

Fig. 2. PFS (a) and overall survival (b) in patients with adenocarcinoma. Thick line = Female patients; thin line = male patients.

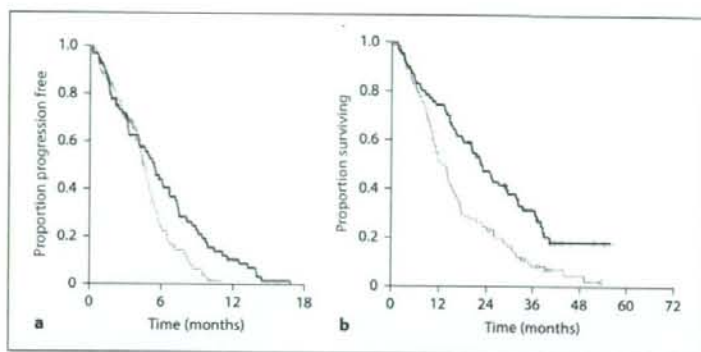
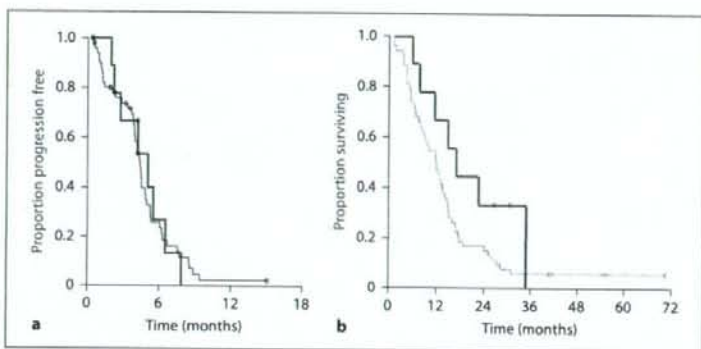


Fig. 3. PFS (a) and overall survival (b) in patients with non-adenocarcinoma. Thick line = Female patients; thin line = male patients.



and smoking status, female gender was a significant factor for a favorable prognosis (hazard ratio 0.49, 95% confidence interval 0.33–0.73; table 3). In the subset analyses, among patients with adenocarcinoma, PFS and MST were better in females than in males (fig. 2), whereas among patients with non-adenocarcinoma, there was no gender difference in PFS or MST (fig. 3).

Discussion

The present study and other previous studies have shown that female gender is a favorable prognostic factor in patients with stage IIIB or IV NSCLC who receive combination chemotherapy [7–12]. The reasons for this gender difference are currently unknown, but there are 5 possibilities. First, men may not have received sufficient cycles and doses of chemotherapy, since they develop more severe toxicity during chemotherapy than women. How-

Table 3. Multivariate analysis of baseline characteristics for overall survival in all patients

Variables	Patients	Hazard ratio
Sex		
Male	147	1
Female	80	0.49 (0.33–0.73)
Stage		
IIIB	71	1
IV	156	1.37 (1.00–1.89)
Performance status		
0	65	1
1	162	1.31 (0.95–1.81)
Histology		
Adenocarcinoma	165	1
Non-adenocarcinoma	72	1.03 (0.73–1.45)
Smoking		
Never-smoker	77	1
Smoker	150	0.96 (0.65–1.42)

Figures in parentheses are 95% confidence intervals.

ever, in the present study, the number of chemotherapy cycles was the same for both male and female patients, and hematological toxicity was more severe in females than in males. Of note, treatment-related death was observed only in male patients, but the number of deaths was very small (2.7%). The second possibility may be that chemotherapy was more effective in females than in males. However, there was no difference in the response rates by gender in the present study and in previous studies [7, 11, 12]. In 1 study, the duration of response was also found to be the same in male and female patients [11]. The PFS was longer in females than in males in this and in 1 previous study [7], but the PFS can be affected by several factors other than chemotherapy-induced responses. Thus, the second scenario is not likely. The third reason may be that more men die from diseases other than lung cancer. However, in the present study, 95% of male patients and 93% of female patients died of lung cancer progression.

The fourth possibility is that males may have a more aggressive tumor that grows more rapidly than in females. In the present study, there was a higher percentage of never-smokers among female compared with male patients, especially in patients with adenocarcinoma. Large case series studies have found that patients with lung adenocarcinoma who had never smoked had a better survival than those who had a smoking history [19, 20]. Thus, the higher frequency of never-smokers among female patients may explain the better prognosis of female patients in the present study. Recent developments in the molecular pathogenesis of lung cancer suggest that the origins of adenocarcinomas may involve different pathways: a K-RAS mutation-dependent pathway in smokers and an epidermal growth factor receptor mutation-dependent pathway in never-smokers [21]. Lung adenocarcinomas arising by these distinct pathways may have a different potential for progression. Thus, adenocarcinoma in females arising through the epidermal growth factor receptor mutation-dependent pathway may be less aggressive than adenocarcinoma in males, which may arise mainly through the K-RAS mutation-dependent pathway. Carcinogenesis pathways in NSCLC other than adenocarcinoma are unknown, but they are not likely to differ by gender because these tumors are associated with a heavy smoking habit in both genders. These hypotheses are consistent with the results of the present study that there are gender differences in patients with adenocarcinoma, but that the gender differences were small, if any, in those with non-adenocarcinoma.

Finally, gefitinib administration may be associated with a gender difference in overall survival. In the present study,

more female patients received gefitinib monotherapy, and the treatment duration was 4 times longer in female than in male patients. Thus, gefitinib treatment probably contributed to the improved survival of female patients.

The present study found that females had more chemotherapy-related hematological toxicity than males during treatment, while there was no gender difference in neurological toxicity. More severe hematological toxicity in females was also noted among patients with SCLC treated with combinations of cyclophosphamide, vincristine, doxorubicin, etoposide and cisplatin [22]. This can be explained by decreased clearance of cyclophosphamide, vincristine, doxorubicin and etoposide due to a 2.4-fold lower expression of hepatic P-glycoprotein, which is a transporter of these agents [23]. The mechanism that could explain the gender difference in toxicity associated with carboplatin and paclitaxel in the present study is unknown, but decreased clearance of paclitaxel is not likely, because neurological toxicity did not differ by gender. Since DNA repair capacity measured using peripheral blood lymphocytes is lower in female lung cancer patients than in male patients [24], increased susceptibility to carboplatin-induced DNA damage may be one factor related to increased chemotherapy-related toxicities in female patients. A recent large-scale study did not show an association between the severity of toxicity and polymorphisms of 16 key genes for drug-metabolizing enzymes, transporters and DNA repair in 914 patients with ovarian cancer who received combination chemotherapy consisting of carboplatin with paclitaxel or docetaxel [25]. However, our understanding of the true regulation of chemotherapy action is very limited at present, and the possibility remains that gender differences in chemotherapy outcome may be based on pharmacogenomic differences between the genders. The lower DNA repair capacity in females may also influence tumor DNA repair after exposure to cytotoxic chemotherapy, and therefore, it may have implications for the significantly longer PFS in female patients after first-line chemotherapy with carboplatin and paclitaxel.

In conclusion, female gender was associated with a favorable prognosis in patients with NSCLC who received combination carboplatin and paclitaxel chemotherapy, even though response rates did not differ by gender. Hematological toxicity was more severe in female patients.

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EGFR Mutations Predict Survival Benefit From Gefitinib in Patients With Advanced Lung Adenocarcinoma: A Historical Comparison of Patients Treated Before and After Gefitinib Approval in Japan

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ABSTRACT

Purpose

This study evaluated whether the presence of *epidermal growth factor receptor* (*EGFR*) mutations is a predictive marker for survival benefit from gefitinib and/or a prognostic marker in patients with advanced lung adenocarcinoma.

Patients and Methods

Overall survival (OS) was compared between patients with advanced lung adenocarcinoma who began first-line systemic therapy before and after gefitinib approval in Japan (January 1999 to July 2001 and July 2002 to December 2004, respectively). Deletional mutations in exon 19 or the L858R mutation in exon 21 of *EGFR* were evaluated using high-resolution melting analysis.

Results

EGFR mutations were detected in 136 (41%) of the 330 patients included in this study. OS was significantly longer among the *EGFR*-mutant patients treated after gefitinib approval compared with the OS of patients treated before gefitinib approval (median survival time [MST], 27.2 v 13.6 months, respectively; $P < .001$), whereas no significant survival improvement was observed in patients without *EGFR* mutations (MST, 13.2 v 10.4 months, respectively; $P = .13$). A significant interaction between the presence of *EGFR* mutations and a survival improvement was seen ($P = .045$). Among patients treated before gefitinib approval, those with *EGFR* mutations lived longer than those without *EGFR* mutations (MST, 13.6 v 10.4 months, respectively; $P = .034$). The response rates to first-line cytotoxic chemotherapy were not significantly different between patients with and without *EGFR* mutations (31% v 28%, respectively; $P = .50$).

Conclusion

EGFR mutations significantly predict both a survival benefit from gefitinib and a favorable prognosis in patients with advanced lung adenocarcinoma.

