Table 3 Non-hematological toxicity

Group A (Ccr ≥60 mL/min)	Dose level (mg/m^2), (number of patients)									
	80 ($n = 7$) Grade					100 ($n = 15$) Grade				
Event	0	1	2	3	4	0	1	2	3	4
Nausea	5	1	1	0	0	3	9	3	0	0
Vomiting	6	1	0	0	0	15	0	0	0	0
Anorexia	5	1	1	0	0	7	4	4	0	0
Diarrhea	6	1	0	0	0	14	1	0	0	0
Stomatitis	7	0	0	0	0	15	0	0	0	0
Hyperbilirubinemia	6	0	1	0	0	15	0	0	0	0
AST increase	6	1	0	0	0	13	2	0	0	0
ALT increase	6	1	0	0	0	13	2	0	0	0
ALP increase	7	0	0	0	0	15	0	0	0	0
Cr increase	7	0	0	0	0	15	0	0	0	0

Group B (40 ≤ Ccr < 60 mL/min)	Dose level (mg/m^2), (number of patients)														
	60 ($n = 6$) Grade					80 ($n = 6$) Grade					100 ($n = 5$) Grade				
Event	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Nausea	1	4	1	0	0	1	3	2	0	0	1	1	3	0	0
Vomiting	6	0	0	0	0	5	1	0	0	0	5	0	0	0	0
Anorexia	4	2	0	0	0	1	3	2	0	0	1	1	3	0	0
Diarrhea	6	0	0	0	0	5	1	0	0	0	5	0	0	0	0
Stomatitis	6	0	0	0	0	6	0	0	0	0	5	0	0	0	0
Hyperbilirubinemia	6	0	0	0	0	6	0	0	0	0	4	0	1	0	0
AST increase	4	2	0	0	0	5	0	1	0	0	4	0	1	0	0
ALT increase	5	1	0	0	0	5	0	1	0	0	4	0	1	0	0
ALP increase	6	0	0	0	0	5	1	0	0	0	5	0	0	0	0
Cr increase	6	0	0	0	0	4	2	0	0	0	4	0	1	0	0

AST asparatate aminotransferase, *ALT* serum alanine aminotransferase, *ALP* alkaline phosphatase, *Cr* creatinine

Discussion

In this dose-finding study, we evaluated the toxicities, pharmacokinetics as well as antitumor activity, and determined the recommended doses of nedaplatin for elderly patients with advanced NSCLC based on renal function. The predominant toxicities were hematological, such as leukopenia, neutropenia and thrombocytopenia, in both groups. These hematological toxicities tended to increase in severity with the increased dose level of nedaplatin. Non-hematological toxicities were acceptable and those were not dose limiting in either group. The recommended dose was determined as 100 mg/m^2 every 4 weeks in elderly patients with a renal function of Ccr ≥ 60 mL/min, which is the same dose recommended for patients aged ≤70 years. On the other hand, for elderly patients with a renal function of 40 ≤ Ccr < 60 mL/min, the recommended dose was 80 mg/m^2 every 4 weeks. In this study,

Table 4 Response

Group	Dose level (mg/m^2)	No. of patients	Response				PR	
			CR	PR	SD	PD	Sq.	Non–sq.
Group A (Ccr ≥60 mL/min)	80	7	0	2	3	2	2	0
	100	15	0	4	6	5	4	0
Group B (40 ≤ Ccr < 60 mL/min)	60	6	0	3	2	1	2	1
	80	6	0	3	1	2	3	0
	100	5	0	1	1	3	1	0
Total		39	0	13	13	13	12	1

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *Sq.* squamous cell carcinoma, *Non-sq.* non-squamous cell carcinoma

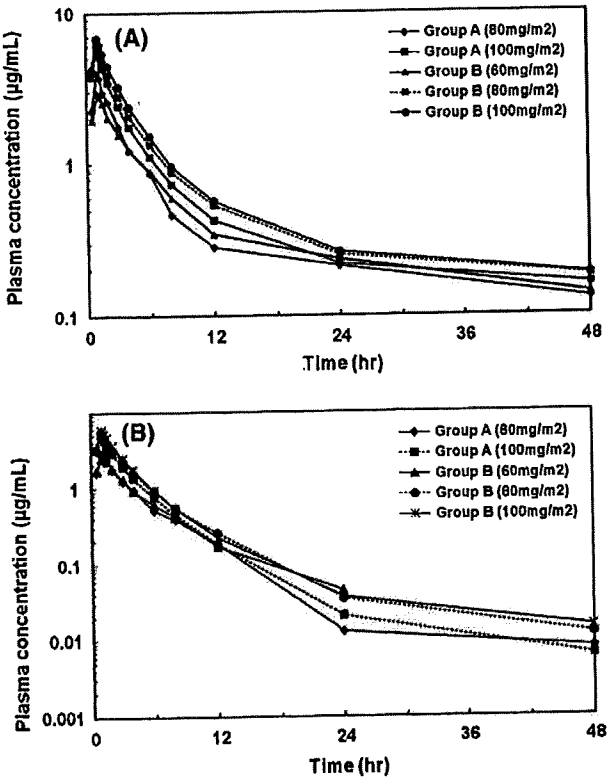

Fig. 1 Mean plasma concentration–time profiles for: a total-Pt and b free-Pt of nedaplatin

an additional nine patients were enrolled at the dose level of 100 mg/m^2 in Group A. First, the favorable antitumor response was observed in squamous cell carcinoma and we intended to evaluate the antitumor response mainly for squamous cell carcinoma. Then, five of nine additional patients enrolled had squamous cell carcinoma. Second, the recommended dose was determined as 100 mg/m^2 in Group A, which was the same dose in younger patients. We intended to confirm the toxicity and pharmacokinetic profiles in this elderly subgroup.

In the development of chemotherapy for elderly patients, the selection of appropriate agents is extremely important. Candidate agents must have confirmed antitumor activities and acceptable toxicity profiles in younger patients (e.g., aged ≤70 years). In this study, we investigated nedaplatin as it had a lower incidence of associated emesis and nephrotoxicity, compared with cisplatin, and favorable antitumor activity in NSCLC patients aged ≤70 years. Furthermore, the current standard treatment for elderly patients with advanced NSCLC, that is, third-generation single-agent chemotherapy such as vinorelbine, gemcitabine or docetaxel, had not been established at the time of planning of the study [15–17]. The DLT of nedaplatin in patients aged ≤70 years was reported to be thrombocytopenia, which is correlated with renal function; therefore, we expected that nedaplatin could be safely administered to elderly patients by stratifying the patients according to renal function. Patients with a Ccr ≥40 mL/min were eligible for inclusion in this study based on the results of a previous PK analysis examining the correlation between the nadir platelet count and renal function (described in "Introduction") [11]. When younger patients with a Ccr ≥40 mL/min were treated with 100 mg/m^2 of nedaplatin, the predicted nadir platelet count was ≥50,000/mm^3. Therefore, the initial doses of nedaplatin in Group A (Ccr ≥60 mL/min) and Group B (40 ≤ Ccr < 60 mL/min) were determined to be 80 and 60 mg/m^2, respectively. The dose escalation over 100 mg/m^2 was not planned, because the recommended dose in younger patients (aged ≤70 years) had already been determined at 100 mg/m^2.

In this study, milder criteria of DLT was applied, compared with that used in conventional phase I studies. In this developmental strategy, we pursued "the recommended dose with moderate and acceptable toxicities for the majority of elderly patients", instead of "the recommended dose with the severe toxicities in a small and limited number of patients, as per most conventional phase I studies", because the physiological and pharmacological function of elderly patients is highly variable.

