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ORIGINAL PAPER

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

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

Keywords EGFR · Lung cancer · Polymorphism · R497K

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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. In 2004, two reports have shown that *EGFR* mutation statuses at tyrosine kinase (TK) domain in NSCLC patients were correlated with the clinico-pathological features related to good response to gefitinib (Paez et al. 2004; Lynch et al. 2004). *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy in vivo and in vitro (Paez et al. 2004; Lynch et al. 2004; Pao et al. 2004). Genomic

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

Materials and methods

Patients and samples

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

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

PCR assays for *EGFR* polymorphism

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

Statistical analysis

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

Results

EGFR gene mutation status

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

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

EGFR polymorphism at exon 13

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

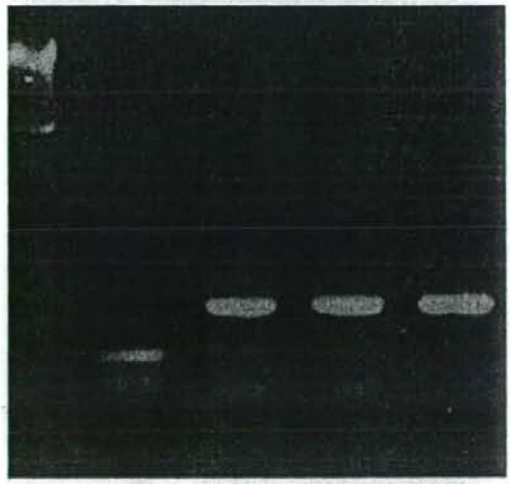

Fig. 1 Representative PCR–RELP patterns of different EGFR codon 497 status. PCR products after being digested by BstN1 were separated by agarose gel electrophoresis

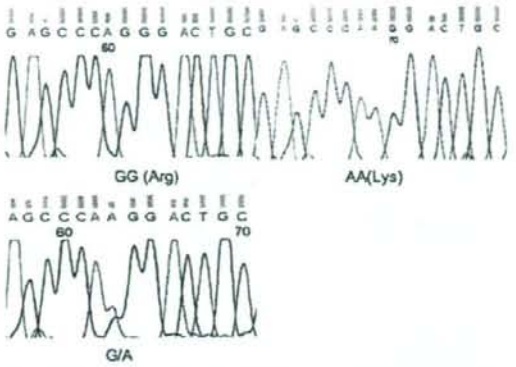

Fig. 2 The sequence results of *EGFR* exon 13. *Left upper* wild type (GG). *Right upper* heterozygous change (GA). *Left lower* homozygous change (AA)

Table 1 Clinico-pathological data of 225 lung cancer patients

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

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

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

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

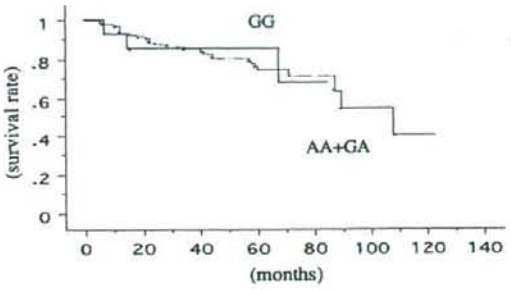

Fig. 3 The overall survival of node-negative lung cancer patients was studied in reference to the *EGFR* (R497K) status. There was no difference of survival between the patient with EGFR wild type (GG) ($n = 14$, 3 were dead) and the patient with R497K *EGFR* (GA or AA) ($n = 119$, 28 were dead) (Log-rank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

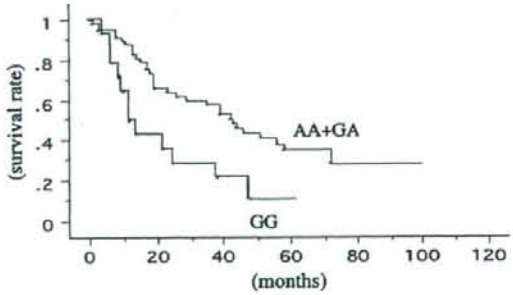

Fig. 4 The overall survival of node-positive lung cancer patients was studied in reference to the *EGFR* (R497K) status. The patients with EGFR wild type (GG) ($n = 14$, 12 were dead, median follow up = 21.7 months) had significantly worse prognosis than the patients with R497K *EGFR* (GA or AA) ($n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

