



Low T790M relative allele frequency indicates concurrent resistance mechanisms and poor responsiveness to osimertinib

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Background: T790M relative allele frequency (RAF) in plasma, calculated by the ratio of T790M to epidermal growth factor receptor (EGFR)-sensitizing mutation allele frequencies (AF), is associated with osimertinib response in patients with progressive non-small cell lung cancer (NSCLC) post 1st generation EGFR-tyrosine kinase inhibitor (TKI) treatment. However, which subgroup of patients carry concurrent resistance mechanisms and have poor responsiveness to osimertinib remains unknown.

Methods: Matched re-biopsy tissue and plasma samples obtained from 32 patients who had progression following 1st generation EGFR-TKI treatment were genotyped using next-generation sequencing (NGS) to investigate which subgroup of patients, classified by plasma position 790 (T790M) RAF, were more likely to carry concurrent resistance mechanisms. In another independent cohort, consisting of 21 T790M-positive patients, we validated whether these patients had a poor response to osimertinib treatment.

Results: In the discovery cohort, patients with T790M RAF less than 20% were more likely to harbor concurrent resistance mechanisms (P=0.018), such as MET or ERBB2 amplification, and small cell lung cancer transformation. In the validation cohort, we found that patients with low T790M RAF (<20%) had significantly lower objective response rates (ORRs) (0 *vs.* 68.8%, P=0.03) and disease control rates (DCRs) (60% *vs.* 100%, P=0.048) in response to osimertinib compared to patients with high T790M RAF.

Conclusions: In patients with progressive NSCLC post 1st generation EGFR-TKI treatment, plasma T790M RAFs of less than 20% can be used to identify patients who carry concurrent resistance mechanisms, and can predict a poorer response to osimertinib.

Trial registration: This study was registered on <http://www.chictr.org.cn> (registration number: ChiCTR-DDD-16007900).

Keywords: Epidermal growth factor receptor position 790 (EGFR T790M); next-generation sequencing (NGS); non-small cell lung cancer (NSCLC); liquid biopsy; tyrosine kinase inhibitor

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Introduction

The treatment of patients with non-small cell lung cancer (NSCLC) has been revolutionized by the development of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) (1-5). For patients harboring EGFR-sensitizing mutations treated with first-generation (gefitinib/erlotinib) or second-generation EGFR-TKIs (afatinib/dacomitinib), the objective response rates (ORRs) range from 56% to 74%, which are significantly higher than those of patients receiving first-line platinum-based doublet chemotherapy (1,2,5). However, drug resistance eventually develops, which requires subsequent treatment with third-generation EGFR TKIs or chemotherapy. Approximately 50% of patients with an EGFR-sensitizing mutation develop a secondary mutation in EGFR at position 790 (T790M), which impairs the binding of TKIs to the EGFR kinase domain, leading to EGFR-TKI resistance (6). EGFR-TKI resistance may result in a median progression-free survival (PFS) of less than 1 year (3,4). Osimertinib, a 3rd generation irreversible EGFR-TKI designed to overcome such resistance, has been approved by the US Food and Drug Administration for the treatment of metastatic EGFR^{T790M+} NSCLC patients who have experienced disease progression from prior EGFR-TKI therapy (7). EGFR T790M testing has become a standard procedure when patients develop resistance to first- or second-generation EGFR-TKIs (8).

Due to patients' unwillingness to undergo re-biopsy, or risk of biopsy complications (included pneumothorax and pulmonary hemorrhage), obtaining tissue samples remains challenging (9). Thus, minimally invasive liquid biopsy, which captures circulating biomarkers in patient fluid samples such as plasma, urine, and saliva using Cobas, digital PCR (ddPCR), or next-generation sequencing (NGS), has rapidly developed as an alternative for tissue biopsy in recent years (10-12). A companion study of the AURA2 trial reported that patients with EGFR^{T790M+} plasma, identified by BEAMing ddPCR, may achieve comparable ORRs to osimertinib compared to those with EGFR^{T790M+} tumor tissues (13), suggesting that liquid biopsy might be a reliable substitute for tumor rebiopsy. Certain patients with EGFR^{T790M+} plasma are unresponsive to osimertinib therapy possibly due to a low allelic frequency (AF) of plasma EGFR T790M (13). However, the abundance of T790M does not appear to be a predictive factor of the response to osimertinib (14), as it is also significantly associated with circulating tumor DNA (ctDNA) levels which vary markedly among individuals and is influenced by multiple

factors (15). Therefore, osimertinib treatment response may be correlated with the relative allelic frequency (RAF) of EGFR T790M, calculated as the ratio of the AF of T790M to the AF of the sensitizing mutation (16). Previous studies have shown that multiple resistant subclones, in addition to T790M, can arise simultaneously following EGFR-TKI exposure in approximately 50% of patients in whom targeting T790M alone is ineffective (10,11). Some studies have proposed the RAF of T790M as a predictor of patient response to osimertinib or another 3rd generation TKI, rociletinib (17-19). A lower ratio of the allele fraction for EGFR T790M to activating EGFR mutations was reported in NSCLC that associated with lower efficacy of osimertinib in subsequent lines of treatment using tissues (20). However, these studies did not elucidate which subgroup of patients with T790M positive plasma are less responsive to 3rd generation TKIs, and what is the concurrent resistance mechanisms.

