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Origin of the T790M mutation and its impact on the clinical outcomes of patients with lung adenocarcinoma receiving EGFR-TKIs

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ABSTRACT

Objective: Recently, a low frequency of *de novo* T790M mutations existing in tumor tissues before TKIs therapy has been reported. However, the origin of T790M and its impact on clinical outcomes is still being debated. This study aimed to use highly sensitive methods to detect T790M before and after TKIs therapy and investigated the correlation of T790M with clinical prognosis.

Patients and methods: Matched tumor samples before and after treatment were collected from 61 lung adenocarcinoma (LAC) patients in Beijing Chest Hospital between June 2014 to October 2017. Presence of the T790M mutation was simultaneously detected using amplification refractory mutation system-PCR (ARMS-PCR) assay and droplet digital PCR (ddPCR) assay.

Results: Of the 61 enrolled patients, 46 were candidates for and received TKIs treatment based on their EGFR mutation status. When these samples were assayed, ddPCR identified significantly more T790M mutations than ARMS-PCR (before TKIs treatment: 19.6% (9/46) vs. 2.2% (1/46), $P = 0.040$; after TKIs treatment: 78.3% (36/46) vs. 50% (23/46), $P < 0.001$, respectively). Patients with first-line TKIs treatment harboring *de novo* T790M mutations showed a shorter PFS compared to those without *de novo* T790M mutations (median, 7.0 months vs. 11.7 months, $p = 0.013$). In multivariate analyses, *de novo* T790M mutation was an independent predictor of PFS in EGFR-mutant patients who received TKIs treatment ($p = 0.031$, HR 0.310, 95% CI: 0.107-0.900).

Conclusion: The ddPCR assay is an ultra-sensitive method to detect a minor amount of *de novo* T790M mutations in tumor samples. The *de novo* T790M mutation is a relatively unfavorable prognosis factor for patients receiving first-line TKIs treatment.

1. Introduction

In recent years, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have improved clinical outcomes for advanced lung adenocarcinoma patients harboring EGFR-activating mutations, resulting in higher tumor response rates and longer progression-free survival (PFS) [1]. However, the majority of patients who have an initial response to therapy eventually develop resistance to TKIs [2]. The most common resistance mechanism is the substitution of threonine to methionine at codon 790 (T790M), which accounts for approximately 50% of acquired drug resistance [3]. Third-generation EGFR-TKIs, such as Osimertinib, are highly effective in patients with a T790M mutation who experience tumor progression.

Previous studies have investigated the role of T790M in acquired

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resistance to TKIs treatment [3–6]. However, some studies suggest that the accumulation of *de novo* T790M mutations during TKIs therapy may be the primary driver of resistance [7,8]. The development of the highly sensitive ddPCR assay has made it possible to detect low levels of T790M mutations in tumor samples. The frequency of *de novo* T790M mutations has been reported in several studies, but the mutation rates varied broadly from 0%–79.9% [9–15]. Moreover, findings regarding the prognostic value of *de novo* T790M were contradictory. Some studies reported poor PFS of EGFR-mutant patients harboring *de novo* T790M who received TKIs therapy, while another study suggested outcomes were favorable [9,11,12,14].

In this study, we retrospectively investigated *de novo* versus acquired T790M mutations in matched tumor samples from LAC patients before and after TKIs therapy. We analyzed the contribution of *de novo* versus acquired T790M mutations to TKIs resistance, and the impact of *de novo* T790M on PFS of patients with first-line TKIs treatment.

2. Material and methods

2.1. Patients and tumor samples

A total of 61 patients with lung adenocarcinomas (LACs) treated at Beijing Chest Hospital between June 2014 and October 2017 were included. Each patient had available tumor samples that were taken before and after treatment. Clinical data collected for analyses included gender, age, smoking status, disease stage, and T790M mutation status.

Tumor tissues were obtained either by biopsy (before treatment, n = 54, 88.5%; after treatment, n = 61, 100%) or by surgical resection (before treatment, n = 7, 11.5%). All tumor samples were analyzed for EGFR mutations that included T790M. Progression-free survival (PFS) was defined as the time from the first treatment to the time of radiological confirmation of disease progression during follow-up. The last follow-up occurred on December 1 st, 2017. The medical ethics committee of the Beijing Chest Hospital at Capital Medical University approved this study. Informed consent was obtained from all the participants.

