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Quantitative Detection of *EGFR* Mutations in Circulating Tumor DNA Derived from Lung Adenocarcinomas

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Abstract

Purpose: Examination of somatic epidermal growth factor receptor (*EGFR)* mutations is now a diagnostic routine for treatment of cancer using EGFR tyrosine kinase inhibitors (EGFR-TKI). Circulating tumor DNA is a promising target for noninvasive diagnostics. We evaluated its utility by quantitatively detecting activating and resistant mutations, which were measured with BEAMing (beads, emulsion, amplification, and magnetics).

Experimental Design: Twenty-three patients with lung cancer with progressive disease after EGFR-TKI treatment and 21 patients who had never been treated with EGFR-TKIs were studied. Their primary tumors were confirmed to have activating mutations. In the plasma DNA of each patient, the activating mutation found in the corresponding primary tumor and the T790M resistance mutation were quantified by BEAMing.

Results: In 32 of 44 patients, activating mutations were detected in the plasma DNA [72.7%; 95% confidence interval (CI), 58.0%–83.6%]. The T790M mutation was detected in 10 of 23 patients in the first group (43.5%; 95% CI, 25.6%–53.4%). The ratio of T790M to activating mutations ranged from 13.3% to 94.0%. The peak of the distribution of the mutation allele fraction in the plasma DNA was in the 0.1% to 1% range.

Conclusions: The major advantage of BEAMing is its ability to calculate the fraction of T790M-positive alleles from the alleles with activating mutations. This feature enables the detection of increases and decreases in the number of T790M mutations in cancer cells, regardless of normal cell DNA contamination, which may be useful for monitoring disease progression. Circulating tumor DNA could potentially be used as an alternative method for *EGFR* mutation detection. *Clin Cancer Res; 17(24); 7808–15. ©2011 AACR.*

Introduction

The strong effects of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKI; i.e., gefitinib and erlotinib) on non–small cell lung cancer (NSCLC) are correlated with activating somatic mutations in the epidermal growth factor receptor (*EGFR*; refs. 1–3). Patients subjected to these drugs are currently selected on the basis of the presence of these activating mutations. In addition, a mutation known as T790M has been identified as a cause of gefitinib resistance (4, 5). The T790M mutation appears in about half of the cases of acquired resistance to EGFR-TKIs. Detection of T790M may have prognostic value in the patients with acquired resistance to EGFR-TKIs, because the presence of T790M defines a clinical subset with a relatively favorable prognosis and more indolent progression (6).

Detecting *EGFR* mutations using tumor tissues obtained via a biopsy or surgical resection is now routinely used to diagnose NSCLC. Because a biopsy is an invasive procedure, it is desirable to replace it with a noninvasive procedure. In particular, noninvasive tests allow the frequent monitoring of disease progression in patients with the T790M mutation (7).

For some time, circulating nucleic acids in the plasma or serum have been considered to be candidates for noninvasive cancer diagnostics (8, 9). In particular, circulating tumor DNA (ctDNA) has been explored to detect somatic mutations derived from malignant tumors. For example, in 2 studies, somatic mutations in ctDNA were used to monitor disease status with the appearance of target mutations (10, 11). One major problem is that detecting rare mutant alleles is technically difficult. Diehl and colleagues used their proprietary technique called BEAMing (beads, emulsion, amplification and magnetics; ref. 12) to measure somatic mutations in ctDNA and monitor the tumor burden during the course of the disease. In BEAMing, PCR

