Multiplex picoliter-droplet digital PCR for quantitative assessment of *EGFR* mutations in circulating cell-free DNA derived from advanced non-small cell lung cancer patients

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Abstract. To explore the possible diagnostic value of liquid biopsy, two multiplex panels using picoliter-droplet digital polymerase chain reaction (ddPCR) were established to quantitatively assess the epidermal growth factor receptor (*EGFR*) mutations in cell-free DNA (cfDNA) extracted from the plasma of advanced non-small cell lung cancer (NSCLC) patients. Plasma samples derived from 22 patients with stage IIIB/IV NSCLC harboring *EGFR* mutations in matched tumor tissues confirmed by amplification refractory mutation system (ARMS) analysis were subjected to two multiplex ddPCR panels to assess the abundance of tyrosine kinase inhibitor (TKI) -sensitive (19DEL, L858R) and TKI-resistant (T790 M) mutations. Fluctuations in *EGFR* mutant abundance were monitored by either of the multiplex ddPCR panels for three patients undergoing EGFR-TKI treatment, with serial plasma sample collections over 2 months. The multiplex ddPCR panels applied to plasma cfDNA from advanced NSCLC patients achieved a total concordance rate of 80% with the *EGFR* mutation profiles obtained by ARMS from matched biopsy tumor specimens (90% for 19DEL, 95% for L858R, 95% for T790M, respectively) and revealed additional mutant alleles in two subjects. The respective sensitivity and specificity were 90.9 and 88.9% for 19DEL, 87.5 and 100% for L858R, 100 and 93.8% for T790M. The fluctuations of *EGFR* mutant abundance in serial plasma cfDNA were in accordance with the changes in tumor size as assessed by imaging scans. The authors demonstrated the utility of multiplex ddPCR panels with ultra-sensitivity for quantitative analysis of *EGFR* mutations in plasma cfDNA and obtained promising usefulness in EGFR-TKI decision-making for advanced NSCLC patients.

Introduction

Non-small cell lung cancers (NSCLCs) account for >80% of all lung cancers and represent the leading cause of cancer mortality worldwide, and in China alone (1,2). Most NSCLC patients are initially diagnosed with advanced-stage disease, which is defined as an unresectable, widely metastatic tumor and is followed by poor prognosis.

Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) are helpful for patients with *EGFR*-activating mutations, especially exon 19 deletions (19DEL) and exon 21 L858R mutations (L858R), which account for 85% of all clinically important mutations (progression-free survival (PFS) was 13.1 months with erlotinib compared to 4.6 months with platinum-based chemotherapy and 8.0 months with gefitinib compared to 6.3 months with platinum-based chemotherapy) (3,4). However, on average, most patients undergoing EGFR-TKI treatment develop resistance within 1 year (5,6), and >50% of them carry the exon 20 T790M mutation (T790 M) in *EGFR* (7). Recently, the efficiency of AZD9291, a third-generation EGFR-TKI, was demonstrated in lung cancer patients resistant to prior therapy with EGFR-TKIs who harbored T790M (PFS was 9.6 months in T790M-positive patients and 2.8 months in T790M-negative patients) (8). Increasing evidence has suggested that there may be a low-abundance,

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intrinsic T790M mutation prior to EGFR-TKI therapy (9-11), which enforces the need for exploring T790M status before treatment. American Society of Clinical Oncology, European Society for Medical Oncology and National Comprehensive Cancer Network guidelines recommend clarifying *EGFR* mutation profiles before TKI therapy or after the emergence of TKI resistance to provide a precise and effective therapeutic regimen (12-14).

DNA extracted from tumor tissue remains the major source for mutation analysis in clinical settings. Circulating cell-free DNA (cfDNA) in plasma provides a homogeneous representation of all tumor DNA and can help implement non-invasive, continual monitoring of tumor mutation profiles while obviating the reliance on invasive biopsies, tissue archives and tumor heterogeneity. However technical challenges remain in analyzing plasma cfDNA for *EGFR* mutations, such as the low quantity and variability in the tumor-derived cfDNA fraction between individuals.

Picoliter-droplet digital polymerase chain reaction (ddPCR) is a promising approach with ultra-sensitive detection and absolute quantification. This method compartmentalizes samples into millions of picoliter droplets containing single DNA molecules and analyses the terminal fluorescence of each droplet after parallel amplification. Multiplex ddPCR can effectuate multiple mutation assays simultaneously in one well with minimal DNA sample consumption (15).

This study developed two multiplex ddPCR panels for quantitative analysis of treatment-related *EGFR* mutations in plasma cfDNA and compared the results generated by multiplex ddPCR assays on plasma samples and by amplification refractory mutation system (ARMS) assays on matched tumor tissue specimens from advanced NSCLC patients.

Materials and methods

Design of the multiplex ddPCR panels. The authors developed two independent multiplex ddPCR panels for clinical application, a 4-plex panel for 19DEL and T790M mutations plus the corresponding wild-type assays and a 5-plex panel to identify L858R (L858R-1 and L858R-2) and T790M mutations.

Tables I and II present the sequences and concentrations of primers and TaqMan® probes (Thermo Fisher Scientific, Inc., Waltham, MA, USA) used (16,17). Probes for 19DEL were targeted at in-frame deletions in exon 19 of the *EGFR* polypeptide. The probes recognized c. 2573T>G for L858R-1, c. 2573T>G and c. 2574G>T for L858R-2, and c. 2369C>T for T790M. The PCR mixture for each multiplex panel consisted of 20 μl TaqMan® Genotyping Master Mix (Thermo Fisher Scientific, Inc.), 1.6 μl Droplet Stabilizer (RainDance Technologies, Lexington, MA, USA), forward and reverse primers, and probes in different concentrations and contained at least 50 ng of target DNA template to a final reaction volume of 40 μl.