In this study, we investigated which subgroup of patients, classified by T790M RAF, might have concurrent resistance mechanisms in addition to T790M and poor response to osimertinib. A cutoff value of T790M RAF was established for the prediction of the coexistence of T790M and additional alterations in predicting response to osimertinib, highlighting NGS-determined plasma T790M RAF as a potential prognostic predictor for patients with progressive NSCLC.

We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/tlcr-20-915>).

Methods

Patients and study design

This study was approved by the Institutional Review Board of West China hospital, Sichuan University, Chengdu, China. A written informed consent was provided by each patient. This study was registered on <http://www.chictr.org.cn> (registration number: ChiCTR-DDD-16007900). All procedures performed in studies involving human participants were in accordance with the Helsinki declaration (as revised in 2013).

Two independent cohorts of patients with NSCLC, a discovery cohort (n=32) and a validation cohort (n=21), admitted to West China Hospital from August 2015 to April 2017 were included in this study. In the discovery cohort, patients with progressive NSCLC post EGFR-

TKI resistance were included if both the repeat biopsy tissue and blood samples were obtained concurrently. The genomic profiles derived from tissue (conventional methods and/or NGS) and plasma (NGS) were compared to find which subgroup of patients carried concurrent resistance mechanisms in addition to T790M. In the validation cohort, patients resistant to first-line EGFR-TKIs were included if they were positive for T790M, determined using an NGS-based plasma assay, and treated with osimertinib. We tested whether patients with concurrent resistance mechanisms and T790M RAF had poor response rates to osimertinib.

Tumor tissue DNA extraction and driver gene testing

DNA was extracted from tissue samples using a QIAamp DNA FFPE tissue kit (Qiagen, CA, USA) according to the manufacturer's instructions. DNA concentration was measured using a Qubit dsDNA assay kit (Life Technologies, Carlsbad, CA, USA). ARMS-PCR was performed to detect EGFR-sensitizing mutations and T790M. This was carried out using Amplification Refractory Mutation System technology as the ADx EGFR Mutations Detection Kit (Amoy Diagnostics, China) had been approved for clinical application by the State Food and Drug Administration in China. Fluorescence *in situ* hybridization (FISH) was performed to assess EGFR amplification and MET amplification as previously described (20-23).

Preparation of plasma cell-free DNA (cfDNA)

Whole blood (10 mL) was collected in K3EDTA-containing and cfDNA blood collection tubes (Streck, La Vista, Nebraska, USA) and centrifuged at 2,000 g for 10 minutes at 4 °C within 72 hours after collection. The supernatant was transferred to a fresh 15 mL centrifuge tube without disturbing the buffy coat layer, and was subjected to an additional centrifugation at 16,000 g for 10 minutes at 4 °C. The supernatant was then transferred to a new tube and stored at -80 °C until further analysis. Circulating cfDNA was extracted from the plasma samples using a QIAamp circulating nucleic acid kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. Quantification of cfDNA was performed using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California, USA) and dsDNA HS assay kits (Life Technologies).

NGS library preparation and capture-based targeted DNA sequencing

DNA shearing was performed using a Covaris M220, followed by end repair, phosphorylation and adaptor ligation. DNA concentration and genomic DNA quality were measured using a Qubit dsDNA assay and 260 nm/280 nm absorption ratios, respectively. Fragments of size 200–400 bp were selected using Agencourt AMPure XP beads (Beckman Coulter, CA, USA) followed by hybridization with capture probes baits, hybrid selection with magnetic beads and PCR amplification. A bioanalyzer high-sensitivity DNA assay was then performed to assess the quality and size of the fragments, and indexed samples were sequenced on a Nextseq500 sequencer (Illumina, CA, USA) with pair-end reads.

