Rapid point-of-care testing for epidermal growth factor receptor gene mutations in patients with lung cancer using cell-free DNA from cytology specimen supernatants

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Received November 25, 2017; Accepted February 16, 2018

DOI: 10.3892/ijo.2018.4334

Abstract. Epidermal growth factor receptor (*EGFR*) mutations are associated with responses to EGFR tyrosine kinase inhibitors (EGFR-TKIs) in non-small-cell lung cancer (NSCLC). Our previous study revealed a rapid point-of-care system for detecting *EGFR* mutations. This system analyzes cell pellets from cytology specimens using droplet-polymerase chain reaction (d-PCR), and has a reaction time of 10 min. The present study aimed to validate the performance of the *EGFR* d-PCR assay using cell-free DNA (cfDNA) from supernatants obtained from cytology specimens. Assay results from cfDNA supernatant analyses were compared with those from cell pellets for 90 patients who were clinically diagnosed with, or suspected of having, lung cancer (80 bronchial lavage fluid samples, nine pleural effusion samples and one spinal fluid sample). *EGFR* mutations were identified in 12 and 15 cases using cfDNA supernatants and cell pellets, respectively. The concordance rates between cfDNA-supernatant and cell-pellet assay results were 96.7% [kappa coefficient (K)=0.87], 98.9% (K=0.94), 98.9% (K=0.79) and 98.9% (K=0.79) for total *EGFR* mutations, L858R, E746_A750del and T790M, respectively. All 15 patients with *EGFR* mutation-positive results, as determined by *EGFR* d-PCR assay using cfDNA supernatants or cell pellets, also displayed positive results by conventional *EGFR* assays using tumor tissue or cytology specimens. Notably, *EGFR* mutations were even detected in five cfDNA supernatants for which the cytological diagnoses of the corresponding cell pellets were 'suspicious for malignancy', 'atypical' or 'negative for malignancy.' In conclusion, this rapid point-of-care system may be considered a promising novel screening method that may enable patients with NSCLC to receive EGFR-TKI therapy more rapidly, whilst also reserving cell pellets for additional morphological and molecular analyses.

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Abbreviations: NSCLC, non-small cell lung cancer; *EGFR*, epidermal growth factor receptor; EGFR-TKIs, EGFR tyrosine kinase inhibitors; *EGFR* d-PCR assay, *EGFR* droplet-polymerase chain reaction; cfDNA, cell-free DNA; BLF, bronchial lavage fluid; PE, pleural effusion; SF, spinal fluid; FFPE, formalin-fixed paraffin-embedded

Key words: lung cancer, *EGFR*, point-of-care testing, droplet-PCR, cytology, cell pellet, supernatant, cell-free DNA, bronchial lavage fluid, pleural effusion

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide; in 2012, 1.8 million new cases of lung cancer were diagnosed and it was responsible for 1.6 million deaths (1). In the past decade, epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), including gefitinib, erlotinib and afatinib, have been widely used in the treatment of patients with non-small cell lung cancer (NSCLC). The *EGFR* gene encodes a pharmacologically targetable tyrosine kinase, and patients with NSCLC harboring *EGFR* mutations, such as deletions in exon 19 and L858R in exon 21, exhibit notable responses to EGFR-TKIs, resulting in improved prognoses compared to those achieved with standard chemotherapies (2-7). In addition, osimertinib, which is a third-generation TKI that specifically targets the T790M secondary *EGFR* mutation, has been demonstrated to exhibit clinical efficacy (8). Since the therapeutic effect of EGFR-TKIs is strongly dependent upon the *EGFR* mutation status of patients (9), reliable mutation detection methods are required to facilitate personalized lung cancer treatments.

At present, in clinical practice, the molecular testing guidelines (10) for the selection of patients with lung cancer for EGFR-TKI administration recommend using validated polymerase chain reaction (PCR)-based assays and specimens containing sufficient cancer cells to provide accurate results. Although tissue specimens, such as transbronchial biopsy or surgically resected specimens should be prioritized, the guidelines also recommend using cytological specimens, as it is often difficult to obtain sufficient tissue specimens for mutation analysis. Numerous studies have reported the sensitivity and reliability of using cytology specimens for EGFR testing (11-13). Furthermore, recent advances in highly sensitive genotyping have allowed the development of liquid biopsies, which examine circulating tumor cells or freely circulating cell-free DNA (cfDNA) isolated from the serum or plasma (14-16). A liquid biopsy is a useful and minimally invasive method, particularly for patients who require rebiopsy to confirm acquired *EGFR* T790M mutation (16). However, the amount of cfDNA in the bloodstream is extremely low; therefore, liquid biopsies require highly sensitive assay platforms, which are often slow to perform and yield limited detection rates (17-19).

