Evaluation of the epidermal growth factor receptor gene mutation and copy number in non-small cell lung cancer with gefitinib therapy

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Abstract. Several studies have suggested that epidermal growth factor receptor (EGFR) gene mutation, EGFR gene amplification, and some other biomarkers may be predictors of gefitinib sensitivity. We analyzed EGFR mutation and EGFR copy number in 22 gefitinib-treated non-small cell lung cancer (NSCLC) cases and their relation to the survival of patients. We also studied 143 gefitinib-naïve Japanese NSCLC cases. The ErbB2 copy number was also studied in 59 gefitinib-naïve NSCLC cases. In gefitinib-treated patients, the presence of EGFR mutation was associated with a higher response rate to gefitinib and a longer overall survival, but the increased EGFR gene copy number was not. In gefitinib-naïve cases, EGFR mutation but not EGFR gene copy number was significantly correlated with gender, pathological subtypes, and smoking status. The ErbB2 copy number was not significantly correlated with the EGFR mutation or EGFR copy number in 59 cases. In conclusion, EGFR mutation was a better predictor of clinical outcome in gefitinib-treated patients than the EGFR gene copy number.

Introduction

Lung cancer has a high mortality rate in many developed countries. Gefitinib inhibits tyrosine kinase (TK) activity of EGFR by reversibly competing with ATP at the ATP-binding cleft within the EGFR protein. Good clinical responses have been observed most frequently in women, in nonsmokers, in patients with adenocarcinomas, and in Japanese patients (1-4). Unfortunately, the addition of gefitinib to traditional chemotherapy did not add any benefit to patient survival for advanced NSCLC (3), although overexpression of EGFR protein was seen in relatively high frequencies (5). We and others have shown that the somatic mutation in the TK domain of the EGFR is associated with the sensitivity of NSCLC to gefitinib (6-8). The most frequently reported EGFR mutations are either deletion or single amino acid substitutions in exon 18, 19, or 21 clustered around the ATP-binding pocket of the TK domain. *In vitro*, EGFR mutation has been reported to confer enhanced TK activity in response to the epidermal growth factor (EGF) and increased sensitivity to the inhibition by gefitinib (6,7,9,10). Thus, it is highly likely that EGFR mutation is a critical determinant of the patient's response to gefitinib.

Other biomarkers of NSCLC have also been studied. Some studies reported that downstream signaling molecules (particularly, the Akt signaling pathway), EGFR gene amplification, and the expression of the other ErbB receptors were the other predictors of gefitinib sensitivity. The EGFR and chromosome 7 copy numbers in NSCLC were assessed using fluorescence *in situ* hybridization (FISH), and more than 3 EGFR copies per cell (balanced polysomy or gene amplification) were detected in 39 (22%) of 183 patients (11). A correlation between an increased EGFR copy number and gefitinib sensitivity was also proposed in another study from USA (12). Determining the EGFR gene mutation status and EGFR gene amplification may bring important information whether gefitinib is a therapeutic option for Japanese NSCLC patients. Therefore, we evaluated EGFR mutation and copy number as predictors of gefitinib sensitivity in patients with NSCLC in this study.

Patients and methods

Patients and genomic DNA. NSCLC tissues were obtained at Nagoya City University Hospital by surgical excision from 22 patients who were subsequently treated with gefitinib (Iressa®, Astra Zeneca, London, UK) for their recurrent disease. The EGFR mutation and EGFR copy number were analyzed in these 22 gefitinib-treated samples. We also analyzed 143 additional gefitinib-naïve NSCLC cases operated between 1997 and 2000 at Nagoya City University Hospital. We evaluated the ErbB2 copy number in 59 of the additional 143 gefitinib-naïve NSCLC cases. The research

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was approved by the Institutional Review Board of the hospital. All the patients consented to the use of their tissues for the present analysis. The tissues were placed in liquid nitrogen immediately after resection or were formalin-fixed and paraffin-embedded. Genomic DNA was extracted using the Wizard SV Genomic DNA purification system (Promega) according to the manufacturer's instructions.

Analysis of EGFR mutation. Twenty-two gefitinib-treated samples and the additional 143 gefitinib-naïve NSCLC samples were analyzed by the TaqMan PCR assay (14) and direct sequencing (13). The primers and TaqMan® MGB probes were designed with Primer Express 2.0 software (Applied Biosystems). We designed 13 sets of specific TaqMan probes (14). TaqMan PCR and genotyping analysis were performed on 7500 Real Time PCR system (Applied Biosystems) according to the manufacturer's instructions.