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

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

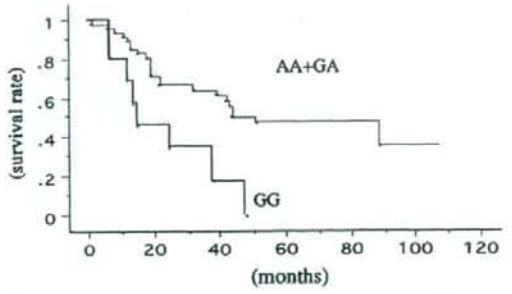

Fig. 5 The overall survival of adjuvant or neo-adjuvant chemo-untreated lung cancer patients was studied in reference to the *EGFR* (R497K) status. The patients with EGFR wild type (GG) ($n = 10$, 8 were dead, median follow up = 23.7 months) had significantly worse prognosis than the patients with R497K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

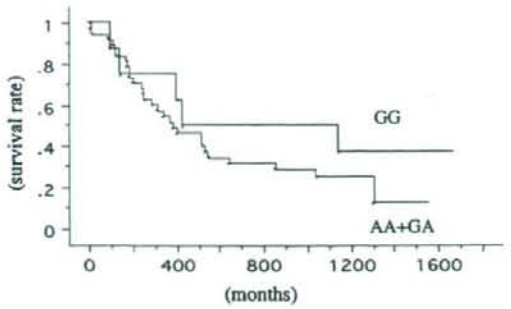

Fig. 6 The overall survival of 46 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* (R497K) status. There was no difference of survival between the patients with EGFR wild type (GG) ($n = 8$, 5 were dead) and the patients with R497K *EGFR* (GA or AA) ($n = 38$, 28 were dead)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

Discussion

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

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

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

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

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

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Conflict of interest statement None declared.

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

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

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

Introduction

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

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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 à 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, $P < 0.0001$).

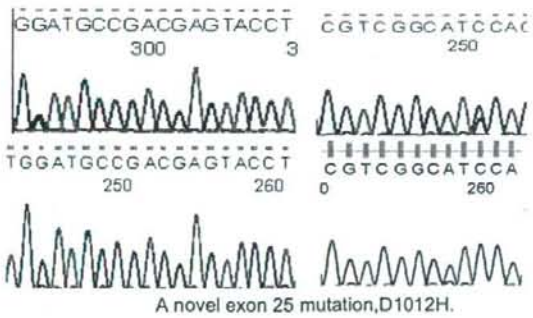

Fig. 1 A novel mutation, D1012H, at exon 25. *Left upper*; forward sequence from lung cancer samples. *Left lower*; forward sequence from adjacent normal lung tissue. *Right upper*; reverse sequence from lung cancer samples. *Right lower*; reverse sequence from adjacent normal lung tissue

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

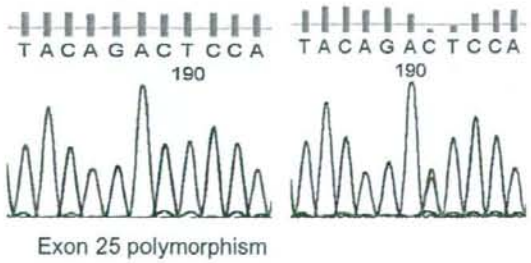

Fig. 3 The sequence results of *EGFR* exon 25. *Left*; wild type (CC). *Right upper*; heterozygous SNP (CT)

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

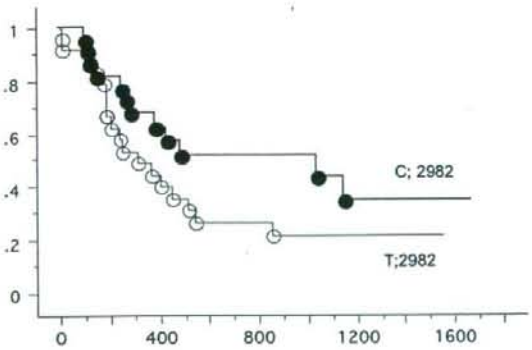

Fig. 4 The overall survival of 46 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* polymorphism (C2982T) status. The prognosis was not significantly different between the patient with EGFR wild type (CC) ($n = 22$, 12 were dead) than the patient with *EGFR* polymorphism (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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Efficacy, toxicity and cost analysis for non-platinum triplet (gemcitabine and vinorelbine, followed by docetaxel) vs. platinum-based chemotherapy in IIIB/IV non-small-cell lung cancer: single-institution experience

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Efficacy, toxicity and cost analysis for non-platinum triplet (gemcitabine and vinorelbine, followed by docetaxel) vs. platinum-based chemotherapy in IIIB/IV non-small-cell lung cancer: single-institution experience