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Translational Relevance

For therapies using EGFR-TKIs (e.g., gefitinib and erlotinib), it is essential to determine the epidermal growth factor receptor mutation status of lung cancer lesions. Although a biopsy of the primary lesion is indispensable, noninvasive diagnostics are desirable because they allow repeated testing. In particular, it is useful to follow the disease progression by monitoring the T790M status. In contrast to other techniques, beads, emulsion, amplification, and magnetics (BEAMing) can estimate the extent to which the activating mutation alleles have been converted into resistant alleles, regardless of normal DNA contamination. This information should be more suitable for monitoring the disease status. Because BEAMing also detects activating mutations with a moderate success rate, examining the ctDNA may support a diagnosis via a biopsy. It should be noted that BEAMing and next-generation sequencers are based on the same technological principle. With this study, we can predict how next generation sequencers will detect mutations in circulating tumor DNA.

products amplified from a single molecule are fixed to a single magnetic bead using emulsion PCR. The mutation site is labeled with a fluorescent probe or primer extension, and the mutated allele is quantitatively detected by counting the fluorescently labeled beads. Simply by increasing the number of beads that are analyzed, BEAMing can be more sensitive than other PCR-based techniques (13).

In this report, we used BEAMing to detect activating and resistant *EGFR* mutations in ctDNA derived from lung cancer. The results suggest that ctDNA may complement the biopsy of primary lesions as a source of *EGFR* mutation detection. Its major advantage over other techniques is its ability to calculate the fraction of T790M-positive alleles in cancer cells, regardless of normal cell DNA contamination. In particular, this approach would enable the monitoring of disease progression during EGFR-TKI therapy via the T790M mutation.

Materials and Methods

Patient characteristics

Patients with activating *EGFR* mutations in tumor tissues were selected following a biopsy examination between June 2010 and April 2011. We recruited 23 patients with progressive disease (PD) after EGFR-TKI treatment as group 1. PD is defined as the appearance of a new lesion or a 20% increase in tumor size. The duration between the detection of PD and blood sampling for BEAMing was variable. We recruited 21 patients who had never been treated with EGFR-TKIs as group 2. In all of the patients, activating EGFR mutations were found in biopsy samples using the PNA-LNA PCR clamp method (14).

Plasma samples and DNA extraction

DNA was purified from plasma obtained from 5 mL of heparin-treated blood using Agencourt Genfind version 2 (Beckman Coulter). The DNA concentration was determined by measuring the copy number of *LINE-1* (15). It should be noted that the calibration was done using intact human genomic DNA, whereas the plasma DNA was in fragments of approximately 200 bp or less. Thus, the deduced measurement may be biased to be too low.

BEAMing

BEAMing was done as described previously (16, 17), except for the use of locked nucleic acids (LNA) as the hybridization probes for single-base substitutions. Primer and probe sequences are shown in Table 1. In the initial PCR step, the target region (~100 bp) was amplified using gene-specific primers with tag sequences. Amplification was done in a 100-μL reaction mixture containing genomic DNA obtained from 400 μL of plasma, 600 pmol of primers and 2 units of KOD -Plus- DNA polymerase (Toyobo). The product was purified with a MinElute PCR Purification Kit (Qiagen).

To prepare the magnetic beads for BEAMing, a common oligonucleotide, the sequence of which was identical to the forward primer for emulsion PCR (Table 1), was synthesized using a dual biotin group at the 5′ end and a spacer 18 polyethylene glycol between the biotin group and the terminal thymidine (Integrated DNA Technologies). One nanomole of the common oligonucleotide was attached to 100 μg of MyOne streptavidin-coated magnetic beads (Dynal), as described previously (12). The beads were finally suspended in 100 μL of TK buffer (20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl). To prepare the emulsifier oil, 7% ABIL WE09 (Degussa), 20% mineral oil (Sigma-Aldrich) and 73% Tegosoft DEC (Degussa) were mixed by vortexing and allowed to settle for 30 minutes.

