Lung cancers with concomitant EGFR mutations and ALK rearrangements: diverse responses to EGFR-TKI and crizotinib in relation to diverse receptors phosphorylation

Jin-Ji Yang¹*, Xu-Chao Zhang¹,²*, Jian Su², Chong-Rui Xu¹, Qing Zhou¹, Hong-Xia Tian², Zhi Xie², Hua-Jun Chen¹, Yi-Sheng Huang¹, Ben-Yuan Jiang¹, Zhen Wang¹, Bin-Chao Wang¹, Xue-Ning Yang¹, Wen-Zhao Zhong¹, Qiang Nie¹, Ri-Qiang Liao¹, Tony S. Mok³ and Yi-Long Wu¹,²

¹Division of Pulmonary Oncology, Cancer Center, Guangdong Lung Cancer Institute, Guangdong General Hospital & Guangdong Academy of Medical Sciences, Guangzhou, P. R. China

² Medical Research Center, Guangdong General Hospital & Guangdong Academy of Medical Sciences, Guangzhou, P. R. China

³ State Key Laboratory of Southern China, The Chinese University of Hong Kong, Sir YK Pau Cancer Center, Prince of Wales Hospital, Hong Kong, China

* These authors contributed equally to this work

Financial support: Financial support was provided by the following grants: National Natural Science Foundation of China Grants No. 81071699 (to Xu-chao Zhang) and No. 30772531 (to Yi-Long Wu) and No. 81172090 (to Qing Zhou); Guangzhou Science and Technology Project No. 11BppZXaa6040020 (to Yi-Long Wu)
Wu); Wu Jieping Medical Foundation Special for Tumor Targeted Therapy Research. No. 3206720.10001 (to Qing Zhou)

Corresponding author:
Yi-Long Wu, M.D., Guangdong Lung Cancer Institute, Guangdong General Hospital & Guangdong Academy of Medical Sciences, 106 Zhongshan 2nd Road, Guangzhou 510080, China. Phone: +86 20 83877855; Fax: +86 20 83827712; e-mail: syylwu@live.cn

Tony S. Mok, M.D., State Key Laboratory of Southern China, The Chinese University of Hong Kong, Sir YK Pau Cancer Center, Prince of Wales Hospital, Hong Kong, China. Telephone: 86-00852-91987497; Fax: 86-00852-91987497; e-mail: tony@clo.cuhk.edu.hk

Running title: NSCLC with concomitant EGFR mutations and ALK rearrangements

Key words: Lung cancer, Tyrosine kinase inhibitors, EGFR, ALK, EML4-ALK

Disclosures of potential conflicts of interest: All authors completed the disclosure declaration. None of the authors have potential conflicts of interest.

Word count: 3185 (excluding references)
Translational Relevance

Both \textit{EGFR} mutation and \textit{ALK} rearrangement define molecular subgroups of non-small cell lung cancer (NSCLC) that can significantly benefit from EGFR TKI (gefitinib and erlotinib) and ALK TKI (crizotinib). With increased sensitivity of molecular assays and expanded list of driver gene mutations in clinical diagnostic workup, more and more co-altered driver genes could be found. This study describes the co-altered \textit{EGFR} and \textit{ALK} in a large cohort of NSCLC, finding that 3.9\% (13/336) of \textit{EGFR} mutant and 18.6\% (13/70) of \textit{ALK} rearranged tumors have co-alterations. ALK fusion proteins and EGFR mutant proteins co-existed in the same tumor cells. Tumors harboring co-altered \textit{EGFR} and \textit{ALK} could have diverse responses to first-line EGFR-TKIs, which were associated with phospho-EGFR levels. Phospho-ALK levels correlated efficacy of subsequent crizotinib treatment. In clinical practice we should pay attention to the specific biological behavior and corresponding management of NSCLC with dual altered genes of \textit{EGFR} and \textit{ALK}. 
ABSTRACT

Purpose:

We investigated the incidence of concomitant *EGFR* mutations and *ALK* rearrangements in Chinese patients with non-small-cell lung cancer (NSCLC), and assessed responses to EGFR tyrosine kinase inhibitors (EGFR-TKIs) and crizotinib in such tumors.

Experimental Design:

We screened 977 consecutive patients with NSCLC for the presence of concomitant *EGFR* mutations and *ALK* rearrangements by rapid amplification of cDNA ends-coupled polymerase chain reaction sequencing and fluorescence *in situ* hybridization. Immunohistochemistry (IHC) and Western blotting were used to correlate the activation of EGFR, ALK and downstream proteins with responses to EGFR-TKIs and crizotinib.

Results:

The overall frequency of concomitant *EGFR* mutations and *ALK* rearrangements was 1.3% (13/977). *EGFR/ALK* co-alterations were found in 3.9% (13/336) *EGFR*-mutant and 18.6% (13/70) *ALK*-rearranged patients. Ten tumors were treated with first-line EGFR-TKIs, with a response rate of 80% (8/10). Two tumors with high phospho-ALK levels and low phospho-EGFR levels achieved stable and progressive disease, respectively. Median progression-free survival was 11.2 months. Co-expression of mutant EGFR and ALK fusion proteins in the same tumor cell populations was detected by IHC. Two cases with high phospho-ALK
levels treated with crizotinib achieved partial responses; two cases with low phospho-ALK levels had progressive or stable disease.

