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Safia El Messaoudi, Florent Mouliere, Stanislas Du Manoir, Caroline Bascoul-Mollevi, Brigitte Gillet, et al.. Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care. *Clinical Cancer Research*, 2016, 22 (12), pp.3067 - 3077. 10.1158/1078-0432.CCR-15-0297 . hal-01826197

**HAL Id: hal-01826197**

**<https://unilim.hal.science/hal-01826197v1>**

Submitted on 28 Nov 2018

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

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## 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.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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**doi:** 10.1158/1078-0432.CCR-15-0297

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## 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

### 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 care of colorectal cancer patients.

noninvasive, quantitative, and qualitative analysis of the tumor genome. Tumor cells release ccfDNA, which exhibits the genetic and epigenetic alterations of the tumor of origin (4). The clinical significance of tumor-derived ccfDNA released in the blood of patients with colorectal cancer has already been investigated as a prognostic tool in previous studies using various technologic approaches (5–9). In a recent large meta-analysis, a high correlation between ccfDNA concentration and mCRC patient survival was observed, revealing that patients with relatively low levels of ccfDNA lived significantly longer than patients with higher levels (6). The relevance of ccfDNA levels for the prognosis of other cancer types has also been described, including advanced breast cancer (10), lung cancer (11, 12), prostate cancer (13), pancreatic cancer (14), and other cancer types (15).

However, the majority of these studies have focused on the total ccfDNA concentration in the blood or on the detection of genetic or epigenetic alterations (16–20). Moreover, the relationship between total ccfDNA concentration and outcome may be biased because an increase in the level of total ccfDNA might also be indicative of non-cancerous disease, such as inflammation or trauma (16). Thus, recent studies on the clinical application of ccfDNA have mainly focused on qualitative analysis such as the presence of mutations (5). The ccfDNA fragmentation level has been also investigated as a predictive tool for cancer progression (21–23).

On the basis of our observations on their structures and origins, we designed Intplex, an allele-specific, Q-PCR-based system specifically adapted for ccfDNA analysis (24). With using this method, we reported the first clinical validation in oncology by testing the presence of *KRAS*/*BRAF* point mutations in a cohort of 106 mCRC patients (25). The Intplex method enables simultaneous determination of the total ccfDNA concentration, point mutation detection, mutant ccfDNA concentration, mutation load (% of mutant ccfDNA among total ccfDNA), and ccfDNA integrity.

Here, we examined overall survival (OS) of 97 mCRC patients; this is the largest cohort of mCRC patients studied for potential prognostic interest of ccfDNA analysis. The five Intplex parameters were simultaneously determined for the first time in all patients. We investigated the value of these parameters according to OS by

univariate and multivariate analysis. The results were compared with the prognostic value of the CEA.

## 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  $\geq 18$  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  $1,200 \times g$  and a temperature of  $4^{\circ}\text{C}$ . 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  $4^{\circ}\text{C}$  and with a spin speed of  $16,000 \times g$ . Plasma supernatant was then transferred in a 1.5 mL tube, extracted immediately after or stored at  $-20^{\circ}\text{C}$ .

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  $\mu\text{L}$  of elution buffer. Eluted ccfDNA was stored at  $-20^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$ .

### 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 ( $60\text{--}100 \text{ bp} \pm 10 \text{ bp}$ ) are implemented among a larger amplicon ( $300 \pm 10 \text{ bp}$ ). 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

(DII) were determined with an analysis flowchart detailed in a previous study by our team (24, 25).

Quality control was one of the assets of the method used. First, a stringent pre-analytic guideline ensured the integrity of plasma DNA and potentially excluded samples due to non-proper blood or plasma handling (26). Numeric traceability guaranteed proper sample archiving in a specific blood collection bank for colorectal cancer plasma samples at Dr. Thierry's Laboratory (no. DC-2013-1931). 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 between CEA analysis and blood drawing for ccfDNA analysis. Data were summarized by frequency for categorical variables, and by median and range values for continuous variables. OS was calculated from the date of first metastatic diagnosis to the date of death. 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.

Of note, 106 mCRC patients were initially included in this study in the period between July 2010 and December 2012. Eight 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.

