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

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Running title: Quantitative cfDNA and KRAS in metastatic colorectal cancer

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Additional figures and tables for online only: Tables 2, figures 3
Statement of translational relevance

Additional predictors for outcome of cetuximab and irinotecan in mCRC 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 showed 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 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 colorectal cancer patients.
Abstract

Purpose The present study investigated the levels of circulating cell-free DNA (cfDNA) in plasma from metastatic colorectal cancer (mCRC) patients 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 PS and organ function. Treatment consisted of irinotecan 350 mg/m² q3w and weekly cetuximab 250 mg/m² until progression or unacceptable toxicity. A quantitative polymerase chain reaction (qPCR) method was developed to assess the number of cfDNA alleles, KRAS and BRAF mutation alleles plasma at baseline.

Results The study included 108 patients. Only 3 patients were positive for BRAF mutations. The majority of KRAS mutations detected in tumors were also found in plasma (32/41 (78%)). Plasma cfDNA and plasma mutant KRAS levels (pmKRAS) were strongly correlated (r = 0.85, p<10⁻⁴). The disease control (DC) rate was 77% in patients with low cfDNA (<25 percentile) and 30% in patients with high cfDNA (>75% percentile (p=0.009)). Patients with pmKRAS levels higher than the 75% percentile had a DC rate of 0% compared to 42% in patients with pmKRAS below (p=0.048). Cox analysis confirmed the prognostic importance of both cfDNA and pmKRAS. High levels were clear indicators of a poor outcome.

Conclusion 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.
Introduction

The overall outcome of metastatic colorectal cancer (mCRC) has been improved by the use of monoclonal antibodies (MoABs) 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 search for predictive and prognostic markers in this setting has successfully identified downstream KRAS and BRAF mutations as responsible for tumour resistance to treatment (6-8). A recent meta analysis investigated 22 studies, including 2188 patients who were treated with anti-EGFR MoABs and concluded that overall KRAS mutational status was associated with lack of response, shorter progression free survival (PFS) and overall survival (OS) (9). Thus, the emerging data on the association between KRAS mutational status and non-responsiveness has led to restriction of the use of these drugs to KRAS wildtype (wtKRAS) patients only. BRAF mutational status has not yet been established as a selection criteria, but seems to be equally important to outcome, although less frequent. However, colorectal tumours are known to be heterogeneous in nature, which is illustrated by the fact that approximately >60% of the wtKRAS patients fail to achieve a radiological response to EGFR inhibitors. Even more intriguing, a subgroup of patients hosting KRAS mutant disease achieves a prolonged stabilisation of disease. Therefore, additional reliable markers for outcome are still needed.

In general KRAS mutations are considered an early event in colorectal carcinogenesis, and the use of tissue from the primary tumour for pre-treatment testing has been accepted as a basis for treatment of metastatic disease (10-12). However, the absence of detectable mutations in the primary tumor cannot formally exclude the presence of mutant metastatic disease. Tumor heterogeneity and mutational selection
during disease progression are aspects that need further elucidation (13;14). Unfortunately metastatic tissue is rarely available for testing due to practical and ethical reasons and alternative methods for mutational testing could become of great clinical value.

The presence of circulating nucleic acids in plasma and serum of cancer patients was identified more than 60 years ago, and studies have suggested a predictive and prognostic role in different settings, including colorectal cancer (15;16). However, results have not been translated into clinical practice, but efforts during the last decade have led to significant progress in the development of highly sensitive and reproducible methods (17). The factors influencing the quantitative as well as qualitative changes of the cell-free DNA (cfDNA) in cancer patients are multiple and not yet fully explored, but a substantial proportion of circulating cfDNA in plasma is believed to originate from tumour cells, and can therefore be tested for tumour specific genetic alterations such as KRAS or BRAF mutations (18;19). Clearly this opens up for investigations of a number of highly relevant issues such as: methodology, correlation to clinical outcome, dynamic changes of mutational status during EGFR inhibitor treatment and potential solutions of practical issues.