**Conclusion:**

ALK rearrangements and EGFR mutations could co-exist in a small subgroup of NSCLC. Advanced pulmonary adenocarcinomas with such co-alterations could have diverse responses to EGFR-TKIs and crizotinib. Relative phospho-ALK and phospho-EGFR levels could predict the efficacy of EGFR-TKI and crizotinib.
INTRODUCTION

Lung cancer accounts for a large number of deaths caused by cancer worldwide (1). Similar to tumors with epidermal growth factor receptor (EGFR) mutations, non-small-cell lung cancer (NSCLC) with anaplastic lymphoma kinase (ALK) rearrangements are a molecular subgroup that could benefit from crizotinib (2). Fusion of ALK with the echinoderm microtubule-associated protein-like 4 (EML4) gene was first identified in 2007 and the incidence of ALK rearrangements ranged from approximately 3%~13% in unselected or selected patients with NSCLC (3-5). ALK rearrangements and EGFR mutations have largely been reported to be mutually exclusive (3-5), and as mutual causes of resistance to EGFR tyrosine kinase inhibitors (TKIs) or ALK-TKIs (6, 7). However, such co-alterations did co-exist in some clinical cases (3, 8, 9). Whereas the EGFR mutation rate is higher in East Asian patients as compared with Caucasians (10, 11), co-existence of ALK rearrangements might be more common in East Asian EGFR mutant patients. Both EGFR-TKIs and ALK-TKI have been approved as standards of care for EGFR- or ALK- altered disease. NSCLC patients with such co-alterations deserve more attention than before. The prevalence and clinical relevance of co-alterations in these two driver genes require detailed investigation.

First-line EGFR-TKIs in EGFR mutant NSCLC have been shown to be superior to chemotherapy in terms of response rate, progression-free survival (PFS), and quality of life (12-19). Patients with ALK rearrangements could greatly benefit from crizotinib in terms of response (2). However, for patients with
concomitant *EGFR* mutations and *ALK* fusions, few data are available regarding the clinical activity of EGFR-TKIs and ALK-TKIs, except for limited studies showing conflicting results in terms of the response to EGFR-TKI (20-22). The present study was performed to determine the prevalence of *EGFR/ALK* co-alterations in NSCLC. In addition, we sought to evaluate the clinical activity of EGFR-TKIs and crizotinib and the possible mechanisms in patients with co-alterations.

**MATERIALS AND METHODS**

**Study design**

We prospectively screened consecutive patients from January 2010 through November 2011, for *EGFR* and *KRAS* mutations and *ALK* rearrangements at Guangdong Lung Cancer Institute (GLCI), Guangdong General Hospital (GGH). Histologically proven NSCLC patients with sufficient tissue were eligible to be enrolled in this study. The prevalence of *EGFR/ALK* co-alterations and protein expression levels of mutant *EGFR*, rearranged *ALK*, phospho-EGFR, phospho-ALK, and downstream molecules were investigated. Objective responses to EGFR-TKI and crizotinib and PFS were also assessed. This study was approved by the Institutional Review Board at GLCI of GGH, and all patients provided specimens with written informed consents.

**Treatment and evaluation**
All advanced patients harboring \textit{EGFR/ALK} co-alterations received first-line EGFR-TKIs, except for one case enrolled in the crizotinib trial after first-line platinum-based chemotherapy. EGFR-TKIs included gefitinib (250 mg, po, qd), erlotinib (150 mg, po, qd) and afatinib (50mg, po, qd). Objective responses were assessed every 6 to 8 weeks according to RECIST (Response Evaluation Criteria In Solid Tumors) \cite{23,24}. PFS was measured from the initiation of EGFR-TKI or crizotinib treatment until radiologic or clinical progression. Four patients were recruited into the A8081005 (NCT0093245) or A8081007 (NCT0093289) trial evaluating crizotinib.

\textbf{EGFR and KRAS mutation analysis by direct sequencing}

Genomic DNA from each sample was used for sequence analysis of \textit{EGFR} exons 18, 19, 20, and 21, and \textit{KRAS} exons 2 and 3. These exons were amplified by PCR as previously described \cite{25}, and the resulting PCR products were purified and labeled for sequencing using the BigDye 3.1 kit (Applied Biosystems) according to the manufacturer’s protocol.

\textbf{RT-PCR and RACE-PCR sequencing for ALK fusion analysis}

Total RNA was extracted from lung tissue samples using the RNeasy kit (QIAGEN, Valencia, CA). Reverse-transcriptase polymerase chain reaction (RT-PCR) and 5’ rapid amplification c-DNA ends (RACE)-coupled PCR plus sequencing was conducted as reported previously \cite{8}. PCR products were then
sequenced using a 3730XL Genetic Analyzer (Applied Biosystems). Target sequences of interest were aligned with the ALK reference sequence (NM_004304.3) to determine if a fusion with another gene was present.

