DCR, respectively) in this study. The RR and DCR also did not differ significantly between never-smokers and smokers (Fisher's exact test, $P = 0.37$ for RR, $P = 0.062$ for DCR, respectively). Patients with squamous histology ($n = 2$) showed no response to the treatment.

Table 1. Patient characteristics

Characteristics	Number of patients (%)
Total number of patients	26
Gender	
Male	11 (42)
Female	15 (58)
Age: median, years (range)	68 (51–79)
Performance status	
0	16 (61)
1	8 (31)
2	2 (8)
Histology	
Adenocarcinoma	24 (92)
Squamous cell carcinoma	2 (8)
Stage	
IIIB	3 (11)
IV	22 (85)
Recurrence	1 (4)
Smoking status	
Smoker (current/former)	6 (23)
Non-smoker (never)	20 (77)
EGFR mutation type	
Exon 19	19 (73)
Exon 21	7 (27)
No. of prior chemotherapy	
1	25 (96)
2	1 (4)

At the point of data cutoff (January 2012), after a median follow-up time of 17.3 months (range: 5.8–29.5 months), the median PFS period was 9.3 months (95% CI 7.6–11.6 months; Fig. 1). We also analyzed PFS according to several clinical characteristics including *EGFR* mutation type, gender, histology, smoking status and PS. As shown in Fig. 2, PFS was longer in the patients who had never smoked, and appeared longer in the patients with exon 21 mutations. Furthermore, we analyzed the relationship between efficacy and skin toxicity, but no obvious correlation was found (Table 3). To date, only nine patients have died due to disease progression, and therefore, it is not yet possible to determine the median OS.

Safety and Toxicity

Toxicity was evaluated in all eligible patients (Table 4). The most frequent toxicity was skin disorder including rash, pruritus, desquamation, xeroderma and paronychia. Four patients developed grade 3 skin disorder, but this toxicity was reversible with either an appropriate treatment interruption or dose reduction, and only one patient had to terminate erlotinib treatment for this reason. Liver dysfunction was also common, but generally mild. Two patients experienced ≥grade 3 elevation of the AST or ALT level, but this subsided after erlotinib treatment had been suspended for about a week. Other toxicities were also generally tolerable, and no unexpected toxicities were observed. Diarrhea was less common than we expected.

ILD was not observed in any of the study patients, and no treatment-related deaths occurred.

DISCUSSION

We performed a multicenter phase II trial to evaluate the efficacy and toxicity of erlotinib for pretreated Japanese

Table 2. Tumor response by baseline characteristics

	Overall	Type of *EGFR* mutation		Histology		Smoking status	
		Exon 19	Exon 21	Ad	Sq	Never	Current/former
PR, *n* (%)	14 (53.8)	9 (47.4)	5 (71.4)	14 (58.3)	0 (0)	12 (60)	2 (33.3)
SD, *n* (%)	7 (26.9)	5 (26.3)	2 (28.6)	7 (29.2)	0 (0)	6 (30)	1 (16.7)
PD, *n* (%)	4 (15.4)	4 (21.1)	0 (0)	2 (8.3)	2 (100)	2 (10)	2 (33.3)
NE[a], *n* (%)	1 (3.8)	1 (5.3)	0 (0)	1 (4.2)	0 (0)	0 (0)	1 (16.7)
ORR, % (95% CI)	53.8% (33.4–73.4)	47.4% (24.5–71.1)	71.4% (29.0–96.3)	58.3% (36.6–77.9)	0% (0–84.2)	60% (36.1–80.9)	33.3% (4.3–77.7)
DCR, % (95% CI)	80.8% (60.7–93.5)	73.7% (48.8–91.0)	100% (59.4–100)	87.5% (67.6–97.3)	0% (0–84.2)	90% (68.3–98.8)	50% (11.8–88.2)

PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; ORR, objective response rate; DCR, disease control rate; CI, confidence interval; Ad, adenocarcinoma; Sq, squamous cell carcinoma.
[a]One patient was not evaluable because of poor evaluation of efficacy.

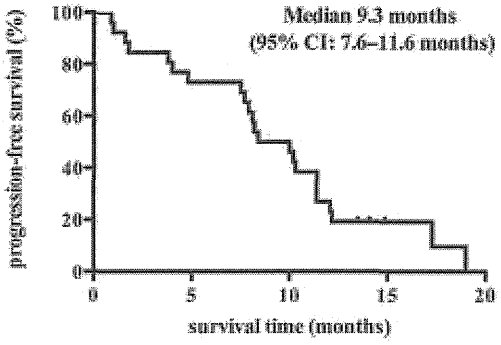

Figure 1. Progression-free survival ($n = 26$).

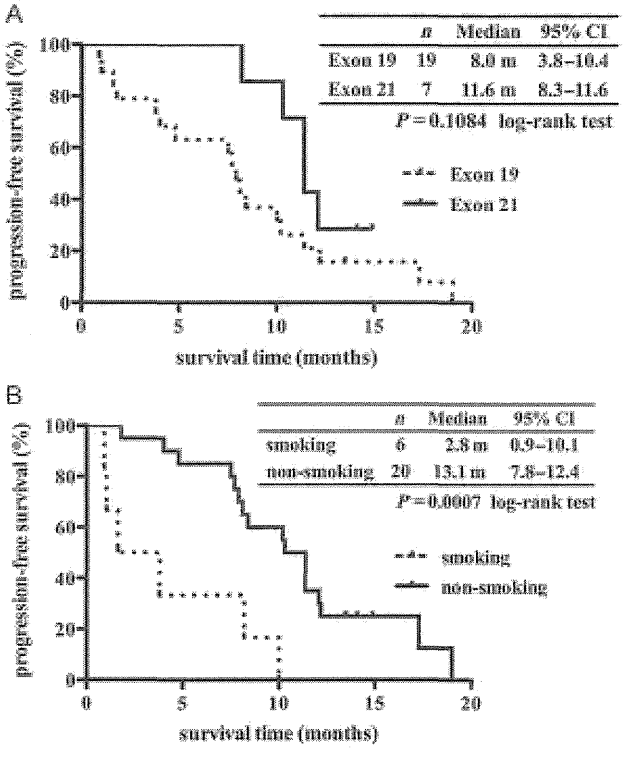

Figure 2. Progression-free survival by *EGFR* mutation type (A) and smoking status (B). m, months; NR, not reached; CI, confidence interval.

NSCLC patients with *EGFR-mt* tumors. In this trial, we obtained an ORR of 53.8%, a DCR of 80.8% and a median PFS period of 9.3 months, along with manageable toxicity, mainly skin disorder.

To date, four phase III trials (10–13) and several phase II trials of gefitinib or erlotinib (8,17–20) in patients with NSCLC harboring *EGFR* mutations have been reported. These demonstrated a higher response rate of around 70% and a longer PFS of approximately 10 months than for patients who received the standard platinum-doublet chemotherapy. We initially expected that the ORR of erlotinib, the primary endpoint of this trial, would be at least 80% based on reports from a Spanish group in 2006 (21) and several sets of Japanese data for gefitinib (17–19), and for this reason the sample size of this trial was small. As a result, although our current data barely exceeded the response rate threshold, this trial could not meet the primary endpoint. However, the DCR, one of the secondary endpoints, attained 80.8% overall, and was 87.5% for adenocarcinoma alone. Furthermore, the median PFS was as long as 9.3 months. These data were comparable with the reported prospective trials of gefitinib and erlotinib in NSCLC patients with *EGFR-mt* tumors.

Why did the PFS reach 9.3 months in this study, despite the relatively lower response rate than that in previously reported trials? Patients having SD may have made a substantial contribution to the result because although these seven patients could not achieve PR unfortunately, all of them showed tumor shrinkage. In addition, the median PFS of these patients was a long as 11.5 months, whereas that of patients having PR was 10.4 months. Several studies have reported that the rate of response to gefitinib or erlotinib was lower in patients who had received previous chemotherapy than in those who were chemotherapy-naïve, but the OS was unaffected by whether these agents were used for first-line therapy or later (10,22–25). Although the impact of cytotoxic chemotherapy on the subsequent response to EGFR-TKIs in *EGFR-mt* patients remains unclear, it is suggested that cytotoxic chemotherapy may induce some biological effects that lead to reduction of EGFR-TKIs sensitivity (26), while not affecting survival. This might be one of the reasons why the PFS in our series was comparable, despite the relatively low response rate. Furthermore, in recent years, it has been considered that EGFR-TKIs may still have potential for disease control even if PD, as defined by RECIST, occurs, and a trial to test this hypothesis is currently ongoing in Asia. In future, the endpoint of efficacy for molecular-targeting agents other than EGFR-TKIs may have to be reconsidered.

In subgroup analysis, response rates in patients with *EGFR* mutation in exon 19 or smokers were especially low. While it remains unclear, previously published studies have suggested that smoking affected treatment outcomes in patients with NSCLC (27,28). In a study by Johnson et al. (29), comparison of smokers and non-smokers in erlotinib treatment showed a strong effect of smoking status favoring non-smokers. Furthermore, Hamilton et al. (30) have shown that smoking could significantly influence the pharmacokinetics of erlotinib. Because all smokers except one had *EGFR* mutation in exon 19 in our cohort, this may affect our result.

Two patients with squamous cell carcinoma were enrolled in our trial, but unfortunately neither showed a response. Some reports have demonstrated that the RR and median PFS of squamous cell carcinoma patients with *EGFR*-mt tumor were around 20–30% and approximately 1.5–3 months, respectively (31,32). These results are clearly inferior to the data for adenocarcinoma patients with *EGFR*-mt tumor. Although resistant mechanism to squamous cell carcinoma with *EGFR*-mt tumor remains unclear, the possible

Table 3. Relationship between skin disorder and efficacy

Grade of skin disorder	*n*	PR	SD	PD	NE	ORR, % (95% CI)	*P*	DCR, % (95% CI)	*P*	Median PFS, months (95% CI)	*P*
Grade 0/1	7	3	2	1	1	42.9 (0.10–0.82)	0.69[a]	71.4 (0.29–0.96)	0.59[a]	10.4 (0.9–19.2)	0.46[b]
Grade 2/3	19	11	5	3	0	57.9 (0.34–0.80)		84.2 (0.60–0.97)		8.3 (4.9–11.6)	

PFS, progression-free survival.
[a]Fisher's exact test.
[b]Log-rank test.

