tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (16).

Pharmacokinetics

Blood samples (4 mL) were collected on days 1 and 2, and 29 and 30 before and 0.5, 1, 2, 3, 4, 6, 8, 10, and 24 hours after dosing. Predose blood samples to determine trough pharmacokinetic values and the attainment of a steady state of BIBF 1120 were collected on days 8, 15, 22, and 29 in the first treatment course. For pharmacokinetic reasons, BIBF 1120 was given only once daily on days 1 and 29 in the first treatment course. During repeated treatment courses (2–6), trough pharmacokinetic samples were taken on days 15 and 29. Plasma concentrations of BIBF 1120 were analyzed, and the pharmacokinetic variables were calculated in the same manner as the previously conducted phase I study (12).

Biomarker evaluation

The concentration of sVEGFR2 in plasma were measured by enzyme-linked immunosorbent assay on days 1, 2, 8, and 29 after BIBF 1120 treatment according to the manufacture's instructions (R&D System).

CD117/c-KIT–positive BMD progenitor cell subsets were measured with the use of flow cytometry. Peripheral blood was collected before starting, and after 2, 8, and 29 days of BIBF 1120 treatment. The 800 uL of whole blood was supplemented with 4.5 mL of 0.2% bovine serum albumin (BSA)-PBS and centrifuged for 5 minutes (1,500 rpm). After the removal of supernatant by aspiration, 4.5 mL of 0.2% BSA-PBS was added and centrifuged. Cell pellet was mixed with 50 μL of human γ-globulin. Antibodies (CD34-FITC, CD117-PE, and CD45-PerCP) were added and kept for 45 minutes at 4°C. Hemolytic agent (4.5 mL) was added and incubated for 10 minutes. After centrifugation (1,500 rpm, 5 min), supernatant was washed twice. Subsequently, 0.2% BSA-PBS (4.5 mL) was added, and supernatant was removed by centrifugation (1,500 rpm, 5 min). Cell pellet was filled up to 800 uL by BSA-PBS and

Table 1. Patient characteristics

Characteristic	No. of patients
Median (range) age (y)	62 (41-81)
Sex	
Male	11 (52%)
Female	10 (48%)
Performance status (ECOG)	
0	5 (24%)
1	16 (76%)
Previous therapy	
Surgery	18 (86%)
Chemotherapy	19 (91%)
Radiotherapy	6 (29%)
Tumor types	
Colorectal cancer	14 (67%)
Non–small cell lung cancer	1 (4.8%)
Small cell lung cancer	1 (4.8%)
Esophagus sarcoma	1 (4.8%)
Adrenal carcinoma	1 (4.8%)
Renal cell carcinoma	1 (4.8%)
Adenoid cystic carcinoma	1 (4.8%)
Unknown primary site	1 (4.8%)

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

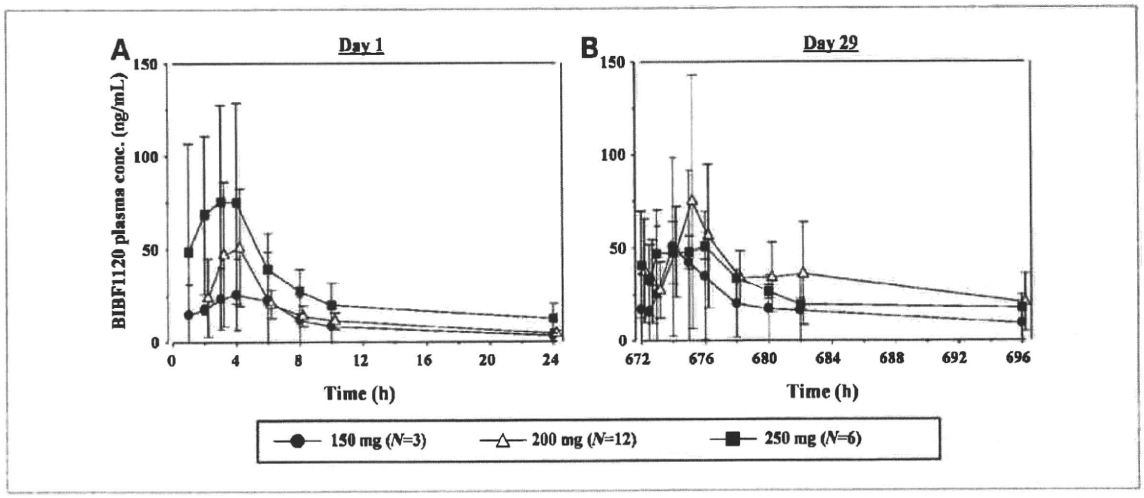

Figure 2. Mean (± SD) plasma concentration–time profiles of BIBF 1120 after single (A; day 1) and multiple (B; day 29) administration of 150, 200, and 250 mg BIBF 1120 twice daily.

Table 2. Dose-escalation scheme and DLT

BIBF 1120 dose (mg bid)	No. of patients		DLTs
	Total	DLT in first course	
150	3	0	
200	12	3	ALT and γ-GT increase; ALT increase; AST, ALT, and γ-GT increase
250	6	3	AST and ALT increase; ALT increase; γ-GT increase

Abbreviations: bid, twice daily; γ-GT, γ–glutamyl transferase.

analyzed by FACSCalibur flow cytometer (BD Biosciences). Cell surface markers of CD133 and CD117 were further identified from the $CD34^{+}CD45^{dim}$ cells in peripheral blood with the use of flow cytometry (Fig. 4A). The cell phenotype data of $CD133^{+/-}$ $CD117^{+/-}$ cells were calculated by the percentage of cell numbers of the target quadrant/those of all quadrants ($CD34^{+}CD45^{dim}$ cells).

Statistical analysis

Student's paired *t*-test was used to compare plasma sVEGFR2 levels or circulating $CD45^{dim}CD34^{+}CD117^{+}$ cell numbers between day 8 and before treatment, as well as between day 29 and before treatment, to evaluate the significance of changes induced by BIBF 1120 treatment (Microsoft Excel). A *P*-value of <0.05 was considered statistically significant.

Results

Patient demographics

Twenty-one patients with advanced refractory solid tumors were recruited between June 2006 and July 2007. The demographic and clinical characteristics of the patients are listed in Table 1. The median number of cycles given per patient was three (range, 1-7 cycles), and 10 patients received at least 4 cycles.

Table 3. Adverse events (≥10% incidence) related to BIBF 1120 in all treatment courses

BIBF 1120 dose	150 bid (*N* = 3)		200 bid (*N* = 12)		250 bid (*N* = 6)		Total (*N* = 21)	
CTCAE grade	1/2	3/4	1/2	3/4	1/2	3/4	All	
	N	N	N	N	N	N	N	(%)
ALT increased	0	0	4	4	3	2	13	61.9
AST increased	0	0	6	2	3	1	12	57.1
γ-GT increased	0	0	4	4	2	2	12	57.1
Vomiting	1	0	9	0	2	0	12	57.1
Anorexia	1	0	8	0	2	0	11	52.4
Fatigue	2	0	6	0	2	1	11	52.4
ALP increased	0	0	5	1	3	0	9	42.9
Nausea	1	0	5	0	2	0	8	38.1
Diarrhea	0	0	5	0	2	0	7	33.3
Hemoptysis	1	0	3	0	0	0	4	19.0
Upper abdominal pain	1	0	1	0	2	0	4	19.0
Weight decreased	0	0	4	0	0	0	4	19.0
Abdominal pain	1	0	2	0	0	0	3	14.3
Hypertension	1	1	1	0	0	0	3	14.3
Rash	0	0	2	0	1	0	3	14.3
Proteinuria	1	0	2	0	0	0	3	14.3
LDH increased	0	0	2	0	1	0	3	14.3

NOTE: Presented is the highest ever reached CTCAE grade. One patient may have experienced >1 event.
Abbreviations: CTCAE, Common Terminology Criteria for Adverse Events; bid, twice daily; γ-GT, γ–glutamyl transferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.

Dose escalation and MTD

No DLT was observed at the starting dose of 150 mg twice daily in the first three patients (Table 2), so the dose was escalated to the second dose level of 200 mg twice daily. Because one of the first three patients experienced a DLT of grade 3, an increasein alanine aminotransferase (ALT) and γ–glutamyl transpeptidase levels at 200 mg twice daily, three patients were additionally treated at this dose according to the protocol definition. Among the first six patients treated at 200 mg twice daily, two patients experienced a DLT of grade 3 (ALT and γ–glutamyl transpeptidase increases in one patient, ALT increase in one patient). Given that these increases in hepatic enzyme levels were fully reversible, the investigators and independent data monitoring committee agreed to add four more patients to confirm the judgment of dose escalation/reduction of the dose level. The four additional patients did not experience a DLT, and overall, 2 of 10 patients at this dose level experienced a DLT; therefore, dose escalation proceeded to 250 mg twice daily. At this dose level, three of six patients showed DLTs [aspartate aminotransferase (AST) and ALT elevations of grade 3 in one patient, ALT elevation of grade 3 in one patient, and γ–glutamyl transpeptidase elevation of grade 3 in one patient], and the MTD had been exceeded. The next lower dose of 200 mg twice daily was therefore identified as the MTD. According to the protocol definition, two additional patients were further evaluated at the MTD cohort. Among the total of 12 patients who received 200 mg twice daily, 3 patients experienced a reversible grade 3 or 4 AST, ALT, and γ–glutamyl transpeptidase elevation, which correspond to DLT, and 200 mg twice daily BIBF 1120 was thus confirmed as the MTD.

