FULL PAPER

BJC

British Journal of Cancer (2017) 117, 704–709 | doi: 10.1038/bjc.2017.215 BJC OPEN

Keywords: circulating tumour DNA; circulating cell-free DNA; tumour volume; positron emission tomography; tumour lesion glycolysis; non-small cell lung cancer; next-generation sequencing; survival

Correlation between circulating mutant DNA and metabolic tumour burden in advanced non-small cell lung cancer patients

Anne Winther-Larsen*[,1], Christina Demuth[1], Joan Fledelius[2], Anne Tranberg Madsen[1], Karin Hjorthaug[3], Peter Meldgaard[4] and Boe Sandahl Sorensen[1]

[1]*Department of Clinical Biochemistry, Aarhus University Hospital, 8000 Aarhus, Denmark;* [2]*Department of Nuclear Medicine, Herning Regional Hospital, 7400 Herning, Denmark;* [3]*Department of Nuclear Medicine and PET-Centre, Aarhus University Hospital, 8000 Aarhus, Denmark and* [4]*Department of Oncology, Aarhus University Hospital, 8000 Aarhus, Denmark*

Background: Mutated circulating cell-free DNA (cfDNA) has been suggested as a surrogate marker of tumour burden and aggressiveness of disease. We examined the association between the level of plasma mutant cfDNA and metabolic tumour burden (MTB) measured by ^{18}F-fluoro-D-glucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT). Furthermore, the presence of mutant cfDNA was correlated with patient survival.

Methods: Forty-six advanced non-small cell lung cancer (NSCLC) patients were included. At the time of inclusion, blood sampling and a PET/CT scan were performed. cfDNA was isolated and next-generation sequencing (NGS) was performed (Ion AmpliSeq Colon and Lung Cancer panel v2). MTB was defined by a volumetric PET parameter.

Results: NGS succeeded in 41 patients. Mutations were detected in the blood of 24 patients. A significant correlation between the allele frequency of the most frequent mutation and MTB was found ($P = 0.001$). Patients with detectable mutated cfDNA had a significantly shorter median overall survival compared with patients without (3.7 *versus* 10.6 months, $P = 0.019$). This impact on survival was independent of the MTB.

Conclusions: Level of mutated cfDNA tends to correlate with MTB in advanced-stage NSCLC patients. Patients with detectable mutant DNA in plasma had an inferior survival, indicating that this could be an important predictor of survival.

Circulating cell-free DNA (cfDNA) is small DNA fragments circulating in the blood stream (Jahr *et al*, 2001). Elevated levels of cfDNA have been identified in cancer patients compared with healthy individuals (Leon *et al*, 1977). This elevation is believed to origin from both malignant tumour cells as well as from non-malignant cells constituting the tumour microenviroment (Mouliere and Thierry, 2012; Thierry *et al*, 2016). The precise mechanism for release of the DNA into the blood stream is not fully elucidated. It has been demonstrated that the amount of DNA in the circulation increases when cells are undergoing apoptosis or necrosis (Jahr *et al*, 2001). Other alternative mechanisms such as active secretion from tumour cells (Schwarzenbach *et al*, 2011) and release from circulating tumour cells (Bettegowda *et al*, 2014) have been suggested and may contribute to the total cfDNA level. In the recent years, it has been revealed that the contribution of cfDNA origin from tumour cells can be identified in the total cfDNA level by measuring cancer-specific mutations in the cfDNA also known to be present in the original tumour (Wan *et al*, 2017).

It has been proposed that the level of mutated cfDNA correlates with tumour burden. The level of tumour necrosis is thought to increase when tumour cells are rapidly proliferating as enlarging tumours are more likely to outgrow their blood supply (Leek *et al*, 1999). This causes tumour areas that are poorly vascularised and ischaemic, and if this ischaemia is sustained, ultimately necrosis

*Correspondence: A Winther-Larsen; E-mail: anlarsen@rm.dk

Received 12 May 2017; revised 7 June 2017; accepted 13 June 2017; published online 6 July 2017

will develop. As these necrotic areas are believed to release tumour DNA into the circulation, the amount of mutated cfDNA could be related to tumour burden (Diehl *et al*, 2005). Previous studies have supported this hypothesis by showing increased mutated cfDNA levels in advanced-stage patients compared with early-stage patients (Fleischhacker and Schmidt, 2007; Bettegowda *et al*, 2014; Newman *et al*, 2014). Moreover, correlations have been identified between changes in the amount of mutated cfDNA and changes in treatment response (Dawson *et al*, 2013; Newman *et al*, 2014; Sorensen *et al*, 2014) as well as relapse of disease (Tie *et al*, 2015). In addition to tumour volume, tumour aggressiveness may also be an important factor affecting the level of mutated cfDNA. An association between increasing necrosis grade and higher tumour grade has been identified, indicating that the amount of mutated cfDNA is associated with an aggressive tumour phenotype (Leek *et al*, 1999).

