Clinicopathological and prognostic features of surgically resected pathological stage I lung adenocarcinoma harboring epidermal growth factor receptor and K-ras mutation

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Abstract
Background: This study aimed to evaluate mutations of the epidermal growth factor receptor (EGFR) and K-ras genes and their clinicopathological and prognostic features in patients with resected pathological stage I adenocarcinoma.

Methods: We examined 224 patients with surgically resected lung adenocarcinoma and analyzed the prognostic and predictive value of these mutations in 162 patients with pathological stage I adenocarcinoma.

Results: Mutations of the EGFR and K-ras genes were detected in 100 (44.6%) and 19 (8.5%) of all tumors, and in 81 (50.0%) and 17 (10.5%) of the pathological stage I tumors, respectively. EGFR mutations were significantly associated with female gender, smoking habit (never smoker), and low grade. By contrast, K-ras mutations were significantly associated with male gender, smoking habit (ever smoker), and the presence of mucinous components. No significant differences were observed in recurrence-free or overall survival between the EGFR-mutant, K-ras-mutant, and wild-type groups (five-year recurrence-free survival 77.8% vs. 87.8% vs. 79.5%; five-year overall survival 82.8% vs. 82.4% vs. 79.2%, respectively). Multivariate analysis showed that neither EGFR nor K-ras mutation was an independent prognostic factor.

Conclusions: The present study demonstrated that pathological stage I adenocarcinoma harboring EGFR and K-ras gene mutations have distinct clinicopathological features. The presence of these mutations alone were not prognostic factors in patients with resected pathological stage I adenocarcinoma.

Introduction
Lung cancer remains the leading cause of death among all cancers, and a relationship between tumor node metastasis (TNM) stage and survival has been reported. Over the past decade, the overall survival (OS) of lung cancer patients has greatly improved. This progress is largely a result of the introduction of new drugs and individualized therapy based on different histological subtypes and driver mutations that determine the biology of lung cancers and can be used to predict drug efficacy. The epidermal growth factor receptor (EGFR) gene is currently the most promising and “druggable” oncogene in non-small cell lung cancer (NSCLC). The targeting of EGFRs, especially by using EGFR-tyrosine kinase inhibitors (TKIs), has played a central role in advancing NSCLC research, treatment, and outcome prediction. Recently, EGFR-TKIs have also been shown to improve OS in certain EGFR mutations. Some specific EGFR mutations are associated with sensitivity to EGFR-TKIs. Small exon 19 deletion (del 19) and exon 21-point mutation (L858R) are the two most common mutations associated with improved outcomes after EGFR-TKI therapy. K-ras is another oncogene, in
which mutations occur more frequently in smokers. Compared with an approximate 50% mutation rate of the gene encoding \( \text{EGFR} \) in Asian patients, the mutation rate of \( \text{EGFR} \) is only 10–15% in white populations. K-\( \text{ras} \) is the most commonly mutated oncogene in lung cancers in Western countries, with activating point mutations in 15–20% of all NSCLCs and 25–35% of all adenocarcinomas. Many studies have suggested that mutated K-\( \text{ras} \) is associated with poorer OS in patients with NSCLC.

Anti-\( \text{EGFR} \) therapies are ineffective for K-\( \text{ras} \) mutant tumors, which are associated with a lack of sensitivity and poorer clinical outcomes when treated with \( \text{EGFR} \)-TKIs or chemotherapy. It is worth noting that \( \text{EGFR} \) and K-\( \text{ras} \) mutations are rarely found in the same tumor, suggesting that they may drive functionally different carcinogenetic processes. Direct targeting of K-\( \text{ras} \) has recently raised some concern, as this represents a key transduction pathway in both normal and tumor tissues. Moreover, several parallel escape mechanisms have been identified. Moving from these considerations, alternative targeting of K-\( \text{ras} \) is currently under evaluation.

The aims of the present study were to evaluate mutations of the \( \text{EGFR} \) and K-\( \text{ras} \) genes at the time of surgery and to analyze the clinical significance of these mutations in terms of their prognostic and predictive value in pathological stage I adenocarcinoma patients.