We have developed a highly sensitive method to analyse plasma samples for cfDNA with KRAS or BRAF mutations in colorectal cancer patients with chemotherapy resistant metastatic disease during treatment with third line cetuximab and irinotecan. The purpose was to assess the correlation between baseline plasma- and tumour- mutational status and to investigate the predictive and prognostic value of quantitative estimates of cfDNA and mutated KRAS in plasma (pmKRAS) at baseline.
Patients and Methods

Patient material

A prospective biomarker study was conducted at Department of Oncology, Vejle Hospital to investigate predictive 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, ECOG performance status (PS) 0 to 2, and adequate organ function. Treatment consisted of irinotecan 350 mg/m$^2$ every three week combined with weekly cetuximab 250 mg/m$^2$, (initial loading dose was 400 mg/m$^2$). 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 was 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 performed every 9 weeks, with clinical and radiological examination by computer tomography (CT) scan of the chest and abdomen and/or magnetic resonance scan (MR). 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 SD and PD were defined as non-responders. Disease control (DC) included patients who achieved a response or SD. The study was conducted in accordance with 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 micro dissection was performed to increase the percentage of tumor cells. Three 15 μM tissue sections were de-paraffinated by xylen and ethanol extractions and subjected to a Protease K digestion overnight at 56°C. DNA was then purified using a QIAamp DNA Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacture’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 2000 g for 10 min within 2 hours and stored at -80°C until use.

Total nucleic acid was purified from 1.2 mL plasma using a QIAsymphony virus/bacteria midi-kit on a QIAsymphony robot (Qiagen) according to the manufacturer’s instructions. Plasma samples with inadequate plasma volume were added water to 1.2 mL prior to purification. Since both DNA and RNA was co-purified and plasma DNA is fragmented, often as multiples of 180 bp, the amount and integrity of DNA was determined functionally by 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 (table 5 online). The gCYC qPCR results were used to normalize plasma sample DNA to number of DNA alleles per mL.

KRAS mutational analysis
KRAS analysis of primary tumor and metastasises were performed using a KRAS DxS kit (Qiagen) according to the manufactures 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 (figure 5 and table 5-6) with the use of the OLIGO 7 software (Molecular Biology Insights Inc, Cascade, CO). The in house assays utilize an Amplification Refractory Mutation System-Quantitatine PCR (ARMS-qPCR) methodology and detects 6 mutations in KRAS codon 12 (Gly12Ala, Gly12 Arg, Gly12Asp, Gly12Cys, Gly12Ser, Gly12Val), one mutation in codon 13 (Gly13Asp), and the most frequent BRAF mutation (V600E).

A number of refractory primers were tested on DNA from mutation positive colorectal cancer patient samples as well as normal donor DNA. In order 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 HyNA nucleotides (Pentabase Aps, Soendersoe, Denmark) which increased the melting temperature and blocked extension. The final primer mixtures resulting in amplicon lengths between 118 bp and 122 bp are shown in table 5 (online). All qPCR reactions were performed in a volume of 25 µL in duplicates on an ABI Prism7900HT (Applied Biosystems, Foster City, CA) using ABI Universal Mastermix with UNG (Applied Biosystems). In all assay rounds, a mixture of patient samples containing DNA which represented all mutations was included as positive controls. Water controls and wild type donor DNA controls were used as negative control. The qPCR reaction conditions were: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 60 s 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 performed using the DxS KRAS kit. Nineteen of these samples had a poor quality DNA or lack of 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 (Steffensen et al, in press). The stability and reproducibility of the in house KRAS assays were validated over a 4 month period on 3 mixtures of patient sample DNA from FFPE colorectal cancers containing the 7 KRAS mutations. As shown in figure 6 (online), the assay demonstrated 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 analysed by Sanger sequencing, were all positive with the in house BRAF assay.