Sequence data analysis

Sequence data were mapped to the human genome (hg19) using the BWA aligner 0.7.10. Local alignment optimization, variant calling and annotation were performed using GATK 3.2, MuTect, and VarScan, respectively. Plasma samples were compared against their own white blood cells to identify somatic variants. Variants were filtered using the VarScan ffilter pipeline, and loci with depth less than 100 were filtered out. At least 2 and 5 supporting reads were needed for INDELs in plasma and tissue samples, respectively, while 8 supporting reads were needed for SNVs to be called in both plasma and tissue samples. According to the ExAC, 1,000 Genomes, dbSNP, ESP6500SI-V2 databases, variants with population frequency over 0.1% were grouped as SNPs and excluded from further analysis. Remaining variants were annotated with ANNOVAR and SnpEff v3.6. DNA translocation analysis was performed using Tophat2 and Factera.

Statistical analysis

Continuous data were presented as mean ± standard deviation (normal distribution) or median, range (skewed distribution), while categorical data were presented as frequency and percentage. Comparisons of response rates and disease control rates (DCRs) were conducted using the continuity correction method. Progression free survival rates were estimated using the Kaplan-Meier method. A

Table 1 Patient demographic and clinical characteristics

Characteristics	Discovery cohort (n=32)	Validation cohort (n=21)
Mean age [range] years	58.5 [27–79]	60.4 [44–83]
Gender		
Male	12	5
Female	20	16
EGFR-sensitizing mutation		
Exon 19 deletion	14	10
L858R	18	11
Median PFS 1	9	13
Rebiopsy origin		
Tumor	32	12
Plasma	32	21
Progression type		
Symptomatic	14	7
Asymptomatic	18	14
Treatment regimen		
Gefitinib	22	14
Erlotinib	3	1
Icotinib	7	6

PFS, progression free survival; EGFR, epidermal growth factor receptor.

value of $P < 0.05$ was considered statistically significant. Statistical analysis was carried out using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

The demographic and clinical characteristics of the two cohorts are shown in *Table 1*. In the discovery cohort, 32 patients (12 males and 20 females; mean age = 58.5 years) harboring EGFR sensitizing mutations 19del (n=14) or L858R (n=18) with both re-biopsy tumor and ctDNA genomic profiling were included. All patients were treated with the first-generation TKIs gefitinib (n=22), erlotinib (n=3), or icotinib (n=7). The median PFS of the 1st generation TKIs in this cohort was 9 months. Among the discovery cohort, 14 patients had symptomatic progression and the remaining 18 patients had asymptomatic progression. In the validation cohort, 21 patients (5

males and 16 females; mean age =60.4 years) harboring 19del (n=10) or L858R (n=11) and resistant to TKIs were included. All of them had T790M positive plasma as determined by NGS, and were treated with osimertinib at the time of progression, and 12 patients were subjected to re-biopsy. The median PFS of 1st generation TKIs in this cohort was 13 months.

Concordance and heterogeneity observed in the mutation spectrum between tissue and plasma

To evaluate the sensitivity and reliability of genomic mutation detection, we performed ARMS-PCR for EGFR mutations on re-biopsy tissue samples from 32 TKI-treated progressive NSCLC patients, and performed capture-based targeted deep-sequencing on matched plasma and tissue samples obtained from 30 patients. Two patients (P22 and P31) who had inadequate tissue samples for NGS were excluded. EGFR and MET amplifications were successfully evaluated by FISH in 28 patients (*Figure S1*). For EGFR

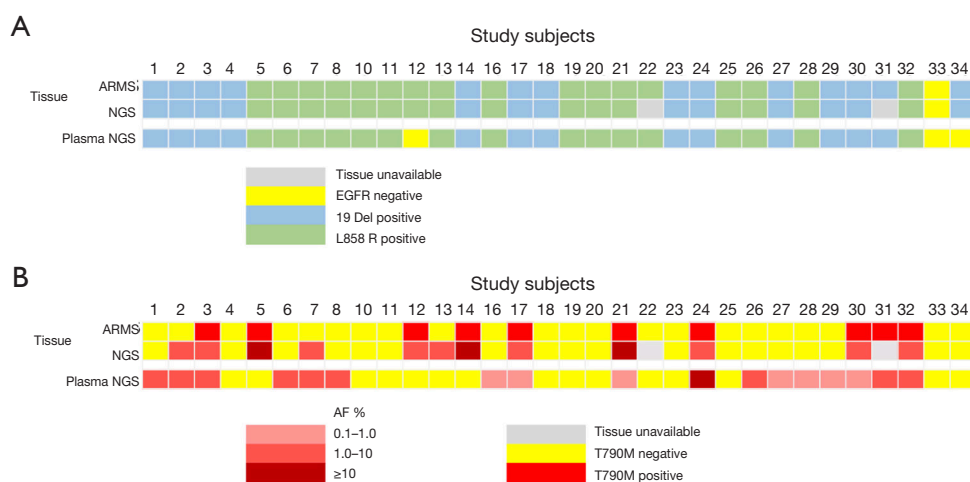


Figure 1 EGFR T790M and sensitizing mutations in paired tissue and plasma samples from patients. EGFR-sensitizing mutations [19Del and L858R (A)] and T790M (B) were detected in paired plasma and tissue samples (n=34) obtained from patients with progressive NSCLC post first-generation TKI therapy using ARMS-PCR and NGS. By-variant comparison was performed. Two patients (P22 and P31) with inadequate tissue samples for NGS were excluded from the comparison. AF, allele frequency; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor; ARMS-PCR, amplification refractory mutation system-polymerase chain reaction; NGS, next-generation sequencing.

