Statistical Analysis

To analyze the background factors, differences in continuous or categorical variables were analyzed by analysis of variance (ANOVA) and the χ^2 test, respectively.

As the primary analysis, the serum BDNF level was analyzed by ANOVA and analysis of covariance (ANCOVA). Background variables that were statistically significantly different between the two groups were examined as independent variables, with the serum BDNF level as the dependent variable, using the Spearman rank correlation coefficient (for continuous variables) or ANOVA (for categorical variables). Only factors that were related to both the background and the BDNF levels were used as covariates in the ANCOVA. As a secondary analysis, stratified analyses according to sex were also performed. All tests were two-tailed, with P values <0.05 indicating statistical significance. The statistical analyses were performed using the statistical software package SPSS for Windows (Version 16.0J, SPSS Japan Institute Inc.)

RESULTS

Participants

During the period of the study, 30 patients refused to participate, while 829 patients provided blood samples and completed self-reported questionnaires. Based on the inclusion/exclusion criteria, 717 patients were found to be eligible for enrollment in the present study (13). Of the 717 subjects, 81 had high HADS-D scores (>10) and were selected as the subjects of the depression group. Of the remaining 319 subjects with HADS-D scores of 4 or under, 81 subjects matched for age and sex were enrolled as controls in the non-depression group.

Group Backgrounds

Table 1 shows the background characteristics of the two groups, including some data that were reported in our previous study (12). The depression group contained more subjects with breathlessness than the non-depression group. Except for the breathlessness, no other variable differed significantly between the groups. The mean and standard deviation in the interval between completion of the HADS questionnaire and the blood sampling in all the subjects were 3.6 and 5.0 days, respectively; these values were similar for both groups [depression group; 3.9 (5.0) days; mean (SD), non-depression group; 3.8 (5.9) days] ($F = 0.04$, $P = 0.85$). The serum BDNF levels showed no significant differences between the subjects with breathlessness [$n = 82$; 28.7 (11.3) ng/ml; mean (SD)] and those without breathlessness [$n = 78$; 31.9 (13.0) ng/ml] ($F = 2.66$, $P = 0.11$).

Table 1. Background of all subjects ($n = 162$)

	Depression	Non-depression	χ^2 or F^a	P-value
HADS-D (score)	11–21	0–4		
Number	81	81		
Sex (male)	57 (70%)	57 (70%)	0.0	1.00
Age (y.o.)	65.1 ± 8.3	65.0 ± 8.3	0.003[a]	0.96
Performance Status (0/1)[b]	23/58	23/58	0.0	1.00
Clinical stage				
Ia–IIIa[c]	34 (42%)	34 (42%)	0.0	1.00
IIIb–IV[c]	47 (58%)	47 (58%)		
Educational level (>9 years)	52 (64%)	56 (69%)	0.46	0.50
Alcohol (>45 g/day)	14 (17%)	12 (15%)	0.38	0.54
Current smoker	33 (41%)	30 (37%)	0.23	0.63
Pathology				
Adenocarcinoma	42	45	1.49	0.83
Squamous cell	19	20		
Small cell	6	7		
Large cell	8	6		
Other	6	3		
Breathlessness (presence)	49 (60%)	33 (41%)	5.61	0.018
Pain (presence)	28 (35%)	31 (38%)	0.19	0.67
Body mass index (kg/m^2)	22.0 ± 3.5	22.1 ± 3.2	0.06[a]	0.80
Platelet ($10^4 \times \mu l$)	27.7 ± 9.3	28.3 ± 9.4	0.20[a]	0.66

Age, body mass index and platelet: mean ± SD. PS: number. Others: number and percentage.
[a]F-value.
[b]Defined by Eastern Cooperative Oncology Groups.
[c]Defined by TNM Classification, International Union Against Cancer.

Serum BDNF Levels in the Two Groups

Figure 1 illustrates the absence of any significant difference in the serum BDNF levels between the depression group and the non-depression group (ANOVA). The serum BDNF levels were normally distributed. Since no covariates were detected as statistically significant variables in the background analyses, ANCOVA was not performed. In the stratified analyses by gender, no significant differences were seen between the two groups among the men. The mean serum BDNF level was lower in the women with depression than in the women without depression, but the difference was not statistically significant.

DISCUSSION

This is the first study, to the best of our knowledge, conducted to investigate the association between serum BDNF levels and major depression in the oncologic setting.

Unlike in previous studies (9–11), the serum BDNF levels were not lower in the subjects with major depression in the

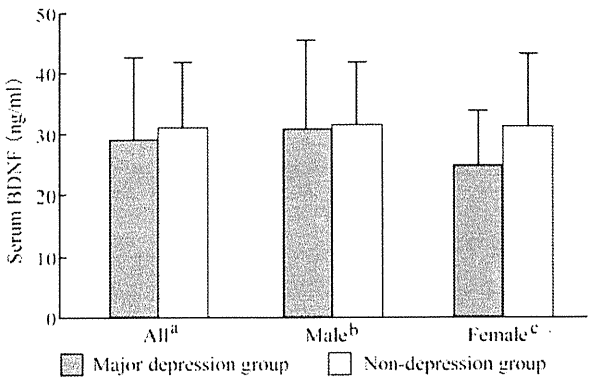

Figure 1. Serum levels of brain-derived neurotrophic factor (BDNF) in the depression group and in the non-depression group. [a]The primary analysis showed the absence of any statistically significant differences in the serum BDNF levels between the subjects in the depression group [$n = 81$; 29.1 (13.6) ng/ml; mean (SD)] and the non-depression group [$n = 81$; 31.4 (10.6) ng/ml] ($F = 1.53$, $P = 0.22$). [b]A stratified analysis by gender showed the absence of any statistically significant difference in the levels between the depression group [$n = 57$; 30.9 (14.8) ng/ml; mean (SD)] and the non-depression group [$n = 57$; 31.8 (10.2) ng/ml] ($F = 0.13$, $P = 0.72$) among men. [c]A stratified analysis by gender also showed the absence of any statistically significant difference between the depression group [$n = 24$; 24.7 (9.1) ng/ml; mean (SD)] and the non-depression group [$n = 24$; 30.7 (11.7) ng/ml] ($F = 3.87$, $P = 0.06$) among women.

present study. The lack of difference in the serum BDNF in our study might be related to the characteristics of depression in oncologic settings, which tends to be reactive to stressful event, mild and of short duration (3,16). In a previous study in which psychiatric patients without cancer were examined, the mean durations of depressive episodes were 0.78 years (9). Of the 81 cancer patients with major depression in the present study, 60 completed the HADS questionnaire within 1 month of the disclosure of their lung cancer diagnosis. None of the subjects in the major depression group visited the clinical psychiatric service or received antidepressants before or after their enrollment in this study. Although the duration of major depression was not directly assessed, the subjects with major depression in the present study might have had mild depression of short duration that remitted by themselves without antidepressants. The associations between peripheral BDNF and severity or duration of depressive episode were not concluded (17). Further study may be needed.

In the present study, depression was defined using the cut-off scores of the HADS-D and not by a structured psychiatric interview (such as the Structured Clinical Interview for DSM-IV). The one-point assessment of HADS-D might not always indicate a major depressive episode defined by DSM-IV; this could be a reason why the present result differ from previous studies' (9–11).

Although the P value did not reach statistical significance, our secondary analysis showed that women with major depression tended to have a lower serum BDNF level than women without depression. This result may support the result of a previous study suggesting an important role of reduced serum BDNF in depressive women, but not in men (11). Other studies reported an association between BDNF and the menstrual cycles in humans (18) and sex hormones in animals (19). Further studies examining these factors may be useful for elucidating the association between BDNF and major depression.

This study had the following limitations: (i) subjects with severe depression might have been excluded from this study because subjects with poor physical activity and cognitive impairment were ineligible and 30 subjects refused to participate in this study. (ii) Although peripheral BDNF was suggested to partly reflect the BDNF levels in cerebral spinal fluids (18,20), serum BDNF was mainly stored in platelets. Relation of serum BDNF levels to BDNF in hippocampus was uncertain. Further studies may be needed to reach definitive conclusions.

Acknowledgements

The authors thank Drs Nobuya Akizuki, Eisho Yoshikawa and Eiji Shimizu.

Funding

This work was supported in part by the Third-Term Comprehensive 10-Year Strategy for Cancer Control and Research, Japanese Ministry of Health, Labour, and Welfare.

Conflict of interest statement

None declared.

References

1. Derogatis LR, Morrow GR, Fetting J, Penman D, Piasetsky S, Schmale AM, et al. The prevalence of psychiatric disorders among cancer patients. *JAMA* 1983;249:751–7.
2. Kendler KS, Karkowski LM, Prescott CA. Causal relationship between stressful life events and the onset of major depression. *Am J Psychiatry* 1999;156:837–41.
3. Chochinov HM. Depression in cancer patients. *Lancet Oncol* 2001;2:499–505.
4. Duman RS, Heninger GR, Nestler EJ. A molecular and cellular theory of depression. *Arch Gen Psychiatry* 1997;54:597–606.
5. Vaidya VA, Marek GJ, Aghajanian GK, Duman RS. 5-HT2A receptor-mediated regulation of brain-derived neurotrophic factor mRNA in the hippocampus and the neocortex. *J Neurosci* 1997;17:2785–95.
6. Smith MA, Makino S, Kvetnansky R, Post RM. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci* 1995;15:1768–77.
7. Nibuya M, Morinobu S, Duman RS. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 1995;15:7539–47.
8. Altar CA, Whitehead RE, Chen R, Wortwein G, Madsen TM. Effects of electroconvulsive seizures and antidepressant drugs on brain-derived neurotrophic factor protein in rat brain. *Biol Psychiatry* 2003;54:703–9.

9. Shimizu E, Hashimoto K, Okamura N, Koike K, Komatsu N, Kumakiri C, et al. Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol Psychiatry* 2003;54:70–5.
10. Karege F, Bondolfi G, Gervasoni N, Schwald M, Aubry JM, Bertschy G. Low brain-derived neurotrophic factor (BDNF) levels in serum of depressed patients probably results from lowered platelet BDNF release unrelated to platelet reactivity. *Biol Psychiatry* 2005;57:1068–72.
11. Huang T-L, Lee C-T, Liu Y-L. Serum brain-derived neurotrophic factor levels in patients with major depression: effects of antidepressants. *J Psychiatr Res* 2008;42:521–5.
12. Kobayakawa M, Yamawaki S, Hamazaki K, Akechi T, Inagaki M, Uchitomi Y. Levels of omega-3 fatty acid in serum phospholipids and depression in patients with lung cancer. *Br J Cancer* 2005;93:1329–33.
13. Nakaya N, Goto K, Saito-Nakaya K, Inagaki M, Otani T, Akechi T, et al. The Lung Cancer Database Project at the National Cancer Center, Japan: study design, corresponding rate, and profiles of cohort. *Jpn J Clin Oncol* 2006;36:280–4.
14. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983;67:361–70.
15. Kugaya A, Akechi T, Okuyama T, Okamura H, Uchitomi Y. Screening for psychological distress in Japanese cancer patients. *Jpn J Clin Oncol* 1998;28:333–8.
16. Inagaki M, Matsuoka Y, Sugahara Y, Nakano T, Akechi T, Fujimori M, et al. Hippocampal volume and first major depressive episode after cancer diagnosis in breast cancer survivors. *Am J Psychiatry* 2004;161:2263–70.
17. Molendijk ML, Bus BA, Spinhoven P, Penninx BW, Kenis G, Prickaerts J, et al. Serum levels of brain-derived neurotrophic factor in major depressive disorder: state-trait issues, clinical features and pharmacological treatment. *Mol Psychiatry* advance online publication 21 September 2010; doi:10.1038/mp.2010.98 (in press).
18. Lommatzsch M, Zingler D, Schuhbaeck K, Schloetcke K, Zingler C, Schuff-werner P, et al. The impact of age, weight and gender on BDNF levels in human platelets and plasma. *Neurobiol Aging* 2005;26:115–23.
19. Cavus I, Duman RS. Influence of estradiol, stress, and 5-HT2A agonist treatment on brain-derived neurotrophic factor expression in female rats. *Biol Psychiatry* 2003;54:59–69.
20. Pan W, Banks WA, Fasold MB, Bluth J, Kastin AJ. Transport of brain-derived neurotrophic factor across the blood–brain barrier. *Neuropharmacology* 1998;37:1553–61.

