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Research paper

Concurrent Driver Gene Mutations as Negative Predictive Factors in Epidermal Growth Factor Receptor-Positive Non-Small Cell Lung Cancer

Minjiang Chen [a], Yan Xu [a], Jing Zhao [a], Wei Zhong [a], Li Zhang [a], Yalan Bi [b], Mengzhao Wang [a,*]

[a] Department of Respiratory and Critical Care Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, China
[b] Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, China

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ABSTRACT

Background: Tyrosine kinase inhibitors (TKIs) are clinically effective in non-small cell lung cancer (NSCLC) patients harbouring epidermal growth factor receptor (*EGFR*) oncogene mutations. Genetic factors, other than *EGFR* sensitive mutations, that allow prognosis of TKI treatment remain undefined.
Methods: We retrospectively screened 423 consecutive patients with advanced NSCLC and *EGFR* 19del or 21L858R mutations. A total of 71 patients whose progression-free survivals (PFS) were shorter than 6 months or longer than 24 months were included and stratified into separate groups. Genetic background discrepancy was analysed in the two groups using next generation sequencing (NGS).
Findings: Sensitive *EGFR* mutations of 19del or 21L858R were detected by NGS in all patients; the 21L858R mutation was the major type. The most frequent accompanying somatic mutations were *TP53*, *RB1*, *MAP2K*. *ALK* fusion, *MET* amplification, and *BRAF V600E* were found only in the short PFS group. Concurrent pretreament T790 M mutation was found in both groups, but was proportionally higher in the short PFS group. In the short PFS group, patients had significantly more driver gene mutations than in long PFS group ($P = 0{\cdot}018$). The numbers of concomitant somatic mutations, *EGFR* pathway-related mutations, and tumor mutation burden (TMB) were not significantly different between the two groups.
Interpretation: Co-occuring driver gene mutations were negative predictive factors of TKI therapy in *EGFR*-mutated patients. This study highlights the importance of exploring co-occuring genomic alterations before initiation of *EGFR*-TKIs.

1. Introduction

Tyrosine kinase inhibitors (TKIs) are clinically effective in non-small cell lung cancer (NSCLC) patients who have epidermal growth factor receptor (*EGFR*) oncogene mutations [1]. Among the variety of *EGFR* oncogenic mutations, in-frame microdeletions around the Leu-Arg-Glu-Ala (LREA) residues of exon 19, and the L858R substitution in exon 21 of *EGFR* comprise approximately 90% of all *EGFR* mutations detected in advanced NSCLC patients. The aforementioned mutations were described as sensitizing *EGFR* mutations and were reported to be predictive markers of tumor responses to EGFR-TKIs. During the last two decades, randomized trials have demonstrated a median progression-free survival (PFS) of 9·3–14·4 months in patients harbouring sensitizing *EGFR* mutations treated with first-generation EGFR-TKIs [2–6]. Despite the high efficacy of TKIs, some patients with EGFR-mutant lung cancer acquire resistance to the drug in <6 months, while others' PFS are longer than 24 months. As such, there may be factors other than *EGFR* mutations that contribute to disease progression. Although previous studies have investigated the association of concomitant genetic alterations with response and survival [7–10], the results are conflicting.

We hypothesized that other molecular markers could contribute to the prediction of EGFR-TKI efficacy. To explore this hypothesis, we retrospectively screened our centre's patient database and performed next generation sequencing (NGS) of archival tissue from EGFR-mutant lung cancers patients. To better identify the differences between groups, we only selected patients with long or short PFS.

2. Materials and methods

2.1. Patient enrolment

A total of 423 consecutive patients with advanced NSCLC (stage IIIB or IV) possessing 19del or 21L858R *EGFR* mutations who had received first generation EGFR-TKI therapy (gefitinib, erlotinib, or icotinib) at Peking Union Medical College Hospital between 2013 and 2018 were screened. Of these, 71 patients were included in the study.

* Corresponding author at: Peking Union Medical College Hospital, No. 1 Shuaifuyuan, Wangfujing, Dongcheng District, Beijing 100730, China.
E-mail address: mengzhaowang@sina.com (M. Wang).

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Research in context

Evidence before this study

We searched PubMed for the articles published up to October 2018 using the terms "non-small cell lung cancer (NSCLC)", "epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI)", "predictive factor", and "co-occurring mutation". We found that a few studies have investigated the associations between co-occurring somatic gene mutations and EGFR-TKI efficacy, with inconsistent results. Some studies indicated that concomitant somatic mutations were negative predictive factors of EGFR-TKI therapy, while the other studies indicated that the tumor-suppressor genes and oncogenes rather than the number of mutations were associated with poorer efficacy of EGFR-TKI. However, these relations have not been demonstrated in real world datasets.

Added value of this study

In this study, we collected two groups of *EGFR* positive patients with comparable clinical characteristics and different progression-free survival spans who were treated with first generation EGFR-TKIs. To the best of our knowledge, it is the first real-world cohort to demonstrate the relationship between co-occurring somatic gene mutations and EGFR-TKI efficacy. We investigated genetic discrepancy using next generation sequencing. We also compared the differences in mutation number, oncogenes, and tumor mutation burden between the two groups.

Implications of all the available evidence

Significantly more co-occurring oncogene mutations and higher T790 M to *EGFR* mutation abundance ratio were found in patients in the short PFS group. Our data confirmed the impact of co-occurring oncogenes in EGFR-TKI treatment. Thus, multiple oncogene mutations besides *EGFR* mutations could be tested prospectively to provide better predictions of EGFR-TKI efficacy and disease progression.