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

In order to obtain unlimited amount 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, (table 6 online) 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 performed using
DNA from the BRAF V600E mutated colorectal adenocarcinoma cell line HT29 (DSMZ, Braunschweig, Germany). 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 (figure 4A online). The Y-intercept corresponding to one DNA copy of the target DNA were estimated and set to a cycle threshold (Ct) of 41 (gCYC and BRAF) or 41-42 (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, (figure 4B online). 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 (KRAS wt for Dxs, gCYC for in house KRAS assay and BRAF wt for the 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 non-conclusive.

Quantification of cfDNA was done by calculating the copy number of gCYC alleles as $10^{((Y\text{-intercept(gCYC)}-\text{meanCt(gCYC)})/\text{slope(gCYC)})}$ and normalizing this to the plasma volume. Quantification of KRAS was done by calculating the copy number of mutated KRAS alleles as $10^{((Y\text{-intercept(KRAS)}-\text{meanCt(KRAS)})/\text{slope(KRAS)})}$ 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 like e.g. 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 alleles and pmKRAS alleles was investigated with Spearman rank correlation. Patients who reached the first objective tumour evaluation after 3 cycles or experienced clinical progression prior to this point were considered evaluable for response according to RECIST. Progression free survival (PFS) was defined as time from start of treatment until documented tumour progression or death. Overall survival (OS) was calculated from date of first treatment until death by any course. Survival analyses were performed according to the Kaplan-Meier method and progression free survival and overall survival curves were compared by log-rank test. A multivariate Cox regression analysis was performed using a backwards stepwise elimination process which eliminates the predictor with the largest p-value in each step until all predictors in the final model had a \(p<0.2\). 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 \(\leq 0.05\). (No correction for multiple testing was applied). Statistics were carried out using the NCSS statistical software 2007 v.07.1.5 (NCSS Statistical Software, Utah 84037, USA, www.ncss.com) except the test of the proportional hazards assumption which was performed in SPSS v. 15.0.
Results

Patients characteristics

Patient’s 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 due to an anaphylactic reaction (3) or patients 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 1 cycle of treatment, showed clinical progression before the first evaluation scan and were subsequently included in analysis as having progressive disease. The rate of partial response was 20% (20/101), stable disease 34% (34/101) and progressive disease 47% (47/101). 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 three cases, leaving 95 assessable for comparison of tissue analyses and peripheral blood (table 2). The majority of KRAS mutations detected in the tumour were
also found in the peripheral blood; 32/41 (78%). Interestingly, one of the patients had a primary KRAS mutant tumour but wild type metastatic disease, which was confirmed in the peripheral blood. Unfortunately, no metastatic tissue was available for testing in the remaining 8 patients with disconcordant results. Of note, none of the patients without mutations detected in the primary tumour tissue were tested positive in the baseline plasma sample.

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

As presented in figure 1 there was a clear correlation between the level of KRAS mutations and cfDNA in the plasma (Spearmans rank correlation 0.85, P<0.0001). The median level of cfDNA was 23000 alleles per mL plasma (range 2000-4616000). There was no significant difference in the levels of cfDNA between patients with KRAS mutant (median 19500, range 2000-4600000) and wt disease (median 25000 range 2600-610000, p>0.05). The median level of pmKRAS was 3300 (range 50-180000). Only two patients revealed BRAF mutation alleles in the plasma and consequently no further analysis was performed. 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 prior to treatment, whereas all other parameters showed non-significant differences. This suggested that PS and disease status possibly influences the level of cfDNA in the peripheral circulation. The median cfDNA in PS 0 patients was 15000 alleles/mL plasma (range 2000-1000000) and 52000 alleles/mL plasma (range 14000-420000) in patients with PS 2 at baseline, p=0.03 (not corrected for multiple comparisons).
Baseline cfDNA levels and correlation to tumour response.

There was a significant difference between the groups of patients who achieved disease control (DC), (29600 alleles/mL plasma) and those who progressed early (130000 alleles/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 patient with low cfDNA (<25% percentile) compared to 30% in the patients with high levels (>75% percentile (p=0.009)). A descriptive receiver operating curve analysis was performed to test the performance of cfDNA for predicting disease stabilisation. The AUC was 0.69 (95% CI 0.56-0.77, p<0.001). A cut off at the 75% level of cfDNA performed with a sensitivity of 87%, whereas the 90 percentile produced a sensitivity of 96% for early disease progression.

