Compound EGFR mutation is frequently detected with co-mutations of actionable genes and associated with poor clinical outcome in lung adenocarcinoma

Eun Young Kim*, Eun Na Cho, Heae Surng Park, Ji Young Hong, Seri Lim**, Jong Pil Youn**, Seung Yong Hwang***, and Yoon Soo Chang***

*Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Republic of Korea; **Pathology, Yonsei University College of Medicine, Seoul, Republic of Korea; ***Department of Bio-Nanotechnology, Hanyang University, Sangnok-gu, Ansan, Gyeonggi-do, Republic of Korea

**Present Address: Department of Molecular and Life Science, Hanyang University, Sangnok-gu, Ansan, Gyeonggi-do, Republic of Korea

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Introduction

Despite relentless efforts to decrease the mortality of lung cancer, it remains a common and leading cause of cancer-related death worldwide. In the year 2012, 1,824,701 new cases were diagnosed and 1,590,000 patients died of lung cancer worldwide (WHO annual report). During the same period, 21,753 new Korean cases were diagnosed and 16,654 Korean patients died of this devastating disease.

Oncogenic driver mutations include multiple types of genomic changes that are critical for cancer development and maintenance. The identification of actionable oncogenic driver mutations that guide selection of appropriate target agents has improved clinical outcomes of lung cancer patients by incorporating tumor genotyping into therapeutic decision making.

Activating EGFR mutations are more frequently identified in lung adenocarcinoma in East Asian patients than in other populations, and advances in tumor genotyping facilitate discovery of such mutations in small population samples. The most common type of EGFR mutation is in-frame deletion of exon 19 (E19del) around the LREA motif (amino acid residues 747 to 750; ~45% of EGFR mutations), followed by L858R point mutation of exon 21 (~40% of EGFR mutations). Tumors with these activating EGFR mutations or less frequent mutations, such as point mutations in exon 18 at position G719 (~3% of EGFR mutations) and the exon 21 L861Q mutant (~2% of EGFR mutations), show sensitivity to EGFR-tyrosine kinase inhibitors (TKIs).

With the clinical application of more sensitive and precise tumor genotyping systems, rare EGFR mutations of unknown biological and clinical significance are frequently encountered in routine clinical practice. Different responses to EGFR-TKI are reported even for mutations at the same approximate location within the genomic DNA.
For example, among the in-frame insertions within \textit{EGFR} exon 20, which were originally considered EGFR-TKI resistance mutations with a low response rate (<5%) and short interval of disease control, A763_Y764insFQEA is now reported to be a sensitizing mutation to EGFR-TKI.\textsuperscript{14,15} These findings indicate that more attention and collaborative efforts are required to elucidate the biological and clinical significance of these rare compound mutations.

Compound \textit{EGFR} mutations are defined as double or multiple independent mutations of the EGFR tyrosine kinase domain (TKD), in which an EGFR-TKI-sensitizing or other mutation is identified together with a mutation of unclarified clinical significance.\textsuperscript{16} Recent advances in tumor genotyping techniques provide not only accurate data, but also a higher probability of identifying atypical and multiple mutations in the EGFR-TKD in a single sample. Kobayashi et al. reported compound \textit{EGFR} mutations in which an EGFR-TKI-sensitizing mutation (such as G719X, E19del, L858R, or L861Q) coexists with uncommon mutations involving other residues of the EGFR-TKD and show some sensitivity to EGFR-TKI. In \textit{EGFR} mutant non-small cell lung cancer (NSCLC), double mutations in \textit{EGFR} were detected in 14–18% of cases using Sanger method based sequencing techniques, but their biologic behavior and clinical significance have not been well characterized.\textsuperscript{16,17}

In this study, we identified \textit{EGFR} compound mutations in lung adenocarcinomas from patients who underwent surgical curative resection using next-generation sequencing (NGS)-based repeated deep sequencing of \textit{EGFR} together with 15 other genes containing actionable oncogenic mutations. This study shows that the compound \textit{EGFR} mutation is common in lung adenocarcinoma and imparts a new meaning of compound \textit{EGFR} mutation.