**Methods**

**Patient eligibility**

Between April 2007 and December 2013, 332 consecutive patients underwent pulmonary resection for lung cancer at the Sagamihara Kyodo Hospital, Kanagawa, Japan. We reviewed the data of 162 of these patients who were diagnosed with pathological stage I adenocarcinoma according to the seventh edition of the TNM Staging Classification for Lung Cancer. Patients who underwent incomplete resection or neoadjuvant chemotherapy/radiotherapy were excluded.

We reviewed the medical records of each patient for the following clinicopathological information: age, gender, smoking habit, serum carcinoembryonic antigen (CEA), extent of pulmonary resection, tumor location, maximum standardized uptake value (\( \text{SUV}_{\text{max}} \)) of the primary tumor, tumor size (cm), grade, pleural invasion, mucinous components, \( \text{EGFR} \) mutation status, K-\( \text{ras} \) mutation status, and pathological stage. All clinical, intraoperative, radiological, and pathological findings from two hospitals in Kanagawa, Japan (Sagamihara Kyodo Hospital and Yuai Clinic) were reviewed. The patients’ characteristics and preoperative and postoperative tumor evaluations are shown in Table 1. Histological classification of NSCLC was based on the World Health Organization classification. Preoperative and postoperative staging were based on the TNM staging system. Data collection and analyses were approved, and the need to obtain written informed consent from each patient was waived by the first author’s institutional review board.

**Computed tomography**

Diagnostic quality contrast-enhanced computed tomography (CT) of the chest with a slice thickness of 5 mm was performed for all patients. A tumor was deemed central if
its center was located in the inner one-third of the lung parenchyma (adjacent to the mediastinum) on transverse CT. Peripherally located tumors were identified as those centered in the outer two-thirds of the lung parenchyma on transverse CT. The maximal diameter of the lung nodules was measured on contrast-enhanced chest CT. All imaging was performed within four weeks of surgery.

**Integrated ¹⁸F-fluorodeoxyglucose positron emission tomography imaging**

Each patient underwent integrated ¹⁸F-fluorodeoxyglucose positron emission tomography/CT (FDG-PET/CT) imaging before surgical resection. All integrated FDG-PET/CT imaging was performed within four weeks of surgery. After fasting for six hours, FDG (3.5 MBq/kg body weight) was intravenously injected if the patient’s blood sugar level was lower than 200 mg/dL. Image acquisition commenced 60 minutes after the injection using a single PET/CT combined scanner (Eminence-SOPHIA; Shimadzu, Kyoto, Japan).²² Image emission data from the eyes to the mid-thigh area were continuously acquired over a period of approximately 20 minutes. After attenuation corrections were made for the resultimg image data, reconstruction was performed using a dynamic row-action expectation maximization algorithm.²² The reconstructed sectional images were then evaluated both visually and quantitatively using the SUVₘₐₓ inside a volume of interest (VOI) placed on the lesions. The SUVₘₐₓ was calculated as follows: ([maximum activity in VOI] / [volume of VOI]) / ([injected FDG dose] / [patient weight]). The quality of radiation measurements of the PET/CT scanner was assured by calibration in accordance with National Electrical Manufacturers Association NU-2 2001 standards.²³

Nodal uptake with an SUVₘₐₓ > 2.5 was considered positive. To determine the SUV, a cylindrical region of interest (ROI) was placed over the tumor site manually on the transaxial slice. The activity concentration within the (ROI) was placed over the tumor site manually on the hot spot transaxial slice. The activity concentration within the ROI was determined and expressed as the SUV, where SUV is the ratio of the activity in the tissue to the decay-corrected activity injected into the patient. All SUV measurements were normalized for patient body weight. SUVₘₐₓ within an ROI was used as the reference measurement.²⁴

Three experienced radiologists individually analyzed the integrated FDG-PET/CT images. Final assessment was made by consensus if the initial assessments differed.

**Surgical resection**

All patients underwent anatomical lung resection and radical lymphadenectomy or sublobar resection in our hospital. Thoracic surgeons at Sagamihara Kyodo Hospital performed all surgical resections and all techniques were standardized. Systematic lymph node dissection was performed in all patients according to American Thoracic Society criteria, removing at least three hilar and three mediastinal stations.

**Pathological examination**

Experienced pulmonary pathologists examined all resected tumor specimens. Histological classification of NSCLC was based on the World Health Organization classification. Dissected lymph nodes were histologically examined following hematoxylin and eosin staining.

