Single-Molecule Detection of Epidermal Growth Factor Receptor Mutations in Plasma by Microfluidics Digital PCR in Non–Small Cell Lung Cancer Patients

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Abstract

Purpose: We aim to develop a digital PCR-based method for the quantitative detection of the two common epidermal growth factor receptor (*EGFR*) mutations (in-frame deletion at exon 19 and L858R at exon 21) in the plasma and tumor tissues of patients suffering from non-small cell lung cancers. These two mutations account for ›85% of clinically important *EGFR* mutations associated with responsiveness to tyrosine kinase inhibitors.

Experimental Design: DNA samples were analyzed using a microfluidics system that simultaneously performed 9,180 PCRs at nanoliter scale. A single-mutant DNA molecule in a clinical specimen could be detected and the quantities of mutant and wild-type sequences were precisely determined.

Results: Exon 19 deletion and L858R mutation were detectable in 6 (17%) and 9 (26%) of 35 pretreatment plasma samples, respectively. When compared with the sequencing results of the tumor samples, the sensitivity and specificity of plasma *EGFR* mutation analysis were 92% and 100%, respectively. The plasma concentration of the mutant sequences correlated well with the clinical response. Decreased concentration was observed in all patients with partial or complete clinical remission, whereas persistence of mutation was observed in a patient with cancer progression. In one patient, tyrosine kinase inhibitor was stopped after an initial response and the tumor-associated *EGFR* mutation reemerged 4 weeks after stopping treatment.

Conclusion: The sensitive detection and accurate quantification of low abundance *EGFR* mutations in tumor tissues and plasma by microfluidics digital PCR would be useful for predicting treatment response, monitoring disease progression and early detection of treatment failure associated with acquired drug resistance.

Non-small cell lung cancer (NSCLC), accounting for 80% of all lung cancers, is a leading cause of cancer deaths worldwide. Most NSCLC patients present with advanced-stage disease. Thus, even if treated with the best cytotoxic chemotherapy, median survival is generally <12 months (1). In previous clinical trials, reversible inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase have been shown to effectively retard disease progression in a subgroup of patients with advanced NSCLC and to improve survival (2–5). The disease responsiveness to tyrosine kinase inhibitors (TKI) was subsequently found to be associated with the presence of activating mutations in the *EGFR* kinase domain (6–8), which is encoded by exons 18 to 21 of the *EGFR* gene. The missense mutation L858R in exon 21 and the in-frame deletion in exon 19, nested around the amino acid residues 747 to 750 of the EGFR polypeptide, account for >85% of all clinically important mutations related to TKI sensitivity (9). Making use of the association between *EGFR* mutations and clinical response to TKI, the detection of *EGFR* mutations in tumor tissues has been applied for predicting the response of TKI treatment and hence guiding the treatment for advanced NSCLC. However, tumor tissues may not always be available in patients with inoperable NSCLC. Therefore, other bodily specimens including blood (10–12), pleural fluid (13) and sputum (14, 15) have also been used for *EGFR* mutational analysis.

For the detection of *EGFR* mutations, direct sequencing was commonly used in the early studies, as this method is useful for identifying known as well as novel mutations (4–6). However, as direct sequencing can only detect mutant sequences constituting >30% of the total genetic content (16), it is not useful for the detection of *EGFR* mutations in body fluids in which only a small fraction of the *EGFR* sequences are mutants. Alternatively, specific detection of the most common mutations at exons 19 and 21 can be done (11, 13, 17–19). Scorpion amplification refractory mutation system analysis has been applied for the detection of circulating *EGFR* mutations in

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Translational Relevance

The presence of activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (*EGFR*) gene is associated with the clinical response to EGFR tyrosine kinase inhibitors (TKI) in patients with non-small cell lung cancers. Therefore, the detection of *EGFR* mutation is useful for predicting the treatment response for EGFR TKI in non-small cell lung cancer patients. However, good quality tumor tissues are available only in ‹50% of patients with inoperable lung cancer for mutation analysis. In this study, we have developed a digital PCR-based method for the sensitive detection and quantification of two common *EGFR* mutations in plasma, which account for ›85% of clinically important *EGFR* mutations. In addition to identifying patients potentially responding to EGFR TKI treatment, the serial measurement of cancer-derived *EGFR* mutations in plasma would also provide a means for monitoring disease progression, as well as treatment response.

previous studies (10, 12). However, the detection of *EGFR* mutations by scorpion amplification refractory mutation system was qualitative only and not able to measure the concentration of circulating mutations. In the two previous studies using scorpion amplification refractory mutation system analysis, the L858R mutation was only detected in the serum of 3.7% (1 of 27 patients; ref. 10) and 4.8% (2 of 42 patients; ref. 12) of the patients, whereas deletions in exon 19 were detected in the serum of 44% (12 of 27 patients; ref. 10) and 9.5% (4 of 42 patients; ref. 12) of the patients. These figures differ significantly from those previously reported in tumor tissues. The relatively low detection rate of the L858R mutation in serum suggests that the scorpion amplification refractory mutation system method may be less sensitive in detecting point mutations present at low concentrations in the presence of a large amount of background wild-type sequences. Furthermore, the detection and quantification of low abundance *EGFR* mutations in tumor tissues is also valuable. In a recent study, the analysis of *EGFR* mutations using massively parallel sequencing of 22 lung adenocarcinoma specimens revealed that the *EGFR* mutations can be very heterogeneous in a single tumor sample and some mutations are only present in <10% of total sequences (20). The significance of detecting low abundance mutations in tumor tissues is currently unclear. It can be due to the presence of a low proportion of tumor cells in the tissue sample or, alternatively, can indicate that the mutant allele is only present in a subset of tumor cells (19). The latter situation can occur during cancer evolution (20). For example, the T790M mutation can emerge during the treatment course with TKI, thus conferring acquired drug resistance to the tumor cells (20–22).

