Clinical Cancer Research

Mutation-Enrichment Next-Generation Sequencing for Quantitative Detection of *KRAS* Mutations in Urine Cell-Free DNA from Patients with Advanced Cancers

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Abstract

Purpose: Tumor-derived cell-free DNA (cfDNA) from urine of patients with cancer offers noninvasive biological material for detection of cancer-related molecular abnormalities such as mutations in Exon 2 of *KRAS*.

Experimental Design: A quantitative, mutation-enrichment next-generation sequencing test for detecting $KRAS^{G12/G13}$ mutations in urine cfDNA was developed, and results were compared with clinical testing of archival tumor tissue and plasma cfDNA from patients with advanced cancer.

Results: With 90 to 110 mL of urine, the $KRAS^{G12/G13}$ cfDNA test had an analytical sensitivity of 0.002% to 0.006% mutant copies in wild-type background. In 71 patients, the concordance between urine cfDNA and tumor was 73% (sensitivity, 63%; specificity, 96%) for all patients and 89% (sensitivity, 80%; specificity, 100%) for patients with urine samples of 90 to 110 mL. Patients had significantly fewer $KRAS^{G12/G13}$ copies in urine cfDNA during systemic therapy than at baseline or disease progression ($P = 0.002$). Compared with no changes or increases in urine cfDNA $KRAS^{G12/G13}$ copies during therapy, decreases in these measures were associated with longer median time to treatment failure ($P = 0.03$).

Conclusions: A quantitative, mutation-enrichment next-generation sequencing test for detecting $KRAS^{G12/G13}$ mutations in urine cfDNA had good concordance with testing of archival tumor tissue. Changes in mutated urine cfDNA were associated with time to treatment failure. *Clin Cancer Res; 23(14); 3657–66. ©2017 AACR.*

Introduction

Detecting molecular alterations can provide guidance for personalized cancer therapy in patients with melanoma, non–small cell lung cancer (NSCLC), colorectal cancer, and other cancers (1–5). *KRAS* mutations are associated with poor prognosis in diverse cancer types and with lack of benefit from anti–EGFR-targeted monoclonal antibodies in colorectal cancer (3, 6–8). Currently, oncogenic alterations such as *KRAS* mutations are assessed in archival tumor tissue, but the tissue availability is often a limiting factor that precludes molecular analysis (9, 10). In addition, mutation assessment of primary tumor tissue or an isolated metastasis does not necessarily reflect the genetic make-up of metastatic disease owing to tumor heterogeneity (11–13). Different oncogenic mutations occur in different areas of a primary tumor, and the mutation statuses of the primary tumor and distant metastases are discrepant in approximately 20% to 30% of cases (12, 14). In addition, translational studies in *EGFR*-mutated NSCLC suggest that cancer genotype can change over time; for example, Sequist and colleagues demonstrated in a group of 37 patients with *EGFR*-mutant NSCLC who had pretreatment and

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

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Translational Relevance

In patients with advanced cancers, mutation-enrichment next-generation sequencing detection of *KRAS*$^{G12/G13}$ mutations in urine cell-free DNA has good concordance with conventional clinical testing of archival tumor tissue, provided that the volume of collected urine is sufficient. Changes in mutated cell-free DNA correspond with time to treatment failure on systemic anticancer therapy.

postprogression tumor biopsies that some mutations can occur and disappear over time (15). Tumor cells undergoing apoptosis or necrosis release small fragments of cell-free (cf) DNA, which can be identified in blood, urine, and other biological materials and offers an alternative source of material for genomic testing (16). Unlike performing tissue biopsies, obtaining samples of urine or plasma cfDNA is less invasive, with less risk to patients at a lower cost, and can be repeated at different times and provide valuable information about genetic changes that occur during the disease evolution. In colorectal cancer, sensitive techniques such as BEAMing (beads, emulsion, amplification, magnetics) PCR, droplet digital PCR, and next-generation sequencing (NGS) detected low-frequency clones with *KRAS* mutations in plasma cfDNA not detected by standard clinical molecular testing, and these clones ultimately led to resistance to EGFR antibodies (17–20).

Preliminary data suggest that molecular testing of urine cfDNA is feasible in patients with advanced cancers (10, 21, 22). The purpose of this study was to develop and validate molecular detection and quantification of exon 2 *KRAS* mutations (*KRAS*$^{G12/G13}$) in urine and plasma cfDNA specimens from patients with advanced cancers and determine whether this approach has acceptable concordance, sensitivity, and specificity with conventional clinical testing of archival tumor samples. In addition, this study sought to determine whether changes in *KRAS*$^{G12/G13}$ copy numbers in urine or plasma cfDNA are correlated with treatment outcomes.

Materials and Methods

Patients

Patients with progressing advanced cancers and known *KRAS* mutation statuses from conventional clinical testing of their archival formalin-fixed, paraffin-embedded (FFPE) tumor tissue specimens (described in Supplementary Methods) treated at The University of Texas MD Anderson, Niguarda Cancer Center, and the University of Southern California Norris Comprehensive Cancer Center were enrolled for urine and plasma collection from December 2012 to November 2015. Patients had the option of providing longitudinally collected samples during the course of their therapy. The study was conducted in accordance with the approval of the participating institutions' Institutional Review Boards and/or with the guidelines of their Ethical Committees.

