Quantitative Cell-Free DNA, KRAS, and BRAF Mutations in Plasma from Patients with Metastatic Colorectal Cancer during Treatment with Cetuximab and Irinotecan

Karen-Lise Garm Spindler1, Niels Pallisgaard2, Ivan Vogelius1,3, and Anders Jakobsen1

Abstract

Purpose: The present study investigated the levels of circulating cell-free DNA (cfDNA) in plasma from patients with metastatic colorectal cancer (mCRC) in relation to third-line treatment with cetuximab and irinotecan and the quantitative relationship of cfDNA with tumor-specific mutations in plasma.

Experimental Design: Inclusion criteria were histopathologically verified chemotherapy-resistant mCRC, adequate Eastern Cooperative Oncology Group performance status, and organ function. Treatment consisted of irinotecan being administered at 350 mg/m2 for 3 weeks and weekly administration of 250 mg/m2 cetuximab until progression or unacceptable toxicity. A quantitative PCR method was developed to assess the number of cfDNA alleles and KRAS and BRAF mutation alleles in plasma at baseline.

Results: The study included 108 patients. Only three patients were positive for BRAF mutations. The majority of KRAS mutations detected in tumors were also found in the plasma [32 of 41 (78%)]. Plasma cfDNA and plasma mutant KRAS levels (pmKRAS) were strongly correlated (r = 0.85, P < 10−4). The disease control rate was 77% in patients with low cfDNA (<25% quartile) and 30% in patients with high cfDNA (≥75% quartile (P = 0.009)). Patients with pmKRAS levels higher than 75% had a disease control rate of 0% compared with 42% in patients with lower pmKRAS (P = 0.048). Cox analysis confirmed the prognostic importance of both cfDNA and pmKRAS. High levels were clear indicators of a poor outcome.

Conclusions: KRAS analysis in plasma is a viable alternative to tissue analysis. Quantitative levels of cfDNA and pmKRAS are strongly correlated and hold promise of clinical application. Clin Cancer Res; 18(4); 1177–85. ©2012 AACR.

Introduction

The overall outcome of metastatic colorectal cancer (mCRC) has been improved by the use of monoclonal antibodies (mAb) targeting the epidermal growth factor receptor (EGFR), but these drugs are associated with a specific toxicity profile along with major costs (1–5). The intensive toxicity profile along with major costs (1–5). The intensive

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Results: The study included 108 patients. Only three patients were positive for BRAF mutations. The majority of KRAS mutations detected in tumors were also found in the plasma [32 of 41 (78%)]. Plasma cfDNA and plasma mutant KRAS levels (pmKRAS) were strongly correlated (r = 0.85, P < 10−4). The disease control rate was 77% in patients with low cfDNA (<25% quartile) and 30% in patients with high cfDNA (≥75% quartile (P = 0.009)). Patients with pmKRAS levels higher than 75% had a disease control rate of 0% compared with 42% in patients with lower pmKRAS (P = 0.048). Cox analysis confirmed the prognostic importance of both cfDNA and pmKRAS. High levels were clear indicators of a poor outcome.

Conclusions: KRAS analysis in plasma is a viable alternative to tissue analysis. Quantitative levels of cfDNA and pmKRAS are strongly correlated and hold promise of clinical application. Clin Cancer Res; 18(4); 1177–85. ©2012 AACR.
Additional predictors for outcome of cetuximab and irinotecan in metastatic colorectal cancer are needed and many aspects of KRAS testing still remain unsolved. Recent technological developments have enabled us to develop a highly sensitive method for detection of DNA in the peripheral circulation and test this DNA for tumor-specific mutations. The present data show that KRAS analysis in plasma is a feasible alternative to tissue analysis and may help overcome the challenges of KRAS mutational testing on heterogeneous tumors. There is a clear difference in the outcome between patients with high levels of KRAS mutations and those with low concentrations. The plasma concentration of cell-free DNA is strongly correlated to clinical outcome in the total cohort, which must lead to further investigations in patients with colorectal cancer.