Lung Cancer 74 (2011) 85–88

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Phase II study of S-1 monotherapy in platinum-refractory, advanced non-small cell lung cancer

Takayuki Shiroyama [a,*], Kiyoshi Komuta [a], Fumio Imamura [b], Tomonori Hirashima [c], Takashi Kijima [d], Isao Tachibana [d], Ichiro Kawase [d]

[a] Department of Respiratory Medicine, Osaka Police Hospital, 10-31 Kitayama-chou, Tennoji-ku, Osaka 543-0035, Japan
[b] Department of Pulmonary Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan
[c] Department of Thoracic Malignancy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Osaka, Japan
[d] Department of Respiratory, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, Osaka, Japan

ARTICLE INFO

Article history:
Received 8 October 2010
Received in revised form 14 January 2011
Accepted 23 January 2011

Keywords:
Lung cancer
Second line
Platinum-refractory
S-1 monotherapy
Ambulatory setting
Oral medication

ABSTRACT

Objective: The aim of this study was to evaluate the efficacy and toxicity of a novel oral 5-fluorouracil formulation (S-1) as second-line therapy after platinum agent chemotherapy for advanced non-small cell lung cancer (NSCLC).
Methods: S-1 was administered orally at a dose of 80 mg/m^2 for 28 days, followed by 14 days of rest (1 cycle); treatment was repeated until disease progression, unacceptable toxicity, or patient refusal.
Results: Of the 46 patients enrolled in this study, 44 were evaluable. Six patients (14%) exhibited a partial response and 28 (64%) showed stable disease. Disease-control rate was 77.3% (34/44) (95% CI, 64.9–89.7%). The overall response rate was 14% (6/44) (95% CI, 3.5–23.8%). Median progression-free survival was 4.2 months. The median survival time was 16.4 months, and the one-year survival rate 60.3%. Grade 3/4 hematological toxicities were minor. All of those adverse reactions were tolerable and reversible.
Conclusion: This study demonstrated the efficacy of S-1 monotherapy as second-line treatment for advanced NSCLC.

1. Introduction

Lung cancer is the most common cancer worldwide, with an estimated 1.2 million new cases globally (12.3% of all cases of cancer) and 1.1 million deaths (17.8% of all cancer deaths) in 2000 [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer. For chemotherapy-naive patients with good performance status (PS) at a stage of IIIB (with pleural effusion) or IV, platinum-based chemotherapy offers a modest survival advantage over best supportive care (BSC) alone [2–4]. To improve outcome, however, there is a need for novel regimens. A recent phase III clinical study of conventional second-line therapies reported median survival times (MST) of approximately 6–8 months for docetaxel, gefitinib, and erlotinib [5–8]. On the other hand, in Japanese trials targeting this population have showed more favorable MST of approximately 12–17 months [9,10]. Doublet chemotherapy as second-line treatment for advanced NSCLC significantly elevates response rate and prolongs progression-free survival (PFS), but it is more toxic and does not improve overall survival rate compared to single-agent treatment [11].

S-1 is a novel, orally administered drug that is a combination of tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP), and oteracil potassium (Oxo) in a 1:0.4:1 molar concentration ratio [12]. CDHP is a competitive inhibitor of dihydropyrimidine dehydrogenase, which is involved in the degradation of 5-FU, and acts to maintain efficacious concentrations of 5-FU in plasma and tumor tissues. Oxo, a competitive inhibitor of orotate phosphoribosyltransferase, inhibits the phosphorylation of 5-FU in the gastrointestinal tract, reducing the serious gastrointestinal toxicity associated with 5-FU. The antitumor effect of S-1 has already been demonstrated in a variety of solid tumors such as advanced gastric cancer [13,14], colorectal cancer [15], head and neck cancer [16,17], breast cancer [18], pancreatic cancer [19,20] and biliary tract cancer [21]. A recent phase II study of S-1 for chemo-naive, advanced NSCLC patients yielded promising results, with a response rate of 22.0%, MST of 10.2 months and favorable toxicity profile [22]. S-1 has exhibited promising activity against several tumors. Moreover, treatment with it can be administered on an outpatient basis. Oral agents such as S-1, which feature few adverse events, are appealing in the 2nd or 3rd line setting when patients are in less favorable condition.

* Corresponding author. Tel.: +81 6 6771 6051.
E-mail address: takayuki.s12@hotmail.co.jp (T. Shiroyama).

0169-5002/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.lungcan.2011.01.017

We therefore conducted a prospective study to evaluate the efficacy and feasibility of S-1 monotherapy in patients with progressive, advanced NSCLC under platinum-based, first line chemotherapy.

2. Patients and methods

2.1. Patient eligibility

Patients were required to meet the following eligibility criteria: histologically confirmed NSCLC; at least one measurable lesion; prior platinum-based treatment; Eastern Cooperative Oncology Group performance status of 0–1; age ≥20 years, adequate organ function, defined as a leukocyte count of 3500–12,000 mm^3, neutrophil count of more than 2000 mm^3, platelet count of more than 100,000 mm^3, hemoglobin level of more than 9.0 g/dl, a serum total bilirubin level within 2.0 times the ULN, and serum creatinine level not exceeding the ULN; partial pressure of arterial oxygen ≥60 mm Hg, and an estimated life expectancy of at least 3 months. The exclusion criteria were as follows: pregnancy, serious concomitant disease (brain metastasis, active infection, severe heart disease or uncontrolled diabetes mellitus), concomitant malignancy, pleural effusion requiring treatment, symptomatic cerebral involvement, and obvious interstitial pneumonia or pulmonary fibrosis on chest radiography.

All patients gave written informed consent prior to enrollment in the study, and the protocol was approved by the Institutional Ethics Committee of each participating institution.

2.2. Study treatment

The initial doses of S-1 were assigned on the basis of body surface area (BSA). Accordingly, the patients received one of the following oral doses divided in two and administered daily after meals: 80 mg for patients with BSA < 1.25 m^2, 100 mg for BSA ≥ 1.25 and <1.50 m^2, and 120 mg for BSA ≥ 1.50 m^2. One therapy cycle comprised the administration of single-agent S-1 for 28 consecutive days followed by 14 days of no treatment. This schedule was repeated every 6 weeks until the occurrence of disease progression, unacceptable toxicities, or patient refusal. In the absence of evidence of disease progression, patients were allowed to continue S-1 treatment. A dose reduction of 20 mg/day was recommended if ≥grade 3 hematological or non-hematological toxicity occurred in the previous cycle; dose re-escalation was not allowed. Patients who required more than 4 weeks of rest for recovery from any toxicity other than nausea, vomiting, or anemia, or who required a dose reduction >20 mg/day, were withdrawn from the study.

2.3. Evaluation of response and toxicity

Before entering the study, all patients provided a detailed medical history and underwent a complete physical examination, complete blood cell count, serum chemistry examination, chest computed tomography scan (CT), determination of weight and height, and determination of ECOG PS. A CT scan was performed for tumor assessment within 14 days of initiation of study treatment and was repeated every 1–2 months. Physical examination, symptom evaluation, and routine blood tests and biochemistry blood examination were performed every 2 weeks during the treatment. Objective response was evaluated every 2 months. Ratings of complete response, partial response (PR), stable disease (SD), progressive disease (PD) or not evaluated (NE) were made according to RECIST version 1.0. Toxicity was evaluated every 2 weeks according to the Common Terminology Criteria for Adverse Events (CTCAE), version 3.0.

Table 1
Patient characteristics ($n = 44$).

Characteristic	No. of patients	%
Sex		
Male	35	79.5
Female	9	20.5
Age, years		
Median	64	
Range	47–79	
ECOG performance status		
0	13	29.5
1	31	70.5
Stage		
IIIB	15	34.1
IV	29	65.9
Histology-no. (%)		
Adenocarcinoma	30	68.2
Squamous cell carcinoma	11	25.0
Large cell carcinoma	1	2.3
Others	2	4.5
First line therapy		
Carboplatin/paclitaxel	33	75.0
Carboplatin/gemcitabine	5	11.4
Carboplatin/vinorelbine	3	6.8
Carboplatin/docetaxel	2	2.3
Cisplatin/docetaxel	1	4.5
Response of first line therapy		
PR	15	34.2
SD	6	13.6
PD	13	29.5
NE	10	22.7

2.4. Study design and statistical analysis

This was a phase II single-arm trial. The primary endpoint was evaluation of the activity, defined as the disease control rate at 8 weeks, of S-1 as a single agent in patients with NSCLC. Disease control rate (DCR) was defined as the percentage of patients without disease progression (CR, PR, or SD) and still on treatment at 8 weeks. DCR was confirmed and sustained 8 weeks or longer. Secondary endpoints included PFS, OS, and safety. PFS was defined as the time from the first day of administration of the study drug to disease progression or death for toxicity or disease progression. OS was defined as the time from the first day of study drug administration to death or last contact.

The primary endpoint of the trial was the DCR (CR + PR + SD according to RECIST criteria) at 8 weeks. The target sample size was 45 patients. Given a threshold DCR of 40% and expected DCR of 60%, the required sample size was estimated to be 39 patients, with $\alpha = 0.05$ and power $\beta = 0.8$.

3. Results

3.1. Patients

From June 2005 to May 2007, 46 patients were enrolled. Of these 46 patients, one was found to be ineligible because of 3rd line treatment, and one other patient withdrew consent. Thus, 44 patients remained in the analysis.

A total of 43 patients had received carboplatin-based chemotherapy, and one patient had received cisplatin-based chemotherapy. Of these 44 patients, one had exhibited CR, 14 PR, 6 SD, and 23 PD. The baseline characteristics of these patients and their tumors are in listed in Table 1.

3.2. Treatments

The 44 patients received a total of 70 cycles of chemotherapy, with a median number of cycles of two (range, 1–7). The dose of S-1 was reduced in 2 patients because of either grade 3 diarrhea

Table 2
Patient characteristics in relation to the response.

Response rate *n* (%) (2nd line)	
CR	0
PR	6(13.6)
SD	28(63.6)
PD	5(11.3)
NE	5(11.3)
Response rate	13.6 (95% CI, 5.0–20.0%)
Disease control rate	77.3 (95% CI, 64.9–89.7%)

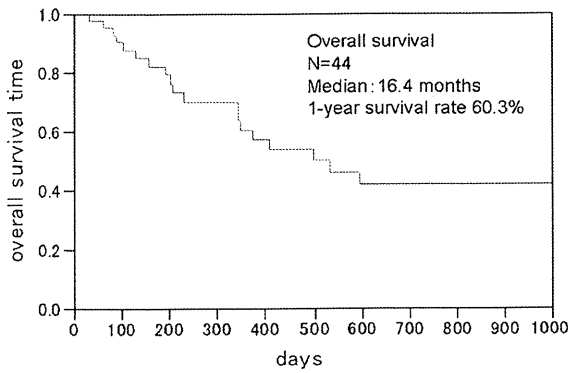

Fig. 1. Overall survival of patient treated with S-1 monotherapy after failure of first-line (platinum-base therapy) treatment.

and abnormal amylase level. The median relative dose intensity for the entire study population was 97.3%, indicating that patient compliance with S-1 chemotherapy was good.

3.3. Efficacy

A total of 44 patients were evaluable for response. Six patients achieved PR, resulting in an overall response rate (RR) of 13.6% (95% CI, 5.0–20%). Twenty-eight patients had SD, yielding an overall DCR of 77.3% (95% CI, 64.9–89.7%). The RR and DCR were evaluated separately from those for the previous first-line chemotherapy (Table 2). At the time of median follow-up interval of 18 months, 20 patients were still alive and censored; MST was calculated for all 44 patients, and OS was 16.4 months, the one-year survival rate 60.3%, and PFS 4.2 months The Kaplan–Meier survival curve is shown in Figs. 1 and 2.

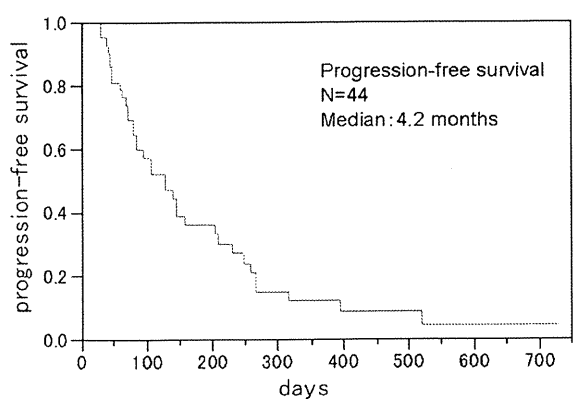

Fig. 2. Progression-free survival of patient treated with S-1 monotherapy after failure of first-line (platinum-base therapy) treatment.

Table 3
Hematologic and non-hematologic toxicities.

