Circulating cell-free plasma tumour DNA shows a higher incidence of *EGFR* mutations in patients with extrathoracic disease progression

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ABSTRACT

Background Non-invasive monitoring of epidermal growth factor receptor (EGFR) mutations conferring sensitivity and resistance to tyrosine kinase inhibitors (TKIs) is vital for efficient therapy of lung adenocarcinoma (LADC). Although plasma circulating cell-free tumour DNA (ctDNA) is detectable at an early stage, the size of the tumour does not strongly correlate with concentration of whole cell-free DNA (cfDNA), including normal leucocyte DNA. We sought to examine the clinical features of patients with LADC whose cfDNA examination held clues for analysis of cancer genomics.

Methods Forty-four plasma samples from 37 patients with LADC receiving EGFR-TKI therapy, including 20 who developed resistance, were prospectively subjected to droplet digital PCR-cfDNA analysis to detect EGFR mutations and analysed according to clinical features.

Results cfDNA samples from 28 (64%) of the 44 samples were positive for TKI-sensitive mutations. Samples from 19 (95%) of the 20 EGFR-TKI-resistant patients were positive for TKI-sensitive mutations. In 24 patients without TKI resistance, 7 (54%) of 13 patients with regional lymph node metastases, 4 (67%) of 6 patients with advanced T stage (T3 or T4) and 8 (57%) of 14 patients with extrathoracic disease progression were also positive for TKI-sensitive mutations. cfDNA analysis from patients with acquired TKI-resistance disease or extrathoracic disease progression correlated with a high detection rate of TKI-sensitive mutations (acquired resistance: risk ratio=2.53, 95% CI 1.50 to 4.29; extrathoracic disease progression: risk ratio=5.71, 95% CI 0.84 to 36.74).

Conclusions cfDNA in patients with EGFR-TKI-resistance or extrathoracic disease progression may be useful for analysis of cancer genomics.

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Key question

What is already known about this subject?

► Epidermal growth factor receptor (*EGFR*)-tyrosine kinase inhibitors (TKIs) for advanced *EGFR*-mutant non-small cell lung cancer (NSCLC) represents a drastic change in treatment paradigms.

► Tumour genotyping using circulating plasma cell-free DNA (cfDNA) has the potential to allow non-invasive assessment of *EGFR* secondary mutation, while many existing assays are cumbersome and vulnerable to false-negative results.

► We previously established a droplet digital PCR system to quantify *EGFR* mutations in cfDNA and documented the clinical characteristics of patients with lung adenocarcinoma (LADC).

What are the new findings?

► We explored the clinical features of patients with LADC whose fraction of ctDNA within the total cfDNA was high.

► We found TKI-sensitive mutations in most of the cfDNA samples obtained after confirming resistance.

► cfDNA obtained from patients who developed extrapleural tumours without EGFR-TKI resistance also exhibited high plasma level of sensitising and resistance *EGFR* mutations.

How might it impact on clinical practice in the foreseeable future?

► cfDNA obtained from patients who developed extrapleural tumours and/or EGFR-TKI resistance also exhibited high detection rates of the EGFR-TKI-sensitising mutation by cfDNA testing.

► Analysis of cfDNA from patients with extrathoracic disease progression and acquired EGFR-TKI resistance may be effective for clarifying the unknown molecular mechanisms of resistance.

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INTRODUCTION

The identification of epidermal growth factor receptor *(EGFR)*-activating mutations and the subsequent development of EGFR tyrosine kinase inhibitors (TKIs) for advanced *EGFR*-mutant non-small cell lung cancer (NSCLC) represents a drastic change in treatment paradigms. Several randomised clinical trials have demonstrated that EGFR-TKI administration results in a superior response rate and longer progression-free

survival than platinum-based chemotherapy for advanced *EGFR*-mutant NSCLC.[1–3] However, patients who initially respond to EGFR-TKIs eventually acquire resistance.

