

Development and Validation of an Ultradeep Next-Generation Sequencing Assay for Testing of Plasma Cell-Free DNA from Patients with Advanced Cancer



Filip Janku¹, Shile Zhang², Jill Waters², Li Liu², Helen J. Huang¹, Vivek Subbiah¹, David S. Hong¹, Daniel D. Karp¹, Siqing Fu¹, Xuyu Cai², Nishma M. Ramzanali¹, Kiran Madwani¹, Goran Cabrilo¹, Debra L. Andrews¹, Yue Zhao², Milind Javle³, E. Scott Kopetz³, Rajyalakshmi Luthra⁴, Hyunsung J. Kim², Sante Gnerre², Ravi Vijaya Satya², Han-Yu Chuang², Kristina M. Kruglyak², Jonathan Toung², Chen Zhao², Richard Shen², John V. Heymach⁵, Funda Meric-Bernstam¹, Gordon B. Mills⁶, Jian-Bing Fan², and Neeraj S. Salathia²

Abstract

Purpose: Tumor-derived cell-free DNA (cfDNA) in plasma can be used for molecular testing and provide an attractive alternative to tumor tissue. Commonly used PCR-based technologies can test for limited number of alterations at the time. Therefore, novel ultrasensitive technologies capable of testing for a broad spectrum of molecular alterations are needed to further personalized cancer therapy.

Experimental Design: We developed a highly sensitive ultra-deep next-generation sequencing (NGS) assay using reagents from TruSeqNano library preparation and NexteraRapid Capture target enrichment kits to generate plasma cfDNA sequencing libraries for mutational analysis in 61 cancer-related genes using common bioinformatics tools. The results were retrospectively compared with molecular testing of archival primary or metastatic tumor tissue obtained at different points of clinical care.

Results: In a study of 55 patients with advanced cancer, the ultradeep NGS assay detected 82% (complete detection) to 87% (complete and partial detection) of the aberrations identified in discordantly collected corresponding archival tumor tissue. Patients with a low variant allele frequency (VAF) of mutant cfDNA survived longer than those with a high VAF did ($P = 0.018$). In patients undergoing systemic therapy, radiological response was positively associated with changes in cfDNA VAF ($P = 0.02$), and compared with unchanged/increased mutant cfDNA VAF, decreased cfDNA VAF was associated with longer time to treatment failure (TTF; $P = 0.03$).

Conclusions: Ultradeep NGS assay has good sensitivity compared with conventional clinical mutation testing of archival specimens. A high VAF in mutant cfDNA corresponded with shorter survival. Changes in VAF of mutated cfDNA were associated with TTF. *Clin Cancer Res*; 23(18); 5648–56. ©2017 AACR.

¹Department of Investigational Cancer Therapeutics (Phase I Clinical Trials Program), The University of Texas MD Anderson Cancer Center, Houston, Texas. ²Illumina, Inc., San Diego, California. ³Department of Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas. ⁴Molecular Diagnostic Laboratory, The University of Texas MD Anderson Cancer Center, Houston, Texas. ⁵Department of Thoracic/Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas. ⁶Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas.

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

Current address for X. Cai and J.-B. Fan: AnchorDx, Guangzhou, China; current address for H.J. Kim, S. Gnerre, R.V. Satya, and J. Toung, GRAIL, Inc., Menlo Park, California; and current address for Y. Zhao and N.S. Salathia, Molecular Stethoscope Inc., San Diego, California.

Corresponding Author: Filip Janku, Department of Investigational Cancer Therapeutics (Phase I Clinical Trials Program), The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 0455, Houston, TX 77030. Phone: 713-563-0308; Fax: 713-745-8056; E-mail: fjanku@me.com

doi: 10.1158/1078-0432.CCR-17-0291

©2017 American Association for Cancer Research.

Introduction

Cell-free DNA (cfDNA) in plasma is a rich resource for the detection of genomic variants in cancer patients, which can be used as a minimally invasive approach for diagnostic and monitoring purposes (1). As tumor DNA comprises a small fraction of total cfDNA (typically $\leq 1\%$), highly sensitive and accurate techniques are required for cancer mutation detection in cfDNA (1). PCR-based technologies, such as droplet digital PCR (ddPCR), BEAMing (beads, emulsions, amplification, and magnetics) PCR, or other quantitative allele-specific PCR methods can detect a low frequency of molecular aberrations in cfDNA, but these approaches cannot sample many target sites and are hampered by the complexities of determining optimal PCR primer sequences, primer combinations, and amplification conditions (2–5). Next-generation sequencing (NGS)-based assays, which are commonly used for tumor tissue testing, are not sufficiently sensitive for plasma cfDNA profiling owing to low tumor DNA fractions in the circulatory system (1). However, the use of targeted panels that can be sequenced to extremely high depth can overcome this issue. Therefore, we developed an assay that

Translational Relevance

We demonstrated the feasibility of performing mutational analysis for 61 cancer-related genes using highly sensitive ultra-deep next-generation sequencing of plasma cell-free DNA from patients with advanced cancers using commonly available consumables and a simplified workflow, which can facilitate adoption of the assays in laboratories with next-generation sequencing expertise. We also demonstrate that our approach allows for robust somatic variant detection, which compares well with standard methods of clinical molecular profiling of archival tumor tissue. Our data suggest that the amount of mutated cell-free DNA is a prognostic biomarker for patient survival and that levels of mutated cell-free DNA are correlated with systemic treatment success.

uses commercially available reagents to detect somatic variants in 61 common cancer-related genes (Supplementary Table S1). The accuracy of this assay was assessed using spike-in experiments of samples with known mutational profiles, and the approach was then applied to a cohort of patients with advanced cancers.

Materials and Methods

Patients

The study enrolled patients with progressing advanced cancers who were referred to Department of Investigational Cancer Therapeutics for experimental therapies from October 2010 to June 2015 and whose tumor mutation status was known from clinical testing of their formalin-fixed, paraffin-embedded (FFPE) specimens. Patients had the option of providing longitudinally collected plasma samples during the course of their therapy. The study was conducted in accordance with MD Anderson's Institutional Review Board guidelines.

Tumor tissue analyses

Archival FFPE specimens of patients' primary and metastatic tumors obtained from routine diagnostic and/or therapeutic procedures were tested for aberrations in common cancer-related genes in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. DNA was extracted from microdissected FFPE tumor sections and analyzed with a PCR-based DNA sequencing method, mass spectrometric detection (MassARRAY, Sequenom), or NGS (Ion Torrent, Thermo Fisher Scientific). We also used the commercially available FoundationOne panel (Foundation Medicine) for molecular aberrations in ≥ 182 cancer-related genes. The lower limit of detection for these technologies is approximately 5% to 10% variant allele frequency (VAF) and is influenced by clonal heterogeneity and the presence of normal tissue.

Plasma collection and cfDNA isolation

Whole blood was collected in ethylenediaminetetraacetic acid-containing tubes and centrifuged and spun twice within 2 hours to yield plasma. For each sample, cfDNA was extracted from 1 to 3 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (cat# 55114, Qiagen). Plasma was incubated with ACL buffer (containing carrier RNA) and Proteinase K for 60 minutes.