Cytology specimens, including bronchial lavage fluid (BLF), are usually obtained directly from the tumor location, and their supernatants are expected to have a higher amount of cfDNA derived from tumor cells than the blood. If cfDNA supernatants from cytology specimens are available for *EGFR* mutation detection, cell pellets can be reserved for additional morphological and molecular analyses. Our previous study revealed a novel rapid point-of-care system for the detection of *EGFR* mutations using droplet-PCR (d-PCR) to assess cell pellets of cytology specimens (*EGFR* d-PCR assay) (20). This *EGFR* d-PCR assay reduced the reaction time to <10 min and exhibited sensitivity as high as that achieved using conventional PCR assays. The purpose of the present study was to validate the performance of the *EGFR* d-PCR assay in assessing cfDNA from supernatants obtained from cytology specimens. Briefly, the results of the assay were compared to those achieved via the *EGFR* d-PCR assay of cytology-specimen cell pellets, as well as those obtained via a cytological diagnosis of the corresponding cell pellets. In addition, conventional EGFR assays were conducted using tissue or cytology specimens in order to confirm the accuracy of the *EGFR* d-PCR assay using cytology specimens.

Materials and methods

Patients. The present study enrolled 90 patients who had been diagnosed with or were radiologically suspected to have lung cancer (including benign disease), and whose cytological specimens were submitted to the Department of Laboratory Medicine at the Shinshu University Hospital (Matsumoto, Japan) between July and November 2016. All patients received medical treatments or follow-up examinations in Shinshu University Hospital following specimen collection. The 90 cytological specimens comprised the following: 80 samples, BLF; nine samples, pleural effusion (PE); and one sample, spinal fluid (SP). BLF specimens were obtained following transbronchial lung biopsies (TBLB), which were performed to either assess an undiagnosed lung mass, or perform T790M screening in patients with diagnosed lung cancer. PE and SP specimens were obtained via fine-needle aspiration from patients with advanced lung cancer. Of the 15 patients that were revealed to be positive for *EGFR* mutations, as determined by the *EGFR* d-PCR assay using cytology specimens, 14 formalin-fixed paraffin-embedded (FFPE) tissue specimens (11 samples, TBLB; three samples, surgical resection) and one FFPE cell block from a cytology specimen (PE) were collected to confirm the mutation status using conventional assays. All patients provided written informed consent for their participation in the present study, which was reviewed and approved by the medical ethics committee of the Shinshu University School of Medicine.

Processing and pathological evaluation of specimens. Processing of the materials is shown in Fig. 1. Briefly, each cytological specimen was immediately centrifuged (400 x g, 5 min, room temperature) upon reception by the department, and was then divided into a cell-pellet and a cfDNA supernatant fraction. The cell pellet was further divided into two portions, one of which was used to prepare the Papanicolaou smear for cytological analysis, whereas the other was used for DNA extraction. Cytological diagnosis was performed by one cytotechnologist, and reviewed by two pathologists. Specimens were classified according to the standardized terminology proposed by the Papanicolaou Society of Cytopathology, as either 'malignant', 'suspicious of malignancy', 'neoplastic, benign neoplasm, low-grade carcinoma', 'atypical', 'negative for malignancy' or 'non-diagnostic' (21). Both fresh cell pellets and cfDNA supernatants were stored at -20˚C.

DNA extraction. DNA was extracted from cell-pellet portions and 1.5 ml cell-free supernatant aliquots using the QIAamp DNA Blood Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. For FFPE tissue and cell block specimens, the QIAamp DNA FFPE Tissue kit (Qiagen, Inc.) was used to extraxt DNA according to the manufacturer's protocol.

For the *EGFR* d-PCR assay, the concentration of extracted DNA from each specimen was quantified via spectrophotometry using a NanoDrop ND100 instrument (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and was adjusted to a concentration of <10 ng/μl with AE Buffer from the QIAamp DNA extraction kit.

