Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care

Safia El Messaoudi, Florent Mouliere, Stanislas Du Manoir, Caroline Bascoul-Mollevi, Brigitte Gillet, Michelle Nouaille, Catherine Fiess, Evelyne Crapez, Frédéric Bibeau, Charles Theillet, et al.

To cite this version:

HAL Id: hal-01826197
https://hal-unilim.archives-ouvertes.fr/hal-01826197
Submitted on 28 Nov 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright
Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care

Safia El Messaoudi¹,²,₃,₄, Florent Mouliere¹,²,₃,₄, Stanislas Du Manoir¹,²,₃,₄, Caroline Bascoul-Mollevi¹,²,₃,₄,⁵, Brigitte Gillet⁶, Michelle Nouailhe⁷, Catherine Fiess⁸, Evelyne Crapez¹,²,₃,⁴, Frederic Bibeau¹,²,₃,⁴,⁹, Charles Theillet¹,²,₃,⁴, Thibault Mazard¹,²,₃,⁴,¹⁰, Denis Pezet⁶, Muriel Mathonnet⁷, Marc Ychou¹,²,₃,⁴,¹⁰, and Alain R. Thierry¹,²,₃,⁴

Abstract

Purpose: Circulating cell-free DNA (ccfDNA) is a valuable source of tumor material obtained from a simple blood sampling that enables noninvasive analysis of the tumor genome. Our goal was to carry out a multiparametric analysis of ccfDNA and evaluate its prognostic value by investigating the overall survival (OS) of 97 metastatic colorectal cancer patients (mCRC).

Experimental Design: Qualitative parameters (determination of the main KRAS exon2 and BRAF V600E mutations) and quantitative parameters (total ccfDNA concentration, mutant ccfDNA concentration, the proportion of mutant ccfDNA, and ccfDNA integrity index) were determined simultaneously in a single run using a unique Q-PCR multimarker approach (100% success rate).

Results: The median follow-up time was 36 months and median OS was 22 months. Patients showing high ccfDNA levels had significantly shorter OS (18.07 months vs. 28.5 months, \( P = 0.0087 \)). Moreover, multivariate analysis revealed that a high ccfDNA level is an independent prognostic factor (\( P = 0.034 \)). All ccfDNA parameters were of prognostic interest: patients with higher levels of mutant ccfDNA and higher mutation loads for the detected mutations had shorter OS (\( P = 0.0089 \) and \( P = 0.05 \), respectively). In addition, the level of ccfDNA fragmentation correlated positively with decreased OS in the exclusive KRAS/BRAF-mutant cohort of patients (\( P = 0.0052 \)) and appeared as a strong independent prognostic factor (\( P = 0.0072 \)), whereas it was not significant in the exclusive KRAS/BRAF WT cohort of patients (\( P = 0.67 \)).

Conclusions: Our data provide for the first time qualitative and quantitative evidence in favor of multiparametric ccfDNA analysis in mCRC patients for prognostic assessment. Clin Cancer Res, 22(12); 3067–77. ©2016 AACR.

Introduction

A strong need exists for a noninvasive tool to improve the prognosis evaluation of colorectal cancer patients, particularly for patients in the early stage as well as for stratifying stage IV patients. The current prognostic gold standard for colorectal cancer patient classification remains the TNM classification (1). Metastatic colorectal cancer patients (mCRC) show a broad range of outcomes. No prognosis-validated biomarker is currently available for mCRC management (2). Several prognostic factors have been reported in the literature as predictors of mCRC patient survival: number of hepatic metastases, node-positive compared with node-negative primary, poorly differentiated compared with well or moderately primary, extra-hepatic disease compared with liver-only disease, tumor diameter, positive compared with negative resection margins, MSI versus MSS status and the carcinoembryonic antigen (CEA) level (3). Nonetheless, the CEA level is measured in current clinical practice at the time of diagnosis to establish disease prognosis and constitutes a tool for disease follow-up. However, CEA is not specific to colorectal tumors or to the tumor process. Hence, there is an urgent need for a colorectal tumor-specific noninvasive biomarker.

Circulating cell-free DNA (ccfDNA) is a potential source of tumor material, obtained by simple blood sampling, enabling
There is an urgent need for a colorectal tumor-specific biomarker beside the TNM classification. Here, we study the prognostic potential of circulating cell-free DNA (ccfDNA) analysis according to a multiparametric approach by a sensitive and specific analytic system we previously validated for its diagnostic and theragnostic capacities. This noninvasive analysis simultaneously provided qualitative (determination of the main KRAS and BRAF mutations) and quantitative (total ccfDNA concentration, mutant ccfDNA concentration, the proportion of mutant ccfDNA, and ccfDNA integrity) information from a simple blood sampling. Each of this parameter significantly correlated with overall survival and showed much higher prognostic value than CEA, revealing that multimarker ccfDNA analysis could bring new insights in the management of colorectal cancer patients.

Translational Relevance

There is an urgent need for a colorectal tumor-specific biomarker beside the TNM classification. Here, we study the prognostic potential of circulating cell-free DNA (ccfDNA) analysis according to a multiparametric approach by a sensitive and specific analytic system we previously validated for its diagnostic and theragnostic capacities. This noninvasive analysis simultaneously provided qualitative (determination of the main KRAS and BRAF mutations) and quantitative (total ccfDNA concentration, mutant ccfDNA concentration, the proportion of mutant ccfDNA, and ccfDNA integrity) information from a simple blood sampling. Each of this parameter significantly correlated with overall survival and showed much higher prognostic value than CEA, revealing that multimarker ccfDNA analysis could bring new insights in the management of colorectal cancer patients.

