Detection of *EGFR* T790M Mutation by Droplet Digital Polymerase Chain Reaction in Lung Carcinoma Cytology Samples

Shara Kamil Nizamaddin, MSc; Meenakshi Mehrotra, PhD; Ari Sami H. Nadhim, MBChB, FICMS; Rajyalakshmi Luthra, PhD; Sinchita Roy-Chowdhuri, MD, PhD

• ***Context.***—**Advanced-stage non–small cell lung carcinoma patients on EGFR-targeted tyrosine kinase inhibitors frequently present with an acquired *EGFR* T790M resistance mutation. Early detection using a high-sensitivity assay is critical to allow patients to switch to third-generation tyrosine kinase inhibitors. The detection of *EGFR* T790M mutation is often challenging because of low tumor fraction in posttreatment specimens. Because a large fraction of non–small cell lung carcinoma patients are given a diagnosis by cytology, evaluating a high-sensitivity technique for *EGFR* T790M detection in these specimens is essential.**

Objective.—**To evaluate a high-sensitivity droplet digital polymerase chain reaction (ddPCR) assay for *EGFR* T790M using different cytologic specimen preparations.**

Design.—**A total of 42 cytology samples, including smears and cell block preparation, were evaluated for *EGFR* T790M using ddPCR. The results of the mutation assay were compared to the patient's known *EGFR* T790M mutation status.**

Results.—**The ddPCR assay successfully determined the *EGFR* T790M mutation status in 36 of 42 samples (86%), including samples with low tumor fraction (≤20%). In 4 cases the results of the ddPCR assay could not be compared because the mutation status was unknown at the time of collection of the cytology sample. There was 1 false-positive result, with borderline positivity, and 1 false-negative result. Overall sensitivity and specificity of the ddPCR assay were 93% and 96%, respectively.**

Conclusions.—**Our results indicate that *EGFR* T790M ddPCR is a highly sensitive and specific mutational assay that can be used reliably in cytologic specimens, including samples with low tumor fraction.**

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The management of non–small cell lung carcinoma (NSCLC), particularly lung adenocarcinoma, has undergone a paradigm shift, with patients harboring sensitizing somatic mutations in *EGFR* showing a dramatic response to targeted therapy by tyrosine kinase inhibitors (TKIs).[1–3] However, a large fraction of these patients eventually develop resistance mechanisms, with an acquired *EGFR* T790M mutation being the most common.[4–6] The early detection of *EGFR* T790M mutation is critical because third-generation TKIs, such as osimertinib, can be used in these patients.[7–11]

Advanced-stage NSCLC patients frequently present with resistance to TKI therapy with recurrence and/or progression of disease in the form of a pleural effusion, increasing tumor size, or a new nodule/metastasis. Because these patients are not candidates for surgical resection, small specimens comprising fine-needle aspiration (FNA) or core needle biopsy using minimally invasive sampling techniques are typically used for the diagnosis and molecular testing to evaluate *EGFR* T790M mutation status. However, posttherapy tumor nodules are often fibrotic and contain large amounts of nonneoplastic stromal tissue with low tumor fraction, making *EGFR* T790M mutation detection challenging.[12–14] The difficulty of detecting *EGFR* T790M is also true for pleural effusion specimens that frequently contain large numbers of nonneoplastic cells, such as macrophages, mesothelial cells, and lymphocytes, that can lower the tumor fraction in the sample. In addition, the resistance mutation may be present in a subclonal population at a lower allelic frequency (AF) than the sensitizing mutation, making the detection of *EGFR* T790M in these already low tumor fraction samples extremely difficult.[12] This has led to the increasing use of liquid biopsy assays in the setting of

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From the Departments of Hematopathology (Drs Nizamaddin, Mehrotra, and Luthra), Pathology (Dr Roy-Chowdhuri), Translational Molecular Pathology (Drs Luthra and Roy-Chowdhuri), and Experimental Radiation Oncology (Dr Nizamaddin), The University of Texas MD Anderson Cancer Center, Houston; and the College of Medicine, University of Sulaimani, Sulaimani, Kurdistan Region, Iraq (Drs Nizamaddin and Nadhim). Dr Mehrotra is currently with the Department of Pathology, The Mount Sinai Hospital, New York, New York. Drs Luthra and Roy-Chowdhuri contributed equally to this work.