The mechanisms of acquired EGFR-TKI resistance have been widely studied and several mechanisms have been identified. The most common mechanism of resistance to TKIs, observed in over 50% of patients, is a threonine-to-methionine substitution within the gatekeeper residue at amino acid position 790 (T790M) of the *EGFR* gene.[4–7] *EGFR*-independent mechanisms include the MET proto-oncogene, receptor tyrosine kinase (*MET*) amplification (about 3%–5%), v-erb-b2 avian erythroblastic leukaemia viral oncogene homolog 2 (*HER2*) amplification (about 8%–13%), phosphatidylinositol 3-kinase catalytic subunit (*PIK3CA*) mutation (about 1%–2%), B-Raf proto-oncogene serine/threonine-protein kinase (*BRAF*) mutation (about 1%), histological transformation from NSCLC to small cell lung cancer (6%) or epithelial-to-mesenchymal transition (EMT) (about 1%–2%).[6–8] With many negative clinical trials in EGFR-TKI-resistant setting,[9] treatment strategies for acquired resistance, until recently, have remained unclear.[10 11]

The standard of care is rapidly changing with the development of third-generation, mutant-selective EGFR-TKIs that have activity against cells harbouring *EGFR* T790M mutations.[11 12] Osimertinib is 30-fold to 100-fold more potent against T790M and less potent against wild-type *EGFR*[13 14] and is approved for patients with T790M mutation-positive NSCLC (as detected from tissue) who have progressed during or after EGFR-TKI therapy. However, tissue genotyping remains a clinical challenge because of the difficulty of tumour rebiopsy after acquisition of resistance in cases with small target lesions. In addition, rebiopsy may also result in insufficient tumour material for genetic analyses due to necrotic or fibrotic changes. Therefore, minimally invasive tests, known as a 'liquid biopsies', represent a promising breakthrough for detection of *EGFR* T790M or other mechanisms when disease progression occurs in distant sites, such as the brain, bone or lungs, that are not involved by the primary tumour.[15–17]

Circulating plasma cell-free tumour DNA (ctDNA), small DNA fragments from apoptotic and necrotic tumour cells or circulating tumour cells (CTCs) into the bloodstream, represents a promising source that inform tumour genetics, mechanisms of progression and drug resistance.[18–20] ctDNA is only the portion of cfDNA specifically released from cancer cells, and most of cfDNA is derived from normal cells, including normal leucocytes that undergo apoptosis or necrosis. cfDNA is released by passive mechanisms, such as lysis of apoptotic and necrotic cells or digestion of tumour cells by macrophages, and also by active mechanisms, such as the release of fragments of tumorous nucleic acid into the circulation by living cells.[17 21] A new technique known as droplet digital PCR (ddPCR) may become a clinical diagnostic tool for assessing mutations in lung adenocarcinoma (LADC).[22 23] Tumour genotyping using cfDNA has the potential to allow non-invasive assessment of tumour biology, while many existing assays are cumbersome and vulnerable to false-negative results. The Roche cobas 4800 system (Roche Molecular Systems, Inc), approved by the US Food and Drug Administration and the Pharmaceuticals and Medical Devices Agency of Japan, is a companion diagnostic system for osimertinib to detect *EGFR* T790M mutations.[24] In addition, comprehensive genetic panel analysis of cfDNA using next-generation sequencing may be useful as a quantitative tool for genomic characterisation to inform choice of therapy.

Although technical advances may further improve the sensitivity of cfDNA analysis, assessment of biological and genomic factors may eventually be limited by the tiny concentrations involved. A range of sensitive sequencing methods is typically implemented in many molecular pathology laboratories. However, very low levels of mutated DNA can lead to a false-positive result and DNA aberrancies do not always represent a cancer clone, or they can produce a false-negative result when the level is below the assay detection limits.[24] Therefore, it is necessary to establish more clinically useful sequencing methods for analysing cfDNA from LADC patients. We previously established a ddPCR system to quantify *EGFR* mutations in cfDNA.[25] We explored the clinical features of patients with LADC whose fraction of ctDNA within the total cfDNA was high, rendering cfDNA examination useful for analysis of cancer genomics with next-generation sequencing.

In this study, we investigated the relationship between patient characteristics and the detection rate of *EGFR* mutations in cfDNA from patients with a histologically confirmed *EGFR* mutation.

