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# Cell-Free DNA Analysis of Epithelial Growth Factor Receptor Mutations in Lung Adenocarcinoma Patients by Droplet Digital PCR

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## ABSTRACT

Cell-free DNA (cfDNA) analysis may provide a non-invasive diagnostic approach for lung adenocarcinoma patients. Recently, droplet digital PCR (ddPCR) has been developed as a highly sensitive detection method for a low mutant allele percentage. The ddPCR detection limit for epithelial growth factor receptor (EGFR) mutations was evaluated using cell lines, NCI-H1975 for EGFR L858R point mutation and PC-9 for EGFR E746-A750del. Subsequently, detection of EGFR mutations by ddPCR was performed in tumor DNA (tDNA) and cfDNA samples of 19 lung adenocarcinoma patients whose tumor biopsies were already evaluated for EGFR mutations by clamp PCR (13 of L858R, 3 of E746-750del, and 3 of EGFR negative). In 12 cases, immunohistochemical analysis was performed to quantify the number of EGFR L858R-positive cells rate. EGFR point mutation or deletion were detected in 16 tumor DNA samples. In the measurable cfDNA samples, the rate of detection by ddPCR in cfDNA was 61.5% (8/13) for L858R and 100% (3/3) for E746-A750del. A relative correlation was found between the allele fraction (AF) of tDNA and the number of EGFR L858R-positive cells rate. No correlation was found between the AF of tDNA and AF of cfDNA. In our study, cfDNA mutation detection was not associated with clinicopathological features, but cases with high AF of cfDNA did have metastatic lesions. Our study shows that ddPCR enables cfDNA analysis for EGFR L858R and E746-A750del, with a high detection rate. Therefore, cfDNA analysis using ddPCR may complement to tumor biopsy and is beneficial for precision medicine in lung adenocarcinoma patients.

***Key words:*** *Cell-free DNA*, *Droplet digital PCR*, *Lung adenocarcinoma*, *EGF*

## INTRODUCTION

Cell-free DNAs (cfDNAs) derived from cancer cells carry genetic information of cancer cells, including tumor-specific mutations, from primary tumor and metastasis sites[3)]. These short double-stranded DNAs circulating in the bloodstream are released from cancer cells that undergo apoptotic and necrotic processes and also from active cancer cells[14)]. cfDNA has been proposed as a minimally invasive tool for cancer diagnosis. Since it is a blood-based biomaterial, it is termed as “liquid biopsy”. Compared to the other biomaterial source for liquid biopsy, cfDNA is relatively easy to be isolated. However, because the fraction of cfDNA derived from cancer cells is often low and it is short in length, a highly sensitive mutation detection tool is required for cfDNA analysis[1,12)].

The allele fraction (AF) in cfDNA may be correlated with the detection limit of liquid biopsy. Droplet digital PCR has recently emerged as a highly sensitive detection tool for rare mutations and degraded DNA[5)]. This study evaluated the utility of droplet digital PCR for cfDNA analysis and compared the results with those of tumor samples to examine the detection limit of liquid biopsy and its correlation with different grades of malignancy.

## METHODS

### Patients and Materials

Nineteen lung adenocarcinoma patients who were treated at the Hiroshima University Hospital from 2010

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**Table 1** Patient Characteristics (N = 19).