Table 5 Pharmacokinetic parameters of total-Pt and free-Pt

Group	Dose level (mg/m^2)	No. of patients	No. of assessables for PK analysis	C_{max} (μg/mL)	AUC (μg/mL h)	V_{dss} (L)	$T_{1/2}$ (h)	CL (L/h)
PK parameters of total-Pt								
Group A (Ccr ≥60 mL/min)	80	7	2[a]	4.02 (3.49, 4.57)	22.58 (13.46, 31.69)	64.24 (35.27, 93.21)	14.15 (3.25, 25.04)	6.00 (3.60, 8.40)
	100	15	13	5.94 ± 1.38	21.65 ± 4.54	31.50 ± 13.40	3.28 ± 1.35	7.63 ± 1.74
Group B (40 ≤ Ccr < 60 mL/min)	60	6	2[a]	3.02 (2.91, 3.12)	19.78 (14.87, 24.68)	57.05 (33.21, 80.89)	10.77 (4.08, 17.46)	5.21 (4.16, 6.25)
	80	6	6	6.35 ± 1.11	25.99 ± 9.68	29.29 ± 13.18	7.88 ± 8.97	6.10 ± 1.13
	100	5	5	6.83 ± 1.20	32.11 ± 7.86	32.84 ± 22.00	6.62 ± 4.55	5.01 ± 1.57
PK parameters of free-Pt								
Group A (Ccr ≥60 mL/min)	80	7	2[a]	2.72 (2.13, 3.31)	10.56 (7.05, 14.06)	42.30 (37.98, 46.62)	3.49 (2.70, 4.28)	12.08 (8.11, 16.04)
	100	15	13	5.11 ± 1.51	16.20 ± 3.34	32.26 ± 11.17	3.51 ± 4.02	10.26 ± 2.46
Group B (40 ≤ Ccr < 60 mL/min)	60	6	2[a]	2.55 (2.46, 2.64)	11.59 (11.38, 11.79)	49.33 (33.22, 65.43)	6.16 (2.98, 9.34)	8.45 (7.89, 9.01)
	80	6	6	5.52 ± 1.25	18.53 ± 7.12	29.51 ± 9.11	3.40 ± 0.65	7.25 ± 2.21
	100	5	5	5.91 ± 1.21	20.69 ± 5.52	29.63 ± 12.32	2.92 ± 0.66	7.87 ± 2.71
Patients ≤70 years [14]	100	5	5		15.9			

Data are shown as mean ± SD excepting the dose level of 80 mg/m^2 in Group A and 60 mg/m^2 in Group B

PK pharmacokinetics, *total-Pt* total platinum, *free-Pt*, free platinum, C_{max} maximum plasma concentration, *AUC* area under the plasma concentration versus time curve, V_{dss} volume of distribution at steady-state, $T_{1/2}$ terminal half life, *CL* systemic clearance

[a] Data are shown as mean (actual data)

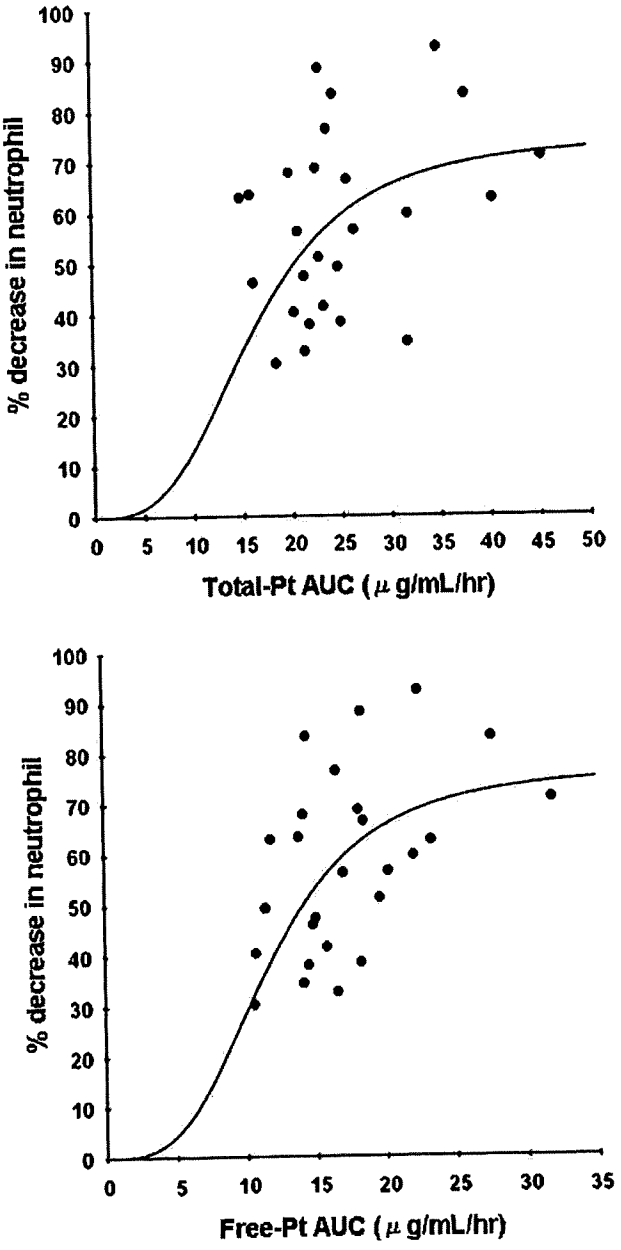

Fig. 2 Relationship between AUCs of total/free-Pt and the percentage decrease in the neutrophil count

In the pharmacokinetic analysis, the free-Pt AUC at a dose of 100 mg/m^2 in Group A seemed similar to that of 80 mg/m^2 in Group B, and there was no significant difference between these two treatment subgroups ($P = 0.336$). These results endorsed an almost equivalent drug exposure in both patient groups, stratified according to renal function. Furthermore, the AUC values in both groups seemed similar to historical data (obtained in a study with a small sample size) for patients aged ≤70 years [14]. However, a significant correlation was not observed between the renal function (i.e., the Ccr value) and the nadir platelet count, as in a previous report examining younger patients. These were possibly attributed to the wide inter-patient physiological and pharmacological variability among elderly patients or just the consequence of the adaptation of dose [11]. For elderly patients, a strict dose calculation of nedaplatin based on renal function, such as the dose calculation for carboplatin using the Calvert formula [18], is not required, and a simple dose selection of nedaplatin stratified according to renal function is considered to be reasonable.

A total of 13 (33%) of the 39 patients achieved partial responses. In this study, 21 patients with squamous cell carcinoma were enrolled, 12 patients achieved PR and the response rate was 57%. The biological mechanism responsible for the antitumor activity of nedaplatin against squamous cell carcinoma of the lung remains unknown. In the pharmacokinetic analysis, no significant differences were observed in responding patients with squamous cell carcinoma compared with non-responding others. However, nedaplatin also has a favorable antitumor activity against head and neck cancer and esophageal cancer, which also have a high frequency of squamous cell histology [19–22]. Although antitumor activity was evaluated only in elderly patients in this study, the development of this activity is worthwhile in the treatment of NSCLC with squamous cell histology. Furthermore, a translational study to identify the biological and/or genetic mechanism responsible for the antitumor activity of nedaplatin against squamous cell carcinoma is also warranted.