Sample collection and processing

Urine and plasma samples for cfDNA isolation were collected at the time of disease progression before treatment initiation and, if feasible, repeatedly during subsequent therapy. The recommended urine collection volume was 90 to 110 mL; however, amounts as small as 10 mL were also accepted. Urine samples were collected in 120-mL containers supplemented with preservative and stored at −70°C. For cfDNA extraction, urine was concentrated to 4 mL using Vivacell 100 concentrators (Sartorius Corp.) and incubated with 700 μL of Q-sepharose Fast Flow quaternary ammonium resin (GE Healthcare). Tubes were spun to collect sepharose and bound DNA. The pellet was resuspended in a buffer containing guanidinium hydrochloride and isopropanol, and the eluted DNA was collected as a flow-through using polypropylene chromatography columns (BioRad Laboratories). The DNA was further purified using QiaQuick columns (Qiagen).

At MD Anderson and Niguarda Cancer Center, whole blood was collected in ethylenediaminetetraacetic acid–containing tubes and centrifuged and spun twice within 2 hours to yield plasma. At the University of Southern California, blood was collected in Cell-Free DNA BCT tubes (Streck), which allow storage for up to 2 weeks. The QIAamp Circulating Nucleic Acid Kit (Qiagen) was used to isolate cfDNA from 1.5 to 4 mL of plasma according to the manufacturer's instructions.

KRAS mutation analysis in cfDNA

We developed a new workflow to create an assay capable of detecting a low abundance of *KRAS*$^{G12/G13}$ mutations [$\leq$0.01% in the wild-type (wt) DNA background] in short, highly fragmented urine cfDNA (Supplementary Fig. S1). The urine cfDNA extraction method was designed to preferentially isolate low-molecular-weight (<400 bp) fragments of cfDNA. Quantitative analysis of 7 common mutations (G12A, G12C, G12D, G12R, G12S, G12V, and G13D) in codons 12 or 13 of exon 2 of the *KRAS* gene (*KRAS*$^{G12/G13}$ mutations) was performed using a mutation-enrichment PCR coupled with NGS (Trovagene). An ultra-short footprint PCR assay (gene-specific footprint 31 bp; overall amplicon length of 75 bp) was used to amplify highly degraded cfDNA *KRAS*$^{G12/G13}$ fragments. The PCR amplification utilized a preferential enrichment of *KRAS*$^{G12/G13}$-mutant cfDNA by using oligonucleotides complementary to wt *KRAS* DNA to block annealing of the PCR primers and to suppress the amplification of wt *KRAS* (Supplementary Fig. S2). PCR primers contained a 3′ gene-specific sequence and a 5′ common sequence that was used in the subsequent sample-barcoding step. The PCR enrichment cycling conditions utilized an initial 98°C denaturation step followed by an assay-specific 5 cycles of pre-amplification PCR and 30 cycles of mutation-enrichment PCR. Custom DNA sequencing libraries were constructed and indexed using the Access Array System for Illumina Sequencing Systems (Fluidigm). The indexed libraries were pooled, diluted to equimolar amounts with buffer and the 5% PhiX Control library, and sequenced on an Illumina MiSeq platform at a high depth (~200,000 reads) using 150-V3 sequencing kits (Illumina). Primary image analysis, secondary base calling, and data quality assessment were performed on the MiSeq instrument using RTAv1.18.54 and MiSeq Reporter v2.6.2.3 software. The analysis output (FASTQ files) from the runs was processed using custom sequencing reads counting and variant calling algorithms to tally the sums of total target gene reads (wt *KRAS* or mutant *KRAS* reads) that passed predetermined sequence quality criteria (qscore $\geq$ 20). A custom quantification algorithm was developed to accurately determine the absolute number of mutant DNA molecules in the source cfDNA sample. The algorithm quantifies the mutational copy number by incorporating into each sequencing run a corresponding reference sample set

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with known copy numbers for each of the seven most common *KRAS*$^{G12/G13}$ mutations. Sequencing result from this reference sample set is used to generate standard curves, and the mutant copy number from the source cfDNA sample is calculated by interpolation. Results are standardized to a 100,000 Genome Equivalents (GEq).

The *KRAS*$^{G12/13}$ mutation detection was determined as the number of *KRAS* mutations detected above a predefined cutpoint which were specific for each of the seven *KRAS* mutations assessed. The predefined cutpoint for each *KRAS* mutation was calculated as the copy number obtained from the mean plus three standard deviations of nonspecific signal (copy number) established by analyzing urine cfDNA samples from 150 healthy volunteers and 24 patients with wt *KRAS*$^{G12/G13}$ metastatic cancer (by tumor tissue analysis). Similarly, assay cut-offs for plasma were established by analyzing plasma cfDNA samples from a separate cohort of 40 healthy volunteers and 80 patients with wt *KRAS*$^{G12/G13}$ metastatic cancer (by tumor tissue analysis). Detection cut-offs were standardized to 100,000 GEq.

Statistical analysis

Concordance between the mutation analyses of urine cfDNA, plasma cfDNA, and archival tumor specimens 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.) or SPSS 23 (SPSS) software programs.

Results

Performance of the assay in detecting *KRAS*$^{G12/G13}$ mutations in urine cfDNA

The performance of mutation-enrichment PCR coupled with NGS for the detection of *KRAS*$^{G12/G13}$ mutations in urine cfDNA was investigated by assessing fold mutation enrichment, lower limit of detection, and assay reproducibility in urine. Fold enrichment was assessed by spiking 5 to 500 copies of mutant DNA into 18,181 GEq of wt DNA (0.027%–2.7%). For the 7 most common *KRAS*$^{G12/G13}$ variants, 2,000- to 3,370-fold enrichment of mutant *KRAS*$^{G12/G13}$fragments was obtained for an input of 5 copies of *KRAS*$^{G12/13}$-mutant DNA within 60 ng (18,181 GEq) of wt DNA (Fig. 1A and B). The resulting sequencing libraries comprised 69.5% to 99.7% mutant reads, thus enabling sensitive mutation detection by NGS (Fig. 1A). Resulting fold-enrichment for *KRAS*$^{G12/G13}$-mutant fragments increased inversely with decreasing amount of mutant copies in the wt background (Fig. 1B).