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4	Grade 3/4 (%)
Leukopenia	1	1	2	0	4.5
Neutropenia	2	1	2	0	4.5
Anemia	5	1	2	0	4.5
Diarrhea	0	2	1	0	2.3
Amylase	0	0	1	0	2.3
Pulmonary fibrosis	0	0	0	1	2.3

3.4. Safety

All patients were assessed for toxicities, which are listed in Table 3. No grade 4 or more severe hematological toxicities were observed; grade 3 events included two episodes each (4.5%) of leucopenia, neutropenia, and anemia. Non-hematological toxicities included one episode each (2.3%) of grade 3 diarrhea, grade 3 anorexia, grade 3 increase in amylase, and grade 4 interstitial pneumonia, each of which recovered with appropriate treatment. The most common hematological and non-hematological toxicities were mild or moderate. There were no treatment-related deaths directly attributable to S-1 chemotherapy.

4. Discussion

Recently, docetaxel has been demonstrated to be useful as second-line therapy for non-small cell lung cancer (NSCLC). Pemetrexed was then found to yield a response rate, overall survival, and PFS similar to those for docetaxel while producing many fewer adverse events; it has therefore been approved as a regimen for second-line therapy in Europe and the United States. Moreover, EGFR-TKI also became a treatment option for second-line therapy for NSCLC, based on the results of comparative studies with docetaxel and BSC. However, it was also reported that pemetrexed was ineffective against squamous cell carcinoma and that EGFR-TKI exhibited only poor efficacy in a group without EGFR gene mutation (exon 19, exon 21) [23–26].

In addition, the combination chemotherapy using two agents exhibited a better response rate and longer PFS than single-agent chemotherapy. On the other hand, since combination chemotherapy produced more toxicity and did not yield prolongation of overall survival compared with single-agent chemotherapy, single-agent chemotherapy has been recognized as effective as second-line therapy. It has also been reported that response rate with second-line or later chemotherapy worsens each time compared with first-line chemotherapy (first 20.9%, second 16.3%, third 2.3%, fourth 0.0%). Therefore, less toxic and highly tolerable single-agent therapy which can be administered on an outpatient basis without impairing QOL, in addition to demonstrating high response rate, is required [27].

Since it was shown in Phase II studies of S-1 in untreated advanced NSCLC that S-1 has a 22.0% anti-tumor effect with low frequency of serious adverse reactions, the present study was conducted to evaluate the efficacy and safety of S-1 in patients with NSCLC previously treated with platinum-based therapy. The median age of eligible subjects in our study was 64 years, and 70% of them had adenocarcinoma. Of the total of 44 evaluable patients, 6 (14%) were responsive to the treatment. Disease control was achieved in 34 patients (77.3%). Grade 3 or more severe toxicities were infrequently observed, including two episodes each (4.5%) of leucopenia, neutropenia, and anemia as hematological toxicities, and one episode each (2.3%) of diarrhea, nausea/vomiting, anorexia, increase in amylase, and interstitial pneumonia as non-hematological toxicities. Throughout all courses in this study, a total of 16% of the patients discontinued, while 4.5% of them was because of violation of discontinuation criteria. Dose inten-

sity exhibited a good percentage of 97.3%, however, change of treatment schedule to two-week dosing followed by one-week wash-out and dose adjustment should be considered in some cases. The major treatment category (inpatient/outpatient) was outpatient therapy, suggesting that this treatment can be safely administered in an ambulatory setting as well. OS, one-year survival rate, and PFS with this treatment were 16.4 months, 57%, and 4.2 months, respectively, suggesting that approximately the same results as obtained with other monotherapies can be achieved with S-1 [28].

On the other hand, Yamamoto et al. reported S-1 may be no difference in efficacy according to histological type [29]. Therefore, we compared squamous cell carcinoma with non-squamous cell carcinoma of PFS (Non-sq;3.6M, Sq: 4.6M) and OS (Non-sq;13.6M, Sq: 17.6M), and no significant differences were observed. However, the patient number of the present study is too little to evaluate the histological deviation in the responders to S-1.

In conclusion, S-1 monotherapy appeared to be effective and highly tolerable as second-line therapy in patients with advanced/recurrent NSCLC previously treated with platinum-based therapy. This therapy could be an option for patients wishing to be treated by oral medication or in an ambulatory setting.

Conflicts of interest statement

None declared.

References

[1] Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.

[2] Stephens R, Fairlamb D, Gower N, Maslove L, Milroy R, Napp V, et al. The Big Lung Trial (BLT): determining the value of cisplatin-based chemotherapy for all patients with non-small cell lung cancer (NSCLC): preliminary results in the supportive care setting. Proc Am Soc Clin Oncol 2002;21:291a (abstr 1161).

[3] Non-Small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer—a meta-analysis using updated data on individual patients from 52 randomized clinical trials. BMJ 1995;311:899–909.

[4] Grilli R, Oxman A, Julian J. Chemotherapy for advanced non-small cell lung cancer: how much benefit is enough? J Clin Oncol 1993;11:1866–72.

[5] Shepherd FA, Dancey J, Ramlau R, Mattson K, Gralla R, O'Rourke M, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000;18:2095–103.

[6] Fossella FV, DeVore R, Kerr RN, Crawford J, Natale RR, Dunphy F, et al. Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-small Cell Lung Cancer Study Group. J Clin Oncol 2000;18:2354–62.

[7] Kim ES, Hirsh V, Mok T, Socinski MA, Gervais R, Wu YL, et al. Gefitinib versus docetaxel in previously treated non-small-cell lung cancer to gefitinib. Lancet 2008;372:1809–18.

[8] Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, et al. Erlotinib in previously treated non-small-cell lung cancer. N Eng J Med 2005;353:123–32.

[9] Maruyama R, Nishiwaki Y, Tamura T, Yamamoto N, Tsuboi M, Nakagawa K, et al. Phase III study V-15-32, of gefitinib versus docetaxel in previously treated Japanese patients with non-small-cell lung cancer. J Clin Oncol 2008;26:4244–52.

[10] Ohe Y, Ichinose Y, Nakagawa K, Tamura T, Kubota K, Yamamoto N, et al. Efficacy and safety of two doses of pemetrexed supplemented with folic acid and vitamin B12 in previously treated patients with non-small cell lung cancer. Clin Cancer Res 2008;14:4206–12.

[11] Di Maio M, Chiodini P, Georgoulias V, Hatzidaki D, Takeda K, Wachters FM, et al. Meta-analysis of single-agent chemotherapy compared with combination chemotherapy as second-line treatment of advanced non-small-cell lung cancer. J Clin Oncol 2009;27:1836–43.

[12] Shirasaka T, Shimamoto Y, Ohshimo H, Yamaguchi M, Kato T, Yonekura K, et al. Development of a novel form of 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor-selective cytotoxicity of 5-fluorouracil by two biochemical modulators. Anticancer Drugs 1996;7:548–57.

[13] Sugimachi K, Maehara Y, Horikoshi N, Shimada Y, Sakata Y, Mitachi Y, et al. An early phase II study of oral S-1 a newly developed 5-fluorouracil derivative for advanced and recurrent gastrointestinal cancers. Oncology 1999;57:202–10.

[14] Koizumi W, Kurihara M, Nakano S, Hasegawa K. Phase II study of S-1, a novel oral derivative of 5-fluorouracil in advanced gastric cancer. Oncology 2000;58:191–7.

[15] Ohtsu A, Baba H, Sakata Y, Mitachi Y, Horikoshi N, Sugimachi K, et al. Phase II study of S-1, a novel oral fluoropyrimidine derivative, in patients with metastatic colorectal carcinoma. Br J Cancer 2000;83:141–5.

[16] Inuyama M, Kida A, Tsukuda M, Kohno N, Satake B. Early phase II study of S-1 in patients with advanced head and neck cancer. Jpn J Cancer Chemother 1998;25:1151–8.

[17] Inuyama M, Kida A, Tsukuda M, Kohno N, Satake B. Late phase II study of S-1 in patients with advanced head and neck cancer. Jpn J Cancer Chemother 2001;28:1381–90.

[18] Saeki T, Takashima S, Sano M, Horikoshi N, Miura S, Shimizu S, et al. A phase II study of S-1 in patients with metastatic breast cancer—A Japanese Trial by the S-1 Cooperative Study Group, Breast Cancer Working Group. Breast Cancer 2004;11:194–202.

[19] Saeki T, Takashima S, Sano M, Horikoshi N, Miura S, Shimizu S, et al. A late phase II clinical study of S-1 in patients with progressed, refractory breast cancer. Jpn J Cancer Chemother 2004;31:539–47.

[20] Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane C. An early phase II study of S-1 in patients with metastatic pancreatic cancer. Oncology 2005;68:171–8.

[21] Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane. Phase II study of S-1 in patients with advanced biliary tract cancer. Br J Cancer 2004;91:1769–74.

[22] Kawahara M, Furuse K, Segawa Y, Yoshimori K, Matsui K, Kudoh S, et al. Phase II study of S-1 a novel oral fluorouracil in advanced non-small-cell lung cancer. Br J Cancer 2001;85:939–43.

[23] Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, von Pawel J, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. J Clin Oncol 2004;22:1589–97.

[24] Scagliotti GV, Parkih P, von Pawel J, Biesma B, Vansteenkiste J, Manegold C, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. J Clin Oncol 2008;26:3543–51.

[25] Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Eng J Med 2009;361:947–57.

[26] Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin docetaxel in patients with non-small lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomized phase 3 trial. Lancet Oncol 2010;11:121–8.

[27] Massarelli E, Andre F, Liu DD, Lee JJ, Wolf M, Fandi A, et al. A retrospective analysis of the outcome of patients who have received two prior chemotherapy regimens including platinum and docetaxel for recurrent non-small-cell lung cancer. Lung Cancer 2003;39:55–61.

[28] Totani Y, Saito Y, Hayashi M, Tada T, Kohashi Y, Mieno Y, et al. A phase II study of S-1 monotherapy as second-line treatment for advanced non-small cell lung cancer. Cancer Chemother Pharmacol 2009;64:1181–5.

[29] Yamamoto N, Yamanaka T, Ichinose Y, Kubota K, Sakai H, Genma A, et al. Pooled analysis of S-1 trials in non-small cell lung cancer according to histological type. Anticancer Res 2010;30:2985–90.

MET Tyrosine Kinase Inhibitor Crizotinib (PF-02341066) Shows Differential Antitumor Effects in Non-small Cell Lung Cancer According to *MET* Alterations

Junko Tanizaki, MD, Isamu Okamoto, MD, PhD, Kunio Okamoto, MD, PhD, Ken Takezawa, MD, PhD, Kiyoko Kuwata, PhD, Haruka Yamaguchi, PhD, and Kazuhiko Nakagawa, MD, PhD

Introduction: Tyrosine kinase inhibitors (TKIs) targeted to MET are undergoing clinical trials in patients with solid tumors, but the precise mechanism of the antitumor activity of these drugs remains unclear. We examined the antitumor action of the MET-TKI crizotinib (PF-02341066) in lung cancer cells that are positive or negative for *MET* amplification or mutation.

Methods: The antitumor action of crizotinib was evaluated on the basis of signal transduction, cell proliferation, apoptosis, and progression of tumor xenografts.

Results: Inhibition of MET signaling by crizotinib or by RNA interference-mediated MET depletion resulted in the induction of apoptosis accompanied by inhibition of AKT and extracellular signal-regulated kinase phosphorylation in lung cancer cells with *MET* amplification but not in cells with a *MET* mutation or in those without amplification or mutation of *MET*. These results suggest that MET signaling is essential for the survival of cells with *MET* amplification but not for that of cells without this genetic change, including those with a *MET* mutation. Crizotinib up-regulated the expression of BIM, a proapoptotic member of the Bcl-2 family, and down-regulated that of survivin, a member of the inhibitor of apoptosis protein family, in cells with *MET* amplification. Forced depletion of BIM and expression of survivin each inhibited crizotinib-induced apoptosis, suggesting that both up-regulation of BIM and down-regulation of survivin contribute to the proapoptotic effect of crizotinib.

Conclusions: Crizotinib shows a marked antitumor action in *MET* amplification-positive lung cancer cells but not in cells without *MET* amplification, including those with a *MET* mutation.

Key Words: MET, Lung cancer, Crizotinib, BIM, Survivin.

