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ORIGINAL ARTICLE

Multiplex *KRAS*$^{G12/G13}$ mutation testing of unamplified cell-free DNA from the plasma of patients with advanced cancers using droplet digital polymerase chain reaction

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Background: Cell-free DNA (cfDNA) from plasma offers easily obtainable material for *KRAS* mutation analysis. Novel, multiplex, and accurate diagnostic systems using small amounts of DNA are needed to further the use of plasma cfDNA testing in personalized therapy.

Patients and methods: Samples of 16 ng of unamplified plasma cfDNA from 121 patients with diverse progressing advanced cancers were tested with a *KRAS* $^{G12/G13}$ multiplex assay to detect the seven most common mutations in the hotspot of exon 2 using droplet digital polymerase chain reaction (ddPCR). The results were retrospectively compared to mutation analysis of archival primary or metastatic tumor tissue obtained at different points of clinical care.

Results: Eighty-eight patients (73%) had *KRAS*$^{G12/G13}$ mutations in archival tumor specimens collected on average 18.5 months before plasma analysis, and 78 patients (64%) had *KRAS*$^{G12/G13}$ mutations in plasma cfDNA samples. The two methods had initial overall agreement in 103 (85%) patients (kappa, 0.66; ddPCR sensitivity, 84%; ddPCR specificity, 88%). Of the 18 discordant cases, 12 (67%) were resolved by increasing the amount of cfDNA, using mutation-specific probes, or re-testing the tumor tissue, yielding overall agreement in 115 patients (95%; kappa 0.87; ddPCR sensitivity, 96%; ddPCR specificity, 94%). The presence of $\geq$ 6.2% of *KRAS*$^{G12/G13}$ cfDNA in the wild-type background was associated with shorter survival ($P = 0.001$).

Conclusion(s): Multiplex detection of *KRAS*$^{G12/G13}$ mutations in a small amount of unamplified plasma cfDNA using ddPCR has good sensitivity and specificity and good concordance with conventional clinical mutation testing of archival specimens. A higher percentage of mutant *KRAS*$^{G12/G13}$ in cfDNA corresponded with shorter survival.

Key words: cell-free DNA, droplet digital PCR, KRAS, multiplex

Introduction

Mutations in codons 12 and 13 of the *KRAS* gene (*KRAS*$^{G12/G13}$ mutations) are prevalent in colorectal cancer, non-small cell lung cancer (NSCLC), and others and can be associated with less favorable prognosis or a lack of benefit from anti-epidermal growth factor receptor antibodies [1–6]. Furthermore, preclinical and early clinical data suggest that *KRAS* mutations can predict the response of advanced low-grade serous ovarian cancer to combinations of PI3K and MEK inhibitors [7]. Therefore, the accurate assessment of *KRAS* mutation status is critical to therapeutic decisions.

Current practice prescribes *KRAS* mutation testing of archival formalin-fixed, paraffin-embedded (FFPE) tumor tissue and a

lack of adequate samples can preclude mutation analysis in at least 10% of patients with advanced cancers [8]. Also, mutation status can change over time, and discrepancies between the genomic profiles of primary and metastatic tumors may occur [9–11]. Thus, archival FFPE tumor samples, which may be many years old, might not necessarily reflect the pertinent genotype.

Cell-free DNA (cfDNA) is secreted into the circulation by tumor cells and cells in the tumor microenvironment that are undergoing apoptosis or necrosis and can be isolated from plasma as a minimally invasive alternative for determining *KRAS* mutation status by polymerase chain reaction (PCR) or next-generation-sequencing (NGS) methods [12]. Droplet digital PCR (ddPCR) has better sensitivity than standard quantitative PCR or NGS and simpler workflow than other digital PCR such as BEAMing [12]. The purpose of the present study of patients with advanced cancer was to determine whether the detection and quantification of *KRAS*$^{G12/G13}$ mutations in unamplified plasma cfDNA by multiplexed ddPCR has an acceptable level of sensitivity, specificity and concordance with conventional clinical testing for *KRAS* mutations in FFPE tumor samples performed by a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. We also sought to determine whether the number of *KRAS*$^{G12/G13}$ mutant alleles in the wild-type background (mutant allele frequency [MAF] or copy number) is associated with outcomes.

Methods

Patients

The study enrolled patients with progressing advanced cancers and known *KRAS*$^{G12/G13}$ mutation status from clinical testing of their FFPE specimens (supplementary Methods, available at *Annals of Oncology* online) who were referred to MD Anderson's Department of Investigational Cancer Therapeutics for experimental therapies from October 2010 to June 2015. Patients had the option of providing longitudinally collected plasma samples during the course of their therapy (at baseline, then every 3–4 weeks if feasible). This retrospective study was conducted in accordance with MD Anderson's Institutional Review Board guidelines.

