

Liquid Biopsies Using Plasma Exosomal Nucleic Acids and Plasma Cell-Free DNA Compared with Clinical Outcomes of Patients with Advanced Cancers



Lino Möhrmann^{1,2}, Helen J. Huang¹, David S. Hong¹, Apostolia M. Tsimberidou¹, Siqing Fu¹, Sarina A. Piha-Paul¹, Vivek Subbiah¹, Daniel D. Karp¹, Aung Naing¹, Anne Krug³, Daniel Enderle³, Tina Priewasser³, Mikkel Noerholm³, Erez Eitan³, Christine Coticchia³, Georg Stoll³, Lisa-Marie Jordan³, Cathy Eng⁴, E. Scott Kopetz⁴, Johan Skog³, Funda Meric-Bernstam¹, and Filip Janku¹

Abstract

Purpose: Blood-based liquid biopsies offer easy access to genomic material for molecular diagnostics in cancer. Commonly used cell-free DNA (cfDNA) originates from dying cells. Exosomal nucleic acids (exoNAs) originate from living cells, which can better reflect underlying cancer biology.

Experimental Design: Next-generation sequencing (NGS) was used to test exoNA, and droplet digital PCR (ddPCR) and BEAMing PCR were used to test cfDNA for *BRAF*^{V600}, *KRAS*^{G12/G13}, and *EGFR*^{exon19del/L858R} mutations in 43 patients with progressing advanced cancers. Results were compared with clinical testing of archival tumor tissue and clinical outcomes.

Results: Forty-one patients had *BRAF*, *KRAS*, or *EGFR* mutations in tumor tissue. These mutations were detected by NGS in 95% of plasma exoNA samples, by ddPCR in 92% of cfDNA

samples, and by BEAMing in 97% cfDNA samples. NGS of exoNA did not detect any mutations not present in tumor, whereas ddPCR and BEAMing detected one and two such mutations, respectively. Compared with patients with high exoNA mutation allelic frequency (MAF), patients with low MAF had longer median survival (11.8 vs. 5.9 months; $P = 0.006$) and time to treatment failure (7.4 vs. 2.3 months; $P = 0.009$). A low amount of exoNA was associated with partial response and stable disease ≥ 6 months ($P = 0.006$).

Conclusions: NGS of plasma exoNA for common *BRAF*, *KRAS*, and *EGFR* mutations has high sensitivity compared with clinical testing of archival tumor and testing of plasma cfDNA. Low exoNA MAF is an independent prognostic factor for longer survival. *Clin Cancer Res*; 24(1); 181–8. ©2017 AACR.

Introduction

Mutations in the *KRAS*, *BRAF*, and *EGFR* genes are prevalent in many cancer types and can predict outcomes of targeted therapies (1–4). Therefore, accurate assessment of the mutation status is of utmost importance. Mutation testing is usually done using archival formalin-fixed, paraffin-embedded (FFPE) tumor tissue, which is not always available or is of inadequate quality (5). Furthermore, the molecular profiles of primary and metastatic sites differ

(6, 7). In addition, the tumor molecular profile can evolve over time, which can be difficult to monitor in clinical practice, as serial tissue biopsies add to the cost of care and can lead to complications (8). Liquid biopsy-based approaches utilizing circulating sources of tumor DNA can provide alternative materials for molecular testing in cancer (9, 10). Plasma-derived cell-free DNA (cfDNA) is most commonly used for genomic testing; however, it originates from cells undergoing apoptosis or necrosis, which may not reflect the viable cell population of the tumor. In contrast, exosomal nucleic acids (exoNAs) such as DNA and RNA are actively secreted from living cells and might better correspond with tumor dynamics (11, 12). Molecular testing of plasma exosomal RNA (exoRNA) was described by Skog and colleagues, who demonstrated the presence of *EGFRvIII* mutations in serum exoRNA from patients with glioblastoma (13). In addition, Kahlert and colleagues reported detecting *KRAS* mutations in serum exosomal DNA (exoDNA) from patients with pancreatic cancer (11). San Lucas and colleagues demonstrated the next-generation sequencing (NGS)-based genomic and transcriptomic profiling of plasma exoDNA and exoRNA from patients with advanced pancreatic or biliary cancers (14). Finally, Allenson and colleagues detected *KRAS*-mutant plasma exoDNA in 80% to 85% of patients with locally advanced/metastatic pancreatic cancer and 44% of patients with early pancreatic cancer (15).

¹Department of Investigational Cancer Therapeutics (Phase I Clinical Trials Program), The University of Texas MD Anderson Cancer Center, Houston, Texas.

²Department of Translational Oncology, National Center for Tumor Diseases (NCT) Heidelberg and German Cancer Research Center (DKFZ), Heidelberg, Germany. ³Exosome Diagnostics, Waltham, Massachusetts. ⁴Department of Gastrointestinal Medical Oncology, 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/>).

Corresponding Author: Filip Janku, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit 0455, Houston, TX 77030. Phone: 713-563-0308; Fax: 713-745-8056; E-mail: fjanku@mdanderson.org

doi: 10.1158/1078-0432.CCR-17-2007

©2017 American Association for Cancer Research.

Translational Relevance

Molecular testing for common oncogenic hotspot mutations in *BRAF*, *KRAS*, and *EGFR* in plasma exosomal nucleic acids and cell-free DNA is feasible and has good agreement with molecular testing of discordantly collected archival tumor samples. The amount of mutated exosomal nucleic acids and cell-free DNA can predict clinical outcomes.

