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ORIGINAL ARTICLE

Comparison of the Amplification Refractory Mutation System, Super Amplification Refractory Mutation System, and Droplet Digital PCR for T790 M Mutation Detection in Non-small Cell Lung Cancer after Failure of Tyrosine Kinase Inhibitor Treatment

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Abstract Plasma mutation detection has the advantages of non-invasiveness and accessibility. Here, we evaluated three methods, the amplification refractory mutation system (ARMS), second-generation ARMS (SuperARMS), and droplet digital PCR (ddPCR), to assess their concordance and feasibility for the detection of mutations in plasma samples. Non-small lung cancer patients with stage IIIB/IV that were resistant to epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) treatment were enrolled. Blood samples were collected within 14 days after TKI resistance. Each sample was simultaneously assessed by the three methods. In total, 169 patients were enrolled; 54.4% were female, 72.2% were diagnosed with stage IV disease; and 97.6% had adenocarcinoma. T790 M mutations were detected in 42 (24.8%) of the 169 samples using ARMS, one of which carried the T790 M alone, 22 that also encoded exon 19 deletions, and 19 with L858R mutations. For the SuperARMS assay, 59 (34.9%) samples exhibited the T790 M mutation,

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and 110 (65.1%) showed no detectable T790 M mutation. ddPCR showed that 61 (36.1%) samples contained the T790 M mutation, whereas 108 (63.9%) were not positive. T790 M abundance ranged from 0.04% to 38.2%. The median T790 M abundance was 0.15% for total samples and 2.98% for T790 M mutation samples. The overall concordance was 78.7% (133/169) among ARMS, SuperARMS, and ddPCR. Compared with patients with stage III disease, patients with stage IV disease exhibited a higher T790 M mutation detection rate (28.7% vs. 14.9% by ARMS; 37.7% vs. 27.7% by SuperARMS; and 41.8% vs. 21.3% by ddPCR). Liquid biopsy showed promise and has the advantages of non-invasiveness and accessibility. T790 M detection based on circulating tumor DNA showed high concordance. Compared with non-digital platforms, ddPCR showed higher sensitivity and provided both frequency and abundance information, which might be important for treatment decisions.

Keywords T790 M · ctDNA · ARMS · ddPCR · SuperARMS · TKI resistance

Introduction

Clinically, the application of epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) in patients with non-small cell lung cancer (NSCLC) is associated with a positive objective response in approximately 70% of cases and greatly prolongs overall survival [1–5]. However, the development of drug resistance remains an important challenge to successful EGFR-TKI therapy in the maintenance of disease control. EGFR T790 M mutation occurs in half of TKI-resistant patients, and other molecular changes, including c-met amplification, HER2, and PI3KCA mutations, have also been observed [6–8].

Several strategies to address TKI-related resistance have been pursued, including switching to chemotherapy and continuing TKI with or without local therapy, based on the progression mode. Recently, the new generation TKI, osimertinib (AZD9291), showed promising activity in patients with the T790 M mutation, and the reported response rate was 61% [9]. Despite the promising efficacy, many patients in this setting cannot undergo re-biopsy due to limited tissue availability and procedural feasibility [10]. This, in turn, stimulates the development of EGFR mutation assessment using circulating tumor DNA (ctDNA) as surrogate samples.

Mutation detection in plasma shows promise in conquering the clinical challenges of re-biopsy, with the advantages of non-invasiveness, accessibility and the facilitation of post-progression analysis. However, another challenge is the limited number of methodologies available to detect T790 M mutation [11, 12]. Currently, the reported detection methods to evaluate T790 M mutation status include non-digital platforms (e.g., TherascreenTM EGFR amplification refractory mutation system, ARMS), digital platforms (e.g., droplet digital PCR, ddPCR), and next-generation sequencing (NGS) systems (e.g., single-molecule amplification and re-sequencing technology, SMART) [13–16]. Additionally, recent research using NGS with ctDNA from lung cancer patients identified concordant mutations between the ctDNA and primary tumor DNA (tDNA). However, each of these approaches has its own advantages and disadvantages [14, 17–21]. Here, we evaluated the performance of three methods, ARMS, SuperARMS, and ddPCR, to assess their concordance and feasibility for detecting T790 M mutations in plasma samples.

Materials and Methods

Study Population

This prospective, observational, multi-institution study was performed between March 2015 and March 2016. The protocol was approved by the Institutional Review Board at each site, and all of the patients signed informed consent.