Plasma collection and cfDNA *KRAS*$^{G12/G13}$ mutation testing

Whole blood was collected in EDTA-containing tubes and centrifuged and spun twice within 2 h to yield plasma. The QIAamp Circulating Nucleic Acid kit (Qiagen, Valencia, CA) was used to isolate cfDNA according to the manufacturer's instructions, and 16 ng of unamplified cfDNA was tested with a multiplex ddPCR *KRAS* $^{G12/G13}$ Screening Kit (Bio-Rad, Pleasanton, CA) to distinguish the wild-type allele from the seven most common mutations in the exon 2 (G12A, G12C, G12D, G12R, G12S, G12V, and G13D) using the QX200 Droplet Digital PCR platform (Bio-Rad) according to the manufacturer's standard protocol (supplementary Table S1, available at *Annals of Oncology* online). Mutation-specific assays (e.g. G12V, G12D) or more cfDNA (24–100 ng) were used for re-testing for patients whose FFPE specimens but not plasma cfDNA showed a *KRAS*$^{G12/G13}$ mutation. The investigators performing the mutation analysis of the cfDNA samples were blinded to the results of the FFPE specimens and used appropriate positive and negative controls. The lower limit of detection is approximately 0.2% MAF for the multiplexed screening assay and <0.1% MAF per single well for the mutation-specific assays.

Statistical analysis

Concordance between the mutation analyses of FFPE specimens and plasma cfDNA was calculated using a kappa coefficient. Overall survival (OS) was defined as the time from the date of study entry 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 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 or TTF. The Spearman rank coefficient was used to assess correlations. 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., La Jolla, CA) or SPSS 23 (SPSS, Chicago, IL) software programs.

Results

Patients

The study enrolled 121 patients with diverse advanced cancers and known *KRAS*$^{G12/G13}$ mutation statuses of archival FFPE specimens (Table 1). The patients' median age was 56 years (range, 20–84 years). Most patients were white (N = 86; 71%) and male (N = 68; 56%). The most common tumor type was colorectal cancer (N = 71; 59%), NSCLC (N = 14; 12%), and melanoma (N = 10; 8%). The median time from tissue to blood sampling was 18.5 months (range, 1.1–134.4 months). The median amount of cfDNA isolated per 1 ml of plasma was 42 ng (range, 8–3093 ng).

KRAS$^{G12/G13}$ mutations in FFPE specimens and plasma cfDNA

Of the 121 patients, 88 (73%) had *KRAS*$^{G12/G13}$ mutations in FFPE specimens, and 78 (64%) had *KRAS*$^{G12/G13}$ mutations detectable by multiplex ddPCR in 16 ng of unamplified cfDNA. There was overall agreement between cfDNA and FFPE specimens in 103 cases (85%; kappa, 0.66; standard error [SE], 0.07; 95% confidence interval [CI], 0.52–0.80). The cfDNA test had a sensitivity of 84% (95% CI, 0.75–0.91), specificity of 88% (95% CI, 0.72–0.97), positive predictive value (PPV) of 95% (95% CI, 0.87–0.99), and negative predictive value (NPV) of 67% (95% CI, 0.51–0.81; Table 2). Results were similar irrespective of the method used by the CLIA laboratory for tissue *KRAS* testing (supplementary Table S2 and File S1, available at *Annals of Oncology* online).

Sixteen nanograms of cfDNA contain only about 5000 genomic equivalents, which enabled us to reliably detect *KRAS*$^{G12/G13}$ mutations in samples with a MAF of ≥0.2%. In the 14 cases with known *KRAS*$^{G12/G13}$ mutations in FFPE specimens but wild-type *KRAS* in cfDNA, we were able to detect *KRAS*$^{G12/G13}$ mutations in the cfDNA in seven cases by increasing the amount of cfDNA (median, 74 ng; range, 24–100 ng) and in an additional three cases using mutation-specific probes (G12V, G12D) and 49–100 ng of cfDNA. This yielded overall agreement between cfDNA and FFPE specimens in 113 cases (93%; kappa, 0.83; SE, 0.06; 95% CI, 0.72–0.95), and the cfDNA test had a sensitivity of 95% (95% CI, 0.89–0.99), specificity of 88% (95% CI, 0.72–0.97), PPV of 95% (95% CI, 0.89–0.99), and NPV of 88% (95% CI, 0.72–0.97; Table 2).

Table 1. Characteristics of 121 patients with advanced cancers

Characteristic	Total No. of patients	No. of patients with *KRAS*$^{G12/G13}$ mutation in FFPE tumor (%)	No. of patients with *KRAS*$^{G12/G13}$ mutation in plasma cfDNA* (%)
All	121	88 (73)	78 (64)
Sex			
Male	68	53 (78)	47 (69)
Female	53	35 (66)	31 (58)
Race			
Caucasian	86	60 (70)	56 (65)
African American	21	18 (86)	13 (62)
Hispanic	13	10 (77)	9 (69)
Asian	1	0 (0)	0 (0)
Disease			
Colorectal cancer	71	59 (83)	53 (75)
Non-small cell lung cancer	14	10 (71)	7 (50)
Melanoma	10	1 (10)	2 (20)
Appendiceal cancer	6	6 (100)	4 (67)
Pancreatic cancer	5	5 (100)	5 (100)
Ovarian cancer	5	3 (60)	3 (60)
Uterine cancer	3	2 (67)	2 (67)
Breast cancer	3	1 (33)	2 (67)
Duodenal cancer	1	1 (100)	0 (0)
Squamous head and neck cancer	1	0 (0)	0 (0)
Papillary thyroid cancer	1	0 (0)	0 (0)
Erdheim–Chester histiocytosis	1	0 (0)	0 (0)
Method of tumor *KRAS*$^{G12/G13}$ testing			
PCR	58	42 (72)	37 (64)
NGS	50	36 (72)	33 (66)
MassARRAY	13	10 (77)	8 (62)

FFPE, formalin-fixed, paraffin-embedded; cfDNA, cell-free DNA; PCR, polymerase chain reaction; NGS, next-generation sequencing.
*Mutations were detected by testing 16 ng of cfDNA using the *KRAS*$^{G12/G13}$ multiplex probe.

