A novel, rapid point-of-care test for lung cancer patients to detect epidermal growth factor receptor gene mutations by using real-time droplet-PCR and fresh liquid cytology specimens

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Abstract. Epidermal growth factor receptor gene (*EGFR*) mutations are associated with response to tyrosine kinase inhibitors (TKIs) in patients with non-small cell lung cancer (NSCLC). We developed a novel, rapid *EGFR* mutation assay using a real-time droplet-polymerase chain reaction machine (*EGFR* d-PCR assay). The purpose of this study was to validate the performance of the *EGFR* d-PCR assay using fresh liquid cytology specimens. We analyzed three major *EGFR* mutations (L858R in exon 21, E746_A750del in exon 19 and T790M in exon 20) in 80 fresh liquid cytology specimens of adenocarcinoma (ADC) or NSCLC-not otherwise specified (NOS) via the *EGFR* d-PCR assay and conventional real-time PCR assay using the *therascreen*® EGFR RGQ PCR kit (Therascreen assay). In addition, we performed sensitivity assays using cell lines with *EGFR* mutations. The *EGFR* d-PCR assay detected 16 L858Rs, 8 E746_A750dels and 1 T790M mutation and the Therascreen assay detected 16 L858Rs, 11 deletions in exon 19 and 1 T790M mutation. The results were concordant between the two assays. The reaction time of the *EGFR* d-PCR assay was 8 min and 10 sec, but that of the Therascreen assay was 1 h and 45 min. Sensitivity, as assessed by the detection limit of the *EGFR* d-PCR assay was 0.5, 0.05 and 0.5% for L858R, E746_A750del and T790M, respectively. The *EGFR* d-PCR assay markedly reduced the detection time of major *EGFR* mutations with high sensitivity compared with the conventional Therascreen assay and is expected to expedite *EGFR*-TKI therapy for lung cancer patients, especially those in advanced stages.

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Abbreviations: NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; *EGFR*, epidermal growth factor receptor gene; *ALK*, anaplastic lymphoma kinase gene; *EGFR*-TKIs, *EGFR* tyrosine kinase inhibitors; TAT, turnaround time; PCR, polymerase chain reaction; *PML*, promyelocytic leukemia; *RARA*, retinoic acid receptor α; *BCR*, breakpoint cluster region; *ABL*, Abelson murine leukemia viral oncogene; *EGFR* d-PCR assay, rapid *EGFR* mutation assay by using real-time droplet-PCR machine; BLF, bronchial lavage fluid; PE, pleural effusion; CE, cardiac effusion; FFPE, formalin-fixed paraffin-embedded; ML, malignant lymphoma; RCC, renal cell carcinoma; SCC, small cell carcinoma; SQCC, squamous cell carcinoma; NOS, not otherwise specified; ARMS, amplification-refractory mutation system; ROC, receiver operating characteristic; PCR-SSCP, PCR-based assays such as single-strand conformation polymorphism; PNA-LNA, peptide nucleic acid-locked nucleic acid

Key words: real-time droplet-PCR, point-of-care testing, lung cancer, *EGFR*, rapid assay, fresh liquid cytology specimen, bronchial lavage fluid, pleural effusion, cardiac effusion

Introduction

Lung cancer is the most common cause of cancer-related deaths worldwide, accounting for 1.8 million new cases and 1.6 million deaths in 2012 (1). During the past decade, major progress has been achieved in the treatment and management of non-small cell lung cancer (NSCLC), especially for adenocarcinomas (ADC), with the discovery of its biological and therapeutic importance of acquired genomic alterations, such as mutations in the epidermal growth factor receptor gene (*EGFR*) (2,3) or inversions involving the anaplastic lymphoma kinase gene (*ALK*) (4,5). *EGFR* encodes pharmacologically targetable tyrosine kinases and it has been recognized that NSCLC patients with *EGFR* mutations such as deletions in exon 19 and L858R in exon 21 show dramatic prolonged response to *EGFR* tyrosine kinase inhibitors (*EGFR*-TKIs), such as gefitinib or erlotinib, in clinical trials of advanced-stage lung cancer (6-8). The reliable detection of *EGFR* mutations

is necessary for the personalized treatment of lung cancer patients.