Baseline pmKRAS levels and tumour response

The patients with high pmKRAS levels (>75%) had a DC rate of 0% compared to 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 to 4.5 month (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 month (95% CI 2.1-2.8), respectively p<0.001. The Kaplan Major survival curves according to baseline cfDNA are shown in figure 2, which also includes survival analysis according to pmKRAS. In brief, an unfavourable survival time was revealed with increasing baseline levels of cfDNA and pmKRAS.
For the initial multivariate Cox regression analysis we included ECOG performance status and cfDNA quartiles (values 1-4) as numeric predictors and pre-treatment KRAS mutational status, number of metastatic sites at baseline, age, gender and tumor location as categorical predictors. By entering performance status and cfDNA quartile as numeric predictor 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 performed separate multivariate analysis by entering cfDNA as a dichotomized covariate (above vs. below median and above vs. below 75% percentile) which both 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 analysed as numeric variable in quartiles. However, using the median or 75% percentile as cut off did not alter the highly significant correlation to outcome (data not shown). A multivariate model build using cfDNA quartiles and the established prognostic markers of KRAS status, number of metastatic sites and age yielded hazard ratio 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 since the sample size for KRAS mutant patients was limited to 35 patients and a strong correlation to cfDNA 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 three 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 cancer patients originates from normal non-malignant cells as well as necrotic and apoptotic tumor cells (20), but neither the origin nor fate of the circulating DNA is fully explored (18). A recent review has summarised 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 to 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 hypothesise that the increasing levels of cfDNA in cancer patients primarily is of tumor origin. Cell-free DNA 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 (21). Tissue availability and selection of specimens with a sufficient number of tumor cells together with tumor heterogeneity are 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 et al. reported that the detection rate of mutations in the peripheral blood ranged between 9% (3/16) and 100% (5/5). However, most prior studies have included a very low number of patients (18). Recently, Yen et al investigated 76 patients using a different method and found a detection rate similar to that of our study (84.4%) (22). The few disconcordant results from our study should be further investigated.

No conclusion can be drawn from our data regarding *pBRAF* detection due to the low sample size and marginally low frequency compared to the literature (23-24), 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 pre-treatment pm*KRAS* had a poor prognosis, compared to the subgroup of patients harbouring *KRAS* mutations at low levels, who achieved a prolonged stabilisation of disease. We present data which indicate that it may not only be the *KRAS* status itself but rather the quantitative amount of this mutation which influences disease behaviour. Consequently, a quantitative measure at baseline will potentially help to select patients with primary *KRAS* dominant disease, who have undetectable or very low levels of the mutation and therefore potentially could benefit from anti-EGFR therapy. Testing in other clinical settings will reveal if this solely applies to anti-EGFR therapy, but we find it likely that the high levels reflect aggressive disease behaviour.
Of broader interest are our results which showed that the cfDNA quantitative levels were related to outcome in terms of response, PFS and OS regardless of cut off used. When entered in a multivariate Cox model an independent prognostic value of cfDNA was confirmed. To our best knowledge there are no other studies investigating the quantitative levels of cfDNA and in a similar clinical setting. Interesting observation were performed by Diehl et al 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 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 colorectal cancer. It is questionable if pmKRAS analysis will provide additional predictive or prognostic information to cfDNA quantification in this specific setting due to the strong correlation and thereby surrogate effect of the two markers. However, our data suggest that patients with a low KRAS allele count at baseline could still benefit from treatment, but at present these patients are not considered candidates for EGFR inhibitor treatment since they harbour 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 furthermore, that 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 individualised pre-treatment testing and could improve selection of therapy. Further studies along that line seem justified.

References


Table 1 Patients baseline characteristics.

