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 non-invasive biologic 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 to clinical testing of archival tumor tissue and plasma cfDNA from patients with advanced cancer.

Results: With 90-110 mL of urine, the $KRAS^{G12/G13}$ cfDNA test had an analytical sensitivity of 0.002%-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 90-110 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.

STATEMENT OF SIGNIFICANCE

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.

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–epidermal growth factor receptor (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 20–30% of cases (12, 14). In addition, translational studies in *EGFR*-mutated NSCLC suggest that cancer genotype can change over time; for example, Sequist et al. demonstrated in a group of 37 patients with *EGFR*-mutant NSCLC who had pre-treatment and post-progression 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 biologic 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) polymerase chain reaction (PCR), droplet digital PCR, and next-generation sequencing (NGS) detected low-frequency clones with *KRAS* mutations in plasma cell-free DNA (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.

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 the 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 90–110 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, Bohemia, NY) and incubated with 700 μL of Q-sepharose Fast Flow quaternary ammonium resin (GE Healthcare, Pittsburg, PA). 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, Irvine, CA). The DNA was further purified using QiaQuick columns (Qiagen, Germany).

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, Omaha, NE), which allow storage for up to 2 weeks. The QIAamp Circulating Nucleic Acid kit (Qiagen, Valencia, CA) was used to isolate cfDNA from 1.5–4 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 (≤ 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, San Diego, CA). 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, San Francisco,

CA). 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, San Diego, CA). 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 ≥ 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 with known copy numbers for each of the seven most common *KRAS*$^{G12/G13}$ mutations. Sequencing results 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 pre-defined cutpoint which were specific for each of the seven *KRAS* mutations assessed. The pre-defined cutpoint for each KRAS mutation was calculated as the copy number obtained from the mean plus three standard deviations of non-specific 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 log1 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 2-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

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 5–500 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 1B). The resulting sequencing libraries comprised 69.5%–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, 20–80 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*$^{G12/G13}$ mutations 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. Intra-patient 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 inter-patient reproducibility (CV%) was 9.7% (Table 1).

Concordance, Sensitivity and Specificity of *KRAS*$^{G12/13}$ Mutation Detection in Urine cfDNA Compared to Tumor

This blinded study with prospectively collected liquid biopsy samples enrolled 71 patients with diverse advanced cancers and archival formalin-fixed paraffin-embedded (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; standard error [SE], 0.09; 95% confidence

interval [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 90–110 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 specimens of 90–110 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,

329 0.36–0.79), specificity of 82% (95% CI, 0.48–0.98), and PPV of 87% (95% CI, 0.60–
330 0.98; Table 4; Supplementary Table S2).
331
332 **_KRAS_$^{G12/G13}$-Mutant Copy Number and cfDNA Concentration and Survival**
333 To determine whether the number of _KRAS_$^{G12/G13}$-mutant copies in urine cfDNA
334 was associated with OS, we first divided the 71 patients into 2 groups: those with < 26.3
335 _KRAS_$^{G12/G13}$-mutant copies and those with ≥ 26.3 _KRAS_$^{G12/G13}$-mutant copies. The
336 threshold was selected based on a 5% trimmed mean value of _KRAS_$^{G12/G13}$-mutant
337 cfDNA. This was deemed to be appropriate as the median percentage of _KRAS_$^{G12/G13}$-
338 mutant cfDNA was 0% because 40 of the 71 patients had no _KRAS_$^{G12/G13}$ mutations in
339 urine cfDNA. The median OS duration of the 57 patients with < 26.3 _KRAS_$^{G12/G13}$-mutant
340 copies (11.1 months; 95% CI, 7.5–14.7 months) and that of the 14 patients with ≥ 26.3 of
341 _KRAS_$^{G12/G13}$-mutant copies (16.5 months; 95% CI, 5.3–27.7 months) did not differ
342 significantly (P = 0.63; Supplementary Fig. S3A). Similarly, again using a threshold
343 selected based on a 5% trimmed mean, we found that the median OS duration of the 23
344 patients with < 198.8 _KRAS_$^{G12/G13}$-mutant copies in plasma cfDNA (18.7 months; 95%
345 CI, 3.5–33.9 months) and that of the 10 patients with ≥ 198.8 _KRAS_$^{G12/G13}$-mutant copies
346 in plasma cfDNA (12.6 months; 95% CI, 11.6–13.4 months) did not differ significantly (P
347 = 0.90; Supplementary Fig. S3B).
348 We next analyzed whether cfDNA concentrations in urine or plasma were
349 associated with OS using thresholds selected based on median values. For the 69 of 71
350 patients for whom urine cfDNA data were available, the median OS duration of the 35
351 patients with < 9.1 ng of cfDNA/mL (13.0 months; 95% CI, 7.2–18.8 months) and that of
352 the 34 patients with ≥ 9.1 ng of cfDNA/mL (11.1 months; 95% CI, 7.4–14.8 months) did
353 not differ significantly (P = 0.31; Supplementary Fig. S4A). Similarly, for the 33 patients
354 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 Response Evaluation Criteria in Solid Tumors (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) (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 towards 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 pre-treatment *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 90–110 mL. Furthermore, in a 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 et al. (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 vs. placebo, Tabernero et al. (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 cancer. Thierry et al. (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 et al. (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 non-invasive method and urine collection does not need to be done by a 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; $P = 0.008$) (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, $P = 0.005$) (31). Similarly, the phase III randomized trial of regorafenib vs. 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 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 it is 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, $P = 0.045$) ((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 impacted concordance and could have impacted 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 7 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.