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INTRODUCTION

Gefitinib (Iressa; AstraZeneca, Osaka, Japan) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI). Gefitinib was approved for the treatment of patients with advanced non-small-cell lung carcinoma (NSCLC) in Japan in July 2002, after its antitumor activity had been demonstrated in two phase II studies.^{1,2} The response rate to gefitinib was higher among women, patients with adenocarcinoma, never-smokers, and Japanese or East Asians.¹⁻³ In April 2004, somatic mutations in the kinase domain of *EGFR*, mainly in-frame deletions including amino acids at codons 747 to 749 (DEL) in exon 19

and a missense mutation at codon 858 (L858R) in exon 21, were suggested to be determinants of gefitinib sensitivity.^{4,5} Since then, retrospective studies have consistently revealed a strong association between *EGFR* mutations and clinical outcomes in NSCLC patients treated with gefitinib.⁶⁻⁹ Although these studies showed that overall survival (OS) was much longer among patients with *EGFR* mutations, they did not intrinsically prove a survival benefit of gefitinib in patients with *EGFR* mutations because there remained the possibility that the differences in OS were merely caused by prognostic differences independent of gefitinib treatment.

Eight large-scale, randomized, phase III trials were conducted to evaluate the survival benefits of

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gefitinib or erlotinib (Tarceva; OSI Pharmaceuticals Inc, Melville, NY), another EGFR-TKI, in patients with advanced NSCLC. The Iressa NSCLC Trial Assessing Combination Treatment (INTACT)-1, INTACT-2, Tarceva Responses in Conjunction with Paclitaxel and Carboplatin (TRIBUTE), and Tarceva Lung Cancer Investigation (TALENT) trials tested the concurrent combination of platinum-based chemotherapy and EGFR-TKIs in a first-line setting but failed to show a survival benefit from the addition of the EGFR-TKIs.¹⁰⁻¹³ The Iressa Survival Evaluation in Lung Cancer (ISEL) trial tested the role of second- or third-line gefitinib monotherapy but also failed to show a significant survival benefit over a placebo,¹⁴ whereas the BR.21 trial showed a significant survival benefit of second- or third-line erlotinib monotherapy.¹⁵ The Iressa NSCLC Trial Evaluating Response and Survival against Taxotere (INTEREST) and V15-32 trials compared OS after second-line gefitinib monotherapy and docetaxel monotherapy, which is a standard second-line treatment; the former study proved the noninferiority of gefitinib to docetaxel, whereas the latter study failed to do so.^{16,17}

In subgroup analyses of some of these trials, significant survival benefits were observed for never-smokers^{12,14} and Asian patients.¹⁴ In the BR.21 trial, no history of smoking was a significant predictor of a survival benefit from erlotinib.¹⁵ Because never-smokers and Asian patients are known to have higher frequencies of *EGFR* mutations,^{4-9,18,19} these results suggested an association between *EGFR* mutations and a survival benefit from EGFR-TKIs. However, in all of these trials, mutational analyses failed to show a significant survival benefit from EGFR-TKIs in *EGFR*-mutant patients,²⁰⁻²³ partly because of the small sample sizes that were used.

In the INTACT and TRIBUTE trials, patients with *EGFR* mutations lived longer than those without *EGFR* mutations, irrespective of treatment with EGFR-TKIs^{20,21}; this result suggested that *EGFR* mutations may have prognostic value in patients with advanced NSCLC who were treated with standard chemotherapy. However, these trials were inconclusive regarding this point because of the small number of *EGFR*-mutant patients who were examined. As for early-stage NSCLC patients, several large-scale retrospective studies have been reported; some studies showed no significant association between the presence of *EGFR* mutations and OS after surgery,^{19,24} whereas others showed that the presence of *EGFR* mutations was associated with a favorable prognosis in a univariate analyses, but the association disappeared when adjustments for patient characteristics like sex and smoking history were made.^{25,26}

To evaluate whether gefitinib provides a survival benefit to patients with lung adenocarcinoma and whether the mutational status of *EGFR* is a predictor of a survival benefit from gefitinib and/or a prognostic factor, we analyzed data obtained on patients with advanced lung adenocarcinoma who were treated before and after gefitinib approval.

PATIENTS AND METHODS

Patients

We performed all the analyses in this study using a protocol approved by the institutional review board of the National Cancer Center Hospital (NCCH; Tokyo, Japan). Consecutive patients with advanced lung adenocarcinoma who had been pathologically diagnosed at NCCH and began first-line systemic therapy without thoracic radiotherapy between July 2002 and December 2004 (after gefitinib approval; group A) or between January 1999 and July 2001 (at

least 1 year before gefitinib approval; group B) were identified using the databases of NCCH. Patients for whom appropriate pathologic samples were available and a mutational analysis could be successfully performed were included in this study.

Mutational Analysis

DNA was extracted from archived paraffin-embedded tissues and/or Papanicolaou-stained cytologic slides, and the two major hotspots of *EGFR* mutations, DEL and L858R, were analyzed using high-resolution melting analysis according to a previously described method.^{9,27} Briefly, polymerase chain reaction (PCR) was performed using primers designed to amplify a region containing E746-1759 or L858 of *EGFR* and the dye LCGreen I (Roche Diagnostics, Indianapolis, IN). Melting curves were obtained using HR-1 (Idaho Technology, Salt Lake City, UT), and the curves of the samples and controls were compared. All of the mutational analyses were performed in a blinded fashion.

Clinical Outcomes

OS was defined as the time from the start of first-line systemic therapy until death. In patients with measurable lesions, tumor response to first-line cytotoxic chemotherapy, including second-line therapy after first-line gefitinib therapy, was evaluated using standard bidimensional measurements.²⁸ The response rate was defined as the proportion of complete and partial responses compared with the total number of patients.

Statistical Analysis

The differences in OS for the patients in group A and those in group B were compared using Kaplan-Meier curves and log-rank tests. To assess the interaction between the groups and the mutational status of *EGFR*, interaction terms were included in the Cox proportional hazards models. The interaction was considered significant if $P < .10$. The impact of *EGFR* mutations on tumor response to chemotherapy and prognosis was assessed using a χ^2 test and a log-rank test, respectively. These analyses were performed with or without adjustments for the following baseline characteristics: age, sex, smoking history (never-smokers v others), performance status (PS), and disease stage (recurrence after surgery v stage III/IV). All the statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC).

RESULTS

Mutational Analysis

Medical and pathologic records were reviewed for 414 clinically eligible patients (255 in group A and 159 in group B), and the mutational status was successfully determined in 330 patients (200 in group A and 130 in group B). Appropriate pathologic samples were not available in 68 patients (49 in group A and 19 in group B), and indeterminate results were obtained because of incomplete PCR in 16 patients (six in group A and 10 in group B). Of the 330 successfully analyzed patients, 193 were analyzed using only cytology samples, 106 were analyzed using only tissue samples, and 31 were analyzed using both samples. DEL and L858R mutations were detected in 77 (23%) and 59 patients (18%), respectively, and these mutations were mutually exclusive.

Patient Characteristics

The patient characteristics of the 330 patients are listed in Table 1. All of the patients were Japanese except for one Korean patient and one Chinese patient. When groups A and B were compared, group A had a significantly higher percentage of patients with recurrence after surgery and patients with a poor PS. Age, sex, and smoking history were similar between the two groups. In group A, most of the patients were treated with EGFR-TKIs. However, 15 patients (8%) were not treated with EGFR-TKIs, and in 12 patients (6%), the EGFR-TKI

Table 1. Patient Characteristics

Characteristic	Group A: July 2002 to December 2004 (n = 200)		Group B: January 1999 to July 2001 (n = 130)		P
	No. of Patients	%	No. of Patients	%	
Age, years					.47
Median	62		62		
Range	27-84		37-84		
Sex					.52
Female	84	42	50	38	
Male	116	58	80	62	
Smoking history*					.70†
Never-smoker	92	46	57	44	
Former smoker	42	21	33	25	
Current smoker	66	33	40	31	
Histologic diagnosis					—
Adenocarcinoma	200	100	130	100	
Other	0	0	0	0	
Performance status					.049‡
0	70	35	46	35	
1	113	57	80	62	
2	13	7	4	3	
3	4	3	0	0	
Stage					.001§
IIIb	37	19	29	22	
IV	79	40	70	54	
Recurrence after surgery	84	42	31	24	
First-line cytotoxic chemotherapy					—
Platinum + third-generation drug¶	140	70	88	68	
Other platinum-based regimen	0	0	8	6	
Non-platinum-based regimen	14	7	34	26	
No cytotoxic chemotherapy	46	23	0	0	
EGFR-TKI therapy					—
First line	81	41	0	0	
Second line	63	32	9	7	
Third or more line	29	15	10	8	
Never	15	8	111	85	
Unknown	12	6	0	0	
EGFR mutation status					
DEL	46	23	31	24	
L858R	32	16	27	21	
Wild type	122	61	72	55	

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; DEL, deletional mutations in exon 19.

*Never-smokers were defined as patients who had never had a smoking habit, and former smokers were defined as patients who had stopped smoking at least 1 year before diagnosis.

†Never-smokers v others.