In conclusion, the recommended doses of nedaplatin for elderly patients with NSCLC were determined based on renal function, a dose of 100 mg/m^2 every 4 weeks was recommended for patients with a Ccr ≥60 mL/min, and a dose of 80 mg/m^2 every 4 weeks was recommended for patients with 40 ≤ Ccr < 60 mL/min. Nedaplatin can be safely administered to elderly patients with an acceptable level of toxicity and favorable antitumor activities against NSCLC, especially squamous cell carcinoma.

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implicated in the proliferation and survival of cancer cells.[6] In two phase II trials in patients with pretreated advanced NSCLC (Iressa Dose Evaluation in Advanced Lung Cancer [IDEAL] 1 and 2), gefitinib 250 mg/d showed response rates of 12% and 18% and a median survival time (MST) of 7.0 and 7.6 months in IDEAL1 and 2, respectively; in addition, the toxicity profile was not severe.[7,8] This favorable tolerability profile, coupled with a mechanism of action that is distinct from that of cytotoxic agents, provides a strong rationale for use of gefitinib in combination with standard cytotoxic regimens. Platinum-doublet chemotherapy added to gefitinib in untreated patients with NSCLC was evaluated in two large-scale, placebo-controlled, randomized trials (INTACT-1 and -2).[9,10] Gefitinib showed no survival benefit over placebo when combined with standard platinum-doublet chemotherapy in both trials. Furthermore, gefitinib did not improve time to progression or objective tumor response over chemotherapy alone. These results were disappointing and surprising because of the significant antitumor activity of gefitinib when given alone to pretreated patients with NSCLC.

First, it is possible that each of the agents is working against a susceptible subpopulation of tumor cells so that the effect is redundant rather than additive, or that one agent results in the loss of an intermediary molecule that is essential to the function of the other agent, resulting in an antagonistic effect. Second, patients included in these studies were not selected on the basis of a specific biomarker, such as target EGFR expression, gene amplification, or mutations. Clinical profiles of females, never smokers, adenocarcinoma histology, and Asian ethnicity have all been recognized as favorable subgroups that respond to gefitinib.[11-14]

Because no additive effect was observed by administering gefitinib continuously in combination with chemotherapy, possible alternatives could be the administration of gefitinib in the interval between chemotherapy cycles or as sequential treatment after chemotherapy. This could also potentially prevent the problem of drug interference or antagonism. We conducted a randomized phase III trial to evaluate whether gefitinib improves survival as sequential therapy after platinum-doublet chemotherapy in chemotherapy-naive patents with NSCLC.

PATIENTS AND METHODS

Patients

Eligible patients were 20 to 75 years of age, with histologically or cytologically confirmed stage IIIB (with malignant pleural effusion or contralateral hilar lymph node metastases) or stage IV NSCLC who had not previously received any chemotherapy. Patients who had recurrence after complete surgical resection were permitted. Patients treated with either adjuvant or neoadjuvant chemotherapy were excluded in this trial. Additional criteria included a Eastern Cooperative Oncology Group performance status of 0 to 1, and adequate organ function as indicated by WBC count $\geq$ 4,000/μL, absolute neutrophil count $\geq$ 2,000/μL, hemoglobin $\geq$ 9.5 g/dL, platelets $\geq$ 100,000/μL, AST/ALT $\leq$ 2.5 times the upper limit of normal, total bilirubin $\leq$ 1.5 mg/dL, serum creatinine $\leq$ 1.2 mg/dL, and PaO_2 in arterial blood $\geq$ 70 mmHg. Asymptomatic brain metastases were allowed provided that they had been irradiated and were clinically and radiologically stable. Patients were excluded from the study if they had radiologically and clinically apparent interstitial pneumonitis or pulmonary fibrosis. All patients provided written informed consent, and the study protocol was approved by the West Japan Thoracic Oncology Group Protocol Review Committee and the institutional review board of each participating institution.

Treatment Plan

Eligible patients were centrally registered at West Japan Thoracic Oncology Group Data Center and were randomly assigned to receive either platinum-doublet chemotherapy up to six cycles (arm A) or three cycles of platinum doublet followed by gefitinib 250 mg/d orally, until disease progression (arm B). Patients who achieved disease control (response or stable disease) treated with three cycles of platinum-doublet went for gefitinib treatment phase in arm B. Each physician selected his/her chemotherapy options before randomization. Platinum-doublet chemotherapy options included any of the following: (1) carboplatin area under the curve 6, day 1, and paclitaxel 200 mg/m^2, day 1, every 3 weeks; (2) cisplatin 80 mg/m^2, day 1, and irinotecan 60 mg/m^2, days 1, 8, 15, every 4 weeks; (3) cisplatin 80 mg/m^2, day 1, and vinorelbine 25 mg/m^2, days 1, 8, every 3 weeks; (4) cisplatin 80 mg/m^2, day 1, and gemcitabine 1,000 mg/m^2 days 1, 8, every 3 weeks; or (5) cisplatin 80 mg/m^2, day 1, and docetaxel 60 mg/m^2 day 1, every 3 weeks. The dose of carboplatin was calculated using Calvert's formula, and the glomerular filtration rate was estimated by the Cockcroft-Gaut formula. These treatment schedules and doses are used as standard platinum-doublet regimens for advanced NSCLC in Japan.[15,16]

Randomization was stratified according to the institution, type of histology (adenocarcinoma *v* nonadenocarcinoma), clinical stage (IIIB *v* IV), and selected platinum-doublet regimens with the use of a minimization procedure. Patients receiving platinum-doublet chemotherapy received standard supportive treatments, including hydration and antiemetics, according to each institutional standard guideline. After withdrawing from the trial as a result of disease progression or intolerable toxicity, any systemic treatment, including with EGFR-TKI, was permitted in both arms.

Baseline and Follow-Up Assessments

Pretreatment evaluation included a complete medical history and physical examination, a CBC with differential and platelet count, standard biochemical profile, ECG, chest radiographs, computed tomography (CT) scans of the chest, abdomen, and brain, magnetic resonance imaging, and a whole-body bone scan. During treatment, a CBC and biochemical tests were performed at least every 2 weeks. A detailed medical history was taken and a complete physical examination with clinical assessment was performed every 2 weeks to assess disease symptoms and treatment toxicity, and chest

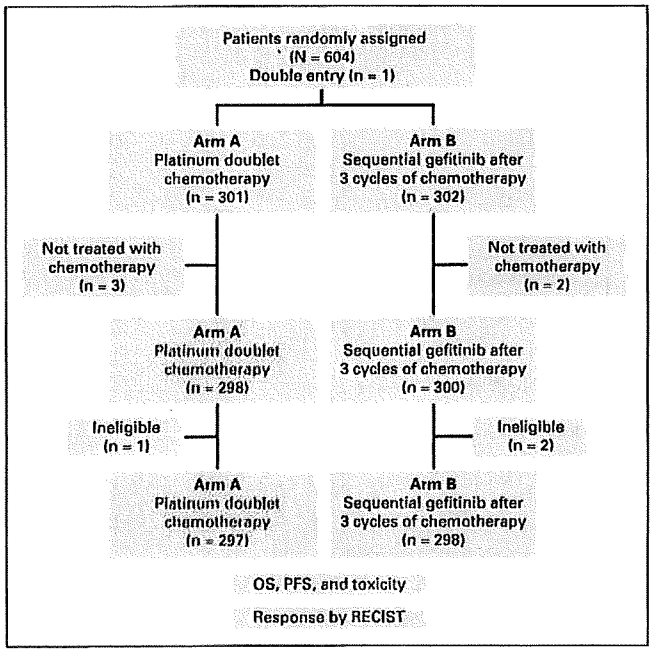

Fig 1. CONSORT diagram for the study. OS, overall survival; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors.

radiographs were done every treatment cycle. Toxicity was evaluated according to the National Cancer Institute Cancer Common Toxicity Criteria (NCI-CTC) version2.[17]

All patients were assessed for response by CT scans monthly during treatment. Response Evaluation Criteria in Solid Tumors (RECIST) were used for the evaluation of response.[18]

Disease-related symptoms were assessed using the Lung Cancer Subscale (LCS) of the Functional Assessment of Cancer Therapy-Lung quality of life instrument (version 4.0).[19] Patients were asked to complete the instrument at the time of enrollment and at 12 weeks and 18 weeks after initiation of treatment. The maximum attainable score on the LCS was 28, where the patient was considered asymptomatic.