sensitizing mutations and T790M in tissue samples, the concordance rates between ARMS-PCR and NGS were 100% and 87%, respectively. For the 4 discordant tissue samples, NGS detected T790M that was missed by ARMS-PCR. The concordance between tissue and plasma was 93.8% (30/32) for EGFR sensitizing mutations compared to only 62.5% (20/32) for T790M. The detection rates of T790M from ctDNA and tissue biopsy were 53.1% (17/32) and 40.6% (13/32), respectively (Figure 1). These findings suggest that NGS may be more sensitive than ARMS-PCR in capturing T790M in tissue samples, and might detect additional T790M⁺ cases in plasma that are missing in tissue samples.

In addition, 130 genetic aberrations involving 27 genes were identified from 30 tissue biopsy samples which were adequate for NGS (Figure S2A). In these aberrations, *MET* amplification, another resistance mechanism to EGFR-TKI, was observed in 25.8% (8/31) patients in combination with other mutations (Figure S2A). Other frequently occurring mutations included, but were not limited to, TP53, RB1, and PTEN in 59%, 19% and 13% patients, respectively. Furthermore, 135 genetic variants involving 36 genes were identified in plasma samples (Figure S2B). Using the mutation spectrum derived from tissue samples as a

reference, we achieved 78% sensitivity excluding CNVs.

Patients with a low RAF of EGFR T790M are more likely to harbor concomitant resistance mechanisms

In the discovery cohort, 59% patients (20/34) harbored EGFR T790M, of whom 60% (12/20) harbored additional resistance mechanisms (Figure 2A). As shown in Figure 2B, the resistance mechanisms in 25 patients (81%) were identified, whereas those in the remaining 19% patients (6/31) were unidentified. EGFR T790M was found in the majority of patients (20/31) either as a solo resistance mechanism or in combination with others. *MET* and *ERBB2* amplification were the other two mechanisms that existed as solo resistance mechanisms. In total, 42% (13/31) of patients had solo resistance mechanisms, including EGFR T790M (n=9), *MET* amplification (n=3) and *ERBB2* amplification (n=1), whereas 39% (12/31) of patients harbored concomitant resistance mechanisms. We next sought to investigate the association between T790M and the presence of concomitant resistance mechanisms. The RAF (ranging from 1.3% to 100%) was plotted in descending order along with concomitant resistance mechanisms. As shown in Figure 2A, 12 patients harbored

