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Quantifying *EGFR* Alterations in the Lung Cancer Genome with Nanofluidic Digital PCR Arrays

Jun Wang,[1] Ramesh Ramakrishnan,[1] Zhe Tang,[2] Weiwen Fan,[2] Amy Kluge,[2] Afshin Dowlati,[2] Robert C. Jones,[1] and Patrick C. Ma[2*]

BACKGROUND: The *EGFR* [epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)] gene is known to harbor mutations in advanced lung cancer involving gene amplification and kinase mutations that predict the clinical response to EGFR-targeted inhibitors. Methods for detecting such molecular changes in lung cancer tumors are desirable.

METHODS: We used a nanofluidic digital PCR array platform and 16 cell lines and 20 samples of genomic DNA from resected tumors (stages I–III) to quantify the relative numbers of copies of the *EGFR* gene and to detect mutated *EGFR* alleles in lung cancer. We assessed the relative number of *EGFR* gene copies by calculating the ratio of the number of *EGFR* molecules (measured with a 6-carboxyfluorescein–labeled Scorpion™ assay) to the number of molecules of the single-copy gene *RPP30* (ribonuclease P/MRP 30kDa subunit) (measured with a 6-carboxy-X-rhodamine–labeled TaqMan™ assay) in each panel. To assay for the *EGFR* L858R (exon 21) mutation and exon 19 in-frame deletions, we used the ARMS™ and Scorpion technologies in a DxS/Qiagen EGFR29 Mutation Test Kit for the digital PCR array.

RESULTS: The digital array detected and quantified rare gefitinib/erlotinib-sensitizing EGFR mutations (0.02%–9.26% abundance) that were present in formalin-fixed, paraffin-embedded samples of early-stage resectable lung tumors without an associated increase in gene number. Our results also demonstrated the presence of intratumor molecular heterogeneity for the clinically relevant *EGFR* mutated alleles in these early-stage lung tumors.

CONCLUSIONS: The digital PCR array platform allows characterization and quantification of oncogenes, such as *EGFR*, at the single-molecule level. Use of this nanofluidics platform may provide deeper insight into the specific roles of clinically relevant kinase mutations during different stages of lung tumor progression and may be useful in predicting the clinical response to EGFR-targeted inhibitors.

Lung cancer has a high incidence and is the leading cause of cancer death in the US, with 161 840 deaths in 2008—approximately 30% of all cancer deaths *(1)*. Non–small-cell lung cancer (NSCLC),[3] which consists of the subtypes adenocarcinoma, bronchioloalveolar carcinoma, squamous cell carcinoma, and large cell carcinoma, accounts for the majority of lung cancer cases. Traditional cytotoxic chemotherapy has reached a plateau with respect to its impact on patient survival, but novel targeted therapies with small-molecule inhibitors of the epidermal growth factor receptor (EGFR) represents a major therapeutic advance in lung cancer treatment *(2–4)*. EGFR-targeted therapy with the small-molecule tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib has been approved for the treatment of advanced NSCLC. These inhibitors target the receptor kinase of EGFR (also known as ERBB1) by competitive binding at the kinase domain's ATP-binding cleft, thereby blocking kinase activation and subsequent downstream signal transduction. EGFR/ERBB1 represents the first identified member of the HER/ERBB family of receptors, which also includes ERBB2/HER2-neu, ERBB3/HER3, and ERBB4/HER4 *(5)*. EGFR has several ligands, including EGF, trans-

[1] Fluidigm Corporation, South San Francisco, CA; [2] Division of Hematology/Oncology, Department of Medicine, Case Western Reserve University, University Hospitals Case Medical Center, Ireland Cancer Center, and Case Comprehensive Cancer Center, Cleveland, OH.
* Address correspondence to this author at: Division of Hematology/Oncology, Department of Medicine, Case Western Reserve University, University Hospitals Case Medical Center, Ireland Cancer Center, and Case Comprehensive Cancer Center, 10900 Euclid Ave., WRB 2-123, Cleveland, OH 44106. Fax +216-368-1166; e-mail patrick.ma@case.edu.
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[3] Nonstandard abbreviations: NSCLC, non–small-cell lung cancer; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; MARVEL, Marker Validation for Erlotinib in Lung Cancer; ARMS, amplification-refractory mutation system; SCLC, small-cell lung cancer; FAM, 6-carboxyfluorescein; ROX, 6-carboxy-X-rhodamine; IPASS, Iressa Pan-Asia Survival Study.

forming growth factor α, and heparin-binding EGF, and is mutated in approximately 10% of NSCLC patients in the US. Global mutational-analysis studies and clinical trials of the *EGFR*[4] [epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)] gene and its role in lung cancer have established that mutations in the EGFR kinase domain are more likely to occur in female NSCLC patients with an adenocarcinoma subtype, a history of no or light cigarette smoking, and an Asian ethnicity *(2, 5)*.

