with response to gefitinib treatment (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; Pao et al. 2004; Shigematsu et al. 2005) or European patients (Shigematsu et al. 2005; Marchetti et al. 2005). Although original two groups have sequenced whole *EGFR* gene, they found no mutation within C-terminal of *EGFR* (Paez et al. 2004; Lynch et al. 2004).

C-terminal domain of the EGFR plays an integral role in regulation of the kinase. In particular, kinetic analyses of the EGFR indicated that the C-terminal domain modulated receptor function by virtue of repressing kinase activity in the absence of autophosphorylation (Bertics and Gill 1985; Bertics et al. 1988). To determine *EGFR* mutation status at C-terminal domain in Japanese lung carcinoma, we investigated *EGFR* gene status by direct sequences. The findings were compared to the clinico-pathologic features of lung cancer.

Materials and methods

Patients and samples

The study group included 374 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2006. Mean age was 65.1 years old and median age was 67 years old. 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. We have also investigated *EGFR* SNP status for 24 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. The clinical and pathological characteristics of the 398 lung cancer patients were as follows; 270 (67.8%) were male 128 were female. Two hundred and sixty-eight (67.3%) were diagnosed as adenocarcinoma, and 130 were diagnosed as other types of carcinoma. Two hundred and sixty (65.3%) were smoker and 138 were non-smoker. Of 374 patients from Nagoya City University, 218 (57.8%) were stage I.

PCR assays for *EGFR* mutations

Total RNA was extracted from lung cancer tissues and adjacent non-malignant lung tissues using Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturers' instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/ml. About ten cases were excluded because tumor cells were too few to sufficiently extract tumor RNA. 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 reaction mixture was incubated at 42°C for 50 min and then at 72°C for 15 min. We then used 1 μl of each DNA for PCR analyses. The PCR reactions were performed using LA-Taq kit (Takara Bio Inc., Shiga, Japan) in a 25-μl reaction volume. The primer sequences for *EGFR* gene for C-terminal domain (exon 23–28) were as follows: the forward primer, 5-GGGAGTTGATGACCTTTGGA-3 and the reverse primer, 5-TTCTGCATTTTCAGCTGTGG-3 (875 bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 45 s, 58°C for 45 s, 72°C for 60 s. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). Genomic DNA was extracted from lung cancer tissues ($n = 91$) and adjacent peripheral leukocyte ($n = 20$) using Wizard SV Genomic DNA purification Systems (Promega) according to the manufacturers' instructions. The primer sequences for *EGFR* gene at exon 25 were as follows: the forward primer, 5-TAAGGC ACCCACATCATGTCA-3 and the reverse primer, 5-TGG ACCTAAAAGGCTTACATCAA-3 (Paez et al. 2004). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 45 s, 64°C for 45 s, 72°C for 45 s. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review. The results of *EGFR* mutation statuses at kinase domain were already reported (Endo et al. 2005; Sasaki et al. 2005, 2006).

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, USA), and was considered significant when the *P* value was less than 0.05.

Results

EGFR gene mutation status in Japanese lung cancer patients

We have sequenced for C-terminal of *EGFR* gene from 286 NSCLC samples. Of 286 patients, from direct sequencing using cDNA samples, we found only one mutation at exon 25 (G3034C, D1012H). Matched normal lung tissues showed wild type sequence suggested this mutation was somatic (Fig. 1). This patient was male, non-smoker with well differentiated adenocarcinoma. Pathological stage was T2N0 (stage Ib). The patient also had the deletion type mutation in exon 19. We have additionally sequenced at exon 25 of *EGFR* gene from 88 NSCLC samples. However, from direct sequencing using genomic DNA samples, no mutation was found. Comparison of protein sequences indicated that D1012 was highly conserved with other erbB family protein, such as Her2 and erbB4 (Fig. 2).

In exon 18 or exon 21, 52 patients had the missense point mutations (1 G719S, 3 G719C, 48 L858R and 2 L861Q). Four patients had exon 20 insertion mutations, and 52 patients had exon 19 deletion mutations. Of these 111 patients, 40 were male and 71 were female. Seventy-nine were non-smokers and 32 were smokers. One hundred and four patients had adenocarcinoma, four had squamous cell carcinoma 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, $P < 0.0001$).

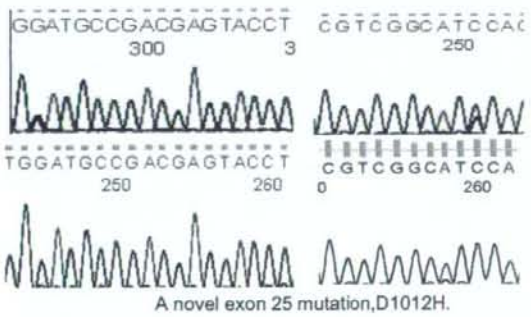

Fig. 1 A novel mutation, D1012H, at exon 25. *Left upper*; forward sequence from lung cancer samples. *Left lower*; forward sequence from adjacent normal lung tissue. *Right upper*; reverse sequence from lung cancer samples. *Right lower*; reverse sequence from adjacent normal lung tissue

