negative for both paclitaxel and carboplatin, suggesting that the exanthematous rashes were caused by drug allergy to sorafenib rather than by dose-dependent toxicity. Indeed, serious erythema multiforme was not observed in any of the seven patients in cohort 2, for whom sorafenib was administered at 400 mg twice daily in cycle 2 and subsequent cycles. The only differences between the treatment regimen in cohort 1 and that of cycle 2 and subsequent cycles in cohort 2 were the dose (AUC) and infusion time of carboplatin, which were 6 mg min mL^{-1} over 30 min and 5 mg min mL^{-1} over 60 min, respectively, and pharmacokinetic analysis revealed that the triplet regimen had no significant effects on the pharmacokinetics of the individual agents. These data thus suggest that the sorafenib-related erythema multiforme observed in cohort 1 was likely the result of classic skin hypersensitivity to the drug.

Two additional DLTs (hand-foot skin reaction and elevation of ALT, both of grade 3) were observed in cohort 1, both of which were manageable and resolved by treatment interruption and remedial therapy. Although the study treatment was discontinued after the first cycle in the four patients with DLTs in cohort 1, one patient showing a partial response received three cycles of carboplatin-paclitaxel-sorafenib and an additional 13 cycles of sorafenib maintenance monotherapy, and another patient showing a complete response received four cycles of the combination therapy and an additional 23 cycles of sorafenib monotherapy. A previous phase I study of sorafenib combined with paclitaxel and carboplatin for advanced solid tumors (mostly malignant melanoma) recommended doses for future trials of sorafenib at 400 mg twice daily, carboplatin at an AUC of 6 mg min mL^{-1}, and paclitaxel at 225 mg/m^2. In a recently completed randomized phase III study of advanced NSCLC, patients were randomly assigned to treatment either with sorafenib at 400 mg twice daily plus carboplatin (AUC of 6 mg min mL^{-1}) and paclitaxel (200 mg/m^2) or with carboplatin and paclitaxel alone [24]. The present study suggests that the dose of sorafenib tolerated by Japanese patients is likely to be lower than that tolerated by Western patients when this agent is combined with standard doses of carboplatin and paclitaxel.

We examined the pharmacokinetics of paclitaxel, carboplatin, and sorafenib in order to detect any relevant drug-drug interactions. The pharmacokinetics of sorafenib in the present combination study were similar to those described in previous monotherapy [7, 17] and combination [16] trials, in which there was no evidence of drug-drug interactions. Neither of the carboplatin doses administered in the present study (AUC of 5 or 6 mg min mL^{-1}) appeared to affect the pharmacokinetics of sorafenib. Furthermore, we have shown for the first time that administration of sorafenib at 400 mg twice daily had no effect on the pharmacokinetics of carboplatin. Whereas small increases in the AUC and C_{max} values of paclitaxel and 6-hydroxy-paclitaxel were observed after sorafenib administration at 400 mg twice daily, these increases were not statistically significant. Paclitaxel is primarily metabolized in the liver by the CYP2C8 pathway to 6-hydroxy-paclitaxel and is also metabolized by CYP3A4 [25]. Although we are not able to exclude possible inhibition by sorafenib of the metabolic clearance of paclitaxel, the observed increase in paclitaxel exposure was not associated with increased clinical toxicity. Together, our pharmacokinetic results suggest that concomitant administration of sorafenib, carboplatin, and paclitaxel had no significant impact on the pharmacokinetics of any of these three drugs in this treatment schedule, although our finding on pharmacokinetics will need to be reproduced in larger cohort of patients treated with this combination.

Although tumor evaluation was not the primary objective of our study, the combination treatment yielded promising results, with one complete response and six partial responses observed among the 12 evaluable patients. Despite this substantial antitumor activity observed in the present study, a phase III trial (ESCAPE: Evaluation of Sorafenib, Carboplatin, and Paclitaxel Efficacy) of 926 patients with advanced NSCLC receiving first-line therapy with paclitaxel and carboplatin in the absence or presence of sorafenib failed to show an improvement in efficacy with the addition of sorafenib to the standard combination chemotherapy [24]. Indeed, a subset analysis of the 219 patients with squamous histology was suggestive of a detrimental effect of sorafenib inclusion. The complete response and all partial responses in our phase I study occurred in patients with non-squamous NSCLC. Although the biological basis for a possible ethnic difference in sorafenib efficacy and toxicity remains unknown, further investigation are warranted to identify the patients who are more likely to benefit from this agent.

In conclusion, in combination with carboplatin AUC 5 mg min mL^{-1} and paclitaxel 200 mg/m^2, administration of sorafenib at 400 mg once daily was confirmed to be feasible in Japanese patients with advanced NSCLC. There was no relevant pharmacokinetic interaction and the observed antitumor activity was encouraging in this study.

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Conflicts of interest Two of the co-authors, Koichi Fukino and Takahiko Tanigawa, are employees of Bayer Yakuhin Ltd.

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Dose-escalation study of pemetrexed in combination with carboplatin followed by pemetrexed maintenance therapy for advanced non-small cell lung cancer

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ABSTRACT

Introduction: The primary objectives of this study were to determine the recommended dose of pemetrexed and carboplatin in patients with chemo-naive advanced non-small cell lung cancer (NSCLC).
Methods: Patients received escalated doses of carboplatin area under the concentration–time curve (AUC) of 5 (cohort 1) or 6 (cohort 2) and pemetrexed 500 mg/m^2 every 3 weeks for six cycles. For patients with objective response and stable disease, pemetrexed were continued until disease progression or unacceptable toxicity.
Results: In cohort 1, a dose-limiting toxicity (DLT) was observed in one of the six patients: grade 4 thrombocytopenia. No DLTs were seen in the first 6 patients of cohort 2, and thus the combination of pemetrexed 500 mg/m^2 plus carboplatin at AUC 6 was determined as the recommended dose. Among a total of 20 patients, 8 patients received a median of four cycles of pemetrexed monotherapy in a maintenance setting without unexpected or cumulative toxicities. No complete responses and 12 partial responses were observed, giving an overall response rate of 60.0% [95% confidence interval (CI), 36.1–80.9%]. Median progression-free survival time for all patients was 7.6 months (95% CI: 4.8–8.0 months).
Conclusions: Pemetrexed 500 mg/m^2 plus carboplatin AUC 6 combination therapy followed by pemetrexed maintenance therapy, is generally tolerable, and shows encouraging antitumor activity in chemotherapy-naive patients with advanced NSCLC.

1. Introduction

Lung cancer is the most common cancer in the world and the leading cause of cancer-related mortality. Approximately 85% of lung cancers are non-small cell, and approximately 70% of patients with NSCLC present with inoperable, locally advanced (Stage IIIB) or metastatic (Stage IV) disease. Platinum-based chemotherapy is the standard first-line treatment for advanced NSCLC on the basis of moderate improvement in survival and quality of life it confers compared with best supportive care alone [1–3]. The poor outlook even for patients with advanced NSCLC who receive such treatment has prompted a search for new chemotherapeutic agents and combination regimens.

Pemetrexed is a multi-targeted antifolate cytotoxic agent. Randomized phase III clinical studies have demonstrated that pemetrexed is efficacious as a single agent in second-line treatment of NSCLC [4], and in combination with cisplatin for first-line treatment of nonsquamous NSCLC [5]. The latter phase III study reported noninferior efficacy and better tolerability for cisplatin/pemetrexed than for cisplatin/gemcitabine in the first-line setting [5]. In addition, overall survival was statistically superior for cisplatin/pemetrexed versus cisplatin/gemcitabine in patients with nonsquamous NSCLC [6]. Another phase III clinical study demonstrated superior overall survival (OS) when pemetrexed was used in a maintenance setting following 4 cycles of non-pemetrexed induction therapy containing a platinum doublet [7]. These study results indicate that pemetrexed-based induction therapy followed by pemetrexed maintenance therapy would be a possible treatment option for patients with nonsquamous NSCLC.

Because carboplatin-based regimens have been shown to be less toxic, convenient, and capable of being administered on an outpatient basis, they have been widely used as a substitute for cisplatin regimens in clinical practice. In wake of the above results, there has been interest in studying the substitution of carboplatin for

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cisplatin in cisplatin plus pemetrexed regimens for NSCLC [8–11]. In previous studies of pemetrexed and carboplatin combination therapy in a first-line setting, different carboplatin doses (AUC 5 and 6) were used. To date, carboplatin dose escalation has not been evaluated in chemotherapy-naive patients with advanced NSCLC. Therefore, we conducted this study to determine the recommended dose of carboplatin combined with pemetrexed and assess the feasibility of pemetrexed monotherapy in a maintenance setting.

2. Materials and methods

2.1. Study design

This phase I dose-escalation study was conducted to examine the safety of the pemetrexed and carboplatin combination in chemotherapy-naive patients with advanced NSCLC. The primary objectives of the study were to evaluate incidence, type and severity of adverse events and to determine the recommended dose of carboplatin used in combination with pemetrexed.

Patients were divided into 2 cohorts. In the first cohort, 3 patients received pemetrexed 500 mg/m^2 plus carboplatin at AUC 5 according to the Calvert formula [12] (cohort 1). If dose-limiting toxicity (DLT) was not observed in any of three patients, an escalated dose of carboplatin (AUC 6) was administered to the first 6 patients in cohort 2. If DLT was observed in 1 out of the first 3 patients, additional 3 patients were enrolled to assess the tolerability of this dose level. If DLT occurred in only 1 out of the 6 patients in the cohort 1, dose escalation of carboplatin (AUC 6) was made. If DLT was observed in two or more of first three patients, a reduced dose of carboplatin (AUC 4) was administered. Dose escalation was decided by the toxic data only in the first cycle of chemotherapy.

The recommended dose was determined based on these initial results from cohorts 1 and 2. Following this and additional number of patients, up to maximum of 20 patients, were enrolled to receive the recommended dose of study treatment.

