Detection of epidermal growth factor receptor mutations in exhaled breath condensate using droplet digital polymerase chain reaction

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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 RTube™ 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.

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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

Table I. Patient characteristics.

Patient number	Sex	Age (years)	Smoking	Stage[b]	Baseline *EGFR* mts	Biopsy site
1	F	60	Never	rec	L858R	–[a]
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

[a]Surgical tissue was used for EGFR testing. [b]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.

exhaled breath condensate (EBC) and urine, have also been tested as alternatives to liquid biopsy (9-12).

We previously reported the detection of *EGFR* exon 19 deletion (Ex19del) mutations in EBC by conventional polymerase chain reaction (PCR) (13). Recently, a highly sensitive droplet digital PCR (ddPCR) was developed for liquid biopsy (14-16). In this study, we investigated whether EBC testing using ddPCR (EBC-ddPCR) for *EGFR* mutations is viable in patients with NSCLC.

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 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).

Droplet digital PCR assay for EGFR mutation detection. DNA was extracted using the QIAamp DNA Mini kit (Qiagen GmbH) according to the manufacturer's protocol. DNA qualification was performed with a NanoDrop spectrometer (Thermo Fisher Scientific, Inc.). The following primer and probe kits were purchased from Bio-Rad): ddPCR Mutation assay: EGFR p.L858R c.2573T>G (#10049550); ddPCR EGFR Exon 19 Deletions Screening kit (#12002392) and ddPCR Mutation assay: EGFR p.T790M, Human (#10049550).