### Translational Relevance

Additional predictors for outcome of cetuximab and irinotecan in mCRC. Inclusion criteria were histopathologically verified mCRC, treatment failure after exposure to fluoropyrimidine, oxaliplatin, and irinotecan, indication for third-line treatment with cetuximab and irinotecan, Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 to 2, and adequate organ function. Treatment consisted of irinotecan 350 mg/m² every 3 weeks combined with weekly cetuximab 250 mg/m² (initial loading dose was 400 mg/m²). Archival paraffin-embedded tissue samples from primary tumor and/or metastatic tissues were collected and blood samples for marker analysis were drawn at baseline prior to cycle one. Response and toxicity were evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 and Common Terminology Criteria for Adverse Events (CTC) version 3.0, respectively. Response evaluation was conducted every 9 weeks with clinical and radiological examination by computed tomographic scan of the chest and abdomen and/or magnetic resonance scan. Patients were classified as having complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Only patients with CR and PR could be classified as responders, whereas those with SD and PD were defined as nonresponders. Disease control (DC) included patients who achieved a response or SD. The study was conducted in accordance with the Danish law after approval by the Regional Ethics Committee. Written informed consent was obtained from all patients.

### Sample collection and DNA purification

Formalin-fixed, paraffin-embedded (FFPE) tumor tissue was evaluated histologically by a dedicated pathologist to confirm the number of viable tumor cells. In some cases, a microdissection was carried out to increase the percentage of tumor cells. Three 15-μm tissue sections were deparaffinated by xylene and ethanol extractions and subjected to a proteinase K digestion overnight at 56°C. DNA was then purified using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s recommendations.

A 9-mL peripheral blood sample was collected in EDTA tubes from patients at baseline (i.e., before starting third-line chemotherapy). After collection, plasma was obtained by centrifugation at 2,000 × g for 10 minutes within 2 hours and stored at −80°C until use.

Total nucleic acid was purified from 1.2 mL plasma using a QIAasympy virus/bacteria midi-kit on a QIAasympy robot (Qiagen) according to the manufacturer’s instructions. Plasma samples with inadequate plasma volume were added to 1.2 mL water prior to purification. Because both DNA and RNA were copurified and plasma DNA is fragmented, often as multiples of 180 bp, the amount and integrity of DNA were determined functionally by quantitative PCR (qPCR) using an in-house assay for the housekeeping gene cyclophilin (gCyc; which is a gene not known to be involved in cancer) through amplification of a 132-bp PCR fragment (Supplementary Table S5). The gCyc qPCR results and prognostic markers in third-line treatment with cetuximab and irinotecan for mCRC. Inclusion criteria were histopathologically verified mCRC, treatment failure after exposure to fluoropyrimidine, oxaliplatin, and irinotecan, indication for third-line treatment with cetuximab and irinotecan, Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 to 2, and adequate organ function. Treatment consisted of irinotecan 350 mg/m² every 3 weeks combined with weekly cetuximab 250 mg/m² (initial loading dose was 400 mg/m²). Archival paraffin-embedded tissue samples from primary tumor and/or metastatic tissues were collected and blood samples for marker analysis were drawn at baseline prior to cycle one. Response and toxicity were evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 and Common Terminology Criteria for Adverse Events (CTC) version 3.0, respectively. Response evaluation was conducted every 9 weeks with clinical and radiological examination by computed tomographic scan of the chest and abdomen and/or magnetic resonance scan. Patients were classified as having complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Only patients with CR and PR could be classified as responders, whereas those with SD and PD were defined as nonresponders. Disease control (DC) included patients who achieved a response or SD. The study was conducted in accordance with the Danish law after approval by the Regional Ethics Committee. Written informed consent was obtained from all patients.

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were used to normalize plasma sample DNA to number of DNA alleles per mL.