‡0 or 1 v 2 or 3.

§IIIb or IV v recurrence after surgery.

||Including second-line therapy after first-line gefitinib therapy.

¶Third-generation drug indicates paclitaxel, docetaxel, gemcitabine, vinorelbine, or irinotecan.

treatment history was unknown because the patients had been transferred to another hospital and the subsequent treatment data was not available. In group B, all but 19 patients (15%) had no history of EGFR-TKI treatment; six patients had been treated with gefitinib in clinical trials before gefitinib approval, one patient had been treated with erlotinib in a phase II trial, and 12 patients had been treated with gefitinib in a clinical practice setting after gefitinib approval.

Historical Comparison Before and After Gefitinib Approval

The median follow-up time for 46 survivors in group A was 30.8 months (range, 10.7 to 49.8 months), and the follow-up times for two

survivors in group B were 65.7 and 85.0 months. OS was significantly longer in group A than in group B (median survival time [MST], 18.1 v 12.5 months, respectively; hazard ratio [HR] = 0.66; 95% CI, 0.52 to 0.84; $P < .001$; Fig 1A). In group A versus group B, a significant improvement in survival was observed in patients with EGFR mutations (MST, 27.2 v 13.6 months, respectively; HR = 0.48; 95% CI, 0.32 to 0.71; $P < .001$; Fig 1B), whereas no significant improvement in survival was observed in patients without EGFR mutations (MST, 13.2 v 10.4 months, respectively; HR = 0.79; 95% CI, 0.59 to 1.07; $P = .13$; Fig 1C). The improvement in survival was similar among patients with DEL (Fig 1D) and those with L858R (Fig 1E). A significant interaction between the mutational status of EGFR

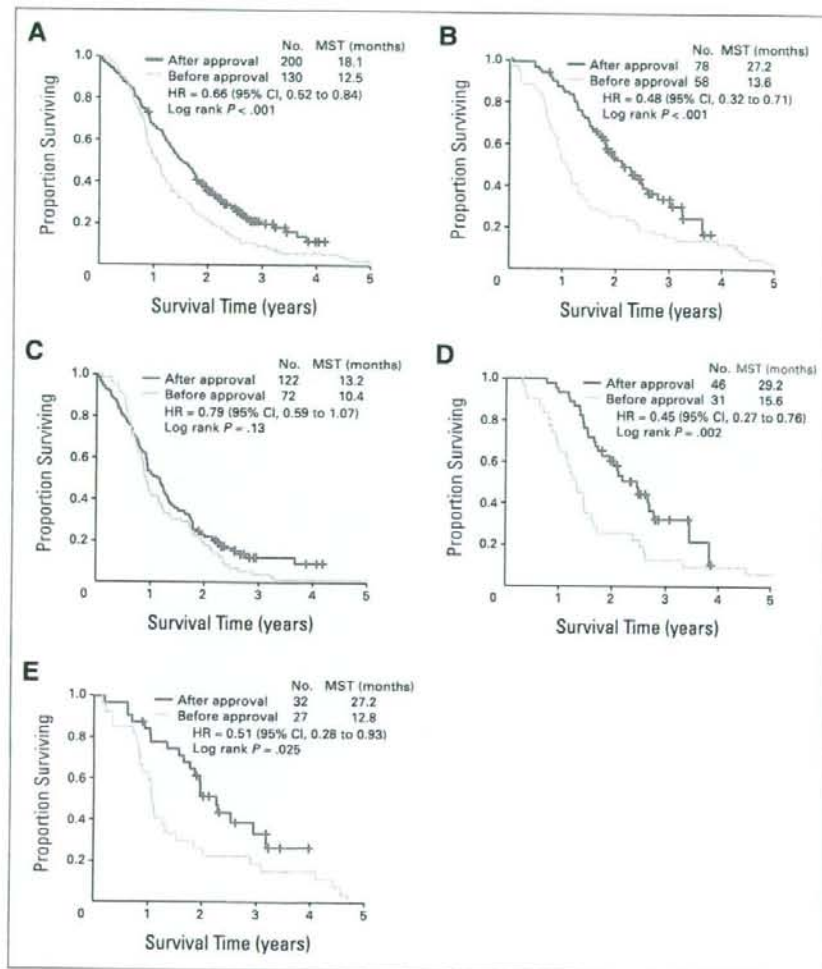


Fig 1. Comparison of overall survival between patients who began first-line systemic therapy after gefitinib approval and patients who began treatment before gefitinib approval. (A) All patients included in the current study. (B) Patients with epidermal growth factor receptor (*EGFR*) mutations. (C) Patients without *EGFR* mutations. (D) Patients with deletional mutations in exon 19. (E) Patients with L858R mutation. MST, median survival time; HR, hazard ratio.

(mutant ν wild type) and the improvement in survival was observed ($P = .045$). After adjusting for age, sex, smoking history, PS, and disease stage, the HR of after to before gefitinib approval was 0.47 (95% CI, 0.31 to 0.70; $P < .001$) among patients with *EGFR* mutations and 0.76 (95% CI, 0.55 to 1.04; $P = .088$) among patients without *EGFR* mutations. The interaction was also significant after the adjustment ($P = .035$).

Prognosis in Patients Before Gefitinib Approval

When patients with and without *EGFR* mutations were compared in group B (patients treated before gefitinib approval), the patients with *EGFR* mutations lived significantly longer than patients without *EGFR* mutations (MST, 13.6 ν 10.4 months, respectively; HR = 0.68; 95% CI, 0.48 to 0.97; $P = .034$; Fig 2A), and this finding persisted after adjustments for age, sex, smoking history, PS, and disease stage (HR = 0.65; 95% CI, 0.44 to 0.96; $P = .028$). However, this result may be affected by *EGFR*-TKI treatment administered to 19

patients (12 with *EGFR* mutations and seven without *EGFR* mutations). When the start of *EGFR*-TKI administration in the 19 patients was treated as a censoring event to exclude the effect, the difference in OS was not significant (HR = 0.74; 95% CI, 0.50 to 1.08; $P = .12$; Fig 2B). Between patients with DEL and those with L858R, the difference in OS was not significant (MST, 15.6 ν 12.8 months, respectively; HR = 0.86; 95% CI, 0.51 to 1.46; $P = .58$).

Response to Cytotoxic Chemotherapy

The response to cytotoxic chemotherapy was evaluated in 279 of the 330 patients. The other 51 patients were excluded because no chemotherapy other than gefitinib was administered ($n = 46$) or they had no measurable lesions ($n = 5$). As shown in Table 2, the total response rate was 29%, and the response rates were not significantly different between patients with and without *EGFR* mutations (31% ν 28%, respectively; $P = .50$). These findings were similar for patients with DEL and with L858R (29% ν 35%, respectively; $P = .49$). *EGFR*

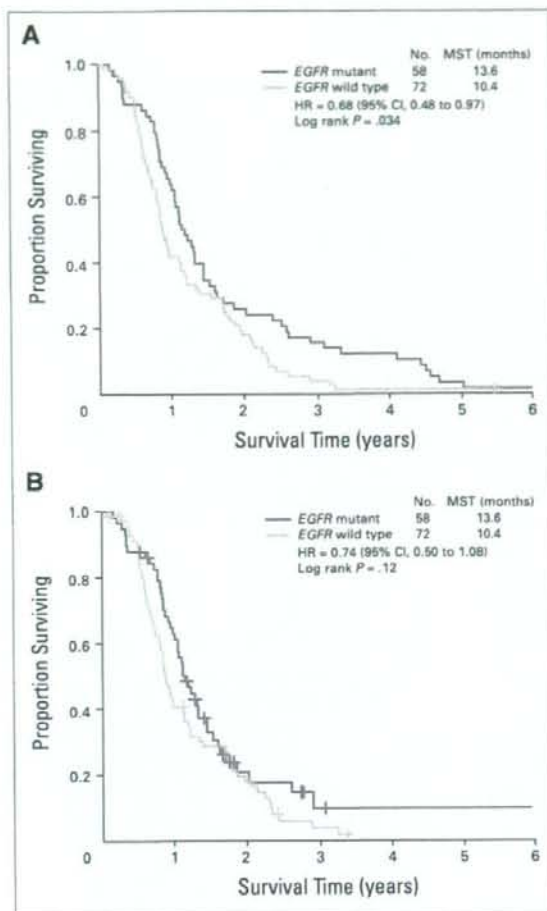


Fig 2. (A) Comparison of overall survival between patients with and without epidermal growth factor receptor (*EGFR*) mutations among patients treated before gefitinib approval, and (B) the same comparison when the start of *EGFR* tyrosine kinase inhibitor administration is treated as a censoring event. MST, median survival time; HR, hazard ratio.

mutations were not significantly associated with response to any specific regimen, although the response rate to taxane monotherapy tended to be higher among patients with *EGFR* mutations than in patients without *EGFR* mutations (31% v 13%, respectively; $P = .17$).

