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ORIGINAL RESEARCH

Conventional real-time PCR-based detection of T790M using tumor tissue or blood in patients with EGFR TKI-resistant NSCLC

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Abstract: Blood biopsy has many advantages over tissue biopsy for diagnosing acquired T790M mutation in patients with non-small-cell lung cancer, such as being less risky and painful. New techniques with high sensitivity (eg, droplet digital PCR) show promising results during blood biopsy, but the positive rates of identification are still quite unclear. Whether there are other factors, except technology, affecting the results of blood biopsy is unclear. In this study, we used conventional amplification refractory mutation system to detect tumor tissue or blood for T790M mutation in patients clinically resistant to tyrosine kinase inhibitors. A total of 45 patients treated at West China Hospital between 2014 and 2016 were analyzed. The positive rate of T790M mutation was 70.8% based on tissue biopsy and 37.5% based on blood biopsy. Of the 24 patients whose epidermal growth factor receptor gene was genotyped through tissue and blood biopsy, 10 (41.7%) were concordant for T790M mutation status (κ=0.006). Of the 17 patients positive for T790M by tissue biopsy, 7 (41.2%) were positive for T790M by blood biopsy, and 3 of these 7 were only weakly positive. Of the 7 patients negative for T790M by tissue biopsy, 2 (28.6%) were positive by blood biopsy. Our T790M detection rate is higher than that reported by other studies using digital droplet PCR. These results suggest that other factors (eg, clinical features), intrinsically connected with circulating tumor DNA level, also affect the results of blood biopsy, and thus cannot be controlled through technological optimization.

Keywords: non-small-cell lung cancer, T790M mutation, re-biopsy, liquid biopsy

Introduction

Certain *EGFR* mutations in patients with non-small-cell lung cancer (NSCLC) are associated with acquired resistance to first-line epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs). The most common resistance-conferring mutation is T790M. Patients with this mutation often respond to the EGFR TKI known as AZD9291, so the mutation must first be confirmed through re-biopsy.[1–3] This normally involves re-biopsy of the tumor tissue for subsequent sequencing, but biopsy is associated with safety risks and pain.

Blood biopsy has been proposed as a less risky and painful alternative. Blood contains circulating tumor DNA (ctDNA) within tumor cells as well as circulating free DNA.[4] These DNA can then be sequenced. New techniques with higher sensitivity (eg, droplet digital PCR [ddPCR]), compared with conventional amplification refractory mutation system (ARMS), show promising results while testing ctDNA, but the positive rates are still quite varied and unclear.[5–9] This forces blood biopsy by ctDNA to be an alternative approach,[10] and tissue-based molecular analysis remains the recommended standard for evaluating resistance to TKIs.[11] Finding out the factors affecting

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the performance and reliability of the tests is necessary for the optimization of blood biopsy by ctDNA. Other factors that affect the tests, except technology, are still unclear.

Here, we genotyped *EGFR* from either tissue or blood biopsy samples for detecting various *EGFR* mutations linked to TKI resistance in patients with NSCLC. In particular, we focused on the mutation T790M. DNA extracted from both types of biopsies was sequenced using the conventional ARMS. We chose this system for two reasons: first, it is still the standard system used for tissue biopsy at our hospital; second, we wanted to know whether there are other factors, except technology, affecting the results. The findings of this study may shed light on the optimization of the performance and reliability of blood biopsy, especially relative to tissue biopsy, for diagnosing acquired EGFR TKI resistance associated with T790M.

Materials and methods

Study design

This observational study was conducted on patients treated at West China Hospital (Chengdu, People's Republic of China) between 2014 and 2016. Patients were eligible if they had advanced NSCLC involving *EGFR* mutation(s), were clinically resistant to EGFR TKIs (gefitinib, erlotinib, icotinib, or afatinib), and were undergoing re-biopsy for *EGFR* genotyping as part of their routine clinical care. The study was approved by the Biomedical Research Ethics Committee of West China Hospital of Sichuan University, and all patients signed the informed consent form.