EGFR d-PCR assay. The *EGFR* d-PCR assay was performed using a d-PCR machine (Seiko Epson Corporation, Suwa, Japan) as previously described (20). Briefly, the *EGFR* d-PCR assay is designed to detect three major *EGFR* mutations: L858R in exon 21, E746_A750del in exon 19 and T790M in exon 20, in 8 min and 10 sec. The primers and probes used in the present study were as follows: L858R, forward 5'-GCT TGGTGCACCGCGACCTG-3',reverse5'-CGCACCCAGCAG TTTGGCAC-3', probe 5'-6FAM-AGCCAGGAACGTACTG GTGAAAACACCGCA-BHQ-1-3'; E746_A750del, forward 5'-GGCAGCATGTGGCACCATC-3', reverse 5'-GTTGGCTT TCGGAGATGTAT-3', probe 5'-6FAM-TCTCACCTTCTG GGATCCAGAGTCCCT-BHQ-1-3'; T790M, forward 5'-CCC CACGTGTGCCGCCTG-3', reverse 5'-GCCGAAGGGCAT GAGCTGTA-3', and probe 5'-6FAM-TGGGCATCTGCCTCA

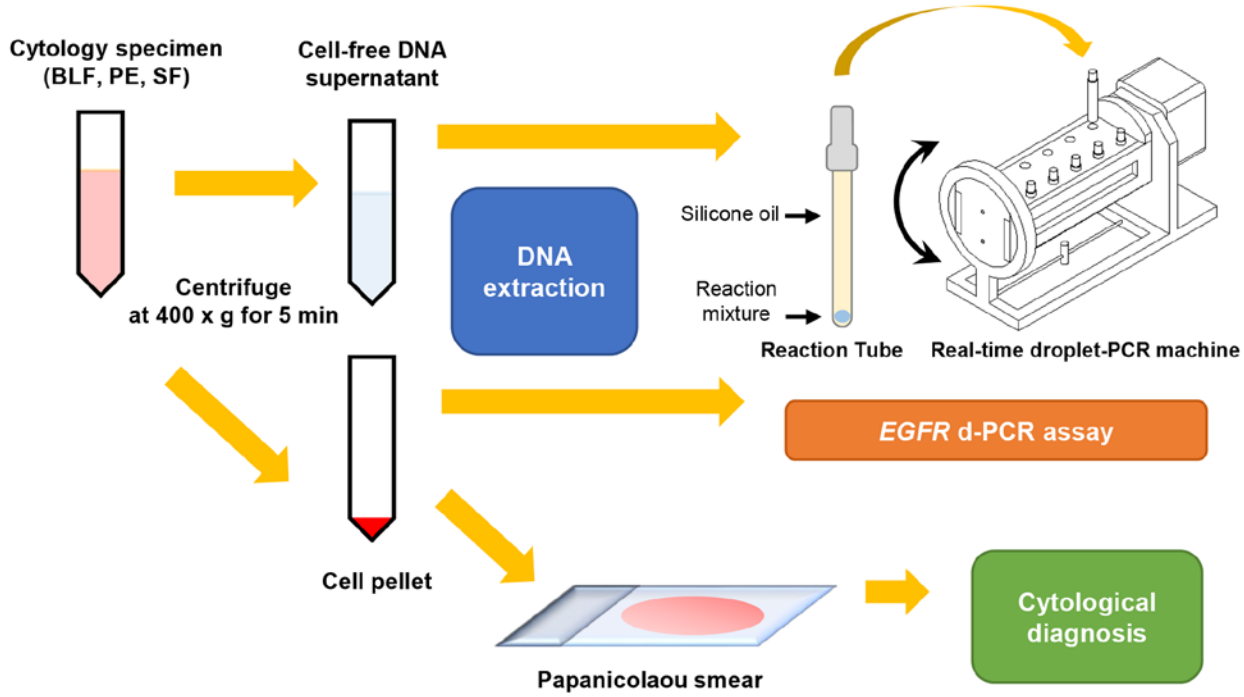

Figure 1. Specimen processing for PCR analysis and cytological diagnosis. Each cytology specimen was centrifuged to separate the cell pellet from the cell-free DNA supernatant. After generating a Papanicolaou smear (for cytological diagnosis) from the first portion of the cell pellet, DNA was extracted from the remaining portion of the cell pellet, and from the 1.5 ml of cell-free DNA supernatant aliquots, and subjected to *EGFR* d-PCR assay. BLF, bronchial lavage fluid; d-PCR, droplet PCR; *EGFR*, epidermal growth factor receptor; PCR, polymerase chain reaction; PE, pleural effusion; SF, spinal fluid.