Table 4. Toxicities ($n = 26$)

Event	Number of patients (%) (All grade)	Number of patients (%) (grade 3/4)	Grade			
			1	2	3	4
Hematologic						
Anemia	21 (80.8)	2 (7.7)	15	4	2	0
Leukopenia	5 (19.2)	0 (0)	3	2	0	0
Neutropenia	4 (15.4)	0 (0)	4	0	0	0
Thrombocytopenia	5 (19.2)	0 (0)	5	0	0	0
Non-hematologic						
Rash	25 (96.2)	2 (7.7)	9	14	2	0
Pruritus	21 (80.8)	2 (7.7)	16	3	2	0
Desquamation	18 (69.2)	1 (3.8)	12	5	1	0
Hand foot reaction	17 (65.4)	0 (0)	10	7	0	0
Xeroderma	16 (61.5)	1 (3.8)	6	10	1	0
Anorexia	14 (53.8)	0 (0)	11	3	0	0
Erythema multiforme	13 (50)	0 (0)	5	8	0	0
Paronychia	11 (42.3)	2 (7.7)	3	6	2	0
Fatigue	10 (38.5)	2 (7.7)	5	3	2	0
Stomatitis	9 (34.6)	0 (0)	7	2	0	0
Nausea	6 (23.1)	1 (3.8)	4	1	1	0
Diarrhea	3 (11.5)	0 (0)	3	0	0	0
Vomiting	1 (3.8)	1 (3.8)	0	0	1	0
Purpura	1 (3.8)	1 (3.8)	0	0	1	0
Elevated AST	8 (30.8)	1 (3.8)	6	1	1	0
Elevated ALT	10 (38.5)	2 (7.7)	8	0	1	1
Elevated total bilirubin	13 (50)	0 (0)	11	2	0	0
Elevated creatinine	13 (50)	0 (0)	11	2	0	0

explanation is the presence of the other pathway activation. Previous studies have shown that *PIK3CA*, encoding a subunit of phosphatidylinositol 3-kinase (PI3K), mutations and copy number gain are more frequent in squamous cell carcinoma than in adenocarcinoma (33–35). It is considered that this alteration leads to PI3K/Akt pathway activation, and is one of the mechanisms of EGFR-TKI resistance. While a small population of patients with squamous cell carcinomas harboring *EGFR* mutations may benefit from EGFR-TKIs, some genetic alterations downstream of EGFR, such as those stated above, may have induced resistance to EGFR-TKIs.

The toxicities observed in this trial were mostly tolerable. Skin disorders such as rash, pruritus, xeroderma and desquamation were the main form of erlotinib toxicity, as reported in previous studies (12,13). Especially, rash and pruritus were observed in almost all patients. Because it was shown that the plasma trough concentration of erlotinib was approximately 3.5 times higher than that of gefitinib when administered at the respective approved dose (36,37), the toxicity of erlotinib seemed stronger than that of gefitinib. However, in this trial, no clear differences compared with gefitinib were evident, probably due to small sample size.

In conclusion, although the response rate was relatively lower than that in previously reported studies, erlotinib treatment for Japanese patients with previously treated NSCLC harboring *EGFR* mutations demonstrated promising DCR, PFS and good tolerability. Therefore, erlotinib appears to be an effective option in this population, similar to gefitinib.

Acknowledgments

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Conflict of interest statement

Y.N. has received honoraria and research grant from Chugai Pharmaceutical Co. Ltd. K.T. is a principal investigator in his hospital for the funded clinical trial of Chugai Pharmaceutical Co. Ltd., and K.T. has received a lecture fee from Chugai Pharmaceutical Co. Ltd.

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Kinase Activity of Protein Kinase Cα in Serum as a Diagnostic Biomarker of Human Lung Cancer

JEONG-HUN KANG[1,†,*], TAKESHI MORI[1,2,*], HIROTARO KITAZAKI[3,*], TAKURO NIIDOME[1,2,3], KOICHI TAKAYAMA[4], YOICHI NAKANISHI[4] and YOSHIKI KATAYAMA[1,2,3,5]

[1]*Department of Applied Chemistry,* [2]*Center for Future Chemistry,*
[3]*Graduate School of Systems Life Sciences,*
[4]*Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, and*
[5]*International Research Center for Molecular Systems, Fukuoka, Japan*

Abstract. *Background: Recently, we reported on the existence of activated protein kinase Cα (PKCα) in blood and the possibility for its use in cancer diagnosis. Materials and Methods: In the present study, serum samples collected from patients with different lung cancer types (small-cell cancer, adenocarcinoma, and anaplastic cancer) were phosphorylated with a PKCα-specific peptide substrate and the phosphorylation ratio was detected by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Results: When 13 patient serum samples were phosphorylated with peptide substrates, phosphorylated peaks were obtained in eight samples. However, no peak associated with the phosphorylated peptide was observed using serum samples obtained from 10 healthy persons. Moreover, broadly used cancer biomarkers (progastrin-releasing peptide, carcino-embryonic antigen, and cytokeratin-19 fragment) were identified in eight samples among the 13 samples studied. Conclusion: These results suggest that serum activated PKCα is a reliable biomarker, applicable to lung cancer diagnosis.*

The use of cancer biomarkers in body fluids (*e.g.* blood, urine, and saliva) has several advantages, such as easier sampling and handling, less pain in patients, and non-invasive detection. Several biomarkers, such as antigens, soluble proteins, metabolites and genes, are broadly used for the diagnosis of cancer and for monitoring the recurrence of cancer after surgical resection, or for evaluating the effect of radiation or anticancer drug therapies (1, 2).

Cancer cells have numerous signal transduction pathways that respond to the extracellular signals (ligands) required to regulate downstream gene expression. Among the signal transduction pathways, phosphorylation of the target proteins by protein kinases and proteolytic cleavage by proteases play a key role in cancer cell motility, differentiation, proliferation, and survival (1, 2).

Protein kinase C (PKC) isozymes, which are phospholipid-dependent serine/threonine kinases, play key roles in differentiation, growth, and survival of cancer cells. PKC isozymes are classified into three subfamilies, based on their structural and activational characteristics: conventional or classic PKCs (α, βI, βII, and γ), novel or non-classic PKCs (δ, ε, η, and θ) and atypical PKCs (ζ, ι, and λ). The activation of classic PKCs requires diacylglycerol (DAG) as an activator and phosphatidylserine (PS) and Ca^{2+} as activation co-factors. The non-classic PKCs are regulated by DAG and PS, but do not require Ca^{2+} for activation. In the case of atypical PKCs, their activity is stimulated only by PS, and not by DAG and Ca^{2+} (3, 4).

Among PKC isozymes, PKCα participates in differentiation, growth, and survival of cancer cells. PKCα is hyperactivated in several cancer cell lines and tissues, but has negligible activity in normal cells and tissues (3, 4). Recently, our group found that activated PKCα existing in the blood is a useful biomarker for cancer diagnosis. The level of PKCα was significantly increased in blood samples of cancer-bearing mice compared with that in normal mice (5).

In the present study, serum samples prepared from patients were studied for cancer diagnosis. Each sample was reacted with a PKCα-specific peptide substrate (Alphatomega) (6) and the phosphorylation ratio was detected by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS).

*These Authors contributed equally to this work.

†*Present address:* Department of Biomedical Engineering, National Cerebral and Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan.

Correspondence to: Yoshiki Katayama, Ph.D., Professor, Department of Applied Chemistry, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan. Tel: +81 928022850, Fax: +81 928022850, e-mail: ykatatcm@mail.cstm.kyushu-u.ac.jp

Key Words: Diagnosis, protein kinase, blood, peptide substrate, phosphorylation.

Table I. *Demographic and clinical characteristics of patients and detection of phosphorylated peak in samples.*

Case	Cancer Type (histological type)	Age, years	Gender	Clinical stage[a]	Biomakers[b]	Phosphorylated peak detected[d]
1	Adenocarcinoma	57	M	4	CEA	○
2	Adenocarcinoma	70	F	4	CEA	×
3	Adenocarcinoma	58	M	4	CEA	○
4	Adenocarcinoma	68	M	4	CEA	×
5	Anaplastic cancer	72	F	4	CEA	○
6	Anaplastic cancer	54	M	4	CYFRA 21-1	○
7	Small-cell cancer	69	M	4	ProGRP	○
8	Small-cell cancer	61	M	4	ProGRP	○
9	Small-cell cancer	63	M	3A	-[c]	×
10	Small-cell cancer	67	M	4	-	○
11	Small-cell cancer	55	M	4	-	×
12	Small-cell cancer	71	M	4	-	○
13	Small-cell cancer	49	M	4	-	×

[a]Clinical stage was classified according to the tumor, node, metastasis (TNM) staging system (11). [b]CEA, carcinoembryonic antigen; ProGRP, progastrin-releasing peptide; CYFRA 21-1, cytokeratin-19 fragment. [c]–, Not detected. [d]○, Phosphorylated peak detected; ×, no phosphorylated peak detected.

Materials and Methods

Peptide synthesis. A peptide substrate (FKKQGSFAKKK) was synthesized and purified, as described previously (5, 6). The purity of the synthetic peptide was identified by high-performance liquid chromatography and MALDI-TOF MS, and the peptide with >95% purity was used for the phosphorylation reaction.

MALDI-TOF MS analysis. α-Cyano-4-hydroxycinnamic acid matrix (10 mg/ml) was prepared in 50% water/acetonitrile and 0.1% trifluoroacetic acid. The matrix and serum samples collected from patients and healthy persons were mixed at a ratio of 20:1. A total volume of 1 μl of the analyte/matrix mixture was then applied to the MALDI plate and then allowed to dry to induce crystallization.