Somatic alterations in the *EGFR* gene, predominantly in exon 19 (short in-frame deletions that involve the protein's LREA motif) and exon 21 (most commonly producing the L858R substitution), have been identified as mutation hotspots in the catalytic kinase domain in advanced NSCLC and as having potential prognostic value. These mutations also have therapeutic relevance, because they predict the response to EGFR-targeting inhibitor therapy (such as with gefitinib or erlotinib) in patients with advanced disease *(6, 7)*. The current large and ongoing collaborative effort in validating *EGFR* mutations as predictive biomarkers for EGFR-targeted inhibitors is best represented by the MARVEL (Marker Validation for Erlotinib in Lung Cancer) trial. On the other hand, the clinical implications and impact of the *EGFR* mutations in EGFR inhibitor–naive patients with resectable disease at an earlier stage who have undergone curative-intent lung resection is much less well established at this time. Detection of an increase in *EGFR* gene copy number by fluorescence in situ hybridization is a strong predictor of survival benefit in patients with advanced NSCLC treated with EGFR inhibitors *(8)*. The standard DNA-sequencing method detects tumor mutations only within an abundance range of 10%–25%, depending on the quality of the tumor and genomic material *(9)*. A number of newer platforms for studying the cancer genome have recently been developed with the goal of improving mutation detection. For instance, genotyping based on MALDI-TOF mass spectroscopy is estimated to have a 5% detection limit *(10)*, and the Scorpion™ amplification-refractory mutation system (ARMS™) technique has a detection limit down to approximately 1% *(11)*, yet the question of which mutation-detection method represents the best platform remains somewhat controversial *(9)*. Regarding assays for gene copy number, fluorescence in situ hybridization, although considered by many to be a gold standard, is labor intensive with a potential sampling bias in determining the mean number of gene copies within a tumor. Therefore, methods based on real-time PCR have been developed to evaluate the degree of *EGFR* gene amplification *(12)*.

We present a method that quantifies alterations in the *EGFR* gene in lung cancer at the single-molecule level, including the relative number of gene copies and activating mutant alleles (L858R and deletions in exon 19) that predict the therapeutic response to TKIs. This method uses the nanofluidic digital PCR array with *EGFR*-specific PCR assays. The digital array has previously been used for a variety of different applications, including absolute quantification *(13)*, mutation detection *(14)*, and studies of variation in copy number *(15, 16)*. Whereas conventional digital PCR uses sequential limiting dilutions of target DNA followed by PCR amplification *(13, 17, 18)*, the digital array performs the same function by partitioning DNA molecules instead of diluting them.

Materials and Methods

LUNG CANCER CELL LINES AND CELL CULTURE

The lung cancer cell lines (NSCLC cell lines A549, Calu-1, Calu-6, H157, H1838, H1975, H1993, H322, H358, H441, H520, H596, and HCC827; small-cell lung cancer (SCLC) cell lines H69, H128, and H345) were obtained from the ATCC and cultured in HyQPAK RPMI-1640 media containing 2.05 mmol/L L-glutamine (HyClone), 1% (v/v) penicillin/streptomycin, and 100 mL/L fetal bovine serum (HyClone) under standard conditions at 37 °C in a humidified cell culture incubator with 5% CO_2.

EXTRACTION AND SEQUENCING OF LUNG CANCER GENOMIC DNA

Resected NSCLC tumor tissues (stages I–IIIA) obtained from patients between 1997 and 2000 were collected from the University Hospitals Case Medical Center/Case Comprehensive Cancer Center Tissue Procurement Core Facility with Institutional Review Board approval. Archival formalin-fixed, paraffin-embedded samples of resected tumor tissues were dissected manually and estimated histologically to have a tumor content of ≥70%. The DNAeasy Kit (Qiagen) was used according to the manufacturer's instructions to extract genomic DNA from lung cancer cell lines and resected tumor tissues. Sequencing of *EGFR* exons 19 and 21 in DNA prepared from resected NSCLC samples was carried out in both the forward and reverse directions, as previously described *(19)*.

[4] Human genes: *EGFR*, epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian; *RPP30*, ribonuclease P/MRP 30kDa subunit; *PIK3CA*, phosphoinositide-3-kinase, catalytic, alpha polypeptide; *MET*, met proto-oncogene (hepatocyte growth factor receptor); *KRAS*, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog.

NANOFLUIDIC DIGITAL PCR ARRAY: MOLECULAR DETECTION OF THE RELATIVE NUMBERS OF GENE COPIES

The nanofluidic digital array uses nanoscale valves to deliver up to 12 mixtures of sample and PCR reagents into 12 individual panels. Each panel contains 765 independent 6-nL reaction chambers. Accurate quantification of DNA samples with the digital array is based on the random distribution of single DNA molecules into >9000 reaction chambers and their subsequent amplification by the PCR. The concentration of any sequence in a DNA sample (in copies per microliter) can be calculated from the numbers of chambers that contain at least 1 copy of that sequence. Our method uses the digital PCR concept *(13, 17, 18)* and an integrated nanofluidics system *(16, 20)*. Digital PCR quantifies the number of molecules in the test sample by counting them directly, in contrast to inferring the amount of starting material from the amplified product, as in the case of conventional analog PCR. To detect *EGFR* alterations, we used the BioMark™ Real-Time PCR System and Digital Array Chip (Fluidigm Corporation) *(16)* in conjunction with the ARMS and Scorpion PCR (DxS/Qiagen) specific for the wild-type or mutant allele.

The relative numbers of copies of a gene are calculated per haploid genome and can be expressed as the ratio of the copy number for a target gene (e.g., *EGFR*) to the copy number for a single-copy reference gene (in our case, *RPP30*, ribonuclease P/MRP 30kDa subunit) in a DNA sample, which is assumed to be 1 per haploid genome. The use on the same digital array of 2 assays for the 2 genes, each of which is detected with a different fluorescent dye, allows both genes to be quantified simultaneously in the same DNA sample. To quantify the *EGFR* copy number, we used the *EGFR* control assay from a DxS EGFR29 Mutation Test Kit (DxS/Qiagen). Each sample was tested in duplicate. The control 6-carboxyfluorescein (FAM)-labeled Scorpion assay was used to amplify an *EGFR* region of exon 2 for which no polymorphism has been reported.