When quantifying rare DNA fragments, the frequency distribution of the number of DNA molecules that will be present in each PCR tube upon repeated measurements can be predicted by the Poisson distribution. Herein, the lower limit of detection was defined as the lowest number of copies for which the frequency distribution of the copy-number events upon repeated measurements fell within the 95% confidence interval (CI) of expected frequency distribution determined by Poisson statistics. For lower limit of detection verification, 20 to 80 repeated measurements were performed on a single multiplexed NGS run for a target spike-in level of 1 mutant *KRAS*$^{G12/G13}$ copy within 18,181 GEq (60 ng) of wt *KRAS* DNA or for a target spike-in level of 2 mutant *KRAS*$^{G12/G13}$copies within 100,000 GEq (330 ng) of wt *KRAS* DNA. Replicates were subjected to mutation-enrichment NGS analysis. The observed distribution of positive and negative hits in our experiments matched the theoretical hit rate of an ideal Poisson distribution for these replicates, confirming 1 copy detection sensitivity of the *KRAS*$^{G12/G13}$ assay in the background of 18,181 wt GEq (0.006%; Fig. 1C) and 2 copies detection sensitivity in a background of 100,000 wt GEq (0.002%; Supplementary Table S1).

The reproducibility of quantitative *KRAS*$^{G12/G13}$ mutation detection was analyzed using urine samples from patients with advanced cancers. Two to three cups (each 90–120 mL) of urine were obtained at a single time point from 3 patients with tumor biopsy specimens positive for *KRAS*$^{G12/G13}$ mutations. Intrapatient reproducibility of the urine *KRAS*$^{G12/G13}$ testing, calculated as the coefficient variation percent (CV%) for repeat measurements, varied from 2.3% to 19.6%. The average interpatient reproducibility (CV%) was 9.7% (Table 1).

Concordance, sensitivity, and specificity of *KRAS*$^{G12/13}$ mutation detection in urine cfDNA compared with tumor

This blinded study with prospectively collected liquid biopsy samples enrolled 71 patients with diverse advanced cancers and archival FFPE tumor specimens with known *KRAS*$^{G12/G13}$ mutation status (Table 2). The patients' median age was 59 years (range, 36–85 years). Most patients were white (n = 51; 72%) and male (n = 38; 54%). The most common tumor type was colorectal cancer (n = 56; 79%), followed by breast cancer (n = 4; 6%) and NSCLC (n = 3; 4%). The median time from tissue to urine sampling was 23.0 months (range, 0.7–91.3 months), and the median time from tissue to plasma sampling was 16.9 months (range, 0.9–80.2 months). The median amount of cfDNA isolated per 1 mL of urine was 9.1 ng (range, 0.2–2057.0 ng) and that isolated per 1 mL of plasma was 18 ng (range, 3.1–605.4 ng).

Of the 71 patients, 49 (69%) had archival tumor specimens with *KRAS*$^{G12/G13}$ mutations, and 31 (44%) had detectable *KRAS*$^{G12/G13}$ mutations in urine cfDNA. There was overall concordance in *KRAS*$^{G12/G13}$ mutation status between urine cfDNA and tumor specimens in 52 cases (73%; kappa, 0.49; SE, 0.09; 95% CI, 0.31–0.66). The urine cfDNA test had a sensitivity of 63% (95% CI, 0.47–0.76), specificity of 96% (95% CI, 0.78–1.00), and positive predictive value (PPV) of 97% (95% CI, 0.83–1.00; Table 3; Supplementary Table S2).

Although the recommended volume for urine specimen collection was 90 to 110 mL, urine specimens with smaller volumes were also collected (median, 60 mL; range, 20–150 mL). Therefore, we investigated whether the collected amount of urine affected the concordance, sensitivity, and specificity of the urine cfDNA test. Among the 43 patients who had urine specimens of >50 mL, there was overall concordance in *KRAS*$^{G12/G13}$ mutation status between urine cfDNA and tumor specimens in 33 cases (77%; kappa, 0.55; SE, 0.11; 95% CI, 0.34–0.77), and the urine cfDNA test had a sensitivity of 66% (95% CI, 0.46–0.82), specificity of 100% (95% CI, 0.77–1.00), and PPV of 100% (95% CI, 0.82–1.00; Table 3). Among the 19 patients who had urine

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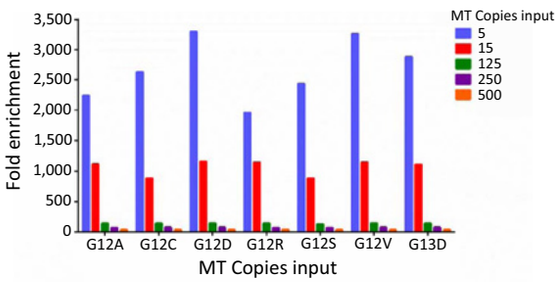
A

Input MT Copies/WT Copies (% Mutant)	Output mutant sequencing reads/wild-type reads (% mutant reads)						
	KRAS G12A	*KRAS* G12C	*KRAS* G12D	*KRAS* G12R	*KRAS* G12S	*KRAS* G12V	*KRAS* G13D
5/18,181 (0.027%)	4151/1381 (62%)	6661/2928 (72%)	13447/858 (91%)	4100/1570 (54%)	2440/882 (67%)	4269/410 (90%)	2318/748 (79%)
15/18,181 (0.082%)	14365/1133 (92%)	2586/864 (74%)	34363/1155 (96%)	37445/2050 (95%)	4614/1774 (73%)	9068/423 (95%)	15726/1053 (92%)
125/18,181 (0.68%)	133074/2662 (98%)	72469/1392 (97%)	156863/1855 (99%)	195110/3634 (98%)	15486/1572 (88%)	144666/1821 (99%)	170503/1348 (99%)
250/18,181 (1.36%)	161048/3353 (98%)	112052/1406 (99%)	309123/2307 (99%)	281142/5513 (98%)	27344/760 (97%)	267933/2452 (99%)	331498/2216 (99%)
500/18,181 (2.7%)	229638/3190 (99%)	194430/3085 (98%)	508045/1442 (100%)	372965/3168 (99%)	41137/632 (98%)	472491/2836 (99%)	585254/1807 (100%)

