Concurrent gene alterations with EGFR mutation and treatment efficacy of EGFR-TKIs in Chinese patients with non-small cell lung cancer

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ABSTRACT

Purpose: We investigated the frequency of concurrent genes in EGFR-mutant non-small cell lung cancer patients and determined its value in predicting the efficacy of EGFR-TKIs treatment.

Methods: Three hundred and twenty patients, who harbored EGFR activating mutations and received EGFR-TKIs treatment, were examined for another eight genes including KRAS, NRAS, PIK3CA, BRAF, and HER2 mutations and ALK, ROS1, and RET fusion genes based on reverse transcription PCR. Progression-free survival and overall survival with EGFR-TKIs treatment were evaluated using Kaplan-Meier methods and compared between different patients using log-rank tests.

Results: Twenty-one (6.6%) of 320 EGFR mutant samples with additional gene alterations were identified. The most common concurrent gene was PIK3CA mutation (n = 9), followed by EML4-ALK rearrangement (n = 6), HER2 mutation (n = 3), RET rearrangement (n = 1), ROS1 rearrangement (n = 1) and KRAS mutation (n = 1). Patients with single EGFR mutation had a significantly longer progression-free survival than those with concurrent genes (10.9 vs. 6.0 months, P = 0.002). Among the 21 cases, patients with PIK3CA mutation had the longest median progression-free survival (7.6 months), followed by ALK rearrangement (5.0 months) and other gene types (1.2 months). No overall survival difference was found between patients with single EGFR mutation and concurrent gene alterations (21.0 vs.17.6 months, P = 0.17).

Conclusion: We demonstrated that concurrent gene alterations occurred in some patients with EGFR mutations. Concurrent gene alterations decreased the efficacy of EGFR-TKIs.

INTRODUCTION

Epidermal growth factor receptor (EGFR) mutations occur in about 40% to 50% of lung adenocarcinoma patients of East Asian descent [1, 2]. The median progression-free survival (PFS) is approximately 9 to 13 months and the objective response rate of 60% to 70% in patients carrying EGFR mutations treated with EGFR-TKIs [3–6].

Drug resistance is a big issue for most patients with clinically evident non-small cell lung cancer (NSCLC). T790M mutation, MET amplification and PIK3CA mutations contributed to secondary resistance to EGFR-TKIs and several new drugs targeting resistance have emerged [7–12]. Primary resistance is another challenge in clinical practice, however, the mechanism is not well investigated currently. Coexistent genetic alterations in cancer-driving genes, i.e., KRAS mutations, PTEN loss and BIM polymorphisms were identified to be associated with primary resistance for EGFR-TKIs treatment [13–14]. But, most studies focused on concurrent ALK and EGFR mutations [15–16]. Other genes such as PIK3CA and HER2 were not well reported. The
efficacy of EGFR-TKIs for NSCLC patients with coexisting genetic alterations remains unclear.

In the present study, we used multiple gene screening of 320 NSCLC patients harboring EGFR-sensitive mutations and evaluated the frequency of concomitant genetic alterations, further to investigate the efficacy of EGFR-TKIs treatment in these patients.

RESULTS

Patient characteristics

The characteristics of the 320 patients are shown in Table 1. Twenty-one patients with EGFR mutation that harbored a concurrent driver gene were identified. The clinical and molecular characteristics of the 21 patients are shown in Table 2. There were 11 male and 10 female with a median age of 62 years. Twenty patients presented with a histology of adenocarcinoma and one typical of adenosquamous carcinoma. Seven patients included former or current smokers and 14 were never-smokers. No clinical or pathological differences were observed between patients with single EGFR mutation and those who harbored concurrent genes (Table 3).

Gene results

Among the 320 patients with EGFR mutations, 157 were with deletion in exon 19, 142 with L858R point mutation in exon 21, 13 with L861Q mutation in exon 21, one with G719X mutation in exon 18 and one with S768I). All the 320 patients were analyzed for KRAS, NRAS, PIK3CA, ALK, ROS1, and RET fusion genes. Coexisting mutations or fusions were identified in 21 patients (6.6%). This analysis included PIK3CA mutation (n = 9, 42.9%), followed by EML4-ALK rearrangement (n = 6, 28.6%), HER2 mutation (n = 3, 14.3%), KRAS mutation (n = 1, 4.8%), RET rearrangement (n = 1, 4.8%), ROS1 rearrangement (n = 1, 4.8%), BRAF mutation (n = 0, 0%), and NRAS mutation (n = 0, 0%). The coexisting mutations are listed in Table 2. Among the 21 patients, 14 included those with deletion in exon 19, 4 with L858R mutation in exon 21, one with G719X mutation in exon 18 and one with L861Q mutation in exons 21. More frequency of coexisting mutations in deletion in exon 19 was observed than L858R mutation in exon 21 (8.9% vs. 2.8%, P = 0.028).