**Epidermal growth factor receptor (EGFR) and K-ras mutation analysis**

Genomic DNA was extracted and purified from tumors embedded in paraffin blocks using the Takara DEXPAT kit (Takara Bio Inc., Kusatsu, Shiga, Japan) from materials macro-dissected from the paraffin-embedded sections. Quantification of the extracted nucleic acids and measurement of the A260/A280 ratio were performed using an ultraviolet spectrophotometer (Beckman Coulter DU800, Koto-ku, Tokyo, Japan). A common fragment analysis was used for screening to detect the deletion in exon 19 of the *EGFR* gene. Sample DNA was amplified with a FAM-labeled primer set: 5’-TGGCACCATCTCACAATTCG-3’ (forward) and 5’-AGGATGGAGATGACAGG-3’ (reverse). PCR products were separated by electrophoresis using an ABI PRISM 310 (Thermo Fisher Scientific, Yokohama, Kanagawa, Japan). When a deletion mutation was present, PCR was used to amplify the shorter DNA segment, thereby creating a new peak in the electropherogram. The deletion in exon 19 was confirmed using primers constructed to make a 147 bp product when the allele was wild type. The primer sequences were 5’-TGGCACCATCTCACAATTCG-3’ (forward) and 5’-GAAAAGGTGGC CCTGAGGTTC-3’ (reverse). A PCR-based restriction fragment length polymorphism analysis was performed to detect the K-ras mutations in codons 12 and 13. All direct sequencing was performed to detect K-ras (codons 12 and 13) mutations according to the manufacturer’s protocol for the BigDye v1.1 kit (Applied
Biosystems, Foster City, CA, USA). Sequencing was performed using the 310 Genetic Analyzer (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using SPSS version 23.0 (IBM Corporation, Armonk, NY, USA). Survival curves were constructed using the Kaplan–Meier method. Recurrence-free survival (RFS) probabilities and OS rates were compared using the log-rank test. The Cox proportional hazard model was used to estimate hazard ratios (HRs) with 95% confidence intervals (CIs) for the univariate and multivariate analyses. All tests were two-sided, and P values <0.05 were considered statistically significant. Factors found to be significant in univariate analysis (P < 0.05) were included in multivariate analysis.

Results

Patient characteristics

The clinicopathological features of the 162 patients (79 women, 83 men; mean age, 68.9 years; age range 40–86 years) are listed in Table 1. Eighty-two of the patients were never smokers. The median tumor size was 2.7 cm, and the median SUV_{max} of the primary tumor was 2.3. EGFR and K-ras mutations were detected in 81 (50.0%) and 17 (10.5%) of 162 tumors, respectively. Forty-one patients with EGFR gene mutations showed an exon 19 deletion, and 40 showed an exon 21-point mutation. Seventeen patients with K-ras gene mutations showed a codon 12-point mutation, while no patients showed a codon 13-point mutation. The EGFR and K-ras gene mutations were mutually exclusive.

Correlations between the mutations and clinicopathological features were analyzed (Table 2). EGFR mutations were significantly associated with female gender, smoking habit (never smoker), and low grade. By contrast, K-ras mutations were significantly associated with male gender, smoking habit (ever smoker), and the presence of mucinous components.

Survival analysis of patients with pathological stage I adenocarcinoma after surgical resection

Among the 162 patients, five-year RFS and OS were 79.6% and 81.3%, respectively. In the survival analyses, the five-year RFS rates were 77.8% vs. 87.8% vs. 79.2% for patients with an EGFR mutation, K-ras mutation, and wild-type status, respectively (Fig 1a). The five-year OS rates were 82.8 vs. 82.4 vs. 79.2 for patients with an EGFR mutation, K-ras mutation, and wild-type status, respectively (Fig 1b). Significant differences were observed in both RFS and OS between patients with an EGFR mutation and those with wild-type genes (RFS P = 0.903, OS P = 0.883), and between patients with an EGFR mutation and those with a K-ras mutation (RFS P = 0.317, OS P = 0.952).

