Plasma *EGFR* mutation abundance affects clinical response to first-line EGFR-TKIs in patients with advanced non-small cell lung cancer

Xiaohong Wang[1], Yonggang Liu[1], Zhiying Meng[1], Yun Wu[2], Shubin Wang[2], Gaowa Jin[3], Yingchun Qin[3], Fengyun Wang[4], Jing Wang[4], Haifei Zhou[5], Xiaoxing Su[5], Xiuhua Fu[6], Xiaolan Wang[7], Xiaoyu Shi[8], Zhenping Wen[9], Xiaoqiong Jia[9], Qiong Qin[10], Yongqiang Gao[10], Weidong Guo[11], Shun Lu[12]

[1]Chest Oncology Medicine, Baotou Cancer Hospital, Baotou, China; [2]Department of Oncology, Baotou Central Hospital, Baotou, China; [3]Oncology Division II, The Inner Mongolia Autonomous Region People's Hospital, Hohhot, China; [4]Oncology Department, The Third Affiliated Hospital of Baotou Medical College, Baotou, China; [5]Berry Oncology Corporation, Beijing, China; [6]Department of Respiratory Medicine, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China; [7]Department of Oncology Medicine, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China; [8]Department of Oncology, Bayan Nur Hospital, Bayan Nur, China; [9]Department of Oncology, The Inner Mongolia Cancer Hospital, Hohhot, China; [10]Department of Oncology, The People's Hospital of DaLaTe Banner, Ordos, China; [11]Oncology Department, Baogang Hospital, Baotou, China; [12]Shanghai Lung Cancer Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China

Contributions: (I) Conception and design: S Lu, X Wang; (II) Administrative support: X Wang, S Lu; (III) Provision of study materials or patients: X Wang, Y Liu, Z Meng, Y Wu, S Wang, G Jin, Y Qin, F Wang, J Wang, X Fu, X Wang, X Shi, Z Wen, X Jia, Q Qin, Y Gao, W Guo; (IV) Collection and assembly of data: X Wang, Y Liu, Z Meng, Y Wu, S Wang, G Jin, Y Qin, F Wang, J Wang, H Zhou, X Su, X Fu, X Wang, X Shi, Z Wen, X Jia, Q Qin, Y Gao, W Guo; (V) Data analysis and interpretation: H Zhou, X Su, S Lu, X Wang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Dr. Shun Lu. Shanghai Lung Cancer Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, 241 West Huaihai Road, Xuhui District, Shanghai 200030, China. Email: shunlu@sjtu.edu.cn.

Background: Activated epidermal growth factor receptor (*EGFR*) mutation is the main pathogenic cause of non-small cell lung cancer (NSCLC) in Asia. However, the impact of plasma *EGFR* mutation abundance, especially of the ultra-low abundance of *EGFR* mutation detected by highly sensitive techniques on clinical outcomes of first-line EGFR tyrosine kinase inhibitors (TKIs) for advanced NSCLC patients remains unclear.

Methods: We qualitatively detected baseline *EGFR* status of NSCLC tissues using amplification-refractory mutation system and quantified the plasma abundance of *EGFR* mutations through next-generation sequencing (NGS). Every 8–12 weeks, we performed dynamic detection of plasma mutation abundance and imaging evaluation. We analyzed the association between plasma abundance of *EGFR* sensitizing mutations, tumor size, tumor shrinkage percentage, concomitant *TP53* mutations, and clinical response to TKIs.

Results: This prospective study enrolled 135 patients with advanced NSCLC. The objective response rate (ORR) and disease control rate (DCR) for *EGFR* mutation–positive patients were 50.0% and 87.0%, respectively. When the cutoff value of plasma *EGFR* mutation abundance was 0.1%, the ORRs of TKI-treated patients were significantly different (60.0% for the >0.1% group *vs.* 21.4% for the ≤0.1% group, P=0.028). Median progression-free survival (PFS) was significantly longer for participants with a mutation abundance above 0.1% compared to those with a 0.01–0.1% abundance (log rank, P=0.0115). There was no significant association between plasma abundance of *EGFR* sensitizing mutations and tumor size, tumor shrinkage percentage, or concomitant *TP53* mutations. Cox multivariate analysis demonstrated that plasma mutation abundance was an independent predictive factor for PFS [hazard ratio (HR) 2.41, 95% confidence interval (CI): 1.12–5.20; P=0.025]. We identified 11 participants with the acquired T790M resistance mutation according to serial dynamic plasma samples.

Conclusions: Liquid biopsy screening based on highly sensitive NGS is reliable for detecting drug

resistance and actionable somatic mutations. The plasma abundance of the *EGFR* driver mutation affected clinical response to EGFR-TKIs in advanced NSCLC patients; prolongation of PFS was also observed in patients with an ultra-low abundance of *EGFR* sensitizing mutations.

Introduction

Lung cancer is a leading cause of cancer-related incidence and mortality worldwide (1). Activated epidermal growth factor receptor (EGFR) mutation is the main cause of non-small cell lung cancer (NSCLC) among those of Asian ethnicity (2). It has been shown that EGFR tyrosine kinase inhibitors (TKIs) significantly prolong the progression-free survival (PFS) of NSCLC patients with TKI-sensitive *EGFR* mutations compared with traditional platinum-based doublet chemotherapy (3-6). However, approximately 20–30% of NSCLC patients with sensitive *EGFR* mutations do not respond to EGFR-TKI therapy (primary resistance), or they develop early progressive disease (PD) after treatment initiation (3,4,7).

Previous studies have found that gene polymorphism, concurrent genomic mutations, or tumor size can affect EGFR-TKI efficacy (8-10). Zhou *et al.* first demonstrated that the abundance of *EGFR* mutations could predict the extent of benefit from EGFR-TKI treatment for advanced NSCLC by using qualitative approaches with different sensitivities (11). Their results revealed that patients with a low abundance of *EGFR* mutations benefit more from EGFR-TKI therapy compared to those with wild-type *EGFR*, while patients with a high abundance of *EGFR* mutations benefit more than those with a low abundance of *EGFR* mutations. Later, Li *et al.* quantitatively analyzed the abundance of *EGFR* mutations using an amplification-refractory mutation system (ARMS+) method and corroborated the effect of *EGFR* abundance (12). Meanwhile, Li *et al.* revealed that the PFS of patients with a low abundance of *EGFR* mutations was similar to that of patients with wild-type *EGFR*. Thus, the impact of *EGFR* mutation abundance on clinical outcomes of advanced NSCLC patients, especially those with a very low abundance of *EGFR* mutations (13,14), remains unclear.

