J Cancer Res Clin Oncol (2008) 134:569–577
DOI 10.1007/s00432-007-0320-z

ORIGINAL PAPER

Epidermal growth factor receptor gene amplification and gefitinib sensitivity in patients with recurrent lung cancer

Hidefumi Sasaki · Katsuhiko Endo · Katsuhiro Okuda · Osamu Kawano · Naoto Kitahara · Hisaichi Tanaka · Akihide Matsumura · Keiji Iuchi · Minoru Takada · Masaaki Kawahara · Tomoya Kawaguchi · Haruhiro Yukiue · Tomoki Yokoyama · Motoki Yano · Yoshitaka Fujii

Received: 1 August 2007 / Accepted: 21 September 2007 / Published online: 12 October 2007

Abstract To evaluate the epidermal growth factor receptor (EGFR) protein expression, gene mutations and amplification as predictors of clinical outcome in patients with non-small-cell lung cancer (NSCLC) receiving gefitinib, we have performed fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC). We investigated the *EGFR* amplification and EGFR protein expression statuses in 27 surgically treated non-small-cell lung cancer (NSCLC) cases. These patients experienced relapse after surgery and received gefitinib 250 mg/day. The presence or absence of *EGFR* mutations of kinase domains was analyzed by genotyping analysis and sequences, and already reported. *EGFR* mutations were found from 15/27 lung cancer patients. *EGFR* mutation status was significantly correlated with better prognosis (log-rank test $P = 0.0023$). Smoking status (never smoker vs. smoker, $P = 0.0032$), and pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, $P = 0.0011$), but not *EGFR* amplification ($P = 0.1278$), were correlated with survival of lung cancers. EGFR IHC results were correlated with FISH results ($P = 0.0125$), but not correlated with prognosis ($P = 0.7921$). Thus, the *EGFR* gene amplification or protein expression is not a predictor of gefitinib efficacy in Japanese patients with NSCLC. We have also evaluated the *EGFR* mutation status and clinico-pathological features for 27 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. The *EGFR* mutation status, especially exon19 mutation was correlated with good response to gefitinib than exon 21 point mutation.

Keywords *EGFR* · Lung cancer · Mutations · Amplification · Exon19

H. Sasaki (✉) · K. Endo · K. Okuda · O. Kawano · H. Yukiue · T. Yokoyama · M. Yano · Y. Fujii
Department of Surgery II,
Nagoya City University Medical School,
1 Kawasumi, Mizuho-cho, Mizuho-ku,
Nagoya 467-8601, Japan
e-mail: hisasaki@med.nagoya-cu.ac.jp

N. Kitahara · H. Tanaka · A. Matsumura · K. Iuchi
Department of Surgery,
Kinki-chuo Chest Medical Center, Sakai, Japan

M. Takada · M. Kawahara · T. Kawaguchi
Department of Internal Medicine,
Kinki-chuo Chest Medical Center, Sakai, Japan

Introduction

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy (Ginsberg et al. 1993). There are much accumulated evidences that epidermal growth factor receptor (*EGFR*) and its family member are strongly implicated in the development and progression of numerous human tumors, including lung cancer (Nicolson et al. 2001; Onn et al. 2004). The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of non-small-cell lung cancer (NSCLC) since 2002. Phase II and III trials have shown partial responses in 8–12% of unselected patients with progressive NSCLC after chemotherapy (Kris et al. 2003; Thatcher et al. 2005), especially higher response in never smokers, females and Asian ethnicity (more than 20%) (Fukuoka et al. 2003; Miller et al. 2004). Two original reports showed that *EGFR* mutations status at ATP binding pockets in NSCLC patients was correlated with the clinico-pathological features related

to good response to gefitinib (Paez et al. 2004; Lynch et al. 2004). These *EGFR* mutations were predominantly found in Japanese lung cancer patients (about 25–40%) (Paez et al. 2004; Kosaka et al. 2004; Shigematsu et al. 2005; Tokumo et al. 2005; Endo et al. 2005) when compared to USA patients (about 8–10%) (Paez et al. 2004; Lynch et al. 2004; Shigematsu et al. 2005; Pao et al. 2004) or European patients (Shigematsu et al. 2005; Marchetti et al. 2005). Actually, *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy (Paez et al. 2004; Lynch et al. 2004; Pao et al. 2004; Mitsudomi et al. 2005). On the other hands, Cappuzzo et al. (2005) reported that *EGFR* amplification by fluorescence in situ hybridization (FISH) and high *EGFR* protein expression has been associated with responsiveness to gefitinib. Takano et al. (2005) showed that both *EGFR* gene mutation and increased copy numbers predicted gefitinib sensitivity in patients with recurrent NSCLC. However, this Japanese report is based on polymerase chain reaction (PCR) assay.

To determine the *EGFR* amplification and *EGFR* mutation statuses and correlation with clinico-pathological features in Japanese gefitinib-treated lung carcinoma, we retrospectively performed FISH and immunohistochemistry. The findings were compared to the clinico-pathologic features of lung cancer.

Materials and methods

Patients and samples

This was a retrospective study and the study group included 27 lung cancer patients who were treated with gefitinib for their recurrent diseases after they had undergone surgery at the Department of Surgery II, Nagoya City University Medical School. Written informed consent was obtained and the institutional ethics committee of the Nagoya City University Medical School approved the study. We have also investigated *EGFR* mutation status for 27 NSCLC patients who were treated with gefitinib for their recurrent diseases after they had undergone surgery at the National Hospital Organization, Kinki-chuo Chest Medical Center (Endo et al. 2005). The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan. All tumor samples were immediately frozen and stored at −80°C until assayed. The clinical and pathological characteristics of the 27 lung cancer patients are as follows; 14 (67.7%) were male and 13 were female. Twenty-two (63%) were diagnosed as adenocarcinoma, and five were diagnosed as other types of carcinoma. Fourteen (52%) were never smokers and 13 were smokers.

PCR assays for *EGFR* and *K-ras* mutations

Genomic DNA was extracted using Wizard SV Genomic DNA purification Systems (Promega) according to the manufacturers' instructions. The primers and TaqMan MGB probe were designed with Primer Express 2.0 software (Applied Biosystems). The sequences of 13 allele-specific probes and primer sets used in the TaqMan PCR assay are already shown (Endo et al. 2005). The results of TaqMan PCR assays were already reported (Endo et al. 2005). *K-ras* codon 12/13 mutation status was investigated by direct sequencing using the primers reported by Krypuy et al. (2006). Total RNA was extracted from the lung cancer tissues using Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturers' instructions. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD, USA) with 0.5 μg oligo $(dT)_{12-16}$ (Amersham Pharmacia Biotech Inc. Piscataway, NJ, USA). The direct sequencing for *EGFR* genes was performed from genomic DNA (Paez et al. 2004) or cDNA (Sasaki et al. 2006). Some cases were genotyped using LightCycler (Sasaki et al. 2005) and confirmed.

FISH analysis

Tumor specimens were obtained at surgical operation and embedded in paraffin. Serial sections (6 μm) containing representative malignant cell were stained with hematoxylin and eosin. Gene copy number per cell was investigated by FISH using the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Vysis, Abbott laboratories, IL, USA) according to a published protocol (Hirsch et al. 2003). Sections were incubated at 56°C overnight, deparaffinized and dehydrated. After incubation in 2× saline sodium citrate buffer (2× SSC; pH 7.0) at 75°C for 15–25 min, sections were digested with protein K (0.25 mg/ml in 2× SSC; pH 7.0) at 37°C for 15–25 min, rinsed in 2× SSC at room temperature for 5 min, and dehydrated using ethanol in a series of increasing concentrations. The EGFR/CEP 7 probe set was applied per the manufacture's instructions onto the selected area based on the presence of tumor foci on each slide. The slides were incubated at 80°C for 8–10 min for codenaturation of chromosomal and probe DNA and then placed in a humidified chamber at 37°C for 20–24 h to allow hybridization to occur. Post hybridization washes were performed in 1.5 M urea and 0.1× SSC at 45°C for 30 min and in 2× SSC for 2 min at room temperature. Pathologist who was blinded to the patients' clinical characteristics and all other molecular variables performed FISH analysis independently. Patients were classified according to the Cappuzzo et al. (2005) criteria with ascending number of copies of the *EGFR* gene

per cell and the frequency of tumor cells with specific number of copies of the *EGFR* gene and chromosome 7 centromere: high polysomy (≥4 copies in ≥40% of cells) and gene amplification (defined by presence of tight *EGFR* gene clusters and a ratio of *EGFR* gene to chromosome of ≥2 or ≥copies of *EGFR* per cell in ≥10% of analyzed cells) were considered as FISH positive. Disomy (≤2 copies in >90% of cells); low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10–40% of cells, 4 ≥copies in <10% of cells); high trisomy (≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells) and low polysomy (≥4 copies in 10–40% of cells) were considered as FISH negative.

Immunohistochemistry

EGFR protein expression was evaluated by immunohistochemistry using the mouse anti-human EGFR, clone 2-18C9 monoclonal antibody (Dako NorthAmerica, Inc., Via Real, Carpinteria, CA, USA). Four micrometer sections were made from paraffin tissue blocks from lung tumors. The slides were treated with xylenes, and then dehydrated in alcohol. After treated with proteinase K for 5 min, endogenous peroxidase was blocked with Peroxidase (H_2O_2) Block. After washed with Wash Buffer (Dako NorthAmerica Inc., USA), the slides were incubated with the monoclonal antibody against EGFR (ready-to use) for 30 min at room temperature. Labeled Polymer, HRP (30 min) and 3,3-diaminobenzidine (DAB) substrate (10 min) were used to visualize the antibody binding, and the sections were counterstained with hematoxylin. The intensity score was defined according to Cappuzzo et al. (2005); 1 = barely detectable, 2 = readily appreciable brown staining, 3 = dark brown staining, 4 = very strong staining. The total score was calculated by multiplying the intensity score and the fraction score (positive cells; 0–100%). Scores of 201–400 were considered positive.

Statistical methods

Statistical analyses were done using the Mann–Whitney *U* test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between the variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and χ^2 test. The overall survival of lung cancer patients was examined by the Kaplan–Meier methods, and differences were examined by the log-rank test. All analyses were done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA, USA), and were considered significant when the *P*-value was less than 0.05.

Results

EGFR gene copy number and clinical outcome

First we assessed *EGFR* copy number by FISH according to Cappuzzo et al. criteria (2005). High polysomy for the *EGFR* gene was present in 44.4% ($n = 12$), and low polysomy in 11.1% ($n = 3$) (Fig.1). However no association was observed between gene amplification and clinical characteristics (Table 1). Smoking status (never smoker vs. smoker, $P = 0.1283$), pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, $P = 0.6280$), or gender (male vs. female, $P = 0.2519$) did not correlate with the *EGFR* amplification status. FISH positive results were obtained in 40% of the patients with *EGFR* mutations. Three other patients with *EGFR* mutations had low polysomy.

A partial response (PR) was achieved in 14 patients, 5 patients had stable disease (SD), and 8 had progressive disease (PD). *EGFR* amplification status was not associated with gefitinib response ($P = 0.7036$). *EGFR* amplification status was not significantly correlated with prognosis (log-rank test, $P = 0.1278$; Breslow–Gehan–Wilcoxon test, $P = 0.0528$) (Fig. 2).

