Prognostic value of absolute quantification of mutated KRAS in circulating tumour DNA in lung adenocarcinoma patients prior to therapy

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Abstract

Droplet digital polymerase chain reaction (ddPCR) is a highly sensitive and accurate method for quantification of nucleic acid sequences. We used absolute quantification of mutated v-Ki-ras2 Kirsten rat sarcoma viral oncogene homology gene (KRAS) by ddPCR to investigate the prognostic role of mutated KRAS in patients with KRAS-mutated lung adenocarcinomas. Pre-treatment plasma samples from 60 patients with stages I–IV KRAS-mutated lung adenocarcinomas were analysed for KRAS mutations. The associations between survival, detectable KRAS mutations in plasma, and the plasma concentration of mutated KRAS were assessed. Overall, 23 of 60 (38%) patients had detectable KRAS mutation in plasma. The percentage of patients with detectable mutation was 8% in stage I, 30% in stage II, 71% in stage III, and 73% in stage IV. Estimated overall median progression-free survival (PFS) and overall survival (OS) were 26.2 months [95% confidence interval (CI) 12.5–39.9] and 50.8 months (95% CI 0–107.3), respectively. Patients with detectable mutations in plasma had significantly worse median PFS compared to patients with undetectable mutation (13.1 versus 70.1 months) and shorter median OS (20.7 versus not reached). High circulating tumour DNA (ctDNA) concentrations of mutated KRAS were significantly associated with shorter PFS [hazard ratio (HR) 1.008, 95% CI 1.004–1.012] and OS (HR 1.007, 95% CI 1.003–1.011). All associations remained statistically significant in multivariable analyses. In conclusion, ddPCR is an accurate and easily feasible technique for quantification of KRAS mutations in ctDNA. The presence of detectable KRAS mutation in plasma at baseline was associated with worse PFS and OS. High concentration of mutated KRAS in ctDNA was an independent negative prognostic factor for both PFS and OS.

Keywords: non-small cell lung cancer; liquid biopsy; plasma analyses; droplet digital PCR; ctDNA levels

Introduction

Despite recent advances in the treatment of non-small cell lung cancer (NSCLC) with the introduction of tyrosine kinase inhibitors and immune checkpoint inhibitors, lung cancer is still the leading cause of death worldwide. Next-generation sequencing (NGS) technologies have become widely accessible and have improved the knowledge of druggable genetic aberrations in tumour DNA, as well as genetic aberrations interfering with targeted treatment strategies. However, there is still a need for easily assessable biomarkers that can be used for better prognostication of individual patients. Analyses of circulating cell-free DNA (cfDNA) and circulating tumour DNA (ctDNA) are promising approaches. ctDNA comprises DNA released from non-malignant cells and a smaller ctDNA fraction derived from apoptotic and necrotic tumour cells [1] and possibly by active release from...
tumour cells [2]. ctDNA may include information from genetically heterogeneous areas of the primary tumour and metastatic sites, possibly reflecting the whole tumour genome [3]. ctDNA analyses have a growing number of clinical applications. In lung adenocarcinoma patients, ctDNA analyses for the detection of druggable genetic aberrations, or mutations associated with treatment resistance mechanisms, have become an important alternative when tissue is not available for analysis [4–9]. Studies also indicate that detectable ctDNA and high pre-treatment ctDNA concentrations are associated with a negative impact on survival in various cancer types [10–13]. Detectable ctDNA after treatment for localised cancer may predict recurrence [14], while an increase in ctDNA concentration during follow-up may indicate relapse or progression, enabling disease monitoring and detection of progression in advance of image detection [15–17].

Hot-spot mutations in codons 12 and 13 in exon 2 of the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homology gene (KRAS) are the most common driver mutations in lung adenocarcinomas and are found in approximately 25–30% of non-Asian patients [18,19]. Thus, we considered KRAS a relevant biomarker for investigating the prognostic role of ctDNA in patients with lung adenocarcinomas harbouring KRAS mutations. Using droplet digital polymerase chain reaction (ddPCR) technology for accurate quantification of mutated KRAS, we aimed to explore whether there was a difference in progression-free survival (PFS) and overall survival (OS) between patients with and without detectable KRAS mutations in plasma at baseline. We also explored whether there was an association between the plasma concentration of mutated KRAS and PFS and OS.