For the detection of rare point mutations in a background of wild-type sequences, digital PCR technology represents an attractive approach (23). Thus, digital PCR analysis has been shown to sensitively detect and quantify low abundance tumor-associated point mutations in the plasma of patients suffering from colorectal cancers (24, 25). In digital PCR, very dilute template DNA is distributed into different reaction vessels such that the average template concentration is <1 molecule per vessel (23, 26). The quantities of different templates were then inferred from the number of wells showing a positive signal after mathematical correction (23, 26). Because each PCR vessel only contains an average of <1 molecule, this method is particularly useful for the detection of mutant DNA sequences in a large quantity of background wild-type sequences. Moreover, this physical counting method can also provide highly accurate quantitative information of the minor sequence (24–26). Despite the strengths of accurate detection and quantification of mutations, conventional digital PCR experiments require significant labor input and reagent consumption to perform hundreds of PCR for each clinical sample, thus reducing its attractiveness for routine diagnostic usage. This problem is overcome by the development of microfluidics digital PCR (24, 25), which in one incarnation can perform 9,180 reactions at the nanoliter scale in a single PCR run (27). The application of this high-throughput microfluidics digital PCR system for the quantification of *EGFR* mutations was investigated in this study. Digital PCR assays for the two common types of *EGFR* mutations, L858R and exon 19 deletion, were developed and used for analysis of both tumor tissues and plasma samples. In this study, we investigated the application of digital PCR analysis for the more sensitive detection and quantification of *EGFR* mutations in the tumor tissues and cell-free plasma of NSCLC patients.

Materials and Methods

Sample collection and processing

Patients with confirmed diagnosis of NSCLC attending the Prince of Wales Hospital, Hong Kong were recruited with informed consent. Ethical approval was obtained from the Joint New Territories East Cluster, Chinese University of Hong Kong Clinical Research Ethics Committee. The formalin-fixed, paraffin-embedded tissues were retrieved from the tissue bank in the Department of Anatomical and Cellular Pathology, Prince of Wales Hospital. Tumor tissues were isolated from the paraffin-embedded tissues by manual microdissection. Tumor content in each microdissected sample was ≥50%. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) and eluted with 100 μL H_2O.

For plasma samples, 2 to 6 mL peripheral blood was collected into EDTA tubes. The blood samples were processed to fractionate the plasma and buffy coat as described previously (28, 29). DNA was extracted from 800 μL plasma with the QIAamp DSP DNA Blood Mini Kit (Qiagen) and was eluted with 50 μL H_2O.

Analysis of EGFR mutations in tumor tissues by sequencing after conformation-sensitive gel electrophoresis

PCR amplification of tumor DNA was done using primers specific to the exons 19 and 21 of the *EGFR* gene as described previously (6). PCR product (5 μL) was mixed with 1 μL loading dye, denatured, and immediately loaded onto a 15% polyacrylamide gel. Electrophoresis was carried out in 0.5× TBE at 40 W at room temperature for 9 h. After electrophoresis, the gel was stained with SYBR Gold and visualized under UV illumination. The shifted bands were cut out and reamplified using the corresponding primers. The PCR products were purified and then sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analyzed using an Applied Biosystems 3130 Genetic Analyzer.

Design of the digital PCR assays

Detection of the variant types of exon 19 deletion. The digital PCR assay for the detection of exon 19 deletion consisted of a pair of primers and two TaqMan probes (Fig. 1). One probe was wild-type specific. It

annealed to the antisense strand of the *EGFR* exon 19 where deletion most frequently occurs. When the region was deleted, the probe would not be able to anneal to the template, hence giving rise to no fluorescence signal. The other probe (reference probe) annealed to the sense strand of a nearby region where mutation/deletion has not been reported. It acted as a reference signal to indicate the presence of a DNA molecule regardless of the mutational status of the molecule. The primer sequences for the assays are 5′-GAAAGTTAAAATTCCCGTCGCTAT-3′ (forward primer) and 5′-ACCCCCACACAGCAAAGC-3′ (reverse primer). The sequences of the two TaqMan minor groove binding (MGB) probes are as follows: 5′-FAM-AATTAAGAGAAGCAACATC-MGB-3′ (wild-type-specific probe) and 5′-VIC-ACATCGAG-GATTTCCTTGT-MGB-3′ (reference probe).

Detection of L858R mutation. The digital PCR assay for the detection of L858R consisted of a pair of primers and three TaqMan probes. One probe was specific to the wild-type sequence. The other two probes were specific to the two variants of the L858R mutation. The most common form of L858R was the 2753T>G mutation, which was detected using the mutant-1 probe. Another variant of the L858R mutation (2573T>G and 2574G>T) was detected using the mutant-2 probe. The primers for the L858R detection assay include 5′-CCGCAGCATGTCAAGATCAC-3′ and 5′-TCCTTCTGCATGGTATTCTTTCTCT-3′. The three TaqMan MGB probes are 5′-VIC-TTGGCCAGCCCAA-MGB-3′ (wild-type), 5′-FAM-TTGGCCCGCCCAA-MGB-3′ (mutant-1), and 5′-FAM-TTTGGCACGCC-CAA-MGB-3′ (mutant-2).

Digital PCR analysis for plasma and tumor samples

Digital PCRs were done using the BioMark system (Fluidigm) with the BioMark 12.765 Digital Array Chip (Fluidigm; ref. 30). Each chip consisted of 12 panels, and each panel was further partitioned into 765 reaction chambers. The number of positive signals in each panel was used for quantifying the different DNA sequences. To determine the sensitivity of these digital PCR assays for mutant sequence detection, DNA extracted from cell lines carrying the L858R mutation and exon 19 deletion (ATCC NCI-H1975 and NCI-H1650, respectively) were mixed with wild-type DNA sequences extracted from buffy coat to produce DNA mixtures with different fractional concentration of mutant DNA (10%, 5%, 1%, and 0.1%). These DNA mixtures were then diluted to achieve different absolute concentrations. The two assays were able to detect the mutant molecules in samples containing ~1,000 molecules with fractional concentration of the mutant of 0.1% (Fig. 2A and B).

The PCR mix for one panel was set up by mixing 3.5 μL DNA sample, 0.5 μL assay loading buffer (Fluidigm), 0.5 μL sample loading buffer (Fluidigm), 5 μL TaqMan Universal PCR Master Mix (Applied Biosystems), and the primer and probe mix in a reaction volume of 10 μL. For the L858R assay, each reaction contained 1 μmol/L of each primer, 81.25 nmol/L wild-type probe, and 81.25 nmol/L mutant-1 or mutant-2 probe. For the deletion assay, each reaction contained 2.29 μmol/L of each primer, 375 nmol/L of the wild-type-specific probe, and 37.5 nmol/L of the reference probe.