\section*{Materials and methods}

\subsection*{Patient characteristics and tumor DNA samples}

A total of 143 patients with a pathologically confirmed diagnosis of pStage IB~III A lung adenocarcinoma who underwent curative surgical resection and platinum-based adjuvant chemotherapy and provided informed consent for tissue collection were randomly selected from tissue archives of affiliated hospitals of Yonsei University Medical Center. Among them, 61 patients with \textit{EGFR} mutations who had not received EGFR-TKI before tumor genotyping were enrolled in this study. All paraffin-embedded samples were loaded onto silanated slides as 4-µm-thick sections. One slide of every block was stained with H&E and re-examined for the presence of cancer cells. The enriched area was marked by an independent lung pathologist to validate the presence of tumor cells. These cancer cell-enriched areas were microdissected, and DNA was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA). Institutional Review Board (IRB) approval was obtained for this study (IRB #3-2013-0298).

\subsection*{Library preparation, NGS with IonTorrent, and variant calling}

Ten micrograms of genomic DNA were amplified by the Ion AmpliSeq\textsuperscript{TM} Custom Panel (Life Technologies, Carlsbad, CA). This panel contains 16 genes that contain actionable mutations; AKT1, ALK, BCL2L11, BRAF, DDR2, EGFR, ERBB2, FGFR1, KRAS, MAP2K1, MET, NRAS, PIK3CA, PTEN, ROSI, and RET. ALK fusion was detected by FISH using Abbott Vysis ALK break apart FISH probe kit (Abbott, Abbott Park, IL). Multiplex pools were purified with Agencourt AMPure XP beads (Beckman Coulter Inc.) and ligated with Ion Xpress barcode adapters (Life Technologies). The fragment size and quantity of each library were analyzed by a BioAnalyzer using a High Sensitivity Chip (Agilent, Santa Clara, CA). The library was diluted, and emulsion PCR was performed with the OneTouch\textsuperscript{TM} reagent kit (Life Technologies). The emulsion PCR product was enriched using Dynabeads\textsuperscript{®} MyOne\textsuperscript{TM} Streptavidin C1 beads (Life Technologies). The final enriched ion spheres were mixed with a sequencing primer and polymerase and loaded onto 5 318v2 chips. The libraries were sequenced with the Ion Torrent PGM sequencer at deep coverage (aiming for 1,000×) using the Ion OneTouch 200 Template Kit v2 DL and Ion PGM Sequencing 200 Kit v2 with the 318 v2 chip kits (all from Life Technologies). The sequencing reads were aligned to the human reference GRCh37 genome, and base calling was performed using the Ion Torrent Suite V3.4.2 using map-f3 on the Ion Torrent server. The Ion Torrent Variant Caller (ITVC) v3.4 was used for the detection of mutations, requiring a frequency greater than 5% for a variant to be called. Bam (Binary sequence Alignment/Map format) and FASTQ files (alignment) were generated based on the base calling results and were used to report the variant calling, including single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs).

\section*{Statistical analysis}

Categorical variables are expressed as percentages and compared using \(\chi^2\)-tests. Differences in distribution of continuous variables between 2 independent samples were assessed by Mann–Whitney U test, and the Kaplan–Meier estimator was used for survival analysis. All analyses were performed with IBM SPSS Statistics version 20 (IBM Corp). All statistical tests were 2-sided, and a \(P\) value <0.05 was considered to indicate statistical significance.

