Objective: The purpose of our study was to define the prognostic impact of increased copies of epidermal growth factor receptor (EGFR) gene in lung adenocarcinoma patients receiving adjuvant chemotherapy after surgery.

Methods: The study included 95 adenocarcinoma patients who received curative resection for non-small cell lung cancer. Patients received adjuvant chemotherapy composed of paclitaxel and carboplatin. We performed fluorescent in situ hybridization on tissue microarray in duplicate to detect EGFR copy number change.

Results: The EGFR fluorescent in situ hybridization result was available in 93 patients with a positive rate of 32.6%. EGFR copy number change did not correlate with age, gender or smoking history. However, EGFR copy number gain was related to high tumor standardized uptake value at diagnosis ($P = 0.042$). An increase in EGFR copy number was a negative prognostic factor in terms of disease-free survival (median disease-free survival not reached versus 23.6 months, $P = 0.037$) and overall survival (median overall survival 74.6 versus 43.5 months, $P = 0.032$). An increase in EGFR copy number was independently related to short disease-free survival in multivariate analysis (hazard ratio 2.039, $P = 0.025$).

Conclusions: EGFR copy number gain is associated with aggressive tumor biology and is a poor prognostic factor for tumor relapse in resected lung adenocarcinoma patients receiving adjuvant chemotherapy of paclitaxel and carboplatin.

Key words: lung adenocarcinoma – non-small cell lung cancer – FISH – EGFR – prognosis
increased EGFR copy number are sensitive to EGFR TKI and tend to have a greater chance of longer survival with EGFR TKI treatment (11–13). However, because an increased EGFR copy number frequently accompanies EGFR mutation (14), it is doubtful whether an increase in EGFR copy number gain per se contributes to EGFR TKI sensitivity. Rather, a recent biomarker analysis in an IPASS study reported that EGFR copy number gain without EGFR mutation is not related to gefitinib sensitivity (15,16). On the while, there are reports that EGFR copy number gain is related to aggressive tumor biology (17) and shows tendency for frequent relapse after resection (18–21). These complicating results necessitate further investigation of an increased EGFR copy number.

Recently, several studies investigated the prognostic role of EGFR pathway molecules in surgically resected NSCLC. These reports consistently state that resected NSCLC with EGFR fluorescent in situ hybridization (FISH) positivity by Cappuzzo et al.’s criteria (13) has a tendency for poor survival (18–21). Hence, the proportion of patients receiving adjuvant chemotherapy was <5% in these four studies, and it is unknown whether this poor tendency for the short survival of EGFR copy number-increased NSCLC can be overcome by adjuvant cytotoxic chemotherapy. In fact, the role of EGFR FISH as a predictive marker for cytotoxic chemotherapy is unknown.

So, we planned to investigate whether the tendency for the poor survival of EGFR copy number-increased NSCLC patients who receive resection could be overcome by adjuvant cytotoxic chemotherapy. Because the interaction between EGFR copy number increase and tumor histology is unknown, we confined our study population to patients with adenocarcinoma histology to avoid confusion. In addition, we compared a maximum standardized uptake value (SUV) in positron emission tomography (PET) scanning according to the EGFR FISH status to confirm the aggressiveness of EGFR copy number-increased tumor.

PATIENTS AND METHODS

Patients

A cohort of consecutive patients who had undergone curative surgery for lung adenocarcinoma at Seoul National University Hospital, received adjuvant chemotherapy after surgery and had available formalin-fixed paraffin-embedded samples taken in adequate amounts for analysis, were selected for this study. The patient cohort was composed of 95 patients who had undergone surgery for clinically operable lung adenocarcinoma between January 2003 and December 2008. The patients received adjuvant chemotherapy, consisting of four cycles of tri-weekly paclitaxel (175 mg/m²) and carboplatin (area under the curve = 5).

All data regarding patient demographics and clinicopathologic characteristics including age, gender, smoking history, histologic subtype, pathologic stage, disease-free survival (DFS) and overall survival (OS) were obtained by a medical record review. Patients who had smoked more than 100 cigarettes during their lifetime were defined as smokers. DFS was defined as a period from curative resection to the documentation of disease recurrence or death from any cause. OS was calculated from curative resection to death from any cause.

The institutional review board of Seoul National University Hospital reviewed and approved the study protocol. In addition, we followed the Declaration of Helsinki recommendations for biomedical research involving human subjects.

Tissue Microarray Construction

Slides of tumor samples stained with hematoxylin and eosin were independently reviewed by two trained pathologists (B.J. and D.H.C.) and the representative areas were marked. We obtained core tissue biopsy specimens (2 mm in diameter) in duplicate from individual paraffin-embedded samples (donor blocks), which we arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (SuperBioChips, Seoul, Korea). Each tissue array block contained up to 60 specimens, which allowed all 190 specimens (duplicate specimens of 95 cases) to be contained in four array blocks. We analyzed serial sections from formalin-fixed paraffin blocks for FISH.