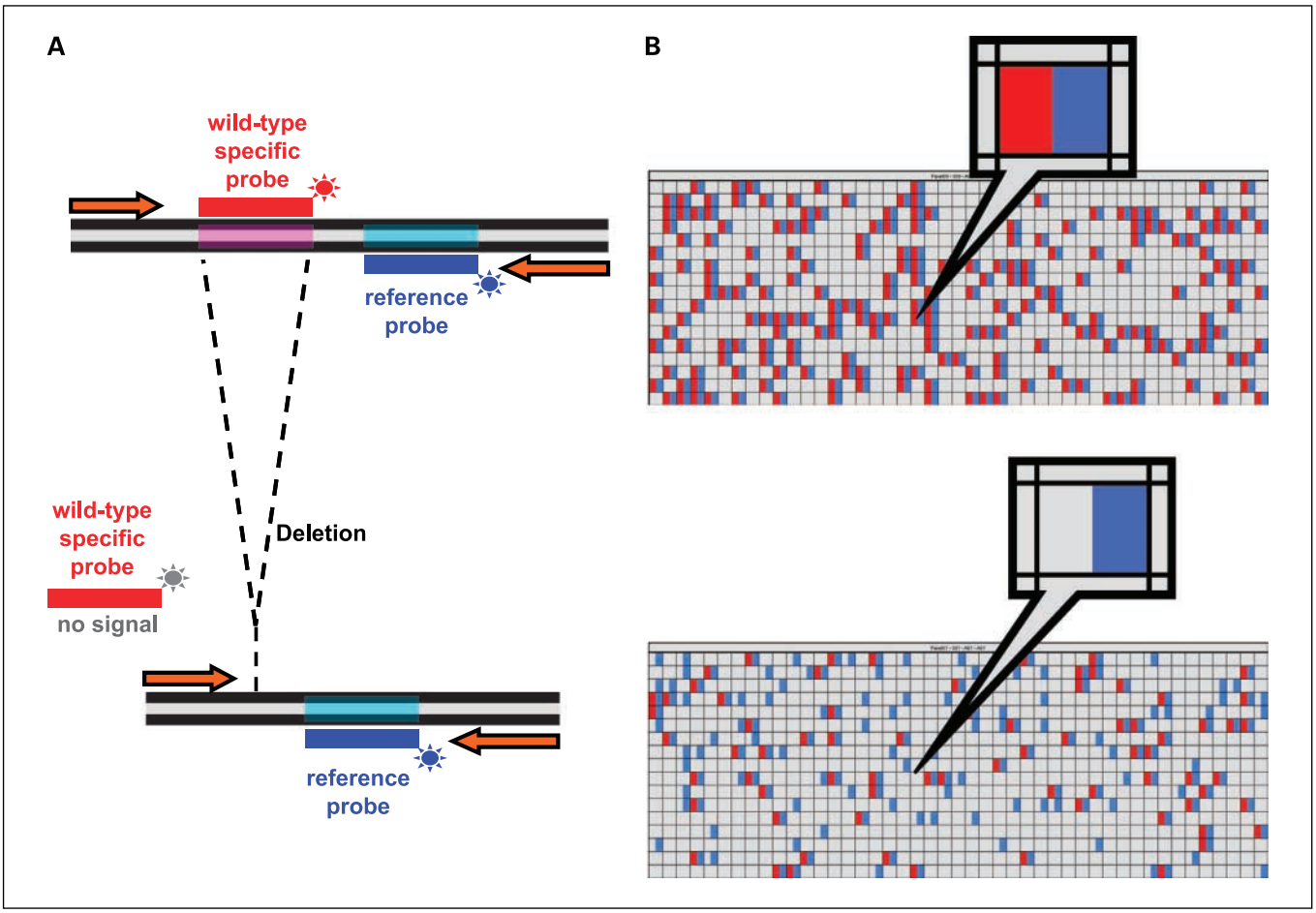

Fig. 1. *EGFR* exon 19 deletion digital PCR assay. *A,* in the assay design, when a wild-type DNA molecule is amplified (*top*), signals from the wild-type-specific probe (*red*) and the reference probe (*blue*) can both be detected. If a mutant molecule with deletion is amplified (*bottom*), only the signal from the reference probe can be detected. *B,* schematic representation of two digital array panels, each with 765 cells. *Top,* all cells with amplified DNA molecules have dual-probe signals, denoting wild-type DNA; *bottom,* a heterogeneous mutant sample, in which some cells have dual-probe signal denoting wild-type DNA and some cells have only the reference probe signal denoting DNA molecule with an exon 19 deletion.