**KRAS mutational analysis**

KRAS analysis of primary tumor and metastases was conducted using a KRAS DxS kit (Qiagen) according to the manufacturer’s recommendations, as previously published (12). Primers and probes for in-house KRAS and BRAF assays as well as KRAS mutation control PCR fragments were generated by site-directed mutagenesis (Supplementary Fig. S5 and Tables S5 and S6) with the use of the OLI GOh 7 software (Molecular Biology Insights Inc.). The in-house assays use an amplification refractory mutation system qPCR (ARMS-qPCR) methodology and detect 6 mutations in KRAS codon 12 (Gly12Ala, Gly12 Arg, Gly12Asp, Gly12Cys, Gly12Ser, and Gly12Val), one mutation in codon 13 (Gly13Asp), and the most frequent BRAF mutation (V600E).

A number of refractory primers were tested on DNA samples from patients with mutation-positive colorectal cancer as well as normal donor DNA. To increase the specificity of the qPCR reactions, a wild-type blocking oligo was added in some reactions. The blocking oligos were modified by including HyNna nucleotides (Penta-base ApS), which increased the melting temperature and blocked extension. The final primer mixtures resulting in amplicon lengths between 118 and 122 bp are shown in Supplementary Fig. S6. The in-house qPCR reaction conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

**Validation of the in-house assays**

The in-house KRAS assay was validated on 294 FFPE ovarian cancer samples, where KRAS analysis had been conducted using the KRAS DxS Kit (Qiagen). Nineteen of these samples had a poor quality DNA or lacked DNA and were not included in the study. Analysis of the remaining 275 samples showed a 100% concordance between the DxS Kit and the in-house KRAS assay (20). The stability and reproducibility of the in-house KRAS assays were validated over a 4-month period on 3 mixtures of DNA sample of patients with FFPE CRCs containing the 7 KRAS mutations. As shown in Supplementary Fig. S6, the assay showed very little variation. The in-house BRAF V600E assay was validated on the primary CRC tumors and a 100% agreement with the results of the DxS Kit was revealed. In addition, 40 FFPE colorectal tumor samples, which were BRAF V600E positive when analyzed by Sanger sequencing, were all positive with the in-house BRAF assay.

**Positive controls by KRAS site-directed PCR mutagenesis**

To obtain unlimited amounts of positive control material for the KRAS assay, a site-directed PCR mutagenesis strategy was used. Seven PCR products of 381 bp were generated, each carrying a KRAS mutation, (Supplementary Table S6) and were used to generate standard curves.

**Quantification of cfDNA and KRAS in plasma**

Standard curves were generated by spiking dilutions of KRAS site-directed mutated PCR product in 5-fold decrements into 100 ng normal donor DNA (pool of DNA purified from normal blood samples). For BRAF, similar spikings were carried out using DNA from the BRAF V600E–mutated colorectal adenocarcinoma cell line H1729 (DSM2). From the standard curves, the slopes were calculated for the gCYC (3.4), KRAS (3.4–3.6), and BRAF V600E (3.4) primer sets (Supplementary Fig. S4A). The y-intercept corresponding to one DNA copy of the target DNA was estimated and set to a cycle threshold (Ct) of 41 (gCYC and BRAF) or 41 to 42 (KRAS) using a threshold of 0.2. The specificity of the different in-house assays were tested in 100 ng normal donor DNA using gCYC as reference and from the standard curves calculated to 0.025% for Gly13Asp, 0.004% for Gly12Ser, and better than 0.001% for Gly12Ala, Gly12Arg, Gly12Asp, Gly12Cys, Gly12Val, and BRAF V600E (Supplementary Fig. S4B). However, for routine use, the maximum sensitivity of the assays was set to 10-fold less than the specificity. For all KRAS mutation-negative samples, the number of DNA alleles was calculated from the internal positive control (WTKRAS for DxS, gCYC for the in-house KRAS assay, and WTBRaf for V600E), and the sensitivity of a negative sample was determined by what was reached first: the allele number or the maximum sensitivity. In samples with a low allele number, this number was compared with the number of tumor cells found by the pathologist, and if the alleles were less than 10-fold higher than the percentage of tumor cells, the sample was considered nonconclusive.