FISH for EGFR

FISH for EGFR was performed on all samples as described previously (22). EGFR FISH was considered positive when either high polysomy (≥4 copies in ≥40% of cells) or gene amplification (presence of tight EGFR gene clusters and a ratio of the EGFR gene to chromosome 7 of ≥2, or ≥15 copies of EGFR per cell in ≥10% of cells) was observed (13).

PET Scanning

PET examination was performed using Gemini (Philips, Milpitas, CA, USA) PET/CT system. All the patients fasted at least 6 h before undergoing PET examination and only hydration with glucose-free water was allowed. Before the injection of FDG, normal blood glucose levels were verified. An intravenous injection of 5.18 MBq of FDG per 1 kg of patient’s weight was done, and then patients rested for 60 min before imaging. PET/CT data were obtained with the patient in the supine position, and the image acquisition time was 2 min 30 s per bed position. Attenuation correction was done based on the CT data, and CT data were resized from a 512 × 512 matrix to a 128 × 128 matrix to match PET emission data to allow image fusion. PET data were reconstructed by using the 3D-RAMLA (row action maximum likelihood algorithm) iterative reconstruction algorithm.
STATISTICAL ANALYSIS

The variables included for analysis in this study were age, gender, smoking history, stage, EGFR FISH status, DFS and OS. Statistical analysis of 2 \times 2 contingency tables of categorical variables were performed using Pearson’s \( \chi^2 \) test or Fisher’s exact test, as appropriate. For the analysis of SUV, non-parametric test was used, where the Mann–Whitney or Kruskal–Wallis test was used as appropriate. Median durations of DFS and OS were calculated using the Kaplan–Meier method and comparisons between groups were made using log-rank tests. Multivariate analysis was performed using Cox’s regression models for DFS and OS. Factors with \( P \) values <0.10 in univariate analysis were examined with proportional hazard regression model. All statistical tests were two-sided, with significance defined as \( P < 0.05 \). All analysis was performed using Statistical Package for the Social Sciences for Windows Version 12.0 (IBM, Chicago, IL, USA).

RESULTS

PATIENT CHARACTERISTICS

This study included 50 males and 45 females with a median age of 63 years. Other patient characteristics include the following: 48 patients (50.5%) had a history of smoking; 31 patients (32.6%) were diagnosed as Stage IB; 9 patients (9.5%) were diagnosed as Stage IIA; 16 patients (16.8%) were diagnosed as Stage IIB; 26 (27.4%) patients were diagnosed as having Stage IIIA; and 13 (13.7%) patients were diagnosed as having Stage IIIB disease. Characteristics of patients are summarized in Table 1.

CLINICOPATHOLOGIC FEATURES OF PATIENTS WITH EGFR COPY NUMBER GAIN

The EGFR FISH result was available in 93 patients. Positive EGFR FISH was observed in 31 patients (31/93 = 32.6%). The EGFR FISH result did not demonstrate a correlation with age, gender, smoking status and tumor stage at surgery. A comparison of clinicopathologic characteristics between EGFR FISH positive and negative patients is summarized in Table 2.

IMPACT OF EGFR FISH POSITIVITY ON DFS AND OS

Patients were followed up for a median of 33.4 months. During the period, 40 patients experienced relapse and 12 patients died. DFS was different according to the tumor stage (\( P = 0.023 \); Fig. 1A). Age, gender and smoking status did not influence DFS. EGFR FISH status significantly influenced DFS. Patients with increased EGFR copy number had shorter DFS compared with those without increased EGFR copy number in univariate analysis (median DFS 23.6 months versus not reached, \( P = 0.037 \); Fig. 1C). When multivariate analysis was performed considering stage and EGFR FISH status, both stage (\( P = 0.028 \)) and EGFR FISH status (\( P = 0.025 \), hazard ratio 2.039) were independent prognostic factors for DFS.

