

ORIGINAL RESEARCH

A Comparison of ddPCR and ARMS for detecting EGFR T790M status in ctDNA from advanced NSCLC patients with acquired EGFR-TKI resistance

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ARMS, ddPCR, EGFR-TKI, non-small cell lung cancer, T790M

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Abstract

A sensitive and convenient method for detecting epidermal growth factor receptor (EGFR) T790M mutations from circulating tumor DNA (ctDNA) in advanced non-small cell lung cancer (NSCLC) patients with acquired EGFR-TKI resistance would be desirable to direct patient sequential treatment strategy. A comparison of two platforms for detecting EGFR mutations in plasma ctDNA was undertaken. Plasma samples and tumor samples were collected from patients with acquired EGFR-TKI resistance in Zhejiang Cancer Hospital from December 2014 to December 2015. Extracted ctDNA was analyzed using two platforms (Droplet Digital PCR and ARMS [dPCR]). A total of 108 patients were enrolled in this study. One hundred and eight patient plasma samples were detected by ddPCR and 75 were detected by ARMS. And 16 patients obtained tissue re-biopsy, using ARMS assay for detecting EGFR T790M mutation. In all, 43.7% (47/108) had acquired T790M mutation by ddPCR. In 75 patient plasma samples, comparing ddPCR with ARMS, the rates of T790M mutation were 46.7% (35/75) and 25.3% (19/75) by ddPCR and ARMS, respectively. Of all, 16 patients both had tumor and plasma samples, the T790M mutation rates were 56.3% (9/16) by ARMS in tissue and 50.5% (8/16) by ddPCR in plasma ctDNA. The progression mode tended to gradual progression in T790M mutation patients (40.4%), but the T790M negative was inclined to the mode of dramatic progression (39.3%). The patients with T790M-positive tumors had a longer time to disease progression after treatment with EGFR-TKIs (median, 13.1 months vs. 10.8 months; $P = 0.010$) and overall survival (median, 35.3 months vs. 30.3 months; $P = 0.214$) compared with those with T790M-negative patients. Our study demonstrates ddPCR assay may provide a highly sensitive method to detect EGFR T790M gene in plasma. And T790M-positive patients have better clinical outcomes to EGFR-TKIs than T790M-negative patients.

Introduction

In recent years, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are clinically effective in patients with non-small cell lung cancer (NSCLC) harboring sensitizing EGFR mutations. Eight randomized trials have demonstrated a significantly higher tumor response rate and longer progression-free survival (PFS) in EGFR-mutant patients treated with first-line TKI [1–8]. However,

a majority of patients eventually acquire resistance to the drug and experience disease progression [9, 10]. The T790M mutation in the EGFR gene is regarded as the most common cause of acquired resistance to EGFR-TKIs [11].

This T790M resistant mutation was found in approximately 50% of rebiopsy samples obtained from patients with acquired resistance to EGFR-TKI therapy [11]. However, this is challenging in clinical practice to obtain serial tumor rebiopsies

following progression disease due to the invasiveness of the procedure, and also carries risk due to the limitations of the biopsy to reflect tumor heterogeneity and the evolution of genetic modifications [12–15]. Several studies showed tumor genome in plasma had homogeneity. [16, 17]. Moreover, mutation detection in plasma has shown promise in terms of accessibility, convenience, and practicality compared with analysis of isolated circulating tumor cells [18, 19].

Detection rates of T790M ctDNA in plasma from NSCLC patients with acquired TKI resistance ranged from 30% to 50% using qualitative PCR-based assays [20–23].

Therefore, finding a sensitive detecting assay is important. We first used ddPCR assay and ARMS assay to evaluate the acquired T790M status in advanced NSCLC patients plasma. Nowadays, the association between acquired T790M status and the patients' clinical outcome is still controversial. Furthermore, our study shows the clinical efficacy and sequential treatment strategy between EGFR T790M-positive and -negative patients.