MATERIALS AND METHODS
Study population
Peripheral blood samples were collected from 13 patients with LADC with *EGFR* exon 19 in-frame deletions or 24 patients with exon 21 L858R mutations after obtaining written informed consent. In addition, peripheral blood samples from two patients with LADC harbouring the 4-anaplastic lymphoma receptor tyrosine kinase (*EML4-ALK* fusion) gene were also collected for *EGFR* mutation-free controls, because of the exclusivity with *EGFR* mutations in lung cancer with *ALK* fusion. Blood samples from each patient were collected in two 5 mL EDTA-containing Vacutainers and spun to separate plasma within 30 min of collection. Plasma samples were kept frozen at −80°C until DNA extraction. We evaluated the incidence of TKI-sensitivity in patients with histologically confirmed *EGFR* mutations. Seventeen patients provided cfDNA before EGFR-TKI therapy and 20 provided cfDNA after developing resistance to EGFR-TKIs. Seven of 20 patients also provided blood samples before administration of EGFR-TKI; therefore, a total of 44 blood samples were collected from the 37 patients who developed resistance (17+20+7=44). Most of these patients were subjects in our previous study.[25]

We collected information about patient characteristics for follow-up analysis. We examined the samples using picoliter-ddPCR to determine the fraction of cfDNA with *EGFR* mutations and the concordance of T790M mutation status between cfDNA and rebiopsied tumour tissues.

Measures and definitions
Picoliter-ddPCR
cfDNA was extracted from 2 mL of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen, USA) and quantitated using a NanoDrop spectrophotometer (Thermo Fisher Scientific/Life Technologies) and a Qubit fluorometer (Invitrogen, Carlsbad, California, USA). Reaction mixtures containing 4 ng of cfDNA were subjected to picoliter-ddPCR. Detection of representative exon 19 in-frame deletions, and L858R and T790M mutations was conducted using an assay (TaqMan SNP genotyping assay, Life Technologies) as previously reported.[25] Digital PCR was performed (RainDrop Digital PCR System, RainDance Technologies, Billerica, Massachusetts, USA) in which PCR takes place in millions of droplets with volumes of approximately 5 pL.[26–28] Compensation factors and the respective thresholds were set based on data from positive-control cell lines (II-18, NCI-H1975, PC-9) to define droplets positive for exon 19 deletions and the L858R and T790M mutations.

Threshold setting for positive detection
To determine the threshold for detecting *EGFR* mutations in patient's cfDNA, cfDNA from two patients with LADC harbouring the *EML4-ALK* fusion was used as negative controls because of the exclusivity of *EGFR* mutations in lung cancer with *ALK* fusion. Only a few droplets among millions were positive for *EGFR* mutations. Due to the possibility that a few *EGFR* mutations could be present even in *ALK* fusion-positive patients with LADC, the threshold for a positive result was tentatively set to 10 droplets based on the negative control data. Using this threshold, the rate of false-positive droplet detection was predicted to be <0.0002%. The picoliter-ddPCR analysis complies with the essential requirements listed on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for ddPCR.[29] This procedure was described in our previous study.[25]

Statistical analysis
Correlation between the ability to detect EGFR mutations in plasma and patient characteristics was calculated using risk ratios and a logistic regression model with the following covariates: tumour stage (postoperative relapse vs stage IV), tumour, node and metastasis (TNM) stage according to the seventh edition of the UICC TNM classification at the time of plasma sampling (Tx/1/2 vs T3/4, N0 vs N1/2/3, M0/M1a vs M1b, postoperative recurrence vs stage IV disease), extrathoracic disease progression at latest imaging (negative vs positive) and resistance to EGFR-TKI (negative vs positive) based on the distribution

for the number of patients. Fisher exact test with a significance level of P<0.05 was used to assess the association between the detection rate and patient characteristics. We also calculated concordance rates of T790M mutation status (T790M-positive or T790M-negative) between tissue and plasma. Analyses were performed using commercial software (IBM SPSS Statistics V.24.0).