^{18}F-fluoro-D-glucose (^{18}F-FDG) positron emission tomography/computed tomography (PET/CT) is a well-established nuclear imaging modality for use in cancer patients. ^{18}F-FDG is a radiolabelled glucose analogue accumulating in tissue with high glucose utilisation. FDG activity thereby reflects the metabolic activity and aggressiveness of a tumour. In addition, data on tumour volume can be assessed on an ^{18}F-FDG-PET/CT scan. New volume-based PET parameters, as tumour lesion glycolysis (TLG), have been introduced capable of measuring the entire metabolic tumour burden (MTB) by incorporating both metabolic activity and volumetric data on all tumours in the patient (Larson *et al*, 1999). Thus TLG assessment on an ^{18}F-FDG-PET/CT scan reflects both tumour burden and, to some extent, tumour aggressiveness of the disease and could be a more informative parameter for assessing the total active tumour burden in patients than tumour burden assessment on a CT scan.

Identification of oncogenic alterations in cfDNA is challenging due to the low concentration of target cfDNA in plasma. With the emergence of next-generation sequencing (NGS), it has become possible to assess a large amount of different mutations from a low input of DNA. NGS-based targeted panels directed against the most frequent alterations in cancer have been designed. In non-small cell lung cancer (NSCLC), a 22-gene NGS panel has been developed, which includes the most frequent mutations in NSCLC (Ion AmpliSeq Colon and Lung Cancer Panel v2). This panel has proven useful for mutation detection in plasma cfDNA (Frenel *et al*, 2015; Zonta *et al*, 2016).

In the present study, we evaluated the correlation between the amount of mutated cfDNA in plasma, and the MTB was measured by TLG in advanced-stage NSCLC patients. Moreover, we evaluated whether the presence of mutated cfDNA was correlated with patient survival.

MATERIALS AND METHODS

Patients. In this retrospective study, advanced-stage NSCLC patients prospectively enrolled in a single-centre study from April 2013 to August 2015 at the Department of Oncology, Aarhus University Hospital, Denmark were evaluated. Details on inclusion and exclusion criteria have been described previously (Winther-Larsen *et al*, 2016). At the time of inclusion, patients underwent an $_{18}$F-FDG-PET/CT scan and a blood sample was drawn. All patients were treated with erlotinib in a palliative setting. Testing for somatic *epidermal growth factor receptor* (*EGFR*) mutations had been performed on the diagnostic tumour biopsy in all adenocarcinoma patients as part of the routine diagnostic work-up (see Supplementary S1). For the purpose of this study, we included only patients from the total cohort who were *EGFR* mutation wild type (wt), had progression on first or second line of chemotherapy, and in whom both an ^{18}F-FDG-PET/CT scan and a blood sample were available and evaluable. Selection of patients is illustrated in Supplementary S2. All patients gave informed written consent before inclusion and the study was approved by the Central Denmark Region Committees on Biomedical Research Ethics (no. 1-10-72-19-12). The study was reported to Clinical-Trial.gov (NCT02043002).

^{18}F-fluoro-D-glucose positron emission tomography/computed tomography. All ^{18}F-FDG-PET/CT scans were performed on a combined PET/CT scanner (Siemens Biograph TruePoint 40, Siemens Healthcare GMbH, Erlangen, Germany) at the Department of Nuclear Medicine and PET-Centre, Aarhus University Hospital, Denmark. The imaging protocol is described in Supplementary S1. Same scanner model, protocol for acquisition, and reconstruction software was used for all patients. An experienced nuclear medicine physician blinded to the patient outcome analysed all PET/CT scans using the Siemens Syngo.via software, Siemens Healthcare GMbH. In all scans, TLG was calculated for all evaluable lesions according to the Positron Emission Tomography Response Criteria in Solid Tumours 1.0 guideline (Wahl *et al*, 2009) (described in Supplementary S1 and illustrated in Supplementary S3). The sum of TLG for all evaluable lesions was defined as the MTB.

DNA extraction from blood. A peripheral blood sample of 10 ml was collected in an EDTA-containing tube for each patient. Within 2 h of withdrawal, samples were centrifuged (1400 g for 15 min) and plasma was isolated from the peripheral blood cells. Plasma was subsequently frozen at $-80\,^{\circ}$C until further analysis. cfDNA was purified from 2 ml of plasma by use of the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and eluted in a volume of 100 μl TE buffer. The amount of cfDNA was quantified by measuring the *beta-2-microglobulin* gene by droplet digital PCR (Pallisgaard *et al*, 2015) as described in Supplementary S1.