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Corresponding author: Sinchita Roy-Chowdhuri, MD, PhD, Department of Pathology, Unit 85, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030 (email: sroy2@mdanderson.org).

secondary clinical resistance, where circulating tumor DNA isolated from the patient's plasma is evaluated for *EGFR* T790M when tissue is inadequate or unavailable.[12,15–21] Although there is a distinct role for liquid biopsy using plasma cell-free DNA (cfDNA) in such situations, the sensitivity of detection of *EGFR* T790M is relatively low and often depends on factors such as burden of disease and prevalence of the mutation in cfDNA.[12,22,23] Therefore, the lung molecular testing guidelines from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology (CAP/IASLC/AMP) recommend that a tissue-based tumor sample testing for *EGFR* T790M be performed using a high-sensitivity assay (capable of detecting *EGFR* T790M mutation from a sample containing as little as 5% viable tumor cells), if plasma-based liquid biopsy results are negative.[12]

Next-generation sequencing (NGS) serves as a highly multiplexed multigene platform for performing comprehensive mutational analyses of NSCLC-related genes, including *EGFR*. The analytic sensitivity of an NGS assay is 5% to 10%, and it may not always provide the analytic sensitivity necessary to detect *EGFR* T790M in patients presenting with secondary resistance to TKI therapy, especially in tissue samples with low tumor fraction. In addition, the cost and turnaround time of performing NGS analysis in scenarios where the clinical question specifically revolves around the presence of an *EGFR* T790M mutation argue for the need for a rapid, cost-effective, and high-sensitivity assay that can interrogate these samples for *EGFR* T790M. Previous studies have shown that a high-sensitivity droplet digital polymerase chain reaction (ddPCR) assay can rapidly and accurately detect *EGFR* T790M mutation in formalin-fixed, paraffin-embedded tissue blocks (unpublished data). Because a large fraction of NSCLC samples that are sent for *EGFR* T790M testing are cytology samples that may not necessarily have formalin-fixed, paraffin-embedded blocks, in the present study we evaluate different cytologic specimen preparations, including direct smears and formalin-fixed, paraffin-embedded cell blocks, for determining *EGFR* T790M mutation status using ddPCR.

MATERIALS AND METHODS

Patient Samples

A total of 42 cytology samples from 36 patients were included in this study. The patient demographics are summarized in Table 1. The samples were selected as follows: (1) a validation set of consecutive in-house cytologic samples (n = 21) from NSCLC patients that had *EGFR* mutational analysis by NGS between May 2016 and April 2017 and had leftover DNA to perform *EGFR* T790M ddPCR; (2) 5 non-NSCLC samples with *EGFR* mutational analysis by NGS and leftover DNA, included as a negative control; and (3) an additional 16 cytologic samples of metastatic lung adenocarcinoma with a known *EGFR* T790M mutation status based on mutation testing of a prior/subsequent tissue sample, included as the test set. All samples had a known cytologic diagnosis, estimated tumor percentage, and known *EGFR* T790M mutation status based on sequencing of a concurrent/prior/subsequent tissue sample (Table 2 and Supplementary Table 1 [see supplemental digital content, containing 1 table and 1 figure at www.archivesofpathology.org in the August 2020 table of contents]). Tumor fraction in specimens was estimated as the proportion of tumor cells relative to the total number of nucleated cells (benign and malignant cells). Samples with 3000 cells or more on smears and/or cell block sections were considered adequate for testing.

Table 1. Patient Demographics and Clinicopathologic Correlates

	Value
Age, y, median (range)	66.5 (34–91)
Sex, No. (n = 36)	
Male	22
Female	14
Cytology specimen source (n = 42)	
Pleural effusion	13
Lymph node FNA	10
Lung FNA	8
Soft tissue FNA	6
Bronchoalveolar lavage	2
Kidney FNA	1
Thyroid gland FNA	1
Bronchial brushing	1

Abbreviation: FNA, fine-needle aspiration.