DISCUSSION

To assess the survival benefit of gefitinib in patients with lung adenocarcinoma, we compared the OS of patients treated after gefitinib approval (group A) with a historical control (group B). As the historical control, we selected patients treated between January 1999 and July 2001 because most of these patients routinely received a combination of platinum and a third-generation drug and were also administered second-line cytotoxic chemotherapy, as indicated; thus, their cytotoxic chemotherapy regimens were sim-

ilar to those of the patients in group A. Actually, fewer cytotoxic chemotherapy regimens were used in group A because some cytotoxic chemotherapy options were replaced with gefitinib therapy. Because the most essential difference between the two groups was the availability of gefitinib, the survival improvement observed in this historical comparison can be interpreted as reflecting a survival benefit from the addition of gefitinib monotherapy or the replacement of cytotoxic chemotherapy with gefitinib monotherapy. Although there was a small number of patients who were not treated with *EGFR*-TKIs in group A or who were treated with *EGFR*-TKIs in group B, we included all consecutive patients in the analysis to avoid biases. Some imbalances in the baseline patient characteristics of the two groups were noted; however, all of the results described in the present study were similar even after adjustments were made for the baseline patient characteristics.

In this study, we clearly showed an improvement in the survival of patients with *EGFR* mutations after gefitinib approval. In fact, the MST doubled (13.6 to 27.2 months), a feat that has never before been achieved in the history of NSCLC treatment. Even in patients without *EGFR* mutations, a nonsignificant improvement in survival was obtained (MST, 10.4 to 13.2 months); this result might be a result of the efficacy of gefitinib, period effects other than the approval of gefitinib therapy, or selection biases. Nevertheless, a significant interaction between the presence of *EGFR* mutations and an improvement in survival was obtained, meaning that the mutational status of *EGFR* is a predictor of a survival benefit from gefitinib.

To our knowledge, this is the first study to show a significant interaction between *EGFR* mutations and a survival benefit from *EGFR*-TKI therapy. Although this study was a retrospective historical comparison conducted only in East Asian patients and some biases could not be excluded, the number of patients with *EGFR* mutations analyzed in this study ($n = 136$) was much larger than those in phase III trials (INTACT, $n = 32$; TRIBUTE, $n = 29$; ISEL, $n = 26$; BR.21, $n = 34$),^{20-22,29} and we believe that the results of this study have a certain amount of importance to clinical practice.

The current study also showed that, among the patients treated with chemotherapy before gefitinib approval (group B), the OS was significantly longer in the patients with *EGFR* mutations than in those without *EGFR* mutations. As with the INTACT and TRIBUTE trials,^{20,21} this result suggested that the presence of *EGFR* mutations was a favorable prognostic factor in patients with advanced NSCLC. However, this result is not conclusive because the difference was marginal when the effects of *EGFR*-TKIs, which were used in a small number of patients, were excluded.

As for the patients who were treated after gefitinib approval (group A), the difference in OS between the patients with and without *EGFR* mutations can be partly explained by the prognostic value of the *EGFR* mutations themselves. However, this study indicated that the difference was mainly caused by the mutations' predictive value for a survival benefit from gefitinib.

The difference in OS according to the mutational status of *EGFR* in group B can also be explained by the predictive value for chemotherapy efficacy other than the pure prognostic value. In INTEREST and V15-32, which were phase III trials comparing docetaxel and gefitinib, the HRs for OS were almost the same between patients with and without *EGFR* mutations,^{16,30} suggesting that *EGFR* mutations might be a predictive factor for a survival benefit from both docetaxel

Table 2. EGFR Mutations and Tumor Response to Cytotoxic Chemotherapy

Therapy	Mutant EGFR		Wild-Type EGFR		P	Total	
	No. of Patients	Response Rate (%)	No. of Patients	Response Rate (%)		No. of Patients	Response Rate (%)
Total	112	31	167	28		279	29
Regimens							
Platinum + taxane	54	37	97	34	.71	151	35
Platinum + other third-generation drug*	35	26	39	26	.99	74	26
Taxane† monotherapy	16	31	23	13	.17	39	21
Other regimen	7	14	8	0	.27	15	7
Treatment line							
First line	95	33	147	27	.37	242	29
Second-line therapy after first-line gefitinib therapy	17	24	20	30	.66	37	27

Abbreviation: EGFR, epidermal growth factor receptor.

*Other third-generation drug indicates gemcitabine, vinorelbine, or irinotecan.

†Taxane indicates paclitaxel or docetaxel.

and gefitinib. In the current study, response rate to taxane monotherapy tended to be higher in patients with EGFR mutations, although the number of patients was small. These results are inconclusive, and further investigation is needed.

We detected no significant difference in the predictive and prognostic values of DEL and L858R in the current study. Some researchers, including ourselves, have reported that patients with DEL had better outcomes after EGFR-TKI treatment than those with L858R^{9,31,32}; however, the current study showed that gefitinib yielded almost the same survival benefit to both patients with DEL and patients with L858R, and we think that the two EGFR mutations should be treated equally when making clinical decisions.

In the ISEL and BR.21 trials, the EGFR copy number (evaluated using fluorescence in situ hybridization), rather than the EGFR mutation status, was suggested to predict a survival benefit from EGFR-TKIs,^{22,23,29} and the authors concluded that a mutational analysis was not necessary to select patients for treatment with EGFR-TKIs. In contrast, the current study indicated that the EGFR mutation status was a determinant of a survival benefit from gefitinib, although EGFR copy numbers were not evaluated in this study. Our previous study showed that the EGFR copy number, as evaluated using quantitative PCR, was associated with a response to gefitinib; however, an increased EGFR copy number tended to be seen in patients with EGFR mutations and was not an independent predictor of response or OS in gefitinib-treated patients.⁶ These discrepancies may be a result of the ethnic difference, the methodologic difference between fluorescence in situ hybridization and quantitative PCR, or the accuracy of biomarker analyses. Although controversy still remains, we believe that the EGFR mutation status is the most useful biomarker for patient selection, at least in East Asian patients who have EGFR mutations more frequently than non-Asian patients.

In conclusion, gefitinib yielded a survival benefit among Japanese patients with lung adenocarcinoma, and the survival benefit was significantly greater in patients with EGFR mutations than in those without EGFR mutations. The presence of EGFR mutations may also be a favorable prognostic factor in advanced lung adenocarcinoma

independent of gefitinib treatment. We need to consider appropriate treatment strategies for patients with NSCLC based on their EGFR mutation status.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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thus influenced the results including those assessed (overall survival) and not assessed (disease free survival and time to progression). Future studies on the efficacy of docetaxel as a second line agent should serve to address issues like the optimal dose regimen and intensity as well as adjust for potential confounders.

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Reply: Higher Intensity Does Not Necessary Yield Better Survival in Second-Line Chemotherapy for NSCLC

To the Editor:

We would like to thank Singh et al. for suggesting that the dose of docetaxel and previous treatment modality may have an impact on second-line therapy in non-small cell lung cancer (NSCLC). Herein, we discuss the dose of docetaxel and the influence of previous chemotherapy in relation to second-line treatment of NSCLC.

In second-line chemotherapy for NSCLC, whether a higher dose of an anticancer agent would inevitably yield a longer survival is open to question. In a study comparing docetaxel 100 mg/m², docetaxel 75 mg/m² and best supportive care, the overall survivals were 5.9, 7.5, and 7.0 months, respectively.¹ Docetaxel 100 mg/m² was also found to be inferior to docetaxel 75 mg/m² in terms of the 1-year survival rate in another phase III study.² A similar tendency was also observed for another agent in the second-line setting; pemetrexed 500 mg/m² and 900 mg/m² were compared, and the overall median survivals were 6.7 and 6.9 months, respectively, and the hazard ratio was 1.013 (95% confidence interval, 0.837-1.226).³ Even the response rate in the 900 mg/m² arm did not exceed that in the 500 mg/m². Thus, finding the optimal dose of docetaxel or other agents for second-line chemotherapy may be an intriguing issue.⁴

Meanwhile, docetaxel 60 mg/m² is the standard therapeutic dose in Japan, since a Japanese phase I trial determined the maximum tolerated dose to be 70 mg/m².⁵ Even though this dose of docetaxel is lesser than that used in other countries,

this may be the optimal dose for Japanese. In a phase II study of docetaxel for previously untreated NSCLC conducted in Japan, the response rate to docetaxel 60 mg/m² was 19%, no less than that to the higher doses used in other countries.⁶ A retrospective study evaluating docetaxel 60 mg/m² for previously treated NSCLC also showed a response rate of 18.5%, comparable with that reported for higher doses.⁷ This difference in the dose requirement in Japanese may be attributed to ethnic differences between the Japanese and other populations, but the issue remains under debate.

The previously employed treatment modality differed between those who had received a combination of carboplatin and paclitaxel (group P) and those who had received a combination of a platinum and an agent other than paclitaxel [group nonpaclitaxel (NP)] in our study. We consider, however, that this difference had only a small impact on our study results, for three reasons. Firstly, all the patients in our study had metastatic disease at the time of recurrence and start of docetaxel therapy. Secondly, although 29% of patients in group NP had received radiotherapy, the response rate to the previous treatment in group NP was the same as that in group P (45.0 versus 44.9%, respectively). In general, the response rate to chemoradiotherapy is higher than that to chemotherapy alone. This difference may have disappeared in our study, probably because we only recruited patients who developed recurrence after chemoradiotherapy. Finally, no previous studies of second-line chemotherapy for NSCLC have dealt with these issues. Even though multiple modalities may have been used in previous treatment, we can only evaluate the integrated result of the treatment. It is impossible to distinguish between the efficacy of chemotherapy and radiotherapy if both are undertaken simultaneously.

