Abstract. The detection of certain oncogenic driver mutations, including those of epidermal growth factor receptor (EGFR), is essential for determining treatment strategies for advanced non-small cell lung cancer (NSCLC). The current study assessed the feasibility of testing exhaled breath condensate (EBC) for EGFR mutations by droplet digital PCR (ddPCR). Samples were collected from 12 patients with NSCLC harboring EGFR mutations that were admitted to Okayama University Hospital between June 1, 2014 and December 31, 2017. A total of 21 EBC samples were collected using the R Tube™ method and EGFR mutations (L858R, exon 19 deletions or T790M) were assessed through ddPCR analysis (EBC-ddPCR). A total of 3 healthy volunteer samples were also tested to determine a threshold value for each mutation. Various patient characteristics were determined, including sex (3 males and 9 females), age (range 54-81 years; median, 66 years), smoking history (10 had never smoked; 2 were former smokers), histology (12 patients exhibited adenocarcinoma), clinical stage (9 patients were stage IV; 3 exhibited post-operative recurrence) and EGFR mutation type (4 had L858R; 8 had exon 19 deletions; 8 had T790M). EBC-ddPCR demonstrated positive droplets in 8 of the 12 patients. The sensitivity and specificity of each mutation was as follows: 27.3 and 80.0% for EGFR L858R, 30.0 and 90.9% for EGFR Ex19del, and 22.2 and 100% for EGFR T790M. EBC-ddPCR analysis of EGFR mutations exhibited modest sensitivity and acceptable specificity. EBC-ddPCR is a minimally invasive and replicable procedure and may be a complementary method for EGFR testing in patients where blood or tissue sampling proves difficult.

Introduction

The detection of oncogenic driver mutations, such as epidermal growth factor receptor (EGFR) mutations, is essential for determining treatment strategies for advanced non-small cell lung cancer (NSCLC). The EGFR-tyrosine kinase inhibitor (TKI) is among the most successful treatments for NSCLC with EGFR mutations, as it extends the median overall survival by over 30 months (1,2). A third-generation EGFR-TKI, osimertinib, has been clinically approved for lung cancers harboring the acquired resistance mutation EGFR T790M (3). However, the invasive tissue biopsy required for EGFR testing is often challenging. Indeed, data show that re-biopsy was not performed in 20-50% of patients treated with EGFR-TKIs, raising questions on the viability of this method (4-6).

Liquid biopsies that assess circulating tumor DNA from blood samples have been developed, providing an alternative for biomarker identification (7,8). Other samples, such as...
methods in Japan by practically examining surgical tissues, biopsy specimens, or cytology samples. Approximately 1-2 ml of EBC was collected using RTube™ (Respiratory Research), according to the manufacturer's protocol. After collecting the EBC, 1-ml aliquots were dispensed and stored at -80°C. This study was approved by the Ethics Committee of Okayama University (Authorization number: 2221).

The lung cancer cell line H3255 harboring EGFR L858R was kindly provided by Dr William Pao (Vanderbilt University, Nashville, TN, USA) (18). The gefitinib-resistant lung cancer cell lines RPC-9 harboring EGFR Ex19del and T790M were previously established in our laboratory (19).

**Patients and methods**

**Clinical samples and lung cancer cell lines.** Patients with lung cancer harboring the EGFR mutations L858R, Ex19del, or T790M were enrolled in this study between June 1, 2014 and December 31, 2017 after obtaining written informed consent. All patients were diagnosed with NSCLC using surgical tissue samples, biopsy specimens, or cytology samples. The diagnosis was based on the General Rules for the Clinical and Pathological Classification of Lung Cancer of the Japan Lung Cancer Society (8th edition) and TNM staging system of the International Association for the Study of Lung Cancer (8th edition) (17). Written informed consent was also obtained from three healthy volunteers. The baseline EGFR mutations of lung cancers were confirmed by clinically approved

| Table I. Patient characteristics. |
|----------------------------------|
| Patient number | Sex | Age (years) | Smoking | Stage | Baseline EGFR mts | Biopsy site |
|----------------|-----|-------------|---------|-------|------------------|-------------|
| 1              | F   | 60          | Never   | rec   | L858R            | -           |
| 2              | M   | 68          | Former  | IVB   | Ex19del          | RL lobe     |
| 3              | F   | 81          | Never   | IVB   | Ex19del          | Pleural fluid |
| 4              | F   | 74          | Never   | IVB   | Ex19del          | RU lobe     |
| 5              | F   | 66          | Never   | rec   | Ex19del          | RU lobe     |
| 6              | F   | 58          | Never   | IVB   | Ex19del          | Pleural fluid |
| 7              | M   | 76          | Never   | IVA   | L858R            | Pleura      |
| 8              | F   | 67          | Never   | rec   | L858R            | Middle lobe |
| 9              | F   | 70          | Former  | IVB   | L858R            | Pleural fluid |
| 10             | M   | 56          | Never   | IVB   | Ex19del          | RU lobe     |
| 11             | F   | 56          | Never   | IVB   | Ex19del          | LL lobe     |
| 12             | F   | 54          | Never   | IVB   | Ex19del          | RL lobe     |