Materials and Methods

Patients

Ninety-seven mCRC patients recruited from 3 clinical centers were analyzed to investigate the prognostic value of qualitative and quantitative parameters determined from ccfDNA analysis. Specific characteristics of this patient cohort were described previously under the STARD criteria (25). Eligible patients were male or female, age ≥ 18 years, with histologically confirmed mCRC. Patients had measurable disease as defined by the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1) and were not treated by chemotherapy or radiotherapy in the 4 weeks before the enrollment. Written, informed consent was obtained from all participants before the onset of this noninterventional study. According to the French Public Health Code Article L1131-1 and the following articles, no specific ethical approval is required for this type of study.

Specimen characteristics and preparation

Samples were handled accordingly with a pre-analytic guideline previously established by our group (26). Briefly, 4 mL blood samples were collected in K3 EDTA tubes. Plasma was isolated within 1 hour after drawing blood. The isolation process consisted in a two-step centrifugation. First, blood tubes were centrifuged for 10 minutes in a Heraeus Multifuge LR centrifuge with a speed spin of 1,200 × g and a temperature of 4°C. Supernatant was collected, and the buffy coat was avoided with precaution. The collected supernatant was centrifuged a second time to remove any possible remaining cells. This second centrifugation step was performed for 10 minutes at 4°C and with a spin speed of 16,000 × g. Plasma supernatant was then transferred in a 1.5 mL tube, extracted immediately after or stored at −20°C.

CcfdNA extraction was performed with the Qiagen Blood Mini Kit, and by following the main steps of the “Blood and body fluid protocol.” However, we modified two elements: during the extraction, 1 mL of plasma was processed sequentially in one column. Then, ccfDNA was eluted in 130 μL of elution buffer. Eluted ccfDNA was stored at −20°C before Q-PCR analysis. Freeze-thawing was avoided to reduce fragmentation of the eluted ccfDNA, and no extracts were conserved for more than three months at −20°C.

Assay methods

Intplex is a Q-PCR-based method specifically aimed at analyzing ccfDNA. Intplex is based on a nested design, where two short amplicons (60–100 bp ± 10 bp) are implemented among a larger amplicon (300 ± 10 bp). One of the short amplicons targets a specific locus hotspot of interest (KRAS codon 12, 13 or BRAF codon 600 in our experiments, but it is applicable to other point mutations). The other short amplicon is designed for amplifying a wild-type (WT) sequence. This amplicon quantification provides an estimation of the total concentration of ccfDNA fragment sequences either when targeting KRAS or BRAF sequence (Ref A KRAS and Refa BRAF). Intplex methodology, primer design and validation have been described previously (24, 25).

In all plasma samples, total ccfDNA concentration (ref A), positivity for a mutation, mutant fragments concentration (mA), mutated allele frequency (mA%), and the DNA integrity index were analyzed to investigate the prognostic value of qualitative and quantitative parameters determined from ccfDNA analysis. Specific characteristics of this patient cohort were described previously under the STARD criteria (25). Eligible patients were male or female, age ≥ 18 years, with histologically confirmed mCRC. Patients had measurable disease as defined by the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1) and were not treated by chemotherapy or radiotherapy in the 4 weeks before the enrollment. Written, informed consent was obtained from all participants before the onset of this noninterventional study. According to the French Public Health Code Article L1131-1 and the following articles, no specific ethical approval is required for this type of study.

Materials and Methods

Patients

Ninety-seven mCRC patients recruited from 3 clinical centers were analyzed to investigate the prognostic value of qualitative and quantitative parameters determined from ccfDNA analysis. Specific characteristics of this patient cohort were described previously under the STARD criteria (25). Eligible patients were male or female, age ≥ 18 years, with histologically confirmed mCRC. Patients had measurable disease as defined by the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1) and were not treated by chemotherapy or radiotherapy in the 4 weeks before the enrollment. Written, informed consent was obtained from all participants before the onset of this noninterventional study. According to the French Public Health Code Article L1131-1 and the following articles, no specific ethical approval is required for this type of study.

Specimen characteristics and preparation

Samples were handled accordingly with a pre-analytic guideline previously established by our group (26). Briefly, 4 mL blood samples were collected in K3 EDTA tubes. Plasma was isolated within 1 hour after drawing blood. The isolation process consisted in a two-step centrifugation. First, blood tubes were centrifuged for 10 minutes in a Heraeus Multifuge LR centrifuge with a speed spin of 1,200 × g and a temperature of 4°C. Supernatant was collected, and the buffy coat was avoided with precaution. The collected supernatant was centrifuged a second time to remove any possible remaining cells. This second centrifugation step was performed for 10 minutes at 4°C and with a spin speed of 16,000 × g. Plasma supernatant was then transferred in a 1.5 mL tube, extracted immediately after or stored at −20°C.

CcfdNA extraction was performed with the Qiagen Blood Mini Kit, and by following the main steps of the “Blood and body fluid protocol.” However, we modified two elements: during the extraction, 1 mL of plasma was processed sequentially in one column. Then, ccfDNA was eluted in 130 μL of elution buffer. Eluted ccfDNA was stored at −20°C before Q-PCR analysis. Freeze-thawing was avoided to reduce fragmentation of the eluted ccfDNA, and no extracts were conserved for more than three months at −20°C.