Next-generation sequencing. NGS was carried out on all cfDNA samples. The Oncomine Solid Tumor DNA Kit was used to prepare libraries on 1.1–10 ng of cfDNA following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). NGS was conducted using the Ion Chef Instrument and the Ion Personal Genome Machine (PGM) System (Thermo Fisher Scientific). Sequencing was performed using Ion 316 v2 BC chips containing eight cfDNA samples per chip. Primary data processing was carried out using the Torrent Suite Software (version 5.0.4) on a Torrent Server and the Ion AmpliSeq Colon and Lung Cancer panel v2 template (all Thermo Fisher Scientific). Variant calling was performed using the Ion Reporter Software (version 5.0) and the AmpliSeq CHPv2 peripheral/CTC/CF DNA single sample workflow (Thermo Fisher Scientific). Default settings were used except from the reference and hotspot BED files, which were supplied in the kit. Sequencing was considered successful if the mean sequencing depth was $\geqslant$2000. If this criterion could not be met, the sample was disqualified. Called variants were only accepted if the allele frequency (AF) was $\geqslant$1%. The Integrative Genomics Viewer (Broad Institute, Cambridge, MA, USA) was used for visualisation of variants (Thorvaldsdóttir *et al*, 2013).

Statistical analysis. Correlations between detection of mutated cfDNA and clinical characteristics were calculated using χ^2 or Fisher's exact tests, where appropriate. Kruskal–Wallis test was used to calculate the correlation between the presence of mutated cfDNA and the levels of TLG and total cfDNA. Correlation between TLG and concentration of mutated cfDNA was evaluated using the Pearson's correlation coefficient after log-transformation of both variables. Follow-up time was calculated using the reverse Kaplan–Meier method. Overall survival (OS) was measured from the start of enrolment until death of any cause or last follow-up

date (19 April 2016). If patients were still alive on the last follow-up date, they were censored at that day. Estimates of median OS were calculated by Kaplan–Meier method and compared by the log-rank test. The Cox proportional hazards model was used to calculate crude and adjusted hazard ratios (HRs). Clinical variables were dichotomised except for age (continuous). All tests were two-sided, and *P*-values <0.05 were considered to be statistically significant. Statistical analyses were performed using SPSS statistics version 20.0 for windows (IBM SPSS Statistics, Chicago, IL, USA). STATA version 13 (Stata Corporation, College Station, TX, USA) was used for preparation of Kaplan–Meier curve and GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) for preparation of scatter plot.

RESULTS

Patients. A total of 46 patients were analysed. Patient characteristics are shown in Table 1. All patients had stage IV disease except for three patients with stage III disease. The majority of patients had progression on first-line chemotherapy. No patients were lost to follow-up. The median time interval between blood sampling and PET/CT scanning was 2 days (range: 0–9 days). After a median follow-up time of 20.9 months (95% CI: 11.2–30.6 month), five patients were still alive. The median OS in all patients was 5.9 months (95% CI: 2.9–8.9 months).

Correlation between the level of mutated cfDNA and MTB. cfDNA isolation was successful in all patients and a median value of 4587 copies ml^{-1} (range: 297–170 720 copies ml^{-1}) was measured. Sequencing of cfDNA succeeded in 41 out of 46 patients (89%). A least one mutation was detected in 24 patients (59%). The number of identified mutations in each patient ranged from 1 to 4 with a median of 1 mutation. In total, 43 mutations were identified. The most frequently mutated genes were: *TP53* (18 patients), *KRAS* (12 patients), and *EGFR* (2 patients; Table 2). The median AF of the most frequent mutation in each patient was 2.7% (range: 1.1–62.5%).

TLG was assessable in all FDG-PET/CT. A significant correlation between the AF of the most frequent mutation (%) and the level of TLG was found as shown in Figure 1 ($r=0.628$, $P=0.001$). By contrast, no significant correlation could be identified between MTB and the number of mutations identified in each patient (data not shown).