Highly sensitive techniques like droplet digital polymerase chain reaction (ddPCR) (15,16), quantitative polymerase chain reaction (qPCR)-Invader (17), and allele-targeted next-generation sequencing (NGS) (18-22) offer alternative methods for plasma *EGFR* mutation analysis, which can lower the detection limit to below 0.1%. In particular, NGS-based circulating tumor DNA (ctDNA) sequencing can detect multiple mutations in parallel fashion and has been increasingly used in clinical practice (23). However, neoplastic tissue biopsy remains the gold standard for tumor genotyping in NSCLC patients. Whether or not naïve NSCLC patients with a low abundance *EGFR* mutation in plasma as detected by highly sensitive techniques should be treated with EGFR-TKIs still requires elucidation.

In this study, we used the circulating single-molecule amplification and resequencing technology (cSMART) method to prospectively evaluate plasma *EGFR* mutation status at baseline and track the dynamic *EGFR* change during TKI therapy. The cSMART assay, a highly-sensitive detection platform based on NGS, is suitable for the quantitation of the *EGFR* mutations (24-26). The primary aim of this study was to further explore the relationship between low abundance plasma *EGFR* mutations as determined by high-sensitivity methods and the therapeutic efficacy of first-generation EGFR-TKIs. The secondary objective was to explore the dynamic changes in mutation status during therapy in relation to clinical outcomes. This study is registered at ClinicalTrials.gov (NCT02980536) in 2016.

We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/atm-20-7155).

Methods

Participants and clinical samples

Newly diagnosed patients with advanced stage lung

adenocarcinoma at 9 participating hospitals in Inner Mongolia, China from November 2016 to December 2018 were recruited into this observational study. All participants were diagnosed pathologically with lung adenocarcinoma according to World Health Organization (WHO) criteria (2015). Clinical disease staging was determined using the tumor, node, metastasis (TNM) staging system of the International Association for the Study of Lung Cancer (version 7). The key inclusion criteria were as follows: initial, nonoperative NSCLC adenocarcinoma patients; stage III and IV; over 18 years old; Eastern Cooperative Oncology Group (ECOG) performance status score (PS) less than 2; clear measurable tumor lesion in the lung according to the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1) (27). Meanwhile, the exclusion criteria were as follows: major organ dysfunction and severe cardiopathy; brain metastasis–related syndrome; bone metastasis–related complications; allergy to TKIs; already received radiotherapy at the site of curative effect observation; receiving allogenic blood transfusion within 14 days of recruitment.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by institutional ethics committee of Baotou Cancer Hospital, Inner Mongolia, China. Written informed consent was taken from all individual participants included in the study. Participant information on sex, age, smoking history, *EGFR* mutational sites and mutation abundance, and treatment with first-generation first-line EGFR-TKIs or chemotherapy was collected.

Tissue and plasma mutation assays

Mutation detections in tissue and plasma were determined by the gold standard ARMS-PCR assay and cSMART assay, respectively. Tumor tissue specimens at baseline were prepared as formalin-fixed, paraffin-embedded (FFPE) sections and DNA was extracted with QIAamp DNA FFPE kit (Qiagen, Hilden, Germany). The DNA from FFPE tissue was analyzed for *EGFR* mutations in exon 18 to 21 by ARMS method using the commercially available AmoyDx kit (Amoy Diagnostics Co., Ltd., Xiamen, Fujian, China). The plasma DNA was extracted according to the manufacturer's protocol formulated for MagMAX™ Cell-free DNA Isolation Kit (Applied Biosystems, Foster City, CA, USA). The cSMART assay, designed specifically for detection of hot spot oncogenic mutations in plasma, was performed as previously described (24-26). The known

mutations specifically targeted in this study were *EGFR* mutations, including G719X (3 variants), exon 19 deletions (16 variants), exon 20 insertions (3 variants), S768I, T790M, C797S, and exon 21 point mutations, L858R and L861Q; Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations, including G12X (6 variants), G13D, Q61X (6 variants), and A146X (3 variants); *ERBB2* mutations, including exon 20 insertions (5 variants); *BRAF* mutations, including V600X (4 variants); *PIK3CA* mutations, including R88Q, E542K, E545K/D, and A1047R/L; *TP53* mutations, including R175C/H, R248W/Q, and R273C/H; and MET exon 14 skipping. Apart from *EGFR* mutations, mutations of *KRAS*, *ERBB2*, *BRAF*, *TP53*, *PIK3CA*, *ROS1*, *cMET*, and *ALK* genes were specifically targeted in the panel of the present study (28). The cSMART assay was also designed for detection of novel mutations within the nearby region picked up by the primers. The mutation abundance was defined as mutant allele read numbers over total allele read numbers.

Treatment and evaluation of objective response rate (ORR) and PFS

At baseline, all participants underwent physical examination, laboratory tests, and computed tomography (CT) scans of the chest and abdomen 1 week before treatment. Brain magnetic resonance imaging (MRI) was required only in participants with known or suspected brain metastasis.

Participants harboring sensitizing mutations detected by ARMS or cSMART at baseline received gefitinib (250 mg QD), icotinib (125 mg TID) or afatinib (40 mg BID) as first-line therapy throughout the course of the disease until disease progression, intolerable toxicity, participant refusal, or death. Dynamic changes of mutations in peripheral blood were detected every 8–12 weeks by cSMART assay. The participants without *EGFR* and other targeted gene mutations received a platinum-based first-line combined chemotherapy regimen, monotherapy S-1 (tegafur, gimeracil, or oteracil potassium) (40 mg BID), or combined therapy with bevacizumab monoclonal antibody according to guidelines. Those with *ALK* mutations were treated with crizotinib (250 mg BID).