Safety

Twenty-one patients received at least one dose of study treatment and were evaluated for safety. As shown in Table 3, the most frequent BIBF 1120–related side effects were increased hepatic enzymes [ALT (61.9% of patients), AST (57.1%), and γ–glutamyl transpeptidase (57.1%)], vomiting (57.1%), anorexia (52.4%), fatigue (52.4%), alkaline phosphatase increase (42.9%), nausea (38.1%), and diarrhea (33.3%). Most of these events were of mild-to-moderate intensity and of Common Toxicity Criteria for Adverse Events grade 1 or 2, fully reversible and clinically manageable over all doses. The predominant Common Toxicity Criteria for Adverse Events grades 3 and 4 adverse events were reversible liver enzyme elevations occurring at BIBF 1120 at 200 mg twice daily and BIBF 1120 at 250 mg twice daily in a total of eight patients. Except for one patient with combined grade 4 AST and ALT elevations, all elevations were of grade 3 intensity. One patient in the BIBF 1120 150 mg twice daily cohort reported grade 3 hypertension, and another patient in the BIBF 1120 250 mg twice daily cohort reported grade 3 fatigue. Drug-related increases in hepatic enzymes occurred within the 1st week after treatment initiation and were fully reversible on

Table 4. Pharmacokinetic variables of BIBF 1120 after a single dose (day 1) and multiple dosing for 29 days

Single dose	**BIBF 1120 dose (mg)**		
	150 (*N* = 3)	**200 (*N* = 12)**	**250 (*N* = 6)**
C_{max}, ng/mL	28.9 (61.5)	52.0 (64.3)	99.8 (70.3)
t_{max}*, h	2.00 (1.00-6.00)	2.98 (1.98-4.00)	2.98 (1.00-4.07)
$t_{1/2}$, h	10.3 (15.8)	10.2 (30.4)	9.53 (10.8)†
AUC_{0-12}, ng·h/mL	145 (88.3)	233 (40.9)	399 (64.9)
Multiple dosing	**150 (*N* = 3)**	**200 (*N* = 7)**	**250 (*N* = 3)**
$C_{max,ss}$, ng/mL	38.8 (107)	67.6 (74.3)	62.9 (14.4)
$t_{max,ss}$, h	2.00 (1.98-4.00)	2.97 (1.98-3.98)	2.00 (1.00-4.00)
$t_{1/2,ss}$, h	20.4 (55.3)	19.9 (75.5)‡	23.8 (39.4)§
AUC_{ss}, ng·h/mL	207 (135)	423 (66.2)	411 (9.15)
Rac	1.42 (35.4)	1.70 (40.9)	1.50 (79.0)

NOTE: Geometric mean (geometric coefficient of variation %).

Abbreviations: $t_{max,ss}$, time to reach maximum plasma concentrations at steady state; AUC, area under the curve.

*Median (range).

†*N* = 5.

‡*N* = 6.

§*N* = 2.

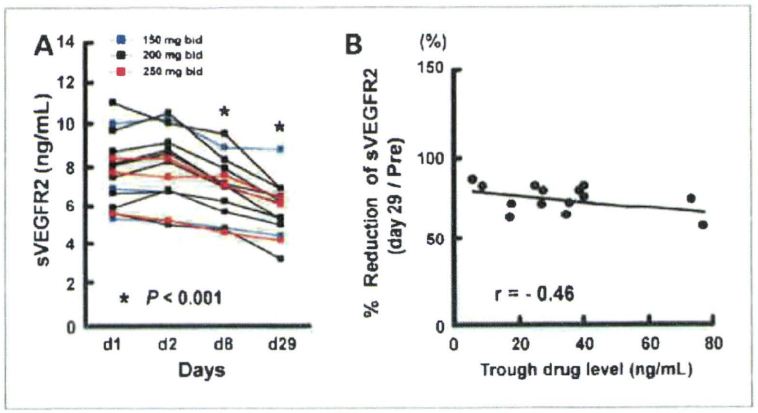

Figure 3. sVEGFR2 levels in plasma after BIBF 1120 treatment. A, plasma sVEGFR2 levels decreased during the 4-week treatment period. B, the decrease in sVEGFR2 at cycle 1, day 29 showed a modest inverse correlation with trough plasma drug levels of BIBF 1120 ($r = -0.46$).

cessation of treatment. There were no bleeding events or clinically relevant hematologic toxicities during all treatment courses throughout the study. Due to adverse events or DLTs, four patients in the BIBF 1120 200 mg twice daily and three patients in the BIBF 1120 250 mg twice daily dose cohorts required dose reduction.

Pharmacokinetics

The pharmacokinetic variables after a single oral dose and multiple oral doses of BIBF 1120 (150-250 mg twice daily) are shown in Table 4. Maximum plasma concentrations [$C_{max,(ss)}$] were reached at 2 to 3 hours after dosing after single and multiple dosing of BIBF 1120 (Fig. 2A and B; Table 4). After attaining C_{max}, the plasma concentration declined in an apparent biexponential manner with the terminal half-life of ~10 hours. Of note, the terminal half-life of BIBF 1120 was calculated from samples obtained during the first 24 hours post dose. After multiple dosing of BIBF 1120, C_{max} were reached at 2 to 3 hours after dosing (Fig. 2B; Table 4). The accumulation ratio (Rac) values based on area under the curve were 1.42 to 1.7, and accumulation was consistent with the terminal half-life observed after single doses. Steady-state plasma concentrations were attained at least on day 8 of repeated twice daily oral dosing based on visual inspection of the trough plasma concentration. In general, C_{max} and area under the curve were increased with increasing dose. Trough plasma concentrations of BIBF 1120 during repeated treatment courses were

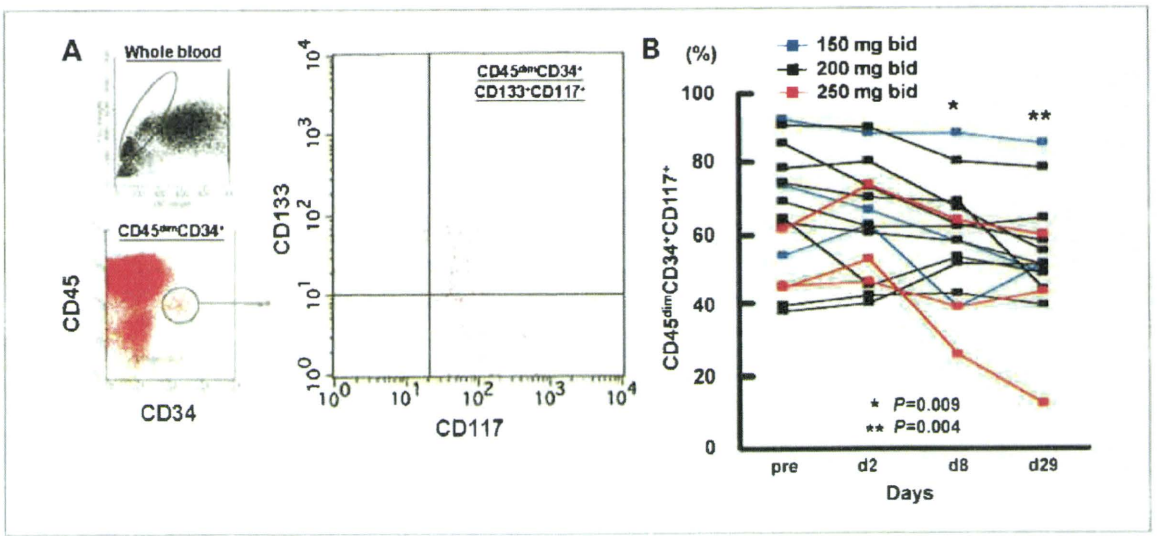

Figure 4. Levels of circulating CD117-BMD progenitor cells after BIBF 1120 treatment. A, representative flow cytometric analysis for determining the number of CD117-positive–BMD progenitor cells defined as $CD45^{dim}CD34^{+}CD117^{+}$. B, circulating levels of $CD45^{dim}CD34^{+}CD117^{+}$ cells decreased during the 4-week treatment period.

almost at the same level within each dose group. The range of the geometric mean of the trough concentration was 14.4 to 38.4 nmol/L for the 150 mg twice daily group and 28.2 to 84.6 nmol/L for the 200 mg twice daily group. In the 250 mg twice daily group, the number of trough concentrations collected during repeated treatment courses was very limited due to the occurrence of dose reduction in this group.