A new non-platinum sequential triplet combination chemotherapy regimen, comprising gemcitabine (1000 mg/m^2) and vinorelbine (25 mg/m^2), followed by docetaxel (60 mg/m^2), was compared in terms of efficacy, toxicity and cost with platinum-based chemotherapy regimens (comprising cisplatin plus one or more other anti-tumour drugs) for the treatment of advanced non-small-cell lung cancer in a matched, small-sample size, case–control study. Patients were selected from a single institution. Patients in the platinum and non-platinum groups were matched for clinical stage (IIIB/IV), performance status (0/1), age and sex. For the non-platinum and platinum groups, the overall response rates were 40% and 47%, and the median survival times were 14 and 14.5 months respectively. The most common grade 3–4 toxicity was neutropenia (27%) in the non-platinum group and nausea/vomiting (67%) in the platinum group. The total treatment cost did not differ significantly between the two groups. The non-platinum sequential triplet combination chemotherapy regimen studied was shown to be as effective as the traditional cisplatin-based combination chemotherapy regimen, and was associated with less toxicity.

Keywords: non-small-cell lung cancer, vinorelbine, gemcitabine, docetaxel, cisplatin.

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INTRODUCTION

Lung cancer is a major cause of deaths from cancer in Japan, the USA and the European Union. Non-small-cell lung cancer (NSCLC) accounts for about 80% of patients with lung cancer in Japan (Ministry of Health and Welfare 2000). Cisplatin (CDDP)-based chemotherapy has been shown to confer a certain survival benefit for patients with advanced NSCLC (Non-small Cell Lung Cancer Collaborative Group 1995), and use of CDDP has been found to be an independent predictor of survival (Grilli *et al.* 1993). A drawback of CDDP-based chemotherapy, however, is its serious toxicity; its side effects include severe nausea, vomiting, renal toxicity requiring adequate hydration, and neuropathy, which increases the difficulty associated with treating elderly patients and outpatients.

Recently, new chemotherapeutic agents, such as the taxanes, vinorelbine (VNR), gemcitabine (GEM), and several non-platinum combinations have been developed for the treatment of NSCLC. The new non-platinum combination of GEM plus VNR has been shown to be active for the treatment of NSCLC, and seems to be less toxic than platinum-based combinations, including those involving CDDP (Non-small Cell Lung Cancer Collaborative Group 1995; Lorusso *et al.* 1998; Feliu *et al.* 1999; Isokangas *et al.* 1999; Beretta *et al.* 2000; Chen *et al.* 2000; Frasci *et al.* 2000; Lorusso *et al.* 2000; Krajnik *et al.* 2000; Herbst *et al.* 2002; Gridelli *et al.* 2003). Treatment with docetaxel (DOC) alone has also been shown to confer a survival benefit, especially as a second-line treatment (Roszkowski *et al.* 2000). Recently, a new non-platinum sequential triplet combination, GEM plus VNR, followed by DOC, was evaluated for 44 chemotherapy-naïve patients with advanced NSCLC in a phase II study conducted by the Japan Multinational Trial Organization (JMTO). The response rate in that study was 47.7%, and the median survival time (MST) was 15.7 months, with a 1-year survival rate of 59%. Grade 3–4 neutropenia was seen in approximately 36% of patients during the GEM/VNR cycles, and in 39% of patients during the DOC cycles. Overall, only 2.3% of patients experienced grade 3–4 thrombocytopenia, and 4.5% experienced grade 3–4 anaemia (Hosoe *et al.* 2003). Given that this non-platinum combination has been found to be very active and well tolerated by patients, it is likely that the regimen will also be suitable for elderly patients and outpatients.

On the basis of these observations, we conducted a case-matched retrospective study as a part of the aforementioned phase II study to assess the non-platinum sequential triplet combination in terms of efficacy, safety and cost relative to platinum-based combinations.

PATIENTS AND METHODS

Patient selection

This study was performed as a part of the phase II trial (JMTO LC00-02) conducted by JMTO (Hosoe *et al.* 2003). The criteria for patient selection (eligibility and exclusion criteria) are summarized in Table 1. Fifteen patients who were enrolled in the phase II trial at the National Hospital Organization Kinki-chuo Chest Medical Center, who received non-platinum triplet chemotherapy (GEM and VNR, followed by DOC) during the period between May 2000 and February 2001, comprised the non-platinum group. For the platinum group, in order to ensure that the two groups were comparable, we selected 15 eligible patients from the pool of all NSCLC patients ($n = 124$) who received CDDP-based chemotherapy between April 1998 and February 2001 at the same institution, by matching each patient in the non-platinum group for stage (IIIB/IV), performance status (0/1), age and sex. The protocol of this study was approved by ethical committees at Kyoto University and the National Hospital Organization Kinki-chuo Chest Medical Center.

