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The predictive and prognostic significance of liquid biopsy in advanced epidermal growth factor receptor-mutated non-small cell lung cancer: A prospective study

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ABSTRACT

Objective: To determine the predictive and prognostic roles of three blood-based biomarkers: circulating tumour DNA (ctDNA), circulating tumour cells (CTC) and carcinoembryonic antigen (CEA), in patients with advanced epidermal growth factor receptor-mutated (EGFR+) lung cancer.

Materials and methods: We recruited 28 patients with 103 serial blood samples. We performed mutational analyses for EGFR mutations using droplet digital PCR (ddPCR) on ctDNA. We evaluated the accuracy of EGFR mutation detection in ctDNA compared with tissue biopsy. We also quantified CTCs, ctDNA and CEA in serially collected blood samples, and evaluated the baseline and changes in these blood-based biomarkers with clinical outcomes.

Results: EGFR mutation detection in plasma was highly concordant as compared with tissue biopsy. Detectable baseline ctDNA was associated with higher disease burden ($p < 0.01$). Early disappearance of ctDNA at 4 weeks was associated with radiological response at 12 weeks of treatment ($p = 0.01$) and improved progression free survival (PFS) (HR 5.47, 95%CI 1.32–22.72, $p = 0.02$) and overall survival (OS) (HR 5.46, 95%CI 1.28–23.22, $p = 0.02$). A decrease in CTC count at 4 weeks was associated with improved PFS (HR 3.81, 95%CI 1.13–12.79, $p = 0.03$) but not OS. 85% of patients with radiological progression had a ctDNA rise compared with 22% of patients with stable disease ($p = 0.01$). ctDNA rise was seen on average 170 days prior to radiological progression. There is a significant association between the rise of CEA level with radiological progression ($p = 0.001$).

Conclusion: Early change in ctDNA, CTC and CEA levels may be long-term predictors of treatment benefit and failure prior to availability of radiological response data.

1. Introduction

One of the many breakthroughs in the field of thoracic oncology in the last two decades is the identification of patients with oncogenic-driven subtypes of lung cancer such as epidermal growth factor receptor-mutated (EGFR+) non-small cell lung cancer (NSCLC), and the development of EGFR tyrosine kinase inhibitors (EGFR-TKI) which have improved the symptom burden, quality of life and survival of patients [1,2]. However, patients will eventually develop acquired resistance to EGFR-TKI with a median progression-free survival (PFS) of 11 months [3]. The most common acquired resistance mechanism is the development of a secondary EGFR mutation (T790M). In this setting, there is clinical data to support the use of third generation EGFR-TKI, such as osimertinib, with response rates of 66% and median PFS of 9.9 months [4]. Identification of the initial driver mutations and subsequent resistance-conferring mutations is essential to guide further systemic

Abbreviations: EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; ctDNA, circulating tumour DNA; CTC, circulating tumour cells; CEA, carcinoembryonic antigen; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; TKI, tyrosine kinase inhibitors; ECOG, Eastern Cooperative Oncology Group; CT, computed tomography; ddPCR, droplet digital polymerase chain reaction; NGS, next generation sequencing

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therapy, thereby necessitating initial and repeat tissue biopsies. In many patients with lung cancer, poor performance status, inaccessibility of some tumours, and insufficient tumour material for successful molecular testing is a major obstacle. Another obstacle in the treatment of patients with EGFR + NSCLC is the variable response rate to EGFR-TKI and prognosis; complicated by the lack of reliable predictive and prognostic biomarkers to guide clinical decision making.

Liquid biopsy with analysis of circulating tumour DNA (ctDNA) and circulating tumour cells (CTC) can potentially overcome these challenges. CTC and ctDNA are valuable sources of tumour DNA which are accessed with simple blood draws to clarify tumour mutation status and tumour load. Liquid biopsy could be performed repeatedly throughout the disease course, offering real-time monitoring of disease status to allow timely change in the management of these patients. Liquid biopsy methodologies also reflect a more comprehensive molecular landscape assessment of the tumour, as the blood provides aggregate information from all tumour sites, and avoids sampling error. Previous studies have shown that identification of EGFR mutations from CTC of EGFR + NSCLC patients was feasible [5] and change in CTC count was prognostic for several advanced solid tumours [6–8]. Further, plasma ctDNA analysis has been shown to be a sensitive alternative to tissue biopsy for mutation detection, and was approved by the FDA recently as a diagnostic test for EGFR activating and resistance mutations in patients with advanced NSCLC [9,10]. Before the availability of assays for CTC and ctDNA, the only blood based biomarker which was promising as a predictive and prognostic biomarker for lung cancer was carcinoembryonic antigen (CEA) [11]. However, the use of CEA is widely debated and is not a standard test being used in current clinical practice.