Quantification of cfDNA was done by calculating the copy number of gCYC alleles as 10[\(\text{y-intercept(gCYC)} - \text{mean}(gCYC)\)/slope(gCYC)] and normalizing this to the plasma volume. Quantification of KRAS was done by calculating the copy number of mutated KRAS alleles as 10[\(\text{y-intercept(KRAS)} - \text{mean}(\text{KRAS})\)/slope(\text{KRAS})] and normalizing this to the plasma volume. Similar method was used to quantify the BRAF mutations.

The high specificity of this new qPCR assay enables detection of KRAS or BRAF mutations in a high background of normal DNA, which is not achieved with other methods such as Sanger or next-generation sequencing.

**Statistics**

The association between marker status and objective response rates, baseline characteristics, and skin toxicity rates was determined by Wilcoxon rank-sum or \(\chi^2\) test, where appropriate. The correlation between cfDNA and
pmKRAS alleles was investigated with Spearman rank correlation. Patients who reached the first objective tumor evaluation after 3 cycles or experienced clinical progression prior to this point were considered evaluable for response according to RECIST. PFS was defined as the time from start of the treatment until documented tumor progression or death. OS was calculated from the date of first treatment until death by any cause. Survival analyses were conducted according to the Kaplan-Meier method, and PFS and OS curves were compared by log-rank test. A multivariate Cox regression analysis was conducted using a backward stepwise elimination process, which eliminates the predictor with the largest P-value in each step until all predictors in the final model had P < 0.2. The proportional hazards assumption was tested by visual inspection of the log[−log (survival)] versus log(time) curves. Two-sided P-values were considered significant when P ≤ 0.05. (No correction for multiple testing was applied.) Statistics were carried out using the NCSS Statistical Software 2007 v.07.1.5 (NCSS Statistical Software; www.ncss.com) except the test of the proportional hazards assumption, which was conducted in SPSS v. 15.0.

Results

Patient characteristics

Patients’ baseline characteristics have been listed in Table 1. The study included patients during the period of April 2005 until April 2008. The median follow-up was 7 months and ended by November 2010. The median number of cycles was 3.4 (range, 0–21). Three patients deteriorated before receiving the first cycle and were not treated. Four patients commenced the first cycle but stopped because of an anaphylactic reaction (3) or patient’s wish (1). Consequently, 7 of the patients were not evaluable for response according to RECIST but were still included in survival analysis according to intention to treat. Seventeen patients having received at least one cycle of treatment showed clinical progression before the first evaluation scan and were subsequently included in analysis as having PD. The rate of PR was 20% (20 of 101), SD 34% (34 of 101), and PD 47% (47 of 101). Outcome according to mutational status was previously presented (12).

Correlation between KRAS status in tissue and plasma

A total of 98 patients had primary tissue available for mutational testing. All patients had blood samples available for testing, but the analysis showed inconclusive results in 3 cases, leaving 95 assessable for comparison of tissue analyses and peripheral blood (Table 2). The majority of KRAS mutations detected in the tumor were also found in the peripheral blood; 32 of 41 (78%). Interestingly, one of the patients had a primary KRAS mutant tumor, but wild-type metastatic disease, which was confined to the peripheral blood. Unfortunately, no metastatic tissue was available for testing in the remaining 8 patients with discordant results. Of note, none of the patients without mutations detected in the primary tumor tissue were tested positive in the baseline plasma sample.