2.2. Eligibility

Patients with histologically or cytologically confirmed advanced NSCLC were eligible for the study. Each patient was required to meet the following criteria: (1) clinical stage IIIB, IV, or post-operative recurrent disease; (2) lesion not amendable for curative radiation; (3) no prior chemotherapy; (4) aged 20–75 years old; (5) ECOG PS 0 or 1; (6) adequate function of major organs (lung: $SpO_2 \geq 90\%$, heart: normal 12 lead ECG, bone marrow: hemoglobin ≥ 9.0 g/dL, neutrophil $\geq 1500/mm^3$, platelet $\geq 100{,}000/mm^3$, liver: AST (GOT)/ALT(GPT) ≤ 2.5 times upper limit of normal, total bilirubin ≤ 1.5 mg/dL, kidney: serum creatinine ≤ 1.2 mg/dL, predicted creatinine clearance or 24-h creatinine clearance ≥ 45 mL/min as estimated by the Cockcroft and Gault formula [13]); (7) life expectancy of at least 12 weeks.

This study followed the ethical principles in the Declaration of Helsinki, and the study protocol was approved by the institutional review board at each participating center. All patients provided written informed consent before study-related procedures were performed.

2.3. Study treatment

All patients received pemetrexed 500 mg/m^2 by 10 min intravenous infusion followed by intravenous infusion of carboplatin over at least 30 min (AUC 5 or 6) on day 1 of 21-day cycle. Combination chemotherapy was repeated every 3 weeks for a maximum of six cycles. After completion of six cycles it was possible to continue pemetrexed monotherapy at the discretion of the investigator until progressive disease (PD). Subsequent cycles of treatment were withheld until the following criteria were satisfied: the neutrophil count $\geq 1500/mm^3$, the platelet count $\geq 100{,}000/mm^3$, hemoglobin >8.0 g/dL, PS ≤ 1, $SpO_2 \geq 90\%$, AST/ALT ≤ 2.5 times upper limit of normal, total bilirubin ≤ 1.5 mg/dL, other nonhematological toxicity $\leq$grade 2, and a decision by the physician. If these criteria were not satisfied within 29 days from the date of dose administration in the cycle, treatment doses were modified as follows: in case of nonhematological toxicity, pemetrexed dose was to be reduced from 500 to 400 mg/m^2 and in case of hematological toxicity, carboplatin dose was to be reduced from AUC 6 to 5 (or 5 to 4). If the toxicity had not resolved within 43 days, the patient was excluded from the study.

While on study, patients received folic acid and vitamin B_{12}. All patients underwent comprehensive baseline assessments including clinical laboratory tests and imaging studies. Patients also received follow-up assessments and monitoring at regular intervals. Toxicity evaluations were based on the Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v3.0).

2.4. Definition of DLT

A DLT was defined as a toxicity occurring in cycle 1 that met one of the following criteria and for which a causal relationship with the study drugs could not be ruled out: grade 4 neutropenia prolonged ≥ 7 days, febrile neutropenia, grade 4 thrombocytopenia, grade 3 thrombocytopenia that required platelet transfusion or was associated with bleeding, or grade 3 nonhematological toxicity (following events were to be DLT if the event does not recover $\leq$grade 2 despite standard/optimal supportive treatment: nausea, vomiting, anorexia, fatigue, constipation, diarrhea, transient increase in AST/ALT, or transient electrolytes abnormally). In case that the patients experienced toxicities which met DLT criteria, treatment doses were modified in subsequent courses.

2.5. Efficacy measures

The efficacy endpoints were tumor response, progression-free survival time and overall survival. Tumor response was evaluated every 6 weeks according to the RECIST guideline [14]. Progression-free survival was defined as the time from enrollment to the date of confirmation of progressive disease (PD) or the date of death from any cause, which is earlier. Overall survival was defined as the time from registration until death from any cause. For patients not known to have died and to have had progression, the patients were censored at the date of the last progression-free assessment.

2.6. Statistical analysis

All patients who received at least one dose of study treatment were included in the safety and efficacy analysis. A maximum of 20 patients were to be enrolled in our study to evaluate the safety of combination therapy with pemetrexed and carboplatin at AUC 5 or AUC 6. At least 14 patients were to be treated at the recommended doses. The probability of adverse events with incidences equal to or greater than 20% not being detected in any of the 14 patients was 4.4%.

The incidence of the adverse events was calculated for each dose group. The distribution of best overall response was summarized in the patients who had target lesions. PFS was estimated using the Kaplan–Meier (K–M) method [15]. This included generating the K–M curve and determining the median with 95% confidence interval.

Table 1
Patient characteristics.

Number of patients	20
Median age, year (range)	64 (46–75)
Gender	
Male	13
Female	7
Performance status	
0	4
1	16
Disease stage	
IIIB	3
IV	16
Relapse after surgery	1
Histology	
Adenocarcinoma	17
Squamous cell carcinoma	2
Other	1

3. Results

3.1. Patients

This study was carried out from January 2008 to August 2009 at 2 study centers in Japan. Twenty-one patients were enrolled, and one patient withdrew consent to participate in the study before the treatment. Table 1 shows the demographics and characteristics of the 20 patients. Seven patients were female and 13 were male. The median age was 64 years (range: 46–75). Histologically 17 patients had adenocarcinomas and 2 had squamous cell carcinomas. One patient who had unspecified NSCLC was classified as "other".

3.2. Determination of recommended dose

In cohort 1, a DLT was observed in one of the first 3 patients: grade 4 thrombocytopenia. Following treatment with blood platelet transfusion, the platelet count in this DLT patient recovered rapidly and the thrombocytopenia severity dropped to grade 0. Additional 3 patients were enrolled in cohort 1, but none of these patients developed DLTs. The dose of carboplatin was then escalated to AUC 6 (cohort 2). No DLTs were seen in the first 6 patients of cohort 2, and thus the combination of pemetrexed 500 mg/m^2 plus carboplatin at AUC 6 was determined as the recommended dose. An additional 8 patients were assigned to this dose level. In total, 20 patients were administered the combination of pemetrexed and carboplatin.

3.3. Treatment delivery

The data of treatment delivery was shown in Table 2.

For 6 patients who received carboplatin at AUC 5, the mean relative dose intensities were 90.3% for pemetrexed and 86.1% for carboplatin. Patients received a median of 8.0 cycles of treatment (range, 2–11) including maintenance pemetrexed monotherapy. A total 43 cycles of treatment was delivered overall. Study protocol requirements stipulated dose reductions in 3 cycles (7% of total cycles) and dose delays in 10 cycles (23% of total cycles).

In 14 patients receiving carboplatin at AUC 6, the mean relative dose intensities were 84.6% for pemetrexed and 82.1% for carboplatin. Patients received a median of 5.5 cycles of treatment (range, 1–10). A total 82 cycles of treatment was delivered overall. Study protocol requirements stipulated dose reductions in 12 cycles (15% of total cycles) and dose delays in 29 cycles (35% of total cycles).

As shown in Table 2, hematologic toxicities were a major cause of both dose reductions and dose delays.

Eleven patients (4 patients in cohort 1 [$n = 6$] and 7 patients in cohort 2 [$n = 14$]) completed 6 cycles of the combination therapy. Three of these patients were discontinued due to disease progression (1 patient in cohort 1) and adverse events (2 patients in cohort 2) before maintenance therapy began. The other eight patients (3 patients in cohort 1 and 5 patients in cohort 2) continued pemetrexed monotherapy in a maintenance setting. Six out of 8 patients were discontinued due to disease progression, and 2 out of 8 patients were discontinued due to adverse events (blood creatinine increased and bronchitis, respectively) during maintenance therapy. In maintenance therapy, only one cycle delay due to adverse event was observed, however no dose reductions were observed; the median number of cycles was 4.0 cycles (range, 2–5).

3.4. Safety

The major adverse events during the entire treatment period are shown in Tables 3 and 4. The hematological adverse events reaching ≥ grade 3 were neutropenia (75%), anemia (50%), thrombocytopenia (45%) and leukopenia (15%). Of these events, grade 4 thrombocytopenia was observed in four patients, and no grade 4 leukopenia was observed. Nonhematological toxicities ≥ grade

Table 2
Summary of treatment delivery.

Cohort	Cohort 1 ($N = 6$) PEM 500 mg/m^2 + CBDCA AUC 5		Cohort 2 ($N = 14$) PEM 500 mg/m^2 + CBDCA AUC 6	
Total cycles treated	43		82	
Median cycles (range)	8.0 (2–11)		5.5 (1–10)	
	PEM (mg/m^2)	CBDCA	PEM (mg/m^2)	CBDCA
Planned dose per week	166.7	AUC 1.667	166.7	AUC 2.000
Actual dose per week	150.5	AUC 1.435	141.1	AUC 1.642
Relative dose intensity (%)	90.3	86.1	84.6	82.1
Dose reduction because of AE				
Cycles (%)	3 (7%)		12 (15%)	
Reason (AE, cases)	Neutropenia (1) Platelet decreased (1)		Neutropenia (5) Platelet decreased (4) Hemoglobin decreased (2) ALT increased (1)	
Dose delay because of AE				
Cycles (%)	10 (23%)		29 (35%)	
Reason (AE, cases)	Neutropenia (8) Platelet decreased (3) Bronchitis (1)		Neutropenia (20) Platelet decreased (8) Hemoglobin decreased (6) ALT increased (1)	

PEM: pemetrexed; CBDCA: carboplatin.
Note: Include both results of combination therapy and pemetrexed monotherapy.

Table 3
Summary of adverse events by cohort.

Adverse events	Cohort 1		N=6		Cohort 2		N=14	
	Toxicity grade				Toxicity grade			
	G1	G2	G3	G4	G1	G2	G3	G4
Hematological								
Thrombocytopenia	2	1	2	1	5	3	3	3
Anemia	0	3	3	0	2	4	6	1
Leucopenia	1	4	1	0	5	5	2	0
Neutropenia	0	1	4	1	0	1	9	1
Nonhematological								
Anorexia	2	3	1	0	6	4	1	0
Nausea	3	2	0	0	10	2	1	0
Vomiting	3	1	0	0	0	3	1	0
Fatigue	2	4	0	0	6	2	0	0
AST increased	2	1	0	0	6	1	0	0
ALT increased	0	2	0	0	6	1	1	0
Constipation	3	0	0	0	5	1	0	0
Diarrhoea	2	0	0	0	3	0	0	0
Rash	2	2	0	0	1	2	0	0
Alopecia	2	0	–	–	2	0	–	–
LDH increased	2	1	0	0	1	0	0	0
GGT increased	1	0	0	0	3	0	0	0
ALP increased	1	0	0	0	1	0	0	0
Nasopharyngitis	2	0	0	0	4	0	0	0
Fever	3	0	0	0	0	0	0	0

Events were graded according to CTCAE v3.0.
WBC: white blood cell count; RBC: red blood cell count; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyltransferase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase.