Statistical Analysis

The primary end point was OS; secondary end points included PFS, tumor response, safety, and quality of life. Based on previous trials evaluating platinum-doublet chemotherapy, the MST was approximately a range of 8 to 11 months.[3] In IDEAL-1, which was the trial of gefitinib alone in patients with previously treated NSCLC, median time to treatment failure was 98 days.[7] This trial was designed to detect a 3-month difference in MST. To attain 80% power at a two-sided significance level of .05, assuming a MST in the chemotherapy alone arm of 9 months with 2 years of follow-up after 3 years of accrual, 225 patients in each treatment group were required. Both the OS and PFS were estimated with the Kaplan-Meier method. Comparisons of OS and PFS between arms were assessed by the stratified log-rank test. Two interim analyses were planned after half the patients were registered and at the end of registration.

At the first interim analysis, 14% of patients in arm B unexpectedly withdrew from sequential gefitinib treatment after the three cycles of platinum-doublet chemotherapy at their own request because of hearing the news of interstitial lung disease (ILD) as a result of the use of gefitinib in Japan. If 15% of patients treated with sequential gefitinib withdrew, 284 patients in each arm were required to attain an 80% power at a two-sided significance level of .05, assuming a MST of the chemotherapy alone arm of 9 months with 2 years of follow-up after 3 years of accrual. Consequently, a protocol amendment was performed in April 2004.

For symptom analysis, comparisons of LCS between arms were conducted using a linear mixed-effects model in which the missing data depend on the observed LCS, using the MIXED procedure in SAS version 9 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

From March 2003 to May 2005, 604 patients with advanced NSCLC from 39 institutions were enrolled (Appendix, online only). Patients were randomly assigned to platinum-doublet chemotherapy up to 6 cycles (n = 302, arm A) or sequential gefitinib after three cycles of platinum-doublet chemotherapy (n = 302, arm B). One patient was double entry in arm A, and three patients in arm A and two in arm B did not receive any chemotherapy. Therefore, a total of 598 patients (298 in arm A and 300 in arm B) were included in the analysis of patients' profiles and the assessment for toxicity. In addition, three patients did not meet the entry criteria; thus, 297 patients with measurable lesions by RECIST in arm A and 298 eligible patients in arm B were assessable for OS, PFS, and response. Figure 1 shows the CONSORT diagram. Table 1 presents baseline patient characteristics and lists the platinum-doublet chemotherapy regimen selected by each physician.

Table 1. Patients' Characteristics and Selected Platinum-Doublet Chemotherapy Regimens

Parameter	Arm A		Arm B		P
	No. of Patients	%	No. of Patients	%	
Patients enrolled	298		300		—
Median age, years	63		62		.114
Range	35-74		25-74		
Sex					
Male	191	34.6	192	64.0	.981
Female	107	67.8	108	36.0	
ECOG PS					
0	103	30.8	90	30.0	.778
1	195	69.2	210	70.0	
Histology					
Adenocarcinoma	232	77.9	237	79.0	.733
Nonadenocarcinoma	66	22.1	63	21.0	
Clinical stage					
IIIB	54	18.1	55	18.3	.946
IV	244	81.9	245	81.7	
Smoking status					
Smoker	202	67.8	210	70.0	.559
Nonsmoker	96	32.2	90	30.0	
Selected platinum-doublet chemotherapy regimens					
CP	193	64.8	195	65.0	.987
IP	8	2.7	10	3.3	
VP	44	14.8	45	15.0	
GP	45	15.1	42	14.0	
DP	8	2.7	8	2.7	

NOTE. Differences between two arms were tested by χ^2 test, excluding age (Wilcoxon test), ECOG PS.
Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; CP, carboplatin and paclitaxel; IP, irinotecan and cisplatin; VP, vinorelbine and cisplatin; GP, gemcitabine and cisplatin; DP, docetaxel and cisplatin.

Treatment Delivery

The median number of chemotherapy cycles was three (range, 1 to 6) in arm A, and three (range, 1 to 3) in arm B. One hundred seventy-two patients (57.3%) in arm B were treated with gefitinib after completion of three cycles of platinum-doublet. The median treatment duration of gefitinib was 69.5 days, and the maximum treatment duration was 1,324 days. As presented in Figure 2, EGFR-TKIs, which included gefitinib, erlotinib, and vandetanib, were used in 54.5% and 75.2% of patients in arm A and B, respectively, at any time during treatment of NSCLC. In arm B, gefitinib treatment did not take place because of early disease progression before the completion of three cycles of platinum-doublet chemotherapy in 93 patients (31.2%), and 33 (11.1%) in arm B rejected the use of gefitinib after platinum-doublet because of publication of a news report about gefitinib-induced ILD.

Treatment Efficacy

At the time of final analysis, 247 (83.2%) and 232 patients (78.0%) had died in arm A and arm B, respectively. The MST was 12.9 months for chemotherapy alone and 13.7 months for chemotherapy followed by gefitinib (hazard ratio [HR] according to Cox's regression model, 0.86; 95% CI, 0.72 to 1.03; $P = .11$ stratified log-rank test, Fig 3A). The PFS was 4.3 months in arm A and 4.6 months in arm B (HR, 0.68; 95% CI, 0.57 to 0.80; $P < .001$, Fig 3B).

When exploratory subset analysis were performed, sequential therapy with gefitinib after three cycles of platinum-doublet chemotherapy prolonged OS significantly in the subset of patients with adenocarcinoma (HR, 0.79; 95% CI, 0.65 to 0.98; $P = .03$; Fig 4A). There was no significant difference in OS due to the small subset of patients with nonadenocarcinoma (HR, 1.24; 95% CI, 0.85 to 1.79; $P = .25$; Fig 4B). In addition to the OS plots, the PFS plots for adenocarcinoma and nonadenocarcinoma were showed in Figure 4C and 4D, respectively. Furthermore, results of the subset analysis were summarized for forest plots in Figure 5. Another subset of smokers had a survival advantage with chemotherapy followed by gefitinib over chemotherapy alone. There was no difference between the two treatment groups in the subset of never smokers. Never smokers with NSCLC had a prolonged survival of about 23.5 months in arm A and 21.7 months in arm B.

The overall response rate was 29.3% for chemotherapy alone and 34.2% for chemotherapy followed by gefitinib. There was no significant difference between treatment arms ($P = .20$; Fisher's exact test). The overall disease control rate (response and stable disease) were 71.0% and 75.5% in arm A and in arm B, respectively ($P = .22$).