Assay methods

Intplex is a Q-PCR-based method specifically aimed at analyzing ccfDNA. Intplex is based on a nested design, where two short amplicons (60–100 bp ± 10 bp) are implemented among a larger amplicon (300 ± 10 bp). One of the short amplicons targets a specific locus hotspot of interest (KRAS codon 12, 13 or BRAF codon 600 in our experiments, but it is applicable to other point mutations). The other short amplicon is designed for amplifying a wild-type (WT) sequence. This amplicon quantification provides an estimation of the total concentration of ccfDNA fragment sequences either when targeting KRAS or BRAF sequence (Ref A KRAS and Refa BRAF). Intplex methodology, primer design and validation have been described previously (24, 25).

In all plasma samples, total ccfDNA concentration (ref A), positivity for a mutation, mutant fragments concentration (mA), mutated allele frequency (mA%), and the DNA integrity index...
amplifying a short target (3069 bp) to the concentration determined using the primer set amplifying a large target (300 bp) was determined by calculating the ratio of the concentration DNA sample by calculating the DII (DII KRAS and DII BRAF). The system, the Q-PCR reaction and the primer designs are described in the supporting information.

Quality control of the ccfDNA concentration benefited from the determination of two values by targeting two different WT sequences in two different genes (BRAF and KRAS). Samples with concentration values below our quality threshold (concentration below minimal value obtained from plasma samples from healthy donors) revealing sample mishandling following blood collection or for unknown reason were excluded. The coefficient of variation of the ccfDNA concentration from a blood sample was determined as 24% (24). In each single run, negative and positive controls were quantified for each mutations and one standard curve was prepared. Q-PCR amplification was controlled by melt curve differentiation. RT-PCR assay were carried out in duplicate.

The concentration obtained when targeting the mutant sequence corresponded to the concentration of the alleles bearing the mutation (mA). The proportion of mutant allele (mutation load, mA%) was determined by quantifying the relative ratio between mA and Ref A.

The degree of ccfDNA fragmentation was assessed simultaneously by targeting KRAS and BRAF sequences from each plasma DNA sample by calculating the DII (DII KRAS and DII BRAF). The DII was determined by calculating the ratio of the concentration determined using the primer set amplifying a large target (300 ±10 bp) to the concentration determined using the primer set amplifying a short target (<100 bp; ref. 24).

Details of the Q-PCR program, the ccfDNA quantification system, the Q-PCR reaction and the primer designs are described in the supporting information.

Study design and statistics

Sample size justification of the cohort is described in Supplementary Methods. The study was performed according to the REMARK criteria. Blood for ccfDNA analysis was collected around the date of first metastatic diagnosis [median: 1.3 months (0–39)] of delay after first metastatic diagnosis. CEA measurements were taken in the 2 months preceding or following the blood sampling for ccfDNA analysis. None of the patients included in the cohort have been treated by chemotherapy or radiotherapy. Blood samples for ccfDNA analysis were stored at −80°C.

Survival rates were estimated using the Kaplan–Meier method and the log-rank test was used to identify the prognostic variables. Univariate analysis was performed for each ccfDNA parameter, CEA, and clinical parameters. One univariate analysis has been realized on the entire cohort (N = 97) and another has been studied in the exclusive KRAS/BRAF–mutant cohort (N = 43).

Age, localization of the tumor, CEA, Ref A KRAS, RefA BRAF, and mutant status were implemented in a multivariate COX proportional hazards model for analysis on the entire cohort. mA, mA%, mutant status, CEA, Ref A KRAS, DII KRAS, and DII BRAF were included for the multivariate analysis model on the mutant cohort. Statistical analysis was performed using the STATA 11.0 software (StataCorp LP).

Results

Patient characteristics and study design

Patient baseline characteristics, number and localization of metastasis, and number of previous lines of therapy are listed in Table 1. Eighteen patients were excluded from the study because of irrespective inclusion criteria (volume of plasma at least of 2 mL and ccfDNA concentration at least of 5 ng/mL of plasma) and one was lost from sight. Qualitative parameters (determination of the main KRAS exon2 and BRAF V600E mutations) and quantitative parameters (total ccfDNA concentration, mutant ccfDNA concentration, the proportion of mutant ccfDNA, and ccfDNA integrity index) were determined simultaneously in a single run using a unique Q-PCR multimarker approach (100% success rate). Total ccfDNA concentration and DII determined both by targeting KRAS and BRAF WT sequences were available for 97 mCRC patients. We could obtain CEA values in the conditions of delay between CEA analysis and ccfDNA analysis for 83 mCRC patients.

Forty-three patients harbored a KRAS or BRAF mutation and mA and mA% were determined for each of these mutations. The study flowchart is described in Fig. 1.

The median follow-up time was 36 months (0–104 months). Median OS was 22 months, which is consistent with current data on OS of mCRC patients (from 18 to 24 months). OS data are described in Supplementary Fig. S1.
Univariate analysis of clinical variables. Relation between OS and number of metastatic sites was evaluated in 94 mCRC patients. Categories were 1 metastatic site or more than 1 site. Univariate analysis revealed that there was no significant relation between number of metastasis and OS \( P = 0.489; \) HR, 1.071; 95% confidence interval (CI), 0.73–1.96. When dichotomizing patients around 65 years, there was no statistically significant relation with OS \( (P = 0.058; \) HR, 1.64; 95% CI, 0.98–2.76; \( N = 93). \) Regarding the gender, there was no statistical difference between men and women \( (P = 0.77; \) HR, 0.93; 95% CI, 0.56–1.54; \( N = 97). \) Tumor localization seemed to be of prognosis value because there was a statistical relation with OS. Median OS for right colon cancer patients was of 15.2 months compared with 20.9 months for KRAS-mutant mCRC patients and 3.4 months for BRAF-mutant mCRC patients \( (P = 0.033; \) HR, 2.09; 95% CI, 0.99–4.43) and between left colon and rectum cancer groups \( (P = 0.018; \) HR, 1.98; 95% CI, 0.73–1.96). CcfDNA analysis