Quantitative baseline levels of cfDNA, KRAS, and BRAF alleles in plasma

As presented in Fig. 1, there was a clear correlation between the level of KRAS mutations and cfDNA in the plasma (Spearman rank correlation, 0.85; P < 0.0001). The median level of cfDNA was 23,000 alleles per mL plasma (range, 2,000–4,616,000). There was no significant difference in the levels of cfDNA between patients with KRAS mutant (median, 19,500; range, 2,000–4,600,000) and wild-type disease (median, 25,000; range, 2,600–610,000; P > 0.05). The median level of pmKRAS was 3,300 (range, 50–180,000). Only 2 patients revealed BRAF mutation alleles in the plasma and consequently, no further analysis was conducted. The median levels of cfDNA and pmKRAS were tested against baseline characteristics and showed a significantly higher level with poor PS and a tendency of correlation to the number of metastatic sites

### Table 1. Patients’ baseline characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n (%)</th>
<th>N = 108</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>Median (range)</td>
<td>62 (38–82)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>60 (56)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>48 (44)</td>
<td></td>
</tr>
<tr>
<td>PS at inclusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>55 (51)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>42 (39)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9 (8)</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>2 (2)</td>
<td></td>
</tr>
<tr>
<td>Locus primary tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>35 (32)</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>70 (65)</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>3 (3)</td>
<td></td>
</tr>
<tr>
<td>Number of metastatic sites at inclusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–2</td>
<td>56 (52)</td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>49 (45)</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>3 (3)</td>
<td></td>
</tr>
<tr>
<td>Number of CT regimens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>78 (72)</td>
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<tr>
<td>ND</td>
<td>3 (3)</td>
<td></td>
</tr>
<tr>
<td>Previous surgery for primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>97 (90)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>8 (7)</td>
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<tr>
<td>ND</td>
<td>3 (3)</td>
<td></td>
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<tr>
<td>Previous radiotherapy for primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15 (14)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>88 (81)</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>5 (5)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CT, chemotherapy; ND, not determined.
prior to treatment, whereas all other parameters showed nonsignificant differences. This suggested that PS and disease status possibly influence the level of cfDNA in the peripheral circulation. The median cfDNA in PS 0 patients was 15,000 alleles per mL plasma (range, 2,000–1,000,000) and 52,000 alleles per mL plasma (range, 14,000–420,000) in patients with PS 2 at baseline; \( P = 0.03 \) (not corrected for multiple comparisons).

**Baseline cfDNA levels and correlation to tumor response**

There was a significant difference between the groups of patients who achieved DC (29,600 alleles per mL plasma) and those who progressed early (130,000 alleles per mL plasma). The DC rate decreased with increasing level of cfDNA in the plasma, as shown in Table 3. The DC rate was 77% in patients with low cfDNA (<25%) compared with 30% in patients with high levels (>75% \( P = 0.009 \)). A descriptive receiver-operating curve analysis was conducted to test the performance of cfDNA for predicting disease stabilization. The area under the curve was 0.69 [95% confidence interval (CI), 0.56–0.77; \( P < 0.001 \)]. A cutoff point at the 75% level of cfDNA carried out with a sensitivity of 87%, whereas the 90% produced a sensitivity of 96% for early disease progression.

**Baseline pmKRAS levels and tumor response**

The patients with high pmKRAS levels (>75%) had a DC rate of 0% compared with 42% in patients with low pmKRAS levels (<75% percentile; \( P = 0.048 \)).

**The prognostic value of baseline cfDNA levels**

Patients with cfDNA levels below the median had a median OS of 12.2 months (95% CI, 10.2–13.9) compared with 4.5 months (95% CI, 3.9–5.5) in those with high levels; \( P < 0.001 \). The PFS was 5.7 months (95% CI, 4.1–6.9) and 2.2 months (95% CI, 2.1–2.8), respectively; \( P < 0.001 \). The Kaplan–Meier survival curves according to baseline cfDNA are shown in Fig. 2, which also includes survival analysis according to pmKRAS. In brief, an unfavorable survival time was revealed with increasing baseline levels of cfDNA and pmKRAS.