The purpose of the current study was to compare the assessment of common hotspot mutations in *BRAF*, *EGFR*, and *KRAS* using NGS of extracted exoNA and cfDNA with that using standard clinical testing of FFPE archival tumor samples in a Clinical Laboratory Improvement Amendments–certified laboratory and with that using droplet digital PCR (ddPCR) and BEAMing digital PCR testing of plasma cfDNA. In addition, we sought to determine whether the quantity of mutant exoNA and/or cfDNA is correlated with clinical outcomes and survival.

Materials and Methods

Patients and sample collection

The study enrolled consecutive patients with progressing advanced cancers and *BRAF*^{V600}, *KRAS*^{G12/G13}, or *EGFR*^{exon19del/L858R} mutations (except for two patients without mutations, who were enrolled for specificity purposes) detected with clinical testing of their FFPE archival tumor specimens (Supplementary Methods) who were referred to MD Anderson's Department of Investigational Cancer Therapeutics (Houston, TX) for experimental therapies from February 2010 to April 2014 and consented to the protocol LAB10-0334. Whole blood was collected in EDTA-containing tubes and centrifuged twice within 2 hours to yield plasma. The study was conducted in accordance with MD Anderson's Institutional Review Board guidelines and the U.S. Common Rule.

Isolation and molecular testing of exoNA and cfDNA

Plasma exoNA (exosomal DNA and RNA, along with present cfDNA) were coisolated using the ExoLution Plus Isolation Kit (Exosome Diagnostics; Supplementary Methods, Supplementary Fig. S1). Subsequently, a quantitative NGS method EXO1000 was used to detect any hotspot mutations in *BRAF* exon 15, *KRAS* exon 2, and *EGFR* exons 19–21. The lower limit of detection is a mutation allele frequency (MAF) of approximately 0.05% in the wild-type allele background.

Plasma cfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions. Then, if available, 16 ng of unamplified cfDNA was assessed for *BRAF*^{V600} or *KRAS*^{G12/G13} mutations with a multiplex ddPCR Screening Kit or for *EGFR*^{exon19del} and *EGFR*^{L858R} mutations with mutation-specific assays (Bio-Rad) using the QX200 Droplet Digital PCR platform (Bio-Rad). The lower limit of detection is an MAF of approximately 0.2% for the multiplexed assay and an MAF of <0.1% for the single-well mutation-specific assay. Analysis of plasma cfDNA for common hotspot mutations in *BRAF*, *EGFR*, and *KRAS* using the BEAMing assays was conducted as described previously (16). Briefly, individual DNA molecules were attached to magnetic beads in water-in-oil emulsions and then subjected to compartmentalized PCR amplification. The mutational status of

DNA bound to beads was determined by hybridization to fluorescent allele-specific probes for mutant or wild-type alleles of the gene of interest. Quantification of mutant DNA was performed using flow cytometry. The lower limit of detection is an MAF of approximately 0.02%.

Statistical analysis

Overall survival (OS) was defined as the time from the date of exoNA and cfDNA collection to the date of death or last follow-up. Time to treatment failure (TTF) was defined as the time from the date of systemic therapy initiation to the date the patient was taken off the treatment or last follow-up. 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 or TTF. The Mann–Whitney *U* test was applied to assess the association among the response on imaging during therapy per RECIST 1.1 and plasma mutation status and quantity (17). All tests were two-sided, and *P* values <0.05 were considered statistically significant. All statistical analyses were performed with the SPSS 23 (SPSS) software program.

Results

Patients

Of the 43 patients, 41 (95%) had a mutation of interest in their tumor tissues, including 20 (47%) with a *BRAF*^{V600} mutation, 17 (40%) with a *KRAS*^{G12/G13} mutation, and four (9%) with an *EGFR*^{exon19del/L858R} mutation. Most patients were Caucasian [29 (67%)] and male [24 (56%)]. The patients' median age was 57 years (range, 30–76 years). The most common tumor types were colorectal cancer in 20 patients (47%), melanoma in eight patients (19%), and non–small cell lung cancer (NSCLC) in six patients (14%). The median time from tissue sampling to blood sampling was 20 months (range, 0.1–140.4 months). Detailed patient characteristics are depicted in Table 1 and Supplementary File S1.

BRAF, *EGFR*, and *KRAS* mutations in plasma exoNA and plasma cfDNA

Mutation testing of plasma exoNA from 43 patients using NGS detected *BRAF*^{V600} mutations in 19 of 20 patients with *BRAF*^{V600} mutations in tumor tissue [sensitivity, 95%; 95% confidence interval (CI), 75–100], *KRAS*^{G12/G13} mutations in all 17 patients with *KRAS*^{G12/G13} mutations in tumor tissue (sensitivity, 100%; 95% CI, 80–100), and *EGFR*^{exon19del/L858R} mutations in three of four patients with *EGFR*^{exon19del/L858R} mutations in tumor tissue (sensitivity, 75%; 95% CI, 19–99; Supplementary Table S1). In total, NGS detected 39 of 41 mutations present in tumor tissue for an overall sensitivity of 95% (95% CI, 83–99; Table 2). NGS of plasma exoNA yielded two false-negative results: one for an appendiceal cancer patient with a *BRAF*^{V600E} mutation in tumor and one for NSCLC patients with an *EGFR*^{L858R} mutation in tumor (Table 3). None of the samples from patients with no mutation in tumor tissue detected a mutation in exoNA (specificity, 100%; 95% CIs, 85–100, 87–100, and 91–100 for *BRAF*^{V600}, *KRAS*^{G12/G13}, and *EGFR*^{exon19del/L858R} mutations, respectively; Supplementary Table S1).

