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ORIGINAL ARTICLE

Total circulating cell-free DNA as a prognostic biomarker in metastatic colorectal cancer before first-line oxaliplatin-based chemotherapy

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Background: Metastatic colorectal cancer (mCRC) is a heterogeneous disease where prognosis is dependent both on tumor biology and host factors. Total circulating cell-free DNA (cfDNA) has shown to harbor prognostic information in mCRC, although less is known about the biological correlates of cfDNA levels in this patient group. The primary objective was to evaluate the prognostic value of pretreatment cfDNA in patients receiving the first-line oxaliplatin-based chemotherapy for mCRC, by using a predefined upper limit of normal (ULN) from a cohort of presumed healthy individuals. The secondary objective was to model cfDNA levels as a function of predefined tumor and host factors.

Patients and methods: This was a retrospective post hoc study based on a prospective multicenter phase III trial, the NORDIC-VII study. DNA was purified from 547 plasma samples and cfDNA quantified by a droplet digital PCR assay (*B2M*, *PPIA*) with controls for lymphocyte contamination. Main clinical end point was overall survival (OS).

Results: cfDNA was quantified in 493 patients, 54 were excluded mainly due to lymphocyte contamination. Median cfDNA level was 7673 alleles/ml (1050–1 645 000) for *B2M* and 5959 alleles/ml (555–854 167) for *PPIA*. High cfDNA levels were associated with impaired outcome; median OS of 16.6 months for levels above ULN and 25.9 months for levels below ULN (hazard ratio = 1.83, 95% confidence interval 1.51–2.21, $P < 0.001$). The result was confirmed in multivariate OS analysis adjusting for established clinicopathological characteristics. A linear regression model predicted cfDNA levels from sum of longest tumor diameters by RECIST, the presence of liver metastases and systemic inflammatory response as measured by interleukin 6 ($F(6, 357) = 62.7$, $P < 0.001$).

Conclusion: cfDNA holds promise as a minimally invasive and clinically relevant prognostic biomarker in mCRC before initiating first-line oxaliplatin-based chemotherapy and may be a complex entity associated with tumor burden, liver metastases and systemic inflammatory response.

Trial registration: ClinicalTrials.gov, NCT00145314.

Key words: colorectal cancer, circulating cell-free DNA, interleukin 6, *RAS*, *BRAF*, prognostic biomarker

Introduction

Colorectal cancer is the third most common cancer worldwide, with over 1.8 million new cases and 881 000 deaths every year [1]. Combination chemotherapy is the preferred first-line treatment of metastatic colorectal cancer (mCRC) [2]. Adding anti-epidermal growth factor receptor therapy may provide further clinical benefit in *RAS* wild-type [3] and in particular left-sided *RAS* wild-type cancers [4]. mCRC is a heterogeneous disease where prognosis depends both on tumor biology and host factors [5]. There is a need for reliable biomarkers that can aid in clinical decision making throughout the patient's disease trajectory; selecting patients for optimal oncological and surgical strategies.

Small fragments of total circulating cell-free DNA (cfDNA) can be detected in the blood stream of humans in health and disease [6, 7]. cfDNA originate primarily from cell turnover representing cells dying from apoptosis and necrosis [8] and can readily be detected in patients with advanced cancers [7, 9] and diseases driven by inflammatory processes [10, 11]. cfDNA has a short biological half-life and sampling is minimally invasive, making it an attractive biomarker at multiple decision points.

A negative prognostic significance of elevated cfDNA in patients with mCRC has been described [12, 13]. The prognostic role has mainly been investigated in patients before second and subsequent lines of chemotherapy. It is uncertain if results are transferable to a first-line setting. Most studies lack external validation and no reference levels have been established.

Details regarding the release and possible biological correlates of cfDNA in mCRC still remain unclear. There is an association between tumor burden and cfDNA in human xenograft models [14]. In what way other tumor characteristics influence cfDNA levels is uncertain. Since cfDNA is released from both malignant and non-malignant cells, we hypothesized that additional host factors including systemic inflammatory response may further attenuate cfDNA levels.

The primary objective was to evaluate the prognostic value of cfDNA levels in plasma from mCRC patients before initiating first-line oxaliplatin-based chemotherapy, by using a predefined upper limit of normal (ULN) from a cohort of presumed healthy individuals. cfDNA was assessed alone and in combination with established prognostic clinicopathological and biochemical characteristics used in daily clinical practice [15]. The secondary objective was to model cfDNA levels in mCRC patients as a function of predefined tumor and host factors.