"Never smokers" were defined as patients who had smoked < 100 cigarettes in their lifetime. Disease stage was determined in accordance with the Classification of the Union for International Cancer Control, 7th Edition [16]. Patients were treated with the recommended oral dose of either gefitinib, erlotinib, or icotinib, either as first-line therapy or as second-line therapy after chemotherapy.

Objective tumor responses were evaluated every 6–8 weeks in accordance with the Response Evaluation Criteria in Solid Tumors guidelines [17]. Acquired TKIs resistance was determined based on Jackman's clinical definition [18].

2.2. Detection of EGFR mutations by ARMS-PCR and ddPCR assays

Formalin-fixed, paraffin-embedded (FFPE) sections were stained with hematoxylin and eosin and analyzed by a clinical pathologist. Tissue comprised of ≥50% tumor cells were selected for further analyses. Genomic DNA extraction and detection of EGFR mutations (exons 18–21) by ARMS-PCR (AmoyDx Inc., Xiamen, China) was performed as previously described [19].

The ddPCR assay used to detect EGFR T790M mutations was performed according to the manufacturer's protocols. Yuanma Technologies (Beijing, China) provided the T790M-specific primers and probes. For each reaction assay, 10 u M (1ul) probes and 10 u M (3.6ul) primers were mixed with 2× Droplet PCR Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 10 ng of template DNA, and H_2O to a final volume of 21 μL. The solution was added to the droplet generator with 70 μL of oil to form droplets with a mixture of approximately 35 μL of oil-in-water. The mixture was transferred to a 96-well PCR plate and heat-sealed. The plate was placed in a C1000 Touch thermal cycler (Bio-Rad Laboratories) and amplified to the PCR endpoint. The cycling conditions were as follows: one cycle at 95 °C for 10 min, 40 cycles at 94 °C for 30 s, 58 °C for 1 min, and one cycle at 98 °C for 10 min, with a ramp rate of 1.6 °C per second. The analysis was performed according to the manufacturer's protocols using a QX-200 droplet reader (Bio-Rad Laboratories). A threshold line was determined to separate the two clusters of negative and positive droplets. Quantalife software was used to calculate the Poisson distributions of both mutant and wild-type DNA copies. Positive and negative cut off points were established for controls. For positive controls, the cut off was set at three droplets based on preliminary assays and manufacturer's recommendations.

2.3. Statistical analysis

Crosstab Fisher's exact tests were used to estimate statistical agreement between the two testing methods. Kaplan-Meier methods were used to evaluate survival curves for PFS in univariate analysis, and Cox regression models were used to evaluate the independent prognostic factors associated with PFS. The clinical data variables used in multivariate analyses were T790M mutation status, age, sex, and smoking status. All statistical tests were carried out with SPSS version 21.0 (SPSS, Chicago, IL). A two-sided $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Clinical characteristics of patients

Patient clinicopathological features summarized in Table 1. Of the 61 patients enrolled in this study, 24 (39.3%) were female and 38 (62.3%) were non-smokers. The median age was 61 years old (range, 35–78). Five (8.2%) were stage I lung adenocarcinoma patients, 2

Table 1
Clinical characteristics of 61 patients.

Features	N (%)
Age (years)	
Range	35-78
Median	61
≤60	29(47.5)
>60	32(52.5)
Gender	
Male	24(39.3)
Female	37(60.7)
Smoking status	
Non-smoking	38(62.3)
Smoking	23(37.7)
Stage	
I	5(8.2)
II	2(3.3)
III	8(13.2)
IV	46(75.4)
Type of EGFR mutations	
19del	31(50.9)
L858R	13(21.3)
The other EGFR genotype	5(8.2)
The EGFR wild type	12(19.6)
TKIs*	
Icotinib	27(57.4)
Gefitinib	17(36.2)
Erlotinib	3(6.4)
Therapy	
Treated with first-line TKIs	34 (55.7)
Treated with ≥ second-line TKIs	13 (21.3)
Treated with first-line chemotherapy	14 (23.0)

Note: EGFR, epidermal growth factor receptor; TKIs, tyrosine-kinase-inhibitors.