Patients were considered eligible and were enrolled in this study if they met the following criteria [22]: 1) Histologically confirmed stage IIIB/IV NSCLC; 2) harbors an activating EGFR mutation (G719A/C/S/X; Exon 19 insertion/deletion; L858R; L861Q) or exhibited a response or durable stable disease (≥6 months) on EGFR-TKI followed by progression during TKI treatment; 3) clinically resistant to first-generation EGFR-TKIs according to Jackman's criteria; 4) included simultaneously collected data for clinical characteristics.

Sample Collection and DNA Extraction

Blood collection was performed within 14 days of TKI resistance as judged by the physician's assessment, and 15–20 mL of peripheral blood was collected in cell-free DNA protection vacuum tubes (AmoyDx, Xiamen, China). These tubes contain a cell-free DNA protection reagent that guarantees the stability of the DNA for 7 days at 4–25 °C. The blood samples were transported to the Center for Translational Medicine of Hangzhou First People's Hospital within 36 h of being drawn for further processing.

For DNA extraction, blood samples were centrifuged at 2500 g for 10 min at 4 °C, and the supernatant was isolated. The supernatant was then re-centrifuged at 15,800 g for 15 min at 4 °C, and the plasma supernatant was isolated and stored at −80 °C. Cell-free DNA from 1.5 mL plasma was extracted using the QIAamp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany).

EGFR Mutation Detection in Plasma ctDNA

EGFR mutations in plasma ctDNA were identified using the ADx-ARMS (amplification refractory mutation system) kit (Amoy Diagnostics, Xiamen, China), and all of the experiments and genotype calling procedures were performed according to the manufacturer's instructions [23]. T790 M mutation detection using the ADx-SuperARMS assay was performed according to the manufacturer's instructions. EGFR T790 M mutation was also assessed using ddPCR with the AmoyDx® EGFR Exon20 T790 M Mutation Detection Kit and SuperARMS. Details of the ddPCR assay for EGFR T790 M mutation detection are described in Supplementary Method 1.

Statistical Analysis

The sensitivity and specificity of T790 M mutation detection by ddPCR and SuperARMS were determined by comparing the T790 M status with the ARMS result. The concordance of T790 M mutation was calculated using two methods. Crude agreement was calculated by the formulation (A + D)/(A + B + C + D)*100%, and adjusted agreement was calculated by the formulation 1/4*{1/ [(A/(A + B) + A/(A + C) + D/(C + D) + D/(B + D)]}*100% (A represents patient number of both positive detected by each two methods; B and C represents patient number of discrepancy detected by each two methods; D represents patient number of both negative detected by each two methods). *p* values less than 0.05 were considered significant. All of the tests were performed two-sided. Statistical analyses and data visualization were performed using IBM SPSS version 22.0 (IBM SPSS, Inc., Chicago, IL, USA) and GraphPad Prism, Version 6.01 (GraphPad Software Inc., San Diego, CA, USA).

Table 1 Clinical characteristics (*n* = 169)

Factors	No. (%)
Age, years	
Median	63
Range	32–89
Gender	
Male	77 (45.6)
Female	92 (54.4)
Pathology	
Adenocarcinoma	165 (97.6)
Squamous	1 (0.6)
Adenosquamous	3 (1.8)
Tumor Stage	
IIIA	37 (21.9)
IIIB	10 (5.9)
IV	122 (72.2)
Smoking history	
Never	122 (72.2)
Former/current	47 (27.8)
Pre-TKI EGFR mutation	
Exon 19 deletion	96 (56.8)
Exon 21 L858R	60 (35.5)
Rare mutation	13 (7.7)
Type of TKI	
Gefitinib	78 (46.2)
Erlotinib	15 (8.9)
Icotinib	76 (45.0)
Response	
SD	60 (35.5)
PR	88 (52.1)
CR	21 (12.4)

Results

Patient Characteristics

Part of plasma samples were collected from patients enrolled in our previous clinical trial (NCT02418234). In total, 169 patients with advanced or recurrent NSCLC who had progressed during EGFR-TKI treatment were enrolled. The median age for these patients was 63 years (range, 32–89 years). Most of them (72.2%) were non-smokers, and 54.4% were female. Additionally, 72.2% of the patients were diagnosed as stage IV; 56.8% of the enrolled patients had the EGFR exon 19del mutation; 35.5% had EGFR L858R mutation; 97.6% were classified as adenocarcinoma. The patient response rates to EGFR-TKI treatment were 35.5% for stable disease (*SD*), 52.1% for partial response (*PR*) and 12.4% for complete response (*CR*)(Table 1).