The median total concentrations Ref A KRAS and Ref A BRAF were 26 ng/mL (2.58–1,386.9) and 27.6 ng/mL (1.12–1,227.2) of plasma, respectively \( (P = 0.088; \) Fig. 2D). Statistically significant differences were also determined when comparing with the second tertile value of Ref A KRAS or BRAF \( (P = 0.013 \) and 0.011; HR, 1.89 and 1.92; 95% CI, 1.14–3.15 and 1.15–3.19, respectively; \( N = 38; \) Table 3). We observed also a significant statistical difference when analyzing Ref A BRAF (total ccfDNA concentrations determined with DII primers sets): Patients with Ref A BRAF below 27.6 ng/mL of plasma had a median OS of 24.5 months compared with 20.5 months for patients with higher levels \( (P = 0.0001; \) HR, 6.106; 95% CI, 5.72–6.45). Median OS of WT patients showed a statistically high significant difference when compared with BRAF-mutant patients \( (P < 0.0001; \) HR, 8.93; 95% CI, 3.13–25.4; \( N = 83\) ) and BRAF-mutant mCRC patients \( (P = 0.038; \) HR, 1.55; 95% CI, 1.17–3.20; \( N = 38; \) Table 3). CcfDNA fragmentation and OS. ccfDNA fragmentation was evaluated in 97 mCRC patients on both KRAS and BRAF using the DII. Patients showing higher DII KRAS (DII determined with KRAS primer set) than the median value \( (0.12) \) had a higher
median OS than patients with lower levels (23.07 months vs. 19.2 months; Table 3). This observation was the same when analyzing DII BRAF (DII determined with BRAF primer set); patients with a DII higher than the median (0.119) had a median OS of 23.07 months compared with 17.17 months for patients with highly fragmented DNA. When analyzing the first tertile of DII BRAF (0.07), a significant difference was shown, although not statistically, between the two groups of patients ($P = 0.08; HR, 1.62; 95\% CI, 0.95–2.78; Table 3). It seemed that a higher level of fragmentation had a tendency to be correlated with worse prognosis.

**Multivariate analysis on the entire cohort.** CcfDNA parameters that were found to be highly significant in univariate analysis, BRAF mutant, Ref A KRAS, and Ref A BRAF (total ccfDNA concentration), CEA, tumor localization, and age were included in a multivariate Cox proportional hazards model on the entire cohort. Results showed that total ccfDNA concentration appeared...
Table 2. Median value and range of the five studied parameters as determined by ccfDNA analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>KRAS</th>
<th>BRAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median total ccfDNA concentration</td>
<td>26.0 (25.8–1385)</td>
<td>27.6 (11.2–1227.15)</td>
</tr>
<tr>
<td>(ng/mL of plasma)</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>Mutation frequency in cohort (n)</td>
<td>0.12 (0.0006–0.93)</td>
<td>0.11 (0.003–0.85)</td>
</tr>
<tr>
<td>Median DII</td>
<td>3.60 (0.04–507.6)</td>
<td>10.72 (0.31–64.2)</td>
</tr>
<tr>
<td>Median mutant ccfDNA concentration</td>
<td>16.2 (0.57–9.5797)</td>
<td></td>
</tr>
<tr>
<td>(ng/mL of plasma)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median mutation load (mA%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median CEA concentration (µg/L of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>plasma)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: mA%, mutation load (% of mutant ccfDNA among total ccfDNA).

Statistically as a strong independent prognostic factor (P = 0.034;
HR, 1.73; 95% CI, 2.56–21.2), as well as BRAF-mutant status (P = 0.002;
HR, 7.33; 95% CI, 1.04–2.89).

OS analysis in the mutant cohort

Higher mutant ccfDNA concentrations are statistically correlated
with shorter OS. KRAS or BRAF-mutant mCRC patients with a
mA (mutant ccfDNA concentration) below the median of 3.06
ng/mL of plasma had a median OS of 31.6 months (Fig. 3A).
For mutant mCRC patients with higher levels, the median OS
was 11.3 months (P = 0.0089; HR, 2.7; 95% CI, 1.25–5.93).
When studying the second tertile, this observation was con-
firmed (P = 0.0071; HR, 2.7; 95% CI, 1.28–5.78; Table 3). The
presence of BRAF-V600E mutation is known to be strongly
 correlated with a decrease in patient survival (27). To avoid
the influence of BRAF V600E mutation poor prognosis on the
analysis, we also analyzed this parameter exclusively in
KRAS-mutant mCRC patients (N = 38). We found that mA
was still correlated with outcome: Patients with lower mA than
the second tertile presented a median OS of 31.6 compared
with 11.4 months in patients with a higher mA (P = 0.0089;
HR, 2.72; Supplementary Fig. S3).