Each panel of a digital array chip contained a total of 4.59 μL (6 nL × 765 chambers) of the PCR reaction mix; however, for each panel we typically prepared 10-μL reaction mixes, which contained 8 μL of master mix and 2 μL of DNA sample. For the 12 samples tested on each chip, we prepared 13 reactions of 104 μL of master mix (i.e., 8 μL per reaction), which contained 83.2 μL *EGFR* control mix from the DxS EGFR29 Mutation Test Kit, 3.1 μL (15.5 U) AmpliTaq Gold DNA polymerase (Applied Biosystems), 6.5 μL of 20× Sample Loading Reagent (Fluidigm), 1.3 μL of a 50× 6-carboxy-X-rhodamine (ROX) stock solution (Invitrogen), 3.3 μL of a *RPP30* TaqMan assay [900 nmol/L primers and 200 nmol/L probe, as described previously *(16)*], and 6.6 μL PCR-certified water. We then aliquoted 8 μL of the master mix into 12 individual tubes, mixed the contents of each tube with 2 μL sample DNA, and loaded the reaction mixture onto a digital array. The reaction mixture was uniformly partitioned into a 4.59-μL sample panel of 765 reaction chambers containing 6 nL each. The digital array was then thermocycled on the BioMark system. The thermocycling conditions consisted of 30 s at 50 °C, a hot start at 95 °C for 10 min, and 50 cycles of 30 s of denaturation at 95 °C and 1 min of annealing and extension at 60 °C. The FAM (*EGFR*), VIC, and ROX (*RPP30*) signals of all chambers in the same digital array were recorded at the beginning of each annealing step (as suggested by DxS). After the reaction was completed, the Digital PCR Analysis Software (Fluidigm) was used to process the data. The BioMark software then generated a PCR-amplification curve from the signals from each cycle. We counted the number of FAM-positive chambers (*EGFR*) and ROX-positive chambers (*RPP30*) in each panel and used the software to calculate the *EGFR*/*RPP30* ratio. VIC signals were used for the DxS internal positive PCR control to make sure the PCR was working and no inhibition was present.

QUANTITATIVE DETECTION OF RARE MUTATIONS

The digital array had 12 sample panels with nanoscale channels and valves to partition each sample mix into 765 reaction chambers. Partitioning before the PCR thereby provided improvement in the detection of rare mutated alleles. For example, if a mixture containing 1 molecule of the *EGFR* L858R mutant in 5000 molecules of nonmutated *EGFR* is partitioned into 765 independent chambers, the chamber containing the single mutant molecule now contains only approximately 6–7 molecules of nonmutated *EGFR*. This 765-fold increase in relative concentration should allow a 765-fold improvement in the detection sensitivity of PCR reactions, thereby facilitating the detection of a rare copy of a mutated allele in a limited amount of patient sample. We used the digital array to detect *EGFR* mutations in genomic DNA from resected lung cancer tumor tissues. Each sample was tested in duplicate. We used the ARMS and Scorpion assays from a DxS EGFR29 Mutation Test Kit to detect the *EGFR* L858R mutant (exon 21) and in-frame exon 19 deletions. The assay for exon 19 deletions detected all 19 common short in-frame deletions previously reported for *EGFR* exon 19. The mutation assays were labeled with FAM, and each reaction mixture also contained a positive PCR control labeled with hexachloro-6-carboxyfluorescein (detected in the VIC channel on our system).

Similar to the assay for detecting copy number, the assay for quantitative detection of mutations consisted of 10-μL reaction mixes containing 8 μL of master mix and 2 μL of DNA sample, which typically were pre-

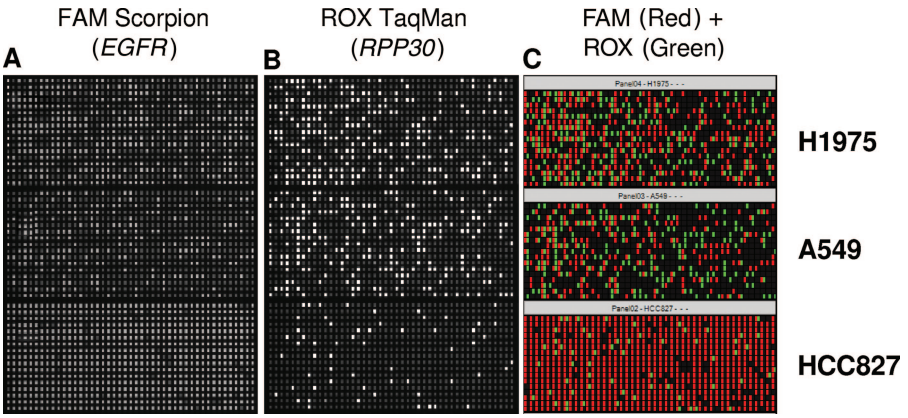

Fig. 1. Quantification of relative *EGFR* copy number in lung cancer cell lines with the nanofluidic digital array.
The relative numbers of *EGFR* copies in genomic DNA was measured by calculating the ratio of the number of *EGFR* molecules (FAM Scorpion assay) to the number of *RPP30* molecules (ROX TaqMan assay) in each panel. (A and B), FAM (*EGFR*) and ROX (*RPP30*) images of the same digital array taken at the cycle 40 of the PCR. (*C*), Software-generated composite color map of both images (red for FAM and green for ROX). Cell line origin of the DNA samples is indicated in each panel: H1975, A549, and HCC827.

pared for each digital array panel. For the 12 samples tested on each chip, we prepared 13 reactions of 8 μL per reaction for a total master mix volume of 104 μL, which contained 83.2 μL of the DxS *EGFR* L858R mix from the EGFR29 Mutation Test Kit, 3.1 μL (15.5 U) of AmpliTaq Gold DNA polymerase, 6.5 μL of the 20× Sample Loading Reagent, 1.3 μL of the 50× ROX stock solution, 2.2 U Perfect Match (1 U/μL; Stratagene), and 7.7 μL PCR-certified water. We then aliquoted 8 μL of the master mix into 12 individual tubes, mixed the contents of each tube with 2 μL sample DNA, and loaded the reaction mixture onto a digital array. The reaction mixture was uniformly partitioned into a 4.59-μL sample panel of 765 reaction chambers containing 6 nL each. To detect deletions in *EGFR* exon 19, we used the DxS deletion mix with the same preparation as for DxS *EGFR* L858R mutant, except that we did not use Perfect Match.

