Barcodesequence identifies resistant mechanisms to epidermal growth factor receptor inhibitors in circulating tumor DNA of lung cancer patients

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Abstract
Most patients with epidermal growth factor receptor (EGFR) mutation-positive non-small cell lung cancer (NSCLC) will inevitably develop acquired resistance induced by treatment with EGFR tyrosine kinase inhibitors (EGFR-TKI). The mechanisms of resistance to EGFR-TKI are multifactorial, and the detection of these mechanisms is critical for treatment choices in patients who have progressed after EGFR-TKI therapy. We evaluated the feasibility of a molecular barcode method using next-generation sequencing to detect multifactorial resistance mechanisms in circulating tumor DNA and compared the results with those obtained using other technologies. Plasma samples were collected from 25 EGFR mutation-positive NSCLC patients after the development of EGFR-TKI resistance. Somatic mutation profiles of these samples were assessed using two methods of next-generation sequencing and droplet digital PCR (ddPCR). The positive rate for EGFR-sensitizing mutations was 18/25 (72.0%) using ddPCR, 17/25 (68.0%) using amplicon sequencing, and 19/25 (76.0%) using molecular barcode sequencing. Rate of the EGFR T790M resistance mutation among patients with EGFR-sensitizing mutations was shown to be 7/18 (38.9%) using ddPCR, 6/17 (35.3%) using amplicon sequencing, and 8/19 (42.1%) using molecular barcode sequencing. Copy number gain in the MET gene was detected in three cases using ddPCR. PIK3CA, KRAS and TP53 mutations were detected using amplicon sequencing. Molecular barcode sequencing detected PIK3CA, TP53, KRAS, and MAP2K1 mutations. Results of the three assays were comparable; however, in cell-free DNA, molecular barcode sequencing detected mutations causing multifactorial resistance more sensitively than did the other assays.

KEYWORDS
circulating tumor DNA, droplet digital PCR, epidermal growth factor receptor, molecular barcode sequencing, non-small cell lung cancer
1 | INTRODUCTION

Lung cancer is the leading cause of cancer death. Targeted therapies are the first treatment option for advanced non-small cell lung cancer (NSCLC) harboring driver mutations. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) such as gefitinib, erlotinib, afatinib and osimertinib are the most important targeted therapies for NSCLC. However, tumor tissue samples are not always available for detecting EGFR mutations in clinical practice. In these cases, liquid biopsies can be used instead of tissue specimens. Plasma cell-free DNA (cfDNA), collected as one type of liquid biopsy, can reflect the tumor genotype to some extent.

Actionable somatic EGFR mutations are associated with the therapeutic response to EGFR-TKI in individuals with advanced NSCLC. However, the majority of tumors develop acquired resistance to EGFR-TKI within 10-16 months after therapy initiation. Multifactorial mechanisms of resistance have been identified, including a secondary point mutation site where methionine is substituted for threonine at position 790 (T790M) in EGFR, amplification of the mesenchymal-to-epithelial transition factor receptor (MET), mutation of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), transformation to small cell lung cancer, loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), an epithelial-mesenchymal transition, and MET exon 14 skipping. Of these mechanisms, T790M mutation is the most common cause of acquired resistance to EGFR-TKI, found in up to 50% of patients after treatment with EGFR-TKI. To overcome the acquired resistance to EGFR-TKI caused by T790M, next-generation EGFR-TKI have been developed: osimertinib is an irreversibly binding EGFR-TKI with specific, robust activity against the T790M mutant and only minimal activity against wild-type EGFR. However, detecting the T790M mutation has proven challenging in clinical practice as a result of the difficulty in obtaining post-relapse samples by re-biopsy.

Liquid biopsy using cell-free DNA (cfDNA) from the blood of cancer patients has been shown to be a promising means of detecting EGFR-activating mutations. The most commonly used tests include both non-diagnostic platforms (cobas EGFR Mutation test, Roche Molecular Systems, Pleasanton, CA, USA) and digital platforms (droplet digital PCR [ddPCR] and BEAMing digital PCR). Droplet digital PCR devices (QX100 ddPCR system; Bio-Rad Laboratories, Hercules, CA, USA) can generate ~20 000 droplets and can successfully detect EGFR mutations in cfDNA, whereas next-generation sequencing (NGS) technologies using ultra-deep sequencing can extensively and simultaneously analyze multiple genes to different types of genetic aberrations, including mutations, copy number variants, and gene rearrangements. Although the application of NGS to cfDNA is feasible, the minimum detection limit is too low to detect very rare mutated alleles.