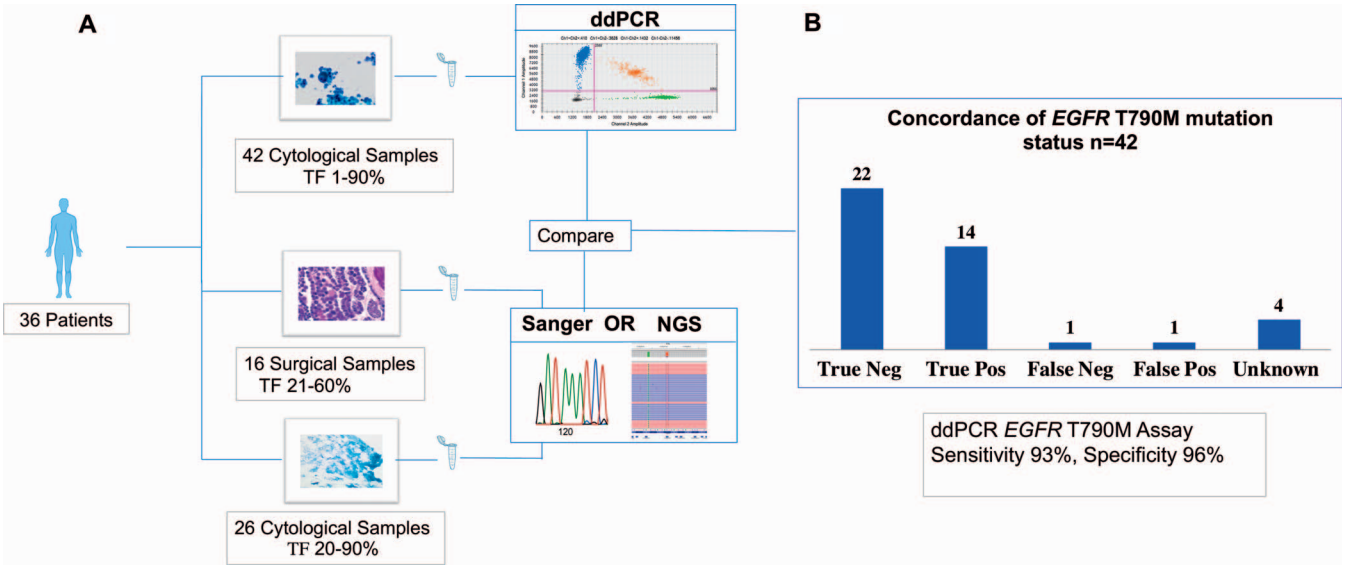
Extraction.—DNA was extracted from patient samples and cell lines using the PicoPure DNA extraction kit (Arcturus, Mountain View, California) and the Agentcourt AMPure XP kit (Agentcourt Biosciences, Beverly, Massachusetts), respectively, as per the manufacturer's instructions. DNA was quantified by a Qubit 2.0 fluorometer using the dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, Massachusetts).

Droplet Digital PCR Assay.—The ddPCR assay was performed using a total of 10 ng of genomic DNA on the QX100 Droplet Digital PCR System (Bio-Rad, Hercules, California), as per the manufacturer's protocol. The ddPCR assay was optimized using serially diluted T790M-positive cell lines (1% and 0.5% dilutions; see Supplementary Material and Supplementary Figure 1). Two cell lines, H1975 (ATCC CRL-5908) and DLD1 (ATCC CCL-221) ATCC (Manassas, Virginia), were used as positive and negative controls, respectively. All samples were run in duplicate. A threshold of more than 10 000 total droplets was set for any samples to be included in the analysis. Two-dimensional amplitude plots on control wells (ie, negative control) were used to establish the threshold for the x-axis and y-axis. Significant amplitude differences and concentration differences between replicates were used as quality indicators for poor handling or mixing of samples. Based on our prior experience using formalin-fixed, paraffin-embedded tissue samples, a set cutoff threshold of more than 10 000 total droplets, 5 or more positive droplets, and AF of 0.1 or more was used to determine positivity.

RESULTS

The 42 samples analyzed in this study included FNA samples (n = 26) from lymph node (n = 10), lung (n = 8), soft tissue (n = 6), kidney (n = 1), and thyroid gland (n = 1), and pleural effusions (n = 13), bronchoalveolar lavage (n = 2), and bronchial brushing (n = 1; Table 1). Of these, 37 samples were from lung carcinoma patients with diagnoses of lung adenocarcinoma (n = 36) and NSCLC not otherwise specified (n = 1); 5 additional samples of nonlung origin, included as negative control, had diagnoses of gastric adenocarcinoma, breast adenocarcinoma, high-grade serous carcinoma, oropharyngeal squamous cell carcinoma, and small cell carcinoma of gastrointestinal origin (Table 2).

The median estimated tumor fraction in these 42 cases was 30% (range, 1%–90%). Of these, 28 cases (66.7%) had direct smears that were used for DNA extraction, 12 cases (28.6%) had cell block sections, and 2 cases (4.7%) had a combination of smears and cell block sections that were used. In cases where direct smears were employed for



Study design and result. A, DNA extracted from 42 cytologic samples was assessed for the samples' EGFR *T790M mutation status by droplet digital polymerase chain reaction (ddPCR) and then compared to their prior/concurrent surgical or cytologic samples tested by either Sanger sequencing or next-generation sequencing (NGS). B, High concordance was observed among the tested samples, with a single false-positive and a single false-negative result for detecting* EGFR *T790M mutation and an overall sensitivity and specificity of 93% and 96%, respectively. Abbreviations: TF, tumor fraction; Neg, negative; Pos, positive.*

extraction, the median number of slides used was 1 smear (range, 1–5), with a combination of both Diff Quik–stained and Papanicolaou-stained smears. The median DNA concentration in these samples was 17.6 ng/μL (range, 2.1–136 ng/μL). No difference in DNA yield was seen between cases that used direct smears versus those that used cell block sections.

