Table 3. Discrimination performance of LDA and c-logistic models using concentrations as explanatory variables.

Model	Subjects		LC	GC	CRC	BC	PC	Pooled
LDA	All	AUC	0.802	0.849	0.874	0.778	0.783	0.796
		CI	(0.766~0.836)	(0.816~0.882)	(0.842~0.906)	(0.741~0.815)	(0.740~0.826)	(0.779~0.814)
	LOOCV	AUC	0.792	0.845	0.868	0.769	0.767	0.793
	Stage 0 patients	AUC	-	-	0.903	0.813		
		CI			(0.807~1.00)	(0.726~0.900)		
	Stage I patients	AUC	0.752	0.859	0.859	0.754		
		CI	(0.698~0.805)	(0.820~0.898)	(0.800~0.918)	(0.692~0.817)		
	Stage II(B) patients	AUC	0.870	0.829	0.921	0.786	0.764	
		CI	(0.772~0.969)	(0.726~0.933)	(0.877~0.954)	(0.727~0.847)	(0.710~0.819)	
	Stage III(C) patients	AUC	0.844	0.834	0.817	0.755	0.777	
		CI	(0.780~0.908)	(0.748~0.920)	(0.743~0.892)	(0.621~0.889)	(0.669~0.885)	
	Stage IV(D) patients	AUC	0.901	0.843	0.950	-	0.873	
		CI	(0.837~0.966)	(0.734~0.951)	(0.895~1.00)		(0.771~0.974)	
C-logit	All	AUC	0.806	0.850	0.876	0.776	0.786	0.798
		CI	(0.771~0.841)	(0.816~0.883)	(0.845~0.907)	(0.739~0.812)	(0.743~0.829)	(0.780~0.815)

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Nevertheless, it remains unclear how the metabolic changes occurring in cancer patients affect the PFAA profile of the whole body, even in patients with early-stage tumors. To clarify the relationship between carcinogenesis and changes in PFAA profiles, we are further investigating the contribution of local effects caused by cancer cell metabolism and the systemic responses of the immune system against tumors or factors released by cancer cells.

Changes in metabolism can be detected in cancer cells even in early-stage patients. Hirayama *et al.* reported no significant correlation between the levels of cancer cell metabolites, including several amino acids, and the tumor stage [13]. The metabolism of Trp is of particular interest because it was identified as one of the most important amino acids in relation to cancer progression in our study. Overexpression of indoleamine-2,3- dioxygenase (IDO), the first enzyme in the kynurenine Trp metabolism pathway in humans, has been reported in cancer cells [47]. IDO is induced in many different tumors and has been suggested to play a role in cancer-mediated evasion of the immune system [47,48,49,50].

Arg, Orn, Cit, and Pro are known to be closely related to immune function. For example, Qiu *et al.* reported an association between the urea cycle and metabolic alterations in CRC patients and found no correlation between the metabolite profile and cancer progression [43]. Cancer cells also release factors that can alter general physical conditions. For example, the transcriptional regulatory molecule high-mobility group B1 (HMGB1) was recently shown to regulate cancer-cell tumorigenesis, expansion, and invasion [51,52,53].

Further elucidation of these mechanisms might allow for the development of both static and dynamic models of carcinogenesis through system analysis [31]. Recently, computer-aided studies have been reported that integrate hierarchical 'omics' datasets for the systemic understanding of metabolic phenotypes to reconstruct the regulatory network from physiological data by means of system analysis. System analysis of cancer patients based on whole body amino acid metabolism could reveal information concerning the nature of a disease and help to establish strategies for its prevention, early detection, prognosis, monitoring, and treatment.

In contrast to many similar efforts to detect biomarkers of disease as single specific molecules (DNA, microRNA, proteins, peptides, or metabolites) in peripheral blood, our approach was to focus on the metabolic status, which is indicative of multivariate function, using non-specific metabolites. Therefore, we believe that our method is superior to those used in other studies, both in versatility and efficiency, because only one amino acid measurement can be applied for detection of various disease states (i.e., renal failure, hepatic failure, and nutritional status).