Patients with high mutation load have statistically reduced OS.
Mutant mCRC patients with mutation loads (mA%) lower than
10.72% (median mA% of the full cohort) had a median OS of
31.6 months compared with 11.4 months for patients with higher
levels (P = 0.15; HR, 2.8; 95% CI, 0.82–3.62). Although the
median OS of the latter group was less than half than that of the
former group, there was no statistical difference. This tendency
was confirmed with different thresholds. When studying the first
tertile (4.14%), the median OS of patients with low mA was
34.6 months compared with 13.9 months for patients with higher
levels (P = 0.05; HR, 2.29; 95% CI, 0.97–5.44; Fig. 3B). When
analyzing the second tertile (15.9%), patients with low mA
presented a median OS of 22.1 months compared with 11.3
months for patients with higher levels (P = 0.08; HR, 1.93;
95% CI, 0.9–4.21; Table 3). When the 5 patients exhibiting a
BRAF V600E mutation were removed from the evaluated cohort,
we observed that there was a trend showing a difference in OS for
patients with low mA% with a median OS of 31.6 months
compared with patients showing higher levels as the median OS
decreased to 17.3 months (P = 0.11; HR, 1.827; Supplementary
Fig. S4).

Higher total ccfDNA concentration and fragmentation are correlated
with decreased OS. Ref A KRAS and DII KRAS (total ccfDNA
concentration and DII determined with KRAS primer sets) were
highly significant in univariate analysis in the mutant cohort (P =
0.016 and 0.0052; HR, 2.58 and 0.26; 95% CI, 1.16–5.78 and 0.1–
0.71, respectively, N = 45; Fig. 3C and D) whereas CEA was not
significant (P = 0.81; HR, 0.89; 95% CI, 0.35–2.28; Fig. 3E).

Multivariate analysis

Implementation in a multivariate Cox proportional hazards model revealed that Ref A KRAS appeared as an independent prognostic factor (P = 0.057; HR, 3.67; N = 43) and that DII KRAS appeared as a strong independent prognostic factor (P = 0.0072; HR, 3.57; N = 43). Note that when studying DII KRAS in the exclusive WT patients cohort, it did not appear of prognostic value (P = 0.67, N = 54, data not shown).

Discussion

Several reports have already described the potential impact of
ccfDNA on the prognosis of patient outcome in terms of either OS
or progression-free survival (6–9, 28, 29). Most of these previous
publications were oriented to analyze the correlation between an
increase in the total level of ccfDNA with a decrease in PFS and OS.
Other analytic parameters exhibited by ccfDNA, such as fragmenta-
tion levels, epigenetic alterations, or allelic frequencies for a
particular mutation have not been studied extensively. In this
study, simultaneous determination of five parameters by ccfDNA
analysis on a cohort of 97 mCRC patients allowed us unprece-
dently to assess the strong prognostic significance of multi-
parametric ccfDNA analysis. Analysis of the prognostic signifi-
cance of each parameter was studied by dichotomizing the pop-
ulation to the median value in univariate analysis. Multivariate
analysis was realized by assessing each parameter found statisti-
cally significant in univariate analysis.

First of all, we confirm the usefulness of point mutation
detection by ccfDNA analysis as a prognostic tool. Our data
showed the previously established prognosis influence of
BRAF V600E (P < 0.0001; HR, 8.93; 95% CI, 3.13–25.4; ref. 30).
However, there was no difference, in our cohort, in OS between
WT and KRAS-mutant patients (P = 0.675; HR, 1.11; 95% CI,
0.67–1.85), although some authors have reported that the
presence of KRAS mutation is indicative of poor outcome
(31). Nevertheless, literature analysis tends to show that there
is sometimes confusion around the terms prognostic and
prediction of therapeutic response. Indeed, KRAS point
mutations are more correlated to anti-EGFR resistance than to
outcome (32).

Next, we showed that high levels of total ccfDNA concentration
were strongly correlated with poor outcome in mCRC patients.
This observation has already been reported (6). Nevertheless, it is
the first time, to our knowledge, that this parameter has been
studied simultaneously on two different genes by targeting either
a KRAS or a BRAF WT sequence highlighting the robustness of our
analytic process and the robustness of the prognostic significance of total ccfDNA concentration. Previous observations of our group on xenografted mice have shown the strong correlation between tumor volume and ccfDNA concentration (33), and other works have showed that ccfDNA concentration is positively correlated to tumor burden (7, 28, 34). Spindler and colleagues (8) revealed the correlation between the concentration of mutant ccfDNA (mA) and OS. Our study