3 were anorexia (10%), nausea, vomiting and increased ALT (5% each).

There were no treatment related deaths. The adverse events observed in our study were predictable from safety profiles of pemetrexed and carboplatin, with all events well managed. Most of the patients recovered from such adverse events by dose adjustment or discontinuing the study treatment.

Table 4
Summary of adverse events.

Adverse events	N=20			
	Toxicity grade			
	G1	G2	G3	G4
Hematological				
Neutropenia	0	2	13	2
Anemia	2	7	9	1
Thrombocytopenia	7	4	5	4
Leukopenia	6	9	3	0
Nonhematological				
Anorexia	8	7	2	0
Nausea	13	4	1	0
Vomiting	3	4	1	0
Fatigue	8	6	0	0
AST increased	8	2	0	0
ALT increased	6	3	1	0
Constipation	8	1	0	0
Diarrhoea	5	0	0	0
Rash	3	4	0	0
Alopecia	4	0	–	–
LDH increased	3	1	0	0
GGT increased	4	0	0	0
ALP increased	2	0	0	0
Nasopharyngitis	6	0	0	0
Fever	3	0	0	0

Events were graded according to CTCAE v3.0.
WBC: white blood cell count; RBC: red blood cell count; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyltransferase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase.

Table 5
Overall response.

	Cohort 1 (N=6) n (%)	Cohort 2 (N=14) n (%)	Total (N=20) n (%)
Overall response rate	4(66.7)	8(57.1)	12(60.0)
Complete response	0(0.0)	0(0.0)	0(0.0)
Partial response	4(66.7)	8(57.1)	12(60.0)
Stable disease	0(0.0)	4(28.6)	4(20.0)
Progressive disease	1(16.7)	1(7.1)	2(10.0)
Not evaluable	1[a] (16.7)	1(7.1)	2(10.0)

[a] One patient had no target lesion.

3.5. Efficacy

There were 12 partial responses (4 patients in cohort 1 and 8 patients in cohort 2) and no complete responses, yielding an overall response rate of 60.0% (95% CI: 36.1–80.9%, Table 5). All 20 treated patients were assessable for progression-free survival and overall survival. With a median follow-up time of 13 months (range of 2.4–16.7 months), 13 patients were still alive. Median progression-free survival for all patients was 7.6 months (95% CI: 4.8–8.0 months; Fig. 1), whereas median overall survival was not reached.

4. Discussion

This was the first dose-escalation study which examined the recommended dose of pemetrexed and carboplatin combination therapy as a first-line therapy for treatment of advanced NSCLC. Six patients were treated with pemetrexed 500 mg/m^2 and carboplatin at AUC 5 (cohort 1), and 14 patients were treated with pemetrexed 500 mg/m^2 and carboplatin at AUC 6 (cohort 2).

DLT was observed in 1 patient in cohort 1. This was grade 4 thrombocytopenia, however the low platelet baseline of this patient (13.6×10^4/mm^3, grade 1) might have made this patient more susceptible to this DLT. This indicates the need for suitable precautions in clinical practice prior to starting treatment, especially for patients who have a low platelet count. No DLT was

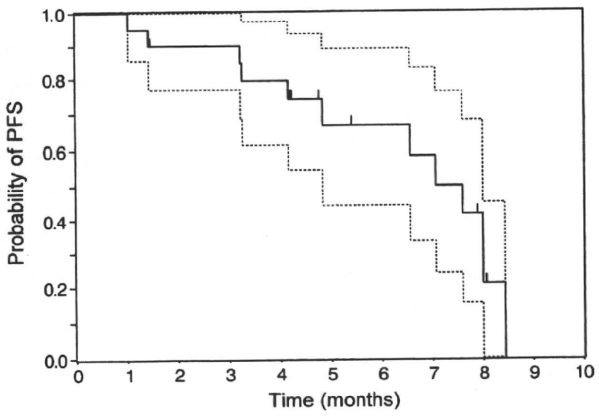

Fig. 1. Progression-free survival time. Solid line shows Kaplan–Meier curve and dashed line shows 95% CI, and events were observed in 12 patients out of 20 patients.

observed in the first 6 patients of cohort 2 (pemetrexed 500 mg/m^2 and carboplatin AUC 6). Other grade 3/4 hematological toxicities were also observed, but these were manageable with dose delays and reductions, or supportive care. Dose delays and reductions were made in both cohorts, however mean weekly dose intensities of pemetrexed and carboplatin exceeded 80% of the planned dose intensities in both cohorts. This demonstrates that pemetrexed and carboplatin can be combined in this population of NSCLC patients without compromising the dose of either agent.

For first-line and maintenance therapy, phase III studies have demonstrated pemetrexed to be efficacious. This has resulted in FDA approvals for pemetrexed as a first-line therapy for NSCLC in 2008 and as a maintenance therapy for NSCLC in 2009. Therefore pemetrexed-based platinum therapy followed by pemetrexed maintenance therapy may be an alternative treatment option for patients with nonsquamous NSCLC, although survival benefit of this regimen was not shown then further clinical study is ongoing [11].

In the present study, 8 patients (cohort 1: 3 patients, cohort 2: 5 patients) started pemetrexed monotherapy in a maintenance setting after 6 cycles of pemetrexed and carboplatin. The median number of cycles was 4.0 cycles of maintenance therapy without dose reduction. Only two of the 8 patients discontinued treatment due to adverse events, and there were no unexpected adverse events or cumulative toxicity in the maintenance phase. This showed that pemetrexed maintenance therapy was tolerable following pemetrexed-based combination therapy. In a recent pemetrexed maintenance phase III study, which demonstrated superior OS, the induction therapy consisted of 4 cycles of a non-pemetrexed agent containing a platinum doublet. In our study, 85% (17/20) of patients were able to complete the first 4 cycles of the pemetrexed and carboplatin combination therapy, and 60% (12/20) were able to continue this combination to at least the fifth cycle. This means that if the combination therapy was limited to 4 cycles, more patients would be able to continue pemetrexed monotherapy as maintenance therapy.

The response rate of 60.0% (95% CI: 36.1–80.9%) and the median PFS of 7.6 months (95% CI: 4.8–8.0 months) are certainly encouraging even though it was a small sample size. Two previous phase II studies of combination therapy with pemetrexed and carboplatin showed lower response rates of 24.0% and 31.6% [8,9]. One possible reason for this difference is the histological type of NSCLC. Recent studies have shown superior efficacy for pemetrexed in nonsquamous NSCLC and inferior efficacy in squamous NSCLC. The phase II studies described above included 12.0% and 30.8% of squamous NSCLC patients, respectively. However, all but two of the patients in our study were nonsquamous NSCLC patients, and thus the higher response rate was not surprising.

Another possible reason is ethnic difference. There are no confirmed data showing that ethnic difference is related to different patient responses to pemetrexed. However, the results of subgroup analyses in the phase III study of pemetrexed and cisplatin as a first-line therapy for NSCLC have shown higher response rates in East Asian (Korea and Taiwan only) populations, 42.6% [16] compared with 29.5% (Data on file) in non-East Asian populations. Response rates for East Asian patients were also comparatively high in a Japan phase II study of second or third line pemetrexed monotherapy [17]. In that study, Ohe et al. reported an 18.5% response rate in the pemetrexed 500 mg/m^2 group. On the other hand, Hanna et al. reported only a 9.1% response rate in the pemetrexed arm from a randomized phase III study of pemetrexed versus docetaxel that included a comparatively small number of Asian patients [4]. Although there are limitations when comparing the results from different studies, the efficacy results in our study compare favorably with those reported in the above first-line studies. The reason for this apparent difference between non-East Asian and East Asian populations remains unknown, however pemetrexed does seem to have better efficacy in East Asian patients with nonsquamous NSCLC.

There is the possibility that further pemetrexed studies might be restricted to patients with nonsquamous NSCLC patients because of the pemetrexed label indications. However, in present study, one responder was observed among the squamous NSCLC patients, and in other studies there were also some responders in the squamous population. Pemetrexed is now in clinical development for head and neck cancer treatment, the major histological tumor type being squamous cell carcinoma. Preclinical data suggest that tumoral expression of thymidylate synthase (TS), which is usually lower in nonsquamous compared with squamous NSCLC, may be responsible for the differential activity of pemetrexed. Results such as these, especially with the number of therapeutic choices steadily increasing, have shown that the development of predictive biomarkers like tumoral TS expression is more important than ever.

In the present study, the combination of pemetrexed 500 mg/m^2 and carboplatin AUC 5 or 6 was confirmed to be feasible and effective for chemotherapy-naive Japanese patients with advanced NSCLC. According to the protocol definition, we concluded that carboplatin at AUC 6 is recommended for use in combination with pemetrexed 500 mg/m^2. And pemetrexed maintenance monotherapy continued after this combination was safe and tolerable. A larger-scale study is needed to confirm these findings overall for advanced NSCLC patients. In addition, large-scale phase III studies of a pemetrexed, carboplatin plus molecular-target drugs combination are currently underway in both Japan and abroad.

Conflicts of interest

I. Okamoto, K. Takeda and K. Nakagawa disclose consultant or advisory relationship with Eli Lilly Japan K.K. R. Sekiguchi, K. Tominaga, S. Enatsu and Y. Nambu have been full-time employees of Eli Lilly Japan K.K. H. Daga, M. Miyazaki, K. Yonesaka, H. Kiyota, J. Tsurutani, S. Ueda, Y. Ichikawa and M. Takeda have no conflict of interest to disclose.