Comparison of Three Methods

Amplification Refractory Mutation System

EGFR mutations were detected in 100 of 169 (59.2%) patients. The details are summarized in Table 2. Of the 169 evaluable samples, 55 (32.5%) harbored sensitizing mutations alone, 3 (1.8%) had resistance mutations alone, and 41 (24.3%) had a combination of activating and resistance mutations. The most common mutations detected were the exon 19 deletion (deletion alone: 17.8% [30 of 169]; in combination with T790 M: 11.8% [20 of 169]) and the L858R point mutation in exon 21 (L858R alone: 11.2% [19 of 169]; in combination with T790 M: 10.1% [17 of 169]). Tumors from 42 patients (24.9%) harbored T790 M resistance mutations, among which one (0.6%) had T790 M alone.

Table 2 Summary of individual EGFR mutation types (Including Multiple Mutations)

	N	%
Patients with an evaluable EGFR mutation test	169	100
Sensitizing mutations alone	55	32.5
Exon 19 deletion	30	17.8
Exon 21 L858R	19	11.2
Exon 21 L861Q	1	0.6
Exon 20 S768I	1	0.6
Exon 18 G719X	4	2.4
Combination of sensitizing mutations	1	0.6
L858R + 19del	1	0.6
Resistance mutations alone	3	1.8
Exon 20 T790 M	1	0.6
Exon 20 insertion	2	1.2
Combination of sensitizing and resistance mutations	41	24.3
19del + T790 M	20	11.8
L858R + T790 M	17	10.1
19del + T790 M + S768I	2	1.2
L858R + T790 M + S768I	2	1.2
Patients with a negative EGFR mutation test	69	40.8

Super Amplification Refractory Mutation System

SuperARMS is an ARMS assay optimized for T790 M detection; thus, it achieves higher sensitivity. Additionally, 59 of 169 samples (34.9%) were identified as T790 M-positive, and 110 (65.1%) samples were not positive for the mutation. The sensitivity and specificity of ddPCR were 85.2% and 88.7% when refer to ARMS, respectively.

Droplet Digital PCR

In this study, the median DNA copy number detected in the plasma samples was 594 copies/reaction, which, ranged from 152 to 17,681 copies/reaction, reflecting the wide variety of ctDNA in plasma isolated from different patients (Fig. 1). Our ddPCR results showed that 61 (36.1%) samples exhibited a detectable T790 M mutation and 108 (63.9%) samples were not positive for the T790 M mutation. The sensitivity and specificity were 96.3% and 65.2%, respectively. The results also revealed that the median T790 M abundance was 0.15% for total samples and 2.98% for T790 M mutation samples (Fig. 2). T790 M abundance ranged from 0.04% to 38.2%. In addition, the percentages of samples with different T790 M abundance values detected across all 169 samples were 45.6% for 0%, 3.6% for 0–0.1%, 22.5% for 0.1–1%, 14.8% for 1–5%, 6.5% for 5–10%, and 7.1% for above 10%.

The overall concordance was 78.7% (133/169) among ARMS, SuperARMS, and ddPCR, indicating that T790 M detection from ctDNA was robust (Fig. 3). The crude and adjusted agreements between ARMS and SuperARMS were 87.6% and 86.1% (Kappa = 0.721, 0.610 to 0.832), 88.8% and 87.7% (Kappa = 0.750, 0.645 to 0.855) between ARMS and ddPCR, and 85.8% and 84.5% (Kappa = 0.690, 0.576 to 0.804) between SuperARMS and ddPCR (Table 3). Forty-eight patients exhibited more than 1% T790 M abundance by ddPCR; 37 (77.1%) of these patients showed T790 M mutations by ARMS, and 42 (87.5%) showed T790 M mutations by SuperARMS. The detailed distributions are shown in Supplementary Fig. S1.