All patients had been pathologically diagnosed with NSCLC. *EGFR* mutations were detected using an amplification refractory mutation system (ARMS). Patients were followed up regularly. Computed tomography (CT) of the thorax and abdomen were performed one month after the initiation of TKIs and every two months subsequently. Magnetic Resonance Imaging (MRI) was performed at baseline and every two months in patients with central nervous system (CNS) metastasis. Patients with symptomatic CNS metastasis or leptomeningeal metastasis were not included in the study. Objective response and progression of disease was assessed according to the response evaluation criteria in solid tumours (RECIST) 1·1 criteria [11]. Patients with PFS longer than 24 months or shorter than six months were included and stratified into two groups. PFS was calculated based on the start of EGFR-TKI therapy to disease progression or death. Overall survival (OS) was calculated from the start of EGFR-TKI therapy to death.

Demographic characters were reviewed based on patients' clinic record files. Age, sex, smoking status, clinical stage, Eastern Cooperative Oncology Group (ECOG) performance status (PS), and TKI treatment were included in analysis.

This study was approved by the Peking Union Medical College Hospital review board. Informed consent was obtained from all patients for use of tissue in genetic analysis. Pre-TKI treatment tumor tissue were collected and evaluated for NGS. Matched blood samples were collected as normal controls (Fig. 1). Genetic test using a panel of 416 cancer-related genes (Supplementary Table 1) were performed at Nanjing Shihe Jiyin Biotechnology Inc. (Jiangsu, China).

3. Sequence analysis

3.1. Sample preparation

Tissue blocks with adequate tumor cellularity were selected by the pathologists who made the diagnosis. Formalin-fixed paraffin-embedded (FFPE) tissues from 10 to 15 unstained slides of 5-μm thick sections were prepared using PCR precautions. DNA was isolated and purified using the QIAamp DNA FFPE Kit (QIAGEN). Quantity of DNA was measured usingQubit 3·0 with a dsDNA HS Assay Kit (Life Technologies).

3.2. Library preparation and sequencing

Sequencing libraries were prepared using the KAPA Hyper Prep kit (KAPA Biosystems) with an optimised manufacturer's protocol. In brief, 1 μg of genomic DNA was sheared into fragments using the Covaris M220 instrument (Covaris), and then subjected to end-repairing, A-tailing, and ligation with indexed adapters sequentially, followed by size selection using Agencourt AMPure XP beads (Beckman Coulter). Finally, libraries were amplified by PCR and purified for target enrichment.

For target enrichment, indexed DNA libraries were pooled together for hybridization with customized biotinylated-DNA probes (GeneseeqOne, Nanjing Geneseeq Technology Inc.) targeting 416 cancer-relevant genes (exons and selected introns for fusion detection).

Enriched libraries were amplified and subjected for NGS on Illumina Hiseq4000 NGS platforms (Illumina) according to manufacturer's instructions. For blood samples, sequencing depth was at least 100× mean coverage by non-PCR duplicate read pairs. For tumor specimens, sequencing depth was 500× mean coverage by non-PCR duplicate read pairs.

3.3. Data processing

Trimmomatic was used for FASTQ file quality control (QC) [12]. Sequencingreads were mapped to the reference sequence Human Genome (hg19) using a Burrows-Wheeler Aligner (BWA-mem, v 0·7·12) [13]. Local realignment around indels and base quality score recalibration were applied using the Genome Analysis Toolkit (GATK 3·4·0) [14]. VarScan2 was employed for detection of somatic mutations [15]. We required VAF ≥2% (≥ 8 variant supporting reads) with somatic p-value = 0·1, minimum quality score = 20 and variant supporting reads mapped to both strands with strand bias no >10%. The mutation list was further filtered through an internally collected list (~2000 normal samples) of recurrent artifacts on the same sequencing platform. Mutations were also removed if they were present in >1% population frequency in the 1000 Genomes Project or 65,000 exomes project (ExAC). Annotation of mutations was performed using ANNOVAR [16]. Copy number variations (CNVs) were detected using ADTEx with default parameters. Genomic fusions were identified by FACTERAwith default parameters [17].

TMB was defined as the number of somatic, coding, base substitution, and indel mutations per megabase of genome examined. For our panel TMB calculation, all non-synonymous alterations and indels in the coding region of targeted genes were considered with the exception of known hotspot mutations in oncogenic drivers and truncations in tumor suppressors. The validation of TMB calculation using our panel is shown in Supplementary Fig. 1.