Furthermore, we were able to retrieve archival FFPE specimens for 2 of 4 cases with *KRAS*$^{G12/G13}$ mutations in cfDNA but wild-type *KRAS* in FFPE specimens and using ddPCR identified low-frequency *KRAS*$^{G12/G13}$ mutations in both of them (0.08% and 0.14%, respectively). This yielded overall agreement between cfDNA and FFPE specimens in 115 cases (95%; kappa, 0.87; SE, 0.05; 95% CI, 0.77–0.97), and the cfDNA test had a sensitivity of 96% (95% CI, 0.89–0.99), specificity of 94% (95% CI, 0.79–0.99), PPV of 98% (95% CI, 0.92–1.00), and NPV of 88% (95% CI, 0.72–0.97; Table 2). Of interest, 1 of 4 patients who had *KRAS*$^{G12/G13}$ mutations in cfDNA but wild-type *KRAS* in FFPE specimens had advanced colorectal cancer and received chemotherapy with cetuximab, which resulted in accelerated disease progression (supplementary Figure S1, available at *Annals of Oncology* online).

KRAS$^{G12/G10}$ mutations in cfDNA and survival

To determine whether the baseline MAF of *KRAS*$^{G12/G13}$-mutated cfDNA was associated with OS, we divided the 121 patients into two groups according to the percentage of *KRAS*$^{G12/G13}$-mutated cfDNA (MAF <6.2% versus MAF ≥6.2%). These thresholds were selected based on a 5% trimmed mean value of *KRAS*$^{G12/G13}$-mutated cfDNA. This was deemed to be representative, as the median percentage of *KRAS*$^{G12/G13}$-mutant cfDNA was only 0.5% because 33 of 121 patients had no *KRAS*$^{G12/G13}$ mutations in cfDNA. The median OS of the 82 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA MAF <6.2% (7.5 months; 95% CI, 5.6–9.4 months) was significantly longer than that of the 39 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA MAF ≥6.2% (5.4 months; 95% CI, 3.7–7.1 months; $P = 0.001$; Figure 1A). Similarly, when we used a median percentage of *KRAS*$^{G12/G13}$-mutant cfDNA (<0.5% versus ≥0.5%) as the cut-off value, the median OS of the 60 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA MAF <0.5% (8.4 months; 95% CI, 5.5–11.3 months) was significantly longer than that of the 61 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA MAF ≥0.5% (5.5 months; 95% CI, 4.7–6.3 months; $P = 0.004$; supplementary Figure S2A, available at *Annals of Oncology* online). We also performed a separate analysis for patients with colorectal cancer ($N = 71$, the most frequent cancer type) and found that the median OS of the 41 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA MAF <6.2% (8.4 months; 95% CI, 5.1–11.7 months) was significantly longer than that of the 30 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA MAF ≥6.2% (5.7 months; 95% CI, 3.8–7.6 months; $P = 0.007$; supplementary Figure S2B, available at *Annals of Oncology* online). In contrast, neither the amount of total cfDNA in plasma (ng/ml) nor the cfDNA concentration (ng/μl) was associated with OS (supplementary Figure S2C and D, available at *Annals of Oncology* online). Finally, in a

Table 2. Concordance assessment of *KRAS*$^{G12/G13}$ mutations in formalin-fixed, paraffin-embedded (FFPE) tumor tissue and plasma cell-free DNA (cfDNA) from 121 patients with advanced cancers