Analysis of EGFR and ErbB2 copy number. EGFR copy number was analyzed for the 22 gefitinib-treated patients and for the additional 143 gefitinib-naïve NSCLC patients by quantitative real-time PCR, performed on 7500 Real Time PCR System using a QuantiTect SYBR-Green kit (Qiagen, Inc., Valencia, CA) as previously described (14). The ErbB2 copy number was also analyzed for the 59 gefitinib-naïve NSCLC patients by quantitative real-time PCR. An increased EGFR and an ErbB2 copy number was defined as more than three copies.

Clinical evaluation of the response to gefitinib. Tumor size was determined by computed tomographic (CT) scan of the chest before treatment. Tumor response was assessed by a CT scan 4 weeks after treatment. A complete response (CR) was defined as the disappearance of all clinical and radiological evidence of tumor for >4 weeks. A partial response (PR) required a >50% reduction in the sum of the products of the perpendicular diameters of all measurable lesions for at least 4 weeks. Progressive disease (PD) was defined as the appearance of an unequivocal new lesion or an increase of >25% in the sum of the products of the perpendicular diameters of any measured lesions. No change (NC) or stable disease was a change insufficient for PR or PD for at least 4 weeks after the start of therapy (15). Responders were defined as patients with CR or PR, and non-responders were defined as those with NC or PD. As patients with recurrent lung cancer often do not have measurable disease, we also included a change in the serum carcinoembryonic antigen (CEA) level (cut-off, 5 ng/ml) as an evaluation criterion to avoid underestimating the effectiveness of gefitinib. When the elevated CEA decreased to a level less than half of the baseline level or within normal limit, gefitinib treatment was judged as effective or as partial response (PR) (16). Overall survival (OS) was calculated as the time from the start of gefitinib administration to death from any cause or last contact.

Statistical analysis. For comparison of proportions, the Fisher's Exact test was used. The overall survival was examined by the Kaplan-Meier method, and differences were examined by the log-rank test. The association of risk factors associated with survival was evaluated using the Cox proportional hazards regression model. Only those variables with significant results in univariate analysis were included in the multivariate analysis. The analyses were done using a Stat View (version 5, SAS Institute Inc., Cary, NC) software and considered significant at p-value <0.05.

Table I. Relationship between the EGFR copy number and clinicopathological factors in 22 gefitinib-treated patients.

Factors	EGFR gene		P-value
	Copy number ≥3	Copy number <3	
Gender			
Male	4 (30.8%)	9	0.1150
Female	0 (0%)	9	
Age			
≤64	2 (20%)	8	>0.9999
>64	2 (16.7%)	10	
Smoking status			
Never-smokers	0 (0%)	4	0.5055
Ever-smokers	3 (30%)	7	
Differentiation			
Well	2 (13.3%)	13	>0.9999
Moderately or poorly	1 (16.7%)	5	
Pathological subtypes			
Adeno	4 (22.2%)	14	0.5538
Non-adeno	0 (0%)	4	

Adeno, adenocarcinoma.

Results

EGFR mutation status in 22 gefitinib-treated patients. Using direct sequencing or the TaqMan PCR assay, 11 cases (50%) were detected with an EGFR mutation. Four patients had deletion in exon 19, 5 patients had L858R mutation, one patient had G719S mutation, and one patient had a novel insertion in exon 20 (2316-2317 insertion GGT, P772_H773 ins V). The relationship between EGFR mutation and clinicopathological factors in 22 gefitinib-treated patients was evaluated. EGFR mutation was found in 5/13 (38.5%) of males and 6/9 (66.7%) of females; 6/10 (60%) of patients who were ≤64 years old and 5/12 (41.7%) of patients who were >64 years old; 11/18 (61.1%) of adenocarcinoma and 0/4 (0%) of non-adenocarcinoma; 3/4 (75%) of never-smokers and 2/10 (20%) of ever-smokers; 9/15 (60%) of well-differentiated and 2/6 (33.3%) of moderately or poorly differentiated NSCLC. None of the trends shown above reached statistical significance probably due to small sample size.

EGFR copy number in 22 gefitinib-treated patients. The EGFR copy number of 22 samples from patients who were

Table II. Relationships among EGFR mutation, the EGFR copy number and gefitinib sensitivity.

