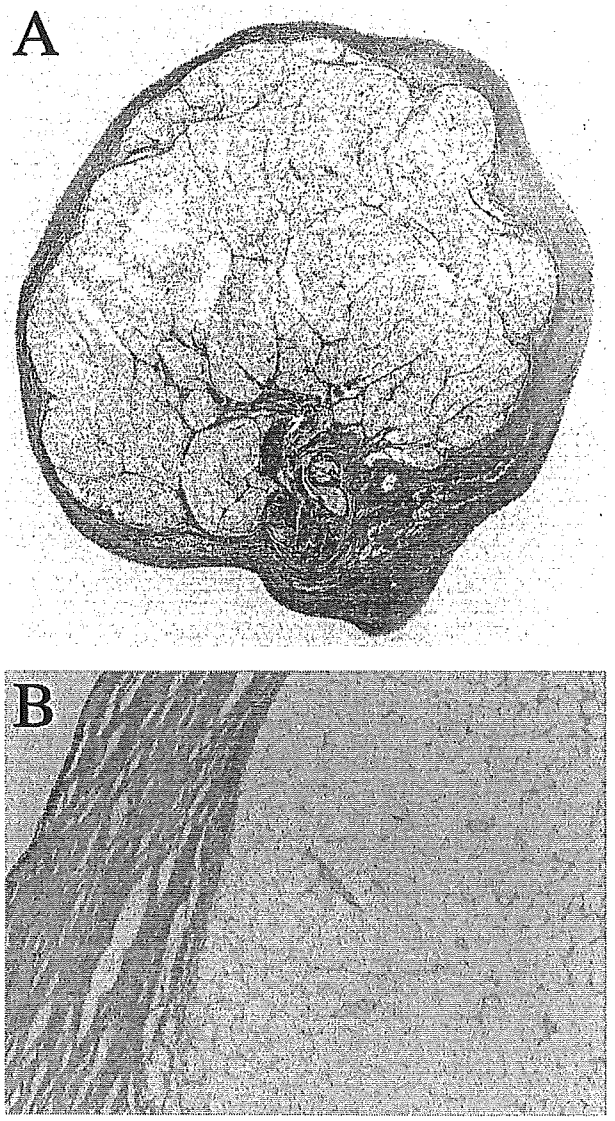

Fig. 3

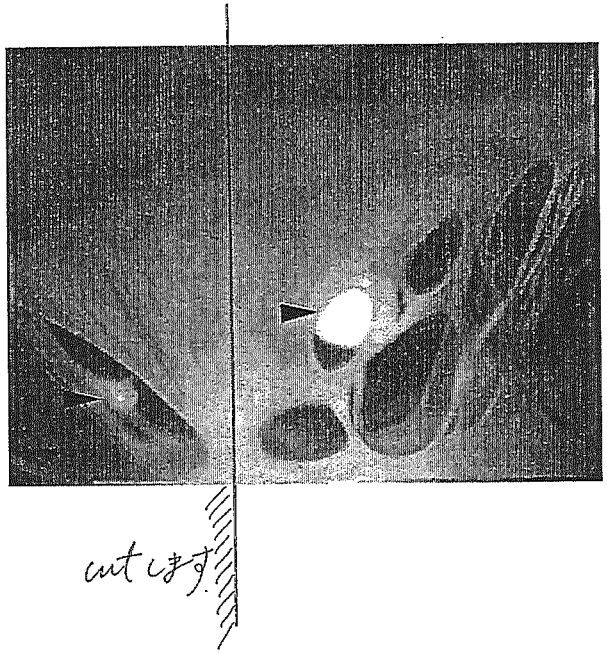

Fig. 4

Fig. 5

Significance of the Number of Positive Lymph Nodes in Resected Non-small Cell Lung Cancer

Takayuki Fukui, MD,† Shoichi Mori, MD,* Kohei Yokoi, MD,† and Tetsuya Mitsudomi, MD**

Background: In the current tumor, node, metastasis (TNM) classification of lung cancer, N status is defined by the anatomic extent of lymph node metastases. In this study, we evaluated the prognostic significance of the number of positive lymph nodes in resected non-small cell lung cancer.

Methods: We retrospectively studied 289 patients with non-small cell lung cancer who underwent surgery, and we compared the prognostic significance of the number of positive nodes with the pN number by using multivariate analysis. Patients were classified into four groups according to the number of positive nodes: those without nodal metastasis were n^0, those with one to three positive nodes were n^{1-3}, those with four to six were n^{4-6}, and those with more than seven were $n^{\geq 7}$.

Results: The 5-year survival rate was 77% in the n^0 patients, 58% in n^{1-3}, 42% in n^{4-6}, and 6% in $n^{\geq 7}$, which indicates that an increased number of positive lymph nodes was associated with poor prognosis. Among the pN2 patients, the n^{1-3} group had a better survival rate than the n^{4-6} and $n^{\geq 7}$ groups. Multivariate analysis showed that the number of positive nodes was a significant prognostic factor, equal to the currently used pN number. Hazard ratios for pN1 and pN2 with respect to pN0 were 2.13 and 3.49; and 2.07, 3.03, and 10.4 for n^{1-3}, n^{4-6}, and $n^{\geq 7}$ with respect to n^0. In addition, we found that our classification could reflect the better prognoses of skip or single-station nodal metastases.

Conclusion: The number of positive lymph nodes is a strong independent prognostic factor in non-small cell lung cancer and may add new information to the pN categories of the current TNM classification.

Key Words: TNM classification, Skip metastasis, Single-station metastasis.

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The presence and anatomic extent of histologically confirmed lymph node metastases are important prognostic factors for many malignancies. These factors, expressed in terms of the N categories, are essential components of the widely used tumor, node, metastasis (TNM) stage classification.[1] In the classification of lung cancer, only the anatomic extent of lymph node metastasis defines the pathological N (pN) categories, in which ipsilateral peribronchial and/or hilar lymph node metastasis are defined as pN1; ipsilateral mediastinal and/or subcarinal metastasis as pN2; and contralateral mediastinal, contralateral hilar, or supraclavicular metastasis as pN3. However, in the latest TNM classifications,[2] the number of positive lymph nodes is included in the definition of the pN categories in breast, gastric, and colorectal cancer, and the pN status shows significant correlation with patient prognosis.[3–7]

In non-small cell lung cancer (NSCLC), the number of nodal stations with metastases has been suggested to be of significance; however, few studies on the numbers of positive lymph nodes have been reported.[8,9] In this retrospective study, we analyzed the clinicopathological data of 289 patients with NSCLC treated in a single institution, the Aichi Cancer Center Hospital, to evaluate the prognostic significance of the number of positive lymph nodes in surgically resected NSCLC.

In addition, recent studies have suggested that patients with "skip lymph node metastasis," consisting of N2 disease without N1 involvement (negative N1 and positive N2), tend to have a better prognosis than other pN2 patients (positive N1 and positive N2).[10–19] Furthermore, the total number of nodal stations with metastases seems to be an independent prognostic indicator in patients with pN1 or pN2 NSCLC.[8,20] We therefore analyzed whether the number of positive lymph nodes could better reflect the prognosis in pN2 patients with skip lymph node metastasis or single node-station metastasis.

PATIENTS AND METHODS

Patient Cohort

During the 5-year period between 1992 and 1996, 289 patients with primary NSCLC were enrolled in this study. The patients with clinical stage IA to IIIA, including cN2 patients with single-station nodal metastasis, underwent surgery. Fifty-three patients with clinical stage IIIA were included in the cohort. All patients underwent pulmonary resection with systematic nodal dissection of the hilum and mediastinum at the Aichi Cancer Center Hospital. The pa-

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tients with bulky N2 disease with extranodal invasion were excluded. In this study, we regarded bulky N2 with extranodal invasion as big lymph nodes that involved adjacent structures including large vessels, trachea, bronchus, and pleura with unclear boundaries. Neither induction therapy nor adjuvant therapy was given to this series of patients. Patient characteristics are shown in Table 1. The patients comprised 193 (67%) men and 96 (33%) women, ranging in age from 24 to 81 years (median ± standard deviation, 61 ± 9.7 years). There were 178 (62%) adenocarcinomas, 83 (29%) squamous cell carcinomas, and 28 (9%) other types of cancer. The surgical procedures performed were lobectomy in 230 patients, bilobectomy in 23, pneumonectomy in 33, and segmentectomy in three.

Analysis of Lymph Node Metastasis

Lymph nodes were dissected from the adipose connective tissue of the corresponding anatomic regions, as subdivided by the surgeon immediately after the operation. The lymph nodes classified in this way were sent for histopathological examination after hematoxylin and eosin staining. The numbers of positive lymph nodes from each defined anatomic region were recorded. Based on the data obtained, stratifications were performed according to two different modes of lymph node status assessment: the absence or presence and anatomic extent of nodal metastases (pN categories) as defined by the TNM classification according to the lymph node mapping; and the number of regional lymph nodes with metastases—the number categories—in which stratification was performed based only on the numbers of positive nodes, ignoring the anatomic extent of the regional lymph node involvement.