Patients and methods

Study designs

We used a retrospective post hoc study design based on a prospective multicenter phase III trial, the NORDIC-VII study (NCT00145314), of which the design, conduct and overall results have been reported [16]. In short, NORDIC-VII investigated the effects of combining cetuximab with the Nordic FLOX regimen with bolus 5-fluorouracil/folinic acid and oxaliplatin in the first-line therapy of mCRC. There were no statistically significant differences in outcome between the treatment arms [16, 17]; in the present study, data were analyzed across all arms. Clinical end points were progression-free survival (PFS) and overall survival (OS), additionally overall response rate (ORR) and number of patients with complete surgical resection of metastases during the study period. Description of tumor tissue *RAS*/*BRAF* mutation analyses and biochemical serum analyses for alkaline phosphatase (ALP), carcinoembryonic antigen (CEA) and interleukin 6 (IL-6) is specified in supplementary Methods, available at *Annals of Oncology* online.

Clinicopathological characteristics

Clinicopathological characteristics were included as recommended for phase III trials of systemic treatment in mCRC [15], which in this study included location of primary tumor, resection status of primary tumor, synchronous versus metachronous metastases, number of metastatic sites, metastatic location, tumor tissue *RAS*/*BRAF* mutation status, age, gender, body mass index and WHO performance status. Sidedness of primary tumor was assigned retrospectively for a subset of patients as described in supplementary Methods, available at *Annals of Oncology* online.

cfDNA purification and quantification

cfDNA was purified from ~480 µl of EDTA-plasma and quantified by droplet digital PCR (ddPCR) using a multiplex assay of gPPIA (132 base pair amplicon) of the peptidylprolyl isomerase A gene (*PPIA*) and gB2M (72 base pair amplicon) of the beta-2-microglobulin gene (*B2M*) as described in supplementary Methods, available at *Annals of Oncology* online.

Control for lymphocyte contamination

A ddPCR assay for detecting immunoglobulin heavy chain rearrangements in B cells was carried out in duplicates for all samples as described in supplementary Methods, available at *Annals of Oncology* online.

Defining ULN for cfDNA in a healthy cohort

The cohort of presumed healthy individuals consisted of random plasma samples ($N = 93$) from the Lolland-Falster Health Study (NCT02482896). The ULN of gPPIA was estimated to be 4663 alleles/ml plasma and the ULN of gB2M was 6418 alleles/ml plasma (see supplementary Methods, available at *Annals of Oncology* online).

Statistical analyses

Values were summarized as median and range for continuous variables and proportions and percentages for categorical variables. Blood analyte levels were not normally distributed and hence log transformed. Levels in different groups were statistically compared using the one-way analysis of variance. Correlations were investigated using the Spearman's rho test. Associations between categorical variables were evaluated using the chi-square test.

The prognostic value of cfDNA level was initially assessed by log-rank test and unadjusted univariate Cox proportional hazards model. Clinicopathological characteristics, CEA and ALP were evaluated for their prognostic value in combination with cfDNA in bivariate and subsequent multivariate analyses.

A linear regression model was established to predict cfDNA levels from clinicopathological characteristics, systemic inflammatory response (SIR) as reflected by IL-6 and sum of longest tumor diameters (SLD) at baseline by RECIST 1.0 [16]. Explanatory variables were arranged belonging to the domains of tumor burden ($N = 3$), tumor characteristics ($N = 8$) and host characteristics ($N = 5$). A similar logistic regression model was established to assess the likelihood of having cfDNA above ULN.

Univariate, bivariate or multivariate regression models refer to regression analyses with one, two or multiple explanatory variables, respectively. Explanatory variables in multivariate regression models were chosen using a stepwise approach, including significant covariates in models

with one or two covariates. An unadjusted *P*-value threshold of $P < 0.003$ was used to call significance, corresponding to a Bonferroni adjusted $P < 0.05$ after correction for $N = 16$ comparisons.

Statistical analyses were computed using SPSS version 25 (IBM Corp., Armonk, NY) and R version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria). Results are reported according to the Recommendations for Tumor Marker Prognostic Studies (REMARK) checklist.

Ethics

The NORDIC-VII study and the Lolland-Falster Health Study were approved by the national ethics committees and governmental authorities in each country and conducted in accordance with the Declaration of Helsinki. All patients and healthy donors gave written informed consent.

Results

Methodological considerations

The median and range of cfDNA as measured by gPPIA was 5959 alleles/ml plasma (555–854 167), whereas there was a tendency of higher values as measured by gB2M with a median of 7673 alleles/mL plasma (1050–1 645 000). The two measures were strongly correlated (Spearman's rho 0.98, $P < 0.001$). Hence, further statistical analyses used the level of cfDNA as measured by gB2M due to high correlation to gPPIA and lower detection limit. Results indicate that some patients have gained a *PPIA* allele or lost a *B2M* allele which could affect cfDNA count, details are specified in supplementary Results, available at *Annals of Oncology* online.