| Case No. | Age | Sex | Tumor Size (T) | Lymph Node Metastasis (N) | Metastasis (M) | Stage | Origin | Biopsy Procedure | EGFR Mutation | Tumor DNA AF ddPCR (%) | cfDNA AF ddPCR (%) | EGFR L858R-Positive Cells Rate (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 62 | M | 1b | 2 | 1 | 4 | Primary site | TBLB | L858R | 16.1 | 5.12 | 3.4 |
| 2 | 65 | M | 4 | 1 | 1 | 4 | Primary site | TBLB | L858R | 52.6 | 4.21 | 32.1 |
| 3 | 78 | F | 2a | 1 | 1 | 4 | Primary site | TBLB | L858R | 5.68 | 0.55 | 21.2 |
| 4 | 73 | M | 1a | 3 | 1 | 4 | Metastasis site | TBLB | L858R | 33.3 | 0.21 | 93 |
| 5 | 85 | F | 1b | 3 | 1 | 4 | Primary site | TBLB | L858R | 15 | 0.09 | 19.1 |
| 6 | 71 | F | 2a | 3 | 1 | 4 | Primary site | TBLB | L858R | 8.32 | 0.08 | 18.9 |
| 7 | 80 | M | 2a | 0 | 1 | 4 | Primary site | Surgical | L858R | 51 | 0.05 | 50.2 |
| 8 | 72 | F | 2a | 0 | 0 | 1B | Primary site | Surgical | L858R | 29.8 | 0.04 | 73.2 |
| 9 | 75 | F | 2b | 2 | 1 | 4 | Primary site | TBLB | L858R | 42.8 | 0 | 67.7 |
| 10 | 77 | M | 4 | 0 | 1 | 4 | Primary site | TBLB | L858R | 71 | 0 | 52.1 |
| 11 | 87 | F | 2a | 3 | 0 | 3B | Primary site | TBLB | L858R | 34.3 | 0 | N/A |
| 12 | 69 | M | 1a | 3 | 1 | 4 | Metastasis site | Surgical | E746-A750del | 16.1 | 18.60 | N/A |
| 13 | 62 | F | 2a | 3 | 1 | 4 | Primary site | TBLB | E746-A750del | 34.7 | 0.15 | N/A |
| 14 | 57 | F | 4 | 3 | 1 | 4 | Primary site | TBLB | E746-A750del | 7.45 | 0.07 | N/A |
| 15 | 66 | F | 4 | 3 | 1 | 4 | Primary site | TBLB | Negative | 0 | 0 | N/A |
| 16 | 72 | M | 2a | 0 | 0 | 1B | Primary site | Surgical | Negative | 0 | 0 | N/A |
| 17 | 67 | M | 3 | 3 | 1 | 4 | Primary site | TBLB | Negative | 0 | 0 | N/A |
| 18 | 80 | F | 2a | 0 | 0 | 1B | Primary site | TBLB | L858R | 11.8 | N/A | 15.2 |
| 19 | 81 | M | 2a | 0 | 0 | 1B | Primary site | Surgical | L858R | 5.7 | N/A | 33.6 |

N/A: not available; Stage: According to International Association for the Study of Lung Cancer (IASLC) TNM staging system 7th edition, AF: allele fraction; tDNA: tumor DNA; cfDNA: cell-free DNA, TBLB: transbronchial lung biopsy.

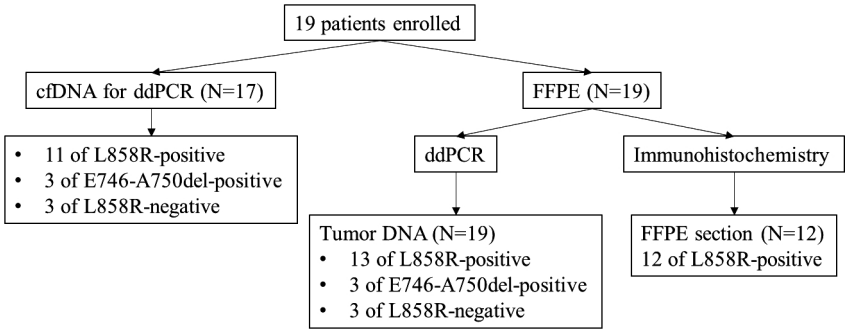


**Figure 1** Research workflow. cfDNA: cell-free DNA, ddPCR: droplet digital PCR, FFPE: formalin-fixed paraffin-embedded.

to 2017 were enrolled in this study. All patients had undergone biopsy and were diagnosed with lung adenocarcinoma. EGFR gene status in the biopsy specimens was evaluated by clamp PCR. Patient characteristics are described in Table 1. The stages of cancer in patients were determined based on the International Association for the Study of Lung Cancer (IASLC) TNM staging system 7$^{th}$ edition. Tumor DNA (tDNA) samples were isolated from 19 formalin-fixed paraffin-embedded (FFPE) samples obtained from primary or metastasis sites of tumors. 14/19 samples were obtained by the transbronchial lung biopsy (TBLB) procedure and 5/19 samples were obtained by surgical procedures. All biopsy samples were obtained before the patients underwent chemotherapy. Immunohistochemistry was performed in 12 FFPE samples. cfDNA was isolated from 17 serum samples taken at the time of diagnosis before starting chemotherapy. The research workflow is illustrated in Figure 1. Additionally, cfDNA isolated from 4 plasma samples of healthy volunteers and placental DNA were used for setting up detection limits of ddPCR assays. Moreover, cfDNA of healthy volunteers was used to establish standards for false positive signals. The detection limit was determined by the fractional abundance of the DNA of the diluted cell line that was above the fractional abundance of cfDNA of healthy volunteers; placental DNA was used as a negative control. For ddPCR, DNA isolated from NCI-H1975 and PC-9 cell lines were used as positive controls for L858R and E746-A750del, respectively, and DNA isolated from Lc-Ad1 cell line was used as a negative control. For immunohistochemical studies, NCI-H1975 and Lc-Ad1 cell lines were used as positive and negative controls, respectively.