Analyses were conducted using a Voyager DE RP BioSpectrometry Workstation (Applied Biosystems, Framingham, MA, USA) in positive ion reflectron mode. All spectra were analyzed using the Data Explorer software (Applied Biosystems). The phosphorylation ratio is defined as the ion intensity ratio of phosphorylated to unphosphorylated material, and was calculated using the formula: [phosphorylated peptide intensity/(phosphorylated peptide intensity + non-phosphorylated peptide intensity) ×100].

Phosphorylation of peptide substrate with serum sample. Patient blood samples (200-500 μl) were collected at the Kyushu University Hospital between June 1 and June 25, 2010 using sterile tubes that contain no additive, heparin, or ethylenediamine tetraacetic acid. Blood samples from 10 healthy persons were obtained as normal controls. Blood samples were centrifuged (3,000 rpm) for 10 min after maintaining them at room temperature for 30 min. The supernatant (serum) was used for the phosphorylation reaction with the peptide substrate. The total protein concentration was determined using the method of Bradford (Coomassie Brilliant Blue G-250 reagent; BIO-RAD Lab., Hercules, CA, USA) and detected by absorbance at 595 nm. The phosphorylation reaction of the peptide substrate was carried out in 30 μl buffer (20 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, and 100 μM ATP) containing 30 μM of the synthetic peptide and serum (2 mg/ml of protein). After incubation for 60 minutes at 37°C, the sample was analyzed by MALDI-TOF MS. Triplicate samples were prepared and each was analyzed twice.

Results

Serum samples from 13 patients with different lung cancer types (small-cell cancer, adenocarcinoma, and anaplastic cancer) were prepared in order to determine the existence of activated PKCα in the serum of these patients and its possibility for use in cancer diagnosis. Serum samples collected from 10 healthy persons were used as controls. Broadly used cancer biomarkers [progastrin-releasing peptide (ProGRP), carcinoembryonic antigen (CEA), and cytokeratin-19 fragment (CYFRA 21-1)] were identified in eight samples out of the 13 samples. When the serum samples were phosphorylated with PKCα-specific peptide, phosphorylated peaks were obtained in eight of the 13 samples (Table I; Figures 1 and 2). The peak associated with the phosphorylated peptide showed an increase in the *m/z* value of 80 Da (Figure 1). However, no peak associated with the phosphorylated peptide was observed in the serum samples of 10 healthy persons (Figure 1).

Discussion

Several lung cancer biomarkers have been reported and used for lung cancer diagnosis. ProGRP, produced by lung cancer, such as small-cell lung carcinoma has high stability in blood and is used as a biomarker for lung cancer diagnosis (7, 8). Serum CEA is a glycoprotein involved in normal glandular and mucosal cells and its serum levels increase in several

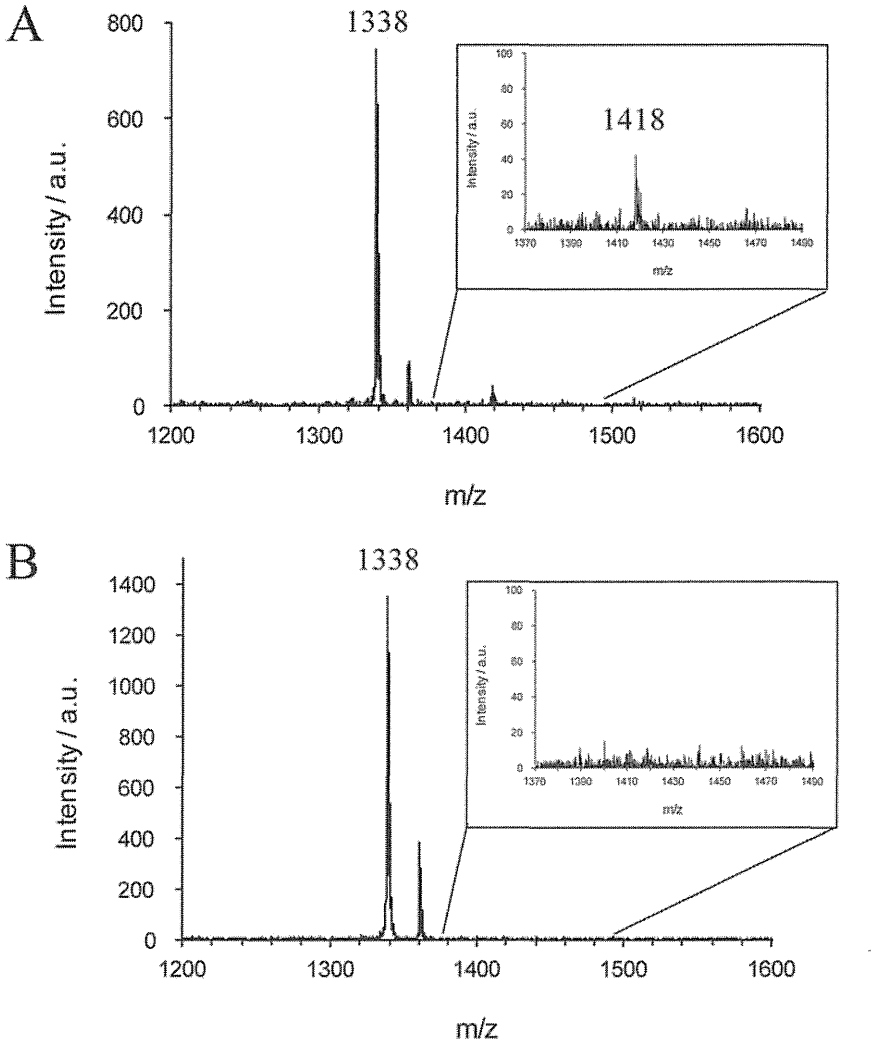

Figure 1. *Typical matrix-assisted laser desorption/ionization-time-of-flight mass spectrometric spectra obtained from the phosphorylation reaction of peptide substrate with serum sample of (A) patient or (B) healthy person.*

lines of cancer cells (*e.g.* lung, gastric, colonic, and breast), and thus it is used as a tumor biomarker for lung cancer diagnosis (7-10). Moreover, serum CYFRA 21-1 levels are also used as an indicator for lung cancer diagnosis (7-10). An increase in serum levels of these biomarkers has been associated with tumor progression and low survival rates. In spite of several useful clinical reports for these biomarkers, the diagnostic accuracy for lung cancer is different from research group to research group, and differs depending on the type and the stage of lung cancer (7-9). Thus, the combination of these biomarkers is encouraged to increase the diagnostic accuracy of lung cancer (8, 9).

In the present study, we detected the presence of phosphorylated peptide in eight serum samples collected from a group of 13 patients with lung cancer after the

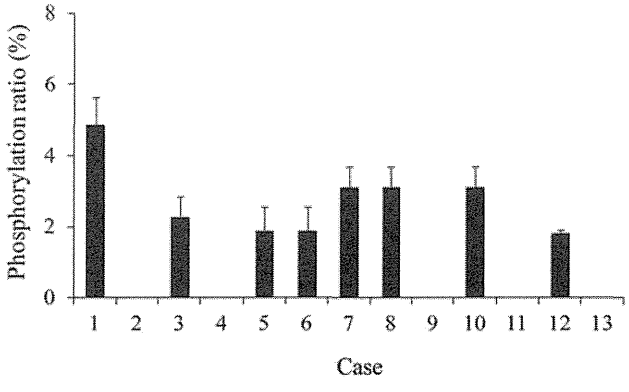

Figure 2. *Phosphorylation ratio is altered after reaction with peptide substrate of serum samples. Data are means±standard deviation of three independent experiments.*

phosphorylation reaction. However, no detection of phosphorylated peptide was detected in the serum samples taken from 10 healthy persons. The diagnostic accuracy for lung cancer by serum PKCα was not lower compared with that of other biomarkers (ProGRP, CEA, and CYFRA 21-1). Thus, our study suggests that serum activated PKCα may be a good biomarker applicable to cancer diagnosis using small samples of patient serum. In spite of these positive results, however, there are two limitations regarding the present study. The first limitation is relatively few cases and the second limitation is that patients with early-stage (stage 1 and 2) lung cancer are not contained.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Acknowledgements

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Sensitivity and kinase activity of epidermal growth factor receptor (EGFR) exon 19 and others to EGFR-tyrosine kinase inhibitors

Kazuto Furuyama,[1] Taishi Harada,[1,3] Eiji Iwama,[1] Yoshimasa Shiraishi,[1] Kyoko Okamura,[1] Kayo Ijichi,[1] Akiko Fujii,[1] Keiichi Ota,[1] Shuo Wang,[1] Heyan Li,[1] Koichi Takayama,[1] Giuseppe Giaccone[2,4] and Yoichi Nakanishi[1]

[1]Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

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The presence of epidermal growth factor receptor (EGFR) somatic mutations in non-small-cell lung cancer patients is associated with response to treatment with EGFR-tyrosine kinase inhibitors, such as gefitinib and erlotinib. More than 100 mutations in the kinase domain of EGFR have been identified. In particular there are many variations of deletion mutations in exon 19. In this study, using yellow fluorescent protein-tagged fragments of the EGFR intracellular domain, we examined the differences in sensitivity to gefitinib, erlotinib and afatinib between several exon 19 mutants and other common EGFR mutations. We also used serum of patients undergoing treatment with EGFR-tyrosine kinase inhibitors in this system. In addition, we examined the relative kinase activity of these mutants by measuring relative fluorescent intensity after immunofluorescence staining. We found that both sensitivity to EGFR-tyrosine kinase inhibitors and relative kinase activity differed among several EGFR mutations found in the same region of the kinase domain. This study underscores the importance of reporting the clinical outcome of treatment in relation to different EGFR mutations. (*Cancer Sci* 2013; 104: 584–589)