Patient characteristics and cfDNA levels

cfDNA was quantified in 547 baseline samples. Fifty-three samples were excluded due to contamination of lymphocytes and one sample due to failed ddPCR assay, leaving 493 patients with a valid result for further analyses (Figure 1). Clinicopathological and biochemical characteristics of relevant cohorts are presented in supplementary Table S1, available at *Annals of Oncology* online.

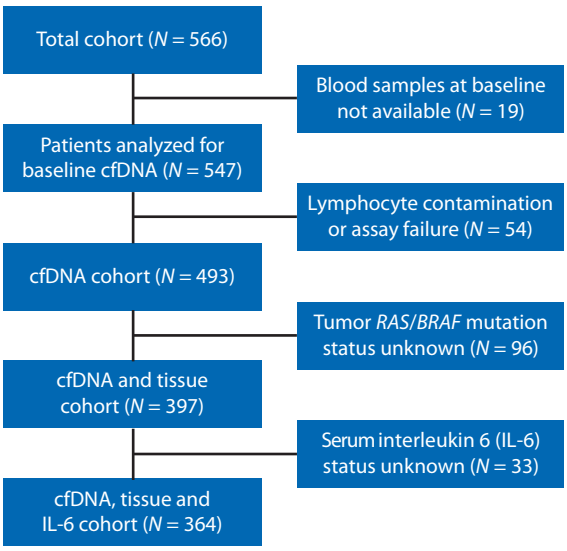

Figure 1. CONSORT diagram.

Elevated cfDNA levels were associated with poor performance status, intact primary tumor, synchronous disease, liver metastases and elevated levels of CEA and ALP ($P < 0.001$; Table 1). There was no statistically significant association with respect to age, gender, body mass index, location of primary tumor (colon versus rectum), sidedness of primary tumor (right versus left), number of metastatic sites, *RAS*/*BRAF* tumor mutation status or treatment arms (Table 1; supplementary Table S2, available at *Annals of Oncology* online).

Clinical outcome and cfDNA levels

High cfDNA levels were associated with impaired outcome, with median PFS of 7.7 months for levels above ULN and 8.3 months for levels below ULN (hazard ratio = 1.43, 95% confidence interval 1.18–1.73, $P < 0.001$) and median OS of 16.6 for levels above ULN and 25.9 months for levels below ULN (hazard ratio = 1.83, 95% confidence interval 1.51–2.21, $P < 0.001$). Results were comparable when using an external ULN or internal cohort quartiles (Figure 2). The same prognostic OS trend was observed when stratifying for *RAS*/*BRAF* mutation status (supplementary Figure S1, available at *Annals of Oncology* online). The independent prognostic role of cfDNA was confirmed in bivariate (supplementary Table S3, available at *Annals of Oncology* online) and a subsequent multivariate Cox model for OS (Table 2). Numerically more secondary metastasectomies were seen in patients with cfDNA levels below ULN ($N = 23/213$, 10.8%) than in patients with levels above ULN ($N = 16/280$, 5.7%, $P = 0.04$), although the result did not reach the adjusted significance threshold. Confirmed ORR did not differ between patients with cfDNA levels below versus above ULN ($N = 90/213$, 42.3% versus $N = 137/280$, 48.9%, $P = 0.14$).

Modelling cfDNA levels as a function of 16 predefined variables belonging to the domains of tumor burden, tumor characteristics and host characteristics

Regression models were established as described in Figure 3. Six of the explanatory variables remained significantly associated with cfDNA in univariate regression analyses. A multivariate linear regression model significantly predicted cfDNA levels from SLD by RECIST, the presence of liver metastases and SIR as measured by IL-6. These variables in combination accounted for 51% of the explained variability ($F(6, 357) = 62.7$, $P < 0.001$), and represented each predefined domain (supplementary Table S4, available at *Annals of Oncology* online). A multivariate logistic regression model identified the same variables to significantly account for the likelihood of having cfDNA above ULN (supplementary Table S5, available at *Annals of Oncology* online; Figure 3C).

Discussion

We have previously reported in a meta-analysis that high cfDNA levels are associated with poor prognosis in mCRC [13]. The meta-analysis included 10 variously sized cohorts, mainly reporting data before second or subsequent treatment lines. We hereby confirm these findings in a large cohort before initiating first-line

Table 1. Total circulating cell-free DNA (cfDNA) levels (alleles/ml plasma) as measured by gPPIA and gB2M for different clinicopathological characteristics in the cfDNA cohort ($N = 493$) of patients with mCRC