Tumor response

Twenty patients were evaluated for tumor response. Although no complete or partial responses were observed, 16 (76.2%) patients had stable disease for at least two treatment courses (56 d). The disease stabilization was observed across all the tested doses: BIBF 1120 150 mg, all patients (100%) of 3; 200 mg, 9 (75%) of 12; 250 mg, 4 (67%) of 6. Median progression-free survival for all patients was 113 days (95% confidence interval, 77-119 d).

Plasma levels of sVEGFR2 during treatment with BIBF 1120

At baseline, the mean plasma level of sVEGFR2 obtained from 15 patients [150 mg twice daily (n = 3), 200 mg twice daily (n = 9), and 250 mg twice daily (n = 3)] was 7.7 ± 1.7 ng/mL (range, 5.3-11.0 ng/mL). Plasma concentrations of sVEGFR2 decreased significantly over the first 4 weeks of treatment to a level of 5.8 ± 1.3 ng/mL (range, 3.2-8.8; $P < 0.001$, t-test; Fig. 3A). The decreases in sVEGFR2 levels were seen across all doses tested. As shown in Fig. 3B, the decrease in sVEGFR2 showed an inverse linear correlation with the trough plasma drug levels of BIBF 1120 ($r = -0.46$).

Levels of circulating CD117/C-KIT$^+$–BMD progenitors during treatment with BIBF 1120

Subsets of CD117-positive–BMD progenitor cells were measured in progenitor-enriched ($CD45^{dim}CD34^+$) whole blood of 15 patients [150 mg twice daily (n = 3), 200 mg twice daily (n = 9), and 250 mg twice daily (n = 3)]. CD117 was expressed in the $CD45^{dim}CD34^+$ subset with a level of 60% to 80%, and representative data are shown in Fig. 4A. $CD45^{dim}CD34^+CD117^+$ cells significantly decreased over all BIBF 1120 dose cohorts during the 1st cycle of therapy ($P = 0.009$ on day 8 and $P = 0.004$ on day 29, t-test; Fig. 4B).

Discussion

This phase I study showed that BIBF 1120 can be safely given to Japanese patients with advanced solid tumors, and the MTD was determined as 200 mg twice daily, which was one dose lower than in Caucasian patients (12). Biomarker investigations revealed that the plasma concentration levels of the sVEGFR2 and the $CD45^{dim}CD34^+CD117^+$ cells significantly decreased over the first 4 weeks of treatment with BIBF 1120.

As has been observed in previous phase I and phase II studies with BIBF 1120, gastrointestinal side effects, such as vomiting, fatigue, nausea, and diarrhea, were the most frequent adverse events (12, 15) and have also been observed with other VEGFR inhibitors, such as sorafenib or sunitinib (4, 5, 17). These side effects of mostly mild or moderate intensity occurred predominantly at the MTD of BIBF 1120 or at higher doses, and were easy to monitor and manageable with standard supportive treatment. Hypertension has also been reported with several other VEGF and VEGFR inhibitors (4, 5), and was observed in three patients in this study. All cases were controllable with appropriate antihypertensive treatment.

The pharmacokinetic analysis revealed that there was a dose linear increase for C_{max} and area under the curve. C_{max} values were reached within 3 hours after administration, and steady state was reached at least on day 8. All pharmacokinetic variables displayed a moderate-to-high variability as expected for an oral compound. In addition, different patients with various anticancer pretreatments have been enrolled in this study; thus, differences in pretreatment and other intrinsic factors, such as age and status, might have influenced the variability of these variables, too. Overall, there was no difference in the pharmacokinetic behavior of BIBF 1120 between Japanese and Caucasian patients (12, 18). Based on the trough plasma concentrations for BIBF 1120 at dose levels ≥150 mg twice daily, sufficient exposure has been reached to block the target structures of the molecule according to the IC_{50} values (8, 11).

All DLTs observed in this study were liver enzyme elevations (grade 3 or 4 ALT, AST, and γ-glutamyl transpeptidase). These liver enzyme elevations were fully reversible, responded within 2 weeks to treatment discontinuation or dose reduction, indicating reversible liver side effects, and were not accompanied by an increase of bilirubin. However, at 200 mg twice daily of BIBF 1120 in Caucasian patients, no such liver enzyme elevations were observed in a previous phase I study (12). We cannot exclude the possibility of ethnic differences, although there were no pharmacokinetic differences between Japanese and Caucasian patients. From the exploratory data evaluation, the body weight of all three patients who experienced DLTs at 200 mg twice daily as MTD was below 50 kg, whereas that of the remaining nine patients treated without DLTs was ≥50 kg. This finding suggested that body size, such as body weight or body surface area, might confer liver enzyme elevations on BIBF 1120, with further investigation of possible dose dependency being warranted.

Evaluation of novel targeted agents, such as VEGF signaling inhibitors, may be supported by the identification of suitable biomarkers of biological activity. The most intuitive method to measure the effect of any anticancer drug is to evaluate the tumor tissue. Tumor biopsy strategies provide a way to thoroughly characterize tumor histology and molecular processes with immunohistochemistry, DNA microarray, and proteomics analyses. Indeed, several considerable biomarkers of angiogenesis, such as microvessel density or tumor VEGF expression,

have been extensively investigated with the use of tumor tissue specimens. On the other hand, identifying circulating biomarkers of angiogenesis would have the advantage of being minimally invasive, allowing repetitive sampling throughout treatment without the ethical and technical complications of multiple biopsy. Circulating levels of sVEGFR2 were previously found to be decreased by other VEGFR2 inhibitors that directly target this receptor, such as AZD2171 (8) and SU11248 (9), although the mechanism behind the consistent decrease in sVEGFR2 levels is not entirely understood (4, 5, 19–21). In the present study, plasma sVEGFR2 levels showed time-dependent decrease at all dose levels studied, and the changes in sVEGFR2 were inversely associated with trough plasma concentration of BIBF 1120, suggesting that sVEGFR2 is a useful pharmacodynamic marker of drug exposure, with similar findings reported for other agents.

Circulating endothelial cells have emerged as a potentially useful surrogate marker of antiangiogenic drug activity (4, 10, 19–21). They comprise two distinct populations: mature circulating endothelial cells, which originate from vessel walls and have a limited growth capability, and BMD circulating endothelial cells, which are responsible for most endothelial proliferative potential. Circulating BMD endothelial progenitors have been reported to contribute to tumor vasculogenesis in animal models as well as in humans (18, 21–23). However, the variable degrees of incorporation of circulating endothelial cells shown in different tumor models have led to controversy about the extent of their actual involvement in tumor vascularization. The identification of circulating endothelial cells is highly complex and has been hampered by the overlapping antigenic similarities, with a lack of consensus about the definition of these endothelial cells (4, 24). The pan-hematopoietic marker CD45 has been widely used to first exclude hematopoietic cells (22). CD34 was chosen as a colabel because it is reported to be present on endothelial progenitors, and $CD34^+$ cells alone can repopulate bone marrow *in vivo* (23). This present study reported the first quantitative analysis of subsets of circulating CD117-BMD progenitor cells, characterized as $CD45^{dim}CD34^+CD117^+$, after treatment with BIBF 1120. Results show that levels of circulating CD117-BMD progenitor cells were significantly decreased after BIBF 1120 treatment in time-dependent fashion. One possible explanation for the BIBF 1120–induced decrease in CD117-BMD progenitor cells is that CD117/C-KIT^+ is one of the target receptors of BIBF 1120 as well as many other VEGFR tyrosine kinase inhibitors, resulting in the impaired growth of CD117/C-KIT^+ cells or inhibitory effects of differentiation/mobilization on peripheral blood. This study further showed that the patients who responded (stable disease) to BIBF 1120 had a larger decrease in CD117-BMD progenitor cells after the initial 4 weeks of the study treatment compared with patients who did not (progressive disease; Supplementary Fig. S1) although, given the sample size, there was limited power to detect a significant difference. This observation suggests that a reduction in CD117-BMD progenitor cells would be associated with a higher degree of target inhibition and greater clinical efficacy after BIBF 1120 treatment. This is the first study to show evidence of decreased levels of circulating CD117-BMD progenitor cells during treatment with antiangiogenic agents. Meanwhile, the main limitations in evaluating the circulating endothelial progenitor cells for surrogate biomarkers are "nonstandardized protocols" or "labor-intensiveness." Further investigation to validate whether it will be useful for monitoring the response to antiangiogenic therapy is warranted.