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REFERENCES

1. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med. 2009;361:958-67.
2. 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.
3. 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.
4. Janku F, Wheler JJ, Westin SN, Moulder SL, Naing A, Tsimberidou AM, et al. PI3K/AKT/mTOR inhibitors in patients with breast and gynecologic malignancies harboring PIK3CA mutations. J Clin Oncol. 2012;30:777-82.
5. Sartore-Bianchi A, Trusolino L, Martino C, Bencardino K, Lonardi S, Bergamo F, et al. Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial. Lancet Oncol. 2016;17:738-46.
6. Said R, Ye Y, Falchook GS, Janku F, Naing A, Zinner R, et al. Outcomes of patients with advanced cancer and KRAS mutations in phase I clinical trials. Oncotarget. 2014;5:8937-46.
7. Ihle NT, Byers LA, Kim ES, Saintigny P, Lee JJ, Blumenschein GR, et al. Effect of KRAS oncogene substitutions on protein behavior: implications for signaling and clinical outcome. J Natl Cancer Inst. 2012;104:228-39.
8. Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, Zanon C, Moroni M, Veronese S, et al. Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res. 2007;67:2643-8.
9. Tsimberidou AM, Hong DS, Wheler JJ, Fu S, Piha-Paul S, Naing A, et al. Profile-related evidence to determine individualized cancer therapy (PREDICT): Preliminary results of the Personalized Phase I Clinical Trials program at MD Anderson Cancer Center Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research. 2011:1287.
10. Janku F, Vibat CR, Kosco K, Holley VR, Cabrilo G, Meric-Bernstam F, et al. BRAF V600E mutations in urine and plasma cell-free DNA from patients with Erdheim-Chester disease. Oncotarget. 2014;5:3607-10.
11. Tsimberidou AM, Iskander NG, Hong DS, Wheler JJ, Fu S, Piha-Paul SA, et al. Personalized medicine in a phase I clinical trials program: The M. D. Anderson Cancer Center Initiative. J Clin Oncol. 2011;29:abstr CRA2500.
12. Dupont Jensen J, Laenkholm AV, Knoop A, Ewertz M, Bandaru R, Weihua L, et al. PIK3CA mutations may be discordant between primary and corresponding metastatic disease in Breast Cancer. Clin Cancer Res. 2010.
13. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012;366:883-92.

14. Gonzalez-Angulo AM, Ferrer-Lozano J, Stemke-Hale K, Sahin A, Liu S, Barrera JA, et al. PI3K pathway mutations and PTEN levels in primary and metastatic breast cancer. Mol Cancer Ther. 2011;10:1093-101.
15. 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.
16. 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 review of molecular diagnostics. 2015;15:1631-44.
17. Diaz LA, Jr., Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;486:537-40.
18. Morelli MP, Overman MJ, Dasari A, Kazmi SM, Mazard T, Vilar E, et al. Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment. Ann Oncol. 2015;26:731-6.
19. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;486:532-6.
20. 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.
21. Hyman DM, Diamond EL, Vibat CR, Hassaine L, Poole JC, Patel M, et al. Prospective blinded study of BRAFV600E mutation detection in cell-free DNA of patients with systemic histiocytic disorders. Cancer Discov. 2015;5:64-71.
22. Reckamp KL, Melnikova VO, Karlovich C, Sequist LV, Camidge DR, Wakelee H, et al. A Highly Sensitive and Quantitative Test Platform for Detection of NSCLC EGFR Mutations in Urine and Plasma. J Thorac Oncol. 2016.
23. 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.
24. 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.
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. 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.
27. Thierry AR, Mouliere F, El Messaoudi S, Mollevi C, Lopez-Crapez E, Rolet F, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med. 2014;20:430-5.

