EGFR Array: Uses in the Detection of Plasma EGFR Mutations in Non–Small Cell Lung Cancer Patients

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Introduction: We aim to develop a simple and sensitive array-based method for the detection of epidermal growth factor receptor (*EGFR*) gene mutations in the plasma of non–small-cell lung cancer patients and determine its use in the follow-up of those on tyrosine-kinase inhibitor (TKI) therapy.

Method: DNA from 100 μl of plasma was amplified in the presence of peptide nucleic acid clamp to provide single-stranded template for the allele-specific arrayed primer extension reaction, incorporating cyanine-5-deoxycytidine triphosphate in the newly synthesized strands. The fluorescent product was visualized by laser at 670 nm.

Results: Eleven different types of *EGFR* TKI drug-sensitive mutants (SM) were identified in plasma-DNA from 46 of 51 patients. Five patients carried only wild-type sequence. Plasma-DNA finding was concordant in 36 of 37 cases with tumor-sequencing data. This method could detect as little as 62.5 copies of mutant L858R; 125 copies of E709K + G719A or 625 copies of del 746–750 in the presence of 100,000 copies of wild-type EGFR. In 21 patients on longitudinal follow-up for up to 18 months, SM was found in all initial plasma samples, except for three samples collected after recent chemotherapy. Nine of 16 patients (56%) who responded to TKI had undetectable plasma *EGFR* mutant. SM was present concurrently with drug-resistant mutant in 44% of patients with disease progression while on TKI, the remaining 56% might have other mechanisms of resistance.

Conclusion: The *EGFR* array provides a sensitive, inexpensive, and robust method for monitoring non–small-cell lung cancer patients' response to TKI, and obviates the need of repeated lung biopsy.

Key Words: Non–small-cell lung cancer, Plasma *EGFR* mutations, Tyrosine kinase inhibitor therapy, Follow-up, *EGFR* array.

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Carcinoma of the lung is the leading cause of cancer death worldwide. There are two main types, of which non–small-cell lung cancer (NSCLC) constitutes 80% of cases. Patients with NSCLC usually present with advanced, metastatic disease. If left untreated, they have a median survival of less than 5 months. Even with chemotherapy, which is often associated with significant toxicity, survival is usually prolonged by less than 6 months.[1] The introduction of targeted therapy such as tyrosine kinase inhibitors (TKIs) has improved survival in a subset of NSCLC patients.[2,3] It is now known that patients who respond to TKI harbor activating mutations, notably in the kinase domain (Exons 18–21) of the epidermal growth factor receptor (*EGFR*) gene.[4–6] It is therefore advisable to sequence *EGFR* gene in tumor specimens as a prediction of response to TKI therapy. It has also been observed that despite initial good response, over time (median, 6–12 months), patients develop resistance to EGFR-TKIs because of the acquisition of secondary *EGFR* mutation.[7,8] Clinically it would be important to monitor changes in molecular profiling of biological targets and be able to predict relapse early, so as to make a timely switch to second-generation or irreversible TKIs or combination TKI/chemotherapy.[9,10] However, tumor samples are not always available, particularly during the course of TKI therapy when tumor shrinkage in response to therapy would make biopsy difficult.

Many groups have advocated the measurement of circulating tumor DNA in plasma or serum samples, both at diagnosis and during follow-up. Detection methods ranging from polymerase chain reaction (PCR) plus direct sequencing to mass spectrometry genotyping[11–17] have been employed, but their sensitivity and specificity are variable. For example, although the scorpion-amplification refractory mutation system has a higher detection rate for deletional defects,[13] it can only detect 3.7% to 4.8% of the L858R mutation in plasma samples.[12,13] Given that exon 19 deletions and the exon 21 L858R mutation account for approximately 85% of clinically important *EGFR* mutations, the need to detect point mutations present in minute quantities in plasma or serum against a background of wild-type (wt) sequences is imperative. Theoretically digital PCR[16] in which single molecules of mutants (mts) can be amplified, seems the most promising. However, it requires both expensive hardware and consumables. Previously, using cyanine 5-deoxycytidine triphosphate in an allele-specific arrayed primer extension (AS-APEX) reaction, it was possible to detect the presence of paternal mutation and paternal single-nucleotide polymorphisms in maternal plasma down to one genome equivalent.[18] Others have shown that the use of peptide nucleic acid-locked nucleic

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acid (PNA-LNA) PCR clamp could enhance the sensitivity of detection by at least 100-fold.[19] In this study, a PNA clamp was used in the PCR of the template, and an array based on AS-APEX[20,21] was used to detect mutations of the *EGFR* gene in plasma samples from NSCLC patients at diagnosis and during subsequent follow-up.

PATIENTS AND METHODS

Fifty-one patients with confirmed diagnosis of NSCLC, attending the Respiratory Oncology Clinic at the Department of Medicine, Queen Mary Hospital or the 813 Medical Centre, Hong Kong were recruited. Ten patients without any malignancy, but age and sex matched to the NSCLC group, were recruited as a negative control. All patients gave informed consent, and the study was conducted with approval of the Institutional Review Board.

TKI was started for patients whose lung tumor showed a sensitive *EGFR* mutation, or for those without the benefit of tumor sequencing on clinical criteria alone, i.e., nonsmoker or ex-smoker with adenocarcinoma (only one had squamous-cell carcinoma [no. 15 Table 2], who was started on TKI after chemotherapy). Some NSCLC patients received partial or full sponsorship for TKI therapy from pharmaceutical companies (as TKI therapy is not subsidized by the government health care system in Hong Kong). The availability of such commercial sponsorship was not considered a criterion for recruitment into this project.