B



C

Number of mutant copies	0 (Not detected)	1+ (Detected)
Expected (95% CI) [1 copy/replicate]	7 (2–14)	13 (6–20)
Observed:		
G12A	12	8
G12C	5	15
G12D	3	17
G12R	10	10
G12S	6	14
G12V	4	16
G13D	3	17

Figure 1.

Mutation-enrichment NGS platform for the analysis of cfDNA from urine and plasma. **A,** Comparison between the input ratio of mutant/wt *KRAS*$^{G12/G13}$ copies and the output ratio of mutant/wt *KRAS*$^{G12/G13}$ sequencing reads for 5 to 500 input mutant copies of the 7 most common *KRAS*$^{G12/G13}$ variants diluted in 60 ng (~18,180 genome equivalents) of wt DNA (mutation abundance, 0.0275%–2.75%). The output sequencing reads are the means of 18 replicates from 6 independent NGS dilution series experiments performed on 3 different days by 2 operators on 2 MiSeq instruments. **B,** Fold enrichment was calculated as the percentage of input mutant *KRAS*$^{G12/G13}$ molecules divided by the percentage of output mutant *KRAS*$^{G12/G13}$ sequencing reads in **A**. **C,** Verification of the analytical sensitivity (lower limit of detection, 1) of the *KRAS*$^{G12/G13}$ mutation-enrichment NGS assay. A DNA blend with 20 mutant copies in a background of approximately 363,620 wt genome equivalents (0.006%) was prepared and distributed over 20 wells to achieve a target concentration of 1 mutant copy/18,181 genome equivalents per well. Following mutation-enrichment NGS, the observed distribution frequency of the counts of 0 or ≥1 copies across 20 replicates was compared with theoretical Poisson expectations (95% CIs).

specimens of 90 to 110 mL, there was overall concordance in *KRAS*$^{G12/G13}$ mutation status between cfDNA and tumor specimens in 17 cases (89%; kappa, 0.79; SE, 0.14; 95% CI, 0.52–1.00), and the urine cfDNA test had a sensitivity of 80% (95% CI, 0.44–0.97), specificity of 100% (95% CI, 0.66–1.00), and PPV of 100% (95% CI, 0.63–1.00; Table 3).

Of the 71 patients, 33 (46%) had simultaneous collection of plasma cfDNA and urine cfDNA. Among these 33 patients, there was overall concordance in *KRAS*$^{G12/G13}$ mutation status between plasma cfDNA and tumor specimens in 31 cases (94%; kappa, 0.86; SE, 0.10; 95% CI, 0.67–1.00). The plasma cfDNA test had a sensitivity of 92% (95% CI, 0.73–0.99), specificity of 100% (95% CI, 0.66–1.00), and PPV of 100% (95% CI, 0.85–1.00; Table 4; Supplementary Table S2). In addition, there was overall concordance in *KRAS*$^{G12/G13}$ mutation status between urine cfDNA and plasma cfDNA specimens in 22 cases (67%; kappa, 0.35; SE, 0.15; 95% CI, 0.07–0.64). Using plasma as the reference, the urine cfDNA test (10–110 mL) had a sensitivity of 59% (95% CI, 0.36–0.79), specificity of 82% (95% CI, 0.48–0.98), and PPV of 87% (95% CI, 0.60–0.98; Table 4; Supplementary Table S2).

Table 1. Reproducibility of the detection of *KRAS*$^{G12/G13}$ mutations in urine cfDNA from patients with advanced cancer

Patient, replicate	*KRAS* variant	*KRAS*$^{G12/G13}$ copies	CV%	Average CV%
1, 1		18.29		
1, 2	G12S	17.81	2.3	
1, 3		18.66		
2, 1	G13D	195.02	7.0	9.7
2, 2		176.57		
3, 1		10.43		
3, 2	G12D	7.26	19.6	
3, 3		7.91		

NOTE: Two to three urine cups (each 90–120 mL) were collected at a single time point from 3 patients with known *KRAS* mutational status in tumor biopsies. Following urine extraction, cfDNA was assayed by mutation-enrichment NGS. Intra- and interpatient reproducibility was calculated as CV%.

KRAS$^{G12/G13}$-mutant copy number and cfDNA concentration and survival

To determine whether the number of *KRAS*$^{G12/G13}$-mutant copies in urine cfDNA was associated with OS, we first divided the 71 patients into 2 groups: those with <26.3 *KRAS*$^{G12/G13}$-mutant copies and those with ≥26.3 *KRAS*$^{G12/G13}$-mutant copies. The threshold was selected based on a 5% trimmed mean value of *KRAS*$^{G12/G13}$-mutant cfDNA. This was deemed to be appropriate as the median percentage of *KRAS*$^{G12/G13}$-mutant cfDNA was 0% because 40 of the 71 patients had no *KRAS*$^{G12/G13}$ mutations in urine cfDNA. The median OS duration of the 57 patients with <26.3 *KRAS*$^{G12/G13}$-mutant copies (11.1 months; 95% CI, 7.5–14.7 months) and that of the 14 patients with ≥26.3 of