Chemotherapy regimens

Patients in the non-platinum group were first treated with both GEM (1000 mg/m^2) and VNR (25 mg/m^2) on days 1 and 8 of three cycles of 21 days each, followed by a further three cycles of 21 days each, during which DOC (60 mg/m^2) was administered on day 1 of each cycle (Hosoe *et al.* 2003). Patients in the platinum group received CDDP-

Table 1. Criteria for patient selection

Eligibility criteria
- Eastern Cooperative Oncology Group performance status 0–1
- Over 18 years old (no upper age limit)
- Stage IV or IIIB non-small-cell lung cancer [with malignant pleural effusion and/or pulmonary nodule(s) in the same lobe as the primary lesion]
- Unidimensionally measurable disease

Exclusion criteria
- Presence of apparent interstitial pneumonitis, massive pleural effusion requiring thoracentesis, uncontrollable diabetes mellitus, heart diseases, history of another cancer (excluding non-melanomatous skin cancer and *in situ* cervical cancer)
- Reduced bone marrow, pulmonary, renal or hepatic function
- Stage IIIB disease with pulmonary nodule(s) at the same lobe of the primary lesion (if they could be considered to indicate that the patient had undergone radiation therapy or operation)
- Presence of asymptomatic central nervous system metastases was not considered an exclusion criterion

based chemotherapy without any restrictions on other concomitant drugs, amount of medication, or number of treatment cycles.

Endpoints

The endpoints of this study were tumour response to chemotherapy, recurrence-free survival time, toxicity and cost of treatment. Haematological and non-haematological toxicity were evaluated using the National Cancer Institute's Common Toxicity Criteria, version 2.0. For patients who underwent two or more cycles of chemotherapy, the response was evaluated using the Response Evaluation Criteria in Solid Tumors (Therasse *et al.* 2000). The recurrence-free survival time refers to the period of time between the day treatments began and the day of recurrence or death. The recurrence-free survival time was censored at the end of follow-up. The total cost of treatment was evaluated using patients' receipts, and comprised the cost of chemotherapy (cost of drugs plus costs associated with drug administration) plus the cost of hospitalization, ambulatory care and supportive care for other adverse events or complications. The cost of granulocyte-colony stimulating factor (G-CSF) for each patient was also calculated. The average cost per month was calculated. The endpoints were evaluated during the period between the month in which the chemotherapy began and 1 month after the completion of chemotherapy.

Statistical methods

Differences between the characteristics of patients in the two groups were evaluated using the *t*-test for quantitative variables and Fisher's exact test for categorical variables. Comparisons of the response rate and the incidence of toxicity events between groups were carried out using Fisher's exact test. The survival rate was estimated for each group using the Kaplan-Meier method. Comparisons of survival between groups were performed using the log rank test. Monthly medical costs were compared using the Wilcoxon rank-sum test. Subgroup analyses were carried by dividing the patients into elderly (65 years or older) and non-elderly groups. Statistical analyses were performed using SAS version 8.0 (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics

The characteristics of patients participating in this study are summarized in Table 2. The distribution of these factors is almost identical between groups, because each patient in the platinum group was selected by matching each patient in the non-platinum group for stage (IIIB/IV), performance status (0/1), age and sex. With respect to histological diagnosis, the non-platinum group included a smaller number of patients with adenocarcinomas but a greater number of patients with large cell carcinomas than the platinum group. The dosage of CDDP in the platinum group was 70–80 mg/m^2 per day. The drugs combined with CDDP for treatment of patients in the platinum group are shown in Table 3. Eight patients received new anticancer agents (DOC: 6 patients; VNR: 2 patients) combined with CDDP, accounting for 53% of the platinum group as a whole. The mean number of chemotherapy cycles per patient was 3.9 for the non-platinum group and 3.1 for the platinum group.

The major reasons for discontinuing chemotherapy were patient refusal and the mental burden caused by adverse reactions in the platinum group, and disease progression in the non-platinum group. Adverse reactions were not a major factor for discontinuation of chemotherapy in the non-platinum group.