Characteristics	gPPIA, median (IQR), alleles/ml	*P* value	gB2M, median (IQR), alleles/ml	*P* value
Age				
Below median	6083 (18 417)	0.835	7750 (18 917)	0.856
Above median	5938 (17 108)		7663 (19 420)	
Gender				
Male	6167 (19 571)	0.552	7712 (20 676)	0.760
Female	5917 (16 166)		7489 (17 566)	
Body mass index				
<18.5	7958 (8314)	0.053	9208 (11 850)	0.115
18.5–24.9	6633 (28 125)		8375 (27 813)	
25.0–29.9	6167 (18 387)		7837 (18 500)	
≥30	4783 (4441)		6292 (6057)	
WHO performance status				
0	5011 (11 619)	<0.001	6625 (12 560)	<0.001
1–2	11 167 (38 449)		15 500 (43 206)	
Location primary tumor				
Colon	6612 (19 500)	0.134	9366 (21 222)	0.038
Rectum	5083 (14 660)		6515 (15 826)	
Sidedness primary tumor[a]				
Right	6333 (10 500)	0.436	8500 (12 583)	0.788
Left	5125 (14 833)		6845 (16 729)	
Resection status primary tumor				
Resected	4333 (9324)	<0.001	6042 (12 080)	< 0.001
Not resected	13 417 (56 182)		16 833 (60 625)	
Time of metastases				
Synchronous	7667 (29 639)	<0.001	9702 (31 012)	<0.001
Metachronous	3625 (5872)		5277 (7638)	
Number of metastatic sites				
1 site	5366 (11 917)	0.168	6809 (12 592)	0.101
>1 site	6250 (21 748)		8000 (21 614)	
Metastatic location				
Non-liver	3083 (3256)	<0.001	4291 (4590)	<0.001
Liver + other site	8681 (32 073)		10 833 (34 917)	
Liver only	9047 (27 688)		10 750 (27 850)	
Tissue mutation status[b]				
RAS/BRAF wild-type	6417 (17 245)	0.060	7917 (16 333)	0.133
RAS mutation	4589 (9454)		6542 (12 395)	
BRAF mutation	4986 (11 622)		7241 (14 780)	
Alkaline phosphatase				
Below ULN	3417 (3781)	<0.001	5082 (4713)	<0.001
Above ULN	18 667 (53 390)		20 500 (53 679)	
Carcinoembryonic antigen[c]				
Below ULN	3250 (2917)	<0.001	4833 (3917)	<0.001
Above ULN	7333 (25 301)		9667 (27 167)	

Levels in different groups were statistically compared using the analysis of variance test with log transformed values.
[a]Sidedness primary tumor analyzed for $N = 363$ patients.
[b]Tissue mutation status analyzed for $N = 397$ patients.
[c]Carcinoembryonic antigen analyzed for $N = 492$ patients.
IQR, interquartile range; ULN, upper limit of normal.

treatment. The long-term follow-up of this study (median exceeding 7 years) furthermore enabled us to show that normal cfDNA levels predict long-term survival in this patient group (5-year survival rate ~17%) compared with patients with elevated levels (5-year survival rate ~4.5%). We identified numerically more metastatectomies in patients with normal cfDNA upfront, suggesting an enrichment of patients fit for surgery with limited tumor burden in this group. Based on these findings, we suggest