Patients and Methods

Patients

All patients with pathologically confirmed advanced or recurrent stage IV NSCLC with EGFR mutation, an Eastern Cooperative Oncology Group performance status of 0–2, acquired resistance to EGFR-TKI (gefitinib, erlotinib, or icotinib) therapy. All patients received erlotinib, gefitinib, or icotinib orally at a recommended dose, either at first-line therapy or after first-line standard chemotherapy. Some patients received also second-line chemotherapy before treatment with the TKI. Objective tumor responses were evaluated every 6–8 weeks in accordance with the Response Evaluation Criteria in Solid Tumors guidelines (version 1.1). And the acquired resistance to EGFR-TKI was based on Jackman's clinical definition [24]. The study was approved by the Ethic Committee of Zhejiang Cancer Hospital. Patients all have informed verbal consent to participate in the study.

Plasma samples and tumor samples collection

Plasma samples (10 mL) were collected from all patients enrolled in our study, following progression on EGFR-TKI treatment. We collected plasma samples when progression disease after EGFR-TKI was observed according to RECIST 1.1 but a subsequent treatment did not begin. Plasma DNA was purified using a plasma cell-free DNA kit (AmoyDx, Xiamen, China). Plasma DNA was detected by Droplet Digital PCR (ddPCR) (AmoyDx, Xiamen, China) and amplification refractory mutation system (ARMS) (AmoyDx, Xiamen, China) assays, respectively.

DNA was extracted from re-biopsy tumor tissue (FFPE DNA kit, AmoyDx, Xiamen, China) and used ARMS assay to detect EGFR T790M mutation. All rebiopsy samples of DNA were extracted from tumor specimens as per standard protocols (FFPE DNA kit, AmoyDx, Xiamen, China) and were used for ARMS.

TKI use after acquired resistance to initial EGFR-TKI

After disease progression, we adopted the criteria defined by Yang et al. and divided the patients into three groups: local disease progression, gradual progression, and dramatic progression based on the duration of disease control, evaluation of tumor burden, and clinical symptoms [25]. Local disease progression was defined as solitary progression with no more than three lesions, gradual progression was defined as minor increment of tumor burden, and dramatic progression was defined as rapid increment of tumor burden or with obvious cancer-related symptoms.

Statistical analysis

PFS1 was defined as the time from the start of EGFR-TKI treatment to the first documentation of progressive disease (PD) or death from any cause, while PFS2 was defined as time from the date of the first PD by RECIST version 1.1 to the second PD or death. OS was defined as the period from diagnosed with advanced NSCLC to the date of death by any cause, or the date when the patient was last known to be alive. All time-to-event outcomes were estimated using the Kaplan–Meier method and compared across groups with the log-rank test or the Cox proportional hazards model. Chi-square and Fisher's exact tests were used to analyze correlations between EGFR status and statistical analysis was carried out with SPSS version 19.0 (SPSS, Inc., Chicago, IL). All statistical tests were two sided and results were considered significantly different if $P < 0.05$.

Follow-up

All patients were followed up at the outpatient clinic or over phone and the last follow-up time was in 26 February 2016

Results

Patients characteristics

From December 2014 to December 2015, 108 patients with activating EGFR mutation developed acquired resistance to EGFR-TKI in our study. Seventy patients were with deletion in exon 19 and 33 patients had mutation

Table 1. Clinical characteristics and EGFR T790M ctDNA status in plasma of 108 patients.

Features	ddPCR-T790M (+) (n = 47)	ddPCR-T790M (-) (n = 61)	P
Sex			0.115
Male	28 (50.9%)	27 (49.1%)	
Female	19 (35.8%)	34 (64.2%)	
Age			0.639
<65 years	11 (47.8%)	12 (52.2%)	
≥65 years	36 (42.4%)	49 (57.6%)	
Smoking			0.713
Yes	17 (45.9%)	20 (54.1%)	
No	30 (42.3%)	41 (57.7%)	
Stage			0.123
IIIB	0 (0%)	3 (100%)	
IV	47 (44.8%)	58 (55.2%)	
Pathology			0.172
Adenocarcinoma	46 (45.1%)	56 (54.9%)	
Nonadenocarcinoma	1 (16.7%)	5 (83.3%)	
Types of TKI			0.146
Erlotinib	6 (66.7%)	3 (33.3%)	
Gefitinib	9 (56.3%)	7 (43.8%)	
Icotinib	32 (38.6%)	51 (61.4%)	
Response to TKI			0.176
CR+PR	30 (49.2%)	31 (50.8%)	
SD	17 (36.2%)	30 (63.8%)	
PS			0.931
0–1	39 (43.3%)	51 (56.7%)	
2	8 (44.4%)	10 (55.6%)	
Types of EGFR mutation			0.709
19	31 (44.3%)	39 (55.7%)	
21L858R	14 (42.4%)	19 (57.6%)	
Others	2 (40.0%)	3 (60.0%)	
Progression modes			0.160
Dramatic progression	12 (33.3%)	24 (66.7%)	
Gradual progression	19 (55.9%)	15 (44.1%)	
Local progression	16 (42.1%)	22 (57.9%)	
Tumor size of progression			0.266
<4 cm	17 (51.5%)	16 (48.5%)	
≥4 cm	30 (40.0%)	45 (60.0%)	
Distant metastasis when resistance to TKI			0.935
Yes	12 (42.9%)	16 (57.1%)	
No	35 (43.8%)	45 (56.3%)	