About half of lung adenocarcinoma patients in Japan have somatic mutations in the kinase domain of epidermal growth factor receptor (EGFR),[1,2] and the presence of these mutations is known to be associated with increased response to treatment with EGFR-tyrosine kinase inhibitors (EFGR-TKI).[3–5] To date, more than 100 EGFR somatic mutations have been identified in lung cancer patients, as detailed in the COSMIC database (www.sanger.ac.uk/genetics/CGP/cosmic/). About 90% of EGFR mutations consist of either short deletion mutations in exon 19 or a point mutation in exon 21 (L858R). Mitsudomi *et al.*[6] reported the response rate to gefitinib was higher in patients with exon 19 deletion mutations (81%) than in those with an L858R mutation (71%) in exon 21. The L861Q point mutation in exon 21 accounts for 1–2% of EGFR mutations,[2,7] and the response rate of affected patients to EGFR-TKI is reportedly 60%.[8] G719X is a point mutation in exon 18 of EGFR, in which the glycine at codon 719 is substituted with cysteine, alanine or serine; it comprises <5% of all EGFR mutations. Patients with these mutations are reported to be less sensitive to EGFR-TKI, and the response rate is around 56%.[6] Mutations in exon 20, such as the T790M second mutation, are known to be resistant to EGFR-TKI. Thus, there is variability in sensitivity of different EGFR somatic mutations to these drugs.[9] Furthermore limited data exist regarding the more uncommon EGFR mutations, including the less frequent variants in exon 19. In this study, using yellow fluorescent protein (YFP)-tagged fragments of the EGFR intracellular domain (YFP-EGFR-ICD), we examined the sensitivity of various exon 19 mutations and other common EGFR mutations to EGFR-TKI.[10,11] We then compared the autophosphorylation levels of the kinase domains of the different mutants by measuring fluorescent intensity.

Materials and Methods

Plasmid construction and site-directed mutagenesis. The mutant YFP-EGFR-ICD constructs were generated as described previously.[10,11] A KOD -Plus- Mutagenesis kit (TOYOBO, Tokyo, Japan) was used to construct EGFR mutants according to the manufacturer's protocol with WT YFP-EGFR-ICD as a template. Primers for each mutation are described in Data S1.

Cell culture, transfections and drug treatments. The human breast cancer cell line Michigan Cancer Foundation (MCF)-7 was grown in DMEM supplemented with 10% FBS, 100-U/mL penicillin and 100-μg/mL streptomycin (Gibco, Carlsbad, CA, USA). Cells were seeded onto sterile glass coverslips in six-well plates and transfected with 1-μg plasmid DNA using the X-tremeGENE 9 Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Gefitinib, erlotinib and afatinib were added at the indicated concentrations at 24 h after transfection, and the cells were incubated for 12 h before they were processed for immunofluorescence analyses. Drug treatments were always performed in standard culture medium containing 10% FBS. Gefitinib and erlotinib were purchased from Cayman Chemical (Ann Arbor, MI, USA), and afatinib was purchased from Selleck Chemicals (Houston, TX, USA).

Immunofluorescence and microscopy analysis. To evaluate EGFR autophosphorylation, a rabbit anti-phosphorylated EGFR-Y1068 antibody (#3777S, diluted 1:200, Cell Signaling Technology, Danvers, CO, USA) was used. The immunostaining procedure was used as previously described.[12] Briefly, cells were fixed using 4% formaldehyde in PBS for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min. After a blocking step with Blocking One Histo (Nakarai, Kyoto, Japan) for 30 min, primary antibody diluted in PBS with 0.1% Tween20 was applied for 1 h. After being washed with PBS, samples were incubated with Alexa Fluor 594 (AF-594)-conjugated anti-rabbit secondary antibody (diluted 1:400, Life Technologies, Carlsbad, CA, USA) for 1 h. Finally, the coverslips were mounted onto microscopic slides

[3]To whom correspondence should be addressed.
E-mail: harada-t@kokyu.med.kyushu-u.ac.jp
[4]Present address: Department of Oncology and Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, District of Columbia, USA.

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with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

Slides were examined with a Keyence BZ-8100 fluorescence microscope (Osaka, Japan). BZ Analyzer software (Keyence) was used to collect images, and exposure time was kept constant to allow for comparison of the signal intensities among different samples.

Semi-quantitative comparison of YFP-EGFR-ICD autophosphorylation levels was performed with computer-assisted image analysis. With a ×20 objective lens, images of several transfected cells were taken, and the fluorescent intensity in the green and red channels was measured within a cytoplasmic area (YFP signal and AF-594 signal) and within an area outside the cells (background). The intensity of the YFP and AF-594 signals for each cell was plotted on a scatter plot and an approximation straight lines was obtained. Then, the angles of inclination were compared on a bar graph. All plotted signals in each group were subjected to the analysis of covariance. *P*-values less than 0.05 were considered to be statistically significant. Excel 2008 (Microsoft Corporation, Redmond, WA, USA) was used for these analyses.

Results

Sensitivity to EGFR-TKI. Michigan Cancer Foundation-7 cells were transfected with plasmids encoding YFP-EGFR-ICD for WT EGFR or mutant variants of EGFR. Twenty four hours post-transfection gefitinib or erlotinib at a final concentration ranging from 10 nM to 10 μM and afatinib at a final concentration ranging from 10 to 500 nM were added to the culture medium. As reported previously, the YFP-tagged EGFR fragments used in this study lacked the extracellular and juxtamembrane domains of the receptor.[10,11] Thus, we could reduce interference from the experimental context and introduce mutagenesis more efficiently to shorter EGFR fragments. When treated with EGFR-TKI, these YFP-EGFR-ICD fusion proteins relocate to fibril-like formation. Although the mechanism of this relocation is unclear, it parallels the sensitivity to EGFR-TKI, and correlates with decreasing downstream phosphor-PKB signal.[10,11] In this study, when 70% of cells changed the fusion protein location, we determined there was sensitivity to the EGFR-TKI. Gefitinib had no effect on WT YFP-EGFR-ICD and del746–750/T790M YFP-EGFR-ICD double mutant-transfected cells (Fig. 1a). However, low concentrations of gefitinib (20–100 nM) induced relocation of exon 19 deletion mutant YFP-EGFR-ICD and L858R YFP-EGFR-ICD (Figs 1a, 2). Among various mutations in exon 19, there was a small difference in sensitivity ranging from 20- to 50-nM gefitinib. All exon 19 deletion mutants and/or insertion mutants were more sensitive to EGFR-TKI than to L858R (Table 1). However, an exon 19 insertion variant (745–746 ins VPVAIK [insertion mutation valine-proline-valine-alanine-isoleucine-lysine]) was less sensitive to gefitinib (500 nM) than other exon 19 deletion mutants (Table 1, Fig. 2).

Serum obtained from patients who underwent treatment with gefitinib or erlotinib for at least 1 month were diluted and added to del746–750 YFP-EGFR-ICD-transfected cells. The serum from patient 1, who had received gefitinib treatment, induced YFP signal relocation at a dilution ratio below 1:100. The serum from patient 2, who had received erlotinib treatment, induced relocation at a 1:1000 dilution (Fig. 1b). Erlotinib has a higher potency than gefitinib and is commonly administered at the maximum tolerated dose of 150 mg/day. In contrast, gefitinib is commonly used at a dose (250 mg /day) that is less than half of the maximum tolerated dose. The trough serum concentrations of 250-mg/day gefitinib and 150-mg/day erlotinib are reported to be approximately 400 nM and 1.5–3.0 μM, respectively.[13,14] We examined the serum of

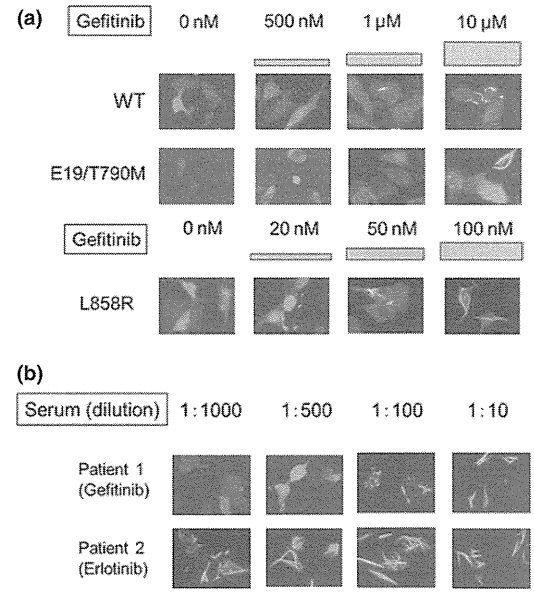

Fig. 1. Evaluation of the sensitivity of EGFR mutations to gefitinib. YFP-EGFR-ICD-transfected cells were treated with gefitinib at the indicated concentrations for 12 h, and cells were then subjected to image analysis by fluorescence microscopy. (a) The WT and del746–750 /T790M YFP-EGFR-ICD-transfected cells showed only partial relocation of the YFP signal at 10-μM gefitinib. (b) Serum from lung cancer patients treated with gefitinib or erlotinib was diluted at the indicated ratios and then added to del746–750 YFP-EGFR-ICD-transfected cells. Del, deletion mutation; E19, exon 19; EGFR, epidermal growth factor receptor; YFP, yellow fluorescent protein; YFP-EGFR-ICD, YFP-tagged fragments of the EGFR intracellular domain.

34 additional patients; all except one sample was of sufficiently high concentration to induce relocation at 1:10–1:1000 dilution (Table S1).

L861Q and G719X mutations are known to be moderately sensitive to EGFR-TKI.[15,16] Gefitinib at 200 nM induced relocation in L861Q YFP-EGFR-ICD-transfected cells (Fig. 2). A relatively high gefitinib concentration (500 nM) was needed to induce relocation of G719X (Fig. 2). We also evaluated the effect of erlotinib or afatinib (BIBW 2992) treatment (Table 1). Erlotinib treatment showed similar results to gefitinib. Afatinib is an irreversible EGFR and human epidermal growth factor receptor type 2 (HER2) inhibitor, predicted to overcome the acquired resistance caused by T790M that covalently binds in the catalytic pocket of EGFR.[17] In a phase I clinical trial, the trough serum concentration of afatinib at a dose of 40–50 mg/day was under 100 nM.[18] The del746–750/T790M YFP-EGFR-ICD-transfected cells showed fibril-like formation of YFP signals at an afatinib concentration of 100 nM or more. Cells transfected with intermediately-sensitive mutants (L861Q, G719X) all presented a similar response to afatinib at 50 nM.