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Table 2. Characteristics of 71 patients enrolled in the study

Characteristic	Number of patients (%)[a]
Median age (range), years	59 (36–85)
Gender	
Male	38 (54)
Female	33 (46)
Ethnicity	
Caucasian	51 (72)
Hispanic	12 (17)
African American	5 (7)
Asian	3 (4)
Cancer type	
Colorectal cancer	56 (79)
Breast cancer	4 (6)
NSCLC	3 (4)
Pancreatic cancer	2 (<3)
Ovarian cancer	2 (<3)
Other cancers	4 (6)
KRAS status in the tissue	
G12C	7 (10)
G12D	24 (34)
G12R	2 (3)
G12S	6 (8)
G12V	6 (8)
G13D	3 (4)
Wild-type	23 (32)
KRAS status in urine cfDNA	
G12C	4 (6)
G12D	17 (24)
G12R	1 (<1)
G12S	4 (6)
G12V	3 (4)
G13D	2 (<3)
Wild-type	40 (56)
KRAS status in plasma cfDNA ($N = 33$)	
G12C	2 (6)
G12D	12 (36)
G12S	2 (6)
G12V	3 (9)
G13D	3 (9)
Wild-type	11 (33)

[a]Unless otherwise indicated.

$KRAS^{G12/G13}$-mutant copies (16.5 months; 95% CI, 5.3–27.7 months) did not differ significantly ($P = 0.63$; Supplementary Fig. S3A). Similarly, again using a threshold selected based on a 5% trimmed mean, we found that the median OS duration of the 23 patients with <198.8 $KRAS^{G12/G13}$-mutant copies in plasma cfDNA (18.7 months; 95% CI, 3.5–33.9 months) and that of the 10 patients with ≥198.8 $KRAS^{G12/G13}$-mutant copies in plasma cfDNA (12.6 months; 95% CI, 11.6–13.4 months) did not differ significantly ($P = 0.90$; Supplementary Fig. S3B).

We next analyzed whether cfDNA concentrations in urine or plasma were associated with OS using thresholds selected based on median values. For the 69 of 71 patients for whom urine cfDNA data were available, the median OS duration of the 35 patients with <9.1 ng of cfDNA/mL (13.0 months; 95% CI, 7.2–18.8 months) and that of the 34 patients with ≥9.1 ng of cfDNA/mL (11.1 months; 95% CI, 7.4–14.8 months) did not differ significantly ($P = 0.31$; Supplementary Fig. S4A). Similarly, for the 33 patients for whom plasma cfDNA data were available, the median OS duration of the 16 patients with <18.0 ng of cfDNA/mL (12.6 months; 95% CI, 5.9–19.2 months) and that of the 17 patients with ≥18 ng of cfDNA/mL (20.6 months; 95% CI, 5.9–35.3 months) did not differ significantly ($P = 0.19$; Supplementary Fig. S4B).

Serial monitoring for $KRAS^{G12/13}$ mutations in the cfDNA of cancer patients on therapy

At least 2 (median, 6; range, 2–13) longitudinal serial urine collections were obtained before and during patients' systemic therapy, which ranged from first-line therapies to experimental therapies after all standard treatment had failed, from 21 patients with $KRAS^{G12/G13}$ mutations in tumor tissue. Of these 21 patients, 17 (81%) had detectable $KRAS^{G12/G13}$ mutations in cfDNA in ≥1 urine specimen. The median $KRAS^{G12/G13}$ copy numbers in specimens collected at baseline (8.6), during therapy (0), and at disease progression (6.9) differed significantly ($P = 0.002$; Fig. 2A). The patients received 21 diverse systemic therapies (Supplementary Table S3). The best response to therapy [complete response (CR) or partial response (PR) or stable disease (SD) ≥ 6 months vs. SD < 6 months or progressive disease (PD)] on imaging per RECIST was not associated with the best change in $KRAS^{G12/G13}$ copy numbers (median change percentage, −100% for patients with CR/PR/SD ≥ 6 months vs. −100% for patients with SD < 6 months/PD; $P = 0.24$; ref. 23). Of the 21 therapies, 16 decreased the $KRAS^{G12/G13}$ copy numbers, and 5 caused no change or increased the $KRAS^{G12/G13}$ copy numbers. The median TTF of the patients with a decrease in $KRAS^{G12/G13}$ copy numbers (4.7 months; 95% CI, 2.6–6.8 months) was significantly longer than that of the patients with no change or an increase in copy numbers (2.8 months; 95% CI, 2.6–3.0 months; $P = 0.03$; Fig. 3A).

At least 2 (median, 5.5; range, 3–14) serial plasma collections were obtained before and during systemic therapy from 18 patients with $KRAS^{G12/G13}$ mutations in tumor tissue. All 18 patients had detectable $KRAS^{G12/G13}$ mutations in cfDNA in ≥1 plasma specimen. The median $KRAS^{G12/G13}$ copy numbers at baseline (488.5), during therapy (11.0), and at disease progression (258.6) differed significantly ($P < 0.001$; Fig. 2B). The patients received 20 diverse systemic therapies (Supplementary Table S3). The best response to therapy (CR, PR, or SD ≥ 6 months vs. SD < 6 months or PD) on imaging per RECIST showed a trend toward association with the best change in copy numbers (median change percentage, −100% for CR/PR/SD ≥ 6 months vs. −36% in SD < 6 months/PD; $P = 0.09$). Of the 18 therapies (2 therapies were excluded because of missing pretreatment $KRAS^{G12/G13}$ copy-number values), 12 decreased the $KRAS^{G12/G13}$ copy numbers, and 6 caused no change or increased $KRAS^{G12/G13}$ copy numbers. The median TTF of the patients with a decrease in $KRAS^{G12/G13}$ copy numbers (5.7 months; 95% CI, 2.8–8.6 months) was significantly longer than that of patients with no change or an increase in copy numbers (3.2 months; 95% CI, 2.1–4.3 months; $P = 0.04$; Fig. 3B).