Mutation testing of plasma cfDNA from 41 of 43 patients (no samples were available for two patients) using ddPCR detected mutations in 17 of 19 patients with *BRAF*^{V600} mutations in tumor

Table 1. Characteristics of 43 patients with advanced cancers

Characteristics	Total No. of patients	No. of patients with <i>BRAF</i> ^{V600} , <i>KRAS</i> ^{G12/G13} , or <i>EGFR</i> ^{exon19del/L858R} mutations in FFPE tumor (%)
All	43	41 (95)
Sex		
Male	24	23 (96)
Female	19	18 (95)
Race		
Caucasian	31	29 (94)
African American	5	5 (100)
Hispanic	5	5 (100)
Asian	1	1 (100)
Unknown	1	1 (100)
Disease		
Colorectal cancer	20	20 (100)
Melanoma	8	8 (100)
NSCLC	6	6 (100)
Ovarian cancer	2	1 (50)
Papillary thyroid cancer	2	2 (100)
Appendiceal cancer	1	1 (100)
CUP	1	1 (100)
Endometrial cancer	1	1 (100)
Erdheim-Chester histiocytosis	1	1 (100)
Prostate cancer	1	0 (0)
Tissue molecular testing method		
PCR	21	19 (90)
Sequenom	5	5 (100)
Targeted next-generation sequencing	17	17 (100)

Abbreviation: CUP, cancer of unknown primary.

tissue (sensitivity, 89%; 95% CI, 67–99), *KRAS*^{G12/G13} mutations in all 17 patients with *KRAS*^{G12/G13} mutations in tumor tissue (sensitivity, 100%; 95% CI, 80–100), and *EGFR*^{exon19del/L858R} mutations in two of three patients with *EGFR*^{exon19del/L858R} mutations in tumor tissue (sensitivity, 67%; 95% CI, 9–99; Supplementary Table S1). In total, ddPCR detected 36 of 39 mutations present in tumor tissue for an overall sensitivity of 92% (95% CI, 79–98; Table 2). Testing of cfDNA using ddPCR yielded three false-negative results: One for a patient with Erdheim-Chester disease and a *BRAF*^{V600E} mutation in the tumor tissue, one for a patient with NSCLC and an *EGFR*^{19del} mutation in the tumor tissue, and one for a patient with rectal carcinoma and a *BRAF*^{V600E} mutation in the tumor tissue (Table 3). Testing with ddPCR detected a *KRAS* mutation in a prostate cancer patient that was not present in the patient's tumor tissue. The respective speci-

cities were 100% (95% CI, 85–100) for *BRAF*^{V600} mutations, 96% (95% CI, 79–100) for *KRAS*^{G12/G13} mutations, and 100% (95% CI, 90–100) for *EGFR*^{exon19del/L858R} mutations (Supplementary Table S1).

Testing of plasma cfDNA from 37 of 43 patients (no samples were available for six patients) using BEAMing detected *BRAF*^{V600} mutations in 13 of 14 patients with *BRAF*^{V600} mutations in tumor tissue (sensitivity, 93%; 95% CI, 66–100), *KRAS*^{G12/G13} mutations in all 17 patients with *KRAS*^{G12/G13} mutations in tumor tissue (sensitivity, 100%; 95% CI, 80–100), and *EGFR*^{exon19del/L858R} mutations in all four patients with *EGFR*^{exon19del/L858R} mutations in tumor tissue (sensitivity, 100%; 95% CI, 40–100; Supplementary Table S1). In total, BEAMing detected 34 of 35 mutations present in tumor tissue for an overall sensitivity of 97% (95% CI, 85–100; Table 2). BEAMing of cfDNA yielded only one false-negative result for an appendiceal cancer patient with a *BRAF*^{V600E} mutation in tumor but yielded two false-positive *KRAS* mutation results: one for a patient with prostate cancer and one for a patient with ovarian cancer (Table 3). The respective specificities were 96% (95% CI, 78–100) for *BRAF*^{V600} mutations, 85% (95% CI, 62–97) for *KRAS*^{G12/G13} mutations, and 100% (95% CI, 88–100) for *EGFR*^{exon19del/L858R} mutations (Supplementary Table S1).

***BRAF*, *EGFR*, and *KRAS* mutations in exoNA or cfDNA and survival**

We analyzed whether the amount of mutant plasma exoNA or cfDNA was associated with OS. For plasma exoNA tested with NGS, the median OS duration of 21 patients with an MAF lower than or equal to the median MAF of 4.22% (11.8 months; 95% CI, 2.4–21.2 months) was significantly longer than that of 22 patients with an MAF higher than the median MAF (5.9 months; 95% CI, 4.7–7.1 months; $P = 0.006$; Fig. 1A). For plasma cfDNA tested with ddPCR, the median OS duration of 21 patients with an MAF lower than or equal to the median MAF of 6.1% (8.5 months; 95% CI, 2.1–14.9) was significantly longer than that of 20 patients with an MAF higher than the median MAF (5.9 months; 95% CI, 3.6–8.2 months; $P = 0.023$; Fig. 1B). For plasma cfDNA tested with BEAMing, there was a trend toward a longer median OS duration in 19 patients with an MAF lower than or equal to the median MAF of 7.22% (7.4 months; 95% CI, 4.3–10.5) compared to 18 patients with an MAF higher than the median MAF (6.5 months; 95% CI, 4.8–8.2 months; $P = 0.066$; Fig. 1C).

