Concomitant ALK translocation and EGFR mutation in lung cancer: a comparison of direct sequencing and sensitive assays and the impact on responsiveness to tyrosine kinase inhibitor


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Background: Epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) translocation are considered mutually exclusive in nonsmall-cell lung cancer (NSCLC). However, sporadic cases having concomitant EGFR and ALK alterations have been reported. The present study aimed to assess the prevalence of NSCLCs with concomitant EGFR and ALK alterations using mutation detection methods with different sensitivity and to propose an effective diagnostic and therapeutic strategy.

Patients and methods: A total of 1458 cases of lung cancer were screened for EGFR and ALK alterations by direct sequencing and fluorescence in situ hybridization (FISH), respectively. For the 91 patients identified as having an ALK translocation, peptide nucleic acid (PNA)-clamping real-time PCR, targeted next-generation sequencing (NGS), and mutant-enriched NGS assays were carried out to detect EGFR mutation.

Results: EGFR mutations and ALK translocations were observed in 42.4% (612/1445) and 6.3% (91/1445) of NSCLCs by direct sequencing and FISH, respectively. Concomitant EGFR and ALK alterations were detected in four cases, which accounted for 4.4% (4/91) of ALK-translocated NSCLCs. Additional analyses for EGFR using PNA real-time PCR and ultra-deep sequencing by NGS, mutant-enriched NGS assays were carried out to detect EGFR mutation.

Conclusions: A portion of NSCLC patients have concomitant EGFR and ALK alterations and the frequency of co-alteration detection increases when sensitive detection methods for EGFR mutation are applied. ALK inhibitors appear to be effective for patients with co-alterations.

Key words: EGFR, ALK, lung cancer, next-generation sequencing, targeted therapy

Introduction

Recently, the discovery of genetic alterations in nonsmall-cell lung cancer (NSCLC) has led to the development of targeted therapeutic agents and survival prolongation in patients with epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) translocation. Therefore, molecular tests for EGFR and ALK are prerequisites for the selection of patients eligible for EGFR or ALK inhibitors, and various methods for detecting EGFR mutations and ALK translocations have been developed [1].

Direct sequencing based on the Sanger method has long been used for detecting EGFR mutations and remains the most useful validated method [1, 2], but is sufficiently sensitive to detect ~25% of mutant cells. Thus, many assays with increased sensitivity are actively being investigated. The peptide nucleic acid (PNA)-clamping real-time PCR (PNA-RT PCR) adapts PNA-clamping technology against the wild-type EGFR allele.
to enrich the mutant allele and has been validated to detect at least 1% of mutant cells [3]. Targeted next-generation sequencing (NGS) have been reported to effectively identify EGFR mutations using routine diagnostic samples with sensitivity to detect <1% of mutants [4, 5]. Moreover, the low burden of EGFR mutation detected by sensitive assays was predictive of responsiveness to EGFR tyrosine kinase inhibitors (TKIs) in NSCLC patients [3, 5].

Although EGFR mutations and ALK translocations are generally considered mutually exclusive [6], sporadic cases with concomitant EGFR and ALK alteration (referred to as ‘dual-positive’ hereafter) have been reported [7–16]. The prevalence of dual-positive differed [14–17] and their responses to EGFR and/or ALK inhibitors were conflicting [9, 11, 13–16]. Moreover, previous studies were carried out using direct sequencing for EGFR.

Considering that the recently introduced EGFR tests have a substantially higher sensitivity compared with direct sequencing, it is possible that additional lung cancers with a dual-positive status may be identified in the future. Therefore, we comprehensively investigated the prevalence of dual-positive NSCLCs using various EGFR assays and analyzed the responsiveness of these patients to EGFR and/or ALK inhibitors. In doing so, we aimed to help determine an appropriate diagnostic and therapeutic strategy for dual-positive patients in the present era of sensitive assays.

materials and methods

patients and samples

A total of 1458 lung cancer patients who had been tested for EGFR mutation by direct sequencing and ALK translocation by fluorescence in situ hybridization (FISH) between 2008 and 2013 at Seoul National University Hospital (SNUH) were enrolled. The details of study population and molecular tests are summarized in Figure 1 and supplementary Table S1, available at Annals of Oncology online. This study was approved by the Institutional Review Board of SNUH (H-1404-101-572).