In conclusion, further investigation of the optimal dose of chemotherapeutic agents for second-line chemotherapy of NSCLC is warranted. The efficacy of previous chemotherapy, whether or not administered in combination with radiotherapy, is a useful reference for subsequent docetaxel therapy.

Disclosure: The authors declare no conflicts of interest.

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Tracheo-Esophageal Fistula with Bevacizumab after Mediastinal Radiation

To the Editor:

We report here a case of a young man who developed a trachea-esophageal

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fistula 4 months following thoracic radiation while being treated with bevacizumab and chemotherapy. A 28-year-old gentleman was diagnosed with non-small cell lung cancer (NSCLC) when he presented with a large right sided mediastinal mass. Transbronchial biopsy results were consistent with adenocarcinoma. Staging evaluation with computerized tomography, flourodeoxyglucose positron emission tomography, and mediastinoscopy confirmed stage IIIB (T2N2M0) disease. He was treated with definitive radiation (74 gray) and concurrent cisplatin with etoposide. One month after completing radiotherapy, he developed progressive disease with enlargement of cervical lymph nodes. Biopsy of a cervical lymph node was consistent with adenocarcinoma. Two months after radiotherapy had been completed, he began systemic treatment with carboplatin, paclitaxel, and bevacizumab (15 mg/kg) every 3 weeks. After two cycles, he had a partial response.

One week prior to his third cycle, he developed progressive odynophagia, then severe coughing with swallowing. An endobronchial evaluation was performed with visualization of a fistulous communication between the esophagus and the trachea, extending into the right mainstem bronchus. An endotracheal stent was placed, but after 2 weeks he had no relief of his respiratory symptoms and was referred to our institution. Bronchoscopy revealed a persistent tracheoesophageal fistula which was not excluded by the endotracheal stent. This endotracheal stent was removed and the fistula was visualized as seen in Figure 1A. At that time, a covered esophageal stent

(18-mm diameter, 120-mm length, Alveolus) was placed in the esophagus to exclude gastric and oral secretions from the airway (Figure 1B). Biopsies of the fistulous tract showed no evidence of malignancy. As the computed tomography scan of the chest and abdomen revealed progressive disease in the mediastinum and liver, an attempt at surgical correction was not considered appropriate. A jejunal feeding tube was placed for nutrition, and he was discharged home with supportive care.

Bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF), has been approved for the treatment of advanced NSCLC in combination with paclitaxel and carboplatin.^{1,2} Bevacizumab has been associated with bleeding complications, hypertension and gastrointestinal tract perforation.² When administered in combination with thoracic radiation, bevacizumab has recently been associated with tracheo-esophageal fistulas. The manufacturer issued a warning based on the development of tracheo-esophageal fistulas in 3 of 29 patients with limited stage small cell lung cancer being treated with definitive radiation, concurrent with irinotecan, carboplatin, and bevacizumab. Data from the manufacturer (as of March 2007) refer to six other instances in which patients with lung and esophageal malignancies developed tracheo-esophageal fistulas while being treated with bevacizumab.³ A black box warning regarding this complication was mandated by the Food and Drug Administration in April 2007;² however, no such reports are available at this time

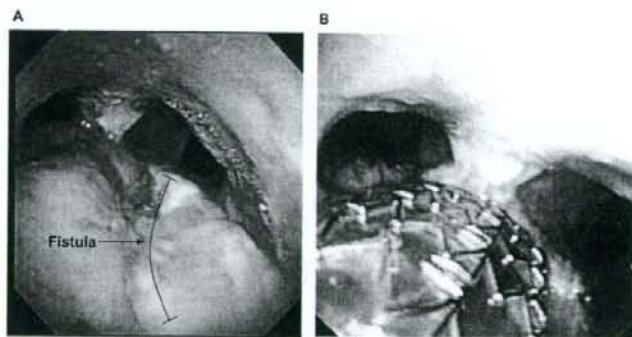


FIGURE 1. A, Tracheo-esophageal fistula in patient treated with bevacizumab. B, Coated stent in the esophagus, as visualized through the large posterior airway defect.

Prospective Study of the Accuracy of *EGFR* Mutational Analysis by High-Resolution Melting Analysis in Small Samples Obtained from Patients with Non-Small Cell Lung Cancer

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Abstract Purpose: Epidermal growth factor receptor (*EGFR*) mutations, especially in-frame deletions in exon 19 (DEL) and a point mutation in exon 21 (L858R), predict gefitinib sensitivity in patients with non-small cell lung cancer (NSCLC). In this study, we verified the accuracy of *EGFR* mutation analysis in small samples by high-resolution melting analysis (HRMA), which is a rapid method using PCR amplification with a dye to analyze the melting curves in NSCLC.

Experimental Design: We designed a prospective study to compare the sensitivity and specificity of HRMA and DNA sequencing with laser capture microdissection. Eligible patients with lung lesions were screened by bronchoscopy or percutaneous needle biopsy to histologically confirm the diagnosis, followed by surgical resection of the NSCLC. Small diagnostic specimens were analyzed for *EGFR* mutations by HRMA, and the surgically resected specimens were examined for mutations by HRMA and DNA sequencing.

Results: The analyses for *EGFR* mutations were conducted in 52 eligible cases of the 92 enrolled patients. *EGFR* mutations were detected in 18 (34.6%) patients. The results of HRMA from surgically resected specimens as well as DNA sequencing revealed 100% sensitivity and specificity. On the other hand, the sensitivity and specificity of HRMA from the small diagnostic specimens were 83.3% and 100%, respectively.

Conclusions: In this study, we showed that HRMA is a highly accurate method for detecting DEL and L858R mutations in patients with NSCLC, although it is necessary to consider the identification of patients with a false-negative result when the analysis is conducted using small samples.

Somatic mutations in the kinase domain of the epidermal growth factor receptor (*EGFR*) have been reported in patients with non-small cell lung cancer (NSCLC; refs. 1–3). Although many types of *EGFR* mutations have been identified, they seem to be concentrated in exons 18 to 21 of *EGFR*; ~85% to

90% of *EGFR*-mutant patients have mutations in two hotspots: a short in-frame deletion in exon 19 (DEL) and a point mutation at codon 858 in exon 21 (L858R; ref. 4). Several studies have revealed that *EGFR* mutations are strongly associated with the tumor response and clinical outcome in patients with NSCLC receiving treatment with *EGFR* tyrosine kinase inhibitors, such as gefitinib (Iressa, AstraZeneca; refs. 5–7). The mutational status of *EGFR*, especially the presence/absence of DEL and L858R, is a strong predictor of the sensitivity to *EGFR* tyrosine kinase inhibitor, and the detection of *EGFR* mutations is useful for decision-making by both patients and physicians (4, 8). Recently, a laboratory test for *EGFR* mutations has become clinically available for guiding treatment decisions.

Until now, screening for these mutations has most commonly been conducted using DNA sequencing methods. In our previous study, we used methanol-fixed, paraffin-embedded surgical specimens and performed direct sequencing and pyrosequencing with laser capture microdissection (LCM) to ensure high-quality genetic analysis of archived tissues (5, 9). However, these approaches are not useful in clinical practice for two reasons. First, although the sequencing methods require a high ratio of tumor-to-normal tissue DNA for optimal results, the diagnostic specimens obtained from cases of advanced NSCLC may contain only a small amount of tumor cells and

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are highly contaminated with normal cells. Secondly, *EGFR* mutation analysis based on DNA sequencing requires special instruments and is also time-consuming and expensive. Therefore, some simple and highly sensitive nonsequencing methods to detect *EGFR* mutations have been reported (10–22). However, the accuracy of these methods for clinical use have not been assessed in prospective studies.

High-resolution melting analysis (HRMA) using the LCGreen I (Idaho Technology) dye was introduced as an easy, quick, and inexpensive method for the screening of mutations (23), and we established and validated the HRMA method to detect DEL and L858R mutations in cases of NSCLC (9, 10). Our cell line study revealed that DEL and L858R mutations could be detected using HRMA in the presence of 10% and 0.1% of mutant cells, respectively (10). We also showed that the two major mutations could be identified by HRMA retrospectively using DNA extracted from archived Papanicolaou-stained cytologic slides with 88% sensitivity and 100% specificity (9). Furthermore, it was shown that among patients treated with gefitinib, the response rate (78% versus 8%), time-to-progression (median, 9.2 versus 1.6 months), and overall survival (median, 21.7 versus 8.7 months) were significantly better in patients with *EGFR* mutations than with wild-type *EGFR* ($P < 0.001$), as detected by HRMA (9). These results suggest that this easy, quick, and inexpensive method which was done using diagnostic small samples of advanced NSCLC tumors is one of the most useful and precise methods to detect *EGFR* mutations in clinical practice.