## DNA Isolation

cfDNA was isolated from 1.5 ml of serum and 1 ml plasma using Circulating Nucleic Acid kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Genomic DNA was extracted from FFPE samples using QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany). DNA from cell lines was isolated using QIAamp DNA Micro Kit (Qiagen, Hilden, Germany)

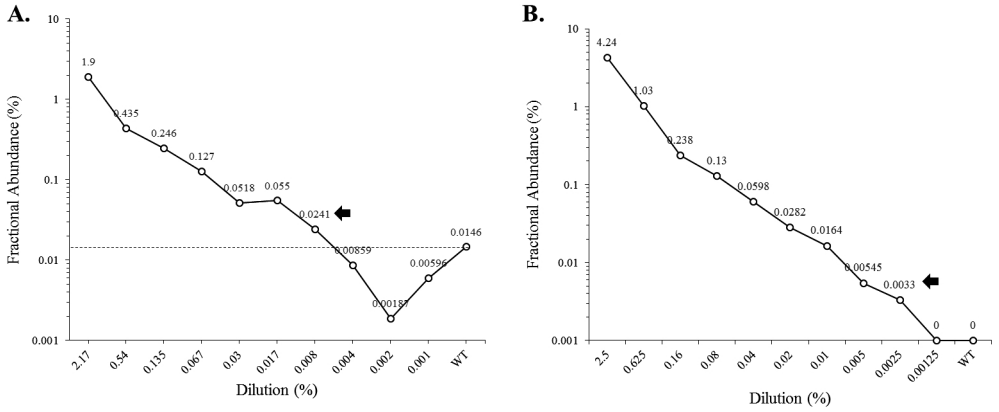


**Figure 2** Detection limit of ddPCR assays indicated as the lowest fractional abundance of diluted cell lines above the fractional abundance of plasma of healthy volunteers. A. Detection limit for EGFR L858R point mutation assay using serial dilutions of DNA from NCI-H1975 cell line is 0.024%. B. Detection limit for EGFR E746-750 deletion assay using serial dilutions of DNA PC-9 cell lines is 0.003%. WT: plasma of healthy volunteers, Arrow: the detection limit of the mutation and deletion.

according to the manufacturer's instructions. All DNA quantification was performed using Qubit 2.0 fluorometer (Thermo Fisher, Waltham, MA, USA) and the isolated DNA samples were stored at 4°C until use.

### Droplet Digital PCR

Allele fractions (AFs) were measured using the QX100 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). Commercially available EGFR L858R point mutation and E746-A750del detection assays were used (Bio-Rad, Hercules, CA, USA). The ddPCR reaction sample contained a final reaction volume of 22 μL consisting of approximately 30 ng cfDNA or 10 ng tDNA, 10 μL 2X ddPCR supermix for probes (No dUTP), 1 μL EFGR assay FAM-labeled primer-probe for the mutated allele, 1 μL EGFR assay HEX-labeled primer-probe for wild type allele, and nuclease-free water. The QX100 droplet generator partitioned the samples into approximately 20,000 uniform droplets per sample. Later, 40 μL of ddPCR reaction mixtures were run in a 96-well plate on the ProFlex PCR system (Life Technologies, Carlsbad, CA, USA) using previously described PCR conditions[8]. Data were analyzed using QuantaSoft v1.3.2.0 Software (Bio-Rad, Hercules, CA, USA). ddPCR of serial dilutions of NCI H1975 and PC9 cell lines DNA with wild-type human genomic DNA was performed to determine the detection limit of ddPCR assays.

### Immunohistochemical analysis

Immunohistochemistry was performed on 5-μm-thick sections cut from FFPE samples using EGF receptor L858R mutant-specific rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA). EGFR L858R-positive cell rate was calculated based on the number of stained cells compared to the total number of cells, both EGFR L858R-positive and -negative, observed under a microscope at 20x magnification. The microscopic fields in which the cells were counted were randomly selected. These cells were manually counted and supported by ImageJ software (NIH and LOCI, WI, USA).