**Fluorescent in situ hybridization (FISH) assays for ALK rearrangement**

Tumor histology was classified using the World Health Organization (WHO) criteria. Interphase molecular cytogenetic studies using a commercially available ALK probe (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular, Abbott Park, IL) were performed on 4-μm-thick paraffin-embedded sections. Samples were deemed to be FISH-positive if more than 15% of scored tumor cells had split ALK $5'$ and $3'$ probe signals or isolated $3'$ signals (26).

**Immunohistochemistry (IHC) for mutant EGFR, ALK, and downstream molecules**

IHC was conducted to detect the protein expression in serial sections from formalin-fixed paraffin-embedded (FFPE) tumor samples, according to the protocols recommended by the manufacturer of the anti-mutant-EGFR and anti-ALK antibodies (Cell Signaling Technology, Danvers, MA). Rabbit monoclonal anti-human ALK antibody (#3633 WP1-01; clone D5F3) was applied at a dilution of 1:100. Staining intensity was scored from 0 to 3+. Tumors with 1+, 2+, or 3+ expression were deemed to be positive for ALK protein expression; tumors with no expression (0) were deemed to be negative(27, 28).
Western blotting for signaling proteins

Fresh tumor tissues were homogenized and re-suspended in lysis buffer (20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM EGTA), incubated on ice for 10 min, and centrifuged for 5 min (15,000 rpm). Protein concentration determination and immunoblotting were performed according to the manufacturer’s protocol using antibodies against total EGFR, phospho-EGFR (p-EGFR Y1068), total ALK, phospho-ALK (p-ALK Y1604), total AKT, phospho-AKT (p-AKT S473/T308), total ERK, and phospho-ERK1/2 (p-ERK T202/Y204) (Cell Signaling Technology, Danvers, MA, USA).

Statistical Analysis

The $\chi^2$ test was used to compare frequencies of molecular alterations. $P < 0.05$ was deemed statistical significance. Kaplan-Meier curves were used to estimate PFS. General data analysis was conducted using SPSS version 13.0 (SPSS Institute, Cary, NC).

RESULTS

Patient Characteristics

A total of 977 NSCLCs were screened and 336 (32.7%), 70 (6.8%), and 40 (3.9%) patients had EGFR mutations, ALK fusions or rearrangements and KRAS mutations respectively. Thirteen patients harbored concomitant EGFR mutations and ALK fusions. All of these 13 cases were adenocarcinomas, never or light
smokers, of advanced stage, and as old as patients positive for ALK rearrangements alone ($P = 0.218$) (Table 1). Five cases had acinar growth patterns and two had solid growth patterns of adenocarcinoma, with 42.8% (3/7) having signet cells. RT-PCR or RACE-PCR followed by sequencing identified EML4-ALK variants in 10 cases with sufficient tissues, with 5 of E13:A20 (V1), 2 of E6a/E6b:A20 (V3a/V3b), 1 of E14:ins124A20 (V4b), 1 of E2:A20 (V5), and 1 of E13:ins90A20 (V6b) (Supplementary Figure 1).

The overall frequency of $EGFR/ALK$ co-alterations was 1.3% (13/977). Of note, the prevalence of co-alterations was 3.9% (13/336) in $EGFR$ mutant patients and 18.6% (13/70) in ALK-positive patients respectively. A single representative patient with co-alterations in $EGFR$ and $ALK$ is shown in Figure 1. These data indicate that driver alterations of $EGFR$ and $ALK$ could co-exist in a small group of NSCLC, and more frequent in ALK-positive tumors.

**Efficacy of EGFR-TKI and crizotinib in NSCLCs with $EGFR/ALK$ co-alterations**

Eleven of the 13 cases with $EGFR/ALK$ co-alterations had evaluable clinical data. Of the 10 patients receiving first-line EGFR-TKIs, 8 achieved partial response (PR) (4, 3, and 1 treated with erlotinib, gefitinib and afatinib respectively); 1 attained stable disease (SD) after afatinib treatment; and 1 patient treated with erlotinib had progressive disease (PD). The objective response rate was 80% (8/10). The last follow-up date was January 5, 2012 and the median follow-up
duration was 29 months (range, 17.5~40.2 months). Eight patients had PD and then stopped EGFR-TKI treatment. Median PFS for first-line EGFR-TKIs was 11.2 months [95% confidence interval (CI), 6.6-15.8] (Figure 2).

Four patients entered trials to receive crizotinib therapy. Three cases having experienced first-line EGFR-TKIs were treated with crizotinib later during disease course. Among them one was de novo resistant to EGFR-TKI, but responsive to crizotinib, while two were responsive to EGFR-TKI but not responsive to crizotinib. One case achieved PR and 15.1 months of PFS after the initiation of crizotinib, but did not respond to subsequent EGFR-TKI (Table 2).