Our aim was to determine the accuracy of using liquid biopsies to identify EGFR sensitising mutations pre-treatment when compared with tissue biopsies. We also hypothesized that change in ctDNA concentration, CTC count and CEA level post-treatment could predict response and treatment resistance, and may have prognostic value.

2. Materials and methods

2.1. Patients

28 consecutive patients diagnosed with advanced NSCLC and established EGFR mutations determined from tissue samples (L858R, exon 19 deletion, L861Q, S768I, T790M) were recruited at diagnosis and treated with the first generation EGFR inhibitors gefitinib or erlotinib, at Liverpool Hospital (NSW, Australia) between December 2015 and December 2017. This prospective study (ACTRN12617000985381) is registered with the Australian New Zealand Clinical Trials Registry (ANZCTR). Written informed consent was obtained from all patients under approved Human Research ethics committee (HREC) protocols from Liverpool Hospital (Project number 13/097, HREC/13/LPOOL/158).

Blood samples were collected into 3 × 9 ml EDTA vacutainer tubes (Greiner Bio-one, Australia) at baseline, one month, three months, six months and every six months subsequently until disease progression. Blood samples were also collected at disease progression. Radiological imaging was performed to assess disease response to treatment at three and six months as per local treatment guidelines, with subsequent scans performed as per treating clinician's discretion (Supplementary Fig. 1). Two patients were excluded from treatment response and survival analyses due to rapid disease progression within one month; as only baseline blood samples were available.

2.2. Disease characteristics and response assessment

Patient demographics and disease characteristics including Eastern Cooperative Oncology Group (ECOG) performance status, ethnicity, and smoking status were collected. Baseline disease burden was determined by the number of sites of metastasis, with ≥4 sites considered as high disease burden and < 4 sites of metastasis considered as low disease burden (Table 1).

Table 1
Patient demographics and disease characteristics.

Patient/Disease characteristics	N = 28 (%)
Age (Median)	67
Gender	
Female	16 (57)
Male	12 (43)
ECOG PS	
ECOG 0-1	22 (79)
ECOG ≥2	6 (21)
Ethnicity	
Asian	15 (54)
Caucasian	11 (39)
Middle Eastern	2 (7)
Smoking status	
Current/former smoker	7 (25)
Never smoker	21 (75)
EGFR mutation status	
Exon 19 deletion	12 (43)
L858R	7 (25)
S768I	5 (18)
L861Q	3 (11)
T790M	1 (13)
Burden of disease (number of sites of metastasis)	
1–3 sites	13 (46)
≥4 sites	15 (54)
Brain metastasis	
Baseline	7 (25)

Abbreviation: ECOG PS, Eastern Cooperative Oncology Group performance status.

Investigator-determined objective response was assessed radiologically by computed tomography (CT) scans or with magnetic resonance imaging (MRI) of the brain or spine when indicated, at approximately three-month intervals for the first six months using RECIST 1.1 criteria [3]. Patients with significant clinical symptoms consistent with disease progression without restaging imaging were classified as having progressive disease (PD).

2.3. Statistical analysis

Patient demographics, disease and treatment information were analysed descriptively. Fisher's exact test was used to determine the association between two categorical variables (eg. baseline ctDNA detectability and disease burden; ctDNA and CTC response/progression and radiological response/progression). Cox regression was used to determine whether baseline ctDNA detectability and ctDNA or CTC response were associated with progression-free survival (PFS) and overall survival (OS). The log-rank test stratified according to different variables was used to compare the duration of PFS and OS between two groups. The Kaplan–Meier method was used to summarise the results for PFS and OS. Data for patients who did not have a progression event or had not died at the time of the analysis were censored at the time of the last clinic follow up.

All analyses were performed using IBM SPSS Statistics (Version 25) and GraphPad Prism (Version 6.01) and p-values were calculated based on deviation from zero with $p < 0.05$ being statistically significant. Graphs were generated in GraphPad Prism.

