Lung Cancers with Concomitant EGFR Mutations and ALK Rearrangements: Diverse Responses to EGFR-TKI and Crizotinib in Relation to Diverse Receptors Phosphorylation

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Abstract

Purpose: We investigated the incidence of concomitant epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (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 PCR sequencing and FISH. 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. Coexpression 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 coexist 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. Clin Cancer Res; 20(5); 1383–92. ©2014 AACR.
Clinical Cancer Research

Translational Relevance

Both epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (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 EGFR and ALK in a large cohort of NSCLC, finding that 3.9% (13/336) of EGFR mutant and 18.6% (13/70) of ALK rearranged tumors have co-alterations. ALK fusion proteins and EGFR mutant proteins coexisted in the same tumor cells. Tumors harboring co-altered EGFR and 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 EGFR and ALK.

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 about 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). This 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 to November 2011, for EGFR and KRAS mutations and ALK rearrangements at Guangdong Lung Cancer Institute (GLCI), Guangdong General Hospital (GGH). Histologically proven patients with NSCLC 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 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, per os, every day), erlotinib (150 mg, per os, every day), and afatinib (50 mg, per os, every day). Objective responses were assessed every 6 to 8 weeks according to Response Evaluation Criteria In Solid Tumors (RECIST; refs. 23 and 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.

EGFR and KRAS mutation analysis by direct sequencing

Genomic DNA from each sample was used for sequence analysis of EGFR exons 18 to 21 and KRAS exons 2 and 3. These exons were amplified by PCR as previously described (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.

RT-PCR and RACE-PCR sequencing for ALK fusion analysis

Total RNA was extracted from lung tissue samples using the RNeasy Kit (Qiagen). Reverse-transcriptase PCR (RT-PCR) and 5’ rapid amplification c-DNA ends (RACE)-coupled PCR plus sequencing was conducted as reported previously (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.

FISH assays for ALK rearrangement

Tumor histology was classified using the World Health Organization criteria. Interphase molecular cytogenetic studies using a commercially available ALK probe (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular) 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 for mutant EGFR, ALK, and downstream molecules

Immunohistochemistry (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). Rabbit monoclonal anti-human ALK antibody (#6533 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 resuspended in lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1% Nonidet P-40, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA), incubated on ice for 10 minutes, and centrifuged for 5 minutes (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).

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).

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 2 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 Fig. S1).

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 Fig. 1. These data indicate that driver alterations of EGFR and ALK could coexist 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 (4, 3, and 1 treated with erlotinib, gefitinib, and afatinib, respectively).

### 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>
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<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>
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<td>Sex</td>
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<tr>
<td>Female (%)</td>
<td>178 (55%)</td>
<td>26 (46%)</td>
<td>8 (62%)</td>
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<td>Male (%)</td>
<td>146 (45%)</td>
<td>31 (54%)</td>
<td>5 (38%)</td>
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<tr>
<td>Smoking habit</td>
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<td>Never (%)</td>
<td>251 (77%)</td>
<td>45 (79%)</td>
<td>12 (92%)</td>
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<td>73 (23%)</td>
<td>12 (21%)</td>
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<tr>
<td>0–1 (%)</td>
<td>309 (95%)</td>
<td>52 (91%)</td>
<td>12 (92%)</td>
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<td>2–3 (%)</td>
<td>15 (5%)</td>
<td>5 (9%)</td>
<td>1 (8%)</td>
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<td>Adenocarcinoma (%)</td>
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<td>54 (95%)</td>
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<td>Other NSCLC (%)</td>
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<td>p-Stage</td>
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<td>I–II (%)</td>
<td>71 (22%)</td>
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<td>III–IV (%)</td>
<td>253 (78%)</td>
<td>53 (93%)</td>
<td>13 (100%)</td>
<td></td>
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Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; 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 (\( P = 1.000 \)).
respectively); 1 attained stable disease after afatinib treatment; and 1 patient treated with erlotinib had progressive disease. 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 progressive disease 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; Fig. 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 1 was de novo resistant to EGFR-TKI, but responsive to crizotinib, whereas 2 were responsive to EGFR-TKI but not responsive to crizotinib. One case achieved partial response and 15.1 months of PFS after the initiation of crizotinib, but did not respond to subsequent EGFR-TKI (Table 2).