Plasmid DNA preparation and DNA controls. The performance of each ddPCR assay was assessed using fragmented plasmid DNA containing wild-type or mutant *EGFR* sequence. Plasmids carrying wild-type (Plasmid #11011) or 19DEL mutant (Plasmid #32062) sequences were purchased from Addgene (http://www.addgene.org). The point

mutations L858R-1, L858R-2 and T790M were introduced using a Phusion Site-Directed Mutagenesis kit (Thermo Fisher Scientific, Inc.), following the manufacturer's procedure (18), and were confirmed by Sanger sequencing. Plasmid containing mutant sequence was serially diluted with plasmid containing wild-type sequence to yield a mixture in which the mutant copy number was approximately 0.01-10% of the wild-type copy number.

Patients and sample collection. The *EGFR* mutation status of 33 plasma samples was assessed from 25 NSCLC patients with stage IIIB/IV cancers that were enrolled from Zhongshan Hospital (Shanghai, China) from January 2015 to December 2015 and harbored *EGFR* mutations, confirmed by ARMS analysis of tumor tissue. Tumor specimens in the present study were taken for clinical diagnostic purpose, which from the 19 patients were obtained before EGFR-TKI therapy and re-biopsies from 3 patients were taken after acquiring resistance to EGFR-TKIs. Contemporaneous plasma samples were subjected to the multiplex ddPCR panels. Another 3 patients were treated with gefitinib at the physician's discretion and were recruited for serial plasma samples collection at 2-month intervals to monitor the fluctuations in *EGFR* mutation abundance by ddPCR assays. These patients also received the appropriate imaging diagnoses, including computed tomography scans performed for Response Evaluation Criteria in Solid Tumors-based treatment response evaluation (19). The study was approved by the Ethics Committee of Fudan University (Shanghai, China) and informed consent was obtained from the all for use of the blood sample and tumor tissue.

DNA extraction. Peripheral blood samples (10 ml) were collected into EDTA-K$_2$ containing tubes. Blood samples were centrifuged for 10 min at 1,900 x g to obtain preliminary supernatants within 2 h following sampling. Afterwards, another centrifugation at 16,000 x g for 10 min at 4°C separated the plasma samples from the blood cell pellet. Circulating cfDNA was extracted using a QIAamp Circulating Nucleic Acid kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and was eluted with 50 μl Tris-EDTA buffer. All tumor specimens were collected for diagnostic purpose by tracheobronchoscopy, percutaneous puncture biopsy or metastatic lymph node biopsies and reviewed by a pathologist for tumor cell content. The tumor tissue DNA was extracted from formalin-fixed and paraffin-embedded diagnostic specimens using a QIAamp DNA FFPE Tissue kit (Qiagen GmbH) following the manufacturer's instructions and was eluted with 100 μl Buffer AE (Qiagen GmbH, Hilden, Germany). The extracted cfDNA and tumor tissue DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

EGFR mutations in tumor tissue specimens detected by ARMS. *EGFR* mutations in tumor tissue specimens were detected using an ADx-ARMS kit (Amoy Diagnostics, Xiamen, China), which was approved by the Chinese Food and Drug Administration for *in vitro* diagnostic use. All experiments and genotype determinations were performed according to the manufacturer's instructions and as previously described (20).

Table I. Primers used for the multiplex droplet digital PCR. The total volume of the PCR mixture was 40 μl, with 0.5 μM each of forward and reverse primers included in each assay.

Targeted sequences	Forward primer (5'-3')	Reverse primer (5'-3')
19DEL[a] (c.746-c.750)	GAAAGTTAAAATTCCCGTCGCTAT	ACCCCCACACAGCAAAGC
L858R-1 (c.2573T>G)	GCAGCATGTCAAGATCACAGATT	CCTCCTTCTGCATGGTATTCTTTCT
L858R-2 (c.2573T>G, c.2574G>T)		
T790M (c.2369C>T)	CCTCACCTCCACCGTGCA	AGGCAGCCGAAGGGCA

[a]This assay was designed to detect 34 representative exon 19 deletions covering >95% of deletion mutations: ΔK745_E749, K745_T751>K, ΔE746_A750, E746_R748>E, E746_A750>IP, E746_T751>IP, E746_T751>I, E746_T751>V, E746_T751>A, E746_T751>VA, E746_S752>I, E746_751T>E, E746_S752>A, E746_S752>V, E746_P753>VS, E746_A755>E, ΔE746_T751, ΔE746_S752, E746_S752>D, ΔE746_S753, ΔL747_E749, ΔL747_A750, L747_A750>P, ΔL747_T751, L747_T751>Q, L747_T751>P, L747_T751>S, ΔL747_S752, L747_S752>Q, ΔL747_P753, L747_P753>V, L747_P753>S, L747_P753>Q, and S752-I 759del. PCR, polymerase chain reaction; 19DEL, exon 19 deletions; L858R, exon 21 L858R mutation; T790M, exon 20 T790M mutation.

Table II. TaqMan® probes used for the multiplex ddPCR.