Next, to analyze the prognostic impact of exoNA and cfDNA MAFs on OS, we used a multivariable analysis that included

Table 2. Agreement assessment of *BRAF*, *KRAS*, or *EGFR* mutations in archival tumor tissue, plasma exoNA, and plasma cfDNA from patients with advanced cancers **Agreement in mutations detection for plasma samples collected before systemic experimental therapy versus FFPE tumor samples tested in the CLIA-certified laboratory**

	Mutation in tumor	Wild type in tumor
Mutation in exoNA, No. of patients	39	0
Wild type in exoNA, No. of patients	2	2
Observed agreements	41 (95%)	
Sensitivity	95% (95% CI, 83–99)	
Mutation in cfDNA ddPCR, No. of patients	36	1
Wild type in cfDNA ddPCR, No. of patients	3	1
Observed agreements	37 (90%)	
Sensitivity	92% (95% CI, 79–98)	
Mutation in cfDNA BEAMing, No. of patients	34	2
Wild type in cfDNA BEAMing, No. of patients	1	0
Observed agreements	34 (92%)	
Sensitivity	97% (95% CI, 85–100)	

Table 3. Discrepancies between mutation testing of archival tumor tissue and plasma exoNA and cfDNA

Characteristics	exoNA	cfDNA ddPCR	cfDNA BEAMing
Samples with tumor tissue mutations	41	39	35
Tissue mutations not detected in plasma			
Appendiceal cancer	<i>BRAF</i> ^{V600E}	Not done	<i>BRAF</i> ^{V600E}
NSCLC		<i>EGFR</i> ^{L858R}	
NSCLC	<i>EGFR</i> ^{L858R}		
Rectal cancer		<i>BRAF</i> ^{V600E}	
Erdheim-Chester histiocytosis		<i>BRAF</i> ^{V600E}	
Plasma mutations not detected in tissue			
Prostate cancer		<i>KRAS</i> ^{G12/G13}	<i>KRAS</i> ^{G12/G13}
Ovarian cancer			<i>KRAS</i> ^{G12/G13}

Royal Marsden Hospital (RMH) prognostic scores (18). The RMH score is a prospectively validated prognostic tool for patients with advanced cancers who are referred for early-phase clinical trials, which is calculated using lactate dehydrogenase levels (greater than the upper limit of normal vs. normal), albumin levels (<3.5 g/mL vs. ≥3.5 g/mL), and the number of metastatic sites (>2 sites vs. ≤2 sites). Scores of 0 or 1 are associated with longer OS than scores of 2 or 3. The median survival duration of 25 patients with RMH scores of 0 or 1 (8.4 months; 95% CI, 6.0–10.8) was significantly longer than that of 18 patients with RMH scores of 2 or 3 (6.0 months; 95% CI, 2.3–9.7 months; $P = 0.017$; Supplementary Fig. S2). A multivariable Cox regression analysis that included RMH scores as well as exoNA, ddPCR cfDNA, and BEAMing cfDNA results demonstrated that an exoNA MAF of ≤4.22% was the only independent prognostic factor for OS (HR, 0.15; $P = 0.026$; Table 4).

BRAF, *EGFR*, and *KRAS* mutations in exoNA or cfDNA and TTF

We analyzed whether the amount of mutant plasma exoNA or cfDNA was associated with TTF in 32 patients who received systemic therapy. For exoNA tested with NGS, the median TTF of 15 patients with an MAF lower than or equal to the median MAF of 4.22% (7.4 months; 95% CI, 1.5–13.3) was significantly longer than that of 17 patients with an MAF higher than the median MAF (2.3 months; 95% CI, 1.2–3.4; $P = 0.009$; Fig. 2A). For plasma cfDNA tested with ddPCR, the median TTF of 14 patients with an MAF lower than or equal to the median MAF of 6.1% (8.6 months; 95% CI, 2.4–14.8) was significantly longer than that of 17 patients with an MAF higher than the median MAF (2.3 months; 95% CI,

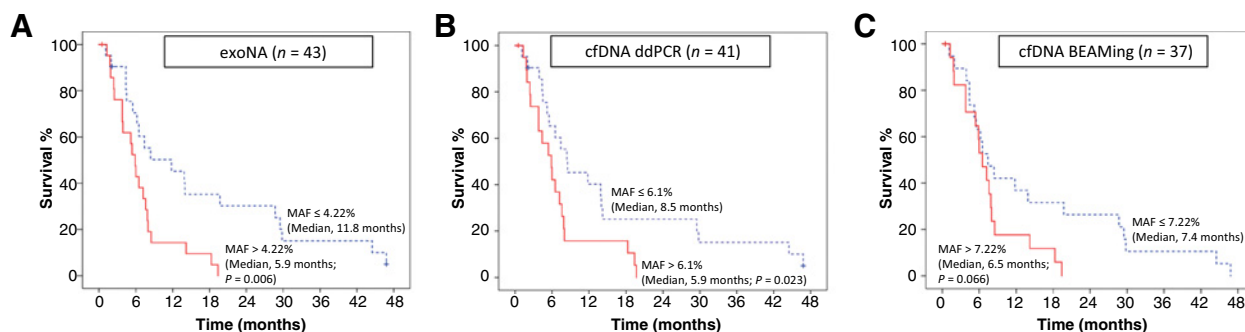
1.2–3.4; $P = 0.001$; Fig. 2B). For plasma cfDNA tested with BEAMing, there was a trend toward longer median TTF in 12 patients with an MAF lower than or equal to the median MAF of 7.22% (3.7 months; 95% CI, 1.8–5.6) compared to 15 patients with an MAF higher than the median MAF (2.3 months; 95% CI, 1.3–3.3; $P = 0.086$; Fig. 2C).