Univariate analysis showed that serum CEA, SUV_{max} of the tumor, pleural invasion, and pathological stage were significant unfavorable prognostic factors for RFS (P < 0.05), and that age at operation, serum CEA, and SUV_{max} of the tumor were significant unfavorable prognostic factors for OS (P < 0.3). In multivariate analysis

| Variables                            | EGFR (n = 81) | K-ras (n = 17) | Wild (n = 64) | P   |
|--------------------------------------|--------------|---------------|--------------|-----|
| Age at operation (year)              |              |               |              |     |
| <70                                  | 39 (48.1%)   | 8 (47.0%)     | 30 (46.9%)   | 0.988|
| ≥70                                  | 42 (51.9%)   | 9 (53.0%)     | 34 (53.1%)   |     |
| Gender                               |              |               |              |     |
| Female                               | 56 (69.1%)   | 5 (29.4%)     | 22 (34.3%)   | <0.001|
| Male                                 | 25 (30.9%)   | 12 (70.6%)    | 42 (65.7%)   |     |
| Smoking habit                        |              |               |              |     |
| Never smoking                        | 54 (66.7%)   | 5 (29.4%)     | 21 (32.8%)   | <0.001|
| Ever smoking                         | 27 (33.3%)   | 12 (70.6%)    | 43 (67.2%)   |     |
| Serum CEA (ng/mL)                    |              |               |              |     |
| ≤5                                   | 69 (85.2%)   | 15 (82.4%)    | 44 (68.8%)   | 0.033|
| >5                                   | 12 (14.8%)   | 2 (11.8%)     | 20 (31.2%)   |     |
| Extent of pulmonary resection        |              |               |              |     |
| Sublobar resection                   | 26 (32.1%)   | 4 (23.5%)     | 21 (32.8%)   | 0.754|
| Lobectomy or more                    | 55 (67.9%)   | 13 (76.5%)    | 43 (67.2%)   |     |
| Tumor location                       |              |               |              |     |
| Central                              | 4 (4.9%)     | 0 (0.0%)      | 4 (6.2%)     | 0.572|
| Non-central                          | 77 (95.1%)   | 17 (100.0%)   | 60 (93.8%)   |     |
| SUV_{max} of primary tumor           |              |               |              |     |
| ≤2.3                                 | 46 (56.8%)   | 12 (70.6%)    | 40 (62.5%)   | 0.015|
| >2.3                                 | 35 (43.2%)   | 5 (29.4%)     | 40 (62.5%)   |     |
| Tumor size (cm)                      |              |               |              |     |
| ≤3                                   | 59 (72.8%)   | 14 (82.4%)    | 42 (65.7%)   | 0.351|
| >3                                   | 22 (27.2%)   | 3 (17.6%)     | 22 (34.3%)   |     |
| Grade                                |              |               |              |     |
| 1                                    | 72 (88.9%)   | 12 (70.6%)    | 37 (57.8%)   | <0.001|
| 2–4                                  | 9 (11.1%)    | 5 (29.4%)     | 27 (42.2%)   |     |
| Pleural invasion                     |              |               |              |     |
| Absent                               | 74 (91.4%)   | 17 (100.0%)   | 54 (84.4%)   | 0.130|
| Present                              | 7 (8.6%)     | 0 (0.0%)      | 10 (15.6%)   |     |
| Mucinous components                  |              |               |              |     |
| Absent                               | 74 (91.4%)   | 5 (29.4%)     | 59 (92.2%)   | <0.001|
| Present                              | 7 (8.6%)     | 12 (70.6%)    | 7 (7.8%)     |     |
| Pathological stage                   |              |               |              |     |
| Stage I A                            | 55 (67.9%)   | 14 (82.4%)    | 34 (53.1%)   | 0.044|
| Stage IB                             | 26 (32.1%)   | 3 (17.6%)     | 30 (46.9%)   |     |

CEA, carcinoembryonic antigen; EGFR, epidermal growth factor receptor; SUV_{max}, maximum standardized uptake value.

