On progression, multiple new alterations appeared including *EGFR/ERBB2/ KRAS/PIK3CA* amplifications as well as *EGFR* S492R, *BRAF* G469A, and activating *KRAS* mutations.
#30	**ctDNA** (-94 days) None detected **Tissue NGS** (-82 days) *AKT1* E17K *BRAF* V600E *CDKN2A* p16INK4a R80* and p14ARF P94L *TP53* R156P *APC* T1556fs*3 *TERT* promoter -124C>T	Irinotecan plus cetuximab PFS: 5.8 months (175 days) Best response = SD (+14%) Off treatment due to progression	**ctDNA** (169 days) *AKT1* E17K (2.0%) *BRAF* V600E (2.8%) *CDKN2A* R80*(1.0%)		Tissue NGS prior to anti-EGFR-based therapy showed alterations in *AKT1*, *BRAF* and *CDKN2A*. These alterations were not apparent in ctDNA. However, upon progression on anti-EGFR-based therapy, *AKT1*, *BRAF* and *CDKN2A* alterations were seen in ctDNA.
#31	**Tissue NGS** (-806 days) *TP53* G245S *APC* S1400fs*1 *MUTYH* G382D	Capecitabine plus cetuximab, followed by irinotecan plus cetuximab PFS: 4.1 months (126 days) Best response = SD (0%) Off treatment due to progression	**ctDNA** (589 days) *TP53* G245S (6.2%)		Tissue NGS and ctDNA analysis did not reveal alterations associated with resistance to anti-EGFR treatment. However, genomic analysis was done > 500 days after cessation of anti-EGFR-based therapy.
#36	**Tissue NGS** (-127 days) *FANCA* splice site 1006+1G>A *FBXW7* R367* *TP53* P152fs*18, R158H *APC* H1349fs*5, R213* *NOTCH1*	FOLFOX plus panitumumab PFS: 1.4 months (42 days) Best response = SD (0%), however, had clinical progression	**ctDNA** (174 days) *APC* R213* (11.1%) *TP53* R158H (11.8%)		

	F930fs*254	Off treatment due to progression.		
#38	**Tissue NGS** (-331 days) *POLE* R446Q *TP53* G266V *APC* E1306* *SPEN* splice site 1750-2 1750-1 ins A *SPTA1* R891*	FOLFOX plus cetuximab PFS: 6.7 months (202 days) Best response = PR (-31%) Off treatment due to mixed response (Lymph node metastases were responding, however, progression seen in adrenal metastasis).	**ctDNA** (232 days) *APC* E1306* (15.0%) *APC* G471* (10.5%) *BRCA2* S1064* (0.7%) *ERBB2* amplification *KRAS* amplification *TP53* G266V (26.5%)	ctDNA on progression from anti-EGFR-based therapy showed new *ERBB2* and *KRAS* alterations.
#67	**Tissue NGS** (-88 days) *BRAF* V600E	FOLFOX plus panitumumab PFS: 1.4 months (43 days) Best response = PD with new bone metastases. Off treatment due to progression.	**ctDNA** (365 days) *BRAF* V600E (1.6%) *SMAD4* R361C (0.8%) *TP53* R249W (1.3%)	Baseline tissue NGS and follow up ctDNA showed persistent *BRAF* V600E mutation which could explain the resistance to anti-EGFR-based therapy.
#68	**Tissue NGS** (-345 days) *CTNNB1* splice site 14-1_118del106 *TP53* R175H	Single agent cetuximab PFS: 2.5+ months (75+ days) CT on 7/1/2016 with stable disease. Lost to follow up.	**ctDNA** (75 days) *BRAF* amplification *CCNE1* amplification *EGFR* S492R (0.7%) *EGFR* amplification *FGFR2* amplification *MET* amplification *PIK3CA* amplification *RHOA* Y42S (2.3%) *TP53* R175H (42.5%)	While patient was on anti-EGFR therapy, ctDNA showed emerging alterations including *BRAF/EGFR/FGFR2/MET/PIK3CA* amplifications as well as resistant *EGFR* S492R mutation.

ctDNA = circulating tumor DNA, FOLFIRI = folinic acid, fluorouracil, irinotecan, FOLFOX = folinic acid, fluorouracil, oxaliplatin, NGS = next-generation sequencing, PD = progressive disease, PFS = progression-free survival, PR = partial response, SD = stable disease.

FIGURE LEGEND

Figure 1, Panel A. Frequency of genomic alterations among patients with colorectal cancer (N = 78)

Includes alterations with > 5% frequency.
* Multiple alterations indicate that the patient had > 1 type of alteration in the same gene (substitutions, amplifications, VUS etc).
Abbreviations: VUS = variant of unknown significance

Figure 1, Panel B. Percent ctDNA among frequently altered genes in patients with colorectal cancer

Depicted are seven frequently altered genes (not including amplification). The median of % ctDNA with standard error is depicted. Presented here are characterized alterations that were detectable.