For the initial multivariate Cox regression analysis, we included ECOG PS and cfDNA quartiles (values 1–4) as numeric predictors and pretreatment KRAS mutational status, respectively, and number of metastatic sites at baseline, age, gender, and tumor location as categorical predictors. By entering PS and cfDNA quartile as numeric predictors in the Cox model, we implicitly assume linearity of the effect on a log-risk scale. The inclusion of quadratic terms did not improve the model, thus supporting the validity of the linearity assumption. Furthermore, we conducted separate multivariate analyses by entering cfDNA as

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**Table 2. Comparison of KRAS detection in tissue and plasma**

<table>
<thead>
<tr>
<th>Tumor KRAS mutation</th>
<th>Tumor WTKRAS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma KRAS mutation</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Plasma WTKRAS</td>
<td>9</td>
<td>54</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>54</td>
</tr>
</tbody>
</table>

NOTE: Sensitivity, 78%; specificity, 100%; positive predictive value, 100%; negative predictive value, 86%.

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Figure 1. Correlation between concentrations of KRAS mutations and cfDNA in plasma. KRAS mutational alleles per mL plasma are plotted against the number of cfDNA alleles per mL plasma. Because of the broad range of values, a logarithmic scale was used. The Spearman rank correlation was 0.85; \( P < 0.0001 \).
A dichotomized covariate (above vs. below median and above vs. below 75% percentile), both of which retained the highly significant correlation to outcome (data not shown). Table 4 shows the final model for PFS and OS after the stepwise elimination process. cfDNA remained a strong prognostic factor for PFS and OS when analyzed as a dichotomized covariate (above vs. below median and above vs. below 75% percentile), both of which retained the highly significant correlation to outcome (data not shown). A multivariate model built using cfDNA quartiles and the established prognostic markers of KRAS status, number of metastatic sites, and age yielded HRs of 1.4 (95% CI, 1.1–1.7) and 1.8 (1.4–2.2) for cfDNA quartiles and PFS and OS, respectively. Quantitative levels of pmKRAS were not entered in the model because the sample size for KRAS mutant patients was limited to 35 patients and a strong correlation to cfDNA was limited to 35 patients and a strong correlation to cfDNA was observed (1.4–2.2) for cfDNA quartiles and PFS and OS, respectively. Quantitative levels of pmKRAS were not entered in the model because the sample size for KRAS mutant patients was limited to 35 patients and a strong correlation to cfDNA was observed.
levels had been revealed. With this in mind, an isolated multivariate analysis including pmKRAS levels grouped as quartiles, PS, and number of metastatic sites confirmed the independent prognostic value of pmKRAS (data not shown). In brief, high levels of cfDNA and/or pmKRAS were both strong predictors of a poor outcome.

Discussion

The present study contributes to our knowledge in 3 major aspects: the importance of circulating nucleic acids, the quantitative measures of tumor-specific KRAS mutation alleles in plasma, and the possibility of improved selection of therapy in mCRC by aids of the above markers.

The cfDNA in plasma from patients with cancer originates from normal nonmalignant cells as well as necrotic and apoptotic tumor cells (21), but neither the origin nor fate of the circulating DNA is fully explored (18). A recent review has summarized the few older clinical studies in CRC, but although the results were promising, the studies were small and primarily based on pre/post-surgical measurements or comparison with healthy individuals.

We report a correlation between the quantitative measures of cfDNA and the tumor-specific KRAS mutation in plasma, which has led us to hypothesize that the increasing levels of cfDNA in patients with cancer are primarily of tumor origin. The cfDNA may therefore have its greatest potential in advanced disease.

pmKRAS analysis may help overcome some of the obvious limitations of tissue analysis for KRAS mutations, which are underlined in a recent review addressing KRAS for clinical oncological practice (22). Tissue availability and selection of specimens with a sufficient number of tumor cells together with tumor heterogeneity are the major challenges affecting the quality and liability of DNA extracted. We found a high concordance between KRAS status in primary tumor and plasma (detection rate of 78% and overall concordance of 91%), which is supported by the literature. Lecomte and colleagues reported that the detection rate of mutations in the peripheral blood ranged between 9% (3 of 16) and 100% (5 of 5). However, most prior studies have included a very low number of patients (18). Recently, Yen and colleagues investigated 76 patients using a different method and found a detection rate similar to that of our study (84.4%; ref. 23). The few discordant results from our study should be further investigated.