CCTCCACCGTGCA-BHQ-1-3'. Each reaction mixture contained genomic DNA (<10 ng/μl), Platinum Taq DNA polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA), appropriate primers (800 nmol/l), TaqMan probe (300 nmol/l) and sufficient reaction buffer [comprised of Tris-HCl (pH 9.0), KCl and $MgCl_2$] to reach a total volume of 10 μl. A 1.6-μl aliquot of each reaction mixture was placed in each reaction tube filled with silicone oil, and subjected to the following reaction conditions: 98˚C for 10 sec, followed by 40 cycles at 98˚C for 5 sec, and either 60˚C for 6 sec (L858R) or 55˚C for 6 sec (E746_A750del and T790M). PCR results were determined to be either *EGFR*-mutation positive or negative according to threshold fluorescence level values of 4.7, 4.7 and 6.8 for L858R, E746_A750del and T790M, respectively. The *EGFR* d-PCR assay was previously shown to exhibit high sensitivity, and concordance with the commercial PCR therascreen assay, which uses the Scorpions-amplification-refractory mutation system method, as performed using the therascreen® EGFR RGQ PCR kit (Qiagen, Inc.) (20).

Conventional EGFR mutation PCR assays. Conventional assays were performed using FFPE tissue or cell block specimens using either a Rotor-Gene Q 5plex HRM instrument with the therascreen® EGFR RGQ PCR kit (Qiagen, Inc.) or a Roche Cobas® EGFR mutation test v2 (Roche Molecular Diagnostics, Branchburg, NJ, USA) according to manufacturer's protocols. Both assays are approved as companion diagnostics in the United States, Europe and Japan (20,22). According to the manufacturer's protocols, the therascreen assay detects 29 types of somatic mutation in *EGFR* in 1 h and 45 min, whereas the Cobas v2 assay detects 42 types in 1.5-2 h. The detection limits for L858R, E756_A750del and T790M are 1.26, 1.64 and 7.02% of mutant DNA in the therascreen assay, and 3.96-5.32, 1.39-2.53 and 2.04-3.03% of mutant DNA in the Cobas v2 assay, respectively.

Statistical analysis. Concordance rates and Cohen's kappa coefficients were used to examine the agreement of the assay results achieved via an assessment of the two different specimen types. A kappa coefficient (*K*) value of zero was considered to indicate that there was no agreement beyond that which occurred by chance, whereas a *K* value of 1.00 was considered to indicate perfect agreement. The following *K* value ranges: 0-0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80, and 0.81-1 were considered to represent slight, fair, moderate, substantial and almost perfect agreement between the compared results, respectively (23). $P<0.05$ was considered to indicate a statistically significant difference. All data were statistically analyzed using JMP® 13 software (SAS Institute Inc., Cary, NC, USA).

Results

Characteristics of patients and specimens. Patient characteristics are shown in Table I. The mean age of the 90 patients was 71.1 years (range, 42-86 years), all patients were Japanese, and of East Asian ethnicity, and 55 (61.1%) and 35 (38.9%) of the patients were male and female, respectively. The final diagnoses by comprehensive clinical, radiological and pathological analysis were lung cancer for 74 (82.2%), metastases from other organs for three (3.3%), malignant lymphoma for one (1.1%), cancer of unknown primary origin for two (2.2%), benign disease for three (3.3%), and unknown for seven (7.8%) patients. At the time of diagnosis, 27 (36.5%), five (6.6%),

Table I. Clinical characteristics of the 90 patients.

Characteristic	Values
Age, years	
Mean	71.1
Range	42-86
Male/female, n (%)	55 (61.1)/35 (38.9)
Final diagnosis, n. (%)	
Lung cancer	74 (82.2)
Metastasis from other organs	3 (3.3)
Malignant lymphoma	1 (1.1)
Cancer of unknown primary origin	2 (2.2)
Benign disease	3 (3.3)
Unknown	7 (7.8)
Stage of lung cancer, n (%)	
I	27 (36.5%)
II	5 (6.6%)
III	15 (20.3%)
IV	25 (33.8%)
Unknown	2 (2.7%)

Table II. Results of the *EGFR* d-PCR assay in 90 patients using cell-free DNA supernatants and cell pellets.

EGFR mutations	Cell-free DNA supernatants	Cell pellets
Positive	12 (13.3%)	15 (16.7%)
Exon 21 L858R	9[a,b]	10[a,b]
Exon 19 E746_A750del	4[a]	6[a]
Exon 20 T790M	2[a,b]	3[a,b]
Negative	78 (86.7%)	75 (83.3%)
Total	90	90

[a]There was one patient with a triple mutation (L858R, E746_A750del and T790M) in the assays using cell-free DNA supernatants and cell pellets. [b]There was one patient in the assay using cell-free supernatants, and two patients in the assay using cell pellets, who had double mutations (L858R and T790M).