Molecular barcode DNA sequencing is expected to increase the minimum detection limit for alleles with minor mutation. However, the feasibility of applying molecular barcode NGS to cfDNA is unclear. The present study highlights the technical feasibility and potential clinical utility of three assays for detecting somatic mutations related to EGFR-TKI resistance in cfDNA derived from clinical plasma samples.

Copy number gains are frequently detected in malignant tumors, including lung cancer. MET or ERBB2 oncogene amplifications have been shown to evolve during the development of resistance to EGFR-TKI in NSCLC. ERBB2 genomic amplification is rare in lung cancer but is more frequent in patients with breast cancer, and can be detected in cfDNA. We previously detected oncogene copy number gains in the plasma cfDNA of colorectal cancer patients using ddPCR. In the present study, we attempted to detect multiple gene alterations affecting resistance, including mutations and copy number gains, using ddPCR and NGS in cfDNA.

2 | MATERIALS AND METHODS

2.1 | Patients

Our study cohort comprised patients with pathologically confirmed advanced (stage IIIb or IV) or recurrent NSCLC who had been treated with EGFR-TKI at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research between October 2015 and March 2016. Using medical records, we retrospectively reviewed the patients’ EGFR T790M status and clinical characteristics. The present study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by the Institutional Review Board of the Cancer Institute Hospital of the Japanese Foundation for Cancer Research and the Kindai University Faculty of Medicine.

2.2 | Blood sample collection

EDTA anticoagulated whole blood (7 mL) was obtained from patients with activating EGFR mutation-positive NSCLC identified using commercial assays, such as therascreen EGFR assay (Qiagen) or the cobas EGFR Mutation Test (Roche Molecular Systems, Pleasanton, CA, USA), using formalin-fixed, paraffin-embedded tissues obtained at the time of diagnosis. Blood samples were centrifuged at 1400 x g for 10 minutes, and the plasma supernatant was stored at -80°C until analysis. Circulating cfDNA was purified using a MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s procedure. DNA concentration of the extracted cfDNA was determined using an RNaseP Copy Number Assay (Thermo Fisher Scientific). The extracted DNA was stored at -80°C until analysis.

2.3 | Digital PCR analysis

Mutant allele frequency was measured using the QX100 Droplet Digital PCR System in accordance with the manufacturer’s instructions (Bio-Rad). The primers and probes for detecting EGFR exon 19 deletion, L858R, and T790M were purchased from Bio-Rad. The PCR reaction was carried out using the
following cycling conditions: 95°C for 10 minutes, 40 cycles at 94°C for 30 seconds and at 55°C for 60 seconds, followed by enzyme deactivation at 98°C for 10 minutes. For the MET copy number assay, the primer sequences were as follows: MET forward, 5'-TTAGTTGCTACGATGCAAGAG-3'; MET reverse, 5'-GGCTTGCACTCTCCGGGACT-3'; MET probe, 5'/-FAM/CACACTCCT/GEN/CATTGGATAGGGCTTG/3IABkFQ/-3'; RPP30 forward, 5'-GATTGGACCTGGACGCG-3'; RPP30 reverse, 5'-GCGGCCTGTCTCCACACAGT-3'; and RPP30 probe, 5'/-HEX/CTGACCTGAAGGCTCT/3IABkFQ/-3'. The PCR reaction was carried out using the following cycling conditions: 95°C for 10 minutes, 40 cycles at 94°C for 30 seconds and at 60°C for 90 seconds, followed by enzyme deactivation at 98°C for 10 minutes. After thermal cycling, the plates were transferred to a droplet reader. The ddPCR data were analyzed using the QuantaSoft analytical software package (Bio-Rad). Plasmid fragments encoding wild-type EGFR, E746-A750 deletion, L858R, and T790M were used as controls. The cut-off values were set at 3.0 copies each for EGFR E746-A750, EGFR L858R, and EGFR T790M. The cut-off value for a MET copy number gain was set at 2.5 copies, as previously described.\textsuperscript{4,18}