RESULTS
Detection rate and risk ratio by subgroup in all patients
We examined the relationship between clinical characteristics and detection rates for TKI-sensitising mutations in plasma DNA. In total, we examined cfDNA samples from 37 patients with LADC who received EGFR-TKI therapy: 24 provided cfDNA before EGFR-TKI therapy and 20 provided cfDNA after developing resistance to EGFR-TKIs. In seven patients, samples that acquired both before and after acquisition of EGFR-TKI resistance were analysed. We used picoliter-ddPCR to detect EGFR-TKI-sensitising and T790M mutations in the samples (table 1). Other clinical characteristics of the patients are shown in the online supplementary tables 1 and 2.

The sensitivity of plasma genotyping was 64% (95% CI 56.8% to 65%) for the sensitising mutation for all samples (28 of 44), 60% for exon 19 deletions (18 of 30) and 71% for L858R (10 of 14). The detection rates for TKI-sensitising mutations in plasma were not significantly correlated with sex, smoking status, EGFR mutation status at diagnosis, tumour stage, immediate prior treatment regimen or the type of EGFR-TKI at first administration. The detection rate of TKI-sensitive *EGFR*-mutations in cfDNA from patients with acquired EGFR-TKI resistance (95%, 19/20, risk ratio=2.53, 95% CI=1.50 to 4.29) was correlated with a higher detection rate of *EGFR* mutations, as previously reported (figures 1 and 2, table 2).

Detection rate and risk ratio by subgroup in patients without EGFR-TKI resistance
Even in 24 patients before EGFR-TKI therapy, individuals with advanced T stage (67%, 4/6, risk ratio=2.40, 95% CI 0.94 to 6.12), any lymph node metastases (54%, 7/13, risk ratio=2.96, 95% CI 0.77 to 11.43) or UICC stage IV disease (60%, 6/10, risk ratio=2.80, 95% CI 0.91 to 8.61), sensitive mutations were also more likely to be detected mutation but not significant (figure 2, table 2). The detection rate was notably higher in patients who developed extrathoracic disease progression (57%, 8/14, risk ratio=5.71, 95% CI 0.84 to 38.74) than in those who did not. Additionally, the adjusted risk ratio for mutation detection according to extrathoracic disease progression using the multivariate generalised linear model was 7.14 (95% CI 1.08 to 47.22, P=0.041) (table 3). There was a strong relationship between TKI-sensitive mutation detection and extrathoracic disease progression.

Tumour tissues from 12 of the 20 patients with TKI resistance were rebiopsied and subjected to genomic analysis (patients 1–10, 19 and 20). The results from 10

Table 1 Study subjects for picoliter-ddPCR analysis of cfDNA

Age	Sex	EGFR TKI-sensitive mutations in tumour	TNM classification at first sampling				cfDNA before resistance acquisition		cfDNA after resistance acquisition		T790M mutation in rebiopsied tumour tissue	Rebiopsied organ
			T stage	N stage	M stage	Stage	TKI-sensitive mut	T790M mut	Sensitive mut	T790M mut		
Cases with resistance to EGFR-TKI therapy (TKI-resistant cohort)												
62	Female	19DEL	4	2	1b	IV	Positive	Negative	Positive	Positive	Positive	Primary disease
74	Female	19DEL	(2)	(3)	(1b)	IV	–	–	Positive	Positive	Positive	Primary disease
67	Female	L858R	(1)	(3)	(1b)	IV	–	–	Positive	Positive	Positive	Liver metastasis
64	Male	19DEL	(4)	(3)	(1b)	IV	–	–	Positive	Positive	Positive	Mediastinal lymph node
71	Female	L858R	(4)	(2)	(1b)	IV	–	–	Positive	Positive	Positive	Liver metastasis
57	Male	19DEL	(2)	(0)	(1b)	IV	–	–	Negative	Negative	Positive	Primary disease
65	Female	19DEL	(3)	(0)	(1b)	IV	–	–	Positive	Negative	Positive	Primary disease
65	Male	19DEL	2	3	1b	IV	Negative	Negative	Positive	Negative	Negative	Primary disease
64	Male	L858R	x	x	(1b)	Rec	–	–	Positive	Negative	Negative	Pleural effusion
58	Male	L858R	4	2	1b	IV	Negative	Negative	Positive	Negative	Negative	Pleural effusion
47	Female	19DEL	4	3	1b	IV	Positive	Negative	Positive	Negative	–	–
69	Female	19DEL	x	2	1b	IV	Positive	Negative	Positive	Negative	–	–
68	Male	19DEL	(4)	(0)	(1b)	IV	–	–	Positive	Negative	–	–
65	Female	L858R	(4)	(3)	(1a)	IV	–	–	Positive	Positive	–	–
53	Female	19DEL	(3)	(2)	(1b)	IV	–	–	Positive	Positive	–	–
51	Female	19DEL	(4)	(2)	(1b)	IV	–	–	Positive	Negative	–	–
63	Male	19DEL	2	x	1b	Rec	Negative	Negative	Positive	Negative	–	–
52	Female	19DEL	1	2	1b	Rec	Negative	Negative	Positive	Negative	–	–
63	Female	L858R	(2)	(1)	(1a)	IV	–	–	Positive	Negative	Positive	Pleural effusion