Efficacy analysis

One hundred and eighty-six patients with single EGFR mutation showed partial responses (62.2%), one with complete response (0.3%) and 67 showed stable disease (22.4%); 46 patients had progressive disease. The ORR was 62.5% and DCR was 84.9%. In patients with concurrent gene alterations, the ORR and DCR were 47.6% and 66.7%, respectively. The efficacy comparisons are shown in Table 4.

The median PFS in all the 320 patients was 10.8 months (95% CI, 9.9–11.6). The PFS in the group with single EGFR mutation and concurrent gene alterations group were 10.9 months (95% CI, 10.0–11.5) and 6.0 months (95% CI, 3.8–8.2), respectively (P = 0.002) (Figure 1). The median PFS in patients carrying PIK3CA, ALK and other genes were 7.6 months, 5.0 months and 1.2 months, respectively (P = 0.880). No PFS difference was found between EGFR/PIK3CA mutation and EGFR/other gene concurrent patients (P = 0.881).

The PFS in the group with single EGFR exon 19 deletion mutation and concurrent gene alterations group were 11.4 months (95% CI, 10.4–12.5) and 6.0 months (95% CI, 4.1–7.8) (P = 0.001). The PFS in the group with single EGFR exon 21 L858R mutation and concurrent gene alterations group were 9.5 months (95% CI, 8.3–10.8) and 2.2 months (95% CI, 0.0–5.9) (P = 0.009).

A multivariate Cox regression model was constructed with the incorporation of age, gender, performance status, and mutation types (single vs. concurrence) to evaluate the PFS. Mutation types (P = 0.032) remained as independent factor for PFS.

The median survival time of all the patients was 21.0 months (95% CI, 19.5–25.4). The OS in the single EGFR mutation and concurrent gene alterations group was 21.0 months, and 17.6 months, respectively (P = 0.170) (Figure 2).

DISCUSSION

Our data demonstrated that the frequency of co-alterations between EGFR and other driver genes (ALK, ROS1, RET, PIK3CA, BRAF, KRAS, NRAS, BRAF) in NSCLC was 6.6%. Patients of NSCLC without concurrent gene had a significantly longer PFS with EGFR-TKIs treatment. To the best of our knowledge, this is the first study demonstrating the presence of EGFR mutations concurrent with multiple gene mutations and the therapeutic efficacy of EGFR-TKIs.

Although driver genes in NSCLC were reported to be mutually exclusive [18–20], several studies have shown that driver genes occur concurrently with EGFR mutations [21–22]. In the current cohort, the frequency of concurrent EGFR/ALK mutations is 1.9%, which is consistent with previous studies reporting in the range of 0.0% to 6% [23–24]. The phosphatidylinositol 3-kinase (PI3K) plays an important role in cancer cell metabolism and proliferation. PIK3CA mutations are commonly found in a variety of cancers, with a prevalence of about 2% to 4% in NSCLC [22–25]. PIK3CA mutations co-exist mostly with KRAS mutations in lung cancer. However, the underlying mechanisms involving EGFR mutation are unclear [22]. A report by Chaft et al. included 23 lung adenocarcinoma patients with PIK3CA and 3 with EGFR concurrent mutations [26]. In the present study, 2.8% of NSCLC patients in China with EGFR mutations harbored...
Table 1: Demographic characteristics of the study population (n=320)

|                              | Number |
|------------------------------|--------|
| Gender                       |        |
| Male                         | 176    |
| Female                       | 144    |
| Age                          |        |
| Range                        | 31-78  |
| Median                       | 59     |
| <60                          | 196    |
| ≥60                          | 124    |
| Smoking status               |        |
| Never                        | 197    |
| Former/current               | 123    |
| Histology                    |        |
| Adenocarcinoma               | 302    |
| No-adenocarcinoma            | 18     |
| Stage at EGFR-TKI treatment  |        |
| IIIB                         | 5      |
| IV                           | 315    |
| Surgical history             |        |
| Yes                          | 135    |
| No                           | 185    |
| Type of EGFR-activating mutation |    |
| Exon 19 deletion             | 157    |
| Exon 21 L858R                | 142    |
| Exon 18 G719X                | 8      |
| Exon 21 L861Q                | 5      |
| Other mutation               | 8      |
| Concurrent mutation          |        |
| Yes                          | 21     |
| No                           | 299    |
| EGFR-TKIs                    |        |
| Erlotinib                    | 43     |
| Gefitinib                    | 56     |
| Icotinib                     | 220    |
| Afatinib                     | 1      |
| EGFR-TKIs in which line      |        |
| First-line                   | 76     |
| Second-line                  | 189    |
| Third-line or further-line   | 55     |
| Performance score at EGFR-TKI treatment | |
| 0-1                          | 249    |
| 2-3                          | 71     |
PIK3CA mutations. With the emerging of next generation sequencing (NGS), more and more concurrent genes were observed. One study reported by Kim et al. showed that compound EGFR mutation was frequently detected with co-mutations of EGFR actionable genes by NGS [27].