EGFR gene	Responders		Non-responders		Responders/ total patients	Response rates (%)
	CR	PR	NC	PD		
Mutant	0	9	1	1	9/11	81.8[a]
Wild-type	0	2	4	5	2/11	18.2[a]
Copy number ≥3	0	1	0	3	1/4	25[b]
Copy number <3	0	10	5	3	10/18	55.6[b]
Total	0	11	5	6	11/22	50

[a]p=0.0089; [b]p=0.5865 (Fisher's exact test); CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

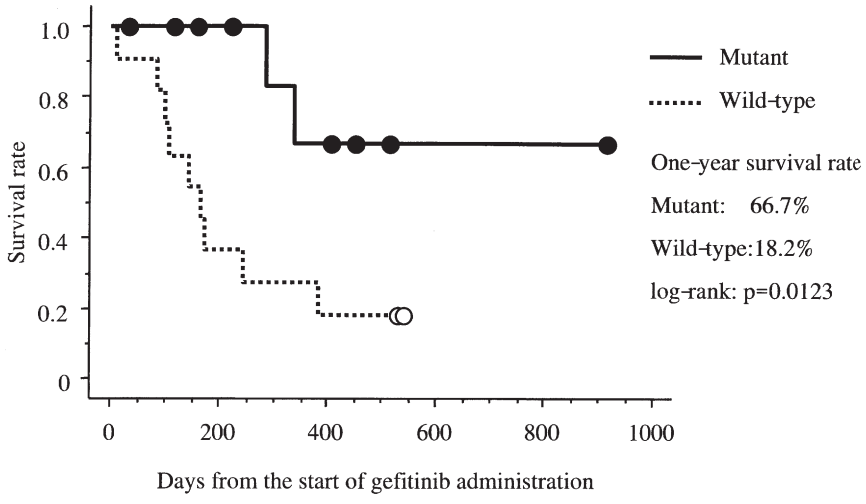

Figure 1. Kaplan-Meier curves of overall survival according to EGFR mutation in 22 gefitinib-treated patients. One-year survival rate of the patients with EGFR mutation was 66.7% vs 18.2% of the patients with wild-type EGFR. Patients with EGFR mutation had a statistically significantly longer OS compared with those with wild-type EGFR (log-rank p=0.0123).

treated with gefitinib was analyzed by quantitative real-time PCR. Four of 22 cases were found to have an increased EGFR copy number. Relationship between EGFR copy number and clinicopathological factors in 22 gefitinib-treated patients is shown in Table I. An increased EGFR copy number was found in 4/13 (30.8%) of males and 0/9 (0.0%) of females; 2/10 (20%) of patients who were ≤64 years old and 2/12 (16.7%) of those who were >64 years old; 4/18 (22.2%) of adenocarcinoma and 0/4 (0%) of non-adenocarcinoma; 0/4 (0%) of never-smokers and 3/10 (30%) of ever-smokers; 2/15 (13.3%) of well-differentiated and 1/6 (16.7%) of moderately or poorly differentiated NSCLC. The EGFR copy number was not significantly correlated with any of clinicopathological factors. In the 11 cases with EGFR mutation in this cohort, only one case had an increased EGFR copy number. The EGFR copy number did not correlate with the EGFR mutation status (p=0.5865).

Gefitinib sensitivity in 22 gefitinib-treated patients. Relationship between gefitinib sensitivity and clinicopathological factors in 22 gefitinib-treated patients was evaluated. Responders (CR or PR) to gefitinib were 5/13 (38.5%) of males and 6/9 (66.7%) of females; 5/10 (50%) of patients who were ≤64 years old and 6/12 (50%) of those who were >64 years old; 10/18 (55.6%) of adenocarcinoma and 1/4 (25%) of non-adenocarcinoma; 3/4 (75%) of never-smokers and 3/10 (30%) of ever-smokers; 9/15 (60%) of well-differentiated and 2/6 (33.3%) of moderately or poorly differentiated NSCLC. There was no significant correlation between the response to gefitinib and these clinicopathological factors.