Citrated blood of 4.5 ml was collected from each subject, and these samples were centrifuged twice at 1800 *g* to separate the plasma and buffy coat. Plasma was stored in 1-ml aliquots and frozen until DNA extraction using the Qiagen DNA mini-kit (Qiagen, Hilden, Germany) according to the Blood and Body Fluid protocol. A single blood sample was obtained from 30 patients as a cross-sectional arm. In addition, 21 patients participated in the longitudinal study. Fifteen were treatment naive with TKI as first-line therapy, the remaining six had received chemotherapy or radiotherapy and changed to TKI upon disease progression. In all cases, blood was collected before the initiation of TKI therapy, and on two to 11 occasions during therapy at their follow-up clinic. The clinical data of the entire patient cohort showed a male:female ratio of 24:27; age range, 38 to 94 years (median 62 years); nonsmoker: ex-smoker or current smoker = 36:15; clinical staging = IB:IIA:IIIA:IIIB or IV = 1:1:3:46; histology of adenocarcinoma: non–small-cell or squamous = 46:4 (one with no histology because tumor biopsy was not performed in view of her advanced age, but staging of her disease was made based on computer-tomography findings).

These patients were observed for tumor responses and survival outcomes. Only 32 of the 51 patients were confirmed as sensitive to TKI through direct sequencing of their biopsied tumor specimen for presence of *EGFR* drug-sensitive mutation (SM) or absence of *KRAS* gene mutation. Of the remaining patients, five had no *EGFR* gene mutation in their tumor-DNA, and for the other 14 cases, TKI therapy was initiated without the benefit of *EGFR* or *KRAS* gene sequencing of their tumor.

Preparation of EGFR Array

Glass slides (75 × 25 mm dimension) were treated as described previously[22] and left for at least 1 month before spotting. Oligonucleotide primers (5 μM) pertaining to both the wt and mt sequences in Exons 18–21 of the *EGFR* gene were designed and spotted on the precoated glass slides by contact printing with a chipmaker pin (Cartesian, Irvine, CA) on the arrayer (Prosys Gantry 5510, Cartesian). The slides were left in a humid chamber for 3 hours, then allowed to dry, and stored at room temperature ready for use. Each slide contains four sections of oligonucleotides for the analysis of four different test samples. In addition, in each section, each oligonucleotide was spotted six times. Table 1 shows the mutations in Exons 18–21 of the *EGFR* gene that were spotted on the array. For convenience, separate arrays were prepared for the detection of exons 18–21 missense and insertional mutations, exons 20–21 mutations, and exon 19 deletional mutations, respectively, each containing in addition primers for the detection of common drug-resistance mutations (RM) on the right.

Preparation of Template and Array Extension

Template for primer extension was prepared by PCR. Exons 18–21 of the *EGFR* gene were amplified individually using one-tenth volume of DNA extracted from 1 ml of patient's plasma, in a 25-μl reaction volume containing 1× Accuprime buffer (containing deoxyribonucleotide triphosphate), 400-nM $MgCl_2$, 200-nM primers, and 0.5 U Accuprime (Life Technologies Corp, Carlsbad, CA). After an initial hot start of 95°C for 5 minutes, 20 cycles' amplification of 95°C for 10 seconds, 74°C for 15 seconds, and 62°C for 20 seconds were followed by another 10 cycles' amplification with an additional 2 seconds' extension time added to each cycle.

Five μl of the first PCR product was used for a second seminested PCR under the same conditions, but with the addition of either 15-pM (exon 18), 100-pM (exon 19), or 50-pM (exon 20 and exon 21) PNA-clamp in the reaction mix of 50 μl and 1 U Accuprime (Life Technologies). An additional 10 cycles' amplification was made with a final extension time of 62°C for 7 minutes.

Three μl of denatured salmon sperm DNA (0.6 μg/ml) was added to the entire amount of the second PCR product, which was precipitated with ammonium acetate and isopropanol, and resuspended in 50 μl of H_20. Five μl of this second PCR product was then used for asymmetric PCR (A-PCR) with a 1:20 ratio of forward:reverse primer, in 100-μl reaction volume and 5U Faststart Taq polymerase (Roche, Mannheim, Germany), yielding essentially A-PCR product. The A-PCR conditions consisted of a hotstart of 95°C for 5 minutes, then 20 cycles of 95°C for 10 seconds, 58°C for 20 seconds, 72°C for 10 seconds followed by another 40 cycles of the same, with 2 seconds' increase in extension time per cycle and a final extension of 72°C for 7 minutes. (Primers sequence and PNAs sequence are available on request.)

The A-PCR product was precipitated with ammonium acetate and isopropanol as described earlier, resuspended in 25 μl of H_2O and column-purified (Auto-Seq G50, Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) to

TABLE 1. List of *EGFR* Mutations Detectable on the *EGFR* Array

	Drug-Sensitive Mutations		
	Missense & Termination Mutations	Deletions and Insertions	Drug-Resistant Mutations
Exon 18	L692P		
	E709A		
	E709G		
	E709K		
	G719A		
	G719C		
	G724S		
Exon 19	W731X	del745-9	D761Y
	W731X	del746-50	
	E734K	del746-51	
	LR747-8FP	del746-52	
	A750P	del747-49	
		del747-50	
		del747-51	
		del747-52	
		del747-53	
		del752-9	
		del746-50DP	
		del746-50IP	
		del746-51A	
		del746-51VP	
		del746-52D	
		del746-52V	
		del746-53VS	
		del747-50P	
		del747-51P	
		del747-51Q	
		del741-53Q	
		del741-53S	
Exon 20	V765M	ins761EAFQ	S768I
	S768C	ins769AS	V769L
	V769M	ins769GS	insN771GT
	H773R	ins769GV	ins770NPG
	V774M	ins770SVD	ins770SVQ
	R776H	insN771GF	T 790M
	G779F	ins772PR1	
	G779S	ins772PR2	
	Q787R	ins773NPH	
	L798H	ins773VH	
	K806E		
	L814P		
Exon 21	L833V		
	V834L		
	H835L		
	L838V		
	A839T		
	K846R		
	L858M		
	L858R		
	L861Q		
	L861R		

Note that oligonucleotide primers for the drug-resistant mutants are located at one end of the array.