Relative kinase activity of various EGFR mutations. We transfected WT YFP-EGFR-ICD and a different YFP-EGFR-ICD mutation into MCF-7 cells. Twenty four hours post-transfection, immunofluorescence staining was performed using a specific primary antibody to detect phosphorylation of EGFR at Y1092. Secondary antibody conjugated to AF-594 was used, and cells were examined with fluorescence microscopy. A YFP signal is a marker of transfection, and non-transfected cells did not contain a detectable AF-594 signal. We used computer-assisted analysis to compare the AF-594 and YFP signal intensity of individual cells. We then evaluated the relative kinase activities (i.e. the levels of autophosphorylated Y1092 at the same YFP level) (Fig. 3a). The phosphorylation

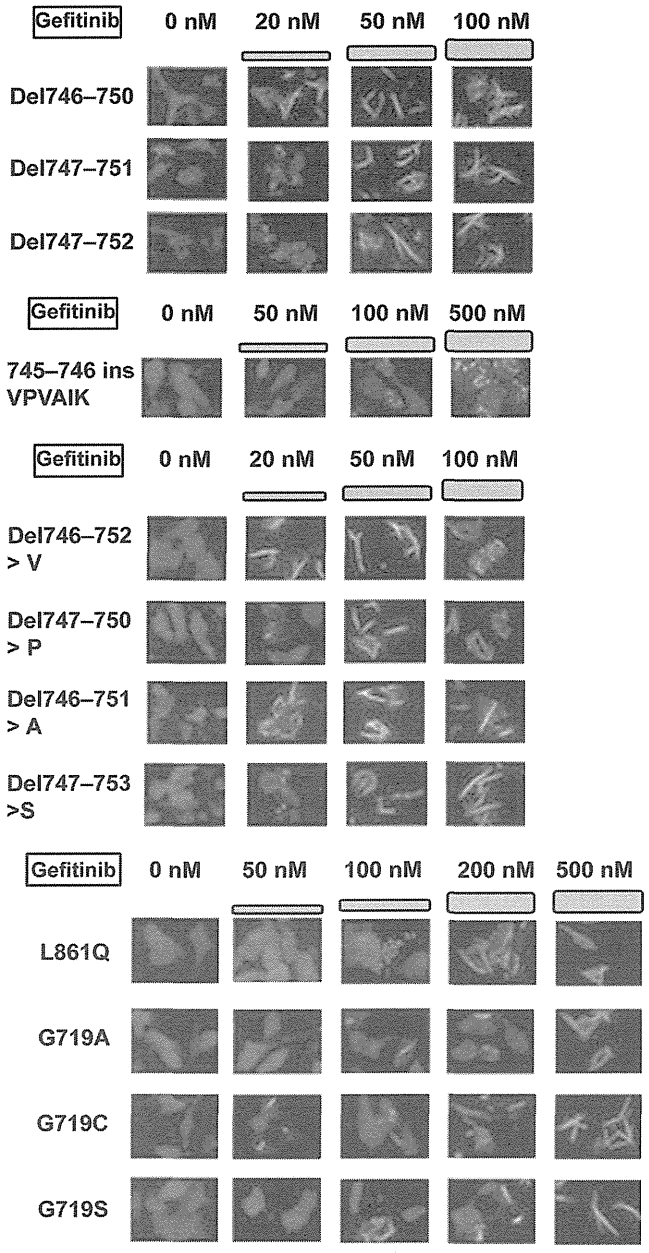

Fig. 2. Sensitivity of various mutations in exon 19 and common EGFR mutations to gefitinib. The various YFP-EGFR-ICD exon 19 mutant-transfected cells showed YFP signal relocation at lower concentrations of gefitinib than WT YFP-EGFR-ICD. Sensitivity to gefitinib was different among these mutations. The L861Q and G719X YFP-EGFR-ICD-transfected cells showed YFP signal relocation at 200–500-nM gefitinib. A, alanine; Del, deletion mutation; EGFR, epidermal growth factor receptor; ins, insertion mutation; P, proline; S, serine; V, valine; VPVAIK, valine-proline-valine-alanine-isoleucine-lysine; YFP, yellow fluorescent protein; YFP-EGFR-ICD, YFP-tagged fragments of the EGFR intracellular domain.

status of WT EGFR was very low. Among the exon 19 mutations, all had similar or significantly higher levels of autophosphorylation than WT EGFR, with the exception of del747–752, which had a much lower autophosphorylation level than the other mutations (Fig. 3b).

With regard to other common EGFR mutations, G719X showed weaker phosphorylation levels than the exon 19 deletion mutants (Fig. 3c). Among G719X mutations, G719C and G719S mutations showed lower levels of autophosphorylation than G719A ($P < 0.01$). The other common mutations (L861Q, L858R and exon 19 del/T790M) showed similarly enhanced phosphorylation to the exon 19 deletion mutations. The results of fluorescent microscopy-based EGFR phosphorylation analysis were confirmed by Western blot analysis (Fig. 3d).

Table 1. Sensitivity of EGFR mutations to EGFR-TKI

	Gefitinib	Erlotinib	Afatinib
Del746–750	20 nM	20 nM	NP
Del747–751	50 nM	20 nM	NP
Del747–752	50 nM	50 nM	NP
Del746–751>A	20 nM	20 nM	NP
Del747–750>P	50 nM	50 nM	NP
Del747–753>S	50 nM	50 nM	NP
Del746–752>V	20 nM	20 nM	NP
E19 ins VPVAIK	500 nM	500 nM	20 nM
L858R	100 nM	50 nM	NP
L861Q	200 nM	200 nM	50 nM
G719C	500 nM	500 nM	50 nM
G719S	500 nM	500 nM	50 nM
G719A	500 nM	500 nM	50 nM
Del746–750/T790M	>10 μM	>10 μM	200 nM
WT	>10 μM	>10 μM	200 nM

EGFR-TKI were added to transfected cells at the iNPicated concentrations. Sensitivities to EGFR-TKI were determined by observation of YFP signal relocation. A, alanine; Del, deletion mutation; E19, exon 19; EGFR, epidermal growth factor receptor; ins, insertion mutation; NP, not performed; P, proline; S, serine; TKI, tyrosine kinase inhibitor; V, valine; VPVAIK, valine-proline-valine-alanine-isoleucine-lysine; YFP, yellow fluorescent protein.

Discussion

Analysis of the COSMIC database revealed that inframe deletion mutations and deletion/insertion mutations comprise 76% and 21% of EGFR mutations in exon 19, respectively. The most frequent mutation is del746–750, which comprises 64% of exon 19 mutations, followed by del747–753>S (6%), del747–751 (4.2%), del747–750>P (4%), del746–752>V (2.4%), del747–752 (2.1%) and del746–751>A (1%), respectively. The frequency is similar to that reported in the Somatic Mutations in EGFR Database. As for insertion mutations in exon 19, this type of mutation is described as less sensitive to EGFR-TKI than other exon 19 deletion mutations.[19] These insertion mutations comprise approximately 1% of all EGFR mutations identified by DNA sequencing of lung tumor specimens in the USA. Although many types of EGFR exon 19 mutations have been reported, little is known about their characteristics. Therefore, we studied several EGFR exon 19 variants using the YFP-EGFR-ICD assay. Previous reports demonstrated that the assay is useful for evaluating the sensitivity of EGFR mutations to EGFR-TKI. We also used HEK/293 and BEAS-2B cell lines in addition to MCF-7 cells, and the results were almost identical (data not shown). We used mainly MCF-7 cells because an irrelevant cell line seemed to be a better method to examine the effect of exogenous EGFR without unknown factors influencing EGFR signaling. Sensitivity to EGFR-TKI was determined by YFP-EGFR fusion protein relocation, not by cell proliferation or apoptosis. The relocation and signaling of YFP-EGFR may be independent of cell addiction to endogenous EGFR signaling. In addition, in our study the assay was used to evaluate the concentration of EGFR-TKI in serum from non-small-cell lung cancer patients undergoing EGFR-TKI treatment. As del746–750 YFP-EGFR-ICD-transfected cells showed YFP relocation at

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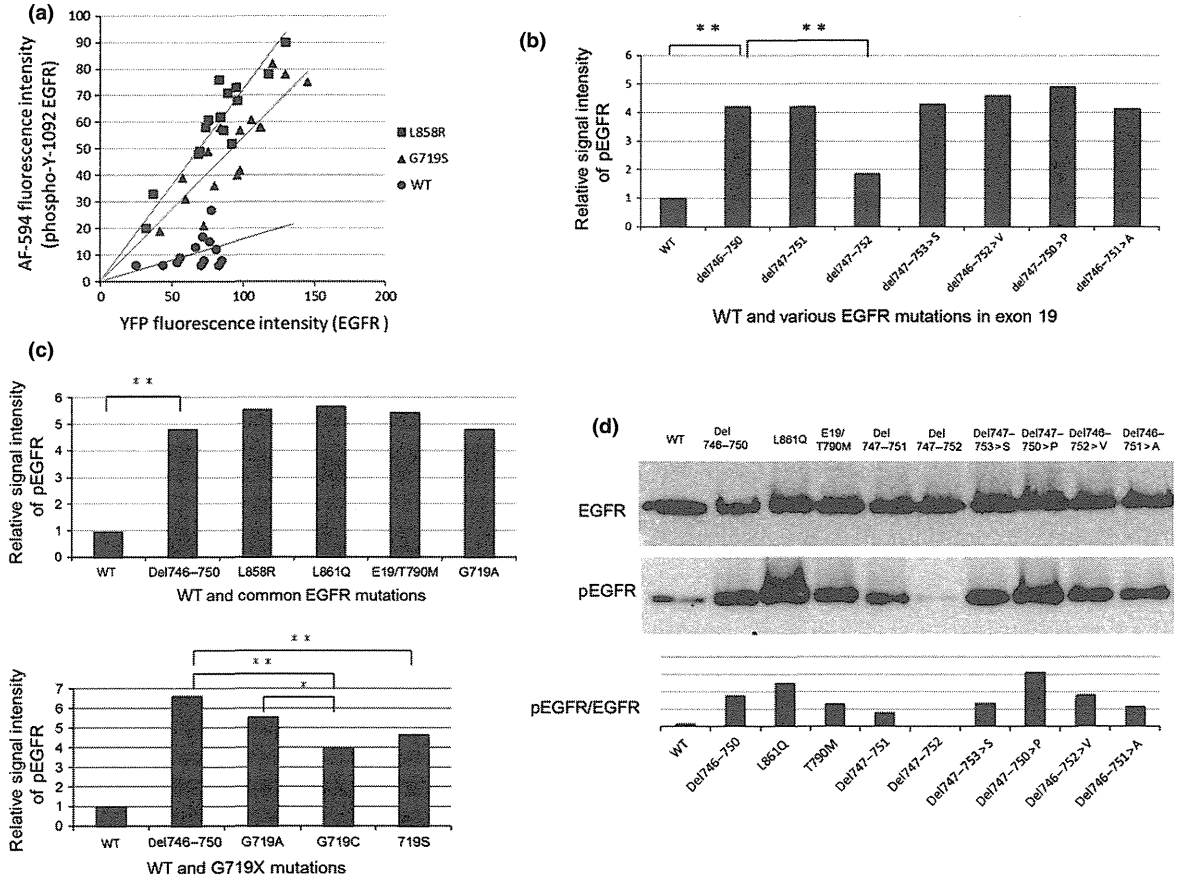

