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

Yoshitaka Seki,1,2 Yutaka Fujiwara,3 Takashi Kohno,1 Kazushi Yoshida,1 Yasushi Goto,3 Hidehito Horinouchi,3 Shintaro Kanda,3 Hiroshi Nokihara,3 Noboru Yamamoto,3 Kazuyoshi Kuwano,2 Yuichiro Ohe3

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-sensitising mutations (acquired resistance: risk ratio=5.71, 95% CI 0.84 to 36.74).

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

Trial registration number  UMIN 000017581.

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 in first-line treatment of advanced EGFR-mutant NSCLC. The incidence of resistance development to EGFR-TKIs is however high, ranging from 10% to 20% in the first line and up to 30% to 40% in the second line or later. EGFR-TKI resistance is also known to be associated with a poor prognosis.

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survival than platinum-based chemotherapy for advanced EGFR-mutant NSCLC. 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. EGFR-independent mechanisms include the MET proto-oncogene, receptor tyrosine kinase (MET) amplification (about 3%–5%), v-erb-b2 avian erythroblastic leukemia 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%). With many negative clinical trials in EGFR-TKI-resistant setting, treatment strategies for acquired resistance, until recently, have remained unclear.

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. Osimertinib is 30-fold to 100-fold more potent against T790M and less potent against wild-type EGFR 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 ‘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.

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. 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. ctDNA 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. A new technique known as droplet digital PCR (ddPCR) may become a clinical diagnostic tool for assessing mutations in lung adenocarcinoma (LADC). 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. 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. 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. 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.
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). The detection rate was notably higher in patients who developed extrathoracic disease progression (57%, 8/14, risk ratio=3.50, 95% CI 0.94 to 6.12) 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 |               |                      |                  |
|      |     |                                      |         |         |         |       |                   |          |                |          |               |                      |                  |
| 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 | Primary disease |
| 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  |                  |

Continued
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** | **Organ** |            |
| 54   | Female | 19DEL                                 | (2) | (3) | (1b) | IV | – | – | Positive | Positive | Positive | Primary disease |
| 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 | – | – | – |            |

Case before EGFR-TKI therapy (pre-TKI cohort)

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, recurrence after surgery or definitive chemoradiotherapy; TKI, tyrosine kinase inhibitor; TNM, tumour, node and metastasis.
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
previous studies. 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. 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. 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. 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. 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. 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.

**Correction notice** The article has been corrected since it first published. The Abstract has been added in.

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**Competing interests** None declared.

**Ethics approval** Institutional Review Board of the National Cancer Center, Japan.

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**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 | 5.00 (0.49 to 50.83) | 0.174 | 7.14 (1.08 to 47.22) | 0.041 |
| Regional lymph node metastasis (any N/N0) | 2.26 (0.79 to 6.49) | 0.131 | 1.50 (0.36 to 6.23) | 0.577 |  |  |
| 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.
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