The objective tumor response was evaluated every 8–12 weeks during treatment by investigators according to the stipulations of RECIST (version 1.1) (27). Tumor shrinkage was expressed as a relative change of the sum of the longest diameters of the target lesions based on computed tomography (CT) examination. Nontarget
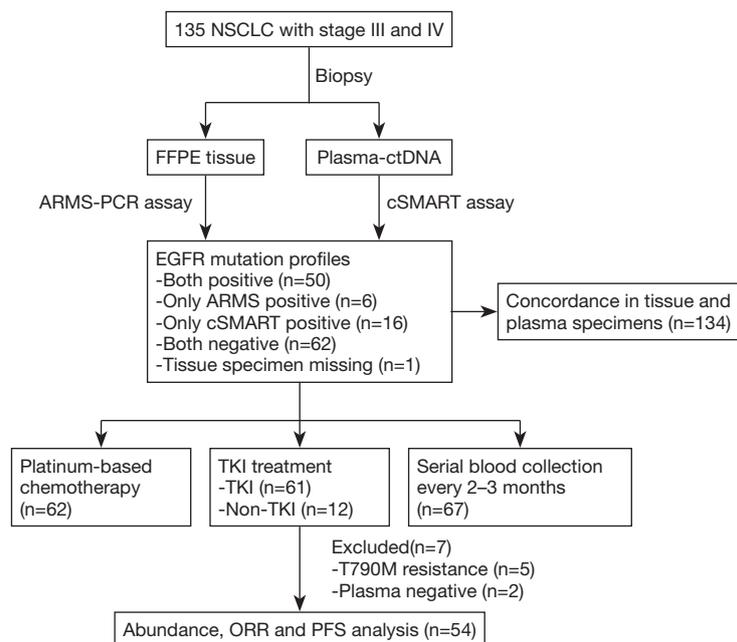
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                          ┌─────────────────────────────┐
                          │ 135 NSCLC with stage III and IV │
                          └─────────────────────────────┘
                                        │ Biopsy
                          ┌─────────────┴─────────────┐
                  ┌──────────────┐            ┌──────────────┐
                  │  FFPE tissue │            │ Plasma-ctDNA │
                  └──────────────┘            └──────────────┘
                   │ ARMS-PCR assay            │ cSMART assay
                   └────────────┬──────────────┘
              ┌────────────────────────────────────┐
              │ EGFR mutation profiles             │          ┌─────────────────────────┐
              │ -Both positive (n=50)              │          │ Concordance in tissue and │
              │ -Only ARMS positive (n=6)          │─────────▶│ plasma specimens (n=134)  │
              │ -Only cSMART positive (n=16)       │          └─────────────────────────┘
              │ -Both negative (n=62)              │
              │ -Tissue specimen missing (n=1)     │
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Figure 1 flowchart:

- 135 NSCLC with stage III and IV → Biopsy
  - FFPE tissue → ARMS-PCR assay
  - Plasma-ctDNA → cSMART assay
- EGFR mutation profiles
  - Both positive (n=50)
  - Only ARMS positive (n=6)
  - Only cSMART positive (n=16)
  - Both negative (n=62)
  - Tissue specimen missing (n=1)
  → Concordance in tissue and plasma specimens (n=134)
- Platinum-based chemotherapy (n=62)
- TKI treatment
  - TKI (n=61)
  - Non-TKI (n=12)
- Serial blood collection every 2–3 months (n=67)
- Excluded (n=7)
  - T790M resistance (n=5)
  - Plasma negative (n=2)
- Abundance, ORR and PFS analysis (n=54)

Figure 1 Patient enrolment and study overview. ORR, objective response rate; PFS, progression-free survival.

lesions and newly occurring lesions were not considered in the measurement of change in tumor size. The therapeutic effect of EGFR-TKI was evaluated as complete response (CR), disappearance of all target lesions; partial response (PR), with at least a 30% decrease in the sum of the longest diameter of target lesions with the baseline sum of the longest diameter as the reference; PD, with an increase of at least 20% from the baseline sum of the longest diameter or the presence of 1 or more new lesions; stable disease (SD), with less than 20% increase or less than 30% decrease in the size of target tumor lesions. The ORR was defined as achieving either CR or PR. The disease control rate (DCR) included CR, PR, and SD. The primary endpoint was PFS, which was defined as the time from initiation of treatment to disease progression according to RECIST criteria or death resulting from any cause, whichever occurred first.

Statistical analyses

The PFS was analyzed by Kaplan-Meier plots and the log-rank test was used to calculate the significance of the difference between groups. Participants with no disease progression or those who were lost to follow-up were censored at the last date of disease assessment for PFS or the last follow-up time. Multivariate Cox proportional hazards regression models were used to evaluate the

association between independent prognosis factors (age, sex, *EGFR* mutation abundance etc.) and PFS. A receiver operating characteristic (ROC) curve analysis was used to determine the optimal cutoff value of mutation abundance, and the area under the curve (AUC) was calculated.

The ORR, DCR, and relationship between *EGFR* mutation abundance and clinical characteristics were calculated by Pearson's chi-square test or Fisher's exact test. Continuous variables were compared using the Mann-Whitney U test.

Data were analyzed using SPSS version 17.0 (IBM Corp., Armonk, NY, USA) or GraphPad Prism 8.1 (GraphPad Software, San Diego, CA, USA). The survival curve was drawn using GraphPad Prism version 8.1. A two-sided P value <0.05 was considered statistically significant. The last follow-up date was January 6, 2019.

Results

Patients characteristics and EGFR mutation status

The outline of the study is diagrammed in *Figure 1*. A total of 135 newly diagnosed advanced NSCLC patients were recruited as the study cohort. Tumor specimens of all but 1 participant were collected at baseline via tissue biopsy and detected by ARMS method. Meanwhile, 135

Table 1 Baseline demographic and clinical characteristics

Characteristics	Patients (n=54)	Percentage
Age in years		
≤65	35	64.8
>65	19	35.2
Gender		
Male	25	46.3
Female	29	53.7
T stage		
T1-2	34	63.0
T3-4	20	37.0
N stage		
N0-1	29	53.7
N2-3	25	46.3
ECOG PS		
0 or 1	42	77.8
>1	12	22.2
EGFR mutations		
Exon 19 deletion	20	37.0
Exon 21 L858R	18	33.4
Uncommon mutations	16	29.6
Smoking status		
Never-smoker	43	79.6
Smoker	11	20.4

plasma samples were collected and analyzed with cSMART array. Overall, there were 134 patients who provided both tumor tissue and matched blood samples at baseline. The 1 participant without a tissue specimen collected had the *EGFR* exon 19 deletion (19Del) mutation in plasma. Of the 135 participants, 58 (43%) were female, and 66 (49%) were over 65 years of age. Totals of 56 (41.8%) and 66 (49.3%) participants were confirmed to harbor *EGFR* mutation in tumor tissues and plasma, respectively. A total of 6 participants were *EGFR* mutation positive in tissue but negative in their matched plasma (ARMS+/cSMART–), whereas 16 cases showed the opposite (ARMS–/cSMART+). In total, 50 participants were *EGFR* mutation positive in both tissue and plasma (ARMS+/cSMART+), 22 were *EGFR* mutation positive in either tissue or plasma (ARMS+/

cSMART–, ARMS–/cSMART+), and 62 were wild-type *EGFR* (ARMS–/cSMART–). The overall, positive, and negative concordance rate of *EGFR* mutation status between tissue and plasma at baseline was 83.6% (112/134), 89.3% (50/56), and 79.5% (62/78), respectively. Among the 73 participants with positive *EGFR* mutations, 12 did not receive first-generation EGFR-TKI therapy, and another 7 either harbored a resistance mutation or were *EGFR* negative in plasma. Eventually, 54 participants who had *EGFR* sensitizing mutations and received first-generation EGFR-TKI therapy as first-line treatment were further assessed for prognosis and clinical outcomes. The demographics and clinical characteristics of the 54 *EGFR* mutation-positive participants are summarized in *Table 1*. Briefly, 35.2% of these participants were over 65 years old, 53.7% were female, 77.8% had an ECOG PS 0 or 1, 63.0% were T1–T2 stage, 53.7% were N0–N1, 20.4% were former or current smokers, and 37.0% carried an 19Del mutation.