Impact of Disease Stage on ctDNA Detection

The ARMS assay detected T790 M mutations in 7 of 47 (14.9%) patients with stage III disease; by contrast, the assay detected 35 of 122 (28.7%) T790 M mutations in patients with stage IV disease. Similar to ARMS, SuperARMS detected

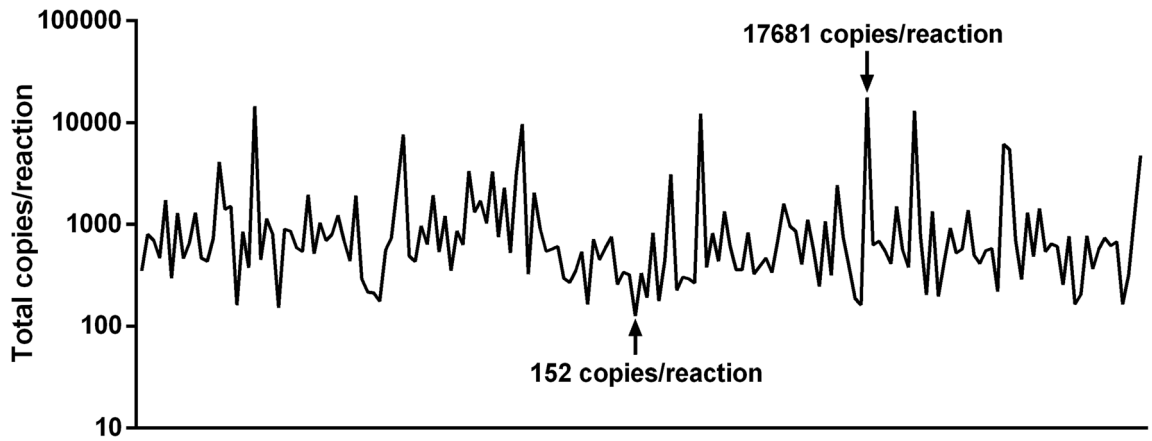

Fig. 1 Plasma DNA of each included sample. Left axis represents the plasma sample DNA input; horizontal axis represents each patient. Down arrow refers to patient with maximum plasma DNA; up arrow refers to patient with minimum plasma DNA

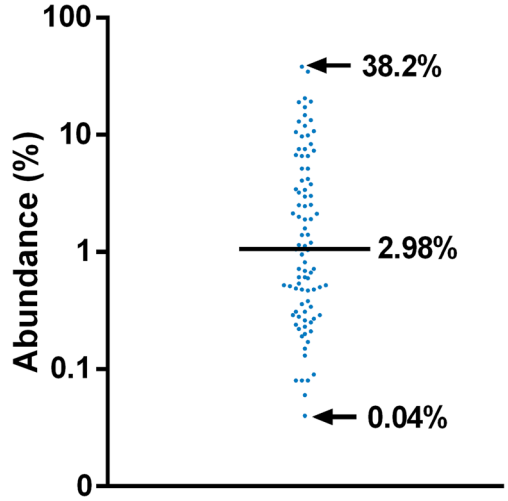

Fig. 2 T790 M abundance by droplet digital PCR in T790 M positive patients. Horizontal line refers to median abundance; left arrows refer to maximum and minimum abundance

T790 M mutations in 13 of 47 (27.7%) and 46 of 122 (37.7%) patients with stage III and stage IV disease, respectively; ddPCR detected T790 M mutations in 10 of 47 (21.3%) and 51 of 122 (41.8%) patients with stage III and stage IV disease, respectively. The overall concordance among the three methods was 83.0% (39/47) for patients with stage III disease and 77.1% (94/122) for patients with stage IV disease, and the positive concordance among three methods was 14.9% (7/47) for patients with stage IV disease and 25.4% (31/122) for patients with stage III disease.

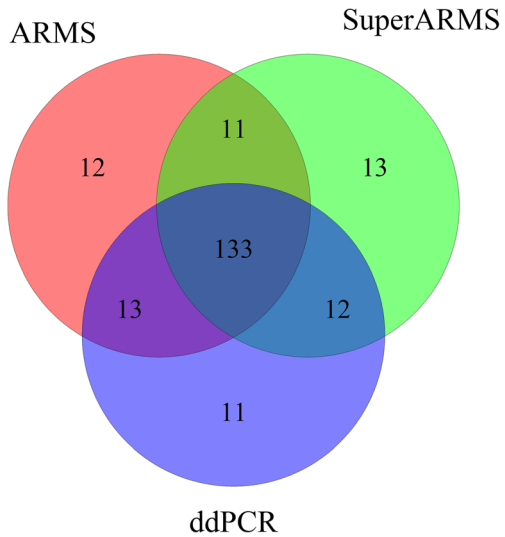

Fig. 3 Patient number of detection concordance and disconcordance between three methods. Pink circle represents patient detected by ARMS; green circle represents patient detected by SuperARMS; Purple circle represents patient detected by ddPCR. Overlapping represents detection concordance of between methods