### Statistical Analysis

Statistical analyses were performed using Fisher's exact test or Spearman's correlation coefficient where appropriate. *P* values < 0.05 were considered to be statistically significant. Statistical analysis was performed using SPSS statistics version 22 (IBM SPSS Statistics, Armonk, NY, USA).

### Ethical Approval

This study was approved by the Institutional Review Boards at Hiroshima University Hospital (IRB 326). All patients provided written informed consent.

## RESULTS

### Detection Limit of ddPCR Assays

The mutant AFs of NCI-H1975 and PC-9 cell lines DNA analyzed by ddPCR were 65% and 87%, respectively. The result of a series of dilutions of the cell lines DNA showed good correlation with the result of fractional abundance obtained by ddPCR. Based on the series of cell line dilution analysis, EGFR mutation assays enabled detection of mutations at a limit of AFs of 0.024% for the L858R point mutation assay and 0.003% for the E746-A750del assay (Figure 2).

### Evaluation of AFs of cfDNA and tDNA

In tDNA samples, EGFR L858R point mutation was detected in all the 13 samples and EGFR E746-A750del was detected in 3 samples. In cfDNA samples, EGFR L858R point mutation was detected in 8 out of 13 (61.5%) cases and EGFR E746-A750del was detected in 3 out of 3 (100%) samples. Among the cases in which mutations were detectable, AFs of EGFR L858R point mutation in cfDNA samples ranged from 0.04% to 5.12% (mean 1.3%), and in EGFR E746-A750del they ranged from 0.07% to 18.6% (mean 6.3%). Compared to cfDNA, AFs in tDNA were high, ranging from 5.7% to 71% (mean 32.7%) for EGFR L858R and from 7.5% to 34.7% (mean 19.4%) for EGFR E746-A750del. However, EGFR L858R point mutation was undetectable in cfDNA samples in three L858R-mutated cases.

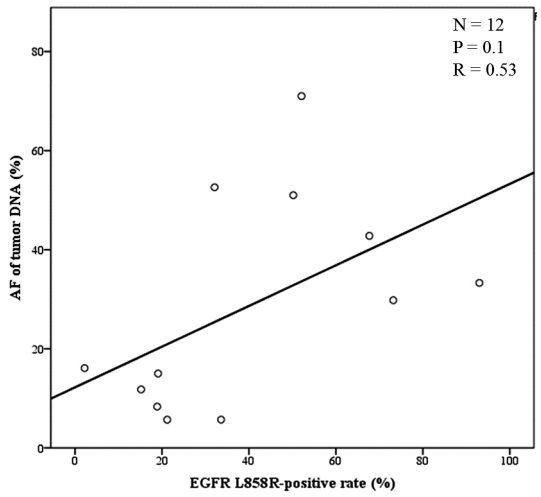


**Figure 3** Correlation of EGFR L858R-positive cell rate and AF of mutations in tDNA (N = 12, r = 0.53, $p$ = 0.1). AF: allele fraction.

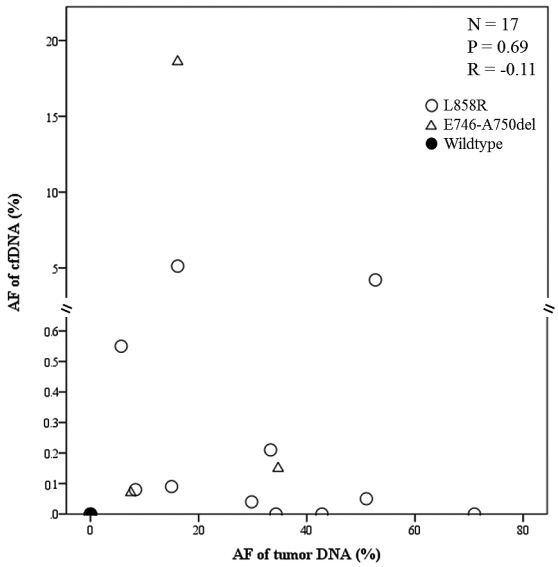


**Figure 4** Correlation of AFs of mutations in tDNA and in cfDNA. There is no significant correlation. (N = 17, r = −0.11, $p$ = 0.69). ○: EGFR L858R point mutated cases, △: EGFR E746-750 deleted cases, ●: wildtype cases, AF: allele fraction, cfDNA: cell-free DNA, tDNA: tumor DNA.