Discussion

Our findings demonstrate that mutation enrichment leads to an approximately 3,000-fold increase of the $KRAS^{G12/G13}$-mutant signal over the wt signal, which allows the detection of low-frequency mutant copies in samples of urine cfDNA. In a blinded study with prospectively collected samples, our assay using mutation-enrichment PCR coupled with NGS detected $KRAS^{G12/G13}$-mutant copies in urine cfDNA from patients with advanced cancers and had acceptable concordance (73%–89%), sensitivity (63%–80%), and specificity (96%–100%) compared with the clinical testing of FFPE tumor tissue obtained at different times during routine care. The concordance increased with the amount of urine collected, which is ideally 90 to 110 mL. Furthermore, in a

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Table 3. Concordance assessment of *KRAS*$^{G12/G13}$ mutations in FFPE tumor tissue and urine cfDNA from patients with advanced cancers

Concordance for urine samples collected before systemic therapy tested for *KRAS*$^{G12/G13}$ mutations versus FFPE tumor samples tested in the clinical laboratory		
Number of patients, $N = 71$	*KRAS*$^{G12/G13}$ mutation in tumor	*KRAS*$^{G12/G13}$ wild-type in tumor
KRAS$^{G12/G13}$ mutation in cfDNA, number of patients	30	1
KRAS$^{G12/G13}$ wild-type in cfDNA, number of patients	18	22
Observed concordance	52 (73%); kappa, 0.49; SE, 0.09; 95% CI, 0.31–0.66	
Sensitivity	63% (95% CI, 0.47–0.76)	
Specificity	96% (95% CI, 0.78–1.00)	
PPV	97% (95% CI, 0.83–1.00)	
Concordance for urine samples (>50 mL of urine) collected before systemic therapy tested for *KRAS*$^{G12/G13}$ mutations versus FFPE tumor samples tested in the clinical laboratory		
Number of patients, $N = 43$	*KRAS*$^{G12/G13}$ mutation in tumor	*KRAS*$^{G12/G13}$ wild-type in tumor
KRAS$^{G12/G13}$ mutation in cfDNA, number of patients	19	0
KRAS$^{G12/G13}$ wild-type in cfDNA, number of patients	10	14
Observed concordance	33 (77%); kappa, 0.55; SE, 0.11; 95% CI, 0.34–0.77	
Sensitivity	66% (95% CI, 0.46–0.82)	
Specificity	100% (95% CI, 0.77–1.00)	
PPV	100% (95% CI, 0.82–1.00)	
Concordance for urine samples (90–110 mL of urine) collected before systemic therapy tested for *KRAS*$^{G12/G13}$ mutations versus FFPE tumor samples tested in the clinical laboratory		
Number of patients, $N = 19$	*KRAS*$^{G12/G13}$ mutation in tumor	*KRAS*$^{G12/G13}$ wild-type in tumor
KRAS$^{G12/G13}$ mutation in cfDNA, number of patients	8	0
KRAS$^{G12/G13}$ wild-type in cfDNA, number of patients	2	9
Observed concordance	17 (89%); kappa, 0.79; SE, 0.14; 95% CI, 0.52–1.00	
Sensitivity	80% (95% CI, 0.44–0.97)	
Specificity	100% (95% CI, 0.66–1.00)	
PPV	100% (95% CI, 0.63–1.00)	

subset of patients for whom plasma cfDNA was available, we demonstrated excellent concordance of 94% with FFPE tumor tissue (sensitivity, 92%; specificity, 100%).

Although preliminary data on the molecular testing of urine cfDNA have been published, to our knowledge, ours is the first report of the development and laboratory and clinical validation of a urine cfDNA assay, whose concordance with testing of clinical samples appears to be similar to previously published data on plasma cfDNA (10, 21). One recent study 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 patients (24). A certain level of discordance can be anticipated if the tumor tissue and plasma are obtained at different times. Higgins and colleagues (25) found 100% concordance between testing plasma cfDNA with BEAMing PCR and testing simultaneously collected tumor tissue with conventional methods for *PIK3CA* mutations 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, which is consistent with our results. In another study of 100 patients with advanced colorectal cancer, droplet digital PCR detection of *RAS* mutations in plasma cfDNA was in concordance with archival tissue in 97% of cases (20). This rate was favorable compared with most other studies; however, the median time from tissue to plasma collection was only 43 days, which could explain the high concordance rate. In a phase III randomized trial of regorafenib versus placebo, Tabernero and colleagues (26), using BEAMing PCR, showed concordant *KRAS* mutation status between plasma-derived cfDNA and archival tumor samples in 76% of tested patients with advanced colorectal

Table 4. Concordance assessment of *KRAS*$^{G12/G13}$ mutations in plasma cfDNA and FFPE tumor tissue or urine cfDNA from patients with advanced cancers