BRAF, *EGFR*, and *KRAS* mutations in exoNA or cfDNA and response to therapy

We analyzed whether mutations in plasma exoNA or cfDNA were associated with response to therapy assessed by imaging using RECIST 1.1 in 32 patients who received systemic therapy (17). For exoNA tested with NGS, the median mutated exoNA MAF of 12 patients with partial response (PR) or stable disease (SD) ≥6 months (0.43%) was significantly lower than that of 20 patients with progressive disease (PD) or SD <6 months (14.74%; $P = 0.006$; Fig. 3A). For plasma cfDNA tested with ddPCR, the median mutated cfDNA MAFs of 12 patients with PR or SD ≥6 months (0.70%) and 19 patients with PD or SD <6 months (16.00%) did not differ significantly ($P = 0.24$; Fig. 3B). For plasma cfDNA tested with BEAMing, the median mutated cfDNA MAFs of 10 patients with PR or SD ≥6 months (5.44%) and 17 patients with PD or SD <6 months (12.88%) did not differ significantly ($P = 0.24$; Fig. 3C).

Discussion

Our findings demonstrate that NGS testing for common hotspot mutations in plasma exoNA from patients with progressing

**Figure 1.**

OS per MAF of *KRAS*, *BRAF*, or *EGFR* mutations in plasma. **A**, Twenty-one patients with a low MAF (≤median; blue dashed line) in plasma exoNA had a significantly longer median OS duration than 22 patients with a high MAF (>median; red line; 11.8 vs. 5.9 months; $P = 0.006$). **B**, Twenty-one patients with a low MAF (blue dashed line) in plasma cfDNA tested with ddPCR had a significantly longer median OS than 20 patients with a high MAF (red line; 8.5 vs. 5.9 months; $P = 0.023$). **C**, There was a trend for 19 patients with a low MAF (blue dashed line) in plasma cfDNA tested with BEAMing to have a longer median OS than 18 patients with a high MAF (red line; 7.4 vs. 6.5 months; $P = 0.066$).

Table 4. Results of a multivariable Cox regression model to evaluate associations of the plasma exoNA MAF, the plasma cfDNA MAF tested with ddPCR, the plasma cfDNA MAF tested with BEAMing, and the RMH score with OS

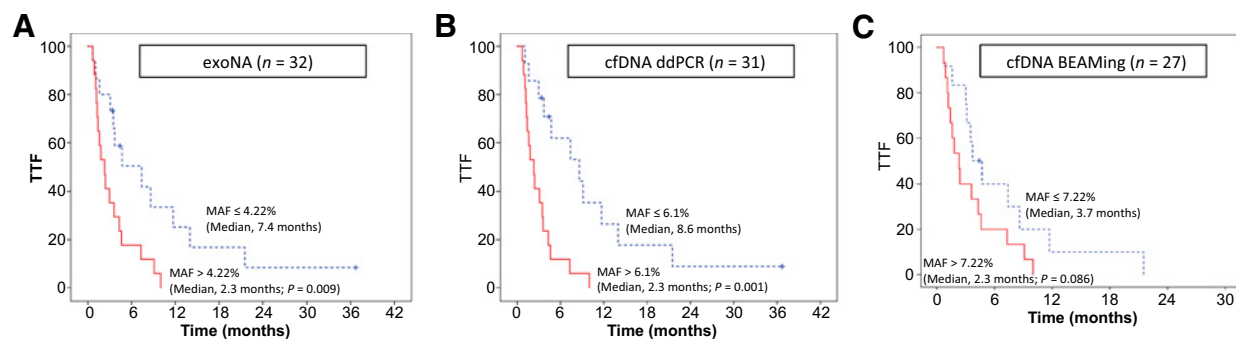
Variable	HR (95% CI)	P
Plasma exoNA MAF ($\leq 4.22\%$ vs. $> 4.22\%$)	0.15 (0.03–0.80)	0.026
Plasma cfDNA MAF with ddPCR ($\leq 6.1\%$ vs. $> 6.1\%$)	0.60 (0.22–1.65)	0.320
Plasma cfDNA MAF with BEAMing ($\leq 7.22\%$ vs. $> 7.22\%$)	4.67 (0.69–31.60)	0.120
RMH score (0 or 1 vs. 2 or 3)	0.84 (0.28–2.49)	0.750

advanced cancers has very good sensitivity overall (95%) compared with the standard testing of archival FFPE samples of discordantly obtained tumor tissue. This sensitivity of plasma exoNA testing was similar to those of simultaneous testing of plasma cfDNA with ddPCR and BEAMing (92% and 97%, respectively). Although BEAMing of plasma cfDNA had the highest sensitivity overall, this was offset by its lower overall specificity; of the three methods used, BEAMing detected the most mutations not detected in the archival tissue.