**Co-expression and co-localization of mutant EGFR and ALK fusion proteins in tumor cells**

To determine the potential expression pattern of EGFR mutant protein in relation to ALK fusion proteins, we tested the two oncoproteins by IHC analysis of serial sections in 10 cases with sufficient FFPE slides. Specific antibodies detected mutant EGFR protein in seven cases, but not in the three cases with mutation types other than exon 19 del of 746E-750A or L858R of EGFR. All seven cases showed EGFR mutant protein co-expressed and co-localized with ALK fusion proteins in the same cell population, although with diverse signal intensities, indicating that these two driver oncoproteins might co-operate in the same cancer cells.

To investigate the activation status of the two driver oncogenes in cancers
with EGFR/ALK co-alterations, EGFR (Y1068) and ALK (Y1604) phosphorylation levels were also assessed by IHC. Three patterns are shown in Figure 3: high p-EGFR and high p-ALK, high p-EGFR and low p-ALK, and low p-EGFR and high p-ALK.

**Correlation of clinical efficacy of EGFR-TKI or ALK-TKI with relative activation of EGFR or ALK**

To identify the molecular characteristics underscoring the efficacy of EGFR-TKI and ALK-TKI in these patients, we carefully checked the relative activation status of EGFR and ALK proteins by IHC analysis of phosphorylated proteins (and Western blotting if there was sufficient tissue) (Figure 3). Of the eight cases treated with first-line EGFR-TKI, six with high levels of p-EGFR had PRs to EGFR-TKI and two with very low levels of p-EGFR (+/-) had PD or SD. Of the four cases treated with crizotinib, two (P7 and P13) had relatively inactivated p-EGFR (-,+) and highly activated p-ALK (+++,+++); one of them showed no benefit from EGFR-TKI, but a PR to third-line crizotinib, and the other was very responsive to crizotinib, but resistant to subsequent EGFR-TKI. In contrast, two cases (P8 and P9) had relative high p-EGFR levels (++,+++), P8 and low p-ALK levels (-,+), corresponding to PR to first-line EGFR-TKI, but no benefit or short-term SD from crizotinib.

Western blotting yielded similar results of IHC in three cases (P4, P6, and P7) (Figures 1 and 4). Expression of both p-EGFR and p-ALK was consistent with the
IHC data in cases P4 and P6. Notably, in treatment-naïve tissue from case P7 there were high levels of p-ALK and relatively low p-EGFR levels. After PD to EGFR-TKI and PR to crizotinib, levels of p-EGFR, p-ALK, and p-AKT in the autopsied pulmonary lesions were increased, although p-ERK levels were significantly reduced (Figure 4). Overall, relative baseline EGFR and ALK activation correlated with the efficacy of EGFR-TKIs or crizotinib in these patients.

DISCUSSION

Although ALK rearrangements and EGFR mutations were previously reported to be mutually exclusive (4, 26, 29-35), several studies have shown that ALK fusions can occur concurrently with EGFR mutations (1/305, 0.3% or 1/103, 1.0% or 4/444, 0.9%) (8, 9, 30). Our data demonstrated that the frequency of EGFR/ALK co-alterations in NSCLC was 1.3% (13/977), which is consistent with our previous study (8). However, the frequency of such co-alterations was not described in three case reports (20-22). Janne et al reported that 6% (3/50) of ALK-positive and crizotinib-naive NSCLCs had concurrent EGFR mutations (6). In contrast, our study showed a frequency of 18.6% (12/70) for such concomitant alterations in ALK-rearranged NSCLCs. Here, we also showed a co-alteration rate of 3.9% (12/336) in patients with EGFR mutations, which is lower than that of 15.8% (15/95) from Rosell’s report at 2012 ESMO conference (36). Thus, the frequency of such co-alterations was considerably high in ALK-positive or EGFR-mutant patients and possibly higher in ALK-positive Chinese patients as
compared with Caucasians. This observation may be of clinical relevance in terms of treatment strategies because this subgroup has a specific genotype with dual therapeutical targets.

Two or more mutations of driver genes could exist concurrently in NSCLC. In Lung Cancer Mutation Consortium (LCMC) project, 5% of driver alterations in lung adenocarcinoma were concurrently double or multiple mutations (37). Lipson et al. also identified 50 alterations in 21 genes, with at least one alteration being present in 83% (20 out of 24) of the lung cancers (with a range of 1–7 alterations) (38). Of note, with the development of more sensitive technologies and parallel testing of multiple molecules, more concomitant alterations will be identified in a single test of a given clinical specimen. Co-existence of multiple driver mutations has been taken into consideration by oncologists to obtain an in-depth understanding of cancer mechanisms and for therapeutic developments, such as combinational targeting the molecular driver “hubs” of a cancer (39, 40). How to treat this subgroup may critically depend on the biological roles of these onco-drivers.