* 47 patients (44 patients with 19del or L858R mutations, one patient with G719X, and one patient with L858R + T790M mutation, one patient with the wild type) received TKIs therapy.

Table 2
Comparison of ARMS-PCR assay with ddPCR assay in detecting T790M mutation before TKIs treatment.

		ddPCR-T790M status		
		mutation detected	No mutation detected	Total
ARMS-PCR T790M status	mutation detected	1	0	1(2.2%)
	No mutation detected	8	37	45(97.8%)
	Total	9(19.6%)	37(80.4%)	46

Note: ddPCR: droplet digital PCR; ARMS: amplification refractory mutation system.

Table 3
Comparison of ARMS assay with ddPCR assay in detecting T790M mutation after TKIs treatment.

		ddPCR-T790M status		
		mutation detected	No mutation detected	Total
ARMS-PCR T790M status	mutation detected	23	0	23(50.0%)
	No mutation detected	13	10	23(50.0%)
	Total	36(78.3%)	10(21.7%)	46

Note: ddPCR: droplet digital PCR; ARMS: amplification refractory mutation system.

(3.3%) were stage II, 8 (13.2%) were stage III, and 46 (75.4%) were stage IV. Of all included patients, 49 had EGFR gene mutations. Forty-six EGFR-mutant patients received TKIs therapy, 27 received icotinib, 17 received gefitinib, and 2 received erlotinib. The median PFS was 11.0 months (range: 0.7–42.0 months).

3.2. Detection of T790M mutation status by ARMS-PCR and ddPCR assays

All of the matched samples were analyzed by ARMS-PCR and ddPCR as shown in Tables 2 and 3 and Fig.1 (Supplementary Table 1). The ddPCR assay was more sensitive for the detection of T790M than ARMS-PCR assay (before TKIs treatment, 19.6% vs. 2.2%, $P = 0.040$; and after TKIs treatment, 78.3% vs. 50%, $P < 0.001$, respectively). After TKIs treatment, 50% (23/46) patients identified T790M mutations by ARMS-PCR assay, which were also positive by ddPCR assay.

3.3. Comparison of T790M mutation status in tumor tissues before and after TKIs therapy

The patient number of T790M mutation before and after treatment is showed in Fig.1. Before TKIs treatment, the *de novo* T790M mutations were detected in nine patients with EGFR mutations. The abundance of the *de novo* T790M mutation ranged from 0.2% to 59.7% (median: 1.2%). After TKIs treatment, the abundance of the *de novo* T790M mutation ranged from 0.05% to 45.7% (median: 16.45%). However, the abundance of the *de novo* T790M mutation in two patients did not increase with the duration of TKIs therapy (59.57% vs. 45.7%; 0.84% vs. 0.05%; respectively). Moreover, the T790M mutation was not found in one EGFR-mutant patient harboring the *de novo* T790M mutation. The abundance of the acquired T790M mutation ranged from 0.71% to 21.4% (median: 4.2%). These results show that T790M is more prevalent in tumors with *de novo* T790M than in those that acquire T790M mutations after TKIs treatment. And, the median abundance of T790M mutation is higher in tumors with the *de novo* T790M mutation then those with the acquired T790M mutation after TKIs therapy.

In addition, when using the ddPCR assay, the *de novo* T790M mutations were also found in four (28.6%) tumor samples with EGFR wild type before chemotherapy, but not in tumor samples after disease progression.

3.4. Survival analysis

The clinical evaluation of TKIs treatment in patients with T790M mutations by ddPCR assay are summarized in Table 4. Of the 61 enrolled patients, 40 patients in stages IIIB-IV were enrolled for survival analysis. Of these, 34 with EGFR mutations received first-line TKIs therapy and six with the wild-type received first-line chemotherapy.