EGFR, epidermal growth factor receptor.

in exon 21L858R, the other 5 patients harboring point mutation in 18G719X. Among them, 16 patients underwent rebiopsy. The median age was 57 years (range, 28–79 years). The percentage of males, never smokers, and patients with adenocarcinoma were 50.9%, 65.7%, and 94.4%, respectively (Table 1). For EGFR-TKI treatment, 40.7% (44/108) patients received TKI as first-line treatment and 59.3%

(64/108) received second-line or more. Of patients, 83 were treated with icotinib, 16 received gefitinb, and 5 with erlotinib. Among them, 38 patients had a local progression, 34 patients had gradual progression, and 36 patients had a dramatic progression. After acquired resistance to EGFR-TKI, 38 patients received continuous TKI, 25 patients received continuous TKI plus chemotherapy, and 28 patients were switched to chemotherapy alone. However, there were 17 patients who did not get information in sequential treatment.

The results of EGFR T790M gene status

Comparing ddPCR assay with ARMS assay in the results of plasma T790M status

In our study, we respectively applied ddPCR and ARMS assays to detect EGFR T790M mutation status in 75 plasma samples. By ddPCR assay, the T790M mutation rate was 46.7% (35/75). However, by ARMS assay, the rate of T790M mutation was only 25.3% (19/75). ARMS assay detected T790M gene positive in 16 plasma samples, which were also positive by ddPCR assay (Table 2). Of the 19 patients, only 1 patient was below 5% abundance (1.44%) and all others were higher than 5% (5.90–27.76%). However, of the 16 patients, 11 patients were below 1% abundance (0.16–0.80%), 3 patients were below 2% but higher than 1% (1.56–1.81%), and 2 patients were higher than 5% abundance (5.09–7.19%).

Comparing ddPCR assay in plasma with ARMS assay in tissue T790M status

Here, we evaluated the detection sensitivity and specificity of plasma T790M mutation using the ddPCR assay in comparison with the T790M status in the paired tumor rebiopsy as standard. The T790M status in 16 pairs of tumor tissue and plasma from EGFR-TKI relapsed NSCLC patients is summarized in Table 3. In ctDNA, the T790M mutation rate was 50.0% (8/16). In tumor tissue, the rate of T790M mutation was 56.3% (9/16). Among these patients, six were positive for T790M in both tumor tissue and plasma, and five were negative in both. Three

Table 2. Comparing detection of T790M status by ddPCR assay with ARMS assay in 75 plasma ctDNA with resistance to EGFR-TKI.

ddPCR-T790M status	ARMS-T790M		
	Positive	Negative	Total
Positive	19	16	35 (46.7%)
Negative	0	40	40 (53.3%)
Total	19 (25.3%)	56 (74.7%)	75

Table 3. Comparing detection of T790M status by ddPCR from plasma ctDNA with ARMS from rebiopsy tissues in 16 patients with resistance to EGFR-TKI.

ddPCR-T790M status	ARMS-T790M in tissues		Total
	Positive	Negative	
Positive	6	2	8 (50.0%)
Negative	3	5	8 (50.0%)
Total	9	7	16

patients were positive for T790M in tumor tissue, but negative in plasma. The other two patients were positive for T790M in plasma, but negative in tumor tissue. Compared with tumor tissue, the sensitivity and specificity for EGFR T790M mutation detection in plasma by ddPCR were 66.7% (6/9) and 71.4% (5/7), respectively.