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Abbreviations; CT, chemotherapy; ND, not determined; PS, ECOG performance status.
Table 2 Comparison of KRAS detection in tissue and plasma

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<th>Tumor KRAS Wild type</th>
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Abbreviations: CI, confidence interval; Sensitivity, 78%; Specificity, 100%; PPV, 100%; NPV, 86%.
• **Figure 1** Correlation between concentrations of *KRAS* mutations and cfDNA in plasma. Cell free DNA alleles/ml plasma is plotted against *KRAS* mutational alleles/ml plasma. Due to the broad range of values a logarithmic scale is used. The Spearman rank correlation 0.85, \( P < 0.0001 \).
KRAS alleles per ml plasma

cfDNA alleles per ml plasma
Table 3 Correlation between disease control and plasma marker levels.

### Quantitative cell free DNA levels in plasma

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<th>Best response</th>
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<th>&lt; 25% Q</th>
<th>25-50% Q</th>
<th>50-70% Q</th>
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<td>52</td>
<td>20 (77%)</td>
<td>14 (56%)</td>
<td>11 (44%)</td>
<td>7 (30%)</td>
<td>0.004</td>
</tr>
<tr>
<td>PD</td>
<td>47</td>
<td>6 (23%)</td>
<td>11 (44%)</td>
<td>14 (56%)</td>
<td>16 (70%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>99</td>
<td>26</td>
<td>25</td>
<td>25</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

| DC, disease control; PD, progressive disease; Q, quartile. Quartiles (generated based on the total cohort) were used for grouping of the patients and analysed by χ²-test. *When dichotomizing the KRAS mutant group by the 75% quartile a significantly higher rate of DC was revealed in the patients with level below the 75% (p=0.049) see text. |

### Quantitative levels of KRAS mutations in plasma

<table>
<thead>
<tr>
<th>Best response</th>
<th>Total</th>
<th>&lt; 25% Q</th>
<th>25-50% Q</th>
<th>50-70% Q</th>
<th>&gt; 75% Q</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>11</td>
<td>5 (56%)</td>
<td>2 (29%)</td>
<td>4 (40%)</td>
<td>0 (0%)</td>
<td>0.16*</td>
</tr>
<tr>
<td>PD</td>
<td>21</td>
<td>4 (44%)</td>
<td>5 (71%)</td>
<td>6 (60%)</td>
<td>6 (100%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>9</td>
<td>7</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 2** Kaplan Major Survival Curves and log rank test according to plasma levels of cell free DNA and KRAS mutations

The sample size for survival analysis was limited to 105 patients, because of invalid inclusion date of one patient and no blood sample available in 2 patients. KRAS mutations were detected in 35 patients. The sum of percentages may not be 100% due to rounding of data. HR hazard ratio, HRs are only presented for data with one chosen cut off for analysis. (p-values were not corrected for multiple comparisons).
<table>
<thead>
<tr>
<th>cfDNA quartiles</th>
<th>cfDNA quartiles</th>
<th>Progression Free Survival</th>
<th>Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,250</td>
<td>0,500</td>
<td>0,250</td>
<td>0,250</td>
</tr>
<tr>
<td>0,500</td>
<td>0,750</td>
<td>0,500</td>
<td>0,500</td>
</tr>
<tr>
<td>0,750</td>
<td>1,000</td>
<td>0,750</td>
<td>0,750</td>
</tr>
</tbody>
</table>

**cfDNA** N* (%) median PFS in Months (95% CI)

- <25% -- 26 (25)              5.9 (4.2-7.7)
- 25-50% - 27 (26)             4.8 (2.3-8.4)
- 50-75% - 26 (25)              2.3 (2.1-4.2)
- >75% -       26 (25)             2.1 (1.9-2.8)

Logrank p=0.0043

**cfDNA** N (%) median OS in Months (95% CI)

- <25% -- 26 (25)             14.5 (12.8-19.5)
- 25-50% - 27 (25)            9.4 (5.7-11.5)
- 50-75% - 27 (25)             5.9 (4.5-9.0)
- >75% -       26 (25)             3.6 (3.3-4.5)