In this study, we designed a prospective study to detect two major *EGFR* mutations by HRMA using small diagnostic cytologic or biopsy specimens and surgically resected specimens, and the results were compared with the results of DNA sequencing methods combined with LCM, which we consider as the "gold standard" for such detection, applied to methanol-fixed, paraffin-embedded surgically resected specimens. We evaluated the diagnostic sensitivity, specificity, predictive values, and accuracy of the detection of *EGFR* mutations using HRMA and revealed that this method is feasible for clinical use to detect *EGFR* mutations in small samples obtained from patients with NSCLC.

Patients and Methods

Patients and materials. Patients with lung lesions, which were suspected clinically to be operable NSCLC, were enrolled in this prospective study. The patients were scheduled for bronchoscopy or percutaneous needle biopsy to establish the histologic diagnosis, and informed consent was obtained from each of the patients prior to these diagnostic procedures. Thereafter, the patients diagnosed with NSCLC underwent lung surgery at our hospital. In this study, mutational analysis of *EGFR* was done by HRMA or DNA sequencing methods combined with LCM in all the patients in which both the preoperatively obtained diagnostic specimens and the resected specimens were histologically confirmed by a certified pathologist to contain malignant cells.

Based on a protocol approved by the Institutional Review Board of the National Cancer Center, we did mutational analyses of *EGFR* to detect DEL and L858R in the eligible patients. The Papanicolaou-stained cytologic slides ($n = 35$), formalin-fixed, paraffin-embedded transbronchial or percutaneous needle biopsy specimens ($n = 34$), and methanol-fixed, paraffin-embedded surgically resected specimens subjected to LCM using a PixCell II LCM system (Arcturus Engineering,

Inc.; $n = 52$) were collected prospectively. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen), as described in our previous report (10).

HRMA. PCR was done to amplify exons 19 or 21 of *EGFR* using LCGreen I (Idaho Technology) on a LightCycler (Roche Diagnostics) and primers designed as previously described (10). If the first PCR products were not available for the mutational analyses of the melting curves, we did a second PCR using the same primers. These PCR products were denatured at 95°C for 10 min and cooled to 40°C to promote the formation of heteroduplexes. The LightCycler capillary was transferred to an HR-1 (Idaho Technology), an HRMA instrument, and heated at a transition rate of 0.3°C/s. Data were acquired and analyzed using the accompanying software (Idaho Technology). After normalization and temperature-adjustment steps, melting curve shapes from 78.5°C to 85.5°C were compared between the tumor samples and control samples. Human Genomic DNA (Roche Diagnostics) was used as the negative control sample with wild-type *EGFR*. Samples revealing skewed or left-shifted curves as compared with the control samples were judged to have mutations without positive controls (9, 10). All analyses were done in a blinded fashion by two researchers (T. Fukui and T. Takano). After independent evaluation by the two researchers, the final judgment was arrived at by consensus after joint viewing of the melting curves from both.

DNA sequencing methods with LCM. In our previous study, we did a direct sequencing or pyrosequencing of *EGFR* in patients with recurrent NSCLC after primary surgery (5). Based on the results of our previous study, we consider direct sequencing with LCM for the detection of DEL and pyrosequencing with LCM for the detection of L858R as the gold standard in relation to *EGFR* mutational analysis. DNA was extracted from methanol-fixed, paraffin-embedded surgical specimens by LCM, according to a previously described method (24). Direct sequencing of the PCR products for DEL was done using ABI PRISM3700 and 3100 DNA sequencers (Applied Biosystems). Pyrosequencing to analyze L858R was done using Pyrosequencing PSQ 96MA (Pyrosequencing; refs. 5, 25). The *EGFR* mutational analysis using DNA sequencing methods was done in a blinded fashion by a researcher (H. Sakamoto) according to a previously described method (5), and then compared with the corresponding results obtained using HRMA.

Statistical analysis. The primary end point of this study was the sensitivity and specificity of the results obtained using HRMA as compared with those of the results obtained using DNA sequencing with LCM. The sample size was calculated using a statistical power level of 0.80 and two-sided α level of 0.1 on the basis of an estimated sensitivity of at least 0.80 and an expected value of 0.95 for HRMA, a minimum of 20 patients with *EGFR*-mutated tumors were required. Because the percentage of NSCLC patients with *EGFR* mutations was expected to be 40% in this study population composed of only Japanese, approximately 50 patients with NSCLC were needed. Therefore, considering a specificity of at least 0.80 and the expected value of 0.95 for HRMA, 30 patients with wild-type tumors showed a statistical power level of 0.90 using a two-sided α level of 0.1.

The associations between mutational status and patient characteristics were assessed by a χ^2 test using the SPSS statistical package (SPSS version 11.0 for Windows; SPCC, Inc.).

Results

Patient characteristics. From December 2005 to December 2006, 92 patients with clinically suspected operable NSCLC were enrolled in this study. The following diagnostic procedures were done preoperatively in 90 patients: bronchoscopy ($n = 57$), percutaneous needle biopsy ($n = 27$), or bronchoscopy followed by percutaneous needle biopsy ($n = 6$). The patient characteristics are shown in Table 1. All the patients were Japanese. Among the patients, a definitive diagnosis was established in 85 patients by bronchoscopy in 43 of 59 patients

Table 1. Patient characteristics**(A) Characteristics of all the patients enrolled in this study (n = 92)**

	All (n = 92)	BF (n = 64)	PNB (n = 34)*
Age, year, median (range)	64 (34-84)	64 (38-84)	62 (41-79)
Gender (male/female)	58/34	41/23	23/11
Smoking history (N/F/C)	29/30/33	23/19/22	7/14/13
Tumor size, mm, average (range)	27.2 (10.2-73.4)	28.3 (13.8-56.6)	24.5 (10.2-73.4)
Accuracy of the diagnostic procedure (%)	66/85 (77.6)	43/59 (72.9)	25/31 (80.6)
Accuracy of the cytologic slides (%)	54/85 (63.5)	31/59 (52.5)	23/30 (76.7)
Accuracy of the biopsy specimens (%)	42/62 (67.7)	35/54 (64.8)	7/9 (77.8)

(B) Characteristics of the patients who underwent analysis of the EGFR mutations in this study (n = 52)

	All (n = 52)	BF (n = 38)	PNB (n = 17) [†]
Age, year, median (range)	64.5 (34-84)	64.5 (34-84)	64 (47-78)
Gender (male/female)	36/16	25/13	14/3
Smoking history (N/F/C)	16/17/19	15/11/12	1/7/9
Tumor size, mm, average (range)	27.0 (11.0-56.6)	28.3 (20.6-56.6)	24.1 (11.0-48.8)
Postoperative diagnosis (Ad/Sq/LCNEC)	45/5/2	34/4/0	12/3/2
Pathologic stage (IA/B, IIA/B, IIIA/B)	19/13, 3/5, 9/2	15/8, 3/2, 8/2	7/5, 0/2, 3/0

NOTE: Never smokers were defined as patients who had never smoked, former smokers were defined as patients who had stopped smoking at least 1 y before the diagnosis, and current smokers were defined as patients who were still smoking at the time of the diagnosis.

Abbreviations: BF, bronchoscopy; PNB, percutaneous needle biopsy; N, never smoker; F, former smoker; C, current smoker; Ad, adenocarcinoma; Sq, squamous cell carcinoma; LCNEC, large cell neuroendocrine carcinoma.

*Including six patients in whom bronchoscopy was done followed by percutaneous needle biopsy.

[†]Including three in whom bronchoscopy was done followed by percutaneous needle biopsy.

(72.9%) and by percutaneous needle biopsy in 25 of 31 patients (80.6%); in 18 of the 85 (21.2%) patients, the histologic diagnosis could not be established preoperatively by bronchoscopy and/or percutaneous needle biopsy, the patients underwent lung surgery for suspicious malignant lung lesion, and examination of the resected specimens revealed the diagnosis of primary NSCLC in 17 and malignant lymphoma in 1 of the 18 patients. Among the 76 patients diagnosed to

have primary NSCLC, 73 consented to undergo lung surgery. Finally, the analysis for EGFR mutations was done on 52 patients with a definitive histologic diagnosis of primary NSCLC, established both by examination of the preoperative diagnostic specimens and of the corresponding resected specimens (Fig. 1).

Mutational analyses. We analyzed 35 cytologic samples and 34 biopsy specimens obtained from 52 patients by HRMA, and

Fig. 1. Flowchart of the analyses conducted in 92 enrolled patients with lung tumors in this study.