To our knowledge, ours is the first study to assess agreement between mutation testing of plasma exoNA and tumor tissue; however, our results with plasma exoNA appear to be similar to those of previously published data with plasma cfDNA (16, 19–24). In a previous study, we demonstrated in a similar patient population that the testing of plasma cfDNA with BEAMing has sensitivities of 76%, 100%, and 80% for *BRAF*, *EGFR*, and *KRAS* mutations, respectively, compared with the mutation analysis of FFPE primary or metastatic tumors (16). Similarly, other studies have reported that ddPCR testing of plasma cfDNA compared with tumor tissue has sensitivities of 84%, 69% to 86%, and 64% to 96% for *BRAF*, *EGFR*, and *KRAS* mutations, respectively (20, 21, 24). A certain level of disagreement between the results of mutation testing of plasma exoNA and testing of tumor tissue in our study could be explained by the discordant collection of both materials, as the median time from tumor tissue acquisition to plasma acquisition was 20 months. A small retrospective study of patients with metastatic breast cancer tested with BEAMing for *PIK3CA* mutations in plasma cfDNA and simultaneously collected tumor tissue demonstrated 100% agreement between the methods; however, the sensitivity decreased to 57% in a prospective cohort when plasma cfDNA and tissue were collected at different points (25). In another study of 100 patients with advanced colorectal cancer, the ddPCR detection of RAS muta-

tions in plasma cfDNA was in agreement with that in archival tissue in 97% of cases (26). This rate was favorable compared with most other studies, including ours; however, the median time from tissue collection to plasma collection was only 43 days, which could explain the high agreement rate. In addition, tumor heterogeneity, clonal evolution, and preanalytic factors such as suboptimal specimen collection can also contribute to discrepancies (6, 27). Of interest, in our study, two of the methods used missed a *BRAF*^{V600E} mutation that was present in the tumor of a patient with appendiceal cancer, but this could have been due to the biology of that specific disease.

We found that patients with a low amount (i.e., that below or equal to the median amount) of mutated plasma exoNA had longer survival than patients with a high amount of mutated exoNA did ($P = 0.006$), which was confirmed in a multivariable analysis. Patients with a low amount of mutated plasma cfDNA tested with ddPCR had significantly longer survival than patients with a high amount of mutated plasma cfDNA ($P = 0.023$), and there was a trend for patients with a low amount of mutated plasma cfDNA tested with BEAMing to have longer survival than patients with a high amount of mutated plasma cfDNA ($P = 0.066$). To our knowledge, this is the first report suggesting a relationship between the amount of mutated plasma exoNA and survival. In our study, exoNA fared better than cfDNA, possibly because, unlike cfDNA, exoNA originates from living cancer cells (11, 12). However, our findings have to be interpreted cautiously, as our sample size was small. An earlier study using BEAMing PCR to detect *KRAS* mutations in plasma cfDNA from patients with advanced cancers found that a low amount of *KRAS*-mutant cfDNA was associated with longer median survival (7.3 vs. 4.8 months; $P = 0.008$; ref. 16). Another study that used the Idylla system to detect *BRAF*^{V600} mutations in plasma cfDNA from patients with advanced cancers showed that a low percentage of

**Figure 2.**

TTF per pretreatment MAF of *KRAS*, *BRAF*, or *EGFR* mutations in plasma. **A**, Fifteen patients with a low MAF (\leq median; blue dashed line) in plasma exoNA had a significantly longer median TTF than 17 patients with a high MAF ($>$ median; red line; 7.4 vs. 2.3 months; $P = 0.009$). **B**, Fourteen patients with a low MAF (blue dashed line) in plasma cfDNA tested with ddPCR had a significantly longer median TTF than 17 patients with a high MAF (red line; 8.6 vs. 2.3 months; $P = 0.001$). **C**, Twelve patients with a low MAF (blue dashed line) in plasma cfDNA tested with BEAMing had a trend toward longer median TTF than 15 patients with a high MAF (red line; 3.7 vs. 2.3 months; $P = 0.086$).

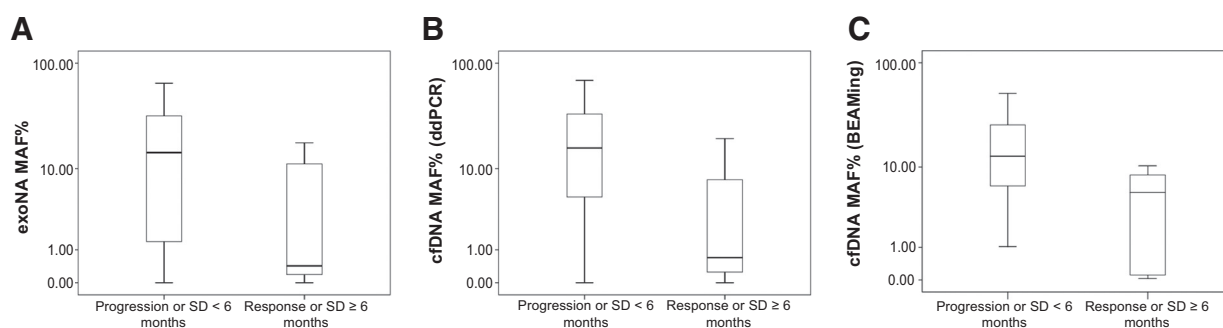


Figure 3.

Pretreatment MAF of *KRAS*, *BRAF*, or *EGFR* mutations in plasma and corresponding response to systemic therapy per RECIST 1.1. **A**, Twelve patients with a PR or SD ≥ 6 months had a significantly lower median mutated exoNA MAF than 20 patients with a PD or SD < 6 months (0.43% vs. 14.74%; $P = 0.006$). **B**, Twelve patients with a PR or SD ≥ 6 months did not have a statistically different median mutated cfDNA MAF (assessed with ddPCR) compared with 19 patients with a PD or SD < 6 months (0.70% vs. 16.00%; $P = 0.24$). **C**, Ten patients with a PR or SD ≥ 6 months did not have a statistically different median mutated cfDNA MAF (assessed with BEAMing) compared with 17 patients with a PD or SD < 6 months (5.44% vs. 12.88%; $P = 0.24$).