A number of methods have been used to detect *EGFR* mutations in NSCLC patients (9). While the sensitivity of *EGFR* mutation assays has improved, the majority of these methods, however, need at least a few hours to detect *EGFR* mutations. In the case of obtaining *EGFR* test results from outside laboratories, it generally requires 7-14 days after tumor sampling. The turnaround time (TAT) for *EGFR* mutation testing has become increasingly important, especially for advanced-stage patients, in order to start *EGFR*-TKI therapy earlier. Therefore, a more rapid and simple *EGFR* mutation assay is needed.

Recently, we developed novel rapid assays using a real-time droplet-polymerase chain reaction (PCR) machine (Seiko Epson Corp. Head Office, Suwa, Japan) for the detection of the human influenza virus (10,11) and the promyelocytic leukemia (*PML*)-retinoic acid receptor α (*RARA*) fusion gene (12,13), for the genotyping of single nucleotide polymorphisms (14,15) and for the detection of bovine respiratory syncytial virus (16) and breakpoint cluster region (*BCR*)-Abelson murine leukemia viral oncogene (*ABL*) fusion mRNA (17). All of these assays provide a much shorter TAT than the conventional assays currently used in clinical settings while demonstrating almost the same reactivity.

The purpose of this study was to develop a novel rapid *EGFR* mutation assay in lung cancer patients by using a real-time droplet-PCR machine (*EGFR* d-PCR assay). In this study, we used fresh liquid cytology specimens, such as bronchial lavage fluid (BLF), pleural effusion (PE) and cardiac effusion (CE) from the patients, which are processed in more simple steps and a shorter time for DNA extraction than formalin-fixed paraffin-embedded (FFPE) specimens. To validate the performance of the *EGFR* d-PCR assay, we compared its reactivity and reaction time to a conventional real-time PCR assay. In addition, we validated the sensitivity of the *EGFR* d-PCR assay by using fresh liquid samples containing variable percentages of cell lines with *EGFR* mutations.

Materials and methods

Patients and samples. This study was reviewed and approved by the Medical Ethics Committee of Shinshu University School of Medicine (Nagano, Japan). BLF, PE and CE specimens were collected from 245 patients who were clinically diagnosed with lung cancer from January to December 2014 and the specimens provided were used for cytological diagnosis at the Department of Laboratory Medicine at the Shinshu University Hospital (Nagano, Japan). BLF specimens were obtained after transbronchial lung biopsies. PE and CE specimens were obtained by fine-needle aspiration from patients with advanced lung cancer. After cytological evaluation of the specimens from the 245 patients, we excluded the specimens that contained no malignant cells (146 patients) or cytologically diagnosed as malignant lymphoma (ML) (1 patient), metastasis of renal cell carcinoma (RCC) (1 patient), small cell carcinoma (SCC) (8 patients) and squamous cell carcinoma (SQCC) (9 patients) (Fig. 1). Specimens from the remaining 80 patients, who were cytologically diagnosed with primary lung ADC or NSCLC-not otherwise specified (NOS),

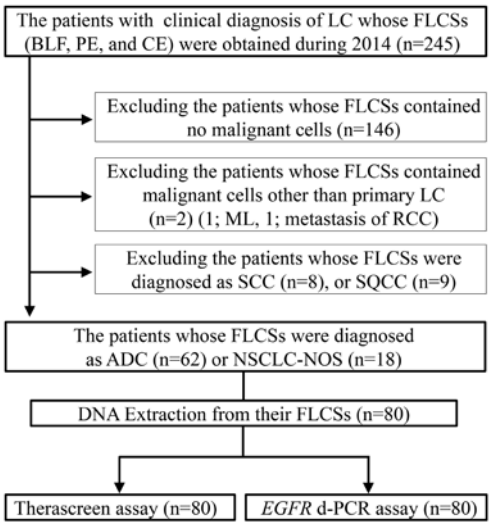

Figure 1. Flow diagram of patients and samples. Fresh liquid cytology specimens (FLCSs), such as bronchial lavage fluid (BLF), pleural effusion (PE) and cardiac effusion (CE), were obtained from 245 patients who were clinically diagnosed with lung cancer (LC). After cytological diagnosis of the specimens, the following specimens were excluded: 146 specimens with no malignant cells, 1 malignant lymphoma (ML), 1 metastasis of renal cell carcinoma (RCC), 8 small-cell carcinoma (SCC) and 9 squamous cell carcinoma (SQCC). Finally, 80 specimens with cytological diagnosis of adenocarcinoma (ADC) or non-small cell lung cancer-not otherwise specified (NSCLC-NOS) were used in this study.