\section*{Results}

\subsection*{Demographic characteristics of the study population}

The 61 patients with mutations in \textit{EGFR-TKD} had a mean age of 59 ± 9.9 years (range; 34–78 years); 17 (27.9%) were male and 44 (72.1%) were female. The difference in age at the time of diagnosis between male and female patients was not significant. The majority of patients (50; 82%) did not have a smoking history, 6 (9.8%) were current smokers, and 5 (8.2%) were ex-smokers; the ever-smokers had a pack-year average of 43 ± 48.2 years. These demographic characteristics are comparable to previous findings of \textit{EGFR} mutation-positive Korean patients with lung adenocarcinoma.\textsuperscript{3,4,18}

\subsection*{Compound \textit{EGFR} mutations}

Determination of the entire sequence of \textit{EGFR} exons 18–21 constituting \textit{EGFR-TKD} revealed that simple mutations were
the more frequent (46 of 61, 75.4%). These were predominantly E19del (24 of 61, 39.3%), followed by L858R point mutation (17 of 61, 27.9%), and EGFR exon 20 insertion mutations (2 out of 61, 3.2%). Point mutations involving exon 20, exon 19 insertions, and L861R were less frequent (Table 1). The remaining 15 cases (24.6%) had compound EGFR mutations, which is composed of double or multiple independent mutations in the EGFR-TKI (Table 1). Most of the compound mutations, (10 of 15, 66.7%) were composed of a rare atypical mutation with EGFR composed of double or multiple independent mutations in the diagnosis were not associated with the type of compound mutation. Smoking status and pStage at the time of distribution between patients with simple mutation and those with compound mutations showed discernible clinical and pathologic characteristics (Table 2). There was no difference in age or gender distribution. Next, we questioned whether the cases with compound EGFR mutation have a poor response to EGFR-TKI. Among 33

Clinical characteristics of cases with compound EGFR mutation

Because the cases with compound EGFR mutation had properties which might be related to poor clinical outcome, we compared the disease-free survival (DFS) and overall survival (OS) of cases with simple and compound mutations (Fig. 1). The median follow-up duration of the study population was 81.9 months (95% confidence interval (CI): 65.7–98.1 months). Of 61 patients, 33 (54.1%) experienced recurrence of the disease and 15 (24.6%) died of same disease during follow-up period. There was no difference in DFS between the groups, but OS was significantly poorer in the cases with compound mutation (simple mutation, 83.7 months vs. compound mutation, 72.8 months, P = 0.020, Breslow test) (Fig. 1A). A multivariate analysis including age, smoking status, EGFR mutation subtypes, stage, and histologic subtypes revealed that smoking history (HR, 11.47; 95% CI, 2.510–54.404; P = 0.002), compound EGFR mutation (HR, 4.030; 95% CI, 1.305–12.446; P = 0.015) were significantly associated with a shorter OS (Table 3). Based on these findings, we hypothesized that cases with compound mutation have a poor response to EGFR-TKI. Among 33

Table 1. Various types of EGFR mutations in exons 18–21 detected by NGS-based repeated deep sequencing.

| EGFR mutation type   | No. | % of total |
|----------------------|-----|------------|
| Simple mutations     |     |            |
| Exon 19 deletions    |     |            |
| Exon 19 insertions   | V738_K739insKIPVAIL | 1 1.6 |
| Exon 20 insertions   | M766_A767insASV | 1 1.6 |
|                      | D770_N771insG+N771T | 1 1.6 |
| Exon 20 mutations    | N771F | 1 1.6 |
| Exon 21 mutations    |     |            |
|                      | L858R | 17 27.9 |
|                      | L861R | 1 1.6 |
| Compound mutations   |     |            |
|                      | L858R + V689L | 1 1.6 |
|                      | L858R + L833V | 1 1.6 |
|                      | L858R + H870R | 1 1.6 |
|                      | L858R + A871G | 1 1.6 |
|                      | L858R + R776H | 1 1.6 |
|                      | L858R + E19del | 1 1.6 |
|                      | G719A + I706T | 1 1.6 |
|                      | G719S + E709K | 1 1.6 |
|                      | G719S + R776H | 1 1.6 |
|                      | E19del + I706T | 1 1.6 |
|                      | D770_N771insNPY +H773Y | 2 3.3 |
|                      | L688F + G824S | 1 1.6 |
|                      | E749Q + A750P | 1 1.6 |
|                      | T785I + Y813H + V845M + V851I + G857R | 1 1.6 |
| Total                | 61  | 100        |
patients that experienced recurrence of lung cancer after curative resection, 24 had taken EGFR-TKI for management of the recurrence. However, when the duration of disease control with EGFR-TKI was analyzed, there was no difference between groups with compound or simple mutations (data not shown).