Acknowledgment

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Tumor and Stem Cell Biology

Cancer Research

Role of Survivin in EGFR Inhibitor–Induced Apoptosis in Non–Small Cell Lung Cancers Positive for *EGFR* Mutations

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Abstract

The molecular mechanism by which epidermal growth factor receptor–tyrosine kinase inhibitors (EGFR-TKI) induce apoptosis in non–small cell-lung cancer (NSCLC) cells that are positive for activating mutations of the EGFR remains unclear. In this study, we report the effects of the EGFR-TKI gefitinib on expression of the antiapoptotic protein survivin that have functional consequences in EGFR mutation–positive NSCLC cells. Immunoblot analysis revealed that gefitinib downregulated survivin expression, likely through inhibition of the PI3K-AKT signaling pathway, in NSCLC cells positive for EGFR mutation. Stable overexpression of survivin attenuated gefitinib-induced apoptosis and also inhibited the antitumor effect of gefitinib in human tumor xenografts. Furthermore, the combination of survivin overexpression with inhibition of the gefitinib-induced upregulation of the proapoptotic protein BIM attenuated gefitinib-induced apoptosis to a greater extent than either approach alone. Our results indicate that downregulation of survivin plays a pivotal role in gefitinib-induced apoptosis in EGFR mutation–positive NSCLC cells. Furthermore, they suggest that simultaneous interruption of the PI3K-AKT-survivin and MEK-ERK-BIM signaling pathways is responsible for EGFR-TKI–induced apoptotic death in these cells. *Cancer Res; 70(24); 10402–10. ©2010 AACR.*

Introduction

Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins and has been shown to inhibit caspases and to prevent caspase-mediated cell death (1–3). Survivin is abundant in many types of cancer cells but not in the corresponding normal cells (4, 5). In nonmalignant proliferating cells, the expression of survivin is regulated in a cell cycle-dependent manner (6, 7). The upregulation of survivin expression in tumors does not seem to be dependent solely on the cell cycle, however, given that it occurs in tumor cells that are not actively cycling (4, 8, 9). Indeed, growth factors have been found to regulate survivin expression in endothelial cells and neuroblastoma cells (10, 11). Although expression of survivin has been demonstrated in non–small cell-lung cancer (NSCLC; refs. 12–14), the mechanism by which such expression is regulated in NSCLC cells has remained unknown.

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The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is abnormally amplified or activated in a variety of tumors including NSCLC, and it has therefore been identified as an important target in cancer treatment (15–17). Inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), which compete with ATP for binding to the tyrosine kinase pocket of the receptor, have been extensively studied in patients with NSCLC (18, 19). Several prospective clinical trials have revealed marked antitumor activity of EGFR-TKIs in NSCLC patients with *EGFR* mutations. The therapeutic benefit of these drugs is much greater than that historically observed with standard cytotoxic chemotherapy for advanced NSCLC. NSCLC cells with *EGFR* mutations manifest activation of the PI3K (phosphatidylinositol 3-kinase)–AKT and MEK–ERK (extracellular signal-regulated kinase) signaling pathways under the control of EGFR, and exposure of such cells to EGFR-TKIs blocks signaling by both pathways and induces apoptosis (20–22). The precise molecular mechanism by which EGFR-TKIs induce apoptosis has remained unclear, however. We have therefore now examined the effect of the EGFR-TKI gefitinib on survivin expression as well as further investigated the mechanism of gefitinib-induced apoptosis in *EGFR* mutation–positive NSCLC cells.

Materials and Methods

Cell culture and reagents

The human NSCLC cell lines PC9, HCC827, NCI-H1975 (H1975), A549, and H1299 were obtained from American Type Culture Collection. The NSCLC line PC9/ZD was obtained as

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described previously (23). All cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum. Gefitinib was obtained from Kemprotec, U0126 and LY294002 were from Cell Signaling Technology and BEZ235 and AZD6244 were from ShangHai Biochempartner.

Immunoblot analysis

Cells were washed twice with ice-cold PBS and then lysed in a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and leupeptin (1 μg/mL). The protein concentration of the cell lysates was determined with the use of the Bradford reagent (Bio-Rad), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 hour at room temperature before incubation overnight at 4°C with primary antibodies. Rabbit polyclonal antibodies to human phosphorylated EGFR (pY1068), to XIAP, to phosphorylated and total AKT, to phosphorylated and total ERK, to poly(ADP-ribose) polymerase (PARP), to caspase-3, and to BIM were obtained from Cell Signaling Technology; those to survivin were from Santa Cruz Biotechnology; those to cIAP-1 were from R&D Systems; and those to β-actin were from Sigma. Mouse monoclonal antibodies to EGFR were obtained from Invitrogen. All antibodies were used at a 1:1,000 dilution, with the exception of those to β-actin (1:200). The nitrocellulose 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 (Sigma) or mouse (Santa Cruz Biotechnology) immunoglobulin G. Immune complexes were finally detected with chemiluminescence reagents (Perkin-Elmer Life Science).

Gene silencing

Cells were plated at 50% to 60% confluence in 6-well plates or 25-cm^2 flasks and then incubated for 24 hours before transient transfection for the indicated times with small interfering RNAs (siRNA) mixed with the Lipofectamine reagent (Invitrogen). The siRNAs specific for AKT (AKT-1, 5′-CCAGGUAUUUUGAUGAGGA-3′; AKT-2, 5′-CAACCGCCAUCCAGACUGU-3′), survivin (survivin-1, 5′-GAAGCAGUUUGAAGAAUUA-3′; survivin-2, 5′-AGAAGCAGUUUGAAGAAUU-3′), or BIM (BIM-1, 5′-GGAGGGUAUUUUUGAAUAA-3′) mRNAs as well as corresponding scrambled (control) siRNAs were obtained from Nippon EGT.

Annexin V binding assay

The binding of Annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche). Cells were harvested by exposure to trypsin-EDTA, 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°C to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson).

Cell cycle analysis

Cells were harvested, washed with PBS, fixed with 70% methanol, washed again with PBS, and stained with propidium iodide (0.05 mg/mL) in a solution containing 0.1% Triton X-100, 0.1 mmol/L EDTA, and RNase A (0.05 mg/mL). The stained cells were then analyzed for DNA content with a flow cytometer and Modfit software (Verity Software House).

Establishment of cells stably overexpressing survivin

A full-length cDNA fragment encoding human survivin was obtained from HCC827 cells by reverse transcription and PCR with the primers survivin-forward (5′-GCGGCCGCGGCGGCATGGGTGCCCCGACGTTG-3′) and survivin-reverse (5′-GGATCCTCAATCCATGGCAGCCAGCTGCTCG-3′). The amplification product was verified by sequencing after its cloning into the pCR-Blunt II-TOPO vector (Invitrogen). The survivin cDNA was excised from pCR-Blunt II-TOPO and transferred to the pQCXIH retroviral vector (Clontech). Retroviruses encoding survivin were then produced and used to infect PC9 and HCC827 cells as described (24). Cells stably expressing survivin were then isolated by selection with hygromycin at 300 μg/mL (Invivogen).

Growth inhibition assay *in vivo*

All animal studies were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University (Osaka, Japan). The ethical procedures followed conformed to the guidelines of the United Kingdom Coordinating Committee on Cancer Prevention Research. Tumors cells (5×10^6) were injected subcutaneously into the axilla of 5- to 6-week-old female athymic nude mice (BALB/c nu/nu; CLEA Japan). Treatment was initiated when tumors in each group of 6 mice achieved an average volume of 200 to 400 mm^3. Treatment groups consisted of vehicle control and gefitinib (10 or 25 mg/kg). Gefitinib was administered by oral gavage daily for 4 weeks, with control animals receiving a 0.5% (w/v) 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.

Statistical analysis

Quantitative data are presented as means ± SE from 3 independent experiments or for 6 animals per group unless indicated otherwise. The significance of differences in the percentage of Annexin V-positive cells was evaluated with the unpaired 2-tailed Student's t test. $P < 0.05$ was considered statistically significant.

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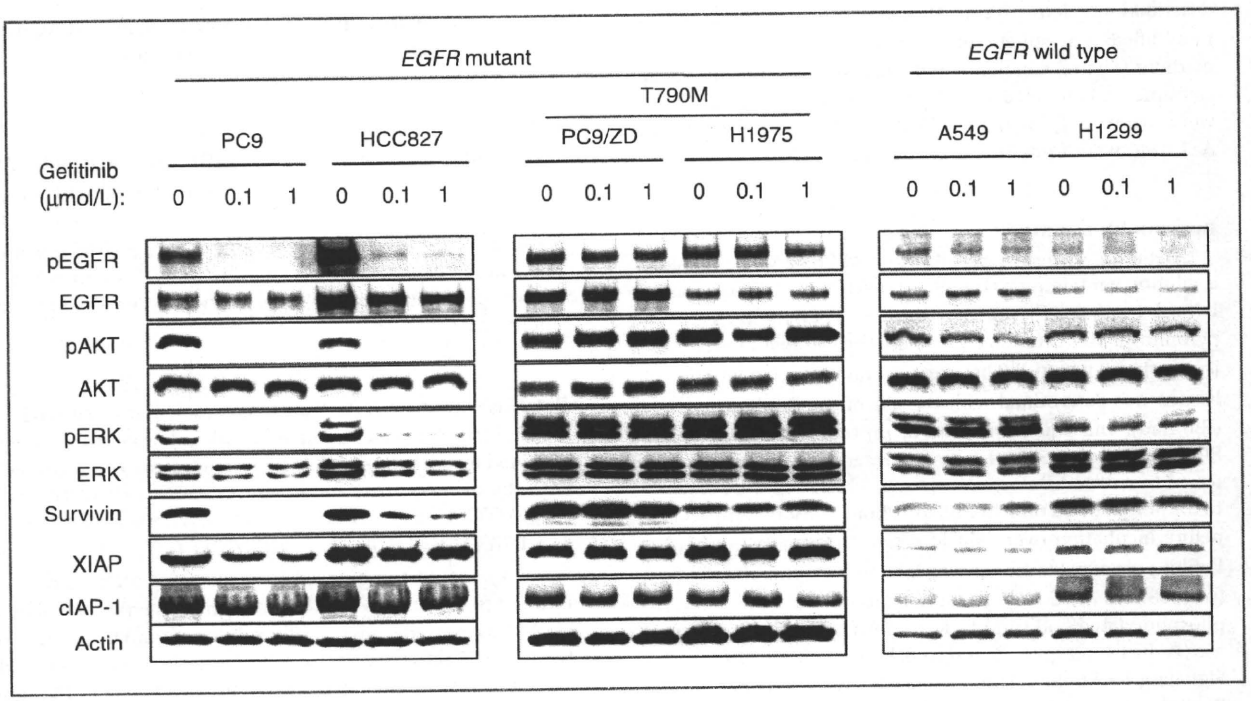

Figure 1. Effects of gefitinib on the expression of IAP family proteins in human NSCLC cells. PC9, HCC827, PC9/ZD, H1975, A549, or H1299 cells were incubated in complete medium and in the presence of the indicated concentrations of gefitinib for 24 hours. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, AKT, or ERK, to survivin, to XIAP, to cIAP-1, or to β-actin (loading control). Data are representative of 3 independent experiments.