	***KRAS*$^{G12/G13}$ mutation in tumor**	***KRAS*$^{G12/G13}$ wild-type in tumor**
Concordance for plasma samples (16 ng of cfDNA) collected before systemic experimental therapy tested with *KRAS*$^{G12/G13}$ multiplex probe versus FFPE tumor samples tested in the CLIA-certified laboratory		
KRAS$^{G12/G13}$ mutation in cfDNA, no. of patients	74	4
KRAS$^{G12/G13}$ wild-type in cfDNA, no. of patients	14	29
Observed agreements	103 (85%); kappa, 0.66; SE, 0.07; 95% CI, 0.52–0.80	
Sensitivity	84% (95% CI, 0.75–0.91)	
Specificity	88% (95% CI, 0.72–0.97)	
Positive predictive value	95% (95% CI, 0.87–0.99)	
Negative predictive value	67% (95% CI, 0.51–0.81)	
Concordance for plasma samples (16–100 ng of cfDNA) collected before systemic experimental therapy tested with *KRAS*$^{G12/G13}$ multiplex and/or mutation specific probes versus FFPE tumor samples tested in the CLIA-certified laboratory		
KRAS$^{G12/G13}$ mutation in cfDNA, no. of patients	84	4
KRAS$^{G12/G13}$ wild-type in cfDNA, no. of patients	4	29
Observed agreements	113 (93%); kappa, 0.83; SE, 0.06; 95% CI, 0.72–0.95	
Sensitivity	95% (95% CI, 0.89–0.99)	
Specificity	88% (95% CI, 0.72–0.97)	
Positive predictive value	95% (95% CI, 0.89–0.99)	
Negative predictive value	88% (95% CI, 0.72–0.97)	
Concordance for plasma samples (16–100 ng of cfDNA) collected before systemic experimental therapy tested with *KRAS*$^{G12/G13}$ multiplex and/or mutation specific probes versus FFPE tumor samples tested in the CLIA-certified laboratory or with droplet digital PCR		
KRAS$^{G12/G13}$ mutation in cfDNA, no. of patients	86	2
KRAS$^{G12/G13}$ wild-type in cfDNA, no. of patients	4	29
Observed agreements	115 (95%); kappa, 0.87; SE, 0.5; 95% CI, 0.77–0.97	
Sensitivity	96% (95% CI, 0.89–0.99)	
Specificity	94% (95% CI, 0.79–0.99)	
Positive predictive value	98% (95% CI, 0.92–1.00)	
Negative predictive value	88% (95% CI, 0.72–0.97)	

SE, standard error; CI, confidence interval.

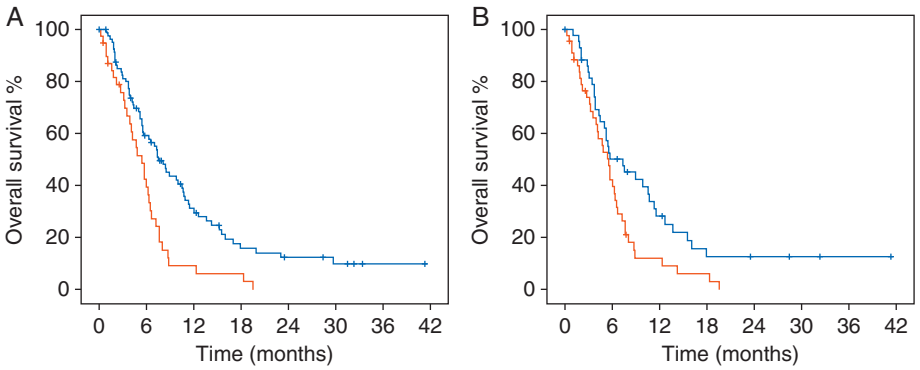

Figure 1. (A) Among the 121 patients whose cfDNA samples were tested for *KRAS*$^{G12/G13}$ mutations, the median overall survival (OS) duration of the 82 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA percentage of <6.2% (7.5 months; 95% confidence interval [CI], 5.6–9.4 months; blue) was significantly longer than that of the 39 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA percentage of ≥6.2% (5.4 months; 95% CI, 3.7–7.1; red; $P = 0.001$). (B) In a separate analysis that included only the 88 patients with *KRAS*$^{G12/G13}$ mutations in formalin-fixed, paraffin-embedded tumor samples, the median OS duration of the 44 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA percentage of <4.0% (7.3 months; 95% CI, 3.6–11.0; blue) was significantly longer than that of the 44 patients with a *KRAS*$^{G12/G13}$-mutant cfDNA percentage of ≥4.0% (5.5 months; 95% CI, 4.3–6.7; red; $P = 0.017$).

Table 3. Multi-variable cox regression models evaluating *KRAS*$^{G12/G13}$-mutant cell-free DNA (cfDNA) percentage and Royal Marsden Hospital (RMH) score with respect to overall survival (OS)

Variable	HR	95% CI	*P* value
OS, all 121 patients			
KRAS$^{G12/G13}$ cfDNA (<6.2% versus ≥6.2%)	0.63	0.39–1.02	0.060
RMH score (0 or 1 versus 2 or 3)	0.57	0.36–0.90	0.015
OS, 88 patients with *KRAS*$^{G12/G13}$ in FFPE			
KRAS$^{G12/G13}$ cfDNA (<4.0% versus ≥4.0%)	0.64	0.39–1.06	0.080
RMH score (0 or 1 versus 2 or 3)	0.68	0.42–1.13	0.140

HR, hazard ratio; CI, confidence interval.

separate analysis of patients with *KRAS*$^{G12/G13}$ mutations in FFPE specimens, the median OS of the 44 patients with a MAF <4% (7.3 months; 95% CI, 3.6–11.0 months) was significantly longer than that of the 44 patients with a MAF ≥4% (5.5 months; 95% CI, 4.3–6.7 months; $P = 0.017$; Figure 1B).