To further investigate the reason for the poor clinical outcome in the cases with compound mutation, we examined co-mutations in the AKT1, BRAF, DDR2, ERBB2, FGFR, KRAS, MAPK2K1, MET1, NRAS, PIK3CA, PTEN, RET, and ROS1 genes, ALK gene rearrangement, and BCL2L11 intron 2 deletion. A total 115 missense mutations were discovered in the tested genes (Table 4). 31 missense mutations were discovered in the cases with simple EGFR mutations whereas 84 were discovered in those with compound EGFR mutation, showing that the cases with compound EGFR mutation have higher chance of harboring multiple missense mutations in the clinically important genes (Table 7) (0.66 mutations/case vs. 6.0 mutations/case, P = 0.001, independent sample t-test). Similarity the cases with compound EGFR mutations have higher chance of co-alteration in the other genes than those with simple EGFR mutations (0.61 vs. 2.2 genes/case). Interestingly, there are a few number of actionable mutations irrelevant to the subtype of EGFR mutations, including ALK rearrangement, BCL2L11 intron 2 deletion, KRAS c.35G>A, PIK3CA c.1633G>A which is possible target mutation of

Table 2. Clinical and pathologic characteristics of the study cases according to subtype of EGFR mutation.

|                      | Simple mutation (n = 46) | Compound mutation (n = 15) | P-value |
|----------------------|-------------------------|---------------------------|---------|
| Age (mean ± SD); yrs | 59.6 ± 10.52            | 58.9 ± 7.93               | 0.778†  |
| Gender               |                         |                           |         |
| Male                 | 10                      | 7                         | 0.061** |
| Female               | 36                      | 8                         |         |
| Smoking status       |                         |                           |         |
| Non-smoker           | 39                      | 11                        | 0.488** |
| Current smoker       | 4                       | 2                         |         |
| Ex-smoker            | 3                       | 2                         |         |
| Stage                |                         |                           |         |
| IB                   | 4                       | 1                         | 0.970** |
| IIA                  | 16                      | 5                         |         |
| IIB                  | 2                       | 1                         |         |
| IIIA                 | 24                      | 8                         |         |
| Maximum tumor diameter |                        |                           |         |
| Histologic subtype   |                         |                           |         |
| Lepidic predominant  | 3                       | 0                         | 0.732** |
| Acinar predominant   | 31                      | 9                         |         |
| Papillary and micropapillary predominant | 7 | 4 | |
| Solid with mucin production | 3 | 1 | |
| Others†               | 2                       | 1                         |         |

*P-value was obtained from t-test
**P-value was obtained from Pearson’s Chi-square test
†Includes invasive mucinous adenocarcinoma and adenosquamous carcinoma

Figure 1. Comparison of overall survival and disease-free survival of patients with lung adenocarcinoma after curative resection according to EGFR mutation type. Kaplan–Meier estimation was used to compare overall survival (A) and disease-free survival (B) of patients with EGFR mutation-positive lung adenocarcinoma according to EGFR mutation subtype. Significant difference in OS were observed between simple and compound EGFR mutation (simple mutation 83.7 months vs. compound mutation 72.8 months, P = 0.020). P-value was obtained by Breslow test.
ally increased from 4% in 2004 to 14% in 2013.16,17,21 In these cases were considered simple mutations. In this study, mutations are accompanied by a continuous, in-frame point mutation or insertion around the deleted motif. In this study, the detection rate of compound mutation in gDNA showed that the frequency of compound mutation in the lung adenocarcinoma. Table 4.