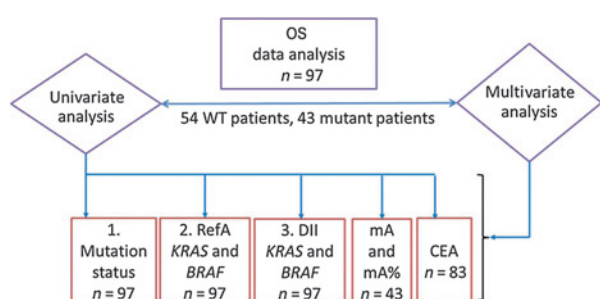
The median follow-up time was 36 months (0–104 months). Median OS was 22 months, which is consistent with current data on OS of mCRC patients (from 18 to 24 months). OS data are described in Supplementary Fig. S1.

**Table 1.** Patient's baseline characteristics

Patient's characteristics		
	<i>N</i> = 97	(%)
Centre		
ICM Montpellier	25	25.8
CHU Clermont-Ferrand	22	22.7
CHU Limoges	50	51.5
Age		
Median (min–max)	66.6	36–87
Missing data	4	
Gender		
Male	58	59.8
Female	39	40.2
Localization tumor primitive		
Right colon	22	22.7
Left colon	41	42.2
Rectum	34	35.1
Primary tumor in place		
No	44	45.4
Yes	53	54.6
Synchronous metastasis		
No	80	82.5
Yes	17	17.5
No. of metastatic sites		
1	51	54.3
>1	43	45.7
Missing data	3	
Chemotherapy		
Naïve	62	63.9
Neoadjuvant/adjuvant	25	71.4
Palliative ( $N = 13$ )		
1 line metastatic	5	38.5
≥2 lines metastatic	8	61.5



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**Figure 1.**

Study flowchart. Abbreviations: WT, wild-type for the *KRAS* and *BRAF* mutations tested; 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 WT *BRAF* sequence; DII *KRAS*, DNA integrity index 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).

**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 (log-rank  $t$  test:  $P = 0.058$ ; HR, 1.64; 95% CI, 0.98–2.76;  $N = 93$ ). Regarding the gender, there was no statistical difference between men and women (log-rank  $t$  test  $P = 0.77$ ; 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 ( $N = 22$ ), it was of 20.03 months for left colon cancer patients ( $N = 41$ ) and of 31.03 months for rectum cancer patients ( $N = 34$ ). Statistical difference was observed for the median OS between right colon cancer and rectum cancer groups ( $P = 0.053$ ; 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 (Table 2). The median DII for *KRAS* and *BRAF* were 0.12 and 0.11, respectively. ccfDNA analysis revealed that 38 mCRC patients (39% of the cohort) were mutant for one of the seven *KRAS* mutations tested and 5% of the cohort exhibited a *BRAFV600E* mutation. The results were fully validated in a blinded study comparing the mutant status determined from tumor tissue using the STARD criteria (25). Concordance rate was 96%. Four percent of samples were discordant; this might be due to intratumoral heterogeneity and clonal heterogeneity between primary tumor and paired metastasis. For those samples, tumor-tissue analysis was realized on primary tumor while blood sampling for ccfDNA analysis was realized after tumor resection. In this cohort, the median mutant ccfDNA concentration determined was 3.06 ng/mL (0.04–507) of plasma. Median mutation load (mA%) was 10.72% (0.51–64.2). Detailed data for each patient are described in Supplementary Table S1. Each of the ccfDNA parameter has been studied for its relation with the following clinical parameters: Tumor localization, number of

metastatic sites, presence of the primary tumor at time of blood sampling, and differentiation of the tumor. Detailed data and figures are presented in Supplementary Fig. S2 and Supplementary Table S2. Note, as illustrated and detailed in Supplementary Data, there is a significant correlation between tumor burden with Ref A *KRAS*, Ref A *BRAF*, mA and CEA (Supplementary Information: relation with tumor burden).

#### CEA value

Median CEA concentration determined from the 83 mCRC patients whom we obtained CEA values in the conditions of delay between CEA analysis and ccfDNA analysis was 16.2  $\mu\text{g/L}$  (0.57–1,999.7) of plasma.