EGFR, epidermal growth factor receptor.

remove unincorporated deoxyribonucleotide triphosphates. The purified A-PCR product was then dried and resuspended in 2.5 µl of H_2O for analysis.

Allele-Specific Arrayed Primer Extension

To reduce nonspecific binding, the arrayed slide was preincubated in 15 µl of 1xTaq buffer (Applied Biosystems Inc., Foster City, CA) under a LifterSlip (Erie Scientific, Portsmouth, NH) in the hybridization cassette (Telechem, Sunnyvale, CA). The entire assembly was submerged in a 63°C water bath for 30 minutes. The slides were rinsed with water, spun dry at 110 *g* for 5 minutes, then overlay with the AS-APEX reaction mix containing A-PCR product and reagents as described previously.[21] For each plasma-DNA sample, A-PCR product pertaining to either Exon 18, 19, or 21 was analyzed together with the A-PCR product from Exon 20 (which harbors the TKI-resistant mutations), with the AS-APEX reaction proceeding at 63°C for 30 minutes. Upon termination of the reaction, the slide was washed, spun dry, and scanned at an emission wavelength of 670 nm and 10-µm resolution (ScanArray 5000; GSI Lumonics, Boston, MA), with the laser power set at 60 and gain at 65. The ScanArray software was used to capture the fluorescent signal but no further manipulation was needed.

Sensitivity of the Array

Lung tumor DNA carrying either Exon 18 E709K and G719A, Exon 19 deletion 746-50 or Exon 21 L858R mutations were cloned into pCR2.1-TOPO vector using reagents from the TOPO cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA of each clone was prepared and used to assess the sensitivity of this array method.

One hundred thousand copies of each mt-cloned DNA together with 100,000 copies of wt *EGFR* from total genomic DNA of a healthy individual (300 ng, based on 6 pg genomic DNA equals one genome equivalent, with two copies of wt *EGFR*) were used as a single sample to generate A-PCR template and analyzed on the array. Subsequent samples containing 10,000, 5000, 2500, 1250, 625, 312.5, 125, 62.5, and 31.25 copies mt in the presence of 100,000 copies of wt were also analyzed. The entire experiment was repeated with the different concentrations of mt in the presence of 10,000 copies of wt (30 ng genomic DNA) only.

RESULTS

Sensitivity of the Array

Using PNA in the second PCR as described earlier, it was possible to detect the presence of 125 copies of Exon 18 and 62.5 copies of Exon 21 mts or 625 copies of Exon 19 deletional mt, respectively in the presence of 100,000 copies of the wt EGFR from 300-ng total genomic DNA (Fig. 1*A*). A lower detection limit was achievable if less genomic DNA (30 ng) was used as background, viz., 31 copies of Exon 18 mt, 10 copies of Exon 21 mt, and 125 copies of Exon 19 deletional mt (Fig. 1*B*). Supplemental figures are available at http://links.lww.com/JTO/A276.