EGFR protein expression and clinical outcome

EGFR protein expression was evaluated by immunohistochemisry (Fig. 3) and the outcome of patients according to the protein score is shown in Fig. 2. Patients with EGFR immunohistochemistry positive ($n = 13$) did not have any advantage for outcomes after treated with gefitinib therapy ($P = 0.7921$).

EGFR gene mutation status in Japanese lung cancer patients

Among 27 patients, 15 had *EGFR* mutations, including four deletion 1a type mutations (2235–2249 del GGAATTAA GAGAAGC), two other types of exon 19 deletion mutations and six L858R mutations. Interestingly, exon 20 insertion mutant patients experienced progressive disease (manuscript submitted). We also compared associations between *EGFR* mutation status, FISH status, and protein expression in each tumor with patient's outcome. Summarized data are shown in Table 2. The overall survival of 27 gefitinib treated-lung cancer patients from Nagoya City University, with follow-up through 30 April 2007, was studied in reference to *EGFR* mutation status. *EGFR* mutations were not associated with FISH+ status, and high protein expression (wild type; 57.1% vs. $P > 0.9999$). Gene mutations were statistically significantly associated with better response ($P = 0.0018$) and longer survival. Patients

Table 1 Clinico-pathological data of 27 lung cancer patients

EGFR gene status			
Factors	FISH positive patients	FISH negative patients	*P* value
Mean age (years) 64.0 ± 11.9	12	15	
Pathological subtypes			
Adeno	9 (40.9%)	13 (59.1%)	0.6260
Non-adeno	3 (60.0%)	2 (40.0%)	
Gender			
Male	8 (57.1%)	6 (42.9%)	0.2519
Female	4 (30.8%)	9 (69.2%)	
Smoking status			
Never smoker	4 (28.6%)	10 (71.4%)	0.1283
Smoker	8 (61.5%)	5 (38.5%)	
Differentiation			
Well	6 (35.3%)	11 (64.7%)	0.2566
Moderately or poorly or Others	6 (60.0%)	4 (40.0%)	
Gefitinib response			
Responder	7 (50.0%)	7 (50.0%)	0.7036
Non-responder	5 (38.5%)	8 (61.5%)	
EGFR mutations			
Wild type	6 (50.0%)	6 (50.0%)	0.8052
Mutant	6 (40.0%)	9 (60.0%)	
IHC			
Positive	9 (69.2%)	4 (30.8%)	0.0213
Negative	3 (21.4%)	11 (78.6%)	

IHC immunohistochemistry, *Adeno* adenocarcinoma

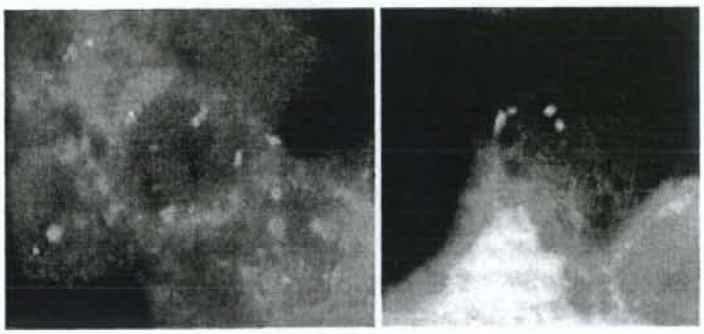

Fig. 1 FISH analysis for lung cancer tissues. *Left* high polysomy case (4 copy numbers in cells >40%), *right* disomy case

with *EGFR* mutations were significantly better in prognosis than the patients with wild type (log-rank test $P = 0.0023$, Breslow–Gehan–Wilcoxon test, $P = 0.0012$) (Fig. 4). Smoking status (never smoker vs. smoker, log-rank test $P = 0.0032$; Breslow–Gehan–Wilcoxon test, $P = 0.0012$), and pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, log-rank test $P = 0.0011$, Breslow–Gehan–Wilcoxon test, $P = 0.0019$), but neither gender (male vs. female, log-rank test $P = 0.0709$, Breslow–Gehan–Wilcoxon test, $P = 0.0353$), nor response (log-rank test $P = 0.2465$, Breslow–Gehan–Wilcoxon test, $P = 0.0588$) were correlated with better prognosis. Using the Cox hazard regression model, *EGFR* mutations ($P = 0.0208$) and smoking status ($P = 0.0218$) were independent prognostic factors, but not pathological subtypes (0.1121). In this analysis, only one *K-ras* codon 12 mutation was found among 27 patients. This patient was wild type for *EGFR* and did not respond to gefitinib therapy.

We have sequenced 27 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center and already reported. We have added these data

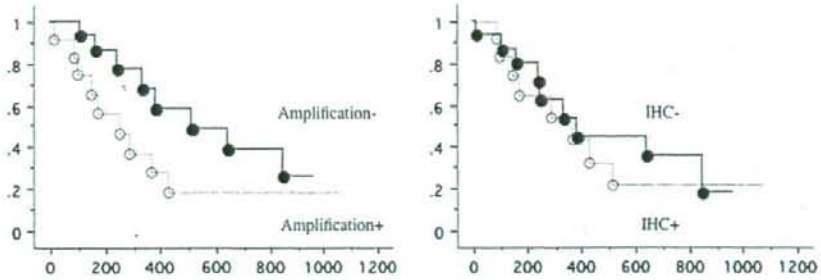

Fig. 2 The overall survival of 27 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* amplification status (*left*) and EGFR protein expression (*right*). Prognosis from patients with EGFR amplification ($n = 12$, 9 were dead) and without *EGFR* amplification ($n = 15$, 8 were dead) was not significantly different (log-rank test, $P = 0.1278$; Breslow–Gehan–Wilcoxon test, $P = 0.0528$). Prognosis from patients with positive EGFR expression ($n = 13$, 8 were dead) and without negative EGFR expression ($n = 14$, 9 were dead) was not significantly different (log-rank test, $P = 0.7921$; Breslow–Gehan–Wilcoxon test; $P = 0.9105$)

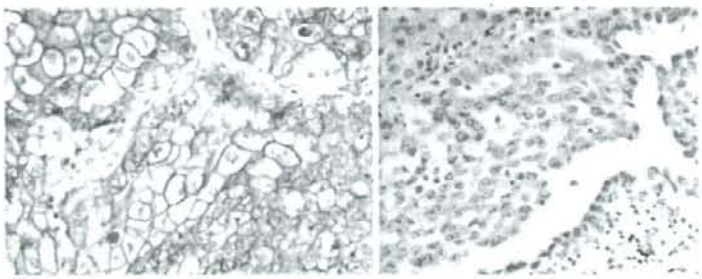

Fig. 3 EGFR protein expression by immunohistochemistry. *Left* positive case, *right* negative case

(Table 3). Ten patients had *EGFR* mutations, including two L858R, one deletion type 1a, and one G719S at exon 18. Three patients had deletion 1b type mutation (2236–2250 del GAATTAAGAGAAGCA). Of 54 patients, 25 were male and 29 female. Twenty-eight were never smokers and 26 were smokers. Forty-eight patients had adenocarcinoma, four had squamous cell carcinoma and one had adenosquamous cell carcinoma. *EGFR* mutation status was significantly correlated with better prognosis (log-rank test $P = 0.0128$, Breslow–Gehan–Wilcoxon test $P = 0.0051$). Patients with *EGFR* mutation at exon 19 deletion 1 types had significantly better prognosis than wild type patients ($P = 0.0032$). However, the prognosis of patients with L858R mutation and wild type was not significantly different ($P = 0.2823$) (Fig. 5).

Discussion

We obtained the findings that the *EGFR* amplification, detected by FISH according to Cappuzzo et al. criteria, was not associated with the response to gefitinib. *EGFR* mutations, smoking history, and pathological subtype of lung cancers were correlated with survival of gefitinib-treated patients. This was in agreement with the recent reports that *EGFR* gene mutations are prognostic factor for gefitinib therapy (Takano et al. 2005; Mitsudomi et al. 2005; Sone et al. 2007). In addition, our analysis also suggested that the deletion type *EGFR* mutation might be more correlated with the survival for gefitinib-treated patients.

Some limitations to the study must be taken into consideration. Our finding is so far based on a single retrospective study with a relatively small number of patients, and the data need to be verified in a large cohort of patients and prospectively. The *EGFR* status was determined on the tumor tissue at the time of primary diagnosis, and possible changes after chemotherapy were not determined in this study (Cappuzzo et al. 2007).

Previous report suggested that NSCLC patients with resected tumors carrying high *EGFR* gene copy number have a tendency to a shorter survival (Hirsch et al. 2003). This might affect the controversial results of Cappuzo et al. (2005) In our analysis, FISH positive population did not correlate with the gender, smoking status and pathological subtypes. The presence of *EGFR* gene amplification did not reach statistical significance. An interesting finding was the association between *EGFR* mutations and increased gene copy number, a phenomenon that was recently described in the human lung cancer cell line H3255 (Tracy et al. 2004) and is probably relevant to gefitinib sensitivity. In fact,

Table 2 EGFR mutation and amplification statuses in 27 gefitinib treated patients

Age	Gender	Smoking	Pathology	EGFR mutation	EGFR amplification	IHC score	Survival (day)
71	F	0	Adeno	Della	High polysomy	220	1,080 (A)
72	M	600	Adeno	L858R	Low polysomy	240	885 (A)
76	M	800	Adeno	WT	High polysomy	90	248 (D)
72	M	0	Adeno	exon 20 ins V	Disomy	80	660 (A)
70	M	1,000	Adeno	L858R	Disomy	210	515 (D)
61	F	0	Adeno	WT	Disomy	160	854 (D)
51	M	500	Adeno	Della	High polysomy	220	286 (D)
76	F	0	Adeno	WT	Disomy	30	640 (D)
57	M	20	Adeno	WT	High polysomy	210	101 (D)
77	M	1,200	Adeno	WT	Disomy	0	168 (D)
38	M	300	Adeno	L858R	High polysomy	210	430 (D)
73	F	0	Adeno	G719S	Disomy	180	339 (D)
42	F	0	Adeno	Del4	High polysomy	100	700 (A)
76	F	920	SCC	WT	High polysomy	220	145 (D)
56	F	0	Adeno	L858R	High polysomy	200	368 (D)
56	M	1,200	Adeno	WT	High polysomy	200	85 (D)
78	M	1200	SCC	WT	High polysomy	250	174 (D)
42	M	400	SCC	WT	Disomy	120	110 (D)
67	M	800	Adeno	WT	Disomy	80	384 (D)
63	M	600	Adsq	WT	High polysomy	90	11 (D)
47	F	0	Adeno	Del5	Disomy	210	945 (A)
62	F	0	Adeno	L858R	Disomy	80	245 (D)
71	F	0	Adeno	L861Q	Low polysomy	210	210 (A)
61	F	0	Adeno	Della	Low polysomy	120	180 (A)
64	F	0	Adeno	WT	Disomy	180	230 (A)
72	M	0	Adeno	L858R	High polysomy	210	110 (A)
77	F	0	Adsq	Della	Disomy	60	210 (A)

F Female, *M* male, *Adeno* adenocarcinoma, *SCC* squamous cell carcinoma, *Adsq* adenosquamous cell carcinoma, *WT* wild type, *IHC* immunohistochemistry, *A* alive, *D* death