Results

Gefitinib downregulates survivin expression in *EGFR* mutation-positive NSCLC cell lines

We first examined the effects of the EGFR-TKI gefitinib on the expression of IAP family members in a subset of NSCLC cell lines (PC9, HCC827, PC9/ZD, H1975, A549, and H1299) by immunoblot analysis (Fig. 1). PC9 and HCC827 cells harbor an *EGFR* allele with an activating mutation, whereas A549 and H1299 cells express wild-type EGFR and PC9/ZD and H1975 cells harbor an *EGFR* allele with both an activating mutation and a mutation (T790M) that confers resistance to EGFR-TKIs. In PC9 and HCC827 cells, gefitinib induced the dephosphorylation of EGFR and reduced the abundance of survivin in a concentration-dependent manner. In contrast, in cells expressing wild-type EGFR or harboring the T790M resistance mutation, gefitinib did not affect the phosphorylation level of EGFR or the expression of survivin. The expression of other IAP family members, including XIAP and cIAP-1, was not substantially affected by gefitinib in any of the cell lines examined. These data thus showed that gefitinib downregulated survivin expression in NSCLC cells with an activating mutation of *EGFR*.

Inhibition of the PI3K-AKT pathway results in survivin downregulation in *EGFR* mutation–positive cells

To identify the signaling pathway (or pathways) responsible for downregulation of survivin by gefitinib, we examined the effects of specific inhibitors of MEK (U0126 and AZD6244) and PI3K (LY294002 and BEZ235) in *EGFR* mutation–positive NSCLC cells (PC9 and HCC827). Each of the PI3K inhibitors reduced the abundance of survivin, whereas the MEK inhibitors had no such effect (Fig. 2A), suggesting that the regulation of survivin expression is mediated by PI3K rather than by MEK in *EGFR* mutation-positive NSCLC cells. Given that the protein kinase AKT is an important downstream target of PI3K, we examined whether the PI3K-dependent survivin expression is also dependent on AKT. Depletion of AKT by transfection of cells with 2 different siRNAs specific for AKT mRNA (AKT-1 and AKT-2 siRNA) resulted in downregulation of survivin expression in both PC9 and HCC827 cells (Fig. 2B). These results thus suggested that gefitinib might regulate survivin expression through inhibition of the PI3K-AKT signaling pathway in *EGFR* mutation–positive NSCLC cells.

Knockdown of survivin expression induces apoptosis in *EGFR* mutation–positive cells

To investigate whether downregulation of survivin by gefitinib is related to gefitinib-induced apoptosis, we transfected PC9 or HCC827 cells with 2 independent siRNA specific for survivin mRNA (survivin-1 and survivin-2 siRNAs). Depletion of survivin resulted in generation of the cleaved forms of both caspase-3 and PARP in both cell lines (Fig. 3A). Staining with Annexin V also revealed that the proportion of apoptotic cells was markedly increased by transfection with the survivin

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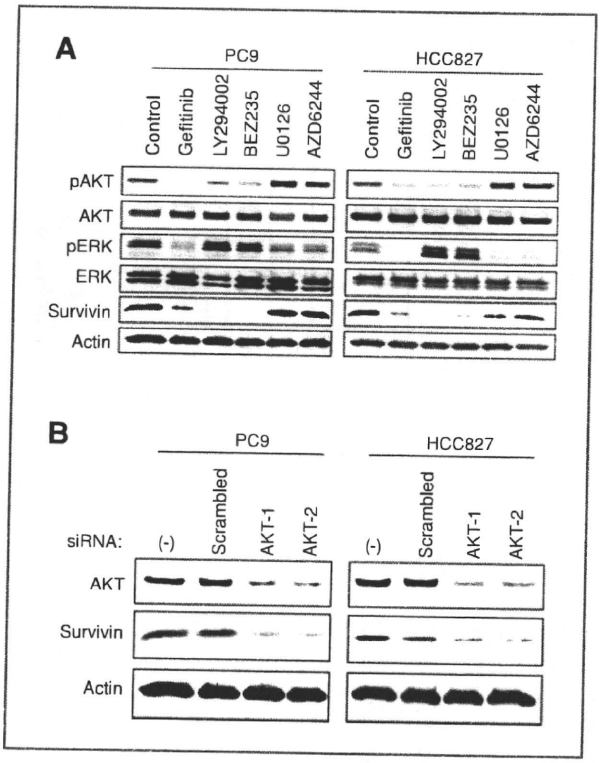

Figure 2. Effects of inhibition of MEK or PI3K signaling pathways on survivin expression in *EGFR* mutation–positive NSCLC cells. A, PC9 or HCC827 cells were incubated in the absence (control, 0.1% dimethyl sulfoxide) or the presence of gefitinib (1 μmol/L), LY294002 (20 μmol/L), BEZ235 (0.2 μmol/L), U0126 (20 μmol/L), or AZD6244 (0.2 μmol/L) for 24 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of AKT or ERK, to survivin, or to β-actin. B, Cells were transfected (or not) with 2 different AKT (AKT-1 or AKT-2) or scrambled (control) siRNAs for 48 hours, lysed, and subjected to immunoblot analysis with antibodies to AKT, to survivin, or to β-actin. All data are representative of 3 independent experiments.

siRNAs (Fig. 3B). In addition, depletion of survivin resulted in an increase in the size of the sub-G_1 (apoptotic) cell population, as revealed by flow cytometry (Fig. 3C). These data suggested that downregulation of survivin induces apoptosis in *EGFR* mutation–positive NSCLC cells.

Overexpression of survivin inhibits gefitinib-induced apoptosis in *EGFR* mutation–positive cells *in vitro*

To examine further the role of survivin in gefitinib-induced apoptosis, we established PC9 and HCC827 sublines (PC9S7, PC9S8, HCC827S6, and HCC827S7) that stably overexpress survivin as a result of retroviral infection. The abundance of survivin in these sublines was substantially greater than that in cells infected with the empty virus (PC9-Mock and HCC827-Mock; Fig. 4A). In addition, gefitinib markedly reduced the level of survivin expression in PC9-Mock and HCC827-Mock cells but not in the corresponding sublines overexpressing survivin (Fig. 4B). Immunoblot analysis of the cleaved forms of caspase-3 and PARP (Fig. 4B) as well as staining with Annexin V (Fig. 4C) also revealed that overexpression of survivin resulted in marked inhibition of gefitinib-induced apoptosis. Examination of the effect of gefitinib on cell cycle distribution revealed that gefitinib increased the proportion of cells in G_0–G_1 phase and reduced that in S phase at 24 hours in a manner independent of survivin overexpression (Fig. 4D). The survivin-overexpressing sublines, however, showed a smaller time-dependent increase in the size of the sub-G_1 cell population than did cells infected with the empty virus. These results thus further indicated that downregulation of survivin by gefitinib contributes to the proapoptotic action of this drug in *EGFR* mutation–positive NSCLC cells.

Overexpression of survivin inhibits the antitumor effect of gefitinib on *EGFR* mutation–positive cells *in vivo*

To investigate whether the antitumor effect of gefitinib on *EGFR* mutation–positive NSCLC cells might be affected by survivin overexpression *in vivo*, we injected HCC827-Mock cells or cells of the survivin-overexpressing subline HCC827S7 into nude mice for elicitation of the formation of solid tumors. When the tumors became palpable (200–400 mm^3), mice were divided into 3 groups and treated with vehicle (control) or gefitinib at a daily dose of 10 or 25 mg/kg by oral gavage for 4 weeks. Gefitinib treatment at either dose eradicated tumors in mice injected with HCC827-Mock cells (Fig. 5A and C). In contrast, tumors in mice injected with survivin-overexpressing cells were not eradicated by gefitinib even at the dose of 25 mg/kg per day, although tumor growth was partially inhibited by gefitinib in a dose-dependent manner (Fig. 5B and C). These results showed that survivin overexpression inhibits the antitumor effect of gefitinib on *EGFR* mutation–positive NSCLC cells *in vivo*.

Effect of attenuation of BIM induction on gefitinib-induced apoptosis in *EGFR* mutation–positive cells overexpressing survivin

Survivin overexpression did not completely eliminate gefitinib-induced apoptosis in PC9 and HCC827 cells, suggesting that other signaling pathways might contribute to this process. Induction of the proapoptotic BH3-only protein BIM has been found to be important for EGFR-TKI–induced apoptosis in *EGFR* mutation–positive lung cancers, and inhibition of the EGFR-MEK-ERK signaling pathway is required for BIM induction (25–27). We therefore examined whether survivin overexpression in combination with specific inhibition of BIM induction results in an additive antiapoptotic effect in *EGFR* mutation–positive NSCLC cells. We transiently transfected survivin-overexpressing sublines of PC9 or HCC827 cells with an siRNA specific for BIM mRNA. Transfection with the BIM siRNA specifically inhibited the induction of BIM expression by gefitinib in both mock-infected and survivin-overexpressing sublines (Fig. 6A). Staining with Annexin V further revealed that the combination of survivin overexpression and attenuation of BIM induction resulted in a greater level of inhibition of gefitinib-induced apoptosis than that observed with either approach alone (Fig. 6B). These data were