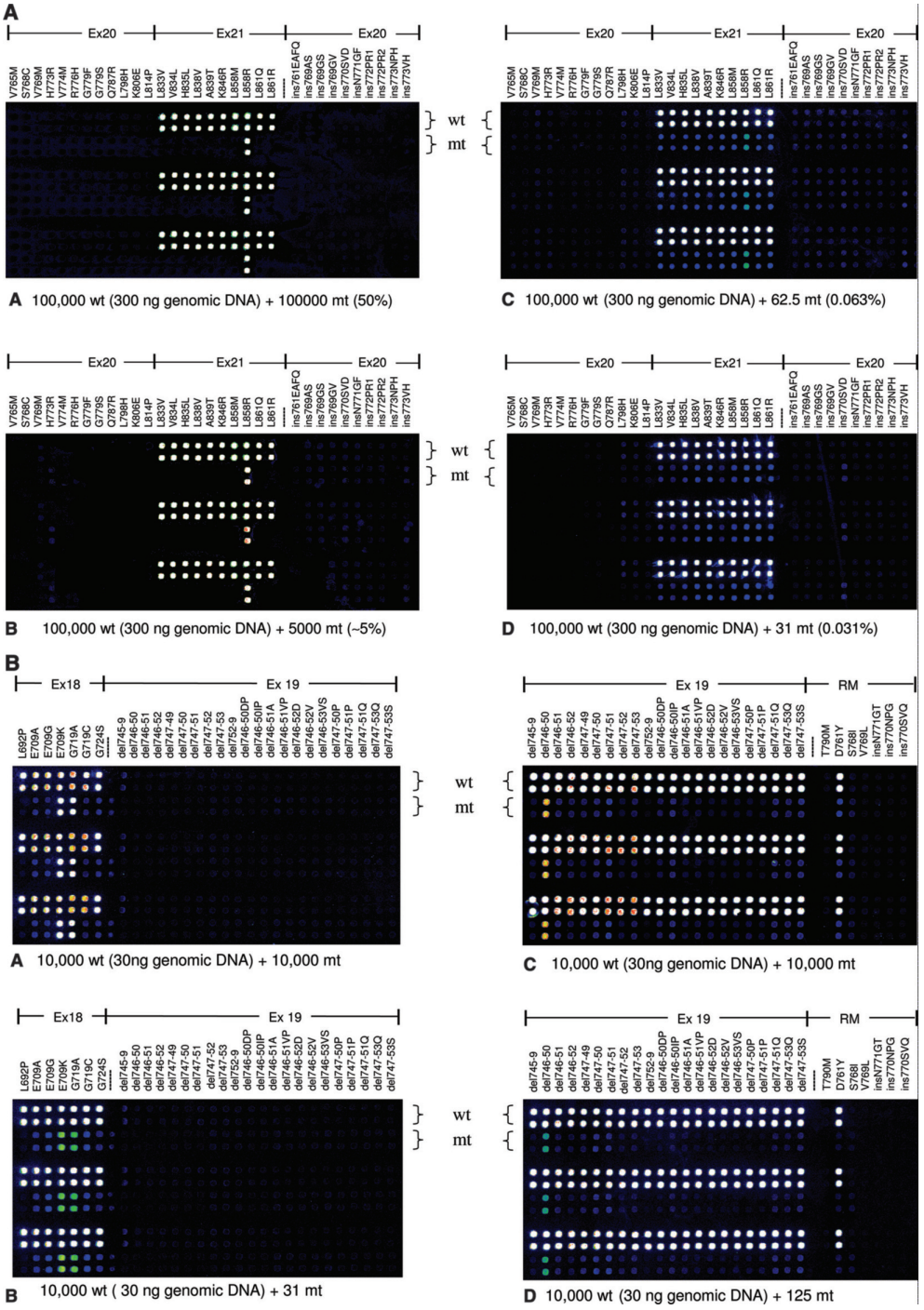

FIGURE 1. *A,* Sensitivity experiment showing the detection of A-PCR products of exon 21 of the *EGFR* gene prepared from (*A*) equal amounts (100,000 copies) of cloned L858R mutant (mt) and wild-type (wt, from 300 ng normal genomic DNA), (*B*) 5000 copies L858R mt in the presence of wt, (*C*) 62.5 copies L858R mt in the presence of wt, and (*D*) 31 copies L858R mt in the presence of wt. Each panel shows a section of the array where oligonucleotide primers for detecting mutations in Exon 20 and Exon 21 of the *EGFR* gene are spotted. Primers are spotted twice, in triplicate rows. Wt = primers for wild type sequence and mt = primer for mutant sequence. The codon position of these sequences along the gene is indicated at the top of each panel. Note that fluorescent signal intensity is white > red > yellow > green, with blue as background. *B*, Sensitivity experiment showing the detection of A-PCR products of exon 18 of the *EGFR* gene prepared from (*A*) equal amounts (10,000) copies of cloned E709K + G719A mt and wt (from 30 ng normal genomic DNA), (*B*) 31 copies of the same Exon 18 mt and 10,000 copies wt, (*C*) equal amounts (10,000 copies) of cloned Exon 19 del 746-50 mt and wt (30 ng genomic DNA), and (*D*) 125 copies Exon 19 mt in the presence of 10,000 copies wt. A-PCR, asymmetric PCR; EGFR, epidermal growth factor receptor; mt, mutant; wt, wild type.

TABLE 2. Clinical Data, Tumor and Plasma Mutation Results of All Patients

No	Code	Sex/Age	Smoking Status	Staging	Histology	Tumor/Biopsy Mutation Results	Previous Treatment & Clinical Status[a]	Plasma Mutation Results
1	L002	F/62	NS (PS)	IIIA	AD	Exon 21 L858R	Chemo-PR	L858R
2	L006	F/84	EX	IV	AD	Exon 21 L833V and L858R	Pre-treatment	L833V, L858R
3	L008	F/60	NS	IV	AD	Exon 21 L858R	Pre-treatment	L858R
4	L009	M/56	NS	IV	AD	Exon 21 L858R	Chemo-SD	L858R
5	L010	F/72	NS	IV	AD	Exon 21 L858R	Pretreatment	L858R
6	L011	F/60	NS	IIIB	AD	Exon 21 L858R	Pretreatment	L858R
7	L013	F/60	NS (PS)	IIIB	AD	Exon 21 L858R	Pretreatment	L858R
8	L014	M/79	SM	IV	AD	Del exon 19 (15bp)	Pretreatment	Del 746–50
9	L016	F/77	NS (PS)	IV	AD	Del exon 19 (15 bp)	Pretreatment	Del 747–51
10	L017	F/51	NS (PS)	IV	AD	Del exon 19	Pretreatment	Del 747–51
11	L018	M/80	EX (PS)	IIIA	AD	Exon 21 L861Q	Pretreatment	L861Q, L861R
12	L019	M/48	NS	IV	AD	Exon 21 L858R	Pretreatment	L858R
13	L001	M/48	SM	IIIB	AD	Wt	Chemo - RT-PR	L858R
14	L004	M/76	EX	IV	AD	ND	Pretreatment	Del 746–50
15	L007	M/65	EX	IIIB	SCC	ND	Chemo-SD	L858R
16	L012	F/86	NS	IV	AD	ND	Pretreatment	Del 745–51
17	TKL001	M/55	NS	IV	AD	Exon 21 L858R	Pretreatment	L858R
18	TKL002	F/69	NS	IV	AD	Del exon 19	RT	Del 747–51
19	TKL003	M/62	NS (PS)	IIIB	AD	Del exon 19 (15bp)	Chemo-PR	Del 746–50
20	TKL005	M/66	NS (PS)	IV	AD	Exon 21 L858R	Pretreatment	L858R
21	TKL006	F/71	NS	IIIB	AD	Exon 21 L858R	Pretreatment	L858R
22	TKC001	F/51	NS	IV	AD	Exon 21 L858R	Pretreatment	L858R
23	TKC002	F/61	NS	IV	AD	Del exon 19	Pretreatment	Del 747–50
24	TKC003	F/93	NS	IV	AD	Exon 18 mutation	TKI-PR	E709K, G719A
25	TKC004	F/62	NS	IV	AD	Del Exon 19 746 - 50	TKI-PD	Del 747–50
26	TKC005	F/76	NS	IV	AD	Exon 21 L858R	TKI-Chemo-PD	L858R
27	TKC006	M/87	EX	IV	AD	Exon 18 G719A	Pretreatment	G719A
28	C003	F/48	NS	IV	SCLC+AD	Del exon 19	Pretreatment	Del 747–51
29	C004	M/57	NS	IV	AD	Exon 21 L858R	Pretreatment	L858R
30	C007	F/74	NS	IV	AD	Del exon 19 (18bp)	Pretreatment	Del 747–51
31	C008	F/52	NS	IV	AD	Exon 21 L858R	Pretreatment	L858R
32	C014	M/63	SM	IV	AD	Exon 21 L858R	Pretreatment	L858R
33	C015	M/62	NS	IIIA	AD	Exon 21 L858R	Pretreatment	L858R
34	C016	M/57	NS	IV	AD	Del exon 19	Pretreatment	Del 747–51
35	C017	F/39	NS	IV	AD	Del exon 19	Pretreatment	Del 747–51
36	C018	M/38	NS	IV	AD	Del exon 19	Pretreatment	Del 747–50
37	C013	F/83	NS	IV	AD	Wt	Pretreatment	Wt
38	C021	M/48	NS	IV	AD	Wt	Chemo-PD	Wt
39	C022	M/48	NS	IV	AD	Wt	Pretreatment	Wt
40	C023	M/46	EX	IV	AD	Wt	Pretreatment	Wt
41	C001	F/81	SM	IB	AD	ND	Pretreatment	Del 747–51
42	C002	M/58	EX	IV	AD	ND	Chemo-RT	Del 747–51
43	C005	M/77	EX	IV	NSCLC	ND	Pretreatment	L858R
44	C006	F/60	SM	IV	NSCLC	ND	TKI-PD	Del 747–51
45	C009	M/55	NS	IV	AD	ND	TKI-GPR	Del 747–51
46	C010	M/62	NS	IV	AD	ND	Pretreatment	L858R
47	C011	F/61	NS	IIIB	AD	ND	Pretreatment	L858R
48	C012	F/75	NS	IV	AD	ND	Pretreatment	Del 747–50, 747–52
49	C019	M/77	EX	IV	NSCLC	ND	Pretreatment	L858R
50	C020	F/63	NS	IIA	AD	ND	Surgery	Wt
51	TKC007	F/94	EX	IIIB	ND	ND	Pretreatment	L858R