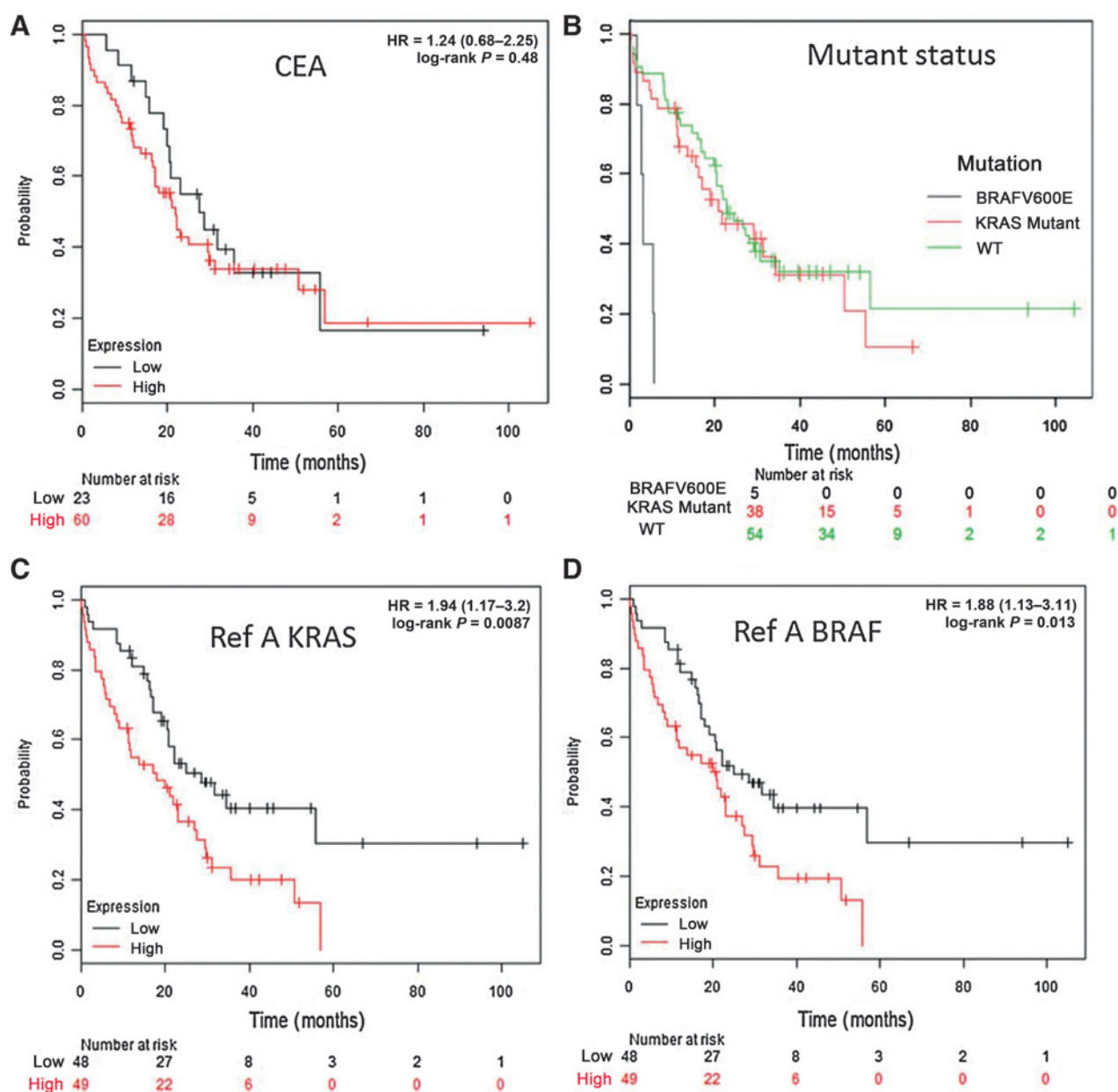
#### OS analysis on the entire cohort

**Relation between CEA and OS.** Patients with lower CEA levels than the median concentration (16.2  $\mu\text{g/L}$ ) presented a median OS of 28.1 months compared with 17.8 months for patients with higher levels ( $P = 0.088$ ; HR, 1.60; 95% CI, 0.99–2.16),  $N = 83$ ; Table 3). Nevertheless, patients with lower CEA levels than the current clinical threshold of significance (5  $\mu\text{g/L}$ ) had a median OS of 27.2 months compared with patients with higher levels with a median OS of 21.7 months ( $P = 0.48$ ; HR, 1.24; 95% CI, 0.68–2.25; Fig. 2A and Table 3).