For calculation of sensitivity, specificity and concordance rate, tissue mutation status was the reference standard with the following formulas being used [12]:

Measures	Formulae
Sensitivity	TP/TP + FN
Specificity	TN/TN + FP
Concordance rate	TP + TN/TP + FP + TN + FN

TP = True positive; FN = False negative; TN = True negative; FP = False positive

2.4. Plasma processing and ctDNA extraction

Two 9 ml whole blood samples per patient and time point were processed within two hours of blood collection. Plasma was separated from the cellular fraction initially by centrifugation at 800*g* at room temperature for ten minutes followed by second centrifugation at 16,000*g* at 4 °C for ten minutes. Supernatant was immediately stored at −80 °C in 1 ml aliquots until ctDNA extraction. ctDNA was extracted from 2 to 3 ml of plasma and eluted in 60 µl elution buffer using QIAamp circulating nucleic acid (CNA) kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions. This kit has the highest efficiency in extracting ctDNA for mutation detection in both plasma and pleural fluid for patients with EGFR + NSCLC [13].

2.5. Droplet digital PCR

Droplet digital PCR (ddPCR) using the QX200 BioRad ddPCR suite (Biorad, Gladesville, Australia) was used for mutation analyses. Assay sensitivity and specificity were performed as described previously [13]. The sequence for primers/probes and the annealing temperatures used for each ddPCR assay are listed in Supplementary Table 1. Amplified PCR products (fluorescent droplets) were detected using the QX200 droplet reader and analysed with QuantaSoft software V1.7.4 (Biorad, Gladesville, Australia). Mutant and wild type control DNAs and no-template controls were included in every run. The automatic call assignments for each data cluster were manually adjusted as required. The number of copies for mutant and wild type alleles were normalised based on the input plasma volume (copies/mL plasma) based on the following formula:

$$\frac{Events\ (copies)\ \times 20}{Input\ ctDNA\ volume\ (\mu L)} \times \frac{Elution\ volume\ (\mu L)}{Plasma\ volume\ (mL)}$$

The ddPCR threshold for positive mutant detection was set at two or more positive droplets (≥10 mutant copies/ml) to ensure specificity. The sensitivity of our ddPCR assay allowed for the detection of a mutant allele fraction of 0.1% or more (i.e. one mutant molecule in a background of 1000 wild type molecules).

2.6. CTC isolation and enumeration

One 9 ml whole blood samples per patient and time point were processed within 24 h of blood collection for CTC enrichment using the IsoFlux CTC isolation platform with the CTC enrichment kit (Fluxion Biosciences, San Francisco, USA) according to the manufacturer's instructions. The kit was adjusted to using 25 ng/µl anti-human EGFR antibody (clone LA1, Millipore, Massachusetts, USA) conjugated to 30 µl immunomagnetic beads (Rare cell isolation kit, Fluxion Biosciences) with mouse anti-EGFR [LA1] antibodies (Millipore, Massachusetts, USA) plus 30 µL anti-human EpCAM conjugated immunomagnetic beads (Fluxion Biosciences, San Francisco, USA) for CTC isolation, as described previously [14].

After isolation using the IsoFlux CTC enrichment system with the standard separation protocol (Fluxion Biosciences), CTCs were immuno-cytostained for cytokeratin (FITC-pan-cytokeratin, clone C-11, 1:250; Sigma, Missouri, USA), CD45 (1:100; Fluxion Biosciences) and nuclei (Hoechst dye, 1:1; Fluxion Biosciences). CTCs mounted on glass slides were visualised and scanned with the CellCelector microscope (Automated Lab Solutions, Jena, Germany). The exposure time for Hoechst, CD45 and cytokeratin were 50, 100 and 100 ms, respectively. CTCs were defined as cytokeratin and Hoechst positive, but CD45 negative cells, and counted manually from scanned images.

2.7. Tissue EGFR mutation testing and assay for CEA

Tissue EGFR mutation testing was performed as part of the routine diagnostic testing by the molecular pathology laboratory at Liverpool Hospital, NSW, Australia. The Qiagen *therascreen*® EGFR RGQ PCR kit (QIAGEN, Hilden, Germany) was used with targeted ARMS (Amplification Refractory Mutation System) technology to detect mutations in exon 18–21 of the EGFR gene.

Serum CEA (normal range ≤3 µg/L) was measured by the biochemistry laboratory at Liverpool Hospital using the Roche Cobas e602 (Hoffmann-La Roche, Basel, Switzerland).

3. Results

103 blood samples from 28 patients were analysed for CTC, ctDNA and CEA at all timepoints. Demographics and characteristics of patients included in this study are listed in Table 1 and a swimmer plot (Fig. 1) indicates the types of mutations for each patient, treatment types and duration as well as survival outcome for each patient after disease progression. The median follow-up duration for all patients was 18 months (range 5.1–34 months). Median duration of treatment with first-line EGFR inhibitors was 13.7 months (range 4.9–33.5 months). Of the 17 patients who progressed on first-line EGFR inhibitors, 11/17 (66%) patients had at least one further line of treatment (Fig. 1). Median PFS was 15.4 months (95% CI 4.3–26.5 months) and median OS was 32.5 months (95% CI 20.0–45.1 months) for the group.