NS, nonsmoker; PS, passive smoker; EX, ex-smoker; SM, current smoker; AD, adenocarcinoma; NSCLC, non–small-cell lung cancer; SCLC, small-cell lung cancer; SCC, squamous cell carcinoma; wt, wild type; ND, not done.

[a]This relates to the patients' condition at the time of the first blood collection.

All Patients Studied

A total of 51 patients were studied, 30 in the cross-sectional group and 21 in the longitudinal study. Table 2 lists the clinical data and the results of lung tumor and plasma *EGFR* mutations in the entire cohort.

Lung tumor specimen from 37 patients had been sequenced, 32 were positive for *EGFR* drug-SM, and five with wt only. The mutation status for tumor and plasma-DNA were concordant in 36 cases. One patient (no. L001), whose tumor was thought to have wt *EGFR*, was found to carry the L858R in his plasma-DNA. This patient also participated in the longitudinal follow-up study.

It was also possible to identify the presence of SM in the plasma-DNA of all but one of the 14 patients whose lung tumor had not been sequenced (Table 2, patient no. 50). The plasma-DNA SM identified in 46 of 51 patients include 23 with exon 21 L858R, 19 with deletions of exon 19 (six were deletions of codon 747–50; 12 were deletions of codon 747–51, and one with both deletions of codon 747–50 and codon 747–52), one patient with exon 18 G719A and three other patients with double mutations (one with exon 18 E709K and G719A, one with exon 21 L833V and L858R and a third case with both exon 21 L861Q and L861R).

To test the specificity of the arrayed oligonucleotides, 10 age- and sex-matched control patients (without malignancy) served as negative control. All were found to have only wt *EGFR* in their plasma-DNA (Table 3).

TABLE 3. Plasma-DNA *EGFR* Analysis in Age-/Sex-Matched Patients Without Malignancy

No.	Code	Sex/ Age	Diagnosis	Plasma Mutation Results
1	MC 001 (12167)	F/35	Polymyositis, interstitial lung disease	Wt
2	MC 002 (12156)	F/46	Iron-deficiency anemia	Wt
3	MC 003 (12149)	F/53	Polycythemia	Wt
4	MC 004 (12169)	F/57	Hypertension	Wt
5	MC 005 (12151)	F/81	Chronic obstructive pulmonary disease	Wt
6	MC 006 (12168)	M/41	Pneumonia with empyema	Wt
7	MC 007 (12162)	M/53	Hypertension	Wt
8	MC 008 (12159)	M/58	Protein S deficiency, diabetes mellitus	Wt
9	MC 009 (12161)	M/60	Old tuberculosis diabetes mellitus	Wt
10	MC 010 (12163)	M/74	Gout, hypertension	Wt

Wt, wild type; M, male; F, female.

Longitudinal Follow-up Study

Eighty-eight serial blood samples, collected from 21 patients during longitudinal follow-up were analyzed (see Appendix, Supplemental Digital Content 1, http://links.lww.com/JTO/A275). All patients were confirmed to have SM *EGFR* mutations either in their tumor or in plasma, before the initiation of TKI (gefitinib or erlotinib) therapy.

Table 4 was derived from the longitudinal crude data (see Appendix, Supplemental Digital Content 1, http://links.lww.com/JTO/A275) of detectable plasma *EGFR* mts, the duration of treatment (grouped at 3-monthly intervals) and the clinical response of the disease at that time interval (classified as good partial response [GPR] with less than 10% residual disease; partial response [PR] with less than 50% residual disease; stable disease [SD] with 50% to 90% residual disease, and progressive disease [PD]). The absence of mutation (ND) or the presence of SM or emergence of RM (T790M only in this study) was computed for each time interval and for each disease status.