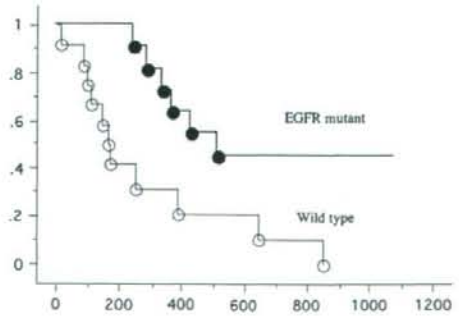

Fig. 4 The overall survival of 27 gefitinib-treated lung cancer patients was studied in reference to the *EGFR* mutation status. Prognosis from patients with EGFR mutations ($n = 15$, 6 were dead) was significantly better than the patients without *EGFR* mutations ($n = 12$, 11 were dead) (log-rank test, $P = 0.0023$, Breslow–Gehan–Wilcoxon test; $P = 0.0012$)

among the 15 patients with *EGFR* mutations who responded to gefitinib therapy, six were also FISH positive (high polysomy) and three were low polysomy. However, between the two non-responding patients with *EGFR* mutations, both were FISH negative. Sone et al. (2007) reported that the *EGFR* mutations and not the gene amplifications were the predictors of gefitinib efficacy in Japanese lung cancers. They evaluated the biopsy specimens and 5/59 samples were small and inadequate for FISH analysis. Another possible explanation for the discrepancies between the findings from the studies described by Cappuzo et al. and our findings is the difference in *EGFR* mutation statuses according to ethnicity. Han et al. (2006) investigated *EGFR* gene mutations, gene amplification, *K-ras* mutation, and Akt phosphorylation in tumor samples from East-Asian patients with NSCLC and demonstrated that *EGFR* mutation was an independent predictor of response and survival

Table 3 Clinico-pathological data of 54 lung cancer patients

EGFR gene status			
Factors	Mutation patients	Wild type patients	*P*-value
Mean age (years) 62.5 ± 11.5	26	28	
Pathological subtypes			
Adeno	25 (52.1%)	23 (47.9%)	0.1938
Non-adeno	1 (16.7%)	5 (83.3%)	
Gender			
Male	11 (44.0%)	14 (56.0%)	0.5952
Female	15 (51.7%)	14 (48.3%)	
Smoking status			
Never smoker	18 (64.3%)	10 (35.7%)	0.0168
Smoker	8 (30.8%)	18 (69.2%)	
Age			
<60	13 (61.9%)	8 (38.1%)	0.1626
>60	13 (39.4%)	20 (60.6%)	
Gefitinib Response			
PR	19 (30.8%)	6 (69.2%)	<0.0001
SD or PD	7 (27.8%)	22 (72.2%)	

PR Progressive disease, *SD* stable disease, *PD* progressive disease

in a multivariate analysis. FISH-positive results were associated with better response rate, the same as *EGFR* mutation in the univariate analysis, but were not associated with prolonged survival (Han et al. 2006).

Although many reports have identified more than 30 different mutation in the tyrosine kinase domains of *EGFR*, the vast majority of which can be grouped into three major types, including in-frame deletion at exon 19, single-nucleotide substitution at exon 18 or 21 and in-frame duplication at exon 20 (Paez et al. 2004; Lynch et al. 2004; Pao et al. 2004; Shigematsu et al. 2005). The L858R missence mutation in exon 21 and deletions in exon 19 have been proven to be activating mutations (Paez et al. 2004; Lynch et al. 2004; Pao et al. 2004). The L858R single-nucleotide substitution mutation located near the conserved Asp-Phe-Gly sequence, stabilizes the activation loop (A-loop) (Paez et al. 2004; Shigematsu et al. 2005). The deletions in exon 19 were located on the side of the alpha-C-helix in the N lobe, which controls the angle of the ATP-binding pocket. This mutation might result in similar conformational changes in *EGFR* that cause a shift in the helical axis that results in the narrowing of the ATP-binding cleft, which leads to increased gene expression and tyrosine kinase inhibitor sensitivity. In vitro analysis, Y845 position of *EGFR* was

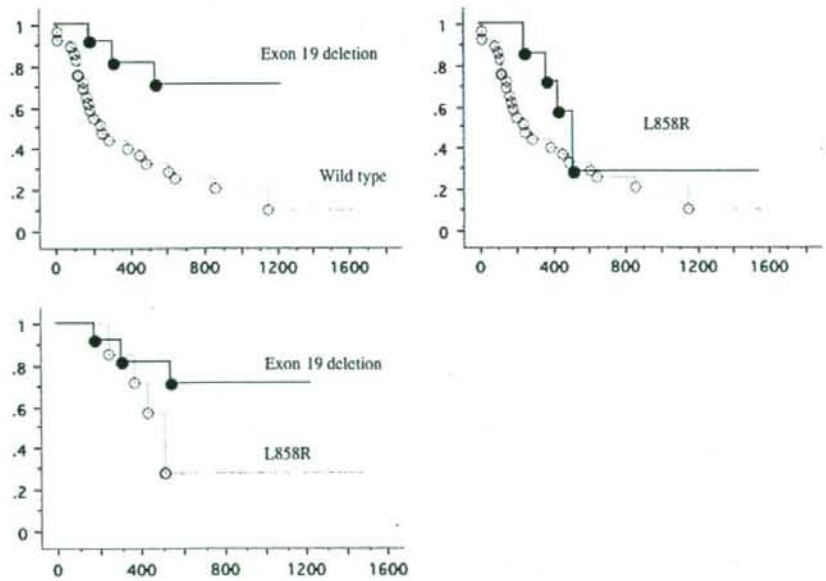

Fig. 5 The overall survival of 54 gefitinib-treated lung cancer patients was studied in reference to the *EGFR* mutation status. *Left upper* prognosis from patients with exon 19 deletion mutations ($n = 12$, 3 were dead) was significantly better than the patients without *EGFR* mutations (Log-rank test, $P = 0.0032$, Breslow–Gehan–Wilcoxon test; $P = 0.006$). *Right upper* prognosis from patients with L858R mutation ($n = 8$, 5 were dead) and patients without *EGFR* mutation was not significantly different (log-rank test, $P = 0.2823$, Breslow–Gehan–Wilcoxon test; $P = 0.142$). *Left lower* there was a tendency towards better prognosis in the patients with exon 19 deletions than in the patients with the L858R mutation (log-rank test, $P = 0.1032$, Breslow–Gehan–Wilcoxon test; $P = 0.1732$)

highly phosphorylated in the L858R mutant, but not in the wild type or the exon 19 deletion mutant, and hence appears to be unique in distinguishing the two types of *EGFR* mutant (Sordella et al. 2005). This might explain the difference in gefitinib response between tumors with L858R and those with deletions. Mitsudomi et al. (2005) noted a 62% (8 of 13) response rate in patients with *EGFR* point mutations compared with 100% (16 of 16) response rate in patients with *EGFR* exon 19 deletion ($P = 0.0019$). Two recent studies reported that patients with *EGFR* exon 19 deletion mutations had a longer median survival than the patients with *EGFR* L858R mutations, although these patients were treated with erlotinib or gefitinib (Jackman et al. 2006; Riely et al. 2006). The findings of the breakdown of *EGFR* mutations among the three exons were interesting, and all the mutations might not be equally correlated with sensitivity for gefitinib.

In summary, our results indicate that high *EGFR* gene amplification identified by FISH may not be an effective molecular predictive marker for gefitinib sensitivity in Japanese patients with NSCLC. Prospective data would be needed to determine if the treatment with gefitinib alters the natural history of patients with *EGFR* mutated Japanese NSCLC.

Acknowledgments We thank Nihon Gene Research Laboratories Inc. (Drs. Narusawa and Shimada) for suggesting that they use fluorescence in situ hybridization to test for epidermal growth factor receptor gene amplification. Grant Sponsor: AstraZeneca Research Grant 2004, Grand-in-Aid for Research in Nagoya City University (2006), and Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science (JSPS) (Nos, 19390367, 18390381,18659407)

References

Cappuzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn Jr PA, Varella-Garcia M (2005) Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 97:643–655

Cappuzzo F, Ligorio C, Toschi L, Rossi E, Trisolini R, Paioli D, Magrini E, Finocchiaro G, Bartolini S, Cancellieri A, Hirsh FR, Crino L, Varella-Garcia M (2007) EGFR and HER2 gene copy number and response to first-line chemotherapy in patients with advanced non-small cell lung cancer (NSCLC). J Thorac Oncol 2:423–429

Endo K, Konishi A, Sasaki H, Takada M, Tanaka H, Okumura M, Kawahara M, Sugiura H, Kuwabara Y, Fukai I, Matsumura A, Yano M, Kobayashi Y, Mizuno K, Haneda H, Suzuki E, Iuchi K, Fujii Y (2005) Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay. Lung Cancer 50:375–384

Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereuslova A, Dong RP, Baselga J (2003) Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 21:2237–2246

Ginsberg RJ, Kris K, Armstrong G (1993) Cancer of the lung. In Principles and practice of oncology. 4th edn. Lippincott, Philadelphia, pp 673–682

Han SW, Kim TY, Jeon YK, Hwang PG, Im SA, Lee KH, Kim JH, Kim DW, Heo DS, Kim NK, Chung DH, Bang YJ (2006) Optimization of patient selection for gefitinib in non-small cell lung cancer by combined analysis of epidermal growth factor receptor mutation, K-ras mutation, and Akt phosphorylation. Clin Cancer Res 12:2538–2544

Hirsch FR, Varella-Garcia M, Bunn PA Jr, Di Maria MV, Veve R, Bremnes RM, Baron AE, Zeng C, Franklin WA (2003) Epidermal growth factor receptor in non-small-cell lung carcinoma: correlation between gene copy number and protein expression and impact on prognosis. J Clin Oncol 21:3798–3807

Jackman DM, Yeap BY, Sequist LV, Lindeman N, Holmes AJ, Joshi VA, Bell DW, Huberman MS, Halmos B, Rabin MS, Haber DA, Lynch TJ, Meyerson M, Johnson BE, Janne PA (2006) Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients with gefitinib or erlotinib. Clin Cancer Res 12:3908–3914

Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T (2004) Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 64:8919–8923

Kris MG, Natale RB, Herbst RS, Lynch TJ Jr, Prager D, Belani CP, Schiller JH, Kelly K, Spiridonidis H, Sandler A, Albain KS, Cella D, Wolf MK, Averbuch SD, Ochs JJ, Kay AC (2003) Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non small cell lung cancer: randomized trial. JAMA 290:2149–2158

Krypuy M, Newnham GM, Thomas DM, Conron M, Dobrovic A (2006) High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. BMC Cancer 6:295

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Eng J Med 350:2129–2139

Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Camplese PP, Iarussi T, Muchilli F, Mezzetti A, Cuccurullo F, Sacco R, Buttitta F (2005) EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 23:857–865

Miller VA, Kris MG, Shah N, Patel J, Azzoli C, Gomez J, Krug LM, Pao W, Rizvi N, Pizzo B, Tyson L, Venkatraman E, Ben-Porat L, Memoli N, Zakowski M, Rusch V, Heelan RT (2004) Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. J Clin Oncol 22:1103–1109

Mitsudomi T, Kosaka T, Endoh, Horio Y, Hida T, Mori S, Hatooka S, Shinoda M, Takahashi T, Yatabe Y (2005) Mutation of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. J Clin Oncol 23:2513–2520