Emulsion PCR was done as follows. A 150-μL reaction mixture consisted of 15 pg of the first aforementioned PCR product, 15 μL of 10× KOD buffer, 75 pmol of the forward primer, 12 μmol of the reverse primer and 6 μL of the magnetic beads, which were prepared as described above. Next, 0.6 mL of the emulsifier oil and 5-mm Zirconia beads were added to the 150-μL reaction mixture. A water–oil emulsion was prepared in a 2-mL Eppendorf tube using a Mixer Mill MM 300 (Qiagen) at 15 Hz for 17 seconds. The reaction mixture was divided into 50-μL aliquots and amplified using the following thermal cycling protocol: 94°C for 2 minutes; 3 cycles of 98°C for 15 seconds, 64°C for 45 seconds, and 72°C for 75 seconds; 3 cycles of 98°C for 15 seconds, 61°C for 45 seconds, and 72°C for 75 seconds; 3 cycles of 98°C for 15 seconds, 58°C for 45 seconds, and 72°C for 75 seconds; and 50 cycles of 98°C for 15 seconds, 57°C for 45 seconds, and 72°C for 75 seconds.

After thermal cycling, the reaction mixture was centrifuged to separate the oil and water. After removing the supernatant, the emulsion was degraded with 400 μL of breaking buffer (5 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100, 1% SDS, 100 mmol/L NaCl, 1 mmol/L EDTA) and by

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Table 1. Primer list

	Name	Sequence	Modification	Target
Primers for exon amplification from plasma DNA				
exon19	Tag119del	TCCCGCGAAATTAATACGACAAGTTAAAATTCCCGTCGCTATC		
	Tag219del	GCTGGAGCTCTGCAGCTAGACCCCCACACAGCAAAG		
exon20	Tag1T790M	TCCCGCGAAATTAATACGACGCATCTGCCTCACCTCCAC		
	Tag2T790M	GCTGGAGCTCTGCAGCTAAGCAGGTACTGGGAGCCAAT		
exon21	Tag1L858R	TCCCGCGAAATTAATACGACAGCCAGGAACGTACTGGTGA		
	Tag2L858R	GCTGGAGCTCTGCAGCTATGCCTCCTTCTGCATGGTAT		
Primers for emulsion PCR				
	Tag1 (forward)	TCCCGCGAAATTAATACGAC	5′ biotin	
	Tag2 (reverse)	GCTGGAGCTCTGCAGCTA	5′ biotin	
Hybridization probes for detection of beads with successful amplification			5′ biotin	
exon19	19del_BEAM_PE_b	AGCAAAGCAGAAACTCACATC		
exon20	T790M_BEAM_PE_b	CGGACATAGTCCAGGAG		
exon21	L858R_BEAM_PE_b	ATGCCTCCTTCTGCATGGTAT		
Hybrization probe for BEAMing				
exon19	19del_35_49_647	GGAGATGTTTTGATAGCG	5′ Alexa647	exon 19 E746-A750del
	19del_36_50_647	CGGAGATGTCTTGATAGC	5′ Alexa647	exon 19 E746-A750del
	19del_40_57_647	TGGCTTTCGATTCCTTGA	5′ Alexa647	exon 19 L747-S752del.P753S
	19del_AATTCC_647	TGTTGCTTCTCTTGGAATT	5′ Alexa647	exon 19 E746-L747del.IP
	19del_36_55_T_647	GCTTTCGGAACCTTGATAG	5′ Alexa647	exon 19 L747-S752del. E746V
	19del_35_53_ACT_647	GGAGAAGTTTTGATAGCG	5′ Alexa647	exon 19 K745-E749del.A750K
	19del_39_56_CAG_647	TTTCGGCTGTTCCTTGAT	5′ Alexa647	exon 19 L747-T751del.S752Q
	19del_39_48_C_647	GAGATGTTGGTTCCTTGAT	5′ Alexa647	exon 19 L747-E749del.A750P
	19del_WT_488	TGTTGCTTCTCTTAATTCC	5′ Alexa488	exon 19 wild-type control
exon20	T790M_Mut_BNA_647	atgagctgcAtgatgag	5′ Alexa647	*T790M* mutation
	T790M_WT_BNA_488	tgagctgcGtgatgag	5′ Alexa488	Wild-type control for T790M
exon21	L858R_Mut_LNA_647	gtttggccCgcccaaat	5′ Alexa647	*L858R* mutation
	L858R_WT_LNA_488	gtttggccAgcccaaat	5′ Alexa488	Wild-type control for L858R
	T2582A_Mut_647	cacccagcTgtttggcc	5′ Alexa647	*T2582A* mutation
	T2582A_WT_488	cacccagcAgtttggcc	5′ Alexa488	Wild-type control for T2582A