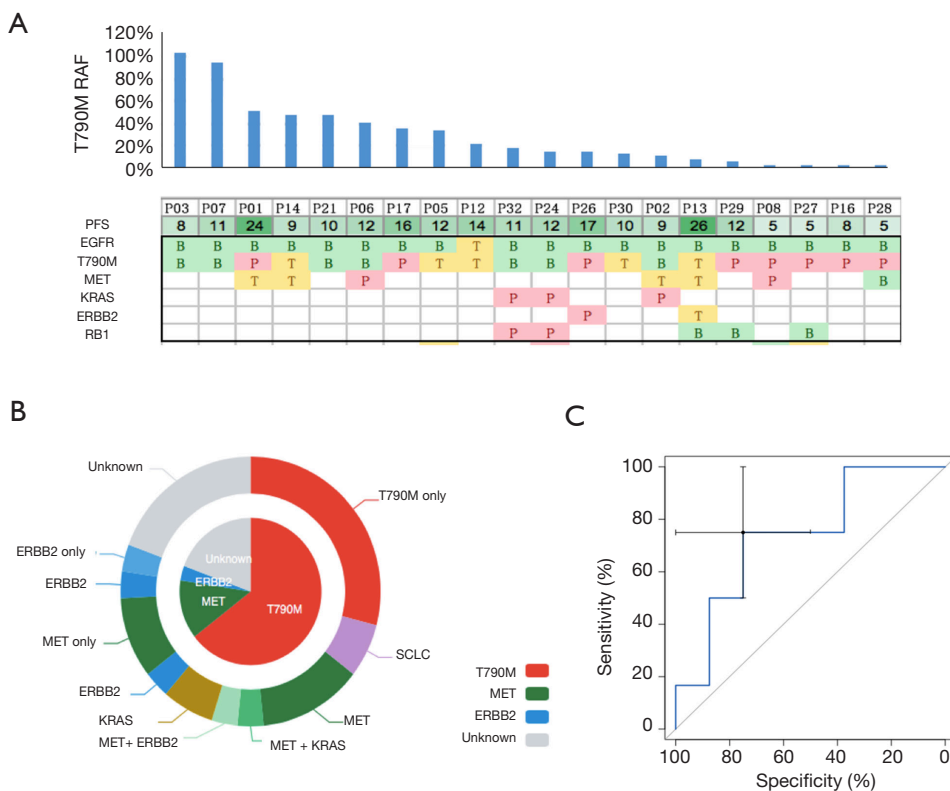


Figure 2 Correlation between EGFR T790M RAF and additional genetic alterations related to TKI-resistance. (A) RAFs of EGFR T790M were plotted in descending order along with representative identified genetic alterations (MET, KRAS, ERBB2, and RB1). (B) A pie chart showing the proportion of NGS-detected genetic alterations in patient plasma samples. Both single (inner pie) and multiple (outer pie) alterations were detected. (C) A ROC analysis was generated to evaluate the RAF threshold in predicting the coexistence of T790M with other alterations. EGFR, epidermal growth factor receptor; RAF, relative allele frequency; TKI, tyrosine kinase inhibitor; NGS, next-generation sequencing; ROC, receiver operating characteristic curve.

concomitant resistance mechanisms in addition to EGFR T790M, among whom 9 had an RAF less than 20%.

To evaluate whether 20% T790M RAF may serve as an optimal cutoff in distinguishing populations who are more likely to harbor concomitant resistance mechanisms, we conducted a receiver operating characteristic (ROC) curve analysis of the presence of concomitant resistance mechanisms against EGFR T790M RAF. As shown in *Figure 2C*, the area under the curve was 74%. Using 20% as a cutoff, patients harboring low RAF (<20%) were more likely to have concomitant resistance mechanisms in addition to T790M (P=0.018), suggesting that RAF (<20%) can be used as a marker to distinguish populations carrying additional genetic alterations other than T790M, and

theoretically, having poorer response rates to osimertinib.

T790M RAF predicted poorer osimertinib response

To explore whether T790M RAF can potentially predict osimertinib response, we recruited an independent cohort consisting of 21 EGFR T790M-positive NSCLC patients who experienced progression from previous 1st generation EGFR-TKI therapy and received osimertinib treatment. Among these patients, 11 experienced partial response (PR), 8 stable disease (SD) and 2 progressive disease (PD). *Figure 3A* demonstrates that absolute T790M AF is not an ideal marker to predict osimertinib efficacy. Patients with T790M AF less than 1% achieved an ORR of 41.5% and a DCR of

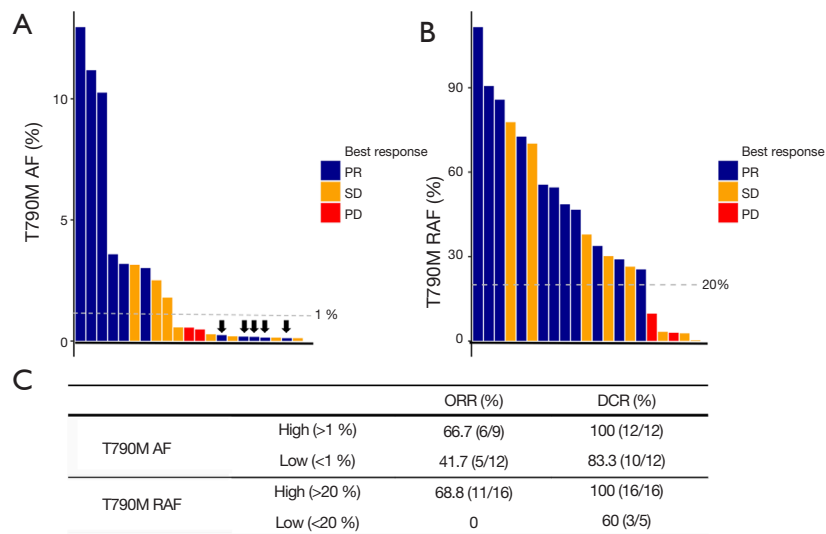


Figure 3 Correlation between T790M RAF/AF and ORRs and DCRs of patients treated with osimertinib. An independent cohort including 21 patients with T790M+ NSCLC receiving osimertinib treatment were recruited. AFs (A) or RAFs (B) of EGFR T790M were plotted in descending order against the response of each patient. The cut-off values of AF and RAF were 1% and 20%, respectively. Of these 21 patients, 11 patients had partial response, 8 stable disease, and 2 progressive disease. (C) ORR was calculated as the percentage of patients achieving PR. DCR was the percentage of patients achieving PR and SD. PR, partial response; SD, stable disease; PD, progressive disease; ORRs, objective response rates; DCRs, disease control rates; AF, allele frequency; RAF, relative allele frequency; NSCLC, non-small cell lung cancer.