Table 1 Continued

Age	Sex	EGFR TKI-sensitive mutations in tumour	TNM classification at first sampling				cfDNA before resistance acquisition		cfDNA after resistance acquisition		T790M mutation in rebiopsied tumour tissue	Rebiopsied organ
			T stage	N stage	M stage	Stage	TKI-sensitive mut	T790M mut	Sensitive mut	T790M mut		
54	Female	19DEL	(2)	(3)	(1b)	IV	–	–	Positive	Positive	Positive	Primary disease
Case before EGFR-TKI therapy (pre-TKI cohort)												
71	Female	L858R	x	x	1a	Rec	Negative	Negative	–	–	–	–
55	Female	L858R	1	3	1b	IV	Negative	Negative	–	–	–	–
49	Male	L858R	x	x	1a	Rec	Negative	Negative	–	–	–	–
62	Male	L858R	x	x	1a	Rec	Negative	Negative	–	–	–	–
58	Female	19DEL+T790M	x	x	1a	Rec	Negative	Negative	–	–	–	–
58	Female	19DEL	1	0	1b	IV	Positive	Negative	–	–	–	–
51	Male	19DEL	x	x	1b	Rec	Positive	Negative	–	–	–	–
53	Female	19DEL	x	x	1b	Rec	Negative	Negative	–	–	–	–
64	Male	19DEL	x	x	1a	Rec	Negative	Negative	–	–	–	–
64	Male	19DEL	x	2	0	Rec	Positive	Negative	–	–	–	–
60	Female	19DEL	x	3	1a	Rec	Negative	Negative	–	–	–	–
63	Female	L858R	x	3	1b	Rec	Negative	Negative	–	–	–	–
50	Female	19DEL	x	x	1b	Rec	Negative	Negative	–	–	–	–
63	Female	L858R	4	x	1b	Rec	Negative	Negative	–	–	–	–
58	Male	L858R	4	2	1b	IV	Positive	Negative	–	–	–	–
55	Female	19DEL	4	3	1b	IV	Positive	Negative	–	–	–	–
54	Male	19DEL	4	3	1b	IV	Positive	Negative	–	–	–	–