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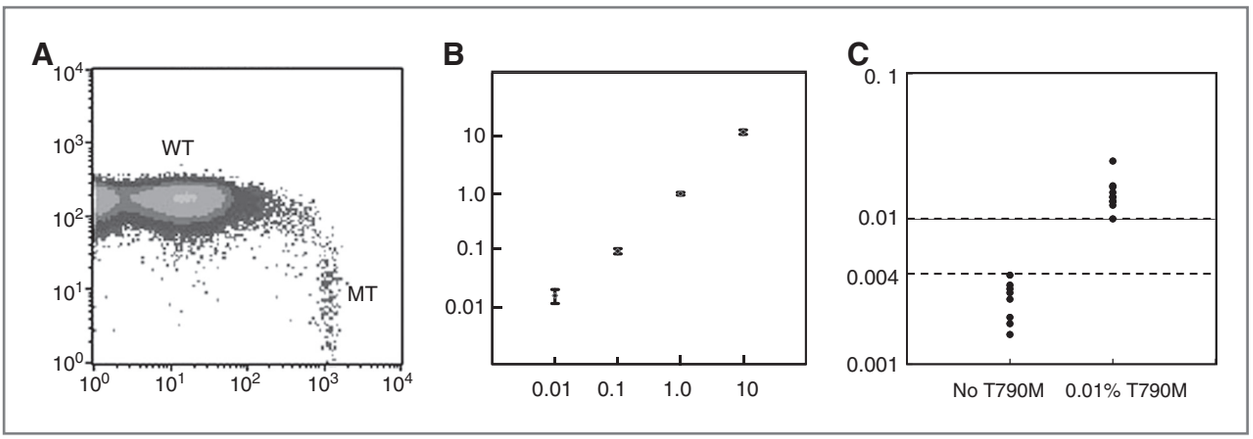

Figure 1. A, flow cytometric profile of BEAMing. The wild-type *EGFR* fragment was mixed with 0.1% of the T790M *EGFR* fragment, and BEAMing was done after PCR amplification. Horizontal axis, the fluorescence intensity of Alexa 647; vertical axis, the fluorescence intensity of Alexa 488. WT, signals from the wild-type *EGFR* fragment; MT, signals from the T789M fragment. B, linear correlation between the inoculated amount of the T790M *EGFR* fragment and the BEAMing measurement. Horizontal axis, the fraction of the T790M fragment inoculated into the wild-type fragment; vertical axis, the fraction of the T790M-positive allele measured using BEAMing. C, repeated measurements of the samples with 0.01% of the T790M fragment and those with no T790M fragment. The vertical axis indicates the fractions of the T790M-positive allele detected using BEAMing.

vortexing. After centrifugation and removal of the supernatant, the beads were washed once. Next, the DNA on the beads was denatured by 2 minutes of incubation at room temperature with 500 μL of 0.1 mol/L NaOH. After washing twice, the beads were suspended in 30 μL of distilled water.