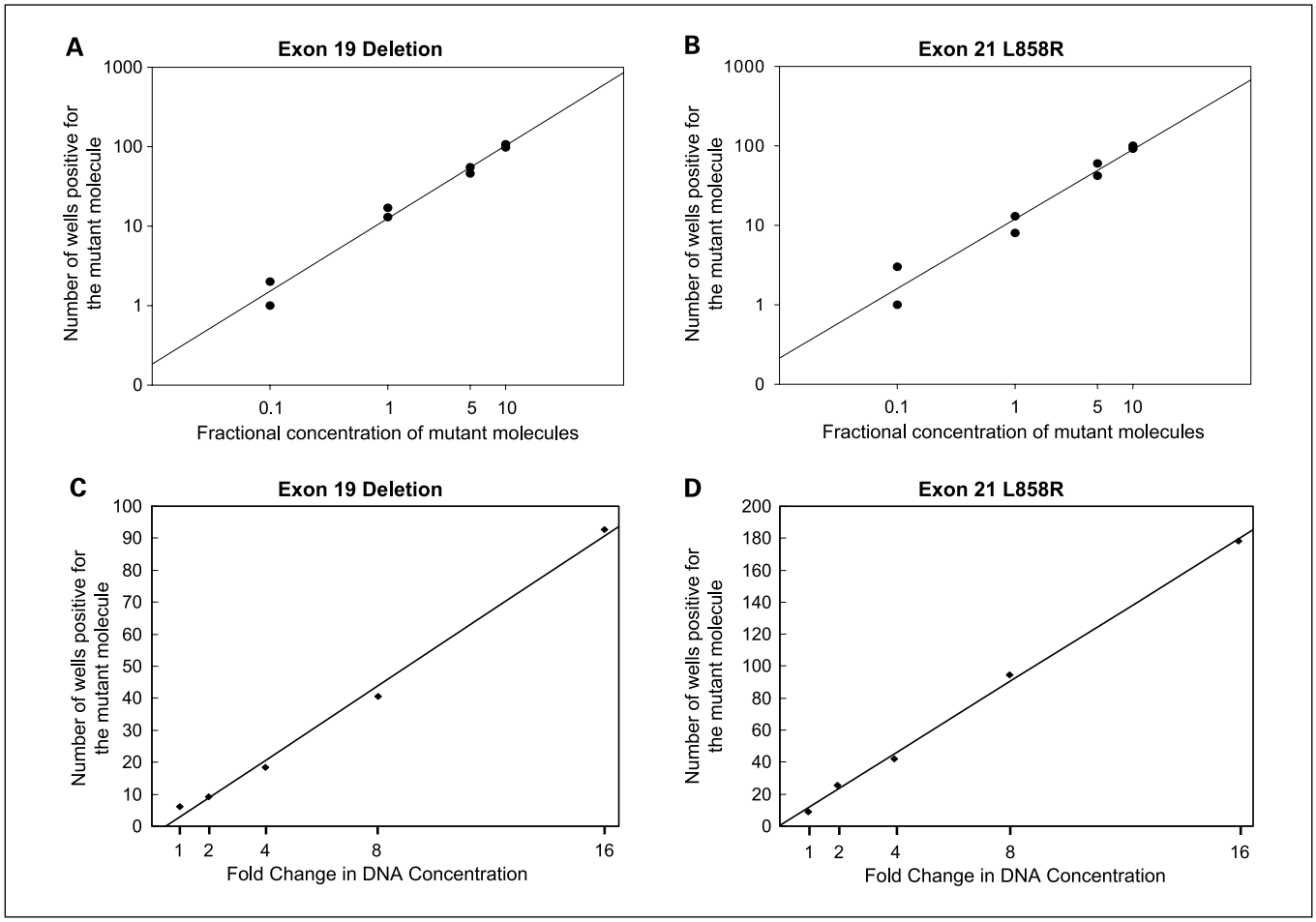

Fig. 2. Quantitative performance of digital PCR analysis for *EGFR* mutations. Mixtures of mutant and wild-type *EGFR* sequences with exon 19 deletion (*A*) and exon 21 L858R (*B*) present at fractional concentrations of 0.1% to 10% were analyzed with digital PCR. The total number of *EGFR* sequences in the sample was ~1,000 in each sample. The number of mutant molecules is plotted against the fractional concentration. Mutant sequences with exon 19 deletion (*C*) and exon 21 L858R (*D*) were serially 2-fold diluted and analyzed by digital PCR. The number of DNA molecules measured by the digital PCR assays is plotted against the input DNA concentration.

The processes of chip priming and sample loading were done on a NanoFlex 4-IFC Controller (Fluidigm). After sample loading, the chip was loaded onto the BioMark Real-time PCR System for thermal cycling and detection of reporter signals. The cycling profile of the exon 19 deletion assay was as follows: initiation at 50°C for 2 min and incubation at 95°C for 10 min followed by 60 cycles of 95°C for 15 s and 55°C for 1 min. The cycling profile of the L858R assay was as follows: initiation at 50°C for 2 min and incubation at 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 56°C for 1 min.

Analysis of the PCR data was done using the BioMark Digital Array software. For the exon 19 deletion assay, a well would be regarded as containing a mutant allele if it is positive for the reference signal but negative for the wild-type signal (Fig. 1) and a well would be regarded as containing a wild-type sequence if both reference and wild-type signals are positive. For the L858R assay, the wild-type and mutant signals represent the presence of wild-type and mutant molecules, respectively.

Results

Quantitative performance of the digital PCR assays. Figure 2 shows the detection of 2-fold serially diluted mutant DNA samples with either an exon 19 deletion (Fig. 2C) or an exon 21 L858R mutation (Fig. 2D). The number of mutant DNA molecules determined by the digital PCR assays is plotted against the fold change in DNA concentrations. A positive linear relationship can be observed between the amount of input mutant sequences and the quantity determined using the digital PCR assays (R^2 = 0.994 and 0.998, linear regression analysis).

EGFR mutations detection in archived tumor tissues using digital PCR and conformation-sensitive gel electrophoresis analyses. The results obtained using digital PCR analysis were compared with those analyzed using conformation-sensitive gel electrophoresis (CSGE) and sequencing (31, 32). First, 10 samples identified to carry an exon 19 deletion by CSGE and sequencing were analyzed with digital PCR analysis. Among these 10 samples, 6 had the most common 15-bp deletion, ΔE746-A750, and the other 4 samples had three variant types of deletion (Table 1). Exon 19 deletion was identified in all 10 cases using the digital PCR analysis, confirming that this assay was able to detect different variants of the exon 19 deletion. Then, 13 samples identified as carrying the L858R mutation by sequencing were analyzed using the digital PCR analysis. Eleven of them had the most common form of L858R mutation (2573T>G) and 2 samples had the variant form (2573T>G and 2574G>T). The digital PCR assays detected the L858R mutation in all 13 samples.

Furthermore, 22 samples, in which no *EGFR* mutation was identified by CSGE and sequencing, were analyzed using the digital PCR assays. Exon 19 deletion and L858R mutations were detected in 1 and 2 of the tumor tissues, respectively, constituting 4.4% to 14% of the total *EGFR* sequences. In another 19 samples, no *EGFR* mutation was detected by both digital PCR analysis and sequencing.

Detection of* EGFR *mutations in prospectively collected tumor samples using digital PCR. Tumor samples were prospectively collected from 35 patients with stage III to IV adenocarcinoma and analyzed using digital PCR and CSGE/sequencing analysis. Twenty (57%) of the patients were male and their median age was 59 years (range, 33-91). CSGE and sequencing analysis was successfully carried out in 29 (83%) samples. L858R mutation and exon 19 deletions were detected in 7 (24%) and 5 (17%) samples, respectively. The representative heat maps of the digital array panels for the tumors with and without deletions in the exon 19 (Fig. 3A and B) and L858R mutation (Fig. 3C and D) are shown in Fig. 3. Table 2 shows the results of the *EGFR* mutation analysis for the tumor samples. In 4 samples where CSGE and sequencing analysis was unsuccessful (cases 18, 26, 30, and 34), *EGFR* mutations were detected using the digital PCR analysis. In 3 tumor samples (cases 3, 4, and 19), CSGE and sequencing analysis revealed only wild-type *EGFR* sequence, but mutations were detected using the digital PCR analysis. The mutant sequences constituted 2% to 4.6% of the total *EGFR* sequences. Interestingly, both exon 19 deletion and L858R were detected in case 18 and the mutant percentages of exon 19 deletion were substantially higher than L858R (56% versus 3.9%). In 16 (46%) samples, neither the exon 19 deletion nor the L858R mutation was detected by digital PCR.