K-ras mutation, and wild-type status, respectively. In multivariate analysis
pathological stage I patients with EGFR
Western populations, pathological stage I adenocarcinoma. Compared with
we retrospectively evaluated the outcomes of patients with
Discussion
Figure 1
Figure 1 (a) Recurrence-free survival curves of pathological stage I patients after pulmonary resection. Data are shown for patients with epidermal growth factor receptor (EGFR) and K-ras mutations and for those who were wild type for both genes. (b) Overall survival curves of pathological stage I patients with EGFR and K-ras mutations or both wild-type genes after pulmonary resection. adjusted for the significant univariate factors, SUVmax of the tumor remained an independent prognostic factor for RFS (P = 0.001), and age at operation and SUVmax of the tumor remained independent prognostic factors for OS (P = 0.029, 0.008; Table 4). EGFR and K-ras mutations did not affect the prognosis of patients with pathological stage I adenocarcinoma.

Discussion
We retrospectively evaluated the outcomes of patients with pathological stage I adenocarcinoma. Compared with Western populations, EGFR mutations are detected more frequently in the lung adenocarcinomas of Japanese patients, ranging from 40% to 60%. On the other hand, compared with Western populations, K-ras mutations are detected less frequently in the lung adenocarcinomas of Japanese patients. The frequency of K-ras mutation ranges from about from 7% to 16% in worldwide populations.5,30,33,34 Similarly, the frequency of K-ras mutations was 10.5% in the current study.

The presence of an EGFR mutation is closely associated with several clinicopathological features, such as gender and smoking habit. This is consistent with previous studies, which reported that EGFR gene mutations are common in lung cancers in never smokers and in women with adenocarcinoma.6,7 Several reports have described the relationship between K-ras mutation status and clinicopathological features such as gender, smoking habit, and pathological type.28,30,33,34 Similar to results reported in previous studies, the current series showed a relationship between K-ras mutation status and gender. Mucinous bronchioloalveolar carcinoma (BAC)/adenocarcinoma with bronchioloalveolar features is found in 48–76% of adenocarcinomas with K-ras mutations, and K-ras mutations are found in 28–86% of adenocarcinomas with mucinous BAC.30,36,40 In the present study, 12 (70.6%) of the 17 cases with K-ras mutations were mucinous BAC/adenocarcinoma with bronchioloalveolar features.

In lung adenocarcinoma simultaneously harboring multiple heterogeneous clones of EGFR and K-ras mutations, the effect of EGFR-TKIs may be limited to the parts carrying EGFR mutations only.51,41 Because both EGFR and K-ras mutations are thought to be early events in lung adenocarcinoma,32 the reported coexistence of EGFR and K-ras mutations only accounts for about 5% of patients with EGFR mutations.43 Takamochi et al. reported coexisting EGFR and K-ras mutations in two (2%) of 82 patients with lung adenocarcinomas.41 A previous study reported that all tumors that had responded to gefitinib had wild type K-ras,44 thereby suggesting that K-ras and EGFR mutations are mutually exclusive.45 None of the patients in our series had concomitant EGFR and K-ras mutations; this result is similar to previous reports, further suggesting that K-ras and EGFR mutations are mutually exclusive. Accordingly, combined EGFR and K-ras mutation analyses may be helpful in selecting treatment strategies for patients with lung adenocarcinomas.

We also investigated the effects of EGFR and K-ras mutation status on survival. Neither EGFR nor K-ras mutations affected the prognosis of patients with pathological stage I adenocarcinoma. The prognostic role of EGFR mutations in patients with resectable NSCLC has not been established. In their study, Mansuet-Lupo et al. did not find a significant effect on OS for patients with EGFR mutations compared with those with wild-type EGFR in
their cohort or in a subset with stage I disease.\textsuperscript{46} Hu et al. found no impact on OS in multivariate analysis when the presence or absence of an \textit{EGFR} mutation was included.\textsuperscript{47} On the other hand, in a smaller study, Russell et al. conducted molecular analysis and assessed survival outcomes in 59 patients who had undergone surgical resection of lung adenocarcinoma with N2 nodal involvement.\textsuperscript{48} Patients with acinar-predominant adenocarcinoma had significantly better survival than those with micropapillary or solid predominant adenocarcinoma. This trend suggests that patients with resected micropapillary tumors harboring an activating \textit{EGFR} mutation have similar survival outcomes to patients with acinar predominant tumors, whereas patients with micropapillary predominant tumors with wild-type \textit{EGFR} have poorer outcomes.