Assay	Probes	Reporter dye	Sequence (5'-3')	Final concentration (μM)
4-plex ddPCR panel assay	WT (19DEL)	VIC	AATTAAGAGAAGCAACATC	0.2
	Exon 19 reference-type	FAM	ACATCGAGGATTTCCTTGT	0.1
	WT (T790M)	TET	T+CATC+A+C+GC/ZEN/A+GCTC	0.2
	T790M	FAM	T+CATC+A+T+GC/ZEN/A+GCTC	0.2
5-plex ddPCR panel assay	WT (L858R)	VIC	AGTTTGGCCAGCCCAA	0.1
	L858R-1	FAM	AGTTTGGCCCGCCCAA	0.1
		VIC	AGTTTGGCCCGCCCAA	0.2
	L858R-2	FAM	AGTTTGGCACGCCCAA	0.2
		VIC	AGTTTGGCACGCCCAA	0.2
	WT (T790M)	TET	T+CATC+A+C+GC/ZEN/A+GCTC	0.2
	T790M	FAM	T+CATC+A+T+GC/ZEN/A+GCTC	0.1

ddPCR, picoliter-droplet digital polymerase chain reaction; WT, wild type; 19DEL, exon 19 deletions; L858R, exon 21 L858R mutation; T790M, exon 20 T790M mutation; VIC, green fluorescent protein; FAM, 6-carboxyfluorescein; TET, tetrachlorofluorescein.

Circulating cfDNA detected by multiplex ddPCR panels. The multiplex ddPCR panels were performed on a RainDrop™ system (RainDance Technologies) with two separate microfluidic chips, each composed of eight panels for droplet generation (Source Chip; RainDance Technologies) and fluorescence detection (Sense Chip; RainDance Technologies). The cycling was performed on a Veriti® Thermal Cycler with a hot lid (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: Initiation at 50°C for 2 min, incubation at 95°C for 10 min, and then 45 cycles of 95°C for 15 sec and 60°C for 1 min (using a 0.5°C/min ramp rate). These cycles were followed by a final step at 98°C for 10 min and a 12°C hold. The endpoint fluorescence signal of each droplet was scanned and analyzed using RainDrop Analyst software II version 1.6.0_43-b01 (RainDance Technologies), following the manufacturer's instructions. Spectral compensation was applied to each assay to eliminate the contamination signals between different fluorophores. To eliminate the crosstalk fluorescence signals from the VIC/TET and FAM fluorophores, a compensation matrix

was established based on the positive control. Positive and negative controls were included in each run, and gates for the identified clusters were manually selected.

Data analysis. Results were considered negative when the number of droplets within the gated region was under the limit of blank (LOB), otherwise positive when the gated region yielded over 20 copies. Samples with results in the grey area (droplet counts between LOB and 20) were assayed in triplicate and the mean value was recorded. Templates with valid droplet counts <100 were re-extracted. Positive mutant assay results were expressed as the percentage of target templates over the sum of both mutant and wild-type templates.

Statistical analysis. Statistical analysis was performed using SPSS software (version, 17.0; SPSS Inc., Chicago, IL, USA). A Poisson model was used to define the LOB for each mutation in the multiplex panels. The consistency between the results obtained from plasma cfDNA by multiplex ddPCR panels
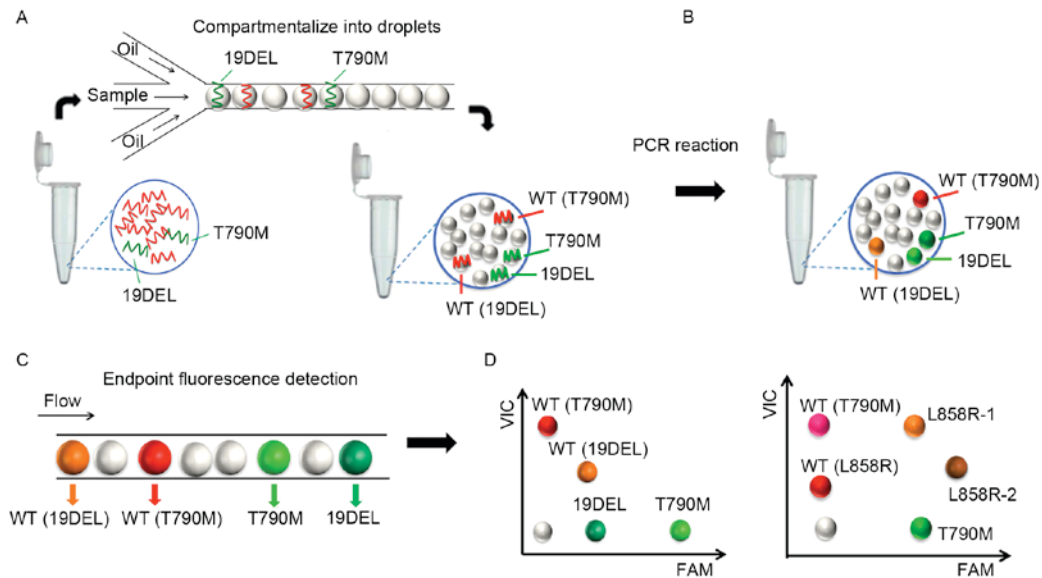
Figure 1. Workflow of the multiplex ddPCR panels. (A) The PCR mixture for each assay consisted of DNA templates, primer pairs, probes, master mix and stabilizer to a final volume of 40 μl and was (B) compartmentalized into >5 million picoliter-sized droplets for independent PCR reactions. (C) The endpoint fluorescence signal of each droplet was scanned and analyzed. (D) Different fluorophores and diverse end point fluorescence intensities distinguished the droplets containing target templates into different clusters. ddPCR, picoliter-droplet digital polymerase chain reaction; WT, wild-type; 19DEL, exon 19 deletions; L858R-1, exon 21 L858R mutation (c.2573T>G); L858R-2, exon 21 L858R mutation (c.2573T>G, c.2574G>T); T790M, exon 20 T790M mutation; FAM, 6-carboxyfluorescein; VIC, green fluorescent protein.

and the results from ARMS assays of matched tumor tissue specimens was calculated using a κ test. A paired Student's t-test was applied to analyze the discrepancy in the abundance of T790M as measured by both multiplex ddPCR panels. The linearity range was assessed using the linear correlation coefficient values (r^2) and slope. P<0.05 was considered to indicate a statistically significant difference.