Table 2. Patient characteristics

Regimen characteristic	Non-platinum ($n = 15$) n (%)	Platinum ($n = 15$) n (%)
Sex*		
Male	11 (73)	11 (73)
Female	4 (27)	4 (27)
Median age in years (range)*	65 (42–74)	64 (48–76)
ECOG performance status*		
0	2 (13)	2 (13)
1	13 (87)	13 (87)
Stage*		
IIIB	3 (20)	3 (20)
IV	12 (80)	12 (80)
Histological diagnosis		
Adenocarcinoma	3 (20)	8 (53)
Squamous cell carcinoma	7 (47)	6 (40)
Large cell carcinoma	5 (33)	1 (7)

ECOG, Eastern Cooperative Oncology Group.
*Matching factor.

Table 3. Drugs apart from cisplatin used in CDDP-based chemotherapy regimens ($n = 15$)

Drug	n
Docetaxel	6
Vindesin	6
Vinorelbine	1
Mitomycin C	1
Mitomycin C + vinorelbine	1

Table 4. Response, toxicity and cost of platinum and non-platinum chemotherapy regimens

	Non-platinum (*n* = 15) *n* (%)	Platinum (*n* = 15) *n* (%)	*P*-value
Response			
Complete response	0	0	
Partial response	6	7	
Stable disease	5	6	
Progressive disease	4	2	
Response rate	6 (40)	7 (47)	1.000
MST (months)	14	14.5	0.264
(95% CI)	(8–14)	(11–31)	
Mean no. cycles administered	3.9	3.1	0.378
Grade 3–4 toxicity experienced	6 (40)	14 (93)	0.005
Haematological			
Neutropenia	4 (27)	2 (13)	0.651
Leukopenia	2 (13)	5 (33)	0.390
Non-haematological			
Nausea, vomiting	0 (0)	10 (67)	<0.001
Fatigue	0 (0)	3 (20)	0.224
Mean total treatment cost (yen/month)	475 372	443 979	0.147

MST, median survival time.

Response, toxicity and cost

All results regarding response, toxicity and cost are summarized in Table 4. The overall response rate was 40% for the non-platinum group and 47% for the platinum group ($P = 1.000$). The MST was 14 months for the non-platinum group and 14.5 months for the platinum group ($P = 0.264$). The frequency of grade 3–4 toxicity was 40% for the non-platinum group and 93% for the platinum group, which was statistically significant ($P = 0.005$). The most frequently observed grade 3–4 haematological toxicity was neutropenia (27%) in the non-platinum group and leucopenia (33%) in the platinum group. There were no grade 3–4 non-haematological toxicity events in the non-platinum group, but 67% of patients suffered from nausea/vomiting in the platinum group ($P < 0.001$). As an index of overall efficacy and toxicity, the number of responders who did not experience grade 3–4 toxicity was 3 (20%) in the non-platinum group and 0 (0%) in the platinum group ($P = 0.100$). The number of non-responders who experienced grade 3–4 toxicity was 3 (20%) in the non-platinum group and 7 (47%) in the platinum group ($P = 0.128$). The average total treatment costs per month were ¥475 372 (approximately US$4080) and ¥443 979 (approximately US$3810) for the non-platinum and platinum groups respectively ($P = 0.141$). The average hospitalization costs per month were ¥265 663 (approximately US$2290) and ¥266 415 (approximately US$2296) for the non-platinum and platinum groups respectively. There was no statistically significant difference between the two groups with respect to cost. Three patients in each group received G-CSF. The average costs for G-CSF per patient were ¥12 797 (approximately US$110) and ¥22 073 (approximately US$190) in the non-platinum and platinum groups respectively ($P = 0.366$).

Table 5. Response and toxicity of platinum and non-platinum chemotherapy regimens for elderly patients

	Non-platinum (*n* = 8) *n* (%)	Platinum (*n* = 6) *n* (%)	*P*-value
Response			
Complete response	0	0	
Partial response	5	5	
Stable disease	2	1	
Progressive disease	1	0	
Response rate	5 (63)	5 (83)	0.580
Grade 3–4 toxicity experienced	5 (63)	5 (83)	0.580
Haematological			
Neutropenia	3 (38)	0 (0)	0.209
Leukopenia	2 (25)	2 (33)	1.000
Non-haematological			
Nausea, vomiting	0 (0)	4 (55)	0.015
Fatigue	0 (0)	1 (17)	0.429