*Surgical tissue was used for EGFR testing.*  
*Clinical stage was determined based on 8th edition of the International Lung Cancer Staging System. EGFR, epidermal growth factor receptor; F, female; M, male; rec, recurrence; Ex19del, EGFR exon 19 deletion; RL, right lower; RU, right upper; LL, left lower.

| Table II. Healthy volunteer characteristics. |
|---------------------------------------------|
| Healthy volunteer | Sex | Age | Smoking |
|-------------------|-----|-----|---------|
| 1                 | M   | 41  | Never   |
| 2                 | M   | 33  | Never   |
| 3                 | M   | 37  | Never   |

M, male.
or elution buffer AE from the QIAamp DNA Mini kit (Qiagen) as negative controls were tested to determine the threshold (Fig. S2A-D). No droplets were detected for **EGFR** L858R or **EGFR** T790M, whereas positive reactions were observed for **EGFR** Ex19del in the negative control samples. Therefore, the threshold for positive results for **EGFR** Ex19del were analyzed with the receiver operating characteristic (ROC) using patient samples (Fig. S3). As a result, the threshold for **EGFR** mutation-positive was defined as follows: **EGFR** L858R (≥0.01 copies/µl), **EGFR** Ex19del (≥0.5 copies/µl), and **EGFR** T790M (≥0.01 copies/µl).

**Statistical analysis.** Statistical analysis was performed using STATA software version 15.1 (StataCorp). ROC analysis was performed to determine the optimal threshold for epidermal growth factor receptor exon 19 deletion. The 95% confidence interval (CI) was calculated by using the Clopper-Pearson exact method for binomial proportions.

**Results**

**Patient characteristics and EBC samples.** Patient and healthy control characteristics are detailed in Tables I and II, respectively. The median patient age was 66 years (range, 54-81), comprising 3 males and 9 females: 10/12 were never-smokers and 9/12 were in stage IV of the disease. Four lung tumors harbored **EGFR** L858R, whereas 8 tumors showed the **EGFR** Ex19del mutation (Table I). In total, 21 samples were collected from the 12 patients. Of these 21 samples, 11 were from patients with lung cancers harboring **EGFR** L858R and 10 were from patients with lung cancers harboring **EGFR** Ex19del. The timing of EBC collection and treatment history are shown in Fig. 1. Nine EBC samples were collected from two patients at the time of initiation of **EGFR**-TKI (Table III). In contrast, 12 EBC samples were collected from ten patients at the time of the second biopsy (Table IV). There were no adverse events due to EBC sampling.

**EBC-ddPCR for **EGFR** mutations in patients.** The 21 EBC samples from 12 patients were analyzed by ddPCR using **EGFR** L858R, Ex19del, or T790M primer sets. In the four patients with lung cancer harboring **EGFR** L858R, 3 of 11 EBC samples (sample nos. 1-1, 8-4, and 9-4) were positive for **EGFR** L858R (Fig. 2A and Tables II and IV). In the 8 patients with lung cancer harboring **EGFR** Ex19del, 3/10 EBC samples (sample nos. 2-1, 4-1, and 11-1) were positive for **EGFR** Ex19del (Fig. 2B and Table IV). In the 8 patients with lung cancer harboring **EGFR** T790M, 2/9 EBC samples (sample nos. 5-1 and 11-1) were positive for **EGFR** T790M (Fig. 2C and Table IV). No association was detected between the detection of mutations and T-factor/tumor localization in the lung or quality/quantity of DNA in the EBC samples (Tables III and IV).

Consequently, the sensitivity of the EBC test for **EGFR** mutations was as follows: 27.3% (95% CI, 6.0-61.0%) for **EGFR** L858R, 30.0% (95% CI, 6.7-65.2%) for **EGFR** Ex19del, and 22.2% (95% CI, 2.8-60.0%) for **EGFR** T790M. The specificity...
Figure 2. Detection of EGFR mutations by EBC-ddPCR. (A) ddPCR was performed using EGFR L858R primer sets. EGFR L858R was amplified in EBC samples 1-1, 8-4 and 9-4. (B) ddPCR was performed using EGFR exon 19 deletion primer sets. EGFR exon 19 deletions were amplified in EBC samples 2-1, 4-1 and 11-1. (C) ddPCR was performed using EGFR T790M primer sets. EGFR T790M was amplified in EBC samples 5-1 and 11-1. The black bars indicate the positive threshold, which was 0 copies/µl for EGFR L858R and EGFR T790M, and 0.5 copies/µl for EGFR exon 19 deletion. EGFR, epidermal growth factor receptor; EBC, exhaled breath condensate; ddPCR, droplet digital PCR; Ex19del, EGFR exon 19 deletions.
was as follows: 80.0% (95% CI, 44.4‑97.7%) for EGFR L858R, 90.9% (95% CI, 58.7‑99.8%) for EGFR Ex19del, and 100% (95% CI, 73.5‑100%) for EGFR T790M (Table V).