BRAF^{V600}-mutant cfDNA was associated with longer survival (10.7 vs. 4.4 months; $P = 0.005$; ref. 28). Similarly, baseline samples collected from patients with advanced colorectal cancer treated in a phase III randomized trial of regorafenib versus placebo showed that low baseline levels of *KRAS*-mutant cfDNA were associated with longer survival durations (29). In addition, low amounts of *KRAS*-mutant cfDNA were associated with longer survival durations in patients with advanced colorectal cancer treated with irinotecan and cetuximab and in patients with advanced NSCLC treated with carboplatin and vinorelbine (30, 31). Similarly, in a combined analysis of clinical trials of *BRAF* and *MEK* inhibitors in patients with advanced melanoma and *BRAF* mutations in their tumors, the absence of *BRAF*^{V600E} mutations in plasma cfDNA was associated with longer median survival (22).

We also demonstrated that patients with a low amount of mutated plasma exoNA identified with NGS or cfDNA identified with ddPCR had a significantly longer TTF than patients with a high amount of mutated exoNA or cfDNA did ($P = 0.009$ and 0.001 , respectively). In addition, patients with a low amount of mutated plasma cfDNA identified with BEAMing had a trend to longer TTF than patients with a high amount of mutated cfDNA did ($P = 0.086$). Allenson and colleagues demonstrated that the amount of mutated exoDNA can be predictive of disease-free survival in patients with localized pancreatic cancer (15). In a previous study, using allele-specific quantitative PCR (Idylla) to test for *BRAF* mutations in plasma cfDNA, we demonstrated that patients with advanced cancers and *BRAF* mutations in their tumors but not plasma cfDNA had a longer median TTF on *BRAF* and/or *MEK* inhibitors than patients with detected *BRAF* mutations in cfDNA did (3.0 vs. 13.1 months; $P = 0.001$). In addition, a retrospective analysis of plasma samples collected from patients with malignant melanoma and *BRAF* mutations in their tumors who were treated with *BRAF* and *MEK* inhibitors in clinical trials also demonstrated that the absence of *BRAF* mutations in cfDNA predicted longer progression-free survival (22).

Moreover, we showed that patients who received systemic therapy who had a PR or SD ≥ 6 months had a significantly lower median amount of mutated plasma exoNA than patients without a PR or SD ≥ 6 months did ($P = 0.006$). This was not observed for cfDNA tested with ddPCR or BEAMing ($P = 0.24$ and 0.24 , respectively). Weiss and colleagues demonstrated that quantitative chromosomal instability in plasma cfDNA can predict immu-

notherapy response in patients with advanced cancers (32). Although some data suggest that changes in mutated cfDNA during therapy can correspond with treatment response, our study is the first to our knowledge to show that pretreatment levels of mutated exoNA and cfDNA can be associated with response to systemic therapy (33).

Our study had several potential limitations. First, the study was retrospective and enrolled relatively few patients. Second, its patient population was heterogeneous, with diverse tumor types that were treated with diverse therapies. Third, we tested for only selected common hotspot mutations in the *BRAF*, *KRAS*, and *EGFR* genes. These mutations are relevant in only a limited number of patients with certain cancer types; thus, whether our findings can be extrapolated to other molecular abnormalities remains unclear. Fourth, nearly half of the patients (47%) had colorectal cancer, which could have influenced the results. Fifth, we used archival tumor tissue, which was not collected at the same time as plasma samples, and this may have impacted our sensitivity assessments. Finally, our observation that increased plasma MAF can be associated with worse outcomes irrespective of disease burden (using the RMH score) is intriguing; however, it needs to be interpreted with caution because our tools to estimate disease burden are not optimal.

In conclusion, our findings suggest that molecular testing of plasma exoNA has potential in cancer molecular diagnostics and can be predictive of clinical outcomes. Thus, future clinical studies to assess its utility in furthering personalized cancer therapy are warranted.

Disclosure of Potential Conflicts of Interest

D.S. Hong has ownership interests (including patents) at OncoResponse, reports receiving speakers bureau honoraria from LOXO and miRNA, is a consultant/advisory board member for Baxter, Bayer, and Guidepoint Global, and reports receiving commercial research grants from Amgen, Bayer, Daichi-Sankyo, Eisai, Genentech, Ignyta, Lilly, LOXO, Merck, Mirati, and Pfizer. V. Subbiah reports receiving commercial research support from Bayer, Blueprint, LOXO, Novartis, and Roche/Genentech. C. Eng reports receiving speakers bureau honoraria from Genentech, and is a consultant/advisory board member for Bayer and Sirtex. F. Meric-Bernstam is a consultant/advisory board member for Clearlight Diagnostics, Darwin Health, Dialecta, GRAIL, Inflection Biosciences, Pieris, and Sumitomo Dainippon and reports receiving commercial research grants from Aileron, AstraZeneca, Bayer, Calithera, Curis, Debiopharma, eFFECTOR, Genentech, Novartis, Pfizer, PUMA, Taiho, and Zymeworks. F. Janku has ownership interests (including patents) at Trovogene, is a

consultant/advisory board member for Deciphera, Guardant Health, Illumina, Trovagene, and reports receiving commercial research grants from Agios, Astellas, BioMed Valley Discoveries, Deciphera, Genentech, Novartis, Piquar, Plexikon, Symphogen, and Upsher-Smith Laboratories. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J. Skog, F. Janku