We carried out further analysis on a subgroup of elderly patients (those aged 65 years or older; the non-platinum group: 8 patients, and the platinum group: 6 patients). The response and toxicity data for the elderly patient subgroups are summarized in Table 5. In this subgroup, the overall response rate was 63% for the non-platinum group and 83% for the platinum group ($P = 0.580$). The frequency of grade 3–4 toxicity was 63% for the non-platinum group and 83% for the platinum group ($P = 0.580$). The most frequently observed grade 3–4 haematological toxicity was neutropenia (38%) in the non-platinum group and leucopenia (33%) in the platinum group. There was no grade 3–4 non-haematological toxicity event in the non-platinum group, but 67% of patients in the platinum group suffered from nausea/vomiting; the difference was statistically significant ($P = 0.015$). The number of responders who did not experience grade 3–4 toxic events was 2 (25%) in the non-platinum group and 0 (0%) in the platinum group. The number of non-responders who experienced grade 3–4 toxic events was 2 (25%) in the non-platinum group and 0 (0%) in the platinum group.

DISCUSSION

Although CDDP-based chemotherapy has become established as a standard therapy for the treatment of patients

with advanced NSCLC with good performance status, it has the drawback of serious toxicity, causing such symptoms as severe nausea, vomiting and renal toxicity, and is thus not suitable for elderly patients and outpatients. Recent trials of new anticancer drugs have indicated that some non-platinum-based combinations are almost as active as CDDP-based chemotherapy regimens, but are less toxic. In particular, the GEM/VNR combination has been shown to be well tolerated by patients, and to be very active (Non-small Cell Lung Cancer Collaborative Group 1995; Lorusso *et al.* 1998; Feliu *et al.* 1999; Isokangas *et al.* 1999; Beretta *et al.* 2000; Chen *et al.* 2000; Frasci *et al.* 2000; Lorusso *et al.* 2000; Krajnik *et al.* 2000; Herbst *et al.* 2002; Gridelli *et al.* 2003), and thus might be a good alternative to CDDP-based chemotherapy regimens. A new non-platinum sequential triplet combination, GEM and VNR, followed by DOC, was recently evaluated in a JMTO phase II study, and was found to be well tolerated, with one of the highest response rates yet reported for treatment of advanced NSCLC (JMTO LC00-02; Hosoe *et al.* 2003). Given these findings, a phase III randomized trial (JMTO LC00-03) began in April 2001 to compare this non-platinum sequential triplet combination with a platinum combination (carboplatin/paclitaxel). This phase III trial is ongoing in collaboration with the Southwest Oncology Group's (SWOG) trial (S0003) (carboplatin/paclitaxel versus carboplatin/paclitaxel + tirapazamine), using the same protocol for the common control arm (carboplatin/paclitaxel) (Williamson *et al.* 2005). We thus conducted a case-matched retrospective study in a single institution as a part of the multi-institutional phase II trial (JMTO LC00-02). The purpose of the present study was, in the context of JMTO LC00-02, to assess this non-platinum sequential triplet combination in terms of efficacy, toxicity and treatment cost relative to platinum-based combinations comprising CDDP plus one or more other anticancer drugs for the treatment of advanced NSCLC. Consequently, the present study provides some of the first results concerning a comparison of the new non-platinum sequential triplet combination with platinum-based combinations.

The non-platinum group in the present study was a subgroup of patients involved in the JMTO LC00-02 phase II trial, which included 44 patients from 17 institutions (response rate of 47.7%, median survival time of 15.7 months) (Hosoe *et al.* 2003). We believe that the selected patients were representative of the phase II study population as a whole, because there was no significant difference in the distribution of outcomes and patient characteristics between the group as a whole and the selected patients.

In order to ensure comparability between the non-platinum and platinum groups, we sourced patients from a single institution, and selected each patient in the platinum group from the pool of all patients who received CDDP-based chemotherapy during the study period by matching for stage (IIIB/IV), performance status (0/1), age and sex, all of which are considered to be important prognostic factors. The resulting number of patients in each group was small.

Differences in the distribution of histological diagnoses between the two groups were found: the non-platinum group included a smaller number of patients with adenocarcinomas but a larger number of patients with large cell carcinomas than the platinum group. We performed subgroup analysis according to histological diagnosis to evaluate the effects of treatment. The overall response rate for patients with adenocarcinoma, squamous cell carcinoma, and large cell carcinoma were 67% (2/3), 29% (2/7) and 40% (2/5) respectively in the non-platinum group, and 50% (4/8), 34% (2/6) and 100% (1/1) respectively in the platinum group. There was no significant difference in overall response rate between the non-platinum and platinum groups when subgroups of patients with similar histological diagnoses were compared.