The mutation loci were detected using allele-specific hybridization probes that consisted of locked nucleic acids and were fluorescence-labeled at their 5′ ends. Alexa 647 and Alexa 488 fluorescent dyes were used for the mutated and wild-type alleles, respectively. A hybridization probe complementary to common sequences in the mutated and wild-type alleles was manufactured via 5′ biotinylation. The hybridization reactions were carried out in a 100-μL reaction mixture consisting of 3 mol/L tetramethylammonium chloride, 50 mmol/L Tris-HCl (pH 7.5), 4 mmol/L EDTA, and 5 pmol each of the aforementioned hybridization probes. The reaction mixture was divided into 50 μL aliquots, incubated at 70°C for 10 seconds, then at 35 °C for 2 minutes after cooling down at a rate of 0.1°C/s, and additionally cooled down to room temperature using the GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems). After removing the supernatant, the beads were incubated at room temperature for 10 minutes in 20 μL of binding buffer (5 mmol/L Tris-HCl, pH 7.5, 1 mol/L NaCl, 1 mmol/L EDTA) containing 2 μg of streptavidin-conjugated phycoerythrin (PE; Invitrogen). After washing, the beads were suspended in 100 μL of TK buffer. Flow cytometric analysis was conducted with FACSCalibur (BD Bioscience) according to the manufacturer's protocol.

Results

Quantitation of the accuracy and sensitivity of BEAMing

We examined the measurement's accuracy and sensitivity using T790M as an example. We prepared normal *EGFR* gene fragments containing the mutated fragment at 10%, 1%, 0.1%, and 0.01%. These preparations were subjected to emulsion PCR. A typical example of a flow cytometric profile separating 1% T790M from the wild-type allele is shown in Fig. 1A. In BEAMing, the fractions of the mutated fragment are estimated by the ratio of the numbers of beads labeled with Alexa 647 (mutant) and those labeled with Alexa 488 (wild-type). There is a good linear correlation between the ratio deduced from the numbers of beads and the fraction of mutated fragments in the initial preparations (Fig. 1B). To determine the detection limit of BEAMing, samples without the T790M mutation and samples with 0.01% T790M mutations were analyzed repeatedly. The measurements of these 2 groups did not overlap (Fig. 1C). To confirm this result in a real experimental setting, we measured exon 19 deletion, L858R, and T790M mutations in the plasma DNA purified from 20 normal individuals. The mutation rates ranged from 0 to 0.0094 (average, 0.0021; 95% CI, 0.0012–0.0030), from 0.0009 to 0.0074 (average, 0.0025; 95% CI, 0.0019–0.0031) and from 0.0011 to 0.0097 (average, 0.0042; 95% CI 0.0030–0.0054), respectively. Thus, we set the detection limit of BEAMing as 1 in 10,000.

Activating and resistant *EGFR* mutations in plasma DNA

Plasma obtained from 44 patients was analyzed by BEAMing for the *T790M* mutation, and activating mutations were determined via a tumor biopsy. The results are shown in Table 2. In group 1, which consisted of patients who developed PD after EGFR-TKI treatment, the detection of activating and *T790M* mutations can be evaluated. In group 2, which consisted of patients who were never treated with EGFR-TKI, only those with activating mutations were evaluated. Most of the cases were in stage IV when their plasma DNA was obtained. In 32 of 44 patients, activating

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Table 2. Allele frequency of activating and resistant *EGFR* mutations