Detection of* EGFR *mutations in plasma samples by digital PCR analysis. The plasma DNA samples of the patients were analyzed for the exon 19 deletion and the L858R mutation using digital PCR (Table 2). For the 12 patients with detectable *EGFR* mutations in tumor by CSGE and sequencing analysis, 11 (92%) had the corresponding mutation detected in plasma. For the patient with a negative plasma result, the mutation only constituted 5.2% of the total *EGFR* sequences in the tumor tissue. In the 4 patients who had unsuccessful CSGE analysis but positive detection of *EGFR* mutations in the tumor tissues by digital PCR, all of them had the corresponding mutations detected in plasma. In the 3 patients who showed low proportion of mutant *EGFR* sequences in tumor tissues by digital PCR but only wild-type sequence in sequencing analysis, no mutation was detected in their plasma samples. For the 16 patients with no *EGFR* mutation detected in tumor tissues using digital PCR analysis and sequencing, none of them had detectable *EGFR* mutation in plasma.

Variations in plasma* EGFR *mutation concentration after TKI treatment. Table 3 shows 10 patients with plasma samples available after commencing TKI treatment. Pretreatment plasma samples were available in 5 of them. *EGFR* mutations were detectable in the pretreatment plasma samples for all 5 patients, and 4 patients showed a marked reduction in the plasma concentration of the mutant sequences after the commencement of TKI treatment. Three of the 4 patients showed partial remission of disease on computed tomography scan and 1 patient had disappearance of all hypermetabolic lesions on positron emission tomography. For the patient with persistent *EGFR* mutations detected in plasma after commencement of TKI treatment, increase in tumor size was observed. For the 5 cases with no available pretreatment plasma, the follow-up samples were taken after a median of 29 weeks of TKI treatment. One patient showed stable disease and 4 patients showed partial remission. No *EGFR* mutation was detected in their follow-up samples. Of note, one of these patients (case 9) developed hepatic dysfunction from TKI and the drug was stopped. At 4 weeks after cessation of treatment, the *EGFR* mutation became detectable in the plasma by digital PCR.

Discussion

Presence of activating mutations involving the tyrosine kinase domain of *EGFR* is an important predictive factor for treatment response to TKI (6–8). However, in a significant proportion of inoperable NSCLC patients, good-quality tumor tissues that allow sequencing of the *EGFR* gene would not be

Table 1. Detection of *EGFR* mutations in archived tumor samples by CSGE and DNA sequencing and digital PCR analyses

No. samples	CSGE and DNA sequencing result	Digital PCR analysis result	
		Mutation*	Mutant percentage †
Concordant mutations detected by sequencing and digital PCR analyses			
6	ΔE746-A750	Del19	31% (interquartile range, 9-49%)
1	ΔE746-S752 (ins V)	Del19	49%
1	ΔE746-S753 (ins VS)	Del19	31%
2	ΔL747-E749 (A750P)	Del19	14%; 24%
11	L858R 2573T>G	L858R 2573T>G	27% (interquartile range, 17-45%)
2	L858R 2573T>G and 2574 G>T	L858R 2573T>G and 2574 G>T	9.3%; 41%
Mutation detected by digital PCR only			
1	Wild-type only	Del19	4.4%
2	Wild-type only	L858R 2573T>G	10%; 14%
No mutation detected by both sequencing and digital PCR analyses			
19	Wild-type only	—	—

*Del19 denotes deletion mutation in exon 19 of the EGFR gene.
†Median (interquartile range) is shown if there are >3 cases having the same combination of results. All values are displayed at two significant figures.