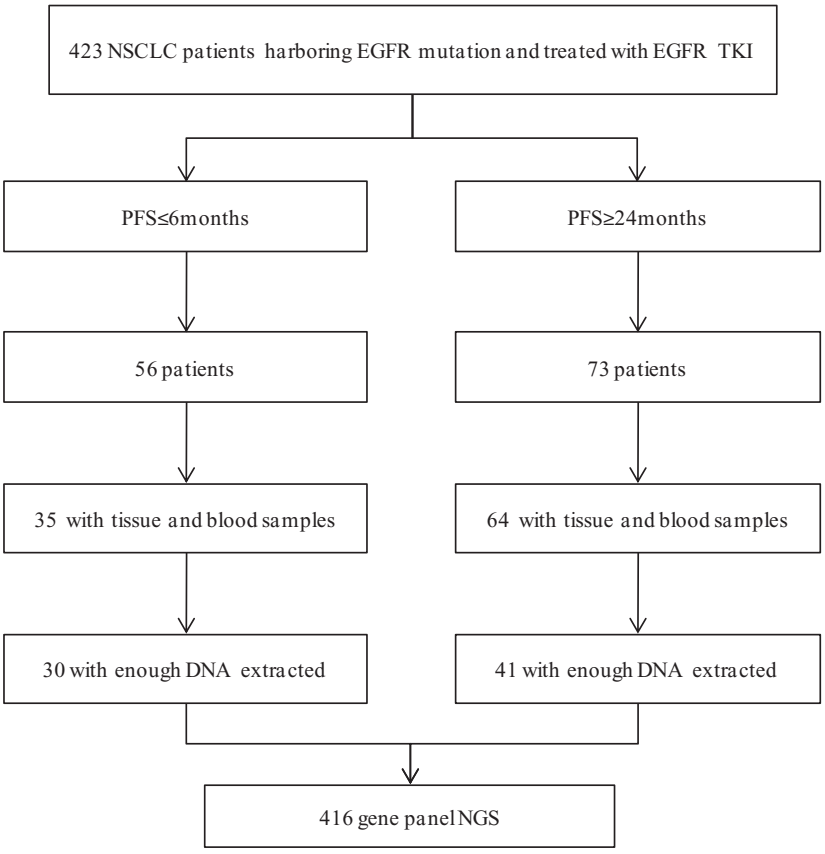

Fig. 1. Flowchart of the study design. NSCLC = non-small cell lung cancer. PFS = progression-free survival. EGFR = epidermal growth factor receptor. TKI = Tyrosine kinase inhibitors. NGS = next-generation sequencing.

3.4. Statistical analysis

The Wilcoxon rank-sum test or χ^2 test and Fisher's exact test was used to test differences in clinical and genetic parameters between patients with different PFS. Non-parametric tests were used to compare mutation number between groups. Survival curves were estimated using the Kaplan-Meier method. The statistical significance level was defined as two-sided $P < 0{\cdot}05$. SPSS statistical software, version 19·0 (IBM Corp., Armonk, NY, USA) was used to perform statistical analysis. Forset plot was drawn by GraphPad Prism, version 8·0·2.

4. Results

4.1. Patient characteristics

Of the 71patients, 41 belonged to the long PFS group and 30 belonged to the short PFS group.The median PFS and OS in the two groups of patients were 36·3 months (95% CI 32·0–40·6) vs. 3·9 (95% CI 3·0–4·8) months, and 63·6 months (95% CI 57·4–69·8) vs. 19·4 months (95% CI 8·6–31·2) (Supplementary Fig. 2A and 2B). The median age of all the patients was 60 years(range, 36-78 years). The distributation of age, gender, smoking status, ECOG score, tumor stage, mutation types, and lines of TKIs were not significantly different between the two groups. Patients' clinical characteristics and *EGFR* mutations detected using ARMS sequencing are given in Table 1.

4.2. Mutations detected by NGS in all patients

Sensitive *EGFR* mutations of 19del or 21L858R were detected in all 71 patients. The *EGFR* mutation types detected by NGS were consistent with the result obtained through ARMS PCR, with the exception of two patients with co-occurring 19del and 21L858R mutations. The 21L858R mutation was the major *EGFR* mutation type in both groups (61% in the short PFS group vs. 60% in the long PFS group). The 19del mutations consisted of several different types. The most frequent 19del mutation type was del745/746-750 (41% in the short PFS group vs. 43% in the

Table 1
Baseline characteristics of patients in long and short PFS groups.

Characteristic	No. of patients	Long PFS group	Short PFS group	*P* value
	71	41	30	
Age>65 years	23	16	7	0·127
Gender				0·125
Male	23	10	13	
Female	48	31	17	
Smoking status				0·780
Never smoker	54	32	22	
Ever smoker	17	9	8	
ECOG score				0·304
0–1	67	40	27	
≥2	4	1	3	
Clinical stage				0·069
IIIb	5	5	0	
IV	66	36	30	
EGFR mutations				1·000
19del	28	16	12	
21L858R	43	25	18	
TKI lines				1·000
1st line	59	34	25	
≥2nd line	12	7	5	
TKI treatments				0·638
Gefitinib	53	29	24	
Erlotinib	13	9	4	
Icotinib	5	3	2	

long PFS group), followed by del746insA, del747insS, S752fs, A750fs, and T751P. Complex *EGFR* mutations were found in 22 patients (31%), including 12 patients with complex exon20 T790M mutation and four patients with more than one EGFR-sensitive mutation of exons 19 or 21. The remaining five patients had co-occurring *EGFR* mutations of exon28 1142-1155del, exon20 R776H, exon18 E709K, exon18T710N, and exon18 L718 M. *EGFR* amplification was found in 18% patients. In the long PFS group, the most frequent accompanying somatic mutations were *TP53* (51%), followed by *MAP2K2* (15%), *NKX2-1* (15%), *CTNNB1* (15%), and *RB1* (12%). In the short PFS group, the most frequent accompanying somatic mutations were *TP53* (63%), followed by *RB1* (17%), *FAT1* (17%), and *ABCB1* (13%) (Supplementary Fig. 3). We further did cluster analysis and gene ontology (GO) analysis of the 71 patients. The results were shown in Supplementary Fig. 4 and Supplementary Fig. 5.

4.3. Differences in mutations between two groups

The percentage of 21L858R and 19del mutations were not significantly different between the two groups ($P = 1{\cdot}000$). Patients in the long PFS group tended to have more exon19 deletion mutations of del745/746-750 (82% vs. 54% in the short PFS group, $P = 0{\cdot}121$), fewer T790M mutations (10% vs. 27% in the short PFS group, $P = 0{\cdot}106$), and fewer complex *EGFR* mutations (22% vs. 43% in the short PFS group; $P = 0{\cdot}071$), but the differences were not statistically significant (Fig. 2).