**Correlation of mutant status with OS.** Patients WT for *KRAS* exon 2 and *BRAFV600E* showed a median OS of 21.9 months compared with 20.9 months for *KRAS*-mutant mCRC patients and 3.4 months for *BRAF*-mutant mCRC patients (Table 3). Kaplan-Meier survival curves were established (Fig. 2B) for each mutant status. No statistical differences in OS was found between the WT ( $N = 54$ ) and *KRAS*-mutant mCRC patients ( $N = 38$ ;  $P = 0.675$ ; HR, 1.11; 95% CI, 0.67–1.95). However, there was a statistically high significant difference between the median OS of *BRAF*-mutant patients ( $N = 5$ ) and *KRAS*-mutant patients ( $N = 38$ ;  $P < 0.0001$ ; 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; Fig. 2B and Table 3).

**Higher total ccfDNA concentration is statistically correlated with a decrease in OS.** Patients with Ref A *KRAS* (total ccfDNA concentration determined with *KRAS* primer set) below the median of 26 ng/mL of plasma had a median OS of 28.5 months compared with 18.07 months for patients with Ref A *KRAS* higher than the median ( $P = 0.0087$ ; HR, 1.94; 95% CI, 1.17–3.20; Fig. 2C). We observed also a significant statistical difference when analyzing Ref A *BRAF* (total ccfDNA concentrations determined with *BRAF* 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 ( $P = 0.013$ ; HR, 1.55; 95% CI, 1.13–3.11; 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; 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

**Figure 2.**

OS analysis on the entire cohort. A, Kaplan-Meier survival curve and log-rank test according to CEA dichotomized around the standard threshold of 5  $\mu\text{g/L}$  ( $N = 83$ ). B, Kaplan-Meier survival curve and log-rank test according to the mutant status determined by ccfDNA analysis ( $N = 97$ ). C, Kaplan-Meier survival curve and log-rank test according to Ref A *KRAS* determined by ccfDNA analysis dichotomized around the median (26 ng/mL,  $N = 97$ ). D, Kaplan-Meier survival curve and log-rank test according to Ref A *BRAF* determined by ccfDNA analysis dichotomized around the median (27.6 ng/mL,  $N = 97$ ). Abbreviations: WT, wild-type for the *KRAS* and *BRAF* mutations tested; 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.

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

	KRAS	BRAF
Median total ccfDNA concentration (ng/mL of plasma)	26.0 (2.58–1,385)	27.6 (1.12–1,227.15)
Mutation frequency in cohort (n)	38	5
Median DII	0.12 (0.0006–0.93)	0.11 (0.003–0.85)
Median mutant ccfDNA concentration (ng/mL of plasma)		3.06 (0.04–507.6)
Median mutation load (mA%)		10.72 (0.51–64.2)
Median CEA concentration (μg/L of plasma)		16.2 (0.57–19,997)

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 confirmed ( $P = 0.0071$ ; 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 OS 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,  $N = 43$ ; 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 fragmentation 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 unprecedentedly to assess the strong prognostic significance of multiparametric ccfDNA analysis. Analysis of the prognostic significance of each parameter was studied by dichotomizing the population to the median value in univariate analysis. Multivariate analysis was realized by assessing each parameter found statistically 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