Of the 40 stage IIIB-IV patients, six EGFR-mutant patients with *de novo* T790M mutations showed significantly worse PFS compared with 28 patients without *de novo* T790M mutations (7.0 months vs. 11.7 months, $p = 0.013$), but these patients had a similar PFS with six patients with the wild type (7.0 months vs. 5.0 months, $p = 0.263$)

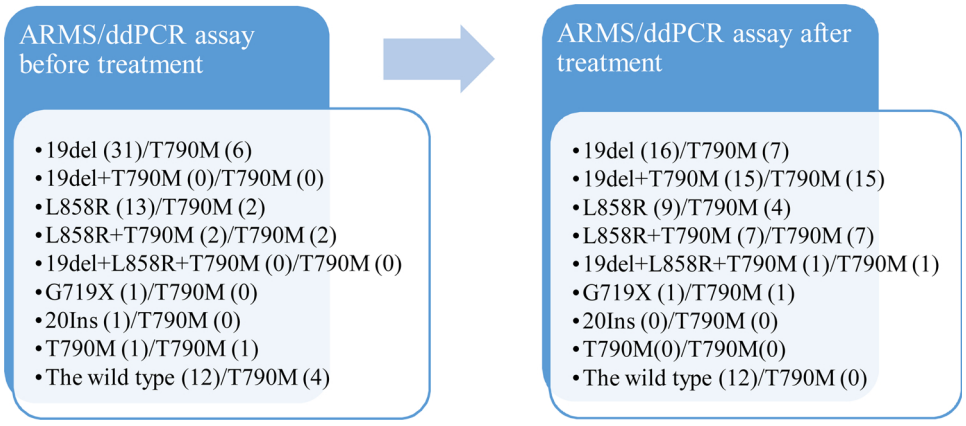

Fig. 1. Comparison of the patient number of each genotype before and after treatment.

Table 4
Clinical evaluation of TKIs treatment in patients with T790M mutations detected by ddPCR assay.

	De novo T790M (+) (n = 9)	Acquired T790M (+) (n = 28*)
Response to TKIs		
PR	3	14
SD	5	13
PD	1	1
Progression model		
Gradual	3	21
Dramatical	6	7
Progression type		
Local recurrence	5	25
Distant metastasis	4	3

Note: PR: partial response; SD: stable disease; PD: progressive disease;
* The T790M mutation was not detected in one tumor sample with *de novo* T790M after TKIs treatment.

(Fig. 2A).

Moreover, after TKIs treatment, 22 patients with acquired T790M mutations had significantly better PFS than those with *de novo* T790M mutations (13.0 months vs. 7.0 months, $p = 0.004$) or those without acquired T790M mutations (13.0 months vs. 7.0 months, $p = 0.010$) (Fig. 2B).

Clinical variables including T790M mutation status, age, sex, and smoking status were used in multivariate analysis. Cox analysis showed that *de novo* T790M mutation ($p = 0.031$, HR 0.310, 95% CI 0.107-0.900) was an independent prognostic factor for PFS. However, there were no significant differences in patient survival depending on sex ($p = 0.848$, HR 1.136, 95% CI 0.307–4.201), age ($p = 0.791$, HR 1.113, 95% CI 0.502–2.469), or smoking status ($p = 0.902$, HR 1.090, 95% CI 0.274–4.335) (Table 5).

4. Discussion

Despite an initial response to therapy, the vast majority of lung adenocarcinoma patients ultimately acquire TKIs resistance around 10–12 months. The EGFR T790M mutation is found in nearly 50% of patients who develop TKIs resistance (5). Nonetheless, it remains unclear whether and how T790M mutation develops during TKIs treatment.

The *de novo* T790M maybe exist at low frequencies within EGFR-mutant tumor cells before TKIs treatment [20,21]. The ARMS-PCR assay is considered to be a stable and sensitive method and is widely applied in clinical practice. However, the sensitivity of ARMS-PCR assay is approximately 1%, so a small frequency of T790M mutations cannot be detected. Our findings confirm previous studies that show ddPCR is more sensitive than ARMS-PCR for the detection of T790M mutation, with a lower limit of 0.01% [22,23]. As a result, ddPCR could detect the presence of very small quantities of T790M mutations in tumor samples.