Figure 2. *KRAS* wild-type colon cancer patient treated with anti-EGFR-based therapy. ctDNA analysis at progression with multiple emerging resistance alterations.

49-year-old woman with metastatic colon cancer. Tissue NGS at diagnosis showed alterations including *TP53* and *APC* (**Table 4**, case ID #38). After progressing on FOLFIRI plus bevacizumab, patient was started on FOLFOX plus cetuximab. Patient initially had response in left adrenal and retroperitoneal lymph node metastases. However, after 6.7 months of therapy, left adrenal node was progressing, thus patient was taken off from therapy. Post-progression ctDNA showed persistent *TP53* and *APC* alterations as well as emerging *KRAS* and *ERBB2* amplifications.

Left: Pre-treatment PET/CT. Upper figure shows left adrenal metastasis. Lower figure shows retroperitoneal lymph node metastases.

Middle: Day 139 from FOLFOX plus cetuximab with response in left adrenal and peritoneal lymph node metastases.

Right: Day 202 from FOLFOX plus cetuximab with worsening left adrenal metastasis.

Figure 1, Panel A. Frequency of genomic alterations among patients with colorectal cancer (N = 78)

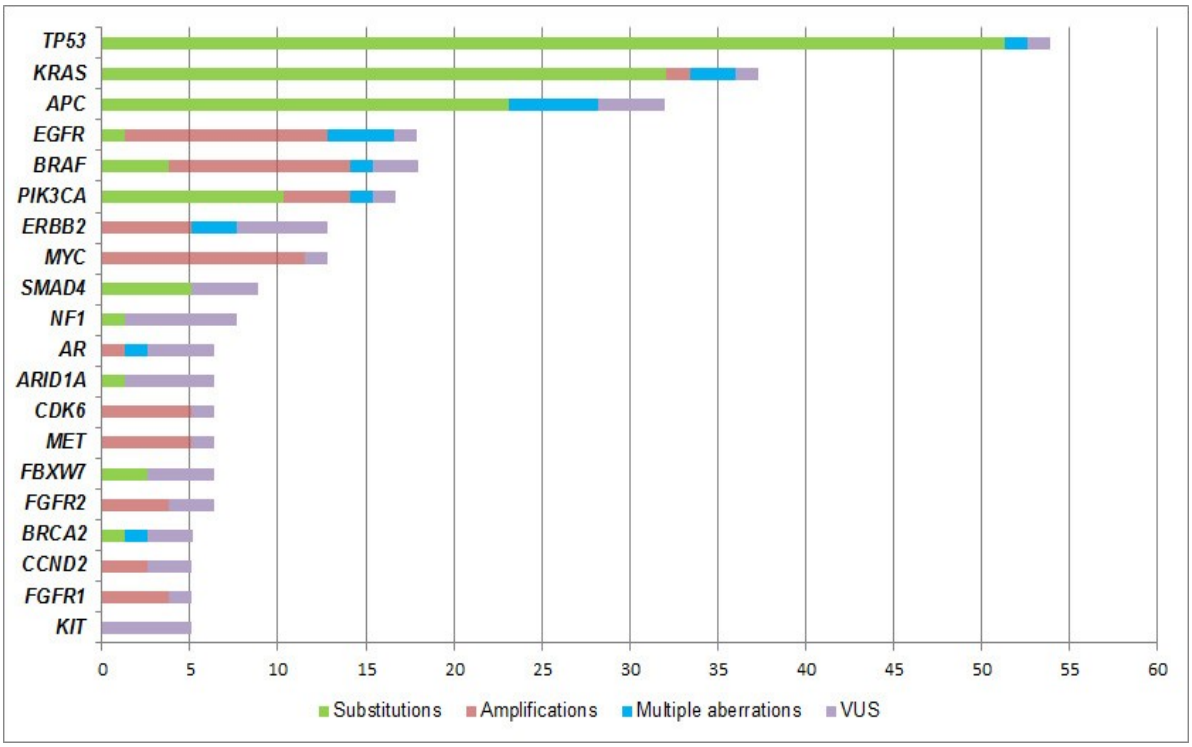


Figure 1, Panel B. Percent ctDNA among frequently altered genes in patients with colorectal cancer