Toxicity

Toxicity was assessed according to NCI-CTC version 2 in all patients who received at least one treatment cycle of platinum-doublet chemotherapy (Table 2). Grade 3 or 4 anemia developed in 21.8% of patients in arm A and 13.3% of patients in arm B. There was a significant difference between the two arms ($P = .006$). Grade 3 or 4

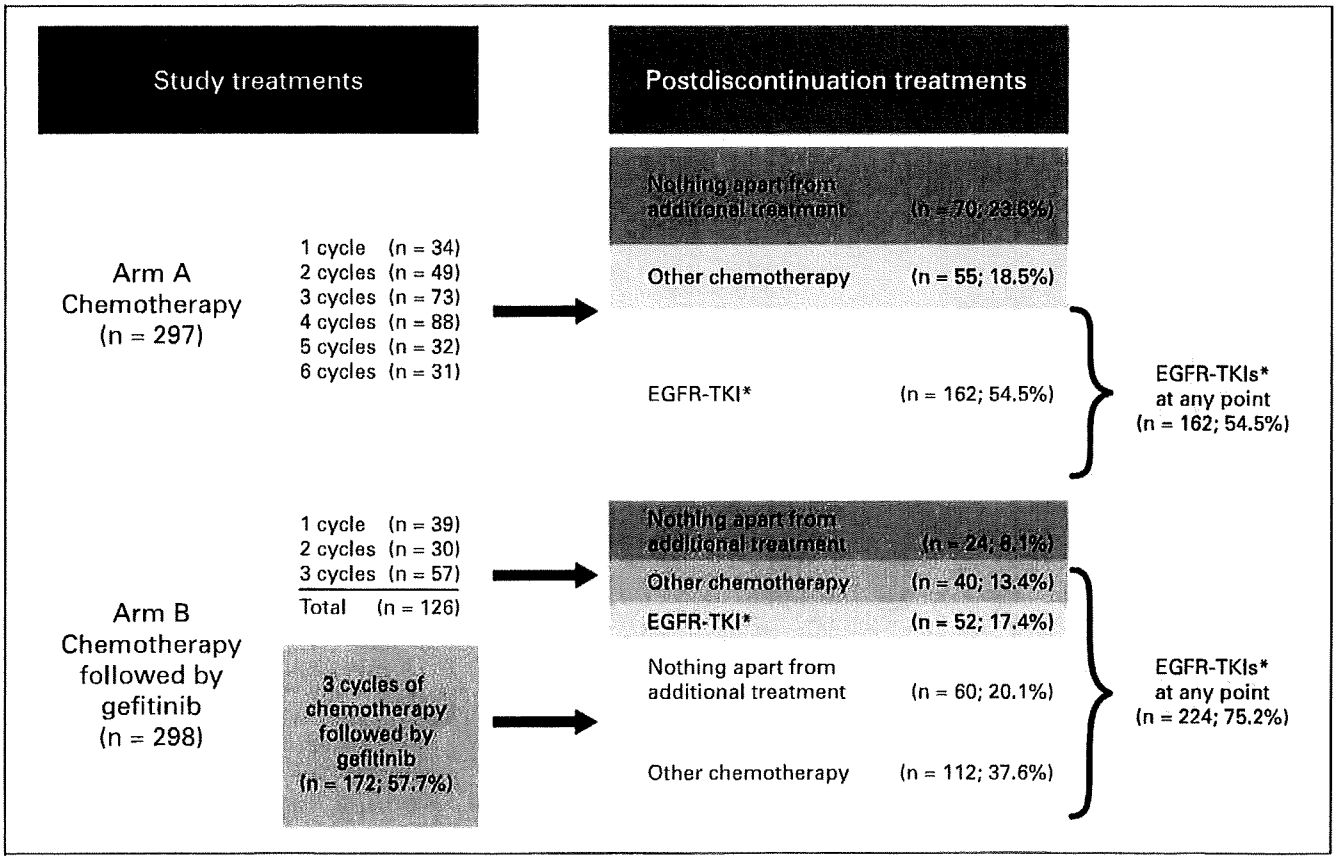

Fig 2. Exposure to active epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), including postdiscontinuation treatments in the full analysis set population (n = 595).

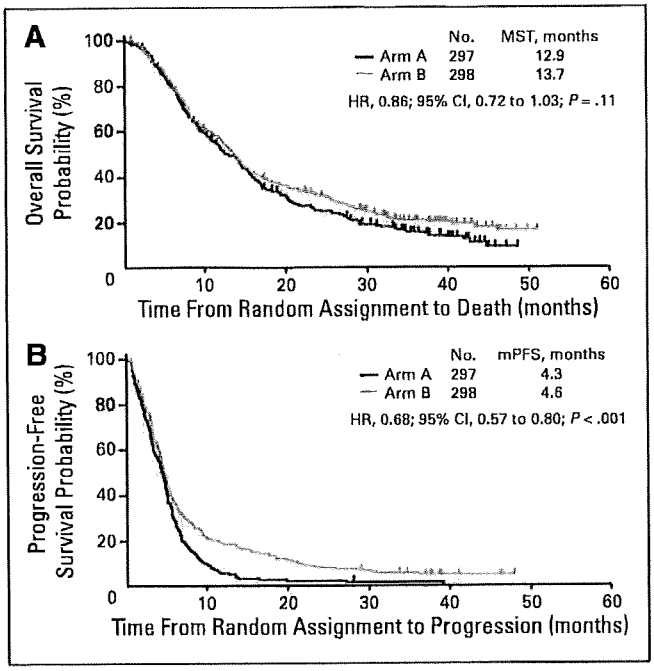

Fig 3. (A) Overall survival and (B) progression-free survival (n = 598). MST, median survival time; HR, hazard ratio; mPFS, median progression-free survival.

thrombocytopenia occurred in 10.7% of patients in arm A and 6.3% of patients in arm B, but differences did not reach significance ($P = .054$). Conversely, grade 3 or 4 AST/ALT elevation in arm B was severer than in arm A ($P = .002$). Severe ILD induced by gefitinib, which many patients feared developing, was observed in two patients in this study.

Disease-Related Symptoms Assessment

All 595 patients completed baseline LCS questionnaires; questionnaire completion rates were 81.0% at 12 weeks and 70.3% at 18 weeks. LCS data were missing in 111 surveys because of death or severe impairment of the patient's general condition; this accounted for 6.2% of the total number of surveys scheduled. The adjusted mean of initial summed scores of LCS were 20.3 for arm A and 20.6 for arm B, respectively. The adjusted LCS scores at 12 and 18 weeks were 21.0 and 20.9 for arm A, and 21.8 and 21.2 for arm B, respectively. Sequential gefitinib seemed to provide better symptom relief, although differences did not reach statistical significance ($P = .10$).