Blood sampling and sequencing

Peripheral blood was collected within 2 weeks of re-biopsy into a 10-mL vacutainer containing ethylenediaminetetraacetic acid. Blood samples were transported to our laboratory within 2 h of being drawn, plasma was isolated by centrifugation at 2,000× *g* for 10 min, and the supernatant was further cleared at 8,000× *g* for an additional 10 min. The Circulating Nucleic Acid Kit (AmoyDx, Xiamen, People's Republic of China) was used to isolate ctDNA according to the manufacturer's protocol. *EGFR* genotyping was performed on tissue biopsies according to standard institutional procedures in a certified laboratory, with conventional ARMS sequencing carried out using the Human EGFR Gene Mutations Fluorescence PCR Diagnostic Kit (AmoyDx). The same kit was used to genotype *EGFR* from ctDNA. This kit has been approved for clinical use by the China Food and Drug Administration since 2010.

Statistical analysis

Diagnostic concordance between ARMS sequencing of tissue-derived DNA or ctDNA was assessed using Cohen's κ, and the concordance was assessed for significance using the McNemar test. Percent agreement values were calculated based on the main diagonal in 2×2 tables. All statistical analyses were performed with SPSS 20.0 (IBM Corp., Armonk, NY, USA), and the significance threshold was set as $P<0.05$.

Results

Patient characteristics

From January 2014 to June 2016, 45 patients were enrolled in the study (Figure 1, Table 1). Of the 45 patients, 4 (8.9%) received two types of EGFR TKI. As the most recent treatment, 8 (17.8%) received chemotherapy, 4 (8.9%) received EGFR TKI plus chemotherapy, and 33 (73.3%) received EGFR TKI.

EGFR genotyping in tissue biopsy and plasma ctDNA

Among patients whose tissue biopsy was sequenced, 25 showed two *EGFR* mutations, 12 showed one mutation, and 1 had three mutations (Table 2). Among patients whose plasma ctDNA was sequenced, 14 had two types of *EGFR* mutation, 12 had no mutation, and 5 showed one mutation.

Of the 24 patients whose tissue and plasma ctDNA were sequenced (Table 3), 18 (75.0%) were concordant for exon 19 deletion (κ=0.385; Figure 2A), 21 (87.5%) were concordant for L858R (κ=0.654; Figure 2B), and 10 (41.7%) were concordant for T790M (κ=0.006; Figure 2C).

The positive rate for T790M mutation was 70.8% based on tissue sequencing and 37.5% based on plasma ctDNA sequencing (Table 3). Of the 24 patients for whom both tissue and plasma ctDNA sequencing results were available,

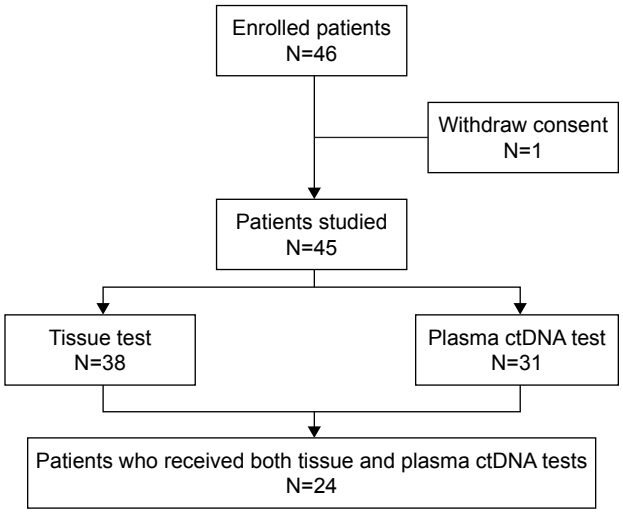

Figure 1 Patient flow diagram.
Abbreviation: ctDNA, circulating tumor DNA.

Table 1 Patient characteristics

Characteristics	N (%)
Age, years	
Median	53.2
Range	36–75
Sex	
Male	23 (51.1)
Female	22 (48.9)
Histology (initial biopsy)	
Adenocarcinoma	42 (93.3)
Squamous carcinoma	3 (6.7)
Other	0 (0.0)
Stage before re-biopsy	
IIIB	2 (4.4)
IV	43 (95.6)
Type of initial *EGFR* mutation	
19 Del	30 (66.7)
L858R	15 (33.3)
Other	0 (0.0)
Type of initial EGFR TKI	
Gefitinib	37 (82.2)
Erlotinib	4 (8.9)
Icotinib	4 (8.9)
Line of initial EGFR TKI	
First	39 (86.7)
Second	6 (13.3)
Response to initial EFGR TKI	
CR/PR/SD	43 (93.3)
PD	2 (6.7)
Second biopsy	
Tissue biopsy	38 (84.4)
Blood biopsy	31 (68.9)
Both	24 (53.3)

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; CR, complete response; PR, partial response; SD, stable disease; PD progressive disease.