The most prevalent *EGFR* mutations were exon19 non-frameshift deletion (24/72, 33.3% in tissue; 22/73, 31.5% in plasma) and *EGFR* L858R mutation (23/72, 31.9% in tissue; 20/73, 27.4% in plasma). The less prevalent mutations were *EGFR* G719X variants (3 tissue and 16 blood samples), S768I mutation [6, 6], L861Q mutation [2, 3], exon 20 insertion [1, 4], and resistance mutation T790M [1, 6]. Complex *EGFR* mutations were found in 4 tissue and 13 blood samples. The overall concordance rate between tissue and plasma was very high for 19Dels (97.0%, 130/134), L858R (96.3%,129/134), and S768I (100.0%, 6/6), but quite low for uncommon mutations, including G719X (18.8%, 3/16), T790M (16.7%, 1/6), and exon 20 insertions (0/5).

Among the 9 genes assayed in plasma by cSMART panel, the most common mutated genes in the *EGFR* mutation–positive cohort were *TP53* (47.9%, 35/73), followed by *KRAS* (11.0%, 8/73), *ALK* (11.0%, 8/73), and *PIK3CA* (9.6%, 7/73). The comutation of *ERBB2* or *BRAF* with *EGFR* was each found only once (1.4%).

Impact of plasma abundance of EGFR sensitizing mutations and response to EGFR-TKIs

Of the participants with *EGFR* sensitizing mutations in plasma, 27 (50%) had PR to TKIs, 20 (37.4%) had SD and 7 (12.6%) exhibited PD. The overall ORR and DCR were 50.0% and 87.0%, respectively (*Table 2*).

The abundance of *EGFR* sensitizing mutations in plasma

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Wang et al. Plasma *EGFR* mutation abundance and efficacy of EGFR-TKIs

Table 2 EGFR mutation abundance and tumor response

EGFR abundance	PR (%)	SD (%)	PD (%)	ORR (%)	P value	DCR (%)	P value
≤0.0622%	3	5	2	30.00	0.293	80.00	0.601
>0.0622%	24	15	5	54.55		88.64	
≤0.1%	3	9	2	21.43	0.028	85.71	>0.999
>0.1%	24	11	5	60.00		87.50	
≤1.14%	10	13	3	38.46	0.173	88.46	>0.999
>1.14%	17	7	4	60.71		85.71	
≤3.36%	18	17	4	48.72	0.544	89.74	0.382
>3.36%	9	3	3	58.33		80.00	
Overall	27	20	7	50.0		87.0	

varied from 0.02% to 47.76% with a median of 1.14%, as revealed by cSMART assay at baseline. The median abundance for participants with PR was higher than those with SD after EGRF-TKI treatment (1.32% *vs.* 0.36%, P=0.058) (*Figure 2A*), suggesting that the abundance of *EGFR* activating mutations might be associated with the objective response to EGFR-TKIs. Therefore, we used 25th percentile (0.10%), the median (1.14%), and 75th percentile (3.36%) of mutation abundance as the cutoff values to compare the response rates of the high and low abundance groups (*Figure 2B*). The difference in DCR between the high and low abundance groups was not significant with any of the cutoff values, but the difference in ORR between the high and low abundance groups was significant when the cutoff value was at 0.1%, (60.00% *vs.* 21.43%, P=0.028) (*Figure 2C*; *Table 2*). Furthermore, we used ROC analysis to estimate the cutoff values of high and low abundance *EGFR* mutations according to objective response, yielding an AUC of 0.733 [95% confidence interval (CI): 0.604–0.861, P=0.005] (*Figure 2D*). The responding cutoff value of mutation abundance was 0.062%. However, no significant difference in ORR or DCR was found at this cutoff (*Table 2*). Finally, 0.10% was used as the lowest threshold of EGFR-mutant abundance in peripheral blood for efficacy evaluation.

The participants were subdivided into two groups based on the 0.10% abundance value in TKI-naïve plasma samples (high: >0.10%; low: ≤0.10%). There was a significant difference in the PFS duration across the high abundance mutation, low abundance mutation, and wild-type *EGFR* groups (log rank, P<0.0001) (*Figure 3A*). The median PFS after EGFR-TKI treatment in the *EGFR* sensitizing mutation group (8.0 months, 95% CI: 5.0–11.0) was superior to that in the wild-type *EGFR* (2.0 months, 95% CI: 1.6–2.4; log rank P<0.0001) (*Figure 3A*). The median PFS for participants with an *EGFR* mutation abundance above the cutoff (9.5 months, 95% CI: 5.6–13.4) was markedly longer than for those with a low abundance of *EGFR* mutation (5.0 months, 95% CI: 1.3–8.7) (log rank P=0.0115). The PFS of participants with a low abundance of *EGFR* sensitizing mutations was significantly longer than that of those in the *EGFR* wild-type group (log rank P=0.0054). These results showed that participants with a high abundance of *EGFR* mutations in plasma demonstrated a better response to TKIs and longer PFS than those with wild-type *EGFR* or a low abundance of *EGFR* mutations. However, participants with a low abundance of *EGFR* mutations could also gain some benefits from TKI treatment compared to those with wild-type *EGFR* in terms of PFS. No significant difference in PFS was found for participants with 19Del or L858R mutations (*Figure 3B*).