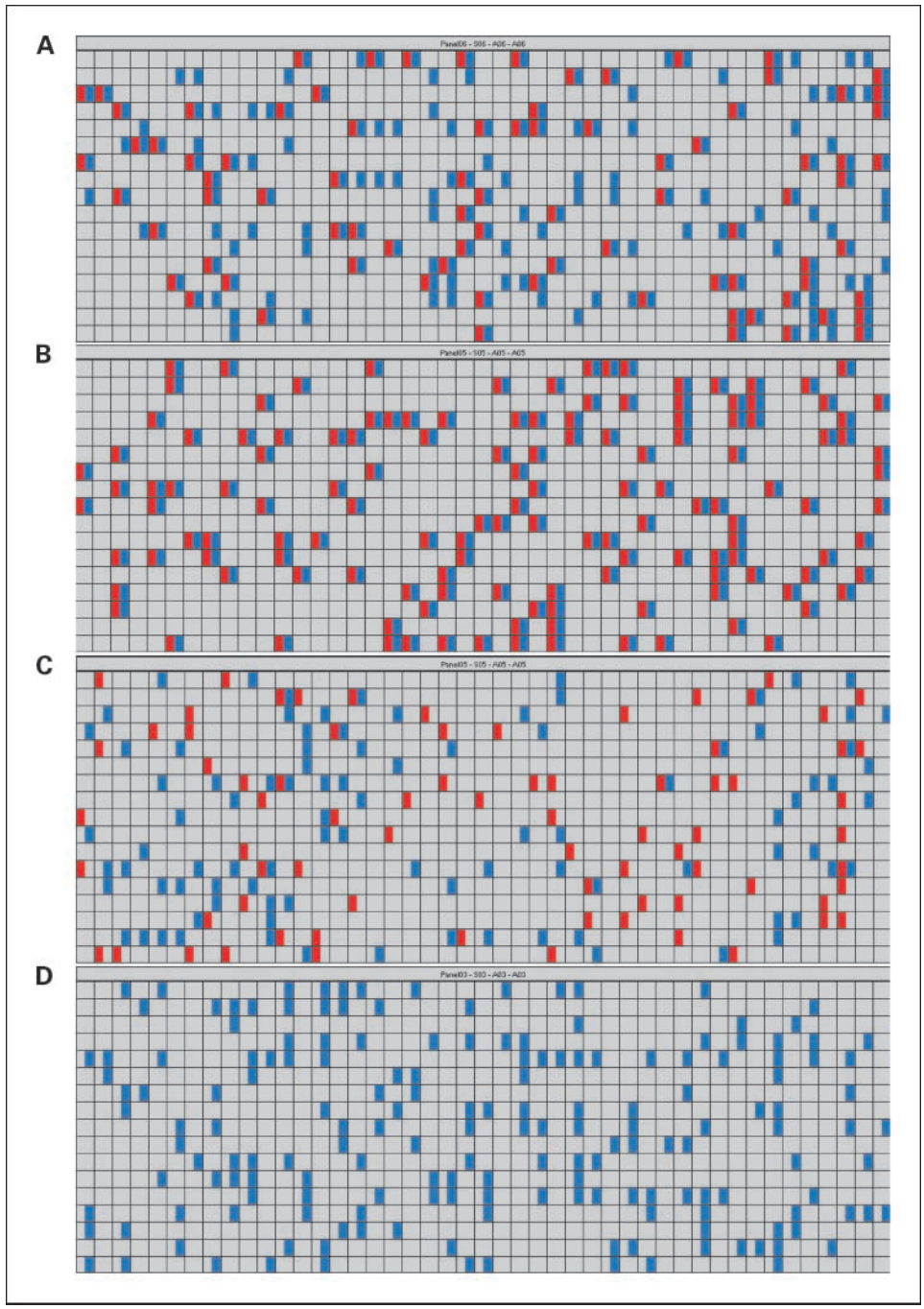

Fig. 3. Representative heat map view of digital array panels. Each panel consists of 765 wells. DNA samples were diluted such that only a fraction of wells contained DNA molecules (red or blue signals). Tumor DNA samples with and without deletions in the exon 19 are shown in *A* and *B*, respectively. The coexistence of blue and red signals in the same well denotes wild-type DNA, whereas blue-only signal denotes DNA with a deletion in exon 19. Tumor DNA samples with and without exon 21 L858R mutation are shown in *C* and *D*, respectively. Blue and red signals denote wild-type and mutant DNA, respectively.

available. Therefore, in this study, we investigated the use of digital PCR analysis for the detection of the two most common *EGFR* mutations, which account for >85% of clinically significant mutations in the tumor tissues and plasma of inoperable NSCLC patients (9). In digital PCR analysis, template DNA molecules are distributed into multiple reaction vessels such that the average number of DNA molecules in each reaction vessel is <1 (23, 26). This method is most useful in the detection and quantification of low abundance mutant sequences in a large amount of background wild-type sequences. Moreover, the quantification of mutant sequence by digital PCR analysis is through the physical counting of the number of positive reactions and hence does not require calibration standards for generating calibration curves. Thus, digital PCR quantification has been shown to be more accurate and precise than other quantitative methods, for example, conventional real-time PCR (24–26, 30). The omission of calibration curve has also advantages when quantification of multiple targets is required.

In the first part of the study, we compared the sensitivity of digital PCR analysis with that of CSGE and DNA sequencing for the detection of *EGFR* mutation in tumor samples. DNA sequencing following CSGE was shown to be more sensitive than direct sequencing for mutation detection (32, 33). In 23 archived and 12 prospectively collected tumor tissues, identical *EGFR* mutations were detected using CSGE and sequencing and

digital PCR analysis. In addition to the most common ΔE746-A750 in exon 19, the digital PCR assays correctly identified all the three variants of the exon 19 deletion involving the amino acids 746 to 750 of the EGFR polypeptide. Theoretically, this assay should be able to detect other sequence alterations involving these amino acids residues. In 3 archived and 3 prospectively collected tumor tissues, *EGFR* mutations were detected using digital PCR but not detected by CSGE and sequencing. In these samples, the mutant sequences constituted a median of 4.5% (range, 2-14%) of the total *EGFR* sequences. The discrepant results were likely due to the better sensitivity of the digital PCR assays rather than false-positive results. In this regard, we have shown that the digital PCR assays were highly sensitive and specific for detecting *EGFR* mutations. These assays were able to detect a mutant molecule present in a background of wild-type sequences at a fractional concentration of 0.1%, whereas up to 10,000 wild-type sequences per reaction would not give rise to a false-positive result. Furthermore, our findings are also consistent with the results of a recent study using massively parallel sequencing for analyzing 22 adenocarcinoma tissues (20). This study revealed that the *EGFR* mutations can be very heterogeneous in a single tumor sample and some mutations are only present in <10% of total sequences (20). The significance of detecting low abundance mutations in tumor tissues is currently unclear. One hypothesis is that new mutations arise during cancer evolution (20); for

Table 2. Digital PCR detection of *EGFR* mutations in plasma collected before treatment

Patient	Tumor tissue			Plasma		Response to TKI treatment if treated with TKI*
	CSGE and sequencing analysis† ‡	Digital PCR analysis§		Digital PCR analysis§		
		Del19 mutant percentage	L858R mutant percentage	Mutation	Concentration (copies/mL)	
(A) Mutations detected by digital PCR and by CSGE and sequencing						
Case 8	L858R	ND	40	L858R	3.2	PR
Case 15	L858R	ND	19	L858R	55	PD
Case 20	L858R	ND	23	L858R	3.2	—
Case 29	L858R	ND	87	L858R	29	—
Case 38	L858R	ND	39	L858R	34,000	CR
Case 39	L858R	ND	26	L858R	9.7	PR
Case 37	L858R	ND	5.2	None	ND	—
Case 11	Del19	65	ND	Del19	21	PR
Case 13	Del19	42	ND	Del19	140	—
Case 17	Del19	38	ND	Del19	890	—
Case 31	Del19	68	ND	Del19	200	—
Case 40	Del19	32	ND	Del19	49	—
(B) Mutations detected by digital PCR (CSGE and sequencing was unsuccessful)						
Case 18	Failed	56	3.9	Del19	22	—
Case 26	Failed	ND	62	L858R	8,300	PR
Case 30	Failed	ND	64	L858R	52	—
Case 34	Failed	ND	45	L858R	500	—
(B) Mutations detected by digital PCR but not by CSGE and sequencing						
Case 3	Wild-type only	ND	3.4	None	ND	—
Case 4	Wild-type only	ND	2.0	None	ND	—
Case 19	Wild-type only	4.6	ND	None	ND	—
(C) No mutation detected in tumor						
Case 5	Wild-type only	ND	ND	None	ND	—
Case 6	Wild-type only	ND	ND	None	ND	—
Case 10	Wild-type only	ND	ND	None	ND	—
Case 14	Wild-type only	ND	ND	None	ND	—
Case 16	Wild-type only	ND	ND	None	ND	—
Case 21	Wild-type only	ND	ND	None	ND	—
Case 22	Wild-type only	ND	ND	None	ND	—
Case 23	Wild-type only	ND	ND	None	ND	—
Case 24	Wild-type only	ND	ND	None	ND	—
Case 25	Wild-type only	ND	ND	None	ND	—
Case 27	Wild-type only	ND	ND	None	ND	—
Case 28	Wild-type only	ND	ND	None	ND	—
Case 32	Wild-type only	ND	ND	None	ND	—
Case 33	Failed	ND	ND	None	ND	—
Case 35	Failed	ND	ND	None	ND	—
Case 36	Wild-type only	ND	ND	None	ND	—