9 (37.5%) showed the same results, although the plasma ctDNA results for one-third of these patients were only weakly positive. Of the 17 patients who were positive for T790M based on the tissue test, only 7 (41.2%) were positive for the plasma ctDNA test, and 3 of those 7 were only weakly positive. Of the 7 patients negative for T790M based on tissue sequencing, 2 were positive based on plasma ctDNA sequencing (Table 4).

Table 2 *EGFR* genotyping of initial and secondary biopsy

EGFR genotyping	Initial	Secondary	
	Tissue N=45 (%)	Tissue N=38 (%)	ctDNA N=31 (%)
Exon 19 deletion	30 (66.7)	28 (73.7)	12 (38.7)
L858R	15 (33.3)	11 (28.9)	6 (19.4)
T790M			
Weakly positive[a]	0 (0.0)	0 (0.0)	4 (12.9)
Positive	0 (0.0)	26 (68.4)	9 (29.0)
S768I	0 (0.0)	0 (0.0)	2 (6.5)
Negative	0 (0.0)	0 (0.0)	12 (38.7)

Note: [a]The curve rises up, but does not reach the peak.
Abbreviation: ctDNA, circulating tumor DNA.

Table 3 *EGFR* genotyping in patients who received both tissue and plasma ctDNA tests

EGFR genotyping	Initial	Secondary	
	Tissue N=24 (%)	Tissue N=24 (%)	ctDNA N=24 (%)
Exon 19 deletion	16 (66.7)	18 (75.0)	10 (41.7)
L858R	8 (33.3)	7 (29.2)	4 (16.7)
T790M			
Weakly positive[a]	0 (0.0)	0 (0.0)	3 (12.5)
Positive	0 (0.0)	17 (70.8)	6 (25.0)
S768I	0 (0.0)	0 (0.0)	2 (8.3)
Negative	0 (0.0)	0 (0.0)	9 (37.5)

Note: [a]The curve rises up, but does not reach the peak.
Abbreviation: ctDNA, circulating tumor DNA.

Discussion

In this study, we used conventional ARMS to sequence the *EGFR* gene of patients with NSCLC for mutations associated with TKIs resistance from re-biopsied tumor DNA or from plasma ctDNA. Our results suggest that ctDNA can be a suitable template for *EGFR* genotyping in these patients, although the positive rate for T790M (37.5%) based on blood biopsy is lower than that based on tissue sequencing (70.8%). These findings support the usefulness of plasma ctDNA for mutation analysis as established in several other disease contexts[9,12–17] and also suggest that there are other factors affecting the performance results of the test.

We sequenced the *EGFR* gene from tissue and blood using conventional ARMS despite its lower sensitivity,[18] rather than using newer techniques such as ddPCR,[12,19–21] because we wanted to analyze both types of biopsy in the same way to identify whether there are other factors, except technology, affecting the results. This should not threaten the validity of our results because studies using newer techniques also show a large variation in T790M-positive rates,[6–9,12–15,19–22] and the same held true even in four prospective studies on NSCLC patients with acquired resistance.[6–8,23] Indeed, the fact that our T790M-positive rate based on tissue sequencing (70.8%) was similar to that of several previous studies (63%, 66%, and 76.5%)[6–8] suggests that our study population was representative of the target patient population.

Some important characteristics of the four prospective studies and this study are listed in Table S1. All these prospective studies used new techniques with higher sensitivity. Mok et al[23] used allele-specific amplification, but the kit (cobas®, Roche Diagnostics Limited, Indianapolis, IN, USA) had as high a sensitivity and specificity as the