The digital array was thermocycled on the BioMark system. The thermocycling conditions consisted of 30 s at 50 °C, a hot start at 95 °C for 10 min, and 50 cycles of 30 s of denaturation at 95 °C and 1 min of annealing and extension at 61 °C. The FAM and VIC signals of all chambers were recorded at the beginning of each annealing step, as suggested by DxS. After the reaction was completed, the Digital PCR Analysis Software was used to process the data, analyze PCR amplification, and count the numbers of FAM-positive chambers (*EGFR*) in each panel. VIC signals were used for the DxS internal positive PCR control to make sure the PCR was working and no inhibition was present.

In the spiking experiment, cell line DNA carrying an *EGFR* mutant [*EGFR* exon 19 deletion (cell line HCC827, with *EGFR*-E746_A750 del) or *EGFR* L858R (H1975)], was mixed with cell line DNA carrying wild-type *EGFR* (A549). Different amounts of mutant DNA were mixed with wild-type DNA to yield 0–40 copies of mutant molecules in 5000 wild-type molecules per panel on the digital array. For the NSCLC samples, 5000 copies of genomic DNA were detected on each panel. The DNA copy number of each sample was previously quantified by the *RPP30* assay during the assay of *EGFR* copy number.

Results

First, we demonstrated the feasibility of applying the nanofluidic digital array system by optimizing the assays with lung cancer cell lines. We quantified the relative numbers of *EGFR* gene copies (Fig. 1) by calculating the ratio of the number of *EGFR* molecules (FAM Scorpion assay, Fig. 1A) to the number of copies of the single-copy gene *RPP30* (ROX TaqMan assay, Fig. 1B) in each panel of the digital array. We used quantification cycle readings of each chamber from both fluorescent channels or from both images of the last PCR cycle (red for FAM and green for ROX, Fig. 1C) with end point reading to generate a composite color map of the positive hits. Cell line genomic DNA was used to adapt the digital array to measure the relative number of *EGFR* copies number in the panel of NSCLC and SCLC lung cancer cell lines (Table 1). Each

Table 1. Relative number of *EGFR* gene copies in lung cancer cell lines, as measured by the nanofluidic digital PCR array.

Cell line	Lung cancer subtype	Relative copy number (*EGFR*/*RPP30* ratio)	*EGFR* genotype
A549	Adenocarcinoma	1.0	WT[a]
Calu-1	Squamous	1.7	WT
Calu-6	Adenocarcinoma	1.0	WT
H128	SCLC	1.7	WT
H157	Squamous	1.8	WT
H1838	Adenocarcinoma	5.9	WT
H1975	Adenocarcinoma	1.8	L858R, T790M
H1993	Adenocarcinoma	1.2	WT
H322	BAC	1.2	WT
H345	SCLC	1.8	WT
H358	BAC	1.7	WT
H441	Adenocarcinoma	1.0	WT
H520	Squamous	1.8	WT
H596	Adenosquamous	3.4	WT
H69	SCLC	2.0	WT
HCC827	Adenocarcinoma	35.5	Exon 19 deletion E746_A750 del

[a] WT, wild type; BAC, bronchioloalveolar carcinoma.

array run also included a no-template control. The HCC827 lung adenocarcinoma cell line showed an increase in *EGFR* gene copy number to 35.5, which was consistent with a previous report with a conventional assay platform *(21)*. Additionally, the H1838, H596, and H69 cells lines were found to have ≥2 *EGFR* copies per haploid genome (5.9, 3.4, and 2.0 copies, respectively; Table 1).

We also investigated lung cancer cell line genomic DNA for the presence of gefitinib/erlotinib-sensitizing, *EGFR*-activating exon 19 deletions (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue4) and the L858R mutation (Fig. 2) with the DxS allele-specific Scorpion (ARMS) PCR assays. For each array panel, we spiked 0–40 copies of DNA from the respective cell lines into 5000 copies of wild-type genomic DNA (from the A549 cell line). The HCC827 cell line *(21)* (with *EGFR*-E746_A750 del) was used for evaluating exon 19 deletions, and the H1975 cell line *(22)* was used for investigating the L858R mutation. We found the nanofluidic digital array to be capable of detecting both the *EGFR* exon 19 deletion (see Fig. 1 in the online Data Supplement) and the exon 21 L858R missense mutation (Fig. 2) against a wild-type genomic background at relative concentrations as low as 0.04%.

To test the application of the digital array molecular-assay platform to human tumor samples in

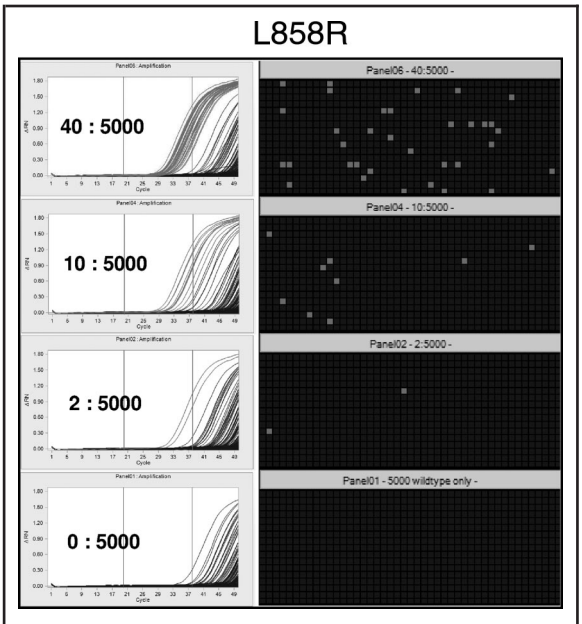

Fig. 2. Nanofluidic digital array detection of the L858R mutation in the *EGFR* kinase domain in spike-in mixtures with lung cancer cell line genomic DNA.