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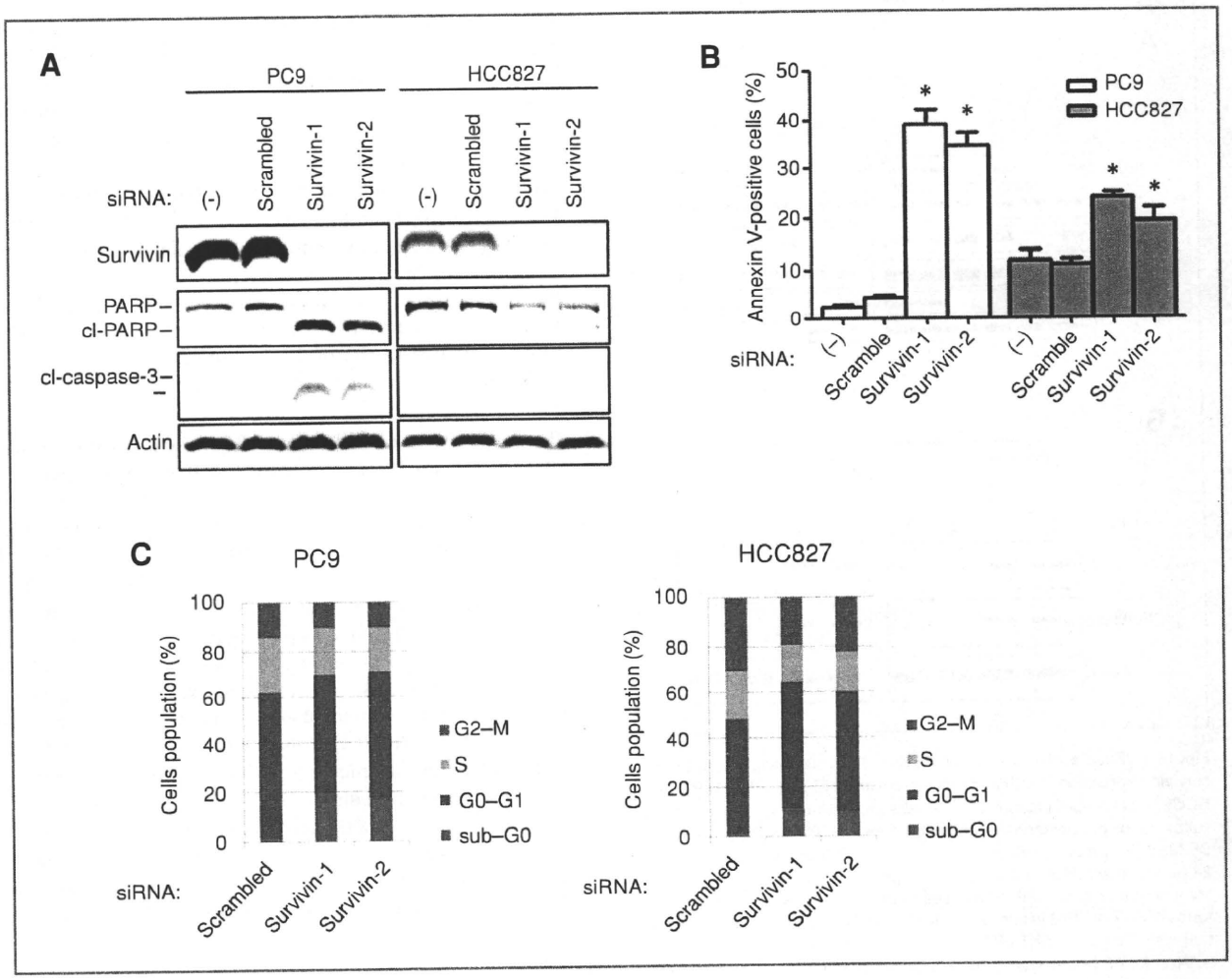

Figure 3. Effect of survivin depletion on apoptosis in *EGFR* mutation–positive NSCLC cells. A, PC9 or HCC827 cells were transfected (or not) with 2 different survivin (survivin-1 or survivin-2) or scrambled (control) siRNAs for 48 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to survivin, to PARP, to caspase-3, or to β-actin. Bands corresponding to the cleaved (cl) forms of caspase-3 and PARP are indicated. Data are representative of 3 independent experiments. B, cells were transfected with survivin or scrambled siRNAs for 72 hours, after which the proportion of apoptotic cells was determined by staining with fluorescein isothiocyanate–conjugated Annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from 3 independent experiments. *, $P < 0.05$ versus the corresponding value for cells transfected with the scrambled siRNA. C, cells were transfected with survivin or scrambled siRNAs for 48 hours, fixed, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. Data are means of triplicates from representative experiments that were repeated 3 times.

confirmed with a second BIM siRNA to rule out off-target effects (Supplementary Fig. 1). These results thus suggested that both survivin downregulation and BIM induction contribute independently to gefitinib-induced apoptosis in *EGFR* mutation–positive NSCLC cells.

Discussion

EGFR-TKIs induce marked clinical responses in patients with NSCLC positive for activating mutations of *EGFR* (1–3). *In vitro* experiments have shown that EGFR-TKIs induce a substantial level of apoptosis in NSCLC cell lines expressing mutant EGFRs (4). However, the key downstream mediators of EGFR-TKI–induced apoptosis in *EGFR* mutation–positive cells have remained unidentified. We have now found that gefitinib downregulated survivin expression in *EGFR* mutation-positive NSCLC cells but not in NSCLC cells expressing wild-type EGFR or EGFR with the T790M resistance mutation. With the use of specific PI3K inhibitors and siRNAs specific for AKT mRNA, we further showed that the downregulation of survivin expression by gefitinib is likely mediated through inhibition of PI3K-AKT signaling. Human epidermal growth factor receptor 2 (HER2)–targeting agents such as lapatinib and trastuzumab were previously found to induce downregulation of survivin through inhibition of the PI3K-AKT pathway in breast cancer cells positive for *HER2* amplification

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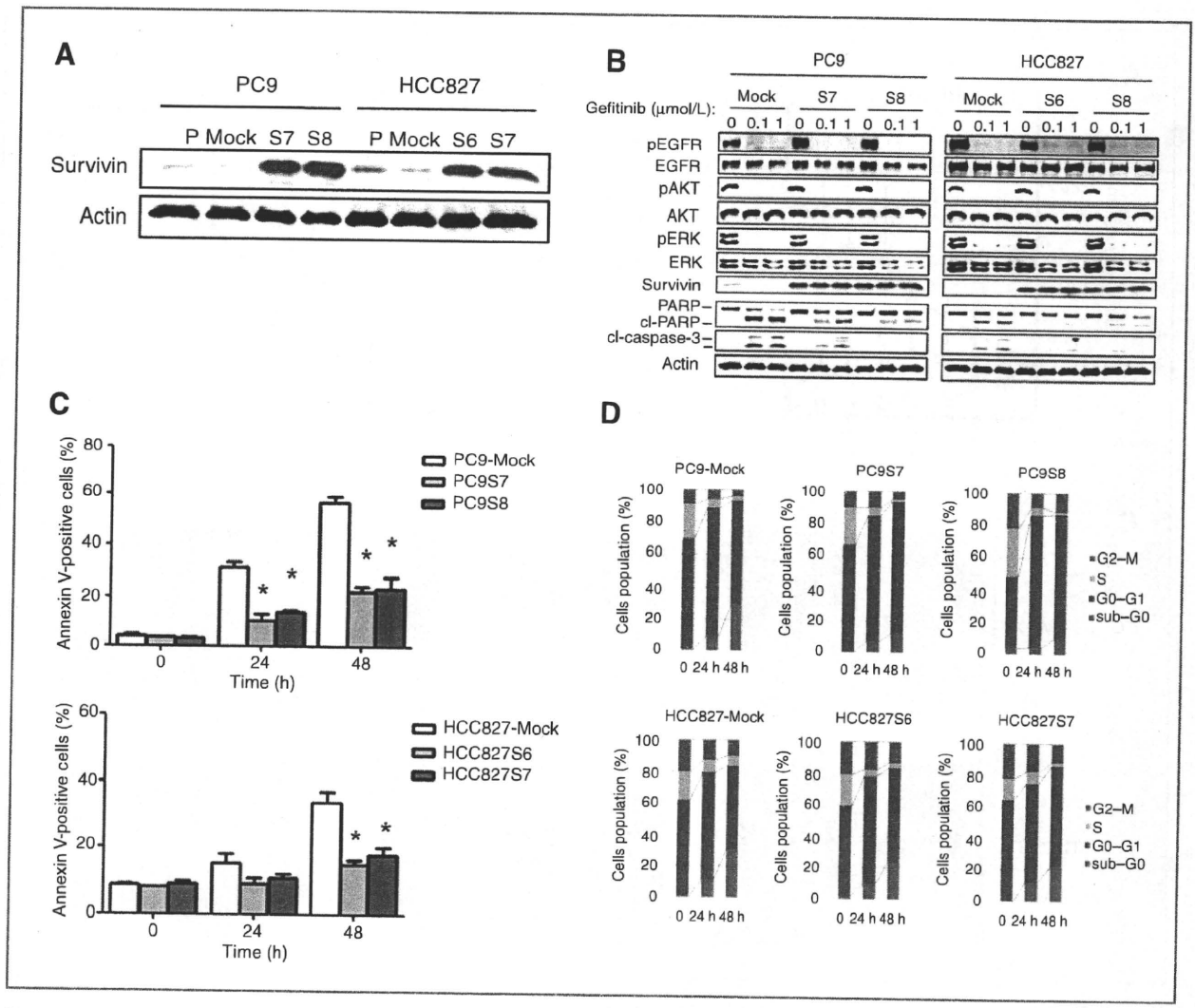

Figure 4. Effect of survivin overexpression on gefitinib-induced apoptosis in *EGFR* mutation–positive NSCLC cells *in vitro*. A, parental (P) PC9 or HCC827 cells or corresponding sublines either stably overexpressing survivin (PC9S7, PC9S8, HCC827S6, and HCC827S7) or infected with the empty retrovirus (PC9-Mock and HCC827-Mock) were cultured overnight in complete medium, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to survivin or to β-actin. B, PC9 or HCC827 isogenic cell lines were incubated in the presence of the indicated concentrations of gefitinib for 48 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, AKT, or ERK, to survivin, to PARP, to caspase-3, or to β-actin. Data in A and B are representative of 3 independent experiments. C, PC9 or HCC827 isogenic cell lines were incubated with gefitinib (0.1 μmol/L) for the indicated times, after which the proportion of apoptotic cells was determined by staining with Annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from 3 independent experiments. *, $P < 0.05$ versus the corresponding value for cells infected with the empty retrovirus. D, PC9 or HCC827 isogenic cell lines were incubated with gefitinib (0.1 μmol/L) for the indicated times and then analyzed for cell cycle distribution by flow cytometry. Data are means of triplicates from representative experiments that were repeated 3 times.