Clinical course of highlighted cases with lung adenocarcinoma, whose EGFR mutations were detected by EBC-ddPCR

Patient number 8. A non‑smoking 67‑year‑old woman was diagnosed with synchronous double primary lung cancer composed of adenocarcinoma harboring EGFR L858R in the right lower lobe of the lung, and adenocarcinoma harboring EGFR Ex19del in the right upper lobe of the lung. A right lower lobectomy (pathological stage T2bN0M0 cStage IIA) and right segment 3a partial resection (pathological stage T1aN0M0 cStage IA1) were performed, with four subsequent courses of adjuvant chemotherapy with a combination of cisplatin and vinorelbine. However, at 12 months post-surgery, a new lesion appeared in the middle lobe. Transbronchial biopsy of the lesion revealed an adenocarcinoma harboring EGFR L858R. The patient's Eastern Cooperative Group Performance Status was grade 1, and thus gefitinib was administered at 250 mg daily. EBC was collected 1 day prior starting gefitinib and at 2, 16 and 62 days after gefitinib initiation. Gefitinib was discontinued from days 41-62 because of liver damage, but later re-administered at a reduced dosage of 250 mg every 2 days. EBC-ddPCR detected EGFR L858R only in the fourth sampling (21 days after gefitinib cessation). The maximum therapeutic effect of gefitinib was a partial response (Response Evaluation Criteria in Solid Tumors version 1.1), with a progression‑free survival of 48 months (Fig. 3A and B).

Patient number 11. A non‑smoking 56‑year‑old woman was diagnosed with adenocarcinoma in the left lower lobe of the lung (clinical stage T3N1M1b cStage IVB, multiple brain metastases, bone metastases). The patient's Eastern Cooperative Group Performance Status was grade 1. EGFR Ex19del was detected in the biopsied tissue, after which erlotinib was administered. However, at 12 months after initiating erlotinib, the primary tumor increased. Two subsequent cycles of cytotoxic chemotherapy with cisplatin and pemetrexed were administered, but the tumor regrew following treatment. Re‑biopsy was performed on primary lung tumor and EBC was collected at the same time. In addition to the baseline EGFR Ex19del mutation, EGFR T790M was also detected in the biopsy sample. Similarly, EGFR Ex19del and T790M

| Patient no. | 2nd-biopsy EGFR mutations | EGFR test | Biopsy site | T-factor of lung tumors | Sample no. | DNA (ng) | 260/280 ratio | EBC-ddPCR |
|------------|----------------------------|-----------|-------------|-------------------------|------------|---------|--------------|-----------|
| 1          | L858R + T790M              | Clamp     | LL lobe     | Tx⁺                     | Peri       | 1-1     | 214.5        | L858R     |
| 2          | Ex19del                   | Clamp     | RL lobe     | 4                       | Center     | 2-1     | 268.5        | Ex19del   |
| 3          | Ex19del + T790M            | Clamp     | Pleural fluid | 2a                     | Peri       | 3-1     | 272.0        | (-)       |
| 4          | Ex19del + T790M            | TaqMan    | Blood       | 2a                     | Peri       | 4-1     | 244.5        | Ex19del   |
| 5          | Ex19del + T790M            | Clamp     | CSF         | Txᵇ                     | Peri       | 5-1     | 277.0        | T790M     |
| 6          | Ex19del + T790M            | Clamp     | Pleural fluid | 1b                     | Peri       | 6-1     | 280.5        | (-)       |
| 7          | L858R                     | Clamp     | Pleural fluid | 2a                     | Peri       | 7-1     | 420.5        | (-)       |
| 10         | Ex19del + T790M            | Clamp     | Pleural fluid | 1b                     | Peri       | 10-1    | 123.5        | (-)       |
| 11         | Ex19del + T790M            | Clamp     | LL lobe     | 3                       | Peri       | 11-1    | 152.0        | (-)       |
| 12         | Ex19del + T790M            | Clamp     | RU lobe     | 2a                     | Peri       | 12-1    | 323.0        | (-)       |

Table IV . EBC-ddPCR.

| Parameter               | L858R       | Ex19del     | T790M       |
|-------------------------|-------------|-------------|-------------|
| Sensitivity (95% CI)    | 27.3 (6.0-61.0) | 30.0 (6.7-65.2) | 22.2 (2.8-60.0) |
| Specificity (95% CI)    | 80.0 (44.4-97.5) | 90.9 (58.7-99.8) | 100 (73.5-100) |
| Positive predictive value (95% CI) | 60.0 (14.7-94.7) | 75.0 (19.4-99.4) | 100 (15.8-100) |
| Negative predictive value (95% CI) | 50.0 (24.7-75.3) | 58.8 (32.9-81.6) | 63.2 (38.4-83.7) |

Table V . Sensitivity, specificity and predictive values of exhaled breath concentrate-droplet digital PCR.