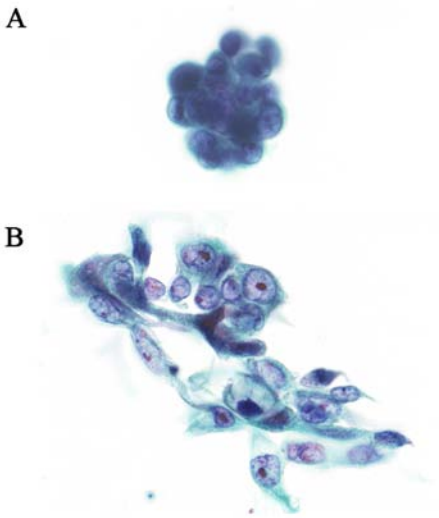

Figure 2. Representative cytological findings of cytology smears in fresh cytology specimens. (A) Specimens showing typical cytological findings of adenocarcinoma (ADC); three-dimensional aggregate, peripherally oriented large nuclei with smooth membrane, finely granular chromatin, distinct small nucleoli and basophilic foamy cytoplasm (Papanicolaou staining; original magnification, x600). (B) Specimens showing no other definite characteristics of ADC, squamous cell carcinoma, nor small-cell carcinoma, which were classified as non-small cell lung cancer-not otherwise specified (NSCLC-NOS) (Papanicolaou staining; original magnification, x600).

were also evaluated (Fig. 2). The fresh liquid cytology specimens were centrifuged to create a cell pellet and after picking up enough of the cell pellet for the routine smear slide for cytological diagnosis, the remainder of the pellet was used for the samples in this study. DNA was extracted from the specimens