Logrank p=0.0000

**pmKRAS** N (%) median PFS in Months (95% CI)

- <25%  -- 9 (26)       4.8 (1.2-5.9)
- 25-50%  –   9 (26)      2.1 (2.0-2.8)
- 50-75% - 10 (29)       2.3 (2.1-2.8)
- >75% -   7 (20)       1.8 (1.3-1.8)

Logrank p=0.018

**pmKRAS** N (%) median OS in Months (95% CI)

- <25% -- 9 (26)         9.8 (5.8-13.0)
- 25-50% - 9 (26)        4.7 (2.4-4.8)
- 50-75% - 10 (29)       4.7 (2.8-6.5)
- >75% -   7 (20)       2.1 (1.3-3.6)

Logrank p=0.0008

**pmKRAS** N (%) median OS in Months (95% CI)

- <25% -- 28 (80)        5.0 (4.7-7.1)
- 25-50% - 28 (80)       5.0 (4.7-7.1)
- 50-75% - 27 (27)       5.9 (4.5-9.0)
- >75% -   7 (20)        2.1 (1.3-3.6)

Logrank p=0.0005
### Table 4. Multivariate Cox Regression analysis of PFS and OS.

<table>
<thead>
<tr>
<th>Variables</th>
<th>PFS</th>
<th></th>
<th>OS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>cfDNA quartile (1 to 4)*</td>
<td>1.3 (1.1-1.6)</td>
<td>0.005</td>
<td>1.7 (1.4-2.1)</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>PS*</td>
<td>1.3 (0.9-1.8)</td>
<td>0.11</td>
<td>1.5 (1.1-2.2)</td>
<td>0.014</td>
</tr>
<tr>
<td>KRAS status in tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type†</td>
<td>1</td>
<td>&lt;0.00001</td>
<td>1</td>
<td>0.047</td>
</tr>
<tr>
<td>Mutation</td>
<td>2.7 (1.7-4.4)</td>
<td></td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>No of metastatic sites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2†</td>
<td>1</td>
<td>0.0024</td>
<td>1</td>
<td>0.001</td>
</tr>
<tr>
<td>&gt;2</td>
<td>2.0 (1.3-3.2)</td>
<td></td>
<td>2.2 (1.4-3.6)</td>
<td></td>
</tr>
<tr>
<td>Anatomic site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>colon†</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0.087</td>
</tr>
<tr>
<td>Rectum</td>
<td>0.7 (0.4-1.06)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: cfDNA, cell-free DNA; CI, confidence interval; HR, Hazard ratio; OS, overall survival; PFS, progression-free survival; PS, ECOG performance status. *Entered in the model as numeric variables. The quartiles (1-4) were used for grouping of cDNA 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). †Reference group.
Figure 1 Correlation between concentrations of KRAS mutations and cfDNA in plasma. KRAS mutational alleles/mL plasma are plotted against the number of cell free DNA alleles/ml plasma. Due to the broad range of values a logarithmic scale is used. The Spearman’s rank correlation was 0.85, P<0.0001.

Figure 2 Kaplan Major Survival Curves and log rank test according to plasma levels of cell free DNA and KRAS mutations
The sample size for survival analysis was limited to 105 patients, because of invalid inclusion date of one patient and no blood sample available in 2 patients. KRAS mutations were detected in 35 patients. The sum of percentages may not be 100% due to rounding of data. HR, hazard ratio, HRs are only presented for data with one chosen cut off for analysis. (p-values were not corrected for multiple comparisons).

Table 1 Patients baseline characteristics.

Table 2 Comparison of KRAS detection in tissue and plasma

Table 3 Correlation between disease control and plasma marker levels.

Table 4. Multivariate Cox Regression analysis of PFS and OS.
Quantitative Cell Free DNA, KRAS and BRAF mutations in Plasma from Patients with Metastatic Colorectal Cancer during Treatment with Cetuximab and Irinotecan


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