### 2.4 | Amplicon targeted sequencing

Amplicon sequencing was carried out using Ion AmpliSeq Colon and Lung Cancer Panel v2 (CLv2; Thermo Fisher Scientific), which targets 22 cancer-associated genes: AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH1, NRAS, PIK3CA, PTEN, SMAD4, STK11, and TP53. For library preparation, cfDNA (maximum of 10 ng) was subjected to multiplex PCR amplification using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific) according to the manufacturer’s protocol. Purified libraries were pooled then sequenced with an Ion Torrent Proton instrument, the Ion PI Hi-Q Chef Kit, and the Ion PI Chip Kit v3 (all from Thermo Fisher Scientific). DNA sequencing data were accessed through the Torrent Suite version 5.10 program (Thermo Fisher Scientific). Reads were aligned with the hg19 human reference genome, and potential mutations were called using a plug-in cfDNA variant caller.

### 2.5 | Molecular barcode sequencing

Molecular barcode sequencing was carried out using the Ion Torrent Oncomine cfDNA Lung Assay (Thermo Fisher Scientific), which targets 11 cancer-associated genes: ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1, and TP53. Library preparation was carried out using cfDNA (maximum of 10 ng) according to the manufacturer’s instructions. Purified libraries were pooled then sequenced with an Ion Torrent Proton instrument, the Ion PI Hi-Q Chef Kit, and the Ion PI Chip Kit v3 (all from Thermo Fisher Scientific). DNA sequencing data were accessed through the Torrent Suite version 5.10 program (Thermo Fisher Scientific). Reads were aligned with the hg19 human reference genome, and potential mutations were called using a plug-in cfDNA variant caller.

### 2.6 | Statistical analysis

Correlations between the presence of a mutation and patient characteristics were evaluated using the chi-squared ($\chi^2$) test. $P$-value $<.05$ was considered statistically significant. All the statistical analyses were carried out using JMP software (ver. 10; SAS Institute).

### 3 | RESULTS

#### 3.1 | Sensitivity of molecular barcode sequencing

We investigated the sensitivity and feasibility of a targeted sequencing method that adds barcode sequences by adaptor ligation using the Ion Torrent Oncomine cfDNA assay (molecular barcode sequencing). A multiplex I cfDNA reference standard (Horizon Discovery, Waterbeach, UK) was used to determine the minimum detection limit. Engineered samples with 0.1%, 1%, and 5% mutant DNA were subjected to the molecular barcode sequencing assay. Frequencies of the detected mutant alleles are summarized in Tables 1 and 2. The $R^2$ values between the designed and estimated mutant allele frequencies for each EGFR mutation were 0.997-1.000. Mutations in PIK3CA, KRAS, and NRAS, adjust for the bias of PCR amplification. The adjusted read depth was log2-transformed, and the median log2 value per gene was used for the copy number analysis. The log2 ratio cut-off value for the copy number gain was set at 1.25 based on a previous study.\textsuperscript{20,21}

### TABLE 1 | Sensitivity of molecular barcode sequencing: EGFR mutations

| Designed mutant frequency (%) | Mutant detection frequency (%) |
|-------------------------------|--------------------------------|
|                               | EGFR.p. L858R  | EGFR.p. E746_A750delELREA | EGFR.p. T790M | EGFR.p. V769_D770insASV |
| 0.10                          | 0.2             | 0.12                        | 0.07          | 0.24                      |
| 1.00                          | 0.82            | 0.78                        | 1.03          | 1.15                      |
| 5.00                          | 5.1             | 4.23                        | 5.26          | 5.06                      |
| $R^2$                         | 0.9968          | 0.9995                      | 1.0000        | 1.0000                    |

Abbreviation: EGFR, epidermal growth factor receptor.
as well as those in EGFR, were correctly called, and the frequencies of these mutant alleles were consistent with the expected values. The $R^2$ values between the designed and estimated mutant allele frequencies for each mutation were 0.990-1.000. This result suggests that the minimum detection limit of the assay is below 0.1%.