Of the 26 samples in the validation set that had a concurrent NGS analysis on the same specimen, 8 samples (31%) had a known *EGFR* T790M mutation, in addition to an *EGFR* sensitizing mutation, 3 samples (11%) had an *EGFR* sensitizing mutation but were negative for *EGFR* T790M, and the remaining 15 samples (58%) were wild-type for *EGFR* mutations (Table 2). Of the 8 cases with the *EGFR* T790M mutation, 7 had prior documentation of an *EGFR* sensitizing mutation and a history of TKI therapy with clinical suspicion for secondary resistance to therapy. The ddPCR assay was able to successfully detect the *EGFR* T790M mutation in all 8 samples (100% concordance). Of the remaining 18 samples that were wild-type for *EGFR* T790M mutation by NGS, 17 (94%) were wild-type by the ddPCR assay, with a single sample (case 23) showing a borderline positive result for *EGFR* T790M mutation (AF, 0.12; 6 positive droplets), just above the cutoff threshold (ie, 5 positive droplets) for the assay. This was an FNA sample of a high-grade serous carcinoma that did not have any known *EGFR* sensitizing mutations or a history of TKI therapy, and the corresponding NGS analysis was negative for the *EGFR* T790M mutation. The ddPCR assay could not be repeated or confirmed on an orthogonal platform because of insufficient DNA for further evaluation.

The 16 samples included in the test set for this study were all cases of metastatic lung adenocarcinoma with known *EGFR* sensitizing mutations that were on TKI therapy and subsequently developed acquired resistance with the *EGFR* T790M mutation. Priority was given to cases with borderline or relatively low tumor fraction, with 67% of the samples showing an estimated tumor fraction of 20% or less. Of these, 4 cases had a tumor fraction of 10%, 3 cases had 5%, and 2 cases had 1%. A single Diff-Quik/Papanicolaou-stained smear from all 16 samples yielded adequate amounts of DNA for the ddPCR assay. The results from the ddPCR assay were compared to those of the patients with known *EGFR* T790M status at the time of the cytologic procedure. Of these, 14 cases (88%) had a known *EGFR* sensitizing mutation, with 7 cases (44%) harboring a known *EGFR* T790M mutation. The ddPCR assay was able to successfully detect the *EGFR* T790M mutation in 6 of these cases (86%), with a single case (case 31) that failed to detect the known *EGFR* T790M mutation (Table 2).

Five cases that were negative for *EGFR* T790M mutation (by sequencing of a prior sample) were also negative by ddPCR, whereas the *EGFR* T790M mutation status was unknown at the time of collection in 4 samples. Of these, 2 cases (cases 27 and 40) had a prior sample that showed only the *EGFR* sensitizing mutation, but testing on subsequent tissue samples revealed an acquired *EGFR* T790M mutation.

Cases 37 to 40 were samples collected from the same patient within 3 months of each other. The first 2 samples (cases 37 and 38) were collected prior to any mutation testing and were negative for *EGFR* T790M mutation by ddPCR. Sample 39, negative for *EGFR* T790M mutation by ddPCR, was collected concurrently at the time of a tissue biopsy which showed only an *EGFR* L858R mutation by NGS analysis. The patient was started on TKI therapy; however, after an initial response the patient showed progression of disease, and a bronchial brushing specimen (case 40) was collected. The tumor fraction of this bronchial brushing specimen was low (20%), thus precluding NGS analysis; however, the ddPCR assay was able to successfully detect the *EGFR* T790M mutation from this specimen (Table 2). The overall sensitivity and specificity of the ddPCR assay for detecting *EGFR* T790M mutation were 93% and 96%, respectively (Figure).