Nicolson RI, Gee JM, Harper ME (2001) EGFR and cancer prognosis. Eur J Cancer 37:S9–S15

Onn A, Correa AM, Gilcrease M, Isobe T, Massarelli E, Bucane CD, O'Reilly MS, Hong WK, Fidler IJ, Putnum JB, Herbst RS (2004) Synchronous overexpression of epidermal growth factor receptor

and HER2-neu protein is a predictor of poor outcome in patients with stage I non-small cell lung cancer. Clin Cancer Res 10:136–143

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Seller WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304:1497–1500

Pao W, Miller V, Zakowski M Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kri M, Varmus H (2004) EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and elrotinib. Proc Natl Acad Sci USA 101:13306–13311

Riely GJ, Pao W, Pham DK, Li AR, Rizvi N, Venkatraman ES, Zakowski MF, Kris MG, Ladanyi M, Miller V (2006) Clinical course of patients with non-small cell lung cancer and epidermal growth factor receptor exon 19 and exon 21 mutations treated with gefitinib or erlotinib. Clin Cancer Res 12:839–844

Sasaki H, Endo K, Konishi A, Takada M, Kawahara M, Iuchi K, Matsumura A, Okumura M, Tanaka H, Kawaguchi T, Shimizu T, Takeuchi H, Yano M, Fukai I, Fujii Y (2005) EGFR mutation status in Japanese lung cancer patients: genotyping analysis using LighyCycler. Clin Cancer Res 11:2924–2929

Sasaki H, Shimizu S, Endo K, Takada M, Kawahara M, Tanaka H, Matsumura A, Iuchi K, Haneda H, Suzuki E, Kobayashi Y, Yano M, Fujii Y (2006) EGFR and erbB2 mutation status in Japanese lung cancer patients. Int J Cancer 118:180–184

Shigematsu H, Lin L, Takahashi T, Numura M, Suzuki M, Wistuba II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Herz J, Minna JD, Gazdar AF (2005) Clinical and biological features associated with epidermal growth factor receptor gene mutation in lung cancers. J Natl Cancer Inst 97:339–346

Sone T, Kasahara K, Kimura H, Nishio K, Mizuguchi M, Nakatsumi Y, Shibata K, Waseda Y, Fujimura M, Nakao S (2007) Comparative analysis of epidermal growth factor receptor mutations and gene amplification as predictors of gefitinib efficacy in Japanese patients with nonsmall cell lung cancer. Cancer 109:1836–1844

Sordella R, Bell DW, Haber DA, Settleman J (2005) Gefitinib-sensitizing EGFR mutations in lung cancer activated anti-apoptotic pathways. Science 305:1163–1167

Takano T, Ohe Y, Sakamoto H, Tsuta K, Matsuno Y, Tateishi U, Yamamoto S, Nokihara H, Yamamoto N, Sekine I, Kunitoh H, Shibata T, Sakiyama T, Yoshida T, Tamura T (2005) Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J Clin Oncol 23:6829–6837

Thatcher N, Chang A, Parikh P, Rodrigues Pereira J, Ciuleanu T, von Pawel J, Thongprasert S, Tan EH, Pemberton K, Archer V, Carroll K (2005) Gefitinib plus best supportive care in previously treated patients with refractory advance non-small-cell lung cancer: result from a randomized, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). Lancet 366:1527–1537

Tokumo M, Toyooka S, Kiura K, Shigematsu H, Tomii K, Aoe M, Ichimura K, Tsuda T, Yano M, Tsukuda K, Tabata M, Ueoka H, Tanimoto M, Date H, Gazder AF, Shimizu N (2005) The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. Clin Cancer Res 11:1167–1173

Tracy S, Mukohara T, Hansen M, Meyerson M, Johnson BE, Janne PA (2004) Gefitinib induces apoptosis in the EGFR (L858R) non-small-cell lung cancer cell line H3255. Cancer Res 64:7241–7244

Journal of Surgical Research **148,** 260–263 (2008)
doi:10.1016/j.jss.2007.09.001

EGFR Polymorphism of the Kinase Domain in Japanese Lung Cancer

Hidefumi Sasaki, M.D., Ph.D.,*[,1] Katsuhiko Endo, M.D., Ph.D.,* Minoru Takada, M.D., Ph.D.,† Masaaki Kawahara, M.D., Ph.D.,† Hisaichi Tanaka, M.D., Ph.D.,‡ Naoto Kitahara, M.D., Ph.D.,‡ Akihide Matsumura, M.D., Ph.D.,‡ Keiji Iuchi, M.D., Ph.D.,‡ Tomoya Kawaguchi, M.D., Ph.D.,† Katsuhiro Okuda, M.D., Ph.D.,* Osamu Kawano, M.D., Ph.D.,* Haruhiro Yukiue, M.D., Ph.D.,* Tomoki Yokoyama, M.D., Ph.D.,* Motoki Yano, M.D., Ph.D,* and Yoshitaka Fujii, M.D., Ph.D.*

*Department of Surgery II, Nagoya City University Medical School, Nagoya, Japan; †Department of Internal Medicine, National Hospital Organization, Kinki-chuo Chest Medical Center, Osaka, Japan; and ‡Department of Surgery, National Hospital Organization, Kinki-chuo Chest Medical Center, Osaka, Japan

Submitted for publication June 14, 2007

Background. **Mutations of the epidermal growth factor receptor (EGFR) gene at kinase domain have been reported in non-small-cell lung cancer (NSCLC), and some common somatic mutations in EGFR have been examined for their ability to predict sensitivity to gefitinib or erlotinib. However, EGFR mutations at exon 20 have been reported to predict resistance to gefitinib therapy.**

Materials and methods. **We investigated the EGFR mutations and/or polymorphism statuses at kinase domain in 303 surgically treated non-small cell lung cancer (NSCLC) cases. One hundred ninety-four adenocarcinoma cases were included. The presence or absence of EGFR polymorphism of kinase domains was analyzed by direct sequences. We have also investigated EGFR polymorphism status at exon 20 for 23 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center.**

Results. **EGFR mutations at kinase domain were found in 75 of 303 lung cancer patients. During sequencing of EGFR tyrosine kinase domain in tumors, 86 EGFR polymorphism (G2607A) cases were identified at exon 20. G2067A polymorphism was significantly higher in nonadenocarcinomas (37.4%) than in adenocarcinoma (25.3%, $P = 0.0415$). The polymorphism status did not correlate with gender, smoking (never smoker *versus* smoker), and EGFR mutations. In 46 total gefitinib treated NSCLC patients, there was a tendency toward better prognosis in EGFR wild type (GG) patients than AG + AA patients. EGFR polymorphism in Japanese lung cancers seemed to be less frequent than Caucasian lung cancers.**

Conclusions. **EGFR-tyrosine kinase polymorphism might be associated with clinicopathological background of lung cancers.**

Key Words: **EGFR; lung cancer; polymorphism; exon 20.**

INTRODUCTION

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior, and lack of major advancements in treatment strategy [1]. There is much accumulated evidence that epidermal growth factor receptor (EGFR) and its family members are strongly implicated in the development and progression of numerous human tumors, including lung cancer [2, 3]. The EGFR tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of non-small cell lung cancer (NSCLC) in 2002. Phase II and III trials have shown partial responses in 8% to 12% of unselected patient with progressive NSCLC after chemotherapy [4, 5], especially a higher response in the never-smoker, female, and of Asian ethnicity (more than 20%) [4, 6, 7]. Original two reports showed that EGFR mutation status at tyrosine kinase (TK) domain in NSCLC patients was correlated with the clinicopathological features related to good response to gefitinib [8, 9]. These EGFR mutations were predominantly found in Japanese lung cancer patients (about 25–40%) [8, 10–13] compared with U.S.A. patients (about 8% to 10%) [8, 9, 11, 14] or European patients [11, 15]. Actually, EGFR mutations in lung cancer have been correlated with clinical response

[1] To whom correspondence and reprint requests should be addressed at Department of Surgery II, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. E-mail: hisasaki@med.nagoya-cu.ac.jp.

0022-4804/08 $34.00

to gefitinib therapy in vivo and in vitro [8, 9, 14]. However, EGFR mutations at exon 20 have been reported to predict resistance to gefitinib therapy [16, 17]. During sequencing of the EGFR tyrosine kinase domain in lung cancers, an EGFR polymorphism (G2607 A) was identified at exon 20 [17]. This EGFR single nucleotide polymorphism (SNP) was significantly higher in lung cancer (83.6%) than control (73.5%) in the Caucasian population [18]. To determine this EGFR polymorphism status and correlation with clinicopathological features in Japanese lung carcinoma, we investigated EGFR gene status by direct sequences. The findings were compared with the clinicopathological features of lung cancer.

MATERIALS AND METHODS

Patients

The study group included 303 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2005. Mean age was 65.2 y and median age was 66 y. We have also investigated EGFR SNP status for 23 NSCLC patients who had undergone surgery followed by treated with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan [19]. All tumor samples were immediately frozen and stored at −80°C until assayed.

The clinical and pathological characteristics of the 303 lung cancer patients were as follows: 209 (69.0%) were male and 94 were female; 194 (64.0%) were diagnosed with adenocarcinoma, and 109 were diagnosed with other types of carcinoma; 205 (67.7%) were smokers and 98 were nonsmokers.

Polymerase Chain Reaction (PCR) Assays for EGFR Mutations

Genomic DNA was extracted using Wizard SV Genomic DNA purification Systems (Promega, Madison, WI) according to the manufacturer's instructions. The primers and TaqMan MGB probe were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The sequences of 13 allele-specific probes and primer sets used in the TaqMan PCR assay were already shown and the results were already reported [13]. The direct sequencing for EGFR genes was performed for 91 cases at Dana-Farber Cancer Institute. Most of the results from sequencing were already reported by Paez *et al.* [8]. Other cases were genotyped using LightCycler and also sequenced [20, 21]. The PCR reactions were performed using LA-Taq kit (Takara Bio Inc., Shiga, Japan) in a 50 μL reaction volume. The primer sequences for EGFR gene at exon 20 were as follows: forward primer, 5-ATCGCATTCATGCGTCTTCA-3 and reverse primer, 5-ATCCCCATGGCAAACTCTTG-3 (378 bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 64°C for 30 s, and 72°C for 45 s. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA). These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review.

Statistical Analysis

Statistical analyses were done using the Mann-Whitney *t*-test for unpaired samples and Wilcoxon's singed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and $\chi 2$ test. The overall survival of lung cancer patients was examined by the Kaplan-Meier method, and differences were examined by the Log-rank test. All analyses were done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA), and a P value < 0.05 was considered significant.

RESULTS

GFR Gene Mutation Status in Japanese Lung Cancer Patients

Of 303 patients from Nagoya City University, in exon 19, 33 patients had the deletion type mutation. In exon 18 or exon 21, 41 patients had the missense point mutations (1 G719S, 2 G719C, 36 L858R, and 2 L861Q). One patient had exon 20 insertion mutation. Of these 75 patients, 26 were male and 49 were female; 55 were nonsmokers and 20 were smokers; 72 patients had adenocarcinoma, 1 had squamous cell carcinoma, and 2 had adenosquamous cell carcinoma. Thus EGFR mutation status at exon 18 to 21 was significantly correlated with gender ($P < 0.0001$), tobacco-smoking ($P < 0.0001$) and pathological subtypes (adenocarcinoma *versus* nonadenocarcinoma, $P < 0.0001$). Of 303 patients from Nagoya City University, 176 (58.1%) were Stage I. There was a tendency toward higher EGFR mutation in Stage I (50/176, 28.4%) than in Stage II to IV (25/127, 19.7%, $P = 0.1052$).