(28, 29). Given that downregulation of survivin through inhibition of the PI3K-AKT pathway was induced by EGFR-TKIs in *EGFR* mutation–positive NSCLC cells and by HER2-targeting agents in breast cancer cells positive for *HER2* amplification, the expression of survivin is likely dependent on PI3K-AKT signaling that operates downstream of receptor tyrosine kinases and is essential for cell survival. This hypothesis is further supported by the observation that transfection of *EGFR* mutation–positive NSCLC cells with an siRNA specific for EGFR mRNA resulted in marked inhibition of survivin expression, whereas transfection of cells expressing wild-type EGFR had no such effect (Supplementary Fig. 2). The PI3K-AKT pathway has been implicated in the regulation of survivin expression by cytokines, growth factors, and chemotherapeutic drugs (8, 10, 30). Although no direct correlation has been established between downregulation of survivin and inhibition of EGFR signaling, these previous findings support the notion that inhibition of the EGFR-PI3K-AKT pathway

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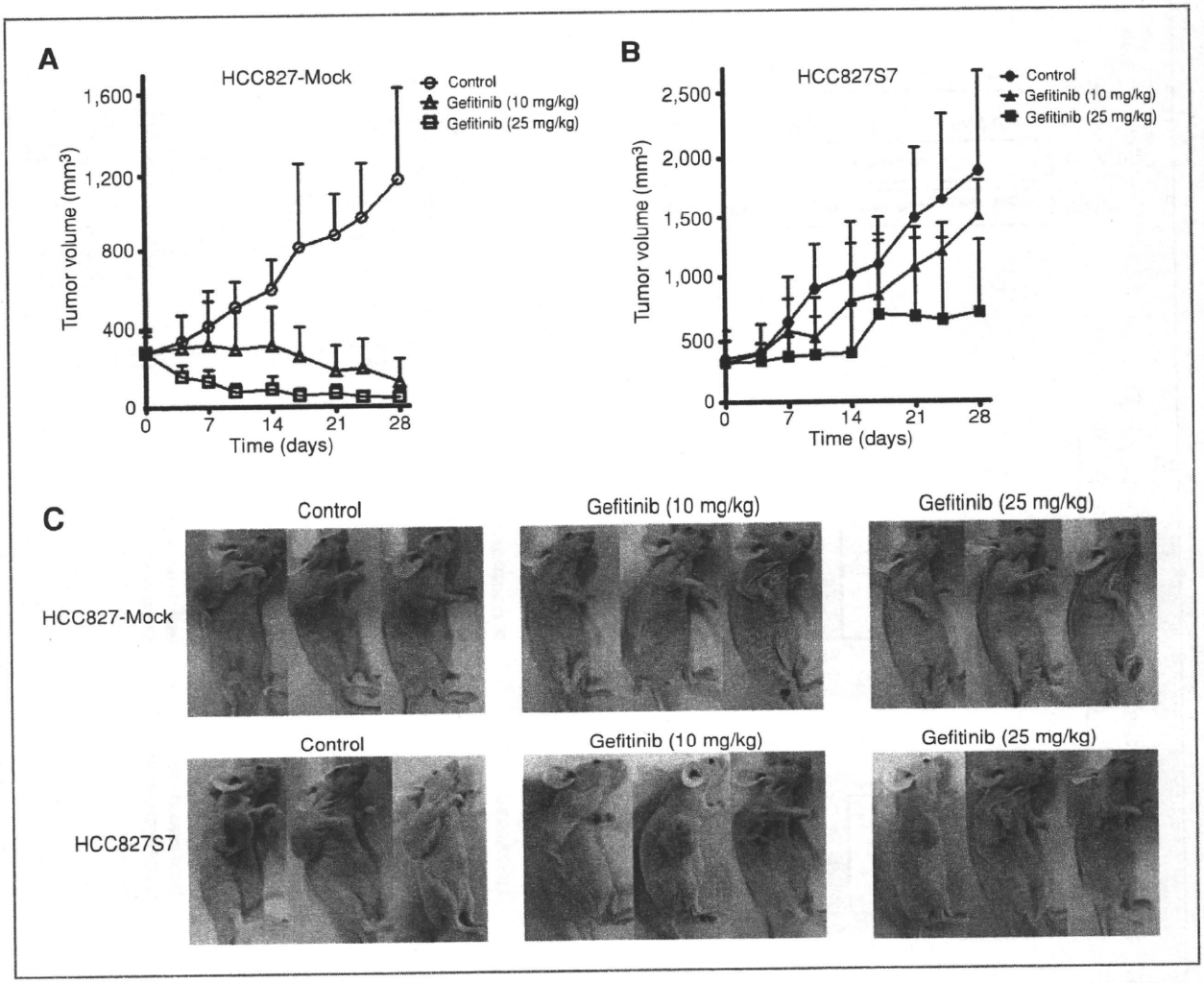

Figure 5. Effect of gefitinib on the growth of *EGFR* mutation–positive NSCLC cells overexpressing survivin *in vivo*. A and B, nude mice with tumor xenografts established by subcutaneous injection of HCC827-Mock or HCC827S7 cells, respectively, were treated daily for 4 weeks with vehicle (control) or gefitinib (10 or 25 mg/kg). Tumor volume was determined at the indicated times after the onset of treatment. Data are means ± SE of values from 6 mice per group. C, representative mice showing tumors at the end of the 4-week treatment period.

contributes to downregulation of survivin expression by EGFR-TKIs in *EGFR* mutation–positive NSCLC cells.

Survivin has been implicated in resistance of cancer cells to apoptosis, although the effect of survivin expression on gefitinib-induced apoptosis in *EGFR* mutation–positive NSCLC cells has not previously been examined. We have now shown that survivin overexpression inhibited gefitinib-induced apoptosis in such cells. Inhibition of the PI3K-AKT and MEK-ERK pathways was previously found to account for much of the proapoptotic activity of EGFR-TKIs in *EGFR* mutation–positive NSCLC cells (31). We further found that overexpression of survivin resulted in inhibition of apoptosis induced by a combination of PI3K and MEK inhibitors in such cells (Supplementary Fig. 3). Increased AKT activity as a result either of the loss of PTEN or of expression of a constitutively active form of AKT was previously found to be associated with a reduced sensitivity to EGFR-TKIs in *EGFR* mutation–positive NSCLC cells (32). However, the principal molecular target underlying the response to inhibition of PI3K-AKT signaling by EGFR-TKIs has remained to be elucidated. In the present study, we show that the sensitivity of *EGFR* mutation–positive NSCLC cells to EGFR-TKIs depends, at least in part, on survivin downregulation through inhibition of the PI3K-AKT pathway. In our xenograft model, we showed that survivin overexpression inhibited the antitumor effect of gefitinib on *EGFR* mutation–positive NSCLC cells. The extent of the clinical benefit of EGFR-TKIs varies among NSCLC patients harboring activating *EGFR* mutations, and the efficacy of these drugs is limited by either *de novo* resistance or resistance acquired after the onset of therapy (33). Although several

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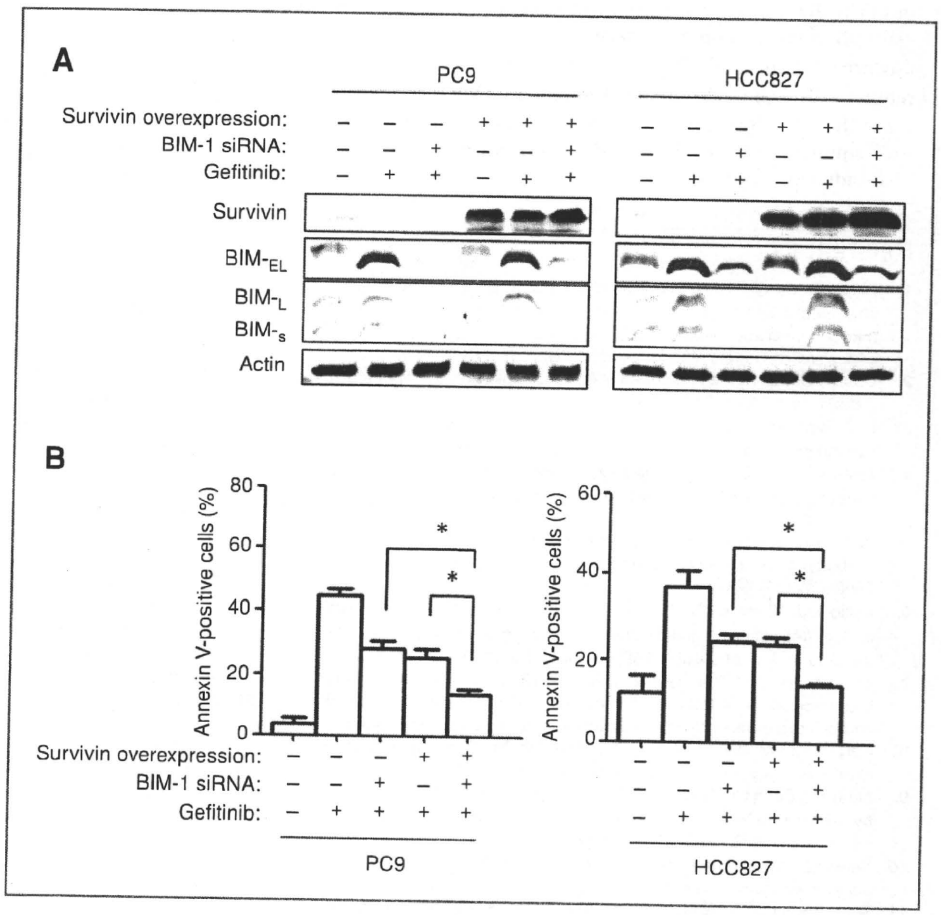

Figure 6. Effect of the combination of survivin overexpression and inhibition of BIM induction on gefitinib-induced apoptosis in *EGFR* mutation–positive NSCLC cells. A, cells stably overexpressing survivin (PC9S7 and HCC827S6) or infected with the empty retrovirus (PC9-Mock and HCC827-Mock) were transfected with BIM (BIM-1) or scrambled siRNAs for 24 hours and then incubated for 24 hours in complete medium with or without gefitinib (0.1 μmol/L). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to survivin, to BIM, or to β-actin. Data are representative of 3 independent experiments. B, cells transfected as in (A) were incubated for 48 hours in the absence or presence of gefitinib (0.1 μmol/L) and then evaluated for the proportion of apoptotic cells by staining with Annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from 3 independent experiments. *, $P < 0.05$ for the indicated comparisons.

mechanisms of acquired resistance have been described, it remains of clinical concern that molecular markers for prediction of *de novo* resistance to these drugs have not been well delineated (23, 34–38). It will therefore be of interest to determine whether increased survivin expression in tumors is clinically useful as a negative predictive marker of sensitivity to EGFR-TKIs in patients with *EGFR* mutation–positive NSCLC.