Table 2. *EGFR* T790M Genotyping by Droplet Digital Polymerase Chain Reaction in Cytology Samples in Patients With Known *EGFR* Mutation Status

							EGFR T790M ddPCR (Cytology)					
Case	Diagnosis	Site	Tumor, %	EGFR Sensitizing Mutation	TKI	EGFR T790M Status (Cytology/Tissue)	T790M Status	T790M FA	No. of Positive Droplets	No. of Total Droplets	Concurrent Specimen	Concordance
1	Metastatic lung ADC	ST	70	Exon 19 del	Yes	Mutant	Mutant	30.45	1605	18 074	Yes	TP
2	Metastatic lung ADC	Kidney	85	Exon 19 del	Yes	Mutant	Mutant	32.65	292	18 808	Yes	TP
3	Lung ADC	Lung	90	Exon 19 del	Yes	Mutant	Mutant	18.25	274	17 566	Yes	TP
4	Metastatic lung ADC	ST	30	Exon 19 del	No	WT	WT	0.08	3	17 859	Yes	TN
5	Metastatic lung ADC	Lung	40	WT	No	WT	WT	0.18	4	18 514	Yes	TN
6	Lung ADC	LN	60	WT	No	WT	WT	0.1	4	17 449	Yes	TN
7	Metastatic lung ADC	LN	30	L858R	No	Mutant[a]	Mutant	14.25	541	16 629	Yes	TP
8	Lung ADC	Lung	60	Exon 19 del	Yes	Mutant	Mutant	16.4	802	17 925	Yes	TP
9	Lung ADC	Lung	65	L858R	Yes	Mutant	Mutant	7.75	424	16 298	Yes	TP
10	Lung ADC	Lung	70	Exon 19 del	Yes	Mutant	Mutant	25.35	1254	10 748	Yes	TP
11	Metastatic lung ADC	ST	70	Exon 19 del	Yes	Mutant	Mutant	10.6	626	17 680	Yes	TP
12	Lung ADC	Lung	21	WT	No	WT	WT	0.12	2	17 016	Yes	TN
13	Lung ADC	Lung	30	WT	No	WT	WT	3.95	2	15 282	Yes	TN
14	Metastatic lung ADC	LN	50	E709K; G719A	No	WT	WT	0.14	1	19 247	Yes	TN
15	Metastatic lung ADC	Pl fluid	50	WT	No	WT	WT	0.06	1	17 444	Yes	TN
16	Metastatic lung ADC	LN	60	WT	No	WT	WT	21.75	0	14 936	Yes	TN
17	Lung ADC	Lung	80	WT	No	WT	WT	0.07	2	16 706	Yes	TN
18	Metastatic lung ADC	ST	80	WT	No	WT	WT	0.49	2	15 896	Yes	TN
19	Metastatic lung ADC	LN	90	WT	No	WT	WT	18.3	3	15 485	Yes	TN
20	Metastatic lung ADC	LN	50	WT	No	WT	WT	0.19	3	10 150	Yes	TN
21	Metastatic NSCLC	LN	70	Exon 19 del	No	WT	WT	0.6	2	13 546	Yes	TN
22	Metastatic gastric ADC	LN	30	WT	No	WT	WT	0.41	0	16 460	Yes	TN
23	Metastatic HG Serous CA	ST	25	WT	No	WT	Mutant[b]	0.12	6	19 132	Yes	FP
24	Metastatic SCC	LN	80	WT	No	WT	WT	0.05	2	18 683	Yes	TN
25	Metastatic breast CA	ST	90	WT	No	WT	WT	0.34	2	17 630	Yes	TN
26	Metastatic SqCell CA	Thyroid	90	WT	No	WT	WT	0.04	1605	18 683	Yes	TN
27	Metastatic lung ADC	Pl fluid	5	Exon 19 del	Yes	Unknown[c]	Mutant	0.45	5	16 430	No	-
28[g]	Metastatic lung ADC	Pl fluid	20	L858R	No	WT	WT	0.25	1	16 285	Yes	TN
29[g]	Metastatic lung ADC	Pl fluid	60	L858R	No	WT	WT	0.7	2	18 070	Yes	TN
30	Metastatic lung ADC	Pl fluid	1	Exon 19 del	No	WT	WT	0.32	2	12 830	No	TN
31	Metastatic lung ADC	Pl fluid	1	Exon 19 del	Yes	Mutant	WT[d]	1.15	1	15 171	No	FN
32[h]	Metastatic lung ADC	Pl fluid	60	Exon 19 del	Yes	Mutant	Mutant	26	60	18 343	Yes	TP
33[h]	Metastatic lung ADC	Pl fluid	5	Exon 19 del	Yes	Mutant	Mutant	36	135	18 356	No	TP
34	Metastatic lung ADC	BAL	10	Exon 19 del	Yes	Mutant	Mutant	7	40	14 829	Yes	TP
35[i]	Metastatic lung ADC	LN	5	G719S	No	Mutant[e]	Mutant	56	485	17 635	No	TP
36[i]	Metastatic lung ADC	BAL	10	G719S	No	Mutant[e]	Mutant	52	617	16 330	No	TP
37[j]	Metastatic lung ADC	Pl fluid	30	unknown	No	Unknown[f]	WT	0.15	1	17 531	No	-
38[j]	Metastatic lung ADC	Pl fluid	40	unknown	No	Unknown[f]	WT	0.15	2	17 510	No	-
39[j]	Metastatic lung ADC	Pl fluid	10	L858R	No	WT	WT	0	0	18 005	Yes	TN