Patient	Age, y	Sex	Histology	Stage	T790M, %	Activating mutation, %	T790M/activating mutation, %	Activating mutation type
A. Group 1 (patients with PD after EGFR-TKI treatment)								
1	56	M	adeno	4	0.029	0.058	50.8	L861Q
2	58	F	adeno	4	0.26	0.28	94.0	L858R
3	70	F	adeno	4	2.61	7.39	35.3	L858R
4	78	M	adeno	3A	0.08	0.13	65.0	L858R
5	65	M	Adeno + Sq	4	0.63	1.10	57.6	L858R
6	20	M	Adeno	4	0.14	1.03	13.3	exon 19 E746-A750del
7	41	F	adeno	4	4.28	10.3	41.6	exon 19 E746-A750del
8	59	F	adeno	4	9.54	42.7	22.3	exon 19 E746-A750del
9	53	M	adeno	4	0.16	0.19	83.6	exon 19 E746-A750del
10	49	F	adeno	4	ND	0.12	0.0	L861Q
11	75	F	adeno	4	ND	2.03	0.0	L858R
12	34	M	adeno	4	ND	0.33	0.0	L858R
13	64	M	adeno	4	ND	12.2	0.0	L858R
14	73	F	adeno	4	ND	0.046	0.0	L858R
15	66	F	adeno	4	ND	0.28	0.0	exon 19 L747-S752del.P753S
16	70	M	adeno	4	ND	11.5	0.0	exon 19 L747-S752del.P753S
17	44	F	adeno	4	ND	0.09	0.0	exon 19 L747-E749del.A750P
18	52	M	adeno	4	ND	0.74	0.0	exon 19 L747-E749del.A750P
19	74	F	adeno	4	ND	0.33	0.0	exon 19 E746-A750del
20	63	F	adeno	4	ND	ND	NA	L858R
21	65	F	adeno	4	0.10	ND	NA	exon 19 E746-A750del
22	51	F	adeno	4	ND	ND	NA	exon 19 E746-A750del
23	62	F	adeno	4	ND	ND	NA	exon 19 E746-A750del
B. Group 2 (patients not treated with EGFR-TKI)								
24	68	M	adeno	4	ND	0.17	0.0	L858R
25	45	F	adeno	4	ND	0.23	0.0	L858R
26	85	F	adeno	2B	ND	0.25	0.0	L858R
27	67	F	adeno	4	ND	0.079	0.0	L858R
28	58	F	adeno	4	ND	0.013	0.0	L858R
29	39	F	adeno	3B	ND	36.4	0.0	L858R
30	36	F	adeno	4	ND	0.11	0.0	exon 19 L747-S752del.E746V
31	56	M	adeno	4	ND	6.47	0.0	exon 19 L747-T751del.S752Q
32	55	F	adeno	3A	ND	6.24	0.0	exon 19 L747-E749del.A750P
33	65	F	adeno	3B	ND	11.8	0.0	exon 19 E746-A750del
34	76	F	adeno	4	ND	1.06	0.0	exon 19 E746-A750del
35	63	F	Sq	4	ND	0.73	0.0	exon 19 E746-A750del
36	63	M	adeno	4	ND	0.030	0.0	exon 19 E746-L747del.IP
37	72	F	adeno	4	ND	ND	NA	L858R
38	70	F	adeno	4	ND	ND	NA	L858R
39	63	M	adeno	4	ND	ND	NA	L858R
40	80	F	adeno	4	ND	ND	NA	L858R
41	70	F	adeno	4	ND	ND	NA	L858R
42	72	M	adeno	4	0.03	ND	NA	exon 19 L747-S752del.P753S
43	47	M	adeno	4	ND	ND	NA	exon 19 E746-A750del
44	54	F	adeno	4	ND	ND	NA	exon 19 E745-E749del.A750K

Abbreviations: NA, not applicable; ND, not detected.

mutations were detected in the plasma DNA (72.7%; 95% CI, 58.0–83.6%). The detection rate was higher in group 1 (group 1, 82.6%; group 2, 61.9%), but this difference was not statistically significant (the Fischer exact test, $P = 0.18$). The detection rates of L858R/L861Q and exon 19 deletion were identical (72.7%). The T790M mutation was detected in 10 of 23 patients in group 1 (43.5%; 95% CI, 25.6%–53.4%). Because T790M accounted for about half of the