EGFR Polymorphism at Exon 20

During sequencing of the EGFR-TK domain in lung cancer samples, a sequence difference in exon 20 (G2607A; Q787Q) was found in tumors that defined a previously identified SNP (refSNP ID: rs 10251977) in the EGFR-TK gene (Fig. 1). Of 303 patients, 86 patients had the EGFR polymorphism; 57 were male and 29 were female; 60 were nonsmokers and 26 were smokers; 49 patients had adenocarcinoma and 37 had

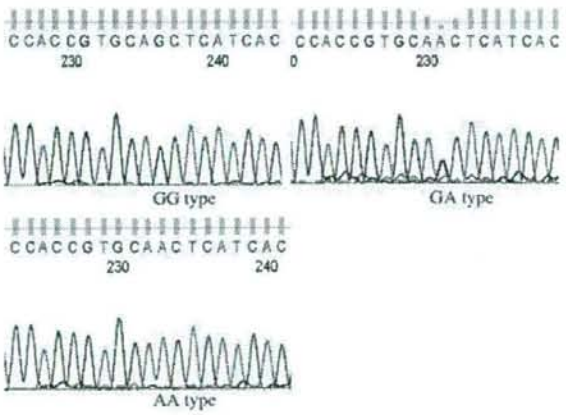

FIG. 1. The sequence results of *EGFR* exon 20. Left upper; wild type (GG). Right upper; heterozygous SNP (GA). Left lower; homozygous SNP (AA). (Color version of figure is available online).

TABLE 1

Clinicopathological Data of 303 Lung Cancer Patients

Factors	EGFR		P value
	GG patients	GA + AA patients	
Mean age (years) 65.2 ± 9.6	86	217	
pStage			
I	47 (26.7%)	129 (73.3%)	0.5187
II–IV	39 (30.7%)	88 (69.3%)	
Smoking			
Nonsmoker	26 (26.5%)	72 (73.5%)	0.6837
Smoker	60 (29.3%)	145 (70.7%)	
Pathological subtype			
Adenocarcinoma	49 (25.3%)	145 (74.7%)	0.0415
Others	37 (37.4%)	72 (62.6%)	
EGFR mutation			
Positive	19 (25.3%)	56 (73.7%)	0.5566
Negative	67 (29.4%)	161 (70.6%)	
Age			
=<60	17 (20.7%)	65 (79.3%)	0.0853
>60	69 (31.2%)	152 (68.8%)	
Gender			
Male	57 (27.3%)	152 (82.7%)	0.3141
Female	29 (20.9%)	65 (79.1%)	

other types of lung cancers. G2607A polymorphism was significantly higher in nonadenocarcinomas (37/109; 37.4%) than in adenocarcinoma (49/194; 25.3%, $P = 0.0415$). However, the polymorphism did not correlate with gender ($P = 0.5820$), smoking status ($P = 0.7789$), pathological stages ($P = 0.5077$), and EGFR-TK mutation status of lung cancer ($P = 0.5566$) (Table 1). Previous report from the United States demonstrated that the G2607A polymorphism was found from 102/122 (83.6%) patients. EGFR polymorphism (G2607A) in our Japanese lung cancers was less frequent than Caucasian lung cancers ($P < 0.0001$).

Relationship Between Clinical Courses of Lung Cancer Patients Treated with Gefitinib and EGFR Polymorphism

The overall survival of gefitinib untreated lung cancer patients from Nagoya City University, with follow-up through December 30, 2006, was studied in reference to the EGFR polymorphism status. Of 303 patients from Nagoya City University, 23 were treated with gefitinib therapy. A total of 46 gefitinib treated patients were investigated for G2607 polymorphism status. In this analysis, 11 patients had EGFR polymorphism (AG or AA). There was a tendency toward better prognosis in EGFR wild type patients (GG; 21/35 were deceased) than in EGFR polymorphism patients (AG + AA; 9/11 were deceased) ($P = 0.0653$) (Fig. 2).

DISCUSSION

We obtained findings that G2607A EGFR polymorphism was significantly higher in nonadenocarcinomas than in adenocarcinomas. In addition, our analysis also suggested that there was a tendency toward better prognosis in EGFR wild type patients (GG) than in EGFR polymorphism patients (GA + AA) who were treated with gefitinib.

In this report, the EGFR SNP(G2607A) is not associated with somatic EGFR-TK mutation. Approximately 563 EGFR-SNPs have been identified in human genome according to the National Cancer for Biotechnology information database. However, there are few studies examining associations between EGFR SNPs and human disease [18, 22–25]. In this study, we detected a polymorphism in exon 20 of the EGFR-TK domain at nucleotide 2607, codon 787 (Gln), which changed nucleotide 2607 from G to A, without amino acid substitution. Previous reports suggested that EGFR exon 20 mutations were critical roles for gefitinib resistance. EGFR containing the exon 20 point mutation T790M were associated with resistance to gefitinib and erlotinib [16]. Greulich *et al.* reported that transformation by the D770_N771insNPG (exon 20) EGFR insertion mutant was remarkably insensitive to gefitinib and erlotinib, as inhibition of colony growth in soft agar required exposure to 100-fold higher concentrations (>1 mM) of these agents than was required to inhibit colony formation by cells expressing the EGFR missense mutants or deletion mutant [17]. Greulich *et al.* also reported that all three lung adenocarcinoma patient with known exon 20 insertion mutants of EGFR have failed to show a clinical response to treatment and have instead achieved only stable disease with erlotinib [17]. Actually, in this report, a weak association between G2607A polymorphism and the prognosis of gefitinib therapy was also found. This

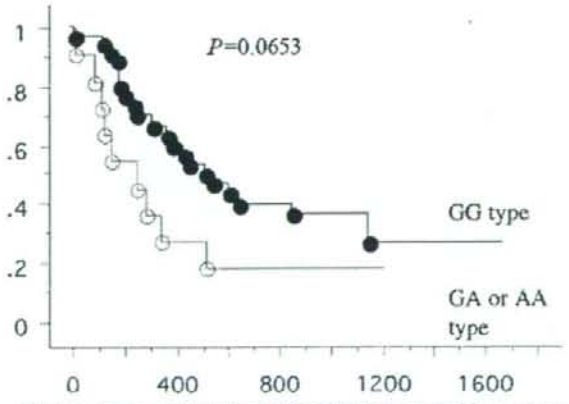

FIG. 2. The overall survival of 46gefitinib untreated lung cancer patients was studied in reference to the EGFR polymorphism (G2607A) status. There was a tendency toward better prognosis from patient with EGFR wild type (GG) ($n = 35$, 21 were deceased) than the patient with EGFR polymorphism (GA or AA) ($n = 11$, 9 were deceased) (log-rank test, $P = 0.0653$, Breslow-Gehan-Wilcoxon test; $P = 0.0174$).

might be explained because of the difference in gefitinib response between adenocarcinomas and other types of carcinomas. In our report, G2607 polymorphisms were lower in adenocarcinomas in the Japanese population. A larger number would help to determine the correlation between the G2607 polymorphism and gefitinib sensitivity.

A previous report showed a different G2607 frequency of distribution between Swiss and Japanese population with glioblastoma [22]. This polymorphism was found at a higher frequency in lung cancer patients than normal control [18]. Zhang *et al.* also suggested that no association was found between the EGFR-TK mutation and the G2607A SNP [18]. It remains to be verified whether the *EGFR* G2607A changes EGFR expression or function [18, 22]. Even if there is no amino acid change, the EGFR polymorphism identified here might lead to difference in EGFR gene transcription, mRNA stability or translation, or could be a genetic marker of another risk-associated genotype. Shintani *et al.* demonstrated that another EGFR-SNP at position 2073 was correlated with truncated EGFR transcription, which might interfere with EGFR three-dimensional structure and EGFR expression [24].

ACKNOWLEDGMENTS

The authors thank Mrs. Emi Sugiyama for her excellent technical assistance. This work was supported by AstraZeneca Research grant 2004, Grant-in-Aid for Research in Nagoya City University 2006, and Grant-in-Aid for Scientific Research, Japan Society for the Promotion of Science (19390367, 18390381, 18659407).

REFERENCES

1. Ginsberg RJ, Kris K, Armstrong G. Cancer of the lung. In Principles and Practice of Oncology, 4th ed. Philadelphia: Lippincott, 1993; p. 673.
2. Nicolson RI, Gee JM, Harper ME. EGFR and cancer prognosis. Eur J Cancer 2001;37:S9.
3. Onn A, Correa AM, Gilcrease M, et al. Synchronous overexpression of epidermal growth factor receptor and HER2-neu protein is a predictor of poor outcome in patients with stage I non-small cell lung cancer. Clin Cancer Res 2004;10:136.
4. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non small cell lung cancer: Randomized trial. JAMA 2003;290:2149.
5. Thatcher N, Chang A, Purvish P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advance non-small-cell lung cancer: Result from a randomized, placebo-controlled, multicenter study (Iressa Survival Evaluation in Lung Cancer). Lancet 2005;366:1527.
6. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 2003;21:2237.
7. Miller VA, Kris MG, Shah N, et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. J Clin Oncol 2004;22:1103.
8. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. Science 2004;304:1497.
9. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. New Engl J Med 2004;350:2129.
10. Kosaka T, Yatabe Y, Endoh H, et al. Mutations of the epidermal growth factor receptor gene in lung cancer: Biological and clinical implications. Cancer Res 2004;64:8919.
11. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutation in lung cancers. J Natl Cancer Inst 2005;97:339.
12. Tokumo M, Toyooka S, Kiura K, et al. The relationship between epidermal growth factor receptor mutation and clinicopathological feature in non-small cell lung cancers. Clin Cancer Res 2005;11:1167.
13. Endo K, Konishi A, Sasaki H, et al. Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay. Lung Cancer 2005;50:375.
14. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci 2004;101:13306.
15. Marchetti A, Martella C, Felicioni L, et al. EGFR mutations in non-small-cell lung cancer: Analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Onlol 2005;23:857.
16. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell-lung cancer to gefitinib. New Engl J Med 2005;352:786.
17. Greulich H, Chen T-H, Feng W, et al. Oncogenic transformation by inhibitor sensitive and resistant EGFR mutants. Plos Med 2005;2:1167.
18. Zhang W, Stabile LP, Keohavong P, et al. Mutation and polymorphism in the EGFR-TK domain associated with lung cancer. J Thorac Oncol 2006;1:635.
19. Japan Lung Cancer Society, General Rule for Clinical and Pathological Record of Lung Cancer, 5th ed. Japan Lung Cancer Society 1999;5:1.
20. Sasaki H, Endo K, Konishi A, et al. EGFR mutation status in Japanese lung cancer patients: Genotyping analysis using LightCycler. Clin Cancer Res 2005;11:2924.
21. Sasaki H, Shimizu S, Endo K, et al. EGFR and erbB2 mutation status in Japanese lung cancer patients. Int J Cancer 2006;118:180.
22. Fukushima T, Favereaux F, Huang H, et al. genetic alterations in primary glioblastomas in Japan. J Neuropathol Exp Neurol 2006;65:12.
23. Kang D, Gridley G, Huang WY, et al. Microsatellite polymorphisms in the epidermal growth factor receptor (EGFR) gene and the transforming growth factor-α (TGFA) gene and risk of oral cancer in Puerto Rico. Pharmacogenet Genomics 2005;15:343.
24. Shintani S, Matsuo K, Crohin CC, et al. Intragenic mutation analysis of the human epidermal growth factor receptor (EGFR) gene in malignant human oral keratinocytes. Cancer Res 1999;59:4142.
25. Liu G, Gurubhagavatula S, Zhou W, et al. Epidermal growth factor receptor polymorphisms and clinical outcomes in non-small-cell lung cancer patients treated with gefitinib. Pharmacogenomics J 2008;8:129.