3.1. High sensitivity and specificity in detecting EGFR activating mutations in plasma

To investigate the accuracy of ctDNA based EGFR activating mutation detection, we compared findings from all 28 baseline plasma samples from patients with known EGFR mutations to the original tissue, as well as 10 plasma control samples from EGFR wild-type metastatic lung cancer patients. Using tissue biopsy EGFR mutation status as the reference standard, the mean sensitivity for detection of EGFR sensitising mutations in baseline plasma was 80% (range 69–100%), specificity was 100%, and overall concordance was 88% (range 81–100%) as shown in Table 2.

3.2. Detectable baseline ctDNA was associated with higher disease burden

EGFR mutations in ctDNA were measured at pre-treatment, one-month, three months, six months and at disease progression in all patients. For patients who had not progressed at one year, further plasma samples were taken every six months until disease progression or end of study. ctDNA was detectable at baseline in 19/28 (68%) patients starting first-line EGFR inhibitors. Detectable baseline ctDNA was associated with higher disease burden, which was defined as ≥4 sites of metastatic disease ($p < 0.0001$, Table 3). There was no correlation between detectability of ctDNA at baseline with survival (Supplementary Fig. 2).

CTCs were isolated and enumerated for 27/28 (96%) patients as one patient did not have sufficient blood collected for CTC analysis. 25/27 (93%) had detectable (≥1 CTC/9 ml blood) CTC at baseline (range 1–343 CTCs/9 ml blood). 19/27 (70%) had an elevated CTC count (≥5 CTCs/9 ml blood) at baseline. Previous studies in breast and prostate cancer patients had shown that patients with ≥5 CTCs/7.5 ml whole blood at baseline had worse prognosis [15,16]. However, there was no correlation between elevated CTC counts at baseline with higher

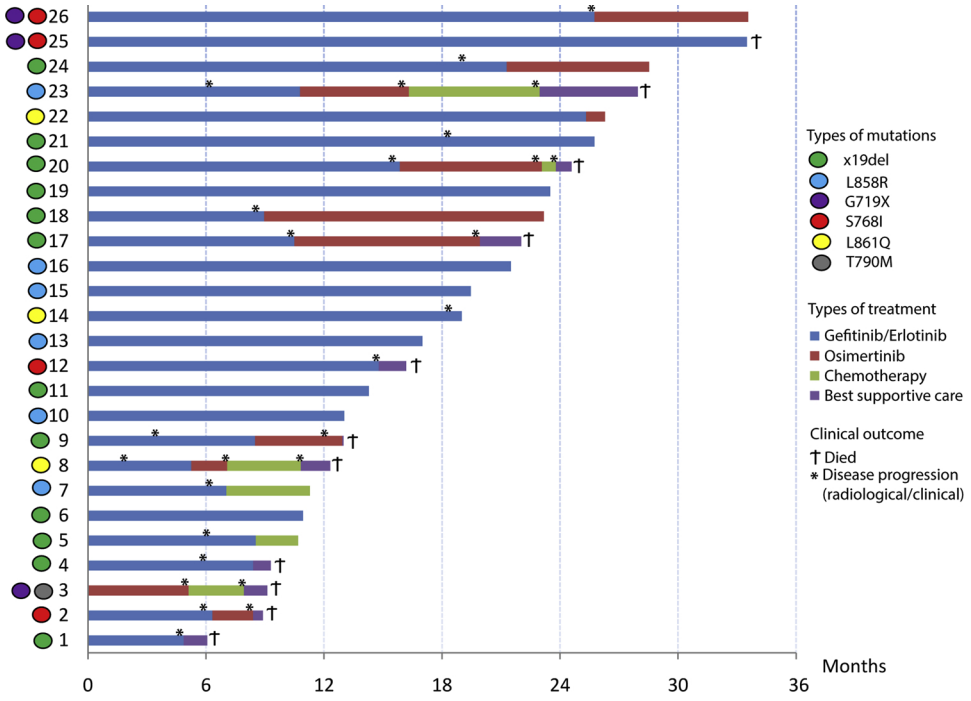

Fig. 1. *Patient mutation status and therapy.* The swimmers plot illustrates mutation type, treatment types, treatment durations and survival outcome of 28 patients. Colour coding of bars indicates the type of treatment for each patient and colour of circles indicates the specific EGFR mutations detected in each patient, * indicates disease progression (radiological/clinical) (see key on right hand side of figure).

disease burden ($p = 1$, Table 3) or survival (Supplementary Fig. 2) in our study.