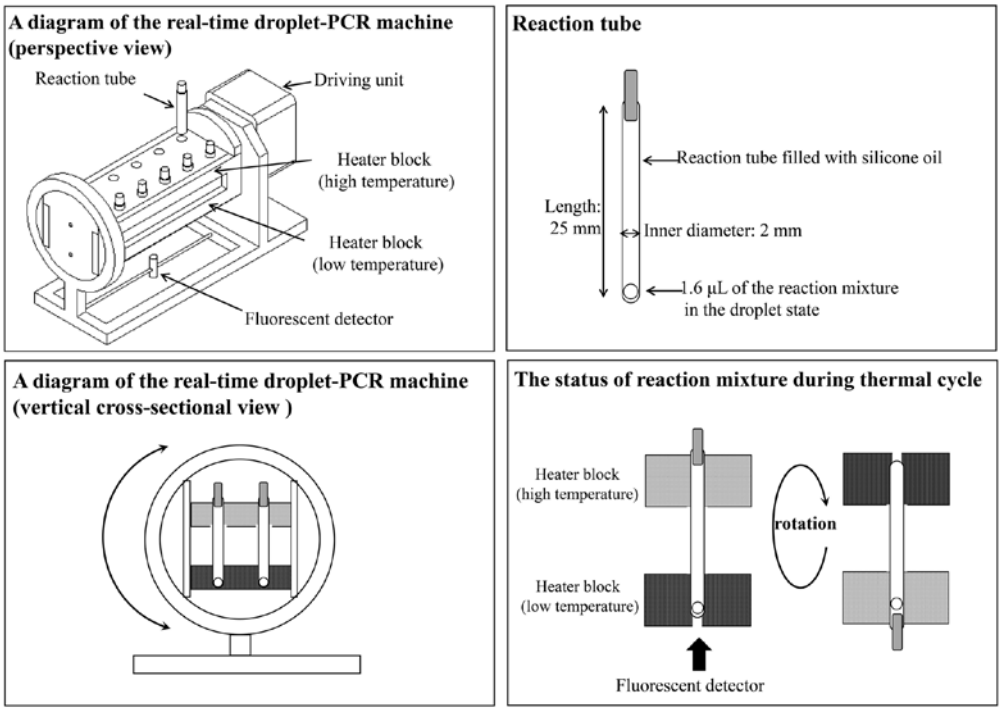

Figure 3. A modified diagram of the real-time droplet-PCR machine from our previous report (14). The real-time droplet-PCR machine has two connected heater blocks, which regulate the temperature of each end of the reaction tube. The reaction temperatures of the two heater blocks are consistently controlled during the denaturation and annealing/extension steps. The reaction tube is filled with silicone oil, allowing the droplet of PCR mixture to move easily in the tube during the mechanical rotation of the machine with the two connected heater blocks. The droplet transfers from one end to another owing to gravitation, and then the machine with the two heater blocks inverts to return the droplet. The machine integrates a fluorescence detector monitoring the amount of PCR products after each extension step.

using the QIAamp DNA Blood Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions.

Conventional real-time PCR assay. For the reference, the conventional method to the *EGFR* d-PCR assay in this study, we used the Rotor-Gene Q 5plex HRM instrument with the *therascreen*® EGFR RGQ PCR kit (Qiagen, Inc.) (Therascreen assay). This assay is approved in the United States, Europe, Japan and China and the kit is based on the amplification-refractory mutation system (ARMS) and Scorpions PCR technologies, which enable sensitive and selective site-specific detection of 29 types of somatic mutations in the *EGFR* gene (18). The reaction conditions of the Therascreen assay were as follows: 95˚C for 15 min for 1 cycle, 95˚C for 30 sec and then 60˚C for 60 sec for 40 cycles. Analysis was performed using the Rotor-Gene Q series software, version 2.0.2 (Qiagen, Inc.) and the manufacturer-supplied ΔCt thresholds were used to analyze the result of each *EGFR* mutation reaction. The reaction time was about 1 h and 45 min.

Real-time droplet-PCR machine used for the EGFR d-PCR assay. The real-time droplet-PCR machine has two connected heater blocks, which regulate the temperature of each end of the reaction tube (Fig. 3). The reaction temperature of the two heater blocks is consistently controlled during the denaturation and annealing/extension steps. To perform a rapid temperature transition, the reaction tube is filled with silicone oil, allowing the droplet of the PCR mixture to move easily in the tube during the mechanical rotation of the machine with the two connected heater blocks. Once the droplet transfers from the one end to the other owing to gravitation, the machine with the two heater blocks inverts to return the droplet. Therefore, the PCR mixture in the droplet is able to perform shuttle PCR in the reaction tube. The real-time droplet-PCR machine integrates a fluorescence detector and it allows monitoring of the amount of PCR products after each extension step.

Target mutations of EGFR. As for the target mutations of *EGFR* in this study, we selected the two most frequent mutations. One was the point mutation L858R (c.2573T>G) in exon 21 and the other was the 15-bp/5-amino acid deletion of E746_A750del (c.2235_2249del and c.2236_2250del) in exon 19. These two major mutations cover about 90% of oncogenic *EGFR* mutations (19). In addition, we also selected the point mutation of T790M (c.2369>T) in exon 20, which is the most common resistance mutation for *EGFR*-TKIs (20).

Reaction conditions of the EGFR d-PCR assay. We designed specific primers based on ARMS that induced a mutation-matched nucleotide in the 3'-end and a template-mismatched nucleotide at the -2 position from the 3'-end. The primers and probes used in this study are as follows (5'-3'): L858R forward, GCTTGGTGCACCGCGACCTG; reverse, CGCACCCAG CAGTTTGGCAC; probe, 6FAM-AGCCAGGAACGTACT GGTGAAAACACCGCA-BHQ-1; E746_A750del forward, GGCAGCATGTGGCACCATC; reverse, GTTGGCTTTCG GAGATGTAT; probe, 6FAM-TCTCACCTTCTGGGATC CAGAGTCCCT-BHQ-1; T790M forward, CCCCACGTGTG

Table I. Clinical characteristics of the patients.