In conclusion, BIBF 1120 shows an acceptable profile for Japanese patients suffering from advanced solid tumors at doses up to 200 mg twice daily. The preliminary evaluation of biological activity of BIBF 1120 with the use of plasma (sVEGFR2) and cellular (CD117-BMD progenitor cells) markers, and disease stabilization data show that this agent is biologically active. BIBF 1120 is currently being investigated in a range of tumor types, and recruitment to a series of randomized, double-blind phase II and III trials is ongoing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Taylor M, Rossler J, Geoerger B, et al. High levels of circulating VEGFR2+ bone marrow-derived progenitor cells correlate with metastatic disease in patients with pediatric solid malignancies. Clin Cancer Res 2009;15:4561–71.
2. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2002;2:727–39.
3. Morabito A, De Maio E, Di Maio M, Normanno N, Perrone F. Tyrosine kinase inhibitors of vascular endothelial growth factor receptors in clinical trials: current status and future directions. Oncologist 2006;11:753–64.
4. Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer 2006;6:835–45.
5. Brown AP, Citrin DE, Camphausen KA. Clinical biomarkers of angiogenesis inhibition. Cancer Metastasis Rev 2008;27:415–34.
6. Agulnik M, Oza AM, Pond GR, Siu LL. Impact and perceptions of mandatory tumor biopsies for correlative studies in clinical trials of novel anticancer agents. J Clin Oncol 2006;24:4801–7.
7. Helft PR, Daugherty CK. Are we taking without giving in return? The ethics of research-related biopsies and the benefits of clinical trial participation. J Clin Oncol 2006;24:4793–5.
8. Drevs J, Siegert P, Medinger M, et al. Phase I clinical study of AZD2171, an oral vascular endothelial growth factor signaling inhibitor, in patients with advanced solid tumors. J Clin Oncol 2007;25:3045–54.
9. Norden-Zfoni A, Desai J, Manola J, et al. Blood-based biomarkers of SU11248 activity and clinical outcome in patients with metastatic imatinib-resistant gastrointestinal stromal tumor. Clin Cancer Res 2007;13:2643–50.
10. Roth GJ, Heckel A, Colbatzky F, et al. Design, synthesis, and evaluation

of indolinones as triple angiokinase inhibitors and the discovery of a highly specific 6-methoxycarbonyl-substituted indolinone (BIBF 1120). J Med Chem 2009;52:4466–80.
11. Hilberg F, Roth GJ, Krssak M, et al. BIBF1120: triple angiokinase inhibitor with sustained receptor blockade and good anti-tumor efficacy. Cancer Res 2008;68:4774–82.
12. Mross K, Stefanic M, Gmehling D, et al. Phase I study of the angiogenesis inhibitor BIBF 1120 in patients with advanced solid tumors. Clin Cancer Res 2010;16:311–9.
13. du Bois A, Huober J, Stopfer P, et al. A phase I open-label dose-escalation study of oral BIBF 1120 combined with standard paclitaxel and carboplatin in patients with advanced gynecological malignancies. Ann Oncol 2010;21:370–5.
14. Ledermann JA, Rustin GJ, Hackshaw A, et al. A randomized phase II placebo-controlled trial using maintenance therapy to evaluate the vascular targeting agent BIBF 1120 following treatment of relapsed ovarian cancer (OC). J Clin Oncol 2009;27:15s (abstr 5501).
15. von Pawel J, Kaiser R, Eschbach C, et al. Efficacy, safety and pharmacokinetic (PK) results of a phase II study with the triple angiokinase inhibitor BIBF 1120 in patients suffering from advanced non small cell lung cancer (NSCLC). J Thorac Oncol 2008;3:S61.
16. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.
17. Faivre S, Djelloul S, Raymond E. New paradigms in anticancer therapy: targeting multiple signaling pathways with kinase inhibitors. Semin Oncol 2006;33:407–20.
18. Beaudry P, Force J, Naumov GN, et al. Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. Clin Cancer Res 2005;11:3514–22.
19. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann Oncol 2004;15:139–45.
20. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. Blood 2001;97:3658–61.
21. Stamper IJ, Byrne HM, Owen MR, Maini PK. Modelling the role of angiogenesis and vasculogenesis in solid tumour growth. Bull Math Biol 2007;69:2737–72.
22. Summer R, Kotton DN, Sun X, Fitzsimmons K, Fine A. Translational physiology: origin and phenotype of lung side population cells. Am J Physiol Lung Cell Mol Physiol 2004;287:L477–83.
23. Blair A, Sutherland HJ. Primitive acute myeloid leukemia cells with long-term proliferative ability *in vitro* and *in vivo* lack surface expression of c-kit (CD117). Exp Hematol 2000;28:660–71.
24. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002;109:337–46.

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ORIGINAL REPORT

Phase III Trial Comparing Oral S-1 Plus Carboplatin With Paclitaxel Plus Carboplatin in Chemotherapy-Naïve Patients With Advanced Non–Small-Cell Lung Cancer: Results of a West Japan Oncology Group Study

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This study is registered with University Hospital Medical Information Network Clinical Trial Registry (http://www.umin.ac.jp/ctr/index.htm; identification number UMIN000000503).

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical Trials repository link available on JCO.org.

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ABSTRACT

Purpose
The primary goal of this open-label, multicenter, randomized phase III trial was to determine whether treatment with carboplatin plus the oral fluoropyrimidine derivative S-1 was noninferior versus that with carboplatin plus paclitaxel with regard to overall survival (OS) in chemotherapy-naive patients with advanced non–small-cell lung cancer (NSCLC).

Patients and Methods
A total of 564 patients were randomly assigned to receive either carboplatin (area under the curve, 5) on day 1 plus oral S-1 (40 mg/m^2 twice per day) on days 1 to 14 or carboplatin (area under the curve, 6) plus paclitaxel (200 mg/m^2) on day 1 every 21 days.

Results
At the planned interim analysis, with a total of 268 death events available, the study passed the O'Brien-Fleming boundary of 0.0080 for a positive result and noninferiority of carboplatin and S-1 compared with carboplatin and paclitaxel was confirmed for OS (hazard ratio, 0.928; 99.2% CI, 0.671 to 1.283). Median OS was 15.2 months in the carboplatin and S-1 arm and 13.3 months in the carboplatin and paclitaxel arm, with 1-year survival rates of 57.3% and 55.5%, respectively. Rates of leukopenia or neutropenia of grade 3/4, febrile neutropenia, alopecia, and neuropathy were more frequent in the carboplatin and paclitaxel arm, whereas thrombocytopenia, nausea, vomiting, and diarrhea were more common in the carboplatin and S-1 arm. The carboplatin and S-1 arm had significantly more dose delays than the carboplatin and paclitaxel arm.

Conclusion
Oral S-1 with carboplatin was noninferior in terms of OS compared with carboplatin and paclitaxel in patients with advanced NSCLC, and is thus a valid treatment option.

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INTRODUCTION

Lung cancer is the leading cause of death related to cancer worldwide,[1] with non–small-cell lung cancer (NSCLC) accounting for 85% of lung cancer cases. For individuals with advanced or metastatic NSCLC, platinum-based chemotherapy is the mainstay of first-line treatment on the basis of the moderate improvement in survival and quality of life it affords compared with best supportive care alone.[2-5] Thus, there is still a need for new treatment regimens to ameliorate symptoms and prolong survival in patients with advanced NSCLC in a manner that is both convenient and safe.

S-1 (TS-1; Taiho Pharmaceutical Co Ltd, Tokyo, Japan) is an oral fluoropyrimidine agent that consists of tegafur, 5-chloro-2,4-dihydroxypyridine, and potassium oxonate in a molar ratio of 1:0.4:1.[6,7] A phase II trial of oral S-1 as a single agent for the treatment of advanced NSCLC yielded a response rate of 22% and a median survival time of 10.2 months in 59 patients without prior chemotherapy.[8] We previously performed a phase I/II study of carboplatin/S-1 combination therapy and found that administration of S-1 (40 mg/m^2 twice per day) on days 1 to 14 in combination with carboplatin (area under the curve [AUC], 5) on day 1 of every 3-week cycle yielded efficacy results similar to those of other platinum doublets.[9] The carboplatin and S-1 combination had a more favorable toxicity profile than that typically seen with platinum-based regimens,

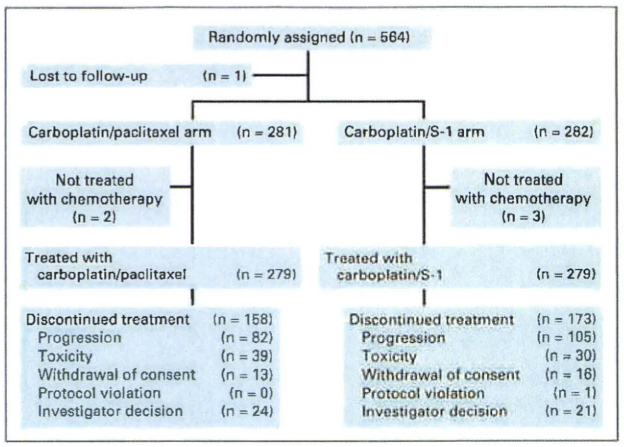

Fig 1. CONSORT diagram for the study

especially with regard to neutropenia, febrile neutropenia, neuropathy, and alopecia.[9] In addition, replacement of paclitaxel with oral S-1 in combination therapy with carboplatin avoids the need for premedication to ameliorate paclitaxel-induced hypersensitivity and the 3-hour infusions required for paclitaxel administration. We therefore undertook and now report the results of the LETS (Lung Cancer Evaluation of TS-1) study, a multicenter, randomized, phase III, noninferiority trial of carboplatin and S-1 in comparison with carboplatin and paclitaxel combination therapy in chemotherapy-naive patients with advanced NSCLC.