ddPCR was performed at Biobank (Okayama University Hospital, Okayama, Japan) using the QX200 Droplet Digital PCR system (Bio-Rad) according to the manufacturer's protocol. The following conditions were used for ddPCR: i) An initial denaturation step at 95˚C for 10 min followed by: ii) 45 cycles at 94˚C for 30 sec; and iii) 45 cycles at ˚C for 1 min, with a 4) final enzyme deactivation step at 98˚C for 10 min. The ramp rate for all steps was 2˚C/sec. PCR products were then subjected to analysis with a QX-200 Droplet reader and QuantaSoft analysis software (Bio-Rad), with a EGFR Specific Multiplex DNA Reference Standard (#HD802, Horizon Discovery) used as a positive control. The accuracy of the ddPCR was confirmed using serially diluted DNA of lung cancer cell lines (Fig. S1A-F). The patient samples and control samples including EBC samples from three healthy-volunteers
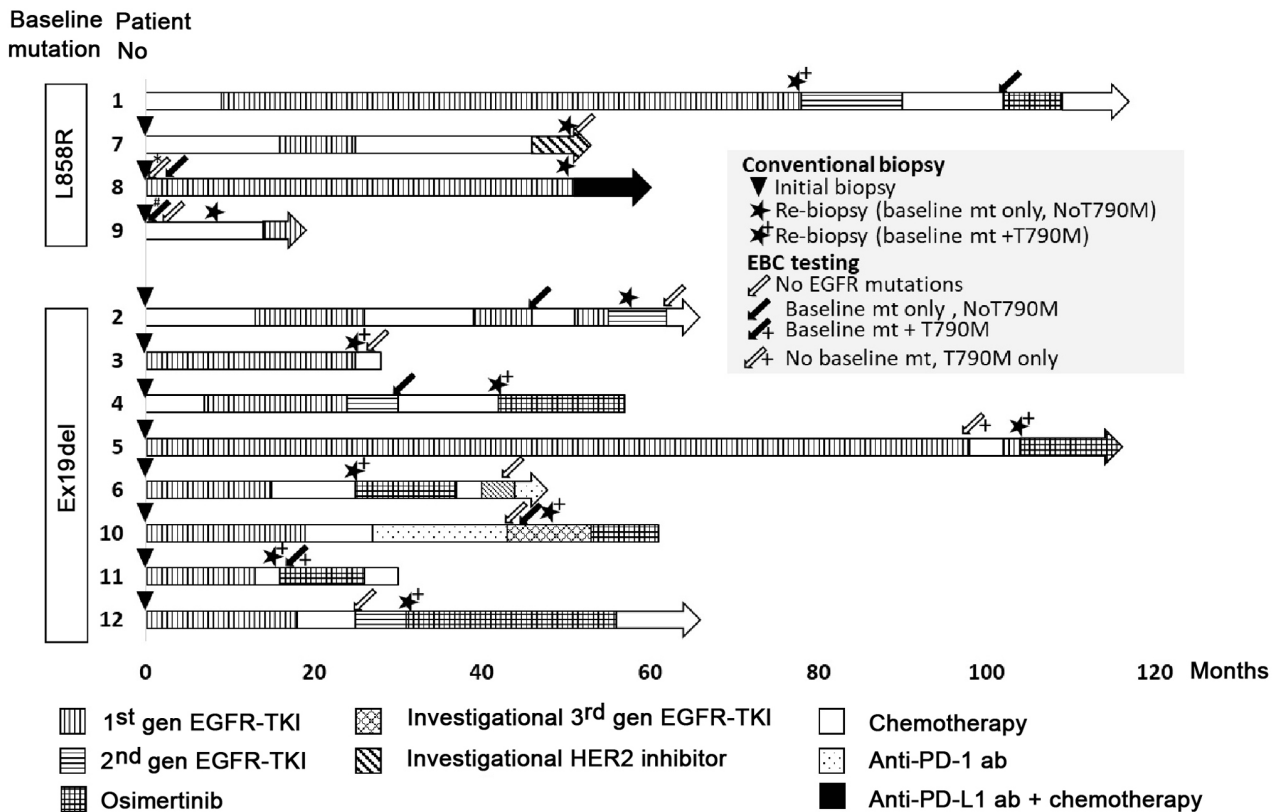
Figure 1. Flow diagram of the clinical course of 12 patients with lung cancer harboring *EGFR* mutations and the time of biopsy or exhaled breath condensate sampling. *Three consecutive samples were collected. #Four consecutive samples were collected. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; Ex19del, *EGFR* exon 19 deletions; Anti-PD-1 ab, anti-programmed cell death-1 antibody; Anti-PD-L1 ab, anti-programmed cell death ligand-1 antibody.