626 28. Sacher AG, Paweletz C, Dahlberg SE, Alden RS, O'Connell A, Feeney N, et al.
627 Prospective Validation of Rapid Plasma Genotyping for the Detection of EGFR and
628 KRAS Mutations in Advanced Lung Cancer. JAMA Oncol. 2016.
629 29. Frenel JS, Carreira S, Goodall J, Roda D, Perez-Lopez R, Tunariu N, et al. Serial
630 Next-Generation Sequencing of Circulating Cell-Free DNA Evaluating Tumor Clone
631 Response To Molecularly Targeted Drug Administration. Clin Cancer Res.
632 2015;21:4586-96.
633 30. Meric-Bernstam F, Frampton GM, Ferrer-Lozano J, Yelensky R, Perez-Fidalgo
634 JA, Wang Y, et al. Concordance of genomic alterations between primary and
635 recurrent breast cancer. Mol Cancer Ther. 2014;13:1382-9.
636 31. Janku F, Huang HJ, Claes B, Falchook GS, Fu S, Hong D, et al. BRAF Mutation
637 Testing in Cell-Free DNA from the Plasma of Patients with Advanced Cancers Using a
638 Rapid, Automated Molecular Diagnostics System. Mol Cancer Ther. 2016.
639 32. Nygaard AD, Garm Spindler KL, Pallisgaard N, Andersen RF, Jakobsen A. The
640 prognostic value of KRAS mutated plasma DNA in advanced non-small cell lung
641 cancer. Lung Cancer. 2013;79:312-7.
642 33. Spindler KL, Pallisgaard N, Vogelius I, Jakobsen A. Quantitative cell-free DNA,
643 KRAS, and BRAF mutations in plasma from patients with metastatic colorectal
644 cancer during treatment with cetuximab and irinotecan. Clin Cancer Res.
645 2012;18:1177-85.
646 34. Santiago-Walker A, Gagnon R, Mazumdar J, Casey M, Long GV, Schadendorf D,
647 et al. Correlation of BRAF Mutation Status in Circulating-Free DNA and Tumor and
648 Association with Clinical Outcome across Four BRAFi and MEKi Clinical Trials. Clin
649 Cancer Res. 2016;22:567-74.
650 35. Karachaliou N, Mayo-de las Casas C, Queralt C, de Aguirre I, Melloni B,
651 Cardenal F, et al. Association of EGFR L858R Mutation in Circulating Free DNA With
652 Survival in the EURTAC Trial. JAMA Oncol. 2015;1:149-57.
653 36. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al.
654 Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci
655 Transl Med. 2014;6:224ra24.
656 37. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of
657 circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med.
658 2013;368:1199-209.
659 38. Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, et al.
660 Noninvasive identification and monitoring of cancer mutations by targeted deep
661 sequencing of plasma DNA. Science translational medicine. 2012;4:136ra68.
662 39. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-
663 invasive analysis of acquired resistance to cancer therapy by sequencing of plasma
664 DNA. Nature. 2013;497:108-12.
665 40. Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al.
666 Noninvasive detection of response and resistance in EGFR-mutant lung cancer using
667 quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res.
668 2014;20:1698-705.
669 41. Mok T, Wu YL, Lee JS, Yu CJ, Sriuranpong V, Sandoval-Tan J, et al. Detection
670 and Dynamic Changes of EGFR Mutations from Circulating Tumor DNA as a