Table 4. Multiclass discriminant analyses of male cancer patients using concentrations as explanatory variables.

		Patients with:			
		LC	GC	CRC	PC
Discriminated as:	LC	**72(69)**	*19(22)*	*12(13)*	*26(26)*
	GC	*18(19)*	**58(52)**	*16(17)*	*25(25)*
	CRC	*13(14)*	*25(28)*	**71(69)**	*16(17)*
	PC	*22(23)*	*24(24)*	*15(15)*	**67(66)**
	Total	125	126	114	134
	Accuracy	**57.6%(55.2%)**	**46.0%(41.3%)**	**62.3%(60.5%)**	**50.0%(49.3%)**

The numbers in the blanket indicate the results of LOOCV.

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Table 5. Multiclass discriminant analyses of female cancer patients using concentrations as explanatory variables.

		Patients with:			
		LC	GC	CRC	BC
Discriminated as:	LC	**41(37)**	*4(6)*	*8(11)*	*43(44)*
	GC	*13(14)*	**40(38)**	*15(16)*	*30(30)*
	CRC	*6(8)*	*13(13)*	**52(47)**	*17(17)*
	BC	*15(16)*	*16(16)*	*10(11)*	**106(105)**
	Total	75	73	85	196
	Accuracy	**54.7%(49.3%)**	**54.8%(52.1%)**	**61.2%(55.2%)**	**54.1(53.6%)**

The numbers in the blanket indicate the results of LOOCV.
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Supporting Information

Figure S1 PFAA profiles of cancer patients stratified by progression stage. The axes show the AUC of ROC for each amino acid for discriminating patients from controls. A. Comparison of concentrations of cancer patients and controls. B. Comparison of ratios of cancer patients and controls. Scale as described for Figure 2. For LC, GC, CRC, and BC, cancer stages were determined according to the International Union Against Cancer TNM Classification of Malignant Tumors, 6th edition [38], and for PC, cancer stages were determined according to Jewett staging system [39].
(TIF)

Table S1 Detailed demographic and clinical characteristics of subjects. a: $p<0.05$, c: $p<0.001$ *: For LC, GC, CRC, and BC, cancer stages were determined according to the International Union Against Cancer TNM Classification of Malignant Tumors, 6th edition [38], and for PC, cancer stages were determined according to Jewett staging system [39].
(XLS)

Table S2 PFAA profiles of cancer patients and controls.
(XLS)

Table S3 AUCs of ROC of each amino acid concentration for discrimination for cancer patients from controls.
(XLS)

Table S4 AUCs of ROC of each amino acid ratio for discrimination for cancer patients from controls. AUCs were calculated using all patients and controls, and patiens and matched controls stratified by cancer stage.
(XLS)

Table S5 Significance values for PFAA profiles for each data set by two-way ANOVA for the effects of cancer existence and other parameters. Column headings indicate Mann-Whitney U-test of cancer existence (None), two-way ANOVA for the effects of cancer existence and gender (Gender), cancer existence and age (Age), and cancer existence and smoking status (Smoking).
(XLS)

Table S6 Variables incorporated into LDA and c-logistic models using ratios as explanatory variables. +, ++, +++: positive coefficients in the model −, −−, −−−: negative coefficients in the model +,−: $p<0.05$, ++,−−: $p<0.01$, +++,−−−: $p<0.001$.
(XLS)

Table S7 Discrimination performance of LDA and c-logistic models using ratios as explanatory variables.
(XLS)

Table S8 Multiclass discriminant analyses of male cancer patients using ratios as explanatory variables. The numbers in the blanket indicate the results of LOOCV.
(XLS)

Table S9 Multiclass discriminant analyses of female cancer patients using ratios as explanatory variables. The numbers in the blanket indicate the results of LOOCV.
(XLS)

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Author Contributions

Conceived and designed the experiments: YM HY MY NO. Performed the experiments: MH AG MA TI T. Miura NS EB HK FI MM II AC FO HM OT T. Mitsushima MY NO. Analyzed the data: YM AI KH. Contributed reagents/materials/analysis tools: HM. Wrote the paper: YM AI KH.