Next, we analyzed the prognostic impact of the MAF of *KRAS*$^{G12/G13}$-mutant cfDNA on OS using a multi-variable analysis, which included the Royal Marsden Hospital (RMH) prognostic score [13]. The RMH score, a prospectively validated tool used to predict OS in patients with advanced cancers who are referred for early phase clinical trials, is calculated on the basis of lactate dehydrogenase levels (greater than the upper limit of normal versus normal), albumin levels (<3.5 g/ml versus ≥3.5 g/ml), and the number of metastatic sites (>2 sites versus ≤2 sites). Scores of 0 or 1 are associated with longer OS than are scores of 2 or 3. The median OS duration of the 70 patients with an RMH score of 0 or 1 (8.4 months; 95% CI, 5.7–11.1 months) was significantly longer than that of the 51 patients with an RMH score of 2 or 3 (4.4 months; 95% CI, 2.4–6.4 months; $P < 0.001$). A multi-variable analysis including all 121 patients revealed that, compared to an RMH score of 2 or 3, an RMH score of 0 or 1 was associated with longer OS (hazard ratio [HR], 0.57; 95% CI, 0.36–0.90; $P = 0.015$; Table 3) and that, compared to a *KRAS*-mutant cfDNA MAF ≥6.2%, a *KRAS*$^{G12/G13}$-mutant cfDNA MAF <6.2% demonstrated a trend towards longer OS (HR, 0.63; 95% CI, 0.39–1.02; $P = 0.06$; Table 3). An additional multi-variable analysis including 88 patients with *KRAS*$^{G12/G13}$ mutations in FFPE (patients with wild-type *KRAS* in cfDNA were excluded), which included RMH score (0 or 1 versus 2 or 3) and *KRAS*$^{G12/G13}$-mutant percentage of cfDNA (≥4.0% versus <4.0%), demonstrated that a *KRAS*$^{G12/G13}$-mutant cfDNA percentage <4.0% (HR, 0.64; 95% CI, 0.39–1.06; $P = 0.08$) but not RMH score was associated with a trend towards longer OS (HR, 0.68; 95% CI, 0.42–1.13; $P = 0.14$; Table 3).

Longitudinal testing for *KRAS*$^{G12/G13}$ mutations in plasma cfDNA

At least two (median, 4; range, 2–12) longitudinal serial plasma collections were obtained before and during systemic therapy from 23 patients with *KRAS*$^{G12/G13}$ mutations in FFPE specimens who underwent 26 diverse experimental systemic therapies (Figure 2 and supplementary Figure S3, available at *Annals of Oncology* online). In all 23 patients, *KRAS*$^{G12/G13}$ mutations were detected in plasma cfDNA at ≥1 time points. Because MAF might be influenced by the number of wild-type copies, which can vary for reasons other than cancer, we also analyzed changes in the number of *KRAS*$^{G12/G13}$ copies per 1 ml of plasma, which did not take into account wild-type copies [12]. The median *KRAS*$^{G12/G13}$ MAFs at baseline (0.50%), during therapy (0.22%), and at disease progression (1.13%) differed significantly ($P = 0.04$), as did the median numbers of *KRAS*$^{G12/G13}$ copies at baseline (14), during therapy (17) and at disease progression (273; $P = 0.03$; Figure 3). The best response to therapy on imaging per Response Evaluation Criteria in Solid Tumors (RECIST) was not correlated with the best change in MAF ($r = 0.26$; $P = 0.21$) but was correlated with the best change in the number of *KRAS*$^{G12/G13}$ copies ($r = 0.42$; $P = 0.04$) [14, 15]. Of the 26 diverse systemic cancer therapies, 12 decreased the *KRAS*$^{G12/G13}$ MAF, 14 caused no change or increased the *KRAS*$^{G12/G13}$ MAF, 11 decreased the number of *KRAS*$^{G12/G13}$ copies, and 15 caused no change or increased the number of *KRAS*$^{G12/G13}$ copies. The median TTFs of patients with a decrease in *KRAS*$^{G12/G13}$ MAF (2.5 months) and those with no change or an increase in *KRAS*$^{G12/G13}$ MAF (2.8 months) did not differ significantly ($P = 0.72$); however, there was a trend towards longer TTF for decrease in number of *KRAS*$^{G12/G13}$ copies compared to no change or increase (3.4 months versus 2.7 months; $P = 0.11$; supplementary Figure S4, available at *Annals of Oncology* online). Nevertheless, decrease in size of target tumor lesions on imaging per RECIST vs. no change or increase was the only variable significantly associated with prolonged median TTF (8.2 months versus 2.5 months; $P = 0.001$; supplementary Figure S4, available at *Annals of Oncology* online).