Relationships among EGFR mutation, the EGFR copy number, gefitinib sensitivity and clinical outcome. Relationships among EGFR mutation, the EGFR copy number and gefitinib sensitivity are shown in Table II. The response rates of patients with mutant and wild-type EGFR were 81.8% and 18.2%, respectively (p=0.0089). Two patients with EGFR mutation were non-responders; one patient was judged as NC and one patient had PD. The patient with PD had a novel exon 20 insertion (P772_H773 insV). The Kaplan-Meier curves of OS according to EGFR mutation in

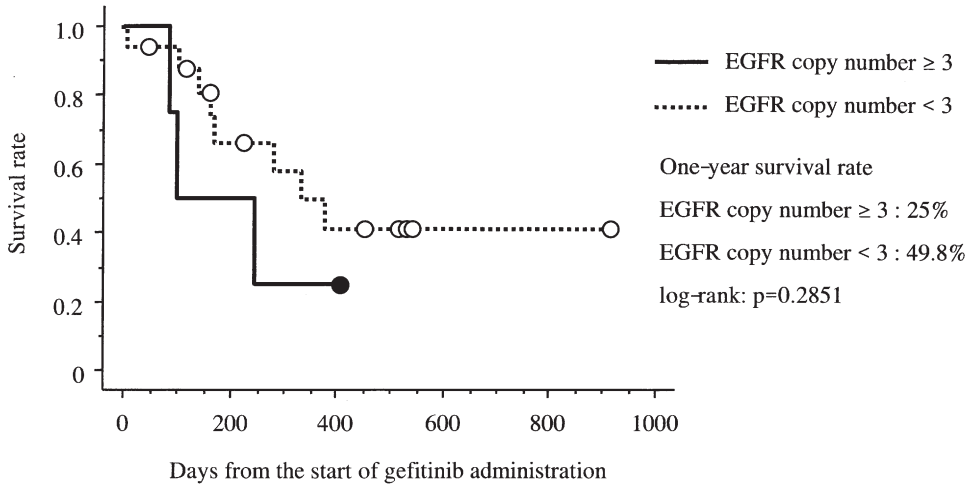

Figure 2. Kaplan-Meier curves of overall survival according to the EGFR copy number in 22 gefitinib-treated patients. One-year survival rate of the patients with an increased EGFR copy number was 25% vs 49.8% of the patients without an increased EGFR copy number. The difference did not reach statistical significance (log-rank p=0.2851).

Table III. Univariate and multivariate analyses of association between clinicopathological factors and overall survival in patients with gefitinib therapy.

	Univariate	Multivariate
Risk factors	P-value	P-value
Gender (male vs female)	0.0084	0.5949
Age (≤64 years vs >64 years)	0.9135	-
Pathological sybtypes (adeno vs non-adeno)	0.0124	0.3525
Smoking status (never-smokers vs ever-smokers)	0.0094	0.6570
Differentiation (well vs moderately or poorly)	0.0250	0.9748
EGFR mutation (mutant vs wild-type)	0.0123	0.3680
EGFR copy number (<3 vs ≥3)	0.2851	-
Gefitinib response (responders vs non-responders)	0.0453	0.1758

Adeno, adenocarcinoma.

22 gefitinib-treated patients are shown in Fig. 1. One-year survival rate of the patients with EGFR mutation was 66.7% vs 18.2% of the patients with wild-type EGFR. Patients with EGFR mutation had a statistically significantly longer OS compared with those with wild-type EGFR (log-rank p=0.0123). The response rates of patients with an increased EGFR copy number (EGFR copy number ≥3) and without an increased EGFR copy number (EGFR copy number <3) were 25% and 55.6%, respectively (p=0.5865). Three patients with an increased EGFR copy number were non-responders; all three patients had PD. The Kaplan-Meier curves of OS according to EGFR copy number in 22 gefitinib-treated patients are shown in Fig. 2. One-year survival rate of the patients with an increased EGFR copy number was 25% vs 49.8% of the patients without an increased EGFR copy number. However, the difference did not reach statistical significance (log-rank p=0.2851). The presence of EGFR mutation was significantly associated with a higher response rate and a longer OS. However, the association of an increased EGFR copy number and lower response rate or a shorter OS was not statistically significant, possibly due to the small number of patients with an increased EGFR copy number. To define which variables were predictive for survival, those factors that were statistically significant in the univariate analysis were included in the multivariate analysis. Univariate and multivariate analyses of association between clinicopathological factors and overall survival in patients with gefitinib therapy are shown in Table III. In the univariate analyses, OS was significantly associated with gender (male vs female, p=0.0084), pathological subtypes (adenocarcinoma vs non-adenocarcinoma, p=0.0124), smoking status (never-smokers vs ever-smokers, p=0.0094), differentiation (well differentiated vs moderately or poorly differentiated, p=0.0250), EGFR mutation (mutant vs wild-

Table IV. Relationship between EGFR mutation and clinicopathological factors in the additional 143 gefitinib-naïve NSCLC patients.