The relationship between T790M mutation by ddPCR assay and clinical features

All 108 patients were detected by ddPCR from plasma ctDNA. In this study, 47 patients were positive for T790M and the frequency of T790M mutation was 43.5%. For 70 patients with EGFR 19 deletion, the mutation rate of T790M was 44.3% (31/70). And for 33 with 21L858R mutation, the rate was 42.4% (14/33). In the group of local progression, 16 patients had T790M-positive (42.1%) and 22 patients had T790M-negative (57.9%) mutations. However, in the group of gradual progression, the rate of mutation (55.9%) was compared to wild type (44.1%). On the contrary, in the group of dramatic progression, the frequency of negative (66.7%) mutation was higher than positive (33.3%).

Association of ddPCR T790M mutation in acquired resistance to TKI plasma ctDNA with survivals on EGFR-TKI

PFS1 and OS were assessed in all 108 patients based on their plasma T790M status and TKI treatments. However, we did not access the subsequent treatment with 17 patients. In all patients, the median PFS1 was 12.3 months and the median OS was 32.8 months. For subgroup analysis, mPFS1 in the T790M-positive group was longer than the negative group (13.1:10.8 months, $P = 0.010$) (Fig. 1). Similarly, the mOS in patients with T790M mutation was 35.3 months, which was significantly longer than 30.3 months in the patients without T790M mutation ($P = 0.214$). On the other hand, we analyzed the clinical features between with T790M mutation and without T790M mutation in the time of PD. We found that the incidence rate of tumor

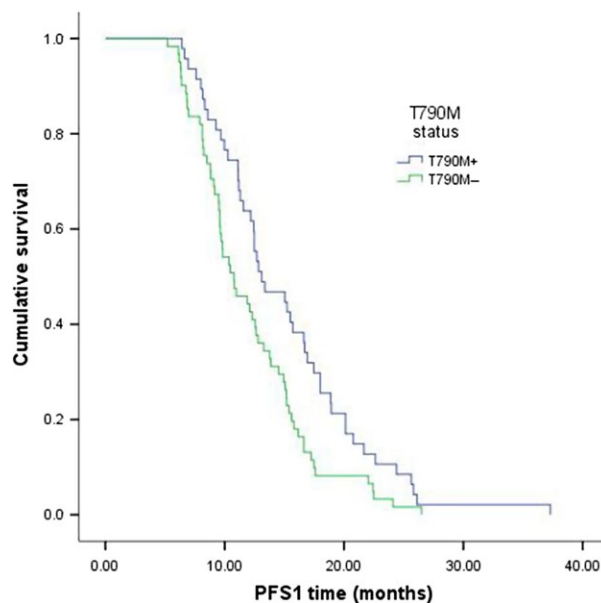


Figure 1. Kaplan–Meier curves showed that patients with T790M mutation had a significantly longer PFS1 than the group without T790M (13.1 months:10.8 months, $P = 0.010$).

size ≥ 4 cm in the patients with T790M-negative mutation was slightly higher than patients with the T790M-positive mutation (60.0%:40.0%, $P = 0.266$). The occurrence rates of distant metastasis were similar (42.9%:43.8%, $P = 0.935$). The difference in the median PFS1 (mPFS1) among the three groups was significant ($P = 0.006$). The mPFS1 of patients in dramatic progression group (10.4 months) was shorter than that in gradual progression group (12.8 months) or local progression group (12.4 months).

Next, we investigated the sequential treatment of 91 patients after PD. The median PFS2 was 3.8 months (95% CI: 2.709–4.631). The PFS2 in the patients with T790M mutation was 4.0 months, which was not significantly longer than 3.1 months in the patients without T790M mutation ($P = 0.709$) (Fig. 2). Among them, the PFS2 of 38 patients receiving continuous TKI was 3.0 months, PFS2 of 25 patients receiving continuous TKI plus chemotherapy was 5.7 months, and PFS2 of 28 patients who switched to chemotherapy alone was 2.9 months (Table 4). Subgroup analysis in group of receiving continuous TKI plus chemotherapy showed that PFS2 of T790M-positive was longer than T790M-negative patients (6.0: 4.3 months, $P = 0.722$) (Table 5). Among the three groups of EGFR-TKI failure modes, the PFS2 of patients in gradual progression group (6.0 months) was longer than that in dramatic progression group (4.9 months) or local progression group (4.3 months) (Table 6).

