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

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A B S T R A C T

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, MAP2K1, ALK fusion, MET amplification, and BRAFV600E were found only in the short PFS group. Concurrent pretreatment T790M 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 = 0.018). 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-occurring driver gene mutations were negative predictive factors of TKI therapy in EGFR-mutated patients. This study highlights the importance of exploring co-occurring genomic alterations before initiation of EGFR-TKIs.

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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 9–13–14.4 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 prospectively 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.

https://doi.org/10.1016/j.ebiom.2019.03.023
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All patients had been pathologically diagnosed with NSCLC. EGFR mutations were detected using an amplification refraction mutation system (ARMS). Patients were followed up regularly. Computed tomography (CT) of the thorax and abdomen were performed once a 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 tumors (RECIST) 1·1 criteria [11]. Patients with PFS longer than 6 months in patients with central nervous system (CNS) metastasis. Patients 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.

**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 concomi-tant 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.
3.4. Statistical analysis

The Wilcoxon rank-sum test or χ² 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 < 0.05. 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 71 patients, 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 months (95% CI 3·0–4·8) (Supplementary Fig. 2A and 2B). The median age of all the patients was 60 years (range, 36-78 years). The distribution of age, gender, smoking status, ECOG score, tumor stage, 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 long PFS group).

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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%), NTK2-1 (15%), CTNNB1 (15%), and RBL1 (12%). In the short PFS group, the most frequent accompanying somatic mutations were TP53 (63%), followed by RBL1 (17%), FAT1 (17%), and ABCB1 (13%) (Supplementary Fig. 4). 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 = 1·000). 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 = 0·013), fewer T790M mutations (10% vs. 27% in the short PFS group, P = 0·121), and fewer complex EGFR mutations (22% vs. 43% in the short PFS group, P = 0·071), 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 = 0·056) but the difference was not significant (Fig. 2).

4.4. Tumor mutation burden (TMB) analysis

TMB for each patient was calculated. The mean TMB was 7·3 ± 5·2 mutations/Mb in the 71 patients. Mean TMBs for the two groups were 6·7 ± 0·7 for the long PFS group vs. 8·2 ± 1·0 for the short PFS group (P = 0·172). 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. 14·3% in the short PFS group (P = 0·18) (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

| Alterations                  | Long PFS group | Short PFS group |
|------------------------------|----------------|-----------------|
| Missense                     | 33%            | 17%             |
| Inframe deletion             | 33%            | 13%             |
| Frame shift                  | 33%            | 13%             |
| Nonsense                     | 33%            | 13%             |
| Copy number amplification    | 33%            | 13%             |
| Copy number deletion         | 33%            | 13%             |
| Splice region                | 33%            | 13%             |

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

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 = 0·018).

Other genetic variants that might be associated with PFS were also analysed, including RBL1, 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 7·63 ± 0·50 and 8·43 ± 0·65 (P = 0·314) in the long and short PFS groups, respectively. Among the somatic mutations, the mean number of EGFR pathway-related mutations were 1·51 ± 0·19 vs. 1·37 ± 0·18 (P = 0·652) 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).
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 mutations 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 genomic 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 selected 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 = 0.018), 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 respond 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 co-occurring 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

| Table 2 Differences in genetic alterations between two groups detected using NGS. |
|----------------|----------------|----------------|----------------|
|                | Long PFS group | Short PFS group | P              |
| No.            | Percent (%)    | No.            | Percent (%)    |
| 21E558R        | 25             | 18             | 60%            | 1·000          |
| 19 Del         | 17             | 13             | 43%            | 1·000          |
| Del746–750     | 14             | 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 ± 8·43 ± | 0·314          |                |
| EGFR pathway related mutations | 1·51 ± 1·37 ± | 0·652          |                |
| Non EGFR pathway mutations | 0·19          | 0·18           |                |
| T7P 53 mutations | 4·83 ± 5·57 ± | 0·337          |                |
| TP53 missense mutation | 22         | 54%            | 20             | 67%            | 0·332          |
| RB1 mutations  | 11             | 27%            | 14             | 47%            | 0·131          |
| PIK3CA missense mutation | 5          | 12%            | 5              | 16%            | 0·733          |
| MAP2K2         | 6              | 15%            | 0              | 10%            | 0·071          |
| EGFR amplification | 6          | 15%            | 7              | 23%            | 0·371          |
| BIM polymorphism | 4             | 10%            | 4              | 13%            | 0·714          |

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 = 0.018). 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 occur 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 = 0.036), but we were unable to conclusively demonstrate its positive prognostic value due to the limited number of cases. We further compared the occurrence 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.

Acknowledgement

We thank the patients for providing their samples in this study. We acknowledge Xiaoe Li (Nanjing Genesseq Technology Inc.) for technical support. We also thank Xiaoyan Liu (Department of Respiratory and Critical Care Medicine, Peking Union Medical College Hospital) and Hua Chen (Department of Rheumatology, Peking Union Medical College Hospital) for supporting bioinformatic analysis.

Funding sources

The study was supported by National Science Foundation of China (Grant No. 81702292) and CAMS Innovation Fund for Medical Sciences (Grant No. 2018-I2M-1-003).

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.

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