PATIENTS AND METHODS

Patients

The criteria for patient eligibility included a diagnosis of NSCLC confirmed either histologically or cytologically; a clinical stage of IIIB not amenable to curative treatment or of stage IV; a measurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST)[10]; no prior chemotherapy; an age of 20 to 74 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; and a projected life expectancy of at least 3 months. Patients had adequate bone marrow reserve and organ function including a calculated creatinine clearance of $\geq$ 60 mL/min based on the standard Cockcroft and Gault formula. Radiation therapy for metastatic disease was permitted if it was completed at least 2 weeks before random assignment. Main exclusion criteria included active concomitant malignancy, symptomatic brain metastasis, interstitial pneumonia, watery diarrhea, heart failure, uncontrolled diabetes mellitus, active infection, and a past history of drug allergy. These inclusion and exclusion criteria are consistent with those of previous studies involving carboplatin and paclitaxel treatment.[11] Written informed consent was obtained from all patients, and the study protocol was approved by the institutional ethics committee of each of the participating institutions.

Treatment Plan

Eligible patients were randomly assigned to receive either carboplatin (AUC, 6) plus paclitaxel (200 mg/m^2) on day 1[11] or carboplatin (AUC, 5) on day 1 plus oral S-1 (40 mg/m^2 twice per day) on days 1 to 14. Chemotherapy was repeated every 3 weeks for a maximum of six cycles unless there was earlier evidence of disease progression or intolerance of the study treatment.

End Points

The primary objective of this open-label, multicenter, randomized phase III trial was to establish the noninferiority of S-1 plus carboplatin compared with paclitaxel plus carboplatin as first-line therapy in terms of overall survival (OS) in patients with advanced NSCLC. Secondary end points included tumor response, treatment safety, quality of life (QOL), and progression-free survival (PFS).

Baseline and Follow-Up Assessments

Baseline evaluations included medical history, physical examination, ECG, tumor status, ECOG performance status, and laboratory analyses. During treatment, blood counts and biochemical tests were performed at least biweekly. A computed tomography scan was performed for tumor assessment within 14 days of initiation of study treatment and was repeated after every 1 to 2 months of planned therapy. All responses were defined according to RECIST. If a patient was documented as having a complete response (CR) or a

Table 1. Patient Demographic and Clinical Characteristics

Characteristic	Carboplatin/Paclitaxel (n = 281) No.	Carboplatin/Paclitaxel (n = 281) %	Carboplatin/S-1 (n = 282) No.	Carboplatin/S-1 (n = 282) %	*P*
Age, years					
Median	63		64		.510
Range	36-74		38-74		
Sex					
Male	215	76.5	217	77.0	.902
Female	66	23.5	65	23.0	
ECOG PS					
0	90	32.0	86	30.5	.695
1	191	68.0	196	69.5	
Histology					
Adenocarcinoma	195	69.4	195	69.1	.560
Nonadenocarcinoma	86	30.6	87	30.9	
Clinical stage					
IIIB	68	24.2	68	24.1	.981
IV	213	75.8	214	75.9	
Smoking status					
Smoker	229	81.5	230	81.6	.984
Nonsmoker	52	18.5	52	18.4	

Abbreviation: ECOG PS, Eastern Cooperative Oncology Group performance status.

partial response (PR), a confirmatory evaluation was performed after an interval of 4 weeks. Disease control was defined as the best tumor response among CR, PR, or stable disease that was confirmed and sustained for 6 weeks or longer. Patients were evaluated for adverse events during therapy and until 42 days after administration of the last dose of the study treatment. Toxicity was evaluated according to the National Cancer Institute Cancer Common Toxicity Criteria, version 3. QOL was assessed with the lung cancer subscale of the Functional Assessment of Cancer Therapy–Lung (FACT-L)[12] and the neurotoxicity subscale of the FACT/Gynecology Oncology Group-Neurotoxicity (GOG-Ntx) version 4.[13] In addition, alopecia was evaluated on the basis of the single item "I have been bothered by hair loss," which was included in the former version of FACT-L. The maximum attainable scores on the lung cancer subscale, neurotoxicity subscale, and alopecia item were 28, 44, and 4, respectively, with which the patient was considered to be asymptomatic. Patients were asked to complete each instrument at the time of enrollment and at 6 and 9 weeks after initiation of treatment.

Statistical Analysis

Eligible patients were randomly assigned according to a 1:1 ratio to receive either carboplatin and paclitaxel or carboplatin and S-1. After a check of patient eligibility, random assignment was performed centrally at the West Japan Oncology Group data center by minimization with stratification factors including disease stage (IIIB *v* IV), type of histology (adenocarcinoma *v* nonadenocarcinoma), sex (male *v* female), and investigator center. The intent-to-treat (ITT) patient population included all patients who underwent random assignment. The per-protocol (PP) population was defined as the ITT population minus patients considered to have major violations of inclusion or exclusion criteria and those who did not receive any protocol treatment. The safety population was defined as all patients receiving at least one dose of study drugs. The primary end point of the study was OS, which was analyzed in the ITT population by estimation of the hazard ratio (HR) and two-sided 95% CI derived from a Cox regression model with adjustment for the stratification factors with the exception of investigator center. Median OS in both treatment arms was assumed to be 14 months on the basis of data from previous clinical trials.[11] Noninferiority of carboplatin and S-1 was to be concluded if the upper limit of the 95% CI of the HR was lower than 1.33; that is, the null hypothesis that the median OS of the carboplatin and S-1 group would be up to 3.48 months shorter than that of the carboplatin and paclitaxel group was analyzed. Demonstration of noninferiority with a statistical power of 85% at a two-sided significance level of .05 and 2 years of follow-up after 2.5 years of accrual would require 263 patients in each treatment group. Given the possibility of variance inflation due to censoring, the sample size was set at 560 (280 per arm). One interim analysis was planned when all the patients had been enrolled. For analysis of the primary end point, adjustment for multiple comparisons was handled by the method of Lan and DeMets, with the use of the O'Brien-Fleming type α spending function. The significance level was set at .008 for the interim analysis, taking the numbers of observed events (n = 268) and expected events (n = 442) into account. Survival curves (PFS and OS) were analyzed by the Kaplan-Meier method and were compared between groups by the Cox regression model. The 95% CI for median PFS and OS was calculated by the method of Brookmeyer and Crowley. Planned subgroup analyses for OS were performed to examine the interaction effect of treatment arm with each of performance status, sex, disease stage, type of histology, and smoking status. Patient characteristics (ie, sex, ECOG PS, histology, clinical stage, and smoking status) as well as response and toxicity incidence were compared between the two treatment arms by the χ^2 test, and age was compared by the Wilcoxon test. Longitudinal QOL data were analyzed with a linear mixed-effects model. All *P* values were two sided. Statistical analyses were performed with SAS for Windows, release 9.1 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

From August 2006 to May 2008, 564 patients from 30 institutions were enrolled in the study. One patient was excluded from the carboplatin and paclitaxel arm because of loss to follow-up. The ITT population thus consisted of 563 patients: 281 individuals randomly assigned to the carboplatin and paclitaxel group and 282 individuals randomly assigned to the carboplatin and S-1 group (Fig 1). The baseline demographic and disease-related characteristics of the study subjects were well-balanced between the two treatment arms (Table 1). Two patients in the carboplatin and paclitaxel arm and three patients in the carboplatin and S-1 arm did not receive any chemotherapy, with the result that 558 patients were eligible for safety analysis (Fig 1).

Delivered Chemotherapy

The number of treatment courses administered was 1,037 in the carboplatin and paclitaxel arm (median, 4; range, 1 to 6) and 987 in the carboplatin and S-1 arm (median, 4; range, 1 to 6). Dose reductions occurred in 90 (8.7%) of the carboplatin and paclitaxel courses and in 49 (5.0%) of the carboplatin and S-1 courses. Carboplatin and paclitaxel dose reductions were mainly due to neuropathy, whereas those for carboplatin and S-1 were most commonly attributable to thrombocytopenia. Dose delays occurred in 47.9% of carboplatin and paclitaxel courses and 68.5% of carboplatin and S-1 courses. Delays due to

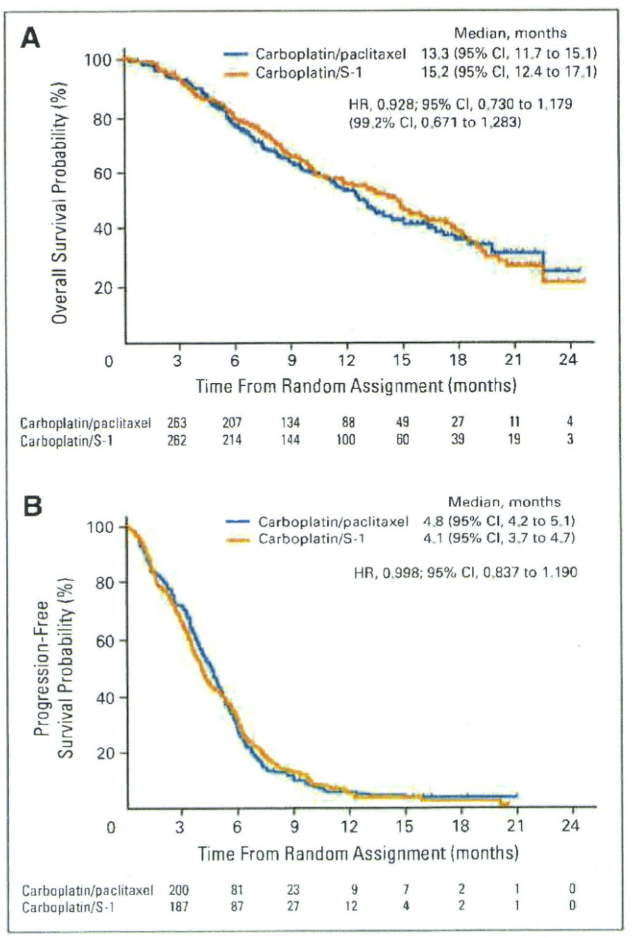

Fig 2. (A) Overall survival and (B) progression-free survival for the intent-to-treat population (n = 563). HR, hazard ratio.