671 Predictor of Survival Outcomes in NSCLC Patients Treated with First-line
672 Intercalated Erlotinib and Chemotherapy. Clin Cancer Res. 2015;21:3196-203.
673 42. Karlovich C, Goldman JW, Sun JM, Mann E, Sequist LV, Konopa K, et al.
674 Assessment of EGFR Mutation Status in Matched Plasma and Tumor Tissue of NSCLC
675 Patients from a Phase I Study of Rociletinib (CO-1686). Clin Cancer Res.
676 2016;22:2386-95.
677 43. Marchetti A, Palma JF, Felicioni L, De Pas TM, Chiari R, Del Grammastro M, et
678 al. Early Prediction of Response to Tyrosine Kinase Inhibitors by Quantification of
679 EGFR Mutations in Plasma of NSCLC Patients. J Thorac Oncol. 2015;10:1437-43.
680 44. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating
681 mutant DNA to assess tumor dynamics. Nat Med. 2008;14:985-90.
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Table 1. Reproducibility of the detection of *KRAS*$^{G12/G13}$ mutations in urine cell-free DNA from patients with advanced cancer. 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 inter-patient reproducibility was calculated as CV%.

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

Abbreviation: CV%, coefficient variation percent.

694 **Table 2**. Characteristics of 71 patients enrolled in the study.

Characteristic	**No. of Patients (%)***
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)
Non-small cell lung cancer	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)

695 *Unless otherwise indicated.

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Table 3. Concordance assessment of *KRAS*$^{G12/G13}$ mutations in formalin-fixed, paraffin-embedded (FFPE) tumor tissue and urine cell-free DNA (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, no. of patients	30	1
KRAS$^{G12/G13}$ wild-type in cfDNA, no. 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)	
Positive predictive value	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, no. of patients	19	0
KRAS$^{G12/G13}$ wild-type in cfDNA, no. 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)	
Positive predictive value	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, no. of patients	8	0
KRAS$^{G12/G13}$ wild-type in cfDNA, no. 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)	
Positive predictive value	100% (95% CI, 0.63-1.00)	

703 **Table 4.** Concordance assessment of *KRAS*$^{G12/G13}$ mutations in plasma cell-free DNA

704 (cfDNA) and formalin-fixed, paraffin-embedded (FFPE) tumor tissue or urine cfDNA from

705 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, no. of patients	22	0
KRAS$^{G12/G13}$ wild-type in plasma, no. 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)	
Positive predictive value	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, no. of patients	13	2
KRAS$^{G12/G13}$ wild-type in urine, no. 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)	
Positive predictive value	87% (95% CI, 0.60-0.98)	

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Figure Legends

Figure 1. Mutation-enrichment next-generation sequencing (NGS) platform for the analysis of cell-free DNA from urine and plasma. **A.** Comparison between the input ratio of mutant/wild-type (wt) *KRAS*$^{G12/G13}$ copies and the output ratio of mutant/wt *KRAS*$^{G12/G13}$ sequencing reads for 5-500 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 percent of input mutant *KRAS*$^{G12/G13}$ molecules divided by the percent 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 ~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 to theoretical Poisson expectations (95% confidence intervals [CIs]).

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 ($P < 0.001$).

Figure 3. Association between changes in cell-free DNA *KRAS*$^{G12/13}$ copies and time to treatment failure (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