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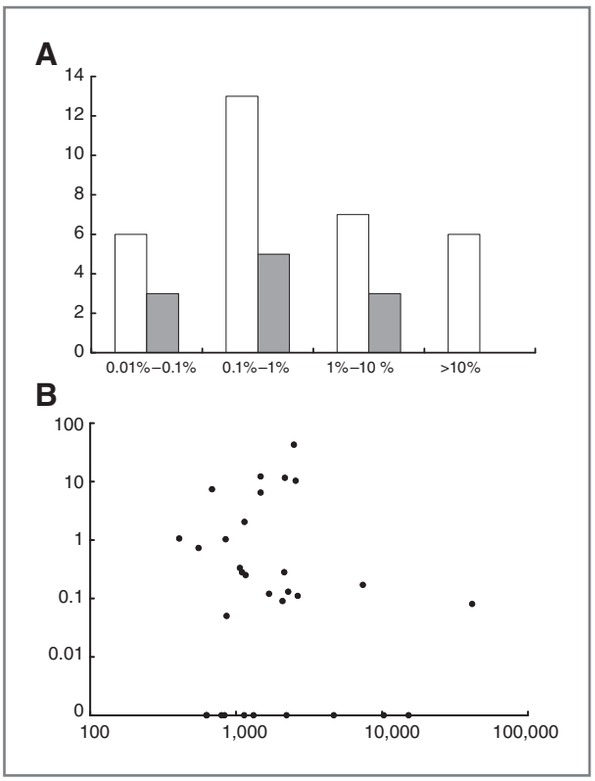

Figure 2. A, distribution of the fraction of *EGFR* molecules with activating (white columns) or resistant (gray columns) mutations in the plasma DNA. Horizontal axis, the percentage of activating EGFR mutations; vertical axis, the number of patients. B, relationship between the amount of recovered plasma DNA and the fraction of activating *EGFR* mutations. Horizontal axis, the amount of recovered plasma DNA (pg) corresponding to 400 μL of plasma; vertical axis, the percentage of activating *EGFR* mutations.

TKI-resistant cases, BEAMing was likely to detect T790M in most of the eligible cases. There were 2 cases of T790M mutations without an activating mutation (i.e., patients 21 and 42).

The fraction of ctDNA in plasma DNA can be estimated from the fraction of *EGFR* mutations [Table 2, activating mutations (%)]. On the basis of the histogram in Fig. 2A, the fraction of activating mutations varied widely across patients, and the peak of the distribution was in the 0.1% to 1% range. The fraction of the *T790M* mutation was distributed similarly but tended to shift toward lower percentages. We also investigated the relationship between the amount of recovered plasma DNA and the ctDNA deduced from the activating mutations, but we found no relationship between them (Fig. 2B). It should be noted that *EGFR* mutations were not detected in some samples with a high plasma DNA recovery.

We can deduce the number of tumor *EGFR* alleles that have been converted into resistant forms (i.e., T790M) by calculating the ratio of T790M to the number of activating mutation fractions. The ratios were within a range of 13.3% to 94.0% (Table 2), in contrast to a much wider range of tumor alleles in the plasma DNA.

Discussion

There have been a number of studies on the analysis of ctDNA to detect *EGFR* mutations in the serum or plasma DNA of NSCLC patients. These studies have mainly used techniques based on selective amplification (18, 19) or digestion (19) of specific alleles and/or high-throughput separation techniques [i.e., MALDI-TOF (20) or denaturing high performance liquid chromatography (DHPLC) (19, 21)]. The sensitivity is restricted by the specificity of the primers and enzymes in the former and by the signal-to-noise ratio in the latter. Because the sensitivity of BEAMing is only restricted by the mutations introduced during PCR (which is common to all techniques), it is theoretically more sensitive than other methods.