Because each T or N status is classified into four subgroups in the current TNM classification, we decided to make four categories. Because all the patients with seven or more positive lymph nodes were included pN2 category, we defined the patients as one group (Figure 1). The patients with one to six positive lymph nodes were just divided into two groups in the middle of the number. Thus, the absence of nodal metastasis was defined as n^0 (that is, identical to pN0); the presence of one to three positive lymph nodes was defined as n^{1-3}; four to six nodes was defined as n^{4-6}, and seven or more nodes was defined as $n^{\geq 7}$.

TABLE 1. Patient characteristics

	No. (%)
Age	
< 61 years	131 (45)
≥ 61 years	158 (55)
Gender	
Male	193 (67)
Female	96 (33)
Smoking history	
< 20 pack-years	117 (40)
≥ 20 pack-years	172 (60)
Histology	
Adenocarcinoma	178 (62)
Squamous cell carcinoma	83 (29)
Other	28 (9)
pT status	
pT1	131 (45)
pT2	125 (43)
pT3	28 (10)
pT4	5 (2)
pN status	
pN0	190 (66)
pN1	35 (12)
pN2	64 (22)
Pathological stage	
IA	99 (34)
IB	72 (25)
IIA	12 (4)
IIB	37 (13)
IIIA	63 (22)
IIIB	6 (2)
Number of positive LNs	
0	190 (66)
1–3	63 (22)
4–6	19 (7)
≥ 7	17 (5)

LN, lymph nodes.

Statistical Analyses

The Kaplan–Meier method was used to plot the survival curves, and the log-rank test was used to evaluate differences among the subgroups. Student's *t* test was used to compare the numbers of lymph nodes with metastases among each group. To evaluate the significance of the number category as an independent prognostic factor, we performed multivariate analysis by using Cox's proportional hazards model with two different models: one that included pN status and one that included the number category. Trend *P* was assessed by score tests. All patients in this study were followed for at least 5 years or until death. *P* values <0.05 were regarded as statistically significant. Statistical calculations were performed using a statistical package (StatView version 5.0; SAS Institute Inc., Cary, NC).

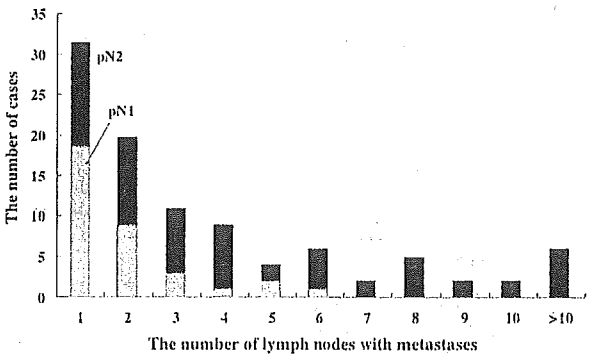

FIGURE 1. Distribution of the number of positive lymph nodes in 99 pN(+) patients. The pN1 and pN2 patients are shown in gray and black, respectively.

RESULTS

The mean (± SD) nodal yield was 22 ± 8.8 per patient (range, 3–59). At least one nodal metastasis was found in 99 (34%) patients. The distribution of the number of lymph node metastases in 99 pN(+) patients is shown in Figure 1. The breakdown by number category was 190 n^0 patients, 63 n^{1-3} patients, 19 n^{4-6} patients, and 17 $n^{\geq 7}$ patients. No significant difference in clinical factors such as age, gender, smoking history, tumor histology, and performed surgical procedure, was observed among the defined subgroups. The mean number of positive lymph nodes in those patients with at least one lymph node metastasis was 3.9 ± 4.2 (range, 1–28). The mean number of positive lymph nodes was 1.9 ± 1.3 (range, 1–6) in patients classified as pN1 and 5.0 ± 4.7 (range, 1–28) in patients classified as pN2, with a significant difference between each of the pN categories ($P < 0.0001$) (Table 2). Thus, the number of positive lymph nodes correlated with the anatomic extent of the metastasis.

The 5-year survival rate of the 190 patients without nodal metastasis (pN0) was 77% in this study, which was significantly better than that of patients classified as pN1 (57%) and pN2 (40%) ($P < 0.0001$) (Figure 2). With respect to the number category, the 5-year survival rate was 77% for n^0, 58% for n^{1-3}, 42% for n^{4-6}, and 6% for $n^{\geq 7}$, which indicates that an increased number of lymph nodes with metastases is associated with poor prognosis ($P < 0.0001$) (Figure 3). Similar differences in survival were observed among pN2 patients who were divided into subgroups n^{1-3} (32 patients), n^{4-6} (15 patients), and $n^{\geq 7}$ (17 patients) ($P < 0.0001$), but not between subgroups n^{1-3} (31 patients) and n^{4-6} (four patients) among pN1 patients (Figure 4). In addition, similar differences were observed in the patients divided into subgroups by pathological type, such as adenocarcinoma and squamous cell carcinoma (data not shown).

Hazard ratios for pN1 and pN2 versus pN0 were 2.13 and 3.49, respectively; and 2.07, 3.03, and 10.4 for n^{1-3}, n^{4-6}, and $n^{\geq 7}$, respectively, versus n^0 by multivariate analysis using Cox's proportional hazards model (Tables 3 and 4). Among the clinicopathological variables analyzed, the number category, determined by the number of positive lymph nodes after lymphadenectomy, was an independent prognostic factor similar to the pathological N category defined by the anatomic extent of lymph node metastases

In the current study cohort, the patients with skip metastasis had a significantly better prognosis, with 5-year survival rates of 55% compared with 31% for the other pN2 patients ($P = 0.04$) (Table 5). Comparing the patients with skip metastasis versus those without skip metastasis in 64 pN2 patients, the numbers of positive lymph nodes were 2.0 ± 1.6 and 6.9 ± 5.1, respectively ($P < 0.0001$). Similarly, among the pN2 group, patients with single N2 lymph node metastasis, including single N2 station metastasis, had a significantly better prognosis than those with multiple lymph node metastases ($P = 0.007$, data not shown).

TABLE 2. Number of Positive Lymph Nodes and pN Status

	Number of positive lymph nodes				
	0	1–3	4–6	≥7	Mean number ± SD
pN1	–	31	4	0	1.9 ± 1.3
pN2	–	32	15	17	5.0 ± 4.7
					$P < 0.0001$

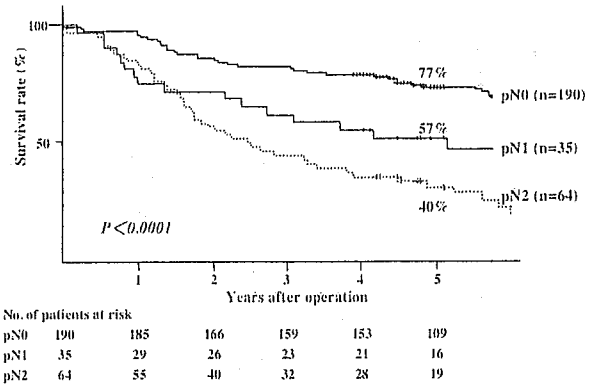

FIGURE 2. Survival curves for subgroups stratified according to pN status. The 5-year survival rate and the number of patients at risk in each subgroup are indicated. The survival curves showed significant stepwise deterioration as the pN number increased.

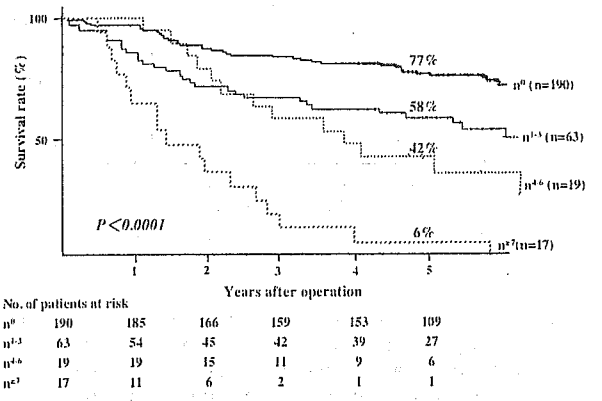

FIGURE 3. Survival curves according to the number of positive lymph nodes. The 5-year survival rate and the number of patients at risk in each subgroup are indicated. The survival curves showed significant stepwise deterioration as the number increased. The feasibility of the application of our classification was confirmed.