Materials and methods

Ethics

This study was approved by the Regional Committee for Medical and Health Research Ethics (REC) in Central Norway. Both tissue and plasma samples were collected from Biobank1®, the regional research biobank in Central Norway. The biobank is approved by the REC in Central Norway, the Ministry of Health and Care Services, and the Norwegian Data Protection Authority. Patients enrolled in the biobank are over 18 years old and have given written informed consent.

Patient inclusion and tumour specimens

A total of 553 patients with NSCLC were included in the biobank from 2007 to 2018. All tumour specimens in the biobank were reviewed and classified according to the World Health Organization (WHO) 2015 classification of lung tumours [20] by two lung pathologists (SGFW and VGD). Clinical and pathological disease stages were restaged according to the Eighth Edition of the TNM Classification for lung cancer. Patients fulfilling the following criteria were included: treatment-naïve KRAS-mutated lung adenocarcinoma, available blood sample collected prior to first treatment, and a time interval of ≥5 years between the final control of any previous cancer and the lung cancer. Sixty patients fulfilled all criteria and were eligible for this study (Figure 1). Clinicopathological baseline characteristics were collected from the hospital medical records. Tumour specimens were tested for KRAS mutations using either the KRAS mutation analysis protocol implemented for routine diagnostics at the Department of Pathology, St. Olavs Hospital, or targeted NGS using the Trusight Tumour 26 panel (Illumina®, San Diego, CA, USA).

Blood samples and DNA extraction from plasma

A total of 10–18 ml peripheral whole blood was collected in ethylenediaminetetraacetic (EDTA) acid tubes. Plasma was prepared within 1 h by centrifugation at 4 °C, either at 2500 × g for 10 min or at 1500 × g for 15 min. Samples collected after 2016 had a second centrifugation step at 10 000 × g for 10 min at 4 °C. The plasma samples were then aliquoted, transferred to cryotubes (Nalgene™ Cryotubes, Thermo Scientific™; Thermo Fisher Scientific Inc., Waltham, MA, USA), and stored at −80 °C until DNA extraction. The plasma samples were thawed at room temperature, ctDNA was extracted from 2 to 4 ml plasma using the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer’s manual and was finally eluted in 50 µl of elution buffer. The DNA concentration was quantified by a Qubit fluorometer (Thermo Fisher Scientific Inc.).

Plasma samples used as KRAS mutation-negative controls were prepared according to the same protocol from deidentified healthy donor blood obtained from the Department of Immunology and Transfusion Medicine at St. Olavs Hospital and from patients included in the biobank.

Validation of ddPCR assays

Before running the patient samples, we performed mutation serial dilutions for each assay to test the ability to detect and quantify specific mutant alleles in a background of excess wild-type (wt) DNA. Mutated KRAS plasmid templates were serially diluted to 1:2 from a
stock solution of 250 copies/μl down to 2 copies/μl for all target KRAS mutations and further down to 0.5 copies/μl for KRAS G12A. A 2-μl plasmid solution was spiked into a background of 4 ng of genomic wt DNA (approximately 1212 copies) extracted from blood samples from healthy individuals. The plasmid titre samples and PCR reagents were loaded onto the QX200 Droplet Digital PCR System (Bio-Rad Laboratories Inc., Hercules, CA, USA) in triplicate or quadruplicate. In addition to each serial dilution assay, we ran the following controls: KRAS wt reference standards (Horizon Discovery, Cambridge, UK) for assessment of assay performance; wt genomic DNA controls extracted from blood samples from healthy individuals (the same DNA used in the KRAS plasmid dilutions) for the detection of false-positive mutant KRAS; and DNA-free, non-template controls (NTCs) for contamination. In addition to each serial dilution assay, we ran the following controls: KRAS wt reference standards (Horizon Discovery, Cambridge, UK) for assessment of assay performance; wt genomic DNA controls extracted from blood samples from healthy individuals (the same DNA used in the KRAS plasmid dilutions) for the detection of false-positive mutant KRAS; and DNA-free, non-template controls (NTCs) for contamination. The PCR and data analyses were performed as described for the KRAS analyses of plasma samples. The wt reference standards, NTCs, and cfDNA from healthy donors were used as controls when running the patient samples. The wt genomic DNA from healthy donors was also used as a control in some of the set-ups. The performance of the ddPCR assays was investigated using regression analysis to assess the linearity of the serial dilutions.