Table 1. Patient and tumour characteristics ($N=46$)

Characteristics	*N* (%)
Age	
Median years (range)	67 (48–81)
Gender	
Female	20 (43)
Male	26 (57)
Performance status, ECOG	
0–1	38 (83)
2	8 (17)
Smoking status	
Never or former[a]	33 (72)
Current	12 (26)
Unknown	1 (2)
Stage	
III	3 (7)
IV	43 (93)
Histology	
Adenocarcinoma	38 (83)
Squamous cell carcinoma	8 (17)
Progression on	
First-line chemotherapy	37 (80)
Second-line chemotherapy	9 (20)
Prior treatment	
First line	
Carboplatin/vinorelbine[b]	26 (56)
Carboplatin/vinorelbine/bevacizumab[c]	20 (44)
Second line	
Pemetrexed	5 (56)
Docetaxel	4 (44)

Abbreviation: ECOG = Eastern Cooperative Oncology Group.
[a]Former smoker was defined as having stopped smoking at the time of diagnosis.
[b]Carboplatin day 1 (AUC 5) and vinorelbine day 1 and day 8 (60–80 mg m^{-2} (p.o.)) every 3 weeks for a maximum of four cycles.
[c]Bevacizumab (7.5 mg m^{-2} i.v. day 1) was given in combination with chemotherapy. Patients with disease control received subsequent maintenance therapy every 3 weeks until progression or toxicity.

Evaluation of patients with and without detectable mutated cfDNA. No mutations could be identified in the cfDNA of 17 patients. A comparison of clinical and tumour characteristics between the mutated cfDNA-positive and the mutated cfDNA-negative group was performed, and no significant differences were identified (Supplementary S4). In addition, no significant difference in the level of total cfDNA was found between the mutated cfDNA-positive (5170 copies ml^{-1}, range 1518–170 720) and the mutated cfDNA-negative (4510 copies ml^{-1}, range 1232–39 160) patients (χ^2 (2) = 0.018, $P=0.895$). However, a significant higher median MTB was found in the mutated cfDNA-positive patients (689 (range 73–7034)) compared with the mutated cfDNA-negative patients (269 (range 67–3958)) ($P=0.047$).

When evaluating OS, patients with mutated cfDNA presence in the blood had a significant shorter median OS (3.7 months (95% CI: 0.0–7.6)) compared with mutated cfDNA-negative patients (10.6 months (95% CI: 7.1–14.1), $P=0.019$; Figure 2). In a univariate Cox regression analysis, the presence of mutated cfDNA together with high level of MTB was found to be associated with decreased OS (Table 3). To evaluate the independent impact of the presence of mutated cfDNA on survival, a multivariate Cox regression analysis was performed. Being mutated cfDNA positive remained an independent predictor of shorter OS with an adjusted HR of 2.11 (95% CI: 1.02–4.38) (Table 3).

No difference in OS was found between mutated cfDNA-positive patients, with only one mutation identified compared with a higher number of mutations ($P=0.355$). Neither was any difference in survival found between mutated cfDNA-positive patients with an AF above or below the median 2.7% ($P=0.949$).

DISCUSSION

Our study shows a significant correlation between the frequency of mutated cfDNA and the MTB measured by PET/CT in advanced-stage NSCLC patients. The study thereby supports the hypothesis of mutated cfDNA as a surrogate marker of tumour burden and tumour aggressiveness. In addition, we observed that mutated cfDNA-positive patients had a higher median MTB than patients without mutated cfDNA present in plasma and could confirm from previous reports showing that mutated cfDNA positivity is correlated to an inferior survival.

To our knowledge, only one previous study has, similar to ours, compared the exact level of mutated cfDNA with radiological tumour burden in NSCLC patients. Newman *et al* (2014) evaluated the correlation between amount of mutated cfDNA and tumour volume, defined as visible tumour on a CT scan, in nine newly diagnosed or recurrent NSCLC patients with different stages (I–IV). In line with ours, they found a significant correlation. In a study by Pécuchet *et al* (2016), the concentration of mutated cfDNA found in 75 advanced-stage NSCLC patients was divided in tertiles and correlated to the tumour burden defined as the sum of

Table 2. Mutations identified in circulating cell-free DNA by use of next-generation sequencing ($N = 24$)