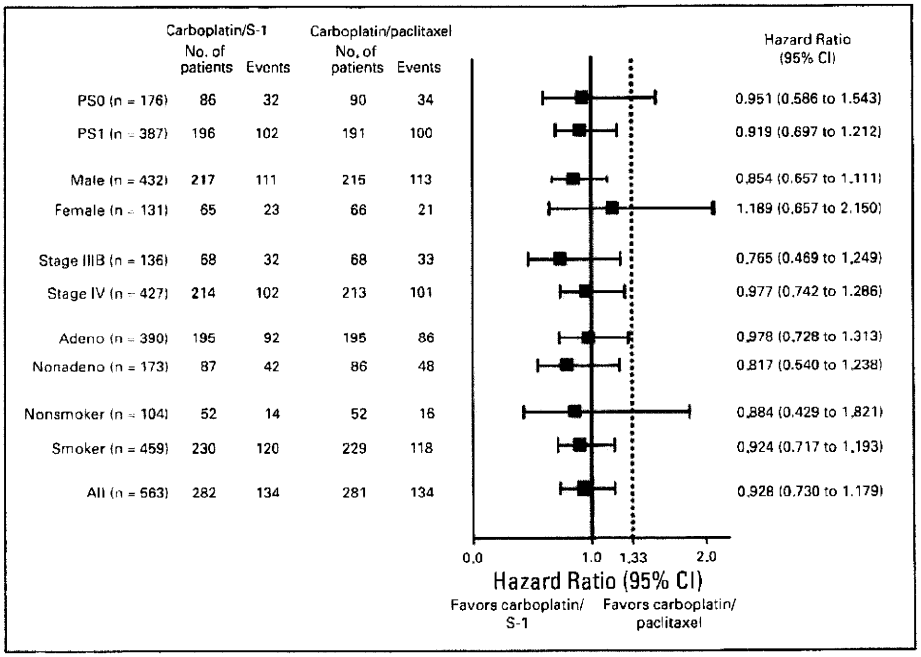

Fig 3. Subgroup analysis of overall survival in the intent-to-treat population (n = 563) PS, performance status; Adeno, adenocarcinoma; Nonadeno, nonadenocarcinoma.

hematologic toxicity occurred in a higher proportion of carboplatin and S-1 courses (51.6%) than carboplatin and paclitaxel courses (9.6%). S-1 was administered for the planned 14 days without interruption in 89.1% of carboplatin and S-1 courses. The median relative dose intensities were high for both carboplatin and paclitaxel (89.6% and 87.6%, respectively) and carboplatin and S-1 arms (83.3% and 94.3%, respectively). The most frequent reason for discontinuation of therapy was disease progression in both arms. Treatment was withdrawn before completion from a similar proportion of patients in each group (13.6% for carboplatin and paclitaxel and 10.7% for carboplatin and S-1) because of adverse events.

Efficacy

At the interim analysis planned for when patient enrollment was completed, 268 death events were available in total. The study passed the O'Brien-Fleming boundary of 0.0080 for a positive result with a *P* value of .002. The HR for OS (carboplatin and S-1 *v* carboplatin and paclitaxel) in the ITT population was 0.928, with a two-sided 99.2% CI after adjustment for multiplicity due to interim analysis of 0.671 to 1.283 (Fig 2A). Noninferiority of carboplatin and S-1 therapy was thus confirmed at the interim analysis by the upper limit of the CI being less than the protocol-specified margin of 1.33. The crude (unadjusted) 95% CI of the HR for OS of 0.928 was 0.730 to 1.179 in the ITT population, and an HR for OS of 0.931 (95% CI, 0.732 to 1.186) was obtained with the PP population. Median OS was 15.2 months (95% CI, 12.4 to 17.1) in the carboplatin and S-1 arm and 13.3 months (95% CI, 11.7 to 15.1) in the carboplatin and paclitaxel arm, with the 1-year survival rates being 57.3% and 55.5%, respectively. Subgroup analysis of OS in the ITT population according to stratification variables and other baseline characteristics were consistent with the primary analysis. A significant interaction effect between treatment arm and subgroups was not observed. The 95% CI for the HR in each subgroup included 1.00 (Fig 3).

The median PFS was 4.1 months in the carboplatin and S-1 arm and 4.8 months in the carboplatin and paclitaxel arm in the ITT population, with a corresponding HR of 0.998 and 95% CI of 0.837 to 1.190 (Fig 2B). In the PP population, the median values of PFS were 4.2 and 4.8 months for the carboplatin and S-1 and carboplatin and paclitaxel arms, respectively, with a corresponding HR of 0.992 and 95% CI of 0.832 to 1.184. Response to treatment was assessed in 279 patients (99.3%) of the carboplatin and paclitaxel group and in 279 patients (98.9%) of the carboplatin and S-1 group. For overall response (CR + PR) rate, carboplatin and paclitaxel was superior to carboplatin and S-1 (29.0% *v* 20.4%; $P = .019$, χ^2 test), whereas the overall disease control (CR + PR + stable disease) rate was similar in both treatment groups (73.5% *v* 71.7%, respectively; $P = .635$).

Safety

The incidence of leukopenia or neutropenia of grade 3 or 4 was significantly lower for patients in the carboplatin and S-1 arm than for those in the carboplatin and paclitaxel arm (leukopenia, 5% *v* 33%; neutropenia, 21% *v* 77%, respectively), as was the incidence of febrile neutropenia (1% *v* 7%; Table 2). Conversely, treatment with carboplatin and S-1 was associated with a higher rate of thrombocytopenia of grade 3 or 4 than was that with carboplatin and paclitaxel (33% *v* 9%, respectively). Platelet transfusion was also necessary for more patients in the carboplatin and S-1 arm than in the carboplatin and paclitaxel arm (8% *v* 2%, respectively; $P = .002$). The overall rates of neuropathy and alopecia were much lower in the carboplatin and S-1 arm (neuropathy, 16% *v* 81%; alopecia, 9% *v* 77%), whereas nausea, vomiting, and diarrhea occurred more frequently in the carboplatin/S-1 arm (Table 2). Death as a result of toxicity occurred in two patients; one death in the carboplatin and S-1 arm was associated with gastrointestinal hemorrhage, and another patient in the carboplatin and paclitaxel arm died of febrile neutropenia and pneumonia.

Table 2. Incidence of Drug-Related Toxicities in Randomly Assigned and Treated Patients

Toxicity	Regimen by Grade (%)						*P*	
	Carboplatin/Paclitaxel (n = 279)			Carboplatin/S-1 (n = 279)				
	All	3	4	All	3	4	All	3 or 4
Hematologic								
Leukopenia	86.0	29.7	2.9	55.4	5.0	0.4	< .001	< .001
Neutropenia	89.6	31.9	44.8	58.3	18.3	2.9	< .001	< .001
Anemia	82.4	14.3	2.5	86.7	15.5	3.6	.165	.680
Thrombocytopenia	63.1	7.2	2.2	87.4	19.4	13.3	< .001	< .001
Nonhematologic								
Febrile neutropenia	7.2	6.8	0.4	1.1	1.1	0	< .001	< .001
Nausea	49.1	2.2	0	62.4	1.8	0	.002	.475
Vomiting	23.7	1.1	0	34.1	1.8	0	.007	.837
Diarrhea	20.8	1.1	0	32.6	3.2	0	.002	.302
Neuropathy: sensory	81.0	2.9	0	15.8	0.4	0	< .001	.668
Arthralgia	67.4	2.5	0	7.9	0	0	< .001	.357
Alopecia	76.7			9.3			< .001	

NOTE. Differences between the two arms were evaluated by the χ^2 test.