### Discussion

The definition of a compound EGFR mutation remains as ambiguous as its clinical significance. A compound mutation is defined as a combination of 2 or more independent mutations in EGFR-TKD. In the case of E19del, approximately half of the mutations are accompanied by a continuous, in-frame point mutation or insertion around the deleted motif. In this study, these cases were considered simple mutations.

The detection rate of compound EGFR mutations has gradually increased from 4% in 2004 to 14% in 2013.16,17,21 In a report from the early era of EGFR sequencing, CDNA of EGFR exon 18–21 was generated by RT-PCR and used as a template for sequencing. In that study of Japanese cohorts, 111 of 277 lung adenocarcinomas showed EGFR mutations, and 4 of 111 EGFR mutation-positive cases (4%) were compound EGFR mutations.21 A study that applied the direct sequencing of EGFR mutation-positive cases, 78 (12.4%) were uncommon EGFR mutations and approximately half of these, 32 cases, were compound EGFR mutations.22 A report that adapted bidirectional direct DNA sequencing showed that the detection rate of compound EGFR mutation was 14% of total EGFR mutations.16 These differences in the frequency of compound EGFR mutations may be attributed to the progress of sequencing technology and the source of sequencing templates. Recent extensive clinical application of PNA clamping-based EGFR mutation detection techniques that focus on detection of the G719X, L858R, L861Q, or E19del and one or more rare atypical partner mutations showed an increased detection rate of EGFR mutations. However, compound EGFR mutations were very rarely encountered in daily practice. This study adopted NGS-based repeated deep sequencing at exon 18–21 of EGFR, and the detection rate of compound EGFR mutations was 24.6%. These technical advances in sequencing provide a higher probability of encountering EGFR compound mutations.

The majority of compound EGFR mutations are composed of one typical EGFR mutation and an atypical partner mutation. Point mutations have a higher chance of harboring an atypical partner mutation. This may be related to the definition of a compound EGFR mutation, in which consecutive mutation around the E19del is defined as a simple mutation. The atypical partner mutations are quite heterogeneous with respect to location in the EGFR gene, and it is difficult to generalize their effects on EGFR-TKI. A report by Peng et al. showed that among the 22 cases of the multiple EGFR mutation 20 (90.1%) had L858R or exon 19 in-frame deletion EGFR mutation.23 The type of compound EGFR mutation is more homogeneous than our findings, which showed 7 (46.7%) out of 15 cases accompanied with L858R or exon 19 in frame deletion. In a report by Kosaka et al., one tumor with a mutation at codon 719 and 3 tumors with mutations at codon 858 contained another mutation at E709H, S768I, R776C, or T790M, respectively.21 This finding is similar to that of Wu et al., who showed that all multiple mutations contained one sensitizing mutation such as G719X, L858R, L861Q, or E19del and one or more rare atypical partner mutations. However, the findings of Kobayashi et al. and the current study indicate that 20–27% of compound EGFR mutations consist of rare atypical mutations.16

The concept that one cancer has single driver mutation is being challenged by the advancement of techniques which...
are capable of sequencing multiple genes at a time. When
the frequency of the co-alteration of EGFR and ALK rear-
rangement was evaluated by EGFR direct sequencing and
ALK FISH, it is 0.27%.24 When the EGFR mutations status
was re-inspected in ALK rearrangement positive and
EGFR mutation negative cases with the mutant enriched NGS, the
co-mutation rate was increased up to 15.4%.24 Another
study that investigated mutation of PIK3CA exon 9 and 20
in 1,117 NSCLC showed that it was detected in 3.9% of
squamous cell cancer and 2.7% of adenocarcinoma.25 Among 34 NSCLC cases that have
PIK3CA mutation, 17 cases had co-mutation in the EGFR exon 18~21 and 4 cases
in the KRAS exon 2~3 showing PIK3CA mutation is frequently
accompanied with EGFR/KRAS mutation.25 In our
study, ALK rearrangement and PIK3CA was observed in 3
cases respectively, suggesting that the representative driver
mutations are not completely mutually exclusive and can occasionally be found at lower frequently. It is worthy of
notice that MET had highest mutational burden among the
genes tested in this panel. However, no mutation was
detected in the exon14 and exon skipping could not be
detected by the applied technique.26,27 Mutations in the
MET kinase domain (c.3166-c.4068; Exon 15~21) were
detected in the 8 cases, but their biologic signifi-
cance is not
confirmed yet.
A few papers have reported that there are differences in
the responses to the EGFR-TKIs among compound EGFR
mutations. Peng et al. revealed that when the clinical out-
come between NSCLC patients with L858 single mutation
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Table 6. Mutations detected in the lung adenocarcinoma with compound EGFR mutation.