Library preparation and NGS

cfDNA yield in the 100–400 base pair range was determined using high-sensitivity DNA reagents (cat# 5067-4626, Agilent Technologies) on a 2100 Bioanalyzer instrument (Agilent Technologies). Libraries were prepared using adapter TruSeq Nano reagents (cat# FC-121-4003, Illumina) with minor changes. No upfront fragmentation of the cfDNA was performed. To minimize DNA sample loss, the bead-based sample cleanup after end-repair reactions was eliminated prior to cfDNA A-tailing. A detailed library preparation protocol and comparison with the standard Illumina TruSeq Nano protocol is provided in Supplementary File S1.

Adapter-ligated cfDNA was PCR amplified, and libraries were enriched for genomic target regions of interest by probe hybridization. For target enrichment of the generated sequencing libraries, 80-mer biotinylated DNA probes tiling 61 genes with relevance to a broad range of cancer types were designed using DesignStudio (Illumina; Supplementary Table S1). cfDNA libraries were enriched for desired genomic content using these biotinylated probes with modifications to the Nextera Rapid Capture Enrichment protocol (cat# FC-140-1007, Illumina). For library enrichment, we used the oligo blocker Capture Target Buffer 3 (cat# 15048799, Illumina) instead of enrichment hybridization buffer. Enrichment wash solution was substituted with enhanced enrichment wash solution (cat# 15065792, kit# FC-144-1000, Illumina).

The analytic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the assay were determined by spiking in DNA of NCI-H1975, a non-small cell lung carcinoma (NSCLC) cell line (ATCC CRL-5908) with 4 to 5 validated mutations covered in our panel (*EGFR* heterozygous c.2369C>T p.T790M, *EGFR* heterozygous c.2573T>G p.L858R, *PIK3CA* heterozygous c.353G>A p.G118D, *TP53* homozygous c.818G>A p.R273H, *NF1* heterozygous c.846G>A) at 0.5% input mass in 30 ng of wild-type NA12878 genomic DNA (Coriell Institute, Camden, NJ). This model therefore represents assay analytic performance in the context of 0.25% and 0.5% VAF (heterozygous and homozygous variants, respectively). DNA was sonicated with an S series sonicator (Covaris) and size-selected using Pippin Prep (Sage Science) to mimic a size of approximately 170 bp. For analytic studies, four independent experiments with technical replicates of each condition were tested in each experiment.

All samples were sequenced on a HiSeq 2500 sequencer (Illumina) in high-throughput mode, typically with 4 to 6 samples sequenced on a single flow cell, using a 100-bp, dual-indexed, paired-end sequencing configuration.

For mutation analysis, Fastq files containing raw sequencing reads were generated using bcl2fastq Conversion Software 2.17.1.13 (Illumina). Fastq files were aligned to a human reference genome (UCSC hg19) using BWA-MEM (v0.7.10-r789) with default parameters. Alignments were converted to BAM format using Samtools (v1.2). Alignment coordinates of raw read alignments were adjusted to include soft-clipped bases. Raw reads that shared identical start and end coordinates were considered to be PCR duplicates and were grouped together for error correction. Grouped reads were aligned to one another, and a consensus sequence was generated by taking the most prevalent base at each position. Consensus base qualities were generated at each position by summing the base qualities that agreed with the consensus and subtracting those that disagreed. Consensus base qualities

were capped at 90. For variant calling, error-corrected consensus sequences were realigned to the hg19 genome using BWA-MEM. Mpileup files were generated using Samtools with the parameters "-Q30 -q 15 -B -A -d10000000." Variant calls were generated using VarScan (v2.3.9) with parameters "-min-var-freq 0.0 -min-reads2 1 -strand-filter 1 -p-value 1 -min-freq-for-hom 1." Variants belonging to dbSNP or ClinVar were annotated using SnpEff (v4.1b).

For gene amplification calling, we used an algorithm to call copy number variants (CNV) in tumor samples. The robustness of the CNV detection algorithm was validated by the identification of amplifications in DNA samples harboring known amplifications using an enrichment-based ligation assay similar to that used in this study. The presence of amplifications in these samples was reconfirmed using ddPCR (personal communication, Matt Friedenber, Oncology, Illumina). In our study, a training dataset was established from sample data from 44 healthy individuals to learn the baseline coverage behavior, and then step-wise normalization was performed on target regions to reduce baseline effect, followed by GC bias removal through loess regression in test samples. The algorithm calculates a fold change for each gene by comparing the median normalized value across its target regions with the genome median. For further stringency in CNV calling, we determined the CNV for gene regions with >15 genomic bins, for which the corrected copy number fold change was >3 standard deviations from the test population's cancer samples.

Statistical analysis

Agreement rates between the mutation analyses of the FFPE specimens and plasma cfDNA were assessed using common genes and their loci queried by both tissue and cfDNA platforms. Overall survival (OS) was defined as the time from the date of baseline blood collection to the date of death or last follow-up. The Royal Marsden Hospital (RMH) score was calculated on the basis of lactate dehydrogenase levels (greater than the upper limit of normal vs. normal), albumin levels (<3.5 g/mL vs. ≥3.5 g/mL), and number of metastatic sites (>2 sites vs. ≤2 sites). Time to treatment failure (TTF) was defined as the time from the date of systemic therapy initiation to the date of removal from the treatment. The Kaplan–Meier method was used to estimate OS and TTF, and a log-rank test was used to compare OS and TTF among patient subgroups. Cox proportional hazards regression models were fit to assess the association between patient characteristics and OS. All tests were two-sided, and P values <0.05 were considered statistically significant. All statistical analyses were performed with the GraphPad (GraphPad Software, Inc.) or SPSS 23 (SPSS) software programs.

Results

Analytic performance

To determine the analytic performance of the assay, we spiked the DNA of the NCI-H1975 cell line, which harbors well-characterized variants, into a wild-type genomic DNA background. After processing these samples using our cfDNA library preparation protocol, we were able to determine the analytic sensitivity, specificity, PPV, and NPV of the assay at different variant sequence read thresholds. A threshold of ≥2 unique variant sequence reads (denoted by nonidentical genome alignment coordinates) yielded an analytic sensitivity of 95% and specificity of >99.99% for known variants at a VAF of 0.25% (Fig. 1). To optimize assay

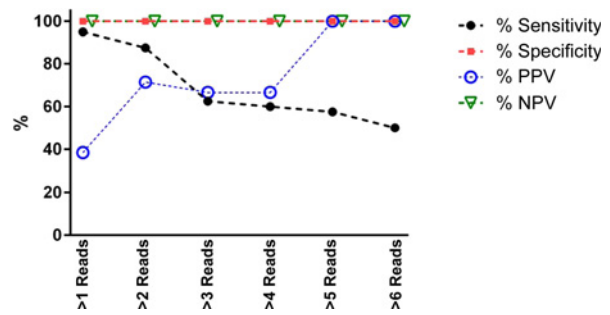


Figure 1. Sensitivity, specificity, PPV, and NPV of the assay. With a requirement of >1 unique sequence reads (denoted by nonidentical genome alignment coordinates), the sensitivity and specificity of the assay were 95% and >99.99%, respectively, for known variants at a variant allele frequency of 0.25%.

sensitivity, we evaluated all clinical samples using a threshold of ≥2 unique variant sequence reads.