Results

Multiplex ddPCR panels detected the three most common EGFR mutations. Droplet digital PCR assays compartmentalize the reaction mixture into millions of picoliter-sized droplets so that each contains only one DNA string as the template, and an independently PCR is then performed within the droplets in parallel. Multiplex ddPCR panels can efficiently detect multiple mutations simultaneously in one assay with minimal sample consumption. The abridged general view of the workflow is described in Fig. 1. Different fluorophores and a diverse end point fluorescence intensity determined by the concentration and combination use of TaqMan® probes distinguished the droplets containing specific target templates into different clusters in a 2-dimensional histogram. The cluster representing 19DEL or L858R is located along the diagonal line for the VIC and FAM co-conjugated probes used (as presented in Fig. 2).

Performance assessments of the multiplex ddPCR panels. As the defining characteristic of an assay, the LOB determines the lower limit of detection. The authors subjected fragmented plasmids containing only wild-type sequence to both multiplex ddPCR panels in 24 replicates to observe the false-positive events in each mutant assay, which allowed us to generate the

LOB (the count distribution is presented in Fig. 3). A Poisson model was applied to fit the false-positive distribution, and the LOB was determined using the 95% confidence interval. The LOBs were 5 for 19DEL, 3 for L858R-1, 3 for L858R-2, and 7 for T790M (the LOB for T790M generated from the two panels was the same, data not shown). To assess the specificity of the panel assays, plasmids containing either the L858R-1 or the L858R-2 sequence, which have only one base difference between them, were subjected to the 5-plex panel in triplicate; no cross-reactivity was observed (all false-positive events were less than the LOB, data not shown).

The quantitative performance of multiplex ddPCR panels was assessed using a series of dilutions from fragmented plasmid sequences (Fig. 4). Each dilution was analyzed in triplicate, and the averaged results were plotted on 2-dimensional histograms (as shown in Fig. 4A and C for each panel). The mutant allele expected copy number was plotted against the actual copy number (Fig. 4B and D), and regression analysis revealed a strong correlation (the slopes were 1.008 for 19DEL, 1.007 for T790M, 0.996 for L858R-1, and 0.992 for L858R-2; all slopes had r^2 values greater than 0.990). Detectable abundance ranged from 0.01 to 10%. There was no significant difference between the T790M abundance in the same sample by either panel (t=1.386, P>0.05). Therefore, it follows that the quantitative analysis of 19DEL, L858R-1, L858R-2 and T790M using these two multiplex ddPCR panels is reliable and has ultra-sensitivity, which allows the detection and quantification of mutant alleles, even at ultra-low ratios (0.01%).