For determination of the limit of detection (LOD), the distribution of false-positive mutant KRAS copies in the wt standards and wt controls per reaction for each assay in both the mutation titration series and patient sample set-ups was recorded. A 95% confidence interval (CI) for false positives per assay was calculated, and the upper limit of the CI was defined as the LOD.

**KRAS analysis of plasma**

KRAS analysis of plasma DNA was performed using the QX200 Droplet Digital PCR System and Bio-Rad’s ddPCR KRAS mutation assays (Bio-Rad Laboratories). In brief, 5 μl of plasma DNA (2–30 ng) was mixed in a total reaction volume of 20 μl with ddPCR Supermix and primers and Taqman probes (Bio-Rad Laboratories) for both wt and mutated KRAS alleles.
Patient characteristics

An overview of patient characteristics is presented in Table 1. Median age was 69 (range 47–83) years; all patients were former or current smokers; 37 (62%) were women; and 25 (42%) had stage I disease, 10 (17%) stage II, 14 (23%) stage III, and 11 (18%) had stage IV disease. Thirty-three patients (55%) had WHO PS 0, 23 (38%) PS 1, and 4 (7%) PS 2. The primary treatment was surgery in 42 patients (70%), curative chemoradiotherapy in 3 (5%), and palliative treatment in 15 (25%). Of the latter, 12 (80%) received platinum-doublet chemotherapy, 1 (7%) received whole-brain radiotherapy only, and 2 (13%) received palliative radiotherapy. The distribution of KRAS mutations was as follows: G12C in 31 (52%) patients, G12V in 13 (22%), G12D in 9 (15%), G12A in 3 (5%), G12S in 2 (3%), and G13D in 2 (3%) patients. Detailed clinical data and tumour characteristics are listed in supplementary material, Table S1.

Assay validation and determination of detection limits

Regression analyses revealed good linearity over the range of dilutions with the following $R^2$ values: 0.9957 for G12V, 0.9906 for G12C, 0.9963 for G12D, 0.9979 for G12S, 0.9884 for G12A, and 0.9933 for G13D (see supplementary material, Figure S1). The average number of false-positive mutated KRAS copies detected per reaction was 0 for G12V, 0.0326 for G12C, 0.392 for G12D, 0.093 for G12S, 0 for G12A, and 0.063 for G13D. The upper limit of the 95% CI for each assay was defined as the LOD and was as follows: 0 for G12V and G12A, 0.69 mutated copies for G12D, 0 for G12A, 0.10 mutated copies for G12C, 0.29 mutated copies for G12S, and 0.19 mutated copies for G13D.

Plasma

The plasma samples were obtained as part of the primary investigation, with a median of 19 (range 0–307) days prior to first treatment. In 38 patients, the plasma samples were collected <38 days before first treatment; in 18 patients, between 30 and 89 days before first treatment; and in 4 patients, >90 days prior to first treatment (see supplementary material, Table S1).
The median cfDNA yield from plasma was 1.04 (range 0.40–6.24) ng/μl for disease stage I, 0.93 (range 0.46–5.03) ng/μl for stage II, 0.75 (range 0.40–1.58) ng/μl for stage III, and 0.76 (range 0.37–1.28) ng/μl for stage IV. There was no difference in the cfDNA yield across the disease stages (p = 0.119). The median DNA yield in patients with detectable KRAS mutations in plasma was 0.89 (range 0.41–2.04) ng/μl and 0.90 (range 0.37–1.58) ng/μl in patients with non-detectable KRAS mutation. The difference in DNA yield between these groups was not significant (p = 0.67). Overall, 23 of 60 (38%) patients had detectable KRAS mutation in plasma. The proportion of patients with detectable KRAS mutation in plasma increased significantly with increasing disease stage: in stage I, 2 of 25 (8%) had detectable mutation; in stage II, 3 of 10 (30%); in stage III, 10 of 14 (71%); and in stage IV, 8 of 11 (73%) (p < 0.001). The concentration of mutated KRAS increased significantly with disease stage, with a median of 0 (range 0–3.3) copies/ml for stage I, 0 (range 0–92.4) for stage II, 11.26 (range 0–217.8) for stage III, and 84.1 (range 0–1208.0) for stage IV (p < 0.001). Within the group of patients with detectable mutations, the median concentration was 37.0 (range 1.1–1208) mutated KRAS copies/ml plasma. Results of the ctDNA analyses are summarised in supplementary material, Table S1.