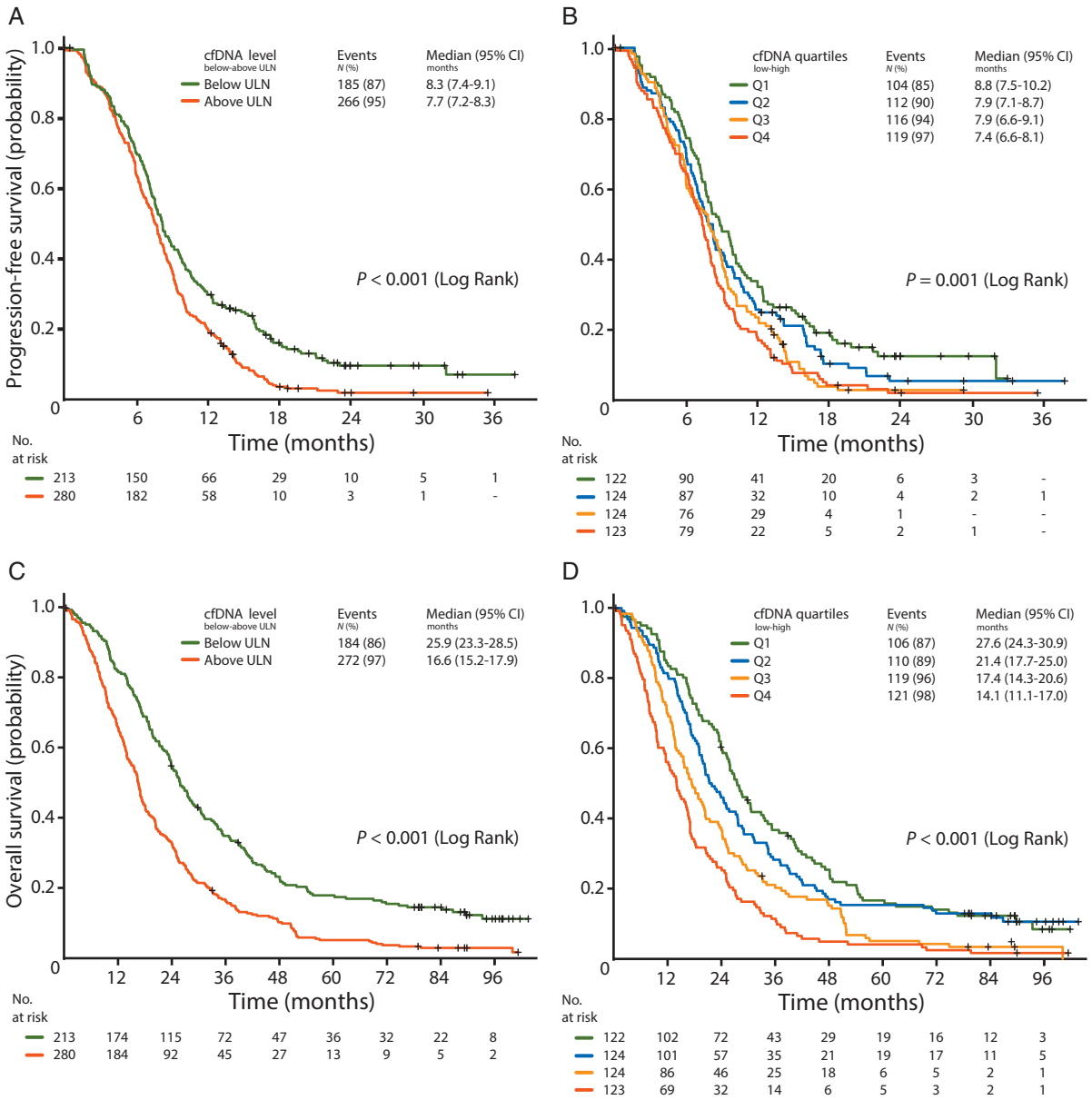

Figure 2. Clinical outcome of patients in the cfDNA cohort ($N = 493$). Progression-free survival as stratified according to total circulating cell-free DNA (cfDNA) below and above upper limit of normal (ULN) (A) and quartiles (B). Overall survival as stratified according to cfDNA below and above ULN (C) and quartiles (D). CI, confidence interval.

that cfDNA measured at baseline reflects fundamental aspects of the tumor and host, rather than predicting the effect of first-line chemotherapy.

Increased cell death and damaged vasculature due to tumor invasiveness may both be relevant underlying processes leading to increased cfDNA. Thus, tumor burden is commonly regarded as a major factor influencing cfDNA, although there is no consensus on how 'burden' should be measured. An experimental model using nude mice xenografted with human CRC cells indeed showed increasing cfDNA with increasing tumor weight [18]. Human studies on local/locally advanced disease indicate that surgical removal of the primary tumor results in lower cfDNA levels [19]. However, the data are conflicting in a metastatic setting. One study on advanced lung cancer found no significant correlation between cfDNA and metabolic tumor volume or total lesion glycolysis as estimated by positron emission tomography/computed tomography [20]. Others have found that baseline cfDNA levels in treatment naive mCRC patients correlate with radiologic disease burden, but this trend could not be observed at time of disease progression and subsequent therapy lines [21].

We established multiple regression models and identified that cfDNA level variability in mCRC can be explained partly by tumor burden, but also by other characteristics of tumor and host. Our findings support that the presence of liver metastases is associated with high cfDNA levels, which could partly be independent from tumor burden. Sprouting angiogenesis with dysfunctional and leaky vasculature is common in liver metastases, and could be a relevant underlying process leading to increased cfDNA [22].

Table 2. Adjusted multivariate Cox regression model for overall survival in the cfDNA and tissue cohort ($N = 397$) including total circulating cell-free DNA (cfDNA) level as measured by gB2M and other prognostic variables significant in bivariate analyses

	HR	L 95% CI	U 95% CI	*P* value
WHO performance status				
0 ($N = 269$)	1			
1–2 ($N = 128$)	1.64	1.31	2.06	<0.001
Tissue mutation status				
RAS/BRAF wild type ($N = 171$)	1			
RAS mutation ($N = 182$)	1.55	1.24	1.93	<0.001
BRAF mutation ($N = 44$)	4.50	3.13	6.46	<0.001
Alkaline phosphatase				
ALP below ULN ($N = 211$)	1			
ALP above ULN ($N = 186$)	1.71	1.35	2.16	<0.001
Carcinoembryonic antigen				
CEA below ULN ($N = 69$)	1			
CEA above ULN ($N = 328$)	1.47	1.08	2.01	0.015
cfDNA level				
gB2M below ULN ($N = 182$)	1			
gB2M above ULN ($N = 215$)	1.54	1.21	1.96	<0.001

HR, hazard ratio; L, lower; U, upper; CI, confidence interval; ULN, upper limit of normal.