In previous studies regarding therapy for NSCLC, the response rate and MST were found to be 13% and 6 months respectively for treatment with DOC alone (Roszkowski *et al.* 2000), and 19% and 6.5 months respectively for treatment with VNR alone (The Elderly Lung Cancer Vinorelbine Italian Study Group 1999). As for combined regimens, the response rate and MST have been found to be 17% and 7.4 months (Schiller *et al.* 2002) and 37% and 11.7 months (Takeda *et al.* 2000) respectively for DOC + CDDP, and 30% and 9.3 months (Le Chevalier *et al.* 1994), and 26% and 8 months (Wozniak *et al.* 1998) respectively for VNR + CDDP. The response rate and MST of the platinum group in the present study (40%, 13.5 months respectively) were greater than those found in previous studies of combination regimens comprising CDDP and new anticancer drugs. In the present study, the MST of the platinum group was comparable to that of the non-platinum group. This might be partly due to additional treatments, such as radiotherapy and/or other chemotherapeutic agents, received by patients in the platinum group.

With respect to toxicity, some patients in the platinum group suffered from adverse reactions accompanied by symptoms such as leucopenia (one-third of patients) and nausea/vomiting (two-thirds or more). In addition, six patients who suffered a physical and/or mental burden from these toxicities refused further chemotherapy and withdrew from treatment early. It should be noted that

because the toxicity information in the platinum group was obtained from medical charts, a certain proportion of toxicity events may not have been reported, and thus the event rates may have been underestimated. In contrast, a thorough reporting system was used for patients in the non-platinum group because they were involved in a phase II trial. In the non-platinum group, one-fourth of patients had grade 3–4 neutropenia, but no patients presented with any severe non-haematological toxicity. In fact, the major reason for interruption of chemotherapy in the non-platinum group was progression of the primary cancer (8 cases). Furthermore, for five patients in the non-platinum group who began receiving chemotherapy in an ambulatory setting in the middle of the follow-up period, no severe adverse events were observed, and emergency hospital admission was not required.

In the subgroup of elderly patients (65 years or older), the overall response rate was higher than that in each group as a whole. No elderly patients in the non-platinum group suffered grade 3–4 non-haematological toxicity events, including nausea and vomiting or fatigue, whereas 55% and 17% of elderly patients in the platinum subgroup experienced these adverse reactions respectively.

As we have already seen, the incidence of toxic events in the non-platinum group was significantly lower than that in the platinum group, and in each group the incidence was similar in the subgroup of elderly patients and the group as a whole. We thus conclude that this new non-platinum regimen could be established as a standard treatment, especially for elderly patients or outpatients.

Because most participants in this study were inpatients, even in the non-platinum group, there was no difference in the cost of treatment between the two groups. The cost of hospitalization was also equal in each group. Because management of adverse events is required to a lesser extent for patients receiving non-platinum regimens, chemotherapy could be administered in an ambulatory setting rather than in an inpatient setting. If chemotherapy can be administered in an ambulatory setting, the medical cost would become substantially lower, much lower than that of CDDP-based chemotherapy, which usually requires hospitalization.

In an overall assessment of efficacy and toxicity, the number of responders who did not experience grade 3–4 toxic events, which represents one of the most positive outcomes for patients, was 3 (20%) in the non-platinum group and 0 (0%) in the platinum group. The number of non-responders who experienced grade 3–4 toxic events, which represents the worst outcome for patients, was 3 (20%) in the non-platinum group and 7 (47%) in the platinum group.

In conclusion, these results indicate that the chemotherapy regimen used for the non-platinum group was equally beneficial and less burdensome than those used for the platinum group. Although this study is retrospective and could be considered a preliminary study, given its limited small sample size, the results suggest that the new non-platinum sequential triplet combination could replace CDDP-based chemotherapy as first-line treatment for advanced NSCLC, and that this regimen would be particularly useful for elderly patients and outpatients.

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Phase I study of TZT-1027, a novel synthetic dolastatin 10 derivative and inhibitor of tubulin polymerization, given weekly to advanced solid tumor patients for 3 weeks