The frequency of concomitant EGFR mutations and other driver genes except ALK and PIK3CA in NSCLC were not well known, and elucidated the case report [28–29]. In our cohort, three patients with HER2, one with RET, one with KRAS and one with ROS1 gene were found, while, no BRAF and NRAS were found coexisting with EGFR mutation.

The efficacy of EGFR-TKIs treatment in patients with concomitant EGFR mutations and other driver genes is not well studied due to their rarity. The median PFS of EGFR-TKIs was 11.2 months in Yang’s study including 10 patients with co-existing EGFR/ALK mutations [30].

Relative levels of phospho-EGFR predicted the efficacy of EGFR-TKI in patients with EGFR/ALK mutations. In the current series, the median PFS of six patients with concurrent EGFR/ALK mutations was 5.0 months, which is shorter than in Yang’s study. The small sample may explain the difference of our cases and previous studies.

A concurrent PIK3CA mutation did not decreased the efficacy of EGFR-TKIs in Eng, et al. study [31], which including 10 patients of EGFR/PIK3CA co-altered. In contrast, in present cohort, we found that patients’ concurrent PIK3CA mutation may decrease the PFS and objective response, consistent with previous preclinical studies [32]. Different from Eng, et al. study, our results indicated no significantly OS difference between patients with single EGFR mutation and concurrent genes. One reason may contribute to the small sample of patients with concurrent genes. Another reason may due to the influence

### Table 2: Clinical profile of concurrent gene alterations in non-small cell lung cancer patients

| Case | Gender | Age | Smoking history | Histology       | Gene type          | EGFR-TKI/ which line | Response | PFS/ month | OS/ month |
|------|--------|-----|-----------------|-----------------|-------------------|----------------------|----------|------------|-----------|
| 1    | Male   | 44  | Yes             | Adenocarcinoma  | 19del+PIK3CA      | Gefitinib/ Second    | PR       | 10.4       | 18.7      |
| 2    | Male   | 75  | No              | Adenocarcinoma  | 19del+PIK3CA      | Icotinib/Third       | PR       | 11.2       | 20.3      |
| 3    | Female | 62  | No              | Adenocarcinoma  | L861Q+PIK3CA      | Icotinib/Second      | PD       | 1.2        | 12.5      |
| 4    | Male   | 59  | Yes             | Adenocarcinoma  | 19del+PIK3CA      | Icotinib/Third       | PR       | 7.6        | 17.6      |
| 5    | Female | 75  | No              | Adenocarcinoma  | 19del+PIK3CA      | Gefitinib/Third      | PR       | 6          | 15.5      |
| 6    | Male   | 62  | No              | Adenocarcinoma  | L858R+PIK3CA      | Icotinib/Second      | SD       | 9.5        | 16.7      |
| 7    | Male   | 67  | Yes             | Adenosquamous    | 19del+PIK3CA      | Icotinib/Third       | PR       | 9.7        | 20.5      |
| 8    | Female | 66  | No              | Adenocarcinoma  | G719X+PIK3CA      | Icotinib/Second      | PD       | 2          | 12.1      |
| 9    | Male   | 44  | Yes             | Adenocarcinoma  | 19del+PIK3CA      | Gefitinib/ Second    | PR       | 7.5        | 17.6      |
| 10   | Female | 64  | No              | Adenocarcinoma  | L858R+ALK         | Gefitinib/First      | SD       | 4.5        | 16.5      |
| 11   | Female | 40  | No              | Adenocarcinoma  | 19del+ALK         | Icotinib/Second      | PR       | 8.9        | 24.3+     |
| 12   | Male   | 64  | No              | Adenocarcinoma  | 19del+ALK         | Erlotinib/First       | PR       | 14         | 28.7      |
| 13   | Female | 59  | No              | Adenocarcinoma  | 19del+ALK         | Icotinib/Third       | PD       | 1.2        | 19.5      |
| 14   | Male   | 45  | No              | Adenocarcinoma  | 19del+ALK         | Erlotinib/First       | SD       | 5          | 18.6      |
| 15   | Male   | 64  | Yes             | Adenocarcinoma  | 19del+ALK         | Icotinib/Second      | SD       | 6.5        | 17.7      |
| 16   | Female | 65  | No              | Adenocarcinoma  | 19del++HER2       | Icotinib/Third       | PD       | 1.2        | 12.5      |
| 17   | Female | 69  | No              | Adenocarcinoma  | L861Q+HER2        | Icotinib/Second      | PD       | 1          | 6.5       |
| 18   | Female | 50  | No              | Adenocarcinoma  | 19del+HER2        | Icotinib/Second      | PR       | 14.4       | 17.7      |
| 19   | Female | 60  | No              | Adenocarcinoma  | L858R+RET         | Gefitinib/Third      | PD       | 2.2        | 10.2      |
| 20   | Male   | 63  | Yes             | Adenocarcinoma  | L858R+KRAS        | Erlotinib/First       | PD       | 1          | 6.5       |
| 21   | Male   | 49  | Yes             | Adenocarcinoma  | 19del+ROS1        | Erlotinib/First       | PR       | 24         | 47.8+     |
Table 3: Comparative analysis of clinical profile between single EGFR mutation and concurrent gene alteration patients