Previous studies revealed that patients with EGFR/ALK co-alterations demonstrated no ALK expression by IHC (6, 21). However, in our study, IHC of serial sections showed co-expression and co-localization of mutant EGFR and ALK fusion proteins in the same cell population in all seven evaluable patients, although staining intensities varied greatly. Our finding of co-localization was consistent with other studies of cell lines, indicating that the two driver alterations could develop in the same clone of tumor cells and might co-operate during cancer
development (30). Clarification of the dominant driver receptor(s) is critical to understanding the disease mechanism and clinical decision-makings. In our study four patients with co-alterations responded only to either of an EGFR TKI or ALK TKI at different time points, suggesting that one of these oncogenes might act as a “dominant” driver. To address this point, phosphorylation of both EGFR and ALK was evaluated by IHC and three patterns could be observed: “high p-EGFR and high p-ALK”, “high p-EGFR and low p-ALK” and “low p-EGFR and high p-ALK”. IHC data showing altered onco-proteins expression and phosphorylation were confirmed by Western blotting in two cases. Differential phosphorylation of EGFR or ALK might contribute to differences in sensitivity to EGFR-TKIs or crizotinib in this subgroup. Therefore, we further correlated the efficacy of TKIs with the relative activation status of these receptor kinases.

The objective response rate (80%, 8/10) and median PFS (11.2 months; 95% CI, 5.6-16.8) for first-line EGFR-TKI in EGFR/ALK co-altered tumors were similar to those in previous studies (12-17). Preclinical studies showed co-expression of altered EGFR and ALK in vitro lead to mutual resistance to single-agent ALK or EGFR TKI (6). In contrast, response to either EGFR or ALK-TKI for our patients with co-alterations was achieved. Interestingly, we found that efficacy of first-line EGFR-TKI was associated with EGFR phosphorylation level. Among the four cases treated with both an EGFR-TKI and an ALK-TKI, P7 and P13, with a baseline “low p-EGFR and high p-ALK”
expression pattern, had de novo or subsequent resistance to EGFR-TKI treatment, but were responsive to ALK-TKI. Alternatively, P8 and P9, with a baseline “high p-EGFR and low p-ALK” expression pattern, achieved PRs to first-line EGFR-TKI, but had PD or SD following crizotinib treatment. Thus the baseline relative activation of ALK and EGFR was associated with the efficacy of EGFR-TKI and ALK-TKI treatment. To our knowledge, this is the first cohort study showing diverse responses to first-line EGFR-TKIs in patients harboring EGFR/ALK co-alterations. In previous studies, five cases with such co-alterations were treated with EGFR-TKIs (one with first-line gefitinib (20), two with second-line erlotinib (21, 22), and two with unspecified-line erlotinib (6)), 80% (4/5) achieved PRs, similar to our results. A Caucasian patient with lung adenosquamous carcinoma harboring such co-alterations was reported resistant to second-line erlotinib treatment.(22) No expression of ALK protein tested by IHC in three cases of these studies might be due to false positive results of FISH testing. (6, 21) In contrast, ALK protein was detected in our study. We suggest that a relative increase in the p-EGFR level would contribute to a favorable response to first-line EGFR-TKI in this subgroup, although whether the level of benefit of EGFR-TKI is similar to that in patients with pure EGFR mutations requires further evidence (41).

The shortcomings of our study were the small sample size concerning crizotinib treatment and p-EGFR/p-ALK testing, non-prospective design, and the fact that it was not multi-institutional. No NSCLC cell lines with EGFR/ALK co-alterations were used to model the relative activation statuses of driver
receptors in relation to the efficacy of targeted therapies. Moreover, re-biopsy at serial time-points would be helpful to clarify the resistance mechanisms and dynamic changes of mutations of these driver molecules in cancer (37, 42).

In summary, the frequency of concomitant EGFR mutations and ALK rearrangements was significantly higher in ALK-rearranged NSCLCs. EGFR/ALK co-alterations could define a specific subgroup that had diverse, although mostly favorable, responses to first-line EGFR-TKIs. Testing of the relative phosphorylation levels of EGFR and ALK might help to guide the selection of TKIs in clinical practice. Molecular mechanisms underlying responsiveness and resistance to EGFR-TKIs and ALK-TKIs, and potential combination or sequential treatment modes, require further investigation in this specific subgroup with co-alterations.

AUTHOR CONTRIBUTIONS

Conception and design: Yi-Long Wu, Tony S. Mok, Jin-Ji Yang, Xu-Chao Zhang

Financial support: Yi-Long Wu

Administrative support: Yi-Long Wu

Provision of study materials or patients: Jin-Ji Yang, Xu-Chao Zhang

Collection and assembly of data: Chong-Rui Xu, Qing Zhou, Hua-Jun Chen, Yi-Sheng Huang, Zhi Xie, Ben-Yuan Jiang, Zhen Wang, Bin-Chao Wang, Xue-Ning Yang, Wen-Zhao Zhong, Qiang Nie, Ri-Qiang Liao, Tony S. Mok

Data analysis and interpretation: Xu-chao Zhang, Jian Su, Hong-Xia Tian, Tony
ACKNOWLEDGEMENTS

We thank Wei-Bang Guo, Shi-Liang Chen, Ying Huang, and Shi-Jiang Fei for their work on biomarker analysis. We also thank Bin Gan, Yan Yang, Xiao-Ying Zheng, Su-Fen Luo, Xuan Li, and Yu-Mei Chen for their assistance in collecting clinical follow-up data.