We further analysed T790M abundance in T790M-positive samples. Mean T790M frequency was 0·77% in the long PFS group and 9·15% in the short PFS group ($P = 0{\cdot}053$). To minimise the impact of different proportions of cancer cells and genetic heterogeneity in each sample, we also compared the ratio of T790M alleles to *EGFR* sensitive mutation alleles. The result indicated that patients in the long PFS group had a relatively lower ratio (mean of 8·09% vs. 109·22% in the short PFS group; $P = 0{\cdot}037$) (Supplementary Table 2).

TP53 mutations were the most frequent mutation in both groups, and the missense mutation occurred more often in the short PFS group (27% vs. 47% in the short PFS group, $P = 0{\cdot}131$). The *PIK3CA* missense mutation was seen only in the short PFS group ($P = 0{\cdot}071$), while the MAP2K2 mutations were seen only in the long PFS group ($P = 0{\cdot}036$).

Co-occurring driver mutations [18] other than *EGFR* mutations were detected only in the short PFS group. Among them, one patient had *ALK* fusion gene, one patient had *MET* amplification,and one patient had *BRAF V600E*. Accompanying driver oncogene mutations were significantly prevalent in the short PFS group (10% vs. 33% in the short PFS group, $P = 0{\cdot}018$).

Other genetic variants that might be associated with PFS were also analysed, including *RB1*, *PIK3CA*, *EGFR* amplification and *BIM* polymorphism, but no statistically significant differences could be found. The number of co-occurring somatic mutations detected by NGS were $7{\cdot}63 \pm 0{\cdot}50$ and $8{\cdot}43 \pm 0{\cdot}65$ ($P = 0{\cdot}314$) in the long and short PFS groups, respectively. Among the somatic mutations, the mean number of EGFR pathway-related mutations were $1{\cdot}51 \pm 0{\cdot}19$ vs. $1{\cdot}37 \pm 0{\cdot}18$ ($P = 0{\cdot}652$) in the two groups (Table 2).

The relationship between genetic heterogeneity and PFS were further analysed. In multivariate analysis, co-occurring driver mutations and *MAP2K2* were independent predictive factors (Supplementary Fig. 6).

4.4. Tumor mutation burden (TMB) analysis

TMB for each patient was calculated. The mean TMB was $7{\cdot}3 \pm 5{\cdot}2$ mutations/Mb in the 71 patients. Mean TMBs for the two groups were $6{\cdot}7 \pm 0{\cdot}7$ for the long PFS group vs. $8{\cdot}2 \pm 1{\cdot}0$ for the short PFS group ($P = 0{\cdot}172$). Patients with TMBs higher than eight mutations/Mb tended to fall into the short PFS group, where 29·3% of patients in the long PFS group vs. 50·0% of patients in the short PFS group had TMBs higher than eight mutations/Mb ($P = 0{\cdot}056$) but the difference was not significant(Fig. 3).

5. Discussion

EGFR mutations have long been recognized as the most important prognostic factor in advanced NSCLC patients in the era of EGFR-TKI. Despite the high efficacy of EGFR-TKI, patients harbouring *EGFR* mutations can have extremely short PFS, but means of identifying patients whose tumours do not respond well to these drugs are lacking. In this study, we collated two groups of advanced NSCLC patients harbouring *EGFR* mutations with different PFS spans and found the concurrent driver gene mutations to be more frequent in short PFS group. This finding

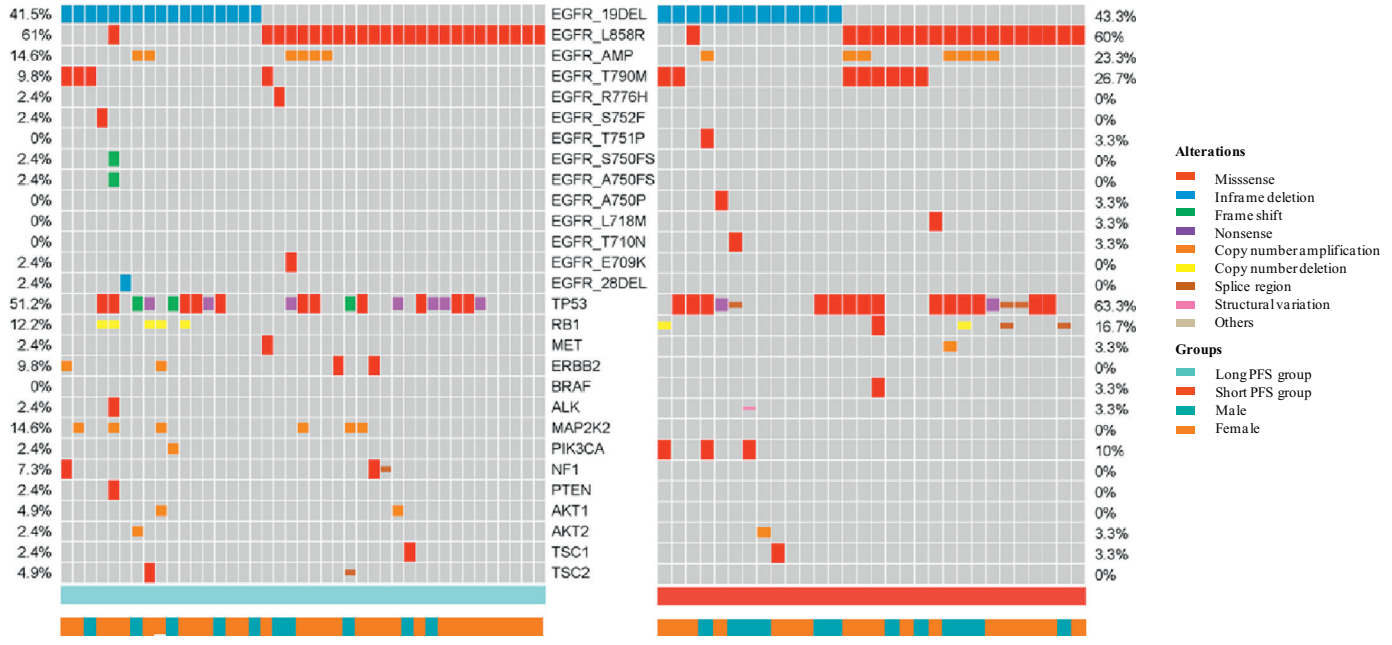

Fig. 2. Gene mutations in pretreated FFPE samples of patients in the two groups.