Testing of clinical samples of plasma cfDNA

To validate the assay, we tested 165 plasma cfDNA samples collected from 55 patients with advanced cancers who had results from clinical molecular testing of archival tumor tissue (Table 1). The median patient age was 58 years (range, 30–82 years). Most patients were white (n = 50; 91%) and men (n = 30; 55%). The most common tumor types were colorectal cancer (n = 14; 25%), NSCLC (n = 11; 20%), and breast cancer (n = 5; 9%; Table 1). The median time from tissue to plasma sampling was 19.5 months

Table 1. Characteristics of the 55 patients enrolled in the study

Characteristics		Number of patients (%)
Age, years - median (range)	58 (30–82)	
Gender	Male	30 (55)
	Female	25 (45)
Ethnicity	White	50 (91)
	Hispanic	2 (4)
	African-American	2 (4)
	Asian	1 (2)
Cancer type	Colorectal cancer	14 (25)
	NSCLC	11 (20)
	Breast cancer	5 (9)
	Cholangiocarcinoma	4 (7)
	Melanoma	4 (7)
	Thyroid carcinoma	3 (5)
	Pancreatic cancer	3 (5)
	Ovarian cancer	3 (5)
	Appendiceal carcinoma	2 (4)
	Other cancers	6 (11)
Molecular testing of tumor tissue	Ion Torrent NGS 46 genes	13 (24)
	Ion Torrent NGS 50 genes	26 (47)
	Foundation One targeted NGS	10 (18)
	Other NGS	2 (4)
	PCR	3 (5)
	Sequenom MassArray	1 (2)
Molecular aberrations in tumor tissue	Patients with molecular aberration	49 (89)
	Patients without molecular aberration	6 (11)
	Total number of molecular aberrations	92

Downloaded from http://aacrjournals.org/clinccancerres/article-pdf/23/18/5650/1038699/5650.pdf by guest on 14 September 2024

(range, 1.0–114.5 months). For 39 patients (71%), archival tumor tissue was tested using an Ion Torrent targeted NGS assay (Thermo Fisher Scientific Inc.), and for 10 patients (18%), archival tumor tissue was tested using a FoundationOne targeted NGS assay (Foundation Medicine). Among the 55 patients, 49 had 92 molecular aberrations in archival tumor tissue, and 6 had no molecular aberrations in archival tumor tissue (Table 1; Supplementary File S2).

The average amount of cfDNA isolated from 3 mL of plasma was 47.1 ng (range, 3–586 ng). Libraries from cfDNA were sequenced with an average of $97 \pm 54 \times 10^6$ pass-filter reads per sample, yielding a mean coverage of 1,456 unique reads (SE, 74 unique reads) per target site. When requiring ≥ 2 uniquely mapped reads at a locus to detect a variant, this coverage depth allows for the detection of variants down to a VAF of 0.33% with 95% confidence.

To enable robust variant calling from cfDNA sequencing data, we created a normal baseline from 44 healthy individuals. By ascertaining VAF distributions at each target site in these healthy individuals, we were able to retain somatic variants with statistically different VAFs relative to the normal baseline ($P \leq 0.01$). To improve sensitivity, we also removed variants supported by only one uniquely mapped read as well as synonymous variants and those in untranslated regions from further consideration. Furthermore, to exclude germline mutations, we removed variants in the dbSNP database (build 142) that had $\geq 20\%$ minor allele frequency (MAF) from further analyses (6). In this manner, we identified a median of 26 mutations per sample. With the requirement of more uniquely mapped reads to call a variant, there was a significant drop in the number of variants detected per sample, with a median of only three variants per sample supported by at least six uniquely mapped reads (Supplementary Fig. S1).

Among the 55 baseline plasma cfDNA samples, 45 (82%) had molecular profiles identical to those of archival tumor tissue (complete detection), and 3 (5%) had molecular profiles that overlapped but were not identical to those of archival tumor tissue (partial detection), resulting in an aggregate complete and partial detection rate of 87% (Table 2). The sensitivity of plasma cfDNA testing was 80% [95% confidence interval (CI), 0.66–0.90] for

Table 2. Concordance between ultra-deep NGS of plasma cfDNA and clinical molecular testing of archival tumor tissue for the 55 patients enrolled in the study

Type of agreement between plasma cfDNA and tumor tissue	Number of patients (%)
Complete detection	45 (82%)
Partial detection	3 (5%)
Aggregate complete and partial detection	48 (87%)
Complete disagreement	7 (13%)

complete detection and 86% (95% CI, 0.73–0.94) for complete and partial detection. Of the 14 variants not detected in cfDNA, 8 (57%) had unknown therapeutic impact and 6 (43%) had proven or possible therapeutic impact (Table 3).

All reported variants in cfDNA are listed in Supplementary File S2. Some of these variants were not reported in archival tumor tissue, and their presence in plasma cfDNA could represent tumor heterogeneity, DNA release from sites not sampled by biopsy, or clonal evolution of the tumor. Therefore, we identified potentially clinically significant genomic variants in cfDNA that had not been characterized in the baseline tumor tissue in 14 patients (Table 4).

Although the assay was not specifically designed or analytically validated to detect gene amplification, we used it to perform a CNV analysis of the cfDNA samples. Of the 55 patients, 10 (18%) had undergone CLIA-certified laboratory testing for amplifications in tumor tissue. Of these 10 patients, 3 had 4 CNV events, 2 of which were confirmed in cfDNA. Furthermore, 2 patients for whom tissue CNV testing had not been performed had an additional 3 gene amplifications in cfDNA (Supplementary Table S2).

cfDNA and survival

To determine whether the abundance of mutant plasma cfDNA was associated with survival, we first calculated the aggregate cfDNA VAF for all variants from the 55 plasma samples collected at baseline. We then divided the 55 patients into two groups according to the aggregate VAF (VAF $\leq 6\%$ vs. VAF $> 6\%$). These thresholds were selected on the basis of a 5% trimmed mean value of mutant cfDNA and were deemed to be representative to minimize the bias from samples with no mutated cfDNA detected. The median OS duration of the 38 patients with a

Table 3. Partial and complete disagreements

Patient number	Tumor type	Aberration in tissue	Aberration in cfDNA (VAF%)
Partial disagreements			
46	NSCLC	<i>KRAS</i> ^{G12V} <i>TP53</i> ^{R248Q} <i>TP53</i> ^{C238S}	<i>KRAS</i> ^{G12V} (0.18%) Not detected Not detected
86	Breast cancer	<i>ERBB2</i> ^{D769H} <i>IDH2</i> ^{R172K}	<i>ERBB2</i> ^{D769H} (0.34%) Not detected
102	NSCLC	<i>KRAS</i> ^{G12C} <i>NOTCH1</i> ^{L2457V}	<i>KRAS</i> ^{G12C} (0.31%) Not detected
Complete disagreements			
44	NSCLC	<i>KRAS</i> ^{G12D}	Not detected
47	Urothelial carcinoma	<i>TP53</i> ^{E286*}	Not detected
54	Appendiceal carcinoma	<i>KRAS</i> ^{G12D} <i>GNAS</i> ^{R201H}	Not detected Not detected
55	NSCLC	<i>BRAF</i> ^{D594G} <i>TP53</i> ^{H193L}	Not detected Not detected
73	NSCLC	<i>KRAS</i> ^{G12C}	Not detected
80	Breast cancer	<i>PIK3CA</i> ^{H1047R} <i>TP53</i> ^{K120*}	Not detected Not detected
111	Breast cancer	<i>AKT1</i> ^{E17K}	Not detected

NOTE: Alterations with proven or possible therapeutic impact are in bold.