83.3%, with no significant differences compared to patients with T790M AF higher than 1%. Using T790M RAFs as a biomarker, *Figure 3B* shows that patients with low T790M RAF achieved 0% ORR and only 60% DCR, significantly lower than those with high T790M RAF ($P=0.012$ and $P=0.048$, respectively), suggesting that low T790M RAF is likely a contraindication for osimertinib treatment. The ORR and DCR of osimertinib for patients classified by T790M AF and RAF are listed in *Figure 3C*.

We further evaluated tumor size changes in this cohort of patients. As shown in *Figure 4*, among the 16 patients with T790M RAF greater than 20%, 14 of them did not experience tumor growth during treatment. Furthermore, tumor size reductions were observed in all patients except 1 at certain points during treatment, suggesting a sensitive response to osimertinib in this group. Contrastingly, among the 5 patients with T790M RAF less than 20%, 2 exhibited continuous tumor growth and 3 experienced a short period of tumor reduction followed by steady tumor growth, which indicates a poor response to osimertinib in this group. These findings further support the positive correlation

between T790M RAF and osimertinib response.

Low T790M RAF indicated poor PFS with osimertinib treatment

We next sought to investigate the correlation between T790M RAF and PFS in the validation cohort by performing a Kaplan-Meier survival analysis. As shown in *Figure 5*, disease progression occurred in all 5 patients with low T790M RAF with a median PFS of 5 months. The median PFS of patients with high T790M RAF was not reached at the end of follow-up, which was markedly longer than the median PFS of patients with low RAF ($P=0.003$, HR =11.37, 95% CI: 2.28–56.59). Taken together, these data suggest that low T790M RAF is an indicator of poor osimertinib efficacy in this population of patients.

Discussion

In the present study, we demonstrated that NGS of liquid biopsy samples can effectively detect the mutation spectrum

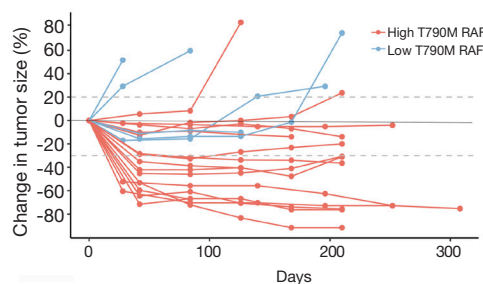


Figure 4 Correlation between T790M RAF and tumor size changes in response to osimertinib. Twenty-one patients with T790M+ NSCLC receiving osimertinib treatment were divided into high RAF ($\geq 20\%$) and low RAF ($< 20\%$) groups and were monitored for tumor size changes from the beginning of treatment. The changes in tumor size (%) were plotted against the duration of treatment. Red and blue dot/lines indicate each patient with high and low RAF, respectively. RAF, relative allele frequency; NSCLC, non-small cell lung cancer.

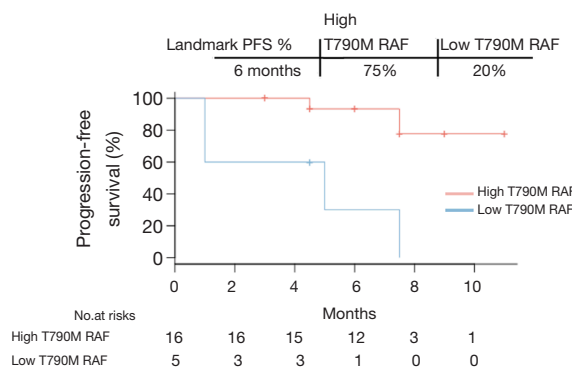


Figure 5 Kaplan-Meier survival analysis. The correlation between T790M RAF and the survival of patients receiving osimertinib was evaluated using the Kaplan-Meier method. Twenty-one patients with T790M+ NSCLC receiving osimertinib treatment were divided into high RAF ($\geq 20\%$) and low RAF ($< 20\%$) groups and followed up 10 months after the start of osimertinib therapy. The progression free survival (%) was plotted against the time of follow-up. The 6-month PFS rate was 20% in patients with low RAF, compared to 75% in high RAF patients (HR: 11.37, 2.28–56.69, $P=0.003$). RAF, relative allele frequency; NSCLC, non-small cell lung cancer.