**Table 3.** OS analysis on the entire cohort

	Death occurrence	Median OS (Mo)	HR (95% CI)	P
CEA ( $\mu\text{g/L}$ )				
≤Median: 16.2 $\mu\text{g/L}$	23/42	28.1	1.00 (0.99–2.16)	0.088
>Median: 16.2 $\mu\text{g/L}$	30/41	17.8	1.30	
≤5 $\mu\text{g/L}$	15/23	27.2	1.00 (0.68–2.25)	0.48
>5 $\mu\text{g/L}$	38/60	21.7	1.24	
KRAS mutant status				
WT	40/59	21.9	1.00 (0.67–1.85)	0.675
Mutant	24/38	20.9	1.11	
BRAF mutant status				
WT	59/92	21.9	1.00 (3.13–25.4)	<0.0001
Mutant	5/5	3.4	8.93	
KRAS or BRAF mutant				
KRAS mutant	24/38	20.9	1.00 (5.72–6.487)	<0.0001
BRAF mutant	5/5	3.4	6.106	
Ref A KRAS (ng/mL)				
≤1st tertile: 15.6	19/32	22.23	1.00 (0.465–1.635)	0.253
>1st tertile: 15.6	45/65	21.17	1.05	
≤Median: 26.0	26/49	28.5	1.00 (1.17–3.20)	0.0087
>Median: 26.0	38/48	18.07	1.94	
≤2nd tertile: 47.5	39/64	23.17	1.00 (1.14–3.15)	0.013
>2nd tertile: 47.5	25/33	13.9	1.89	
Ref A BRAF (ng/mL)				
≤1st tertile: 13.6	19/32	22.23	1.00 (0.465–1.635)	0.2
>1st tertile: 13.6	45/65	21.17	1.05	
≤Median: 27.6	28/49	24.5	1.00 (1.13–3.11)	0.013
>Median: 27.6	36/48	20.5	1.88	
≤2nd tertile: 48	39/64	24.9	1.00 (1.15–3.19)	0.011
>2nd tertile: 48	25/33	13.9	1.92	
DII KRAS				
≤1st tertile: 0.07	20/31	20.8	1.00 (0.65–1.94)	0.72
>1st tertile: 0.07	44/66	22.2	1.1	
≤Median: 0.12	30/47	19.2	1.00 (0.66–1.76)	0.73
>Median: 0.12	34/50	23.07	1.09	
≤2nd tertile: 0.23	44/66	22.03	1.00 (0.77–1.98)	0.52
>2nd tertile: 0.23	20/31	22.23	1.18	
DII BRAF				
≤1st tertile: 0.07	25/34	20.6	1.00 (0.95–2.78)	0.08
>1st tertile: 0.07	39/63	23.07	1.62	
≤Median: 0.119	34/49	17.17	1.00 (0.81–2.25)	0.15
>Median: 0.119	30/48	26.90	1.44	
≤2nd tertile: 0.20	41/64	20.8	1.00 (0.60–1.7)	0.9
>2nd tertile: 0.20	23/33	22.23	1.03	
Mutant ccfDNA concentration (ng/mL)				
≤1st tertile: 1.06	10/14	22.1	1.00 (1.125–2.055)	0.57
>1st tertile: 1.06	19/29	13.9	1.59	
≤Median: 3.06	13/22	31.6	1.00 (1.25–5.93)	0.0089
>Median: 3.06	16/21	11.3	2.72	
≤2nd tertile: 7.53	16/28	22.1	1.00 (1.28–5.78)	0.0071
>2nd tertile: 7.53	13/15	6.83	2.72	
Mutation load (%)				
≤1st tertile: 4.14	7/14	34.53	1.00 (0.97–5.44)	0.053
>1st tertile: 4.14	22/29	13.9	2.29	
≤Median: 10.72	13/22	31.6	1.00 (0.82–3.621)	0.15
>Median: 10.72	16/21	11.4	1.72	
≤2nd tertile: 15.9	17/28	22.1	1.00 (0.9–4.12)	0.08
>2nd tertile: 15.9	12/15	11.3	1.93	

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  $\mu\text{g/L}$ . 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.