In contrast, lung and lymph node metastases more often hijack existing well-functioning vasculature by co-option [23, 24].

Furthermore, we confirm an association between cfDNA and SIR as measured by IL-6. In non-malignant disease there is a positive correlation between cfDNA levels and inflammatory states [10, 11]. An acute phase response increases local and/or systemic vascular permeability, which intuitively could make cell debris including DNA rapidly appear in the blood circulation. Contrary, studies have mechanistically suggested that endogenous cfDNA may enhance an innate immune response through activation of toll-like receptor 9 in dendritic cells, monocytes and macrophages [10, 25]. Despite a strong association in our study, we cannot conclude on a causal or temporal relationship between cfDNA and IL-6/SIR in patients with mCRC.

Our quest for factors influencing cfDNA level variability was limited to the parameters available within the framework of a phase III clinical trial. Variables within the categories tumor burden, tumor characteristics and host characteristics are surrogate markers, with certain overlaps and interactions as highlighted in our analyses. As an example, tumor burden as characterized by RECIST is a rather crude measure and could add uncertainty to our predictions. Furthermore, the molecular orchestra mediating SIR in colorectal cancer patients is complex. We chose circulating IL-6 as a marker since it has been proposed as one of the key mediators of SIR in mCRC as a result of tumor necrosis [26, 27]. Still this is a simplification of reality, and other markers could have strengthened predictions and complemented interpretations. Despite these limitations, our final multivariate model identified one factor from each domain explaining more than half of the observed cfDNA level variability in mCRC patients.

Our findings suggest a relationship between cfDNA and tumor burden, the presence of liver metastases, and SIR, all of which are modes associated with poor prognosis in mCRC. There could also be a more direct link between cfDNA, tumor biology and prognosis. A recent CRC cell line study found that the presence of DNA in the tumor microenvironment promotes tumor cell survival after cytotoxic insults, through induction of autophagy [28]. This suggests that cfDNA could exert a disease-modulating biological function and not only be an innocent bystander. Further pre-clinical and clinical studies are needed to understand the different facets of cfDNA, both as a complex biomarker and potential target during mCRC treatment.

There are several potential clinical implications of our findings. High cfDNA predicts poor survival in mCRC, and patients with high cfDNA fit for therapy may potentially benefit from a more intensive first-line regimen (i.e. triplet chemotherapy; FOLFOXIRI). One of the keys of ensuring appropriate patient selection for metastasectomy is prediction of long-term survival, but current risk scores lack sufficient discriminatory accuracy [29]. Given that normal cfDNA predicts long-term survival, cfDNA could potentially improve established risk scores used for stratifying patients based on their likelihood of recurrence. Despite the fact that the prognostic utility of cfDNA looks promising, its clinical usefulness must be validated in prospective clinical trials.

Conclusion

cfDNA at baseline is a strong prognostic factor for mCRC before initiating first-line oxaliplatin-based chemotherapy, even when adjusting for established clinicopathological and biochemical prognostic markers. Our findings indicate that cfDNA may be a complex marker for tumor burden, the presence of liver metastases and SIR, and that it holds promise as a clinically relevant prognostic biomarker in mCRC.

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Disclosure

The authors have declared no conflicts of interest.