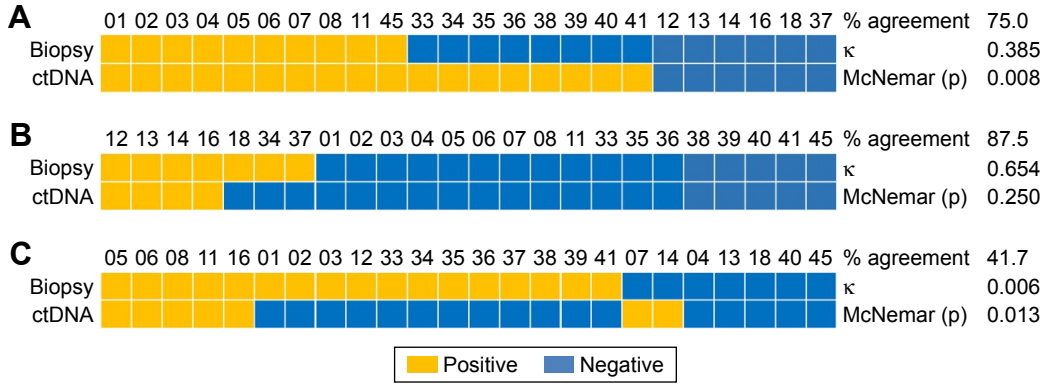

Figure 2 Comparison of *EGFR* mutation status based on tissue biopsy or plasma ctDNA with respect to (**A**) exon 19 deletion, (**B**) L858R mutation, or (**C**) T790M mutation.
Abbreviation: ctDNA, circulating tumor DNA.

ddPCR system.[19] However, our T790M rate was not the lowest one, either in all the patient receiving blood biopsy or in patients confirmed by tissue biopsy. In contrast, a clinical trial[7] reported the lowest T790M rate in both two situations. These results indicate that the less sensitive, conventional ARMS used in the present study does not necessarily perform worse than the sequencing techniques used in other studies. This implies that technology is not the primary reason why the T790M rate of ctDNA sequencing is highly varied.

While some authors have suggested the need to optimize the timing of blood biopsy,[24] this seems unlikely to explain the higher T790M rate, since in the present study and four other prospective studies,[6–8,23] both blood and tissue biopsies were performed at the same time after disease progression. It seems more likely that the higher T790M rate obtained with conventional ARMS is due to high ctDNA levels; in fact, high levels of circulating free DNA are associated with poor prognosis.[25,26] This implies that the risk of false negatives is much higher with blood biopsy than tissue biopsy in patients with good prognosis. We do not know whether the poor prognosis causes the high ctDNA levels in the present study. However, we do know that the disease course of patients in the clinical trial (mentioned earlier),[7] who only received first-line EGFR TKI treatment, is shorter compared to patients who received a multiline treatment in real-world studies.

Table 4 T790M mutation tested by both tumor tissue and plasma ctDNA levels

Tissue biopsy	Plasma ctDNA	
	Positive	Negative
Positive	7	10
Negative	2	5

Abbreviation: ctDNA, circulating tumor DNA.

We believe the factors causing high ctDNA levels are intrinsically connected with clinical features, different from those factors relating to preanalytical plasma processing and ctDNA extraction or detection technique,[10] and cannot be controlled through technological optimization.[10] It is necessary to find out what these factors are, so that patients with high ctDNA levels can be identified to choose blood biopsy as preferred diagnostic approach. In the meantime, and even more importantly, it may be advisable to use tissue biopsy for patients with low ctDNA levels in order to avoid the risk of false-negative by blood biopsy.

Future work should attempt to identify the clinical features relating to ctDNA level as well as additional factors that may affect the sensitivity and specificity of blood biopsy in order to clarify for which patients and at what times in disease progression this type of biopsy can be reliably performed. At the same time, studies should continue to examine how to make re-biopsy as safe as possible, especially since some patients, such as those with low ctDNA levels, may not be suitable for blood biopsy.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Characteristics of the studies

Characteristics	Sundaresan et al[1]	Yanagita et al[2]	Takahama et al[3]	Mok et al[4]	Our research
Type	Prospective	Prospective	Prospective	Prospective	Prospective
Enrolling condition	Real world	Clinical trial	Real world	Real world	Real world
Patient feature	AR	AR	AR	AR	AR
Sequencing method					
Tissue	IS	ddPCR	ddPCR	Cobas® (ASA)	AmoyDx (ARMS)
Plasma ctDNA	AS PCR	ddPCR	ddPCR	Cobas® version 2 (ASA)	AmoyDx (ARMS)
Positive rate of T790M					
In all the patients (%)	50.0	23.0	28.8	52.5	25.0–37.5
In patients CTB (%)	60.0	33.3	64.5	52.5	41.2

Abbreviations: AR, acquired resistance; IS, institution's standard; ASA, allele-specific amplification; AS PCR, allele-specific PCR; ddPCR, droplet digital-PCR; ARMS, amplification refractory mutation system; CTB, confirmed by tissue biopsy.

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