patient treatment and response evaluation

The patients who recurred or had metastatic NSCLC with ALK translocation were treated with crizotinib or ceritinib (LDK378) as part of a clinical trial.
fluorescence in situ hybridization and immunohistochemistry for ALK

The ALK FISH was carried out on formalin-fixed paraffin-embedded (FFPE) tissues using ALK Dual Color, Break Apart Probe (Abbott Molecular, Des Plaines, IL). Patients were diagnosed as ALK FISH-positive when 15% or more of tumor cells showed split and/or isolated 3' signals, whereas cases with 5%–15% tumor cells with split and/or isolated 3' signals were regarded as equivocal. ALK immunohistochemistry (IHC) was carried out using anti-ALK antibody (clone 5A4, Novocastra, Newcastle, UK).

direct sequencing analysis for EGFR and KRAS

Direct Sanger sequencing for EGFR and KRAS was carried out. Briefly, genomic DNA was extracted from FFPE tissues after manual microdissection of tumor area. EGFR exons 18–21 and KRAS exon 2 were amplified by PCR, and the products were purified using the QIAquick PCR purification kits (Qiagen, Hilden, Germany) and subsequently submitted for sequencing using BigDye Terminator v3.1 kits and a 3730XL DNA Analyzer (Applied Biosystems, Vernon Hills, IL).

PNA-clamping real-time PCR for EGFR

The PNA-RT PCR assay to detect EGFR mutations was carried out using the PANAClamp™ EGFR kit (Panagene, Inc., Daejeon, South Korea) as described in the supplementary Methods, available at Annals of Oncology online [3].

conventional targeted next-generation sequencing and mutant-enriched NGS for EGFR

Targeted NGS to detect EGFR mutations was carried out using the Cancer Panel on a Roche 454 GS Junior Sequencer (Roche Diagnostics) by the manufacturer’s protocol. In brief, 10 ng of genomic DNA was used for PCR of the EGFR panel (SeaSun Biomaterials, Daejeon, South Korea). After quantification and normalization of the purified amplicons, parallel pyrosequencing was carried out using the GS Junior System, and the data were analyzed with the GS Amplicon Variant Analyzer (Roche Diagnostics) as described in the supplementary Methods, available at Annals of Oncology online.

To increase the resolution for the detection of low level of EGFR mutants, Insight™Onco Panel for EGFR (SeaSun Biomaterials), which incorporates PNA-clamping before NGS, was used as described in the supplementary Methods, available at Annals of Oncology online.

results

analysis of EGFR and ALK genetic alterations in lung cancers

The EGFR and ALK status in 1458 cases of lung cancer including NSCLC (n = 1445) and small-cell carcinoma (n = 13) was analyzed using direct sequencing and FISH, respectively, and the results are summarized in supplementary Table S1, available at Annals of Oncology online, and Figure 1. Overall, the EGFR mutation and ALK translocation rates in NSCLCs were 42.4% (612/1445) and 6.3% (91/1445), respectively. Of note, concomitant EGFR and ALK alteration was detected in 4 (0.3%) of the 1445 NSCLCs, which constituted 4.4% (4/91) of ALK-translocated NSCLCs and 0.7% (4/612) of EGFR-mutated NSCLCs.

detection rate of concomitant EGFR mutation and ALK translocation in lung cancers depends on the sensitivity of the EGFR genotyping technologies

To clarify the prevalence of a concomitant EGFR and ALK alteration, we sequentially applied several technologies with higher sensitivity for detecting EGFR mutation as schematically shown in Figures 1 and 2.

We first carried out PNA-RT PCR in 97 cases with available tissues out of 115 cases showing ALK FISH-positive (n = 91) and -equivocal (n = 24) status. In doing so, four additional dual-positive cases were found from resection specimens (cases 6, 7, and 8) and small biopsy (case 5) (Figure 2 and Table 1). These cases were confirmed to have an EGFR mutation by NGS (supplementary Table S2, available at Annals of Oncology online). The proportion of EGFR mutants ranged from 0.78% to 27.45% (mean ± standard deviation: 12.11% ± 12.19%).