(*J Thorac Oncol.* 2011;6: 1624–1631)

Department of Medical Oncology, Kinki University Faculty of Medicine, Osaka-Sayama, Osaka, Japan.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Isamu Okamoto, MD, PhD, Department of Medical Oncology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan. E-mail: chi-okamoto@dotd.med.kindai.ac.jp

ISSN: 1556-0864/11/0610-1624

Activation of protein tyrosine kinases (PTKs) plays a key role in oncogenesis, as exemplified by the role of the *BCR-ABL* fusion gene in chronic myeloid leukemia and by that of epidermal growth factor receptor (*EGFR*) gene mutation and the *EML4-ALK* fusion gene in non-small cell lung cancer (NSCLC). Tyrosine kinase inhibitors (TKIs) that target activated PTKs have exhibited marked therapeutic efficacy in patients with these specific molecular alterations.[1–5] The identification of other target kinases or kinase gene alterations would thus be expected to facilitate the development of new molecularly targeted therapies.

Lung cancer is the leading cause of cancer death worldwide. Despite the successful development of EGFR- or EML4-ALK-targeted TKIs, treatment options remain limited for patients with advanced lung cancer, making the identification of new therapeutic targets an important goal. The tyrosine kinase MET is one such potential therapeutic target. Amplification of *MET* occurs in ~5% of lung cancer cases,[6–9] and *MET* mutations have recently been detected in ~10% of patients with this condition.[10,11] Nevertheless, the relationship between the efficacy of MET-TKIs and *MET* status, such as amplification or mutation, has not been well established. We have, therefore, now investigated the effects of the MET-TKI crizotinib (PF-02341066),[12,13] which is currently undergoing clinical studies, on cell survival and signal transduction in lung cancer cells with or without amplification or mutation of *MET*. We further examined the molecular mechanism underlying the antitumor action of this agent.

MATERIALS AND METHODS

Cell Culture and Reagents

The human NSCLC cell lines H1993, H2122, H1437, A549, H1299, PC9, HCC827, and H596 were obtained from American Type Culture Collection (Manassas, VA). The human NSCLC cell line EBC-1 was obtained from the Health Science Research Resources Bank (Tokyo, Japan). The human NSCLC cell line H3122 was obtained as described previously.[14] All cells were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine

serum. Crizotinib was kindly provided by Pfizer Global Research & Development (Groton, CT). EBC-1 and H1993 cell lines were previously shown to manifest high-level amplification of *MET* (copy number, >10).[15] Sequencing of all 21 coding exons of *MET* also previously revealed that H2122 cells contain an N375S mutation and that H1437 and H596 cells harbor a deletion of exon 14 of this gene.[10,11]

Growth Inhibition Assay In Vitro

Cells were plated in 96-well flat-bottomed plates and cultured for 24 hours before exposure to various concentrations of crizotinib for 72 hours. Cell viability was then assessed with the use of a Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of crizotinib resulting in 50% growth inhibition (IC_{50}) was calculated.

Immunoblot Analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (1 μg/ml). The protein concentration of cell lysates was determined with a BCA protein assay kit (Thermo Fischer Scientific, Waltham, MA), and equal amounts of lysate protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5 or 12% gel (Bio-Rad, Hercules, CA). The separated proteins were transferred to a nitrocellulose membrane, which was then incubated with Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 20 minutes at room temperature before incubation overnight at 4°C with primary antibodies. Rabbit polyclonal antibodies to phosphorylated human MET (pY1234/pY1235), to AKT, to phosphorylated AKT, to poly(ADP-ribose) polymerase (PARP), to BIM, to Bcl-x_L, to Mcl-1, and to XIAP were obtained from Cell Signaling Technology (Danvers, MA); those to extracellular signal-regulated kinase (ERK) and to phosphorylated ERK were from Santa Cruz Biotechnology (Santa Cruz, CA); those to MET were from Zymed (South San Francisco, CA); those to survivin were from Novus (Littleton, CO); and those to β-actin were from Sigma. All antibodies were used at a 1:1000 dilution, with the exception of those to β-actin (1:200). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 hour at room temperature with horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (Sigma). Immune complexes were finally detected with chemiluminescence reagents (GE Healthcare, Little Chalfont, UK).

Annexin V Binding Assay

Binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche, Basel, Switzerland). Cells were harvested by exposure to trypsin ethylenediaminetetraacetic acid, washed with PBS, and centrifuged at 200 *g* for 5 minutes. The cell pellets were resuspended in 100 μl of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 minutes at 15 to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

Gene Silencing

Cells were plated at 50 to 60% confluence in six-well plates or 25-cm^2 flasks and then incubated for 24 hours before transient transfection for the indicated times with small interfering RNAs (siRNAs) mixed with the Lipofectamine reagent (Invitrogen, Carlsbad, CA). The siRNAs specific for MET (MET-1, 5′-ACAAGAUCGUCAACAAAAA-3′; MET-2, 5′-CUACAGAAAUGGUUUCAAA-3′) or BIM (BIM-1, 5′-GGAGGGUAUUUUUGAAUAA-3′; BIM-2, 5′-AGGAGGGUAUUUUUGAAUA-3′) messenger RNAs (mRNAs); and nonspecific (control) siRNAs were obtained from Nippon EGT (Toyama, Japan). The data presented for the effects of BIM depletion were obtained with the BIM-1 siRNA, but similar results were obtained with the BIM-2 siRNA.

Forced Expression of Survivin

The pQCXIH-survivin vector was constructed as described previously.[16] The expression vector was introduced into EBC-1 or H1993 cells by transfection for 48 hours with the use of the Lipofectamine 2000 reagent (Invitrogen).

Growth Inhibition Assay In Vivo

Tumor cells (5×10^6) were injected subcutaneously into the axilla of 5- to 6-week-old male athymic nude mice. Treatment was initiated when tumors in each group achieved an average volume of 200 or 600 mm^3. Treatment groups ($n = 5$ mice each) consisted of control and crizotinib (25 or 50 mg/kg of body weight). Crizotinib was administered by oral gavage daily for 28 days; control animals received a 0.5% (wt/vol) aqueous solution of hydroxypropylmethylcellulose as vehicle. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula $LW^2/2$. Both tumor size and body weight were measured twice per week. The in vivo experiments were approved by the appropriate ethics committee.

Statistical Analysis

Unless indicated otherwise, quantitative data are presented as means ± SE from three independent experiments and were analyzed with the unpaired two-tailed Student's t test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Crizotinib Inhibits the Proliferation of Lung Cancer Cells with *MET* Amplification

We first examined the effect of the MET-TKI crizotinib on the proliferation in vitro of lung cancer cells positive or negative for *MET* amplification. Both of two cell lines with *MET* amplification, EBC-1 and H1993, were sensitive to crizotinib, with IC_{50} values of ≤10 nM (Table 1). In contrast, crizotinib did not substantially inhibit the proliferation of lung cancer cells with a *MET* mutation (H2122, H1437, and H596), with an *EGFR* mutation (PC9 and HCC827) or without such gene amplification or mutation (A549 and H1299) (Table 1). These data suggested that crizotinib has a marked antiproliferative effect in lung cancer cells with *MET*

TABLE 1. IC_{50} Values of Crizotinib for Inhibition of the Growth of NSCLC Cells In Vitro

MET and *EGFR* Status	Cell Line	Crizotinib IC_{50} (nM)
MET amplification (+)	EBC-1	5
	H1993	10
MET amplification (−)		
MET mutation (+)	H2122	472
	H1437	1284
	H596	752
EGFR mutation (−)	A549	646
	H1299	642
EGFR mutation (+)	PC9	787
	HCC827	767

Data are means of triplicates from representative experiments that were repeated a total of three times.
EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

amplification but not in those without *MET* amplification, including those with a *MET* mutation.

Effects of Crizotinib on Downstream Signaling of MET and on Apoptosis in Lung Cancer Cells with or without *MET* Amplification

We next examined the effects of crizotinib on phosphorylation of AKT and ERK in lung cancer cell lines. Crizotinib markedly inhibited the phosphorylation of AKT and ERK and that of MET in cells with *MET* amplification (Figure 1*A*). Lung cancer cells with a *MET* mutation manifested a low level of MET phosphorylation that was completely inhibited by crizotinib, whereas this agent had little effect on the phosphorylation of AKT or ERK in these cells (Figure 1*A*). In addition, crizotinib did not inhibit AKT or ERK phosphorylation in lung cancer cells with an *EGFR* mutation or in those without amplification of *MET* or mutation of *MET* or *EGFR* (Figure 1*A*). We further investigated the effect of crizotinib on apoptosis in lung cancer cells. An annexin V binding assay revealed that crizotinib induced a substantial level of apoptosis in *MET* amplification-positive cells but was largely without effect in the other cell lines studied (Figure 1*B*). Consistent with these results, immunoblot analysis showed that crizotinib triggered the generation of the cleaved form of PARP in cells with *MET* amplification but not in those with a *MET* mutation and in those without amplification or mutation of *MET* (Figure 1*C*, data not shown). These data thus suggested that crizotinib inhibits the phosphorylation of AKT and ERK, resulting in induction of apoptosis, in lung cancer cells with *MET* amplification, whereas such effects were not observed in cells without *MET* amplification, including those with a *MET* mutation.

Effects of Depletion of MET in Lung Cancer Cells with *MET* Amplification or a *MET* Mutation

To verify that the antitumor action of crizotinib in *MET* amplification-positive lung cancer cells is indeed mediated by MET inhibition rather than by nonspecific inhibition of other kinases, we transfected lung cancer cells with two independent siRNAs specific for MET mRNA. Transfection with each MET siRNA resulted in a marked decrease in the expression of MET in both cells with *MET* amplification and those with a *MET* mutation (Figure 2*A*). Transfection of *MET* amplification-positive cells with the MET siRNAs also resulted in pronounced inhibition of AKT and ERK phosphorylation, whereas transfection of *MET* mutation-positive cells had little such effect (Figure 2*A*). Depletion of MET markedly increased the proportion of apoptotic cells (Figure 2*B*) and induced generation of the cleaved form of PARP (Figure 2*C*) in cells with *MET* amplification but not in those with a *MET* mutation. These data thus indicated that the antitumor action of crizotinib in lung cancer cells is mediated by inhibition of MET, and they also suggested that the survival of lung cancer cells with a *MET* mutation is not predominantly dependent on MET signaling.

Effects of Crizotinib on the Expression of Apoptosis-Related Proteins in *MET* Amplification-Positive Lung Cancer Cells

Given that crizotinib induced apoptosis in *MET* amplification-positive lung cancer cells, we examined the effects of this drug on the expression of apoptosis-related proteins in such cells. Crizotinib up-regulated the expression of BIM, a proapoptotic member of the Bcl-2 family of proteins, in both of the *MET* amplification-positive cell lines examined, whereas it had little effect on the expression of other Bcl-2 family members including Mcl-1 and Bcl-x_L (Figure 3*A*). Furthermore, crizotinib down-regulated the expression of survivin, a member of the inhibitor of apoptosis protein (IAP) family, in cells with *MET* amplification, whereas the expression of XIAP, another IAP family member, remained unaffected (Figure 3*A*).

Role of BIM Induction and Survivin Down-Regulation in Crizotinib-Induced Apoptosis in Cells with *MET* Amplification

To investigate further whether the up-regulation of BIM is related to the induction of apoptosis by crizotinib, we transfected *MET* amplification-positive lung cancer cells with a siRNA specific for BIM mRNA. Transfection with the BIM siRNA markedly suppressed the up-regulation of BIM by crizotinib without affecting the down-regulation of survivin (Figure 3*B*). The annexin V binding assay revealed that such transfection resulted in inhibition of crizotinib-induced apoptosis (Figure 3*B*), indicating that BIM induction contributes to the proapoptotic effect of crizotinib in lung cancer cells with *MET* amplification. We obtained similar results with a second siRNA targeted to a different sequence within BIM mRNA (data not shown). Given that crizotinib induced down-regulation of survivin and up-regulation of BIM, we next examined the role of survivin down-regulation in crizotinib-induced apoptosis. Transfection of *MET* amplification-positive cells with an expression vector for human survivin resulted in inhibition of the crizotinib-induced down-regulation of survivin (Figure 3*C*). Such overexpression of survivin also inhibited the induction of apoptosis by crizotinib (Figure 3*C*), indicating that down-regulation of survivin by crizotinib