DISCUSSION

The stage grouping of the TNM subsets was developed to provide high specificity for identifying patient groups with similar prognoses and treatment options. Because the extent of lymphatic spread is considered to reflect the aggressiveness of cancer cells and because extensive nodal metastasis implies poor survival, lymph node status assessment continues to be an essential component of the stage classification for many kinds of cancer. In the latest TNM classification, as revised in 1997, the anatomic extent of lymph node metas-

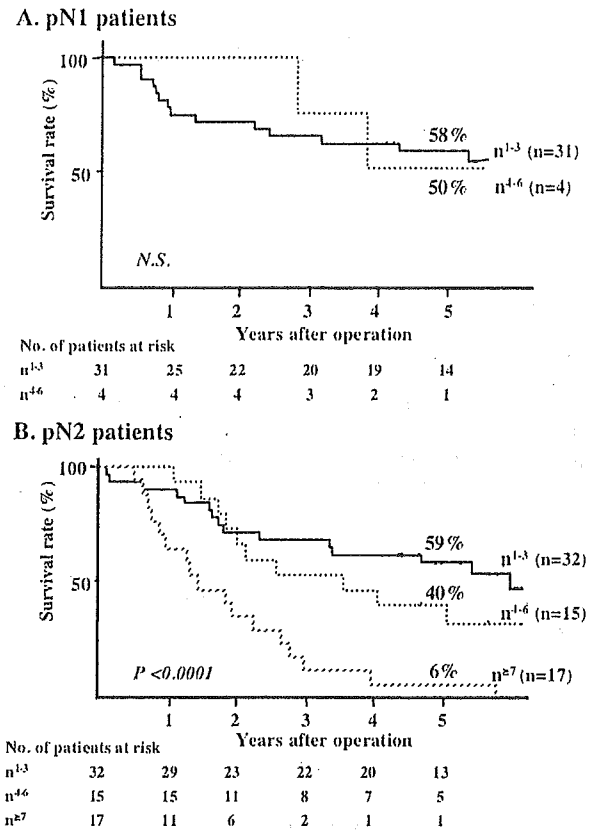

FIGURE 4. Survival curves according to the number categories among pN1 and pN2 patients. The 5-year survival rate and the number of patients at risk in each subgroup are indicated. Significant differences in survival were observed among pN2 patients who were divided into subgroups n^{1-3}, n^{4-6}, and $n^{\geq 7}$ ($P < 0.0001$) (*B*), but not between subgroups n^{1-3} and n^{4-6} for pN1 patients (*A*).

TABLE 3. Multivariate Analysis by Cox's Proportional Hazards Model Including pN Status

Variable	Hazard ratio	95% CI	*P* value
Age (≥61 vs. <61 years)	1.73	1.15–2.60	0.009
Gender (male vs. female)	1.29	0.68–2.46	0.441
Smoking (≥20 vs. <20 pack-years)	1.04	0.56–1.91	0.909
Histology (non-sq vs. sq)	1.02	0.65–1.61	0.925
Tumor size (≥3 vs. <3 cm)	1.87	1.22–2.88	0.004
pN status			
pN1/pN0	2.13	1.20–3.77	0.010
pN2/pN0	3.49	2.28–5.36	<0.0001
	Trend *P* for pN < 0.0001		

Sq, squamous cell carcinoma.

tasis defines pN categories in lung cancer. In contrast, the number of positive lymph nodes is included in the definition of pN categories in breast, gastric, and colorectal cancers.

TABLE 4. Multivariate Analysis by Cox's Proportional Hazard's Model Including the Number of Positive Lymph Nodes

Variable	Hazard ratio	95% CI	*P* value
Age (≥61 vs. <61 years)	1.63	1.07–2.50	0.024
Gender (male vs. female)	1.50	0.78–2.91	0.226
Smoking (≥20 vs. <20 pack-years)	0.89	0.48–1.67	0.719
Histology (non-sq vs. sq)	0.84	0.53–1.34	0.462
Tumor size (≥3 vs. <3 cm)	1.83	1.19–2.81	0.006
The number of positive LNs			
1–3/0	2.07	1.29–3.31	0.003
4–6 / 0	3.03	1.57–5.85	0.001
≥ 7/0	10.4	5.71–19.0	<0.0001
	Trend *P* for the number category <0.0001		

LN, lymph node; sq, squamous cell carcinoma.

TABLE 5. Summary of Skip and Nonskip Groups of pN2 Patients

	Pattern of LN metastasis		
	Skip	Nonskip	*P* value
Patients	25	39	
LNs (mean ± SD)	2.0 ± 1.6	6.9 ± 5.1	<0.0001
5-year survival rate	55.4%	30.8%	0.04

Skip, N1(-) and N2(+); Nonskip, N1(+) and N2(+); LNs, lymph nodes.

To evaluate the prognostic significance of the number of positive lymph nodes in primary NSCLC, we defined the "number" category for 289 patients in NSCLC. The survival curves in accordance with the number categories showed significant stepwise deterioration as the number increased, and the feasibility of applying our classification was confirmed. Multivariate analysis using Cox's proportional hazards model revealed that the number categories might be considered as indicators of prognosis similar to the current TNM classification based on the anatomic extent of nodal metastases. These results suggest that the number of positive lymph nodes is a strong independent prognostic factor in NSCLC. Specifically, remarkable results of the similar survival rate between pN1 and pN2 limited in n^{1-3} patients and the better survival rate in n^{1-3} patients among pN2 versus n^{4-6} among pN1 patients were observed. These results seemed to be a real challenge for current pN category of NSCLC. Consequently, the number category should be useful in determining patients who should receive adjuvant therapy. Meanwhile, similar differences in survival among the subgroups, n^0, n^1, n^{2-4}, and $n^{\geq 5}$, which divided n^{1-3} patients into two categories, were observed. Therefore, further discussion about the pattern of division of positive nodes will be necessary when the system undergoes revision.

A similar stepwise deterioration of survival as the number increased was also observed in 64 patients in the pN2 group. Patients with N2 NSCLC are a heterogeneous sub-

group in regard to their prognosis and treatment. Some authors have proposed new TNM classifications to classify N2 disease into subcategories. For example, homogeneous N2 NSCLC prognostic subgroups determined by combined pretreatment and postoperative staging consisting of diagnostic imaging results, and the lymph node levels involved were suggested as a guide to preoperative staging and to the selection of appropriate treatment options.[10] The number category used in our analysis could also divide the N2 patients into homogeneous subgroups.

Recent studies have suggested that patients with skip lymph node metastases tend to have a better prognosis than other pN2 patients [11–19] and that the total number of stations with metastases is an independent prognostic indicator in patients with completely resected pathological N1 or N2 NSCLC.[8,20] Thus, the group of pN2 patients with NSCLC is also considered heterogeneous on survival, according to the presence of skip or single-station metastasis. The better prognosis of patients with skip metastasis may be attributed to the smaller number of positive lymph node or to subpleural lymphatic channels that can drain directly to the mediastinum. Both possibilities exist. It is very difficult to conclusively determine the mechanism from our current knowledge. Our analyses suggest that number category could reflect the better prognosis of skip or single-station lymph node metastasis in pN2 patients and may have the potential for stratification of patients with lymph node metastases to single or multiple N2 stations.

Despite these benefits of the number category, our category system cannot help to define treatment options for primary lung cancer because preoperative clinical staging consists of imaging studies. To date, neither computed tomography nor positron emission tomography could successfully detect each positive lymph node during preoperative evaluation. However, it may be possible to use the number category for preoperative evaluation if a new device that can identify each lymph node and qualitative diagnosis is developed in the future.

Meanwhile, the extent of lymphadenectomy and the thoroughness of lymph node retrieval decide the total number of resected lymph nodes for each specimen, and the significance of the number categories varies among countries and institutions. In gastric cancer, a higher probability of detecting nodal metastases with increasing nodal yields has been confirmed and follows an exponential model, with extended lymphadenectomy leading to an increase in the number of nodes with metastases.[21] In addition, the number of positive lymph nodes is considered to reflect only the anatomic extent of the lymph node metastases. In fact, the numbers of positive lymph nodes correlated significantly with the pN categories based on the current TNM classification, with stepwise deterioration of survival as the pN number increased (Table 2) (Figure 2).

The adequacy of mediastinal lymph node sampling or the reduction of the extent of lymph node dissection in lung cancer has been discussed.[22,23] Although this trend leads to fewer lymph nodes being harvested, the priority of our number category will be stable as far as less dissection is based on the concept of sentinel lymph node.

Although the number category contains some deficiencies, such as difficulty in determining nodal status based on imaging studies in preoperative evaluation, from the strong correlation between the number and pN categories, we conclude that the number category is a strong independent prognostic factor in NSCLC and can add new information to pN status in breast, gastric, and colorectal cancers. Furthermore, the number category may allow stratification of N2 patients into homogeneous subgroups. These findings should be confirmed by others, and consideration should be given to its inclusion in future pathological staging of NSCLC when the system undergoes revision.

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EGFR Mutation Is Specific for Terminal Respiratory Unit Type Adenocarcinoma

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Abstract: We have previously reported that terminal-respiratory-unit (TRU) type adenocarcinoma is a distinct subset of lung adenocarcinoma in terms of molecular pathway for carcinogenesis and phenotypic profiles. This type of cancer shows TRU features, characterized by distinct cellular morphology and the expression of TTF-1 and surfactant proteins. Recently, two groups published novel mutations of the epidermal growth factor receptor (EGFR) that are closely associated with clinical response to gefitinib. The clinicopathologic features of gefitinib responders overlap with those of TRU-type adenocarcinoma, and the characteristics of TRU are likely to correspond to the bronchioloalveolar features reported as a predictor of gefitinib response. We therefore examined the characteristics of EGFR-mutated pulmonary adenocarcinomas with special reference to TRU-type adenocarcinoma. EGFR mutation was detected in 97 of 195 adenocarcinomas, 91 of 149 TRU-type adenocarcinomas and 6 of 46 tumors of other types. Conversely, 91 of 97 EGFR-mutated adenocarcinomas were categorized as TRU-type adenocarcinomas. This type-specific involvement was confirmed by logistic regression model. In addition, EGFR mutation was detected in some cases of atypical adenomatous hyperplasia, a preinvasive lesion of TRU-type adenocarcinoma. These findings further confirm that TRU-type-adenocarcinoma is a distinct adenocarcinoma subset in which a particular molecular pathway is involved.