Concordance for plasma samples collected before systemic therapy tested for *KRAS*$^{G12/G13}$ mutations versus FFPE tumor samples tested in the clinical laboratory		
Number of patients, $N = 33$	*KRAS*$^{G12/G13}$ mutation in tumor	*KRAS*$^{G12/G13}$ wild-type in tumor
KRAS$^{G12/G13}$ mutation in plasma, number of patients	22	0
KRAS$^{G12/G13}$ wild-type in plasma, number of patients	2	9
Observed concordance	31 (94%); kappa, 0.86; SE, 0.10; 95% CI, 0.67–1.00	
Sensitivity	92% (95% CI, 0.73–0.99)	
Specificity	100% (95% CI, 0.66–1.00)	
PPV	100% (95% CI, 0.85–1.00)	
Concordance for plasma and urine samples collected before systemic therapy tested for *KRAS*$^{G12/G13}$ mutations		
Number of patients, $N = 33$	*KRAS*$^{G12/G13}$ mutation in plasma	*KRAS*$^{G12/G13}$ wild-type in plasma
KRAS$^{G12/G13}$ mutation in urine, number of patients	13	2
KRAS$^{G12/G13}$ wild-type in urine, number of patients	9	9
Observed concordance	22 (67%); kappa, 0.35; SE, 0.15; 95% CI, 0.07–0.64	
Sensitivity	59% (95% CI, 0.36–0.79)	
Specificity	82% (95% CI, 0.48–0.98)	
PPV	87% (95% CI, 0.60–0.98)	

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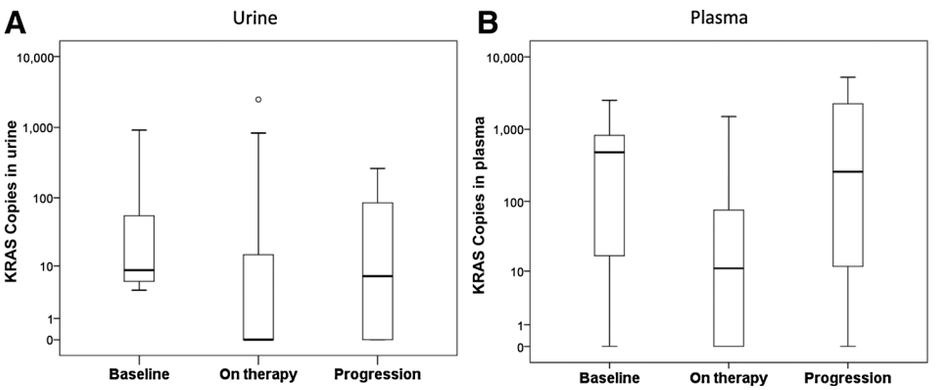

Figure 2.
A, The median *KRAS*$^{G12/G13}$ copy numbers in urine at baseline (8.6), on therapy (0), and at disease progression (6.9) differed significantly ($P = 0.002$). **B,** The median *KRAS*$^{G12/G13}$ copy numbers in plasma at baseline (488.5), during therapy (11.0), and at disease progression (258.6) also differed significantly ($P < 0.001$).

cancer. Thierry and colleagues (27), using allele-specific quantitative PCR of plasma cfDNA and mutation detection in primary or metastatic tissue, demonstrated a 96% concordance for combined *KRAS* and *BRAF* mutation testing. Finally, Sacher and colleagues (28), in the only prospective study to date, demonstrated that digital droplet PCR detected *KRAS*^{G12} mutations in the plasma cfDNA in 64% of patients with known *KRAS*^{G12} mutations in the tumor. Compared with most of these previous studies' findings, our concordance results for *KRAS*$^{G12/G13}$ mutations in urine cfDNA were similar, and those for *KRAS*$^{G12/G13}$ mutations in plasma cfDNA were favorable, despite the fact that the median times between archival tumor tissue collection and urine or plasma collection were relatively long (23.0 months and 16.9 months, respectively) and that fact that urine cfDNA is a far more challenging material because of its short fragments and low mutation allele frequencies (25–29). 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 tumor heterogeneity and evolution, and preanalytical factors such as inadequate specimen collection (28, 30). In addition, testing of urine cfDNA offers a completely noninvasive method, and urine collection does not need to be done by trained personnel, which can expand the use of molecular cfDNA testing.

In our study, we did not find any relationship between OS and *KRAS*$^{G12/G13}$ copy-number values in urine or plasma cfDNA. An earlier study using BEAMing PCR to assess plasma cfDNA for *KRAS*$^{G12/G13}$ mutations in patients with advanced cancers found that a high amount of *KRAS*-mutant cfDNA was associated with shorter OS duration (4.8 months vs. 7.3 months; $P = 0.008$; ref. 24). Another study that used the Idylla system to detect *BRAF*^{V600} mutations in plasma-derived cfDNA from patients with diverse advanced cancers showed that a higher percentage of *BRAF*^{V600}-mutant cfDNA was associated with shorter OS (4.4 months vs. 10.7 months, $P = 0.005$; ref. 31). Similarly, the phase III randomized trial of regorafenib versus placebo showed that high baseline levels of *KRAS*-mutant cfDNA were associated with shorter OS durations in patients with advanced colorectal cancer (26). In other studies, higher amounts of *KRAS*-mutant cfDNA were associated with shorter OS durations in patients with advanced colorectal cancer treated with irinotecan and cetuximab and in patients with advanced NSCLC treated with carboplatin and vinorelbine (32, 33). Similarly, in a combined analysis of clinical trials of BRAF and MEK inhibitors in patients with advanced melanomas, a *BRAF*^{V600E} mutation in cfDNA was associated with shorter OS duration (34). In contrast, in