After relapse, 31 patients received palliative chemotherapy. Among them, 28 patients received either erlotinib or

Table 1. Baseline characteristics of 95 resected lung adenocarcinoma patients who received adjuvant chemotherapy

<table>
<thead>
<tr>
<th>Number of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median, range)</td>
</tr>
<tr>
<td>63 (37–75)</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male 50 (52.6)</td>
</tr>
<tr>
<td>Female 45 (47.4)</td>
</tr>
<tr>
<td>Smoking history</td>
</tr>
<tr>
<td>Yes 48 (50.5)</td>
</tr>
<tr>
<td>No 47 (49.5)</td>
</tr>
<tr>
<td>Pathologic stage</td>
</tr>
<tr>
<td>IB 31 (32.6)</td>
</tr>
<tr>
<td>IIA 9 (9.5)</td>
</tr>
<tr>
<td>IIB 16 (16.8)</td>
</tr>
<tr>
<td>IIIA 26 (27.4)</td>
</tr>
<tr>
<td>IIIB 13 (13.7)</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of 95 patients according to the EGFR FISH status

<table>
<thead>
<tr>
<th></th>
<th>EGFR FISH(+) (n = 31)</th>
<th>EGFR FISH(–) (n = 62)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median, range)</td>
<td>62.1 (39–75)</td>
<td>62.0 (40–75)</td>
<td>0.856</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>29</td>
<td>0.187</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>31</td>
<td>0.883</td>
</tr>
<tr>
<td>No</td>
<td>15</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Pathologic stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>11</td>
<td>19</td>
<td>0.852</td>
</tr>
<tr>
<td>IIA</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>5</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>10</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>IIIB</td>
<td>3</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Pre-operative SUV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean, range)</td>
<td>7.3 (1.6–13.1)</td>
<td>5.8 (1.1–14.4)</td>
<td>0.042</td>
</tr>
</tbody>
</table>

EGFR FISH, epidermal growth factor receptor fluorescent in situ hybridization; SUV, standardized uptake value.

*EGFR FISH result was available in 93 patients.

*Comparison was made with the Mann–Whitney test.
gefitinib as a palliative treatment. Response rate to EGFR TKI was 21.4%. Their median PFS of EGFR TKI treatment was 4.6 months. In OS analysis, EGFR FISH positivity was related to short OS in univariate analysis (median OS 43.5 versus 74.6 months, \( P = 0.032 \); Fig. 1D). Age, gender, smoking status and tumor stage did not influence OS (Fig. 1B).

In addition, 34 among 95 patients had information on EGFR mutation status. When the impact of EGFR mutation status on DFS and OS was analyzed, EGFR mutations were not a predictive factor for DFS (\( P = 0.102 \)). Also for OS, EGFR mutations were not a prognostic factor (\( P = 0.238 \)) (Fig. 2). When we checked OS and DFS according to the SUV, we found that tumor SUV also influences DFS of these patients. Using the median as a cut-off value, we categorized the study patients into two groups: (1) patients with high tumor SUV and (2) patients with low tumor SUV. Then, for DFS, high SUV of tumor was related to short DFS (median DFS 22.7 versus 54.7 months, \( P = 0.030 \)). However, for OS, high SUV of tumor was not related to short OS (\( P = 0.237 \)) (Fig. 4).

**DISCUSSION**

In this study, we showed that EGFR copy number increase was independently related to short DFS in lung adenocarcinoma patients who received curative resection followed by adjuvant cytotoxic chemotherapy. Both univariate and multivariate analyses showed that an increase in EGFR copy number was a poor prognostic factor in these patients. Also, we showed that tumors with EGFR copy number increase had higher pre-operative SUV compared with EGFR FISH negative patients (\( P = 0.042 \) (Table 2 and Fig. 3).

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**ANALYSIS OF PRE-OPERATIVE SUV ACCORDING TO EGFR FISH STATUS**

Seventy-three patients underwent PET testing before operation. A maximum SUV of the patients ranged from 1.09 to 14.40 and the mean and median values were 6.3 and 5.8, respectively. Gender (\( P = 0.710 \)), smoking (\( P = 0.365 \)) and stage at diagnosis (\( P = 0.094 \)) were not related to the maximum SUV. However, the EGFR FISH result influenced maximum SUV and EGFR FISH-positive patients had higher pre-operative SUV compared with EGFR FISH negative patients (\( P = 0.042 \)) (Table 2 and Fig. 3).

Figure 1. Disease-free survival (DFS) (A) and overall survival (OS) (B) according to the stage, and DFS (C) and OS (D) according to the EGFR FISH status. Stage at surgery had a prognostic value in terms of DFS (A). However, stage did not have a prognostic value in terms of OS (B). EGFR FISH positivity was a poor prognostic factor both in terms of DFS (C) and OS (D).
meaning of EGFR FISH in patients receiving adjuvant chemotherapy for the first time.