### Correlation of AFs in cfDNA and tDNA and Mutated Cell Rate

The average mutation-positive cells detected in surgically resected samples and from TBLB samples were 52.3% and 35.9%, respectively. The AFs of tDNA by ddPCR was relatively correlated with the percentage of mutated cells in the tumor section (r = 0.53, $p$ = 0.1, N = 12) (Figure 3). However, no correlation was observed between AFs of cfDNA and those of tDNA (r = −0.11, $p$ = 0.69, N = 17) (Figure 4). Similarly, correlation was not found between AFs of cfDNA and mutation-positive cell rate (r = −0.56, $p$ = 0.09, N = 10).

### Association of EGFR Mutations with Clinicopathological Status and Clinical Features

Comparison between the EGFR L858R mutation AF rate in cfDNA and the EGFR L858R-positive cell rate of tumor biopsy from the primary site, obtained by TBLB procedure, was performed. Surprisingly, the sample with the lowest number of EGFR L858R-positive cell rate has the highest AF of cfDNA (5.12%) (Case 1). In contrast, the mutated allele was not detectable in cfDNA sample derived from the patient with the highest number of EGFR L858R-positive cell rate (Case 9) (Figure 5). Therefore, the AF rates were not considered to reflect the rates of mutated tumor cells in primary tumors.

In the patients with and without detectable mutation in cfDNA, association analyses with the clinicopathological status and clinical features, including outcome, tumor size, lymph node metastasis, distant metastasis, and stage of cancer, were conducted. However, there was no statistically significant difference observed between the tumor characteristics and the clinical parameters. Subsequently, AFs of cfDNA were divided into two groups, ≤ 0.1% and > 0.1%, and association analyses were performed. There was no difference in clinical and tumor characteristics between groups with AFs of cfDNA ≤ 0.1% and > 0.1%. Interestingly, all patients with AFs of cfDNA > 0.1% had lymph node metastasis and distant metastasis (Table 2).

## DISCUSSION

cfDNA derived from tumor cells is diluted with an abundant amount of cfDNA derived from normal cells. Moreover, cfDNA is usually degraded to several hundreds of base pairs. Thus, the fraction of a particular mutant allele is likely to be very low in a of cfDNA[13]. In this study, we showed the ability of ddPCR to detect low levels AFs in cfDNA, as low as 0.04%. Furthermore, our result demonstrated a high detection rate for EGFR mutations in cfDNA by ddPCR. Compared to an alternative cfDNA detection platform, we present ddPCR as a highly sensitive detection method[3]. A previous study that used ddPCR to evaluate EGFR mutation in cfDNA showed a high detection rate using this method (95%)[11]. Based on these results, we suggest that cfDNA analysis offers a complementary detection assay and is beneficial for patients from whom only a limited amount of tumor biopsy sample can be obtained.

In this study, we evaluated mutated cell fractions in tumors using an EGFR L858R mutant-specific antibody. The EGFR L858R-mutated cell rate relatively correlated with AF of tDNA; however, there was no correlation between the AFs in cfDNA and tDNA. Since cfDNA is usually considered to be derived from tumor cells as well as normal cells, the mutant AF is consequently lower in cfDNA and tDNA. Interestingly, our data showed that the highest AF detected in cfDNA had the smallest tumor cellularity based on immunohistochemical studies. Previous studies comparing the detection of specific genes also showed discordance between tDNA and cfDNA[2,4]. Instead of mutated cell numbers, we suggest that AFs of cfDNA may be associated with the characteristics of the tumor, such as grades of malignancy, or vascular invasion, or lymph node metastasis and distant metastasis. In fact, all the cases in which mutated AFs of cfDNA were