The prevalence of *de novo* T790M mutations varies from 0%–79.9%, depending on the sensitivity of detection method being used. In two studies, low copy numbers of *de novo* T790M mutations were identified by next-generation sequencing (NGS), suggesting that tumors acquired T790M mutations over the course of disease progression in patients receiving TKIs treatment [7,8]. In contrast, other studies have demonstrated that there was a high prevalence of *de novo* T790M mutations in tumors before TKIs treatment, with a mutation rate of 18.2% to 25.0% [11,13]. Moreover, the *de novo* T790M mutation rate in some studies was even higher, up to 79.9%, when ultrasensitive methods were used [14,15]. Our present findings show that the ddPCR assay is a highly sensitive quantitative method for the detection of *de novo* T790M in EGFR-mutant tumor samples. Our study also found the mutation rate of *de novo* T790M to be 19.6% (9/46), which is consistent with previous research findings.

The *de novo* T790M mutation may contribute to primary resistance against TKIs treatment, and therefore understanding its mechanism is vital [24]. Lee et al. reported that the *de novo* T790M mutation was identified in 9.1% (1/11) patients who showed primary resistance to TKIs treatment [25]. In this study, *de novo* T790M mutations were detected in 19.6% (9/46) of EGFR-mutant patients who received TKIs therapy, while T790M mutations were detected in 78.3% (36/46) of matched tumor samples after drug resistance developed. These results suggest that both *de novo* and acquired T790M mutations may contribute to the development of TKIs drug resistance.

It was widely believed that the abundance of T790M in tumor samples gradually increases during TKIs therapy [3,5]. In this study, we

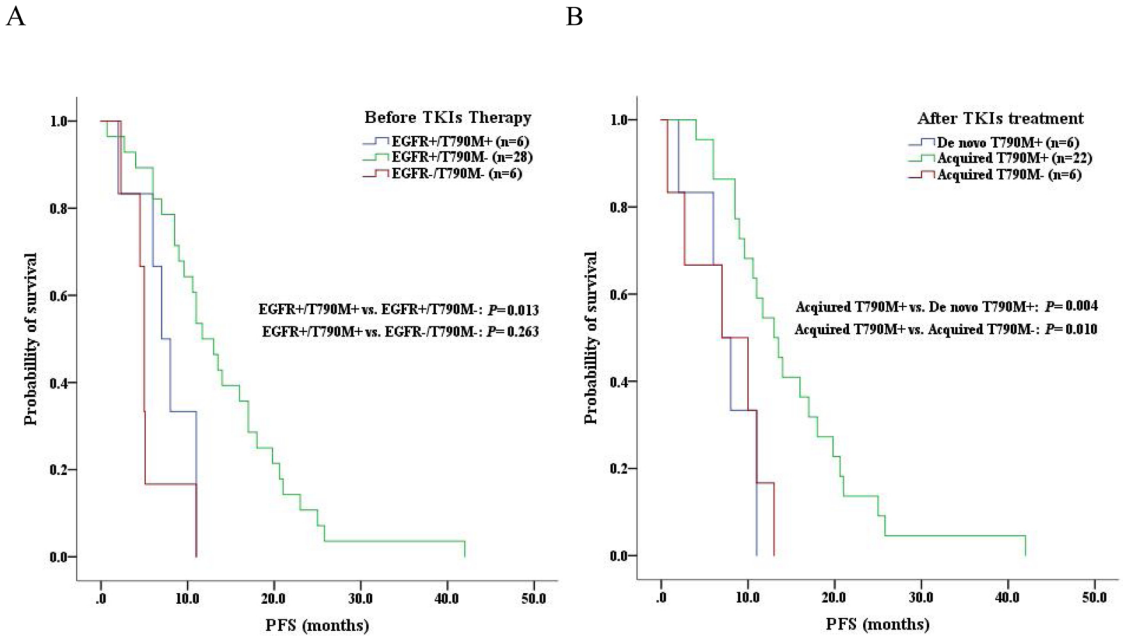

Fig. 2. Kaplan-Meier survival curve for 34 patients harboring EGFR mutations who received first-line TKIs therapy according to T790M status identified by ddPCR assay. (A) before TKIs treatment, the mutations status of T790M, (B) after TKIs treatment, the mutations status of T790M.