J Cancer Res Clin Oncol (2009) 135:313–318
DOI 10.1007/s00432-008-0464-5

ORIGINAL PAPER

EGFR R497K polymorphism is a favorable prognostic factor for advanced lung cancer

Hidefumi Sasaki · Katsuhiro Okuda · Shigeki Shimizu · Minoru Takada · Masaaki Kawahara · Naoto Kitahara · Meinoshin Okumura · Akihide Matsumura · Keiji Iuchi · Tomoya Kawaguchi · Akihito Kubo · Osamu Kawano · Haruhiro Yukiue · Motoki Yano · Yoshitaka Fujii

Received: 19 May 2008 / Accepted: 10 August 2008 / Published online: 23 August 2008

Abstract
Introduction It has been reported that the R497K polymorphism of the epidermal growth factor receptor (*EGFR*) gene has attenuated functions in ligand binding, tyrosine kinase activation, and growth stimulation. On other hand, *EGFR* gene mutations at kinase domain in non-small cell lung cancer (NSCLC) have been examined for their ability to predict sensitivity to gefitinib or erlotinib.
Materials and methods We investigated the *EGFR* mutations and/or R497K polymorphism statuses in 225 surgically treated NSCLC cases. 192 adenocarcinoma cases were included. The presence or absence of *EGFR* polymorphism of exon 13 was analyzed by PCR–RFLP method.
Results *EGFR* mutations at kinase domain were found from 95 of 225 lung cancer patients. In 86.2% of patients, homo- or heterozygous Lys497 allele was present. No correlation existed between R497K *EGFR* genotype and clinico-pathological features, such as gender, smoking status, and pathological subtypes.
Conclusions *EGFR* mutation status was not correlated with R497K*EGFR* genotype of lung cancers. In node-negative patients, R497K*EGFR* genotype was not correlated with disease outcome. In node-positive patients, however, R497K *EGFR* was significantly associated with better overall survival. This association was attributable to neo-adjuvant or adjuvant chemotherapy. In 46 total gefitinib treated NSCLC patients, the prognosis was not different between the *EGFR* wild type (GG) patients and AG+AA patients. R497K*EGFR* polymorphism might be associated with favorable prognosis of advanced lung cancers and correlated with chemosensitivity.

Keywords EGFR · Lung cancer · Polymorphism · R497K

H. Sasaki (✉) · K. Okuda · O. Kawano · H. Yukiue · M. Yano · Y. Fujii
Department of Surgery II, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
e-mail: hisasaki@med.nagoya-cu.ac.jp

S. Shimizu
Department of Pathology II, Nagoya City University Medical School, Nagoya, Japan

M. Takada · M. Kawahara · T. Kawaguchi · A. Kubo
Department of Internal Medicine, National Hospital Organization, Kinki-chuo Chest Medical Center, Sakai, Osaka, Japan

N. Kitahara · M. Okumura · A. Matsumura · K. Iuchi
Department of Surgery, National Hospital Organization, Kinki-chuo Chest Medical Center, Sakai, Osaka, Japan

Introduction

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior, and lack of major advancements in treatment strategy (Ginsberg et al. 1993). There are much accumulated evidences that epidermal growth factor receptor (*EGFR*) and its family member are strongly implicated in the development and progression of numerous human tumors, including lung cancer (Nicolson et al. 2001; Onn et al. 2004). The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of non-small cell lung cancer (NSCLC) since 2002. In 2004, two reports have shown that *EGFR* mutation statuses at tyrosine kinase (TK) domain in NSCLC patients were correlated with the clinico-pathological features related to good response to gefitinib (Paez et al. 2004; Lynch et al. 2004). *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy in vivo and in vitro (Paez et al. 2004; Lynch et al. 2004; Pao et al. 2004). Genomic

profiling of the EGFR signaling is also helpful in identifying lung cancer patients who are at risk of tumor recurrence and those who are more likely to benefit from chemoradiation therapy. For example, the NSCLC patients with more than 35 (CA)n repeats in *EGFR* intron 1 polymorphism had a significantly longer overall survival than the patients with the 35 or fewer (CA)n alleles, who received radiation (RT; 50.4 Gy) or RT concurrent with chemotherapy (CT; four cycles of cisplatin plus etoposide) (Dubey et al. 2006; Keller et al. 2000). *EGFR* intron 1 and −216G/T polymorphisms influenced clinical outcomes in gefitinib-treated NSCLC patients (Liu et al. 2008). A polymorphic variant *EGFR* arising from a single nucleotide change (G–A) leading to an arginine (Arg) to lysine (Lys) substitution in codon 497 (R497K) in the extracellular domain of EGFR has been identified (Moriai et al. 1994). This polymorphism alone or in combination with another polymorphism in the same gene is associated with a lower recurrence of tumor in rectal cancer patients treated with chemoradiation (Zhang et al. 2005). To determine this *EGFR* polymorphism status and correlation with clinicopathological features in Japanese lung carcinoma, we investigated *EGFR* gene status by PCR–RELP method and direct sequencings. The findings were compared to the clinicopathologic features of lung cancer.

Materials and methods

Patients and samples

The study group included 206 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2005. Fifty eight patients were treated with platinum-based neoadjuvant or adjuvant chemotherapy. Twenty seven patients were treated with gefitinib for their recurrence of lung cancer after they had undergone surgery. We have also investigated *EGFR* R497K status for 19 NSCLC patients who had treated with gefitinib for their recurrence of lung cancer after undergone surgery at the National Hospital Organization, Kinki-chuo Chest Medical Center. The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan, as well as WHO classification. All tumor samples were immediately frozen and stored at −80°C until assayed.

The clinical and pathological characteristics of the 225 lung cancer patients were as follows; 132 (58.6%) were male and 93 were female. One hundred and ninety two were diagnosed as adenocarcinoma, and 33 were diagnosed as other types of carcinoma (20 squamous cell carcinomas, eight adenosquamous carcinomas and five large cell carcinomas). One hundred and twenty five (55.6%) were smoker (current smoker or ever smoker) and 100 were non-smoker. Written informed consent was obtained from the patients, and the institutional ethics committee of the Nagoya City University approved the study.

PCR assays for *EGFR* polymorphism

Genomic DNA was extracted using Wizard SV Genomic DNA purification Systems (Promega) according to the manufacturers' instructions. *EGFR* mutation statuses at kinase domain were investigated using TaqMan PCR assay (Applied Biosystems). The sequences of 13 allele-specific TaqMan MGB probes and primer sets used in the TaqMan PCR assay were already shown (Endo et al. 2005). The results of TaqMan PCR assays were already reported. The R497K *EGFR* (G–A) polymorphism was examined by PCR–RELP method as described previously (Zhang et al. 2005; Wang et al. 2007). Briefly, the PCR reactions were performed using LA-Taq kit (Takara Bio Inc, Shiga, Japan) in a 50 μl reaction volume. The primer sequences for *EGFR* gene at exon 13 were as follows: the forward primer, 5′-TGCTGTGACCCACTCTGTCT-3′ and the reverse primer, 5′-CCAGAAGGTTGCACTTGTCC-3′. The cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles at 94°C for 60 s, 59°C for 60 s, 72°C for 60 s. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA), and then digested by BstN1 restriction enzyme (New England Biolabs) at 60°C for 16 h. These samples were separated on 4% ethidium bromide-stained agarose gels. In some cases, direct sequencing were performed and analyzed by BLAST and chromatograms by manual review.

Statistical analysis

Statistical analyses were done using the Mann–Whitney *U* test for unpaired samples and Wilcoxon's singed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and χ^2 test. The overall survival of lung cancer patients was examined by the Kaplan–Meier methods, and differences were examined by the Log-rank test. All analysis was done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA), and was considered significant when the p value was less than 0.05.

Results

EGFR gene mutation status

Of 225 patients, in exon 19, 51 patients had the deletion type mutation. In exon 18 or exon 21, 39 patients had the

missense point mutations (1 G719S, 3 G719C, 34 L858R and 1 L861Q). Five patients had exon 20 insertion mutations (Sasaki et al. 2007). Of these 95 patients, 34 were male and 61 were female. Sixty seven were non-smokers and 28 were smokers. Ninety two patients had adenocarcinoma and three had adenosquamous cell carcinoma. Thus *EGFR* mutation statuses at exon 18–21 were significantly correlated with gender ($p < 0.0001$), tobacco-smoking ($p < 0.0001$), and pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, $p < 0.0001$). Of 206 patients from Nagoya City University, 97 (51.5%) were stage I. There was a higher *EGFR* mutation in stage I (51/97, 28.4%) than in stage II–IV (33/89, 19.7%, $p = 0.0235$).

EGFR polymorphism at exon 13

Using the PCR–RFLP assay, a sequence difference in exon 13 (R497K) was found in tumors that defined in the *EGFR* gene. Example of the *EGFR* gene analyzed by PCR–RELP method was shown in Fig. 1. Same codon 497 polymorphism of *EGFR* was found in both DNAs isolated from several lung cancer samples and adjacent peripheral blood samples. Several samples were also confirmed by direct sequencing (Fig. 2). Of 225 patients, 194 patients had the *EGFR* polymorphism (80 AA and 114 GA), 117 were male and 77 were female, 110 were non-smokers and 84 were smoker, and 166 patients had adenocarcinoma and 28 had other types of lung cancers. The R497K polymorphism did not correlate with gender ($p = 0.2410$), smoking status

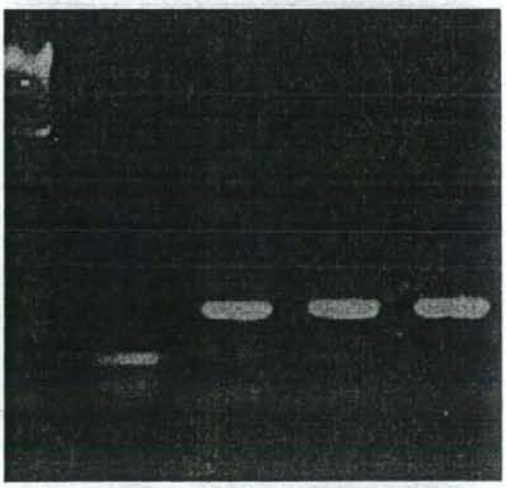

Fig. 1 Representative PCR–RELP patterns of different EGFR codon 497 status. PCR products after being digested by BstN1 were separated by agarose gel electrophoresis