Table 2
Differences in genetic alterations between two groups detected using NGS.

	Long PFS group		Short PFS group		P
	No.	Percent (%)	No.	Percent (%)	
21L858R	25	61%	18	60%	1·000
19 Del	17	41%	13	43%	1·000
Del746–750	14	82%	7	54%	0·121
Complex EGFR mutations	9	22%	13	43%	0·071
T790M mutation	4	10%	8	27%	0·106
Co-occurring driver gene mutation	4	10%	10	33%	0·018
Number of mutations	7.63 ± 0.50		8·43 ± 0·65		0·314
EGFR pathway related mutations	1.51 ± 0.19		1·37 ± 0·18		0·652
Non EGFR pathway mutations	4.83 ± 0.41		5·57 ± 0·59		0·337
TP 53 mutations	22	54%	20	67%	0·332
TP53 missense mutation	11	27%	14	47%	0·131
RB1 mutations	5	12%	5	16%	0·733
PIK3CA missense mutation	0	–	3	10%	0·071
MAP2K2	6	15%	0	–	0·036
EGFR amplification	6	15%	7	23%	0·371
BIM polymorphism	4	10%	4	13%	0·714

indicates that the concurrent driver gene mutations might be a negative predictive factor. To the best of our knowledge, this is the first study comparing genetic differences between short and long PFS patients.

The recent wide spread use of NGS enables us a full view of genetic alteration in NSCLC. In several previous studies, concomitant somatic mutation and complex *EGFR* mutation have been reported to be predictive factors related to the PFS of patients treated with first generation TKI [7,9,19]. We profiled the genetic landscape of patients and explored for possible genomic alterations contributing to differences in TKI efficacy.

In our study, the clinical parameters in the two groups were not significantly different. Patients were mainly female and non-smoker, in accordance with previous reports. Mutation of exon 21 was the predominant mutation in both groups, which might because of the highly selection and limited number of patients. The high prevalence of complex *EGFR* mutations and high incidence of de novo T790M were observed in the study due to the selection of short PFS patients and the high sensitivity of the NGS method.

Acquired resistance of T790M was the most common resistance mutation in patients treated with first generation EGFR-TKIs. Additionally, previous studies suggested that the pre-existing T790M could present as a co-occurring mutation in a small population of *EGFR* positive NSCLC patients. The incidence of de novo T790M is 35%–60% in these patients [20,21]. Pretreatment T790M was reported to be a poor prognostic factor in NSCLC patients [22–24]. While using EGFR-TKI monotherapy, shorter PFS was shown for patients with T790M than without [22]. The combined use of EGFR-TKI and bevacizumab might prolong PFS in the T790M-positive subgroup [25]. Our study also indicated that patients with short PFS tended to have more T790M mutations, although de novo T790M also existed in long PFS patients. The abundance of T790M was higher in the short PFS group and the T790M rate to EGFR was significantly higher in the short PFS group, which was also consistent with previous studies [26]. This may indicate that the abundance of T790M is important in predicting the efficacy of EGFR-TKIs, especially when using highly sensitive detection methods. And combination therapy with bevacizumab might be of choice in these patients [25].

Co-occurring driver gene mutations other than *EGFR* were found only in the short PFS group. These patients had *ALK* fusion, *MET* amplification, or *BRAF V600E* mutation aside from EGFR-sensitive mutations. Previous reports indicated that the co-occurring of driver mutations were rare [27–31] and were associated with resistance to TKI therapy [19,32]. In the current study, patients with co-occurring driver gene mutations were much more in the short PFS group than in the long PFS group (10% vs. 33%, $P = 0{\cdot}018$), which suggests the co-occurrences of driver gene mutations might be the most important factor affecting clinical outcomes.

The reported incidence of concomitant ALK rearrangement and EGFR mutation was 1.3% to 1.6% [28,31]. And the coexistence of *ALK* rearrangement could be predictive of poor response in patients treated with EGFR-TKIs [33,34]. First line ALK-TKIs therapy might bring out a better outcome in these patients [32,35,36]. *MET* amplification is another known resistant mechanism of EGFR-TKI therapy. The baseline *MET* amplification in *EGFR*-mutant patients seems to be small (3.2%) [37]. These patients may response to *MET* inhibitors and crizotinib in previous reports [38–40]. Co-mutation of *BRAF V600E* was also rare. The experience of targeted therapy in these patients still needs to be defined. Due to the limited number of patients, all the experiences of treatment were from case reports. Moving forward, identifying the appropriate therapeutic strategy for co-occurring with *ALK*, *MET*, and *BRAF* mutations in *EGFR*-mutant NSCLC will require increasingly nuanced molecular screening strategies and well-designed clinical trials.