ID	Gene	Coding	Protein	Coverage	Mutation allele coverage	Allele frequency (%)	COSMIC
4	TP53	c.833C>G	p.Pro278Arg	1536	27	1.76	COSM10887
6	KRAS	c.34G>A	p.Gly12Ser	3201	40	1.25	COSM517
9	TP53 KRAS	c.714_715insT c.34G>A	p.Asn239Ter p.Gly12Ser	5734 3387	339 86	5.91 2.54	COSM45870 COSM517
10	TP53	c.313G>T	p.Gly105Cys	2800	32	1.14	COSM44481
12	KRAS	c.34G>T	p.Gly12Cys	3084	66	2.14	COSM516
14	SMAD4 TP53 KRAS	c.1051G>A c.313G>T c.34G>A	p.Asp351Asn p.Gly105Cys p.Gly12Ser	4554 2750 3003	612 343 153	13.44 12.47 5.09	COSM32910 COSM44481 COSM517
15	STK11 TP53	c.766G>T c.799C>T	p.Glu256Ter p.Arg267Trp	6164 3961	168 82	2.73 2.07	COSM5731897 COSM11183
21	KRAS KRAS TP53 TP53	c.34G>A c.35G>A c.478A>G c.491A>C	p.Gly12Ser p.Gly12Asp p.Met160Val p.Lys164Thr	8580 6666 5363 5420	1913 426 105 95	22.30 6.39 1.96 1.75	COSM517 COSM521 COSM44328 COSM44387
23	KRAS	c.34_35delGGinsTT	p.Gly12Phe	3432	45	1.31	COSM512
26	MET TP53	c.3029C>T c.578A>G	p.Thr1010Ile p.His193Arg	5643 2674	1994 662	35.34 24.76	COSM707 COSM10742
29	TP53 EGFR EGFR	c.641A>G c.2235_2249delGGAATTAAGAGAAGC c.2240T>C	p.His214Arg p.Glu746_Ala750del p.Leu747Ser	4574 4149 4178	1214 140 60	26.54 3.37 1.44	COSM43687 COSM6223 COSM26704
30	TP53	c.730G>T	p.Gly244Cys	5693	393	6.90	COSM11524
34	KRAS TP53 ERBB4	c.34G>T c.716A>G c.512G>T	p.Gly12Cys p.Asn239Ser p.Trp171Leu	1517 4539 2605	51 112 61	3.36 2.47 2.34	COSM516 COSM44094 COSM5369807
35	TP53	c.711G>A	p.Met237Ile	4353	60	1.38	COSM10834
36	TP53 PIK3CA	c.404G>A c.1624G>C	p.Cys135Tyr p.Glu542Gln	2745 9973	1716 2250	62.51 22.56	COSM10801 COSM17442
37	TP53	c.743G>A	p.Arg248Gln	8221	129	1.57	COSM10662
38	KRAS	c.34G>T	p.Gly12Cys	3081	40	1.30	COSM516
40	TP53 KRAS TP53 TP53	c.742C>T c.34G>A c.578A>G c.830G>T	p.Arg248Trp p.Gly12Ser p.His193Arg p.Cys277Phe	7006 3743 4570 3210	256 71 60 37	3.65 1.90 1.31 1.15	COSM10656 COSM517 COSM10742 COSM10749
44	TP53	c.469G>T	p.Val157Phe	3714	48	1.29	COSM10670
45	KRAS TP53	c.34G>T c.747G>T	p.Gly12Cys p.Arg249Ser	2020 6103	100 121	4.95 1.98	COSM516 COSM10817
49	EGFR	c.2234_2245delAGGAATTAAGAG	p.Glu746_Ala750del	6360	91	1.43	COSM6223
51	KRAS	c.34G>T	p.Gly12Cys	4237	46	1.09	COSM516
54	KRAS TP53 KRAS	c.34G>A c.488A>G c.47A>G	p.Gly12Ser p.Tyr163Cys p.Lys16Arg	3214 2946 3208	86 50 47	2.68 1.70 1.47	COSM517 COSM10808 COSM1163765
64	TP53	c.844C>G	p.Arg282Gly	1814	53	2.92	COSM10992

Abbreviation: COSMIC = Catalogue of Somatic Mutations in Cancer.

the RECIST target lesions. A significant higher median tumour burden was found in the groups with high and intermediate concentration of mutated cfDNA compared with the low group. In addition, there was also a significantly higher median tumour burden when comparing the group with high concentration of mutated cfDNA to the group with intermediate concentration. Both of these studies used a CT-defined tumour volume and no measurement of tumour aggressiveness was therefore incorporated. In contrast, we measured the MTB by use of the PET parameter TLG. This parameter not only measures the entire tumour burden by incorporating all measurable tumours in the patient (and not only RECIST target lesions) but also integrates the metabolic activity of each tumour. Thereby, the parameter becomes a measurement of both tumour burden and aggressiveness of the tumour. MTB has previously been used for assessment of tumour burden in a study evaluating the correlation between tumour burden and total amount of total cfDNA in 53 advanced NSCLC patients (Nygaard *et al*, 2014). No correlation could be found in this study indicating that total cfDNA is a more unspecific marker of tumour burden than mutated cfDNA. The majority of cfDNA originates from non-malignant tissue. Hence, the total amount of cfDNA not simply reflects tumour burden and tumour aggressiveness but rather reflect much more complicated biological mechanisms yet not fully understood.