Abbreviations: ND, not detected; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
*Clinical response was defined by Response Evaluation Criteria in Solid Tumors.
† Del19 denotes deletion mutation in exon 19 of the *EGFR* gene. All L858R mutations are 2573T>G.
‡ Cases that PCR amplification is failed are denoted as "Failed."
§ All numerical figures are correct to two significant figures.

Table 3. Analysis of plasma samples of follow-up cases

Patient code	Tumor sample		Plasma samples collected before commencement of treatment		Plasma samples collected during or after treatment*		Time after TKI treatment commenced (wk)	Response to TKI treatment
	Mutation	Mutant percentage	Mutation	Mutant concentration (copies/mL)	Mutation	Mutant concentration (copies/mL)		
(A) Cases without pretreatment plasma samples								
Case 1	Del19	47	NA		ND	—	30	SD
Case 2	Del19	53	NA		ND	—	32	PR
Case 7	L858R	33	NA		ND	—	54	PR
Case 9	Del19	9.8	NA		ND	—	20	PR
					Del19	370	26*	SD*
Case 12	L858R	44	NA		ND	—	4	PR
(B) Cases with pretreatment plasma samples								
Case 8	L858R	40	L858R	3.2	ND	—	60	PR
Case 15	L858R	19	L858R	55	L858R	55	26	PD
Case 26	L858R	62	L858R	8,300	L858R	100	14	PR
Case 38	L858R	39	L858R	34,000	L858R	78	4	CR
Case 39	L858R	26	L858R	9.7	ND	—	8	PR

NOTE: Clinical response was defined by Response Evaluation Criteria in Solid Tumors. Cases 1, 2, 7, 9, and 12 had no pretreatment plasma samples. All cases, except case 9, had gefitinib treatment. Case 9 had erlotinib treatment. Del19 denotes deletion in *EGFR* exon 19. ND, not detected. NA, not available. All L858R mutation are 2573T>G. All numerical figures are correct to two significant figures.
Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NA, not applicable.
*Samples were collected during treatment, except for case 9. Erlotinib treatment was stopped in case 9, 4 wk before plasma collection.

example, the T790M mutation can emerge during the treatment course with TKI, thus conferring acquired drug resistance to the tumor cells (20–22). Although massively parallel sequencing is useful for detecting and quantifying low abundance mutations, this method is costly and labor-intensive. In this regard, digital PCR analysis would be an attractive alternative to massively parallel sequencing for identifying and studying the significance of these low abundance mutations. In addition, the accuracy of sequencing analysis is very much dependent on the amount and quality of the tumor tissues. Thus, CSGE and sequencing was unsuccessful in 6 (17%) samples due to PCR failure. Digital PCR analyses were successful in all the 6 cases and identified *EGFR* mutations in 4 cases. The robustness of digital PCR analysis makes it a useful tool for detecting *EGFR* mutations in tumor sample, which DNA may be partially degraded, for example, paraffin-embedded tissues.

In the 35 patients with paired tumor tissues and pretreatment plasma samples, *EGFR* mutations were detected in the tumor tissues of 12 (34%) patients using CSGE and sequencing analysis of which 7 cases carried the L858R mutation and 5 cases carried the exon 19 deletion. Corresponding mutations were detected in the plasma of 11 of these 12 patients. The sensitivity of detecting mutations in plasma by digital PCR was 92%. In 4 (11%) of the 35 patients, CSGE analysis for the tumor samples was unsuccessful due to PCR failure, but *EGFR* mutations were detected using the digital PCR assays. The corresponding mutations were detected in all their pretreatment plasma samples. For the 16 patients in which no *EGFR* mutation was detectable in their tumor tissues by both sequencing and digital PCR analyses, *EGFR* mutation was not detected in their plasma samples, thus representing a specificity of 100%. For the 3 cases with low abundance of *EGFR* mutations detected by digital PCR analysis but not by CSGE and sequencing, corresponding mutations were not detected in their plasma samples. The clinical significance of these findings is unclear, as these cases would have been identified as not carrying any *EGFR* mutations if only less sensitive detection methods, for example, direct sequencing, were used. The clinical response of these patients to TKI treatment warrants further investigation using a larger cohort.

In addition to sensitively detecting the circulating *EGFR* mutations, the digital PCR assays are also able to measure the concentrations of these mutant sequences. In this regard, we have shown that plasma concentrations of *EGFR* mutations would decline after TKI treatment in patients showing clinical response. Of note, in one patient (case 9; Table 3), TKI treatment was stopped because of liver derangement after showing an initial clinical response to TKI treatment. The mutation was not detected in the first plasma sample, which was collected at 19 weeks after TKI treatment. The treatment was stopped at 22 weeks and reemergence of the mutation in plasma was observed 4 weeks later. This correlation between treatment response and plasma mutation concentration suggests that the serial monitoring of plasma concentration of mutated *EGFR* sequences might be useful for the detection of clinical relapse, particularly when acquired resistance to TKI treatment develops.