Characteristics	Data
Age (years)	
Average	68
Range	41-85
Gender, n (%)	
Male	57 (71.3)
Female	23 (28.7)
Diameter of the tumor (mm)	
Average	34.2
Range	7-132
Stage, n (%)	
I	37 (46.3)
II	5 (6.3)
III	9 (11.3)
IV	27 (33.8)
Unknown	2 (2.5)

Table II. Comparison between Therascreen assay and *EGFR* d-PCR assay

EGFR mutation type	Therascreen assay, n (%)	*EGFR* d-PCR assay, n (%)
Positive	28 (35.0)	25 (31.3)
Exon 21		
L858R	16 (20.0)	16 (20.0)
Exon 19	11 (13.8)	8 (10.0)
E746_A750del	[8] (10.0)	8 (10.0)
L747_S752del	[2] (2.5)	N/D
L747_S752del P753S[a]	[1] (1.3)	N/D
Exon 20		
T790M[a]	1 (1.3)	1 (1.3)
Insertions	1 (1.3)	N/D
Negative	52 (65.0)	55 (68.8)
Total	80	80

[a]One patient had double *EGFR* mutations (L747_S752del P753S in exon 19 and T790M in exon 20). [ ] indicate the number of patients whose mutations were confirmed by direct sequencing. N/D, not done.

CCGCCTG; reverse, GCCGAAGGGCATGAGCTGTA; probe, 6FAM-TGGGCATCTGCCTCACCTCCACCGTG CA-BHQ-1.

The reaction mixture contained genomic DNA (10 ng/μl), Platinum® *Taq* DNA polymerase (Life Technologies, Grand Island, NY, USA), 800 nmol/l of primer designed as aforementioned, 300 nmol/l of TaqMan probe and reaction buffer composed of Tris-HCl pH 9.0, KCl and $MgCl_2$, in a total volume of 10 μl. From the reaction mixtures, 1.6 μl was used for one reaction tube.

Each reaction condition was as follows: for L858R, 98˚C for 10 sec, 40 cycles at 98˚C for 5 sec and 60˚C for 6 sec; for E746_A750del and T790M, 98˚C for 10 sec, 40 cycles at 98˚C for 5 sec and 55˚C for 6 sec. The reaction time was 8 min and 10 sec.

Evaluation of the EGFR d-PCR assay results. To evaluate the PCR results as *EGFR* mutation-positive or -negative, we determined the arbitrary cut-off values of fluorescence level in 40 cycles, which were based on the receiver operating characteristic (ROC) curve analysis and using the Therascreen assay as 'gold standard'. Each cut-off value was as follows: 4.7 for L858R, 4.7 for E746_A750del and 6.8 for T790M.

Compared to the *EGFR* d-PCR assay, which was designed to detect only E746_A750del in exon 19, the Therascreen assay can detect 19 types of deletions in exon 19 including E746_A750del, however, it cannot tell which mutation was detected among the 19 types. To examine the exact locus of the deletion in all the specimens determined as deletion positive by Therascreen assay, we performed direct sequencing for those specimens.

Sensitivity analysis of EGFR d-PCR assay. To evaluate the sensitivity of the *EGFR* d-PCR assay, we performed sensitivity assays using DNA mixtures extracted from the following cell lines: PC9 [E746_A750del (c.2235_2249del)], H1975 [L858R (c.2573T>G) and T790M (c.2369>T)] and A549 [*EGFR* wild-type]. The PC9 cell line was obtained directly from the RIKEN Cell Bank (Tsukuba, Japan). The H1975 cell line was obtained directly from the American Type Culture Collection (ATCC, Rockville, MD, USA). The A549 cell line was obtained directly from the Japanese Cancer Research Bank (Tokyo, Japan). PC9 and A549 and H1975 and A549 were mixed respectively in ratios of 1:0, 0.5:0.5, 0.1:0.9, 0.05:0.95, 0.01:0.99, 0.005:0.995, 0.001:0.999, 0.0005:0.9995, 0.0001:0.9999 and 0:1. As a result, each percentage of *EGFR* mutant cells in these mixtures was 100, 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01 and 0%. *EGFR* mutations of these mixtures were analyzed by the *EGFR* d-PCR assay.

