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Cancer Chemother Pharmacol (2008) 62:465–470
DOI 10.1007/s00280-007-0626-1

ORIGINAL ARTICLE

Phase II study of nedaplatin and irinotecan followed by gefitinib for elderly patients with unresectable non-small cell lung cancer

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Received: 10 July 2007 / Accepted: 5 October 2007 / Published online: 25 October 2007

Abstract
Purpose We conducted a phase II study of combination chemotherapy with nedaplatin (NP) and irinotecan (CPT) followed by gefitinib to determine the effects and toxicities in patients 70 years or older with unresectable non-small cell lung cancer (NSCLC).
Methods Eligible patients were entered to receive 3 courses of 50 mg/m^2 NP and 60 mg/m^2 CPT on days 1 and 8 every 4 weeks and sequential gefitinib 250 mg po once a day was followed until tumor progression.
Results Twenty-eight patients received NP and CPT combination chemotherapy. One patient achieved CR, 10 PR, 14 SD and 3 PD, and the response rate was 39.3%. Twenty-one patients received gefitinib 250 mg per day until tumor progression after completion of the NP and CPT chemotherapy. Two patients with SD after NP and CPT chemotherapy achieved PR. For the 3-drug combination, there were 13 responders and the overall response rate was 42.9%. Of the toxicities associated with NP and CPT chemotherapy, grade 4 neutropenia, and grade 3 febrile neutropenia were observed in 24 (33.8%) and 3 (4.2%) courses, respectively. Of the toxicities associated with gefitinib treatment, grade 3 anemia, and SGOT and SGPT elevation were observed in one patient (4.8%) each, respectively. The median survival time was 8.7 months, and the 1- and 2-year survival rates were 42.9 and 32.1%, respectively.
Conclusion NP and CPT followed by gefitinib is feasible for elderly patients with unresectable NSCLC.

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Keywords Nedaplatin · Irinotecan · Gefitinib · Lung cancer · Elderly

Introduction

Current chemotherapy regimens for metastatic non-small cell lung cancer (NSCLC) are not particularly effective. Regimens based on combinations of new anticancer agents such as vinorelbine, gemcitabine, docetaxel and paclitaxel with platinum compounds have emerged as a gold standard for such patients [1].

In a subset analysis of randomized trials, the response rate, toxicity and survival rates in fit, elderly patients with NSCLC receiving platinum-based treatment appeared to be similar to those in younger patients [2]. However, elderly patients with normal organ function had been selected as subjects for the analysis. A feasibility study of standard cisplatin-based chemotherapy in elderly lung cancer patients with normal organ function showed that only 29% satisfied the eligibility criteria, and that these patients experienced severe neutropenia after cisplatin-based chemotherapy [3]. It is generally believed that elderly patients are less able to tolerate aggressive chemotherapy than their younger counterparts. The randomized Elderly Lung Cancer Vinorelbine Study Group trial demonstrated that elderly patients treated with vinorelbine—in combination with best supportive care (BSC)—have a significantly improved chance of survival and quality of life in comparison with patients treated with BSC alone [4]. The Multicenter Italian Lung Cancer in the Elderly Study trial demonstrated that the use of a combination of gemcitabine plus vinorelbine in this patient population did not further improve the survival rate or quality of life in comparison with either vinorelbine or gemcitabine monotherapy [5]. Thus, standard combination chemotherapy

has not been established for elderly patients with advanced NSCLC.

Three-dimensional analysis models have demonstrated a remarkable synergistic interaction of concurrently administered nedaplatin (NP) and irinotecan (CPT) [6]. In our previous phase I/II study, a combination of NP and CPT showed high activity against NSCLC: the response rate was 31.0%, and the 1-year survival rate was 45.2% [7]. A phase II study of combination chemotherapy with NP and CPT in 38 patients aged 70 years or older with advanced NSCLC demonstrated a 65.8% response rate, a median survival time of 418 days, and a 1-year survival rate of 55.3% [8]. However, seven of the 38 patients could not receive a second cycle of the chemotherapy because of toxicities such as vomiting, diarrhea and febrile neutropenia. Dose or schedule modifications are therefore required to make the NP and CPT combination safe for elderly patients.