HumanEGFR	1007	MDDVVDADEYL	1017
MouseEGFR	1007	MEDVVDADEYL	1017
Her2	1015	MGDLVDAEEYL	1025
ErbB4	1013	LEDMMDAEEYL	1023

Fig. 2 Comparison of protein sequences indicated that D1012 was highly conserved with other erbB family protein, such as Her2 and erbB4

EGFR polymorphism at exon 25

During sequencing of the *EGFR* C-terminal domain in lung cancer samples, a sequence difference in exon 25 (C2982T; D994D) was found (Fig. 3). Of 398 patients, 194 patients had the *EGFR* polymorphism. The sequencing results from adjacent peripheral leukocyte showed the same results. One hundred and thirty-six were male and 58 were female. Sixty-seven were non-smokers and 127 were smoker. One hundred and twenty-eight patients had adenocarcinoma and 66 had other types of lung cancers. Of 374 patients from Nagoya City University, 180 (48.1%) had the polymorphism. The polymorphism did not correlate with pathological stages ($P = 0.5400$). The *EGFR* polymorphism ratio was significantly higher in lymph node positive NSCLC (66/115, 57.4%) than in lymph node negative NSCLC (114/259, 44%, $P = 0.0168$).

The polymorphism did not correlate with gender ($P = 0.3457$), smoking status ($P = 0.9552$), pathological subtypes ($P = 0.5734$) and *EGFR*-TK mutation status of lung cancer ($P = 0.7447$) (Table 1). The *EGFR* polymorphism ratio was significantly higher in younger NSCLC (≤60, 56.8%) than in older NSCLC (>60, 45.6%, $P = 0.0467$), although the P value was marginal.

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

The overall survival of gefitinib treated lung cancer patients with follow-up through December 30, 2006, was studied in reference to the *EGFR* polymorphism status. Of 377

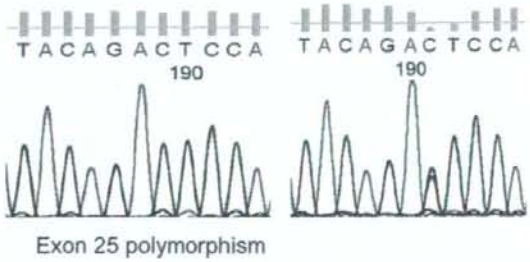

Fig. 3 The sequence results of *EGFR* exon 25. *Left*; wild type (CC). *Right upper*; heterozygous SNP (CT)

Table 1 Clinico-pathological data of 398 lung cancer patients

Factors	EGFR		*P* value
	CC patients (%)	CT + TT patients (%)	
pStage			
I	116 (59.8)	102 (56.7)	0.5400
II–IV	78 (40.2)	78 (43.3)	
Lymph node (meta)			
Negative	145 (74.7)	114 (63.3)	0.0168
Positive	49 (25.3)	66 (36.7)	
Smoking			
Non-smoker	71 (34.8)	67 (34.5)	0.9552
Smoker	133 (65.2)	127 (65.5)	
Pathological subtype			
Adenocarcinoma	140 (68.6)	128 (66.0)	0.5734
Others	64 (31.4)	66 (34.0)	
EGFR mutation			
Positive	63 (30.8)	57 (29.4)	0.7447
Negative	141 (69.2)	137 (70.6)	
Age			
≤60	48 (23.5)	63 (29.9)	0.0467
>60	156 (76.5)	131 (70.1)	
Gender			
Male	134 (65.7)	136 (70.1)	0.3457
Female	70 (34.3)	58 (29.9)	

NS not significant, *Adeno* adenocarcinoma

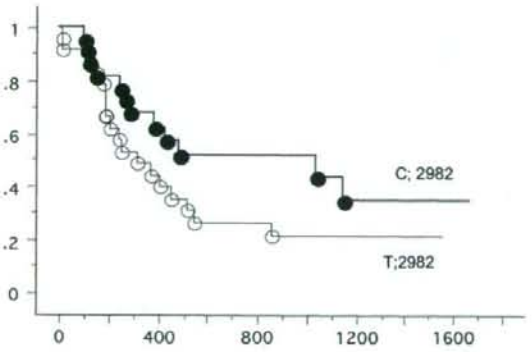

Fig. 4 The overall survival of 46 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* polymorphism (C2982T) status. The prognosis was not significantly different between the patient with EGFR wild type (CC) ($n = 22$, 12 were dead) than the patient with *EGFR* polymorphism (CT or TT) ($n = 24$, 18 were dead) (Log-rank test, $P = 0.1471$)

patients from Nagoya City University, 22 were treated with gefitinib therapy. Total 46 gefitinib treated patients were investigated with the C2982T polymorphism statuses. In this analysis, 24 patients had *EGFR* polymorphism (CT or TT). The prognosis was not different between in *EGFR* wild type patients (CC; 12/22 were dead) and in *EGFR* polymorphism patients (CT + TT; 18/24 were dead) ($P = 0.1471$) (Fig. 4).