Factors	EGFR gene		P-value
	Mutant	Wild-type	
Gender			
Male	12 (11%)	97	<0.0001
Female	19 (55.9%)	15	
Age			
≤64	16 (24.6%)	49	0.5416
>64	15 (19.2%)	63	
Smoking status			
Never-smokers	17 (48.6%)	18	<0.0001
Ever-smokers	12 (12.4%)	83	
Lymph node metastasis			
N0	21 (23.3%)	69	0.6749
N+	10 (18.9%)	43	
Differentiation			
Well	18 (29%)	44	0.1020
Moderately or poorly	12 (16.9%)	59	
Pathological subtypes			
Adeno	27 (31%)	60	0.0007
Non-adeno	4 (7.1%)	52	
pStage			
I	17 (27%)	46	0.2230
II-IV	14 (17.5%)	66	

N+, lymph node metastasis-positive; adeno, adenocarcinoma.

Table V. Relationship between the EGFR copy number and clinicopathological factors in the additional 143 gefitinib-naïve NSCLC patients.

Factors	EGFR gene		P-value
	Copy number ≥3	Copy number <3	
Gender			
Male	9 (8.2%)	100	0.4519
Female	1 (2.9%)	33	
Age			
≤64	8 (12.3%)	57	0.0433
>64	2 (2.6%)	76	
Smoking status			
Never-smokers	3 (8.6%)	32	0.7014
Ever-smokers	6 (6.3%)	89	
Lymph node metastasis			
N0	8 (8.9%)	82	0.3230
N+	2 (3.8%)	51	
Differentiation			
Well	3 (4.8%)	59	0.3365
Moderately or poorly	7 (9.9%)	64	
Pathological subtypes			
Adeno	4 (4.6%)	83	0.1899
Non-adeno	6 (10.7%)	50	
pStage			
I	5 (7.9%)	58	0.7494
II-IV	5 (6.3%)	75	

N+, lymph node metastasis-positive; adeno, adenocarcinoma.

type, p=0.0123), and gefitinib response (responders vs non-responders, p=0.0453), but not the EGFR copy number (p=0.2851). However, in the multivariate analyses, none of these was a significant prognostic factor, including the EGFR mutation (p=0.3680).

EGFR mutation status in the additional 143 gefitinib-naïve NCSLC patients. EGFR mutation was analyzed for the additional 143 gefitinib-naïve NSCLC patients who were not treated by gefitinib using direct sequencing or the TaqMan PCR assay. Thirty-one mutations were detected; 18 patients had the deletion in exon 19, 11 patients had L858R mutation, one patient had G719S mutation, and one patient had a novel insertion mutation in exon 20 (P772_H773 ins V). The relationship between EGFR mutation and clinicopathological factors in the additional 143 gefitinib-naïve patients is shown in Table IV. EGFR mutation status was significantly correlated with gender (male vs female, p<0.0001), pathological subtypes (adenocarcinoma vs non-adenocarcinoma, p=0.0007), and smoking status (never-smokers vs ever-smokers, p<0.0001). Patients with EGFR mutation did not have a statistically significantly longer OS compared with those with wild-type EGFR (log-rank p=0.9787, data not shown).

EGFR copy number in the additional 143 gefitinib-naïve NCSLC patients. The EGFR copy number was analyzed for the additional 143 gefitinib-naïve NSCLC patients by quantitative real-time PCR. Ten of the 143 cases (7.0%) were found to have an increased EGFR copy number. The relationship between the EGFR copy number and clinicopathological factors in the additional 143 gefitinib-naïve NSCLC patients is shown in Table V. An increased EGFR copy number was found in 9/109 (8.2%) of males and 1/34 (2.9%) of females; 8/65 (12.3%) of those who were ≤64 years old and 2/78 (2.6%) of those who were >64 years old; 4/87 (4.6%) of adenocarcinoma and 6/56 (10.7%) of non-