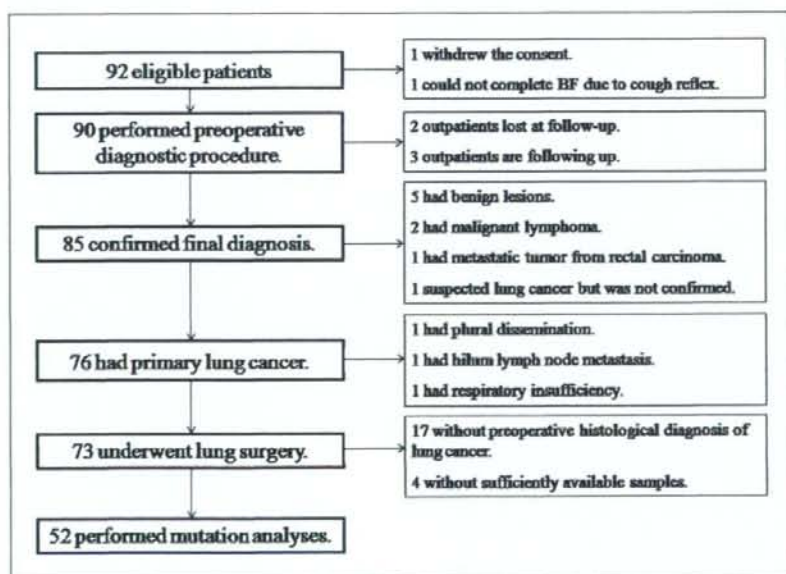


Table 2. EGFR mutation status among the patient subgroups

	n	EGFR mutations*			%	P
		DEL	L858R	Total		
Total	52	5	13	18	34.6	—
Gender						
Women	16	2	9	11	68.8	0.001
Men	36	3	4	7	19.4	
Smoking history						
Never	16	3	8	11	68.8	0.001 [†]
Former	17	2	4	6	35.3	
Current	19	0	1	1	5.3	
Histology						
Ad	44	5	13	18	100	0.025 [‡]
Sq	6	0	0	0	0	
LCNEC	2	0	0	0	0	

Abbreviations: DEL, deletional mutations in exon 19; L858R, a point mutation at codon 858 in exon 21; Ad, adenocarcinoma; Sq, squamous cell carcinoma; LCNEC, large cell neuroendocrine carcinoma.

*The EGFR mutations were analyzed by DNA sequencing with LCM.

[†]Comparison between never smokers and others.

[‡]Comparison between adenocarcinoma and others.

did both HRMA and DNA sequencing with LCM in the 52 resected specimens corresponding to the 52 patients. Among the 52 surgically resected specimens analyzed by DNA sequencing with LCM, there were 18 (34.6%) samples with EGFR mutations, 5 with DEL mutations, and 13 with L858R mutations. As shown in Table 2, the EGFR mutations were detected more frequently in women, never-smokers, and patients with a histologic diagnosis of adenocarcinoma. All results from HRMA done in a blinded fashion by two researchers (T. Fukui and T. Takano) were consistent.

HRMA could be conducted using small diagnostic samples from all 52 patients, although the analysis needed to be conducted using the second PCR product in 15 cases. In the analysis of exon 19, 5 samples revealed different curves from the control and 47 samples revealed almost the same curves as the control; therefore, we judged that the five former patients had DEL mutations (Fig. 2A). In the analysis of exon 21, 10 samples revealed a left-shift from the control and 42 samples revealed almost the same curves as the control; therefore, we judged that the 10 former patients had L858R mutations

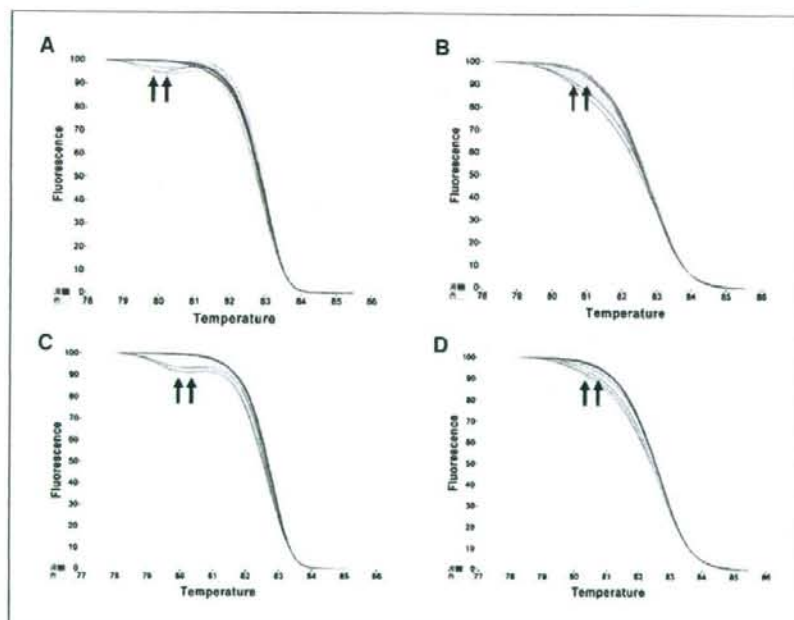


Fig. 2. Adjusted melting curves obtained by HRMA of the samples in this study to detect EGFR mutations (↑), in-frame deletions in exon 19 (A, small samples; C, resected specimens) and a point mutation in exon 21 (B, small samples; D, resected specimens). Each sample that revealed a skewed or left-shifted curve from those of the control sample was judged to have a mutation.

Table 3. Results of the EGFR mutation analyses in patients with EGFR mutation-positive tumors

No. of patients	Small samples	Surgically resected specimens	
	HRMA	HRMA	Sequence with LCM
13	DEL	DEL	DEL1*
26	DEL	DEL	DEL1*
32	DEL	DEL	DEL2†
40	DEL	DEL	DEL2†
47	DEL	DEL	DEL1*
5	L858R‡	L858R	L858R
6	Wild-type	L858R	L858R
12	L858R	L858R	L858R
18	L858R	L858R	L858R
21	L858R	L858R	L858R
23	L858R‡	L858R	L858R
25	Wild-type	L858R	L858R
27	L858R‡	L858R	L858R
28	L858R	L858R	L858R
31	Wild-type‡	L858R	L858R
41	L858R‡	L858R	L858R
53	L858R	L858R	L858R
54	L858R‡	L858R	L858R

Abbreviations: DEL, deletional mutations in exon 19; L858R, a point mutation at codon 858 in exon 21.

*DEL1: del E746-A750 (del 2235-2249).

†DEL2: del E746-A750 (del 2236-2250).

‡The analyses by HRMA were done using second PCR products.

(Fig. 2B). All the 52 surgically resected specimens analyzed by DNA sequencing with LCM could also be analyzed by HRMA, although the analysis needed to be conducted using the second PCR product in two cases. DEL mutations were detected in 5 patients (Fig. 2C) and L858R mutations in 13 patients (Fig. 2D) among the 52 patients. Of the 52 specimens, both cytologic slides and biopsy specimens were analyzed in 17 cases. Discrepant results were obtained by HRMA in one of the cases, with L858R mutation being detected in the cytologic slides but not in the biopsy specimens. We included this patient in the population with L858R mutations.

The results of HRMA were consistent with the results of DNA sequencing with LCM in all the surgically resected specimens analyzed by the two methods. On the other hand, HRMA using small diagnostic specimens revealed the wild-type curve in three cases, although analysis of the corresponding surgically resected specimens analyzed by pyrosequencing with LCM revealed the L858R mutation (Table 3). Thus, the results for these samples obtained by HRMA were considered as false-negative results. Neither method of analysis yielded any false-positive cases. The results of the EGFR mutational analysis by HRMA compared with DNA sequencing with LCM using surgically resected specimens were shown in Table 4. The sensitivity, specificity, and accuracy of HRMA using small diagnostic specimens were 83.3%, 100%, and 94.2%, respectively. Using surgically resected specimens, those of HRMA were all 100%.

Discussion

In this prospective study, we showed the high accuracy of the HRMA method for detecting two major EGFR mutations, DEL

and L858R in patients with NSCLC. The accuracy of HRMA was clearly equal to that of DNA sequencing with LCM for the detection of mutations in surgically resected specimens. On the other hand, the sensitivity and specificity of HRMA were 83.3% (90% confidence interval: 68.9-97.7%) and 100%, respectively, when the small diagnostic samples were analyzed. Although the sensitivity of HRMA which was estimated to be at least 0.80 did not reach statistical significance, we consider HRMA as one of the available methods for the detection of EGFR mutations in clinical practice because the specificity, which is important for clinical decision-making, of HRMA was 100% and the EGFR mutation rate was less than the expected 40% to secure enough statistical power in this study.