Downloaded from http://aacrjournals.org/clinccancerres/article-pdf/23/18/5648/2038699/5648.pdf by guest on 14 September 2024

Table 4. Potentially clinically significant molecular aberrations in plasma cfDNA not reported in the archival tumor tissue and the clinical context

Patient number	Cancer	Molecular profile in tumor tissue	Molecular profile in plasma cfDNA (VAF)	Possible clinical relevance
24	Colorectal cancer	None	<i>KRAS</i> ^{G12C} (2.54%)	Presence of <i>KRAS</i> ^{G12C} and <i>PTEN</i> ^{R130Q} mutations plausibly explains prior resistance to EGFR antibody
25	Melanoma	<i>BRAF</i> ^{V600E} <i>TP53</i> ^{P278S} <i>TP53</i> ^{R110C}	<i>PTEN</i> ^{R130Q} (0.21%) <i>BRAF</i> ^{V600E} (1.99%) <i>TP53</i> ^{P278S} (1.00%) <i>TP53</i> ^{R110C} (0.59%) <i>IDH1</i> ^{R132H} (0.22%)	Emergence of <i>IDH1</i> ^{R132H} mutation on therapy with BRAF inhibitor
26	Melanoma	<i>BRAF</i> ^{V600E}	<i>KRAS</i> ^{G61L} (26.67%)	Disappearance of <i>BRAF</i> ^{V600E} and emergence of <i>PIK3CA</i> ^{E545K} on therapy with BRAF and MET inhibitors
33	Anaplastic thyroid cancer	<i>BRAF</i> ^{V600E}	<i>PIK3CA</i> ^{E545K} (1.38%) <i>BRAF</i> ^{V600E} (2.45%)	Presence of inactivating mutation <i>BRAF</i> ^{D594G} increases signaling through MAPK pathway and plausibly explains progression to BRAF and MEK inhibitors
38	Colorectal cancer	<i>KRAS</i> ^{G12D}	<i>TP53</i> ^{K132M} (4.48%) <i>BRAF</i> ^{D594G} (0.13%) <i>KRAS</i> ^{G12D} (7.89%)	<i>PIK3CA</i> ^{E545A} mutation offers additional target for cancer therapy selection
51	Colorectal cancer	<i>KRAS</i> ^{G12D}	<i>PIK3CA</i> ^{E545A} (0.66%) <i>KRAS</i> ^{G12D} (42.66%)	Emergence of <i>EGFR</i> ^{L858M} and <i>HRAS</i> ^{Q61H} mutations at progression on panRAF inhibitor
52	Colorectal cancer	None	<i>EGFR</i> ^{L858M} (0.05%) <i>HRAS</i> ^{Q61H} (0.05%) <i>KRAS</i> ^{G12C} (0.53%)	<i>KRAS</i> ^{G12C} mutation offers target for cancer therapy selection (resistance to EGFR antibodies)
73	NSCLC	<i>KRAS</i> ^{G12C}	<i>PIK3CA</i> ^{E545K} (0.18%)	<i>PIK3CA</i> ^{E545K} offers additional target for cancer therapy selection
77	Head and neck squamous cancer	<i>PIK3CA</i> ^{E545K}	<i>PIK3CA</i> ^{E545K} (1.06%) <i>BRAF</i> ^{D594G} (0.11%) <i>KRAS</i> ^{G13V} (0.12%)	<i>BRAF</i> ^{D594G} and <i>KRAS</i> ^{G13V} mutations can plausibly explain resistance to mTOR inhibitor based therapy
86	Breast cancer	<i>ERBB2</i> ^{D769H} <i>IDH2</i> ^{R172K}	<i>ERBB2</i> ^{D769H} (0.03%) <i>KRAS</i> ^{G12D} (0.04%) <i>PIK3CA</i> ^{E545K} (1.21%) <i>PIK3CA</i> ^{E542K} (0.46%) <i>KRAS</i> ^{Q61H} (2.78%)	<i>KRAS</i> ^{G12D} , <i>PIK3CA</i> ^{E545K} and <i>PIK3CA</i> ^{E542K} mutations emerged at progression to ERBB2 and mTOR inhibitors
87	Colorectal cancer	None	<i>KRAS</i> ^{Q61H} (2.78%)	Presence of <i>KRAS</i> ^{Q61H} mutation plausibly explains prior resistance to EGFR antibody
94	Cholangiocarcinoma	<i>TP53</i> ^{C141*}	<i>PIK3CA</i> ^{E542K} (0.39%) <i>IDH1</i> ^{R132L} (0.39%)	<i>PIK3CA</i> ^{E545K} and <i>IDH1</i> ^{R132L} mutations offer additional target for cancer therapy selection
95	NSCLC	None	<i>PIK3CA</i> ^{E545Q} (0.33%)	<i>PIK3CA</i> ^{E545Q} and <i>KRAS</i> ^{G12A} mutations offer additional target for cancer therapy selection
112	NSCLC	<i>BRAF</i> ^{V600E}	<i>KRAS</i> ^{G12A} (1.44%) <i>BRAF</i> ^{V600E} (4.73%) <i>IDH2</i> ^{R172G} (0.12%)	<i>IDH2</i> ^{R172G} mutation emerged at progression to BRAF inhibitor

mutant cfDNA VAF of $\leq 6\%$ (10.4 months; 95% CI, not estimated) was significantly longer than that of 17 patients with a mutated cfDNA VAF of $>6\%$ (5.4 months; 95% CI, 3.0–7.8 months; $P = 0.018$; Fig. 2A).

Next, we analyzed the prognostic impact of mutant cfDNA VAF on OS using a multi covariable analysis that included patients' RMH prognostic scores (RMH score is a prospectively validated tool used to predict the OS of patients with advanced cancers who are referred for early-phase clinical trials; scores of 0 or 1 are associated with longer OS than scores of 2 or 3; ref. 7). As expected, the median OS duration of the 35 patients with an RMH score of 0 or 1 (12.6 months; 95% CI, not estimated) was significantly longer than that of the 20 patients with an RMH score of 2 or 3 (3.8 months; 95% CI, 0.5–7.1 months; $P = 0.008$; Fig. 2B). A multivariable analysis revealed that, compared with an RMH score of 2 or 3, an RMH score of 0 or 1 was associated with longer OS duration (HR, 0.45; 95% CI, 0.22–0.94; $P = 0.033$) and that, compared with a mutated cfDNA MAF of $>6\%$, a mutated cfDNA

MAF of $\leq 6\%$ demonstrated a trend to longer OS (HR, 0.50; 95% CI, 0.24–1.06; $P = 0.07$).