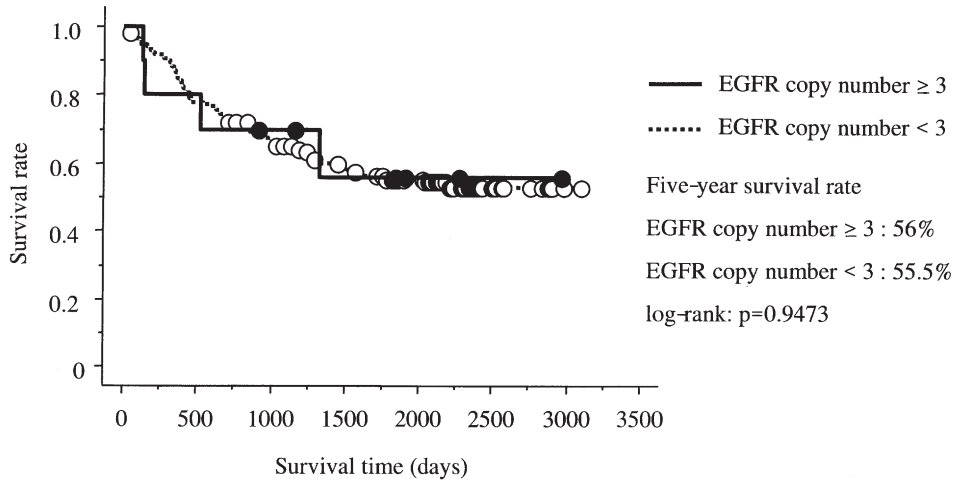

Figure 3. Kaplan-Meier curves of overall survival according to the EGFR copy number in the additional 143 gefitinib-naïve NSCLC patients. Survival time was calculated as the time from operation to death from any cause or last contact. Five-year survival rate of the patients with an increased EGFR copy number was 56% vs 55.5% of the patients without an increased EGFR copy number. The difference did not reach statistical significance (log-rank p=0.9473).

Table VI. Relationship between the ErbB2 copy number and clinicopathological factors in 59 gefitinib-naïve NSCLC patients.

Factors	ErbB2 gene		P-value
	Copy number ≥3	Copy number <3	
Gender			
Male	7 (16.3%)	36	>0.9999
Female	2 (12.5%)	14	
Age			
≤64	5 (17.9%)	23	0.7231
>64	4 (12.9%)	27	
Smoking status			
Never-smokers	2 (11.1%)	16	0.7080
Ever-smokers	7 (17.1%)	34	
Differentiation			
Well	4 (13.3%)	26	0.7220
Moderately or poorly	5 (18.5%)	22	
Pathological subtypes			
Adeno	8 (20.0%)	32	0.2473
Non-adeno	1 (5.3%)	18	

Adeno, adenocarcinoma.

adenocarcinoma; 3/35 (8.6%) of never-smokers and 6/95 (6.3%) of ever-smokers; 8/90 (8.9%) of those with no lymph node metastasis and 2/53 (3.8%) of those with lymph node metastasis; 3/62 (4.8%) of well differentiated and 7/71 (9.9%) of moderately or poorly differentiated NSCLC; 5/63 (7.9%) of pStage I and 5/80 (6.3%) of pStage II-IV patients. The EGFR copy number was significantly correlated with age (≤64 years vs >64 years, p=0.0433), but not with any other clinicopathologic factor. In the 31 cases with EGFR mutation in this cohort, only two cases had an increased EGFR copy number. The EGFR copy number did not correlate with EGFR mutation status (p>0.9999). The Kaplan-Meier curves of OS according to EGFR copy number in the additional 143 gefitinib-naïve NSCLC patients are shown in Fig. 3. The five-year survival rate of the patients with an increased EGFR copy number was 56% vs 55.5% of the patients without an increased EGFR copy number. The difference did not reach statistical significance (log-rank p=0.9473).

ErbB2 copy number in 59 gefitinib-naïve NCSLC patients. In the 59 gefitinib-naïve NSCLC samples, ErbB2 copy number was analyzed. The relationship between the ErbB2 copy number and the clinicopathologic factors in the 59 gefitinib-naïve NSCLC patients is shown in Table VI. An increased ErbB2 copy number was found in 7/43 (16.3%) of males and 2/16 (12.5%) of females; 5/28 (17.9%) of those who were ≤64 years old and 4/31 (12.9%) of those who were >64 years old; 8/40 (20%) of adenocarcinoma and 1/19 (5.3%) of non-adenocarcinoma; 2/18 (11.1%) of never-smokers and 7/41 (17.1%) of ever-smokers; 4/30 (13.3%) of well-differentiated and 5/27 (18.5%) of moderately or poorly differentiated NSCLC. The ErbB2 copy number was not significantly correlated with any of clinicopathological factors. The Kaplan-Meier curves of OS according to ErbB2 copy number in the 59 gefitinib-naïve NSCLC patients are shown in Fig. 4. The five-year survival rate of the patients with an increased ErbB2 copy number was 63.5% vs 55.1% of the patients without an increased ErbB2 copy number. The difference did not reach statistical significance (log-rank p=0.4133).