DISCUSSION

Sequential gefitinib therapy after three cycles of standard platinum-doublet chemotherapy showed no survival benefit over platinum-doublet chemotherapy up to six cycles in previously untreated patients with advanced NSCLC. However, sequential gefitinib was associated with significantly prolonged PFS. Recently, positive results with maintenance or sequential chemotherapy have been reported in clinical trials in PFS or time to progression; however, OS was not significantly lengthened.[20,21] More recently, pemetrexed administered to NSCLC patients without progression after four cycles of first-line treatment with platinum-doublet provided significant improvement in PFS compared with placebo (HR, 0.60; 95% CI, 0.49 to 0.73; $P < .00001$).[22]

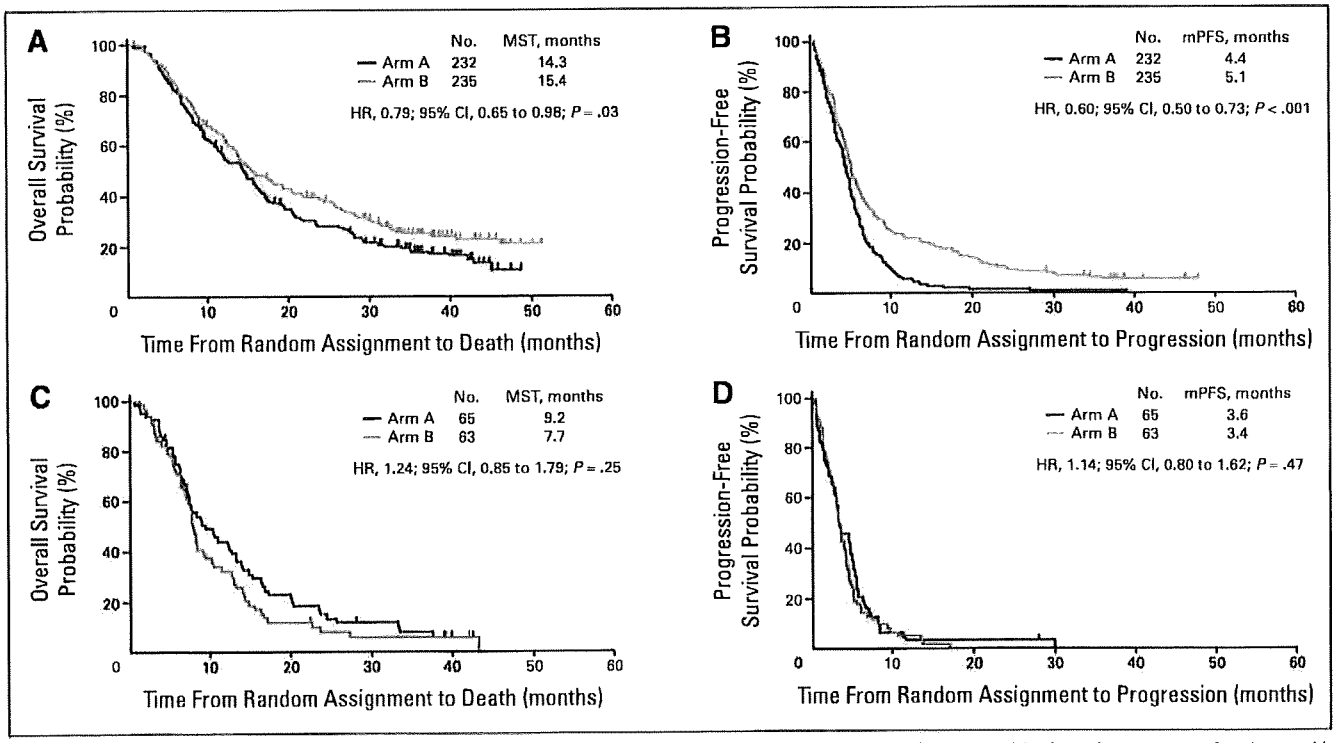

Fig 4. (A) Overall survival in the subset groups of patients with adenocarcinoma (n = 467), (B) progression-free survival in the subset groups of patients with adenocarcinoma (n = 467), (C) overall survival in the subset groups of patients with nonadenocarcinoma (n = 128), and (D) progression-free survival in the subset groups of patients with nonadenocarcinoma (n = 128). MST, median survival time; HR, hazard ratio; mPFS, median progression-free survival.

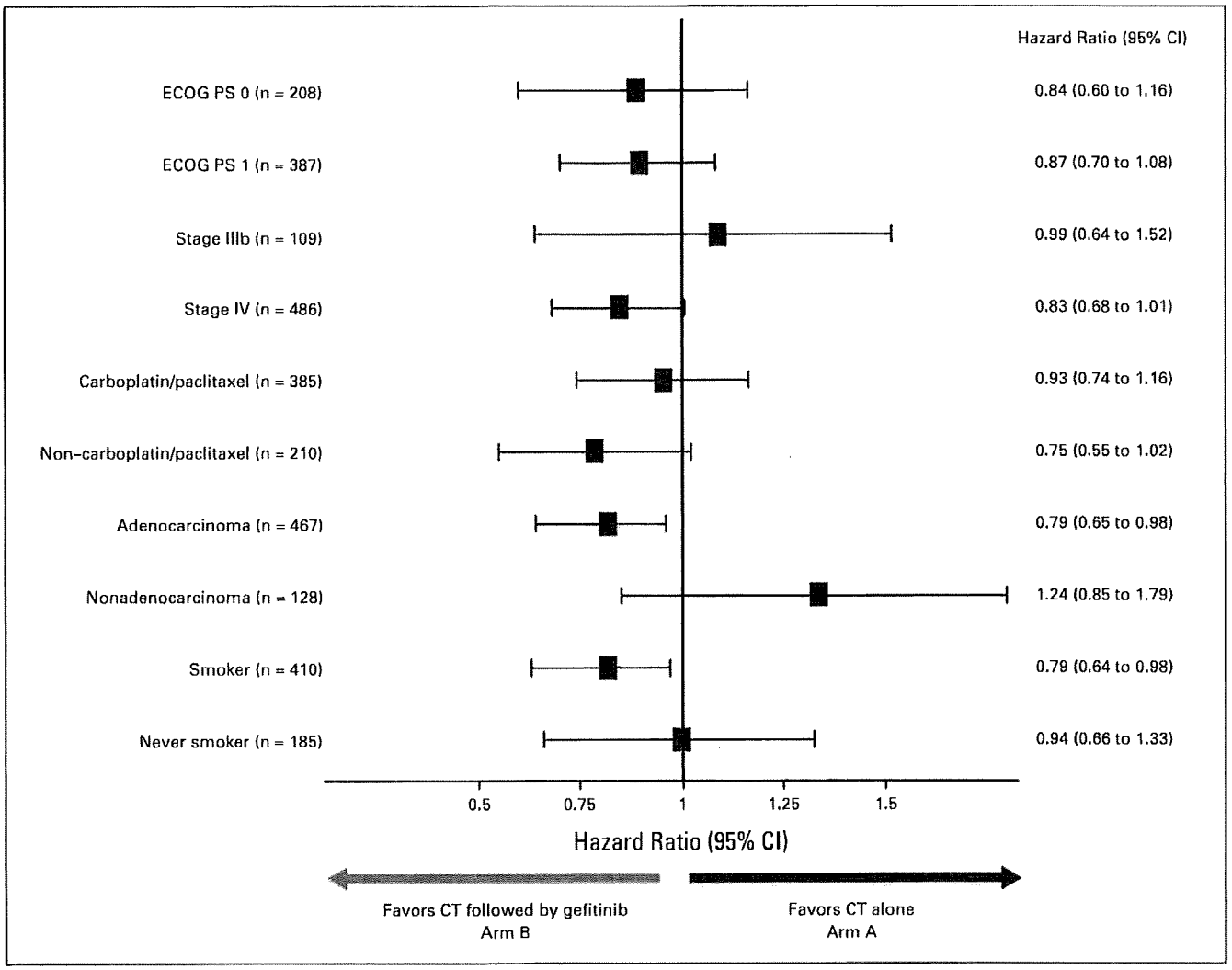

Fig 5. Forest plot subgroup analysis according to patients' backgrounds. CT, chemotherapy; ECOG PS, Eastern Cooperative Oncology Group performance status.