Several previous studies have investigated the association of concomitant genetic alterations with response and survival [7–10]. These studies challenged the single driver oncogene view and revealed the potential function of co-occurring genetic alterations as co-drivers of tumor progression [10]. In the current study, the percentage of patients

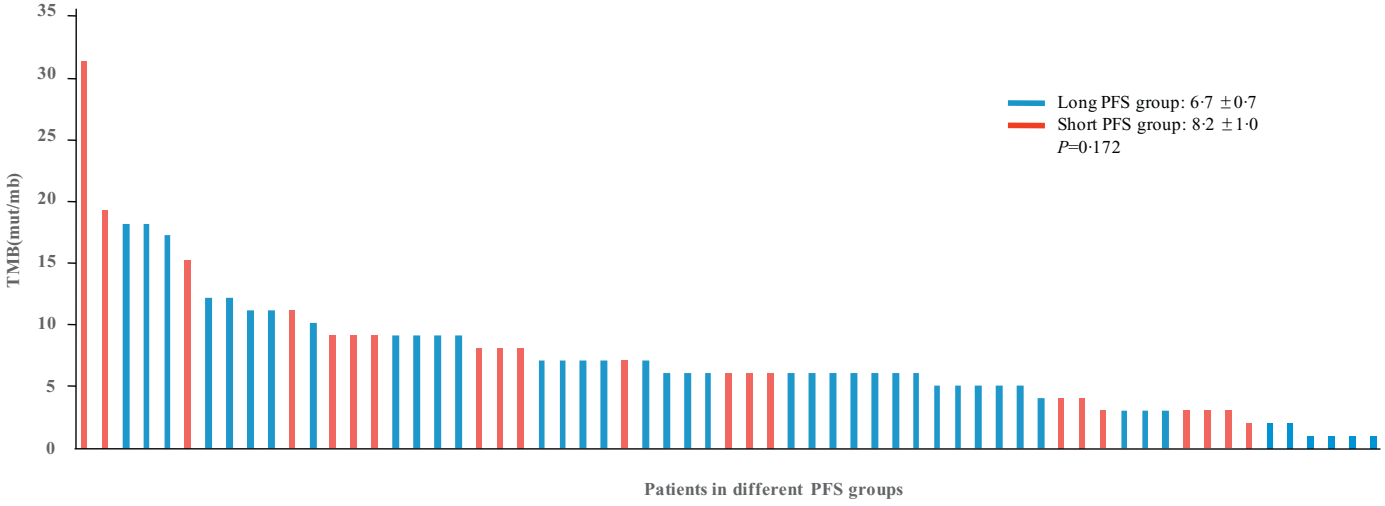

Fig. 3. Distribution of tumor mutation burden in the 71 patients. TMB = tumor mutation burden. PFS = progression-free survival.

with co-occurring resistance mutations were higher in the short PFS group than in the long PFS group ($P = 0{\cdot}018$). The result is consistent with previous studies and suggests that co-occurring driver gene mutations play an important role in tumor progression and drug resistance and might be a very important factor affecting clinical outcomes.

Co-existence of somatic gene mutations was also analysed. The results indicated that concomitant mutation of *TP53* was the most frequent somatic mutation in advanced NSCLC [32,41], in line with previous reports [42]. The missense mutation of *TP53* was more prevalent in the short PFS group, but the difference between the two groups was not significant.

RB1 is a tumor suppressor gene, whose mutations occurs in a variety of cancers and emerges as a cause of acquired resistance to EGFR antagonists [43]. Niederst et al. had described the association between *RB1* loss and the transformation to SCLC in EGFR-mutant NSCLC [44]. Aurora B kinase inhibitor was reported to be efficacious in *RB1* loss SCLC models [43]. But the impact of pretreatment *RB1* in NSCLC was unknown. In previous report, the median interval between the initiation of EGFR-TKI to small cell lung cancer (SCLC) transformation was 15·8 months [45]. Thus, we speculated that the *RB1* loss might be a relatively indolent mutation. In our study, *RB1* were found in both groups. No different of frequency were seen between the two groups. Specified treatments for these patients need further clinical researches and investigations.

MAP2K2 amplification was found only in the long PFS group ($P = 0{\cdot}036$), but we were unable to conclusively demonstrate its positive prognostic value due to the limited number of cases.

We further compared the occurance of concomitant mutations, the number of genes in the EGFR-related pathway and the number of total somatic gene mutations between the two groups, but found no significant differences, in contrast to previous studies [7]. This indicated that co-occurring somatic mutations that were not driver mutations were not strong predictive factors for patients treated by EGFR-TKIs.

In addition, our study analysed the differences of concomitant mutation number and TMB in the two groups. The result showed that the short PFS group had more patients whose TMB was higher than eight. However, neither the mean number of mutations nor the TMB level were significantly different between the two groups. There are several possible mechanisms for this. TMBs of patients developing T790M resistance were lower, and TMB was not affected in patients with SCLC transformation after developing TKI resistance [46]. This suggests an explicit resistance gene or mechanism serving as a single oncogene and leading to lower heterogeneity of lung cancer. In our study, the incidence of pretreatment-resistant mutations, including T790M, was high in the short PFS group; this may have contributed to the lower mean TMB. However, this hypothesis requires additional further study for confirmation.

There are several limitations in this study. First, since this was a retrospective analysis with all patients at advanced stages of disease, the number of patients and contributing tissue samples were limited. Only approximately 55% of the patients had sufficient DNA extracted for NGS. Second, we included patients receiving different lines of TKI treatment. The last, due to the insufficiency of remaining tissue, PD-L1 immunohistochemistry was not performed. As conflicting data are present in the literature on the prognostic role of TMB and concomitant somatic mutations in EGFR-positive NSCLC [7,41], additional prospective studies including more patients should be performed.