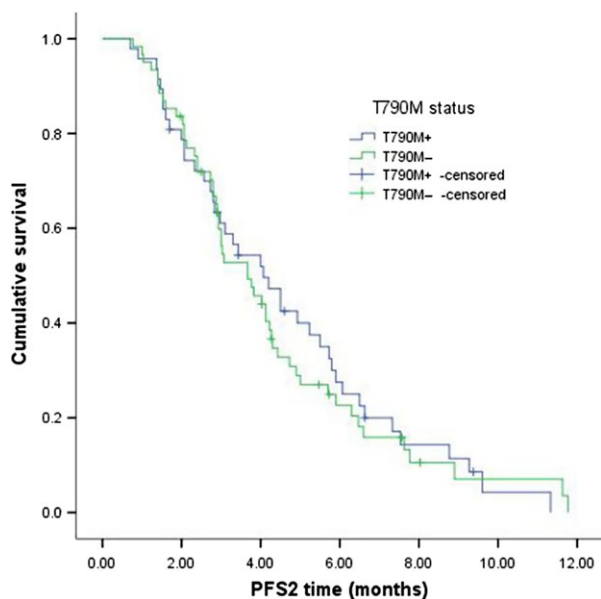


Figure 2. Kaplan-Meier curves showed that patients with T790M mutation had a slightly longer PFS2 than the subgroup without T790M mutation (4.0 months:3.1 months, $P = 0.709$).

Discussion

Recently, with the study of overcoming acquired TKI resistance, it is becoming imperative to accurately and quickly detect molecular resistance mechanisms in individual patients. The secondary EGFR T790M mutation has been discovered in rebiopsy tumor tissue from about 50% of NSCLC patients with acquired resistance to EGFR-TKI therapy [26, 27]. Although detecting rebiopsy tumor tissue is a gold standard, the tissue may be difficult to obtain from resistance patients in some times. Therefore, detecting T790M status in patients' plasma is an available method and a highly sensitive and convenient assay is needed, such as ARMS, ddPCR, and so on. Studies have showed that the sensitivity of ddPCR is 0.01% [28] and the sensitivity of the ARMS is 0.1% [29]. Nowadays, a more standard and appropriate assay, to detect EGFR T790M status in plasma, is controversial. Therefore, we compared ddPCR with ARMS to clear that if ddPCR could provide an alternative approach in detecting plasma T790M status. On the other hand, the relationship between T790M mutation and patients' prognosis is still controversial. Some researches demonstrated that T790M-mutated patients were associated with poor survivals [30–32]. However, other studies showed that NSCLC patients who acquired TKI resistance due to T790M gene mutation had longer survival than patients without T790M [33, 34]. Therefore, we also analyzed ddPCR assay to detect plasma ctDNA T790M and T790M status associated with clinical outcome.

Table 4. The PFS2 of the sequential treatment in 91 patients after progression disease.

Regimens	<i>n</i>	PFS (95% CI)	<i>P</i>
Continued TKI	38 (35.2%)	3.0 months (2.758–3.242)	
Chemotherapy	28 (25.9%)	2.9 months (2.579–3.361)	
Chemotherapy plus TKI	25 (23.1%)	5.7 months (3.053–8.407)	0.001

PFS, progression-free survival.

Table 5. The relationship between acquired T790M status and sequential treatment with 91 patients.

Therapeutic regimen	PFS2-T790M (+)	PFS2-T790M (–)	<i>P</i>
Continuous TKI	3.1 months	2.9 months	0.834
Chemotherapy	2.9 months	2.8 months	0.772
Continuous TKI plus chemotherapy	6.0 months	4.3 months	0.722

PFS, progression-free survival.

Table 6. The relationship between progression mode and sequential treatment in 91 patients.