Relationships among EGFR copy number, the ErbB2 copy number and EGFR mutation. In the 59 gefitinib-naïve

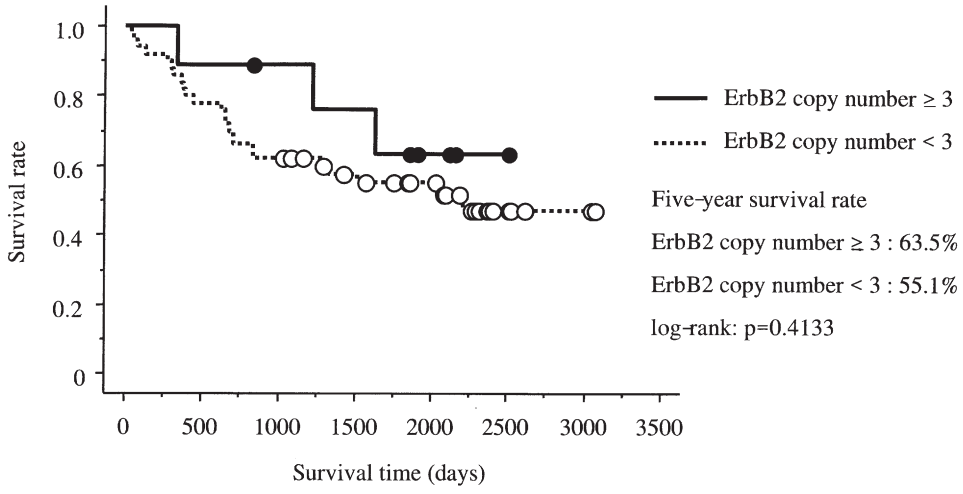

Figure 4. Kaplan-Meier curves of overall survival according to the ErbB2 copy number in 59 gefitinib-naïve NSCLC patients. Survival time was calculated as the time from operation to death from any cause or last contact. Five-year survival rate of the patients with an increased ErbB2 copy number was 63.5% vs 55.1% of the patients without an increased ErbB2 copy number. The difference did not reach statistical significance (log-rank p=0.4133).

NSCLC patients, 16 EGFR mutations were detected. Five of the 59 cases were found to have an increased EGFR copy number and nine of 59 cases were found to have an increased ErbB2 copy number. Only one patient had both an increased ErbB2 copy number and an EGFR mutation. Only one patient had both increased ErbB2 copy number and increased EGFR copy number. The ErbB2 copy number was not significantly correlated with the EGFR mutation (p=0.4206) or the EGFR copy number (p=0.5768).

Discussion

In our study, EGFR mutation, but not the EGFR copy number, correlated with survival of patients treated with gefitinib. Gefitinib showed a promising effect on a few types of cancer in a phase I trial (1). Subsequently, however, in phase II randomized trials in which the drug was used in combination with traditional chemotherapy, the effect was marginal in patients with NSCLC (7). Our group and others have reported on identification of genetic mutations in the EGFR kinase domain (6,7). EGFR mutation was seen in a subset of NSCLC with a good response to gefitinib. These reports triggered further studies on EGFR mutation and the tumor's response to gefitinib and erlotinib (6-8). All the groups identified recurrent mutations in the same region around the ATP-binding pocket in the EGFR tyrosine kinase domain. *In vitro* studies have reported that the kinase activity of EGFR or the sensitivity to gefitinib showed a strong association with EGFR gene mutation (7,9). In our analysis, the response rates of patients with mutant and wild-type EGFR were 81.8% and 18.2%, respectively (p=0.0089). The presence of EGFR mutation was significantly associated with a higher response rate. Nine of 11 (81.8%) gefitinib-responders had an EGFR mutation and only two of the 11 (18.2%) patients with wild-type EGFR were gefitinib-responders. The presence of two gefitinib-responding tumors with wild-type EGFR may be because i) the EGFR mutation analysis was not sensitive enough, ii) the EGFR mutation occurred in the tumors after the primary surgery (whose sample we analyzed) or iii) other factors of gefitinib sensitivity were present (17). There was a statistically significantly correlation between EGFR mutation and gefitinib sensitivity or overall survival in our analysis and from the analysis of the other groups (16,18-20). Thus, these results demonstrated that EGFR mutation was a critical determinant of gefitinib sensitivity, at least in the Japanese population. One EGFR-mutant patient was resistant to gefitinib therapy. This patient had a novel insertion at exon 20 (P772_H773 ins V). Several recent reports show that exon 20 mutations, such as T790M and D770_N771 ins NPG, are correlated with gefitinib resistance (21,22).