In conclusion, our study highlights the co-occurring driver gene mutations, which were significantly more frequent in short PFS group, may serve as important negative predictive factors for TKI therapy in patients with *EGFR* 19del or 21L858R mutations. A high T790M ratio to *EGFR* mutation ratio rather than existence of T790M predicted a shorter PFS. Co-occurring genomic alterations as well as EGFR sensitive mutations should be detected prior to treatment to identify subgroups of patients with less favourable outcomes of TKI therapy.

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Declaration of interests

The authors declare no potential conflicts of interest.

Author contributions

MW designed the study. MW and MC contributed to data analysis and preparation of the report. YB, YX, JZ, and LZ collected the samples assembled the clinical data. WZ conducted the statistical analysis. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.023.

References

[1] Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non- small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
[2] MaemondoM Inoue A, Kobayashi K, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010;362:2380–8.
[3] Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012;13:239–46.
[4] Zhou C, Wu YL, Chen G, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open- label, randomised, phase 3 study. Lancet Oncol 2011;12:735–42.
[5] Mitsudomi T, Morita S, Yatabe Y, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol 2010;11(2):121–8.
[6] Yan X, Wang H, Li P, et al. Efficacy of first-line treatment with epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) alone or in combination with chemotherapy for advanced non-small cell lung cancer (NSCLC) with low-abundance mutation. Lung Cancer 2019;128:6–12.
[7] Hong S, Gao F, Fu S, et al. Concomitant genetic alterations with response to treatment and epidermal growth factor receptor tyrosine kinase inhibitors in patients with EGFR-mutant advanced non-small cell lung cancer. JAMA Oncol 2018. https://doi.org/10.1001/jamaoncol.2018.0049 [Epub ahead of print] PubMed PMID: 29596544.
[8] Jin Y, Shi X, Zhao J, et al. Mechanisms of primary resistance to EGFR targeted therapy in advanced lung adenocarcinomas. Lung Cancer 2018;124:110–6.
[9] Wang Z, Cheng Y, An T, et al. Detection of EGFR mutations in plasma circulating tumour DNA as a selection criterion for first-line gefitinib treatment in patients with advanced lung adenocarcinoma (BENEFIT): a phase 2, single-arm, multicentre clinical trial. Lancet Respir Med 2018;6(9):681–90. https://doi.org/10.1016/S2213-2600(18)30264-9 Epub 2018 Jul 17.*Erratum in: Lancet Respiratory Medicine* 2018;6(9):e50. PubMed PMID: 30017884.
[10] Blakely CM, Watkins TBK, Wu W, et al. Evolution and clinical impact of co-occurring genetic alterations in advanced-stage EGFR-mutant lung cancers. Nat Genet 2017;49(12):1693–704.
[11] Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009;45(2):228–47.
[12] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30(15):2114–20.
[13] Li H, Durbin R. Fast and accurate short read alignment with burrows-wheeler transform. Bioinformatics 2009;25(14):1754–60.