Discussion

Our findings demonstrate that the ddPCR *KRAS* multiplex assay of a small amount of unamplified, plasma cfDNA from patients with advanced cancers can detect *KRAS*$^{G12/G13}$ mutations and has acceptable concordance (85%), sensitivity (84%), and specificity (88%) compared with the CLIA-certified laboratory-based testing of FFPE tumor tissue obtained at different times during routine care. The addition of more cfDNA with or without the use of mutation-specific probes, as well as retesting discrepant FFPE specimens with ddPCR, resolved the majority of discrepancies between cfDNA and tumor tissue and increased concordance, sensitivity, and specificity to 95%, 96%, and 94%, respectively. We recently demonstrated in a similar patient population that the testing of plasma cfDNA for *KRAS*$^{G12/G13}$ mutations with BEAMing PCR is concordant with the standard-of-care mutation analysis of FFPE primary or metastatic tumor in 83% of cases, which is similar to the results obtained with the ddPCR KRAS multiplex assay in the present study [16]. A certain level of discordance can be anticipated if the tumor tissue and plasma are not obtained at the same time. Higgins et al. [17] found 100% agreement between *PIK3CA* mutation testing of plasma cfDNA with BEAMing PCR and tumor tissue collected in a cohort of patients with advanced breast cancer and simultaneous collection. 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, which is consistent with

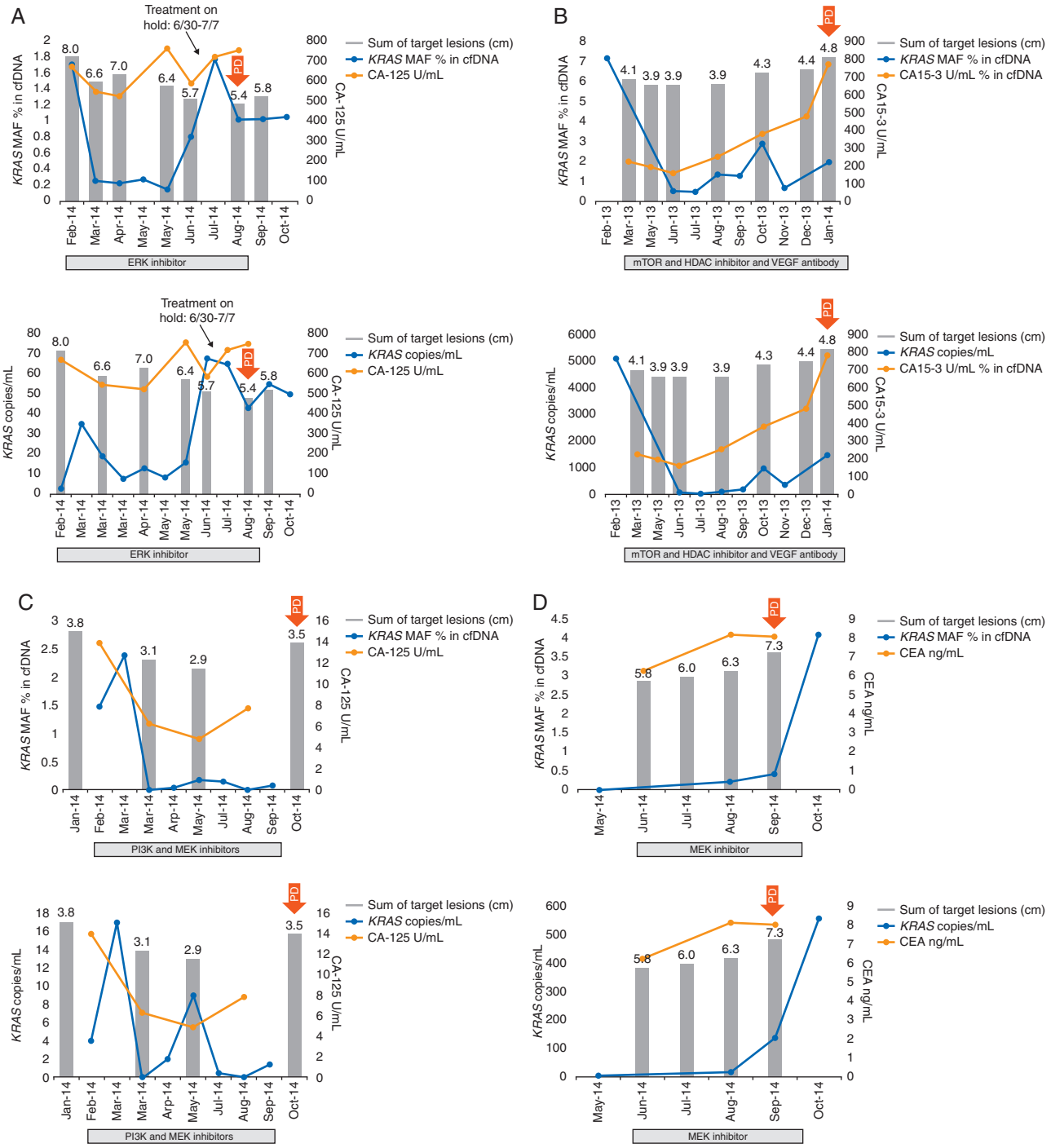

Figure 2. Dynamic changes in *KRAS*$^{G12/G13}$-mutated cfDNA in various patients with heavily pretreated *KRAS*$^{G12/G13}$-mutated advanced cancers. In each panel, the top graph depicts the *KRAS*$^{G12/G13}$ mutation allelic frequency (MAF) in the wild-type background, and the bottom graph depicts the number of *KRAS*$^{G12/G13}$ copies per 1 ml of plasma. Red arrows indicate the onset of progressive disease. Shown are sequential measurements of *KRAS*$^{G12/G13}$ (blue); cancer antigen 125 (CA125) levels (panels A and C), cancer antigen 15-3 (CA15-3) levels (panel B), carcinoembryonic antigen (CEA) levels (panels D–F; orange); and the sum (cm) of target lesions on imaging (grey bars) in (A) a patient with metastatic ovarian carcinoma who received targeted therapy with an ERK inhibitor; (B) a patient with metastatic breast carcinoma who received therapy with an mTOR inhibitor, HDAC inhibitor, and anti-VEGF antibody; (C) a patient with metastatic ovarian carcinoma who received targeted therapy with PI3K and MEK inhibitors; (D) a patient with metastatic ampullary carcinoma who received targeted therapy with a MEK inhibitor.