Discussion

We have found a novel D1012H (G3034C) mutation at C-terminal domain of *EGFR* gene. This mutation was very rare (0.2%) somatic mutation. We also obtained findings that C2982T *EGFR* polymorphism was existed in 49% of Japanese lung cancer, and the *EGFR* polymorphism ratio was significantly higher in lymph node positive NSCLC (57.4%) than in lymph node negative NSCLC (44%). However, none of other clinico-pathological factors were correlated with the polymorphism. The *EGFR* polymorphism ratio was significantly higher in younger NSCLC, although the *P* value was marginal.

In this report, we found a novel somatic *EGFR* mutation (D1012H) within *EGFR*-C-terminal domain. The-C-terminal phosphorylation domain of the EGFR is believed to regulate protein kinase activity as well as mediate the assembly of signal transduction complexes (Lee et al. 2006). It was shown that truncation of the C-terminal domain enhanced the affinity of the nucleotide binding site for TNP-ATP, suggesting that the C-terminal autophosphorylation domain of the EGFR modulates the nucleotide-binding properties of the protein TK domain (Cheng and Koland 1996). In addition, the computational analyses, based on the three-dimensional structure of EGFR's kinase domain suggested that direct contact between the kinase and a segment from the C-terminal regulatory domains inhibits enzymatic activity (Landau et al. 2004). More recently, it has been reported that EGFR C-terminal sequences 1005–1017 and di-leucine motif (1,010) LL (1,011) are essential in EGFR endocytosis (Wang et al. 2007). Graduate truncation within 991–1044 of EGFR showed progressively lower EGF-induced EGFR endocytosis with most significant effects observed for residues 1005–1017 (Wang et al. 2007). The residues 1005–1017 were also required for EGFR internalization triggered by non-ligand-induced receptor internalization. Comparison of protein sequences indicated that D1012 was highly conserved with other erbB family protein, such as Her2 and erbB4, suggested that the sequence was important. However, D1012H mutation was found in only one patient from our cohort and this patient also had deletion mutation in exon 19. This finding would indicate that D1012H mutation was lacking of strong impact in EGFR function in Japanese lung cancers.

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 (Zhang et al. 2006; Fukushima et al. 2006; Kang et al. 2005; Shintani et al. 1999; Liu et al. 2008). In this study, we detected a polymorphism in exon 25 of the *EGFR* C-terminal domain at nucleotide 2982, codon 994 (Asp), which changed nucleotide 2982 from C to T, without amino acid substitution. The *EGFR* polymorphism ratio was significantly higher in lymph node positive NSCLC than in lymph node negative NSCLC. Thus C2982T polymorphism might be associated with the aggressive behavior of lung cancers. It remains verified whether the *EGFR* C2982T changes *EGFR* expression or function (Zhang et al. 2006; Fukushima et al. 2006). Even if there is no amino acid change, the *EGFR* polymorphism identified here might led to difference in *EGFR* gene transcription, mRNA stability, or translation, or could be a genetic marker of another risk-associated genotype. 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. These might be explanation for higher *EGFR* polymorphism ratio in younger NSCLC, probably correlated with early onset of lung cancers. However, if we used cut-off value of 65 or 66 years old, the *EGFR* polymorphism ratio was not different between old and young patients.

In summary, *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.

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Conflict of interest None declared.

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ORIGINAL PAPER

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

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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

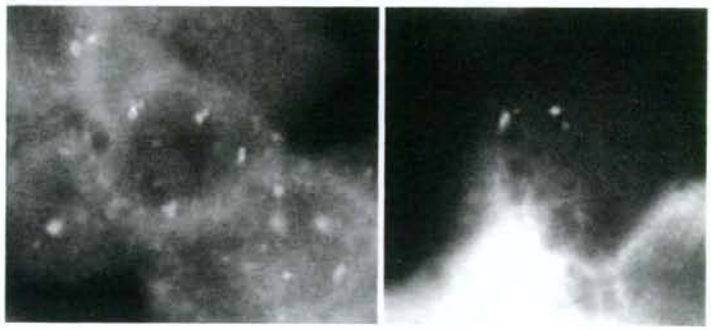

Fig. 1 FISH analysis for lung cancer tissues. *Left* high polysomy case (4 copy numbers in cells >40%), *right* disomy case

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

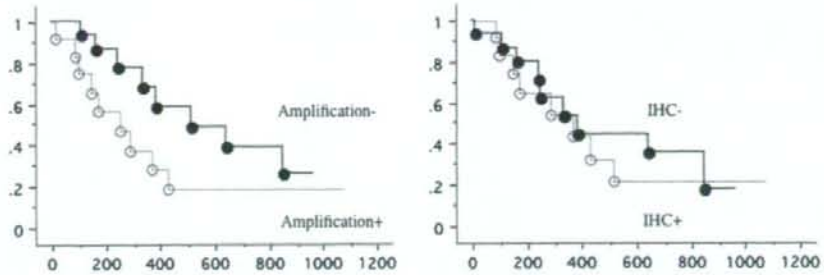

Fig. 2 The overall survival of 27 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* amplification status (*left*) and EGFR protein expression (*right*). Prognosis from patients with EGFR amplification ($n = 12$, 9 were dead) and without *EGFR* amplification ($n = 15$, 8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