| Characteristics                         | Single EGFR mutation | Concurrent alteration | P    |
|-----------------------------------------|----------------------|-----------------------|------|
| Gender                                  |                      |                       | 0.80 |
| Male                                    | 165                  | 11                    |      |
| Female                                  | 134                  | 10                    |      |
| Age                                     |                      |                       | 0.02 |
| <60                                     | 188                  | 8                     |      |
| ≥60                                     | 111                  | 13                    |      |
| Smoking status                          |                      |                       | 0.62 |
| Never                                   | 183                  | 14                    |      |
| Former/current                          | 116                  | 7                     |      |
| Histology                               |                      |                       | 0.73 |
| Adenocarcinoma                          | 288                  | 20                    |      |
| No-adenocarcinoma                       | 11                   | 1                     |      |
| Stage at EGFR-TKI treatment             |                      |                       | 0.75 |
| IIIB                                    | 5                    | 0                     |      |
| IV                                      | 294                  | 21                    |      |
| EGFR mutation type                      |                      |                       | 0.57 |
| Exon 19 deletion+Exon 21 L858R          | 280                  | 19                    |      |
| Other types                             | 19                   | 2                     |      |
| Performance score at EGFR-TKI treatment |                      |                       | 0.20 |
| 0-1                                     | 235                  | 14                    |      |
| 2-3                                     | 64                   | 7                     |      |

Table 4: Clinical efficacy comparison of EGFR-TKI in single EGFR mutation and concurrent gene alterations

| Best response | Single EGFR mutation (n=299) | Concurrent gene alterations (n=21) | P    |
|---------------|------------------------------|----------------------------------|------|
| CR            | 1(0.3%)                      | 0(0.0%)                          | 0.79 |
| PR            | 186(62.2%)                   | 10(47.6%)                        | 0.18 |
| SD            | 67(22.4%)                    | 4(19.0%)                         | 0.25 |
| PD            | 46(15.4%)                    | 7(33.3%)                         | 0.03 |
| ORR           | 62.5%                        | 47.6%                            | 0.17 |
| DCR           | 84.9%                        | 66.7%                            | 0.03 |
| Median PFS(month) | 10.9                    | 6.0                               | 0.002|
| Median OS(month)    | 21.0                        | 17.6                             | 0.17 |
of additional treatment after failure of EGFR-TKI therapy. In our cohort, four of six patients with ALK rearrangement received the crizotinib treatment after failure of EGFR-TKI treatment and three with partial response. However, no further treatment data were provided in Eng, et al study. The influence of concurrent genes to overall survival should be validated with large number patients in the future studies.

The most remarkable shortcomings of our study were related to the small sample size of concurrent genes. Secondly, MET amplification, mutation and other genes like NTRK1 and PTEN, which may coexist with EGFR

Figure 1: Comparison of progression free survival with EGFR-TKI treatment between single EGFR mutation and concurrent gene alterations patients (10.9 vs. 6.0 months, *P* = 0.002).