Development of methodology: H.J. Huang, V. Subbiah, A. Krug, D. Enderle, M. Noerholm, C. Coticchia, J. Skog, F. Janku

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Möhrmann, H.J. Huang, D.S. Hong, A.M. Tsimberidou, S. Fu, S.A. Piha-Paul, V. Subbiah, D.D. Karp, A. Naing, D. Enderle, M. Noerholm, E. Eitan, G. Stoll, L.-M. Jordan, E.S. Kopetz, J. Skog, F. Meric-Bernstam, F. Janku

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Möhrmann, H.J. Huang, D.S. Hong, A.M. Tsimberidou, S. Fu, V. Subbiah, A. Naing, A. Krug, D. Enderle, T. Priewasser, M. Noerholm, E.S. Kopetz, J. Skog, F. Meric-Bernstam, F. Janku

Writing, review, and/or revision of the manuscript: L. Möhrmann, D.S. Hong, A.M. Tsimberidou, S. Fu, S.A. Piha-Paul, V. Subbiah, D.D. Karp, A. Naing, D. Enderle, T. Priewasser, M. Noerholm, L.-M. Jordan, C. Eng, E.S. Kopetz, J. Skog, F. Meric-Bernstam, F. Janku

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Möhrmann, A.M. Tsimberidou, V. Subbiah, M. Noerholm, J. Skog, F. Janku

Study supervision: J. Skog, F. Janku

References

- Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med* 2013;369:1023–34.
- Hong DS, Morris VK, El Osta B, Sorokin AV, Janku F, Fu S, et al. Phase IB study of vemurafenib in combination with irinotecan and cetuximab in patients with metastatic colorectal cancer with BRAFV600E mutation. *Cancer Discov* 2016;6:1352–65.
- Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–57.
- Falchook GS, Long GV, Kurzrock R, Kim KB, Arkenau TH, Brown MP, et al. Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: a phase 1 dose-escalation trial. *Lancet* 2012;379:1893–901.
- Tsimberidou AM, Iskander NG, Hong DS, Wheler JJ, Falchook GS, Fu S, et al. Personalized medicine in a phase I clinical trials program: the MD anderson cancer center initiative. *Clin Cancer Res* 2012;18:6373–83.
- Meric-Bernstam F, Frampton GM, Ferrer-Lozano J, Yelensky R, Perez-Fidalgo JA, Wang Y, et al. Concordance of genomic alterations between primary and recurrent breast cancer. *Mol Cancer Ther* 2014;13:1382–9.
- Vakiani E, Janakiraman M, Shen R, Sinha R, Zeng Z, Shia J, et al. Comparative genomic analysis of primary versus metastatic colorectal carcinomas. *J Clin Oncol* 2012;30:2956–62.
- Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* 2011;3:75ra26.
- 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.
- Bardelli A, Pantel K. Liquid biopsies, what we do not know (Yet). *Cancer Cell* 2017;31:172–9.
- Kahlert C, Melo SA, Protopopov A, Tang J, Seth S, Koch M, et al. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *J Biol Chem* 2014;289:3869–75.
- Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* 2015;523:177–82.
- Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008;10:1470–6.
- San Lucas FA, Allenson K, Bernard V, Castillo J, Kim DU, Ellis K, et al. Minimally invasive genomic and transcriptomic profiling of visceral cancers by next-generation sequencing of circulating exosomes. *Ann Oncol* 2016;27:635–41.
- Allenson K, Castillo J, San Lucas FA, Scelo G, Kim DU, Bernard V, et al. High prevalence of Mutant KRAS in circulating exosome-derived DNA from early stage pancreatic cancer patients. *Ann Oncol* 2017;28:741–47.
- 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.
- 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.
- 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.
- 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.
- Janku F, Huang HJ, Fujii T, Shelton DN, Madwani K, Fu S, et al. Multiplex KRASG12/G13 mutation testing of unamplified cell-free DNA from the plasma of patients with advanced cancers using droplet digital polymerase chain reaction. *Ann Oncol* 2017;28:642–50.
- Sanmamed MF, Fernandez-Landazuri S, Rodriguez C, Zarate R, Lozano MD, Zubiri L, et al. Quantitative cell-free circulating BRAFV600E mutation analysis by use of droplet digital PCR in the follow-up of patients with melanoma being treated with BRAF inhibitors. *Clin Chem* 2015;61:297–304.
- Santiago-Walker A, Gagnon R, Mazumdar J, Casey M, Long GV, Schadendorf 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.
- Karachaliou N, Mayo-de las Casas C, Queralt C, de Aguirre I, Melloni B, Cardenal F, et al. Association of EGFR L858R mutation in circulating

- free DNA with survival in the EURTAC Trial. *JAMA Oncol* 2015;1:149–57.
24. 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.
 25. 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.
 26. 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.
 27. 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.
 28. 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;15:1379–404.
 29. 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.
 30. 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.
 31. 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.
 32. Weiss GJ, Beck J, Braun DP, Bornemann-Kolatzki K, Barilla H, Cubello R, et al. Tumor cell-free DNA copy number instability predicts therapeutic response to immunotherapy. *Clin Cancer Res* 2017;23:5074–81.
 33. Fujii T, Barzi A, Sartore-Bianchi A, Cassingena A, Siravegna G, Karp DD, et al. Mutation-enrichment next-generation sequencing for quantitative detection of KRAS mutations in urine cell-free DNA from patients with advanced cancers. *Clin Cancer Res* 2017;23:3657–66.