Additionally, we found that patients with detectable mutated cfDNA in plasma had an inferior median survival compared with patients without mutated cfDNA present in plasma. A prognostic value of mutated cfDNA has previously been evaluated in several

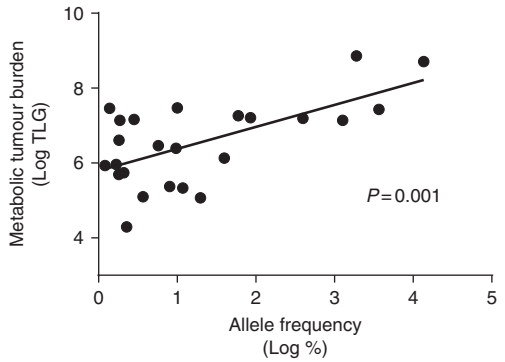

Figure 1. Scatter plot showing the correlation between the allele frequency of the most frequent mutation and the level of TLG in patients with circulating mutated cell-free DNA present in plasma ($N=24$). The *P*-value was calculated using the Pearson's correlation coefficient.

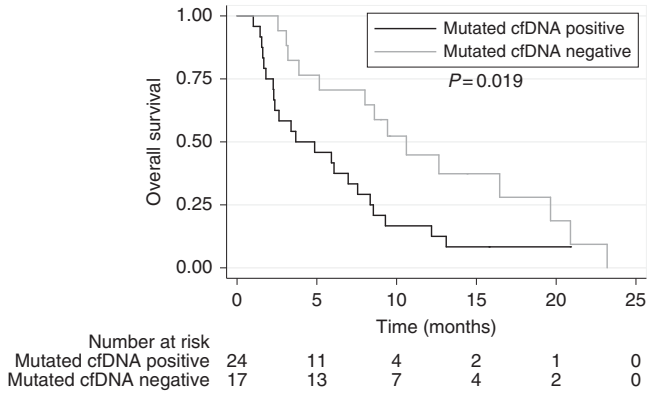

Figure 2. Kaplan–Meier curve for overall survival (OS) according to the presence or absence of circulating mutated cell-free DNA (cfDNA) in plasma. A median OS of 3.7 months (95% confidence interval (CI): 0–7.6 months) was found in patients with detectable mutated cfDNA, while a median OS of 12.6 months (95% CI: 8.4–16.9 months) was found in patients without detectable mutated cfDNA. The difference between the groups was calculated using the log-rank test.

types of cancers, such as colorectal (Lecomte *et al*, 2002), breast (Dawson *et al*, 2013; Bettegowda *et al*, 2014), pancreatic (Hadano *et al*, 2016), and gastric (Gao *et al*, 2016). In NSCLC patients, a study of EGFR mutation-positive patients treated in the EURTAC trail found that the subgroup of L858R-positive patients with detectable mutations in plasma had a significant shorter OS as compared with L858R-positive patients with no plasma mutations, also supporting our finding (Karachaliou *et al*, 2015). However, the importance of mutated cfDNA positivity in EGFR mutation wt patients is less investigated. A recent study performed NGS of cfDNA and tumour samples in 105 unselected advanced NSCLC patients using the same NGS panel as used in the present study (Pécuchet *et al*, 2016). Mutations in cfDNA were identified in 71% of patients and a significantly reduced OS was seen in the mutated cfDNA-positive patients. Though, 43% of the patients harboured an EGFR mutation either in the tumour or in the cfDNA making the patient cohort heterogeneous. In our cohort, all patients had tumours that were EGFR wt and only 2 patients (2%) showed EGFR mutations in plasma. Owing to the homogenous nature of our cohort, we could show that mutated cfDNA positivity is a valuable tool for predicting survival in advanced EGFR mutation wt patients. The prognostic value of mutated cfDNA positivity could in theory be explained by the close correlation to tumour burden. However, we found that the impact of mutated cfDNA on survival was independent of the MTB in the multivariate analysis, indicating that mutated cfDNA positivity is an independent marker of survival.

Table 3. Univariate and multivariate Cox regression analyses of overall survival ($N=46$)

Variables	HR (95% CI)	*P*-value	Adjusted HR (95% CI)	*P*-value
Age				
Continuous	0.99 (0.95–1.04)	0.690		
Gender				
Female	0.84 (0.41–1.74)	0.842		
Male	1.00			
Histology				
Adenocarcinoma	0.36 (0.12–1.10)	0.073		
Squamous cell	1.00			
Smoking				
Never or former	1.37 (0.68–3.01)	0.400		
Current	1.00			
Performance status, ECOG				
0–1	0.60 (0.20–1.74)	0.344		
2	1.00			
Stage				
IV	0.51 (0.15–1.76)	0.287		
III	1.00			
Erlotinib treatment				
Second line	0.69 (0.27–1.75)	0.432		
Third line	1.00			
Presence of mutated cfDNA				
Mutated cfDNA positive	2.26 (1.13–4.55)	0.022	2.11 (1.02–4.38)	0.044
Mutated cfDNA negative	1.00		1.00	
TLG[a]				
>420	2.80 (1.41–5.55)	0.003	3.08 (1.46–6.47)	0.003
⩽420	1.00		1.00	

Abbreviations: cfDNA = cell-free DNA; CI = confidence interval; ECOG = Eastern Cooperative Oncology Group; HR = hazard ratio; TLG = tumour lesion glycolysis.
[a]Divided by the median value.