our results. In addition, Tabernero et al. [18], using BEAMing PCR, showed concordant *KRAS* mutation status between plasma-derived cfDNA and archival tumor samples in 76% of tested patients with advanced colorectal cancer who had participated in a phase III randomized trial of regorafenib or placebo. Finally, Thierry et al. [19] demonstrated a 96% concordance for combined *KRAS* and *BRAF* mutation testing using allele-specific quantitative PCR of plasma cfDNA and mutation detection in primary or metastatic tissue. Our concordance results for *KRAS*$^{G12/G13}$ mutation compare favorably to most of these

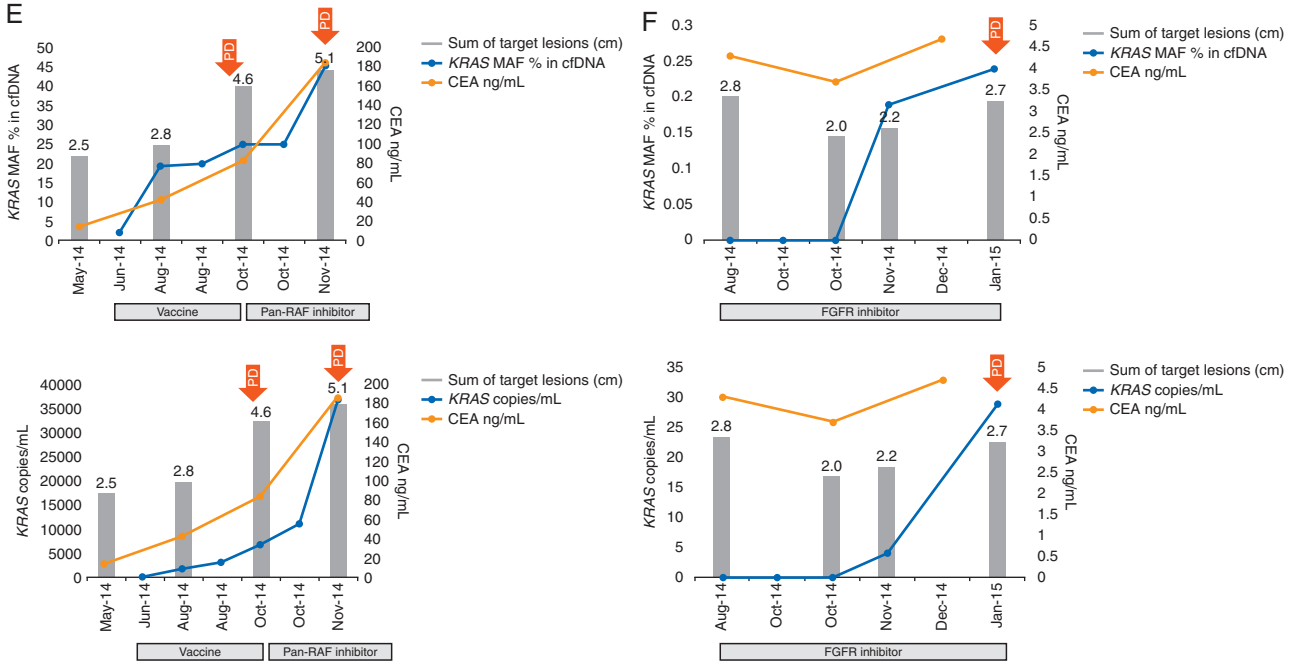

Figure 2. (E) a patient with colorectal cancer who received an intratumoral autologous dendritic cell vaccine; and (F) a patient with non–small cell lung cancer who received targeted therapy with a FGFR inhibitor.

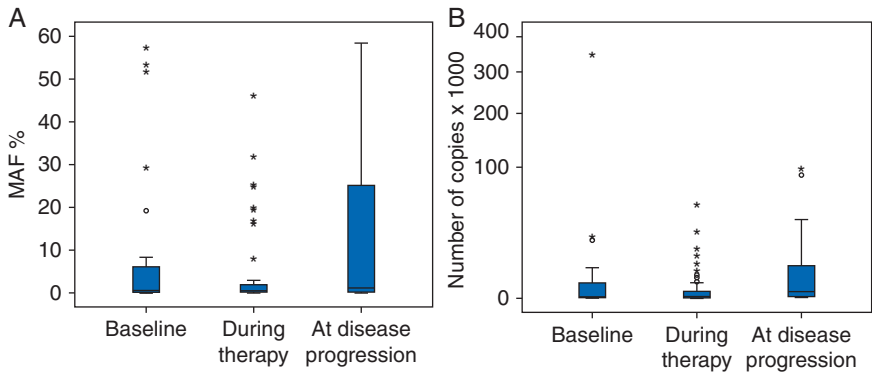

Figure 3. (A) *KRAS*$^{G12/G13}$ mutant allele frequencies (MAFs) at baseline (median, 0.50%), during therapy (median, 0.22%), and at disease progression (median, 1.13%) differed significantly ($P = 0.04$). (B) *KRAS*$^{G12/G13}$ copy numbers at baseline (median, 14), during therapy (median, 17), and at disease progression (median, 273) differed significantly ($P = 0.03$).