It was the first randomized, double-blind, placebo controlled trial to demonstrate a significant OS prolongation for maintenance treatment with pemetrexed in patients with advanced NSCLC (HR, 0.79; 95% CI, 0.65 to 0.95; $P = .012$).[22] The results of the Sequential Erlotinib in Unresectable NSCLC (SATURN) study, which was a randomized, double-blind, placebo controlled trial with erlotinib as maintenance, were presented this year. Erlotinib maintenance treatment had improvement in PFS of 41% compared with placebo.[23] Maintenance or sequential chemotherapy strategy after standard treatment has lately been receiving considerable attention. As a result, our trial was considered a consolidation therapy using other agent without progression after front-line treatment rather than maintenance.

Although the median number of chemotherapy cycles was three in both arms, 47.5% of patients received more than four cycles in Arm A. The number of treatment cycles was lower in Japanese than in whites; however, comparability was to be kept between the two arms in this randomized trial. These results were consistent with Japanese data on the median number of cycles of platinum-doublet chemotherapy.[15]

Toxicity results were consistent with previous Japanese studies of advanced NSCLC patients who received platinum-doublet chemotherapy.[15,16] Furthermore, no significant severe adverse events were seen that were not predictable from the safety profiles of gefitinib in sequential therapy after platinum-doublet chemotherapy. Recently published data suggested that gefitinib might be associated with ILD in Japanese patients[11]; however, in our study, the overall incidence of ILD was less than 1%, and no imbalance was identified between the two treatment arms in terms of ILD.

It was interesting that sequential gefitinib therapy had a significant survival prolongation in patients with adenocarcinoma histology (HR, 0.79; 95% CI, 0.65 to 0.98; $P = .03$). There was no difference also in PFS or OS for patients with nonadenocarcinoma. It was possible that these patients just did not benefit from an ineffective therapy of sequential gefitinib. In patients with NSCLC, adenocarcinoma histology, nonsmoker, and Japanese or Asian ethnicity are favorable predictive factors for a response to gefitinib treatment.[11-14] When the analysis was performed in the most favorable subset population that responded to gefitinib—that is, among those with both adenocarcinoma histology and nonsmokers—the MST was 23.5 months in arm A and 25.1 months and in arm B, respectively. Indeed, more than three quarters of the patients with favorable profiles in arm A received gefitinib after the protocol treatment, because physicians recognized

Table 2. Toxicity According to National Cancer Institute Common Toxicity Criteria Version 2

Toxicity	Arm A (n = 298)				Arm B (n = 300)				χ^2 Test P for Grade 3 + 4
	Grade 3		Grade 4		Grade 3		Grade 4		
	No.	%	No.	%	No.	%	No.	%	
Hematologic									
Leukopenia	98	32.9	21	7.0	97	32.3	14	4.7	.461
Neutropenia	90	30.2	136	45.6	79	26.3	133	44.3	.153
Febrile neutropenia	33	11.1	5	1.7	38	12.8	0	0	.297
Anemia	57	19.1	8	2.7	35	11.7	5	1.7	.006
Thrombocytopenia	32	10.7	0	0	18	6.0	1	0.3	.054
Nonhematologic									
Anorexia	43	14.4	0	0	33	11.0	2	0.7	.316
AST/ALT	11	3.7	1	0.3	32	10.7	0	0	.002
Constipation	25	8.4	0	0	20	6.7	1	0.3	.631
Creatinine	1	0.3	0	0	0	0	0	0	.315
Diarrhea	6	2.0	0	0	5	1.7	0	0	.152
Dyspnea	3	1.0	5	1.7	4	1.3	5	1.7	.816
Fatigue	22	7.4	7	2.3	18	6.0	4	1.3	.294
Hypersensitivity	1	0.3	1	0.3	2	0.7	2	0.7	.417
Infection	36	12.1	1	0.3	26	8.7	0	0	.135
Nausea	38	12.8	0	0	29	9.7	0	0	.232
Neuropathy									
Motor	5	1.7	1	0.3	4	1.3	1	0.3	.991
Sensory	12	4.0	1	0.3	7	2.3	0	0	.260
Performance status	27	9.1	8	2.7	23	7.7	9	3.0	.676
Pneumonitis (ILD)	2	0.7	0	0	4	1.3	0	0	.417
Rash	2	0.7	0	0	1	0.3	0	0	.559
Stomatitis/pharingitis	0	0	0	0	2	0.7	0	0	.482
Vomiting	12	4.0	1	0.3	15	5.0	2	0.7	.465

Abbreviation: ILD, interstitial lung disease.

these patients were more likely to respond to gefitinib. Patients who were nonsmokers with adenocarcinoma in arm A resulted in subsequent gefitinib therapy as well as in arm B.

Activating mutations in the gene for *EGFR* appear in a subset of adenocarcinoma of lung cancer.[24,25] A higher response to EGFR-TKIs is noted in specific subgroups that include females, never smokers, patients with adenocarcinoma histology, and East Asians.[12] Higher EGFR mutation rates are also noted in these subgroups and are also related to a better response to EGFR-TKIs[24,25] and longer survival.[12] Patients with these mutations exhibit objective response rates in the range of 75% to 95%.[12-14,26,27]

Patients included in this study were not selected on the basis of the target *EGFR* mutation status, because when this study was planned, we had not recognized the *EGFR* mutation as a predictive factor to respond to gefitinib. In Japanese patients with adenocarcinoma, a higher incidence of *EFGR* mutations, are estimated compared with white patients. It seems that more than 40% of Japanese patients with adenocarcinoma have an *EGFR* mutation.[12] Complex results in this study can be explained by analyzing the *EGFR* mutation status of participating patients. It may be important to select patients who are known to receive a clinical benefit with treatment using an EGFR-TKI.

In conclusion, this trial failed to meet the primary end point of OS in patients with advanced NSCLC. The exploratory subset analyses demonstrate a possible survival prolongation for sequential therapy of gefitinib, especially for patients with adenocarcinoma. Further investigations are warranted to confirm the best sequential therapy after platinum-based chemotherapy for 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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De Novo Resistance to Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors in *EGFR* Mutation-Positive Patients with Non-small Cell Lung Cancer

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Background: Somatic mutations in the epidermal growth factor receptor (EGFR) gene are a predictor of response to treatment with EGFR tyrosine kinase inhibitors (TKIs) in patients with non-small cell lung cancer (NSCLC). However, mechanisms of de novo resistance to these drugs in patients harboring *EGFR* mutations have remained unclear. We examined whether the mutational status of *KRAS* might be associated with primary resistance to EGFR-TKIs in *EGFR* mutation-positive patients with NSCLC.

Methods: Forty patients with NSCLC with *EGFR* mutations who were treated with gefitinib or erlotinib and had archival tissue specimens available were enrolled in the study. *KRAS* mutations were analyzed by direct sequencing.

Results: Three (7.5%) of the 40 patients had progressive disease, and two (67%) of these three individuals had both *KRAS* and *EGFR* mutations.

Conclusions: Our results suggest that *KRAS* mutation is a negative predictor of response to EGFR-TKIs in *EGFR* mutation-positive patients with NSCLC.

Key Words: Drug resistance, Epidermal growth factor receptor, KRAS, Non-small cell lung cancer, EGFR-TKI.