Recently, many researchers reported establishing simple and highly sensitive nonsequencing methods for detecting EGFR mutations using small tumor samples (11-22), and the results of several mutation analyses were correlated with the clinical outcome of EGFR tyrosine kinase inhibitor treatment (17-19). Using serial dilution studies, some researchers have reported methods that are able to detect mutations in samples containing ~0.1% to 10% mutated DNA (13, 14, 16-18, 20-22), as opposed to direct DNA sequencing which requires the presence of at least 10% to 30% of mutated DNA in the samples (18, 20). Additionally, several novel methods offered higher sensitivity and specificity than DNA sequencing to identify the mutations in clinical samples. But almost none of the methods were validated for diagnostic accuracy in a prospective study, and we therefore consider these methods to still be unsuitable for routine clinical examination. Although these nonsequencing methods were not mutually compared, based on our previous results of retrospectively verifying the accuracy of HRMA (9, 10), we thought to develop in this prospective study an easy, quick (PCR for ~1 hour and HRMA for 2 to 3 minutes), and inexpensive (at a running cost per sample of approximately \$7.50, which consisted of \$5.50 for the DNA extract and less than \$2.00 for PCR using LCGreen I dye) method that might be useful in clinical practice with a great advantage over DNA sequencing, which requires the

Table 4. Comparison of the sensitivity, specificity, predictive values, and accuracy between HRMA and DNA sequencing with LCM (n = 52)

	HRMA using small samples	HRMA using surgically resected specimens
True-positive	15	18
True-negative	34	34
False-positive	0	0
False-negative	3	0
Sensitivity	83.3 (68.9-97.8)	100
Specificity	100	100
NPV	91.9 (84.5-99.3)	100
PPV	100	100
Accuracy	94.2 (88.9-99.5)	100

NOTE: The results of these analyses were compared with those of DNA sequencing with LCM (used as the gold standard in this study). Data are presented as % or % (90% confidence interval). True-positive is defined as the correct detection of DEL in exon 19 or L858R in exon 21. Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

Table 5. Results of HRMA using cytologic slides or biopsy specimens

	Cytologic slides (n = 35)		Biopsy specimens (n = 34)	
	First PCR	Second PCR	First PCR	Second PCR
Successfully analyzed	29 (83.0%)	35 (100%)	5 (15.0%)	34 (100%)
True-positive	7	11	1	10
True-negative	19	21	4	22
False-positive	0	0	0	0
False-negative	3	3	0	2
Sensitivity	70.0% (7/10)	78.6% (11/14)	100% (1/1)	83.3% (10/12)
Specificity	100% (19/19)	100% (21/21)	100% (4/4)	100% (22/22)
NPV	100% (7/7)	100% (11/11)	100% (1/1)	100% (10/10)
PPV	86.4% (19/22)	87.5% (21/24)	100% (4/4)	91.2% (22/24)
Accuracy	89.7% (26/29)	91.4% (32/35)	100% (5/5)	94.1% (32/34)

NOTE: The results of these analyses were compared with those of DNA sequencing with LCM (used as the gold standard in this study). True-positive is defined as the correct detection of DEL in exon 19 or L858R in exon 21. Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

extraction of high-quality DNA from an adequate amount of pure tumor cells, takes a long time, and is expensive.

In this study, the three patients with L858R detected by DNA pyrosequencing with LCM using the surgically resected specimens were labeled as having the wild-type *EGFR* in the analyses conducted using the small diagnostic samples. With regard to these false-negative results, the following three points need to be discussed: first, our previous study, conducted using human lung cancer cell lines, showed that HRMA can detect the mutations, even when samples contain only a small proportion (DEL, 10%; L858R, 0.1%) of mutant cells (10). In this study, the sensitivity of HRMA was also considered to be sufficiently high for the detection of *EGFR* mutations, especially L858R, even when the analysis was conducted using small samples after evaluation by a clinical pathologist to determine if they contained benign or malignant cells. Thus, we assume a higher accuracy of HRMA when using small samples in clinical practice. Although it still needs to be comparatively analyzed with the previously reported non-sequencing methods, HRMA can be considered as one of the sensitive methods available for the detection to *EGFR* mutations in clinical practice.

Second, high-quality DNA should be preserved in clinical samples to obtain the best results. There always remains the risk of an indeterminate or false-negative result because the DNA might have degenerated during sampling or during the preservation of clinical samples. In a comparison between the cytologic slides and biopsy specimens, better results were obtained from analyses of the first PCR products using the cytologic slides rather than the results obtained using the biopsy specimens, regardless of the amount of tumor cells examined (Table 5). This could probably be explained by the differences in the method of sample fixation between the two types of specimens. It has been suggested by a previous report that DNA is preserved better in the methanol-fixed samples than in the formalin-fixed specimens (26). Therefore, if we used methanol for specimen fixation of biopsy specimens, the results of HRMA using the first PCR products from small biopsy samples might improve. Hereafter, we propose to perform mutation analyses using methanol-fixed specimens, if possible.

Finally, we need to consider the possibility of intratumoral heterogeneity, and small diagnostic samples and surgically resected specimens may each represent overlapping but different populations of these tumor cells. A lack of association in the immunohistochemical expression profile between lung biopsy specimens and the corresponding resected tumor specimens has been reported (27). Furthermore, intratumoral heterogeneity was shown not only in terms of microheterogeneity of the tumor cell phenotype (28), but in terms of genetic heterogeneity in cancer (29, 30). In particular, the intratumoral genetic heterogeneity of *EGFR* mutations may explain the variable clinical response of NSCLC to gefitinib. It is also possible that the small diagnostic samples contain only wild-type cells, even if the tumor, overall, shows mutations, because the small samples yield only small part of the tumor. It is always necessary to consider the possibility of a false-negative result of mutational analyses conducted using the small samples.

In the current prospective study, we showed the feasibility and high accuracy of using HRMA for detecting two major *EGFR* mutations, DEL and L858R, in patients with NSCLC. Although HRMA showed high accuracy, the possibility of indeterminate or false-negative results, and because of the sensitivity of this method, the quality of DNA preservation in the clinical samples or intratumoral genetic heterogeneity, must be borne in mind to a certain extent when this analysis is conducted using small diagnostic samples. Therefore, HRMA should not be used to exclude patients from *EGFR* tyrosine kinase inhibitor treatment on the basis of the negative results only. Based on the results of this prospective study, we suggest that this method is very useful for clinical decision-making, especially in patients with a positive result.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Influence of Previous Chemotherapy on the Efficacy of Subsequent Docetaxel Therapy in Advanced Non-small Cell Lung Cancer Patients

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Purpose: To identify factors, particularly the previous use of paclitaxel, that might influence the efficacy of subsequent docetaxel therapy.

Patients and Methods: The patient characteristics, responses, and survivals were compared between the two groups that had received a combination of carboplatin and paclitaxel (group P), and a combination of a platinum and an agent other than paclitaxel (group NP).

Results: A total of 227 patients (127 in group P, and 100 in group NP) were recruited from a hospital-based registry. Two hundred twenty patients were evaluated for the survival, and 210 patients were evaluated for the response of docetaxel therapy. The response rate to docetaxel therapy (14.2% versus 16.0%, $p = 0.702$) or the median survival time (10.9 months versus 11.1 month, $p = 0.567$) did not differ between groups P and NP. The results of multivariate analysis, adjusted for sex, age, and performance status at the start of docetaxel therapy, showed that not the regimen per se, but the response to previous chemotherapy significantly influenced the response rate of docetaxel therapy (odds ratio [OR]: 1.38, 95% confidential interval [CI]: 0.63–3.01; and OR: 2.93, 95% CI: 1.28–6.72, respectively). As for the overall survival, neither the response to nor the previous chemotherapy regimen had any impact (hazard ratio [HR]: 0.90, 95% CI 0.66–1.22; HR 0.88, 95% CI 0.65–1.20, respectively).

Conclusion: The previous use of paclitaxel had no impact on the response or survival to subsequent docetaxel therapy. In contrast, the response to previous chemotherapy had a predictive value in relation to responses to subsequent docetaxel therapy in patients with advanced non-small cell lung cancer.

Key Words: Non-small cell lung cancer, Second-line chemotherapy, Docetaxel.

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Lung cancer is a leading cause of cancer-related deaths worldwide.¹ Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer. For chemotherapy-naïve patients with advanced NSCLC, with a good performance status (PS), platinum-based chemotherapy has been shown to offer a modest survival benefit over best supportive care alone.^{2,3} A high proportion of patients, however, shows disease relapse after initial clinical responses, or progress during the chemotherapy. Thus, a large percentage of patients is moved on to second-line chemotherapy, even though it should only be considered in selected patients with a good PS.⁴

In the landmark study by Shepherd et al., second-line docetaxel therapy was demonstrated to improve the outcome over best supportive care alone in patients with a history of previous chemotherapy.⁵ Since then, a number of agents have been introduced as effective agents for the second-line setting^{6–8}; however, the impact of previous chemotherapy on the efficacy of subsequent chemotherapy has not been established.

In relation to small-cell lung cancer, the response of tumors to first-line therapy and recurrence more than 3 months after completion of the initial therapy is often referred to as “sensitive relapse,” and absence of tumor response, tumor progression through treatment, or tumor recurrence within 3 months of discontinuation of initial therapy is termed “refractory” disease. Although both are grouped together in most second-line clinical trials, their prognosis and response to salvage therapy have been shown to be different.^{9,10} Therefore, in patients with small-cell lung cancer, the efficacy of previous chemotherapy has a significant impact on selection of the subsequent chemotherapy. Whether this relationship between first- and second-line chemotherapy would also apply to cases of NSCLC has not yet been clarified.

In this study, we attempted to identify factors, particularly the previous use of paclitaxel, that might influence the response to subsequent docetaxel therapy in patients with NSCLC. Towards this objective, we divided our patients into two groups according to the previous regimen received.

PATIENTS AND METHODS

We evaluated the patients with histologically or cytologically proven unresectable locally advanced or metastatic