Figure 2: Comparison of overall survival with EGFR-TKI treatment between single EGFR mutation and concurrent gene alterations patients (21.0 months vs. 17.6 months, *P* = 0.170).
gene in EGFR-TKIs treatment-naive samples, were not detected in current study for lack of sufficient tumor tissues. Thirdly, only five cases were treated with inhibitor focus on the coexisting gene (four with ALK and one with ROS1), so, the clinical efficacy of further treatment after failure of EGFR-TKIs could not be fully evaluated. Lastly, age and performance score imbalance were found between the single EGFR mutation and coexisting gene group, which may influence the outcome analysis in present study (Table 3). However, as the first study investigating the role of multiple genes in EGFR mutant patients, the findings are clinically meaningful.

In conclusion, this is the first study to focus on the predictive value of concurrent EGFR and other mutations in driver genes for EGFR-TKIs treatment. It suggests that some of the genes concomitant with EGFR mutation might decrease the efficacy of EGFR-TKIs treatment. In the future, prospective studies must validate the efficacy of different therapies for concurrent gene alterations NSCLC patients.

MATERIALS AND METHODS

Patient selection

Four hundred and twenty-nine consecutive patients who carried sensitive EGFR mutations and underwent EGFR-TKIs treatment for advanced NSCLC at Ningbo First Hospital were screened between 2009 and 2013. Of the 429 patients, 109 were ineligible because of a lack of tumor tissue for analysis of 8 genes. In 320 patients with identified genes, 135 patients had formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks obtained at the time of surgical resection, 157 were tissue biopsies, and 38 with malignant effusion. Among the 320 samples, 270 of the samples used for 8 genes detection were obtained from the remaining tissues of EGFR gene analysis and 50 were re-obtained before EGFR-TKIs treatment. Ethics Committee of Ningbo First Hospital approved this study and a written informed consent was obtained from each participant.

Gene detection

Amplification refractory mutation system (ARMS)-based EGFR mutation detection kit (Amoy, Xiamen, China) was used to detect EGFR mutation in all patients. The ARMS kit is able to detect 29 mutations: three in exon 18 (G719A, G719C and G719S; the kit was unable to distinguish between these subtypes, which are referred to as G719X hereafter), 19 deletions in exon 19, two mutations in exon 20 (T790M, S768I), three insertions in exon 20, and two mutations in exon 21 (L858R, L861Q).

A microscopy was used to patients with EGFR-mutated ensure the tumor tissues analyzed had more than 20% tumor contents. Genomic DNA or RNA was extracted from tumor tissues according to standard protocols (RNase Mini Kit, and QiAamp DNA Mini Kit, Qiagen, Hilden, Germany). Briefly, the isolated RNA samples were used for reverse transcription into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). Either genomic DNA or cDNA was used for PCR amplification and sequencing. HER2, KRAS, NRAS, BRAF, and PIK3CA were PCR amplified using genomic DNA. Cycle sequencing of the purified PCR products was carried out with PCR primers using the commercially available ADx Mutation Detection Kits (Amoy, Xiamen, China).

The ALK, ROS1, and RET fusion mRNAs were detected by PCR with fusion gene detection kit (Amoy, Xiamen, China). In brief, total RNA was extracted with Qia-genRNeasy FFPE Kit. The mRNA was reverse-transcribed to cDNA at 42°C for 1 hour. β-actin was used as the internal control. The RT-PCR conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 95°C for 25 seconds, 64°C for 20 seconds, and 72°C for 20 seconds to ensure the specificity; and 31 cycles at 93°C for 25 seconds, 60°C for 35 seconds, 72°C for 20 seconds were performed for data collection and sensitivity analysis, as detailed in previous study [17]. All of the partners which could be detected were attached as Supplementary Table 1.

Efficacy evaluation

Tumors were evaluated during EGFR-TKIs treatment every 8 weeks, or were evaluated early when significant signs of progression appeared. Objective tumor responses were determined according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Objective responses rate (ORR) includes complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Disease control rate (DCR) is defined as the addition of objective response and stabilization rates (CR+ PR+SD).

Statistical analysis

Survival curves were calculated using the Kaplan-Meier method from the start of diagnosis of advanced NSCLC until death or last follow-up. PFS of EGFR-TKIs was defined as the time from EGFR-TKIs therapy to documented progression or death from any cause. Statistical analysis was performed with the SPSS 16 software (Chicago, IL, US). P < 0.05 was considered statistically significant. The median follow-up period was 23.5 months (7.5-65) and the last follow-up was on April 31, 2015.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.
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