The epidermal growth factor receptor (EGFR) superfamily was identified early on as a potential target for therapy of solid tumors. Given the biological importance of the EGFR molecular network in carcinomas, several molecules that can inhibit the EGFR tyrosine kinase domain have been synthesized. The inhibitor gefitinib at 250 mg per day demonstrated an 18.4% objective response in 103 patients with previously treated advanced NSCLC [9]. Adverse events associated with use of the drug were mainly skin reactions and diarrhea. As no hematological adverse events or infections related to chemotherapy safety in elderly patients with NSCLC were observed in a trial of gefitinib at 250 mg per day, gefitinib treatment is considered to be feasible for such patients.

Here we report a phase II study of combination chemotherapy with NP and CPT followed by sequential gefitinib treatment for elderly patients with advanced NSCLC. We modified the NP arm so that it was divided on days 1 and 8, in order to ensure safety and to allow continuous use of gefitinib after completion of the NP and CPT chemotherapy until tumor progression.

Patients and methods

The Institutional Review Board of Kanagawa Cancer Center reviewed and approved this study prior to commencement.

Patients

Patients with histologically or cytologically proven unresectable NSCLC were registered for the NP and CPT combination followed by gefitinib chemotherapy. Eligibility criteria for the chemotherapy were: no prior chemotherapy, expected survival of at least 6 weeks, age $\geq$ 70 years, Eastern Cooperative Oncology Group PS score $\leq$ 2, leukocyte count $\geq$ 4,000/μl, hemoglobin count $\geq$ 9 g/dl, platelet count $\geq$ 100,000/μl, total serum bilirubin $\leq$ 1.5 mg/dl, aspartate aminotransferase and alanine aminotransferase $\leq$ 90 IU/l, serum creatinine $\leq$ 1.5 mg/dl, and creatinine clearance more than 40 ml/min. We did not attempt their geriatric assessment in the present study. Patients experiencing postoperative recurrence and patients who had received radiotherapy for metastatic lesions were eligible for the present study, and at least 4 weeks' rest was required after prior surgery or radiation therapy. Patients with massive pleural effusion, pericardial effusion, symptomatic brain metastasis, paralytic ileus, severe infection or pneumonitis were excluded. Patients with uncontrolled ischemic heart disease, severe cardiac insufficiency, hypertention or diabetes mellitus were also excluded. Written informed consent was obtained in every case.

Chemotherapy

Patients exhibiting no progression of the disease were treated every 4 weeks with 60 mg/m^2 CPT and 50 mg/m^2 NP on days 1 and 8. Patients received 5-HT$_3$ antagonist IV and 8 mg dexamethasone IV before administration of the anticancer drugs. Both drugs were administered on day 8 when the following criteria were satisfied: leukocyte count $\geq$ 3000/μl, neutrophil count $\geq$ 1,500/μl, platelet count $\geq$ 75,000/μl, non-hematologic toxicity of less than grade 2 except for alopecia, and leukocyte or neutrophil count greater than 1,000/μl or 500/μl respectively during the period between day 2 and 8. Recombinant human granulocyte colony-stimulating factor (G-CSF), 50 mg/m^2 per day or 2 μg/kg per day, was administered subcutaneously once a day when the patient's leukocyte or neutrophil counts were below 1,000 and 500/μl, respectively. Subsequent cycles of chemotherapy were started when patients were able to satisfy the organ function eligibility criteria, with the exceptions of hemoglobin count and creatinine clearance, for entry to the study. The doses of CPT and NP were reduced by 10 mg/m^2 for the subsequent cycle if dose-limiting toxicities (DLT) were observed, such as grade 4 neutropenia lasting $\geqq$4 days or grade 4 neutropenia with fever $\geqq$38°C, grade 4 thrombocytopenia, other grade 4 blood/bone marrow toxicities, except for leukocyte and hemoglobin toxicities, grade 4 vomiting, grade 4 anorexia, grade 4 constipation, grade 4 stomatitis/pharyngitis, grade 4 metabolic/laboratory toxicities, grade 4 coagulation toxicities, or grade 3 or 4 other non-blood/bone marrow toxicities, except for nausea and vomiting. The NP and CPT chemotherapy was repeated for a maximum of three cycles unless the disease progressed, or if severe toxicities developed, such as septic shock, irreversible renal failure, grade 4 hepatic toxicity, grade 4 cardiovascular toxicity, grade 4 pulmonary toxicity, grade 4 diarrhea, grade 4 CNS cerebrovascular