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TZT-1027 is a novel synthetic dolastatin 10 derivative that inhibits tubulin polymerization. A phase I study was conducted to determine the maximum tolerated dose (MTD) of TZT-1027, and to assess its pharmacokinetic profile in Japanese patients with advanced solid tumors following administration of the drug weekly for 3 weeks. Eligible patients had advanced solid tumors that failed to respond to standard therapy or for which no standard therapy was available, and met the following criteria: performance status ≤2 and acceptable organ function. The MTD was defined as the highest dose at which more than two-thirds of the patients experienced grade 4 hematological toxicity or grade 3/4 non-hematological toxicity during weekly TZT-1027 administration for 3 weeks. Forty patients were enrolled in the present study. Twelve doses between 0.3 and 2.1 mg/m² were evaluated. Grade 4 neutropenia was the principal dose-limiting toxicity (DLT). At a dose of 2.1 mg/m², two patients developed DLT: one patient developed grade 4 neutropenia, grade 3 myalgia, and grade 4 constipation, and the other one developed grade 4 neutropenia and grade 3 constipation. At a dose level of 1.8 mg/m², toxicity was acceptable and no DLT was observed. The area under the curve and maximum concentration of TZT-1027 tended to increase linearly with the dose. The DLT observed were neutropenia, myalgia, and constipation, and the MTD was 2.1 mg/m². The recommended dose for a phase II study was determined to be 1.8 mg/m² for the drug administered weekly for 3 weeks. (*Cancer Sci* 2009; 100: 316–321)

TZT-1027 (N^2-[*N*,*N*-dimethyl-L-valyl]-*N*-([1S,2R]-2-methoxy-4-([2S]-2-([1R, 2R]-1-methoxy-2-methyl-3-oxo-3-([2-phenylethyl]-amino)propyl)-1-pyrrolidinyl)-1-([1S]-1-methylpropyl)-4-oxobutyl)-*N*-methyl-L-valinamide) is a synthetic analog of dolastatin 10, a compound isolated from the marine mollusk *Dolabela auricularia*.[1,2] The chemical structures of TZT-1027 and dolastatin 10 are shown in Figure 1.

In *in vitro* studies, TZT-1027 was found to exhibit time-dependent cytotoxicity superior to that of many other antitumor agents against a variety of murine and human tumor cell lines.[3] TZT-1027 exhibited antitumor activity against p-glycoprotein-overexpressing cell lines established from colon cancer H116 and breast cancer-resistant protein-positive cell lines established from lung cancer PC-6, and was more potent than vincristine, paclitaxel, and docetaxel against these cell lines. The efficacy of TZT-1027 has been attributed to its inhibition of tubulin polymerization. TZT-1027, which is believed to interact with the same domain on tubulin as the vinca alkaloid-binding region, inhibits the polymerization of microtubule proteins and the binding of GTP to tubulin.[4] In *in vivo* studies, intravenous injection of TZT-1027 has been shown to potently inhibit the growth of P388 leukemic cells and several solid tumors in mice, and to prolong the survival of the animals, and its antitumor efficacy has been shown to be superior or comparable to that of the reference agents dolastatin 10, cisplatin, vincristine, and 5-fluorouracil.[5] Furthermore, in xenograft models, TZT-1027 reduced intratumoral blood perfusion 1 to >24 h after its administration, thereby producing hemorrhagic necrosis of the tumors.[6–8] Thus, TZT-1027 exerts its antitumor activity both through direct cytotoxicity and by selective blockade of tumor blood flow, resulting in marked antitumor activity. In animal toxicology studies, TZT-1027 exhibited little or no neurotoxic potential, in marked contrast to vincristine and paclitaxel, which are antimicrotubule agents that have been shown in controlled animal studies to exert peripheral neurotoxicity.[9] However, at high doses of TZT-1027, myocardial toxicity was observed in rats and monkeys. It was estimated that the drug exerts its effects in a time-dependent manner because of the pattern of its cytocidal effects. The results of assessment in murine models of P388 leukemia and B16 melanoma indicate that simple dosing at short intervals would be the most suitable dosing schedule.

On the basis of this consideration, single dosing (a session of 1-h intravenous drip infusion followed by a 4-week period of observation) was conducted first in humans as a phase I study, and the present study was planned on the basis of the data from the single-dosing study. The previous single-dose phase I study

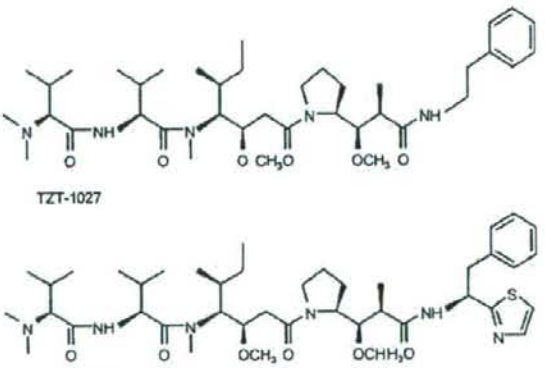

Fig. 1. Structural formulae of TZT-1027 and dolastatin 10.

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