### Progression-free and overall survival

The median follow up for PFS was 38.7 (range 9.4–115.5) months; 27 patients were alive and progression free when the data collection was completed. The median follow-up for OS was 40.9 (range 9.4–142.0) months; 32 patients were alive at the time of data collection completion.

For all patients, the estimated median PFS was 26.2 months (95% CI 12.5–39.9), and the estimated
median OS was 50.8 months (95% CI 0–107.3). Patients with detectable plasma KRAS mutations had significantly worse median PFS compared with the patients with non-detectable KRAS mutation (13.1 versus 70.1 months, p < 0.001) and significantly shorter median OS (20.7 versus not reached, p = 0.002) (Figure 2). These associations remained statistically significant in the multivariable analyses, adjusting for baseline characteristics for both PFS [hazard ratio (HR) 2.76, 95% CI 1.064–7.162, p = 0.037] and OS (HR 3.609, 95% CI 1.261–10.328, p = 0.017).

The concentration of mutated KRAS was significantly associated with both shorter PFS (HR 1.008, 95% CI 1.004–1.012, p < 0.001) and OS (HR 1.007, 95% CI 1.003–1.011, p = 0.001). The associations remained statistically significant in the multivariable analyses (Table 2) for both PFS (HR 1.008, 95% CI 1.002–1.014, p = 0.005) and OS (HR 1.009, 95% CI 1.003–1.016, p = 0.004).

To illustrate the association between mutated KRAS plasma concentration and survival, the patients were split into three groups: (1) those with plasma concentrations of mutated KRAS above the median of 37.0 mutated copies/ml, (2) those with concentrations below 37.0 mutated copies/ml, and (3) those without detectable KRAS mutations. For the three groups, the median PFS was 6.7 versus 16.1 versus 70.1 months, respectively (p < 0.001), and the median OS was 6.7 months, 28.1 months, and not reached, respectively (p = 0.002) (Figure 3).

Figure 2. Association between detectable KRAS mutation in plasma and PFS (A) and OS (B). NR, not reached.

Table 2. Associations between mutant KRAS plasma concentration, PFS, and OS.

|                      | PFS       |      |      |                      | OS        |      |      |
|----------------------|-----------|------|------|----------------------|-----------|------|------|
|                      | HR        | 95% CI | p    |                      | HR        | 95% CI | p    |
| Age (years)          |           |       |      |                      |           |       |      |
| Sex                  |           |       |      |                      |           |       |      |
| Female               | 1.01      | 0.96–1.07 | 0.65 |                      | 1.05      | 1.00–1.11 | 0.06 |
| Male                 | 0.66      | 0.26–1.70 | 0.39 |                      | 0.55      | 0.19–1.55 | 0.26 |
| Clinical stage       |           |       |      |                      |           |       |      |
| I                    | 1.11      | 0.54–8.25 | 0.28 |                      | 1.20      | 0.47–10.40 | 0.32 |
| II                   | 2.70      | 0.71–10.30 | 0.15 |                      | 2.20      | 0.47–10.40 | 0.32 |
| III                  | 2.18      | 0.22–21.94 | 0.51 |                      | 3.58      | 0.28–45.46 | 0.33 |
| IV                   | 2.18      | 0.22–21.94 | 0.51 |                      | 3.58      | 0.28–45.46 | 0.33 |
| WHO PS               |           |       |      |                      |           |       |      |
| 0                    | 1.72      | 0.30–1.73 | 0.47 |                      | 0.84      | 0.33–2.10 | 0.70 |
| 1                    | 4.45      | 0.74–26.68 | 0.10 |                      | 8.31      | 1.24–55.62 | 0.03 |
| 2                    | 1.00     | 1.00–1.01 | 0.005 |                      | 1.00     | 1.00–1.01 | 0.004 |
| Treatment intention  |           |       |      |                      |           |       |      |
| Complete resection   | 1.02      | 0.18–5.72 | 0.99 |                      | 0.61      | 0.06–6.02 | 0.68 |
| Curative radiochemotherapy | 9.76  | 1.55–61.49 | 0.02 |                      | 5.48      | 0.86–34.79 | 0.07 |
| Palliative chemotherapy and/or radiotherapy | 1.008     | 1.002–1.014 | 0.005 |                      | 1.009     | 1.003–1.016 | 0.004 |