In this report, the response rates to gefitinib of patients with an increased EGFR copy number and without an increased EGFR copy number were 25% and 49.8%, respectively (p=0.2851). One of 11 (9.1%) of gefitinib-responders and 3/11 (27.3%) of gefitinib non-responders had an increased EGFR copy number. Ten of 18 (55.6%) patients without an increased EGFR copy number were gefitinib-responders. Some studies reported that an increased EGFR copy number was associated with gefitinib sensitivity. For example, Hirsch *et al* (11) reported that patients with an increased EGFR gene copy number had a trend for higher response rates and a longer time to progression after gefitinib therapy, and Cappuzzo *et al* (12) reported that a high EGFR gene copy number was associated with a better response and better survival. However, there was no statistically significant correlation between an increased EGFR copy number and gefitinib sensitivity or overall survival in our analysis and we were unable to show any differences in increased EGFR copy number between tumors carrying the wild-type EGFR sequence and tumors carrying the mutant EGFR sequence, which is not surprising as it has been convincingly shown that EGFR mutation and not the expression levels is responsible for the clinical response to EGFR tyrosine kinase inhibitors (6,7,23). The difference between the previously published studies and ours might be caused by the difference in ethnicity; Caucasian or Oriental. Thus, whether an increased EGFR copy number is a

predictor of gefitinib sensitivity, remains to be determined. The results of multivariate analyses suggested that none of clinicopathological factors, including EGFR mutation was an independent predictor of prognosis. Further studies will be needed to delineate the relationships among EGFR mutation, EGFR gene copy number, EGFR mRNA expression, gefitinib sensitivity, and prognosis.

In our analysis of the additional 143 gefitinib-naïve cases, EGFR mutation status was significantly correlated with gender, pathological subtypes, and smoking status. This result confirmed and extended the results of previous reports (6,8,9,24). The EGFR copy number was significantly correlated with age only, and did not significantly affect the OS in the additional 143 gefitinib-naïve cases.

Overexpression of EGFR/ErbB2 and ErbB ligands was correlated with advanced disease and poor patient prognosis (25). The EGFR-ErbB2 heterodimers were associated with a stronger and more sustained proliferative signal than the EGFR homodimers (26,27). HER2 mutations were in-frame insertions in exon 20 and were significantly more frequent in never smokers and adenocarcinoma histology (28). Thus, some studies have reported on ErbB2 gene expression and mutation, but very few studies have reported on the association between the ErbB2 gene copy number and lung cancer. Cappuzzo *et al* (29) reported that HER2 gene gain was significantly associated with EGFR gene gain and with EGFR gene mutations, and that EGFR-positive patients who also had increased copy numbers of the HER2 gene had a better response rate, disease control rate, TTP, and survival. Because analysis of the ErbB2 copy number was not performed in gefitinib-treated patients, we were not able to show the relationship between the ErbB2 gene copy number and gefitinib sensitivity. However, we have shown that the ErbB2 copy number was not significantly correlated with the EGFR mutation and EGFR copy number in the 59 gefitinib-naïve NSCLC cases.

In summary, EGFR mutation was significantly associated with a better clinical outcome in gefitinib-treated patients, but an increased EGFR gene copy number was not significantly associated with a better clinical outcome in gefitinib-treated patients and the additional 143 gefitinib-naïve NSCLC cases. The populations with EGFR mutation may be different from the populations with an increased EGFR copy number and the effect of an increased EGFR copy number to gefitinib response may be different from that of EGFR mutation. However, for another TK inhibitor erlotinib (Tarceva®, Genetech), because the dose setting was different from gefitinib therapy, EGFR amplification may still be a better predictor of erlotinib sensitivity for lung cancers (30). In most studies, analysis of the EGFR copy number was performed by FISH or quantitative real-time PCR. FISH analysis was relatively expensive and was usually performed on one or few of the sliced sections. On the other hand, quantitative real-time PCR method is inexpensive, relatively easy, and efficient. Therefore, we adopted the latter method. Although the methods suited for the analysis of the EGFR copy number cannot be determined, standardization of the EGFR copy number analysis is required to evaluate the EGFR copy number accurately.

Further studies are required to evaluate the predictive values of some biomarkers other than EGFR mutation and the EGFR copy number, and future clinical trials may need to incorporate several predictive biomarkers in molecular targeted therapy including gefitinib and erlotinib.

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