Table 5
Univariate and multivariate analysis for progression-free survival of patients with EGFR mutations who received first-line TKIs treatments.

variables	PFS (month)	Univariate		Multivariate		
		95% CI	*p*	HR	HR (95% CI)	*p*
Gender						
Male	11.700	8.463-14.937	0.729	1.136	0.307-4.201	0.848
Female	10.600	9.285-11.915				
Age(years)						
< 60	10.600	3.877-17.323	0.795	1.113	0.502-2.469	0.791
≥60	11.000	10.035-11.965				
Smoking status						
Yes	11.000	6.655-15.345	0.807	1.090	0.274-4.335	0.902
No	11.000	9.910-12.090				
T790M detection by ddPCR assay Before TKIs therapy						
T790M-positive	7.000	4.600-9.400	0.019	0.310	0.107-0.900	0.031
T790M-negative	11.000	8.511-13.489				

Note: EGFR: epidermal growth factor receptor; TKI: tyrosine-kinase-inhibitor; PFS: progression free survival; CI: Confidence interval; HR: Hazard Ratio; ddPCR: droplet digital PCR.

found that the abundance of T790M in seven patients with *de novo* T790M increased during TKIs therapy. But, we also found two exceptions. Moreover, we detected *de novo* T790M mutations in four (4/12, 33.3%) tumor samples with the EGFR wild-types. These findings suggest that the mechanisms of primary resistance against TKIs are likely heterogeneous.

Previous observations indicate that the presence of *de novo* T790M mutations in a small fraction of tumor cells has significant implications for the prognosis of patients with EGFR mutations who are receiving TKIs treatment. Most studies suggest that the *de novo* T790M mutation negatively impacts PFS, but not overall survival (OS) [9–11,26–28]. In contrast, other studies indicate that *de novo* T790M either has no significant impact on or may even be favorable for TKIs treatment outcomes in advanced NSCLC patients [13,14]. Furthermore, the acquired T790M mutation has been found to be as a positive factor for PFS in EGFR-mutant patients receiving TKIs therapy [6,9]. In our study, patients with *de novo* T790M mutations tended to have dramatic disease progression, distant metastases, and a worse PFS compared with those without *de novo* T790M mutations. In contrast, patients with acquired T790M mutations demonstrated gradual progression, local progression, and greater benefit from TKIs treatment than those without acquired T790M mutations. In particular, tumors with *de novo* T790M mutations had a greater abundance of T790M compared to those that acquired T790M mutations over the course of therapy. Thus, we hypothesize that the high T790M abundance of tumors with *de novo* T790M mutations may be attributed to the poor prognosis of patients harboring the *de novo* T790M mutations. One recent report found that use of Osimertinib as a first-line TKIs treatment had superior efficacy to that of first-line TKIs treatments in EGFR-mutant patients with advanced NSCLC [29]. However, it is unclear whether patients with *de novo* T790M mutation could benefit more from Osimertinib as first-line TKIs treatment.

There are several limitations in this study. First, we only used the ddPCR assay to detect T790M because there were insufficient tumor samples for additional testing. Second, due to the short follow-up time, PFS was the only endpoint used in this study, and we were unable to assess whether overall survival was dependent on the *de novo* T790M mutation. Third, most of the samples were biopsy specimens, so there was insufficient tumor tissue to assay for concurrent mutations that might impact patient outcomes, such as PIK3CA、Met、or TP53. Finally, the sample size was small due to the limited availability of matched tumor samples from the same patients before and after TKIs therapy. Despite these limitations, the results of this study reliably show that the *de novo* T790M mutation plays an important role in the clinical outcome of TKIs therapy.

In conclusion, the ddPCR assay is a sensitive method that can effectively detect a low frequency of *de novo* T790M mutations. The *de novo* T790M mutation is a negative marker for TKIs-treated patients harboring EGFR mutations. Detection of *de novo* T790M mutation before TKIs treatment may help to determine the prognosis of lung adenocarcinoma patients and to identify the most suitable therapy for improved clinical outcomes.

Conflict of interest

The authors declare no conflict of interests.

Compliance with ethical standards

This study was approved by the medical ethics committee of Beijing Chest Hospital, Capital Medical University and informed consent was obtained from all participants.

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