Furthermore, another six cases (cases 9–14) showing borderline C1 values in PNA-RT PCR were also tested by conventional targeted NGS and validated by the mutant-enriched NGS (supplementary Table S3, available at Annals of Oncology online). These cases were all from small biopsies and confirmed to have EGFR mutation (Figure 2 and Table 1). Specifically, the proportion of mutants ranged from 0.82% to 8.79% in three patients (cases 10, 12, and 13), but the other three patients (cases 9, 11, and 14) had an EGFR mutant burden lower than the detection limit of conventional NGS (Table 1 and supplementary Tables S3 and S4, available at Annals of Oncology online).

Altogether, the application of a series of sensitive assays enabled us to identify 10 additional dual-positive cases. Thus, the final proportion of dual-positive cases was 15.4% (14/91) of ALK-translocated NSCLCs (Figure 2 and Table 1); the pathologic features of a representative case are displayed in supplementary Figure S1, available at Annals of Oncology online.

clinicopathological characteristics of patients with lung cancers harboring concomitant EGFR mutation and ALK translocation

The characteristics of the dual-positive patients (n = 14) are summarized in Table 1. Of note, cases 1, 3, and 4 showed negative immunoreactivity for ALK by IHC, even in the presence of ALK translocation. However, fusion transcripts of EML4-ALK were identified in these three patients by reverse-transcription PCR (supplementary Figure S2, available at Annals of Oncology online). There were no significant differences in the main clinicopathological features between the dual-positive group and the EGFR-wild-type/ALK-translocated group (supplementary Table S5, available at Annals of Oncology online).

responsiveness to EGFR-TKI and/or ALK inhibitor in patients with adenocarcinoma harboring concomitant EGFR mutation and ALK translocation

Of the 14 patients, 2 patients (cases 1 and 5) were treated with gefitinib, 1 patient (case 2) received gefitinib and crizotinib sequentially, and 7 patients (cases 6, 8–10, 12–14) were treated with ALK inhibitor (Table 1). Although they were positive for the EGFR mutation by direct sequencing and/or PNA-RT PCR, the responses to gefitinib were disappointing, with progressive
Figure 2. Schematic diagram of the study flow for detecting EGFR mutation status in ALK FISH-positive population and subsequent results. Sequential applications of EGFR direct sequencing, PNA-clamping real-time PCR, NGS-based target sequencing and mutant-enriched NGS for ALK rearranged cases (n = 115) are shown. Of the 115 cases, 91 patients showed overt ALK translocation, with ALK rearranged signals in at least 15% of tumor cells. The other 24 patients were equivocal and only exhibited ALK rearranged signals in 5%–15% of tumor cells. Resection included lobectomy, resected or excised specimens and small biopsy included needle and bronchoscopic biopsy specimens. In a stepwise manner, additional EGFR mutants were discovered, leading to an increase in the dual-positive population percentage from 4.4% to 15.4% of ALK-translocated lung cancers. (*While 97 of 115 cases with available FFPE tissues were submitted for PNA-clamping real-time PCR, the remaining 18 cases were also included to calculate numbers and ratios in this figure.)

In this study, we found that a portion of NSCLC patients have co-activation of EGFR, although not through EML4-ALK rearrangement was found to have co-activation of EGFR, although not through EML4-ALK translocation or EML4-ALK mutation was identified after ALK inhibitor treatment [17]. In contrast, a cell line having homogeneous EML4-ALK rearrangement was found to have co-activation of EGFR, although not through EML4-ALK mutation [19], which suggests that multiple oncogenic pathways may be altered in a single clone of tumor cells. Favoring the latter hypothesis, a study by Yang et al. demonstrated immunohistochemical co-localization of EGFR mutant protein and ALK protein in putatively identical tumor cells [16]. In our study, EGFR mutation frequency did not increase following crizotinib failure in case 2, which suggests that dual-positive alteration might exist in same clonal population. The precise mechanism disease (PD) in cases 2 and 5 and a stable disease (SD) in case 1 (PFS for 6 months). In contrast, of the eight patients who received ALK inhibitor, seven patients acquired partial responses (PR, response rate of 87.5%) and one showed SD. With the exception of case 9, who stopped crizotinib due to side-effect, the other seven patients continued to take ALK inhibitor for 5–43 months.

Case 2, who has been previously described [11], had EGFR exon 19 deletion and showed PD after one cycle of EGFR-TKI therapy. He was then treated with crizotinib and showed a PR for 18 months. The re-biopsy specimen after crizotinib failure (case 2-2) contained only 0.18% of exon 19 deletion mutant by NGS.