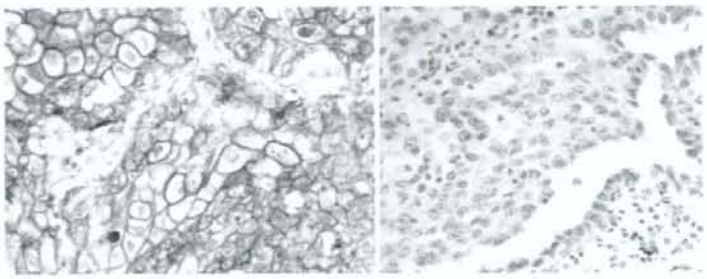

Fig. 3 EGFR protein expression by immunohistochemistry. *Left* positive case, *right* negative case

(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

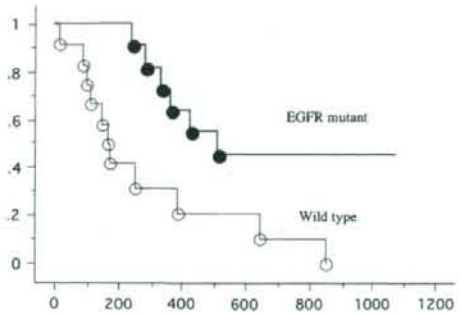

Fig. 4 The overall survival of 27 gefitinib-treated lung cancer patients was studied in reference to the *EGFR* mutation status. Prognosis from patients with EGFR mutations ($n = 15$, 6 were dead) was significantly better than the patients without *EGFR* mutations ($n = 12$, 11 were dead) (log-rank test,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

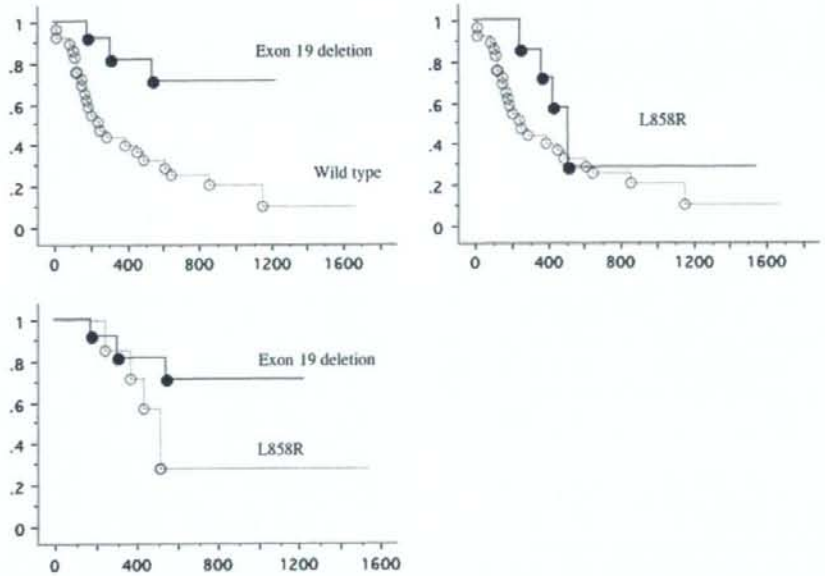

Fig. 5 The overall survival of 54 gefitinib-treated lung cancer patients was studied in reference to the *EGFR* mutation status. *Left upper* prognosis from patients with exon 19 deletion mutations ($n = 12$, 3 were dead) was significantly better than the patients without *EGFR* mutations (Log-rank test,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

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Phase I/II Study of Docetaxel and S-1 in Patients with Previously Treated Non-small Cell Lung Cancer

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Introduction: The aim of this study was to determine and evaluate the recommended dose of docetaxel in combination with a novel oral 5-fluorouracil analogue S-1 and evaluate the efficacy and safety in patients with previously treated non-small cell lung cancer.

Methods: In phase I, patients with previously treated non-small cell lung cancer were treated with docetaxel (starting dose 40 mg/m^2) intravenously on day 1 and oral administration of S-1 at a fixed dose of 80 mg/m^2 on days 1 to14 every 3 weeks. The recommended dose was the dose level preceding the maximum tolerated dose; once determined, patients were enrolled in phase II.

Results: The recommended dose of docetaxel was 40 mg/m^2 in combination with S-1 80 mg/m^2/d. Of 30 patients enrolled in phase II part, 29 patients were eligible and analyzed. No complete response and 7 (24.1%) partial responses were observed, for an overall response rate of 24.1% (95% confidence interval, 10.3–43.5%). Median overall survival was 11.8 months. The 1-year survival rate was 42%. The grade 3 to 4 hematologic toxicities were neutropenia (34.5%), leukopenia (20.6%), and anemia (10.3%). The grade 3 to 4 nonhematological toxicities included fever 2 (6.9%), diarrhea 1 (3.4%), stomatitis 1 (3.4%), cerebral infarction 1 (3.4%), and pneumonitis 1 (3.4%). There was one treatment-related death due to relapse of drug induced pneumonitis.

Conclusions: This combination chemotherapy is highly active and well tolerated in previously treated patients with non-small cell lung cancer. These results are encouraging and warrant additional investigation.