Tumor size change and concomitant mutations were also considered to be related to responsiveness. In order to assess the impact of tumor change and concomitant mutations, we further investigated the relationship between the abundance of *EGFR* sensitizing mutations with baseline tumor size, maximal tumor shrinkage after treatment, and concomitant *TP53* gene mutations. The participants with lower *EGFR* mutation abundance had larger tumor sizes than those with higher *EGFR* mutation abundance [mean ± standard deviation (SD) 52.4±23.7 *vs.* 41.0±19.2 mm)]. However, the difference was not significant (P=0.0797) (*Figure 4A*). The median, 25th, and 75th percentile of the
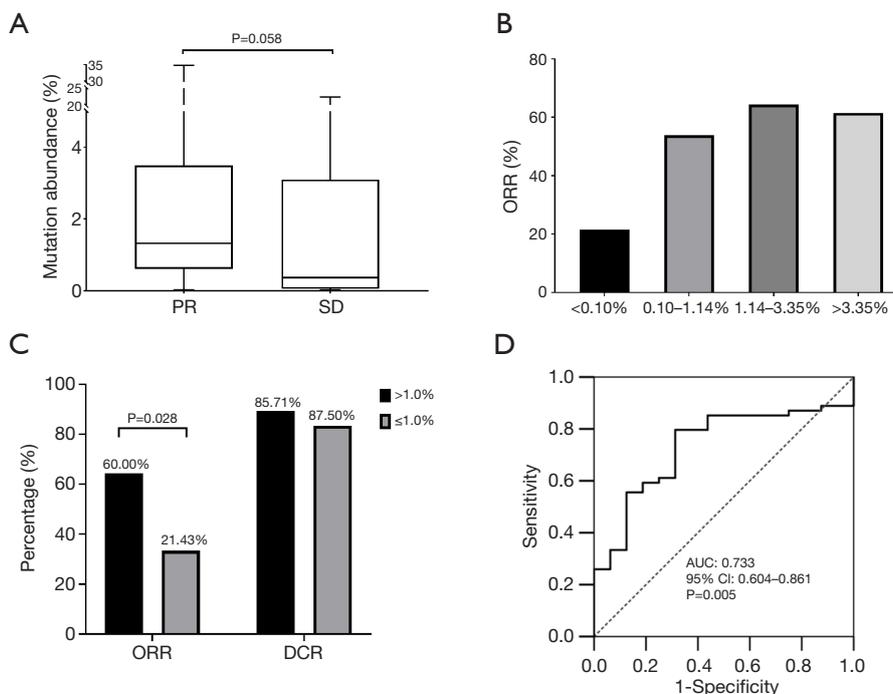
Figure 2 *EGFR* mutation abundance and clinical response. (A) Box plot analysis for mutation abundance in patients with PR or SD. (B) ORR stratified by quantiles of *EGFR* sensitizing mutation abundance in plasma. (C) ORR and DCR of low and high mutation abundance groups. (D) ROC curve and AUC. EGFR, epidermal growth factor receptor; PR, partial response; SD, stable disease; DCR, disease control rate; ORR, objective response rate; ROC, receiver operating characteristic; AUC, area under ROC curve.
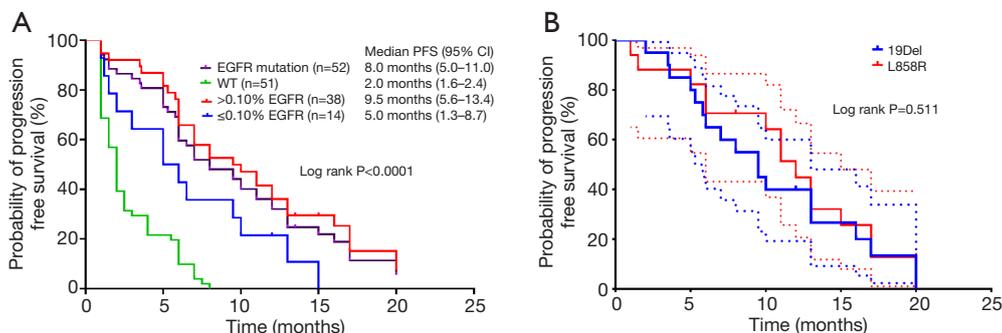
Figure 3 Kaplan-Meier analysis of PFS in patients with (A) high and low mutation abundance or (B) 19Del or L858R mutation (95% CI). The dotted line indicates 95% CI. 19Del, exon 19 deletion; PFS, progression-free survival; CI, confidence interval.

maximal tumor shrinkage (nadir size compared to baseline) was 35.5%, 17.6%, and 50.0%, respectively. Responders were further divided into four groups according to the degree of maximal tumor shrinkage in response: >50%, 35.5–50%, 17.6–35.5%, and <17.6%. No significant association was observed between tumor shrinkage and median PFS (log rank P=0.223) (*Figure 4B*), suggesting that tumor size change was not predictive of PFS in responders.

In addition, there was no significant difference in PFS for participants with or without a concomitant *TP53* mutation (log rank P=0.935) (*Figure 4C*).

Univariate and multivariate analysis of PFS

The results in univariate analysis showed that *EGFR* mutation abundance >0.1% and ECOG PS 0 or 1 were

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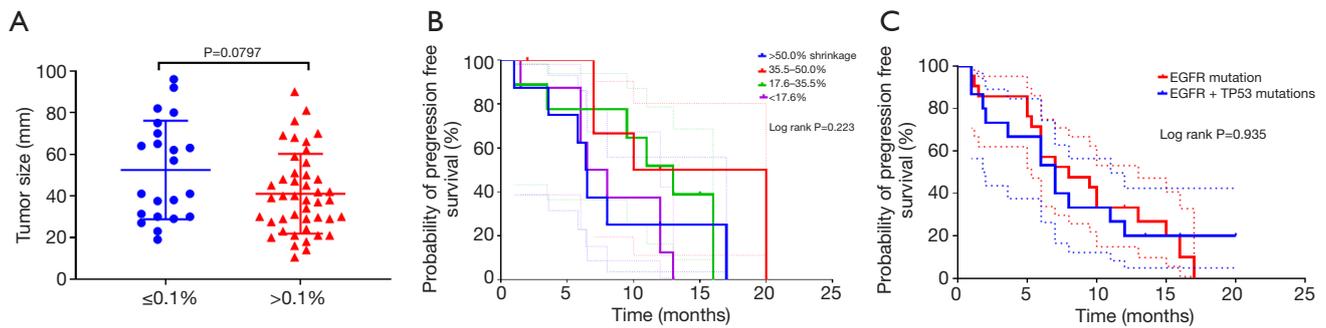Wang et al. Plasma *EGFR* mutation abundance and efficacy of EGFR-TKIs

Figure 4 Association between *EGFR* mutation abundance, tumor size (A), tumor shrinkage (B), and *TP53* concomitant mutation (C). The dotted line indicates 95% CI. CI, confidence interval; EGFR, epidermal growth factor receptor.

Table 3 Univariate and multivariate Cox regression analyses of PFS for patients

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Abundance: ≤0.1% *vs.* >0.1%	2.21 (0.96–4.96)	0.011	2.41 (1.12–5.20)	0.025
ECOG PS: >1 *vs.* 0 or 1	2.29 (0.86–6.12)	0.029	1.83 (1.19–2.82)	0.006
Age: ≤65 *vs.* >65 years	0.83 (0.42–1.53)	0.476		
Female *vs.* male	0.84 (0.45–1.56)	0.561		
T3+4 *vs.* T1+2 stage	1.12 (0.57–2.16)	0.728		
N2+3 *vs.* N0+1 stage	0.97 (0.51–1.88)	0.725		
Smokers *vs.* non-smokers	1.31 (0.59–2.94)	0.446		

significantly associated with better PFS. In the multivariate Cox proportional hazards regression model, mutation abundance, sex, smoking status, TNM stage, and ECOG PS were used as covariates. Multivariate analysis results demonstrated that mutation abundance [hazard ratio (HR) 2.41, 95% CI: 1.12–7.375.20; P=0.025] and ECOG PS (HR 1.83, 95% CI: 1.19–2.82; P=0.006) were independent predictive factors for PFS (*Table 3*).