ischemia, or grade 4 CNS hemorrhage/bleeding. Tumor responses were evaluated according to the RECIST criteria [10]. Toxicities were evaluated according to the NCI-CTC ver.2 criteria [11].

Sequential chemotherapy with gefitinib 250 mg po once a day was started after completion of the NP and CPT combination chemotherapy when the following criteria were satisfied: PS score ≤ 2, leukocyte count $\geq 4{,}000/\mu l$, hemoglobin count ≥ 9 g/dl, platelet count $\geq 100{,}000/\mu l$, total serum bilirubin ≤ 1.5 mg/dl, aspartate aminotransferase and alanine aminotransferase ≤ 90 IU/l, serum creatinine ≤ 1.5 mg/dl. Sequential gefitinib treatment was interrupted for a maximum 14 days until the toxicities became less than grade 2, if grade 4 hematological toxicities, grade 3 skin toxicity, grade 3 diarrhea, or grade 3 other non-hematological toxicities appeared. The sequential chemotherapy was stopped if the disease progressed, toxicities did not recover to grade 0 or 1 within 14 days, 2 breaks of treatment were required due to toxicities, or patients refused the treatment.

Study design

We chose a 60% response rate as a desirable target level for the NP and CPT regimen, and considered a 40% response rate as not significant. The study design had the power to detect responses greater than 90%, with less than 10% error. Therefore, we required 28 assessable patients in the first stage and 13 in the second stage, according to the Minimax design of Simon [12]. We decided to stop the study if less than 11 patients responded to NP and CPT in the first stage. This regimen was defined as active if the number of responders out of 41 patients was $\geqq 21$, and inactive if the number of responders was $\leqq 20$ [12, 13]. Overall survival was estimated using the method of Kaplan and Meier.

We also defined toxic regimen when one-third patients experienced grade 4 thrombocytopenia, grade 3 neutropenic fever or other grade 3 non-hematological toxicities in this study.

Results

Between November 2002 and July 2005, 28 patients were registered in the study. Patient characteristics are summarized in Table 1. Twenty patients were male and 8 were female, with a median age of 74 years (range 70–81 years). Six patients had a performance status (PS) of 0 and the other 22 patients had a PS of 1. Twenty-three patients had adenocarcinoma, 4 had squamous cell carcinoma, and 1 had non-small cell carcinoma. Seven and 21 patients were stage IIIB and stage IV, respectively. All 28 patients were assessed for response, toxicities and survival. Twenty-five patients received 2 or 3 cycles of NP and CPT combination chemotherapy. Three patients dropped out the study after the first cycle of NP and CPT chemotherapy: 1 with disease progression, 1 with febrile neutropena requiring 15 days for improvement, and 1 with grade 2 diarrhea and grade 3 CNS cerebrovascular ischemia. Treatment-related toxicities during the total 71 courses of NP and CPT chemotherapy are listed in Table 2. Of the hematological toxicities, grade 4 anemia and neutropenia were observed during 2 (2.8%) and 24 (33.8%) courses, respectively. There was no grade 4 thrombocytopenia, and none of the patients required transfusion. Of the non-hematological toxicities, grade 3 febrile neutropenia was observed in three courses (4.2%). Grade 3 diarrhea and grade 3 CNS cerebrovascular ischemia was observed in 1 course (1.4%) each, respectively. Other non-hematological toxicities were mild. The outcome of the NP and CPT regimen in 28 patients were 1 CR, 10 PR, 14 SD and three PD, and the response rate was 39.3%. Thus, the study was stopped in the first stage.