Finally, we analyzed the association between the VAF of mutated cfDNA and OS for patients with favorable RMH scores (0 or 1) and for those with unfavorable RMH scores (2 or 3). Among the 35 patients with favorable RMH scores, the median OS duration of 25 patients with a mutated cfDNA VAF of $\leq 6\%$ (not reached) was significantly longer than that of 10 patients with a mutated cfDNA VAF of $>6\%$ (5.4 months; 95% CI, 3.1–7.7 months; $P = 0.001$; Fig. 2C). Among the 20 patients with unfavorable RMH scores, the median OS duration of 13 patients with a mutated cfDNA VAF of $\leq 6\%$ (5.2 months; 95% CI, 0–10.8 months) was longer than that of 7 patients with a mutated cfDNA VAF of $>6\%$ (3.6 months; 95% CI, 2.7–4.5 months), but this difference was not significant ($P = 0.73$; Fig. 2D). We found no association between mutated cfDNA VAF ($\leq 6\%$ vs. $>6\%$) and tumor burden (≤ 2 metastatic sites vs. >2 metastatic sites as per RMH score; $P = 0.53$).

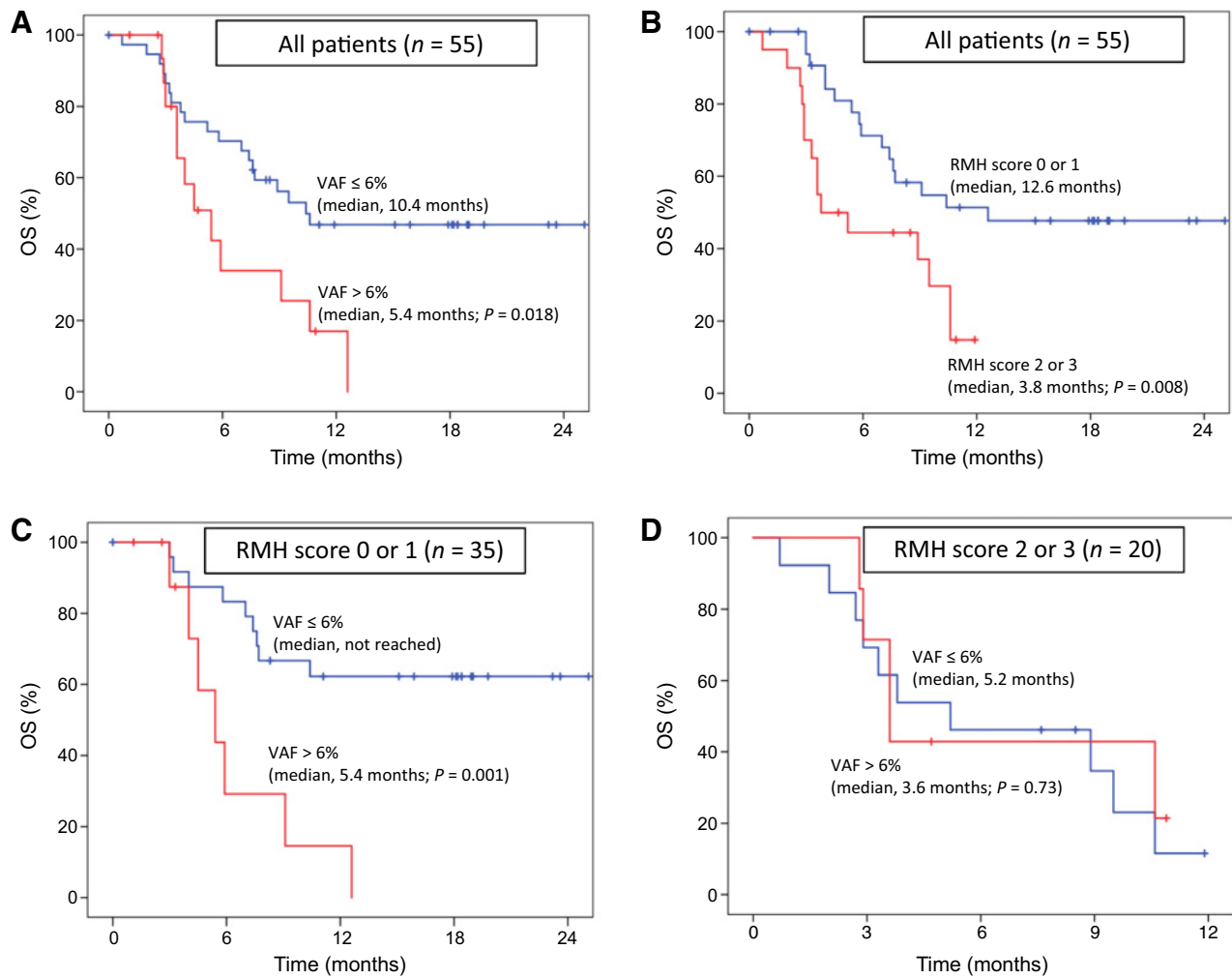


Figure 2.

OS and VAF or RMH score. **A**, The median OS duration of the 38 patients with a mutant cfDNA VAF of $\leq 6\%$ (blue; 10.4 months; 95% CI, not estimated) was longer than that of the 17 patients with a mutant cfDNA VAF of $> 6\%$ (red; 5.4 months; 95% CI, 3.0–7.8 months; $P = 0.018$). **B**, The median OS duration of the 35 patients with an RMH score of 0 or 1 (blue; 12.6 months; 95% CI, not estimated) was significantly longer than that of the 20 patients with an RMH score of 2 or 3 (red; 3.8 months; 95% CI, 0.5–7.1 months; $P = 0.008$). **C**, Among 35 patients with favorable RMH scores (0 or 1), the median OS duration of 25 patients with a mutant cfDNA VAF of $\leq 6\%$ (blue; not reached) was longer than that of 10 patients with a mutant cfDNA VAF of $> 6\%$ (red; 5.4 months; 95% CI, 3.1–7.7 months; $P = 0.001$). **D**, Among 20 patients with unfavorable RMH scores (2 or 3), the median OS duration of 13 patients with a mutant cfDNA VAF of $\leq 6\%$ (blue; 5.2 months; 95% CI, 0–10.8 months) was longer than that of 7 patients with a mutant cfDNA VAF of $> 6\%$ (red; 3.6 months; 95% CI, 2.7–4.5 months), but this difference was not significant ($P = 0.73$).

Serial monitoring of mutated plasma cfDNA

Longitudinal serial plasma specimens obtained before and during systemic therapy were available for 19 patients who received a total of 23 systemic therapies. The median number of cfDNA samples per patient was 4 (range, 2–11 samples). The median aggregate VAF of mutated cfDNA at baseline (0.82%), during therapy (0.48%), and at disease progression (1.06%) did not differ significantly ($P = 0.15$; Fig. 3A). A decrease in the aggregate VAF of mutated cfDNA was associated with the best response to therapy [complete response (CR) or partial response (PR) vs. stable disease (SD) or progressive disease (PD)] on imaging studies as per RECIST (median change, -10.51% for CR/PR vs. 0% for SD/PD; $P = 0.02$; Fig. 3B; ref. 8). Of the 23 diverse systemic cancer therapies these patients received, 13

decreased the VAF of mutated cfDNA and 10 caused no change or increased the VAF of mutated cfDNA. The median TTF of the patients with a decrease in aggregate VAF mutant DNA (3.8 months; 95% CI, 1.3–6.3 months) was significantly longer than that of patients with no change or an increased cfDNA VAF (2.5 months; 95% CI, 2.2–2.8 months; $P = 0.03$; Fig. 3C).