### 3.2 Detection of mutations in cfDNA using molecular barcode sequencing

DNA was extracted from plasma samples collected from EGFR mutation-positive NSCLC patients after the acquisition of resistance to EGFR-TKI ($n = 25$). Patient characteristics are shown in Table 3. Twenty-five patients were treated with afatinib after resistance to EGFR-TKI developed. Median plasma volume was 1.6 mL (range, 0.5-2.0 mL), and the median amount of DNA extracted from the plasma was 7.76 ng per sample (range, 1.75-34.92 ng), suggesting that the plasma samples were successfully processed for DNA extraction. Twenty-five cfDNA samples were tested with three different molecular assays to compare mutation profiles. Amount of input cfDNA is shown in Table S1. Average depths of 25 cfDNA samples measured by molecular barcode sequencing and amplicon sequencing were 88,009 and 42,147 reads per sample, respectively (Table S1). Among the 25 plasma samples obtained from patients after developing acquired resistance to EGFR-TKI, TKI-sensitizing mutations were found in 19 (76.0%) cases by molecular barcode sequencing. An EGFR T790M mutation was detected in eight of the 19 (42.1%) patients whose plasma tested positive for a TKI-sensitizing mutation (Table 4). The response rate was 48% (12/25), this rate is higher than in previous reports, because of the inclusion of cases that were sequentially treated after chemotherapy. No relationship was observed between EGFR mutation status and response to afatinib.

### 3.3 Comparisons of EGFR mutation detection in plasma cfDNA

We compared the frequencies of EGFR mutations in plasma detected using molecular barcode sequencing (Oncomine Lung cfDNA assay), conventional amplicon sequencing (CLv2, colon and lung cancer research panel), and ddPCR (Table 4). Amplicon sequencing detected TKI-sensitizing mutations in 17 of 25 (68.0%) samples and EGFR T790M mutations in six of 17 (35.3%) samples from patients whose plasma tested positive for TKI-sensitizing mutations. Concordances of EGFR TKI-sensitizing and T790M mutation detection between molecular barcode sequencing and ddPCR were 88.0% and 96.0%, respectively (Table S2). In contrast, molecular barcode sequencing detected a TKI-sensitizing mutation in 19 of 25 (76.0%) samples and EGFR T790M mutations in eight of 19 (42.1%) samples from patients whose plasma tested positive for the TKI-sensitizing mutation. Concordances of EGFR TKI-sensitizing and T790M mutation detection between molecular barcode sequencing and ddPCR were 88.0% and 96.0%, respectively (Table S2). Taken together, these results suggest that the frequencies of mutation detection were comparable among the three technologies. In order to examine the amount of input DNA affecting detection sensitivity, samples were divided into two groups; samples with high- and low-input DNA based on the amount of DNA input. Significantly higher detection rates of TKI-sensitizing mutations were observed in the group with high-input DNA compared to the group with low-input DNA in the molecular barcode sequencing assay (Table S2). No correlation was found in the other assays. This result suggests that higher amounts of input DNA may be advantageous for the increased detection rate of mutations in the molecular barcode sequencing assay.

### 3.4 Comparisons of the detection of other gene mutations in plasma cfDNA

Several resistance mechanisms to EGFR-TKI other than the EGFR T790M mutation are known to exist. Variant calling by amplicon

### Table 2: Sensitivity of molecular barcode sequencing: Other mutations

| Designed mutant frequency (%) | Mutant detection frequency (%) |
|-------------------------------|-------------------------------|
| KRAS:p.G12D | NRAS:p.Q61K | NRAS:p.A59T | PIK3CA:p.E545K |
| 0.13 | 0.16 | 0.13 | 0.17 | 0.13 |
| 1.30 | 1.02 | 1.67 | 1.07 | 1.44 |
| 6.30 | 7.19 | 5.58 | 6.93 | 6.36 |
| $R^2$ | 0.9955 | 0.9904 | 0.9968 | 0.9995 |

### Table 3: Characteristics of lung cancer patients ($n = 25$)

| Characteristic | Classification | No. (%) |
|----------------|----------------|---------|
| Age, years median (range) | 67 (48-93) |
| <65 | 10 (40.0) |
| ≥65 | 15 (60.0) |
| Gender | | |
| Male | 9 (36.0) |
| Female | 16 (64.0) |
| Smoking status | | |
| Current or former | 14 (56.0) |
| Never | 11 (44.0) |
| Disease stage | | |
| IVA | 6 (24.0) |
| IVB | 12 (48.0) |
| Postoperative recurrence | 7 (28.0) |
| Response to afatinib | | |
| PR | 12 (48.0) |
| SD | 6 (24.0) |
| PD | 6 (24.0) |
| NE | 1 (4.0) |