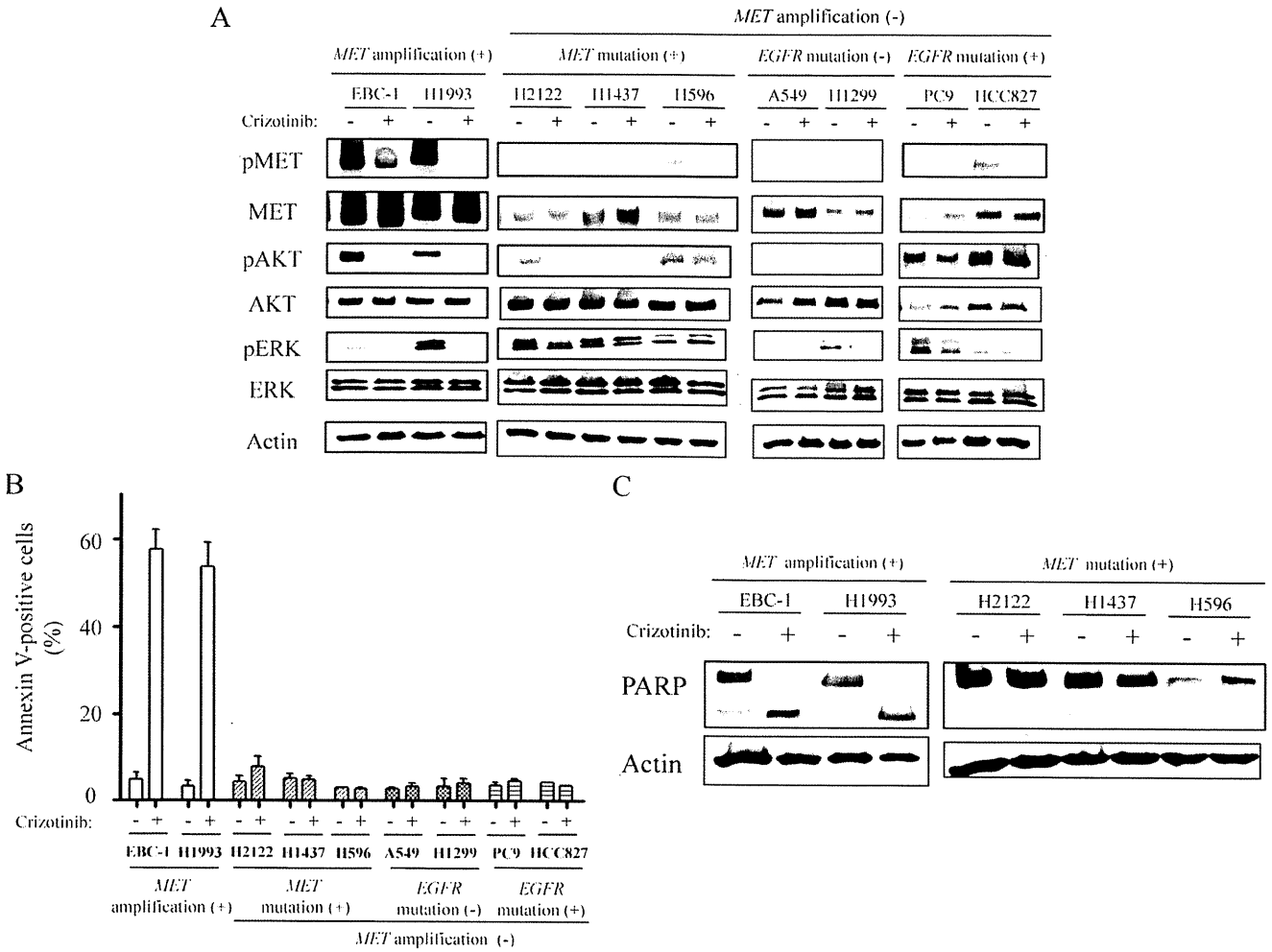

FIGURE 1. Effects of crizotinib on MET, AKT, and extracellular signal-regulated kinase (ERK) phosphorylation and on apoptosis in lung cancer cell lines. *A*, The indicated cell lines were incubated with or without crizotinib (100 nM) for 24 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of MET, AKT, or ERK or to β-actin (loading control). *B*, Cells were incubated for 72 hours with or without crizotinib (100 nM), after which the number of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from three independent experiments. *C*, Cells incubated as in (*B*) were lysed and subjected to immunoblot analysis with antibodies to poly(ADP-ribose) polymerase (PARP) or to β-actin.

contributes to the proapoptotic effect of this agent in lung cancer cells with *MET* amplification. Together, these data thus suggested that the induction of apoptosis by crizotinib in lung cancer cells with *MET* amplification is mediated, at least in part, by BIM up-regulation and survivin down-regulation.

Effect of Crizotinib on the Growth of Lung Cancer Cells In Vivo

To determine whether the antitumor action of crizotinib observed in vitro might also be apparent in vivo, we injected EBC-1 cells (positive for *MET* amplification), H1437 cells (positive for a *MET* mutation), or A549 cells (negative for *MET* amplification and mutation) into nude mice to elicit the formation of solid tumors. After tumor formation, the mice were treated with vehicle (control) or crizotinib at a daily dose of 25 or 50 mg/kg by oral gavage for 4 weeks. Crizotinib at either dose eradicated tumors in mice injected with EBC-1 cells (Figure 4*A*). In contrast, tumors in mice injected with H1437 or A549 cells were not affected by crizotinib treatment even at the dose of 50 mg/kg/d (Figures 4*B*, *C*). Treatment with crizotinib at either dose was well tolerated by the mice, with no signs of toxicity or weight loss during therapy (data not shown). Crizotinib thus exhibited a marked antitumor effect in lung cancer xenografts positive for *MET* amplification, whereas it had little effect on those negative for *MET* amplification, including those with a *MET* mutation, consistent with our results obtained in vitro.

DISCUSSION

Aberrant activation of PTK signaling pathways contributes to the development of various types of cancer. Small-

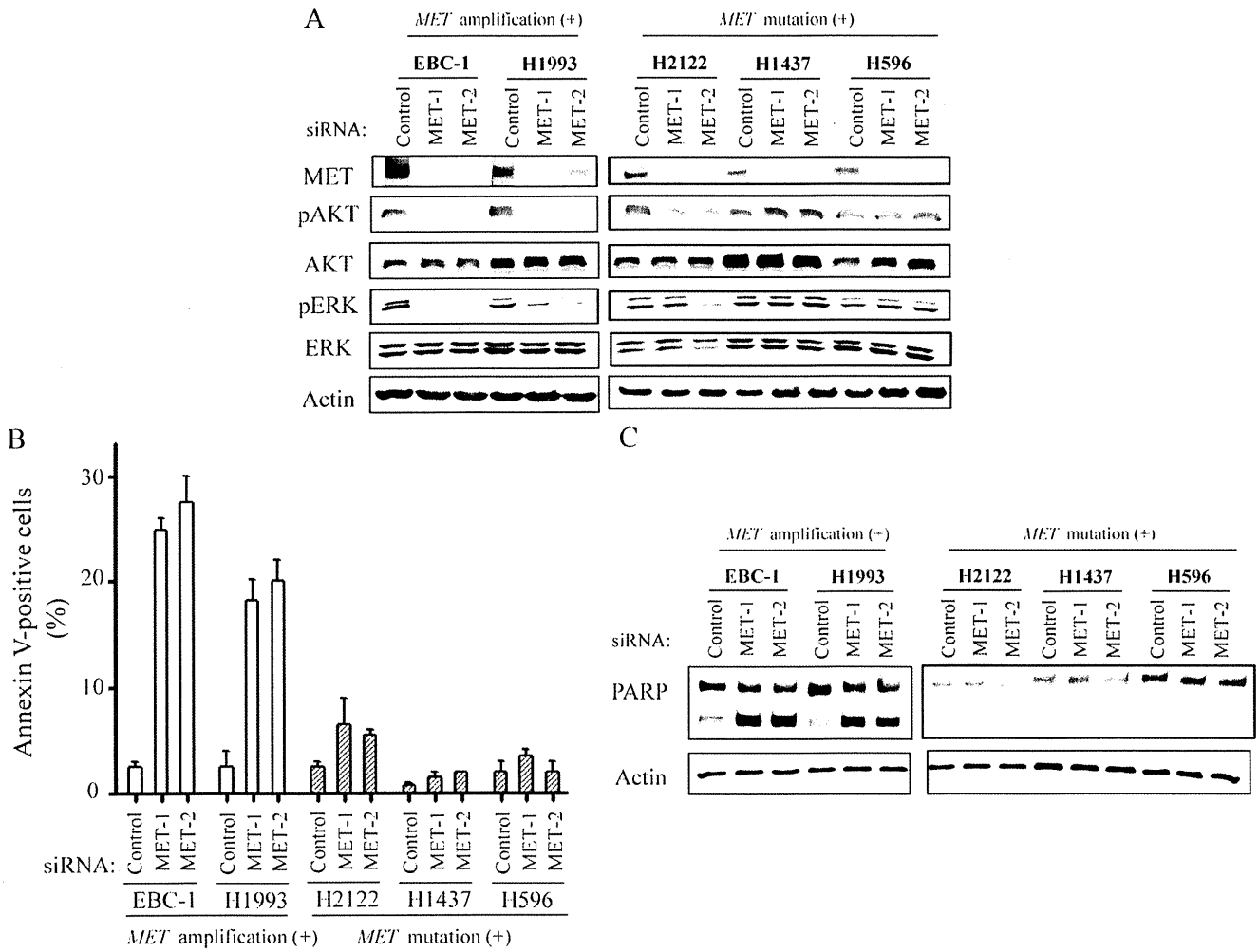

FIGURE 2. Effects of depletion of MET on signal transduction and apoptosis in lung cancer cells. *A*, The indicated cell lines were transfected with nonspecific (control) or MET small interfering RNAs (siRNAs) for 48 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of MET, AKT, or ERK or to β-actin. *B*, Cells were transfected with nonspecific (control) or MET siRNAs for 72 hours, after which the number of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from three independent experiments. *C*, Cells transfected as in *B* were lysed and subjected to immunoblot analysis with antibodies to poly(ADP-ribose) polymerase (PARP) or to β-actin.

molecule inhibitors that target these activated kinases have been developed and have shown substantial efficacy in clinical trials. The receptor tyrosine kinase MET is considered one such potential target in cancer, and several MET-TKIs are currently undergoing clinical trials in humans. The identification of patient subgroups that might actually benefit from treatment with such drugs would be expected to optimize their efficacy. We have now shown that the MET-TKI crizotinib, which is presently undergoing clinical evaluation, exerted a marked antitumor action in lung cancer cells with *MET* amplification but not in those without this genetic change, including those with a *MET* mutation. In lung cancer cells with *MET* amplification, inhibition of MET by either crizotinib or siRNAs specific for MET mRNA resulted in down-regulation of AKT and ERK signaling and the induction of apoptosis. We also found that the secretion of hepatocyte growth factor (HGF), a MET ligand, did not differ substantially between lung cancer cells with or without *MET* amplification (see Figure, Supplemental Digital Content 1, http://links.lww.com/JTO/A98). In addition, exogenous HGF had little effect on the viability of lung cancer cells with *MET* amplification (see Figure, Supplemental Digital Content 1, http://links.lww.com/JTO/A98). Together, these results suggest that *MET* amplification itself results in constitutive activation of MET downstream signaling and that tumors with *MET* amplification are dependent on such signaling for their growth and survival. Targeting of MET signaling by MET-TKIs is thus a potentially valuable therapeutic strategy for patients with lung cancer with *MET* amplification, who account for ~5% of all lung cancer cases.[6–8]

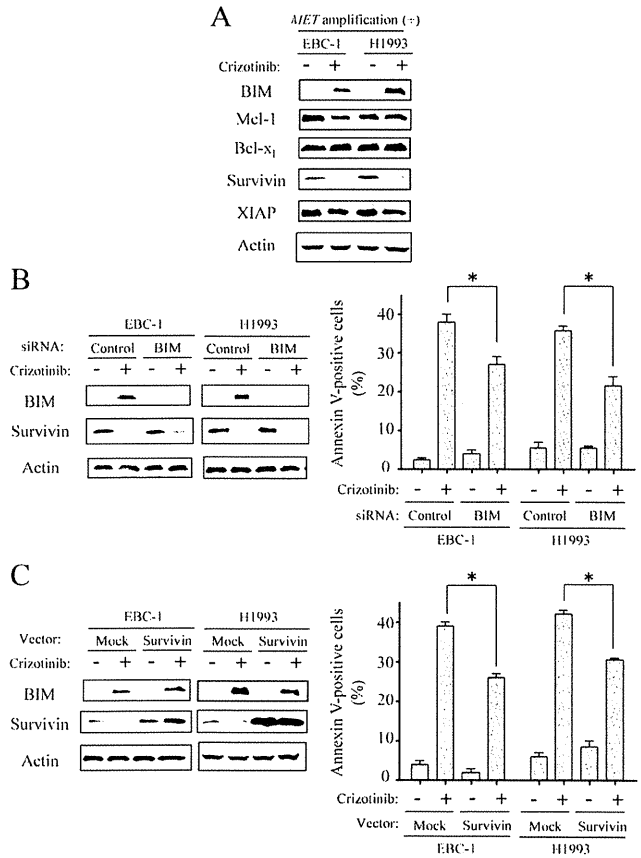

FIGURE 3. Effects of BIM depletion and forced expression of survivin on apoptosis induced by crizotinib in lung cancer cells with *MET* amplification. *A*, EBC-1 and H1993 cells were incubated with or without crizotinib (100 nM) for 48 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins. The position of the band corresponding to BIM_{EL} is indicated. *B*, Cells were transfected with BIM or nonspecific small interfering RNAs (siRNAs) for 24 hours and then incubated for 72 hours with or without crizotinib (100 nM). The cells were then lysed and subjected to immunoblot analysis with antibodies to the indicated proteins (left panel), or they were evaluated for apoptosis by staining with annexin V and propidium iodide followed by flow cytometry (right panel). *C*, Cells were transfected with an expression vector for survivin or with the corresponding empty vector (Mock) for 24 hours, incubated with or without 100 nM crizotinib for 72 hours, and then analyzed as in (*B*). Quantitative data in (*B*) and (*C*) are means ± SE from three independent experiments. *$p < 0.05$ for the indicated comparisons.