Our study is the first of its kind and strengthened by the prospective nature of the original study allowing the PET scanning and the blood sampling to be performed in very close proximity to each other. The same PET scanner model, protocol for acquisition, and reconstruction software was used for all patients, thereby reducing the possible interindividual variability of the scans. Additionally, we had a homogeneous patient cohort receiving the same treatment and with complete clinical data in all patients. On the other hand, our study had some limitations to consider. Even though our study is the largest in the field, the number of patients is limited and our results are primarily hypothesis generating. Furthermore, we performed a targeted sequencing using a 22-gene panel with the risk that rare lung cancer mutations would not be detected. As we used the AF for quantification of mutated DNA, a possibility exist that a mutation could have been rejected (if the AF < 1%) owing to a high amount of non-mutated cfDNA in the sample. Finally, we chose to define the most frequently mutation as the mutation defining the level of mutated cfDNA. This may underestimate the true level, but it would be more problematic to combine the frequency of different mutation types as it is not possible to determine whether different mutations exist in the same clone or arise from several different clones contributing their individual mutations.

In conclusion, we showed for the first time that a correlation exists between the level of mutated cfDNA and MTB in EGFR mutation wt NSCLC patients with advanced-stage disease. This indicates that mutated plasma cfDNA can be used as an indirect measure of tumour biology. Furthermore, we observed an inferior

survival in patients with mutated cfDNA present in plasma compared with patients without. This correlation was independent of tumour burden and, if further validated, mutated cfDNA positivity could be a valuable tool for prediction of survival in patients with advanced NSCLC in future.

ACKNOWLEDGEMENTS

We are thankful to Birgit Westh Mortensen for laboratory help.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong S-M, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SKN, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih L-M, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang T-L, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N, Diaz LA (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* **6**: 224ra24.

Dawson S-J, Tsui DWY, Murtaza M, Biggs H, Rueda OM, Chin S-F, Dunning MJ, Gale D, Forshew T, Mahler-Araujo B, Rajan S, Humphray S, Becq J, Halsall D, Wallis M, Bentley D, Caldas C, Rosenfeld N (2013) Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* **368**: 1199–1209.

Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, Diaz LA, Goodman SN, David KA, Juhl H, Kinzler KW, Vogelstein B (2005) Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci* **102**: 16368–16373.

Fleischhacker M, Schmidt B (2007) Circulating nucleic acids (CNAs) and cancer—a survey. *Biochim Biophys Acta* **1775**: 181–232.

Frenel JS, Carreira S, Goodall J, Roda D, Perez-Lopez R, Tunariu N, Riisnaes R, Miranda S, Figueiredo I, Nava-Rodrigues D, Smith A, Leux C, Garcia-Murillas I, Ferraldeschi R, Lorente D, Mateo J, Ong M, Yap TA, Banerji U, Gasi Tandefelt D, Turner N, Attard G, de Bono JS (2015) Serial next-generation sequencing of circulating cell-free DNA evaluating tumor clone response to molecularly targeted drug administration. *Clin Cancer Res* **21**: 4586–4596.

Gao Y, Zhang K, Xi H, Cai A, Wu X, Cui J, Li J, Qiao Z, Wei B, Chen L (2016) Diagnostic and prognostic value of circulating tumor DNA in gastric cancer: a meta-analysis. *Oncotarget* **8**: 6330–6340.

Hadano N, Murakami Y, Uemura K, Hashimoto Y, Kondo N, Nakagawa N, Sueda T, Hiyama E (2016) Prognostic value of circulating tumour DNA in patients undergoing curative resection for pancreatic cancer. *Br J Cancer* **115**: 59–65.

Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R (2001) DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* **61**: 1659–1665.