3.3. ctDNA and CTC change at 4 weeks predict radiological response and survival

Early disappearance of detectable ctDNA at four weeks post therapy commencement, was associated with radiological response at 12 weeks ($p = 0.01$, Table 3) and improved PFS (median PFS 136 vs 511 days, HR 5.47, 95%CI 1.32–22.72, p = 0.02) and OS (Median OS 311 days vs not reached (NR), HR 5.46, 95%CI 1.28–23.22, $p = 0.02$), which remained significant after adjustment for burden of disease (Fig. 2A). Reduction in CTC count at 4 weeks, which was defined as any decrease in baseline CTC count (≥1 CTC/9 ml blood), was associated with improved PFS (HR 3.81, 95%CI 1.13–12.79, $p = 0.03$) but not OS (HR 4.24, 95% CI 0.82–21.91, $p = 0.08$) (Fig. 2B). There was no association between a reduction in CTC count at 4 weeks with radiological response ($p = 0.08$, Table 3).

3.4. Rise in ctDNA precedes and correlates with radiological progression

We defined 'rise in ctDNA' as any increase in ctDNA mutant copy number (≥1 mutant copy/ml plasma). Of the sixteen patients who progressed on study, 13/16 (81%) had a rise in ctDNA concentration prior to disease progression on imaging, one had a rise in ctDNA at disease progression and two patients with low metastatic disease burden did not have detectable ctDNA at radiological progression. For the eight patients who were still responding to treatment based on radiological imaging, one patient who did not have detectable ctDNA at baseline had a small rise in ctDNA level (< 10 mutant copies/ml plasma). Overall the relationship between rise in ctDNA with radiological progression is statistically significant ($p = 0.01$, Table 3). On average, a ctDNA rise was seen 170 days prior to radiological progression (range 0–395 days). Of note, we also screened all plasma samples at progression for T790M mutations. We detected new T790 M mutations in ctDNA of 6/15 (40%) patients who had disease progression on first generation EGFR-TKI.

Table 2
EGFR assay sensitivity and specificity (A) Patients with advanced lung cancer with EGFR mutation (*Tissue*+) or EGFR wild type (*Tissue*-) were grouped according to EGFR mutation status detected in plasma (*Plasma*+ or *Plasma*-). (B) High level of agreement (sensitivity, specificity and overall concordance) was found for plasma and tissue biopsy for all four EGFR activating mutations tested.

(A)

Plasma/Tissue EGFR mutation status	X19del		L858R		S768I		L861Q	
	Tissue+ n = 16	Tissue- n = 10	Tissue + n = 16	Tissue- n = 10	Tissue + n = 5	Tissue- n = 5	Tissue + n = 3	Tissue- n = 3
Plasma+	11	0	11	0	4	0	3	0
Plasma-	5	10	5	10	1	5	0	3

(B)

% Agreement of plasma and tissue biopsy	X19del	L858R	S768I	L861Q
Sensitivity (%)	69	69	80	100
Specificity (%)	100	100	100	100
Overall concordance (%)	81	81	90	100

Table 3
Correlation of biomarkers with disease parameters. Fisher's exact test was used to assess the significance of association between baseline and changes in CTCs, ctDNA and CEA with metastatic disease burden, radiological response and disease progression.

Independent Variable	Dependent variable	Fisher's exact test OR (95% CI)	*P* values (2 sided)
Metastatic disease burden	Baseline CTC	0.89 (0.19–4.07)	1
	Baseline ctDNA	1.83 (1.44–2.34)	< 0.0001***
Radiological response	Baseline CEA	1.4 (0.14–22.9)	1
	CTC reduction^	6.5 (1.05–37.64)	0.08
	Undetected ctDNA^	24 (2.05–303.1)	0.01*
	CEA reduction^	4 (0.62–23.4)	0.3
Radiological progression	CTC rise#	1.2 (0.26–5.5)	1
	ctDNA rise#	10.5 (1.44–58.9)	0.01*
	CEA rise#	45 (4.07–534.2)	0.001**

Abbreviations: CTC, circulating tumour cells; ctDNA, circulating tumour DNA; CEA, carcinoembryonic antigen; OR, Odds ratio.
^ Any decrease in CTC count/CEA or disappearance of ctDNA at 4 weeks into treatment compared with baseline level.
# Any rise compared with previous timepoint.
* P < 0.05.
** P < 0.01.
*** P < 0.001.