Given that crizotinib showed a proapoptotic effect in *MET* amplification-positive lung cancer cells, we further investigated the mediators of crizotinib-induced apoptosis in these cells. We found that crizotinib induced up-regulation of BIM, a key proapoptotic member of the Bcl-2 family of proteins that initiates apoptosis signaling by binding and antagonizing the function of prosurvival Bcl-2 family members.[17] Furthermore, depletion of BIM by RNA interference resulted in inhibition of crizotinib-induced apoptosis in lung cancer cells with *MET* amplification, suggesting that up-regulation of BIM contributes to the induction of apoptosis by crizotinib in such cells. We also found that crizotinib induced down-regulation of survivin, a member of the IAP family that protects cells against apoptosis by either directly or indirectly inhibiting the activation of effector caspases.[18] Moreover, forced expression of survivin suppressed the induction of apoptosis by crizotinib, suggesting that inhibition of survivin expression plays a key role in the proapoptotic effect of this agent in lung cancer cells with *MET* amplification. Our present data thus suggest that MET inhibitor-induced apoptosis is mediated both by up-regulation of BIM and by down-regulation of survivin in lung cancer cells with *MET* amplification. We and others have previously shown that EGFR-TKIs induce the up-regulation of BIM through inhibition of the MEK-ERK signaling pathway in lung cancer cells with an *EGFR* mutation.[16,19–21] Furthermore, we recently demonstrated that EGFR-TKIs induce down-regulation of survivin expression through inhibition of the phosphoinositide 3-kinase (PI3K)-AKT signaling pathway and that the MEK-ERK-BIM and PI3K-AKT-survivin pathways independently contribute to EGFR-TKI-induced apoptosis in *EGFR* mutation-positive lung cancer cells.[16] Given that the MET-TKI crizotinib inhibited phosphorylation of both ERK and AKT and induced BIM up-regulation and survivin down-regulation in *MET* amplification-positive lung cancer cells, MEK-ERK-BIM and PI3K-AKT-survivin pathways likely also mediate crizotinib-induced apoptosis in these cells.

Several *MET* mutations, including those that affect the kinase domain or other domains (extracellular semaphorin and juxtamembrane domains) of the protein, have been identified in tumors. MET-TKIs have been shown to be active against MET with mutations in the kinase domain,[22] whereas the relationship between the efficacy of such agents and nonkinase domain mutants of MET has been unclear. *MET* mutations occur in ~10% of patients with lung cancer and they cluster in nonkinase domain regions of MET.[10,23] The NSCLC cell line H2122 harbors the N375S mutation of *MET*, which is the most frequent mutation of this gene in lung cancer. This mutation is localized to the ligand-binding semaphorin domain of MET and was found to be associated with loss of affinity of the receptor for HGF,[10] although its biological consequences remain largely unclear. We have now shown that crizotinib had little effect on signal transduction or cell survival in H2122 cells, consistent with the previous observation that transient expression of the N375S mutant of MET did not result in an increased susceptibility of the transfected cells to a MET-TKI compared with that of cells expressing wild-type MET.[10] We also examined the effects of crizotinib in lung cancer cells (H1437 and H596 cells) with another *MET* mutation, deletion of exon 14. Loss of the juxtamembrane domain encoded by this exon was found to result in a reduced level of MET ubiquitination and an increase in the half-life of MET, leading to enhancement of ligand-dependent cell proliferation.[11] Nevertheless, the antitumor effects of MET-TKIs in lung cancers with deletion of exon 14 of *MET* have not been well characterized. We have

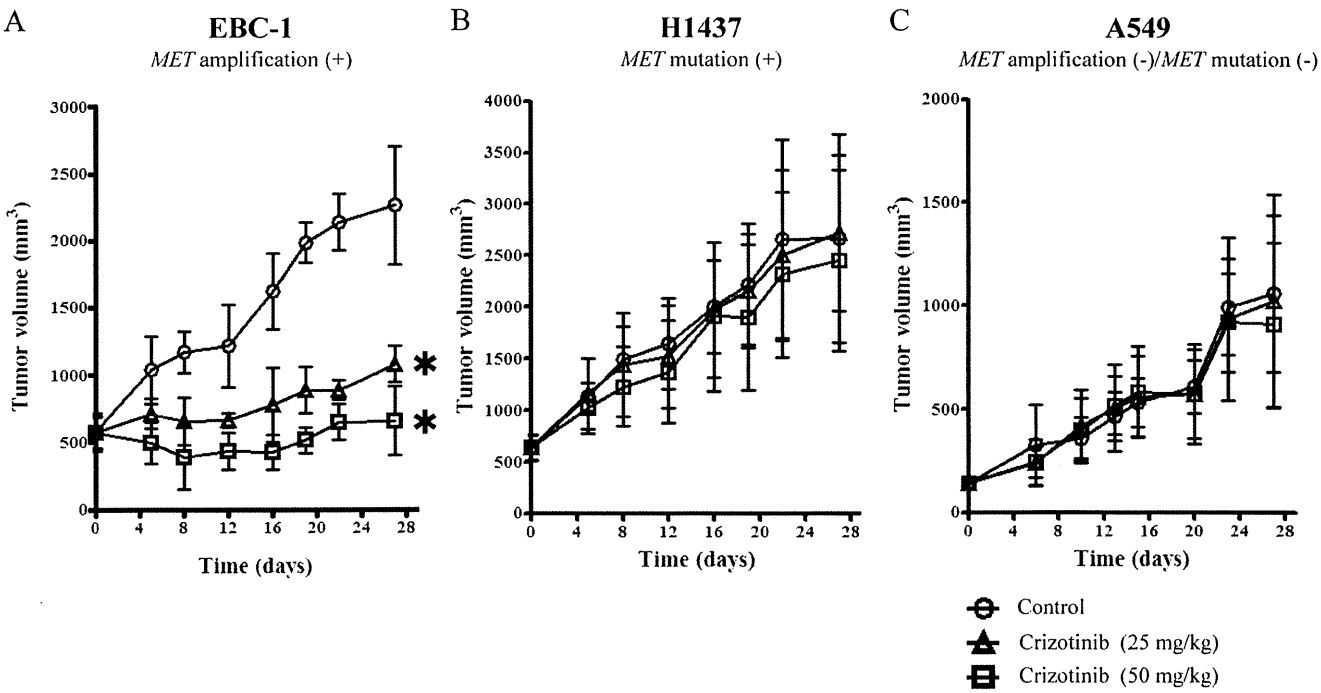

FIGURE 4. Effect of crizotinib on the growth of lung cancer cells in vivo. Nude mice with tumor xenografts established by subcutaneous injection of EBC-1 (*A*), H1437 (*B*), or A549 (*C*) cells were treated daily for 4 weeks with vehicle (control) or crizotinib (25 or 50 mg/kg). Tumor volume was determined at the indicated times after the onset of treatment. Data are means ± SE for 5 mice/group. $*p < 0.05$ for crizotinib (25 or 50 mg/kg) at 28 days versus the corresponding control value.

now shown that inhibition of MET by crizotinib had no substantial antitumor effect on lung cancer cells with an exon 14 deletion either in vitro or in vivo. In contrast to its effects in lung cancer cells with *MET* amplification, depletion of MET by RNA interference did not substantially affect the phosphorylation of AKT or ERK or induce apoptosis in lung cancer cells with either of the *MET* mutations studied. Our present study thus suggests that MET-TKIs may have little clinical efficacy in patients with lung cancer with a *MET* mutation.

Our analysis of the effects of crizotinib on the growth of lung cancer cells in vivo revealed that this agent had a pronounced antitumor effect in cells with *MET* amplification but not in those with a *MET* mutation or in those without amplification or mutation of *MET*. These in vivo data are consistent with our results obtained in vitro. In addition to amplification of *MET*, the MET pathway is also activated by HGF stimulation. Although lung cancer cells with *MET* mutations did not produce substantial amounts of HGF, we found that exogenous HGF stimulation tended to enhance the growth of such cells and that of cells without amplification or mutation of *MET* in vitro (see Figure, Supplemental Digital Content 1, http://links.lww.com/JTO/A98). Furthermore, this HGF-induced growth enhancement was inhibited by crizotinib. These data suggest that crizotinib may also exert antitumor activity in lung cancer without *MET* amplification under conditions of paracrine HGF-induced tumor growth. Our in vivo observations should, therefore, be interpreted with caution given the limitation that the local stromal environment of subcutaneous xenograft tumors in animals differs, at least in part, from that of human cancers in situ.

Crizotinib also inhibits oncogenic fusion variants of the tyrosine kinase ALK in addition to MET.[24] We found that crizotinib markedly inhibited the survival of H3122 lung cancer cells, which are positive for EML4-ALK, in addition to that of *MET* amplification-positive cells (see Figure, Supplemental Digital Content 2, http://links.lww.com/JTO/A99). Given that crizotinib has recently shown marked therapeutic efficacy in patients with lung cancer positive for EML4-ALK,[25] this agent may also have substantial clinical activity and be an attractive therapeutic option for patients with lung cancer with *MET* amplification.

In conclusion, our results have shown that crizotinib has pronounced effects on signal transduction and survival in lung cancer cells with *MET* amplification but not in those without *MET* amplification, including those with a *MET* mutation. Furthermore, both BIM induction and survivin down-regulation were found to mediate the proapoptotic effect of crizotinib in lung cancer cells with *MET* amplification. Our observations thus provide a rationale for clinical evaluation of MET-TKIs in patients with lung cancer with *MET* amplification.

ACKNOWLEDGMENTS

The authors thank E. Hatashita for technical assistance.

REFERENCES

1. Asahina H, Yamazaki K, Kinoshita I, et al. A phase II trial of gefitinib as first-line therapy for advanced non-small cell lung cancer with

epidermal growth factor receptor mutations. *Br J Cancer* 2006;95:998–1004.
2. Sequist LV, Martins RG, Spigel D, et al. First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. *J Clin Oncol* 2008;26:2442–2449.
3. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001;344:1031–1037.
4. Okamoto I. Epidermal growth factor receptor in relation to tumor development: EGFR-targeted anticancer therapy. *FEBS J* 2010;277:309–315.
5. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693–1703.
6. Masuya D, Huang C, Liu D, et al. The tumour-stromal interaction between intratumoral c-Met and stromal hepatocyte growth factor associated with tumour growth and prognosis in non-small-cell lung cancer patients. *Br J Cancer* 2004;90:1555–1562.
7. Nakamura Y, Niki T, Goto A, et al. c-Met activation in lung adenocarcinoma tissues: an immunohistochemical analysis. *Cancer Sci* 2007;98:1006–1013.
8. Zhao X, Weir BA, LaFramboise T, et al. Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. *Cancer Res* 2005;65:5561–5570.
9. Go H, Jeon YK, Park HJ, et al. High MET gene copy number leads to shorter survival in patients with non-small cell lung cancer. *J Thorac Oncol* 2010;5:305–313.
10. Krishnaswamy S, Kanteti R, Duke-Cohan JS, et al. Ethnic differences and functional analysis of MET mutations in lung cancer. *Clin Cancer Res* 2009;15:5714–5723.
11. Kong-Beltran M, Seshagiri S, Zha J, et al. Somatic mutations lead to an oncogenic deletion of met in lung cancer. *Cancer Res* 2006;66:283–289.
12. Zillhardt M, Christensen JG, Lengyel E. An orally available small-molecule inhibitor of c-Met, PF-2341066, reduces tumor burden and metastasis in a preclinical model of ovarian cancer metastasis. *Neoplasia* 2010;12:1–10.
13. Zou HY, Li Q, Lee JH, et al. An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. *Cancer Res* 2007;67:4408–4417.
14. Koivunen JP, Mermel C, Zejnullahu K, et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. *Clin Cancer Res* 2008;14:4275–4283.
15. Lutterbach B, Zeng Q, Davis LJ, et al. Lung cancer cell lines harboring MET gene amplification are dependent on Met for growth and survival. *Cancer Res* 2007;67:2081–2088.
16. Okamoto K, Okamoto I, Okamoto W, et al. Role of survivin in EGFR inhibitor-induced apoptosis in non-small cell lung cancers positive for EGFR mutations. *Cancer Res* 2010;70:10402–10410.
17. Chen L, Willis SN, Wei A, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005;17:393–403.
18. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000;407:770–776.
19. Costa DB, Halmos B, Kumar A, et al. BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. *PLoS Med* 2007;4:1669–1679, discussion 1680.
20. Cragg MS, Kuroda J, Puthalakath H, et al. Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics. *PLoS Med* 2007;4:1681–1689, discussion 1690.
21. Gong Y, Somwar R, Politi K, et al. Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. *PLoS Med* 2007;4:e294.
22. Timofeevski SL, McTigue MA, Ryan K, et al. Enzymatic characterization of c-Met receptor tyrosine kinase oncogenic mutants and kinetic studies with aminopyridine and triazolopyrazine inhibitors. *Biochemistry* 2009;48:5339–5349.
23. Ma PC, Jagadeeswaran R, Jagadeesh S, et al. Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. *Cancer Res* 2005;65:1479–1488.
24. Christensen JG, Zou HY, Arango ME, et al. Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. *Mol Cancer Ther* 2007;6:3314–3322.
25. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693–1703.