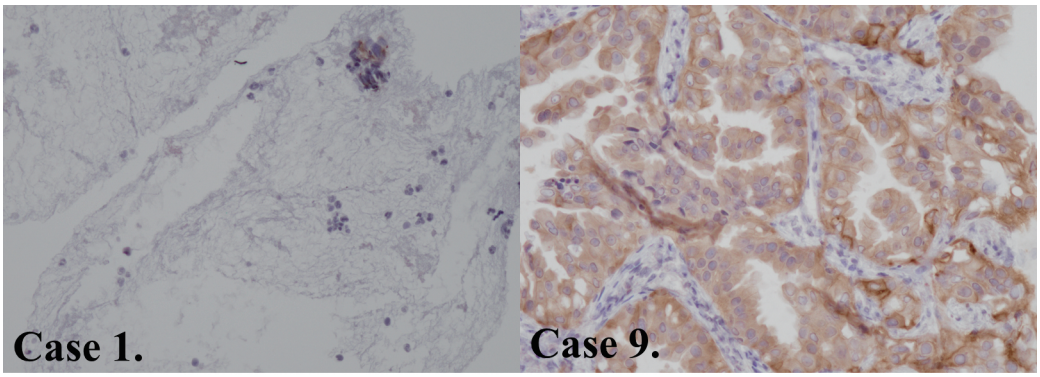


**Figure 5** Immunohistochemical staining of EGFR L858R-positive cells. A. Case 1 with AF of cfDNA was 5.12% and EGFR L858R-positive cell rate was 3.4%. the patient's characteristics were as follows: tumor size of T1b, lymph node metastasis of N2, distant metastasis of M1, and stage IV. B. Case 9 with EGFR L858R-positive cell rate of 67.7% but undetectable in cfDNA. The patient's characteristics were as follows: tumor size of T2b, lymph node metastasis of N2, distant metastasis of M1, and stage IV. AF: allele fraction, cfDNA: cell-free DNA.

**Table 2** Association of *EGFR* Mutations with Clinicopathological Features and Outcome.

| | | Tumor Size | | Lymph Node Metastasis | | Metastasis | | Stage | | Outcome | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | T1/T2 | T3/T4 | Yes | No | Yes | No | IA/IB/IIA/IIB | IIIA/IIIB/ IV | Dead | Alive |
| Detection of cfDNA (N = 14) | Positive | 9 | 2 | 9 | 2 | 10 | 1 | 1 | 10 | 4 | 7 |
| | Negative | 2 | 1 | 1 | 2 | 2 | 1 | 0 | 3 | 0 | 3 |
| AF of cfDNA (N = 14) | ≤ 0.1% | 6 | 2 | 5 | 3 | 6 | 2 | 1 | 7 | 2 | 6 |
| | > 0.1% | 5 | 1 | 6 | 0 | 6 | 0 | 0 | 6 | 2 | 4 |

CfDNA: cell-free DNA, AF: allele fraction.

> 0.1% were found to be metastatic cases.

The presence of mutations of cfDNA in the blood has been correlated with overall survival, progression-free survival, response to therapy, disease progression, and poorer prognosis in previous studies[6,15]. In addition, levels of cfDNA mutations have been correlated with tumor volumes[9] and were demonstrated to be remarkably higher in patients with detectable cfDNA mutations[10]. On the contrary, another study did not find a correlation between mutations found in cfDNA with tumor sizes[7]. In the present study, a comparison of detectability of cfDNA mutations and clinicopathological status and clinical features, including tumor size and patient outcome, revealed that there were no differences between patients with and without detectably mutated cfDNA. Similarly, clinical and tumor characteristics did not differ between groups of patients with high or low mutant AFs of cfDNA. However, we noted that a high number of patients with mutated cfDNA had lymph node metastasis, distant metastasis, and advanced stage of cancer. A similar pattern was observed in the group of patients with AF of cfDNA > 0.1%. In this group, all patients had lymph node metastasis and distant metastasis. These findings strengthen our suggestion that AF of cfDNA may be closely associated with lymph node and distant metastasis.

In conclusion, ddPCR is a promising technique with high detection rate for the molecular analysis of EGFR mutations in cfDNA of lung adenocarcinoma patients. Moreover, patients having limited tumor cell numbers in their biopsy samples could benefit from cfDNA mutation analysis.

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### Author contributions

HKPF and EH designed the study. YH, NH, and NK provided the clinical materials. TK and KT performed cell culture and provided cell lines. HKPF and YU isolated DNA. HKPF, SH, and YU performed ddPCR. HKPF, EY, and YU performed immunohistochemistry. HKPF and YU analyzed the immunohistochemistry

results. HKPF and MK analyzed statistical data. HKPF designed, wrote, and edited the manuscript. EH reviewed and approved the manuscript.

**Conflicts of Interest Statement**

There are no conflicts of interest to declare.

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