of solid tumors, providing an alternative approach to tissue-based gene profiling in revealing the mechanisms underlying drug resistance to NSCLC. Our results showed that genotyping in matched rebiopsy tissue and plasma samples achieved 93.8% concordance for EGFR sensitizing mutations and 62.5% for T790M. In the discovery cohort, patients with low plasma T790M RAFs ($< 20\%$) were more likely to carry concurrent resistance mechanisms. In the independent validation cohort, patients with low plasma T790M RAFs exhibited a poorer response to osimertinib than those with high plasma T790M, suggesting that unresponsiveness to osimertinib may be due to other resistance mechanisms in addition to T790M. Thus, T790M RAF may represent a prognostic stratification in

addition to the current binary assessment of T790M status (positive *vs.* negative) for patients treated with 3rd generation TKIs (24).

NGS-based liquid biopsy is a well-established alternative approach to tissue rebiopsy (10-12). In our study, the results of NGS-detected EGFR sensitizing mutations between matched tissue and plasma samples were highly concordant (93.8%). The by-variant sensitivity was 78% excluding CNVs and the by-patient sensitivity was 93.5%, consistent with previous studies (25,26). This suggests that NGS-based liquid biopsy is a reliable approach for the identification of genetic alterations. In addition, the concordance rate for NGS-detected T790M between tissue and plasma samples was 62.5% because more T790M

cases were captured by NGS in plasma samples than in tissues samples. This suggests that NGS-based liquid biopsy is a highly sensitive method for T790M detection, especially when tissue samples are unavailable. Although EGFR T790M is responsible for over 50% of acquired resistance to first-generation EGFR-TKIs (4,27,28), other genes, such as MET, KRAS, ERBB2, and RB1 (20-22,29,30), have been found to be altered simultaneously with T790M, contributing to the underlying mechanism of TKI resistance. Our data showed that 60% of patients harboring T790M carried other resistance mechanisms concurrently, which is partially consistent with previous studies (31-33). Based on the high sensitivity and reliability of NGS in detecting EGFR sensitizing mutations as well as T790M in plasma, RAF of plasma EGFR T790M, could be used to differentiate subgroups of patients harboring different levels of T790M. By analyzing the T790M RAFs of patients who carried solo T790M or in combination with other resistant genomic alterations, we found 20% was an optimal cutoff to distinguish these two subgroups. Patients in the discovery cohort with low plasma T790M (<20%) were more likely to carry concurrent resistance mechanisms in addition to T790M. This cutoff value was further tested in an independent cohort receiving osimertinib therapy. The results showed that patients with low plasma T790M RAF (<20%) had poorer ORR and PFS than those with high RAF ($\geq 20\%$). These data collectively suggest that a poor response to osimertinib in the patients with low plasma T790M RAF is possibly due to the cooperation of multiple resistance mechanisms in these patients. This finding is consistent with several previous studies showing that quantification of T790M mutant alleles can be used to predict outcome after TKI treatment using 50% as a cutoff value (17-19). However, based on our results, we suggest that patients with T790M RAF between 20–50% should still be prescribed osimertinib as some patients could also benefit from osimertinib treatment and achieve PR or SD. Thus, the cutoff value of 50% may be too high to identify all the patients responding positively to osimertinib therapy. Since our study was conducted using a small sample size, the feasibility of a 20% cutoff should be tested in a larger population in future studies.

Clinical studies have shown that osimertinib treatment may achieve approximately 60% ORR in T790M-positive patients (34,35). Patients who do not respond to osimertinib treatment may harbor other resistance mechanisms in addition to T790M, and the percentage of T790M-positive tumor cells is positively correlated with the response to

osimertinib (14-16,34). Our results suggest that NGS of plasma may serve as an effective approach to quantify the percentage of T790M-positive tumor cells in order to predict the response to osimertinib. T790M RAF in plasma may better reflect the proportion of T790M-positive tumor cells compared to rebiopsy tissue due to tumoral heterogeneity. Therefore, NGS of plasma should also be performed even when tissue biopsy is available, and T790M RAF should be taken into consideration before commencing osimertinib treatment. In addition, plasma-based NGS for detection of MET amplification and RB1 mutations may provide information associated with innate and acquired resistance mechanisms. However, due to the limited sample size in this study, a prospective study involving more patients is needed.

In summary, our results suggest that NGS-detected plasma T790M RAF with an optimal cutoff value of 20% is useful for distinguishing subgroups of patients resistant to 1st generation EGFR-TKIs. Patients with low T790M RAF (<20%) are more likely to carry concurrent resistance mechanisms and are less likely to benefit from osimertinib treatment. T790M RAF determination should be considered a potential standard procedure in NSCLC treatment decisions.