Discussion

We developed a highly sensitive assay that uses protocol changes to commercially available DNA assays and bioinformatics tools widely used for NGS to detect genetic variants in 61 cancer-related genes in plasma cfDNA. Our results demonstrate that our ultra-deep NGS assay confirmed molecular profiles

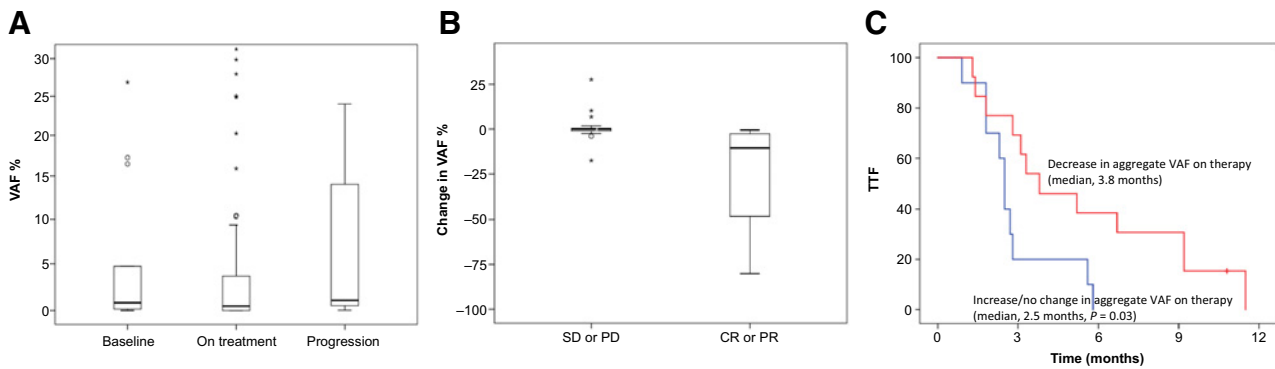


Figure 3.

Serial monitoring of VAF of mutant cfDNA during 23 diverse systemic therapies. **A**, The median aggregate VAF of mutated cfDNA at baseline (0.82%), during therapy (0.48%), and at disease progression (1.06%) did not differ significantly ($P = 0.15$). **B**, The best response to therapy (CR or PR vs. SD or PD) on imaging per RECIST was associated with the best change in the aggregate VAF of mutated cfDNA (median change for CR/PR, -10.51% , vs. median change for SD/PD, 0% ; $P = 0.02$). **C**, The median TTF of patients with decreased aggregate VAF mutant cfDNA (3.8 months; 95% CI, 1.3–6.3 months) was longer than that of patients with unchanged or increased aggregate VAF mutant cfDNA (blue; 2.5 months; 95% CI, 2.2–2.8 months; $P = 0.03$).

previously reported from clinical testing of archival tumor tissue in a CLIA-certified laboratory in 82% (complete detection) to 87% (complete and partial detection) of patients with advanced cancers. These rates corresponded to variant detection sensitivities of 80% and 86%, respectively.

Our results compare favorably to previously published data. Kim and colleagues (9) used a targeted 54-gene digital NGS panel to assess plasma cfDNA samples from 61 patients with advanced cancers and detected molecular variants present in the tumor tissue in 48% (complete detection) to 66% (complete and partial detection) of cases. In another study, using the same technology to assess the plasma cfDNA of NSCLC patients, Thompson and colleagues found that 58% (complete detection) to 74% (complete and partial detection) of plasma cfDNA samples had molecular profiles that matched those of tumor tissue specimens (10). Wang and colleagues (11) used the NGS SafeSeqS Pipeline to detect molecular variants in cfDNA isolated from the cerebral spinal fluid of patients with primary brain and spine tumors and demonstrated agreement between plasma and tumor tissue in 74% of cases. Finally, a small study using the NGS CAPP-Seq method to test molecular variants in plasma cfDNA from patients with any stage of NSCLC confirmed 85% of variants previously detected in tumor tissue (12). The CAPP-Seq method was later modified to use integrated digital error suppression (iDES-enhanced CAPP-Seq) and demonstrated a PPV of 72% for plasma cfDNA compared with archival tissue in 24 patients with NSCLC; however, the time between tumor and tissue collection was not clear (13). Jovelet and colleagues (14) used a standard 50-gene Ion AmpliSeq panel commonly used for archival tumor tissue to test plasma cfDNA from 283 patients with advanced cancer; although the tumor tissue and plasma were obtained around the same time, the sensitivity of the method was 49.9%. The same group later presented an optimized assay with increased sensitivity (15). Compared with NGS, PCR-based methods demonstrate higher concordance rates, which usually range from 85% to 95%; however, these assays have been hindered by their inability to test for multiple aberrations simultaneously (3, 5, 16). A certain level of discordance can be anticipated if the tumor tissue and plasma are not obtained at

the same time. Higgins and colleagues (17) found 100% agreement between *PIK3CA* mutation testing of simultaneously collected plasma cfDNA (assessed with BEAMing PCR) and tumor tissue in a cohort of patients with advanced breast cancer. However, the concordance between the methods decreased to 79% in a cohort of patients whose tumor and plasma cfDNA samples were obtained at different times. In another study of 100 patients with advanced colorectal cancer, ddPCR detection of *RAS* mutations in plasma cfDNA was in agreement with *RAS* mutation status in archival tissue in 97% of cases (18). This compared favorably with most other studies; however, the median time from tissue collection to plasma collection was only 43 days. In the current study, that time was 19.5 months. Indeed, there is increasing evidence that the molecular testing results for cfDNA are highly concordant with those for archival tumor tissue and that concordance decreases with increasing time between tissue and plasma collection (17).

In the current study, we found that patients with a low aggregate VAF of mutant cfDNA have a significantly longer median OS duration than patients with a high aggregate VAF of mutant cfDNA (10.4 months vs. 5.4 months; $P = 0.018$). We previously used BEAMing PCR to assess plasma cfDNA for 21 common mutations in *BRAF*, *EGFR*, *KRAS*, and *PIK3CA* in patients with advanced cancers and found that, irrespective of tumor type, patients with a high amount of mutant cfDNA had shorter OS than patients with a low amount of mutant cfDNA did (5.5 months vs. 9.8 months; $P = 0.001$; ref. 3). In another study, using the Idylla system (Biocartis) to detect *BRAF*^{V600} mutations in plasma-derived cfDNA from patients with diverse advanced cancers, we also found that patients with a high percentage of *BRAF*^{V600}-mutant cfDNA had shorter OS than patients with a low percentage of *BRAF*^{V600}-mutant cfDNA did (4.4 months vs. 10.7 months; $P = 0.005$; ref. 4). Similarly, among patients with advanced colorectal cancer who were treated in a phase III randomized trial of regorafenib versus placebo, high baseline levels of *KRAS*-mutant cfDNA were found to be associated with shorter OS (19). Also, higher amounts of *KRAS*-mutant cfDNA have been associated with shorter progression-free survival and OS in patients with advanced colorectal cancer treated with irinotecan and

cetuximab and in patients with advanced NSCLC treated with carboplatin and vinorelbine (20, 21). Similarly, a *BRAF*^{V600E} mutation in cfDNA was associated with shorter OS in a combined analysis of clinical trials of BRAF and MEK inhibitors in patients with advanced melanomas (22). To our knowledge, our study is the first report showing the prognostic significance of mutation burden tested by the NGS method.