Shown for each panel are the screen shot of the FAM image from the BioMark Digital Analysis Software and the amplification curve.

Table 2. *EGFR* gene alterations in genomic DNA from samples of resected NSCLC tumors, as determined by the nanofluidic digital PCR array.[a]

							EGFR Del 19		*EGFR* L858R	
Sample ID[b]	NSCLC histology subtype	Sex	Age, y	TNM Staging[c]	Stage	EGFR CNV	Sequencing	Mutant molecule count by DID[d]	Sequencing	Mutant molecule count by DID[d]
NSCLC-1	Adenocarcinoma	F	72	T2 N1 M0	IIB	0.7	No	0	No	0
NSCLC-2	Squamous cell	M	69	T2 N0 M0	IB	0.8	No	0	No	0
NSCLC-3	Adenocarcinoma	M	78	T3 N0 M0	IIB	0.8	No	0	No	1
NSCLC-4	Adenocarcinoma	F	69	T2 N2 M0	IIIA	0.7	No	0	No	0
NSCLC-5	Adenocarcinoma	M	66	T2 N0 M0	IB	0.9	No	0	No	0
NSCLC-6	Squamous cell	F	74	T2 N0 M0	IB	0.8	No	0	No	0
NSCLC-7	Adenocarcinoma	F	39	T2 N0 M0	IB	0.7	No	0	No	0
NSCLC-8	Adenocarcinoma	F	52	T2 N1 M0	IIB	0.9	No	0	No	0
NSCLC-9	Squamous cell	F	57	T1 N0 M0	IA	0.6	No	0	No	0
NSCLC-10	Adenocarcinoma	F	74	T2 N1 M0	IIB	0.6	No	0	No	0
NSCLC-11	Squamous cell	M	56	T1 N1 M0	IIA	0.8	No	1	No	0
NSCLC-12	Large cell	F	62	T3 N0 M0	IIB	0.9	No	0	No	0
NSCLC-13	Adenocarcinoma	F	45	T2 N2 M0	IIIA	0.9	No	0	No	1
NSCLC-14	Squamous cell	F	64	T1 N0 M0	IA	0.6	No	0	No	2
NSCLC-15	Adenocarcinoma	F	82	T2 N0 M0	IB	1.3	No	0	Yes/No	103
NSCLC-16	Adenocarcinoma	F	63	T1 N0 M0	IA	0.3	No	0	No	0
NSCLC-17	Squamous cell	F	82	T2 N1 M0	IIB	0.5	No	0	No	0
NSCLC-18	Adenocarcinoma	M	70	T2 N0 M0	IB	1.0	No	0	No	0
NSCLC-19	Adenocarcinoma	F	58	T2 N2 M0	IIIA	1.2	No	0	No	1
NSCLC-20	Adenocarcinoma	M	76	T2 N0 M0	IB	1.3	No	0	Yes	463

[a] Summary of data for relative numbers of *EGFR* copies and EGFR mutations obtained with the nanofluidic digital PCR array. Results of direct DNA sequencing of the corresponding *EGFR* exon in selected tumor samples are illustrated to highlight the ability of the digital array to detect rare or low-abundance mutant alleles that can be missed by the standard DNA-sequencing method.

[b] ID, identification number; CNV, copy number variation; Del 19, deletion in exon 19; DID, ●●●.

[c] American Joint Committee on Cancer (AJCC) TNM staging classification [Greene et al. *(32)*]. T, tumor size stages 1–4; N, nodal stages 0–3; M, distant metastasis stages 0–1.

[d] ●●●.

this study, we first evaluated the relative numbers of *EGFR* copies in DNA prepared from archival formalin-fixed, paraffin-embedded samples of surgically resected NSCLC tumors (stages I–IIIA). We detected no amplification in *EGFR* copy number for any of the 20 lung tumor DNA samples we tested (i.e., the incidence of *EGFR* copy amplification is <5% at early stages of tumor progression; Table 2).

We next focused on the detection of *EGFR* mutations by evaluating for erlotinib/gefitinib-sensitizing deletions in *EGFR* exon 19 (Fig. 3; see Fig. 2 in the online Data Supplement) and the exon 21 L858R mutation (Fig. 4; see Fig. 3 in the online Data Supplement) in the samples of genomic DNA from resected NSCLC tumors used in the gene copy number assay described above. In 1 of the 20 NSCLC tumor DNA samples tested, we identified the presence of 1 molecule of an exon 19 deletion among 5000 genomic DNA copies (0.02%) in a sample of a squamous cell NSCLC tumor [stage IIA (T1 N1)] obtained from a 56-year-old man (Fig. 3, Table 2). In addition, we identified the presence of L858R mutant *EGFR* alleles in 6 (30%) of the 20 samples examined (Fig. 4, Table 2). Three of these samples (all from adenocarcinomas) showed a single mutated allele (0.02%) among 5000 genomic DNA copies, and 1 sample (squamous cell carcinoma; NSCLC-14: T1 N0 M0, stage IA) showed the presence of 2 mutant alleles (0.04%) among 5000 genomic DNA copies. Finally, 2 of the samples (NSCLC-15 and NSCLC-20) showed 103 L858R mutant *EGFR* molecules (2.06%) and 463 mutant molecules (9.26%), respectively, among the 5000 genomic DNA copies tested. Both of these tumors expressing relatively higher numbers of L858R *EGFR*