15 (20.3%) and 25 (33.8%) of the 74 lung cancer cases were clinically staged (TNM classification 7th edition) (24) as stage I, II, III and IV, respectively. The stage of the remaining two (2.7%) lung cancer cases was unknown.

Cytological diagnosis of cell pellets classified the 90 specimens as follows: 'malignant', 28 (31.1%); 'suspicious for malignancy', nine (10.0%); 'neoplastic, benign neoplasm, low-grade carcinoma', none (0%); 'atypical', 12 (13.3%); 'negative for malignancy', 40 (44.4%); and 'non-diagnostic', one (1.1%).

The mean concentration of DNA extracted from the cfDNA supernatants and cell pellets, prior to adjustment, was 7.4 (range 1.2-152.8 ng/μl) and 47.2 ng/μl (1.3-314.1 ng/μl), respectively.

Table III. Comparison of assay results obtained for each epidermal growth factor receptor mutation using cell-free DNA supernatants and cell pellets.

	Cell pellets		
Cell-free DNA supernatants	Positive	Negative	Total
Exon 21 L858R			
Positive	9	0	9
Negative	1	80	81
Total	10	80	90
Exon 19 E746_A750del			
Positive	4	0	4
Negative	2	84	86
Total	6	84	90
Exon 20 T790M			
Positive	2	0	2
Negative	1	87	88
Total	3	87	90

Comparison of cfDNA-supernatant and cell-pellet assay results. The results of the EGFR d-PCR assays performed using cfDNA supernatants and cell pellets were compared (Tables II and III), and concordance rates were determined (Table IV). The total number of patients with *EGFR* mutations detected using cfDNA supernatants was 12 (13.3%), compared to 15 (16.7%) using cell pellets. L858R, E746_A750del and T790M mutations were identified in nine, four and two cfDNA supernatant samples respectively, and in 10, six and three cell pellet samples, respectively. The number of patients negative for *EGFR* mutations was 78 (86.7%) using cfDNA supernatants, and 75 (83.3%) using cell pellets. Three patients had more than one *EGFR* mutation, one had a triple mutation (L858R, E746_A750del and T790M), and two had double mutations (L858R and T790M). The concordance rates between the *EGFR* d-PCR assay results obtained using cfDNA supernatants and cell pellet samples were 96.7% [K=0.87, 95% confidence interval (CI) 0.73-1.00, P<0.0001] for all three EGFR mutations in combination, 98.9% (K=0.94, 95% CI 0.83-1.00, P<0.0001) for L858R alone, 98.9% (K=0.79, 95% CI 0.51-1.00, P<0.0001) for E746_A750del alone, and 98.9% (K=0.79, 95% CI 0.40-1.00, P<0.0001) for T790M alone.

Comparison of the EGFR assay results obtained using cfDNA supernatants and the corresponding cytological diagnosis obtained from cell pellets. The *EGFR* d-PCR assay results achieved using cfDNA supernatant were then compared with the cytological diagnosis of the corresponding cell pellets (Table V). Using cfDNA supernatants as templates, the *EGFR* d-PCR assay detected *EGFR* mutations in 12 samples. The corresponding cytological diagnosis was 'malignant' in seven patients (53.8%), 'suspicious for malignancy' in one patient (7.7%), 'atypical cells' in one patient (7.7%), and 'negative for malignancy' in three patients (30.8%).

Table IV. Concordance rates between the results obtained using cell-free DNA supernatants and cell pellets.

Mutation	Concordance rate (%)	*K* coefficient	95% CI	P-value
EGFR mutation	96.7	0.87	0.73-1.00	<0.0001
L858R	98.9	0.94	0.83-1.00	<0.0001
E746_A750del	98.9	0.79	0.51-1.00	<0.0001
T790M	98.9	0.79	0.40-1.00	<0.0001

CI, confidence interval; *EGFR*, epidermal growth factor receptor.

Table V. Comparison of the cell-free DNA-supernatant assay and corresponding cell-pellet cytological diagnosis results.

	EGFR d-PCR assay results of cell-free DNA supernatants		
Cytological diagnosis of cell pellets	Positive	Negative	Total
Malignant	7	21	28
Suspicious for malignancy	1	8	9
Neoplastic, benign neoplasm, low-grade carcinoma	0	0	0
Atypical	1	11	12
Negative for malignancy	3	37	40
Non-diagnostic	0	1	1
Total	12	78	90

EGFR, epidermal growth factor receptor.