With these results, we conjecture the followings. First, EGFR copy number-increased lung adenocarcinoma is biologically more aggressive than that without EGFR copy number increase. In fact, previous studies support this idea. Both Western and Asian studies state that EGFR FISH positive lung adenocarcinoma is aggressive with high tumor SUV on PET (17,23). In addition, one retrospective study from Japan demonstrated a poor survival of EGFR FISH-positive NSCLC patients receiving surgery (18). The study is distinct in the sense that it demonstrated a statistically significant short survival of EGFR gene-amplified NSCLC patients who receive resection, which does not coincides with the study results by Cappuzzo et al. (19) and Hirsch et al. (20) that showed only tendency for the poor survival of EGFR copy number-increased NSCLC patients receiving resection. However, when we focus on the histology of the patients in those three studies, we find an interesting point. The Japanese study (18) included mainly adenocarcinoma patients (91/109 = 83%), whereas the proportion of adenocarcinoma patients in the other two studies were only 54 (19) and 43% (20), respectively. Hence, we guess that the differences in the proportion of adenocarcinoma in these three studies lead to different results. To summarize, EGFR copy number increase may be related to aggressive tumor biology only in adenocarcinoma, whereas the meaning of increased EGFR copy number would be different in NSCLC with the other histologies.

Secondly, the aggressive tumor biology of EGFR copy number-increased lung adenocarcinoma is not overcome by cytotoxic chemotherapy with paclitaxel and carboplatin.
Rather, there is a chance that the aggressive tumor feature is exaggerated after chemotherapy, resulting in an earlier relapse. There is no firm evidence regarding chemoresistance of EGFR FISH-positive lung adenocarcinoma so far. Rather, subgroup analysis of TRIBUTE (24) and INTEREST (25) trials failed to show firm chemoresistance of EGFR FISH-positive tumors. However, because both TRIBUTE (26) and INTEREST (27) trials enrolled adenocarcinoma and non-adenocarcinoma patients at the same time, we cannot conclude with those studies that EGFR FISH is not a predictive marker for cytotoxic chemotherapy in lung adenocarcinoma. Rather, further studies on a cohort of patients with adenocarcinoma histology receiving palliative cytotoxic chemotherapy are necessary to confirm this issue. Also, establishing EGFR gene-amplified cell lines with adenocarcinoma and non-adenocarcinoma histology, respectively, would reveal the underlying biologic mechanism behind these phenomena.

Also, it is well known that EGFR mutations status has high correlation with EGFR FISH status. Considering this, we also analyzed the impact of EGFR mutations on DFS and OS to differentiate the impact of EGFR mutations from EGFR copy number gain on DFS and OS. And when this was performed, EGFR mutations status was not predictive of DFS and OS. Hence, we confirm that in contrast to EGFR mutations, EGFR copy number gain per se is a poor predictive factor for DFS and OS. However, because only 34 patients had information on EGFR mutations status, we admit that further large-scale study is still necessary for confirmatory data on this issue. In addition, we compared a prognostic value of EGFR FISH with SUV per se. Although high SUV of tumor was related to short DFS, high SUV of tumor was not related to short OS. Referring from the above, we do not think that tumor SUV is a more substantial predictive marker or a better prognostic marker than EGFR FISH status.

Although we showed a poor prognostic impact of EGFR FISH in terms of OS, we focused our analysis on DFS instead of OS for the following reasons. First, only 12 patients died during the follow-up period and the small number of deaths may mislead us to make a bias. Secondly, EGFR FISH positivity is a predictive marker for response in EGFR TKI treatment, and subsequent palliative treatment with EGFR TKI after relapse would alter the natural course of EGFR FISH-positive NSCLC. Hence, we suggest that DFS is a more preferable surrogate marker in our study compared with OS.

A limitation of our study is that the adjuvant chemotherapy regimen applied to the patients was paclitaxel and carboplatin. Although paclitaxel and carboplatin have been administered to many patients as adjuvant chemotherapy historically (28,29) and is still used in some centers and used for specific patients, the regimen is not anymore recommended as a standard regimen by a national comprehensive cancer center in the adjuvant setting. Hence, there is a chance that with recently used regimens, the adverse prognostic impact of EGFR copy number increase in lung adenocarcinoma can be overcome.

In conclusion, EGFR copy number gain is associated with aggressive tumor biology and is a poor prognostic factor for tumor relapse in resected lung adenocarcinoma patients receiving adjuvant chemotherapy of paclitaxel and carboplatin. A biologic meaning of EGFR copy number gain is not well defined yet, and the underlying mechanism of these phenomena mandates further research.

AUTHOR CONTRIBUTIONS

Y.K. performed research, analyzed data and wrote the paper; B.J. performed research and analyzed data; Y.K.I. performed research and contributed analytical tools; T.M.K. performed research; S.-H.L. performed research; D.-W.K. designed research, analyzed data and wrote the paper; D.H.C. performed research and contributed analytical tools; Y.T.K. performed research; Y.W.K. performed research; and D.S.H. performed research and gave administrative support.

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Conflict of interest statement

None declared.

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