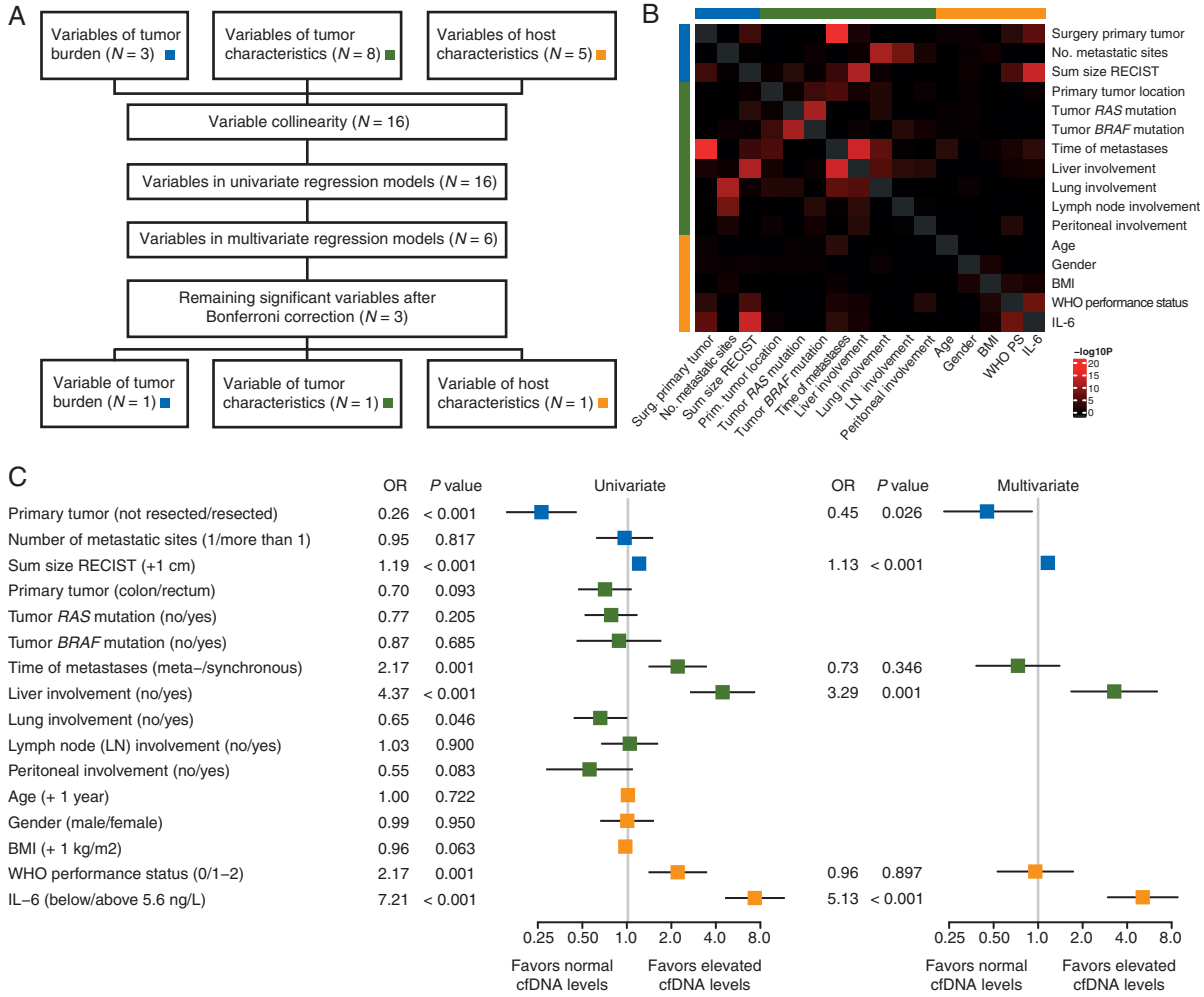

Figure 3. Modelling total circulating cell-free DNA levels as a function of 16 predefined variables. Linear and logistic regression models were established to investigate relationships between cfDNA levels and 16 predefined variables belonging to the domains of tumor burden, tumor characteristics and host characteristics of patients in the cfDNA, tissue and IL-6 cohort ($N = 364$). Variables of tumor burden ($N = 3$) include resection status of primary tumor, number of metastatic sites and sum of longest tumor diameters (SLD) by RECIST; tumor characteristics ($N = 8$) include location of primary tumor, tumor *RAS* and *BRAF* mutation status, time of metastases, and metastatic involvement of the liver, lung, lymph nodes and peritoneum; host characteristics ($N = 5$) include age, gender, body mass index (BMI), WHO performance status and systemic inflammatory response (SIR) as reflected by serum level of interleukin 6 (IL-6) (A). Collinearity between explanatory variables used in the models is illustrated using a heatmap. Strength of associations was evaluated as –log10 *P* values for each variable combination. Fisher's exact test was used for categorical variables, Pearson correlation for continuous variables and Wilcoxon signed-rank test for the combination of categorical and continuous variables (B). The multivariate logistic regression model identified increasing SLD by RECIST, the presence of liver metastases and SIR as measured by IL-6 to significantly account for the likelihood of having cfDNA above ULN (C). OR, odds ratio.

References

1. Bray F, Ferlay J, Soerjomataram I et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J Clin 2018; 68(6): 394–424.
2. de Gramont A, Figer A, Seymour M et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 2000; 18(16): 2938–2947.
3. Bokemeyer C, Bondarenko I, Hartmann JT et al. Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. Ann Oncol 2011; 22(7): 1535–1546.
4. Arnold D, Lueza B, Douillard JY et al. Prognostic and predictive value of primary tumour side in patients with *RAS* wild-type metastatic colorectal cancer treated with chemotherapy and EGFR directed antibodies in six randomized trials. Ann Oncol 2017; 28(8): 1713–1729.
5. Linnekamp JF, Wang X, Medema JP, Vermeulen L. Colorectal cancer heterogeneity and targeted therapy: a case for molecular disease subtypes. Cancer Res 2015; 75(2): 245–249.
6. Mandel P, Metais P. Les acides nucléiques du plasma sanguin chez l'homme. C R Seances Soc Biol Fil 1948; 142(3-4): 241–243.
7. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977; 37(3): 646–650.
8. Jahr S, Hentze H, Englisch S et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001; 61: 1659–1665.
9. Bettegowda C, Sausen M, Leary RJ et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014; 6: 224ra224.
10. Nishimoto S, Fukuda D, Higashikuni Y et al. Obesity-induced DNA released from adipocytes stimulates chronic adipose tissue inflammation and insulin resistance. Sci Adv 2016; 2(3): e1501332.