Twenty-one patients received sequential gefitinib treatment, and 7 patients were unable to do so, 3 because of decreased PS, 3 due to refusal, and 1 because of the need for whole brain irradiation for progressive brain metastasis. The median duration of sequential gefitinib treatment was 68 days (range 21–932 days). Two patients, whose

Table 1 Patient characteristics

	No. of patients
Total	28
Age (years)	
Median	74
Range	70–81
Gender	
Male	20
Female	8
Performance status (ECOG)	
0	6
1	22
Smoker	22
Clinical stage	
IIIB	7
IV	18
Postoperative recurrence	3
Histology	
Adenocarcinoma	23
Others	5
No. of metastatic organs	
1	16
$\geqq 2$	5
Brain metastasis	1

Table 2 Toxicities in NP and CPT combination chemotherapy

	Grade (NC I-CTC ver.2)					
	0	1	2	3	4	Grade 3, 4 (%)
Hemogbbin	7	21	27	14	2	22.5
Leukocytes	10	9	24	23	5	39.4
Neutrophils	10	5	10	22	24	64.8
Platelets	19	27	9	16	0	22.5
Bilirubin	68	1	2	0	0	–
SGOT	56	15	0	0	0	–
SGPT	63	7	1	0	0	–
Creatinine	62	7	2	0	0	–
Fatigue	2	48	16	5	0	7.0
Fever	65	6	0	0	0	–
Alopecia	48	22	1	0	0	–
Rash/desquation	68	3	0	0	0	–
Diarrhea	37	27	6	1	0	1.4
Nausea-vomiting	40	25	6	0	0	–
Febrile neutrophenia	62	6	0	3	0	4.2
CNS cerebrovascular ischemia	70	0	0	1	0	1.4
Neuropathy	71	0	0	0	0	–
Pneumonitis	71	0	0	0	0	–

response to NP and CPT was SD, responded to gefitinib treatment, and the overall response rate for NP and CPT followed by gefitinib was 42.9%. Treatment-related toxicities for the total of 21 patients who received gefitinib treatment are listed in Table 3. Of the hematological toxicities, grade 3 anemia was observed in one patient (4.8%). Of the non-hematological toxicities, infection with grade 3 SGOT and SGPT elevation was observed in one patient (4.8%). Other hematological and non-hematological toxicities were mild.

The overall survival curve is shown in Fig. 1. Five patients survived and the other 23 patients died during the follow-up period. The median survival time was 8.7 months. The 1- and 2-year survival rates were 42.9 and 32.1%, respectively.

Discussion

The combination of NP with CPT followed by gefitinib treatment showed activity against NSCLC in the present study. We chosen 60% response rate as a desirable target level in NP and CPT regimen. The responders in 28 entered patients of first stage were required 12 patients, the responders were 11 and this regimen was concluded inactive. However, two patients, whose response to NP and CPT was SD, responded to gefitinib treatment. Thus, overall response rate 42.9% for NP and CPT followed by gefitinib was considered to be active. A previous study of NP and CPT combination chemotherapy showed that it was effective for elderly patients with advanced NSCLC, although 29% of patients experienced febrile neutropenia [8]. In the present study design, we defined toxic when one third patients experienced grade 4 thrombocytopenia, grade

Table 3 Toxicities in gefitinib treatment

	Grade (NC I-CTC ver.2)					
	0	1	2	3	4	Grade 3, 4 (%)
Hemogbbin	1	12	7	1	0	4.8
Leukocytes	16	4	1	0	0	–
Neutrophils	18	2	1	0	0	–
Platelets	15	4	2	0	0	–
Bilirubin	19	2	0	0	0	–
SGOT	13	7	0	1	0	4.8
SGPT	16	4	0	1	0	4.8
Creatinine	17	3	1	0	0	–
Fatigue	1	18	2	0	0	–
Fever	21	0	0	0	0	–
Alopecia	19	2	0	0	0	–
Dry skin	12	9	0	0	0	–
Nail change	20	1	0	0	0	–
Pruritis	13	8	0	0	0	–
Rash/desquation	7	12	2	0	0	–
Anorexia	20	1	0	0	0	–
Diarrhea	15	6	0	0	0	–
Gastritis	20	1	0	0	0	–
Nausea-Vomiting	19	2	0	0	0	–
Epistaxix	20	1	0	0	0	–
Infection	20	0	1	0	0	–
Neuropathy	21	0	0	0	0	–
Pneumonitis	21	0	0	0	0	–