In the initial 21 plasma samples, SM was detected in 18. The three patients (L001, L002, and TKL003) with baseline negative result were found to have positive SM in their subsequent follow-up plasma samples. They had all received four courses of chemotherapy with or without radiotherapy and were in GPR/PR at the initial blood sampling. At 4 to 6 months after TKI treatment, 16 of 19 patients (84%) had GPR/PR or SD and nine (56%) had undetectable *EGFR* mutation in their plasma. Among the three patients with PD, two had in their plasma-DNA, a drug RM, T790M together with the initial SM (*, Table 4).

During the 18 months' follow-up, nine patients developed PD and four (44%) had T790M detectable in plasma, eight had persistence of the initial SM whereas one was negative for *EGFR*

TABLE 4. Longitudinal Data in 21 Lung Cancer Patients

Duration (month)	Initial				Number of Plasma Samples With *EGFR* Mutations According to Disease Status															
					GPR/PR				SD				PD				Total			
	n	ND	SM	RM	n	ND	SM	RM	n	ND	SM	RM	n	ND	SM	RM	n	ND	SM	RM
0–3	21	3	18	0																
4–6					8	6	2	0	8	3	5	1[a]	3	0	3	2[a]	19	9	10	3[a]
7–9					7	3	3	2[a,b]	3	2	1	0	3	1	2	0	13	6	6	2[a,b]
10–12					4	0	4	0	1	0	0	1[b]	1	0	1	1[a]	6	0	5	2[a,b]
13–18					1	0	1	0	2	0	1	1[b]	2	0	2	1[a]	5	0	3	2[a,b]
				n (%)	20	9 (45)	10 (50)	2 (10)	14	5 (35)	7 (50)	3 (21)	9	1 (11)	8 (88)	4 (44)	43	15	24	9

GPR/PR, good partial remission/partial remission; SD, stable disease; PD, progressive disease; n, number of plasma samples; ND, not detected; SM, sensitive mutation; RM, resistant mutation (T790M); EGFR, epidermal growth factor receptor.

Coexistence of SM and RM.

RM only, e.g., $3^{a,b}$ = all 3 samples with coexistence of SM and RM, 2^{a}=1 sample with coexistence of SM & RM and 1 sample with RM only.

mutation (Table 4). In total, nine plasma samples obtained during various times of follow-up showed T790M, six occurred in association with the initial SM but three occurred alone (Table 4). Using Kaplan-Meier analysis, the median progression-free survival in the 16 responsive patients was 14 ± 2.7 months.

One patient (L002) had received two courses of chemotherapy before TKI; blood collections were made just before TKI and during attendance at follow-up clinic in the subsequent 2 years. The clinical course and results of *EGFR* screening were detailed in Table 5. After chemotherapy, no SM was detected. After 3 months, L858R mt was seen. Blood taken at 7 months showed the emergence of a weak signal for T790M. Clinically, the tumor was downstaged from IIIA to Ib and the patient underwent surgery for removal of her residual tumor at 8 months. However, resected mediastinal lymph node showed persistence of disease. In the blood sample taken a fortnight later, the L858R mt had disappeared and only T790M remained. Post surgery, the patient was taken off TKI but received daily radiotherapy for a fortnight. No circulating *EGFR* mutations were detected during radiotherapy except for T790M. Her TKI was resumed and her plasma samples since then only showed positive signal for L858R. At 21 months (13 months after resection and adjuvant radiotherapy), she was still in clinical remission.

A second case (TKL001) (Table 5) diagnosed as Stage-IV adenocarcinoma of the lung presented with relapse, with bilateral lung masses and right cervical lymph node in February 2009 (time of recruitment into study). On initial diagnosis in March 2007 (22 months ago), he had been treated with Gefitinib for 18 months. Plasma taken before treatment showed L858R mutation only. Initial PR was achieved and upon relapse, he received two courses of chemotherapy in October 2008 and January 2009, respectively. Plasma samples taken between February to July 2009 (at 19–24 months) still showed presence of L858R only (Fig. 2*A*), although he had relapsed with metastasis to the brain. Radiotherapy to the brain and another course of chemotherapy (Pemetrexed and Temozolomide for 3 months) failed to control disease. When his lungs worsened, he was started on Erlotinib in September 2009 (at 25 months); plasma samples showed presence of T790M only at 28 to 30 months (Fig. 2*B*). The patient showed initial improvement with this second TKI and good PR for 4 months. As disease progressed, there were intense signals for both L858R and T790M at 34–37 months (Fig. 2*C*). He was given further chemotherapy and Erlotinib and Sunitinib. He eventually succumbed to allergic alveolitis from Sunitinib, with persistent intense signals for L858R only (Fig. 2*D*). The drug-resistant T790M clone of tumor cells had disappeared.