Multiplex ddPCR analysis of EGFR mutations in circulating cfDNA from patients with advanced NSCLC. To demonstrate the feasibility of applying these two multiplex panels to clinical practice, analysis was performed on plasma samples
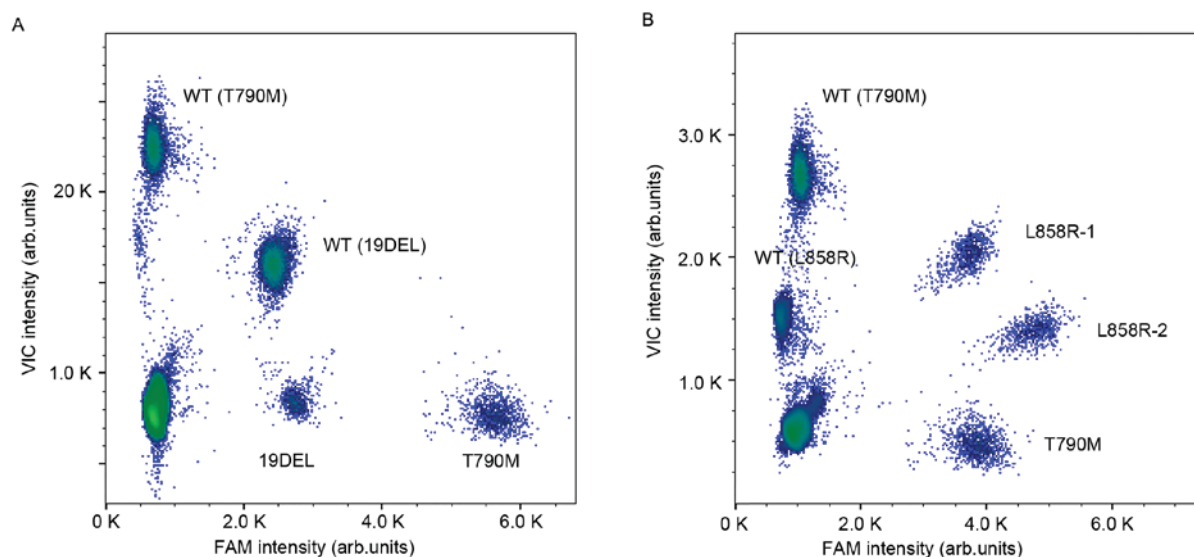
Figure 2. Multiplex picoliter-droplet digital polymerase chain reaction panels for *EGFR* mutation assays. Two-dimensional histograms of the (A) 4-plex and the (B) 5-plex panels. EGFR, epidermal growth factor receptor; FAM, 6-carboxyfluorescein; VIC, green fluorescent protein; WT, wild-type; 19DEL, exon 19 deletions; L858R-1, exon 21 L858R mutation (c.2573T>G); L858R-2, exon 21 L858R mutation (c.2573T>G, c.2574G>T); T790M, exon 20 T790M mutation.
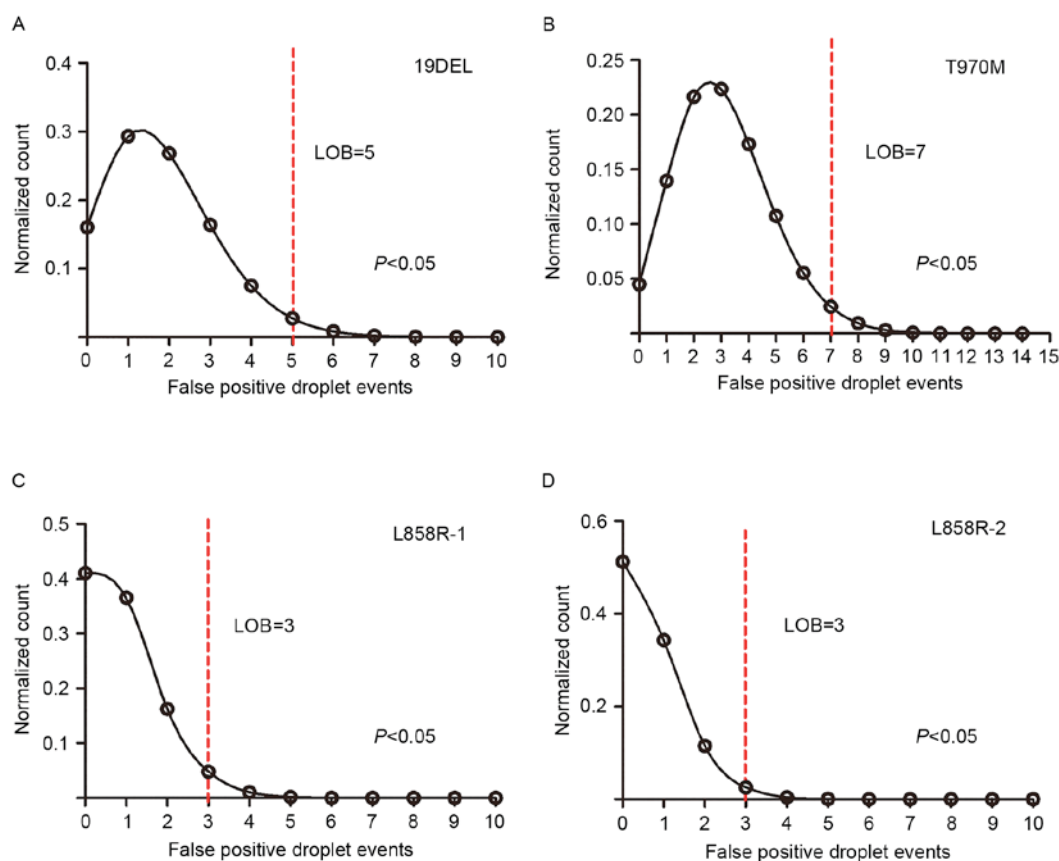

Figure 3. The limit of blank for the multiplex ddPCR panels is generated by observing the false-positive droplet events in assays for each mutant with wild-type templates. Fragmented plasmid containing only wild-type sequence was subjected to the 4-plex and 5-plex panel in 24 replicates. Presented here are the frequency distributions of false-positive events for (A) 19DEL, (B) T790M, (C) L858R-1 and (D) L858R-2. A Poisson model was applied to fit the frequency distribution and LOB for each, as determined by the 95% confidence interval following regression analysis, which is presented by the red cutoff line. 19DEL, exon 19 deletions; L858R, exon 21 L858R mutation; T790M, exon 20 T790M mutation; LOB, limit of blank.