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see:
http://www.textcheck.com/certificate/thiRnD

REFERENCES


Table 1. Baseline clinicopathologic features among patients with *EGFR* mutations, *ALK* rearrangements, and *EGFR/ALK* co-alterations

<table>
<thead>
<tr>
<th>Variable category</th>
<th>EGFR mutation (n = 324)</th>
<th>ALK rearrangement (n = 57)</th>
<th>Co-altered EGFR and ALK (n = 13)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median, range)</td>
<td>59 (29–83)</td>
<td>52 (25–77)</td>
<td>59 (31–71)</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (%)</td>
<td>178 (55%)</td>
<td>26 (46%)</td>
<td>8 (62%)</td>
<td>0.364</td>
</tr>
<tr>
<td>Male (%)</td>
<td>146 (45%)</td>
<td>31 (54%)</td>
<td>5 (38%)</td>
<td>0.364</td>
</tr>
<tr>
<td>Smoking habit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never (%)</td>
<td>251 (77%)</td>
<td>45 (79%)</td>
<td>12 (92%)</td>
<td>0.532</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>73 (23%)</td>
<td>12 (21%)</td>
<td>1 (8%)</td>
<td>0.532</td>
</tr>
<tr>
<td>ECOG PS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1 (%)</td>
<td>309 (95%)</td>
<td>52 (91%)</td>
<td>12 (92%)</td>
<td>0.103</td>
</tr>
<tr>
<td>2-3 (%)</td>
<td>15 (5%)</td>
<td>5 (9%)</td>
<td>1 (8%)</td>
<td>0.103</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma (%)</td>
<td>310 (96%)</td>
<td>54 (95%)</td>
<td>13 (100%)</td>
<td>0.848</td>
</tr>
<tr>
<td>Other NSCLC (%)</td>
<td>14 (4%)</td>
<td>3 (5%)</td>
<td>0 (0%)</td>
<td>0.848</td>
</tr>
<tr>
<td>p-Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II (%)</td>
<td>71 (22%)</td>
<td>4 (7%)</td>
<td>0 (0%)</td>
<td>0.004*</td>
</tr>
<tr>
<td>III–IV (%)</td>
<td>253 (78%)</td>
<td>53 (93%)</td>
<td>13 (100%)</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; NSCLC, non-small-cell lung cancer; p, pathological.

*Age:*  
EGFR mutation vs. *ALK* rearrangement (*P* < 0.001)  
EGFR mutation vs. Co-altered *EGFR* and *ALK* (*P* = 0.409)  
*ALK* rearrangement vs. Co-altered *EGFR* and *ALK* (*P* = 0.218)  

*P-Stage*  
EGFR mutation vs. *ALK* rearrangement (*P* = 0.009)  
EGFR mutant vs. Co-altered *EGFR* and *ALK* (*P* = 0.120)  
*ALK* rearrangement vs. Co-altered *EGFR* and *ALK* (*P* = 1.000)
Table 2. Molecular and demographic characteristics and efficacy of EGFR-TKIs and crizotinib in 13 patients with co-alterations

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
<th>P8</th>
<th>P9</th>
<th>P10</th>
<th>P11</th>
<th>P12</th>
<th>P13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>44</td>
<td>56</td>
<td>59</td>
<td>67</td>
<td>70</td>
<td>40</td>
<td>65</td>
<td>65</td>
<td>54</td>
<td>60</td>
<td>31</td>
<td>66</td>
<td>45</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ECOG PS</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Histology/features</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td></td>
<td>acinar</td>
<td>acinar</td>
<td>acinar</td>
<td>acinar</td>
<td>solid with signet cells</td>
<td>solid with signet cells</td>
<td>acinar</td>
<td>acinar</td>
<td>acinar</td>
<td>acinar</td>
<td>acinar</td>
<td>acinar</td>
<td></td>
</tr>
<tr>
<td>Clinical staging</td>
<td>IV</td>
<td>IV</td>
<td>IV</td>
<td>IIIA</td>
<td>IV</td>
<td>IV</td>
<td>IIIA</td>
<td>IV</td>
<td>IV</td>
<td>IV</td>
<td>IV</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>KRAS</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>EGFR mutation rearrangement</td>
<td>DEL</td>
<td>L858R</td>
<td>L858R</td>
<td>DEL</td>
<td>L858R</td>
<td>DEL</td>
<td>Exon20</td>
<td>DEL</td>
<td>DEL</td>
<td>DEL</td>
<td>K757R</td>
<td>L858R</td>
<td>DEL</td>
</tr>
<tr>
<td>FISH†</td>
<td>+,18%</td>
<td>+,21%</td>
<td>+,31%</td>
<td>+,33%</td>
<td>+,26%</td>
<td>+,19%</td>
<td>+,40%</td>
<td>+,35%</td>
<td>+,28%</td>
<td>+,44%</td>
<td>+,29%</td>
<td>+,22%</td>
<td>+,52%</td>
</tr>
<tr>
<td>IHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 2. Molecular and demographic characteristics and efficacy of EGFR-TKIs and crizotinib in 13 patients with co-alterations.*