Dynamic monitoring for resistance mutation

To identify the development of resistance mutation during TKI treatment, serial blood specimens (222 samples) in 67 patients with mutant *EGFR* were assayed every 8–12 weeks (*Figure 5*). At baseline, T790M resistance mutation was found in 6 cases (Table S1). Of them, only 1 participant (no. NM01ZMD) was identified as harboring concurrent 19Del and T790M resistance mutation in both tissue and plasma. A single participant had a L858R mutation in tissue but a T790M mutation in plasma (abundance: 0.06%).

Two participants with *EGFR* mutation–negative tissue were found to carry T790M in plasma (abundance: 0.02%). Both T790M mutation and *EGFR* sensitizing mutation in plasma were found in 2 participants but the mutation abundance of T790M was very low (0.04%, 0.07%). Three participants with *de novo* T790M mutation received EGFR-TKI therapy, and T790M mutation was cleared from plasma during treatment. However, recurrent T790M was observed in 1 participant (no. NM01ZSH) for a duration of 12 months.

Acquired T790M resistance mutations were detected in 11 participants. The median T790M mutation abundance was 0.34% in the range of 0.01–10.2%. Of these 11 participants, 5 achieved PR and 6 achieved SD in response to TKIs (Table S2). In 2 of these participants with acquired T790M resistance mutation, T790M mutation was unexpectedly detected at the first molecular testing after initiating therapy (abundance 0.16% and 0.01%, respectively; *Figure 5D,K*). The 2 participants were considered as early resistant (1.5 and 2.0 months, respectively). The period of the presence of T790M
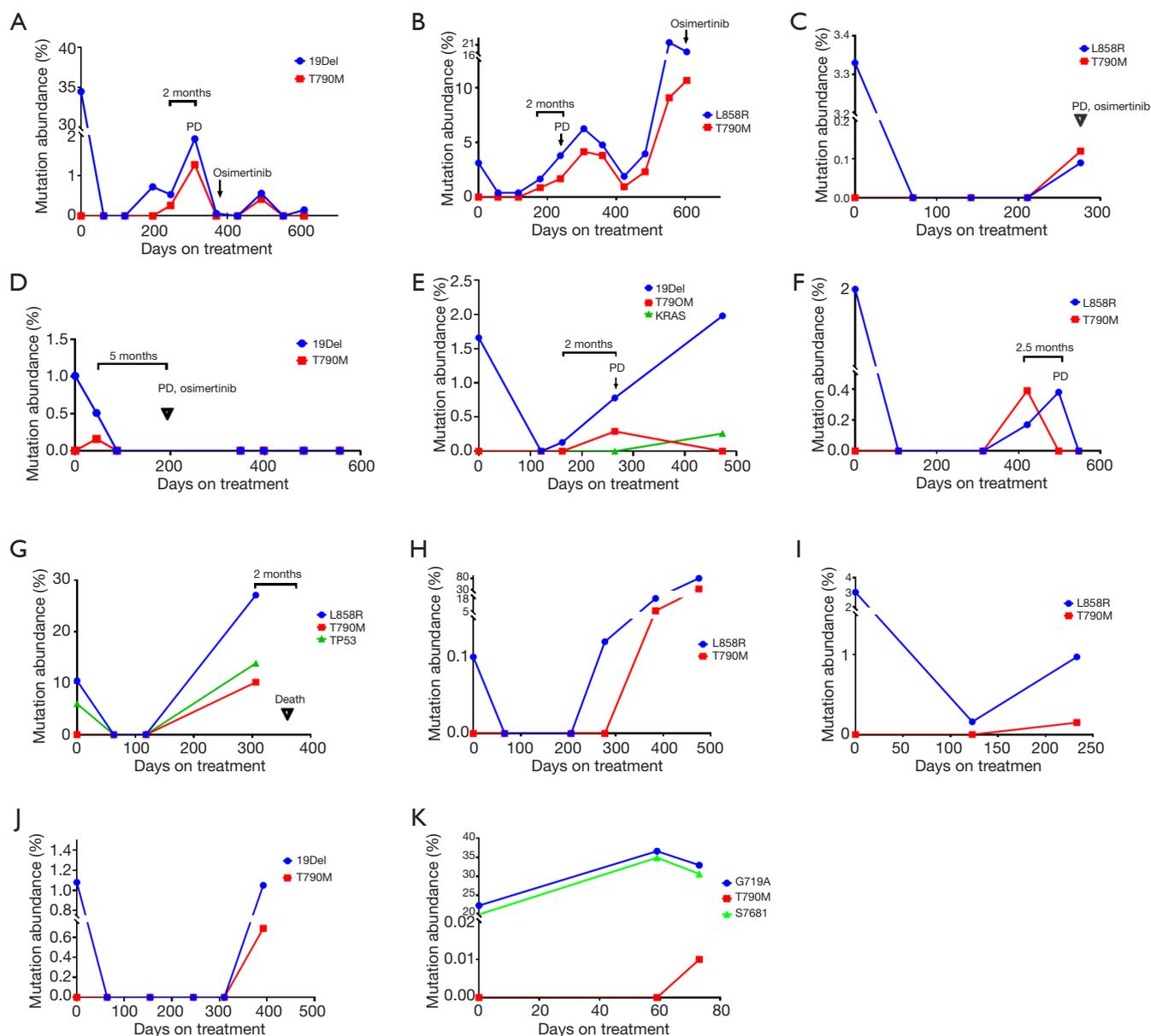
Figure 5 Dynamic monitoring of the T790M resistance mutation during treatment. (A-K) The abundance changes of *EGFR* mutations for 11 patients. EGFR, epidermal growth factor receptor.

mutation in the remaining 9 participants was 4–14 months with a mean of 10 months.

With the exception of 1 participant (*Figure 5K*), *EGFR* sensitizing mutation abundance in plasma decreased in response to first-generation EGFR-TKIs; 8 participants exhibited a complete plasmatic response (*Figure 5A,C,D,E,F,G,H,J*). Subsequently, plasmatic *EGFR* sensitizing mutations reemerged and mutation abundance increased. Plasmatic T790M was identified at a somewhat

lower abundance than the *EGFR* sensitizing mutation. The T790M in plasma was detectable 2–5 months before imaging progression by RECIST criteria. A total of 4 participants were treated with the third-generation EGFR-TKI, osimertinib, after PD (*Figure 5 A,B,C,D*).