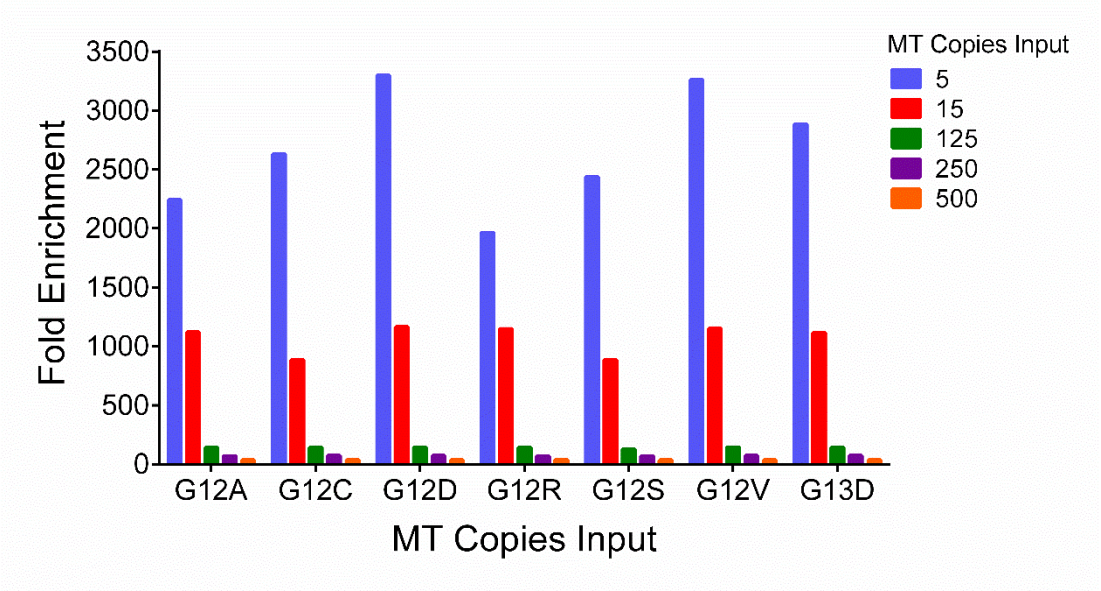
longer than that of patients with no change or an increase in *KRAS*$^{G12/G13}$ copy numbers in urine (2.8 months; 95% CI, 2.6-3.0 months; red; $P = 0.03$). **B.** The median TTF of patients with a decrease in *KRAS*$^{G12/G13}$ copy numbers in plasma (5.7 months; 95% CI, 2.8-8.6 months; blue) was significantly 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; $P = 0.04$).

Figure 1.

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 2.