Fig. 3. Relative kinase activity of EGFR exon 19 mutations. (a) Semi-quantitative comparison of YFP-EGFR-ICD autophosphorylation levels based on computer-assisted image analyses. The intensity of YFP and AF-594 for each cell line was plotted on a scatter plot and an approximation straight lines as obtained. The angles of inclination were compared on a bar graph for each mutation. (b) Comparison of phosphorylation levels of WT EGFR and EGFR exon 19 mutants. (c) Comparison of phosphorylation levels of WT and common EGFR mutations. The *y*-axis indicates the relative phosphorylation levels of EGFR. The phosphorylation of WT EGFR is shown as 1. Statistical analysis was performed as described in the Materials and Methods. (d) EGFR and pEGFR levels of several EGFR mutations were quantified by Western blotting. $*P < 0.01$. $**P < 0.05$. A, alanine; AF-594, Alexa Fluor 594; Del, deletion mutation; E19, exon 19; EGFR, epidermal growth factor receptor; P, proline; pEGFR, phosphorylated epidermal growth factor receptor; S, serine; V, valine; YFP, yellow fluorescent protein; YFP-EGFR-ICD, YFP-tagged fragments of the EGFR intracellular domain.

20-nM gefitinib, we could estimate the approximate serum concentration of gefitinib by the addition of diluted serum. The concentration in serum from patients administered gefitinib treatment was approximately 5–10 times lower than that of patients who received erlotinib treatment (Table S1). The results seemed to confirm the previously reported pharmacokinetics of EGFR-TKI. Therefore, this assay might be useful as an alternative method for monitoring serum concentrations.

Several reports described the sensitivity of exon 19 mutation variants.[15,20] According to the previous data, del746–750 and del747–753>S were a little more sensitive (IC_{50} < 10 nM) than other variants. In one report, del746–752>V-transfected cells were less sensitive (IC_{50} = 306 nM) to gefitinib than other variants (IC_{50} < 100 nM). In our study, del746–750, del746–751>A and del746–752>V were more sensitive (<20 nM) than other variants (<50 nM). Although there is a little discrepancy in results between our data and previous studies, in part because of different methods of detection, the most common mutation, del746–750, generally seems to be the most sensitive. As the amino acid residues at position 747–750 in human EGFR protein (leucine, arginine, glutamic acid and alanine) are highly conserved, it is presumed that single amino acid changes in exon 19 variants may influence sensitivity to EGFR-TKI and then partly contribute to the individual differences in clinical benefit from EGFR-TKI treatment. As variants of exon 19 deletions have not been distinguished in clinical trials, the clinical relevance of these variants remains unclear.

A small difference in sensitivity to EGFR-TKI was also found between del746–750 and L858R. In several clinical trials of gefitinib or erlotinib treatment, lung cancer patients with exon 19 deletion mutations had superior response rates, progression free survival and overall survival rates than patients with L858R.[6,21,22] The differences in clinical outcome might partially reflect the differences in sensitivity.

YFP-tagged fragments of the EGFR intracellular domain L861Q-or G719X-transfected cells showed intermediate sensitivity to gefitinib and erlotinib, and L861Q was more sensitive than G719X. No difference in sensitivity between these mutations was found for afatinib. Kancha *et al.*[23] noted that because L861Q retained high binding affinity for ATP, irreversible inhibitors of EGFR might be more beneficial than reversible inhibitors to patients with L861Q EGFR mutation. In our study, the L861Q mutation was relatively sensitive to reversible and irreversible tyrosine kinase inhibitors. Erlotinib may be more effective than gefitinib in patients with G719X

that need 500 nM of either drug, because trough levels of gefitinib are lower than effective concentrations when the drug is given at standard dose.

In approximately half of patients with acquired resistance after treatment with first generation EGFR-TKI, T790M secondary mutation will occur.[24–27] In the present study, afatinib needed to be administered at a concentration of at least 100 nM to alter the location of YFP-EGFR-ICD fusion protein with the del746–750/T790M mutation, whereas afatinib had a median trough concentration of 30–60 nM in a clinical study.[18] In addition, a recent *in vitro* study showed that T790M secondary mutation was also involved in acquired resistance after treatment with afatinib.[28] Clinical studies targeting T790M mutations are necessary. Higher intermittent doses of afatinib may be more effective than standard continuous dosing in this patient population.

Several lines of evidence indicated that EGFR mutants exhibit differentially enhanced kinase activities. In our study, exon 19 mutants, with the exception of del747–752, presented with almost similar autophosphorylation status. Reflecting previous data,[29] del747–752, which accounts for <2% of mutations in exon 19, presented a low autophosphorylation status comparable to WT EGFR.[2,9] Pao *et al.* evaluated the phosphorylation of this mutation variant using a phospho-Y1092-specific antibody and an anti-phosphotyrosine antibody; phosphorylation was at a low level compared to WT EGFR or L858R. Although it is unclear why only del747–752 is not as highly phosphorylated at Y1092, patients with del747–752 mutation benefit from EGFR-TKI treatment.[29,30] A possible hypothesis is that this EGFR mutation may be activated by heterodimerization with HER2 or human epidermal growth factor receptor type 3 (HER3).

The autophosphorylation levels of G719X were less enhanced than other common mutations. Relatively low response rates in patients with EGFR G719X mutations to inhibitor treatment might indicate a lower dependence on EGFR signaling.

In conclusion, various EGFR exon 19 deletion mutants exhibited similar characteristics, with the exception of del747–752, in terms of phosphorylation of the EGFR tyrosine kinase domain. However, variants of EGFR exon 19 deletions showed differences in sensitivity to EGFR-TKI. The difference of these characteristics of various EGFR mutations may be relevant to clinical outcome. Therefore, further studies to functionally characterize and determine the clinical relevance of EGFR mutations are warranted.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Doc. S1. List of the primers for site-directed mutagenesis.

Table S1. Patients' characteristics and serum concentration of epidermal growth factor receptor-tyrosine kinase inhibitors in this study.

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Short Hydration in Chemotherapy Containing Cisplatin (≥75 mg/m^2) for Patients with Lung Cancer: A Prospective Study

Hidehito Horinouchi*, Kaoru Kubota, Hidetoshi Itani, Tomoko Katsui Taniyama, Shinji Nakamichi, Hiroshi Wakui, Shintaro Kanda, Hiroshi Nokihara, Noboru Yamamoto, Ikuo Sekine and Tomohide Tamura

Division of Thoracic Oncology, National Cancer Center Hospital, Tokyo, Japan

*For reprints and all correspondence: Hidehito Horinouchi, Division of Thoracic Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan. E-mail: hhorinou@ncc.go.jp

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Objective: We previously reported that 22% of lung cancer patients experienced a Grade 2 or 3 elevation in creatinine after chemotherapy containing cisplatin. We conducted a Phase II trial to evaluate the safety and efficacy of short hydration.
Methods: The major eligibility criteria included patients with lung cancer for whom a ≥75 mg/m^2 cisplatin-based regimen was indicated and adequate organ function. Cisplatin was administered with pre- and post-hydration containing 10 mEq of potassium chloride in 500 ml of fluid over a 60-min period. Immediately before the administration of cisplatin, mannitol (20%, 200 ml) was administered as forced diuresis over 30 min. And magnesium sulfate (8 mEq) was added to pre-hydration.
Results: Forty-four patients were enrolled between April and December 2011. The patients included 29 men and 15 women with a median (range) age of 64 (42–74) years. Twenty patients received cisplatin and pemetrexed as their most frequent regimen and 38 patients received three to four cycles of chemotherapy. The median (range) duration and volume of the chemotherapies were 4.0 (3.3–6.8) h and 1600 (1550–2050) ml, respectively. Of the 44 patients, 43 (97.8%) completed the cisplatin-based chemotherapy without Grade 2 or higher renal dysfunction. The only patient who had Grade 2 elevation in creatinine (maximum value 1.7 mg/dl) had prompt improvement in creatinine levels and completed four cycles of chemotherapy.
Conclusions: The short hydration is safe without severe renal toxicities in regimens containing cisplatin (≥75 mg/m^2) for patients with lung cancer.