EGFR status analyzed by conventional EGFR assays using FFPE tissue or cell block specimens. The cytological diagnoses of cell pellets and the results of a conventional *EGFR* assay using FFPE tumor tissue or cell block specimens for the patients whose cytology specimens (cfDNA supernatants or cell pellets) were positive for *EGFR* mutations, as determined using the EGFR d-PCR assay, are shown in Table VI. For the 15 *EGFR* mutation-positive patients, FFPE tumor tissue specimens were available for 14 patients (11 TBLBs and three surgical resections) and a PE FFPE cellblock specimen was available for one patient. *EGFR* mutations were detected in all of the specimens using conventional assays. Only two patients exhibited different results between the *EGFR* d-PCR assay using cell pellets and conventional assays using FFPE tumor tissues or cell block specimens: L858R and T790M vs. L858R in patient #16, and L858R, E746_A750del and T790M vs. exon 19 deletion in patient #29.

Effectiveness of EGFR-TKIs. Of the 12 patients with cfDNA supernatants found to be positive for *EGFR* mutations, eight received EGFR-TKI therapy using gefitinib, erlotinib or osimertinib, based on the conventional *EGFR* assay results achieved using FFPE tumor tissue or cell block specimens: four TBLBs and one PE cell block were analyzed with the Cobas v2 assay, and three TBLBs with the therascreen assay. Six of these eight patients (75%) exhibited a positive response to EGFR-TKI therapy and four patients (50%) were continuing this therapy at the data cut-off point in September 2017.

Discussion

Due to recent advances in molecular targeted therapies and genomic technologies, such as next-generation sequencing (NGS), multiple and parallel molecular testing is now available and is recommended to facilitate improved treatment strategies for patients with lung cancer (25,26). However, these processes often consume extensive resources, and integrating their results into standard clinical care regimes is complex and sometimes challenging (27,28). Furthermore, although obtaining appropriate tumor specimens with a sufficient number of cancer cells from patients is crucial for accurate molecular testing, doing so is often problematic or impossible (26). Therefore, simple, rapid and cost-effective methods of molecular testing, which take full advantage of the limited materials available in the clinical setting, such as cytology specimens, are urgently required. This is particularly important in cases of frequent oncogenic genomic alteration, including *EGFR* mutation. In the present study, the suitability of an *EGFR* d-PCR assay, which has a reaction time of <10 min, for analyzing cfDNA from cytology specimen supernatants was determined. The results of this assay were compared with those of an *EGFR* d-PCR assay using cell pellets, cytological diagnoses and conventional *EGFR* assays using FFPE tumor tissue or cell block specimens. In addition, the results were compared with the observed clinical effectiveness of EGFR-TKI therapy.

The *EGFR* d-PCR assay results produced using cfDNA supernatants exhibited substantial agreement with those obtained using cell pellets, and detected *EGFR* mutations even in specimens cytologically diagnosed as 'suspicious for malignancy', 'atypical' and 'negative for malignant cells.' One reason for this is the difference in detection sensitivity between cytological analysis with the Papanicolaou smear for the cancer cells and the *EGFR* d-PCR assay for target mutations. Since the Papanicolaou smear uses only a few drops from cell pellets derived from specimens such as BLF, which usually do not contain thousands of cells, the detection sensitivity of cytological analyses is limited. Conversely, the detection limits of the *EGFR* d-PCR assay were previously determined using mutation-positive cancer cell line mixtures; the results demonstrated that the detection limit of each mutation was 0.5, 0.05 and 0.5% of mutation-positive cancer cells, for L858R, E756_A750del and T790M, respectively (20).

Table VI. *EGFR* status in patients with *EGFR* mutation-positive results according to the *EGFR* d-PCR assay using tumor tissue or cytology specimens and conventional assays.