Results

Characteristics of the patients and specimens. Fresh liquid cytology specimens from 80 lung cancer patients, including 77 BLF specimens, 2 PE specimens and 1 CE specimen were used in this study and the patient clinical characteristics are shown in Table I. The median age of the 80 patients was 68 years (range, 41-85 years); all of the patients were Japanese and 57 patients (71.3%) were men while 23 patients (28.7%) were women. Cytologically, 62 patients (77.5%) were diagnosed with ADC and 18 patients (22.5%) were diagnosed with NSCLC-NOS. Radiologically, the median diameter of the tumors was 34.2 mm (range, 7-132 mm). The distribution of the clinical stages at the time of diagnosis was as follows: 37 patients (46.3%) had stage I cancer, 5 patients (6.3%) had stage II, 9 patients (11.3%) had stage III and 27 patients (33.8%) had stage IV.

Comparison between the Therascreen assay and the EGFR d-PCR assay. Table II shows the comparison between the Therascreen assay and the *EGFR* d-PCR assay. Among the specimens from 80 patients, the Therascreen assay detected exon 21 L858R point mutations in 16 patients (20.0%),

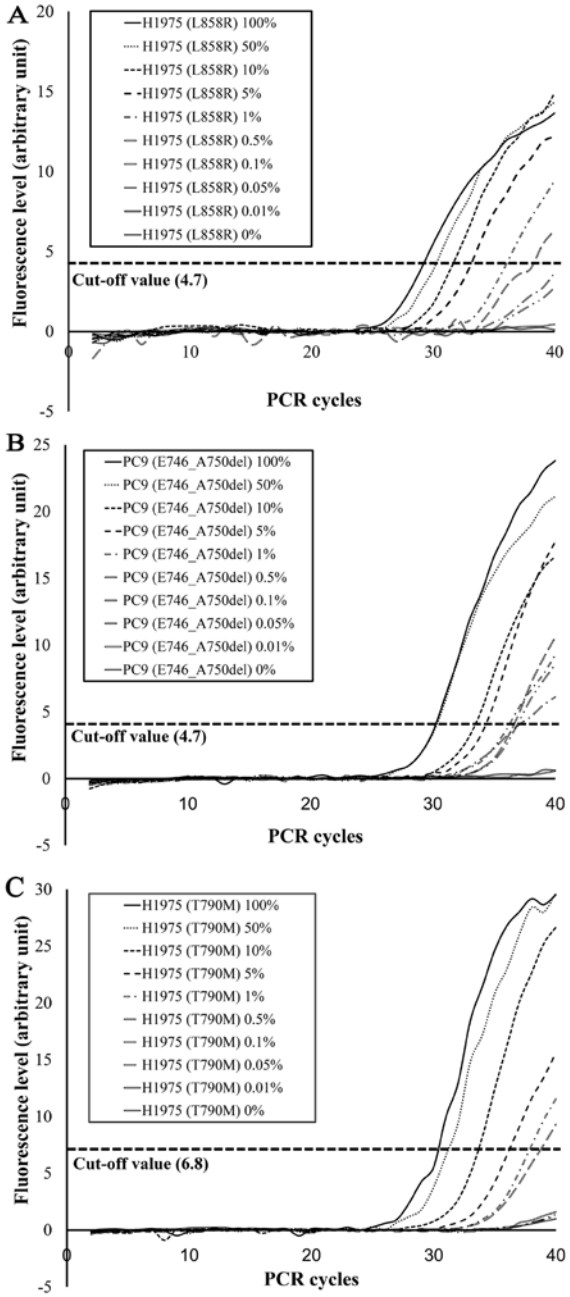

Figure 4. Sensitivity assessment of the EGFR d-PCR assay. (A) L858R, (B) E746_A750del and (C) T790M mutations were detected in mutation-positive cell line mixtures at respective percentages of 100, 50, 10, 5, 1 and 0.5% of H1975 cells for L858R (A), 100, 50, 10, 5, 1, 0.5, 0.1 and 0.05% of PC9 cells for E746_A750del (B), and 100, 50, 10, 5, 1 and 0.5% of H1975 cells for T790M (C).