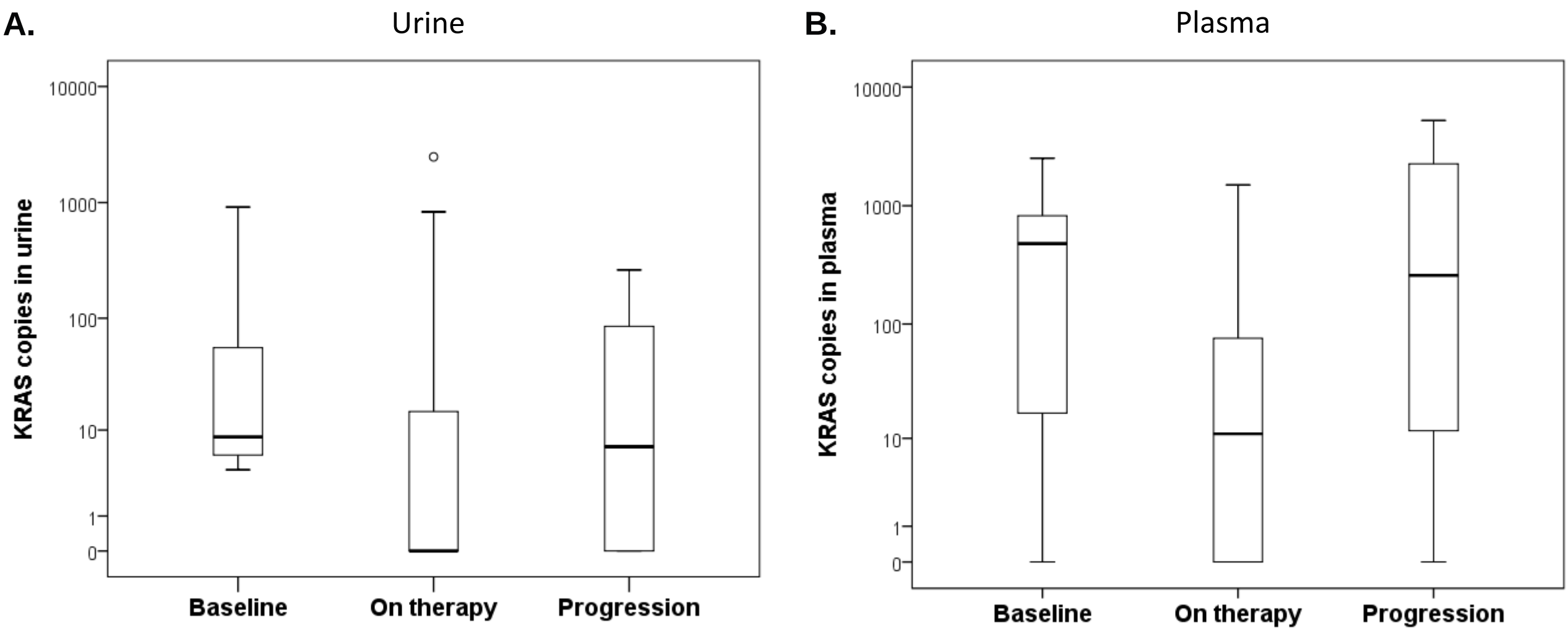
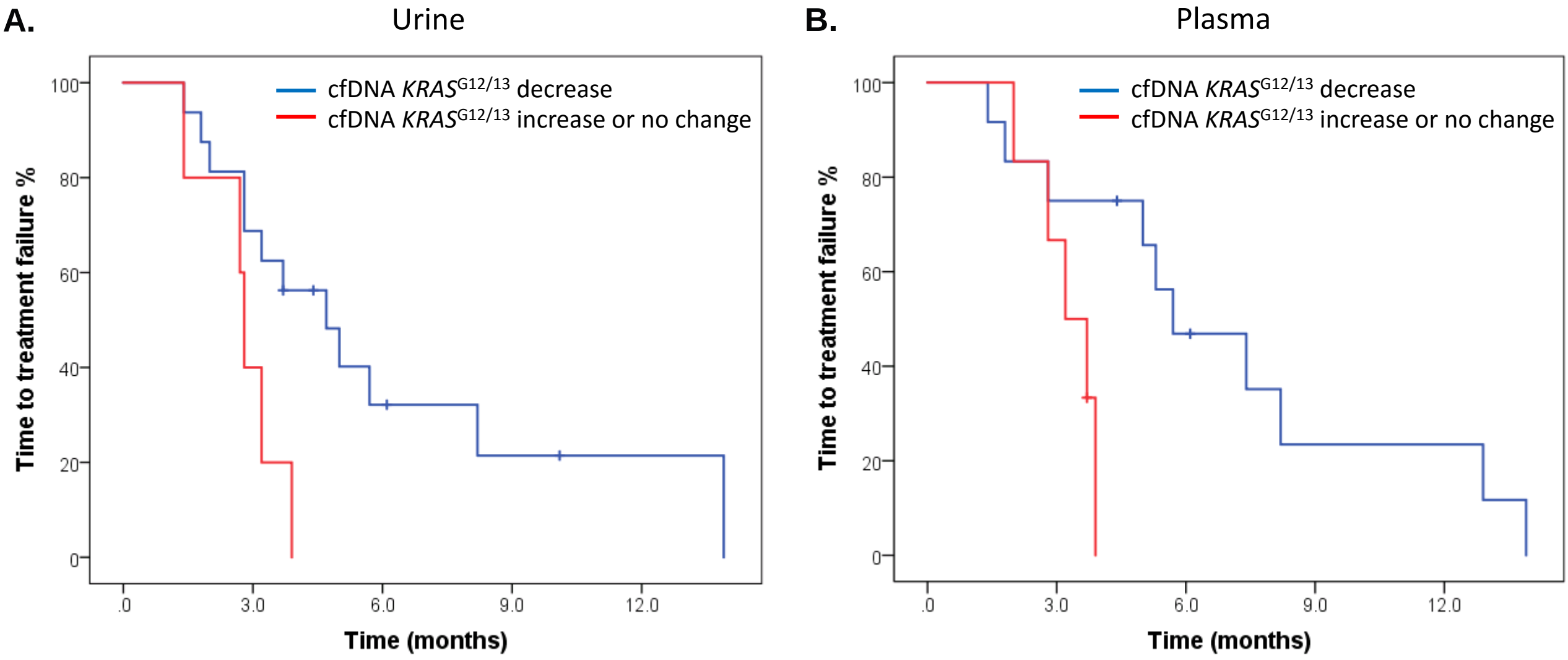

A.
Urine
KRAS copies in urine
10000
1000
100
10
1
0
Baseline
On therapy
Progression
B.
Plasma
KRAS copies in plasma
10000
1000
100
10
1
0
Baseline
On therapy
Progression

Figure 3.


A.
Urine
B.
Plasma
cfDNA KRASG12/13 decrease
cfDNA KRASG12/13 increase or no change
Time to treatment failure %
100
80
60
40
20
0
.0
3.0
6.0
9.0
12.0
Time (months)