3.5. Rise in CTC counts is not related to radiological progression

Fourteen patients in our cohort developed disease progression on treatment and had blood available for CTC isolation. 'CTC rise' was defined as any increase in CTC count (≥1 CTC/9 ml blood) compared with previous time point. 10/14 (71%) had a CTC rise, prior to, or at radiological progression compared with 4/9 (44%) of patients who had a rise in CTC count while still responding to treatment. There was no statistically significant relationship between CTC rise with radiological progression ($p = 1$, Fig. 3B and Table 3).

3.6. Rise in CEA predicts radiological progression

CEA levels were available for 22 patients. Baseline CEA was elevated (> 3 μg/L) in 19/22 (86%) of patients with mean CEA levels of 130 μg/l (range 1.6–1278 μg/L). High baseline CEA values did not predict higher metastatic disease burden ($p = 1$, Table 3) or worse survival (HR 1.65; $p = 0.4$). 'Fall in CEA level' was any decrease in CEA level of ≥0.1 μg/L, whereas 'rise in CEA level' was defined as any increase in CEA level of ≥0.1 μg/L. There was no correlation between fall in CEA level at 4 weeks with treatment response ($p = 0.3$, Table 3) or survival (HR 1.8; $p = 0.3$). In our patient cohort, CEA levels predicted radiological progression with 15/16 (94%) of patients developing a rise in CEA level prior to, and at radiological progression, compared with 2/8 (25%) of patients who had a rise in CEA level while still responding to treatment ($p = 0.001$, Fig. 3C and Table 3). For the 12 patients who had a rise in CEA level prior to radiological progression, CEA rise preceded radiological progression by an average of 88 days (range 28–180 days).

4. Discussion

To the best of our knowledge, this study is unique as it is the first study to prospectively compare the utility of ctDNA, CTC and CEA for monitoring treatment response in a cohort of patients with advanced EGFR + NSCLC from diagnosis to disease progression. ctDNA had a useful dynamic range that correlated with tumour response to treatment, suggesting that it may be an excellent tool for prediction of treatment response and disease progression, and prognosis. Early disappearance of ctDNA at the 4 week timepoint correlates with tumour volume reduction; further, undetectable ctDNA and reduction in CTC counts at 4 weeks predict longer survival. Similarly, a rise in ctDNA occurs in the majority of patients prior to radiological progression by an average of approximately 6 months.

Our findings have important clinical implications for both clinicians and patients. In routine clinical practice, treatment response can only