Abbreviations: NE, not evaluable; PD, progressive disease; PR, partial response; SD, stable disease.
sequencing and molecular barcode sequencing were carried out as described in Materials and Methods. Amplicon sequencing detected TP53 mutations in seven cases (28.0%), an EGFR mutation in one case (4.0%), an ERBB4 mutation in one case (4.0%), a KRAS mutation in one case (4.0%), and a PIK3CA mutation in one case (4.0%). Molecular barcode sequencing detected TP53 mutations in eight cases (32.0%), PIK3CA mutations in two cases (8.0%), a KRAS mutation in one case (4.0%), and a MAP2K1 mutation in one case (4.0%) using a plug-in cfDNA variant caller. We found four cases with mutations called in amplicon sequencing but uncalled in molecular barcode sequencing. We verified the existence of these mutations using the Integrative Genomics Viewer (IGV) and could confirm these mutations in molecular barcode sequencing (Figure S1). The reason for this uncalling is that it is considered to be limited to the hotspot region that was predesigned for variant calling in molecular barcode sequencing. In contrast, mutations in six cases were called by molecular barcode sequencing but not by amplicon sequencing. Variant alleles of these six cases were not confirmed by IGV of amplicon sequencing. One ERBB4 mutation (case 669, p.S341L) detected using amplicon sequencing was not identified by molecular barcode sequencing as ERBB4 is not included in the panel of molecular barcode sequencing. Overall, the concordance of gene mutation detection between molecular barcode sequencing and amplicon sequencing was 79.3% (23/29 see Table 6).

### 3.5 Detection of MET copy number gains in plasma cfDNA

MET gene amplification is a known mechanism of resistance to EGFR-TKI. We tested for MET copy number gains using molecular barcode sequencing, conventional amplicon sequencing, and ddPCR. None of the 25 cases showed a MET copy number gain using the sequencing assay. ddPCR detected a copy number gain in the MET gene in plasma samples from three of 25 cases (12.0%). Both EGFR T790M and a MET copy number gain were detected in one case.

| Type of TKI-sensitizing mutation at diagnosis | Molecular barcode sequencing | Amplicon sequencing | Droplet digital PCR |
|---------------------------------------------|------------------------------|-------------------|--------------------|
| TKI-sensitizing mutation (VAF) | T970M mutation (VAF) | TKI-sensitizing mutation (VAF) | T970M mutation (VAF) | TKI-sensitizing mutation | T970M mutation |
| 645 Del19 | + (1.26) | + (4.07) | - | + | - |
| 646 Del19 | + (3.35) | + (0.65) | + (5.68) | - | + | + |
| 654 Del19 | + (17.92) | + (17.40) | + (11.41) | + (7.33) | + | + |
| 655 Del19 | + (6.71) | + (1.80) | + (9.14) | + (0.94) | + | + |
| 660 Del19 | - | - | - | - | - | - |
| 669 Del19 | + (10.65) | + (11.55) | - | + | - |
| 672 Del19 | + (0.98) | + (0.71) | - | + | - |
| 673 L858R | + (19.34) | + (4.38) | + (19.24) | + (5.18) | + | + |
| 674 Del19 | + (25.85) | - | + (37.53) | - | + | - |
| 675 L858R | + (0.08) | - | - | - | - | - |
| 690 L858R | - | - | - | - | - | - |
| 691 Del19 | + (18.75) | + (13.47) | - | + | - |
| 700 L858R | + (0.87) | + (1.40) | - | + | - |
| 701 Del19 | - | - | - | - | - | - |
| 714 Del19 | + (5.02) | + (3.54) | + (9.87) | + (3.67) | + | + |
| 717 Del19 | + (48.09) | + (23.79) | + (70.59) | + (23.23) | + | + |
| 718 L858R | - | - | - | - | - | - |
| 729 Del19 | - | - | - | - | - | - |
| 730 L858R | + (8.84) | - | + (37.72) | - | + | - |
| 731 Del19 | + (19.67) | - | + (36.50) | - | + | - |
| 733 Del19 | - | - | - | - | - | - |
| 751 Del19 | + (8.60) | - | + (9.06) | - | + | - |
| 756 L858R | + (9.48) | + (13.98) | + (9.80) | + (7.51) | + | + |
| 766 L858R | + (0.24) | - | - | - | - | - |
| 770 L858R | + (1.03) | + (0.94) | + (2.25) | - | + | - |