QOL

At random assignment, 99.6% of patients (562 of 564) completed baseline questionnaires, with the questionnaire completion rates being 93.4% at 6 weeks and 90.1% at 9 weeks. Compliance rates were not significantly different between the treatment arms. QOL data were missing in 38 surveys due to death or severe impairment of the patient's general condition, which accounted for 2.3% of the total number of the surveys scheduled. There was no significant difference in the lung cancer subscale of FACT-L between the treatment arms (Fig 4). Scores on the neurotoxicity subscale of FACT/GOG-Ntx had decreased significantly in the carboplatin and paclitaxel arm after two cycles of chemotherapy (Fig 4); the adjusted mean scores at 6 and 9 weeks were 41.2 and 41.0 for the carboplatin and S-1 arm and 38.2 and 37.1 for the carboplatin and paclitaxel arm. The alopecia score was also significantly worse in the carboplatin and paclitaxel arm than in the carboplatin and S-1 arm ($P < .001$, analysis of variance), with the adjusted means at 6 and 9 weeks being 3.8 and 3.7 for carboplatin and S-1 and 1.7 and 1.9 for carboplatin and paclitaxel ($P < .001$ at both 6 and 9 weeks, Tukey-Kramer multiple-comparison test).

Poststudy Treatment

There were no major differences in poststudy treatment between the two arms. Overall, 69.4% of carboplatin and paclitaxel patients and 75.5% of carboplatin and S-1 patients received an additional line of therapy ($P = .103$, χ^2 test). Docetaxel was administered in 43.4% and 52.0% of patients and epidermal growth factor receptor tyrosine kinase inhibitors were administrated in 24.0% and 27.2% of patients in the carboplatin and paclitaxel and carboplatin and S-1 arms, respectively.

DISCUSSION

Our phase III study is the first to evaluate the efficacy of an S-1–containing regimen in comparison with standard platinum-doublet chemotherapy for first-line treatment of patients with advanced NSCLC. The primary objective of the study—determination of the noninferiority of carboplatin and S-1 compared with carboplatin and paclitaxel in terms of OS—was met at the planned interim analysis.

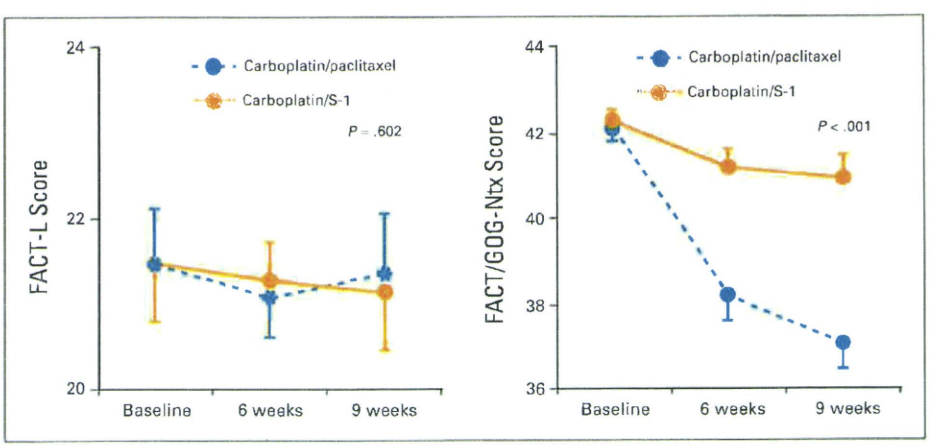

Fig 4. Quality of life assessments with the (left) seven-item Functional Assessment of Cancer Therapy–Lung (FACT-L) and (right) 11-item FACT/Gynecology Oncology Group-Neurotoxicity (GOG-Ntx) scales. Data are least square means ± 95% CI. Higher scores indicate better quality of life. *P* values shown were determined by analysis of variance, with *P* being less than .001 for comparison of FACT/GOG-Ntx scores between the two arms at both 6 and 9 weeks by the Tukey-Kramer multiple-comparison test.

Analysis of OS in the ITT and PP populations as well as in subgroups of the study subjects demonstrated the noninferiority of carboplatin and S-1. Although there was a significant difference in response rate favoring carboplatin and paclitaxel, disease control rate and PFS were similar for carboplatin and S-1 and carboplatin and paclitaxel. Given that subsequent therapies after discontinuation of the study treatment were well-balanced between the treatment groups, it is unlikely that poststudy therapy confounded survival results. Collectively, our secondary data indicate that the findings of the main analysis are robust. Although the protocol-specified noninferiority margin of 1.33 may be large, the survival curves themselves mostly coincided for the two treatment arms and median OS in the carboplatin and S-1 group was noteworthy at approximately 15 months.

The profile of adverse events associated with carboplatin and S-1 and carboplatin and paclitaxel was as expected, but there were marked differences in the incidence of some of these events. Carboplatin and paclitaxel treatment resulted in a typically high incidence of neutropenia of grade 3 or 4 (76.7%) as well as of febrile neutropenia (7.2%), compared with incidences of only 21.1% and 1.1%, respectively, for carboplatin and S-1. These rates of neutropenia associated with carboplatin and paclitaxel treatment are consistent with those observed in previous studies of Japanese patients.[11,14] Carboplatin and S-1 treatment showed a significantly higher rate of thrombocytopenia, which was the most frequent reason for dose delays in the carboplatin and S-1 group. However, this condition was considered manageable because it was associated with bleeding of grade 3 in only one patient. With regard to nonhematologic toxicities, neuropathy, arthralgia, and alopecia were much less frequent in patients treated with carboplatin and S-1 than in those receiving carboplatin and paclitaxel. Consistent with these results, carboplatin and S-1 treatment showed a clinically relevant improvement in QOL as assessed by the FACT/GOG-Ntx scale and alopecia score. Despite these QOL benefits with carboplatin and S-1, however, there was no significant difference in FACT-L score between carboplatin and S-1 and carboplatin and paclitaxel, possibly because of other more toxic effects of carboplatin and S-1. The incidence of nausea, vomiting, and diarrhea of any grade was higher in patients assigned to the carboplatin and S-1 arm than in those assigned to carboplatin and paclitaxel, although grades 3 or 4 of these toxicities were uncommon (< 4%) in both groups. The relative dose intensity of S-1 was 94.3% in the carboplatin and S-1 arm (median of four cycles administered), and treatment was discontinued in only approximately 10% of patients in this arm because of adverse events. Overall, these data indicate that carboplatin and S-1 was well-tolerated, with continuation of treatment as specified in the protocol not being a problem. According to our previous phase I/II study of carboplatin and S-1,[9] this study excluded elderly (≥ 75 years old) patients. Given its efficacy and favorable toxicity profile, the combination of S-1 and carboplatin warrants further evaluation in elderly patients.

In conclusion, our present study demonstrates the noninferiority of carboplatin and S-1 relative to carboplatin and paclitaxel in terms of OS for patients with advanced NSCLC. Carboplatin and S-1 is therefore a valid therapeutic option for the first-line treatment of patients with advanced NSCLC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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REFERENCES

1. Jemal A, Siegel R, Ward E, et al: Cancer statistics, 2009. CA Cancer J Clin 59:225-249, 2009
2. Azzoli CG, Baker S Jr, Temin S, et al: American Society of Clinical Oncology Clinical Practice Guideline update on chemotherapy for stage IV non-small-cell lung cancer. J Clin Oncol 27:6251-6266, 2009
3. Sandler A, Gray R, Perry MC, et al: Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med 355:2542-2550, 2006
4. Scagliotti GV, Parikh P, von Pawel J, 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 26:3543-3551, 2008
5. Reck M, von Pawel J, Zatloukal P, et al: Phase III trial of cisplatin plus gemcitabine with either placebo or bevacizumab as first-line therapy for nonsquamous non-small-cell lung cancer: AVAil. J Clin Oncol 27:1227-1234, 2009
6. Shirasaka T, Nakano K, Takechi T, et al: Antitumor activity of 1 M tegafur-0.4 M 5-chloro-2,4-dihydroxypyridine-1 M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted into nude rats. Cancer Res 56:2602-2606, 1996
7. Okamoto I, Fukuoka M: S-1: A new oral fluoropyrimidine in the treatment of patients with advanced non-small-cell lung cancer. Clin Lung Cancer 10:290-294, 2009
8. Kawahara M, Furuse K, Segawa Y, et al: Phase II study of S-1, a novel oral fluorouracil, in advanced non-small-cell lung cancer. Br J Cancer 85:939-943, 2001

9. Tamura K, Okamoto I, Ozaki T, et al: Phase I/II study of S-1 plus carboplatin in patients with advanced non-small cell lung cancer. Eur J Cancer 45:2132-2137, 2009

10. Therasse P, Arbuck SG, Eisenhauer EA, et al: New guidelines to evaluate the response to treatment in solid tumors: European Organisation for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205-216, 2000

11. Ohe Y, Ohashi Y, Kubota K, et al: Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-arm cooperative study in Japan. Ann Oncol 18:317-323, 2007

12. Cella DF, Bonomi AE, Lloyd SR, et al: Reliability and validity of the Functional Assessment of Cancer Therapy-Lung (FACT-L) quality of life instrument. Lung Cancer 12:199-220, 1995