<table>
<thead>
<tr>
<th>Table 3. OS analysis on the entire cohort</th>
<th>Death occurrence</th>
<th>Median OS (Mo)</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA (µg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ Median: 16.2 µg/L</td>
<td>23/42</td>
<td>28.1</td>
<td>1.00 (0.99–2.16)</td>
<td>0.088</td>
</tr>
<tr>
<td>&gt; Median: 16.2 µg/L</td>
<td>30/41</td>
<td>17.8</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>≤ 5 µg/L</td>
<td>15/23</td>
<td>27.2</td>
<td>1.00 (0.68–2.25)</td>
<td>0.48</td>
</tr>
<tr>
<td>&gt; 5 µg/L</td>
<td>38/60</td>
<td>21.7</td>
<td>1.24</td>
<td></td>
</tr>
<tr>
<td>KRAS mutant status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>40/59</td>
<td>21.9</td>
<td>1.00 (0.67–1.85)</td>
<td>0.675</td>
</tr>
<tr>
<td>Mutant</td>
<td>24/38</td>
<td>20.9</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>BRAF mutant status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>59/92</td>
<td>21.9</td>
<td>1.00 (1.13–25.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mutant</td>
<td>5/5</td>
<td>3.4</td>
<td>8.93</td>
<td></td>
</tr>
<tr>
<td>KRAS or BRAF mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS mutant</td>
<td>24/38</td>
<td>20.9</td>
<td>1.00 (5.72–6.487)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BRAF mutant</td>
<td>5/5</td>
<td>3.4</td>
<td>6.106</td>
<td></td>
</tr>
<tr>
<td>Ref A KRAS (ng/mL)</td>
<td>≤ 1st tertile: 15.6</td>
<td>22.23</td>
<td>1.00 (0.465–1.635)</td>
<td>0.253</td>
</tr>
<tr>
<td></td>
<td>&gt; 1st tertile: 15.6</td>
<td>45/65</td>
<td>21.17</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>≤ Median: 26.0</td>
<td>28.5</td>
<td>1.00 (1.17–3.20)</td>
<td>0.0087</td>
</tr>
<tr>
<td></td>
<td>&gt; Median: 26.0</td>
<td>38/48</td>
<td>18.07</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>≤ 2nd tertile: 47.5</td>
<td>23.17</td>
<td>1.00 (0.14–3.15)</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>&gt; 2nd tertile: 47.5</td>
<td>25/33</td>
<td>13.9</td>
<td>1.89</td>
</tr>
<tr>
<td>Ref A BRAF (ng/mL)</td>
<td>≤ 1st tertile: 13.6</td>
<td>22.23</td>
<td>1.00 (0.465–1.635)</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>&gt; 1st tertile: 13.6</td>
<td>45/65</td>
<td>21.17</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>≤ Median: 27.6</td>
<td>24.5</td>
<td>1.00 (1.13–3.11)</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>&gt; Median: 27.6</td>
<td>36/48</td>
<td>20.5</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>≤ 2nd tertile: 48</td>
<td>24.9</td>
<td>1.00 (1.15–3.19)</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>&gt; 2nd tertile: 48</td>
<td>25/33</td>
<td>13.9</td>
<td>1.92</td>
</tr>
<tr>
<td>DII KRAS</td>
<td>≤ 1st tertile: 0.07</td>
<td>20/31</td>
<td>20.8</td>
<td>1.00 (0.65–1.94)</td>
</tr>
<tr>
<td></td>
<td>&gt; 1st tertile: 0.07</td>
<td>44/66</td>
<td>22.2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>≤ Median: 0.12</td>
<td>19.2</td>
<td>1.00 (0.66–1.76)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>&gt; Median: 0.12</td>
<td>34/50</td>
<td>23.07</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>≤ 2nd tertile: 0.23</td>
<td>44/66</td>
<td>22.03</td>
<td>1.00 (0.77–1.98)</td>
</tr>
<tr>
<td></td>
<td>&gt; 2nd tertile: 0.23</td>
<td>20/31</td>
<td>22.23</td>
<td>1.18</td>
</tr>
<tr>
<td>DII BRAF</td>
<td>≤ 1st tertile: 0.07</td>
<td>25/34</td>
<td>20.6</td>
<td>1.00 (0.95–2.78)</td>
</tr>
<tr>
<td></td>
<td>&gt; 1st tertile: 0.07</td>
<td>39/63</td>
<td>23.07</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>≤ Median: 0.19</td>
<td>17.7</td>
<td>1.00 (0.81–2.25)</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>&gt; Median: 0.19</td>
<td>30/48</td>
<td>26.90</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>≤ 2nd tertile: 0.20</td>
<td>41/64</td>
<td>20.8</td>
<td>1.00 (0.60–1.7)</td>
</tr>
<tr>
<td></td>
<td>&gt; 2nd tertile: 0.20</td>
<td>23/33</td>
<td>22.23</td>
<td>1.05</td>
</tr>
<tr>
<td>Mutant ccfDNA concentration (ng/mL)</td>
<td>≤ 1st tertile: 1.06</td>
<td>10/14</td>
<td>22.1</td>
<td>1.00 (125–2.055)</td>
</tr>
<tr>
<td></td>
<td>&gt; 1st tertile: 1.06</td>
<td>19/29</td>
<td>13.9</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>≤ Median: 3.06</td>
<td>31.6</td>
<td>1.00 (1.25–5.93)</td>
<td>0.0089</td>
</tr>
<tr>
<td></td>
<td>&gt; Median: 3.06</td>
<td>16/21</td>
<td>11.3</td>
<td>2.72</td>
</tr>
<tr>
<td></td>
<td>≤ 2nd tertile: 7.53</td>
<td>22.1</td>
<td>1.00 (1.28–5.78)</td>
<td>0.0071</td>
</tr>
<tr>
<td></td>
<td>&gt; 2nd tertile: 7.53</td>
<td>13/15</td>
<td>6.83</td>
<td>2.72</td>
</tr>
<tr>
<td>Mutation load (%)</td>
<td>≤ 1st tertile: 4.14</td>
<td>34.53</td>
<td>1.00 (0.97–5.44)</td>
<td>0.053</td>
</tr>
<tr>
<td></td>
<td>&gt; 1st tertile: 4.14</td>
<td>22/29</td>
<td>13.9</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td>≤ Median: 10.72</td>
<td>31.6</td>
<td>1.00 (0.82–3.62)</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>&gt; Median: 10.72</td>
<td>16/21</td>
<td>11.4</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>≤ 2nd tertile: 15.9</td>
<td>22.1</td>
<td>1.00 (0.9–4.12)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>&gt; 2nd tertile: 15.9</td>
<td>12/15</td>
<td>11.3</td>
<td>1.93</td>
</tr>
</tbody>
</table>

NOTE: Ref A KRAS, Ref A BRAF, DII KRAS, DII BRAF, and CEA are tested by dichotomization of the entire cohort on the first tertile, the median, and the second tertile. CEA is dichotomized around the median and the standard threshold of 5 µg/L. mA and mA% are tested by dichotomization of the exclusive KRAS/BRAF–mutant cohort around the first tertile, the median and the second tertile.