Abbreviation: Del19, exon 19 deletion mutation; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; VAF, variant allele frequency.
TABLE 5 Concordance of EGFR mutation detection by ddPCR, amplicon sequencing, and molecular barcode sequencing

| Droplet digital PCR | + | − | Total |
|---------------------|---|---|-------|
| **A** Amplicon sequencing | 17 | 0 | 17 |
| + | 17 | 0 | 17 |
| − | 1 | 7 | 8 |
| Total | 18 | 7 | 25 |

| Molecular barcode sequencing | + | − | Total |
|-----------------------------|---|---|-------|
| **B** Amplicon sequencing | 6 | 0 | 6 |
| + | 6 | 0 | 6 |
| − | 1 | 18 | 19 |
| Total | 7 | 18 | 25 |

| Molecular barcode sequencing | + | − | Total |
|-----------------------------|---|---|-------|
| **C** Molecular barcode sequencing | 17 | 2 | 19 |
| + | 17 | 2 | 19 |
| − | 1 | 5 | 6 |
| Total | 18 | 7 | 25 |

| Molecular barcode sequencing | + | − | Total |
|-----------------------------|---|---|-------|
| **D** Molecular barcode sequencing | 7 | 1 | 8 |
| + | 7 | 1 | 8 |
| − | 0 | 17 | 17 |
| Total | 7 | 18 | 25 |

(A) Concordance of active mutation detection between droplet digital PCR (ddPCR) and amplicon sequencing. (B) Concordance of T790M mutation detection between ddPCR and amplicon sequencing. (C) Concordance of active mutation detection between ddPCR and molecular barcode sequencing. (D) Concordance of T790M mutation detection between ddPCR and molecular barcode sequencing. Abbreviation: EGFR, epidermal growth factor receptor.

TABLE 6 Concordance of other mutations between molecular barcode sequencing and amplicon sequencing

| Molecular barcode sequencing | + | − | Total |
|-----------------------------|---|---|-------|
| **A** Amplicon sequencing | 10 | 6 | 16 |
| + | 10 | 6 | 16 |
| − | 0 | 13 | 13 |
| Total | 10 | 19 | 29 |

(A) Concordance of other mutations between molecular barcode sequencing and amplicon sequencing.

These results suggest that ddPCR is more sensitive than the NGS system for detecting copy number gains in cfDNA.

4 | DISCUSSION

In the present study, we compared the detection frequencies of EGFR mutations and other gene mutations in plasma cfDNA using molecular barcode sequencing, conventional amplicon sequencing, and ddPCR. Minimum detection limit of barcode cfDNA sequencing was approximately <0.1%, as estimated using standard samples (Tables 1 and 2), and this assay was deemed to be highly quantitative, with high R² values (Tables 1 and 2). The sensitivity of molecular barcode sequencing (0.1%) seems to be greater than that of conventional amplicon sequencing.12,24

The minimum detection limits of molecular barcode sequencing and ddPCR were approximately 0.1% and 0.01%, respectively. These results suggest that barcode cfDNA sequencing, with its minimum detection limit of ~0.1%, seems to be sufficient to detect mutations in plasma cfDNA. Overall, all of these technologies seem to be a feasible means of detecting mutations in cfDNA. However, each assay has specific features in this regard.

In detecting EGFR mutations, allele frequency of EGFR mutations was correlated between the three assays. Mutant allele frequency (%) was observed between molecular barcode sequencing and ddPCR assays yielding correlation coefficients (R²) of 0.8202 and 0.9911 for TKI-sensitizing mutation and T790M, respectively. Likewise, mutant allele frequency (%) between amplicon sequencing and ddPCR assay showed high correlation coefficients (R²) of 0.9043 and 0.8141 for TKI-sensitizing mutation and T790M, respectively.

In contrast, we found three cases in which these were EGFR mutation-positive in molecular barcode sequencing but negative in ddPCR and amplicon sequencing (case nos 675, 766, and 770). In two of the three cases, we detected 2.0 and 1.6 copies of the EGFR mutation in case nos 766 and 770 by ddPCR, respectively. The allele frequencies of the two cases, estimated as mutant copy number/wild-type copy number were 0.18% and 0.65% by ddPCR, respectively, and this was consistent with the allele frequency of molecular barcode sequencing. Although the probability of false positivity cannot be completely dismissed, it is possible that low-input DNA may have led to dispersion differences of the mutant allele between the three assays thus contributing to the differences in detection.