Key Words: EGFR mutation, lung adenocarcinoma, atypical adenomatous hyperplasia, gefitinib, bronchioloalveolar features

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Lung cancer is the leading cause of cancer deaths for both men and women in United States, Japan, and Western countries.[46] A significant number of clinical trials using chemotherapeutic strategy against this cancer have been attempted, but the effects on advanced lung cancer remain marginal. Recently, small molecules which inhibit receptor protein kinase activity, have been developed. Gefitinib is one of such drug, which targets epidermal growth factor receptor (EGFR) kinase. Although EGFR is expressed in more than 80% of non-small cell lung cancers (NSCLCs), as well as a wide range of epithelial cancers,[2,5,30] clinical trials have shown significant variability in the response to gefitinib: 10% to 20% of patients respond to gefitinib treatment, and in some of the patients the response is dramatic, whereas the remaining patients show no response.[10,13,15,24] Further analysis revealed that nonsmokers, female sex, and histologic subtype of adenocarcinoma, especially with bronchioloalveolar feature, are significantly prevalent in the responders.[10,31] These features overlap with the characteristics of terminal-respiratory-unit (TRU) type-adenocarcinoma, which we have noted previously.[54,55,57]

TRU is composed of alveolar cells and nonciliated bronchiolar epithelium, and its characteristics are highlighted by morphology and expression of thyroid transcription factor-1 (TTF-1) and surfactant proteins. The concept of the TRU is suggested by constant and uniform expression of TTF-1, appearing as a series of cells that represented a certain functional unit or common lineage. TTF-1 is a crucial transcription factor in the lung, required for lung development and the maintenance of lung function. TTF-1 regulates functional molecules of the lung, including surfactant proteins,[6,34,52] and TTF-1-deficient mice resulted in lung aplasia.[21,32] TRU-type adenocarcinoma, which is putatively derived from the TRU, demonstrates a different pattern of alteration of cancer-associated genes, suggesting a distinct molecular pathway of its carcinogenesis. Recently, two groups published novel mutations of the EGFR, which are closely associated with clinical response to gefitinib.[29,35] In this study, we attempted to explore the clinicopathologic significance of the mutations with special reference to the TRU-type adenocarcinoma, using a cohort of 241 patients with NSCLC, a part of which we previously reported on EGFR mutation.[23]

MATERIALS AND METHODS

Patients

A series of 241 consecutive patients with non-small cell carcinoma between 2001 and 2002, from whom frozen tissue was available, were selected for this study from a file held at the Department of Pathology and Molecular Diagnostics, Aichi Cancer Center, Nagoya, Japan. This series included 195 patients with adenocarcinoma, including 5 bronchioloalveolar carcinomas, 34 patients with squamous cell carcinoma, 7 patients with large cell carcinoma, and 5 patients with adenosquamous carcinoma. The cohort was a part of a previous study, and their clinical details are described elsewhere.[23] In

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addition, atypical adenomatous hyperplasia (AAH) and corresponding primary tumors were examined in 5 patients to determine the mutation status in precursors of invasive adenocarcinoma.

Tissue Microarray and Immunohistochemistry

Expression status of TTF-1 and surfactant pro-protein B (SPPB) were addressed as described previously.[54,55] Briefly, using tissue microarray, immunohistochemical examination proceeded according to the standard avidin-biotin-peroxidase complex method. Antibodies used were TTF-1 (8G7G3, DAKO, Copenhagen Denmark) and SPPB (19H7, Novocastra, UK).

Mutation Status of EGFR, p53, and K-ras

All of the mutation data in this cohort have been published previously.[23,56,57] Briefly, frozen tumor specimens were grossly dissected to enrich the tumor cells, and total RNA was extracted using the RNaeasy kit (Qiagen, Valencia, CA). Using a standard RT-PCR procedure, p53 gene from exon 4 to exon 10 was amplified, and the products were directly sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). When no mutation signals were obtained, the result was confirmed using functional assay in yeast.[18,51] In the functional assay, when more than 10% red colonies or a significant deviation of a split assay were observed, RNA was reextracted from microdissected tumor cells using laser capture microdissection, and RT-PCR products were sequenced. Through this procedure, most molecules were derived from tumor cells. Using the same RNA as for the mutational analysis of p53, EGFR and K-*ras* genes were examined by direct sequencing.

For mutation analysis of AAH samples, DNA was extracted from paraffin-embedded sections,[53] followed by PCR amplification with the following primer sets (5′-3′): for mutations of codon 719, forward-GAGGTGACCCTTG-TCTCTGTGT and reverse-CCCAAACACTCAGTGAAA-CAAA; for deletions in exon 19, forward-TGCCAGT-TAACGTCTTCCTTCT and reverse-ATGTGGAGATGAG-CAGGGTCTA; and for mutations of exon 21, forward-GAGCTTCTTCCCATGATGATCT and reverse-GAAAATG-CTGGCTGACCTAAAG. The PCR products were directly sequenced.

Morphologic Definition of TRU Morphology

The details of the morphologic characteristics of TRU-type adenocarcinoma have been described previously.[54,55] Principally, TRU morphology is based on cellular morphology, and we categorized the tumor as TRU-type when morphologic differentiation to type II pneumocytes, Clara cells, and/or nonciliated bronchioles was seen. Differentiation to type II pneumocytes is characterized by a cuboidal or dome-shaped free cell contour, a clear to foamy cytoplasm with occasional fine vacuoles and occasional nuclear inclusions. Clara cell differentiation is recognized as a cuboidal to dome-shaped free cell contour, a pale eosinophilic cytoplasm, frequently with snouts, and an apical location of the nuclei. Transition between differentiation to type II pneumocytes and Clara cells is quite common. Differentiation to nonciliated bronchioles is difficult to distinguish from differentiation to bronchial surface epithelium, and thus we paid most attention to this point. Tumors with either differentiation contain columnar cells but differ in the features of the luminal, free cell border. Adenocarcinomas with bronchiole differentiation show dome-shaped protrusion of each luminal free cell border, but in cases of differentiation to bronchial surface epithelium, the luminal border gives a smooth line.

Statistical Analysis

The χ^2 test and Fisher exact test for independence were used to compare frequencies of clinicopathologic variables. To estimate the interaction of the clinicopathological variables, we generated a logistic regression model using SYSTAT (SYSTAT Software Inc, Richmond, CA). A P value below 0.05 was considered statistically significant.

RESULTS

EGFR Mutations Specific for TRU-Type Adenocarcinoma

EGFR mutations in kinase domain of EGFR gene were detected in 98 of 241 NSCLCs, all except one of which were adenocarcinomas (97 of 195 adenocarcinomas, including 3 of 5 bronchioloalveolar carcinomas). The typical morphologies of adenocarcinomas with and without EGFR mutation are shown in Figures 1 and 2, respectively. The profiles of patients with the mutation were similar to those of gefitinib responders reported previously,[10,23,24] and among the adenocarcinomas, the mutation was preferentially found in females (60 of 98, Fisher exact test, $P < 0.001$) and nonsmokers (65 of 98, Fisher exact test, $P < 0.001$). The mutation status was not associated with pathologic stage (χ^2 test, $P = 0.805$) and the extent of nodal metastasis (χ^2 test, $P = 0.407$), suggesting that the genetic alteration is not associated with tumor progression. Because similar characteristics were observed in TRU adenocarcinomas,[54,55] we compared the mutation with three features of TRU, including expression of TTF-1 and SPPB, and its morphologic characteristics (Table 1). All were significantly associated with EGFR mutation (Fisher exact test, $P < 0.001$). When TRU-type adenocarcinomas were defined as those showing at least two of the three features, the distinction of TRU or non-TRU-type demonstrated the smallest P value (7.0×10^{-9}). We therefore used the criteria to define TRU-type adenocarcinoma

Of 149 TRU-type adenocarcinomas, 91 (61%) harbored an EGFR mutation (Table 2), as did 6 of 46 (13%) non-TRU-type adenocarcinomas. Conversely, 91 of 97 (94%) adenocarcinomas with EGFR mutation were categorized as TRU-type adenocarcinoma, suggesting a specific involvement of EGFR mutation in TRU-type adenocarcinoma. We constructed a multivariate logistic regression model to determine factors that are significantly associated with EGFR mutation in this cohort. The model, using the variables listed in Table 3, revealed that cellular type and smoking status were independent factors that affected EGFR mutational status with high statistical significance ($P < 0.001$ and $P = 0.004$, respectively). This confirms the specific involvement of EGFR mutation in TRU-type adenocarcinoma.