**discussion**

In this study, we found that a portion of NSCLC patients have concomitant EGFR and ALK alteration. When using sensitive detection methods for EGFR mutation, such as real-time PCR, targeted NGS, and mutant-enriched NGS, the co-alteration rate increased from 4.4% to 15.4% of ALK-translocated NSCLC. These dual-positive patients showed gefitinib resistance but were sensitive to ALK inhibitors.

Compared with earlier studies, we exploited diverse modalities with different sensitivities to identify the coexistence of EGFR mutation and ALK translocation. The observation in this study conflicts with that of several large-scale studies from Western countries, which reported the mutual exclusiveness of EGFR and ALK alterations in NSCLC [6, 7]. Not only ethnic differences but also detection sensitivity can affect to different prevalence of dual-positive alteration. In our study, the dual-positive population accounted for 4.4% of ALK-translocated NSCLC using direct sequencing, which was similar to the ratio [6% (3/50)] reported by Sasaki et al., but lower than the ratio [18.6% (12/70)] reported by Yang et al., which used the same detection method [9, 16]. Furthermore, the dual-positive rates gradually increased up to 8.8%–15.4% of ALK-translocated patients in the same cohort when more sensitive assays were applied (Figure 2). Combined, our data suggest that an increased number of dual-positive NSCLC can be found when advanced molecular genetic technologies are used.

To explain the coexistence of multiple oncogenic drivers in NSCLCs, two different hypotheses have been proposed [17]. Mounting evidences have demonstrated that the genetic instabilities of cancer cells cause genetic and phenotypic heterogeneity in the tumor, suggesting that different genetic alterations might occur in different tumor cells rather than single clone. In fact, intratumoral heterogeneity of EGFR mutation in NSCLC was revealed by multifocal microdissection approaches [18]. Moreover, in a study the presence of different tumor cell clones having either ALK translocation or EGFR mutation was identified after ALK inhibitor treatment [17]. In contrast, a cell line having homogeneous EML4-ALK rearrangement was found to have co-activation of EGFR, although not through EML4-ALK mutation [19], which suggests that multiple oncogenic pathways may be altered in a single clone of tumor cells. Favoring the latter hypothesis, a study by Yang et al. demonstrated immunohistochemical co-localization of EGFR mutant protein and ALK protein in putatively identical tumor cells [16]. In our study, EGFR mutation frequency did not increase following crizotinib failure in case 2, which suggests that dual-positive alteration might exist in same clonal population. The precise mechanism...
### Table 1. Clinicopathological features of patients with concomitant EGFR and ALK alterations

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<td>Excision</td>
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<td>E21 (L858R)</td>
<td>n.d.</td>
<td>WT</td>
<td>E19 del</td>
<td>WT</td>
<td>E21 (E868K)</td>
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<td>60%</td>
<td>n.d.</td>
<td>25%</td>
<td>72%</td>
<td>23%</td>
<td>28%</td>
<td>49%</td>
<td>80%</td>
<td>55%</td>
<td>37%</td>
<td>59%</td>
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<td>PFS (months)</td>
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<td>5</td>
<td>1.3+</td>
<td>9+</td>
<td>–</td>
<td>43+</td>
<td>33+</td>
<td>16+</td>
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1. + in PFS denotes censor.
2. Re-biopsy after crizotinib failure.
3. L747-E749 deletion; A750P.
4. EML4-ALK fusion transcript of variant 3 was detected by reverse-transcription PCR analysis.
5. Although case #9 showed response to crizotinib, this patient halted crizotinib treatment due to hemolytic anemia.

ADC, adenocarcinoma; NOS, not otherwise specified; PNA-RT PCR, PNA-clamping-mediated real-time PCR; n.d., not done; NGS, next-generation sequencing; InsightOnco, mutant-enriched next-generation sequencing; OP, operation; PY, pack years; IHC, immunohistochemistry; NED, no evidence of disease; mets, metastasis; TKI, tyrosine kinase inhibitor; E, exon; WT, wild-type; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival.
underlying the co-existence of EGFR and ALK alteration remains to be clarified.