In addition to its high sensitivity, BEAMing allows the digital quantification of mutant alleles. The DNA in blood is derived from both tumor cells and normal cells, but we still do not know how the DNA in blood is generated. The major advantage of BEAMing is its ability to calculate the fraction of T790M-positive alleles from alleles with activating mutations. This feature enables the monitoring of the fractions of the T790M mutation in cancer cells, regardless of normal cell DNA contamination. This information should be more suitable for monitoring disease statuses. Some patients were reported to have cancer cells with T790M as a minor subpopulation before EGFR-TKI treatment (22, 23). With these cases, a qualitative assay to monitor T790M is inappropriate, and it is desirable to monitor amount of the T790M allele quantitatively. The fraction of the T790M allele would increase during the EGFR-TKI therapy and eventually reach a threshold to acquire resistance. Such threshold can be determined only with a quanititative assay. Partly due to difficulty of the biopsy of recurrent cases, clinical features of T790M-based resistance have not been fully understood, but are currently intensively studied. Such studies would find applications of the quantitative assay. For example, detecting the T790M mutation in blood samples would be useful for patient selection for treatment with new EGFR-TKIs for lung cancers that are resistant to gefitinib and erlotinib (24). One of such agents, PF00299804, is effective to a T790M-positive cell line. However, amplification of the T790M allele led to resistant to PF00299804 (25). If PF00299804 acts in patients in the same manner, quantitative monitoring of T790M allele would be useful for detection of resistance. In such patients, a biopsy of the tumor tissue is difficult and noninvasive diagnostics are highly beneficial.

The aim of this study is the initial demonstration of the technique, and has limitations as a clinical study. The patients were not enrolled in this study prospectively, and the timing of blood draws was not consistent such that the results are not directly applicable to distinct clinical situations. In addition, patients did not have serial tumor biopsies to document development of T790M in their cancer after exposure to EGFR-TKI. A well-designed prospective study enrolling more than 200 Japanese patients, a population with high incidence of activating mutation, now

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being planned to validate present observations. It should be noted that specificity, that is, absence of pseudopositive, is most important for new noninvasive diagnostics. The validation study should also be focused on this point as well.

As suggested by a recent review (26), the most problematic aspect of ctDNA analysis is the difficulty in purifying DNA from the blood. As described above, the amount of plasma DNA varies by 2 orders of magnitude. The cause of this variation (i.e., whether it is due to true variation or the low reproducibility of the purification procedure) is unknown. However, it should be noted that unsuccessful mutation detection was not necessarily frequent in the cases with low DNA recovery and that unsuccessful mutation detection was still found among those with abundant DNA recovery. Some cases of low DNA recovery contained the minimum number of *EGFR* copies for detection. In such cases, whole-genome amplification is beneficial for sound PCR and may enhance the detection rate, as seen in a previous study (19).

This study focused on advanced lung cancer (mainly stage IV lung cancer). If ctDNA analysis is effective for early lung cancer, then it may be applicable to early cancer detection. Because ctDNA is also easily detected in the early stages of colorectal cancer (27), it is worthwhile to test ctDNA analysis for early lung cancer.

BEAMing uses the template preparation step of massively parallel sequencers (so-called next-generation sequencers; ref. 28). Therefore, we can predict the outcome when massively parallel sequencers are applied to this problem. The recent development of a new sequencer (29) has addressed the shortcomings of currently available sequencers (i.e., a long runtime for a single assay and high operating costs), and would be suitable for diagnostic purposes. The cost of sequencing is still rapidly decreasing, and will be eventually negligible in the total cost of the assay. In contrast to BEAMing, which analyzes only a single base and requires information about mutations in primary tumors, the massively parallel sequencers obtain information from more than a hundred bases and could replace BEAMing. A recent study pointed out the need for repeated sequencing to overcome the high error rates of the sequencers that are currently used to detect rare mutations (30). However, in the case of *EGFR* mutations, because the mutation sites are already known, rare mutations may be detected with a statistical method without the repeated sequencing. Our study forecasts the outcome of ctDNA analysis using massively parallel sequencers, suggesting that ctDNA analysis could determine the *EGFR* mutation status of more than 70% of advanced lung cancer cases. In addition, there might be cases in which *EGFR* mutations could be detected only with ctDNA analysis, not with a conventional biopsy. Given the noninvasive nature of the ctDNA analysis, it is a worthwhile field for future investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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