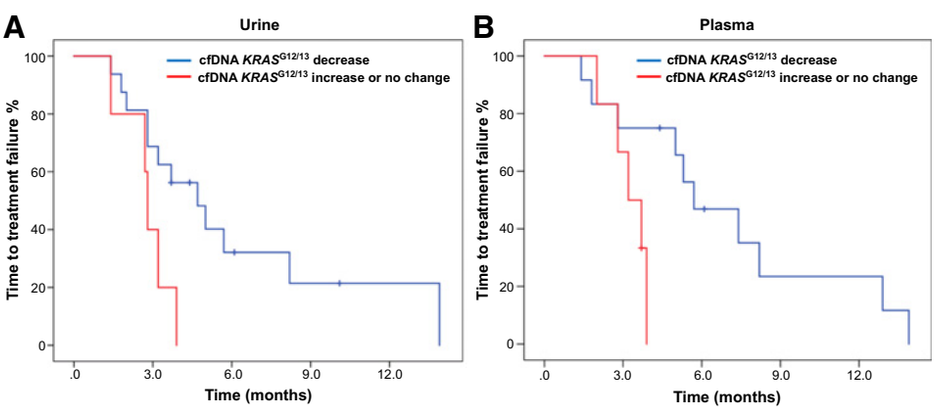

Figure 3.
Association between changes in cfDNA *KRAS*$^{G12/13}$ copies and TTF. **A,** The median TTF of patients with a decrease in *KRAS*$^{G12/G13}$ copy numbers in urine (4.7 months; 95% CI, 2.6–6.8 months; blue) was significantly longer than that of patients with no change or an increase in *KRAS*$^{G12/G13}$ copy numbers in urine (2.8 months; 95% CI, 2.6–3.0 months; red; $P = 0.03$). **B,** The median TTF of patients with a decrease in *KRAS*$^{G12/G13}$ copy numbers in plasma (5.7 months; 95% CI, 2.8–8.6 months; blue) was significantly longer than that of patients with no change or an increase in *KRAS*$^{G12/G13}$ copy numbers in plasma (3.2 months; 95% CI, 2.1–4.3 months; red; $P = 0.04$).

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a study of patients with advanced NSCLC, those with *EGFR* exon 19 deletion in both the tissue and cfDNA had better survival than patients with *EGFR* exon 19 deletion in the tissue only (35). The results of our study may have been affected by the heterogeneity in the tumor types, setting of treatment administration (from first-line to third-line and higher, including clinical trials), and participating institutions and/or by its small sample sizes and large proportion of samples with less-than-optimal urine volumes; these factors may also explain some of the differences between our findings and those of previous studies. A larger prospective study to validate the clinical utility of *KRAS* mutation detection in the urine of patients with advanced colorectal cancer and its association with treatment outcomes is ongoing.

Previous studies have investigated the use of detecting molecular aberrations in cfDNA to monitor response to cancer therapy (19, 21, 36–44). In the present study, we assessed serially collected urine and plasma cfDNA from patients treated with systemic therapies and found that the $KRAS^{G12/G13}$ copy numbers before therapy, during therapy, and at the time of disease progression differed significantly. We also found that patients with a decrease in $KRAS^{G12/G13}$ copy numbers in serially collected urine or plasma cfDNA during therapy had a longer median TTF compared with patients with no change or an increase in copy numbers (4.7 vs. 2.8 months, $P = 0.03$ for urine; 5.7 vs. 3.2 months, $P = 0.04$ for plasma). This observation is consistent with previously published data demonstrating that changes in plasma cfDNA can correspond with treatment outcomes (28, 29, 37–44). In particular, a study using the Idylla system to detect $BRAF^{V600}$ mutations in plasma-derived cfDNA from patients with colorectal or other advanced cancers found that the median TTF of patients who received therapies associated with a decrease in *BRAF*-mutant cfDNA (10.3 months) was significantly longer than that of patients who received therapies associated with an increase or no change in *BRAF*-mutant cfDNA (7.4 months, $P = 0.045$; ref. 31). Overall, however, there is conflicting evidence that such changes in cfDNA can predict or at least correspond with treatment outcomes, and this issue will need to be investigated in future prospective studies.

Our study had several potential limitations. First, the amount of collected urine was suboptimal in many cases, which likely negatively affected concordance and could have affected serial monitoring analysis. Second, our study did not investigate if the timing of urine collection can impact results. Third, the sample size was limited. Fourth, we investigated only $KRAS^{G12/G13}$ mutations, which are clinically relevant to only a limited number of patients with certain tumor types. Finally, because of the heterogeneity in tumor types, systemic therapies, and exploratory nature of the longitudinal analysis, the association between changes in mutant cfDNA and TTF needs to be validated in future prospective studies.

In summary, our study demonstrates that using mutation-enrichment PCR coupled with NGS to molecularly analyze urine cfDNA for the seven most frequent hotspot $KRAS^{G12/G13}$ mutations is feasible and has good concordance with standard mutation testing of discordantly collected FFPE tumor tissue. Our results also suggest that the dynamics of $KRAS^{G12/G13}$-mutant copies in cfDNA corresponds with TTF. The clinical utility of cfDNA mutation testing is gaining increasing acceptance. Regulatory agencies in the United States and European Union have recently approved the use of an *EGFR* mutation plasma cfDNA test for advanced NSCLC when tissue is not available. The clinical utility of serial cfDNA testing is promising and should be further proven in future prospective clinical trials in which therapeutic interventions are tailored based on patients' respective cfDNA mutation statuses.

Disclosure of Potential Conflicts of Interest

G. Siravegna and D. Berz are consultant/advisory board members for Trovagene. A.M. Tsimberidou reports receiving commercial research grants from Foundation Medicine. F. Di Nicolantonio reports receiving commercial research grants from Trovagene. S. Siena is a consultant/advisory board member for Amgen, Bayer, Merck, Roche, and Sanofi. A. Bardelli reports receiving commercial research grants from Trovagene; and is a consultant/advisory board member for Biocartis, Horizon Discovery, and Trovagene. F. Janku reports receiving commercial research grants from Agios, Astellas, BioMed Valley Discoveries, Deciphera, Novartis, Piqur, Roche, Symphogen, and Trovagene; is a consultant/advisory board member for Deciphera and Guardant. No potential conflicts of interest were disclosed by the other authors.

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