\textit{Yoshizawa} et al. did note a statistically and clinically significant improvement in five-year OS rates in patients with \textit{EGFR} mutations, but found no difference in five-year

| Variables                                    | RFS HR (95% CI) | \( P \) | OS HR (95% CI) | \( P \) |
|----------------------------------------------|-----------------|--------|----------------|--------|
| Age at operation (year)                      |                 |        |                |        |
| <70                                          | 1               |        | 2.33 (1.06–5.09) | 0.034 |
| \( \geq 70 \)                                | 1.11 (0.57–2.15) | 0.767  |                |        |
| Gender                                       |                 |        |                |        |
| Female                                       | 1               |        | 1.03 (0.72–1.48) | 0.871 |
| Male                                         | 1.16 (0.59–2.25) | 0.666  |                |        |
| Smoking habit                                |                 |        |                |        |
| Never smoker                                 | 1               |        | 1.25 (0.87–1.81) | 0.224 |
| Ever smoker                                  | 1.20 (0.86–1.68) | 0.278  |                |        |
| Serum CEA (ng/mL)                            |                 |        |                |        |
| \( \leq 5 \)                                 | 1               |        | 2.61 (1.24–5.48) | 0.012 |
| \( > 5 \)                                    | 2.04 (1.01–4.17) | 0.049  |                |        |
| Extent of pulmonary resection                |                 |        |                |        |
| Sublobar resection                           | 1               |        |                |        |
| Lobectomy or more                            | 0.78 (0.53–1.16) | 0.227  | 0.79 (0.52–1.21) | 0.289 |
| Tumor location                               |                 |        |                |        |
| Central                                      | 1               |        |                |        |
| Non-central                                  | 0.93 (0.45–1.89) | 0.833  | 0.57 (0.78–4.21) | 0.584 |
| \( \text{SUV}_{\text{max}} \) of primary tumor \( \leq 2.3 \) | 1             |        |                |        |
| \( \text{SUV}_{\text{max}} \) of primary tumor > 2.3 | 6.08 (3.52–14.65) | 3.85 (1.65–8.98) | 0.002 |
| Tumor size (cm)                              |                 |        |                |        |
| \( \leq 3 \)                                 | 1               |        |                |        |
| \( > 3 \)                                    | 1.61 (0.81–3.19) | <0.001 | 1.58 (0.75–3.33) | 0.225 |
| Grade                                        |                 |        |                |        |
| 1                                           | 1               | 0.175  |                |        |
| 2–4                                         | 1.31 (0.62–2.71) | 0.482  | 1.18 (0.53–2.66) | 0.687 |
| Pleural invasion                             |                 |        |                |        |
| Absent                                       | 1               |        |                |        |
| Present                                      | 2.42 (1.06–5.54) | 0.037  | 1.92 (0.73–5.03) | 0.182 |
| Mucinous components                          |                 |        |                |        |
| Absent                                       | 1               |        |                |        |
| Present                                      | 1.21 (0.71–2.03) | 0.487  | 1.06 (0.62–1.81) | 0.817 |
| EGFR mutation                                |                 |        |                |        |
| Absent                                       | 1               |        |                |        |
| Present                                      | 1.18 (0.61–2.29) | 0.632  | 1.02 (0.71–1.46) | 0.911 |
| K-ras mutation                               |                 |        |                |        |
| Absent                                       | 1               |        |                |        |
| Present                                      | 2.06 (0.49–8.59) | 0.321  | 1.02 (0.56–1.86) | 0.959 |
| Pathological stage                           |                 |        |                |        |
| Stage IA                                     | 1               |        |                |        |
| Stage IB                                     | 2.31 (1.19–4.51) | 0.014  | 1.69 (0.82–3.46) | 0.153 |

CEA, carcinoembryonic antigen; CI, confidence interval; EGFR, epidermal growth factor receptor; HR, hazard ratio; OS, overall survival; RFS, recurrence-free survival; SUV\(_{\text{max}}\), maximum standardized uptake value.
In conclusion, the present study demonstrated that surgically resected pathological stage I adenocarcinoma harboring EGFR and K-ras gene mutations has distinct clinicopathological features. The presence of an EGFR or a K-ras mutation alone was not a prognostic factor in patients with surgically resected pathological stage I adenocarcinoma.

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Disclosure

No authors report any conflict of interest.

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