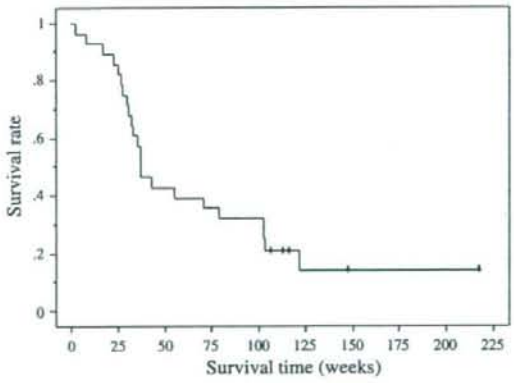

Fig. 1 Survival curves constructed by the Kaplan–Meier method. Five of the 28 patients were alive, the MST was 8.7 months, and the 1- and 2-year survival rates were 42.9 and 32.1%, respectively

3 neutropenic fever or other grade 3 non-hematological toxicities. Only five patients (17.9%) experienced these toxicities and the treatment was concluded to be safe. We also considered the incidence of 33% for grade 4 neutropenia and 4.2% for grade 3 neutropenic fever to be acceptable in this study. Although the response rate of 39.3% for NP and CPT chemotherapy was not high, 25 of 28 patients (89.3%) were able to receive 2–3 cycles of the combination chemotherapy. Division of the NP arm on days 1 and 8 with CPT was confirmed to be safe for elderly patients with NSCLC.

Sequential gefitinib treatment resulted in tumor regression in only 2 of 21 patients (9.5%) achieving SD or PD with NP and CPT treatment. We considered that this small adjuvant effect of gefitinib after NP and CPT treatment may have been due to gefitinib resistance in most of the elderly patients who entered the trial. However, the response rate in the present study was higher than that in a study of gefitinib monotherapy for 40 elderly patients with pretreated NSCLC, which demonstrated a 5% objective response [14]. Responsiveness to gefitinib has been demonstrated in distinct subgroups of patients, such as women, patients who have never smoked, patients with adenocarcinoma, and Asians [15–17]. Twenty-two and 21 of the 28 patients registered in this study were smokers and males, respectively. Only four patients in this study were women who had never smoked, and were sensitive to gefitinib. This may have accounted for the small impact of gefitinib treatment in this study. Median survival time was 8.7 months, but nine patients (32.1%) survived more than 2 years. The presence of such long survivors suggested that gefitinib treatment could be effective for some elderly patients who are gefitinib-sensitive. Although the level of EGFR protein expression is not associated with the response to gefitinib, specific missense and deletion mutations in the tyrosine kinase domain of the EGFR gene have been reported to be associated with gefitinib sensitivity [18, 19]. A retrospective study demonstrated that NSCLC patients with EGFR mutations had a better outcome with gefitinib treatment than patients with the wild-type EGFR gene [20]. Our recent study has also demonstrated a significantly higher gefitinib response in patients with EGFR mutation than in those with wild-type EGFR (90.9 vs. 14.3%), and significantly longer overall and progression-free survivals in patients with EGFR mutation [21]. Unfortunately, the patients in the present study were not analyzed their EGFR genetic status, gefitinib treatment seems to be of some benefit to patients with EGFR mutation.

In conclusion, sequential gefitinib treatment added to NP and CPT combination chemotherapy does not improve the response rate but can have a longer survival benefit for at least some elderly patients with advanced NSCLC. Gefitinib treatment can be considered when candidate patients have EGFR mutation in NSCLC.