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Footnote

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Data Sharing Statement: Available at <http://dx.doi.org/10.21037/tlcr-20-915>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tlcr-20-915>)

[org/10.21037/tlcr-20-915](https://doi.org/10.21037/tlcr-20-915)). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in studies involving human participants were in accordance with the Helsinki declaration (as revised in 2013). This study was approved by the Institutional Review Board of West China hospital, Sichuan University, Chengdu, China. A written informed consent was provided by each patient. This study was registered in <http://www.chictr.org.cn> (register number: ChiCTR-DDD-16007900).

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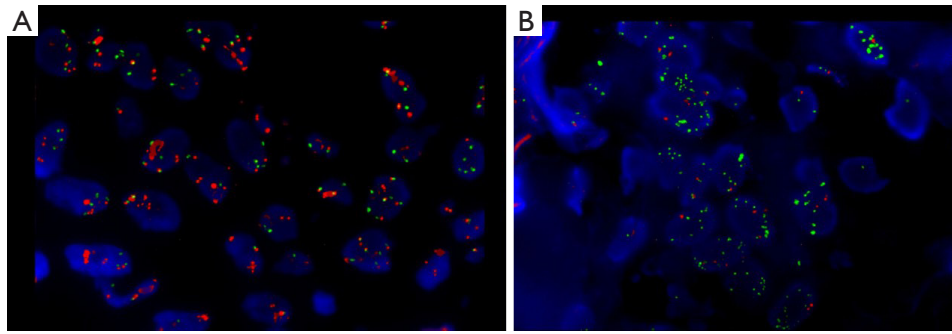


Figure S1 FISH of EGFR amplification and MET amplification: (A) with EGFR probe (red); (B) with MET probe (green). FISH, fluorescence in situ hybridization; EGFR, epidermal growth factor receptor. Magnification, $\times 1,000$.

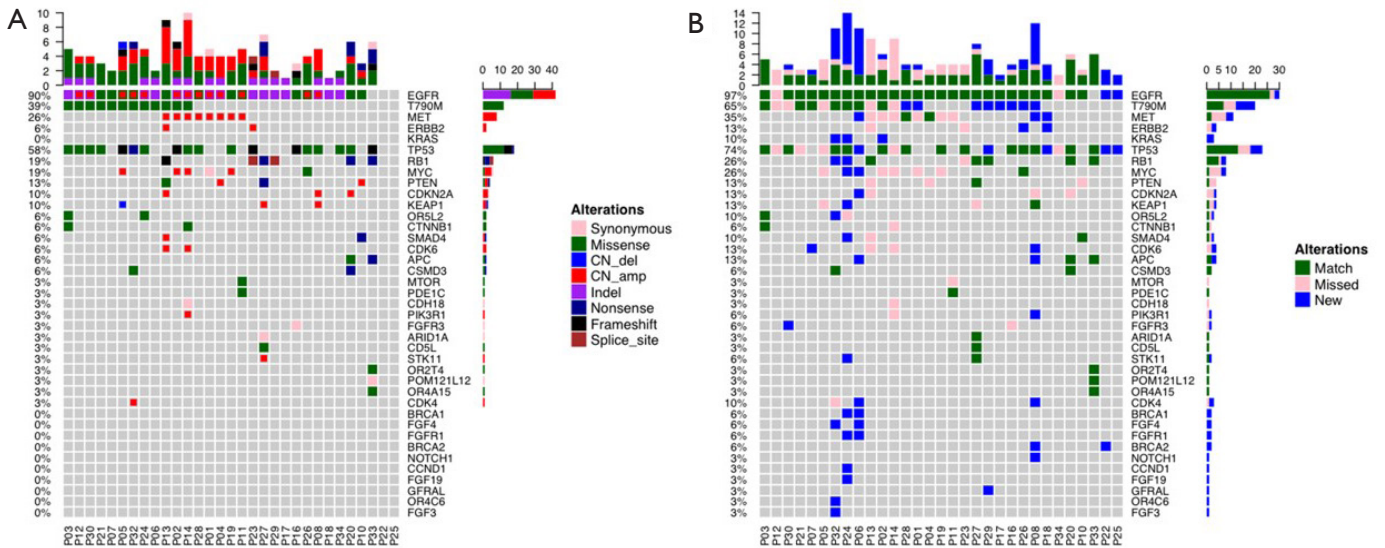


Figure S2 Mutation spectrums of matched tissue and plasma samples. By-variant comparison of somatic mutations detected in tumor tissues (A) and their matching plasma samples (B). Top bar represents the number of mutations in each patient. Left side bar represents the percentage of patients harboring a particular mutation as indicated. (A) Different colors represent different types of mutations. (B) Green represents mutations detectable in both sources, blue represents mutations that are only present in the plasma, and pink represents mutation that are only present in the tissue.