The serial detection of genomic variants in plasma cfDNA can be used to monitor response to cancer therapy (23–33). In the current study, we assessed serially collected plasma cfDNA from patients treated with systemic therapies and found that a decrease in the aggregate VAF of mutant plasma cfDNA was associated with the best response on radiographic imaging per RECIST ($P = 0.02$) and that the median TTF of patients with a decreased aggregate VAF of mutant cfDNA (3.8 months) was longer than that of patients with an unchanged or increased aggregate VAF (2.5 months; $P = 0.03$). This observation is consistent with previously published data demonstrating that changes in plasma cfDNA can correspond with treatment outcomes (2, 26–34). In a study in which the Idylla PCR system was used to detect *BRAF*^{V600} mutations in plasma-derived cfDNA from patients with colorectal and other advanced cancers, therapies associated with a decrease in BRAF-mutant cfDNA produced significantly longer TTF than therapies associated with an increase or no change in BRAF-mutant cfDNA did (10.3 months vs. 7.4 months; $P = 0.045$; ref. 4). Although several previous studies' findings support the concept that changes in cfDNA can predict or at least correspond with treatment outcomes, future prospective studies are needed to address the overall evidence.

Our study had several potential limitations. First, our panel assesses only 61 cancer-related genes and is designed to detect mutations, not amplification or fusion events (apart from *ALK*, *RET*, and *ROS1*, for which all exonic and intronic regions were assayed). Second, the assay does not include unique molecular identifiers, which would be useful for removing potentially false-positive variant calls due to errors in library preparation or PCR (35). Third, the sample size was limited and included patients with diverse advanced cancers, which might impact the sensitivity of our assay, as the amount of released cfDNA can differ across diverse tumor types (25). Finally, the longitudinal and OS analyses were exploratory, and the associations between mutant VAF in cfDNA and TTF and OS need to be validated in future prospective studies.

In summary, our data highlight the feasibility of performing mutational analysis for 61 cancer-related genes using ultra-deep NGS of plasma cfDNA from patients with advanced cancers. To achieve this, we have used commercially available consumables and a simplified workflow. Our sequencing strategy demonstrated good sensitivity and relatively simple workflow, which can facilitate adoption of cfDNA sequencing assays in laboratories with NGS expertise. We also demonstrate that our approach allows for robust somatic variant detection, which compares well with standard methods of clinical molecular profiling of archival tumor tissue. Our data suggest that the aggregate VAF of mutant cfDNA is a prognostic biomarker for patient survival and that cfDNA levels are correlated with systemic treatment success, which to our knowledge has not been reported before using the targeted NGS panels. In the future, broad adoption of cfDNA assays could facilitate personalized therapeutic interventions based on patients' respective cfDNA mutation statuses.

Disclosure of Potential Conflicts of Interest

F. Janku reports receiving commercial research grants from Agios, Astellas, BioMed Valley Discoveries, Deciphera, Novartis, Piquor, Roche, Symphogen, and Upsher-Smith Laboratories; holds ownership interest (including patents) in Trovogene; and is a consultant/advisory board member for Deciphera, Guardant Health, Illumina, and Trovogene. S. Gnerre holds ownership interest (including patents) in Illumina stock. R.V. Satya holds ownership interest (including patents) in Common Stocks of ILMN. F. Meric-Bernstam reports receiving other commercial research support from Aileron, AstraZeneca, Bayer, Calithera, Curis, CytoMx, Debio, Effective Therapeutics, Genentech, Jounce, Novartis, PUMA, Taiho, Verastem, and Zymeworks and is a consultant/advisory board member for Clearlight Diagnostics, Darwin Health, Dialecta, Inflection Biosciences, and Pieris. G.B. Mills reports receiving commercial research grants from Adelson Medical Research Foundation, AstraZeneca, Breast Cancer Research Foundation, Critical Outcome Technologies, Illumina, Karus, Komen Research Foundation, Nanostring, and Takeda/Millennium Pharmaceuticals; reports receiving speakers bureau honoraria from Alloster, AstraZeneca, ImmunoMet, Ionis Pharmaceuticals, Lilly, Medimmune, Novartis, Pfizer, Symphogen, and Tarveda; holds ownership interest (including patents) in Catena Pharmaceuticals, ImmunoMet, Myriad Genetics, PIV Ventures, and Spindletop Ventures; and is a consultant/advisory board member for Adventist Health, Alloster, AstraZeneca, Catena Pharmaceuticals, Critical Outcome Technologies, ImmunoMet, Ionis Pharmaceuticals, Lilly, Medimmune, Novartis, Precision Medicine, Provista Diagnostics, Signalchem Lifesciences, Symphogen, Takeda/Millennium Pharmaceuticals, Tarveda, and Tau Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: F. Janku, S. Zhang, S. Fu, M. Javle, J.V. Heymach, G.B. Mills, J.-B. Fan, N.S. Salathia

Development of methodology: F. Janku, S. Zhang, J. Waters, X. Cai, S. Gnerre, R.V. Satya, H.-Y. Chuang, R. Shen, J.V. Heymach, J.-B. Fan, N.S. Salathia

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Janku, L. Liu, H.J. Huang, V. Subbiah, D.S. Hong, D.D. Karp, S. Fu, N.M. Ramzanali, K. Madwani, G. Cabrilo, E.S. Kopetz, F. Meric-Bernstam, G.B. Mills, N.S. Salathia

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Janku, S. Zhang, H.J. Huang, V. Subbiah, S. Fu, Y. Zhao, E.S. Kopetz, H.J. Kim, S. Gnerre, J. Tounq, C. Zhao, R. Shen, F. Meric-Bernstam, G.B. Mills, N.S. Salathia

Writing, review, and/or revision of the manuscript: F. Janku, S. Zhang, V. Subbiah, D.S. Hong, D.D. Karp, S. Fu, N.M. Ramzanali, G. Cabrilo, M. Javle, E.S. Kopetz, R. Luthra, S. Gnerre, K.M. Kruglyak, J.V. Heymach, F. Meric-Bernstam, G.B. Mills, J.-B. Fan, N.S. Salathia

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Janku, V. Subbiah, D.D. Karp, N.M. Ramzanali, G. Cabrilo, D.L. Andrews, H.J. Kim, R. Shen, J.V. Heymach, N.S. Salathia

Study supervision: F. Janku, J.-B. Fan, N.S. Salathia

Acknowledgments

The authors thank Karen Gutekunst, Paul Van Hummelen, and Matt Friedenberg for intellectual contribution and the sequencing core team at Illumina for sequencing support. The authors also thank Joseph Munch for the editorial assistance.