No conclusion can be drawn from our data about pBRAF detection because of the low sample size and marginally low frequency, compared with the literature (24, 25), which suggest a possible selection bias.

We have presented a feasible alternative method for KRAS testing in plasma and, more importantly, explored the potential value of quantification of the mutated alleles in the clinical setting. We are not aware of similar studies that can be used for direct comparison. In general, patients with a high level of pretreatment pmKRAS had a poor prognosis, compared with the subgroup of patients harboring KRAS mutations at low levels, who achieved a prolonged stabilization of disease. We present data indicating that it may not only be the KRAS status itself but rather the quantitative amount of this mutation that influences the disease behavior. Consequently, a quantitative measure at baseline will reveal whether this solely applies to anti-EGFR therapy, but we find it likely that the high levels reflect aggressive disease behavior.

Of broader interest are our results showing that the cfDNA quantitative levels were related to outcome in terms of response, PFS, and OS regardless of the cutoff point used.

<table>
<thead>
<tr>
<th>Table 4. Multivariate Cox regression analysis of PFS and OS</th>
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<tbody>
<tr>
<td><strong>Variables</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>cfDNA quartile (1–4)</td>
</tr>
<tr>
<td>PS</td>
</tr>
<tr>
<td>KRAS status in tumor</td>
</tr>
<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>Mutation</td>
</tr>
<tr>
<td>Number of metastatic sites</td>
</tr>
<tr>
<td>1–2</td>
</tr>
<tr>
<td>&gt;2</td>
</tr>
<tr>
<td>Anatomic site</td>
</tr>
<tr>
<td>Colon</td>
</tr>
<tr>
<td>Rectum</td>
</tr>
</tbody>
</table>

*Reference group.

aEntered in the model as numeric variables. The quartiles (1–4) were used for grouping of cfDNA levels, and HRs consequently correspond to one-step increase in quartiles. PS was divided into 0, 1, and 2. (No patients with PS 3 were included in the study.)

Quantitative cfDNA and KRAS in Metastatic Colorectal Cancer
When entered in a multivariate Cox model, an independent prognostic value of cfDNA was confirmed. To the best of our knowledge, there are no other studies investigating the quantitative levels of cfDNA and in a similar clinical setting. Interesting observations were conducted by Diehl and colleagues who measured cfDNA with patient-specific mutations such as APC, KRAS, and PIK3CA in consecutive samples during follow-up after surgery and found a correlation between post-surgical mutation levels and the outcome (15).

The present sample size did not allow for a conclusion of the mutual contribution of the pmKRAS and cfDNA levels. Quantification of KRAS in plasma is limited to the subgroup of patients with KRAS mutant disease. Our results of cfDNA should be investigated in larger sample sizes and different clinical settings to clarify its full potential as a marker in CRC. It is questionable whether pmKRAS analysis will provide additional predictive or prognostic information to cfDNA quantification in this specific setting because of the strong correlation and thereby supra effect of the 2 markers. However, our data suggest that patients with a low KRAS allele count at baseline could still benefit from the treatment, but at present, these patients are not considered candidates for EGFR inhibitor treatment because they harbor KRAS mutant disease. We therefore suggest that pmKRAS can be used as supplement to tissue KRAS analysis as a tool for selection prior to treatment and the predictive value of this marker is further investigated in prospective studies in KRAS mutant cohorts.

In conclusion, KRAS mutations can be detected in the peripheral blood as an alternative to tissue analysis, and quantitative levels of cfDNA and pmKRAS were both associated to clinical outcome of third-line treatment of mCRC. Quantification of cfDNA and KRAS mutations in the peripheral circulation has potential value as a clinical tool for more individualized pretreatment testing and could improve selection of therapy. Further studies along that line seem justified.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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