ANTICANCER RESEARCH *31*: 3449-3456 (2011)

Phase II Study of Topotecan with Cisplatin in Japanese Patients with Small Cell Lung Cancer

YUKIO HOSOMI[1], MASAHIKO SHIBUYA[1], SEIJI NIHO[2], YUKITO ICHINOSE[3], KATSUYUKI KIURA[4], HIROSHI SAKAI[5], KOJI TAKEDA[6], SHINZO KUDO[7], KENJI EGUCHI[8], KAORU MATSUI[9], NORIYUKI MASUDA[10], MASAHIRO ANDO[11] and KOSHIRO WATANABE[12]

[1]*Division of Respiratory Medicine, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo, Japan;*
[2]*Department of Thoracic Oncology, National Cancer Center Hospital East, Chiba, Japan;*
[3]*Department of Thoracic Oncology, National Kyushu Cancer Center, Fukuoka, Japan;*
[4]*Department of Allergy and Respiratory Medicine, Okayama University Hospital, Okayama, Japan;*
[5]*Division of Thoracic Oncology, Saitama Cancer Center, Saitama, Japan;*
[6]*Department of Clinical Oncology, Osaka City General Hospital, Osaka, Japan;*
[7]*Department of Respiratory Medicine, Osaka City University Hospital, Osaka, Japan;*
[8]*Division of Medical Oncology, Tokai University Hospital, Kanagawa, Japan;*
[9]*Department of Thoracic Malignancy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Osaka, Japan;*
[10]*Department of Respiratory Medicine, Kitasato University Hospital, Kanagawa, Japan;*
[11]*Division of Internal Medicine, Tsuboi Cancer Center Hospital, Fukushima, Japan;*
[12]*Department of Respiratory Medicine, Yokohama Municipal Citizen's Hospital, Kanagawa, Japan*

Abstract. *Background: We conducted a phase II study of topotecan (Tp) with cisplatin (CDDP) in previously untreated Japanese patients with extensive-disease small cell lung cancer (ED-SCLC). Patients and Methods: In stage 1, a total of 30 patients were allocated to Tp 0.65 mg/m^2 with CDDP 60 mg/m^2 day 1 or Tp 1.00 mg/m^2 with CDDP day 5 following prophylactic granulocyte colony stimulating factor (G-CSF) from day 6. In stage 2, the selective combination in 29 patients was evaluated for response rate, toxicity and overall survival. Results: In stage 1, Tp 1.00 mg/m^2 with CDDP day 5 was selected this schedule had a better hematological profile. In stage 2, the response rate was 83%, and grade 3/4 adverse events were hematological-toxicities. The median survival time was 17.5 months and the 1 year survival rate was 79%. Conclusion: Combination of Tp and CDDP on day 5 with G-CSF support is safe and effective for previously untreated ED-SCLC Japanese patients.*

Correspondence to: Yukio Hosomi, 18-22, Honkomagome 3 chome, Bunkyo-ku, Tokyo, 113-8677, Japan. Tel: +81338232101, Fax: +81338241552, e-mail: yhosomi@cick.jp

Key Words: Cisplatin, small cell lung cancer, topotecan.

Most patients with previously untreated small cell lung cancer (SCLC) are highly sensitive to chemotherapy and radiation therapy. As cisplatin (CDDP) is the most important drug for SCLC chemotherapy, the standard chemotherapy regimen for treatment of extensive-disease (ED)-SCLC has been the combination of CDDP plus etoposide (PE regimen). Although this combination has produced objective response rates as high as 80%, median survival times range from 9 to 11 months, with a 2-year survival rate of less than 10% (1-3). Several novel strategies failed to improve patient survival time. Therefore, investigation of therapy resulting in improvement of survival is still ongoing.

Inhibitors of topoisomerase I (Topo I, an enzyme necessary for DNA replication) are active against SCLC. A randomized study of the Topo I inhibitor, irinotecan, plus CDDP *versus* PE in previously untreated Japanese patients with ED-SCLC indicated survival benefit of Topo I inhibitor (4). Topotecan (Tp), a cytotoxic water-soluble semisymthetic camptothecin analogue, acts as an inhibitor of Topo I. Tp demonstrated anti tumor activity towards human cancer cell lines and animal tumor models, then a combination of Tp and CDDP had synergistic effect *in vitro* studies (5, 6). The combination effect of Tp and CDDP is not influenced by the order of administrating the two drugs. On the contrary, adverse effects are influenced (7). Therefore Tp has been evaluated as useful

for ovarian and uterine cervical carcinoma, besides second-line therapy in SCLC (8-10). The combination regimen of Tp with CDDP has been documented in use for previously untreated ED-SCLC patients (11, 12). In addition, no difference in efficacy and tolerability was recorded in Tp and CDDP combination regimen compared with standard regimen in a phase III study for the first-line therapy of previously untreated ED-SCLC patients (13, 14).

Although the standard regimen for ED-SCLC in North America and Europe is a combination of CDDP plus etoposide (3), the combination of irinotecan plus CDDP is the standard regimen in Japan due to the significant prolongation of survival obtained from the interim analysis of a phase III clinical study by the Japan Clinical Oncology Group (JCOG) (4), with similar results to the phase II clinical trial (15) on this regimen.

Since there are different antitumor efficacies of Topo I inhibitor between Japanese patients and North American and European populations, the present phase II clinical trial was planned to investigate antitumor effects of Tp plus CDDP in Japanese patients with ED-SCLC, for whom irinotecan plus CDDP is the standard regimen.

The recommended schedule was evaluated at stage 1 due to the need for estimation of different schedule dependency on efficacy and safety for Japanese patients. Based on findings from two clinical studies on irinotecan plus CDDP (phase II and phase III), the clinical efficacy of the combination of CDDP plus Tp, which has the same Topo I inhibitory activity with irinotecan, is evaluated in this phase II clinical trial to investigate whether the efficacy of irinotecan plus CDDP is based on the mode of action of Topo I inhibitor plus CDDP in Japanese patients.

In a completed phase I study, we determined the recommended dose of Tp 0.65 mg/m^2 and 1.00 mg/m^2 as in combination with CDDP day 1 and day 5 schedules, respectively, with fixed dose of CDDP at 60 mg/m^2 (16). We found novel potential of the Tp combination regimen with CDDP against previously untreated Japanese patients with ED-SCLC in a phase II study. In present study, the dose of CDDP at 60 mg/m^2 was fixed. A prophylactic G-CSF concomitant treatment was employed from consideration of dose limiting factors of this combination. In addition to efficacy and safety evaluation of Tp plus CDDP combination, the treatment effect of the combination regimen of Topo I inhibitor with CDDP in Japanese patients was also evaluated.

Patients and Methods

Study design. A two-stage study was designed (Figure 1). At stage 1, two arms (arm A and arm B) were compared evaluating tumor response and toxicity to select the superior arm. In stage 2, 15 cases were added to the selected arm. The cases in stage 1 and stage 2 of the selected arm were combined for evaluation of efficacy and safety of this combination.

Eligibility. Japanese patients with histological and/or cytological documented SCLC were eligible for this study. Each patient was required to meet the following criteria: ED-SCLC, previously untreated, having measurable lesion; performance status (Eastern Cooperative Oncology Group: ECOG PS) of 0-1; age 20 to 74 years; adequate organ function (hemoglobin level >9.5 g/dl, leukocyte count 4,000 to 12,000/mm^2, neutrophill cell count >2,000/mm^2, platelet count >100,000/mm^2, aspartate aminotransferase (AST) level <2.5 times of the normal upper limit, total bilirubin value <1.5 mg/dl, serum creatinine below the normal upper limit, resting partial pressure oxygen >60 torr; a life expectancy of at least 3 months; hospitalized; and written informed consent obtained. The protocol and informed consent procedures were reviewed and approved by the Institutional Review Board of each participating institute. This study was subjected to Good Clinical Practice (GCP) and Declaration of Helsinki.

Treatment schedule. Tp of 0.65 mg/m^2 for CDDP day 1 schedule (arm A) or 1.00 mg/m^2 (or 1.2 mg/m^2, if the nadir of the first cycle for leucocytes of >2,000/mm^2 and platelet of >50,000/mm^2, the dose from next cycle could be increased) for CDDP day 5 schedule (arm B) were intravenously administered at over 30 min by drop infusion for the first 5 consequent days within one cycle of 21 days. Tp was provided by Nippon Kayaku Co., Ltd. as 1.1 mg/vial formulation to be dissolved in 500 to 1,000ml of saline. CDDP was also intravenously administered at over 2 h by drop infusion at day 1 (arm A) or day 5 (arm B). Prophylactic G-CSF was administered from day 6 (day after final Tp administration) until recovery from nadir for leucocytes of >10,000/mm^2 or neutrocytes of >5,000 mm^2. For each patient, 4 cycles were planned.

Dose modification. When grade 4 neutropenia, more than grade 3 febrile neutropenia with over 38.5°C or thrombocytopenia (<25,000/mm^2) occurred, the Tp dose was reduced from 0.65 to 0.5 mg/m2 for arm A or 1.0 to 0.8 mg/m^2 for CDDP day 5 schedule (or 1.2 to 1.0 mg/m^2 if applicable), respectively. When the leukocyte count >4,000/mm^2, neutrocyte count >2,000/mm^2, platelet count >100,000/mm^2 and hemoglobin value >8.0g/dl or recovery tendency was observed, the treatment was able to proceed to the next cycle. Treatment could be delayed for up to 30 days from day 1 of the current cycle to allow a patient sufficient time to recover from study drug-related toxicity.

Evaluation. All patients underwent weekly evaluations that included assessment of symptoms (subjective and objective findings), a physical examination, a complete blood cell count, blood chemistry (including measurement of AST, alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (Al-P), total bilirubin, total protein, serum creatinine, blood urea nitrogen (BUN), serum electrolytes) and urinalysis. Toxicity was evaluated according to NCI CTCAE version 3 criteria (17). Tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) (18), assessed by computed tomographic (CT) scanning, as with staging at enrollment. All the observed responses were reviewed by an extramural panel.