Values in parentheses indicate the TNM factor at resistance acquisition.
cfDNA, cell-free DNA; ddPCR, droplet digital PCR; EGFR, epidermal growth factor receptor; mut, mutation; –, not examined; Rec, reccurence after surgery or definitive chemoradiotherapy; TKI, tyrosine kinase inhibitor; TNM, tumour, node and metastasis.
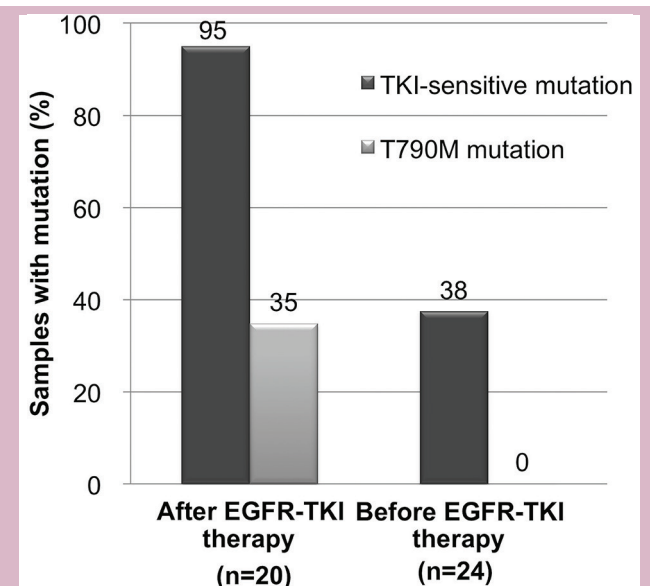
Figure 1 Detection rate for EGFR mutations in cfDNA. Detection rates from cfDNA for TKI-sensitive and T790M mutations are shown. Twenty samples were collected from patients with resistance to EGFR-TKI therapy (patients 1–20), and 24 from patients before EGFR-TKI therapy (patients 1, 8, 10–12, 16, 17 and 21–37). cfDNA, cell-free DNA; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.
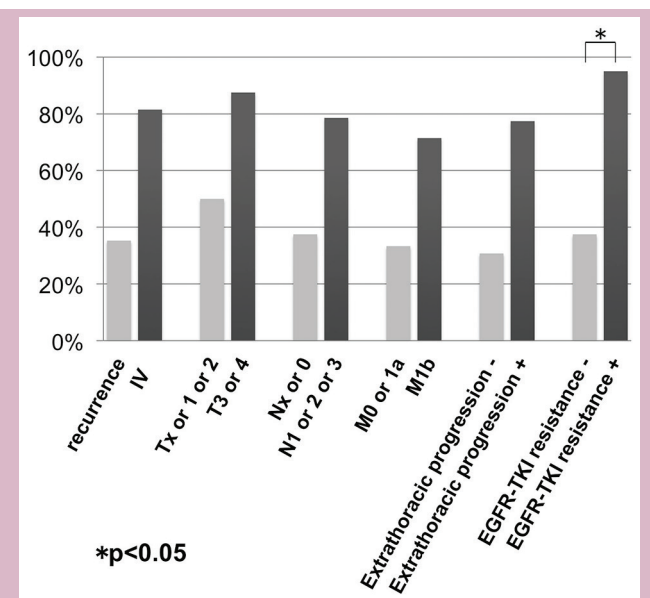

Figure 2 Detection rate of *EGFR* mutations in cfDNA by characteristics. The sensitivity of plasma genotyping for the sensitising mutation was 64% for all samples. Mutation detection rate from patients with UICC stage IV disease, T stage 3/4 disease, any lymph nodal metastases, extrathoracic disease progression and acquired EGFR-TKI-resistant disease was significantly higher than from those without these features. cfDNA, cell-free DNA; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; UICC, Union Internationalis Contra Cancrum .

Table 2	Detection rate and risk ratio by subgroup		
Characteristics	**No**	**Detection (%)**	**Risk ratio (95% CI)**
Total	28/44	64	–
EGFR-TKI resistance			
Negative	9/24	38	1
Positive	19/20	95	2.53 (1.50 to 4.29)
Cases without TKI resistance	9/24	38	
T stage			
X or 1 or 2	5/18	28	1
3 or 4	4/6	67	2.40 (0.94 to 6.12)
N stage			
x or 0	2/11	18	1
1 or 2 or 3	7/13	54	2.96 (0.77 to 11.43)
M stage			
0 or 1a	1/7	14	1
1b or metastatic recurrence	8/17	47	3.29 (0.50 to 21.66)
Stage			
Rec	3/14	21	1
IV	6/10	60	2.80 (0.91 to 8.61)
Extrathoracic progression			
Negative	1/10	10	1
Positive	8/14	57	5.71 (0.84 to 38.74)

EGFR, epidermal growth factor receptor; Rec, recurrence after surgery or definitive chemoradiotherapy; TKI, tyrosine kinase inhibitor.

of these patients were concordant with those from cfDNA analysis (concordance rate, 0.83). While the results from rebiopsied tissue from the remaining two patients were positive for EGFR-TKI-sensitising and T790M mutations, one (patient 6) had a negative result by cfDNA analysis and the other (patient 19) was positive only in the EGFR-TKI-sensitising mutation test.