		EGFR d-PCR assay results		Conventional *EGFR* assay results
Patient # (specimen)	Cytological diagnosis of cell pellets	Cell-free DNA supernatants	Cell pellets	FFPE tumor tissue or cellblock
#4 (BLF)	Malignant	Negative	E746_A750del	Exon 19 Deletion[a] (TBLB, Cobas v2)
#7 (PE)	Malignant	L858R, T790M	L858R, T790M	L858R, T790M (PE cellblock, Cobas v2)
#16 (BLF)	Malignant	L858R	L858R, T790M	L858R (TBLB, therascreen)
#18 (BLF)	Malignant	E746_A750del	E746_A750del	Deletions[a] (TBLB, therascreen)
#24 (BLF)	Negative for malignancy	Negative	L858R	L858R (TBLB, therascreen)
#27 (BLF)	Negative for malignancy	E746_A750del	E746_A750del	Deletions[a] (TBLB, therascreen)
#29 (BLF)	Malignant	L858R, E746_A750del, T790M	L858R, E746_A750del, T790M	Deletions[a] (TBLB, therascreen)
#39 (BLF)	Malignant	L858R	L858R	L858R (TBLB, Cobas v2)
#53 (BLF)	Negative for malignancy	L858R	L858R	L858R (TBLB, Cobas v2)
#58 (BLF)	Negative for malignancy	L858R	L858R	L858R (SR, therascreen)
#67 (BLF)	Malignant	E746_A750del	E746_A750del	Deletions[a] (SR, therascreen)
#74 (BLF)	Malignant	Negative	E746_A750del	Deletions[a] (SR, therascreen)
#75 (BLF)	Atypical	L858R	L858R	L858R (TBLB, therascreen)
#83 (BLF)	Malignant	L858R	L858R, T790M	L858R, T790M (TBLB, Cobas v2)
#87 (BLF)	Suspicious for malignancy	L858R	L858R	L858R (TBLB, therascreen)

[a]Results of 'deletions' in the therascreen assay and 'exon 19 deletion' in the Cobas v2 assay represent a deletion in *EGFR* exon 19 that includes E746_A750del. BLF, bronchial lavage fluid; Cobas v2, Roche cobas® EGFR mutation test v2; *EGFR*, epidermal growth factor receptor; FFPE, formalin-fixed paraffin-embedded; PE, pleural effusion; SR, surgical resection; TBLB, transbronchial lung biopsy; therascreen, therascreen® EGFR RGQ PCR kit.

Furthermore, the PCR assay can detect tumor DNA in the absence of cancer cells, whereas cytological analysis requires cancer cells by definition. These findings indicated that the detection sensitivity of the *EGFR* d-PCR assay is superior to cytological analysis. In addition, cfDNA supernatants from cytology specimens contain a relatively high amount of cancer DNA. While cfDNA from normal cells is derived mainly from apoptotic processes (29), that generated by cancer cells is derived from apoptotic and necrotic processes associated with high cellular turnover (30,31). Therefore, more cfDNA should be isolated from cancer cells than from normal cells in cytology supernatants. Although current guidelines for *EGFR* assays recommend making cell blocks from cell pellets of cytology specimens (10), our previous study demonstrated that cfDNA supernatants had significantly lower quantification cycle values for *EGFR*-mutation detection than cell blocks in conventional PCR assays (32).

Previous studies have demonstrated the use of cfDNA supernatants obtained from cytology specimens for *EGFR* mutation assays in patients with NSCLC, using either PCR or direct sequencing methods (33-36). However, the majority of these studies used PE cytology specimens, which usually contain greater number of cancer cells compared to BLF specimens. Kawahara *et al* (37) and Park *et al* (38) previously compared the *EGFR* mutation status determined by subjecting cfDNA supernatants from bronchial cytology samples (such as BLF, bronchoalveolar washing and bronchial brushing), to that determined by subjecting the corresponding tumor tissue samples to conventional PCR-based assays, for 51 and 20 patients, respectively. The concordance rate between the two types of results was 94.1 (48/51) and 75.0% (9/12) for each study, respectively. To the best of our knowledge, the present study has analyzed the largest number of bronchial cytology-specimen cfDNA using the *EGFR* d-PCR assay of any study conducted to date. A rate of 96.7% concordance was achieved between the results that were obtained using cfDNA supernatants, and those that were obtained using cell pellets, and furthermore, this was achieved using a much shorter reaction time than necessary for conventional PCR-based assays.

In addition to the marked concordance of assay results between cfDNA supernatants and cell pellets, the *EGFR* mutations detected by the *EGFR* d-PCR assay using cytology specimens (cfDNA supernatants or cell pellets) were highly consistent with those detected by conventional assays using tumor tissues or cytology specimens. Our previous study reported complete concordance between BLF cell pellet and FFPE tumor tissue assay results in 49 patients with NSCLC using the current companion diagnostic of EGFR-TKI therapy, the therascreen assay (32). In another study, we also detected complete concordance between the results achieved using the *EGFR* d-PCR assay and the therascreen assay to assess cell pellets of cytology specimens collected from 80 patients with NSCLC (20). Therefore, with respect to L858R, E746_A750del and T790M, the *EGFR* d-PCR assay results appear to be as reliable as current companion diagnostics using FFPE tumor tissues.