Our observations revealed that survivin overexpression did not completely abolish gefitinib-induced apoptosis, suggesting that another proapoptotic regulator activated after EGFR inhibition might contribute to EGFR-TKI–induced apoptotic cell death. Previous studies have shown that gefitinib induces BIM expression via inhibition of the MEK-ERK pathway and that BIM induction plays a key role in EGFR-TKI–induced apoptosis in *EGFR* mutation–positive NSCLC cells (25–27). We have now shown that inhibition of both survivin downregulation and BIM induction attenuated gefitinib-induced apoptosis to a greater extent than did inhibition of either process alone. The recent preclinical study showing that the combination of a PI3K inhibitor and a MEK inhibitor, but neither agent alone, induced substantial growth inhibition in *EGFR* mutation–positive NSCLC cells (31) supports the notion that both the PI3K-AKT-survivin and MEK-ERK-BIM pathways contribute independently to gefitinib-induced apoptosis in such cells (Fig. 7).

In conclusion, we have shown that the EGFR-TKI gefitinib downregulated survivin expression, likely through inhibition

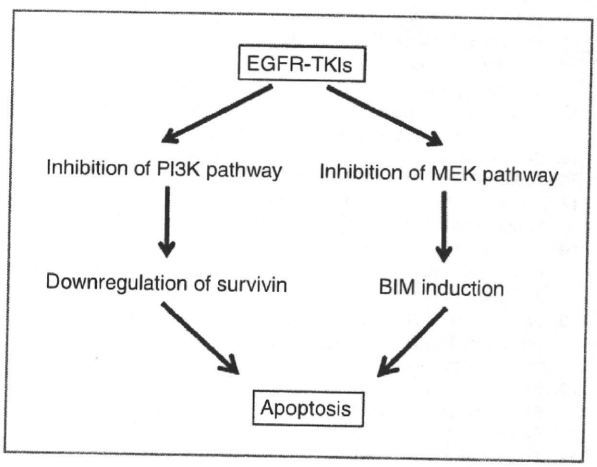

Figure 7. Proposed model for the intracellular signaling underlying EGFR-TKI–induced apoptosis in *EGFR* mutation–positive NSCLC cells.

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of PI3K-AKT signaling, and that this effect plays a key role in gefitinib-induced apoptosis. Moreover, we found that survivin downregulation and BIM induction are independently required for EGFR-TKI–induced apoptosis. Our results thus show that simultaneous upstream interruption of the PI3K-AKT-survivin and MEK-ERK-BIM pathways mediates EGFR-TKI–induced apoptosis.

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Disclosure of Potential Conflicts of Interest

No potential conflicts or interest were disclosed.

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ORIGINAL ARTICLE

Pharmacokinetics of aprepitant and dexamethasone after administration of chemotherapeutic agents and effects of plasma substance P concentration on chemotherapy-induced nausea and vomiting in Japanese cancer patients

Toshiaki Takahashi · Yukiko Nakamura · Asuka Tsuya · Haruyasu Murakami · Masahiro Endo · Nobuyuki Yamamoto

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Abstract

Purpose This study was conducted to determine the pharmacokinetics of aprepitant and dexamethasone as well as the relationship between the plasma concentration of substance P and nausea/vomiting in Japanese cancer patients.

Methods After administration of aprepitant (125/80 mg group [10 patients]: 125 mg on day 1 and 80 mg on days 2–5; 40/25 mg group [10 patients]: 40 mg on day 1 and 25 mg on days 2–5) and dexamethasone (6 mg on day 1 and 4 mg on days 2 and 3 in the 125/80 mg group, and 8 mg on day 1 and 6 mg on days 2 and 3 in the 40/25 mg group) to Japanese cancer patients receiving at least moderately emetogenic antitumor agents, the plasma concentrations of aprepitant, dexamethasone, and substance P were measured.

Results All of 20 patients were treated with the highly emetogenic agent cisplatin (≥70 mg/m^2). The C_{max} and $AUC_{0-24\ h}$ of aprepitant in Japanese cancer patients were similar with those in non-Japanese patients. The clearance of dexamethasone in the 125/80 mg group was approximately one-half of that previously determined in the absence of aprepitant. The substance P concentration in plasma significantly increased only in patients with delayed nausea/vomiting.

Conclusions This study demonstrated similar plasma pharmacokinetics of aprepitant in Japanese and non-Japanese, the validity of reducing dexamethasone dose, and the existence of increased plasma substance P concentration in patients receiving highly emetogenic cisplatin-based chemotherapy.

Keywords Aprepitant · Dexamethasone · Substance P · Pharmacokinetics · Chemotherapy-induced nausea and vomiting

Introduction

Aprepitant is a neurokinin-1 (NK_1) receptor antagonist developed as a treatment for both acute and delayed chemotherapy-induced nausea and vomiting (CINV). It has a novel mechanism of action (i.e., by inhibiting the binding of substance P to the NK_1 receptor in the vomiting center) [1–3]. In the guidelines for management of CINV, aprepitant is recommended to be used in combination with a serotonin antagonist and dexamethasone to prevent nausea/vomiting induced by highly and moderately emetogenic cancer chemotherapy [4–6].

Although aprepitant has no effect on the pharmacokinetics of serotonin antagonists (ondansetron, granisetron, palonosetron) [7, 8], aprepitant inhibits CYP3A4 and in turn inhibits the metabolism of dexamethasone, a substrate of CYP3A4 [9]. It has been shown that the area under the concentration–time curve (AUC) of dexamethasone is increased approximately two times after administration of aprepitant at a dose of 125 mg on day 1 and at a dose of 80 mg on days 2–5 in healthy adults, and so, to maintain

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dexamethasone at the prescribed blood level in the presence of aprepitant, the dose of dexamethasone has to be reduced by 50% [9]. Although a previous population pharmacokinetic study of dexamethasone combined with aprepitant supported the validity of this dose reduction of dexamethasone [10], there has been no full pharmacokinetic study of dexamethasone and aprepitant in cancer patients who receive emetogenic cancer chemotherapy.

While aprepitant may have an antiemetic effect by inhibiting the binding of substance P to the NK_1 receptor in the vomiting center as mentioned above, it is still unclear whether there is any change in the in vivo kinetics of substance P after administration of chemotherapeutic agents, or how the in vivo kinetics of substance P is related to CINV.

This study was conducted to determine the pharmacokinetics of aprepitant as well as dexamethasone in Japanese cancer patients and to verify the dose reduction of dexamethasone used in combination with aprepitant, and furthermore, to evaluate the relationship between CINV and in vivo kinetics of substance P after administration of chemotherapeutic agents.

Patients and methods

Inclusion criteria

Japanese cancer patients aged between 20 and 74 years who received cancer chemotherapy were included in this study. Cancer chemotherapy consisted of at least moderately (Hesketh level ≥ 3 [11]) emetogenic chemotherapeutic agents on day 1 only. With a performance status of 0–2 and an estimated life expectancy of at least 3 months, patients met the following laboratory criteria: white blood cell count $\geq 3{,}000/mm^3$ and neutrophil count $\geq 1{,}500/mm^3$; platelet count $\geq 100{,}000/mm^3$; AST (GOT) and ALT (GPT) $\leq 1.5 \times$ upper limit of the normal range at the facility; ALP $\leq 2.5 \times$ upper limit of the normal range at the facility; total bilirubin ≤ 1.5 mg/dL; and creatinine ≤ 1.5 mg/dL. The following patients were excluded from the study: patients with a risk of vomiting for other reasons (symptomatic brain metastasis, meningeal infiltration, epilepsy, active peptic ulcer, gastrointestinal obstruction, concomitant abdominal or pelvic radiotherapy, etc.); and pregnant, nursing, or possibly pregnant women. After the protocol and informed consent form were approved by the institutional review board (IRB) at the facility, patients who gave written informed consent were enrolled. All studies were conducted in accordance with the principles of Good Clinical Practice (GCP) and the basic principles of the Declaration of Helsinki.

Design and treatment

This was an open-label study. A total of 20 patients were randomized to receive aprepitant at an oral dose of 125/80 mg (125 mg on day 1 and 80 mg on days 2–5; $n = 10$) or 40/25 mg (40 mg on day 1 and 25 mg on days 2–5; $n = 10$). In addition, all patients received standard antiemetic therapy consisting of intravenous granisetron (40 μg/kg on day 1) and intravenous dexamethasone sodium phosphate (on days 1–3). In this study, the dose of intravenous dexamethasone was 6 mg on day 1 and 4 mg on days 2 and 3 in the 125/80-mg group and 8 mg on day 1 and 6 mg on days 2 and 3 in the 40/25 mg group. Although, in the antiemetic guidelines [4–6], it is recommended that dexamethasone is administered at a dose of 12 mg on day 1 and at a dose of 8 mg on day 2 and thereafter in combination with aprepitant 125/80 mg, the dose of dexamethasone in this study was determined in order to compare the clearance of dexamethasone in this study with that obtained from Japanese cancer patients in the absence of aprepitant at a dose of 12 mg dexamethasone on day 1 [10].

Pharmacokinetic evaluation

Blood samples for measurement of plasma aprepitant concentration were collected before administration of aprepitant on days 1–5 and 1, 2, 3, 5, 9, 11, and 24 h after administration of aprepitant on day 1 and on day 5 only. In addition, a separate set of blood samples for measurement of plasma dexamethasone concentration were collected immediately, 15 min, 30 min, and 1.5, 3.5, 7.5, 9.5, and 22.5 h after administration of dexamethasone on day 1.

Methods for measurement of plasma aprepitant and dexamethasone concentrations

For each subject, venous blood was collected in an EDTA 2Na-treated tube at each sampling time point and immediately centrifuged at approximately 1,500*g* (approximately 3,000 rpm) for 10 min at room temperature. Then, the resultant plasma was transferred to a polypropylene tube and stored frozen at −20°C. The plasma concentrations of aprepitant and dexamethasone were measured by liquid chromatography/tandem mass spectrometry (LC/MS/MS). After methanol was added to plasma, the internal standard and carbonate buffer (for aprepitant) or ammonium acetate buffer (for dexamethasone) were added and mixed. Then, *t*-butyl methyl ether (for aprepitant) or diethyl ether (for dexamethasone) was added to and mixed with the plasma sample and centrifuged. After the aqueous layer was frozen in a methanol/dry ice bath, the entire organic layer was collected in a tube and placed under