EGFR mutations observed in our dual-positive cases included exon 19 deletion, L858R, L747P, and E868K. All of these mutations were previously reported recurrent EGFR mutations known to be associated with sensitivity to EGFR-TKI [1, 20, 21]. This raised clinically relevant issues regarding molecular diagnosis and targeted therapy of NSCLC. First, the detection of EGFR mutation does not completely exclude the concomitant ALK translocation and vice versa. This should be taken into consideration to make diagnostic strategies for EGFR and ALK in NSCLC especially when using sensitive assay. Second, appropriate therapeutic strategies should be established for dual-positive patients by determining which genetic alteration is the primary target. In our study, EGFR-TKI was not effective in dual-positive patients, while ALK inhibitors were efficient. Although the reason for the lack of response to gefitinib is uncertain, there may be unknown factors related with primary resistance to EGFR-TKI, which are known to be heterogeneous [22]. Otherwise, it is possible that low burden of EGFR mutation in the dual-positive patients led to the unfavorable response to EGFR-TKI as previously suggested [23].

According to previous reports, the responses to EGFR and/or ALK inhibitor in dual-positive patients were variable, with no predictive biomarkers [9, 11, 13–16]. Yang et al. suggested that the relative activation status of EGFR and ALK, as determined by phosphorylated EGFR and ALK, could be predictive of inhibitor efficacy [16]. However, the number of patients treated with both EGFR and ALK inhibitors in their study was too small to draw definitive conclusions. Likewise, only one patient was treated with both EGFR and ALK inhibitors in our study. Nonetheless, it is notable that the clinical outcome of the dual-positive patients treated with ALK inhibitors was substantially better than those treated with EGFR-TKI. Moreover, the PR rate (87.5%) and PFS (5–43 months) to ALK inhibitors were comparable with those observed in a phase III clinical study for crizotinib in advanced ALK-translocated lung cancer [24].

It is of note that most (7/8) patients treated with ALK inhibitors showed EGFR mutation by PNA-RT PCR and/or NGS, but not by Sanger sequencing, which suggests a low burden of EGFR mutants in these patients. The present data indicate that a minor clone of EGFR mutant may have little influence on the responsiveness to ALK inhibitors in dual-positive patients. However, the minor burden of EGFR mutant clone has also clinical significance when considering the evolutionary dynamics of a tumor cell repopulation [25], and clonal expansion of EGFR-mutated tumor cells during ALK inhibitors [17]. Furthermore, EGFR mutations have been regarded as a resistance mechanism of ALK inhibitors [9, 26], suggesting the need for combination therapy with EGFR-TKIs and ALK inhibitors. Taken together, we suggest that ALK inhibitors could be chosen first in dual-positive patients, particularly those with a low abundance of EGFR mutants. However, the role of EGFR and ALK inhibitor in dual-positive patients in the current and previous studies is not straightforward, thus warranting further study on the appropriate management of dual-positive NSCLC patients.

In conclusion, the present study demonstrates that a portion of NSCLCs have concomitant EGFR and ALK alteration with an increased frequency by the implementation of sensitive molecular genetic techniques. Therefore, both EGFR and ALK status should be evaluated for the appropriate management of patients in the current era of sensitive assays. ALK inhibitors were effective for the treatment of dual-positive patients in this study, but treatment strategies should be optimized for patients with concomitant EGFR and ALK alterations in the future.

funding

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disclosure

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references

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A randomized phase II study of the telomerase inhibitor imetelstat as maintenance therapy for advanced non-small-cell lung cancer


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Background: Continuation or ‘switch’ maintenance therapy is commonly used in patients with advanced non-small-cell lung cancer (NSCLC). Here, we evaluated the efficacy of the telomerase inhibitor, imetelstat, as switch maintenance therapy in patients with advanced NSCLC.

Patients and methods: The primary end point of this open-label, randomized phase II study was progression-free survival (PFS). Patients with non-progressing, advanced NSCLC after platinum-based doublet (first-line) chemotherapy (with or without bevacizumab), any histology, with Eastern Cooperative Oncology Group performance status 0–1 were eligible. Randomization was 2:1 in favor of imetelstat, administered at 9.4 mg/kg on days 1 and 8 of a 21-day cycle, or observation. Telomere length (TL) biomarker exploratory analysis was carried out in tumor tissue by quantitative PCR (qPCR) and telomerase fluorescence in situ hybridization.


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