11. Dwivedi DJ, Toltl LJ, Swystun LL et al. Prognostic utility and characterization of cell-free DNA in patients with severe sepsis. Crit Care 2012; 16(4): R151.
12. El Messaoudi S, Mouliere F, Du Manoir S et al. Circulating DNA as a strong multimarker prognostic tool for metastatic colorectal cancer patient management care. Clin Cancer Res 2016; 22(12): 3067–3077.
13. Spindler KG, Boysen AK, Pallisgard N et al. Cell-free DNA in metastatic colorectal cancer: a systematic review and meta-analysis. Oncologist 2017; 22(9): 1049–1055.
14. Rago C, Huso DL, Diehl F et al. Serial assessment of human tumor burdens in mice by the analysis of circulating DNA. Cancer Res 2007; 67(19): 9364–9370.
15. Goey KKH, Sorbye H, Glimelius B et al. Consensus statement on essential patient characteristics in systemic treatment trials for metastatic colorectal cancer: supported by the ARCAD Group. Eur J Cancer 2018; 100: 35–45.
16. Tveit KM, Guren T, Glimelius B et al. Phase III trial of cetuximab with continuous or intermittent fluorouracil, leucovorin, and oxaliplatin (Nordic FLOX) versus FLOX alone in first-line treatment of metastatic colorectal cancer: the NORDIC-VII study. J Clin Oncol 2012; 30(15): 1755–1762.
17. Guren TK, Thomsen M, Kure EH et al. Cetuximab in treatment of metastatic colorectal cancer: final survival analyses and extended *RAS* data from the NORDIC-VII study. Br J Cancer 2017; 116(10): 1271–1278.
18. Thierry AR, Mouliere F, Gongora C et al. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. Nucleic Acids Res 2010; 38(18): 6159–6175.
19. Frattini M, Gallino G, Signoroni S et al. Quantitative analysis of plasma DNA in colorectal cancer patients: a novel prognostic tool. Ann N Y Acad Sci 2006; 1075(1): 185–190.
20. Nygaard AD, Holdgaard PC, Spindler KL et al. The correlation between cell-free DNA and tumour burden was estimated by PET/CT in patients with advanced NSCLC. Br J Cancer 2014; 110(2): 363–368.
21. Berger AW, Schwerdel D, Welz H et al. Treatment monitoring in metastatic colorectal cancer patients by quantification and *KRAS* genotyping of circulating cell-free DNA. PLoS One 2017; 12(3): e0174308.
22. Lazaris A, Amri A, Petrillo SK et al. Vascularization of colorectal carcinoma liver metastasis: insight into stratification of patients for anti-angiogenic therapies. J Pathol Clin Res 2018; 4(3): 184–192.
23. Jeong HS, Jones D, Liao S et al. Investigation of the lack of angiogenesis in the formation of lymph node metastases. J Natl Cancer Inst 2015; 107(9): djv155.
24. Bridgeman VL, Vermeulen PB, Foo S et al. Vessel co-option is common in human lung metastases and mediates resistance to anti-angiogenic therapy in preclinical lung metastasis models. J Pathol 2017; 241(3): 362–374.
25. Zhang Q, Raoof M, Chen Y et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature 2010; 464(7285): 104–107.
26. Guthrie GJ, Roxburgh CS, Richards CH et al. Circulating IL-6 concentrations link tumour necrosis and systemic and local inflammatory responses in patients undergoing resection for colorectal cancer. Br J Cancer 2013; 109(1): 131–137.
27. Vainer N, Dehlendorff C, Johansen JS. Systematic literature review of IL-6 as a biomarker or treatment target in patients with gastric, bile duct, pancreatic and colorectal cancer. Oncotarget 2018; 9(51): 29820–29841.
28. Anunobi R, Boone BA, Cheh N et al. Extracellular DNA promotes colorectal tumor cell survival after cytotoxic chemotherapy. J Surg Res 2018; 226: 181–191.
29. Roberts KJ, White A, Cockbain A et al. Performance of prognostic scores in predicting long-term outcome following resection of colorectal liver metastases. Br J Surg 2014; 101(7): 856–866.