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Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas

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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 *EGFR* mutations is now a diagnostic routine for treatment of cancer using EGFR tyrosine kinase inhibitors (EGFR-TKI). Circulating tumor DNA (ctDNA) is a promising target for non-invasive 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 lung cancer patients 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 out of 44 patients, activating mutations were detected in the plasma DNA (72.7%; 95% CI, 58.0 – 83.6%). The T790M mutation was detected in 10 out 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 – 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. ctDNA could potentially be used as an alternative method for *EGFR* mutation detection.

Translational relevance

For therapies using EGFR-TKIs (e.g., gefitinib and erlotinib), it is essential to determine the EGFR mutation status of lung cancer lesions. Although a biopsy of the primary lesion is indispensable, non-invasive 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, 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 ctDNA.

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Introduction

The strong effects of epidermal growth factor receptor 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*) (1-3). Patients subjected to these drugs are currently selected based on 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 biopy 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 non-invasive procedure. In particular, non-invasive 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 non-invasive cancer diagnostics (8, 9). In particular, circulating tumor DNA (ctDNA) has been explored to detect somatic mutations derived from malignant tumors. For example, in two 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 et al. used their proprietary technique called BEAMing (beads, emulsion, amplification and magnetics) (12) to measure somatic mutations in ctDNA and monitor the tumor burden during the course of the disease. In BEAMing, PCR 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 performed 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 performed 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 performed 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 nmol 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 mM Tris-HCl, pH 8.4, 50 mM 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 performed as follows. A 150-µl reaction mixture consisted of 15 pg of the first aforementioned PCR product, 15 µl of 10x 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 min; three cycles of 98°C for 15 sec, 64°C for 45 sec, and 72°C for 75 sec; three cycles of 98°C for 15 sec, 61°C for 45 sec, and 72°C for 75 sec; three cycles of 98°C for 15 sec, 58°C for 45 sec, and 72°C for 75 sec; and 50 cycles of 98°C for 15 sec, 57°C for 45 sec, and 72°C for 75 sec.

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 mM Tris-HCl, pH 7.5, 1% Triton-X100, 1% SDS, 100 mM NaCl, 1 mM EDTA) and by 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 M 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 performed in a 100-µl reaction mixture consisting of 3 M tetramethylammonium chloride, 50 mM Tris-HCl (pH 7.5), 4 mM 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 sec, then at 35 °C for 2 minutes after cooling down at a rate of 0.1°C/sec, 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 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA) containing 2 µg of streptavidin-conjugated phycoerythrin (PE) (Invitrogen). After washing, the beads were suspended in 100 µl of TK buffer. Flow cytometry analysis was performed 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 Figure 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 (Figure

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 two groups did not overlap (Figure 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 twenty 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 (0.0025; 0.0019 – 0.0031) and from 0.0011 to 0.0097 (0.0042; 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 out of 44 patients, activating 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 (Fischer's exact test, p=0.18). The detection rates of L858R and exon

19 deletion were identical (72.7%). The T790M mutation was detected in 10 out of 23 patients in group 1 (43.5%; 95% CI, 25.6 – 53.4%). Because T790M accounted for about half of the TKI-resistant cases, BEAMing was likely to detect T790M in most of the eligible cases. There were two 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 (%)). Based on the histogram in Figure 2a, the fraction of activating mutations varied widely across patients, and the peak of the distribution was in the 0.1-1% 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 (Figure 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 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 leaded 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 non-invasive 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 being planned to validate present observations. It

should be noted that specificity, i.e., absence of pseudo-positive, 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 two 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) (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 non-invasive nature of the ctDNA analysis, it is a worthwhile field for future investigation.

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