collected from 22 patients with advanced NSCLC whose *EGFR* mutation profiles had been obtained by ARMS in contemporaneously collected tumor tissues (Table III). The inter-individual concentrations of extracted plasma cfDNA
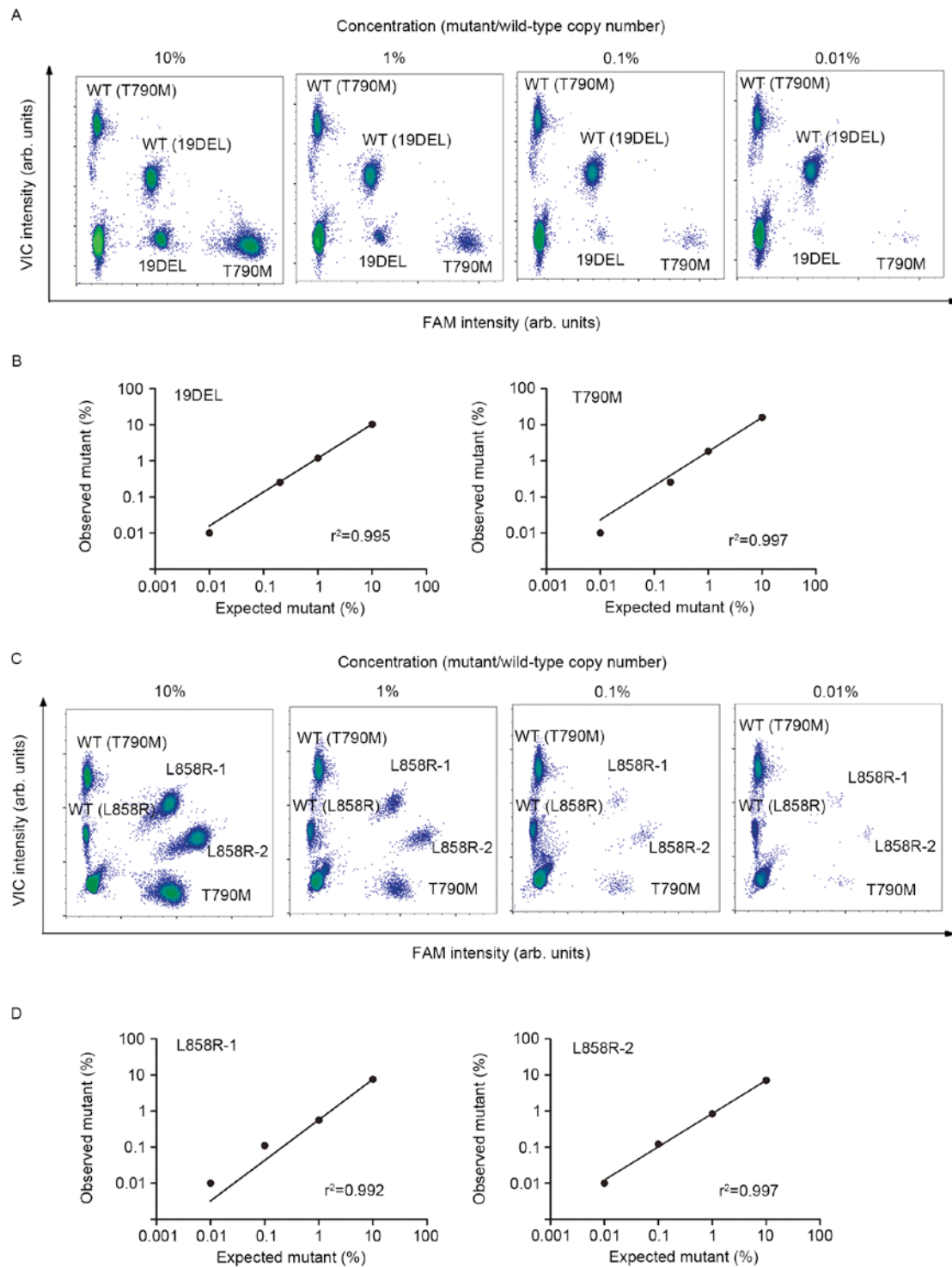
Figure 4. Performance of the multiplex ddPCR panels for quantifying *EGFR* mutants. Samples with descending mutant abundance were subjected to the 4-plex (A) and 5-plex (C) ddPCR panels. Simple linear regression analysis was applied to the expected abundances of mutant alleles and was plotted against the detected abundances of the 4-plex (B) or 5-plex (D) panels. ddPCR, picoliter-droplet digital polymerase chain reaction; EGFR, epidermal growth factor receptor; FAM, 6-carboxyfluorescein; VIC, green fluorescent protein; 19DEL, exon 19 deletions; L858R-1, exon 21 L858R mutation (c.2573T>G); L858R-2, exon 21 L858R mutation (c.2573T>G, c.2574G>T); T790M, exon 20 T790M mutation.

ranged from 16.4 to 102.5 ng/μl, and concentration did not correlate with the content of particular mutant abundance (15). Adequate numbers of valid droplets with >100 counts ensured the validity of each assay; cases 9 and 17, which had no allotment for re-extraction, were excluded because they did not have enough droplets that detected wild-type templates. The overall concordance rate between *EGFR* mutation profiles

obtained from plasma or tumor specimens was 80% (16/20). The concordance rates of the 19DEL, L858R and T790M assays were 90% (k=0.798, P<0.05), 95% (k=0.894, P<0.05), and 95% (k=0.857; P<0.05), respectively, with sensitivities and specificities of 90.9 and 88.9% for 19DEL, 87.5 and 100% for L858R, and 100 and 93.8% for T790M, respectively. The ARMS results from tumor tissue specimens were used as a

Table III. Results generated from plasma cfDNA by multiplex ddPCR panels and tumor tissue DNA by ARMS in advanced NSCLC patients.

Case	Sex	Age (years)	Stage	EGFR-TKI	Site	ARMS on tumor tissue DNA	Multiplex ddPCR panels on plasma cfDNA	
							Mutant	Abundance (%)
1	M	53	IV	No	Lung	19DEL	19DEL[a]	2.00
2	M	53	IV	No	Lung	19DEL	19DEL	15.64
3	F	65	IV	No	Lung	19DEL	19DEL	4.81
4	M	62	IV	No	Lung	19DEL	19DEL	45.68
5	F	41	IV	No	Lung	19DEL	19DEL	5.22
6	F	51	IIIb	No	Lung	19DEL	19DEL	19.62
7	M	46	IV	No	Lung	19DEL	19DEL	12.93
8	F	64	IV	No	Lung	19DEL	N[a]	
9	M	61	IV	No	Lung	19DEL	NE	
10	M	47	IV	No	Lung	L858R	L858R	7.47
11	M	71	IIIb	No	Lung	L858R	L858R[a]	17.74
12	F	63	IV	No	Lung	L858R	L858R[a]	1.32
13	F	58	IV	No	Lung	L858R	L858R	5.78
14	F	69	IV	No	Lung	L858R	L858R[a]	5.63
15	M	35	IIIb	No	Lung	L858R	L858R[a]	3.15
16	F	66	IIIb	No	Lung	L858R	N[a]	
17	F	74	IV	No	Lung	L858R	NE	
18	M	61	IV	Gefitinib	Lung	T790M	T790M[a]	2.91
19	F	48	IV	Gefitinib	Lung	19DEL	19DEL	27.54
						T790M	T790M	23.49
20	F	62	IV	Gefitinib	Lung	19DEL	19DEL	8.64
						T790M	T790M	1.69
21	F	72	IV	No	Lung	19DEL	19DEL	64.02
							T790M[a]	0.91
22	M	68	IV	No	Lung/ lymph node	L858R	19DEL	8.17
						T790M	L858R	4.07
							T790M[a]	3.97