**Note:** The table includes columns for age, sex, smoking history, ECOG PS, histology/features, clinical staging, KRAS, EGFR mutation, ALK rearrangement, RACE-PCR, FISH, and IHC. The data are presented for 13 patients, with each patient having specific values for each characteristic.
<table>
<thead>
<tr>
<th>Mutant EGFR</th>
<th>+++</th>
<th>+++</th>
<th>+</th>
<th>++</th>
<th>ND</th>
<th>NA</th>
<th>NA</th>
<th>NA</th>
<th>++</th>
<th>+++</th>
<th>ND</th>
<th>ND</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK fusion</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>ND</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
<td>+++</td>
</tr>
<tr>
<td>p-EGFR</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>ND</td>
<td>+++</td>
<td>+/-</td>
<td>++</td>
<td>+++</td>
<td>+/-</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>p-ALK</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>ND</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>+++</td>
</tr>
<tr>
<td>First-line</td>
<td>gefitinib</td>
<td>gefitinib</td>
<td>erlotinib</td>
<td>ND</td>
<td>erlotinib</td>
<td>erlotinib</td>
<td>erlotinib</td>
<td>afatinib</td>
<td>erlotinib</td>
<td>afatinib</td>
<td>ND</td>
<td>gefitinib</td>
<td>ND</td>
</tr>
<tr>
<td>EGFR-TKI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Best response</td>
<td>PR</td>
<td>PR</td>
<td>PR</td>
<td>NA</td>
<td>PR</td>
<td>PR</td>
<td>PD</td>
<td>PR</td>
<td>PR</td>
<td>SD</td>
<td>NA</td>
<td>PR</td>
<td>NA</td>
</tr>
<tr>
<td>PFS (months)</td>
<td>9.0</td>
<td>11.2</td>
<td>13.0</td>
<td>NA</td>
<td>27.4*</td>
<td>17.5</td>
<td>1.5</td>
<td>5.0</td>
<td>12.0</td>
<td>7.0</td>
<td>NA</td>
<td>24.5</td>
<td>NA</td>
</tr>
<tr>
<td>Crizotinib</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Best response</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>PR</td>
<td>PD</td>
<td>SD</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>PR</td>
</tr>
<tr>
<td>PFS (months)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.9§</td>
<td>0.4</td>
<td>2.7</td>
<td>NA</td>
<td>NA</td>
<td>15.1</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male; AC, adenocarcinoma; WT, wild-type; DEL, exon 19 deletion; Exon20, exon 20 insertion; K757R, K757R in exon 19. PFS, progression-free survival; ND, not done; NA, not available; PR, partial response; PD, progressive disease; SD, stable disease.

EGFR were tested by direct sequencing. K757R mutation was not readily captured by many commercially available assays.

* P5 was still responsive to erlotinib at the last follow-up appointment.

# P13 received third-line gefitinib treatment, but had PD with a PFS of 1.1 month.

§ P7 took third-line crizotinib for 6 weeks but unfortunately, 15 days later, she died of severe pulmonary infection. So the duration of PFS was only 1.9 months with an initial PR.

* FISH testing was described as positive with “+” along with percentage values of FISH+ tumor cells.

& The duration of SD to EGFR TKI for P10 was 5.6 months though CT scan showed a reduction in size of her target lesions.
Legends of Figures 1-4:

Figure 1. Representative results of EGFR/ALK co-alterations in one case (P4) of pulmonary adenocarcinoma.
(A) Results of a break-apart FISH assay for ALK rearrangements in tumor cells. The green probe hybridizes to the region immediately 5’ to ALK, and the red probe hybridizes to the 3’ region. The separation of red and green probe signals (arrows) indicates a chromosomal rearrangement involving ALK. Close apposition of red and green probe signals indicates an intact wild-type copy of ALK. The probe that was used was the Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe (Abbott Molecular). (B) Graph of the same tumor under light microscopy, revealing adenocarcinoma (hematoxylin and eosin ×200). (C) Immunohistochemical analysis of ALK, showing protein expression in tumor cells (brown) but not in stromal cells (diaminobenzidine). (D) Immunohistochemical analysis of mutant EGFR protein expression in tumor cells (brown) using an anti-EGFR exon 19 Del E746-A750 antibody. (E) Representative sequence electropherogram from a RACE–coupled PCR assay of EML4-ALK. The sequence of a junction between EML4 exon 13 and ALK exon 20 is shown. (F) Wild-type sequence of exons 2 and 3 of the KRAS gene. (G) Del E746-A750 mutation of EGFR exon 19, detected by direct sequencing. (H) Detection of p-EGFR and p-ALK in primary tumor tissue by Western blotting, indicating that both driver receptors might be activated in this tumor.

Abbreviations: EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization; RACE, rapid amplification of c-DNA ends; PCR, polymerase chain reaction; EML4, echinoderm microtubule-associated protein-like 4.

Figure 2. Waterfall plot of the tumor response, CT scan, and PFS curve following first-line EGFR-TKI treatment in patients with EGFR/ALK
co-alterations.