Grant Support

This study was supported by the Sidney Kimmel Foundation for Cancer Research (to F. Janku), the Sheikh Khalifa Al Nahyan Ben Zayed Institute for Personalized Cancer Therapy (to F. Janku), the National Center for Advancing Translational Sciences (grant no. UL1 TR000371), and the NIH through MD Anderson's Cancer Center support grant (P30 CA016672).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 31, 2017; revised March 22, 2017; accepted May 18, 2017; published OnlineFirst May 23, 2017.

References

- Polivka J Jr, Pesta M, Janku F. Testing for oncogenic molecular aberrations in cell-free DNA-based liquid biopsies in the clinic: are we there yet? *Expert Rev Mol Diagn* 2015;15:1631–44.
- Sacher AG, Paweletz C, Dahlberg SE, Alden RS, O'Connell A, Feeney N, et al. Prospective validation of rapid plasma genotyping for the detection of EGFR and KRAS mutations in advanced lung cancer. *JAMA Oncol* 2016; 2:1014–22.
- Janku F, Angenendt P, Tsimberidou AM, Fu S, Naing A, Falchook GS, et al. Actionable mutations in plasma cell-free DNA in patients with advanced cancers referred for experimental targeted therapies. *Oncotarget* 2015; 6:12809–21.
- Janku F, Huang HJ, Claes B, Falchook GS, Fu S, Hong D, et al. BRAF mutation testing in cell-free DNA from the plasma of patients with advanced cancers using a rapid, automated molecular diagnostics system. *Mol Cancer Ther* 2016;15:1397–404.
- Thierry AR, Moulriere F, El Messaoudi S, Mollevi C, Lopez-Crapez E, Rolet F, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. *Nat Med* 2014;20:430–5.
- Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* 2001; 29:308–11.
- Arkenau HT, Barriuso J, Olmos D, Ang JE, de Bono J, Judson I, et al. Prospective validation of a prognostic score to improve patient selection for oncology phase I trials. *J Clin Oncol* 2009;27:2692–6.
- Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009;45:228–47.
- Kim ST, Lee WS, Lanman RB, Mortimer S, Zill OA, Kim KM, et al. Prospective blinded study of somatic mutation detection in cell-free DNA utilizing a targeted 54-gene next generation sequencing panel in metastatic solid tumor patients. *Oncotarget* 2015;6:40360–9.
- Thompson JC, Yee SS, Troxel AB, Savitch SL, Fan R, Balli D, et al. Detection of therapeutically targetable driver and resistance mutations in lung cancer patients by next generation sequencing of cell-free circulating tumor DNA. *Clin Cancer Res* 2016;22:5772–82.
- Wang Y, Springer S, Zhang M, McMahon KW, Kinde I, Dobbyn L, et al. Detection of tumor-derived DNA in cerebrospinal fluid of patients with primary tumors of the brain and spinal cord. *Proc Natl Acad Sci U S A* 2015;112:9704–9.
- Newman AM, Bratman SV, To J, Wynne JF, Eclow NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med* 2014;20:548–54.
- Newman AM, Lovejoy AF, Klass DM, Kurtz DM, Chabon JJ, Scherer F, et al. Integrated digital error suppression for improved detection of circulating tumor DNA. *Nat Biotechnol* 2016;34:547–55.
- Jovelet C, Ileana E, Le Deley MC, Motte N, Rosellini S, Romero A, et al. Circulating Cell-Free Tumor DNA Analysis of 50 Genes by Next-Generation Sequencing in the Prospective MOSCATO Trial. *Clin Cancer Res* 2016; 22:2960–8.
- Fu Y, Jovelet C, Filleron T, Pedrero M, Motte N, Boursin Y, et al. Improving the performance of somatic mutation identification by recovering circulating tumor DNA mutations. *Cancer Res* 2016;76:5954–61.
- Janku F, Huang HJ, Claes B, Falchook GS, Fu S, Hong D, et al. BRAF mutation testing in cell-free DNA from the plasma of patients with advanced cancers using a rapid, automated molecular diagnostics system. *Mol Cancer Ther* 2016;15:1397–404.
- Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater S, Powers P, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. *Clin Cancer Res* 2012;18:3462–9.
- Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nat Med* 2015;21:795–801.
- Tabernero J, Lenz HJ, Siena S, Sobrero A, Falcone A, Ychou M, et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: a retrospective, exploratory analysis of the CORRECT trial. *Lancet Oncol* 2015;16:937–48.
- Nygaard AD, Garm Spindler KL, Pallisgaard N, Andersen RF, Jakobsen A. The prognostic value of KRAS mutated plasma DNA in advanced non-small cell lung cancer. *Lung Cancer* 2013;79:312–7.
- Spindler KL, Pallisgaard N, Vogelius I, Jakobsen A. Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. *Clin Cancer Res* 2012;18:1177–85.
- Santiago-Walker A, Gagnon R, Mazumdar J, Casey M, Long GV, Schandendorf D, et al. Correlation of BRAF mutation status in circulating-free DNA and tumor and association with clinical outcome across four BRAFi and MEKi clinical trials. *Clin Cancer Res* 2016;22:567–74.
- Hyman DM, Diamond EL, Vibat CR, Hassaine L, Poole JC, Patel M, et al. Prospective blinded study of BRAFV600E mutation detection in cell-free DNA of patients with systemic histiocytic disorders. *Cancer Discov* 2015; 5:64–71.
- Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 2012;486:532–6.
- Betegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014;6:224ra24.
- Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* 2013;368:1199–209.
- Forshe T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med* 2012;4:136ra68.
- Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshe T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* 2013;497:108–12.
- Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer Res* 2014;20:1698–705.
- Mok T, Wu YL, Lee JS, Yu CJ, Sriuranpong V, Sandoval-Tan J, et al. Detection and dynamic changes of EGFR mutations from circulating tumor DNA as a predictor of survival outcomes in NSCLC patients treated with first-line intercalated erlotinib and chemotherapy. *Clin Cancer Res* 2015;21:3196–203.
- Karlovich C, Goldman JW, Sun JM, Mann E, Sequist LV, Konopa K, et al. Assessment of EGFR mutation status in matched plasma and tumor tissue of NSCLC patients from a phase I study of rociletinib (CO-1686). *Clin Cancer Res* 2016;22:2386–95.
- Marchetti A, Palma JF, Felicioni L, De Pas TM, Chiari R, Del Grammasio M, et al. Early prediction of response to tyrosine kinase inhibitors by quantification of EGFR mutations in plasma of NSCLC patients. *J Thorac Oncol* 2015;10:1437–43.
- Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med* 2008;14: 985–90.
- Frenel JS, Carreira S, Goodall J, Roda D, Perez-Lopez R, Tunariu N, et al. Serial next-generation sequencing of circulating cell-free DNA evaluating tumor clone response to molecularly targeted drug administration. *Clin Cancer Res* 2015;21:4586–96.
- Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. *Proc Natl Acad Sci U S A* 2011;108:9530–5.