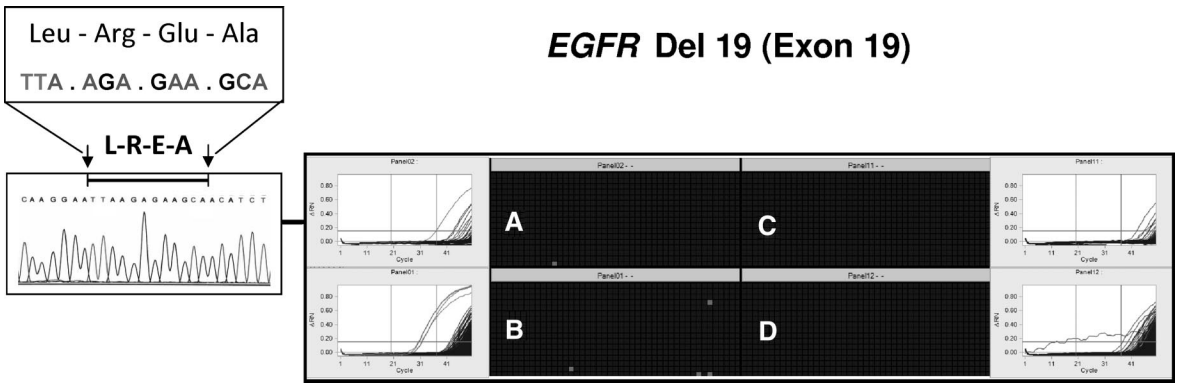

Fig. 3. Detection with the nanofluidic digital array of an erlotinib-sensitizing *EGFR* kinase domain in-frame exon 19 deletion in genomic DNA prepared from resected NSCLC tumor tissue.

Example of digital PCR array detection of gefitinib/erlotinib-sensitizing exon 19 deletions in samples of patient genomic DNA (A and C), with 5000 copies of DNA molecules in each panel. (B), Five copies of exon 19 deletion (Del 19) mutant DNA from cell line HCC827 (as positive control) were spiked into 5000 molecules of wild-type *EGFR* genomic DNA. (D), 5000 copies of wild-type *EGFR* genomic DNA molecules (negative control). Tumor sample NSCLC-11 (DID panel A) shows 1 allele of the *EGFR* exon 19 deletion (0.02%), which is not detectable by direct DNA sequencing. Included for illustration is the corresponding sequencing chromatogram for the wild-type sequence for exon 19 that encodes the Leu-Arg-Glu-Ala peptide motif. See Fig. 2 in the online Data Supplement for the complete view of the DID panel.

mutant alleles were derived from stage IB (T2 N0 M0) lung adenocarcinomas; the *EGFR* copy number was not amplified in either tumor sample (Table 2). Activating *EGFR* mutations that sensitize the patient to erlotinib/gefitinib therapy are most likely to be found in female Asians with advanced NSCLC of the adenocarcinoma subtype and who never or only lightly smoked *(2)*. In our study, we detected L858R *EGFR*–containing tumors from both female and male patients (82 and 76 years of age, respectively) and detected rare mutant alleles in tumors of the squamous cell subtype. Our DNA-sequencing results showed that all of the rare *EGFR* mutant alleles detected by the nanofluidic digital PCR array were indeed beyond the limit of detection by direct sequencing (Figs. 3 and 4; see Figs. 2–4 in the online Data Supplement). Sequencing readily detected the heterozygous L858R mutation in sample NSCLC-20, but the L858R mutated alleles in NSCLC-15 (103 copies in 5000, 2.06%) was only barely detectable by sequencing (see Fig. 3 in the online Data Supplement). Hence, a correlation between the mutant allele percentage detected with the digital PCR array and the height of the chromatograph peak for the mutant nucleotide in the DNA sequencing run was evident.

Discussion

We have described a nanofluidic digital PCR array as a single platform that allows both highly sensitive quantitative measurement of the relative number of *EGFR* copies and the detection of clinically relevant mutated *EGFR* alleles at the single-molecule level, with a minimal amount clinical lung cancer sample. The digital array can detect and quantify the presence of rare erlotinib/gefitinib-sensitizing *EGFR* mutations (0.02%–9.26%) without any associated genomic gain in early-stage resectable lung tumors at a much higher sensitivity than is possible with conventional sequencing. We did not identify amplification in *EGFR* copy number in any of the DNA samples from the 20 resected lung tumors we tested (i.e., <5% incidence at early stages of tumor progression). This finding is probably because genomic amplification of mutated *EGFR* alleles has been reported to occur late in the process of tumor metastatic progression *(23)*, whereas early molecular alterations in the oncogene are often somatic mutations (such as L858R and deletions in exon 19). Yung and coworkers recently reported the feasibility of the digital array in detecting *EGFR* mutations in tumor tissues from patients with advanced metastatic NSCLC *(24)* at a detection limit of 0.1% of the total number of *EGFR* sequences. Our study shows that the nanofluidic digital array allows quantitative measurement of *EGFR* copy number. The nanofluidic digital array can be optimized to quantify rare mutated *EGFR* alleles to a limit of 0.02% in genomic DNA prepared from formalin-fixed, paraffin-embedded samples of early-stage resectable NSCLC tumors.