Table 3 Detection concordance between three methods

	SuperARMS vs. ARMS	ddPCR vs. ARMS	SuperARMS vs. ddPCR
Total concordant N	144/169	146/169	145/169
Crude agreement	85.2%	86.4%	85.8%
Adjusted agreement	83.7%	85.6%	84.5%
Kappa	0.651	0.684	0.690
95% CI	0.526 to 0.770	0.560 to 0.802	0.576 to 0.804

Discussion

After an initial dramatic response, EGFR-TKI resistance will inevitably develop in most patients. Additionally, identification of the resistance mechanism and dynamic monitoring are currently hot topics. To conquer the clinical challenge of re-biopsy, plasma ctDNA has become a preferred surrogate for tumor tissue. ctDNA has clinical applications as a major form of "liquid biopsy" [24], allowing the complementation or even replacement of tissue biopsy with non-invasive blood tests. ctDNA is a carrier that brings solid tumor genetic information to peripheral blood. With the advantages of non-invasiveness and accessibility, T790 M detection in plasma ctDNA is among the most intensively studied in this field. To date, the applications of various technologies have been reported [14, 19, 25, 26]. In our study, the detection rate of T790 M was 36.1%. We did not undertake re-biopsy due to the difficulty in clinical practice. Previous study showed T790 M was detected 13/25 in plasma and 16/25 in matched tissue, and the overall concordance rate of T790 M testing between the paired tumor tissues and plasma was 88.00% by ddPCR (Kappa = 0.757, 95%CI: 0.4996–1.0) [27]. Another study showed T790 M was detected 8/16 in plasma and 9/16 in matched tissue, and the overall concordance rate 68.7% by ddPCR [28].

The ARMS assay has been extensively applied to large clinical trials and is a stable, highly sensitive and specific method to detect EGFR mutations in tumor tissue [23, 29, 30]. This method can detect mutations in samples containing as little as 1% mutated DNA [31–34] and achieves moderate sensitivity but high specificity when using plasma ctDNA to detect EGFR mutations [35, 36]. Compared with tumor tissue, the sensitivity and specificity in plasma were 65.7–75% and 96–100%, respectively [23, 37, 38]. Unlike exon 19 deletion or L858R mutation, T790 M mutation is characterized by low abundance and high frequency, especially in plasma ctDNA. The low sensitivity of ARMS leads to high false-negative results. Thus, negative EGFR mutation-results in plasma should be interpreted with caution, as 40% of patients with EGFR mutant tumors were not identified from plasma ctDNA using ARMS [39]. Many low-abundance cases cannot be detected. *Wang* et al. reported only 34 of 135 (25.2%) patients post-TKI had

detectable T790 M mutations [40]. The abundance in our study was 24.8%, similar to that reported in *Wang* et al.

To improve the sensitivity of plasma detection, other techniques have been applied. ADx-SuperARMS, similar to cobas® EGFR Mutation Test v2, is a second-generation ARMS assay. It was developed by AmoyDx and is one of the key technologies recommended by the Consensus Group for KRAS Gene Mutation Detection in Colorectal Carcinoma (part of the China Pathology Quality Control Center). ADx-SuperARMS detects mutations in blood samples that contain as little as 0.2% mutant DNA in a background of wild-type genomic DNA. The ADx-SuperARMS test is designed to offer highly accurate molecular diagnostic screening for clinical practices. Two clinical trials, RAS mutation in colorectal cancer by Merck and osimertinib for lung cancer by AstraZeneca, will also evaluate the ADx-SuperARMS assay. As a preliminary study, our data showed SuperARMS identified 34.9% samples with T790 M mutations, slightly higher than the ARMS assay.