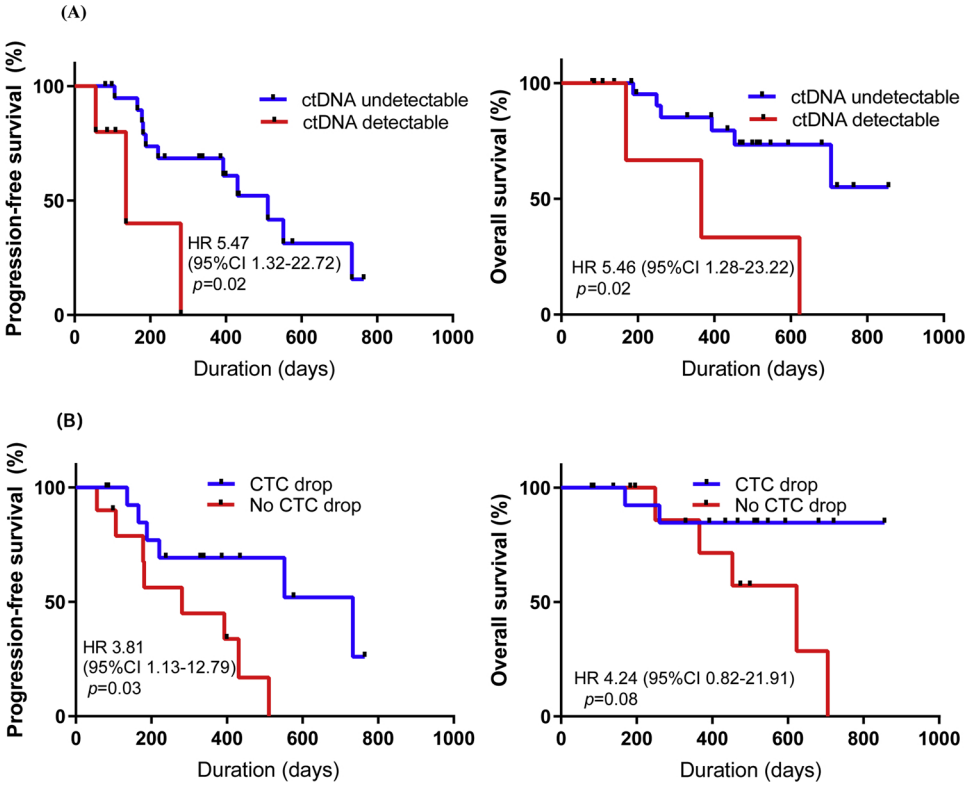

Fig. 2. *Decrease in CTC or ctDNA can predict progression free survival (PFS) and overall survival (OS) in EGFR + NSCLC patients.* (A) Kaplan-Meier survival curves showing PFS (left) and OS (right) for patients based on ctDNA disappearance at 4 weeks post-treatment. Patients with undetectable ctDNA at 4 weeks had improved PFS and OS. p-values and hazard ratio (HR) are indicated on each graph. (B) Kaplan-Meier survival curves showing PFS (left) and OS (right) for patients based on CTC count reduction at 4 weeks post-treatment. Patients with a reduction in CTC count had improved PFS but not OS p-values and hazard ratio (HR) are indicated on each graph.

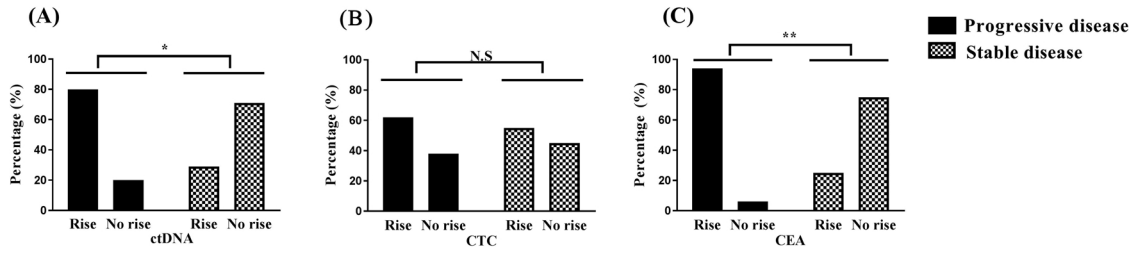

Fig. 3. *Rise in ctDNA and CEA but not CTC number predicts radiological progression in EGFR + NSCLC patients.* (A) Graph showing the percentage of patients with either progressive disease or stable disease which showed a rise in ctDNA during treatment. Fisher's exact test showed a correlation between ctDNA rise and progressive disease (*=$p < 0.05$). (B) Graph showing the percentage of patients with either progressive disease or stable disease which showed a rise in CTC count during treatment. No significant correlation between CTC rise and patient progression was detected. (C) Graph showing the percentage of patients with either progressive disease or stable disease which showed a rise in CEA level during treatment. Fisher's exact test showed a correlation between ctDNA rise and progressive disease (**=$p < 0.01$).

be assessed at around 3 months, since radiological imaging earlier is thought to be uninformative; CT scans in clinical trials are typically conducted at 8 week intervals, but often require confirmatory imaging at least 4 weeks later in many protocols. On the other hand, our data strongly suggests that ctDNA and CTC analyses are considerably more sensitive than radiological imaging, and may be able to guide clinical decision making as early as one month into treatment. This capability could prevent further, unnecessary, treatment-related toxicity and reduce costs for ultimately ineffective therapy. Importantly, it could also facilitate discussion on subsequent treatment plans and prognosis for patients no longer responding to current therapy. This is especially important with the group of patients with EGFR + NSCLC on first-line EGFR inhibitors who are younger (< 70 years old), with good performance status that may benefit from an earlier switch to second-line treatment such as third-generation EGFR-TKI, chemotherapy, or from participation in clinical trials. Since our study showed that patients with undetectable ctDNA at 4 weeks have a three-fold longer median PFS (see Section 3.3) ie median PFS 136 days (95% CI 20–252 days) vs 511 days (95%CI 342–680 days), and that we could detect acquired T790 M mutation in a proportion of patients who developed disease progression with first-generation EGFR-TKI, better personalised treatment options could be offered to patients at a very early timepoint in the disease course: early treatment switches, continuing current therapy but with closer follow-up if patients so choose, and innovative trials with novel approaches to overcome treatment resistance while tumours are smaller. As many patients on EGFR-TKI do not have symptoms suggestive of disease progression, it is sometimes thought that early changes in systemic treatment may not be necessary in the context of advanced lung cancer. However, closer follow-up and more frequent imaging in patients with a rise in ctDNA could be clinically beneficial, as an individual patient data meta-analysis had previously shown that switching to chemotherapy in these patients at disease progression based on conventional radiological imaging would allow a further 'progression-free survival' of approximately 16 months [17]. In addition, determining treatment response with radiological imaging can be challenging if there is a mixed response or if there is insufficient change in tumour dimension based on RECIST criteria that does not agree with clinical suspicion. In these patients, measuring changes in ctDNA may provide essential clarifying information to guide clinicians to improve survival outcomes.