Key Words: Phase I/II, Non-small cell lung cancer, Second-line chemotherapy, S-1, Docetaxel.

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Lung cancer is the leading cause of tumor-related death worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers, and by 2002, there were 1.35 million new cases, representing 12.4% of all new cancers.[1] Surgery offers the best chance for cure in stage I and II NSCLC. However, most patients with NSCLC have advanced disease at diagnosis. Chemotherapy is the mainstay of management. The American Society of Clinical Oncology's clinical guidelines recognize that chemotherapy can prolong the survival of advanced NSCLC and is appropriate for those with good PS.[2] The use of doublet regimens has been widely adopted. The principal agents are platinum analogs, taxanes, gemcitabine, irinotecan, and vinorelbine.[3,4] First-line platinum-based chemotherapy is somewhat effective. However, all patients with advanced NSCLC will ultimately progress or relapse. Therefore, second-line chemotherapy is of importance in the clinical management of the patients who had previously received chemotherapy.

Docetaxel has been proven to show antitumor activity against various cancers, including NSCLC.[5–8] This anticancer agent is a mitotic spindle poison that promotes tubulin polymerization and inhibits the depolymerization of microtubules.[9] Docetaxel is one of the standard drugs in second-line chemotherapy. Two recent studies showed improved survival in patients with NSCLC previously treated with platinum in comparison to best supportive care or other drugs.[10,11]

S-1 is a new oral fluorinated pyrimidine. It is a combination drug consisting of a mixture of futraful, 5-chloro-2,4-dihydrozypyridine, and potassium oxonate (Oxo) in a molar ratio of futraful: 5-chloro-2,4-dihydrozypyridine: Oxo = 1: 0.4: 1, based on the biochemical modulation of 5-FU.[12] In phase II studies for advanced NSCLC conducted in Japan, favorable results of S-1 monotherapy or combination therapy have been reported. Kawahara et al. reported that S-1 monotherapy achieved an overall response rate of 22.0% and a median survival time (MST) of 10.2 months.[13] There were no irreversible, severe or unexpected toxicities. Ichinose et al. reported that S-1 plus cisplatin achieved a 47% response rate and a MST of 11 months.[14] Docetaxel and S-1 have shown synergy in human gastric, and breast cancer xenograft models.[15,16] The expression of thymidylate synthase and dihydrouracil dehydrogenase was lower than compared with con-

trol levels. In in vivo experiments using breast cancer xenografts, significant down-regulation of dihydrouracil dehydrogenase activity was observed in tumors treated with S-1, docetaxel and their combination.[16] However, thymidylate synthase activity was not significantly different from control. We hypothesized that the doublet combination chemotherapy using docetaxel and S-1 would have more effect against NSCLC as compared with the monotherapy of docetaxel. The rationale for this combination is that the drugs have different action mechanisms and safety profiles. To improve upon the efficacy of docetaxel alone as second-line treatment, we conducted a phase I/II study of doublet chemotherapy of docetaxel plus S-1.

PATIENTS AND METHODS

Eligibility

Eligible patients were required to have locally advanced or metastatic NSCLC and had failed one or more prior chemotherapy regimens and had at least one measurable lesion. Other main eligibility criteria were as follows: age 20 years or more; Eastern Cooperative Oncology Group performance status (PS) 0 or 1; estimated life expectancy ≥3 months; one or more prior chemotherapy regimens that did not include docetaxel or 5-FU and that was completed >4 weeks before entry; adequate bone marrow, hepatic, renal, and cardiac function [i.e., WBC count ≥4000/μl, absolute neutrophil count ≥2000/μl, platelet count ≥100,000/μl, hemoglobin ≥9.5 g/dl, serum bilirubine level <1.5 mg/dl, aspartate aminotransferase, and alanine aminotransferase within 2.5 times the upper limit of normal (ULN) for the institution, blood urea nitrogen <25 mg/dl, serum creatinine within the ULN, and creatinine clearance ≥60 ml/min]. Exclusion criteria included the presence of other concomitant or metachronous cancers, severe allergy to drugs, simultaneous infectious disease, interstitial pneumonia, or other serious underling medical conditions. The study was approved by the institutional review board of the participating center and all patients provided written informed consent.

Evaluation

All eligible patients who received any part of the treatment were considered assessable for response and toxicity. The complete blood cell counts and blood chemistry studies were measured weekly. The response was assessed based on weekly chest radiograph or computed tomography scan every 4 weeks findings that initially had been used to define tumor extent during the treatment period. The response was evaluated according to the criteria of response evaluation criteria in solid tumors. A complete response (CR) was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks. A partial response (PR) was defined as an at least 30% decrease in the sum of the longest diameters of the target lesions for more than 4 weeks with no new area of malignant disease. Progressive disease (PD) indicated at least a 20% increase in sum of the longest diameter of the target lesions or a new malignant lesion. Stable disease (SD) was defined as insufficient shrinkage to qualify for PR and insufficient increase to qualify for PD. The best response achieved during the treatment course was reported. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.[17]

Study Design and Treatment Schedule

This was an open-label multicenter, single-arm phase I/II study in patients with previously treated NSCLC. The objective of the phase I part was to determine the dose-limiting toxicity (DLT), maximum-tolerated dose (MTD), and recommended dose (RD) of docetaxel plus a fixed dose of S-1. In the phase II part, the primary objective was to estimate the overall response rate of this combination at the RD. Secondary objectives were to assess overall survival, 1-year survival rate, adverse events, and progression-free survival (PFS).