Statistical analysis. This study was made up of two different stages, one for the CDDP schedule selection (stage 1) and the other for the evaluation of the selected schedule (stage 2). The primary aim of this study was to assess the anti-tumor effect of the combination. Thus, involvement of 15 cases for the two CDDP administration

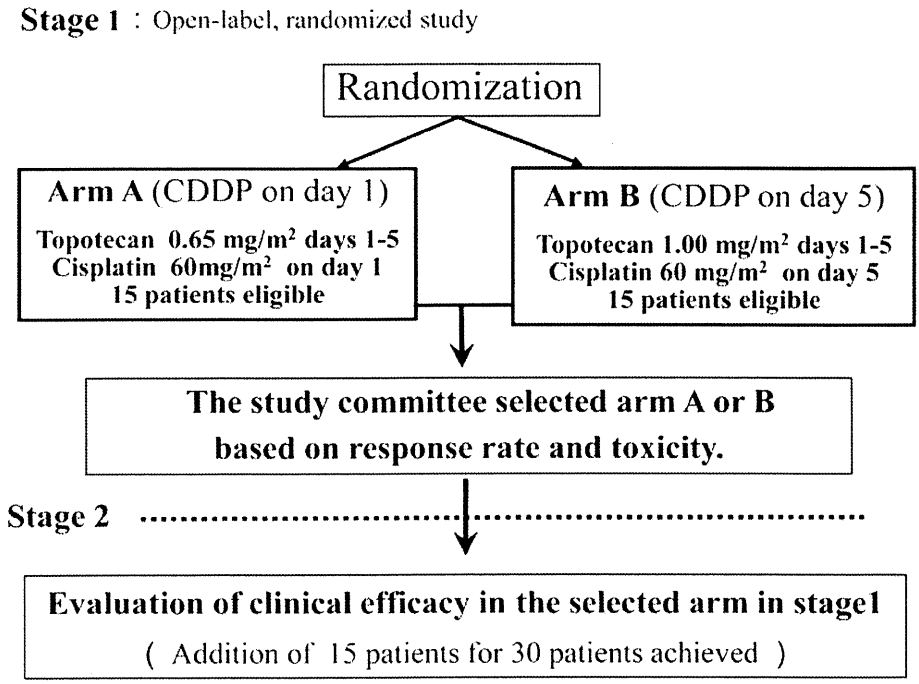

Figure 1. *The study design is shown graphically. Stage 1: comparison phase. Stage 2: evaluation phase.*

schedules (on day 1 and day 5) were adequate based on the following premises: 8 cases of rejection region, 70% expected efficacy rate, 35% threshold response rate, one-sided significance level α=0.042 and power 1-β=0.869.

The anti-tumor activity was estimated by response rate and 95% confidence intervals. Toxicity was estimated by subjective and objective findings and incidence by, in comparison between CDDP day 1 and day 5 schedules. Since the stage 1 study was not statistically powered, the two arms were analyzed separately.

At stage 2, clinically useful threshold and expected efficacy rate were set as 60% and 85% respectively. On this basis, 26 cases were required based on α=0.05 (bilaterally) and β=0.2 (one-sided), thus the target number of cases use 30. In addition to stage 1, 15 cases were planned to be enrolled for the selected superior arm. The primary endpoint of stage 2 was to assess the anti tumor efficacy of Tp in combination with CDDP, taking the toxicity profile into account. The secondary endpoint was overall survival, and the several survival curves were estimated by means of the Kaplan and Meier method.

Results

From August 2005 through July 2008, a total of 44 chemotherapy-naive Japanese ED -SCLC patients were enrolled into this study from 16 institutions. All 44 cases were eligible. In stage 1, 30 patients were randomly assigned to the arms of CDDP day 1 and day 5 schedules. In stage 2, 14 patients were enrolled for arm B, in addition to stage 1 (total 29 cases for this schedule). All enrolled patients were included in analyses of tumor response and toxicity. Survival times of 29 patients for CDDP day 5 schedule were evaluated. Patients' characteristics are listed in Table I. For arm B, 6 female patients were enrolled in stage 1. Median ages for patients in arms A and B in stage 1 were 62 (55 to 74) and 66 (56 to 74) years, respectively. There were no enrolment criteria or protocol violations. Total cycles for each stage are listed in Table II. Median treatment of cycles was 4 in both stage 1 and stage 2.

Response. Clinical response for stage 1 is listed in Table II. Arm A resulted in 12 partial responses (PRs), 1 case of stable disease (SD) and 1 case not evaluated (NE). The response rate was 80% (95% CI of 51.9 to 95.7%). Arm B had 12 PRs, 2 case of SD and 1 case of progressive disease (PD). The response rate of arm B was 80% (95% CI of 51.9 to 95.7%). In stage 1, there was no difference in the response rate between arm A and arm B. It is notable that 1 complete response (CR) was observed in stage 2. Total response for CDDP day 5 schedule (29 cases in stage 1 and 2) was 1 CR, 23 PRs, 3 SDs, and 2 NEs with median response rate of 83% (95% CI of 64.2 to 94.2%). As of November 2009, when the final analysis was conducted, the median overall survival was 17.5 months (95% CI of 14.8 to 20.8 months) for CDDP day 5 schedule. A Kaplan-Meier curve for survival of patients in arm B is indicated in Figure 2. Overall survival rate for this group was 79% (95% CI of 64.6 to 94.1%) at 1 year.

Table I. *Patient characteristics.*

	Stage 1		Stage 2
	CDDP on day 1	CDDP on day 5	CDDP on day 5
No. of patients	15	15	14*
Gender			
Male	15	9	12
Female	0	6	2
Age (years)			
Median	62.0	66.0	64.0
Range	55-74	56-74	46-73
Performance status (ECOG)**			
0	5	4	4
1	10	11	10
Stage			
IIIB	0	3	2
IV	15	12	12
Tumor diameter (mm)			
50-100<	1	2	1
≥100	14	13	13
Metastasis			
Lung	3	3	3
Liver	7	4	2
Brain	6	1	3
Bone	4	5	6

*Additional patients in stage 2. **Eastern cooperative oncology group.

Toxicity. The toxicity profile in stage 1 is listed in Table III. No death or febrile neutropenia was observed for any of the 30 patients in stage 1. The main grade 3/4 adverse events in stage 1 were hematological toxicities: leukopenia, neutropenia, thrombocytopenia and anemia; were observed as 40%, 67%, 67% and 60% for arm A and 7%, 40%, 60% and 47% for arm B, respectively. Arm A had a tendency for higher incidence of adverse events than arm B. Non-hematological events (subjective and objective findings) in stage 1 were nausea, anorexia, constipation, vomiting, fatigue and alopecia, with a range of 53% to 93%. As Grade 3 events, anorexia, fatigue and body weight loss were observed each for 1 patient. There was no difference in non-hematological toxicity profile between the two CDDP schedules. Over 50% of patients in stage 1 experienced increased AST and/or ALT, with 2 cases of grade 3 AST increase. Because no difference in the efficacy between the two arms was observed, the CDDP day 5 schedule was employed for stage 2, taking the hematological toxicity profile into account with lower incidence of grade 3 events in arm B compared with arm A in stage 1. The toxicity profile of stage 2 is listed in Table III. No death or febrile neutropenia was observed in this group. The main adverse events in stage 2 were similar to those of arm B in stage 1, both in nature and grade.

Table II. *Number of treatment cycles and clinical response.*

	Stage 1		Stage 2
	CDDP on day 1	CDDP on day 5	CDDP on day 5
Topotecan (mg/m^2)	0.65	1.00	1.00
No. of patients	15	15	29*
Count of treatment cycles			
Count of total cycles	52	53	110
Median cycle	4	4	4
Clinical response**			
Complete response	0	0	1
Partial response	12	12	23
Stable disease	1	2	3
Progressive disease	1	0	0
Not evaluable	1	1	2
Response rate (%)	80	80	83
95% Confidence interval (%)	51.9-95.7	51.9-95.7	64.2-94.2

*Selected 15 patients of CDDP on day 5 in stage 1 + additional 14 patients in stage 2. **Evaluated according to RECIST.

Discussion

In this study, Tp at 0.65 mg/m^2 and 1.00 mg/m^2 were employed for CDDP day 1 and day 5 schedules, respectively, based on the results of a previous phase I study. In stage 1, CDDP schedules of day 1 and day 5 administration were evaluated to select optimum combination with Tp, taking response and toxic profile into account. In stage 2, additional patients were enrolled onto the superior regimen to evaluate response rate as primary endpoint and overall survival (time and 1-year rate) as secondary endpoints. In stage 1, both arms had similar responses, with different hematological toxicity profiles. In arm A, the incidence of grade 3/4 hematological events were slightly higher than those in arm B. From the safety point of view, the CDDP day 5 schedule was employed for further study.

Although the primary endpoint of stage 1 was the response rate of this combination by schedule, there were no substantial difference between the two schedules, hence the toxicity profile was taken into account for the selection of the superior arm.

In a previous report from North America/Europe, death from sepsis with the CDDP day 1 regimen was shown, indicating the possibility of severe hematological toxicity (19), and then the validity of CDDP day 5 schedule was suggested (7, 12, 19). Although the administration timing of CDDP in the combination is the same for Japanese patients, in arm A in this study, no death or febrile neutropenia was observed, likely due to the contribution of G-CSF from day 6. The response rate for the 29 cases of CDDP day 5

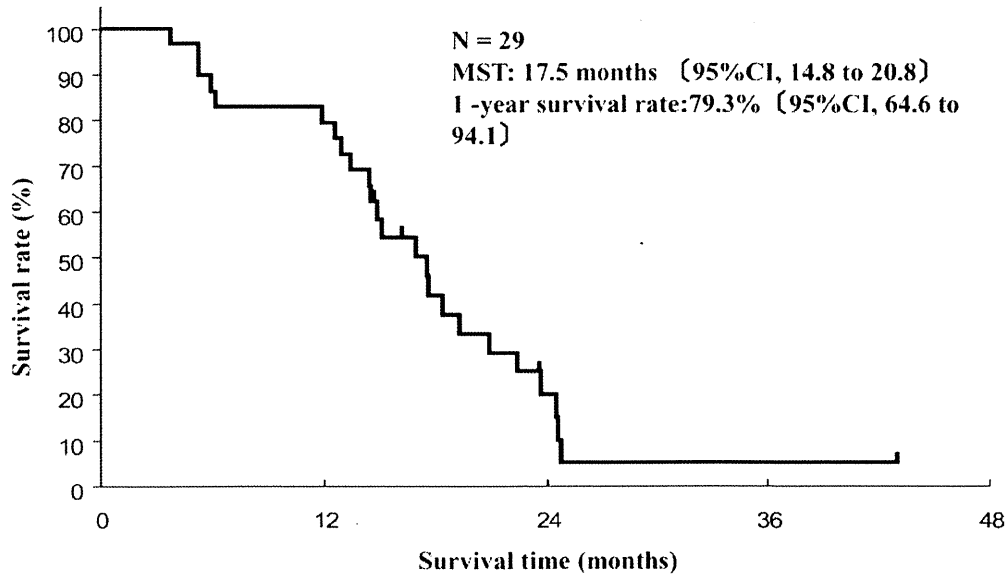

Figure 2. *The survival curve for patients treated with topotecan combination with cisplatin on day 5 is shown. MST, Median survival time; 95% CI, 95% confidence interval.*

Table III. *Adverse events of stage 1 in this phase II study.*

	Toxicity*	CDDP on day 1 (n=15)				CDDP on day 5 (n=15)			
		Grade		Any grade		Grade		Any grade	
		3/4 (n)	(%)	(n)	(%)	3/4 (n)	(%)	(n)	(%)
Hematological	Leukopenia	6/0	40	11	73.3	1/0	7	12	80.0
	Neutropenia	3/7	67	12	80.0	4/2	40	14	93.3
	Thrombocytopenia	6/4	67	14	93.3	3/3	60	15	100.0
	Anemia	7/2	60	14	93.3	5/2	47	15	100.0
Hepatic	AST	0/0	0	2	13.3	2/0	13	10	66.7
	ALT	1/0	7	3	20.0	0/0	0	9	60.0
	T-bilirubin	0/0	0	3	20.0	0/0	0	5	33.3
Gastrointestinal	Anorexia	1/0	7	9	60.0	0/0	0	13	86.7
	Nausea	0/0	0	12	80.0	0/0	0	14	93.3
	Vomiting	0/0	0	8	53.3	0/0	0	9	60.0
	Constipation	0/0	0	8	53.3	0/0	0	10	66.7
	Diarrhoea	0/0	0	1	6.7	0/0	0	5	33.3
	Hiccup	0/0	0	6	40.0	0/0	0	2	13.3
Systemic	Fatigue	0/0	0	10	66.7	1/0	7	10	66.7
	Body weight loss	0/0	0	5	33.3	1/0	7	7	46.7
Fever and other	Fever	0/0	0	2	13.3	0/0	0	1	6.7
	Alopecia	0/0	0	12	80.0	0/0	0	11	73.3

*Severity of each event was evaluated according to CTCAE version 3.0.

schedule group was 83%, including 1 CR. The median survival time was 17.5 months and the 1-year survival rate was 79%. Among observed adverse events, grade 3/4 events were hematological. Major non-hematological adverse events were digestive organ toxicity, (anorexia, nausea, vomiting and constipation), and alopecia, with grade 1/2. These events were also observed in Tp mono therapy (20), thus no CDDP contribution of to stimulation of toxicity was considered. However, AST/ALT increases did occur, in 50% of CDDP day 5 schedule cases. These hepatic events were transient