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

Table III. EBC-ddPCR.

Patient no.	Baseline *EGFR* mts	Biopsy site	T-factor	Localization of lung tumors	Sample no.	DNA (ng)	260/280 ratio	EBC-ddPCR
8	L858R	RL lobe	2b	Peri	8-1	279.5	1.46	(-)
					8-2	133.5	1.37	(-)
					8-3	503.0	1.73	(-)
					8-4	250.0	1.86	L858R
9	L858R	Pleural fluid	4	Center	9-1	138.0	1.66	(-)
					9-2	257.0	1.53	(-)
					9-3	253.5	2.06	(-)
					9-4	267	1.99	L858R
					9-5	181.5	1.37	(-)

EBC, exhaled breath concentrate; ddPCR, droplet digital PCR; EGFR, epidermal growth factor receptor; mts, mutations; T-factor, T-factor of TNM staging system version 8; RL, right lower.
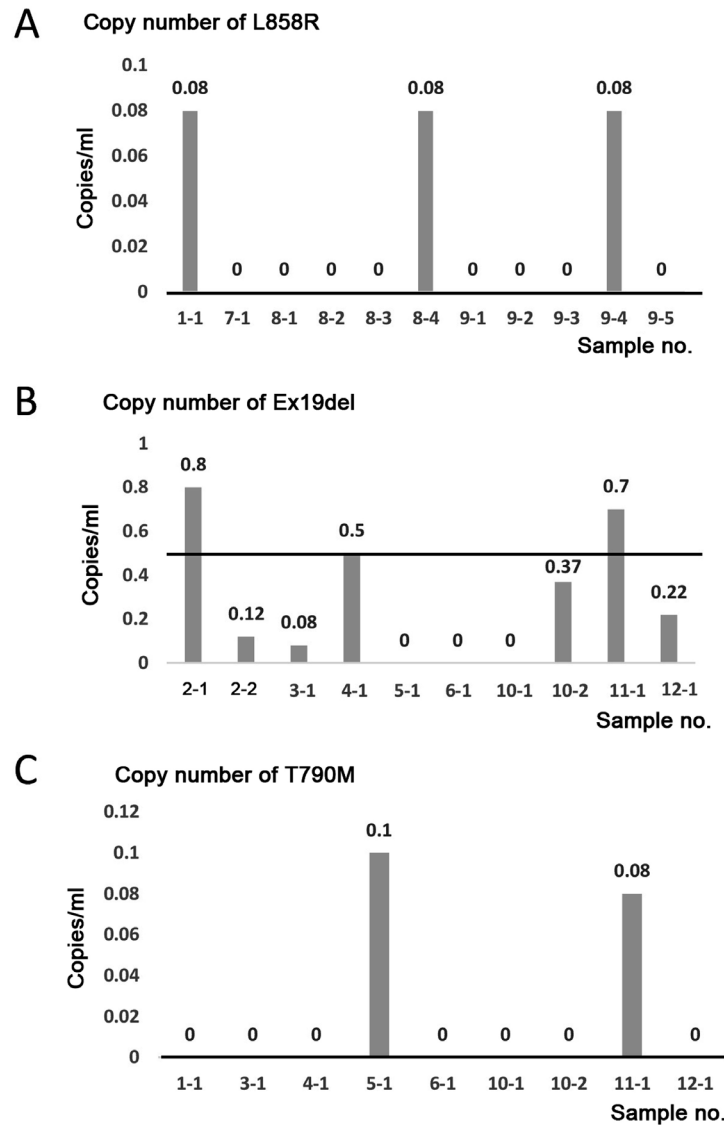

Figure 2. Detection of *EGFR* mutations by EBC-ddPCRA. (A) ddPCR was performed using EGFR L858R primer sets. *EGFR* L858R was amplified in EBC samples 1-1, 8-4 and 9-4. (B) ddPCR was performed using *EGFR* exon 19 deletion primer sets. *EGFR* exon 19 deletions were amplified in EBC samples 2-1, 4-1 and 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.

Table IV. EBC-ddPCR.

Patient no.	2nd-biopsy *EGFR* mutations	EGFR test	Biopsy site	T-factor	Localization of lung tumors	Sample no.	DNA (ng)	260/280 ratio	EBC-ddPCR
1	L858R + T790M	Clamp	LL lobe	Tx[a]	Peri	1-1	214.5	1.37	L858R
2	Ex19del	Clamp	RL lobe	4	Center	2-1	268.5	2.33	Ex19del
						2-2	234.0	1.88	(-)
3	Ex19del + T790M	Clamp	Pleural fluid	2a	Peri	3-1	272.0	192.0	(-)
4	Ex19del + T790M	TaqMan	Blood	2a	Peri	4-1	244.5	2.17	Ex19del
5	Ex19del + T790M	Clamp	CSF	Tx[b]	Peri	5-1	277.0	1.64	T790M
6	Ex19del + T790M	Clamp	Pleural fluid	1b	Peri	6-1	280.5	2.07	(-)
7	L858R	Clamp	Pleural fluid	2a	Peri	7-1	420.5	1.55	(-)
10	Ex19del + T790M	Clamp	Pleural fluid	1b	Peri	10-1	123.5	10.48	(-)
						10-2	163.5	4.41	(-)
11	Ex19del + T790M	Clamp	LL lobe	3	Peri	11-1	152.0	4.01	Ex19del + T790M
12	Ex19del + T790M	Clamp	RU lobe	2a	Peri	12-1	323.0	1.66	(-)