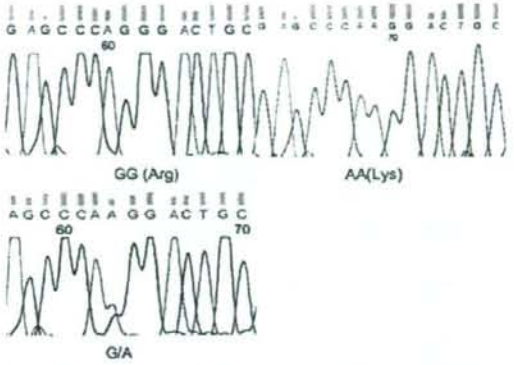

Fig. 2 The sequence results of *EGFR* exon 13. *Left upper* wild type (GG). *Right upper* heterozygous change (GA). *Left lower* homozygous change (AA)

Table 1 Clinico-pathological data of 225 lung cancer patients

Factors	EGFR		*p*-value
	GG	GA+AA	
	Patients	Patients	
Mean age (years) 63.2 ± 10.3	63.4 ± 10.0	62.0 ± 12.0	0.6685
Gender			
Male	15 (48.4%)	117 (60.3%)	0.2410
Female	16 (51.6%)	77 (49.7%)	
Smoking			
Non-smoker	16 (51.6%)	84 (43.3%)	0.4387
Smoker	15 (48.4%)	110 (56.8%)	
Pathological subtype			
Adeno	26 (83.9%)	166 (85.6%)	0.7865
Others	5 (16.1%)	28 (14.4%)	
EGFR mutation			
Positive	14 (45.2%)	81 (41.8%)	0.5566
Negative	17 (54.8%)	113 (58.2%)	
Age			
≤60	12 (38.7%)	72 (39.1%)	>0.9999
>60	19 (61.3%)	112 (60.8%)	
Pathological stages			
I	10 (35.7%)	96 (53.9%)	0.1073
II	4 (14.3%)	29 (16.3%)	
III–IV	14 (50.0%)	53 (29.8%)	
Lymph node metastasis			
Negative	14 (50.0%)	118 (66.3%)	0.1366
Positive	14 (50.0%)	60 (33.7%)	

**EGFR* epidermal growth factor receptor, *Smoker* current smoker or ever smoker, *Adeno* adenocarcinoma

($p = 0.4387$), pathological subtypes ($p = 0.7865$), and *EGFR*-TK mutation status of lung cancer ($p = 0.5566$) (Table 1). Major components of adenocarcinomas with

R497K were as follows; acinar 58.3%, solid 25.0%, and papillary 12.5%. Major components of adenocarcinomas with wild type (Lys/Lys) were as follows; acinar 40.0%, papillary 40.0%, and solid 20.0%. Thus polymorphism status did not correlated with the major components of adenocarcinomas. No significant association between R497K *EGFR* genotype and patient outcome was seen for the 206 patients from Nagoya City University ($p = 0.1121$). Pathological stages ($p < 0.0001$) but not gender ($p = 0.0696$) was a prognostic factor. In node-negative patients, 119 (28 were dead) were R497 K*EGFR* and 14 (three were dead) were wild type *EGFR*. Thus *EGFR* genotype was not correlated with disease outcome (Log-rank test $p = 0.8882$) (Fig. 3). In node-positive patients, however, 59 (33 were dead) were R497K *EGFR* and 14 (12 were dead) were wild type. Thus R497K *EGFR* was significantly associated with better overall survival (Log-rank test, $p = 0.0072$) (Fig. 4). In this cohort, pathological stage (stage II, $n = 17$ vs. stage III–IV, $n = 56$, $p = 0.2932$) or gender (male, $n = 41$ vs. female, $n = 32$, $p = 0.7957$) was not a prognostic factor. Multi-variate analysis showed that R497K status was a prognostic factor ($p = 0.0104$, relative risk 2.4, 1.229–4.689). We also compared associations between *EGFR* polymorphism status and patient outcome who were treated with platinum-based adjuvant or neo-adjuvant chemotherapy who had undergone surgery. The overall survival of 58 lung cancer patients with follow-up through March 1, 2008 was studied in reference to the *EGFR* polymorphism status. Ten were wild type (eight were dead) and 48 were R497K (23 were dead). The prognosis was significantly worse in *EGFR* wild type than in *EGFR* R497K polymorphism ($p = 0.0038$) (Fig. 5). In this cohort, pathological stages (stage I, $n = 11$, stage II, $n = 14$, stage III–IV, $n = 33$, $p = 0.0445$) but not gender (male, $n = 42$ vs. female, $n = 16$, $p = 0.9103$) was a prognostic factor. However, multi-variate analysis showed none of them was a prognostic factor.

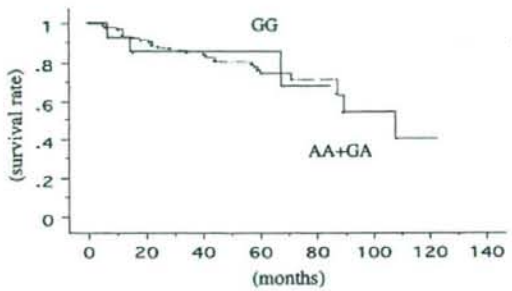

Fig. 3 The overall survival of node-negative lung cancer patients was studied in reference to the *EGFR* (R497K) status. There was no difference of survival between the patient with EGFR wild type (GG) ($n = 14$, 3 were dead) and the patient with R497K *EGFR* (GA or AA) ($n = 119$, 28 were dead) (Log-rank test, $p = 0.8882$)

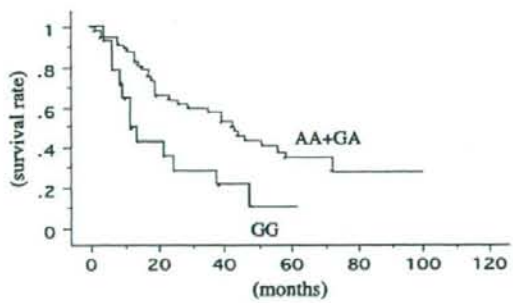

Fig. 4 The overall survival of node-positive lung cancer patients was studied in reference to the *EGFR* (R497K) status. The patients with EGFR wild type (GG) ($n = 14$, 12 were dead, median follow up = 21.7 months) had significantly worse prognosis than the patients with R497K *EGFR* (GA or AA) ($n = 59$, 33 were dead, median follow up = 42.7 months) (Log-rank test, $p = 0.0072$) (relative risk 2.4, 1.229–4.689)

Relationship between clinical courses of lung cancer patients treated with gefitinib and *EGFR*

The overall survival of gefitinib treated lung cancer patients from Nagoya City University, with follow-up through March 1, 2008, was studied in reference to the *EGFR* polymorphism status. Of 206 patients from Nagoya City University, 27 were treated with gefitinib therapy. Total 46 gefitinib treated patients were investigated the R497K polymorphism statuses. In this analysis, 38 patients had *EGFR* polymorphism (AG or GG). The prognosis after gefitinib therapy was not significantly different between *EGFR* wild type patients (GG, 5/8 were dead) and *EGFR* polymorphism patients (AG+GG; 28/38 were dead) ($p = 0.3100$) (Fig. 6).

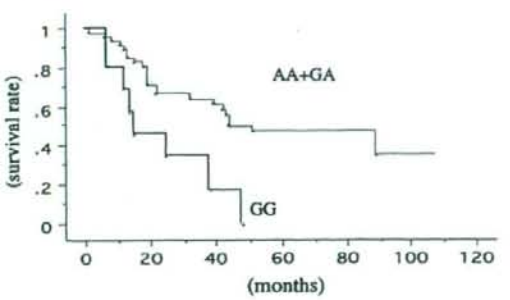

Fig. 5 The overall survival of adjuvant or neo-adjuvant chemo-untreated lung cancer patients was studied in reference to the *EGFR* (R497K) status. The patients with EGFR wild type (GG) ($n = 10$, 8 were dead, median follow up = 23.7 months) had significantly worse prognosis than the patients with R497K *EGFR* (GA or AA) ($n = 48$, 23 were dead, median follow up = 55.1 months) (Log-rank test, $p = 0.0038$)

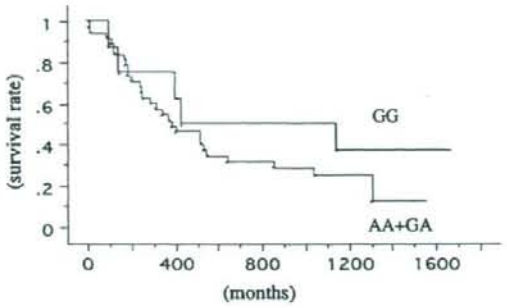

Fig. 6 The overall survival of 46 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* (R497K) status. There was no difference of survival between the patients with EGFR wild type (GG) ($n = 8$, 5 were dead) and the patients with R497K *EGFR* (GA or AA) ($n = 38$, 28 were dead) (Log-rank test, $p = 0.3100$)

Discussion

In the present study, we showed that the R497 polymorphism of EGFR in node-positive lung cancer patients who received curative surgery might account for a longer overall survival. Moreover, this polymorphism was shown to correlate with a better prognosis after platinum-based adjuvant treatment. Although the underlying mechanisms remain unclear, an attenuated ligand interaction and consequential signal transduction might be the main reason for the suboptimal function of this receptor variant (Moriai et al. 1994).

The quantification of certain intratumoral molecules involved in the targeting or metabolism of specific chemotherapeutic agents may be valuable in predicting their efficacies or toxicities in cancer patients. For example, patients with a higher intratumoral level of excision repair cross complementation group 1 (ERCC1), an enzyme involved in nucleotide excision repair, may have a higher resistance to cisplatin-based adjuvant therapy in NSCLC (Olaussen et al. 2006). Moreover, NSCLC patients with a higher class III beta tubulin may have a higher resistance to taxane chemotherapy (Dumontet et al. 2005).

In this report, the R497K *EGFR* SNP(exon 13) is not associated with somatic *EGFR*-TK mutation. Approximately 563 *EGFR*-SNPs have been identified in human genome according to the National Cancer for Biotechnology information database. However, there are few studies examining associations between *EGFR* SNPs and human disease (Shintani et al. 1999; Kang et al. 2005; Fukushima et al. 2006; Zhang et al. 2006; Wang et al. 2007; Liu et al. 2008). In this study, we detected a polymorphism in exon 13 of the *EGFR*-extracellular domain, which changed amino acid Arg (R) to Lys (K), and the K allele seems to decrease the activity of EGFR (Moriai et al. 1994). Previous reports suggested that *EGFR* R497K polymorphism was weakly associated with gefitinib response (Liu et al. 2007). However, in our Japanese cohort, *EGFR* R497K was not associated with response to gefitinib. Although the survival curve of R497K showed higher than *EGFR* wild type (G/G) in our data, the larger number would help to determine the correlation between the R497K polymorphism and gefitinib sensitivity.