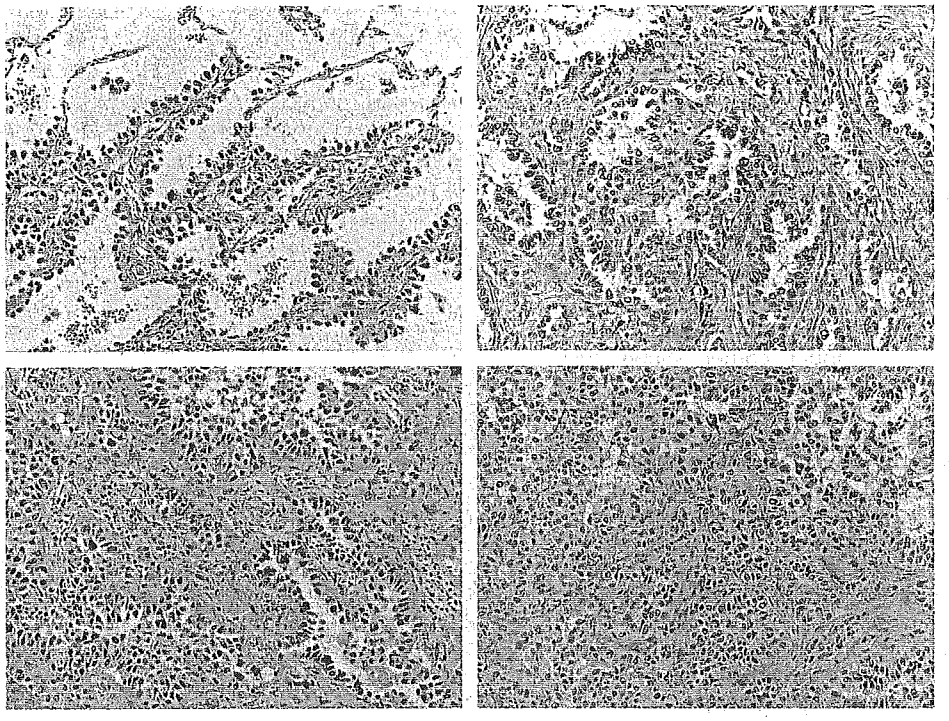

FIGURE 1. Typical feature of TRU-type adenocarcinoma with EGFR mutation. A tumor shows bronchioloalveolar feature in the periphery (left upper) and invasive portion in the center (left lower). In addition to bronchioloalveolar cell carcinomas, both moderately differentiated (right upper) and poorly differentiated adenocarcinomas (right lower) harbor EGFR mutation, while cellular features in both tumors are similar.

Clinicopathologic Features of TRU-Type Adenocarcinoma With Reference to EGFR Mutation

We then examined the clinicopathologic features of TRU-type adenocarcinoma. This type of adenocarcinoma, in which the EGFR gene is specifically mutated, was characterized by its prevalence in females and nonsmokers, and its infrequent mutations of K-*ras* and p53, compared with non-TRU-type adenocarcinoma (Table 2). The characteristics in this cohort were consistent with those we have reported previously,[54,55] which confirmed that they overlapped with EGFR mutation.

None of the tumors in this cohort harbored both EGFR and K-*ras* mutations, as reported.[23] This mutually exclusive relationship suggests a complementary role in the molecular carcinogenesis of lung adenocarcinomas, and either a mutation of EGFR or K-*ras* may contribute to the development of TRU-type adenocarcinoma. For K-*ras* mutation, a strong link with smoking habit has been reported.[1] This close correlation is observed in TRU-type adenocarcinomas in this study. Of the 15 TRU-type adenocarcinomas with K-*ras* mutation, 14 (93%) had developed in smokers, as did 29 of the 91 TRU-type adenocarcinomas with EGFR mutation (without K-*ras* mutation). This difference was statistically significant (Fisher exact test, $P < 0.001$). There were no other characteristics distinguishing TRU-type adenocarcinoma with EGFR mutation from those with K-*ras* mutation, including morphology, pathologic stage, presence or absence of nodal metastasis, and mutational status of p53.

EGFR Mutation in Preneoplastic Lesions

AAH is a preinvasive lesion that is recognized as the earliest lesion in the putative progression scheme of lung adenocarcinoma. Morphologic resemblance to either type II pneumocytes or Clara cells, and constant expression of TTF-1 and SPPB indicate that AAH corresponds to an edge of the spectrum of TRU adenocarcinomas. Because of difficulty obtaining frozen AAH tissues, we examined EGFR mutation status of exon 18, 19, and 21, using paraffin-embedded specimens. This set of examinations was considered to cover more than 90% of EGFR mutations.[23] EGFR mutation was detected in 2 of 5 patients with AAH examined (Table 4). A representative result (patient no. 2) is displayed in Figure 3. It is of note that 1 patient showed EGFR mutation only in the lung adenocarcinomas but not in the AAH (patient no. 3).

DISCUSSION

New identification of a gene alteration often contributes, not only to the understanding of molecular pathogenesis but establishment of the disease entity as well. This is exemplified by specific translocation in soft tissue sarcomas and hematopoietic malignancies. Recent identification of c-kit mutations shed light on gastrointestinal stromal tumors, which are currently considered an entity separate from leiomyogenic tumors and other rare stromal tumors.[14,17,22] Similar clarification might be preceded by the description of EGFR mutations in lung adenocarcinomas. We have previously proposed that the TRU-type adenocarcinoma is distinct from other adenocarcinomas in terms of their molecular carcinogenesis pathways and phenotypic profiles.[54,55,57]

In this study, 91 of 97 (94%) adenocarcinomas with EGFR mutation were categorized as TRU-type adenocarcinoma. This specific involvement of EGFR mutations in TRU-type adenocarcinoma supports that this type is a distinct subset of lung adenocarcinomas. Furthermore, EGFR mutation was detected in AAH, a preinvasive lesion representative of TRU

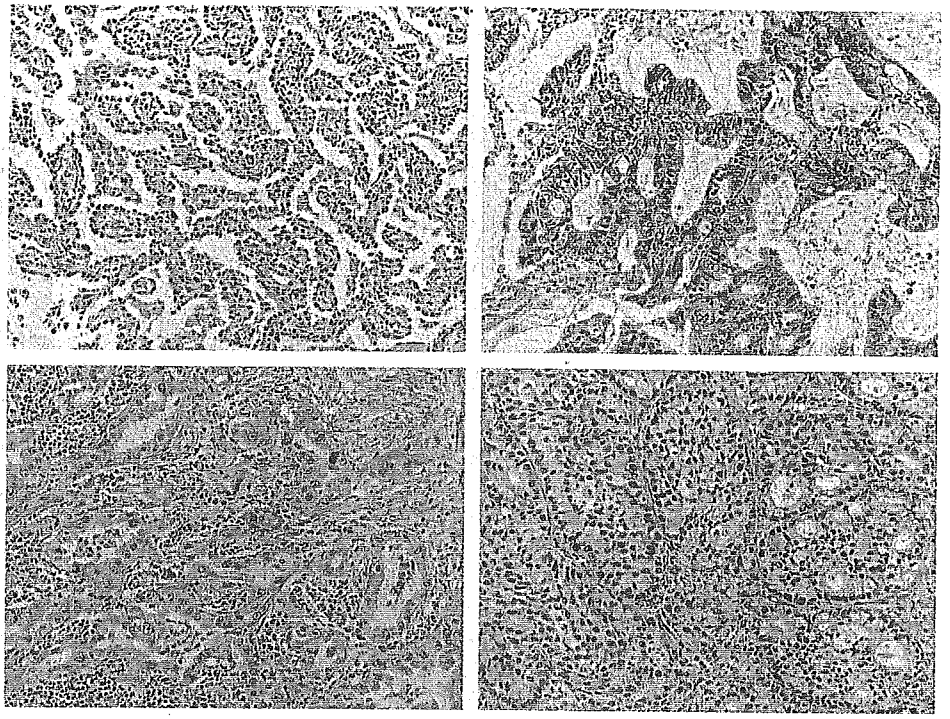

FIGURE 2. TRU-type and non-TRU-type adenocarcinomas that did not harbor EGFR mutations. Frequent mutation has been reported in bronchioloalveolar cell carcinomas, but two of five in our cohort did not harbor the mutation: the picture in the left upper corner shows such a case. Despite their TRU morphology, EGFR mutation was not detected in some TRU-type adenocarcinomas (left lower), which are indistinguishable from TRU-type adenocarcinoma with EGFR mutation. The two pictures on the right show the morphology of non-TRU-type adenocarcinomas without EGFR mutation: adenocarcinoma, resembling bronchial surface epithelium (right upper), and adenocarcinoma with abundant mucin in the cytoplasm (right lower).

neoplasia, suggesting an involvement of the EGFR at an early stage in the pathogenesis of TRU-type adenocarcinoma.