[a]Post-surgery recurrence and multiple lung metastasis; [b]post-surgery recurrence and mediastinum lymphoid metastasis, with no lung nodule observed. EBC, exhaled breath concentrate; ddPCR, droplet digital PCR; EGFR, epidermal growth factor receptor; T-factor, T-factor of TNM staging system version 8; Clamp, the peptide nucleic acid-locked nucleic acid PCR clamp; TaqMan, TaqMan assay; Ex19del, *EGFR* exon 19 deletion; LL, left lower; peri, peripheral; RL, right lower; RU, right upper; CSF, cerebrospinal fluid.

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

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)

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).

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
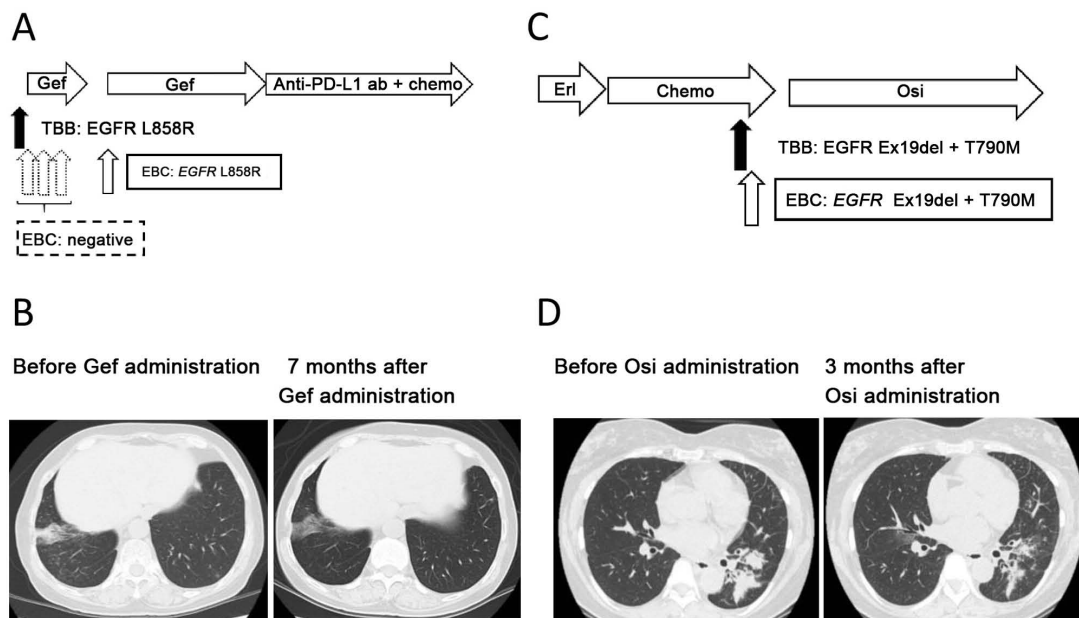
Figure 3. Clinical course of highlighted cases. (A) Clinical course of patient 8. (B) Chest CT image obtained 7 months after the initiation of Gef treatment. A lesion reduction in the right lower lobe can be observed. (C) Clinical course of patient 11. (D) Chest CT image obtained 3 months after the initiation of Osi treatment. Lesion reduction in the left lower lobe is exhibited. Erl, Erlotinib; Chemo, cytotoxic chemotherapy; TBB, transbronchial biopsy; EBC, exhaled breath condensate; Gef, gefitinib; Osi, osimertinib; Anti-PD-L1 ab, anti-programmed cell death ligand 1 antibody; Ex19del, *EGFR* exon 19 deletions. EGFR, epidermal growth factor receptor.

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.

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.

Competing interests

Dr Kadoaki Ohashi reports research funding from Boehringer Ingelheim, Novartis, AstraZeneca, Eli Lilly, MSD, and Daiichi-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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