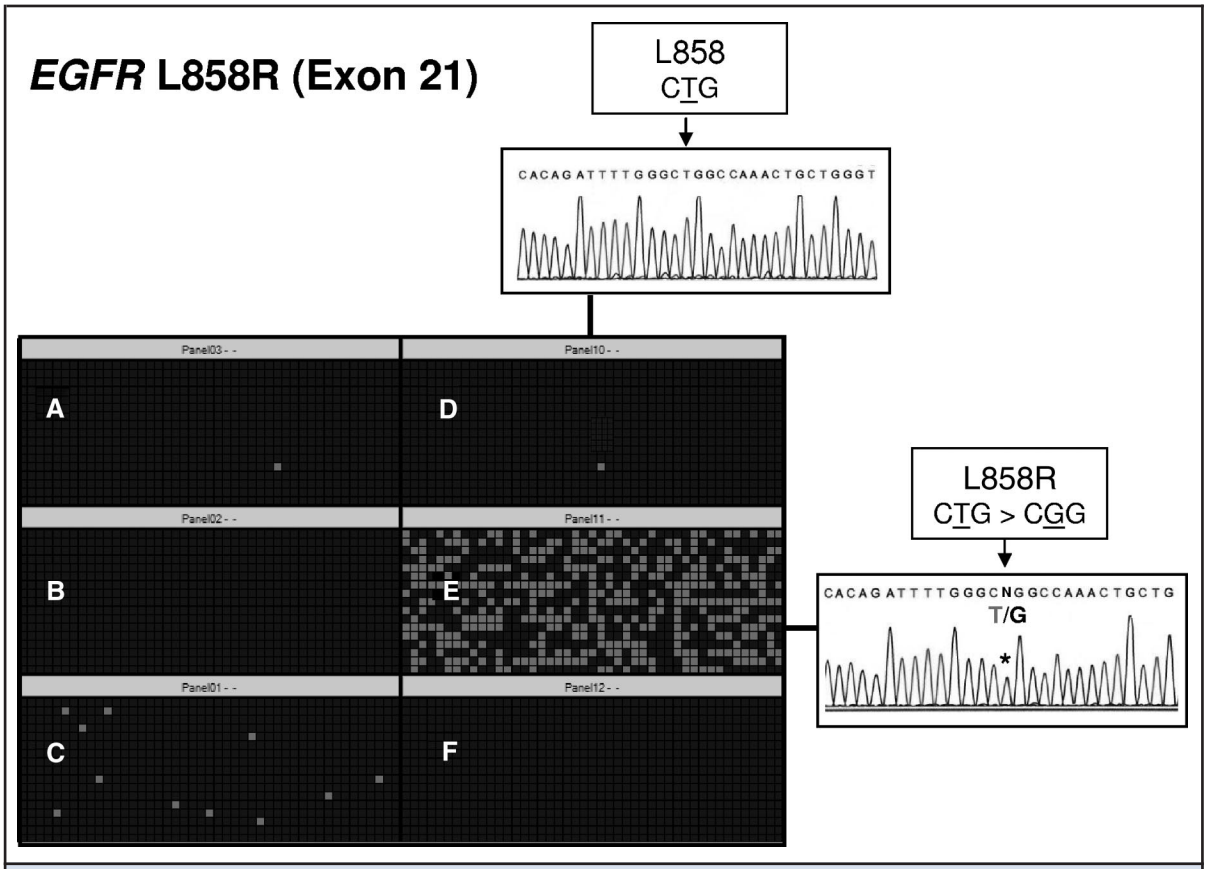

Fig. 4. Detection with the nanofluidic digital array of the erlotinib-sensitizing mutation in the EGFR kinase domain of exon 21 (L858R) in genomic DNA prepared from resected NSCLC tumor tissue.

Shown is an example of digital PCR array detection of the erlotinib-sensitizing L858R mutation in genomic DNA from patients (A, B, D, E), with 5000 copies of DNA molecules for each panel. (C), Ten copies of L858R mutant DNA molecules from cell line H1975 (positive control) were spiked into 5000 molecules of wild-type *EGFR* genomic DNA. (F), Five thousand copies of wild-type *EGFR* genomic DNA molecules (negative control). The NSCLC-20 samples (DID panel E) showed the presence of 463 mutated L858R alleles (i.e., 9.26% mutant in the total genomic DNA from the tumor sample). The mutation was also readily detectable by DNA sequencing in sample NSCLC-20, but not in sample NSCLC-15, in which only 103 mutated L858R alleles (2.06%) were detected among 5000 copies (see Fig. 3 in the online Data Supplement).

One of the advantages of the digital PCR array is its low mutation-detection limit for detecting cancer mutations, compared with conventional methods, such as direct Sanger DNA sequencing or genotyping based on MALDI-TOF mass spectroscopy. The array permits the identification of rare mutated alleles in a high background of wild-type genomic DNA. Thus, the requirement of tumor genomic materials is minimal. A whole-genome amplification methodology to increase the amount genomic materials from scarce tumor biopsy samples for molecular assays, which can have an amplification bias, is not necessary for the use of the digital PCR array. Our results lend further support to the notion of molecular heterogeneity within tumors *(25)*. There are clinically relevant *EGFR* mutated alleles that are activating driver mutations, which exist to varying extents and even at very low abundances in early-stage lung tumors.

Although the EGFR TKIs gefitinib and erlotinib were initially approved for treating advanced metastatic NSCLC in the second- and third-line treatment settings, much of current emphasis in clinical investigations is focused on the potential use of gefitinib/erlotinib in the first-line setting in selected patients enriched with sensitizing *EGFR* mutations *(26)* and as adjuvant therapy in NSCLC patients with tumors resected at an earlier stage (http://clinicaltrials.gov/ct2/show/NCT00567359). The phase 3 randomized Iressa Pan-Asia Survival Study (IPASS) prospectively evaluated and compared gefitinib against standard doublet

cytotoxic platinum-based chemotherapy as first-line treatment in clinically selected advanced-stage (IIIB-IV) lung cancer patients (adenocarcinoma histology, never or lifetime light smokers) to enrich for EGFR kinase mutations. Interestingly, the results of this trial suggest that gefitinib treatment in *EGFR* mutation–positive patients leads to a better response rate, as well as improved overall and progression-free survival, compared with patients in the same subgroup who underwent chemotherapy *(27)*. The investigators concluded that gefitinib is superior to cytotoxic chemotherapy as a first-line treatment in the *EGFR* mutation–positive patients. It is also apparent that knowledge of the actual presence or absence of mutations still has an important impact on the outcome of treatment with EGFR TKIs vs cytotoxic chemotherapy, even within the clinically selected patient population. Knowledge of *EGFR*-activating mutations in early-stage lung tumors that are sensitive to EGFR TKIs may eventually also allow a paradigm change in the use of these kinase inhibitors in lung cancer treatment of curative intent.