Our study deployed ddPCR assays for single mutation analysis for ctDNA detection. This methodology has been shown to provide high accuracy and sensitivity by others [18] as well as in this study. However, ddPCR-based mutation analysis requires the design of personalized assays for each EGFR mutation, which may be rate-limiting and expensive if many different mutations are tested. The sensitivity of our ddPCR assay is approximately 80% across all four EGFR mutations tested, consistent with other studies [19–21], but with exceptionally high specificity of 100% across all mutations tested. Alternatively, other approaches, such as next generation sequencing (NGS) can be used to identify mutations in targeted genes, with similar sensitivity and specificity, but at a higher cost. Given the first FDA approval of ctDNA methodologies, it is likely that ctDNA analysis by ddPCR or NGS will be adopted for routine clinical use across tumour groups in the near future, especially in cases where an initial tissue biopsy is not available for diagnosis. At the initial diagnosis of advanced NSCLC, if EGFR activating mutations are detected in plasma, patients should be started on targeted therapy without delay, as the specificity of ddPCR for detecting EGFR mutations is very high, with no false positives in our study. However, if mutations are not detected, a tissue biopsy should be performed due to the approximately 20% false negative rate for ddPCR assays as shown by our study and other large trials [18,20] and 3–10% of cases developing histological transformation [22] which will not be detected by liquid biopsy.

Prior to the availability of ctDNA and CTC, tumour marker CEA had been widely used as a 'poor man's liquid biopsy' as it is simpler and cheaper to perform. CEA is normally produced in the gastrointestinal tissue during foetal development and stopped before birth. Therefore it is only found at very low levels in the blood of healthy adults [23]. However, levels can be elevated in heavy smokers [24]. The use of CEA as a prognostic and predictive marker for NSCLC is widely debated with studies showing contradictory results [11]. A previous retrospective study showed that CEA level is associated with EGFR mutations in lung cancers and a rise in CEA levels is associated with disease progression [26]. Our study confirmed this finding; a rise in CEA predicts disease progression in our EGFR + patient cohort by approximately 3 months. However, our study also indicated that baseline and early change in CEA levels are not prognostic or predictive of treatment response.

A limitation to our study is related to the accuracy of CTC enumeration. Our study isolated CTCs not only by immuno-magnetic EPCAM but also EGFR targeting, as EPCAM and EGFR are both reported to be expressed in EGFR + lung cancer cell lines and are pivotal for the survival of these cells [27]. Nonetheless, this method may exclude CTCs that have undergone epithelial-to-mesenchymal transition (EMT). We believe that this will be minimal as previous studies showed that EGFR + tumour tissue often show an epithelial phenotype [27,28]. On the other hand, there may be small numbers of false positive 'CTCs' detected by our method, possibly due to the presence of rare blood based cells isolated that express cytokeratin, such as circulating endothelial cells [29]. This limitation may not affect our findings, as we focus on the change in CTC numbers and the proportion of false positive and false negative which will be less likely to fluctuate at each time point.

Another limitation is that our study has a small number of patients, larger studies will be needed to confirm the finding of this small prospective study. Finally, as there is currently no consensus regarding the optimal timing for radiological imaging, blood tests and clinical follow up, we chose to follow local clinical practice: baseline, 4 weeks, 3 months, 6 months, and then every 6 months until disease progression. It is conceivable therefore, that our findings could have been different if

tests and clinical review were conducted at different timepoints.

In summary, we have shown that longitudinal analysis of ctDNA and CTC at baseline and during treatment is an accurate and useful marker that may be able to guide therapy and help to predict patient prognosis. A prospective study of survival in a larger cohort of patients, particularly in randomised Phase III trials designed to change management based on early decision timepoints, is required to validate the findings from this study. Our data also supports the use of liquid biopsy parameters to risk-stratify patients for future clinical trials. With more validated clinical trial data being available, routine use of liquid biopsies with ctDNA and CTC analysis in clinical management of NSCLC patients on EGFR-TKI will guide appropriate management changes in a more timely fashion while potentially avoiding unnecessary treatment and the associated toxicity and cost.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.06.021.

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