DISCUSSION

Since the initial description of the AS-APEX technology,[20] this technique has been successfully applied to the development of a number of diagnostic arrays by our group and others for mutation detection in α and β thalassemias,[18,21,23] hemophilia B,[24] and hepatitis B.[25] The sensitivity of the technique for plasma-DNA had previously been determined as down to one genome equivalent.[18] In this study, to detect *EGFR* mutations in circulating tumor cells, a seminested second PCR was used to ensure specific amplifications of the *EGFR* exon fragments. In addition, to suppress amplification of the genomic wt *EGFR* present, a PNA clamp was used. For a PNA clamp to function effectively, it should be an approximately 13 to 20 base oligonucleotide matching the wt sequence, where the expected mutation would lie in the middle of sequence.[19] This meant that unless mutations are within one to four bases of each other, different PNA clamps would be required for individual mutation. For example, although it is possible to design a single PNA clamp for the exon 19 deletions, two different clamps would be needed for the detection of L858R and L861Q despite both being in exon 21. Thus, prior sequencing of the tumor specimen to identify the *EGFR* mutation would be useful. This may not be possible for every patient because the tumor could be deep-seated and difficult to approach with conventional biopsy techniques. However, given that just two molecular defects, viz., the L858R and exon 19 deletions account for 75% of the *EGFR* SMs found in Asian patients with NSCLC,[26] we were able to identify the mutations in all but one of the 51 patients in this study, despite absence of tumor-sequencing result in 14 of them (Table 2). Because no sequencing had been performed on her tumor sample, this remaining patient (C020, Table 2) with no detectable mutation in her plasma-DNA, may well be carrier of only the wt *EGFR* gene. The sensitivity experiment showed that this array is able to detect the presence of as little as 125 copies of mt Exon 18 E709K + G719A or 62.5 copies of Exon 21 L858R and 625 copies of mt Exon 19 del 746–50 each in the presence of 100,000 copies of wt *EGFR*. This method is thus more sensitive compared with many other plasma- or serum-DNA detection techniques described to date.[11–15,17] The increased sensitivity is a result of the synergistic effects of the PNA clamp, the incorporation of cyanine 5-deoxycytidine triphosphate in the AS-APEX reaction with laser scanning of the signal in the extended strands and the increased number of amplification cycles. For the latter, a high-fidelity polymerase, Accuprime, was used to minimize PCR error. The improved sensitivity of PNA/LNA-PCR had been demonstrated recently in re-biopsy samples of lung cancer patients,[27] enabling the detection of T790M in more patients than by conventional methods. One observation from this study is the need to titrate the amount of PNA required, as too small an amount will not be sufficient to suppress the vast quantity of wt *EGFR* normally present in genomic DNA of the patient, whereas too much may also cause suppression of the mt derived from the circulating tumor DNA.

In the longitudinal study, after several months on TKI, patients with GPR/PR were observed to have lost their SM transiently (56% at 4–6 months and third sample for patient L002, and fifth sample for patient TKL001, Fig. 2*B*). This could be ascribed to the efficacy of therapy or it could be that the amount of mt in plasma was below the detection limit of this array method (determined as 62.5 copies L858R mt/100 μl plasma). In this study, 84% of patients with *EGFR* SM responded to TKI treatment and the median progression-free survival was 14 ± 2.7 months, comparable with previous reports.[7,8,28]

The appearance of the RM T790M predicted for disease progression. Although previous reports indicated that the L858R and T790M mutations are often seen in cis (L858R should always be present despite the development of the secondary RM), they can also occur in trans or in different population of tumor cells,[29,30] as observed in a number of our patients (three of nine

TABLE 5. Correlations of Treatment Response and Plasma-DNA *EGFR* Mutation Status in Two Patients

Patient Number		Prior Treatment	Progression-Free Period Before TKI Treatment if Applicable	0–3 m	4–6 m	7–9 m	10–12 m	13–15 m	16–18 m	19–21 m	22–24 m	25–27 m	28–30 m	31–33 m	34–37 m	38–40 m
L002	Sensitizing mutation			N	L858R	N	N	L858R	L858R	L858R						
	Resistance mutation			N	N	T790M	N	N	N	N						
	Treatment	Pemetrexed/ cisplatin × 3 months	Immediate switch to TKI as maintenance	Erlotinib	Erlotinib	OT + RT	Erlotinib	Erlotinib	Erlotinib	Erlotinib						
	Clinical response	SD			Downstaged	GPR	GPR	GPR	GPR	GPR						
TKL001	Sensitizing mutation			L858R					L858R	L858R	L858R	N	N	L858R wk	L858R++	L858R ++
	Resistance mutation			N					N	N	N	T790M	T790M	T790M wk	T790M++	N
	Treatment	Nil		Gefitinib	Gefitinib	Gefitinib	Gefitinib	Gefitinib	Gefitinib	Chemo	RT Brain + Chemo	Erlotinib	Erlotinib	Erlotinib	Erlotinib	Erlotinib + Sunitinib = Chemo
	Clinical response			—	PR	PR	PR	PR	PD	PR	PR	PD	PR	GPR	PD + Brain	Allergic alveolitis

N, none; wk, weak signal; ++, strong signal; GPR, good partial response; PR, partial response; PD, disease progression; SD, stable disease; OT, surgery; RT, radiotherapy; EGFR, epidermal growth factor receptor; TKI, tyrosine-kinase inhibitor.

samples in Table 4 and both L002 and TKL001). Recent evidence showed that the subset of *EGFR*-mt lung cancer patients harboring T790M mutation after progression on TKI have significantly longer postprogression survival than those with acquired resistance but lacking T790M.[31] The latter group was associated with an earlier development of new metastatic sites and a poor performance status, contributing to their shorter survival. Arcila et al.[27] advocated the detection of T790M mutation as an "acquired" prognostic biomarker. Their use of LNA to augment sequencing of the tumor had enabled the detection of T790M when present in as little as 0.1% of DNA. In the present array method, an even higher sensitivity was achieved. The detection limit is variable for different *EGFR* mutations, ranging from 625 copies of Exon 19 deletion 746–50 to 62.5 copies of L858R in the presence of 100,000 copies of wt. For patient TKL001, with further progression of disease, the use of chemotherapy in combination with erlotinib resulted in the reappearance of L858R and elimination of T790M in later plasma samples (Fig. 2*D*). The presence of a different clone of tumor cells from the original one could explain this finding. Alternatively, this could be an indication for persistence in TKI treatment or combination TKI-chemotherapy despite the development of TKI RM. Oxnard et al.[31] suggested that the beneficial effect of maintaining chronic EGFR-TKI therapy after developing resistance would help prevent the growth of TKI-sensitive clones, and additional cytotoxic therapies would be needed to treat the subset of resistant clones.[32] Recently, through systematic genetic and histological analyses of repeated lung biopsies in 37 patients with drug-resistant NSCLC carrying *EGFR* mutations, Sequist et al.[33] found that T790M could be lost in the absence of continued selective pressure of TKI treatment, and such cancers could then become sensitive to a second round of TKI. Similar finding was noted in both cases L002 and TKL001 described in this study (Table 5 and Fig. 2*D*). In the current study, nine patients developed progressive disease and T790M mutation was found in four (44%). This is similar to that reported by Sequist et al.[33] using second lung biopsy, where T790M occurred in 49% of cases, whereas in the remaining cases, the mechanisms of resistance were *EGFR* and *MET* gene amplification, mutations of the *PIK3CA* gene (a subunit of the phosphatidylinositol-3-kinase), and transformation from NSCLC to small-cell lung cancer.