The objective response rate for EGFR-TKI therapy was 75.0% (6/8) in patients with NSCLC whose cfDNA supernatants were shown to be positive for mutations using the *EGFR* d-PCR assay. This finding suggested that the results of the *EGFR* d-PCR assay obtained using cfDNA supernatants correlate with the clinical effectiveness of EGFR-TKIs. At present, *EGFR* mutations detectable by the *EGFR* d-PCR assay are limited to three mutations: L858R, E746_A750del and T790M. However, L858R and E746_A750del represent ~90% of oncogenic *EGFR* mutations (39). Furthermore, patients with NSCLC with these mutations have been reported to exhibit better responses to EGFR-TKI therapy than those that harbor more minor *EGFR* mutations, including insertions in exon 20, L861Q in exon 21 or exon 19 deletions starting at codon L747 (40,41). Furthermore, application of the *EGFR* d-PCR assay using cfDNA supernatants reserves the cell pellets of cytology specimens, which can thus be subjected to additional comprehensive and detailed genotyping using multiplex PCR assays and/or NGS. Since the *EGFR* d-PCR assay using cfDNA supernatants detects mutations more quickly and with greater sensitively than the cytological diagnosis of cell pellets, it may also be useful as a screening and confirmation method for lung cancer diagnosis.

In two patients, the *EGFR* d-PCR assay using cytology specimens detected more mutations than the conventional *EGFR* assays using FFPE tissue specimens. It is assumed that DNA in FFPE specimens is negatively affected by the formalin-fixation process, including DNA fragmentation and cross-linking formation (42). Notably, several studies (42,43), including our previous study (32), have demonstrated the strong negative impact of fixation on *EGFR* mutation detection efficiency in patients with lung cancer. Furthermore, the *EGFR* d-PCR assay has a notable detection limit compared to conventional assays (20), indicating that mutations detected only by the *EGFR* d-PCR assay may indeed be real mutations. In the present study, there was a rare case of a triple mutation detected only by the *EGFR* d-PCR assay; however, triple *EGFR* mutations have previously been reported in lung cancer (44). Furthermore, since the patient had already received EGFR-TKI treatment for >1 year when the cytology specimen was obtained, it is not surprising that the patient had acquired secondary mutations such as T790M.

A limitation of the present study was that the optimal amount of supernatant that should be used for cfDNA extraction is unknown. While extracting DNA from a greater volume of supernatant would increase the detection sensitivity of the assay, it would also incrementally increase the time and effort required for DNA extraction. Therefore, further study is required to analyze the association between the amount of utilized supernatant and extracted DNA, and to develop more efficient DNA extraction methods for liquid specimens. This may also enable the current *EGFR* d-PCR assay to be further developed into a liquid biopsy using cfDNA from blood serum or plasma. *EGFR* d-PCR liquid biopsy would provide a novel, fast and minimally invasive screening method for detecting *EGFR* mutations in patients with lung cancer.

In conclusion, the present study demonstrated that using the *EGFR* d-PCR assay to analyze cfDNA from cytology specimen supernatants is a rapid, sensitive and reliable method for the detection of *EGFR* mutations, which furthermore reserves cell pellets for use in other morphological and molecular analyses. This assay may therefore be considered a promising novel point-of-care testing method that may enable patients with NSCLC to receive EGFR-TKI therapy as soon as possible.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI; grant no. 25460434). This work was also supported, in-kind, by the Seiko Epson Corporation (Suwa, Nagano, Japan).

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

SA designed the study and wrote the initial draft of the manuscript. AY assisted in study design, data analysis, interpretation and preparation of the manuscript. KS, SA and TN performed DNA extractions and PCR assays using patients' cytological and tissue specimens. YK and RN are cytotechnologists that performed cytological analysis of patients' specimens. SA and AY are pathologists that reviewed the patients' cytological diagnosis. HY contributed to patients' clinical data collection, specimen collection and critically reviewed the manuscript. KM and AY assisted in developing the *EGFR* d-PCR assay and optimized the assay conditions. TH contributed to the acquisition of funding, general supervision, data interpretation and critically reviewed the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent for their participation in the present study, which was reviewed and

approved by the medical ethics committee of the Shinshu University School of Medicine (Matsumoto, Japan).

Consent for publication

All patients provided written informed consent.

Competing interests

Akemi Yamaguchi, one of the authors of this study, was an employee of Seiko Epson Corporation. The remaining authors have no conflicts of interest to disclose.

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