Abbreviations: DII KRAS, DII determined using KRAS primer set; DII BRAF, DII determined using BRAF primer set; mA, mutant ccfDNA concentration; mA%, mutation load (% of mutant ccfDNA among total ccfDNA); mo, months; Ref A KRAS, total ccfDNA concentration as determined by targeting a WT KRAS sequence; Ref A BRAF, total ccfDNA concentration as determined by targeting a BRAF WT sequence; WT, wild-type for the KRAS and BRAF mutations tested.
Figure 3. OS analysis on the KRAS or BRAF mutant cohort. A, Kaplan–Meier survival curve and log-rank test according to mA determined by ccfDNA analysis dichotomized around the median (3.06 ng/mL, N = 43). B, Kaplan–Meier survival curve and log-rank test according to mA% dichotomized to the first (1st) tertile (4.14%) determined by ccfDNA analysis (N = 43). C, Kaplan–Meier survival curve and log-rank test according to Ref A KRAS dichotomized around the second tertile (2nd tertile; 107.0 ng/mL, N = 43). D, Kaplan–Meier survival curve and log-rank test according to DII KRAS determined by ccfDNA analysis dichotomized around the second tertile (2nd tertile; 0.20, N = 43). E, Kaplan–Meier survival curve and log-rank test according to CEA dichotomized around the standard threshold of 5 μg/L (N = 36). Abbreviations: mA, mutant ccfDNA concentration; mA%, mutation load (% of mutant ccfDNA among total ccfDNA); Ref A KRAS, total ccfDNA concentration as determined by targeting a WT KRAS sequence; DII KRAS, DII determined using KRAS primer set.
confirms this observation, as there was a strong statistical decrease in OS for KRAS- or BRAF-mutant patients with mA higher than the median value (3.06 ng/mL). This was consolidated by the high statistical significance of the mutation load (percentage of mutant alleles among total ccfDNA amount, mA%) on prognosis: Patients with lower mutation load than the median value of 10.72% exhibited a median OS of 31.6 months compared with 11.2 months for patients with a higher mutation load. There was a clear trend showing that high mutation load indicated poor prognosis. As for the mutation load, prognosis of patients with high mA is worse whatever the total ccfDNA concentration. However, it has to be considered that mA% and mA in a mutant sample, while being related, do not represent the same meaning: mA% is rather linked to the proportion of malignant cells versus tumor microenvironment cells and could be altered by tumor clonality, whereas mA is rather linked to the total concentration of ccfDNA. We hypothesize that this biomarker might be of great diagnostic potential in regard to individualizing management care. The prognostic value of mutant ccfDNA concentration seems to be stronger than the prognostic value of total ccfDNA concentration ($P = 0.0089$ and 0.016; HR, 2.72 and 2.58; 95% CI, 1.25–5.93 and 1.16–5.73, respectively). We could explain this phenomenon by the fact that mA determination is derived from the quantification of ccfDNA exclusively deriving from malignant cells whereas total ccfDNA concentration is determined from quantifying malignant as well as nonmalignant-derived ccfDNA.

We observed a higher ccfDNA fragmentation level from colorectal cancer patients as compared with healthy individuals butressing the notion that fragmentation could be an interesting parameter for diagnosis (35, 36). In regard to prognosis, patients with a higher fragmentation level showed, in this study, a lower median OS of 17 months as compared with 23 months for patients with a lower fragmentation level. Note, ccfDNA fragmentation index as well as total ccfDNA concentration showed similar values by using either KRAS or BRAF primer sets, confirming the accuracy of our method. Interestingly, univariate analysis as well as multivariate analysis in the exclusive KRAS/BRAF-mutant cohort revealed that DII KRAS (DII determined with KRAS primer set) was highly significant in univariate and multivariate analysis ($P = 0.0052$ and 0.0072 respectively, $N = 43$) whereas it was not significant in the exclusive WT cohort ($P = 0.67$; $N = 54$). This raises questions about possible biomarkers and/or physiopathologic association between mutant status and ccfDNA fragmentation and their link to tumor progression.