Previous report showed that patients with 497 Arg/Arg genotype tended to have a higher risk of local recurrence in chemo-treated rectal cancer patients (Zhang et al. 2005; Brandt et al. 2006). The patients with Arg/Arg genotype showed the highest risk of disease-specificity mortality and none of the patients with the Lys/Lys genotype died throughout the follow-up period of head and neck cancer treated with chemoradiation (Bandres et al. 2007). The mechanism through which the variant human EGFR R497K may account for lower local failures after chemotherapy is unknown (Zhang et al. 2005). A study with Chinese hamster ovary cells, the variant EGFR 497K cell line, showed an attenuated growth response to EGF and transforming growth factor-α, and a reduced induction of the proto-oncogenes fos, jun, and myc (Moriai et al. 1994). It was suggested that the amino acid substitution in the extracellular domain might modulate ligand binding and transmembrane signaling to the intracellular domain (Zhang et al. 2005). Thus, variant EGFR receptor may be less efficient in the recruitment of intracellular substrates and/or cause downstream activation of alternative signaling pathways with decreased proto-oncogene induction or growth stimulation, affecting chemosensitivity. Shintani et al. (1999) demonstrated that another *EGFR*-SNP at position 2073 was correlated with truncated *EGFR* transcription, which might interfere with EGFR three-dimensional structure and EGFR expression.

In summary, R497 polymorphism of EGFR in node-positive lung cancer patients had a better overall survival. R497K*EGFR* polymorphism might be associated with favorable prognosis of advanced lung cancers.

Acknowledgments The authors would like to thank Mrs. Emi Sugiyama for his excellent technical assistances. This work was supported by AstraZeneca Research Grant 2004, Grand-in-Aid for Research in Nagoya City University (2006), and Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science (JSPS) (Nos, 19390367, 18390381,18659407).

Conflict of interest statement None declared.

References

Bandres E, Brarricarte R, Cantero C, Honorato B, Malumbres R, Zarate R, Alcalde J, Garcia-Foncillas J (2007) Epidermal growth

factor receptor (EGFR) polymorphisms and survival in head and neck cancer patients. Oral Oncol 43:713–719

Brandt B, Meyer-Staeckling S, Schmidt H, Agelopoulos K, Buerger H (2006) Mechanisms of egfr gene transcription modulation: relationship to cancer risk and therapy response. Clin Cancer Res 12:7252–7260

Dubey S, Stephenson P, Levy DE, Miller JA, Keller SM, Schiller JH, Johnson DH, Kolesar JM (2006) EGFR dinucleotide repeat polymorphism as a prognostic indicator in non-small cell lung cancer. J Thorac Oncol 1:406–412

Dumontet C, Isaac S, Souquet PJ, Bejui-Thivolet F, Pacheco Y, Peloux N, Frankfurter A, Luduena R, Perol M (2005) Expression of class III beta tubulin in non-small cell lung cancer is correlated with resistance to taxane chemotherapy. Bull Cancer 92:E25–E30

Endo K, Konishi A, Sasaki H, Takada M, Tanaka H, Okumura M, Kawahara M, Sugiura H, Kuwabara Y, Fukai I, Matsumura A, Yano M, Kobayashi Y, Mizuno K, Haneda H, Suzuki E, Iuchi K, Fujii Y (2005) Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay. Lung Cancer 50:375–384

Fukushima T, Favereaux F, Huang H, Shimizu T, Yonezawa Y, Nakazato Y, Ohagki H (2006) Genetic alterations in primary glioblastomas in Japan. J Neuropathol Exp Neurol 65:12–18

Ginsberg RJ, Kris K, Armstrong G (1993) Cancer of the lung. In: Principles and practice of oncology. Lippincott 1993; Fourth Edition, Philadelphia 673–682

Kang D, Gridley G, Huang WY, Engel LS, Winn DM, Brown LM, Bravo-Otero E, Wu T, Diehl SR, Hayes RB (2005) Microsatellite polymorphisms in the epidermal growth factor receptor (EGFR) gene and the transforming growth factor-alpha (TGFA) gene and risk of oral cancer in Puerto Rico. Pharmacogenet Genomics 15:343–347

Keller SM, Adak S, Wagner H, Herskovic A, Komaki R, Brooks BJ, Perry MC, Livingston RB, Johnson DH (2000) A randomized trial of postoperative adjuvant therapy in patients with completely resected stage II or IIIA non-small cell lung cancer. N Engl J Med 343:1217–1222

Liu G, Gurubhagavatula S, Zhou W, Wang Z, Yeap BY, Asomaning K, Su L, Heist R, Lynch TJ, Christiani DC (2008) Epidermal growth factor receptor polymorphisms and clinical outcomes in non-small-cell lung cancer patients treated with gefitinib. Pharmacogenomics J 8:129–138

Liu W, Wu X, Zhang W, Montenegro RC, Fackenthal DL, Spitz JA, Huff LM, Innocenti F, Das S, Cook EH Jr, Cox NJ, Bates SE, Ratain MJ (2007) Relationship of EGFR mutations, expression, amplification, and polymorphisms to epidermal growth factor receptor inhibitors in the NCI60 cell lines. Clin Cancer Res 13:6788–6795

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. New Eng J Med 350:2129–2139

Moriai T, Kobrin MS, Hope C, Speck L, Korc M (1994) A variant epidermal growth factor receptor exhibits altered type a transforming growth factor binding and transmembrane signaling. Proc Natl Acad Sci USA 91:10217–10221

Nicolson RI, Gee JM, Harper ME (2001) EGFR and cancer prognosis. Eur J Cancer 37:S9–S15

Olaussen KA, Dunant A, Fouret P, Brambilla E, Andre F, Haddad Y, Taranchon E, Filipits M, Pirker R, Popper JH, Stahel R, Sabatier L, Pignon JP, Tursz T, Le Chevalier T, Soria JC (2006) DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med 355:983–991

Onn A, Correa AM, Gilcrease M, Isobe T, Massarelli E, Bucana CD, O'Reilly MS, Hong WK, Fidler IJ, Putnum JB, Herbst RS (2004) Synchronous overexpression of epidermal growth factor receptor and HER2-neu protein is a predictor of poor outcome in patients with stage I non-small cell lung cancer. Clin Cancer Res 10:136–143

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Seller WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304:1497–1500

Pao W, Miller V, Zakowski M, Diherty J, Politi K, Sarkaria I, Singth B, Heelman R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kri M, Varmus H (2004) EGF receptor gene mutations are common in lung cancers from 'never smokers'' and are associated with sensitivity of tumors to gefitinib and elrotinib. Proc Natl Acad Sci USA 101:13306–13311

Sasaki H, Endo K, Takada M, Kawahara M, Kitahara H, Tanaka H, Okumura M, Matsumura A, Iuchi K, Kawaguchi T, Kawano O, Yukiue H, Yokoyama T, Yano M, Fujii Y (2007) EGFR exon 20 insertion mutation in Japanese lung cancer. Lung Cancer 58:324–328

Shintani S, Matsuo K, Crohin CC, McBride J, Tsuji T, Donoff RB, Posner M, Todd R, Wong DT (1999) Intragenic mutation analysis of the human epidermal growth factor receptor (EGFR) gene in malignant human oral keratinocytes. Cancer Res 59:4142–4147

Wang WS, Chen PM, Chiou TJ, Liu JH, Lin JK, Lin TC et al (2007) Epidermal growth factor receptor R497K polymorphism is a favorable prognostic factor for patients with colorectal carcinoma. Clin Cancer Res 12:3597–3603

Zhang W, Park DJ, Lu B, Yang DY, Gordon M, Groshen S, Yun J, Press OA, Vallbohmer D, Rhodes K, Lenz HJ (2005) Epidermal growth factor receptor gene polymorphisms predict pelvic recurrence in patients with rectal cancer treated with chemoradiation. Clin Cancer Res 11:600–605

Zhang W, Stabile LP, Keohavong P, Romkes M, Grandis JR, Traynor AM, Siegfried JM (2006) Mutation and polymorphism in the EGFR-TK domain associated with lung cancer. J Thorac Oncol 1:635–647

J Cancer Res Clin Oncol (2008) 134:1371–1376
DOI 10.1007/s00432-008-0411-5

ORIGINAL PAPER

A novel *EGFR* mutation D1012H and polymorphism at exon 25 in Japanese lung cancer

Hidefumi Sasaki · Katsuhiro Okuda · Minoru Takada · Masaaki Kawahara · Naoto Kitahara · Akihide Matsumura · Keiji Iuchi · Tomoya Kawaguchi · Akihiko Kubo · Katsuhiko Endo · Osamu Kawano · Haruhiro Yukiue · Motoki Yano · Yoshitaka Fujii

Received: 12 February 2008 / Accepted: 28 April 2008 / Published online: 14 May 2008

Abstract
Introduction Mutations of the epidermal growth factor receptor (*EGFR*) gene at kinase domain have been reported in non-small-cell lung cancer (NSCLC). However, *EGFR* mutations status at C-terminal domain has not been reported in detail.
Materials and methods We investigated the *EGFR* mutation and polymorphism statuses at C-terminal domain in 398 surgically treated NSCLC cases. Two hundred and sixty-eight adenocarcinoma cases were included. The presence or absence of *EGFR* mutation and polymorphism was analyzed by direct sequences.
Results A novel *EGFR* somatic mutation at exon 25 (G3034, D1012H) was found from 1 of 398 lung cancer patients. During sequencing of *EGFR* C-terminal domain in NSCLC, 194 *EGFR* polymorphism (C2982T) cases were identified at exon 25. The polymorphism statuses were not correlated with gender, smoking status (never smoker vs. smoker), pathological subtypes and *EGFR* mutations. The *EGFR* polymorphism ratio was significantly higher in younger NSCLC (≤60, 56.8%) than in older NSCLC (>60, 45.6%, $P = 0.0467$). The *EGFR* polymorphism ratio was significantly higher in lymph node positive NSCLC (57.4%) than in lymph node negative NSCLC (44%, $P = 0.0168$). In 46 total gefitinib treated NSCLC patients, exon 25 polymorphism was not correlated with prognosis.
Conclusion *EGFR* mutation at C-terminal in lung cancers seemed to be extremely rare, however, this D1012H mutation might be a role in EGFR function. *EGFR* polymorphism at exon 25 might be correlated with progression of NSCLC.

Keywords *EGFR* · D1012H · Lung cancer · Polymorphism · Exon 25

Introduction

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy (Ginsberg et al. 1993). There are much accumulated evidences that epidermal growth factor receptor (*EGFR*) and its family member are strongly implicated in the development and progression of numerous human tumors, including lung cancer (Nicolson et al. 2001; Onn et al. 2004). The *EGFR* tyrosine kinase (TK) inhibitor, gefitinib, was approved in Japan for the treatment of non-small cell lung cancer (NSCLC) since 2002. Phase II and III trial have shown partial responses in 8–12% of unselected patient with progressive NSCLC after chemotherapy (Kris et al. 2003; Thatcher et al. 2005), especially higher response in never smoker, female and Asian ethnicity (more than 20%) (Kris et al. 2003; Fukuoka et al. 2003; Miller et al. 2004). Original two reports showed that *EGFR* mutation statuses at TK domain (exon 18–24) in NSCLC patients were correlated

H. Sasaki (✉) · K. Okuda · K. Endo · O. Kawano · H. Yukiue · M. Yano · Y. Fujii
Department of Surgery II,
Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
e-mail: hisasaki@med.nagoya-cu.ac.jp

M. Takada · M. Kawahara · T. Kawaguchi · A. Kubo
Department of Internal Medicine,
National Hospital Organization,
Kinki-chuo Chest Medical Center, Sakai, Osaka, Japan

N. Kitahara · A. Matsumura · K. Iuchi
Department of Surgery, National Hospital Organization,
Kinki-chuo Chest Medical Center, Sakai, Osaka, Japan