Key words: short hydration – magnesium supplementation – cisplatin – lung cancer – renal toxicity

INTRODUCTION

Cisplatin (≥75 mg/m^2) is an important component of standard therapies for lung cancer, especially in adjuvant chemotherapy, chemoradiotherapy and also in advanced settings (1–3). Ardizzoni et al. (4) conducted an individual patient data meta-analysis and reported that cisplatin-based chemotherapy was associated with a statistically significant better survival compared with carboplatin-based regimens in non-squamous non-small-cell lung cancer. Several liters of hydration over a period of 10 h have been recommended on labels for cisplatin because of prevention of renal toxicity. The introduction of newer-generation antiemetics such as aprepitant and palonosetron significantly reduces nausea and vomiting, resulting in better control of oral intake. However, the conventional long hydration is widely performed because of the nephrotoxicity of cisplatin, preventing the optimal use of a platinum agent (5). We previously reported that 22% of lung cancer patients experienced a Grade 2/3 elevation in creatinine after undergoing conventional long hydration in regimens containing cisplatin (6). To reduce nephrotoxicities of cisplatin, several strategies including magnesium supplementation and forced diuresis using mannitol or furosemide have been reported

(7–10). Because few studies have reported the feasibility and efficacy of short hydration after the introduction of newer-generation antiemetics, we conducted a prospective trial to evaluate the safety of short hydration in regimens containing cisplatin using state-of-the-art protective strategies against renal and gastrointestinal toxicities.

PATIENTS AND METHODS

Patient Selection

The eligibility criteria were as follows: histologically or cytologically proven lung cancer; candidates for platinum-based chemotherapy or chemoradiotherapy with cisplatin ($\geq$75 mg/m^2); no previous treatment including cisplatin; patients aged between 20 and 74 years; an Eastern Cooperative Oncology Group (ECOG) performance status (11) of 0 or 1; adequate bone marrow function [white blood cell (WBC) count $\geq 3.0 \times 10^9$/l, neutrophil count $\geq 1.5 \times 10^9$/l, hemoglobin $\geq$9.0 g/dl and platelet count $\geq 100 \times 10^9$/l], liver function (total bilirubin $\leq$1.5 mg/dl and transaminase $\leq$100 IU/l) and renal function (serum creatinine $\leq$ the upper limit of the normal value and creatinine clearance $\geq$60 ml/min) and an SpO_2 of $\geq$95%. Patients were excluded if they had dysphagia caused by recurrent nerve paralyses or large mediastinal masses, uncontrolled malignant pleural or pericardial effusion, or concomitant serious illness (such as angina pectoris, myocardial infarction in the previous 6 months, heart failure, infection or any other diseases contraindicating chemotherapy or radiotherapy). All the patients gave their written informed consent. The study was approved by the institutional review board of the National Cancer Center Hospital, Tokyo, Japan (UMIN trial registration no. 000004727).

Treatment Schedule

Patients received cisplatin-based chemotherapy with a cisplatin dose of $\geq$75 mg/m^2 every 3 weeks. As a common antiemetic premedication, palonosetron (0.75 mg) and dexamethasone (9.9 mg) were dissolved in 50 ml of normal saline solution and infused, and an oral aprepitant (125 mg on Day 1, 80 mg on Days 2–3) and dexamethasone (8 mg, Days 2–4) were administered before and after chemotherapy. An hour-long infusion of cisplatin dissolved in 250 ml of normal saline solution was inserted between pre-hydration [potassium chloride (10 mEq) and magnesium sulfate (8 mEq) dissolved in 500 ml of one-fourth saline solution] and post-hydration (potassium chloride 10 mEq dissolved in 500 ml of one-fourth saline solution). Mannitol was infused just before the cisplatin administration as an enforced diuresis. The patients received one other cytotoxic agents, including pemetrexed (500 mg/m^2), docetaxel (60 mg/m^2), vinorelbine (20 or 25 mg/m^2), gemcitabine (1000 mg/m^2) or etoposide (100 mg/m^2), with appropriate premedication in combination with cisplatin (see the example in Figure 1).

Antiemetics (0.25hr)	
Palonosetron	0.75mg
Dexamethasone	9.9mg
0.9% saline	50ml
Pemetrexed (10 min)	
Pemetrexed	500mg/m^2
0.9% saline	100ml
Pre-hydration (1hr)	
1/4 saline solution	500ml
Potassium chloride	10mEq
Magnesium sulfate	8mEq
Diuresis (0.5hr)	
20%Mannitol	200ml
Cisplatin (1hr)	
Cisplatin	75mg/m^2
0.9% saline	250ml
Post-hydration (1hr)	
1/4 saline solution	500ml
Potassium chloride	10mEq

Figure 1. An example of chemotherapy regimen: cisplatin and pemetrexed.

Toxicity Assessment and Treatment Modification

Complete blood cell and differential counts were performed and routine chemistry determinants were evaluated on Day 8 of the first cycle and on Day 1 of every cycle. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria (CTCAE) version 4.0 issued in 2009. Although the CTCAE version 4.0 contains two criteria for creatinine, we adopted a classical ULN (upper limit of normal)-based method to evaluate the elevation of creatinine. Subsequent cycles of cisplatin-based chemotherapy were delayed if any of the following toxicities were noted on Day 1: WBC count $<3.0 \times 10^9$/l, neutrophil count $<1.5 \times 10^9$/l, platelet count $<100 \times 10^9$/l, serum creatinine level $>$1.4 mg/dl, elevated hepatic transaminase level $>$100 IU/l and performance status of $\geq$2. The dose of cisplatin was reduced by 25% in all the subsequent cycles if the serum creatinine level increased to Grade 2 or higher.

Statistical Analyses

This study was designed as a nonrandomized, single-center Phase II trial. The primary efficacy variable was the proportion of patients without renal dysfunction, defined as the proportion of patients without a Grade 2 or higher elevation in creatinine from the baseline value after first-cycle cisplatin. The sample size was estimated using a Simon two-stage design to test the null hypothesis for a proportion of patients without renal dysfunction of $\leq$70% versus an alternative hypothesis of a proportion of patients $\geq$88% at a power of 90%. Under the assumption of a Type I error rate of 0.05, with the stated statistical hypothesis, a total of 44 patients were required for the study (12). The successful completion of

Stage I required 24 patients without renal dysfunction among the first 30 patients who received first-cycle cisplatin. The overall proportion of patients without renal dysfunction and its 95% confidence interval (CI) were calculated for the final analysis in all the patients who received the study treatment. The study was considered to have fulfilled the primary end point if 36 out of a total of 44 patients were able to complete the first-cycle cisplatin without a Grade 2 or higher elevation in creatinine. Secondary end points included the number of cycles of chemotherapy, adverse events and the overall response rate in patients who had measurable lesions according to the RECIST criteria (Ver. 1.1) (13). The STATA 11 for Windows software package (StataCorp LP, College Station, TX, USA) was used for the statistical analyses.

RESULTS

Characteristics of the Patients

Forty-four patients were enrolled between April and December 2011. The participants' characteristics were as follows: male/female 29/15; median age (range) 64 (42–74) years and ECOG performance status 0/1 26/18. Of these, 13 received adjuvant chemotherapy, 5 received chemoradiotherapy and 26 received chemotherapy for advanced diseases. Most patients had lung adenocarcinoma ($n = 34$), but seven had squamous cell carcinoma, two had small-cell carcinoma and one had adenosquamous carcinoma. The results of the pretreatment renal function tests [median (range)] were as follows: serum creatinine 0.7 (0.4–1.1) mg/dl and estimated creatinine clearance 85 (50–148) ml/min (Table 1).

Table 1. Patient characteristics

Patient characteristics	$n = 44$
Age (years)	
Median	64
Range	42–74
Sex	
Male	29
Female	15
Performance status	
0	26
1	18
Treatment setting	
Adjuvant therapy	13
Chemoradiotherapy	5
Post-surgical recurrence	9
Advanced disease	17
Comorbidities	
Hypertension	15
Diabetes mellitus	8
Pulmonary disease	7
Cardiac disease	2
Serum creatinine (mg/dl)	
Median	0.7
Range	0.4–1.1
Estimated creatinine clearance (ml/min)[a]	
Median	85
Range	50–148
Measured creatinine clearance (ml/min)	
Median	112
Range	64–169
Histology	
Adenocarcinoma	34
Squamous cell carcinoma	7
Small-cell carcinoma	2
Adenosquamous carcinoma	1

[a]Cockroft–Gault formula.

Post-treatment Renal Function and Other Toxicities

The trial established the primary endpoint with no patient experienced Grade 2 or more creatinine elevation in the first cycle of cisplatin. The proportion of patients without renal dysfunction after all cycles of study treatment was 97.8% (95% CI 88.0–99.9). One patient experienced a Grade 2 elevation in creatinine (pretreatment value 0.7 mg/dl, maximum value 1.7 mg/dl) after three cycles of chemotherapy. With a prompt improvement in the creatinine level (minimum value 1.3 mg/dl), the patient was able to complete another cycle (fourth cycle) of chemotherapy with reduced cisplatin. The results of the post-treatment renal function tests [median (range)] were as follows: serum creatinine 0.75 (0.4–1.4) mg/dl and estimated glomerular filtration rate (GFR) 75 (38–126) ml/min (Table 2). The profiles for toxicities other than renal dysfunction are summarized in Table 3.

Treatment Delivery and Efficacy

Twenty patients received cisplatin and pemetrexed as the most frequent regimen and 38 patients received three to four cycles of chemotherapy. The median (range) duration and volume of the chemotherapies were 4.0 (3.3–6.8) h and 1600 (1550–2050) ml, respectively. The majority of patients (37 patients, 84%) completed preplanned three to four cycles of cisplatin-based chemotherapy. The reasons for early termination of chemotherapy in the other patients were as follows: six (14%) progressive disease and one (2%) patient refusal due to gastrointestinal toxicities. During the study treatment, 13 patients received intravenous hydration and 6 patients required a dose reduction of cisplatin mainly because of gastrointestinal toxicities, except for one patient with a Grade 2 elevation of creatinine (Table 4). The objective response rate was

Table 2. Renal function during all courses of cisplatin administration

	$n = 44$
Maximum grade of Cr elevation (n, %)	
Grade 1	7 (16)
Grade 2	1 (2)
Post-treatment serum Cr (mg/dl)	
Median	0.75
Range	0.4–1.4
Post-treatment estimated GFR (ml/min)	
Median	75
Range	38–126

GFR, glomerular filtration rate using the Cockroft–Gault formula.