Therapeutic regimen	Dramatic progression	Gradual progression	Local progression	<i>P</i>
Continuous TKI	3.3	2.1	3.1	0.066
Chemotherapy	3.0	3.0	2.1	0.512
Continuous TKI plus chemotherapy	4.9	6.0	4.3	0.440

Nowadays, detecting EGFR gene status in plasma is a research focus. And some researchers have reported different assays on detecting EGFR in plasma [4, 21, 35–39]. To further investigate the potential clinical role of detecting T790M gene status in plasmas, we compared two major detecting assays which may be utilized frequently in clinical. The ARMS, which is based on quantitative PCR, identifies EGFR mutation sequences by using specific probes. With its high selectivity and sensitivity, this method can detect mutations in tissue samples containing as little as 1% mutated DNA [4]. And it has been a standard method to detect EGFR gene status in tumor tissue. However, DNA in plasma was less than in tissue which needed more sensitive assay to detect EGFR gene status. Ma et al. [36] reported that EGFR mutation status tested by ARMS in plasma cannot replace a tumor tissue biopsy. The ARMS assay for EGFR mutation detection in plasma ctDNA has moderate sensitivity but high specificity. EGFR mutation positive results detected in plasma are fairly reliable, but negative results are hampered by a high rate of false negatives. ddPCR is a relatively new technology, requiring no external calibrators, for measuring the

absolute and relative copy numbers of target DNA. Rapid microfluidic analysis of thousands of droplets per sample makes ddPCR practical for routine use [37]. For real-time PCR, the concentration of analyte was calculated by Ct comparison to the standard curve. ddPCR was direct quantitative analysis, but ARMS was indirect quantitative analysis. Thress et al. [38] compared four assays to detect EGFR T790M in ctDNA when patients had progression disease. The result showed that the sensitivity of ddPCR was higher than ARMS (71% vs. 29%). In our study, in the group of 75 plasma ctDNA, 19 were detected with T790M gene mutation by two assays and 16 were only detected with T790M mutation by ddPCR assay. And, in 19 patients, only 1 patient was below 5% abundance (1.44%). In the 16 patients, 11 patients were below 1% abundance (0.16%~0.80%), and 3 patients were below 2% but higher than 1% (1.56%~1.81%). Therefore, comparing ARMS assay, ddPCR is a potential method which had a higher precision, sensitivity, and accuracy to detect T790M gene status in plasma.

Takahama et al. [39] evaluated liquid biopsy assays for detection of TKI-sensitizing and T790M mutations of EGFR by ddPCR in EGFR mutation positive NSCLC patients with acquired EGFR-TKI resistance. And the result showed concordance for mutation detection by ddPCR in plasma relative to that in the paired samples with a sensitivity of 64.7% (20/31) and specificity of 70.0% (7/10) for T790M. Zheng et al. [33] studied the ddPCR method to dynamically detect EGFR mutations in patients' plasma. The results demonstrated that T790M ctDNA mutation in plasma was detected in 55 (47%) of the 117 patients. And the overall concordance rate of T790M testing between the paired tumor tissues and plasma was 88.00% (22/25). The sensitivity and specificity of plasma T790M testing by ddPCR assay were 81.25% (13/16) and 100.00% (9/9), respectively. Lee et al. [40] used ddPCR to detect EGFR status in plasma from advanced NSCLC patients. The sensitivity and specificity of plasma EGFR testing by ddPCR assay were 74.1% and 100.00%, respectively. When compared with ARMS assay (25.3%) to detect the ctDNA acquired T790M, ddPCR had a higher sensitivity. Consistent with this, in our study of EGFR mutation NSCLC patient plasma samples, we detected T790M ctDNA by ddPCR in 43.7% (47/108) of patients with resistance to EGFR-TKI therapy. And in our study, although only 16 patients experienced rebiopsy, the result showed that the sensitivity and specificity of plasma T790M testing by ddPCR assay were 66.7% (6/9) and 71.4% (5/7), respectively. Among them, six patients were with T790M-positive mutation from tissues by ARMS assay, and two patients were without T790M mutation from plasma ctDNA by ddPCR method. We believe that biopsy is usually done at single site and it could not show the whole tumor pathological features and

presence of the temporal and spatial heterogeneity of tumor tissue. The genetic information from patients' plasma may be homogeneity in ctDNA. Therefore, detecting rebiopsy and blood samples may be mutual complementation in some cases. Furthermore, considering the feasibility and accessibility of blood sampling, we believe that the comprehensive profiling of TKI resistance to T790M mutation could be better performed using plasma ctDNA analysis of blood samples compared to single tumor rebiopsies. Hence, it needs more prospective studies to compare plasma with tissue in different situations.