The array technique has proven to be a robust method, suitable for use in any routine clinical biochemistry or DNA diagnostic laboratory. Once spotted, the oligonucleotoide primers are very stable and the array can be stored at room temperature for up to 6 months.[21] The arrays can be spotted at one center and shipped to other laboratories for use. This avoids the need to purchase hardware such as the arrayer, and all that would be required are a water bath, bench-top centrifuge, PCR cycler, and a small laser scanner at 670 nm. The outlay would be significantly less than techniques such as digital PCR or mass spectrometry genotyping. With laser scanning, the laser power was set at 60 and gain at 65, using the Scanarray software (GSI Lumonics) supplied with the machine, no further manipulation of the data is needed. This sensitive technique should allow the clinicians to follow up patients' disease progress in plasma, complementing other monitoring parameters, and also allows them to make timely decision in treatment regime, obviating the need of repeated tumor biopsies.

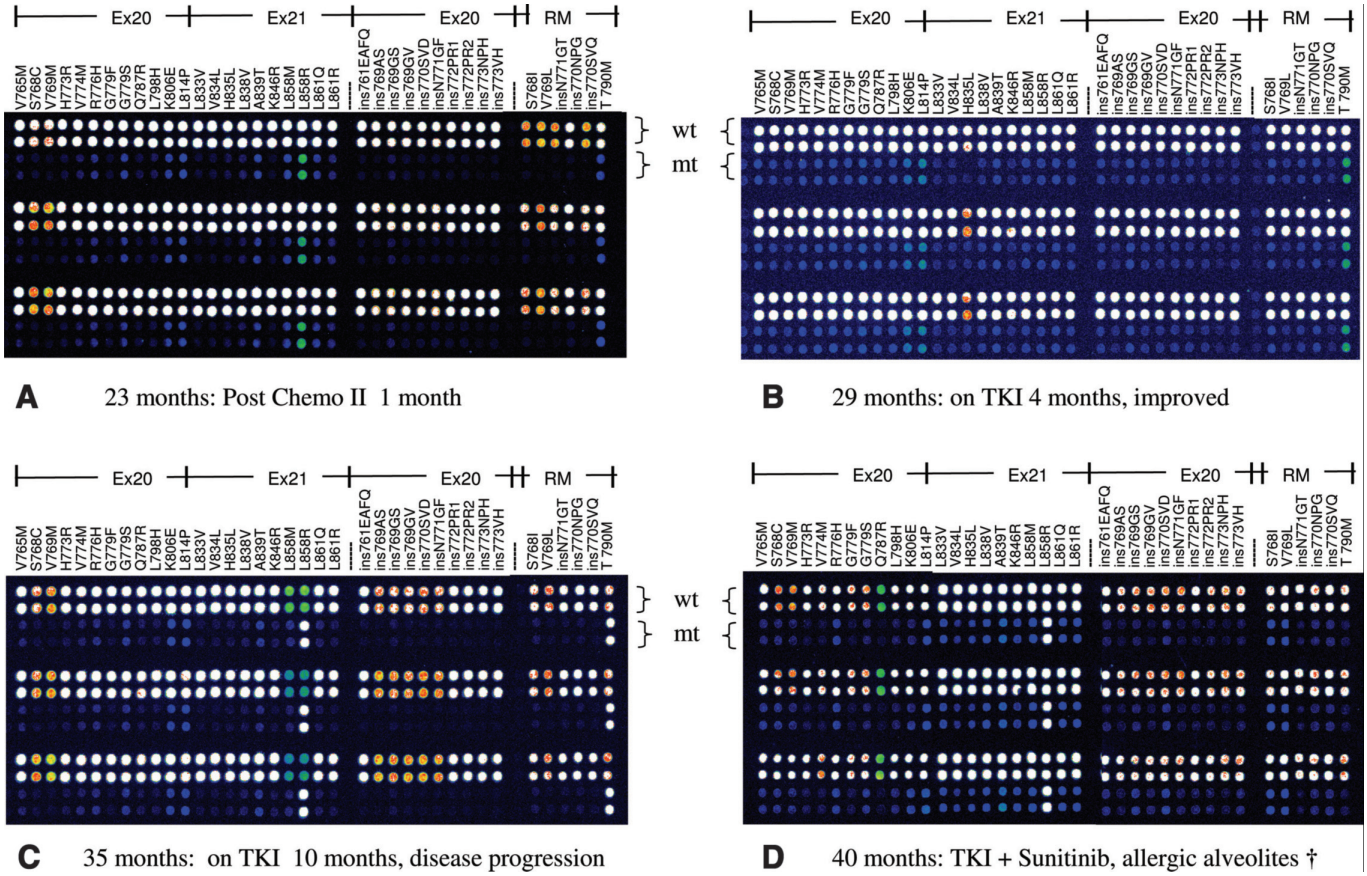

FIGURE 2. Results of plasma-DNA analysis in a patient with NSCLC (TKL001), during 40 months' longitudinal follow-up. Primers for detecting drug-resistant mutations are located at the right of each panel. NSCLC, non–small-cell lung cancer.

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