Table 3. Adverse events other than renal toxicities

Toxicities	Grade 1	Grade 2	Grade 3	Grade 4	Total (%) grade 3/4
Fever	1	1	0	0	0
Fatigue	17	6	0	0	0
Body weight loss	0	0	0	0	0
Anorexia	21	9	1	0	1 (2)
Constipation	20	2	0	0	0
Diarrhea	6	0	0	0	0
Nausea	25	5	1	0	1 (2)
Vomiting	6	0	1	0	1 (2)
Febrile neutropenia	0	0	2	0	2 (5)
Alopecia	11	3	0	0	0

48.0% (95% CI 27.8–68.7) among patients with post-surgical recurrences and advanced non-small-cell lung cancer who had measurable lesions according to the RECIST criteria (version 1.1).

Table 4. Treatment summary

	$n = 44$
Chemotherapy regimens (no. of patients, %)	
Cisplatin + pemetrexed	20 (45)
Cisplatin + vinorelbine	10 (23)
Cisplatin + docetaxel	9 (20)
Cisplatin + gemcitabine	3 (7)
Cisplatin + etoposide	2 (5)
Volume of intravenous fluid for each regimen (ml)	
Cisplatin + pemetrexed	1600
Cisplatin + vinorelbine	1550
Cisplatin + docetaxel	1800
Cisplatin + gemcitabine	1600
Cisplatin + etoposide	2050
Length of time for each regimen (median hour, range)	
Cisplatin + pemetrexed	3.8 (3.3–5.3)
Cisplatin + vinorelbine	3.9 (3.4–5.8)
Cisplatin + docetaxel	4.9 (4.0–5.9)
Cisplatin + gemcitabine	4.0 (3.6–5.3)
Cisplatin + etoposide	6.4 (5.3–6.8)
Number of chemotherapy cycles (no. of patients, %)	
Four cycles	30 (68)
Three cycles	7 (16)
Two cycles	4 (9)
One cycle	3 (7)
Median	4 cycles
Additional intravenous hydration (no. of patients)[a]	13
Total number of cycles (median, range)	1 (1–3)
Total number of days per cycle (median, range)	0 (0–11)
Dose reduction of cisplatin (no. of patients)	6

[a]Intravenous hydration on days other than those on which cisplatin was administered.

DISCUSSION

The prospective trial demonstrated that 97.8% of the participants completed the cisplatin-based chemotherapy without Grade 2 or higher elevation in creatinine using short hydration with magnesium supplementation and appropriate antiemetics.

With cisplatin nephrotoxicities, the major lesion of damage is at the proximal tubules, especially in the S3 segment of the proximal tubule situated in the outer stripe of the outer medulla (14). The pathophysiological mechanism for renal injury is not fully understood; however, high-volume hydration (discussed below) and hyper diuresis were usually employed to prevent cisplatin nephrotoxicities (7,8). Based on the results of basic and clinical research, both mannitol and furosemide seemed to be equally effective in preventing renal dysfunction (15–17). These strategies are intended to lower the concentration and to shorten the period of direct cisplatin exposure. Hypomagnesemia has also been considered as a cause of renal dysfunction and as a target of intervention. Magnesium is associated with the active transport mechanism in the tubular cells of the kidney. Sobrero et al. (18) suggested that hypomagnesemia during cisplatin administration may lead to an elevated concentration of cisplatin in tubular cells, thereby damaging the proximal tubules and resulting in subsequent renal dysfunction. Several studies, including a randomized trial, have demonstrated a favorable effect on the renal dysfunction during cisplatin-based chemotherapies (9,10,19).

Tiseo et al. (20) conducted a retrospective trial examining a short period of hydration during regimens containing high-dose cisplatin using magnesium supplementation and forced diuresis. Based on these findings, the national comprehensive cancer network (NCCN) has provided chemotherapy order templates to improve the safe use of drugs and biologics in cancer care. The cisplatin template of NCCN recommended hyper diuresis using mannitol and magnesium supplementation, which were included in the protocol treatment for the current trial.

Stewart et al. conducted a retrospective analysis examining the effects of pretreatment factors on increase of serum creatinine in 425 patients treated with cisplatin. They found no correlation between nephrotoxicities and the amount of hydration in a logistic regression analysis after adjustments for other parameters associated with cisplatin toxicities (21). Although a few other reports examining the administration of cisplatin with a lower volume and a shorter period of hydration (especially under 2000 ml) have been performed, these studies are now somewhat out of date, especially with regard to the usage of antiemetics, to be directly applied to current clinical practice (22,23).

In this prospective trial using up-to-date chemotherapy and antiemetic regimens, we confirmed the safety of short hydration with regard to renal function in almost all (97.8%) of the participants who received cisplatin-based chemotherapy. The result of the current trial was significantly better than the historical data reported in our retrospective analysis conducted in the same institution with similar patient characteristics (6).

Short hydration is safe and reduces the frequency of severe renal toxicities in regimens containing cisplatin ($\geq$75 mg/m^2) for the treatment of patients with lung cancer. Cisplatin labels with regard to hydration should accept short- and lower-volume hydration as an option based on the result of the present study.

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Conflict of interest statement

Kaoru Kubota received honoraria from Eli Lilly. Hiroshi Nokihara received research funding from Pfizer Inc. Noboru Yamamoto received research funding from Pfizer Inc. and Bristol-Myers Squibb. Ikuo Sekine and Tomohide Tamura received honoraria from Pfizer Inc. Other authors have reported no conflicts of interest in connection with this paper.

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Acute Radiation Esophagitis Caused by High-dose Involved Field Radiotherapy with Concurrent Cisplatin and Vinorelbine for Stage III Non-small Cell Lung Cancer

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Yuuki Kuroda, M.D.[1,3]*
Ikuo Sekine, M.D., Ph.D.[2]
Minako Sumi, M.D., Ph.D.[1]
Shuhei Sekii, M.D.[1]
Kana Takahashi, M.D.[1]
Koji Inaba, M.D.[1]
Hidehito Horinouchi, M.D.[2]
Hiroshi Nokihara, M.D., Ph.D.[2]
Noboru Yamamoto, M.D., Ph.D.[2]
Kaoru Kubota, M.D., Ph.D.[2]
Naoya Murakami, M.D., Ph.D.[1]
Madoka Morota, M.D., Ph.D.[1]
Hiroshi Mayahara, M.D., Ph.D.[1]
Yoshinori Ito, M.D.[1]
Tomohide Tamura, M.D.[2]
Kenji Nemoto, M.D., Ph.D.[3]
Jun Itami, M.D., Ph.D.[1]

[1]Department of Radiation Oncology, National Cancer Center Hospital, Tokyo, Japan
[2]Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tokyo, Japan
[3]Department of Radiation Oncology, Faculty of Medicine, Yamagata University, 5-1-1 Tsukiji Chuo-ku, Tokyo, 104-0045, Japan

*Corresponding author:
Yuuki Kuroda, M.D.
Phone: +81-(3)3542-2511
Fax: +81-(3)3545-3567
E-mail: yuuki.kuroda.96@gmail.com

Purpose of this study is to obtain dose-volume histogram (DVH) predictors and threshold values for radiation esophagitis caused by high-dose involved field radiotherapy (IFRT) with concurrent chemotherapy in patients with stage III non-small cell lung cancer (NSCLC). Thirty-two patients treated by 66 Gy/33 Fr, 72 Gy/36 Fr, and 78 Gy/39 Fr thoracic radiotherapy without elective nodal irradiation plus concurrent cisplatin and vinorelvine were reviewed. Acute radiation esophagitis was evaluated according to common terminology criteria for adverse events version 4.0, and correlations between grade 2 or worse radiation esophagitis and DVH parameters were investigated. Grade 0-1, 2, 3, and 4-5 of radiation esophagitis were seen in 11 (34.4%), 20 (62.5%), 1 (3.1%), and 0 (0%) of the patients, respectively. Multivariate analysis revealed that whole esophagus V35 is a predictor of radiation esophagitis (OR = 0.74 [95%CI; 0.60-0.91], $p = 0.006$). There is a significant difference (38.4% vs. 89.4%, $p = 0.027$) in the cumulative rates of acute esophagitis according to V35 values of more than 20% versus less. As compared with other factors concerning patient and tumor and treatment factors, V35 $\leq$ 20% of the esophagus was an independent predictor (HR = 0.29 [95%CI; 0.09-0.85], $p = 0.025$). In conclusion, whole esophagus V35 $<$ 20% is proposed in high-dose IFRT with concurrent chemotherapy for stage III NSCLC patients.

Key words: NSCLC; CCRT; IFRT; Radiation esophagitis; DVH.

Introduction

Chemoradiotherapy is the standard treatment for patients with locally advanced non-small cell lung cancer (NSCLC) (1-5). It is well known that concurrent chemotherapy with thoracic radiotherapy increases the incidence of pulmonary and esophageal toxicities, which would become significant dose-limiting factors (5-7). In order to reduce such toxicities, involved field radiotherapy (IFRT) without an elective nodal irradiation (ENI) has been developed (8-9), and it has been demonstrated to be capable of dose escalation without increasing toxicities (10-15). Predictive analyses of radiation pneumonitis and esophagitis by using a dose-volume histogram (DVH) have been investigated in many studies (16-26); however, predictive values derived from a standard dose of 60 Gy with an ENI might not be applicable to high-dose IFRT for stage III NSCLC (7). We previously reported that 72 Gy in 36 fractions is the optimal dose that could be attained with maintenance of the normal tissue constraints (14). The purpose of this study was to find potential DVH

Abbreviations: IFRT: Involved Field Radiotherapy; CDDP: Cisplatin; VNR: Vinorelbine; NSCLC: Non-small Cell Lung Cancer; CCRT: Concurrent Chemoradiotherapy; DVH: Dose-volume Histogram.