Because TRU is a new concept, it may be difficult to recognize TRU-type adenocarcinomas. TRU morphology is defined by its resemblance to type II pneumocytes, Clara cells, and/or nonciliated bronchiolar cells. This is quite similar to Shimosato's cytologic classification of lung adenocarcinoma.[25,38] The categories of Clara cell type, type II alveolar epithelial cell type, and mixed-cell or indeterminate cell type in the cytologic classification correspond to TRU-type adenocarcinoma. Similar attempts have been made by many others.[7,8,16,20,27,28,39–41] In the recently published WHO classification,[48] the morphologic characteristics of type II pneumocytes and Clara cells are well described, and the TRU-type adenocarcinoma corresponds in the classification schema to the majority of nonmucinous bronchioloalveolar carcinomas (BACs); most of adenocarcinoma mixed subtype with a BAC component; and a subset of papillary carcinoma. In addition, the majority of so-called sclerosing BACs are included as TRU-type adenocarcinomas. We have introduced TRU-type adenocarcinoma to allow better biologic understanding of pulmonary adenocarcinomas. For example, none of the EGFR mutations was detected in 635 nonpulmonary cancers.[26,29] This may be explained by specific involvement of the EGFR gene in TRU-type adenocarcinoma, of which its putative normal counterpart, ie, the TRU cell, is unique to the pulmonary parenchyma and is not contained in any organs. Furthermore, the distinction of TRU and non-TRU adenocarcinomas appears to be illustrated by hierarchical clustering according to genome-wide expression patterns, as discussed below.

In a practical sense, TTF-1 positivity is quite helpful and serves as the best available marker for identifying a TRU-type adenocarcinoma. The sensitivity of TTF-1 positivity for TRU-type adenocarcinomas reached 97% in this series, although specificity was not as high (76%, Table 2). In contrast, SPPB showed the lowest sensitivity (82%) and the highest specificity (98%) among the three factors examined. Therefore, an adenocarcinoma positive for TTF-1 and SPPB can be readily categorized as TRU-type. Regarding TRU morphology, the individual cell features of a dome-shaped protrusion with a luminal-free cell border are helpful for practical identification. For example, individual cancer cells of a TRU-type adenocarcinoma (Fig. 1; Fig. 2, left panel) appear to bulge out into the lumen or alveolar spaces and show dome-shaped luminal-free borders, whereas the luminal borders of non-TRU-type cancer cells (right two pictures in Fig. 2) appear as smooth lines. In addition to these cellular features, lepidic growth at the tumor periphery is a hallmark of TRU morphology.

Miller et al[31] has reported that adenocarcinoma with bronchioloalveolar features, as well as nonsmoker, serves as a predictor to respond to gefitinib treatment through multivariate analysis of 139 patients with NSCLCs. Because nonmucinous BAC is a prototype of TRU-type adenocarcinoma, it is likely that most of the "adenocarcinomas with non-mucinous BAC features" correspond to TRU-type carcinoma. Indeed, EGFR mutation, which has been reported to correlate with response to gefitinib treatment, is specifically observed in this characteristic subset. Therefore, gefitinib response is associated with EGFR mutation, which occurs in lung adenocarcinoma in a fashion specific for cellular lineage. It is of note that EGFR mutations can also occur in poorly differentiated adenocarcinomas, as long as the tumor belongs to the TRU cellular lineage, as shown Figure 1.

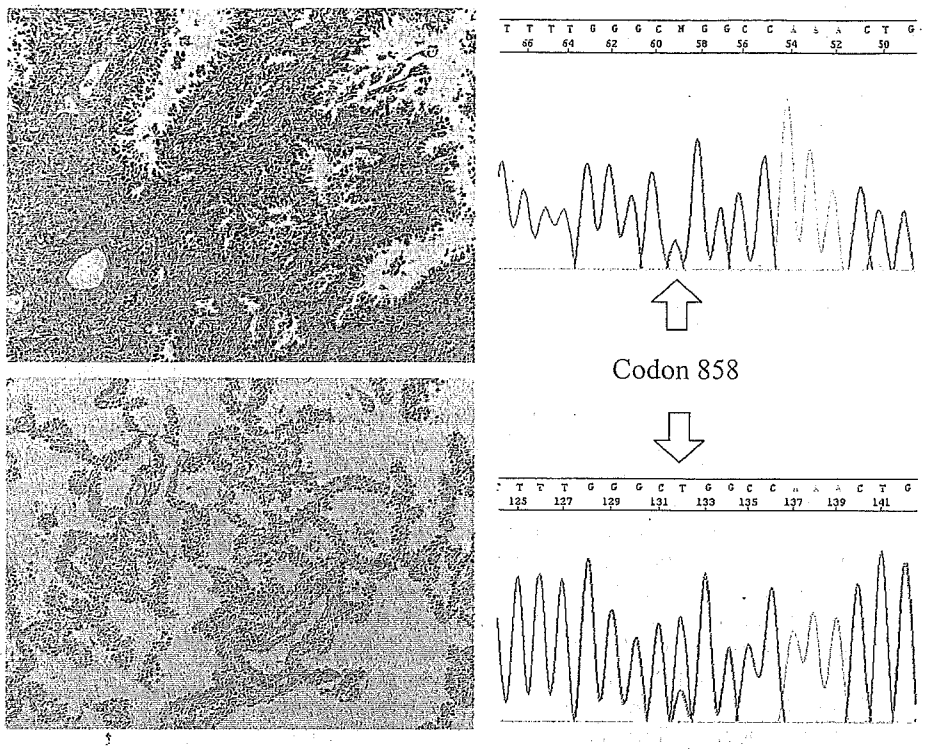

FIGURE 3. A representative result of AAH and its corresponding adenocarcinoma (patient no. 2). The left panel displays the morphologic features of adenocarcinoma (upper) and AAH (lower). Mutation at an identical position (right panel) was noted, although the AAH was found in a different lobe from the adenocarcinoma.

An important insight into the significance of cell lineage in cancers was provided by recent works on breast cancer research. Based on expression profiles, breast cancer is molecularly classified into basal cell-like, luminal cell-like, and HER2/*neu* amplified type,[36,43–45] the first two of which represent cell lineage, like the TRU-type in the lung. The classification was supported by the finding that BRCA1-related breast cancers are clustered specifically in the basal cell-like group[9,44] and that the genome-wide pattern of LOH is correlated with the classification scheme.[50] Recently, it was demonstrated that biologic response against chemotherapy differs between cellular types.[49] Regarding lung cancers, expression profiling analysis also revealed several subsets of adenocarcinoma. It is reasonable to speculate that some of them correspond to TRU-type adenocarcinoma because some subsets were characterized by high expression of TTF-1 and surfactant proteins, eg, adeno-group 1 and 2[12]; C3 and C4[4]; and AD2 to AD4.[47] Identification of properties based on cell lineage may have implications for understanding biologic nature of the tumors.

Recent advances in molecular oncology have revealed a crucial role for the involvement of pathways but not a particular molecule in the development if cancers. For example, about 80% of sporadic colon cancers harbor an alteration of APC, but mutation of β-catenin, a target molecule of APC, could substitute its role in carcinogenesis. Indeed, the mutation has been found in the subset of tumors without APC mutation.[33] Involvement of either c-kit or PDGFRA in gastrointestinal stromal tumors[14] also follows this fashion. Therefore, the mutually exclusive nature of EGFR and K-*ras* mutation

TABLE 1. Correlation of EGFR Mutation and TRU Features In Lung Adenocarcinoma

	EGFR		
	Wild type	Mutated	*P*†
TTF-1 (+/−)	65/33	90/7	<0.001
SPPB (+/−)	47/50	74/21	<0.001
TRU/non-TRU morphology	60/38	91/6	<0.001
TRU-type/non-TRU-type*	58/40	91/6	<0.001†

*TRU-type is defined by being positive for two or more of the three features above.
†*P*-value of this distinction was minimal (7.0×10^{-9}).

TABLE 2. Clinicopathologic Characteristics of TRU Adenocarcinomas

	TRU-type	Non-TRU-type	*P*
N	149	46	
Male/female	67/82	30/16	0.019
Smoker/nonsmoker	65/84	32/14	0.002
pStage (I/II/III/IV)	96/12/38/1	21/7/17/1	0.142
pN(0/1/2)	102/12/33	31/5/9	0.805
TTF-1 (+/−)	144/5	11/35	<0.001
SPPB (+/−)	120/26	1/45	<0.001
TRU/non-TRU morphology	143/6	8/38	<0.001
EGFR (wild-type/mutated)	58/91	40/6	<0.001
Deletion	42	4	
Point mutation	45	2	
Insertion	4	0	
K-ras (wild-type/mutated)	134/15	35/11	0.024
p53 (wild-type/mutated)	94/53	20/26	0.016

TABLE 3. Logistic Regression Model for Estimation of Significant Factors Related to EGFR Mutation

Variable	Category	Odds Ratio	95% CI	P
Age	≥median/<median	1.476	0.767–2.840	0.243
Sex	Female/male	0.783	0.300–2.048	0.618
Smoking status	Nonsmoker/smoker	4.352	1.618–1.710	0.004
Histology	BAC/non-BAC	0.541	0.082–3.546	0.522
Stage	Early-state (IA)/or more	0.769	0.386–1.533	0.455
Cell lineage	TRU-type/non-TRU-type	10.092	3.827–26.61	<0.001

indicates that impairment of this pathway plays an important role in the development of TRU-type adenocarcinomas. This idea suggests further two questions. The first question is what and how many pathways are crucial for the development of this tumor type. When EGFR mutation provides an effect equivalent to K-*ras* mutation, their mutually exclusive nature suggests a crucial role of the RAS-MAPK pathway. However, EGFR has several downstream targets other than the RAS-MAPK pathway, such as the PI3K/AKT and JAK/STAT pathways.[3,19] When EGFR mutation functions to impair the RAS-MAPK and other pathways, the K-*ras* mutation alone is not sufficient to develop TRU-type adenocarcinoma. Indeed, PIK3CA is shown to be mutated, despite low frequency in lung cancers,[37] and a recent study by Sordella et al[42] reported that mutated-EGFR targets the PI3K/AKT and JAK/STAT pathways rather than the RAS-MAPK pathway. Elucidation of these relationships may explain differences in the frequencies of gefitinib responder between Japan and the United States. It is of note that the incidence of K-*ras* mutation is different between the nations[1,11,56]; thus, an international comparative study is needed.