Li et al. [32] demonstrated that patients with T790M mutation after acquired resistance to EGFR-TKI benefit more from EGFR-TKI treatment beyond progression compared to those without T790M mutation (13.0 vs. 10.5 months, $P = 0.894$) and had longer survival than T790M-negative patients (39.8 vs. 23.2 months, $P = 0.044$). However, Zheng et al. [33] suggested patient's plasma T790M status upon TKI failure, which is associated with increased tumor burden and/or metastasis, thereby leading to poor survival (26.9 months vs. unreached, $P = 0.048$). In our study, the PFS of T790M mutation patients was longer than patients without T790M (13.1 vs. 10.8 months, $P = 0.010$) and the OS also was longer than T790M negative (35.3 vs. 30.3 months, $P = 0.214$). Patients with T790M positive tended to local progression and gradual progression, but patients with T790M negative tended to dramatic progression. We also found that the incidence rate of tumor size ≥ 4 cm in the patients with T790M-negative mutation was slightly higher than patients with the T790M-positive mutation (60.0% vs. 40.0%, $P = 0.266$). The tumor without T790M gene mutation may have a more tumor burden and rapid progression, or have other drive gene mutations, therefore would be poor survival.

Our study continued to analyze sequential treatment in patients with acquired resistance to EGFR-TKI. The PFS2 in the patients with T790M mutation was slightly longer than patients without T790M mutation (4.0 vs. 3.1, $P = 0.709$). And the PFS2 of patients receiving continuous TKI plus chemotherapy was better than other treatment agents. Among the three groups of EGFR-TKI failure modes, the PFS2 of patients in gradual progression group (6.0 months) was longer than that in dramatic progression group (4.9 months) or local progression group (4.3 months). Trials including ASPIRATION [41] and IMPRESS [42] explored the treatment strategies for EGFR-TKI failure. Continuing TKI plus chemotherapy might be a potential strategy beyond progression, and the algorithm will be answered by trial IMPRESS. IMPRESS showed that a subgroup analysis evaluating outcomes by T790M mutation status demonstrated a PFS advantage in T790M-negative patients who received chemotherapy (cisplatin

and pemetrexed) plus gefitinib versus only chemotherapy (6.7 vs. 5.4 months, $P = 0.07$), but not in T790M-positive patients. However, in our study, 25 patients receiving chemotherapy plus continued TKI including single chemotherapy regimen was 21 (8 T790M positive and 13 T790M negative) and double chemotherapy regimen was 4. We thought that single chemotherapy not with platinum drugs might reverse TKI drug resistance, single chemotherapy plus TKI had an appropriate therapeutic strategy for T790M-positive patients. Wei et al. [32] also analyzed the subsequent treatment after resistance to EGFR-TKI, the results showed that the PFS2 in the patients with T790M mutation was 6.2 months, which was significantly longer than 2.6 months in the patients without T790M mutation ($P = 0.002$). Nowadays, several promising new agents such as CO1686 and AZD9291 that target T790M showed amazing results. And AZD9291 has been approved by FDA for the treatment of patients with metastatic EGFR T790M mutation-positive NSCLC who have progressed on or after EGFR-TKI therapy [43]. However, in China, there is still no standard therapy for patients with T790M-mediated TKI resistance in clinical practice. Thus, before the approval to use third TKI for T790M mutation, we could choose continuous TKI plus chemotherapy as first treatment agent.

We must mention that we have several limitations. First, because of the deficiency in majority of patient rebiopsy tissues, we had not compared all ctDNA with tumor tissues. Tumor tissues in 16 samples were analyzed by immunohistochemistry (IHC), detected T790M gene status by ARMS, and residual tissues were deficient in detection by ddPCR compared with the plasmas. Second, the nature of retrospective study will induce the statistical bias. And the physicians' decision regarding continuation of TKI or switching to chemotherapy might influence result of observational analysis.

In conclusion, this study indicated the advantages of ddPCR in the associated EGFR T790M mutation after resistance to EGFR-TKI. It is expected that ddPCR may have good application prospects and may play an increasing role in patients with acquired EGFR T790M mutation along with the research development of third EGFR-TKI.

Conflict of Interest

None declared.

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