Discussion

In the present study, we investigated the association of

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EGFR-activating mutation abundance in plasma at baseline and the therapeutic outcomes of first-generation EGFR-TKI treatment in advanced NSCLC. In addition, dynamic changes of *EGFR* mutations from ctDNA during treatment were analyzed to monitor the emergence of drug resistance mutations and predict the clinical efficacy of EGFR-TKIs. We found that not only those participants with highly abundant *EGFR* mutations in pretreatment blood samples have longer PFS on EGFR-TKIs, but that even those with a very low abundance of *EGFR* sensitizing mutations could benefit from EGFR-TKI targeted therapy.

Liquid biopsy based on ctDNA is considered complementary to detection of *EGFR* mutations in tissues, as it has the potential to survey the whole tissue (5,28-31). In this study, we used cSMART assay with high sensitivity to detect *EGFR* mutations and abundance change in plasma. Our results showed that *EGFR* sensitizing mutation abundance in TKI-naïve plasma at baseline was associated with response to EGFR-TKIs regardless of mutation pattern, which was in accordance with prior conclusions (11,12). Importantly, we found that the participants with even a very low abundance (≤0.1%) in plasma had longer median PFS than those with wild-type *EGFR* (5.0 *vs.* 2.0 months). Moreover, the extremely low abundance of *EGFR* mutations was mostly detected in participants who had *EGFR* mutations only in ctDNA. A total of 16 cases in this investigation were found to harbor positive *EGFR* mutations in blood samples but were negative in the matched tissues. The *EGFR* mutation abundance was less than 0.1% in 11 cases (68.8%). Previous studies have suggested that the status or abundance of *EGFR* mutations in the tissues and/or ctDNA may have different predictive values with regard to response to EGFR-TKIs in different therapeutic backgrounds (29,31,32). Therefore, we also assessed EGFR-TKI efficacy in various *EGFR* mutation statuses in tissue and blood samples. The results showed that the median PFS for ARMS+/cSMART+ participants were significantly longer than that of those with ARMS-/cSMART+ (data not shown), which was consistent with another study that used a highly sensitive ddPCR method to assess *EGFR* sensitizing mutation abundance in plasma (31). Undoubtedly, as shown in these studies, participants with *EGFR* sensitizing mutations detected in both tissue and ctDNA (ARMS+/cSMART+) are indicated as bearing a high load of *EGFR* mutations, resulting in a better clinical outcome. Our results demonstrated that NSCLC patients with a low abundance *EGFR* sensitizing mutation, particularly those who were initially mutation negative

by tumor biopsy, could also receive clinical benefit from EGFR-TKIs. Recently, Yan *et al.* demonstrated that, for patients with a low abundance of *EGFR* mutations, the median PFS was significantly longer in a combination group of EGFR-TKI plus chemotherapy than in an EGFR-TKI monotherapy group (median PFS: 7.9 *vs.* 5.9 months) (33). Thus, patients with a low abundance of *EGFR* mutations might achieve better clinical outcomes from EGFR-TKI treatment combined with chemotherapy.

It has been speculated based on comparative studies that the difference in abundance of *EGFR* mutations may be attributed to technical sensitivity (31) or tumor heterogeneity and clonal evolution (34). In terms of technology, cSMART assay has been shown to be highly sensitive and reliable for measuring levels of *EGFR* variants across a wide dynamic range, with the lowest mutation abundance reaching 0.01% (24-26,28). Due to the heterogeneity of tumor tissue, the collected biopsy specimen is not always representative of the whole tumor tissue. Heterogeneity may result in missed detection in some tumor specimens, which can be supplemented by ctDNA from a different region of the tumor (31,34). It is likely that ctDNA is derived from apoptotic or necrotic tumor cells. Therefore, the mutation abundance reflects the proportion of mutated cells in tumor tissue. Although large tumors might theoretically be more heterogeneous than smaller ones because of poor blood supply and hypoxia, we did not find a significant difference in tumor size for participants with high or low mutation abundance. Also, no association between the extent of target lesion shrinking to EGFR-TKI and PFS was found, which further supports previous results (35-37).

In order to exclude the influence of comutated genes, we further confirmed the impact of concomitant *TP53* mutations on EGFR-TKI efficacy. No significant differences were observed in terms of response and PFS in participants with or without concomitant *TP53* mutations, regardless of mutation status of *KRAS* or other actionable genes. The results were not in agreement with a recent study in which *TP53* mutations reduced responsiveness to TKIs and worsened prognosis in EGFR-mutated NSCLC patients (10).

Acquired resistance is an inevitable process during therapy with EGFR-TKIs, usually developing after a median treatment period of 10–12 months (38). Mutation of T790M is the major resistance mechanism to EGFR-TKIs with a prevalence of 49–63% in the most frequent resistance-associated molecular alterations (38,39). Reports on the

frequency of T790M in FFPE specimens for pretreatment patients have varied from 2–40% depending on the type and sensitivity of the detection method employed (40-42). In a study using a highly sensitive assay with a validated sensitivity of 0.1%, T790M mutation was detected in frozen tumor tissue at a rate of 2.8% in TKI-naïve NSCLC patients with *EGFR* sensitizing mutations (43). In our study, the frequency of the primary T790M mutation was found to be 1.8% for tissue (1/56) and 9.1% for blood samples (6/66), which was consistent with previously published results (40-44). Of note, the four T790M-positive samples missed by ARMS-PCR in tissue were found by cSMART assay to have very low T790M levels of 0.02–0.07%.

Wang *et al.* found that patients with pre-TKI plasma samples positive for T790M had significantly inferior PFS compared with pre-TKI–negative patients (45). We did not observe this phenomenon due to the limited samples with *de novo* T790M mutation. However, we observed that the abundance of *EGFR* sensitizing mutations decreased (or reached mutation clearance) in plasma after the initiation of EGFR-TKIs and subsequently started to increase along with the appearance of T790M. Meanwhile, T790M in plasma was detectable before clinical PD and a third-generation EGFR-TKI, osimertinib, was subsequently used to treat participants with acquired T790M mutation. Our findings and previous reports (16,22) were consistent with the hypothesis of the selection of resistant neoplastic clones operated by EGFR-TKI, that grow until becoming clinically relevant. In clinical practice, the abundance of both *EGFR* sensitizing mutations and T790M could vary under the selection pressure of treatment. Therefore, noninvasive dynamic monitoring for the abundance of *EGFR* sensitizing mutations and T790M is necessary, and potentially can be used to guide subsequent treatment by allowing early cessation of ineffective TKIs and commencement of a different TKI drug that counters the resistance mechanism.

There were several limitations in this study. First, the number of *EGFR* mutation–positive participants was limited. The different mutation types, including uncommon *EGFR* mutations, were analyzed together as *EGFR* mutation positive due to the small sample size. For these reasons, the comparison between subgroups should be interpreted with caution. Second, repeat biopsies were not performed to obtain matched tissue samples with blood after disease progression to confirm the presence of T790M. Third, the follow-up time of some participants should be extended.