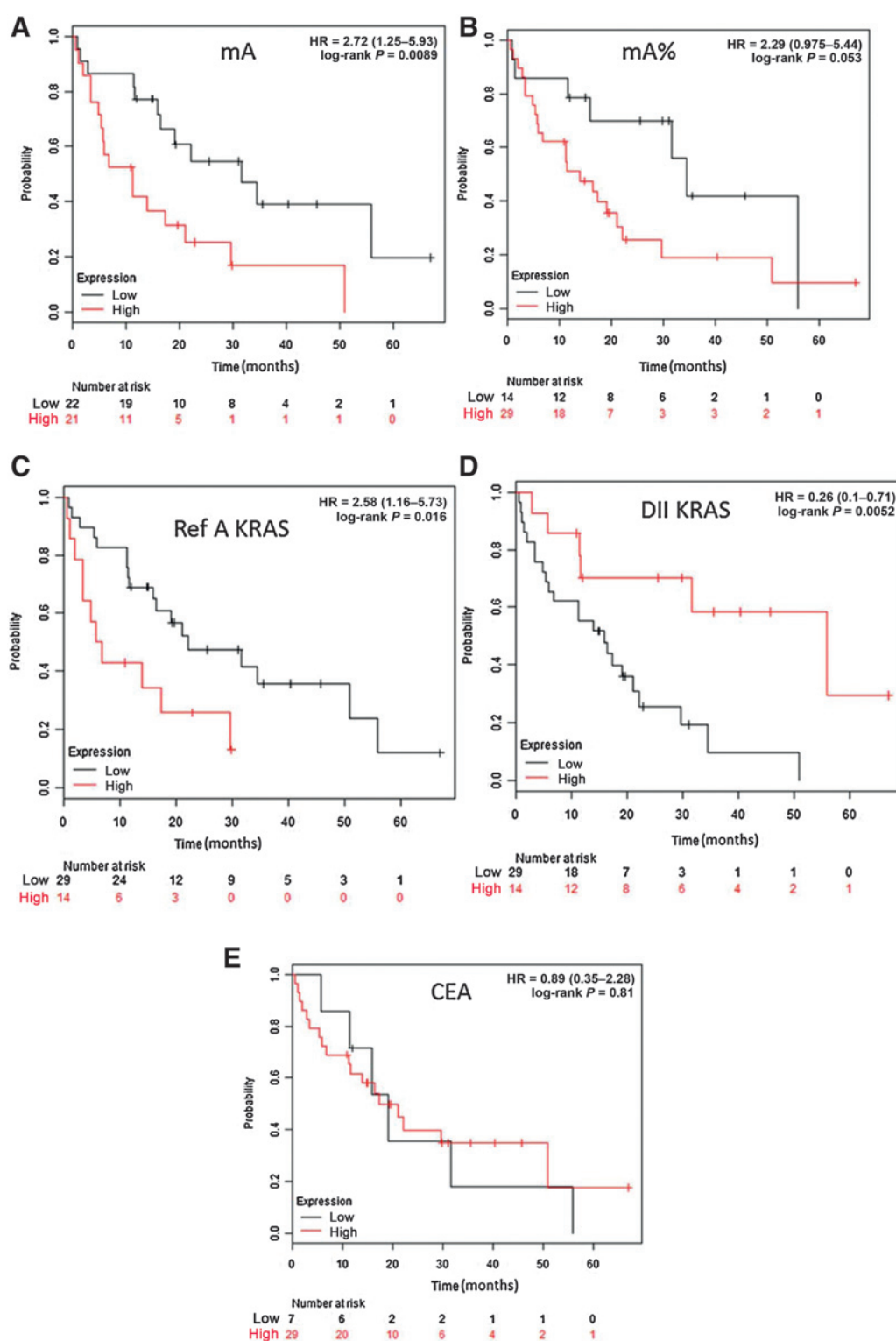
analytic process and the robustness of the prognostic significance of total ccfDNA concentration. Previous observations of our group on xenografted mice have shown the strong correlation between tumor volume and ccfDNA concentration (33), and

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



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**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  $\mu\text{g/L}$  ( $N = 36$ ). 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 but stressing 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 biologic 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  $\geq 0.005\%$  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 I 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).

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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 ccfDNA, 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.

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### 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.

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

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*Clin Cancer Res* 2016;22:3067-3077. Published OnlineFirst February 4, 2016.

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