Table 2. Continued

Case	Diagnosis	Site	Tumor, %	EGFR Sensitizing Mutation	TKI	EGFR T790M Status (Cytology/Tissue)	EGFR T790M ddPCR (Cytology)				Concurrent Specimen	Concordance
							T790M Status	T790M FA	No. of Positive Droplets	No. of Total Droplets		
40[j]	Metastatic lung ADC	BB	20	L858R	Yes	Unknown[f]	Mutant	0.7	5	14 868	No	-
41	Metastatic lung ADC	Pl fluid	10	Exon 19 del	No	WT	WT	0.45	3	14 469	No	TN
42	Metastatic lung ADC	Pl fluid	60	Exon 19 del	Yes	Mutant	Mutant	22.8	286	15 520	No	TP

Abbreviations: ADC, adenocarciNoma; BAL, bronchoalveolar lavage; BB, bronchial brushing; CA, carcinoma; del, deletion; FA, fractional abundance; FN, false negative; FP, false positive; HG, high grade; LN, lymph node; NSCLC, non–small cell lung carcinoma; PCR, polymerase chain reaction; Pl, pleural; SCC, small cell carcinoma; SqCell, squamous cell; ST, soft tissue; TN, true negative; TP, true positive; WT, wild-type.

[a] De novo T790M mutation.

[b] Borderline false positive (allelic frequency, 0.12; 6 positive droplets).

[c] Patient with known EGFR exon 19 deletion on prior biopsy, treated with TKI for 1 year; T790M mutation status unknown at the time of this procedure; however, T790M mutation detected on subsequent surgical biopsy 1 month later.

[d] False-negative result in patient with biopsy-proved T790M mutation.

[e] Germ-line T790M.

[f] Patient with 2 pleural effusion samples (Nos. 37 and 38) prior to any mutation testing; EGFR L858R mutation detected in surgical biopsy collected at the same time as case 39; patient treated with TKI; EGFR T790M mutation status unknown at the time of case 40 but detected on subsequent surgical biopsy (4 months later).

[g,h,i,j] Samples from the same patient at different time points.

DISCUSSION

Advanced-stage NSCLC patients with *EGFR* sensitizing mutations who are being treated with EGFR-targeted TKI therapy frequently present with resistance to TKI therapy, with recurrence and/or progression of disease. The *EGFR* T790M mutation in exon 20 is the most commonly detected acquired resistance mechanism in these patients, and the revised CAP/IASLC/AMP lung cancer molecular testing guideline strongly recommend testing specifically for this mutation using a high-sensitivity mutational assay to select patients for third-generation EGFR-targeted TKIs.[24] However, low tumor fractions in the posttreatment biopsies and effusion specimens frequently make the detection of the *EGFR* T790M mutation quite challenging.[13,14] Therefore, in this study we evaluated the validity and feasibility of using a high-sensitivity ddPCR assay to interrogate different cytologic specimen preparations for *EGFR* T790M mutation.

Our samples included direct smears and cell blocks from a variety of FNA samples, pleural effusions, bronchial brushing, and lavages. All samples yielded adequate amounts of DNA. The validation set of samples, which had NGS analysis performed from the same cytologic specimen, had adequate DNA for both NGS analysis as well as the ddPCR assay. The *EGFR* T790M mutation status detected by ddPCR showed high concordance (96%) with that of the NGS analysis. A single false-positive result with borderline positivity was detected in one of the non-NSCLC cases, indicating the need to exert some caution in samples with borderline positivity, including low AF and low numbers of positive droplets. Repeat analysis or evaluation on a high sensitivity orthogonal platform may be needed in such cases.