The second question is what is reflected in the preference of the involvement between EGFR and K-*ras* even though the resulted features are not different. The different frequency of smokers may provide a clue to the answer. K-*ras* mutation is known to be closely related with smoking,[1] and the frequency of smokers is quite different between EGFR and K-ras-mutated proportions in TRU-type adenocarcinoma. Smoking status may affect the mutation involved, although either mutation results in the development of the same type of adenocarcinoma.

In summary, we demonstrated a specific involvement of EGFR mutation in TRU-type adenocarcinoma. EGFR mutation was also detected in some AAHs, a preinvasive lesion of TRU-type adenocarcinoma. These findings confirmed that TRU-type adenocarcinoma is a distinct subset of lung adenocarcinomas.

TABLE 4. Mutational Status in Atypical Adenomatous Hyperplasia

Patient No.	Tumor	Feature	EGFR Gene
1	Metastatic cancer	Colon cancer metastases	Wild-type
	AAH	Same lobe, 4 mm	Mutation at codon 858
2	AD, poorly differentiated	25 mm in size, pT1N2	Mutation at codon 858
	AAH	6 mm, different lobe	Mutation at codon 858
3	AD, well differentiated	15 mm in size, pT1N0	Deletion at codon 746–750
	AAH	Different segment, 1.3 mm	No mutation
4	AD, moderately differentiated	33 mm in size, pT2N0	Wild-type
	AAH1	Different segment, 6 mm	No mutation
	AAH2	Different segment, 4 mm	No mutation
5	BAC, nonmucinous	9 mm in size, pT1N0	Wild-type
	AAH1	Same segment, 3 mm	No mutation
	AAH2	Different segment, 3 mm	No mutation

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EGFR Mutation and Response of Lung Cancer to Gefitinib

TO THE EDITOR: Kobayashi et al. (Feb. 24 issue)[1] report that a second mutation in the gene encoding the epidermal growth factor receptor (*EGFR*), one resulting in a threonine-to-methionine substitution at amino acid position 790 (T790M), was associated with acquired resistance to gefitinib in their patient and that this mutant gene had been absent from the primary non–small-cell lung cancer. In a reanalysis of the data from the 397 subjects we have previously described,[2,3] we identified two women who had never smoked who had non–small-cell lung cancer and harbored two *EGFR* mutations — T790M and a leucine-to-arginine substitution at amino acid position 858 (L858R) — in resected tumor specimens before treatment with chemotherapy or radiotherapy. Both patients later had recurrent disease and eventually died — outcomes suggesting that tumors with both the L858R and T790M mutations are very aggressive. One patient was treated with gefitinib and had progression.

These findings indicate the existence of cases with inherent double mutations and provide evidence that the T790M mutant genotype is an important factor conferring resistance to gefitinib in non–small-cell lung cancers containing EGFR sensitivity mutations. In addition, detecting T790M may be useful for predicting pretreatment resistance to EGFR tyrosine kinase inhibitors. Our observation, together with data from recent reports,[1,4] may help clarify the role of *EGFR* mutations in the development of EGFR-related non–small-cell lung cancer and help establish effective strategies against specific subtypes of non–small-cell lung cancer.

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THE AUTHORS REPLY: Dr. Toyooka and colleagues describe two patients whose lung tumors harbored a T790M mutation before treatment with chemotherapy or radiotherapy was begun and suggest that this mutation might be a marker of tumor aggressiveness as well as resistance to gefitinib therapy. In the cases we and others[1] have described, the T790M mutation was not found in specimens from untreated patients. Nevertheless, the possibilities do exist that this second mutation might be present in some tumors at a low frequency at the time of diagnosis and that tumor cells harboring the mutation might be enriched over time during treatment with gefitinib or erlotinib. By analogy, imatinib-resistant *BCR-ABL* mutations have, on occasion, been detected in specimens from patients with untreated chronic myeloid leukemia.[2,3] We agree that such interesting findings should motivate further research to improve our understanding of the role of EGFR in non–small-cell lung cancers, to encourage the development of alternative EGFR inhibitors able to overcome such resistance mutations, and to incorporate the knowledge gained into clinical treatment.

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ORIGINAL REPORT

Mutations of the Epidermal Growth Factor Receptor Gene Predict Prolonged Survival After Gefitinib Treatment in Patients With Non–Small-Cell Lung Cancer With Postoperative Recurrence

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose
To evaluate the relationship between mutations of the epidermal growth factor receptor (*EGFR*) gene and the effectiveness of gefitinib treatment in patients with recurrent lung cancer after pulmonary resection.

Patients and Methods
We sequenced exons 18-21 of the *EGFR* gene using total RNA extracted from 59 patients with lung cancer who were treated with gefitinib for recurrent lung cancer. Gefitinib effectiveness was evaluated by both imaging studies and change in serum carcinoembryonic antigen (CEA) levels.

Results
EGFR mutations were found in 33 patients (56%). Of these mutations, 17 were deletions around codons 746-750 and 15 were point mutations (12 at codon 858, three at other codons), and one was an insertion. *EGFR* mutations were significantly more prevalent in females, adenocarcinoma, and never-smokers. Gefitinib treatment resulted in tumor shrinkage and/or CEA decrease to less than half of the baseline level in 26 patients, tumor growth and/or CEA elevation in 24 patients, and gefitinib effect was not assessable in nine patients. Female, never-smoking patients with adenocarcinoma tended to respond better to gefitinib treatment. Gefitinib was effective in 24 of 29 patients with *EGFR* mutations, compared with two of 21 patients without mutations ($P < .0001$). Of note, del746-750 might be superior to L858R mutations for prediction of gefitinib response. Patients with *EGFR* mutations survived for a longer period than those without the mutations after initiation of gefitinib treatment ($P = .0053$).

Conclusion
EGFR mutations were a good predictor of clinical benefit of gefitinib in this setting.

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INTRODUCTION

Lung cancer has long been the leading cause of cancer death in North America. In 1998, it became the leading cause of cancer death in Japan, and now claims more than 55,000 lives annually.[1] Lung cancer is divided into two morphologic types: small-cell lung cancer and non–small-cell lung cancer (NSCLC). NSCLCs are further subdivided into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. Adenocarcinoma is the predominant histologic subtype, and is increasing among patients with lung cancer who are candidates for surgical treatment in Japan. In our institution, adenocarcinoma accounted for 76% of 407 patients who were operated on from 2001 through 2003. Adenocarcinomas are characterized by a high degree of morphologic heterogeneity. Analyses of various cancer-associated genes, including *K-ras*,[2] *p53*,[3,4] cyclin D1,[5] $p27^{Kip1}$,[6] and cyclooxygenase-2,[7]

suggests a different molecular pathway for carcinogenesis in lung adenocarcinomas at least partly accounts for this heterogeneity. In addition, the NSCLC frequently overexpresses receptors of the ErbB family, including the epidermal growth factor receptor (EGFR) encoded by ErbB1 (HER-1).[8,9]

EGFR is a 170 kd receptor tyrosine kinases (TK) that dimerizes and phosphorylates several tyrosine residues upon binding of several specific ligands including epidermal growth factor and transforming growth factor alpha.[8] These phosphorylated tyrosines serve as the binding sites for several signal transducers that initiate multiple signaling pathways resulting in cell proliferation, migration and metastasis, evasion from apoptosis, or angiogenesis, all of which are associated with cancer phenotypes.[8] Downstream pathways include ras-raf-MEK-ERK, phosphatidylinositol-3 kinase-Akt, and PAK-JNKK-JNK.[8]

Gefitinib is an orally bioavailable small molecule that specifically inhibits EGFR tyrosine phosphorylation.[10] Clinical trials revealed that there is significant variability in response to gefitinib. Good clinical responses have been observed most frequently in women, in nonsmokers, in patients with adenocarcinomas, and in Japanese patients.[11,12] However, it was not possible to predict gefitinib sensitivity by levels of EGFR overexpression as determined by immunohistochemistry[13] or immunoblotting.[14] The factors that determine gefitinib sensitivity have long been an enigma. Recently, it has been reported that activating mutations of *EGFR* are present in a subset of pulmonary adenocarcinomas and that tumors with *EGFR* mutations are highly sensitive to gefitinib[15-17] or erlotinib, another EGFR TK inhibitor. Furthermore, the incidence of *EGFR* mutations is significantly higher in female, never-smoking, Japanese patients with adenocarcinoma.[15] These features coincide with those of good responders to gefitinib.