13. Cella D, Peterman A, Hudgens S, et al: Measuring the side effects of taxane therapy in oncology: The functional assesment of cancer therapy-taxane (FACT-taxane). Cancer 98:822-831, 2003

14. Kubota K, Kawahara M, Ogawara M, et al: Vinorelbine plus gemcitabine followed by docetaxel versus carboplatin plus paclitaxel in patients with advanced non-small-cell lung cancer: A randomised, open-label, phase III study. Lancet Oncol 9:1135-1142, 2008

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Cancer Research

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

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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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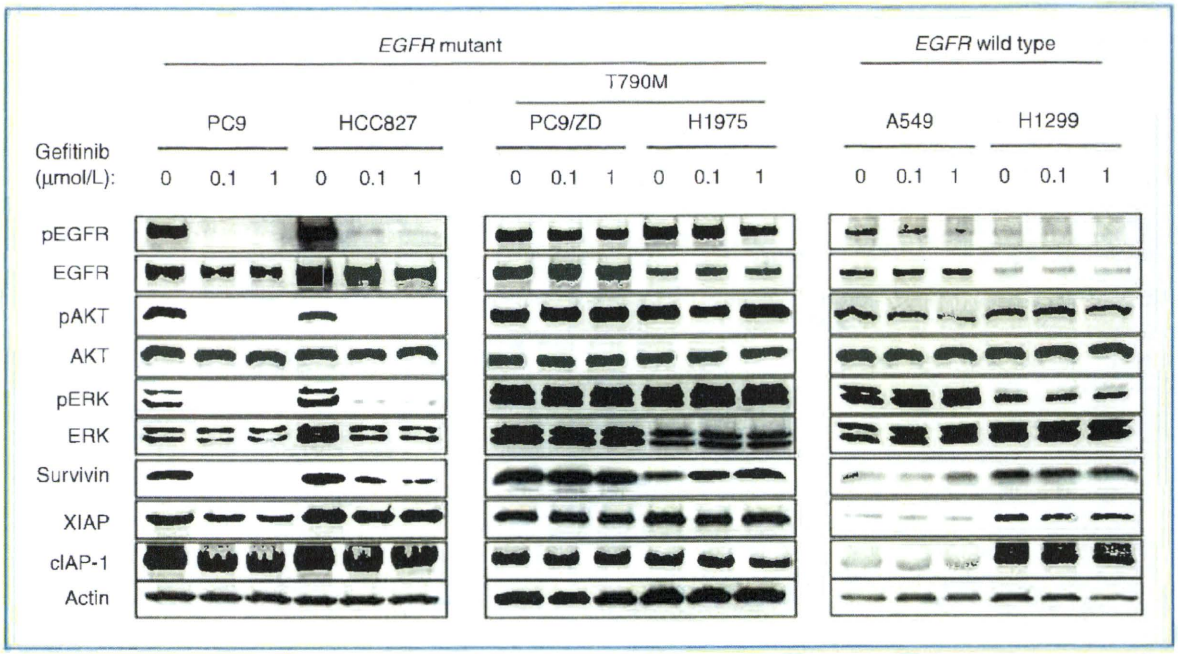

Figure 1. Effects of gefitinib on the expression of IAP family proteins in human NSCLC cells. PC9, HCC827, PC9/ZD, H1975, A549, or H1299 cells were incubated in complete medium and in the presence of the indicated concentrations of gefitinib for 24 hours. Cell lysates were then prepared and subjected 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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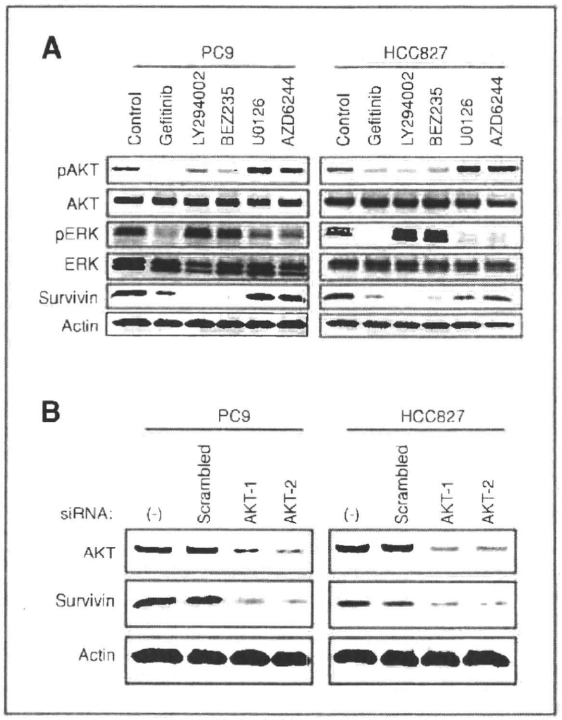

Figure 2. Effects of inhibition of MEK or PI3K signaling pathways on survivin expression in *EGFR* mutation–positive NSCLC cells. A, PC9 or HCC827 cells were incubated in the absence (control, 0.1% dimethyl sulfoxide) or the presence of gefitinib (1 μmol/L), LY294002 (20 μmol/L), BEZ235 (0.2 μmol/L), 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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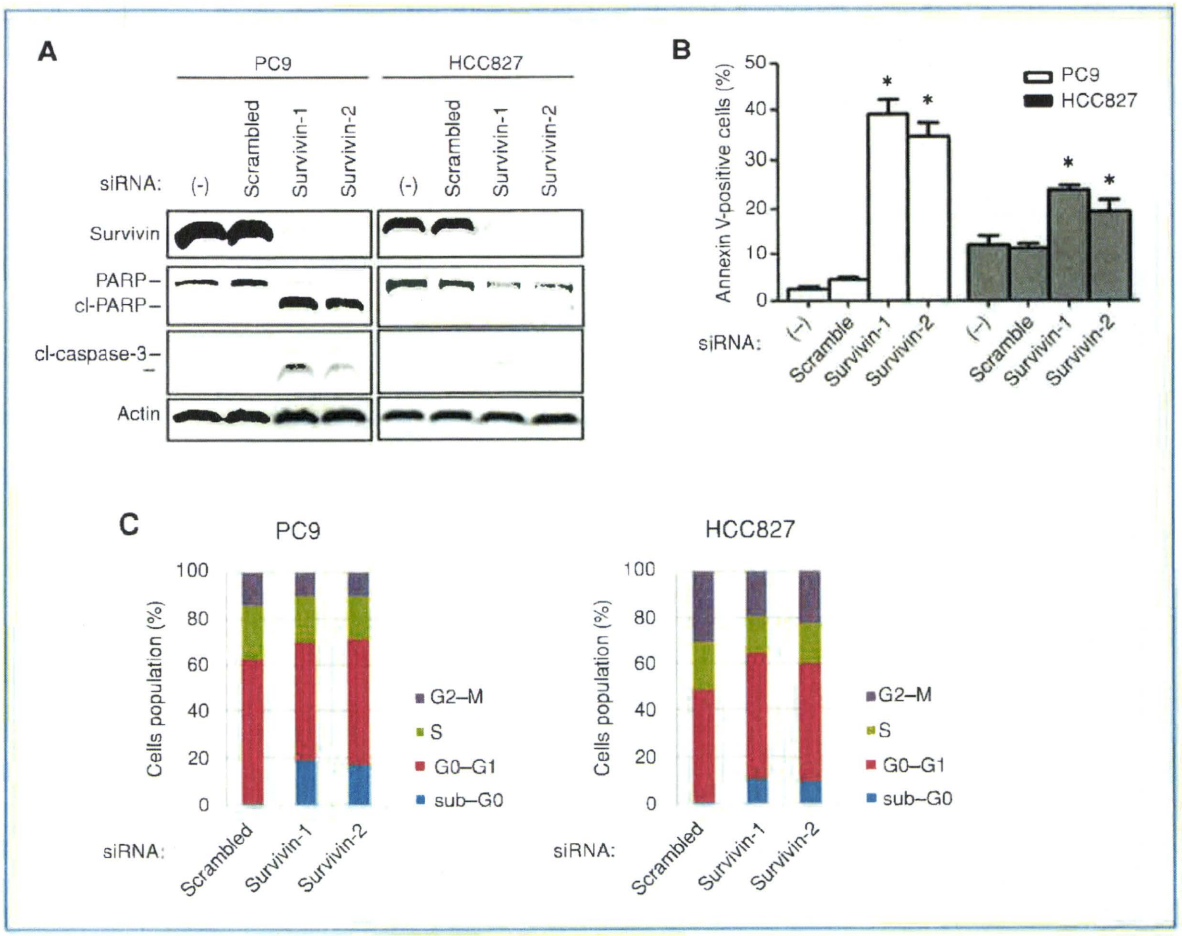

Figure 3. Effect of survivin depletion on apoptosis in *EGFR* mutation–positive NSCLC cells. A, PC9 or HCC827 cells were transfected (or not) with 2 different survivin (survivin-1 or survivin-2) or scrambled (control) siRNAs for 48 hours, after which cell lysates were prepared and subjected to immunoblot 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