In the present cohort, ddPCR detected a MET copy number gain in three of 25 (12%) plasma cfDNA samples. However, copy number gains were not detected in either of the NGS-based assays. The cut-off value was defined based on the log2 ratio of the read depth of NGS.20 We previously reported the detection of copy number gains in HER2 using ddPCR in colorectal cancer patients who were refractory to anti-EGFR antibody therapy.19 Thus, ddPCR might have specific features that influence its ability to sensitively detect copy number gains. However, the mechanisms of resistance to EGFR-TKI are multifactorial. Amplifications of HER2 or the wild-type allele of EGFR both constitute mechanisms of acquired resistance.10,25 HER2 amplification, identified using FISH in tumor tissues, has also been observed in 12% of drug-resistant, EGFR-mutant lung cancers.10 Detection of these copy number abnormalities in the cfDNA of patients who have received EGFR-TKI therapy is important for subsequent treatment. Amplicon NGS sequencing and barcode cfDNA sequencing are characterized by the detection of multiple genes. Indeed, NGS panels can identify several gene mutations, including those in TP53, PIK3CA, KRAS, and ERBB4, in addition to EGFR mutations in cfDNA. PIK3CA
mutations are known mechanisms of resistance to EGFR-TKIs. In the present study, ERBB4 S341L and EGFR T790M mutations were detected in cfDNA using amplicon sequencing. These mutations are variants of uncertain significance. However, in addition to detecting TP53, KARS, and PIK3CA mutations, molecular barcode sequencing was able to identify MAP2K1 Q56P mutations and was the only modality to do so. MAP2K1 Q56P is known as a major actionable mutation of MEK1 in NSCLC. This mutation occurs outside of the kinase domain of MEK1, and preclinical data have shown that it leads to increased MEK1 kinase activity in vitro. In contrast, the presence of MEK1 mutations has been associated with in vitro resistance to EGFR-TKIs. The ability to detect mutations varied between amplicon and barcode-based NGS, possibly because of differences between the two assay systems in the target sites for each gene.

The theoretical minimum detection limit of amplicon sequencing ranged from 12.7% to 0.103% and was <0.5% for 84.4% (1017/1205) of hotspot sites. In contrast, barcode sequencing has sufficient sensitivity (approximately < 0.1%). However, the detection rate for EGFR TKI-sensitizing and T790M mutations was comparable to those using ddPCR and amplicon sequencing. Compared to ddPCR, multi-gene analysis by molecular barcode sequencing as well as amplicon sequencing is beneficial. In the present study, we failed to show the clear benefits of molecular barcode sequencing with regard to the point of detection rate compared to amplicon sequencing. It is generally considered that DNA input is the limiting factor for increased detection rates. Therefore, a higher detection rate could be expected by barcode sequencing with a larger amount of input cfDNA. This is a point that we aim to show in our next study. A disadvantage of barcode sequencing is its high cost and the turnaround time for barcode sequencing assay is 3 days, longer than the time of 5 hours required for ddPCR as in-house assay.

In conclusion, molecular barcode sequencing using samples of circulating blood provides additional information for adaptive treatment strategies and can be used for cfDNA analysis in future studies.

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CONFLICTS OF INTEREST

Atsushi Horiike received research funding from Chugai Pharma, Quintiles, MSD, and Daichi Sankyo, and honoraria from Pfizer, Chugai Pharma, Eli Lilly, and AstraZeneca. Noriko Yanagitani is a consultant for Chugai Pharmaceutical. Makoto Nishio received research funding from Novartis, ONO Pharmaceutical, Chugai Pharmaceutical, Bristol-Myers Squibb, TAIHO Pharmaceutical, Eli Lilly, Pfizer, Astellas Pharma, and AstraZeneca, and honoraria from Pfizer, Bristol-Myers Squibb, ONO Pharmaceutical, Chugai Pharmaceutical, Eli Lilly, TAIHO Pharmaceutical, and AstraZeneca. Kazuto Nishio received honoraria for lectures from Sumitomo Bakelite Co., Ltd, Daichi Sankyo Co., Ltd, Chugai Pharmaceutical Co., Ltd, nippon Boehringer Ingelheim Co., Ltd, and Eisai Co., Ltd, and research funding from Korea Otsuka Pharmaceutical Co., Ltd and Nippon Boehringer Ingelheim Co., Ltd. All remaining authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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