Acknowledgments This work was supported in part by Kanagawa Health Foundation and Kanagawa Prefectural Hospitals Cancer Research Fund.

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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

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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

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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

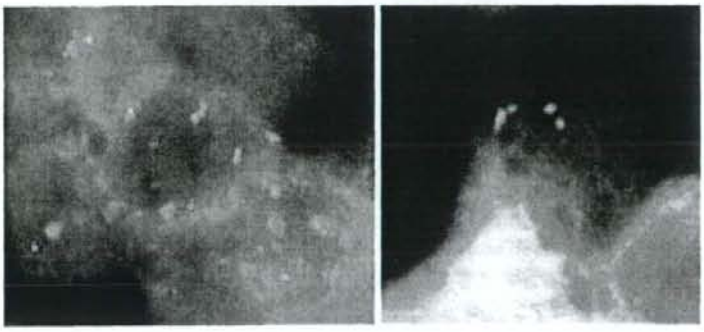

Fig. 1 FISH analysis for lung cancer tissues. *Left* high polysomy case (4 copy numbers in cells >40%), *right* disomy case

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

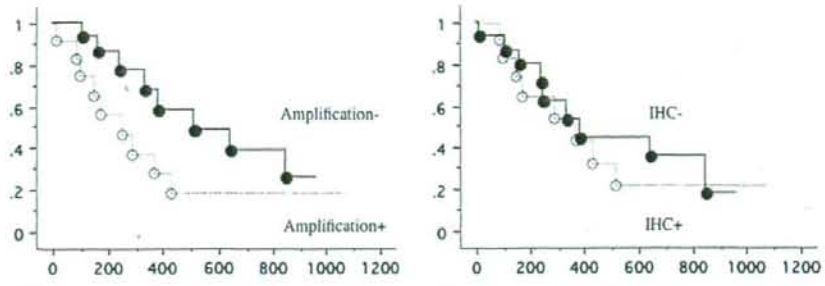

Fig. 2 The overall survival of 27 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* amplification status (*left*) and EGFR protein expression (*right*). Prognosis from patients with EGFR amplification ($n = 12$, 9 were dead) and without *EGFR* amplification ($n = 15$, 8 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; $P = 0.9105$)

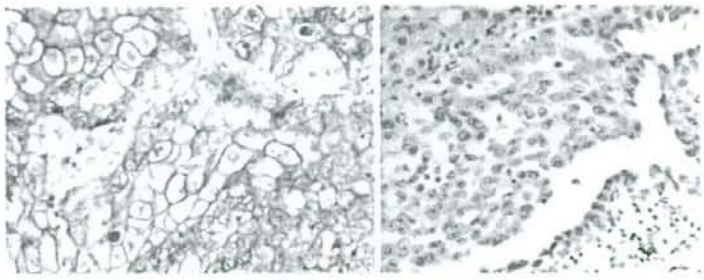

Fig. 3 EGFR protein expression by immunohistochemistry. *Left* positive case, *right* negative case

(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

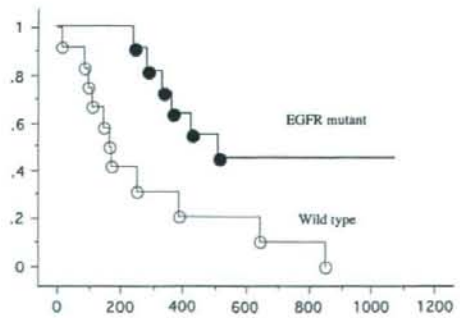

Fig. 4 The overall survival of 27 gefitinib-treated lung cancer patients was studied in reference to the *EGFR* mutation status. Prognosis from patients with EGFR mutations ($n = 15$, 6 were dead) was significantly better than the patients without *EGFR* mutations ($n = 12$, 11 were dead) (log-rank test,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