exon 19 deletions in 11 patients (13.8%), exon 20 insertion in one patient (1.3%) and exon 20 T790M point mutation in one patient (1.3%). In contrast, the *EGFR* d-PCR assay detected L858R in 16 patients (20.0%), E746_A750del in 8 patients (10.0%) and T790M in one patient (1.3%). The Therascreen assay but not the *EGFR* d-PCR assay detected exon 19 deletions in three specimens. Direct sequencing confirmed L747_S752del P753S mutation in one specimen and L747_S752del mutation in two specimens. As a result, in regards to the L858R, E746_A750del and T790M mutations, the results of the *EGFR* d-PCR assay were in complete concordance with those of the Therascreen assay.

Sensitivity of the novel EGFR d-PCR assay. With the *EGFR* d-PCR assay, the L858R mutation was detected in L858R-positive cell line mixtures (H1975 and A549) at 100, 50, 10, 5, 1 and 0.5% H1975 cells (Fig. 4A). Similarly, E746_A750del was detected in E746_A750del-positive cell line mixtures (PC9 and A549) at 100, 50, 10, 5, 1, 0.5, 0.1 and 0.05% PC9 cells (Fig. 4B). T790M was detected in T790M-positive cell line mixtures (H1975 and A549) at 100, 50, 10, 5, 1 and 0.5% H1975 cells (Fig. 4C). These results suggest that the detection limits of the *EGFR* d-PCR assay were 0.5% for L858R, 0.05% for E746_A750del and 0.5% for T790M.

Discussion

Numerous methods are available for *EGFR* mutation testing: direct sequencing, PCR-based assays such as single-strand conformation polymorphism (PCR-SSCP) (21), peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp assays (22), PCR-Invader (23), cycleave PCR (24), the Scorpions amplification-refractory mutation system (ARMS) (25), immunohistochemistry with *EGFR* mutation-specific antibodies (26), real-time PCR methods (27) and next generation sequencing (28). Each method has different sensitivity and specificity and they need at least a few hours to effectively detect *EGFR* mutations. In this study, we demonstrated that the *EGFR* d-PCR assay markedly reduced the detection time of major *EGFR* mutations with high sensitivity compared with conventional methods. To the best of our knowledge, the novel *EGFR* d-PCR assay is the most rapid detection method and a reliable test to assess the dynamics of key *EGFR* mutations.

In 2013, to establish an evidence-based recommendation of the molecular testing of *EGFR* and *ALK* for patients with lung cancer, three professional societies (the College of American Pathologists, the International Associations for the Study of Lung Cancer and the Association for Molecular Pathology) developed a guideline (29). The guideline recommends performing *EGFR* and *ALK* molecular testing for all patients with advanced-stage lung ADC or tumor with an ADC component. Regarding testing samples, the guideline suggested the use of cytology specimens for *EGFR* and *ALK* molecular testing, although FFPE, fresh frozen, or alcohol-fixed tissue specimens should be prioritized. In addition, it recommends that FFPE cell blocks were preferable than smear preparations when cytology specimens were used. However, it requires several days to prepare FFPE specimens for pathological diagnosis and several hours to extract DNA from those specimens because they need deparaffinization and reversal of formaldehyde-induced modification of nucleic acids. Some studies have reported the utility and the reliability of fresh cytology specimens for *EGFR* mutation genotyping in NSCLC patients and their detection sensitivity is similar to or even better than that of FFPE tissue or cell block samples (30-32). Thus, we attempted to use fresh liquid cytology specimens, such as BLF, PE and CE, for the detection of *EGFR* mutations in this study, because it can shorten the time to prepare smear slides for pathological diagnosis to 30 min and the time to extract DNA from the specimens to 30 min.