Finally, the cutoff value to distinguish between participants with a high and low abundance of *EGFR* sensitizing mutations was based on the detection technique in this study. Therefore, larger cohorts are needed to validate the impact of plasma mutation abundance in predicting treatment responsiveness.

Collectively, highly abundant *EGFR* mutations in pretreatment blood samples predicted longer PFS on EGFR-TKIs. The participants with a low abundance of *EGFR* sensitizing mutations quantified by a highly sensitive detection method could benefit from EGFR-TKI targeted therapy. Furthermore, noninvasive, dynamic assessment of plasmatic *EGFR* mutation using a highly sensitive detection method could monitor changes of *EGFR* sensitizing mutation abundance and T790M resistance mutation before clinical resistance, and may potentially be used to guide subsequent treatment.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-7155). Dr. HZ and Dr. X Su reports other from Berry Oncology Corporation, outside the submitted work. The other 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. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by institutional

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ethics committee of Baotou Cancer Hospital, Inner Mongolia, China. Written informed consent was taken from all individual participants included in the study.

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Table S1 Mutation type and abundance in tissue or plasma for EGFR-positive patients

Pt No.	Treatment	Tissue ARMS	Plasma cSMART	Abundance	Comutated genes
1	TKI	19Del	19Del	1.66%	
2	TKI	L858R	L858R	1.32%	
3	TKI	19Del	19Del	1.01%	
4	TKI	L861Q	L861Q	2.62%	KRAS, PIK3CA
5	TKI	negative	G719A	0.03%	TP53
6	TKI	19Del	19Del	0.19%	TP53, ALK, PIK3CA
7	TKI	L861Q	L861Q	0.07%	TP53
8	TKI	19Del	19Del	34.52%	TP53
9	TKI	S768I	G719C	34.88%	TP53
			S768I	31.45%	
10	TKI	negative	L861Q	0.03%	TP53
11	TKI	L858R	L858R	0.97%	TP53
12	TKI	L858R	L858R	3.33%	
13	TKI	19Del	19Del	0.02%	
14	TKI	19Del	19Del	5.74%	TP53
15	TKI	19Del	19Del	0.09%	
16	TKI	G719X	G719A	4.14%	TP53
17	TKI	L858R	L858R	10.48%	TP53
18	TKI	19Del	19Del	4.43%	
19	TKI	L858R	L858R	0.10%	
20	TKI	negative	G719C	0.59%	TP53
21	TKI	19Del	19Del	0.03%	
22	TKI	negative	G719C	0.04%	ERBB2, TP53
23	TKI	negative	G719C	0.02%	TP53
24	TKI	L858R	L858R	1.23%	
25	TKI	undetected	19Del	4.45%	TP53, ALK, KRAS
26	TKI	19Del	19Del	2.00%	TP53
27	TKI	negative	G719S	0.03%	KRAS, PIK3CA, ALK
28	TKI	S768I	S768I	1.94%	TP53
		G719X	G719A	1.86%	
29	TKI	19Del	19Del	1.08%	
30	TKI	L858R	L858R	0.88%	
31	TKI	19Del	19Del	0.61%	
32	TKI	negative	G719A	0.04%	TP53
33	TKI	L858R	L858R	3.44%	
34	TKI	19Del	19Del	0.59%	
35	TKI	L858R	L858R	0.80%	
36	TKI	19Del	19Del	0.22%	TP53
			E20ins	0.06%	
37	TKI	L858R	L858R	0.14%	TP53
38	TKI	negative	G719A	3.47%	
39	TKI	L858R	L858R	3.12%	KRAS
40	TKI	19Del	19Del	1.57%	
41	TKI	negative	19Del	0.88%	TP53, KRAS
42	TKI	L858R	L858R	47.76%	
43	TKI	S768I	G719C	0.05%	
44	TKI	L858R+S768I	L858R	1.19%	TP53
			S768I	1.08%	
45	TKI	S768I	S768I	3.49%	
			G719D	3.02%	
46	TKI	negative	L858R	3.03%	TP53
47	TKI	19Del	19Del	1.19%	PIK3CA
48	TKI	L858R	L858R	13.68%	TP53
49	TKI	19Del	G719A	0.05%	KRAS, MET
50	TKI	L858R	L858R	15.95%	
51	TKI	L858R	L858R	0.07%	ALK
52	TKI	L858R	L858R	3.17%	TP53
53	TKI	19Del	19Del	0.19%	
54	TKI	G719A+ S768I	G719A	22.34%	TP53, PIK3CA
			S768I	19.99%	
55	TKI	19Del	negative		
56	TKI	19Del	19Del	0.05%	TP53, ALK, BRAF
			T790M	0.04%	
57	TKI	L858R	L858R	0.12%	TP53
			T790M	0.07%	
58	TKI	L858R	T790M	0.06%	PIK3CA
59	TKI	19Del+ T790M	19Del	57.02%	
			T790M	15.00%	
60	nonTKI	negative	T790M	0.02%	TP53, ALK
61	TKI	L858R	negative		TP53
62	TKI	L858R	negative		
63	nonTKI	19Del	negative		
64	nonTKI	19Del	19Del	0.17%	TP53
65	nonTKI	negative	G719A	0.03%	
66	nonTKI	L858R	L858R	8.67%	TP53
			G719C	0.03%	
67	nonTKI	negative	E20ins	0.05%	TP53, KRAS
68	nonTKI	19Del	19Del	0.16%	ALK
69	nonTKI	negative	E20ins	0.40%	TP53, ALK
70	nonTKI	negative	T790M	0.02%	TP53, PIK3CA
71	nonTKI	negative	E20ins	0.06%	TP53
72	nonTKI	E20ins	negative		
73	nonTKI	L858R	negative		KRAS

Table S2 Characteristics and response of patients with acquired T790M mutation

Pt No.	Gender	Age	ECOG PS	Response	PFS	Sensitizing mutation		T790M	Period (days)
						Type	Abundance (%)		
1	M	65	1	SD	16	19Del	0.78	0.29	260
2	F	71	2	PR	7	19Del	0.51	0.16	45
3	F	55	1	PR	8	19Del	0.53	0.26	244
4	F	68	0	PR	10	L858R	0.09	0.12	274
5	F	57	1	SD	12	L858R	27.12	10.2	276
6	M	68	1	SD	8	L858R	17.17	5.97	381
7	F	61	1	PR	17	19Del	0.17	0.39	416
8	F	75	2	SD	13	L858R	1.66	0.86	176
9	M	55	1	SD	8	L858R	0.97	0.15	233
10	M	54	4	SD	3.5	G719A	32.92	0.01	69
						S768I	30.65		
11	F	52	0	PR	13	19Del	1.05	0.69	391