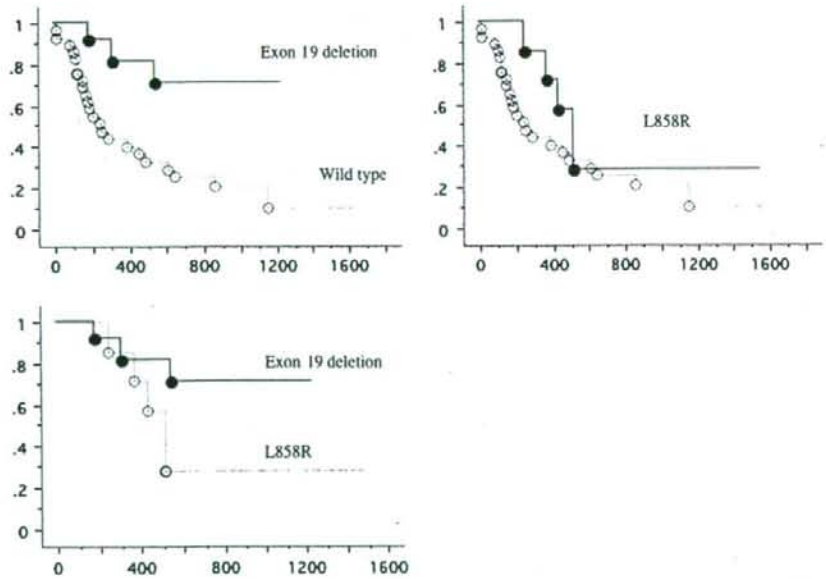

Fig. 5 The overall survival of 54 gefitinib-treated lung cancer patients was studied in reference to the *EGFR* mutation status. *Left upper* prognosis from patients with exon 19 deletion mutations ($n = 12$, 3 were dead) was significantly better than the patients without *EGFR* mutations (Log-rank test, $P = 0.0032$, 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)

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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

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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

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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

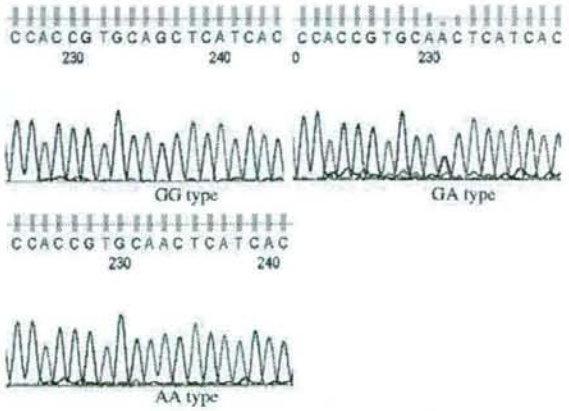

FIG. 1. The sequence results of *EGFR* exon 20. Left upper; wild type (GG). Right upper; heterozygous SNP (GA). Left lower; homozygous SNP (AA). (Color version of figure is available online).

TABLE 1

Clinicopathological Data of 303 Lung Cancer Patients

Factors	EGFR		
	GG patients	GA + AA patients	*P* value
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

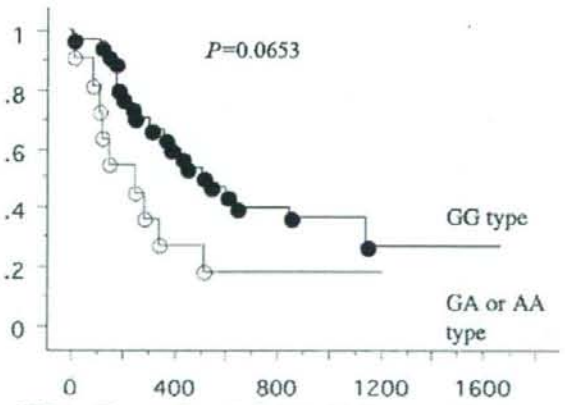

FIG. 2. The overall survival of 46gefitinib untreated lung cancer patients was studied in reference to the EGFR polymorphism (G2607A) status. There was a tendency toward better prognosis from patient with EGFR wild type (GG) ($n = 35$, 21 were deceased) than the patient with EGFR polymorphism (GA or 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).

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