Coexpression and colocalization 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 2 oncoproteins by IHC analysis of serial sections in 10 cases with sufficient FFPE slides. Specific antibodies detected mutant EGFR protein in 7 cases, but not in the 3 cases with mutation types other than exon 19 del of 746E-750A or L858R of EGFR. All 7 cases showed EGFR mutant coexpressed and colocalized with ALK fusion proteins in the same cell population, although with diverse signal intensities, indicating that these 2 driver oncoproteins might co-operate in the same cancer cells.

To investigate the activation status of the 2 driver oncoproteins in cancers with EGFR/ALK co-alterations, EGFR (Y1068) and ALK (Y1604) phosphorylation levels were also assessed by IHC. Three patterns are shown in Fig. 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; Fig. 3). Of the 8 cases treated with first-line EGFR-TKI, 6 with high
levels of p-EGFR had partial responses to EGFR-TKI and 2 with very low levels of p-EGFR (+/−) had progressive disease or stable disease. Of the 4 cases treated with crizotinib, 2 (P7 and P13) had relatively inactivated p-EGFR (−, +) and highly activated p-ALK (+++, ++++); one of them showed no benefit from EGFR-TKI, but a partial response to third-line crizotinib, and the other was very responsive to crizotinib, but resistant to subsequent EGFR-TKI. In contrast, 2 cases (P8 and P9) had relative high p-EGFR levels (+++, ++++) and low p-ALK levels (−, +), corresponding to partial response to first-line EGFR-TKI, but no benefit or short-term stable disease from crizotinib.

Western blotting yielded similar results of IHC in 3 cases (P4, P6, and P7; Figs. 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-naive tissue from case P7, there were high levels of p-ALK and relatively low p-EGFR levels. After progressive disease to EGFR-TKI and partial response 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 (Fig. 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%; refs. 8, 9, and 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 3 case reports (20–22). Janne and colleagues 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 patients with ALK-positive or EGFR-mutant and possibly higher in Chinese patients with ALK-positive 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 and colleagues also identified 50 alterations in 21 genes, with at least one alteration being present in 83% (20 of 24) of the lung cancers (with a range of 1–7 alterations; ref. 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. Coexistence 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...
Table 2. Molecular and demographic characteristics and efficacy of EGFR-TKIs and crizotinib in 13 patients with co-alterations

<table>
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Abbreviations: F, female; M, male; AC, adenocarcinoma; WT, wild-type; DEL, exon 19 deletion; Exon20, exon 20 insertion; K757R, K757R in exon 19. 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.

 فيه: The duration of SD to EGFR TKI for P10 was 5.6 months though computed tomography scan showed a reduction in size of her target lesions.

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

 FISH testing was described as positive with "+" along with percentage values of FISH+ tumor cells.

 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.
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.

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 coexpression of EGFR or ALK in vitro lead to mutual resistance to single-agent ALK or EGFR Tki (6). In contrast, response to 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 4 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," and "low p-EGFR and high p-ALK," respectively, achieved partial responses to first-line EGFR-TKI treatment, as this representative case did (A-D, P6 and E-H, P8). In the L-H panel, 2 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 2 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 4. Differential sensitivities to EGFR-TKIs and crizotinib for the 3 patterns of protein coexpression 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, 2 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 2 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.
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 EGF mutations requires further evidence (41).

The shortcomings of our study were the small sample size concerning crizotinib treatment and p-EGFR/p-ALK testing, nonprospective design, and the fact that it was not multi-institutional. No NSCLC cell lines with EGF/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 EGF mutations and ALK rearrangements was significantly higher in ALK-rearranged NSCLCs. EGF/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.

Disclosure of Potential Conflicts of Interest
T.S.K. Mok has honoraria from the speakers bureau of Roche, BL Eli Lilly, Pfizer, and GSK. T.S.K. Mok is a consultant/advisory board member of AstraZeneca, Roche, BL Eli Lilly, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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