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Discussion

In summary, 23 (38%) of the 60 patients had detectable \textit{KRAS} mutation in their baseline plasma sample. There was no difference in DNA yield across disease stages or between patients with or without detectable \textit{KRAS} mutations in plasma. The fraction of patients with detectable \textit{KRAS} mutations and the concentration of mutated \textit{KRAS} increased with increasing disease stage. Patients with detectable plasma \textit{KRAS} mutations had significantly worse PFS and OS than the patients with undetectable mutations. There was also a negative association between high concentrations of mutated \textit{KRAS} and PFS and OS.

The increase in ctDNA concentration with increasing disease stage is well known and is consistent with the results of other studies [3,10,14]. In addition, the fraction of patients with detectable \textit{KRAS} mutations across disease stages in our study is consistent with other studies on NSCLC using ddPCR for the \textit{KRAS} analyses of plasma [21–23].

A negative association between detectable \textit{KRAS} mutations in baseline plasma and survival has been demonstrated in studies on patients with pancreatic and colorectal cancer [24–26]. Qualitative studies of patients with NSCLC have also demonstrated an association between detectable \textit{KRAS} mutations in serum or plasma at baseline and worse PFS and/or OS [23,27–33]. A recent study, where plasma was analysed for known genetic aberrations in tumour DNA, also showed that detectable baseline ctDNA was associated with shorter OS [34]. However, in three other studies on the detection of \textit{KRAS} mutations in serum or plasma, no associations with survival were found [35–37]. The results of two of these studies might be explained by heterogeneity of the study populations (some included non-adenocarcinoma patients), no prior analyses of corresponding tumour DNA, and a limited number of hot-spot \textit{KRAS} mutations targeted in the ctDNA analyses [35,36]. In the third study [37], only patients with advanced \textit{KRAS} mutated NSCLC were included, but the proportion of patients with detectable \textit{KRAS} mutation in plasma was lower (48%) compared to the detection rate in other, similar studies [21,22]. ctDNA studies of lung adenocarcinoma patients harbouring mutations of the epidermal growth factor receptor (\textit{EGFR}) gene have also shown diverging results, but these might be explained by differences in treatment history [38–40].

Quantitative studies of ctDNA levels, assessing either the concentration of ctDNA or the mutant allele frequency (MAF), have shown a negative correlation between high baseline ctDNA concentration and survival in colorectal cancer [10,41], pancreatic cancer [13,42], breast cancer [11,43], oesophageal cancer [44], and malignant melanoma [12,45]. While many studies on NSCLC have shown a negative association between the levels of ctDNA, as summarised in the meta-analyses by Ai \textit{et al} and Cargnin \textit{et al} [46,47], few have assessed the impact of mutant ctDNA levels before the first treatment. Two recent studies have suggested that

Figure 3. Association between ctDNA concentration of mutated \textit{KRAS} (copies/ml) and (A) PFS and (B) OS. NR, not reached; 37.0 copies/ml was the median concentration of mutated \textit{KRAS} among those with detectable \textit{KRAS} mutations in plasma.
high MAFs of detected alterations are associated with worse PFS and OS also in NSCLC [48,49].

Our work has some limitations. It is a retrospective study with a limited number of patients, especially with respect to patients with stages I and II disease. We detected mutated KRAS in only a small proportion of the patients with stages I and II disease, which may be explained by the low MAF of ctDNA in early-stage disease [3,50]. More comprehensive NGS panels with capturing and sequencing approaches optimised for ctDNA, such as Cancer Personalised Profiling by deep Sequencing (CAPP-Seq) [15], Tracking Cancer Evolution Through Therapy (TRACERx) [3,50], and Targeted Error Correction Sequencing (TEC-Seq) [41], may increase the sensitivity of ctDNA analyses, but detection of ctDNA in early-stage disease remains a challenge.