This digital array may eventually enable future studies to reveal the biology of tumor evolution and its clinicopathologic correlation during tumor progression that the currently available data set obtained via direct DNA sequencing does not provide. Moreover, the impact of mutant-allelic dilution within the tumor mass could be better addressed by applying this digital platform to molecular analysis at the single-molecule level. Our results show that erlotinib/gefitinib-sensitizing *EGFR* mutations are present in early-stage resectable NSCLC tumors that are not associated with *EGFR* genomic gain. Our findings also highlight the presence of molecular heterogeneity in tumors *(25)*, with mutated *EGFR* alleles occurring at 0.02%–9.26% of the total tumor genomic DNA. This digital array can also be applied in studies of other cancer genes *(28)*, such as *PIK3CA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide) *(29)*, *MET* [met proto-oncogene (hepatocyte growth factor receptor)] *(30)*, and *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) *(31)*, to facilitate development toward the new paradigm of personalized targeted cancer therapy.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
2. Shigematsu H, Gazdar AF. Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. Int J Cancer 2006;118:257–62.
3. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer 2007;7:169–81.
4. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. N Engl J Med 2008;358:1160–74.
5. Lynch TJ, Adjei AA, Bunn PA Jr, Eisen TG, Engelman J, Goss GD, et al. Summary statement: novel agents in the treatment of lung cancer: advances in epidermal growth factor receptor-targeted agents. Clin Cancer Res 2006;12:4365s–71s.
6. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, 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. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
8. Hirsch FR, Varella-Garcia M, Bunn PA Jr, Franklin WA, Dziadziuszko R, Thatcher N, et al. Molecular predictors of outcome with gefitinib in a phase III placebo-controlled study in advanced non-small-cell lung cancer. J Clin Oncol 2006;24:5034–42.
9. Pao W, Ladanyi M. Epidermal growth factor receptor mutation testing in lung cancer: searching for the ideal method. Clin Cancer Res 2007;13:4954–5.
10. Thomas RK, Baker AC, Debiasi RM, Winckler W, Laframboise T, Lin WM, et al. High-throughput oncogene mutation profiling in human cancer. Nat Genet 2007;39:347–51.
11. Kimura H, Kasahara K, Kawaishi M, Kunitoh H, Tamura T, Holloway B, Nishio K. 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.
12. Bell DW, Lynch TJ, Haserlat SM, Harris PL, Okimoto RA, Brannigan BW, 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.
13. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96:9236–41.
14. Oehler VG, Qin J, Ramakrishnan R, Facer G, Ananthnarayan S, Cummings C, et al. Absolute quantitative detection of ABL tyrosine kinase domain point mutations in chronic myeloid leukemia using a novel nanofluidic platform and mutation-specific PCR. Leukemia 2009;23:396–9.
15. 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.
16. Qin J, Jones RC, Ramakrishnan R. Studying copy

number variations using a nanofluidic platform. Nucleic Acids Res 2008;36:e116.
17. Simmonds P, Balfe P, Peutherer JF, Ludlam CA, Bishop JO, Brown AJ. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J Virol 1990;64:864–72.
18. Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA. Quantitation of targets for PCR by use of limiting dilution. Biotechniques 1992;13:444–9.
19. Choong NW, Dietrich S, Seiwert TY, Tretiakova MS, Nallasura V, Davies GC, et al. Gefitinib response of erlotinib-refractory lung cancer involving meninges—role of EGFR mutation. Nat Clin Pract Oncol 2006;3:50–7.
20. Spurgeon SL, Jones RC, Ramakrishnan R. High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. PLoS ONE 2008;3:e1662.
21. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
22. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, 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:e73.
23. Nomura M, Shigematsu H, Li L, Suzuki M, Takahashi T, Estess P, et al. Polymorphisms, mutations, and amplification of the EGFR gene in non-small cell lung cancers. PLoS Med 2007;4:e125.
24. Yung TK, Chan KC, Mok TS, Tong J, To KF, Lo YM. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. Clin Cancer Res 2009;15:2076–84.
25. Sakurada A, Lara-Guerra H, Liu N, Shepherd FA, Tsao MS. Tissue heterogeneity of EGFR mutation in lung adenocarcinoma. J Thorac Oncol 2008;3:527–9.
26. Yang CH, Yu CJ, Shih JY, Chang YC, Hu FC, Tsai MC, et al. Specific EGFR mutations predict treatment outcome of stage IIIB/IV patients with chemotherapy-naive non-small-cell lung cancer receiving first-line gefitinib monotherapy. J Clin Oncol 2008;26:2745–53.
27. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361:947–57.
28. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, et al. The genomic landscapes of human breast and colorectal cancers. Science 2007;318:1108–13.
29. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004;304:554.
30. Christensen JG, Burrows J, Salgia R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett 2005;225:1–26.
31. Marks JL, Broderick S, Zhou Q, Chitale D, Li AR, Zakowski MF, et al. Prognostic and therapeutic implications of EGFR and KRAS mutations in resected lung adenocarcinoma. J Thorac Oncol 2008;3:111–6.
32. Greene FL, Page DL, Fleming ID, Fritz A, Balch CM, Haller DG, Morrow M (American Joint Committee on Cancer), eds. AJCC cancer staging manual. 6th ed. New York: Springer; 2002. 435 p.