In the phase I part of this study, patients received variable doses of docetaxel administered as a 1-hour infusion on day 1 and oral S-1 administered at a fixed dose of 80 mg/m on days 1 to 14 every 3 weeks. S-1 is only available in 20-mg or 25-mg capsules. Therefore, it is easier to plan the dose escalation procedure or a dosage adjustment of docetaxl than S-1. The initial starting dose of docetaxel was 40 mg/m^2 (dose level 1), and step-wise dose increases to 50 (dose level 2) and 60 mg/m^2 (dose level 3) were planned for successive patient cohorts. DLT was determined during the first treatment cycle. At least three patients were enrolled at each dose level: (i) the dose was defined as MTD when two or more of three patients developed DLT; (ii) when one of three patients developed DLT, three other patients were enrolled; (iii) when three or more of six patients developed DLT, the dose was defined as MTD; (iv) when one or two of six patients developed DLT, the dose was increased to the next level.

DLT was defined as follows: grade 4 neutropenia; grade 3 or 4 neutropenia associated with a fever ≥38°C; grade 4 thrombocytopenia; or grade 3 or 4 nonhematological toxicities. A DLT was also reported if 7 days or more omission of S-1, or if the second cycle was delayed until after day 29 because the dosing requirements were not satisfied.

S-1 80 mg/m^2 per day was given orally in 2 divided dose after a meal for 2 weeks, after a drug-free interval of 1 week (one cycle). Three doses of S-1 were selected according to body surface area (BSA). So that they would be approximately equivalent to 80 mg/m^2: BSA<1.25 m^2, 40 mg b.i.d.; BSA 1.25, but <1.5 m^2, 50 mg b.i.d.; and BSA ≥1.5 m^2, 60 mg b.i.d. Docetaxel 40 mg/m^2 was diluted in 500 ml of 0.9% salaine and administered as a 1-hour infusion on the morning of day 1 of each cycle (i.e., every 3 weeks). Dexamethasone 8 mg was infused 1 hour before docetaxel administration. Granulocyte colony-stimulating factor was permitted if a patient developed grade 4 neutropenia; primary prophylaxis was not allowed. Antiemetic (ondansetron) treatment was allowed at the discretion of the treatment physician.

In the phase II part of this study, patients received the RD of docetaxel on day 1 and oral S-1 80 mg/m^2 in accordance with the treatment schedule described above. The treatment was repeated every 21 days for at least two cycles unless there was disease progression, unacceptable toxicity, patient refusal, or the physician's decision to stop treatment.

S-1 was stopped if there was a leukocyte count of $<2000/\mu l$, neutrophil count of $<1000/\mu l$, platelet count of $<50{,}000/\mu l$, and a grade 3 or 4 nonhematological toxicity.

The next course of treatment was initiated only when the neutrophil count recovered to $\geq 2000/\mu l$, platelet count to $\geq 100{,}000/\mu l$, creatinine within the ULN, total bilirubin ≤ 1.5 mg/dl, and the level of aspartate aminotransferase/alanine aminotransferase became <2.5 times the ULN. If patients did not recover from these toxicities within 2 weeks of the last administration of S-1, they were withdrawn from this study. If patients experienced grade 4 neutropenia, fever $\geq 38.0°C$ with grade 3 to 4 neutropenia, grade 3 or more thrombocytopenia, the dose of docetaxel was reduced by 10 mg/m^2 in the subsequent cycle. The dose of S-1 was to be reduced by 20 or 30 mg per day if any grade 3 or 4 nonhematological toxicity was recognized including nausea/vomiting, anorexia, and general fatigue.

Statistical Analysis

Based on the assumption that a response rate of higher than 20% would warrant a further investigation of this combination chemotherapy, and a rate of below 5% would make such an investigation unnecessary, a sample size of 27 patients was required with an alpha error of 0.05 and a beta error of 0.2. Therefore, the accrual of 30 patients was planned for a 2-year period since we considered that several ineligible patients might be identified in the course of the study. PFS was defined as the interval from the start of the treatment to the diagnosis of progression or death from any cause. Overall survival was defined as the interval between when treatment was started and death or the final follow-up visit. Median overall survival and median PFS were estimated by the Kaplan-Meier method.[18] Survival time was recorded at the last confirmation date if the patients were alive.

RESULTS

Between January 2005 and May 2006, 33 patients were enrolled on this study. Nine patients (6 in level 1 and 3 in level 2) were enrolled into the phase I part. Of 30 patients enrolled into the phase II part of the study, one patient did not receive either docetaxel or S-1 because his disease had progressed rapidly. This patient was excluded from all analyses. Twenty-nine patients who were given the RD were evaluated for efficacy and detailed safety profile: these patients consisted of 6 and 23 patients who entered into the study at phase I and II, respectively.