ddPCR is another recently introduced technology that is based on sample partitioning into thousands of defined volume micro-reactions [41]. After the PCR reaction, each droplet either does or does not contain the nucleic acid of interest, allowing estimation of the number of molecules in the reaction assuming a Poisson distribution. The results are expressed as target copies per microliter of reaction [42]. ddPCR has some favorable features [35, 43–48], among which are the following: 1) it performs absolute quantification based on the principles of sample partitioning and Poisson statistics, thus overcoming normalization and calibrator issues; 2) it has increased precision and sensitivity at detecting low target copies; 3) it is relatively insensitive to potential PCR inhibitors; 4) it directly expresses the results of the analysis as the number of target copies per microliter of reaction (with confidence intervals); and 5) it provides both mutation abundance and frequency information. With excellent sensitivity, ddPCR detected T790 M mutations in 43.3–47.0% of patients after TKI resistance in plasma ctDNA, significantly higher than the ARMS assay [27, 49]. Our ddPCR results showed that 36.1% of samples had a detectable T790 M mutation. This discrepancy might be due to a high proportion of stage III disease in our study, which accounted for one-third of the total patients. When stage III disease was excluded, the T790 M mutation abundance increased to 41.8%, similar to that of previous publications [10, 27, 50].

Quantitative ctDNA levels generally correlate with tumor burden. More metastatic tumors generate more DNA leakage into the bloodstream, resulting in higher tumor-derived DNA levels. Consistent with this explanation, there was higher sensitivity in patients with stage IV (41.8%) than in patients with stage III disease (21.3%). Notably, although ctDNA is considered a correlate of tumor burden, it is not necessarily correct because these values directly correlate to dying cancer cells. Thus, although cells with a high turnover rate produce more ctDNA than those with a low turnover rate, their population size might not necessarily agree with the ctDNA levels.

We demonstrated here a great concordance between ARMS, SuperARMS, and ddPCR for the detection of plasma T790 M mutations. ddPCR also showed the highest sensitivity and reasonable specificity; thus, our results suggest that ddPCR is a useful tool for the determination of T790 M mutation, aiming for both frequency and abundance, in plasma ctDNA. The relatively low abundance of T790 M mutation observed (median abundance of 0.15%) might reflect the complexity of plasma T790 M detection. Generally, the patients with high abundance of mutation have better clinical response. Previous study showed that patient with EGFR positive detected by both ARMS and 1st sequencing reached the longest PFS than patients with EGFR positive by ARMS alone, because the only the abundance of mutation above 10% can be detected by 1st sequencing [51]. In recent studies, methods with higher sensitive are applied in ctDNA mutation detection. AURA3 showed the response rate was significant better in the osimertinib group (77%, 89/116) than in the platinum-pemetrexed group (39%, 22/56) in ctDNA T790 M-positive patients detected by cobas EGFR Mutation Test V2 (ARMS assay), it was similar in tissue T790 M-positive patients (71%) [52]. Another study showed ORR was 63% and 46% in plasma T790 M+ and T790 M- patients detected by Beaming digital PCR who received osimertinib [53]. Considering the temporal and spatial T790 M heterogeneity observed from repeated and multiple-site re-biopsies, plasma T790 M status might reflect a more representative and informative genetic profiling of patients post progression, because plasma ctDNA is derived from both primary and metastatic lesions and can be collected dynamically. Nevertheless, a detailed understanding how T790 M mutant ctDNA is released into plasma and the clinical significance of plasma ctDNA results are of great interest.

Notably, this is a large-scale, multi-institutional study comparing three methods to detect the T790 M mutation in plasma. However, several limitations existed in this study. First, we only analyzed the T790 M mutation but did not perform survival outcome analysis because of the inadequate follow-up time. Second, molecular profiling was not performed simultaneously on matched tissue biopsies and blood after disease progression, and the analysis of both biopsies would reveal the correlation and discordance between both to elucidate their distinct clinical significance. Third, the dynamic change to ctDNA T790 M status was not monitored during disease progression, which might reveal more relevant associations, especially in terms of changes to T790 M abundance during disease progression. Fourth, only a single EGFR gene

mutation, not multiple gene mutations, was investigated in this study based on the limits of the one-time ddPCR assay and SuperARMS used. Further studies to overcome these limits are urgently needed.

In conclusion, the results from this prospective study indicated the important clinical impact of T790 M detection using plasma samples for NSCLC patients who failed after EGFR-TKI therapy. Well concordance among three assays indicated the feasibility of plasma ctDNA detection. The ddPCR assay had a high sensitivity and might be superior to ARMS and SuperARMS assays.

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Compliance with Ethical Standards

Conflict of Interest All authors declare that there is no conflict of interests.

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