| Rand No. | ALK | BCL2L11 | BRAF | ERBB2 | FGFR1 | KRAS | MET | NRAS | PIK3CA | PTEN | ROS1 | RET |
|----------|-----|---------|------|-------|-------|------|-----|------|--------|------|------|-----|
| E0001    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0012    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0048    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0113    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0140    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0154    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0170    |     |         |      |       |       |      |     |      |        |      |      |     |
| E176     |     |         |      |       |       |      |     |      |        |      |      |     |
| E0214    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0217    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0228    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0231    |     |         |      |       |       |      |     |      |        |      |      |     |
| E0235    |     |         |      |       |       |      |     |      |        |      |      |     |

*Actionable mutations (19).
**BCL2L11 intron 2 deletion mutant (20).
*No mutation was detected in AKT1, DDR2, and MAP2K1.
18–21 was compared, there was no significant differences in OS and PFS.22 Another study addressed the clinical significance of compound EGFR mutations, showing a poorer outcome for patients with rare atypical mutations combined with E19del or L858R (progression-free survival (PFS) 5.3 months, OS 18.8 months) compared with those with single classic mutations (PFS 8.5, OS 19.6 months).17 Compound mutations that contain sensitizing mutations such as G719X or L858R seem to have good responses to EGFR-TKIs. On the other hands, those comprised of rare atypical mutations or L858R seem to have good responses to EGFR-TKIs.17,28 In our study, a homogenous cohort was selected to identify the clinical meaning of compound mutations, and we found that patients with compound EGFR mutations had poorer OS than those with simple EGFR mutations. It is of note that there was no difference in the DFS. These findings suggest that mutation status may be related to the response to drug administered after confirmation of recurrence. The unproved supposition that tumors with a compound EGFR mutation do not respond to EGFR-TKI might cause clinicians to hesitate in positioning EGFR-TKI at the early line of therapy, which may have complicated evaluation of the response to EGFR-TKI in this study cohort. Several other factors such as male predominance, larger tumor size at the time of detection, and aggressive histologic subtype might have acted in combination to influence the poor OS of patients with the compound EGFR mutation.

The biologic significance of co-alteration of EGFR and other genes need to be investigated. In a study that evaluated the response to TKIs in the 14 NSCLC which had EGFR and ALK co-alteration, 3 treated with EGFR-TKI showed poor responses to gefitinib but 8 treated with ALK inhibitors revealed favorable responses, suggesting that signaling from ALK rearrangement override EGFR.24 Others addressed the importance of PIK3CA mutation test by showing that the patients with PIK3CA single mutation showed poorer prognosis than those with co-mutation of PIK3CA and EGFR/KRAS.25

A few mutations in the BCL2L11, ALK, PIK3CA, and KRAS are key driver mutations that can be potentially targeted, while those in the other genes need further validation. It would be interesting to see if the NSCLC patients with EGFR compound mutation or co-alteration with other genes may benefit from 3rd generation EGFR-TKIs when compared to 1st and 2nd generation EGFR-TKIs.29,30

In conclusion, compound EGFR mutation is frequently detected in EGFR-mutant tumors and is related to poor overall survival of patients with lung adenocarcinoma. Because it is expected that such mutations may be more frequently detected with wider adoption of NGS-based tests, more dedicated efforts are needed to clarify their biologic effects on disease course and drug responsiveness.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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