previously published studies despite the fact that we used a very low amount of cfDNA, which in most cases was isolated from much less than 0.5 ml of plasma [17–21]. Collectively, there is increasing evidence that the mutation analysis results for cfDNA are highly concordant with those for archival tumor tissue for concordantly, but not discordantly, collected samples, which may be explained by tumor biology, including heterogeneity and evolution over time [10, 21]. In addition, our *KRAS*$^{G12/G13}$ multiplex assay detects seven of the most frequent *KRAS*$^{G12/G13}$ mutations in one reaction; in other PCR approaches, this would require seven separate tests. At the same time, the *KRAS*$^{G12/G13}$ multiplex ddPCR assay, unlike next-generation sequencing, does not compromise on sensitivity [12].

In the present study, we did not use pre-amplification or other methods of enrichment to detect *KRAS*$^{G12/G13}$-mutant alleles, as we believe that such approaches can skew *KRAS*$^{G12/G13}$ MAF or copy number values, which can have important prognostic significance. Indeed, we found that patients with a low *KRAS*$^{G12/G13}$-mutant cfDNA MAF had a significantly longer median OS duration than patients with a high *KRAS*$^{G12/G13}$-mutant cfDNA MAF did (7.5 months versus 5.4 months; $P = 0.001$) and confirmed these trend in multivariable analysis, which included prospectively validated RMH score. We previously used BEAMing PCR to assess plasma cfDNA for *KRAS*$^{G12/G13}$ mutations in patients with advanced cancers and found that a high amount of *KRAS*-mutant cfDNA was associated with shorter OS (4.8 months versus 7.3 months; $P = 0.008$)[16]. In another study, using the Idylla system (Biocartis, Mechelen, Belgium) to detect *BRAF*^{V600} mutations in plasma-derived cfDNA from patients with diverse advanced cancers, we also found that a higher percentage of *BRAF*^{V600}-mutant cfDNA was associated with shorter OS (4.4 months versus 10.7 months, $P = 0.005$) and, in patients treated with BRAF and/or MEK inhibitors, shorter TTF (3.0 versus 7.4 months, $P = 0.001$) [22]. Similarly, high baseline levels of *KRAS*-mutant cfDNA were found to be

associated with shorter OS in patients with advanced colorectal cancer who were treated in a phase III randomized trial of regorafenib versus placebo [18]. Also, higher amounts of *KRAS*-mutant cfDNA were 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 [23, 24]. 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 [25].

The detection of molecular aberrations can be used to monitor therapy response [26–31]. In the present study, we assessed serially collected plasma cfDNA from patients treated with systemic therapies and found that the $KRAS^{G12/G13}$ MAFs and copy numbers before therapy, during therapy, and at the time of disease progression differed significantly. In addition, the number of $KRAS^{G12/G13}$ copies but not the $KRAS^{G12/G13}$ MAF was correlated with radiographic response, perhaps because $KRAS^{G12/G13}$ MAF is influenced by the amount of wild-type DNA, which can vary for reasons other than cancer (e.g. inflammation, exertion) [12]. We did not find an association between changes in $KRAS^{G12/G13}$ mutations in cfDNA and TTF, which may have been due to the small number of patients in the study and the lack of an effective KRAS inhibitor in clinical testing. Although several previous studies' findings have supported the concept that changes in cfDNA can predict or at least correspond with treatment outcomes overall the evidence remains conflicting [20, 21, 29–32].

In summary, the molecular analysis of a small amount of unamplified cfDNA for $KRAS^{G12/G13}$ mutations using a ddPCR multiplex assay to detect the most frequent seven hotspot mutations is feasible and has good concordance with standard mutation testing of discordantly collected FFPE tumor tissue. Our results also suggest that the number of $KRAS^{G12/G13}$-mutant alleles in cfDNA is a prognostic biomarker for OS. Our study had several potential limitations. First, we investigated only $KRAS^{G12/G13}$ mutations, which are clinically relevant to only a limited number of patients with certain tumor types. Second, because the study retrospectively analyzed OS data, its findings with regard to these measures need to be validated in future prospective studies. Third, we used archival tumor tissue, which was not collected at same time as plasma samples. Fourth, more than half of the patients had colorectal cancer, which could have influenced our results. Finally, despite the clinical utility of cfDNA mutation testing is increasingly accepted additional prospective clinical trials in which therapeutic interventions are tailored on the basis of patients' respective cfDNA mutation statuses are needed.

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Disclosure

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