Phase I

The first cohort of 6 patients received docetaxel 40 mg/m^2 plus S-1 80 mg/m^2 (dose level 1). Among these patients, one experienced cerebral infarction (grade 4 CNS cerebrovascular ischemia). No other DLT was observed at dose level 1. At dose level 2 (docetaxel 50 mg/m^2), 2 of the 3 patients developed grade 4 neutropenia which was considered DLT. From these results, the MTD and RD were determined to be level 2 and level 1, respectively.

Phase II

Baseline characteristics of the 29 patients treated at the RD are shown in Table 1. Ages ranged from 48 to 79 years, with a median of 67 years. There were 23 men and 6 women. Nine patients had Eastern Cooperative Oncology Group PS 0, 20 patients had PS 1. Seven patients had clinical stage IIIB disease and 22 had stage IV disease. Histology consisted of adenocarcinoma in 16 patients, squamous cell carcinoma in 10, large-cell carcinoma in 2, and other in one. A single prior chemotherapy regimen had been given in 23 patients, 2 regimen in 4 patients and 3 in 2 patients. Twenty-eight (96.5%) patients had received a platinum-based chemotherapy.

Response and Survival

Of 29 patients assessable for response, none of the patients achieved a CR; 7 (24.1%) achieved a PR with an overall response rate of 24.1% [95% confidence interval (95% CI), 10.3–44.8%]. Thirteen (44.8%) had SD and 7 patients (24.1%) had PD as best response. Two were unevaluable. The tumor control rate (CR + PR+SD) was 68.9% (95% CI, 49.2–84.7%). Among all 29 patients, the median PFS was 3.9 months. As shown in Figure 1, the MST of all patients was 11.8 months, and the 1-year survival rate was 41.8% (95% CI, 21.8–61.8%).

Toxicity of Treatment

Hematological toxicity and nonhematological toxicity were analyzed during treatment and the follow-up period. The major toxicities during the study period are shown in Tables 2 and 3. The grade 3 to 4 hematological toxicities were neutropenia (34.5%), leukopenia (20.6%), and anemia (10.3%). None of the patients developed grade 2 or more thrombocytopenia. The grade 3 to 4 nonhematological toxicities included fever 2 (6.9%), diarrhea 1 (3.4%), stomatitis 1 (3.4%), cerebral infarction 1 (3.4%), and pneumonitis 1 (3.4%). There was one treatment-related death. The patient died 54 days after the first cycle of chemotherapy due to relapse of drug induced pneumonitis.

Treatment Delivery

The median number of cycles administered was 3 (range, 1–8 cycles).

TABLE 1. Patients' Characteristics

No. patients	30
Eligible	29
Male/Female	23/6
Median age, in yr (range)	67 (48–79)
PS 0/1	9/20
ad/sq/la/other	16/10/2/1
Stage IIIb/IV	7/22
No. previous chemo regimens	
1/2/3	23/4/2
RT	13
Operation	3

PS, performance status; Chemo, chemotherapy; RT, radiotherapy.

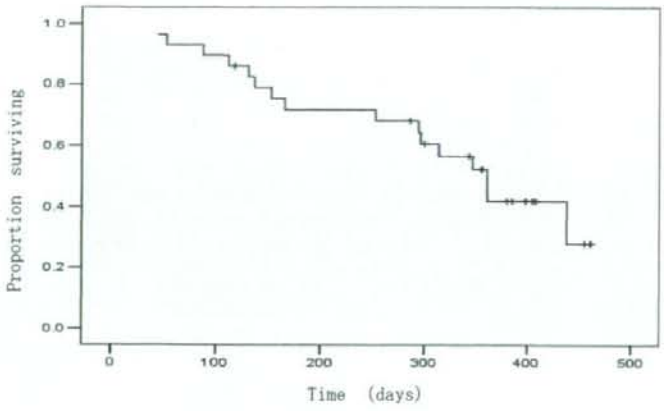

FIGURE 1. Overall survival curve.

TABLE 2. Hematological Toxicity

Grade				
Toxicity	**1**	**2**	**3**	**4**
Leukopenia	3	8	6	0
Neutropenia	1	5	7	3
Thrombocytopenia	2	0	0	0
Hemoglobin	7	7	3	0

TABLE 3. Nonhematological Toxicity

Grade				
Toxicity	**1**	**2**	**3**	**4**
Nausea	5	0	0	0
Vomiting	1	2	0	0
Fatigue	1	5	0	0
Infection	0	0	0	0
Fever	3	0	2	0
Diarrhea	4	0	1	0
Ulcer	0	1	0	0
Cerebrovascular ischemia	0	0	0	1
Skin	2	3	0	0
Stomatitis	3	0	1	0
Pneumonitis	1	0	0	1*

*One patient died from relapse of drug induced pneumonitis.

In all, 20 (62.5%) patients received at least 2 cycles of treatment. The reasons for terminating the chemotherapy before the second treatment cycle were disease progression in seven patients and adverse events in two patients. Five patients each required dose reductions of docetaxel or S-1, respectively.

Poststudy Therapy

Eighteen patients received at least one form of antitumoral treatment after disease progression. Thirteen patients received chemotherapy alone, the most frequently prescribed treatment was carboplatin plus gemcitabine. Ten patients received gefitinib.