95% CIs were calculated using the Clopper-Pearson exact method for binomial proportions. Ex19del, EGFR exon 19 deletion; CI, confidence interval.

was as follows: 80.0% (95% CI, 44.4-97.7%) for EGFR L858R, 90.9% (95% CI, 58.7-99.8%) for EGFR Ex19del, and 100% (95% CI, 73.5-100%) for EGFR T790M (Table V).
were detected by the EBC-ddPCR method. Subsequently osimertinib was administered and continued over 6 months, which caused a partial response (Response Evaluation Criteria in Solid Tumors version 1.1) (Fig. 3C and D).

**Discussion**

This study demonstrated that using EBC-ddPCR to detect *EGFR* mutations is feasible and shows a modest sensitivity (20-30%) and acceptable sensitivity (80-100%) in patients with lung cancers harboring *EGFR* mutations.

Up to 60% of lung adenocarcinomas treated with first- or second-generation EGFR-TKIs develop resistant *EGFR* T790M mutations (20,21). However, third-generation EGFR-TKIs were only administered in 23.7% of patients (22). A negative result for *EGFR* T790M in re-biopsied samples should not prevent physicians from performing a repeat biopsy; however, it is not always possible to carry out repeat biopsies (21,23). The sensitivity of EBC testing for *EGFR* mutations was modest compared to that of blood tests (8); however, EBC testing is much more easily repeated because of its minimal invasiveness. In this study, we performed three or four repeated EBC samplings without any adverse effects in both case 8 and 9. Although we did not assess the concordance between tissue biopsy and EBC testing on a larger scale, gefitinib and osimertinib inhibited the lung tumor in cases 8 and 11, respectively, with both the tissue biopsy and EBC testing detecting *EGFR* L858R or *EGFR* Ex19del and T790M. These cases suggest the potential of EBC testing as a complementary option for patients in whom tissue biopsy is difficult or for those who refuse repeated blood sampling.

Although ddPCR is thought to detect 0.005-0.1% of target DNA (24), this study revealed a modest sensitivity for *EGFR* mutations in EBC samples (20-30%). Smyth et al reported that *EGFR* T790M was detected in 9/10 EBC samples by UltraSEEK™ technology (12). Possible explanations for the discrepancy between the previous report and our study are differences in sample quality and sensitivity of the detection methods.

This study had several limitations. The sample size was small (n=21 in 12 patients) and the number of EBC samples from treatment naïve patients and samples prior to initiating EGFR-TKI were from only 1 case; therefore, patient bias should be considered. Furthermore, concordance among EBC testing, blood testing, and tissue biopsy was not assessed in detail. Therefore, these data should be considered as exploratory.

EBC-ddPCR for *EGFR* mutations by ddPCR was feasible and showed moderate sensitivity and acceptable specificity. EBC sampling is minimally invasive and replicable; therefore, EBC tests could be a complementary option for patients in whom tissue biopsy is difficult or for those who refuse repeated blood sampling. Further studies are needed to explore the potential of the EBC test.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Data interpretation and presentation were the sole responsibility of the authors. KaN and KO had full access to all data and assume responsibility for data integrity and the accuracy of data analysis. KaN and KO contributed to the study design, data collection, analyses, and manuscript writing. TT, KiN, TM, SS, HK, HW, NO, GM, HH, YK, TN, TK, HY, Stom, KH, MT, Stoy, YM, KK collected the data and prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institute Research Ethics Committee of the Okayama University Hospital and written informed consent was obtained from all patients.

Patient consent for publication

Written informed consent regarding publication was obtained from all patients.

Ethics Committee of the Okayama University Hospital and Okayama health foundation (grant no. 33K-2017-03). Chugoku Occupational Health Association (grant no. CRE 17-2) and Chugai Pharmaceutical outside the submitted work.

Competing interests

Written informed consent regarding publication was obtained from all patients.

Authors' contributions

Dr Kadoaki Ohashi reports research funding from Boehringer Ingelheim, Novartis, AstraZeneca, Eli Lilly, MSD, and Daichi-Sankyo outside the submitted work.

Dr Kadoaki Ohashi reports personal fees from AstraZeneca, MSD, and Chugai Pharmaceutical outside the submitted work.

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