This study demonstrated that the *EGFR* d-PCR assay could markedly reduce the reaction time to 8 min and 10 sec, while the conventional method, the Therascreen assay, takes

1 h and 45 min. In the *EGFR* d-PCR assay, the volume of the droplet including the sample and reagents is only 1.6 μl and is surrounded by silicone oil, which has good heat conduction characteristics. Therefore, the temperature of the droplet can quickly reach the temperature regulated by the two heater blocks. In addition, the rapid transition of the droplet between the two set temperatures decreases the time for each cycle in PCR. Few studies have reported rapid assays for detecting *EGFR* gene mutations in lung cancer patients within 10 min. Sakamoto *et al* (33,34) developed a high-speed *EGFR* real-time PCR assay known as the ultra-rapid PCR assay, which can detect L858R and E746_A750del in <10 min. Our *EGFR* d-PCR assay is, however, more rapid and sensitive than the ultra-rapid PCR assay. Moreover, it can also detect the T790M mutation in addition to the L858R and E746_A750del mutations in the same reaction. As a result, the entire procedure of the *EGFR* d-PCR assay is accomplished within 1 h and 30 min; thus, *EGFR* gene mutational status can be reported on the day the specimens are obtained from the patients.

In addition to the benefit of shortening the testing time of *EGFR* mutations, the sample results of the *EGFR* d-PCR assay were highly concordant with those of the Therascreen assay and *EGFR* d-PCR had excellent detection limits. The sensitivity of the Therascreen assay is well documented (18,35) and its detectable mutation percentages are 1.26% for L858R, 1.64% for deletions and 7.02% for T790M according to the manufacturer's database concerning performance characteristics. Our *EGFR* d-PCR assay had detection limits of 0.5% for L858R, 0.05% for E746_A750 and 1.0% for T790M of mutation-positive cells, respectively. These mutation-positive cell lines have two copies of the *EGFR* gene: one is mutated and the other is wild-type. Thus, the *EGFR* d-PCR assay may have much lower detectable mutation percentages than the Therascreen assay.

Regarding the cost of the *EGFR* d-PCR assay, the assay is a cost-effective method compared to conventional assays. This is due to the fact that the reaction mixture volume of *EGFR* d-PCR assay is only 1.6 μl in each reaction tube and it needs a smaller volume of primers and probes. Moreover, it also needs a smaller volume of samples from the patients, so the remnants from the cytology specimens for the diagnosis were sufficient and thus used for the assays in this study. This facet could lead to using the samples from patients for other additional molecular tests.

EGFR mutations detectable by the novel assay were limited to the two major *EGFR* mutations, L858R and E746_A750del and the most common resistance mutation for *EGFR*-TKIs, T790M. However, it has been reported that the response rates for *EGFR*-TKIs in patients with the two major mutations were higher than those with minor mutations such as insertions in exon 20 (36), L861Q in exon 21 and exon 19 deletions starting codon L747 (37); the effectiveness of *EGFR*-TKIs in patients with minor *EGFR* mutations is limited (38,39). Therefore, after examining the major *EGFR* mutations by *EGFR* d-PCR assay, we can examine the minor *EGFR* mutations by multiplex PCR assay or direct sequencing after the precise histological tumor evaluation. Thus, the *EGFR* d-PCR assay may help detect frequent and effective mutations for *EGFR*-TKI therapy right after obtaining specimens and contribute to a more rapid start of therapy for advanced-stage lung cancer patients. Although the *EGFR* d-PCR assay needs two PCR reactions for one sample at this point, once for L858R and once for E746_A750del and T790M, we are planning to adjust the PCR conditions to detect all three *EGFR* mutations simultaneously and currently apply newly designed primers to other *EGFR* mutations to increase the variety of the types it can detect in the future.

In conclusion, our *EGFR* d-PCR assay can detect major *EGFR* mutations rapidly, correctly, sensitively and cost-effectively with a small amount of specimen. By means of using fresh liquid cytology specimens, which require fewer and easier steps to obtain DNA, the TAT can be markedly reduced compared with conventional assays using FFPE specimens. This assay is expected to contribute to an expedited advent of *EGFR*-TKI therapy for NSCLC patients. Furthermore, this assay can be a point-of-care test for such patients in the very near future.

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