The *EGFR* T790M status by ddPCR in the test cases showed 94% concordance with that of the NGS analysis results, with a single sample resulting in a false-negative result. The false-negative result was likely due to the low tumor fraction (estimated at 1%). Although the *EGFR* T790M mutation status in the remaining low–tumor fraction samples, including a second sample with 1% tumor fraction, were concordant with the results of the NGS assay, these results would indicate that one needs to exert some caution in very low tumor fraction samples (<5%) to avoid risking a false-negative result. This caveat has been demonstrated in other studies and recently highlighted in the CAP/IASLC/AMP guidelines that the absence of a mutation in very low tumor fraction samples does not necessarily exclude the possibility of a mutation.[12]

The ddPCR assay also successfully detected an *EGFR* T790M mutation in a low tumor (20%) bronchial brushing specimen (case 40), deemed inadequate for NGS analysis, in a patient with suspected secondary resistance to TKI therapy. The ability of the ddPCR assay to successfully detect the *EGFR* T790M mutation from this specimen highlights the utility of having a high-sensitivity assay for low–tumor fraction samples that can identify patients with acquired resistance and select those eligible for third-generation EGFR-targeted TKIs. This can avoid the need for additional liquid biopsy testing or an invasive repeat biopsy (as done in the case of this patient), thus improving patient morbidity by allowing the patient to rapidly switch to a third-generation TKI.

In addition, the ddPCR assay identified 1 patient (cases 35 and 36) with tumor fractions of 5% and 10% in 2 samples collected within 8 months of each other that demonstrated

EGFR T790M mutations at AFs of 56% and 52%, respectively, raising suspicion for a de novo mutation. A corresponding surgical biopsy evaluated by NGS confirmed the germ-line *EGFR* T790M mutation. Germ-line *EGFR* T790M mutations have been reported in the literature as a cause of possible familial predisposition for the development of lung carcinoma and may prompt the patient to be referred for genetic counseling.[25–27]

In advanced-stage *EGFR*-mutated NSCLC patients who demonstrate progression or relapse after an initial response to EGFR TKI therapy, detecting a low subclonal *EGFR* T790M mutation from a posttreatment low–tumor fraction specimen can be challenging. Therefore, it is imperative that molecular laboratories validate high-sensitivity assays for the detection of *EGFR* T790M mutation from these samples. Several studies have reported the utility of ultrasensitive assays, such as ddPCR, using circulating tumor DNA (ctDNA) and cfDNA from the patient's plasma.[15,16,18,28] The CAP/IASLC/AMP guidelines recommend evaluating for *EGFR* T790M mutation from plasma in the setting of suspected EGFR-TKI resistance when a tissue sample is unavailable or insufficient, because a positive result can guide therapy with osimertinib.[12] However, plasma-based cfDNA analysis for *EGFR* T790M has intermediate sensitivity (0.4–0.78), and a tissue biopsy is recommended if the results are negative.[12] Our results indicate that ddPCR for the detection of *EGFR* T790M mutation is a highly sensitive and specific (sensitivity and specificity of 93% and 96%, respectively) assay that can be used reliably in cytologic specimens, including samples with low tumor fraction, thus providing a higher-sensitivity alternative to plasma-based cfDNA analysis. However, some caution needs to be exerted in samples with very low tumor fraction (<5%) and with borderline positivity results (low AF and/or low numbers of positive droplets) to avoid the risk of false-negative and false-positive results, respectively. Future studies with a larger sample size will be needed to support the findings of this study. The turnaround time of *EGFR* T790M ddPCR assay is significantly lower than that of NGS analysis (2–3 days versus 5–7 days, respectively), thus allowing the patient to rapidly switch to third-generation TKIs, if positive for acquired resistance mutation. The integration of *EGFR* T790M detection by ddPCR into clinical practice will likely reduce the inadequate rate for low–tumor fraction samples, without the need for a repeat biopsy, and provide a rapid and cost-effective way to select patients for third-generation EGFR-targeted TKIs.

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References

1. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304(5676):1497–1500.

2. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A*. 2004; 101(36):13306–13311.

3. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350(21):2129–2139.

4. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med*. 2005;2(3):e73.

5. Engelman JA, Janne PA. Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res*. 2008;14(10):2895–2899.

6. Yu HA, Arcila ME, Rekhtman N, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. *Clin Cancer Res*. 2013;19(8):2240–2247.

7. Remon J, Steuer CE, Ramalingam SS, Felip E. Osimertinib and other third-generation EGFR TKI in EGFR-mutant NSCLC patients. *Ann Oncol*. 2018; 29(suppl 1):i20–i27.

8. Buttner R, Wolf J, Thomas RK, Sos ML. Resistance mechanisms to AZD9291 and rociletinib-response. *Clin Cancer Res*. 2017;23(14):3967–3968.

9. Janne PA, Yang JC, Kim DW, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. *N Engl J Med*. 2015;372(18):1689–1699.