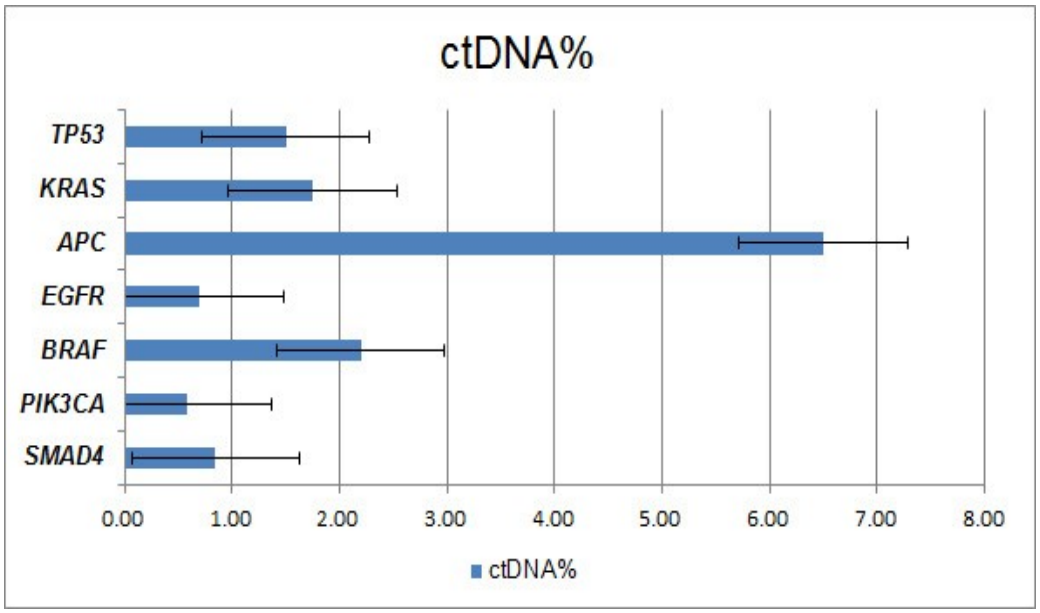
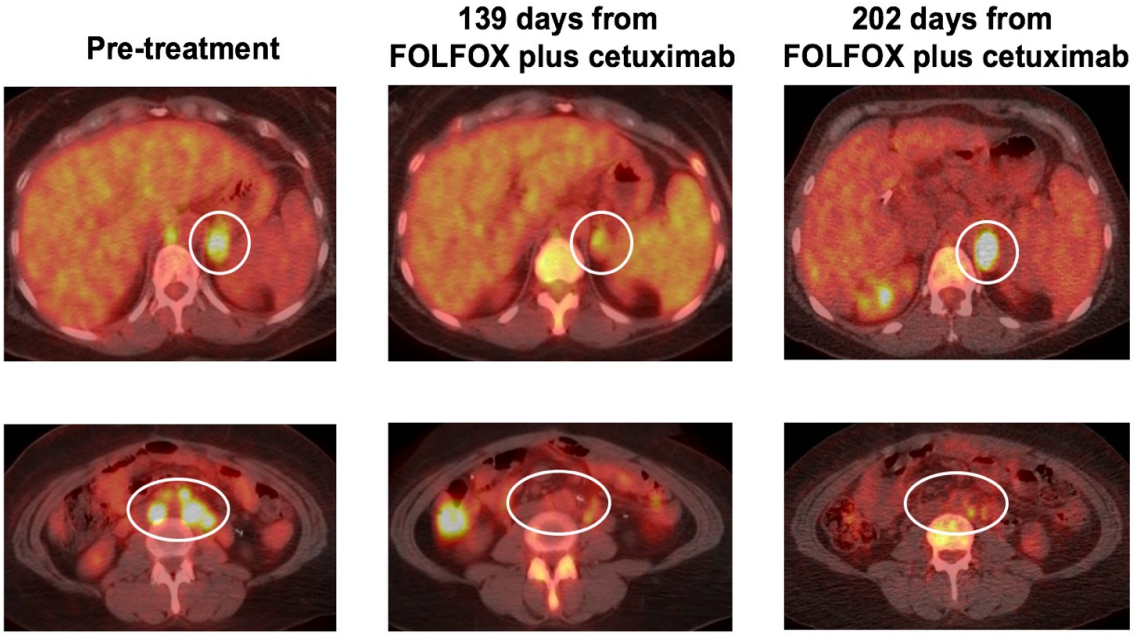


Figure 2. *KRAS* wild-type colon cancer patient treated with anti-EGFR-based therapy. ctDNA analysis at progression with multiple emerging resistance alterations.



Genomic alteration before anti-EGFR-based therapy	Genomic alterations after anti-EGFR-based therapy
Tissue NGS (331 days before FOLFOX plus cetuximab) *POLE* R446Q *TP53* G266V *APC* E1306* *SPEN* splice site 1750-2 1750-1 ins A *SPTA1* R891*	**ctDNA** (232 days after FOLFOX plus cetuximab) *APC* E1306* (15.0%) *APC* G471* (10.5%) *BRCA2* S1064* (0.7%) *ERBB2* amplification *KRAS* amplification *TP53* G266V (26.5%)