In this study, we studied patients who had recurrent disease after pulmonary resection for NSCLC and who were subsequently treated with gefitinib. We searched for mutations of the *EGFR* gene in tumor specimens taken at the time of surgery and we correlated *EGFR* mutations with gefitinib effectiveness, including tumor response and patient survival.

PATIENTS AND METHODS

Patients

Seventy-five patients were treated with gefitinib for their recurrent diseases after they had undergone surgery between 1999 and 2003. We studied 59 patients whose tumors were available for RNA extraction, which was a sole determinant of inclusion into the present study. There were 32 men and 27 women with ages ranging from 48 to 79 years. Fifty patients had adenocarcinomas, five had squamous cell carcinomas, three had large-cell carcinomas, and one had adenosquamous carcinoma. Eight patients had stage IA disease; seven stage IB; three stage IIA; five stage IIB; 24 stage IIIA; eight stage IIIB; and three stage IV at the time of surgery. Lobectomy had been performed in 57, and pneumonectomy and partial resection in one patient each. Four patients received postoperative adjuvant chemotherapy (two with oral uracil/tegafur and two with gemcitabine monotherapy). Forty patients had had chemotherapy before gefitinib treatment (23 patients, platinum doublet; 16 patients, monotherapy with vinorelbine or gemcitabine, one patient, oral uracil/tegafur). Gefitinib treatment with a daily dose of 250 mg was initiated between July 2002 and May 2004, with the median interval between operation and gefitinib treatment being 778 days (range, 107 to 1,931 days). Fifty patients had distant metastatic tumors, eight patients had pleural dissemination and malignant effusion, and one patient had hilar lymph-node metastasis at initiation of gefitinib treatment.

Molecular Analysis of Lung Cancer Specimens

After we obtained appropriate approval from the institution and written informed consent for comprehensive use of molecular and pathologic analysis from the patients, tumor samples were collected during surgery, rapidly frozen in liquid nitrogen and stored at −80°C. A surgical pathologist (Y.Y.) grossly dissected the frozen tumor specimens to enrich the tumor cell population as much as possible. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA).

The first four exons (exons 18-21) of the seven exons (exons 18-24) that code for TK domain of the *EGFR* gene (which includes all the mutations reported so far[15-17]) was amplified with primers F1 (5′-AGCTTGTGGAGCCTCTTACACC-3′) and R1 (5′-TAAAATTGATTCCAATGCCATCC-3′) in a one-step reverse transcription polymerase chain reaction (RT-PCR) using the QIAGEN OneStep RT-PCR Kit (Qiagen). The cDNA sequence of the *EGFR* gene was obtained from GenBank (accession number NM 005228). The RT-PCR conditions were: one cycle of 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 50 seconds, 62°C for 50 seconds, and 72°C for 60 seconds, followed by one cycle of 72°C for 10 minutes.

RT-PCR products were diluted and cycle-sequenced using the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing products were electrophoresed on an ABI PRISM 3100 (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed by BLAST (basic local alignment search tool) and chromatograms by manual review. High-quality sequence variations found in both directions were scored as candidate mutations.

Definition of Effectiveness of Gefitinib

Because this study was a retrospective analysis of the daily clinical practice of oncology, the evaluation of tumor response could not be performed strictly according to predefined criteria, such as Response Evaluation Criteria in Solid Tumors (RECIST).[18] RECIST are not necessarily applicable or complete in such a context and the evaluation may instead be based on a subjective medical judgment that results from clinical and laboratory data.[18] Therefore, gefitinib treatment was judged as effective when the tumors showed at least a 30% decrease in tumor diameter in imaging studies. However, because of the nature of the study, confirmation of tumor response no less than 4 weeks apart, as in RECIST,[18] was not necessarily required.

As patients with recurrent lung cancer often do not have measurable disease, we also included change in serum carcinoembryonic antigen (CEA) level (cut off, 5 ng/mL) as an evaluation

criterion to avoid underestimating gefitinib effectiveness. CEA has been reported as a useful clinical therapeutic marker.[19] When the elevated CEA level decreased to a level less than half of the baseline level, gefitinib treatment was judged as effective. On the other hand, gefitinib treatment was judged as ineffective when the tumors showed any growth or a new lesion appeared in the imaging studies, or when the serum CEA level increased. Any patient who did not fit either of these criteria was classified as not assessable. All these evaluations were done before the *EGFR* gene analysis, without knowledge of mutational status of the *EGFR* gene.

Statistical Analysis

For comparisons of proportions, the χ^2 test or Fisher's exact test was used. The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and survival differences were analyzed by the log-rank test. The two-sided significance level was set at $P < .05$. To identify which independent factors had a joint significant influence on gefitinib effectiveness, the logistic regression modeling technique was used, and for multivariate analysis of the overall survival, the Cox proportional hazards modeling technique was applied. All analyses were performed using StatView version 5 (SAS institute Inc, Cary, NC) software on a Macintosh computer.

RESULTS

EGFR *Mutations*

Mutations of the *EGFR* gene were detected in 33 (56%) of 59 patients. Seventeen were deletions, 15 were point mutations, and one was an insertion. Details of these mutations are shown in Figure 1. As previously reported,[15-17] *EGFR* mutations were significantly associated with adenocarcinoma histology, female sex, and never-smoking status (Table 1). However, the mutations were not associated with the age or stage of the patients. Furthermore, median time from the original surgery to

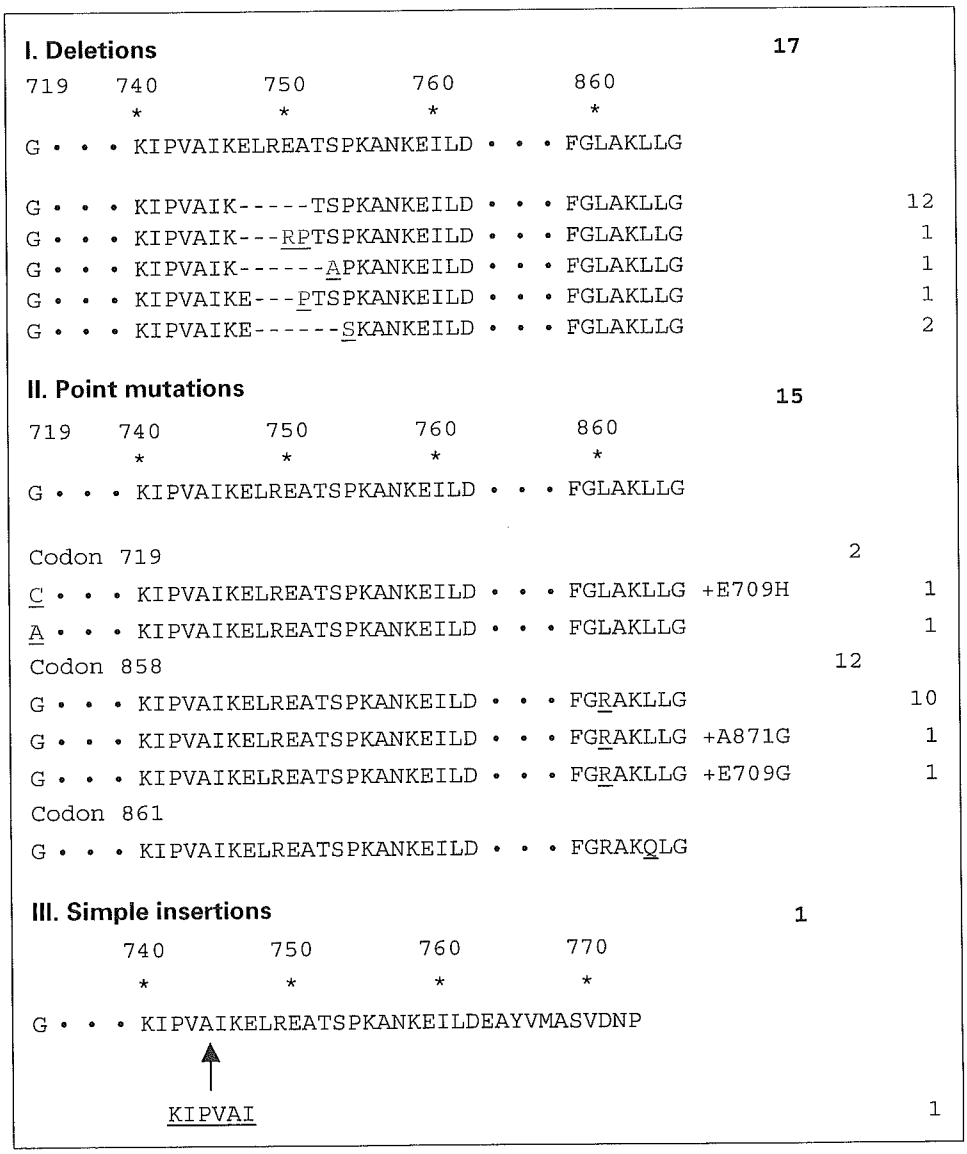

Fig 1. Analysis of 33 epidermal growth factor receptor (EGFR) mutations in tyrosine kinase domain of the *EGFR* gene found in unselected patients with lung cancer.