(A) Waterfall plots for 10 patients with co-alterations of \textit{EGFR} and \textit{ALK} following first-line EGFR-TKI treatment. Eight cases achieved a PR, one had SD, and one had PD. Red line indicates the tumor shrinkage by 30\% according to RECIST 1.0. (B) CT scan of one representative case (P9) before and after erlotinib treatment, showing a good PR following first-line EGFR-TKI treatment. (C) Plot of PFS showing a median PFS of 11.2 months following first-line EGFR-TKI treatment in 10 patients with co-alterations of \textit{EGFR} and \textit{ALK}. All cases underwent biopsy of only one-site for testing genetic alterations before EGFR-TKI treatment. None had a mixed response to EGFR-TKI.

Abbreviations: PR, partial response; PD, progressive disease; PFS, progression-free survival; SD, stable disease.

Figure 3. Expression patterns of mutant \textit{EGFR}, rearranged \textit{ALK}, p-\textit{EGFR}, and p-\textit{ALK} in 10 evaluable cases with \textit{EGFR}/\textit{ALK} co-alterations.

IHC assays were conducted on the serial sections. The 1\textsuperscript{st} and 2\textsuperscript{nd} rows of graphs show the protein expression of mutant EGFR and rearranged ALK. In three cases with EGFR mutation types other than the typical exon 19 del E745-A750 or L858R mutations, E747_S752del ins S for P6 and P7, S768_V769 ins VAS for P8, the EGFR mutant-specific antibodies could not detect the mutant proteins. In the other seven cases, co-expression and co-localization of altered EGFR and ALK proteins were observed in the same tumor cell populations. The third and fourth rows of graphs show levels of phospho-EGFR (p-EGFR Y1068) and phospho-ALK (p-ALK Y1604). Again, we saw IHC staining of both phosphorylated oncoproteins in the same tumor cell populations in all 10 cases, although there was variation in staining intensity. Three patterns could be observed: high p-EGFR and high p-ALK, high p-EGFR and low p-ALK, and low p-EGFR and high p-ALK. For patient 13 (P13), IHC was conducted using a cell block that was made from the cell pellets of malignant pleural effusion.
Note: In this study, “high phosphorylation” of proteins means IHC ++ or +++; “low phosphorylation” of proteins means IHC + or +/-.
NA, not available. “-/NA”, IHC negative because of not available testing.

Figure 4. Differential sensitivities to EGFK-TKIs and crizotinib for the three patterns of protein co-expression of mutant EGFR and rearranged ALK.

H-H, H-L, and L-H indicate “high p-EGFR and high p-ALK,” “high p-EGFR and low p-ALK,” and “low p-EGFR and high p-ALK,” respectively. In the H-H and H-L panels, most patients showed responsiveness to first-line EGFR-TKI treatment, as this representative case did (A-D, P6 and E-H, P8). In the L-H panel, two patients showed PRs to second- or third-line crizotinib treatment (I-L, P7 and M-P, P13). One patient (Q-T, P10) did not receive crizotinib treatment, but showed limited benefit of SD following first-line EGFR-TKI treatment. (U) and (V) The results of Western blotting using fresh tumor tissue from two cases (P6 and P7 corresponding to A-D and I-L). Western blotting results were consistent with IHC data. In P7 (I-L), the level of p-ALK remained high, in contrast to the p-EGFR level and p-ERK and p-AKT levels. With the written consent of the patient, autopsy lung cancer lesions were obtained after third-line ALK-TKI treatment. Western blotting showed that both EGFR and ALK were activated. Notably, ERK was significantly inhibited without AKT inhibition. In the specimens from patient P6 (A-D), levels of both p-EGFR and p-ALK were high.
Figure 1.
Figure 2.

A: Graph showing percent change from baseline with categories PD, SD, and PR.

B: Images of scans showing progression of disease.

C: Bar graph for PFS (months) with patients P5, P12, P6, P3, P9, P2, P1, P10, P8, and P7.
### Figure 3.

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P6</th>
<th>P4</th>
<th>P8</th>
<th>P9</th>
<th>P7</th>
<th>P10</th>
<th>P13</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-/NA</td>
<td>++</td>
<td>-/NA</td>
<td>++</td>
<td>-/NA</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>ALK</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>p-EGFR</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>p-ALK</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

- **high p-EGFR and high p-ALK**
- **high p-EGFR and low p-ALK**
- **low p-EGFR and high p-ALK**
Figure 4.

<table>
<thead>
<tr>
<th></th>
<th>p-EGFR</th>
<th>p-ALK</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>J</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>N</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Q</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>R</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Adjacent tissue
Cancer tissue

U

V Cancer tissue

Baseline Autopsy
Lung cancers with concomitant EGFR mutations and ALK rearrangements: diverse responses to EGFR-TKI and crizotinib in relation to diverse receptors phosphorylation

Jinji Yang, Xu-Chao Zhang, Jian Su, et al.

Clin Cancer Res Published OnlineFirst January 17, 2014.