[a]Assays were performed in triplicate and are presented as the mean values (droplet count between limit of blank and 20). ddPCR, picoliter-droplet digital polymerase chain reaction; cfDNA, circulating cell-free DNA; 19DEL, exon 19 deletions; L858R, exon 21 L858R mutations; T790M, exon 20 T790M mutation; site, biopsy site; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; NE, not evaluated; N, negative for three *EGFR* mutant types; ARMS, amplification refractory mutation system.

reference. Plasma cfDNA assays for cases 21 and 22 revealed additional mutant alleles that were not detected in tumor tissue specimens by ARMS. Therefore, the authors reanalyzed the DNA extracted from these tumor specimens in multiplex ddPCR panels and verified the mutations with ultra-low abundance (0.29% for T790M in case 21 and 0.57% for 19DEL in the metastatic tumor biopsied from the lymph node in case 22; data not shown). However, two patients were negative for the expected mutations in plasma cfDNA, which had been detected by ARMS in tumor tissue specimens (19DEL in case 8 and L858R in case 16).

Therapeutic monitoring via the multiplex ddPCR panels. To investigate whether quantitative analysis of cfDNA *EGFR* mutations by either multiplex ddPCR panel could help

monitor treatment response and reveal therapeutic resistance, 3 patients were analyzed using 11 serially collected plasma samples during gefitinib administration. Fig. 5 demonstrated that the *EGFR* mutant abundance in plasma cfDNA fluctuated according to the change in tumor size as revealed by imaging scans.

Case 23 (Fig. 5A) is a 46-year-old male with no history of smoking with lung adenocarcinoma and brain metastasis harboring a 19DEL mutation, which was confirmed by ARMS in a tumor biopsy. The 4-plex panel (for assaying 19DEL and T790M) was applied to monitor the therapeutic response to gefitinib and noted a persistent decrease in 19DEL mutant abundance that was coincident with the change in the maximum tumor diameter. Case 24 (Fig. 5B) is a 56-year-old female non-smoker with stage IV lung adenocarcinoma who
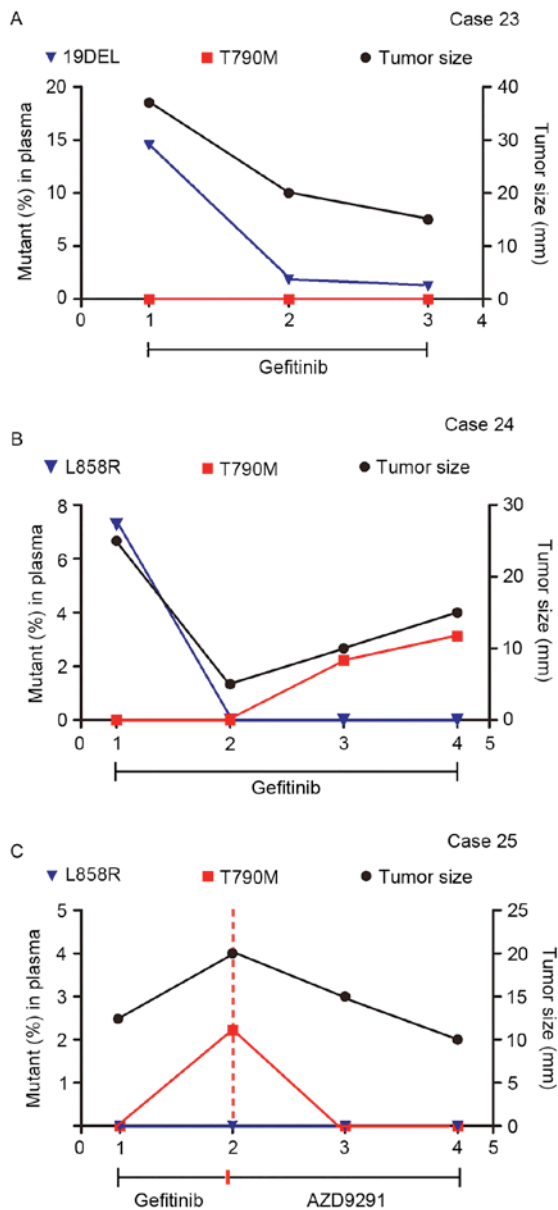
Figure 5. Therapeutic monitoring by multiplex picoliter-droplet digital polymerase chain reaction assays on plasma cfDNA *EGFR* mutations and lung mass imaging scans. Abundance of *EGFR* mutations in plasma cfDNA fluctuated with tumor size, as revealed by imaging scans. (A) 19DEL abundance persistently decreased when tumors shrank in Case 23. (B) T790M rose with the tumor size in Case 24, which indicated an acquired therapeutic resistance to gefitinib. (C) Case 25 presented a change in T790M abundance after receiving AZD9291 treatment. cfDNA, circulating cell-free DNA; EGFR, epidermal growth factor receptor; Mut, mutant; FAM, 6-carboxyfluorescein; VIC, green fluorescent protein; 19DEL, exon 19 deletions; L858R, exon 21 L858R mutation (either L858R-1 or L858R-2); T790M, exon 20 T790M mutation.