10. Sequist LV, Soria JC, Goldman JW, et al. Rociletinib in EGFR-mutated non-small-cell lung cancer. *N Engl J Med*. 2015;372(18):1700–1709.

11. Ariyasu R, Nishikawa S, Uchibori K, et al. High ratio of T790M to EGFR activating mutations correlate with the osimertinib response in non-small-cell lung cancer. *Lung Cancer*. 2018;117:1–6.

12. Lindeman NI, Cagle PT, Aisner DL, et al. Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *Arch Pathol Lab Med*. 2018;142(3):321–334.

13. Arcila ME, Oxnard GR, Nafa K, et al. Rebiopsy of lung cancer patients with acquired resistance to EGFR inhibitors and enhanced detection of the T790M mutation using a locked nucleic acid-based assay. *Clin Cancer Res*. 2011;17(5): 1169–1180.

14. Janne PA. Challenges of detecting EGFR T790M in gefitinib/erlotinib-resistant tumours. *Lung Cancer*. 2008;60(suppl 2):S3–S9.

15. Garcia J, Dusserre E, Cheynet V, et al. Evaluation of pre-analytical conditions and comparison of the performance of several digital PCR assays for the detection of major EGFR mutations in circulating DNA from non-small cell lung cancers: the CIRCAN_0 study. *Oncotarget*. 2017;8(50):87980–87996.

16. Sorber L, Zwaenepoel K, Deschoolmeester V, et al. Circulating cell-free nucleic acids and platelets as a liquid biopsy in the provision of personalized therapy for lung cancer patients. *Lung Cancer*. 2017;107:100–107.

17. Bordi P, Del Re M, Minari R, et al. From the beginning to resistance: study of plasma monitoring and resistance mechanisms in a cohort of patients treated with osimertinib for advanced T790M-positive NSCLC. *Lung Cancer*. 2019;131:78–85.

18. Minari R, Bordi P, Del Re M, et al. Primary resistance to osimertinib due to SCLC transformation: issue of T790M determination on liquid re-biopsy. *Lung Cancer*. 2018;115:21–27.

19. Garcia J, Wozny AS, Geiguer F, et al. Profiling of circulating tumor DNA in plasma of non-small cell lung cancer patients, monitoring of epidermal growth factor receptor p.T790M mutated allelic fraction using beads, emulsion, amplification, and magnetics companion assay and evaluation in future application in mimicking circulating tumor cells. *Cancer Med*. 2019;8(8): 3685–3697.

20. Takahama T, Sakai K, Takeda M, et al. Detection of the T790M mutation of EGFR in plasma of advanced non-small cell lung cancer patients with acquired resistance to tyrosine kinase inhibitors (West Japan oncology group 8014LTR study). *Oncotarget*. 2016;7(36):58492–58499.

21. Malapelle U, Pisapia P, Rocco D, et al. Next generation sequencing techniques in liquid biopsy: focus on non-small cell lung cancer patients. *Transl Lung Cancer Res*. 2016;5(5):505–510.

22. Sakai K, Horiike A, Irwin DL, et al. Detection of epidermal growth factor receptor T790M mutation in plasma DNA from patients refractory to epidermal growth factor receptor tyrosine kinase inhibitor. *Cancer Sci*. 2013;104(9):1198–1204.

23. Wang Z, Chen R, Wang S, et al. Quantification and dynamic monitoring of EGFR T790M in plasma cell-free DNA by digital PCR for prognosis of EGFR-TKI treatment in advanced NSCLC. *PloS One*. 2014;9(11):e110780.

24. Lindeman NI, Cagle PT, Beasley MB, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. *J Mol Diagn*. 2013;15(4):415–453.

25. Sukari A, Nagasaka M, Wakeling E. EGFR-mutant non-small cell lung cancer in the era of precision medicine: importance of germline EGFR T790M testing. *J Natl Compr Canc Netw*. 2017;15(10):1188–1192.

26. Gazdar A, Robinson L, Oliver D, et al. Hereditary lung cancer syndrome targets never smokers with germline EGFR gene T790M mutations. *J Thorac Oncol*. 2014;9(4):456–463.

27. Yu HA, Arcila ME, Harlan Fleischut M, et al. Germline EGFR T790M mutation found in multiple members of a familial cohort. *J Thorac Oncol*. 2014; 9(4):554–558.

28. Vendrell JA, Mazieres J, Senal R, et al. Ultra-sensitive EGFR (T790M) detection as an independent prognostic marker for lung cancer patients harboring EGFR (del19) mutations and treated with first-generation TKIs. *Clin Cancer Res*. 2019;25(14):4280–4289.