Each of the parameters, point mutation detection, total ccfDNA concentration, mutant ccfDNA concentration, mutation load, and ccfDNA fragmentation, shows individually their prognostic potential and promise better prognostic potential than CEA ($P = 0.48$; RR, 1.24 on the entire cohort and $P = 0.81$; RR, 0.89 on the mutant cohort). Nevertheless, we think that it is crucial to investigate all these parameters further (individually or in combination) to assess mCRC patient prognosis. When implementing all these parameters in multivariate analysis for the entire cohort with all clinical standards, such as age, sex, tumor localization, and CEA, the study data revealed that ccfDNA parameters, such as total ccfDNA concentration ($P = 0.034$; HR, 1.73; 95% CI, 1.04–2.89), as well as BRAF-mutant status ($P = 0.002$; HR, 7.33; 95% CI, 3.13–25.4), are strong independent prognostic factors. Quantitative analysis of ccfDNA, especially the mA%, preferentially relies on tumor biology, in particular to the proportion and/or activity of the tumor microenvironment which appears as a significant factor since a low proportion of malignant cells in colorectal cancer is related to poor cancer-specific survival (37). We previously reported the very high variation in mutation load (more than 2,000-fold) among the mCRC patients (35), an observation later confirmed by Bettegowda and colleagues (6). We believe that quantitative analysis of ccfDNA may be of prognostic value in regard to determination of the minimal residual disease, especially post-surgery when deciding on adjuvant therapy. It could be a biologic tool to decipher the prognosis difference between the 5 and 6 colorectal cancer subtypes recently revealed (38). Other approaches involving ccfDNA analysis were taken into consideration in regard to prognosis, such as presence of methylated DNA as independent prognostic factors in mCRC (18). Note that the prognostic value of ccfDNA in severe sepsis (39) and out-of-hospital cardiac arrest patients (40) are under investigation. Prognostic value of quantitative analysis of ccfDNA might be synergistically empowered by analysis of mitochondrial DNA, exosomal RNA, or specific proteins in a “liquid profiling” approach.

We analyzed sex, age, metastasis number, tumor differentiation, and tumor localization in respect to the evaluation of the ccfDNA parameters on prognosis. Our data only highlight statistical difference between of right- and left-sided origin of the primary tumor. Although differences varied with stages, meta-analysis on early-stage colon cancer reported a worse prognosis for right-sided tumors (41, 42). More investigation is needed to better decipher the molecular differences such as RAS or BRAF mutational status between right- and left-sided colon cancers and their association with prognosis, prediction of PFS benefit, and interaction with anti-EGFR therapy.

Although the cohort size of the mutant cohort ($N = 43$) in our study is somewhat a limiting factor, its analysis revealed original observations in respect the prognosis value of mA or mA%. However, work on a larger cohort of patients with mutation should be initiated to assess those observations as well as the ccfDNA fragmentation-based prognostic value in this population. Moreover, we need to be cautious about the values of ccfDNA concentration or fragmentation because those two parameters can vary under specific physiopathologic conditions such as inflammation. In addition, multiparametric analysis on stage II/III patients should be evaluated.

Altogether, the data revealed that ccfDNA analysis should not be limited to the qualitative determination of the presence of genetic alteration, but rather be implemented in the course of patient follow-up by the quantitative determination of others parameters. For instance, we showed the high-diagnostic capacity of total ccfDNA concentration for discriminating healthy individuals and mCRC patients with a statistical significance of $P < 0.001$ (AUC = 0.92; refs. 24, 35). The various qualitative and quantitative parameters could directly be compared in respect to their diagnostic power by this single-run multiparametric method and potentially could synergistically be associated in an algorithm. Furthermore, the observations made in this report benefited from the ultrasensitivity of the method in quantifying a specific sequence based on published and ongoing clinical studies (single-copy detection of variant alleles down to a sensitivity of >0.005% mutant to WT ratio, being the lowest level found in the literature). The success rate of our method for detecting ccfDNA in plasma is 100% in more than 400 stage 1 to IV colorectal cancer and 120 healthy individual plasma samples and the mean data turnaround time is 2 days for multiplex analysis (24, 25, 35).
Achievement of personalized medicine and targeted therapies require further information about the residual risk and the potential benefit of additional treatments. Overcoming the limitation of a restricted number of molecular analyses in tumor tissue by a noninvasive and repetitive blood test, in particular the qualitative and quantitative multimarker analysis of cfDNA, would be an approach necessary for the early identification of high-risk cancer patients, monitoring disease course, and therapy response.

Disclosure of Potential Conflicts of Interest

F. Bibeau reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Amgen and Merck. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: S. El Messaoudi, F. Mouliere, C. Fiess, M. Mathonnet, M. Ychou, A.R. Thierry
Development of methodology: S. El Messaoudi, F. Mouliere, S.D. Manoir, M. Ychou, A.R. Thierry
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. El Messaoudi, F. Mouliere, C. Bascoul-Mollevi, B. Gillet, M. Nouaille, C. Fiess, E. Crapez, F. Bibeau, T. Mazard, D. Pezet, M. Mathonnet, M. Ychou, A.R. Thierry
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. El Messaoudi, F. Mouliere, S.D. Manoir, C. Bascoul-Mollevi, M. Mathonnet, M. Ychou, A.R. Thierry
Writing, review, and/or revision of the manuscript: S. El Messaoudi, F. Mouliere, S.D. Manoir, C. Bascoul-Mollevi, C. Theillet, M. Mathonnet, M. Ychou, A.R. Thierry
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. El Messaoudi, C. Fiess, F. Bibeau, M. Mathonnet, A.R. Thierry
Study supervision: C. Theillet, A.R. Thierry

Acknowledgments

The authors acknowledge the excellent technical assistance of Leigh Kamraoui and Pierre Jean Lamy for helpful discussions.

Grant Support

This work was supported by the SIRIC Montpellier Grant “INCa-DGOS-Inserm 6045.” A.R. Thierry is supported by the INSERM. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 9, 2015; revised December 18, 2015; accepted January 3, 2016, published OnlineFirst February 4, 2016.

References


Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care

Safia El Messaoudi, Florent Mouliere, Stanislas Du Manoir, et al.


Updated version
Access the most recent version of this article at:

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2016/02/04/1078-0432.CCR-15-0297.DC1

Cited articles
This article cites 42 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/22/12/3067.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/22/12/3067.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/22/12/3067.full
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.