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Barcode sequencing 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.[1] 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 (TKI) such as gefitinib, erlotinib, afatinib and osimertinib are the most important targeted therapies for NSCLC.[2,3] However, tumor tissue samples are not always available for detecting *EGFR* mutations in clinical practice. In these cases, liquid biopsies can instead serve as a source of specimens. Plasma cell-free DNA (cfDNA), collected as one type of liquid biopsy, can reflect the tumor genotype to some extent.[4-6]

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.[7-10] 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.[9] 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.[11] 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-digital platforms (cobas EGFR Mutation test, Roche Molecular Systems, Pleasanton, CA, USA and therascreen *EGFR* amplification refractory mutation system assay, Qiagen, Valencia, CA, USA) and digital platforms (droplet digital PCR [ddPCR] and BEAMing digital PCR).[4,6] 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.[12-14] 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.[15,16] 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,[17] and can be detected in cfDNA.[18] We previously detected oncogene copy number gains in the plasma cfDNA of colorectal cancer patients using ddPCR.[19] 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 × *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′-TTAGTTCGCTACGATGCAAGAG-3; *MET* reverse, 5′-GGCTTACACTTCGGGCACT-3′; *MET* probe, 5′-/56-FAM/CACACTCCT/ZEN/CATTTGGATAGGCTTG/3IABkFQ/-3′; RPP30 forward, 5′-GATTTGGACCTGCGAGCG-3′; *RPP30* reverse, 5′-GCGGCTGTCTCCACAAGT-3′; and *RPP30* probe, 5′-/5HEX/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.[4] The cut-off value for a MET copy number gain was set at 2.5 copies, as previously described.[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 and 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 5.0 program (Thermo Fisher Scientific). Reads were aligned with the hg19 human reference genome, and potential mutations were called using Variant Call Format version 5.0, as previously described.[12] For the detection of copy number gain, the read depth of each target region was divided by the average depth of the normal DNA (Promega, Madison, WI, USA) to 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.[20,21]

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 and 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 (χ^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*,

TABLE 1 Sensitivity of molecular barcode sequencing: *EGFR* mutations

	Mutant detection frequency (%)			
Designed mutant 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.

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

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,[22] 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 amplicon sequencing and ddPCR were 96.0% and 96.0%, respectively

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.

(Table 5). 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 the 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 5). 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 4 *EGFR* mutation detection in plasma cfDNA

		Molecular barcode sequencing		Amplicon sequencing		Droplet digital PCR	
	Type of TKI-sensitizing mutation at diagnosis	TKI-sensitizing mutation (VAF)	T790M mutation (VAF)	TKI-sensitizing mutation (VAF)	T790M mutation (VAF)	TKI-sensitizing mutation	T790M 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.

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.[23] 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.

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
−	1	7	8
Total	18	7	25
(B)			
Amplicon sequencing			
+	6	0	6
−	1	18	19
Total	7	18	25
(C)			
Molecular barcode sequencing			
+	17	2	19
−	1	5	6
Total	18	7	25
(D)			
Molecular barcode sequencing			
+	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

	Amplicon sequencing		
	+	−	Total
Molecular barcode sequencing			
+	10	6	16
−	0	13	13
Total	10	19	29

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 R^2 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%,[6] 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 (R^2) 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 (R^2) 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-TKI.[26] In the present study, *ERBB4* S341L and *EGFR* I759M 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.[27] In contrast, the presence of *MEK1* mutations has been associated with in vitro resistance to EGFR-TKI.[28] 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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SUPPORTING INFORMATION

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

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