had taken gefitinib for 6 months before the serial plasma collection started. Considering the L858R mutation detected by ARMS in the biopsied tumor specimens at the time of onset, the 5-plex panel (for assaying L858R and T790M) was applied. A decrease in L858R mutant abundance was observed in the first follow-up period, and T790M mutant abundance then rose, which is in accordance with the tumor size and indicated an acquired therapeutic resistance to gefitinib. Case 25 (Fig. 5C) is a 61-year-old male smoker with lung

adenocarcinoma and bone metastases who had been treated with gefitinib for 10 months and developed resistance. This patient was assessed using the 5-plex panel assay for an *EGFR* activating-mutant profile, which was previously demonstrated by ARMS assays in tumor tissue. Applying the third-generation EGFR-TKI AZD9291 caused a changeover in the T790M mutant abundance and disease progress.

Discussion

In the present study, two multiplex ddPCR panels were established and quantitatively analyzed *EGFR* mutations in plasma cfDNA isolated from advanced NSCLC patients to assess their usefulness in EGFR-TKI decision-making. A previous study demonstrated the high carrying rate of *EGFR* mutations in Asian patients with advanced NSCLC; however, the coexistence of 19DEL and L858R mutants increased in only 0.8% patients (21). Thus, either of the multiplex ddPCR panels (assaying 19DEL and L858R as TKI-sensitive mutants and T790M as a TKI-resistant mutant) is capable of evaluating *EGFR* mutations for current clinical requirements.

Circulating cfDNA assays on tumor genetic information could serve as a 'liquid biopsy' to facilitate dynamic monitoring of *EGFR* mutations during treatment and could allow for constant optimization of personalized therapy (22). Moreover, circulating cfDNA mutant assays can help generate genetic profiles from patients incapable of having or reluctant to accept tumor biopsies. The amount of circulating cfDNA is higher in cancer patients than in healthy individuals due to the release of nucleic acids into the bloodstream by tumor cell apoptosis, necrosis and secretion (23). However, detecting *EGFR* mutations in circulating cfDNA remains a challenge due to its low abundance, which is attenuated in the overall complex genetic background. High-sensitivity methods have been explored in the past year. Scorpion-ARMS, denaturing high-performance liquid chromatography detection, peptide nucleic acid mediated polymerase chain reaction clamping and next-generation sequencing have been evaluated as *EGFR* mutant assays with plasma cfDNA from NSCLC patients and have detection sensitivities ranging from 20.7 to 65.79% of the sensitivities achieved using tumor tissue examination methods (24-27). The detectable abundance of the multiplex ddPCR panels in the current study ranged from 0.01 to 10%. Applying plasma cfDNA assays to a ddPCR platform has greater detection sensitivity than the existing technologies for seeking specific mutations with ultra-low abundance.

The overall concordance rate in the present study between results generated from plasma samples by ddPCR and tumor tissues by ARMS was 80%, which is similar to other studies, which have ranged from 80.0 to 94.19% concordance (19,28-30). Interestingly, the ddPCR assays performed on plasma cfDNA have noted additional *EGFR* mutants that were absent from the tumor tissue in two cases. Despite detection sensitivity issues, the contradiction in results could be due to the inter- or intra-tumor heterogeneity in tumor biopsy specimens (31,32). The *EGFR* mutation status between primary tumors and metastatic tumors is reportedly different (33,34). ddPCR assays were performed on a metastatic tumor collected from the lymph node of one patient and detected an additional 19DEL mutation. Plasma

cfDNA containing genetic information from both primary and metastatic tumors can help overcome the boundedness due to tumor heterogeneity and selection bias in biopsy and can acquire more genetic profiling that is absent from a single snapshot. Plasma cfDNA may degrade without timely separation when collected in EDTA-K$_2$-containing tubes without nuclease inhibitors (35); the authors suspected this limitation was the reason for the positive mutations detected in tumor tissues with negative results in plasma cfDNA in the study.

Several studies have demonstrated that changes in mutant abundance in plasma cfDNA are associated with the tumor burden and malignant progression (30,36). Similarly, the results of our study inferred that *EGFR* mutant abundance in plasma cfDNA fluctuated according to changes in tumor size, which certified the practicality of our multiplex ddPCR panels in therapeutic evaluation and resistance monitoring. Either panel can be chosen flexibly, according to the mutant alleles generated by routine assays on tumor tissue specimens in a clinical setting, and can be applied to patients receiving TKIs to continually inspect *EGFR* mutation abundance in plasma cfDNA. With its very short retention period in plasma, circulating cfDNA can reflect immediate tumor-associated changes (23). Thus, it can be monitored at short intervals, even less than the 2 months described in the present study, and can serve as the evidence for therapeutic regimen adjustments before tumor progression imaging is performed. By monitoring the changes in *EGFR* mutant abundance in plasma cfDNA, our multiplex ddPCR panels can provide an early indication of EGFR-TKI resistance. Patients in whom the post-treatment T790M mutation arose would then benefit from a new treatment plan using the new TKI, AZD9291 (37,38).

In this study, two multiplex ddPCR panels were put forward for analysis of multiple *EGFR* mutant alleles with therapeutic significance that are integrated into a single assay consuming minimal plasma sample from NSCLC patients. The application of these two panels in clinical settings will be practical, flexible, and time- and cost-saving. However, it should be noted that an LOB series was determined for each mutant based on our multiplex ddPCR panels developed in-house; these results should not be used to determine cutoffs for clinical decisions. The utility, feasibility and robustness of the multiplex ddPCR panels should be further examined in a larger-scale clinical trial.

Acknowledgements

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