[14] DePristo MA, Banks E, Poplin R, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011;43(5):491–8.
[15] Koboldt DC, Zhang Q, Larson DE, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res 2012;22(3):568–76.
[16] Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-through put sequencing data. Nucleic Acids Res 2010;38(16):e164.
[17] Newman AM, Bratman SV, Stehr H, et al. FACTERA: a practical method forthe discovery of genomic rearrangements at breakpoint resolution. Bioinformatics 2014;30(23):3390–3.
[18] National Comprehensive Cancer Network. (NCCN). Clinical Practice Guidelines in Oncology. Non-Small Cell Lung Cancer, Version 1; 2019.
[19] Yu HA, Suzawa K, Jordan E, et al. Concurrent alterations in EGFR-mutant lung cancers associated with resistance to EGFR kinase inhibitors and characterization of MTOR as a mediator of resistance. Clin Cancer Res 2018;24(13):3108–18.
[20] Watanabe M, Kawaguchi T, Isa S, et al. Ultra-sensitive detection of the pretreatment EGFR T790M mutation in non-small cell lung cancer patients with an EGFR-activating mutation using droplet digital PCR. Clin Cancer Res 2015;21:3552–60.
[21] Chen L-Y, Molina-Vila MA, Ruan S-Y, et al. Coexistence of EGFR T790M mutation and common activating mutations in pretreatment non-small cell lung cancer: a systematic review and meta-analysis. Lung Cancer 2016;94:46–53.
[22] Su KY, Chen HY, Li KC, et al. Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. J Clin Oncol 2012;30(4):433–40.
[23] Rosell R, Molina MA, Costa C, et al. Pretreatment EGFR T790M mutation and BRCA1 mRNA expression in erlotinib-treated advanced non-small-cell lung cancer patients with EGFR mutations. Clin Cancer Res 2011;17(5):1160–8.
[24] Costa C, Molina MA, Drozdowskyj A, et al. The impact of EGFR T790M mutations and BIM mRNA expression on outcome inpatients with EGFR-mutant NSCLC treated with erlotinib or chemotherapy in therandomized phase III EURTAC trial. Clin Cancer Res 2014;20(7):2001–10.
[25] Rosell R, Dafni U, Felip E, et al. Erlotinib and bevacizumab in patients with advanced non-small-cell lung cancer and activating EGFRmutations (BELIEF): an international, multicentre, single-arm, phase 2 trial. Lancet Respir Med 2017;5(5):435–44.
[26] Li X, Cai W, Yang G, et al. Comprehensive analysis of EGFR-mutant abundance and its effect on efficacy of EGFR TKIs in advanced NSCLC with EGFR mutations. J Thorac Oncol 2017;12(9):1388–97. https://doi.org/10.1016/j.jtho.2017.06.006.
[27] Brega E, Brandao G. Non-small cell lung carcinoma biomarker testing: the pathologist's perspective. Front Oncol 2014;4:182. https://doi.org/10.3389/fonc.2014.00182.
[28] Yang JJ, Zhang XC, Su J, et al. Lung cancers with concomitant EGFR mutations and ALK rearrangements: diverse responses to EGFR-TKI and crizotinib in relation to diverse receptors phosphorylation. Clin Cancer Res 2014;20(5):1383–92.
[29] Lee JK, Kim TM, Koh Y, et al. Differential sensitivities to tyrosine kinase inhibitors in NSCLC harboring EGFR mutation and ALK translocation. Lung Cancer 2012;77(2):460–3.
[30] Li S, Li L, Zhu Y, et al. Coexistence of EGFR with KRAS, or BRAF, or PIK3CA somatic mutations in lung cancer: a comprehensive mutation profiling from 5125 Chinese cohorts. Br J Cancer 2014;110(11):2812–20.
[31] Ulivi P, Chiadini E, Dazzi C, et al. Nonsquamous, non-small-cell lung cancer patients who carry a double mutation of EGFR, EML4-ALK or KRAS: frequency, clinicopathological characteristics, and response to therapy. Clin Lung Cancer 2016;17:384–90.
[32] Lin L, Bivona TG. Mechanisms of resistance to epidermal growth factor receptor inhibitors and novel therapeutic strategies to overcome resistance in NSCLC patients. Chemother Res Pract 2012;2012:817297.
[33] Galetta D, Catino A, Misino A. Concomitant EGFR mutations/ALK rearrangements: beyond a simple dual target. Transl Lung Cancer Res 2016;5:143–4.
[34] Lo Russo G, Imbimbo M, Corrao G, et al. Concomitant EML4-ALK rearrangement and EGFR mutation in non-small cell lung cancer patients: a literature review of 100 cases. Oncotarget 2017;8(35):59889–900.
[35] Won JK, Keam B, Koh J, et al. Concomitant ALK translocation and EGFR mutation in lung cancer: a comparison of direct sequencing and sensitive assays and the impact on responsiveness to tyrosine kinase inhibitor. Ann Oncol 2015;26:348–54.
[36] Lee T, Lee B, Choi YL, et al. Non-small cell lung cancer with concomitant EGFR, KRAS, and ALK mutation: clinicopathologic features of 12 cases. J Pathol Transl Med 2016;50:197–203.
[37] Lai GGY, Lim TH, Lim J, et al. Clonal MET amplification as a determinant of tyrosine kinase inhibitor resistance in epidermal growth factor receptor-mutant non-small-cell lung cancer. J Clin Oncol 2019:JCO1800177. https://doi.org/10.1200/JCO.18.00177 [Epub ahead of print].
[38] Li Y, Zhang R, Zhou Y, et al. Combined use of crizotinib and gefitinib in advanced lung adenocarcinoma with leptomeningeal metastases harboring MET amplification after the development of gefitinib resistance: a case report and literature review. https://www.ncbi.nlm.nih.gov/pubmed/30638795 Clin Lung Cancer 2018 pii: S1525-7304(18)30328-0. [Epub ahead of print].
[39] Ahn MJ, Han JY, Sequist LV, et al. OA 09.03 TATTON Ph Ib expansion cohort: osimertinib plus savolitinib for pts with EGFR-mutant MET-amplified NSCLC after progression on prior EGFR-TKI. J Thorac Oncol 2017;12(11). https://doi.org/10.1016/j.jtho.2017.09.377 Supplement 2:S1768.
[40] Gainor JF, Niederst MJ, Lennerz JK, et al. Dramatic response to combination erlotinib and crizotinib in a patient with advanced, EGFR-mutant lung cancer harboring De novo MET amplification. J Thorac Oncol 2016;11(7):e83–5.
[41] Jiao XD, Qin BD, You P, Cai J, Zang YS. The prognostic value of TP53 and its correlation with EGFR mutation in advanced non-small cell lung cancer, analysis based on cBioPortal data base. Lung Cancer 2018;123:70–5. https://doi.org/10.1016/j.lungcan.2018.07.003.
[42] Xu Y, Tong X, Yan J, Wu X, Shao YW, Fan Y. Short-term responders of non-small cell lung cancer patients to EGFR tyrosine kinase inhibitors display high prevalence of TP53 mutations and primary resistance mechanisms. Transl Oncol 2018;11(6):1364–9.
[43] Oser MG, Fonseca R, Chakraborty AA, et al. Cells lacking the RB1 tumor suppressor gene are hyperdependent on Aurora B kinase for survival. Cancer Discov 2018. https://doi.org/10.1158/2159-8290.CD-18-0389 [Epub ahead of print].
[44] Niederst MJ, Sequist LV, Poirier JT, et al. RB loss in resistant EGFR mutant lung adenocarcinomas that transform to small-cell lung cancer. Nat Commun 2015;6:6377.
[45] Marcoux N, Gettinger SN, O'Kane G, et al. EGFR-mutant adenocarcinomas that transform to small-cell lung cancer and other neuroendocrine carcinomas: clinical outcomes. J Clin Oncol 2019;37(4):278–85.
[46] Offin M, Rizvi H, Tenet M, et al. Tumor mutation burden and efficacy of EGFR-tyrosine kinase inhibitors in patients with EGFR-mutant lung cancers. Clin Cancer Res 2018. https://doi.org/10.1158/1078-0432.CCR-18-1102 [Epub ahead of print] PubMed PMID: 30045933.