Karachaliou N, Mayo-de las Casas C, Queralt C, de Aguirre I, Melloni B, Cardenal F, Garcia-Gomez R, Massuti B, Sánchez JM, Porta R, Ponce-Aix S, Moran T, Carcereny E, Felip E, Bover I, Insa A, Reguart N, Isla D, Vergnenegre A, de Marinis F, Gervais R, Corre R, Paz-Ares L, Morales-Espinosa D, Viteri S, Drozdowskyj A, Jordana-Ariza N, Ramirez-Serrano JL, Molina-Vila MA, Rosell R, Spanish Lung Cancer Group (2015) Association of EGFR L858R mutation in circulating free DNA with survival in the EURTAC trial. *JAMA Oncol* **1**: 149–157.

Larson SM, Erdi Y, Akhurst T, Mazumdar M, Macapinlac HA, Finn RD, Casilla C, Fazzari M, Srivastava N, Yeung HWD, Humm JL, Guillem J, Downey R, Karpeh M, Cohen AE, Ginsberg R (1999) Tumor treatment response based on visual and quantitative changes in global tumor glycolysis using PET-FDG imaging. The visual response score and the change in total lesion glycolysis. *Clin Positron Imaging* **2**: 159–171.

Lecomte T, Berger A, Zinzindohoué F, Micard S, Landi B, Blons H, Beaune P, Cugnenc P-H, Laurent-Puig P (2002) Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. *Int J cancer* **100**: 542–548.

Leek RD, Landers RJ, Harris AL, Lewis CE (1999) Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. *Br J Cancer* **79**: 991–995.

Leon SA, Shapiro B, Sklaroff DM, Yaros MJ (1977) Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* **37**: 646–650.

Mouliere F, Thierry AR (2012) The importance of examining the proportion of circulating DNA originating from tumor, microenvironment and normal cells in colorectal cancer patients. *Expert Opin Biol Ther* **12**(12 Suppl 1): S209–S215.

Newman AM, Bratman SV, To J, Wynne JF, Eclov NCW, Modlin LA, Liu CL, Neal JW, Wakelee HA, Merritt RE, Shrager JB, Loo BW, Alizadeh AA, Diehn M (2014) An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med* **20**: 548–554.

Nygaard AD, Holdgaard PC, Spindler K-LG, Pallisgaard N, Jakobsen A (2014) The correlation between cell-free DNA and tumour burden was estimated by PET/CT in patients with advanced NSCLC. *Br J Cancer* **110**: 363–368.

Pallisgaard N, Spindler K-LG, Andersen RF, Brandslund I, Jakobsen A (2015) Controls to validate plasma samples for cell free DNA quantification. *Clin Chim Acta* **446**: 141–146.

Pécuchet N, Zonta E, Didelot A, Combe P, Thibault C, Gibault L, Lours C, Rozenholc Y, Taly V, Laurent-Puig P, Blons H, Fabre E (2016) Base-Position error rate analysis of next-generation sequencing applied to circulating tumor DNA in non-small cell lung cancer: a prospective study. *PLOS Med* **13**: e1002199.

Schwarzenbach H, Hoon DSB, Pantel K (2011) Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* **11**: 426–437.

Sorensen BS, Wu L, Wei W, Tsai J, Weber B, Nexo E, Meldgaard P (2014) Monitoring of epidermal growth factor receptor tyrosine kinase inhibitor-sensitizing and resistance mutations in the plasma DNA of patients with advanced non-small cell lung cancer during treatment with erlotinib. *Cancer* **120**: 3896–3901.

Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M (2016) Origins, structures, and functions of circulating DNA in oncology. *Cancer Metastasis Rev* **35**: 347–376.

Thorvaldsdóttir H, Robinson JT, Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* **14**: 178–192.

Tie J, Kinde I, Wang Y, Wong HL, Roebert J, Christie M, Tacey M, Wong R, Singh M, Karapetis CS, Desai J, Tran B, Strausberg RL, Diaz LA, Papadopoulos N, Kinzler KW, Vogelstein B, Gibbs P (2015) Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. *Ann Oncol* **26**: 1715–1722.

Wahl RL, Jacene H, Kasamon Y, Lodge MA (2009) From RECIST to PERCIST: evolving considerations for PET response criteria in solid tumors. *J Nucl Med* **50**(Suppl 1): 122S–150S.

Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R, Rosenfeld N (2017) Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* **17**: 223–238.

Winther-Larsen A, Fledelius J, Sorensen BS, Meldgaard P (2016) Metabolic tumor burden as marker of outcome in advanced EGFR wild-type NSCLC patients treated with erlotinib. *Lung Cancer* **94**: 81–87.

Zonta E, Garlan F, Pécuchet N, Perez-Toralla K, Caen O, Milbury C, Didelot A, Fabre E, Blons H, Laurent-Puig P, Taly V (2016) Multiplex detection of rare mutations by picoliter droplet based digital PCR: sensitivity and specificity considerations. *PLoS One* **11**: e0159094.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)