As aforementioned, baseline cfDNA levels in plasma or serum may have prognostic value [46,47] and may also be used in disease monitoring [51]. We did not conduct any additional analyses of cfDNA beyond measuring the DNA yield from plasma prior to ddPCR, although further analyses of the cfDNA levels using reference genes as markers for cfDNA would have been of interest. However, standardised methods for analyses of cfDNA are needed [52,53].

Another potential limitation of our study is the range of days between blood sample collection and commencement of treatment (0–307 days). In four patients, the blood samples were collected >90 days before treatment due to intercurrent diseases or difficulties in obtaining tissue specimens (patient IDs 30, 31, 52, and 57; see supplementary material, Table S1). During the diagnostic work-up, one of these patients had radiological progression from stage IB to IIB, while there was no radiological progression in the other three patients. The results of the survival analyses were not altered when running sensitivity analyses excluding these four patients (data not shown).

The storage time of the plasma samples may also be a potential limitation. The amount of cfDNA in a plasma sample may decrease during storage [54,55] and could have an impact on the detection rate. We did not, however, find any significant difference in the total KRAS concentration between plasma samples with different storage times (data not shown).

Using a single gene or few genes approach targeting known tumour-specific mutations in ctDNA by ddPCR has some advantages over NGS. It may minimise the risk of falsely misinterpreting somatic mutations in haematopoietic stem cells associated with clonal haematopoiesis as tumour derived [56,57]. In addition, for monitoring treatment and disease course, accurate quantification of nucleic acids combined with simple workflow, low cost, and less complex data analysis make ddPCR a feasible technique in the daily routine of a diagnostic laboratory.

Although the assays we used have been validated by the manufacturer, we performed in-house validation with tests for linearity and establishment of LODs for each assay. In our experience, comparing detection rates of ctDNA across different studies is challenging, even if the same platforms for ctDNA analyses have been used. Data on false-positive rates and how the LOD was determined is important information that should be available for cross-study comparison. Lack of concordance between validated commercial kits for KRAS mutation detection, which have the same LOD according to the manufacturers, has been reported [58]. Therefore, the false-positive rate and LOD should be established in house for the assays used, even if the assays are pre-validated by the manufacturer.

In conclusion, patients with detectable KRAS mutation in their baseline plasma sample had worse PFS and OS than patients who did not have detectable KRAS mutations. Our findings also suggest that the baseline plasma concentration of mutated KRAS before the first treatment may be an important, independent prognostic factor in KRAS-positive lung adenocarcinomas. Hence, a quantitative analysis assessing the plasma concentration of mutated KRAS improves the prognostic value compared to qualitative analysis only. These findings are confirmed in other studies, but the value of ctDNA baseline levels in treatment decision-making must be evaluated in larger prospective clinical trials. Finally, ddPCR for ctDNA analyses is a highly sensitive and accurate technique that can be implemented in a daily diagnostic routine.

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Author contributions statement

SGFW participated in planning and designing the study, evaluated all histological material, collected the
clinical data, performed statistical analyses, participated in interpretation of laboratory and statistical analyses, and drafted and wrote the manuscript. HYD designed and planned the study and participated in the laboratory set-up, performed molecular analyses of tissue and plasma, interpreted the molecular analyses, and contributed to manuscript preparation. EFE optimised the laboratory protocol, participated in the laboratory set-up, performed some of the tissue analyses and all the plasma analyses, interpreted the plasma analyses, and contributed to manuscript preparation. BHG was responsible for the performance and interpretation of statistical analyses, and contributed to manuscript preparation. TOH gave statistical advice, participated in manuscript preparation. ER contributed to planning the study and participated in interpreting all histological material and contributed to manuscript preparation. VGD evaluated all histological material and contributed to manuscript preparation. ALO performed some of the tissue analyses and contributed to manuscript preparation. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Linear regression analyses of serial dilution series of mutated KRAS plasmid templates
Table S1. Patient characteristics and results of ctDNA analyses