DISCUSSION

We evaluated the relationship between patient characteristics and the positive detection rate of *EGFR* mutations in cfDNA from patients with histologically confirmed *EGFR* mutations. TKI-sensitive mutations were detected in most (95%, 19/20) (figure 1) of the cfDNA samples obtained after confirming resistance. Notably, cfDNA obtained from patients who developed extrapleural tumours without EGFR-TKI resistance also exhibited high detection rates of the EGFR-TKI-sensitising mutation by cfDNA testing. This finding confirms the usefulness of examining cfDNA to deduce tumour burden and certain amounts of ctDNA in progressed cases, as suggested by

Table 3 Generalised linear model assessing predictors of mutation detection

Variable	Univariate model		Model 2		Final model	
	Risk ratio (95% CI)	P value	Risk ratio (95% CI)	P value	Risk ratio (95% CI)	P value
Advanced T stage (T3 or T4/Tx or T1 or T2)	1.71 (0.76 to 3.85)	0.192				
Regional lymph node metastasis (any N/N0)	2.26 (0.79 to 6.49)	0.131				
Distant metastasis (M1b or metastatic Rec/M0 M1a)	3.33 (0.53 to 20.91)	0.381				
Stage (stage IV / postoperative Rec)	3.81 (1.03 to 14.04)	0.045	1.50 (0.36 to 6.23)	0.577		
Extrathoracic disease progression (+/−)	6.00 (0.914 to 39.41)	0.062	5.00 (0.49 to 50.83)	0.174	7.14 (1.08 to 47.22)	0.041

Rec, recurrence after surgery or definitive chemoradiotherapy.

previous studies.[30 31] On the other hand, only a subset of cfDNA samples obtained before EGFR-TKI therapy was positive for TKI-sensitive mutations (38%, 9/24). One possible explanation for this difference is that the EMT of tumour cells, which often plays a role in resistance to EGFR-TKIs, permits tumour-initiating cells to invade blood vessels, which might increase the amount of plasma ctDNA by spreading tumour cells into the blood.[32 33] A large cfDNA study found that the sensitivity of plasma genotyping for a known sensitising mutation was 82% from patients with acquired EGFR-TKI resistance.[34] It is reassuring that the imperfect sensitivity of cfDNA analysis mitigates its value as a non-invasive alternative to a biopsy. Several systems have been approved as companion diagnostic systems for third-generation EGFR-TKI to detect EGFR T790M mutations from cfDNA all over the world.[34] Given the wide range of reported sensitivities and specificities of the different platforms, it will be essential for prospective therapeutic trials to mandate the collection of plasma cfDNA to establish reproducible concordance rates with tissue for clinical validation.

Notably, our analysis found a lower risk of false-negative results and similar risk of extrapleural disease progression following previous therapy compared with prior studies that examined larger populations.[22 35] Even in a smaller cohort such as ours, these analyses suggest the same trend towards reducing false-negative results. Therefore, selecting specific subgroups could increase the usefulness of cfDNA genotyping as a diagnostic test in response to individual preferences, to increase the true-positivity rate. This may be attributable to the increased use of next-generation sequencing for analysis, which can be used to analyse patient samples that cannot be genotyped because of insufficient tissue or tissue sampling errors related to tumour heterogeneity.[36] To fully exploit the potential usefulness of next-generation sequencing of liquid biopsies, suitable patients must be selected for cfDNA analysis. Therefore, in order to validate the analysis results, we should select patients whose cfDNA contains a sufficient fraction of tumour DNA to screen mutations.

Limitations of this study include its small sample size. Additionally, not all individuals with a positive cfDNA result underwent a diagnostic rebiopsy; therefore, some mutations might have been clinical false-positives. Therefore, to validate the sensitivity, specificity and accuracy of cfDNA analysis, it is important to test and conduct standardised cfDNA-based large-scale studies with samples that contain certain amounts of ctDNA.

CONCLUSIONS

Analysis of cfDNA from patients with extrathoracic disease progression and acquired EGFR-TKI resistance may be efficient for clarifying the molecular mechanisms of resistance. Further analysis of cfDNA from patients with these features is required to validate tumour molecular profiling and treatment modification.

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