DISCUSSION

The benefit of second-line chemotherapy has been substantiated by randomized trials using docetaxel, pemetrexed, topotecan, and erlotinib.[10,11,19–21] The response rate was reported to be 6.7 to 10.8% for docetaxel, 9.1% for pemetrexed, 5% for topotecan, and 8.9% for erlotinib. The 1-year survival rate of these reports ranges from 25 to 37%. It is clear that there is an urgent need for more active treatment regimens to patients with relapsed or refractory NSCLC. On the other hand, second-line chemotherapy is a palliative treatment. Therefore, pretreated patients have poorer tolerance to second-line chemotherapy, lower toxicity, and efficacy, which is important when considering the second-line chemotherapy.

To improve the efficacy of second-line chemotherapy, a number of studies have conducted two-drug second-line therapy combinations.[22–25] Georgoulias et al. reported a randomized phase II study that compared single agent irinotecan with a combination of irinotecan plus gemcitabine.[24] Their results failed to demonstrate a statistically significant survival advantage of the combination of irinotecan and gemcitabine over irinotecan alone, although the combination regimen was better in terms of response rate and QOL. A phase III study by Takeda et al. comparing docetaxel alone versus docetaxel plus gemcitabine was terminated early with unexpected incidence of interstitial lung disease and treatment-related deaths due to interstitial lung disease, only in the combination chemotherapy group.[25] Indeed, a comparison of combination chemotherapy versus monotherapy in patients with previously treated NSCLC failed to demonstrate any difference in terms of overall survival. For the moment, single-agent therapy remains the standard option for patients with relapsed or refractory NSCLC.

In the present study, we administered S-1 plus docetaxel to previously treated patients with NSCLC. Seven of the 29 patients (24.1%) achieved a PR as a result. The MST of this regimen was 11.8 months and the 1-year survival rate was 41.8% (Figure 1). The results of the present study are promising, suggesting that the survival of patients treated

with combination therapy could be improved compared with the survival of those treated with docetaxel alone as a second-line treatment. However, we can not exclude the possibility that the poststudy treatment such as gefitinib or selection bias might also have played a role in prolonging the survival times. Various combination chemotherapy regimens including oral fluoropyrimidine, such as UFUR and capecitabine, have been investigated in NSCLC.[26–28] Kindwall-Keller et al. reported a phase II study of docetaxel and capecitabine in previously treated patients with NSCLC.[27] The response rate was 26% with the MST and 1-year survival rate of 9.1 month and 37%. Chen et al. used UFUR with gemcitabine for 45 patients who failed previous platinum-based chemotherapy.[28] Their patients were treated with 1000 mg/m^2 gemcitabine on days 1 and 8, plus oral UFUR 200 mg/m^2/d from days 1 to 14 of every 3 weeks. They reported that 7 patients (15.6%) had a PR. The MST was 13.2 months.

Our study used 40 mg/m^2 of docetaxel every 3 weeks is lower than that commonly using docetaxel alone at the dose of 75 mg/m^2 as second-line setting in the United States and Europe. By combining docetaxel at 40 mg/m^2 on day 1 with S-1 at 80 mg/m^2/d on days 1 to 14 every 3 weeks, we expected less toxicity, with preserved efficacy. In Japan, docetaxel 60 mg/m^2 every 3 weeks is the commonly used dose. In a phase I study of docetaxel plus S-1, the RD of docetaxel was determined to be 40 mg/m^2 in combination with S-1 80 mg/m^2/d on days 1 to 14. This combination chemotherapy has been evaluated in gastric cancer in Japan.[29–31] The RD of docetaxel was 40 mg/m^2 in combination with S-1 80 mg/m^2/d in the gastric cancer which was the same is our study as a second-line setting. Yamaguchi et al. speculate that the reason for the lower dose of docetaxel may be that the pharmacokinetic parameters (AUC and C_{max}) of 5-FU increase according to the dose of docetaxel.[31]

In our study, the main toxicity was myelosuppression. The most common hematological toxicities were neutropenia and leukopenia. Grade 3 or 4 neutropenia occurred in 34.5% and grade 3 or 4 anemia occurred in 10.3%. In phase III studies of docetaxel 75 mg/m^2 given as a single agent, grade 3 or 4 neutropenia occurred in 40.2 to 67.3% and grade 3 or 4 anemia occurred in 4.3 to 10%.[10,19,20] It seemed that the incidence of grade 3 or 4 neutropenia were lower in our study than in those phase III studies. The majority of nonhematological toxicities were relatively mild. However, grade 4 cerebral infarction and pneumonitis were observed. It is unclear whether this adverse CNS event was related to this combination chemotherapy. This may be due to the hypercoagulability associated with lung cancer. Clotting activation and thromboembolic manifestations are common features in patients with cancer. Therefore, this CNS event might have occurred by chance.[32]

In conclusion, our study indicates that the combination of docetaxel pulse S-1 is an effective and well-tolerated regimen for the treatment of patients with previously treated NSCLC. This regimen seems suitable as a second-line treatment for patients with NSCLC. The response rate and median survival are encouraging and warrant additional investigation.

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