In this study, we have shown that digital PCR analysis is a useful tool for low abundance *EGFR* mutations in both tumor tissues and plasma samples. When compared with other qualitative methods of detecting circulating *EGFR* mutations (10, 12), digital PCR analysis can also provide precise quantitative information of the mutations. Thus, the serial measurement of the concentration of *EGFR* mutations in plasma represents a useful marker for the monitoring of disease progression. For the 15 patients with *EGFR* mutations detected in plasma, 9 patients had the L858R mutation and 6 patients had the in-frame deletion in exon 19. The relative proportion of these two types of mutations is similar to that reported previously on tumor tissues. In contrast, the two previous studies, which applied scorpion amplification refractory

mutation system for detecting circulating *EGFR* mutations, reported a relatively low proportion of the L858R mutation, suggesting that allele-specific PCR may be less sensitive in detecting point mutations.

In a very recent study, the capturing of circulating tumor cells by anti-epithelial cell adhesion molecule antibody has been shown to effectively improve the detection rate of *EGFR* mutations in the circulation (11). The concentration of circulating tumor cells can also be estimated as the number of cells expressing the epithelial cell adhesion molecules. However, circulating tumor cells analysis can only be carried out on fresh blood samples. Thus, the digital PCR analysis proposed in this study represents an attractive alternative that can be carried out in fresh as well as frozen archived plasma samples. In addition, digital PCR analysis using the present microfluidics platform is very robust. The simultaneous analysis of 12 samples (>9,000 PCRs) can be finished in 2 h with only 20 min of operator time. The digital array chip is sealed before the PCR process and hence would minimize the chance of carryover contamination. This would be a major advantage for being applied as a routine clinical service. Finally, this technology can also be applied for the detection and quantification of other mutations, for example, the T790M mutation (21, 34, 35) and MET gene amplification (36), which are associated with acquired TKI resistance. With the increasing availability of high-throughput microfluidics systems, we expect that digital PCR analysis may play an increasing role in the field of molecular cancer diagnostics (37).

Disclosure of Potential Conflicts of Interest

Y.M.D. Lo, T.K.F. Yung, and K.C.A. Chan, patents filed on technology described in the article.

References

1. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 2002;346:92–8.
2. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003;290:2149–58.
3. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial). J Clin Oncol 2003;21:2237–46.
4. Bell DW, Lynch TJ, Haserlat SM, et al. Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. J Clin Oncol 2005;23:8081–92.
5. Han SW, Kim TY, Hwang PG, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. J Clin Oncol 2005;23:2493–501.
6. 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;350:2129–39.
7. 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;101:13306–11.
8. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
9. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer 2007;7:169–81.
10. Kimura H, Kasahara K, Kawaishi M, et al. Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small-cell lung cancer. Clin Cancer Res 2006;12:3915–21.
11. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 2008;359:366–77.
12. Kimura H, Suminoe M, Kasahara K, et al. Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). Br J Cancer 2007;97:778–84.
13. Asano H, Toyooka S, Tokumo M, et al. Detection of EGFR gene mutation in lung cancer by mutant-enriched polymerase chain reaction assay. Clin Cancer Res 2006;12:43–8.
14. Tanaka T, Nagai Y, Miyazawa H, et al. Reliability of the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based test for epidermal growth factor receptor mutations integrated into the clinical practice for non-small cell lung cancers. Cancer Sci 2007;98:246–52.
15. Oshita F, Matsukuma S, Yoshihara M, et al. Novel heteroduplex method using small cytology specimens with a remarkably high success rate for analysing EGFR gene mutations with a significant correlation to gefitinib efficacy in non-small-cell lung cancer. Br J Cancer 2006;95:1070–5.
16. Fan X, Furnari FB, Cavenee WK, Castresana JS. Non-isotopic silver-stained SSCP is more sensitive than automated direct sequencing for the detection of PTEN mutations in a mixture of DNA extracted from normal and tumor cells. Int J Oncol 2001;18:1023–6.
17. Hoshi K, Takakura H, Mitani Y, et al. Rapid detection of epidermal growth factor receptor mutations in lung cancer by the SMart-Amplification Process. Clin Cancer Res 2007;13:4974–83.
18. Endo K, Konishi A, Sasaki H, et al. Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay. Lung Cancer 2005;50:375–84.
19. Nagai Y, Miyazawa H, Huqun, et al. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. Cancer Res 2005;65:7276–82.
20. Thomas RK, Nickerson E, Simons JF, et al. Sensitive mutation detection in heterogeneous cancer specimens by massively parallel picoliter reactor sequencing. Nat Med 2006;12:852–5.
21. Kosaka T, Yatabe Y, Endoh H, et al. Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. Clin Cancer Res 2006;12:5764–9.
22. Kwak EL, Sordella R, Bell DW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci U S A 2005;102:7665–70.
23. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96:9236–41.
24. Diehl F, Li M, Dressman D, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci U S A 2005;102:16368–73.
25. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985–90.
26. Lo YMD, Lun FMF, Chan KCA, et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proc Natl Acad Sci U S A 2007;104:13116–21.
27. Dube S, Qin J, Ramakrishnan R. Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device. PLoS ONE 2008;3:e2876.
28. Chan KCA, Chan ATC, Leung SF, et al. Investigation into the origin and tumoral mass correlation of plasma Epstein-Barr virus DNA in nasopharyngeal carcinoma. Clin Chem 2005;51:2192–5.
29. Chan KCA, Yeung SW, Lui WB, Rainer TH, Lo YMD. Effects of preanalytical factors on the molecular size of cell-free DNA in blood. Clin Chem 2005;51:781–4.
30. Lun FMF, Chiu RWK, Chan KCA, Leung TY, Lau TK, Lo YMD. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008;54:1664–72.
31. Korkko J, Annunen S, Pihlajamaa T, Prockop DJ, Ala-Kokko L. Conformation sensitive gel electrophoresis for simple and accurate detection of mutations: comparison with denaturing gradient gel electrophoresis and nucleotide sequencing. Proc Natl Acad Sci U S A 1998;95:1681–5.
32. Korkko J, Kaitila I, Lonnqvist L, Peltonen L, Ala-Kokko L. Sensitivity of conformation sensitive gel electrophoresis in detecting mutations in Marfan syndrome and related conditions. J Med Genet 2002;39:34–41.
33. Ganguly A. An update on conformation sensitive gel electrophoresis. Hum Mutat 2002;19:334–42.
34. Bell DW, Gore I, Okimoto RA, et al. Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. Nat Genet 2005;37:1315–6.
35. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
36. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
37. Chan KCA, Lo YMD. Circulating tumour-derived nucleic acids in cancer patients: potential applications as tumour markers. Br J Cancer 2007;96:681–5.