(*J Thorac Oncol.* 2010;5: 399–400)

A total of 40 patients with non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (*EGFR*) mutations were treated with gefitinib (n = 36) or erlotinib (n = 4) between September 2002 and January 2009, and three patients exhibited resistance to EGFR-tyrosine kinase inhibitor (TKIs). (i) Case 1 was a 63-year-old man who had never smoked and was diagnosed with lung adenocarcinoma of stage IV. Molecular screening identified a deletion mutation in exon 19 of *EGFR*, and he had received gefitinib as the second-line therapy. Although he tolerated gefitinib well, the primary lung lesion showed slow but steady growth, and he was removed from therapy because of his progressive disease (PD) at day 58 (Figure 1*A*). (ii) Case 2 was a 69-year-old man who had never smoked, had adenocarcinoma of stage IIIB, harbored a deletion in exon 19 of *EGFR*, and was treated with erlotinib as the third-line therapy. A chest computed tomography scan on day 28 revealed enlargement of the primary lung lesion, and the case was subsequently classified as PD (Figure 1*B*). (iii) Case 3 was a 52-year-old man who was a current smoker, had lung adenocarcinoma of stage IV with left adrenal metastasis, harbored a deletion in exon 19 of *EGFR*, and received erlotinib as the fourth-line therapy. Chest computed tomography on day 32 showed enlargement of the left adrenal metastasis, resulting in a classification of PD (Figure 1*C*). Thus, these clinical data demonstrated the existence of de novo resistance to EGFR-TKIs in *EGFR* mutation-positive patients. We examined the mutational status of *KRAS* in the three patients who showed PD as their best response. An amino acid substitution at codon 12 (G12D) of *KRAS* was identified in two of these three patients (Figure 1*D–F*).

DISCUSSION

Somatic mutations of the *EGFR* gene are associated with an increased response to EGFR-TKI in patients with NSCLC. Several prospective clinical trials of EGFR-TKI treatment for patients with NSCLC with *EGFR* mutations have subsequently revealed radiographic response rates of 55 to 91%. It remains of clinical concern, however, that a small proportion of patients with NSCLC with *EGFR* mutations show de novo resistance to EGFR-TKIs and that molecular markers to predict a lack of response to these drugs remain to be identified. We have now examined *KRAS* mutation status in *EGFR* mutation-positive patients with NSCLC treated with EGFR-TKIs and found a high incidence of concomitant *KRAS* mutation in individuals who did not respond to these drugs. Our results indicate that *KRAS* mutation may be clinically useful as a negative predictive marker of sensitivity to EGFR-TKIs in patients with NSCLC with *EGFR* mutations. Previous studies have also shown that *KRAS* mutations are associated with resistance to EGFR-TKIs in patients with NSCLC and that *EGFR* and *KRAS* mutations

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Disclosure: The authors declare no conflict of interest.

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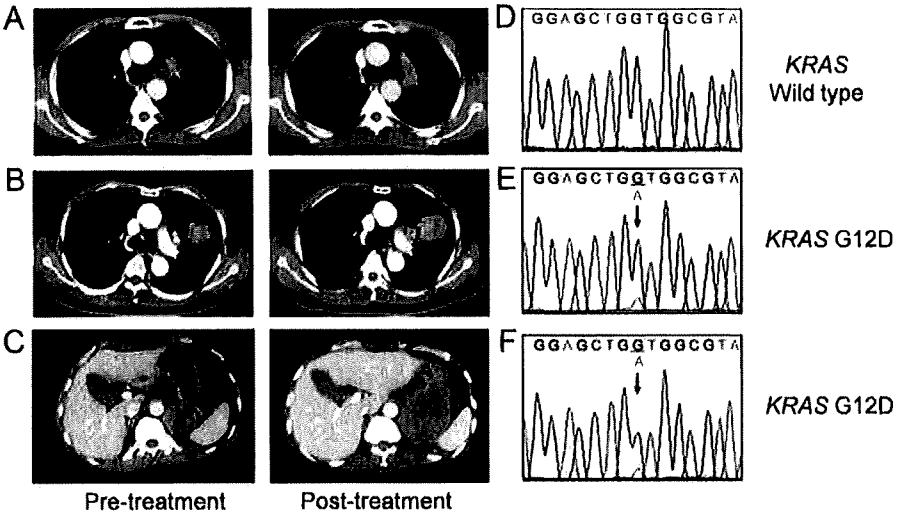

FIGURE 1. Clinical and molecular characteristics of patients with non-small cell lung cancer (NSCLC) who showed de novo resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs). *A–C*, Computed tomography (CT) images obtained before and after EGFR-TKI treatment for cases 1 to 3, respectively. *D–F*, Sequence chromatographs of *KRAS* mutation status determined with tumor tissue isolated before EGFR-TKI treatment for cases 1 to 3, respectively. Arrows indicate the mutated nucleotide (G→A) in codon 12 for cases 2 and 3.

appear to be mutually exclusive in such patients,[1–3] suggesting that *KRAS* mutations are predictors of unresponsiveness to EGFR-TKIs in patients with NSCLC with wild-type *EGFR*. The mutual exclusivity of *EGFR* and *KRAS* mutations combined with their prevalence patterns in lung adenocarcinoma, with *KRAS* mutations being preferentially found in smokers and *EGFR* mutations in nonsmokers, suggests that the mutations in these two genes might arise through different pathogenic pathways. Conversely, some studies have shown that *KRAS* mutations do sometimes coexist with *EGFR* mutations in patients with NSCLC.[4,5] The extent of coexistence of *EGFR* and *KRAS* mutations in NSCLC thus remains unclear, in large part as a result of the low frequency of *KRAS* mutations, and the clinical relevance of *KRAS* mutations in *EGFR* mutation-positive patients has remained unknown. We have now shown that patients with NSCLC harboring *EGFR* mutations who exhibit de novo resistance to EGFR-TKIs have a high incidence of *KRAS* mutation, suggesting that the presence of *KRAS* mutations might provide a basis for the identification of *EGFR* mutation-positive patients who are unlikely to benefit from EGFR-TKI treatment. Our clinical findings are consistent with preclinical data showing that forced expression of mutant KRAS in PC-9 human NSCLC cells, which harbor an activating mutation of *EGFR*, resulted in a reduction in the sensitivity of these cells to gefitinib.[6] Gefitinib shuts down both PI3K-AKT and RAS-RAF-MEK-ERK signaling pathways in PC-9 cells; however, expression of the KRAS mutant resulted in constitutive activation of these signaling pathways in a manner independent of EGFR activation, leading to continued cell growth and survival.

In July 2009, gefitinib received a license from the European Medicines Agency for all lines of therapy in patients with locally advanced or metastatic NSCLC positive for activating mutations of *EGFR*. More patients with *EGFR* mutation-positive tumors will thus now receive EGFR-TKIs. Our present results suggest that EGFR-TKIs should not be given routinely to patients harboring concomitant *KRAS* and *EGFR* mutations. In the event that such patients do receive treatment with EGFR-TKIs, they should be followed up after a short interval to obtain early evidence of possible tumor progression.

KRAS mutations cannot account for all cases of de novo resistance to EGFR-TKIs in *EGFR* mutation-positive patients with NSCLC. A recent study showed that loss of *PTEN* contributes to erlotinib resistance in an *EGFR* mutation-positive NSCLC cell line.[7] Loss of *PTEN* resulted in partial uncoupling of the mutant EGFR from downstream signaling and further activated the receptor, leading to erlotinib resistance. Both homozygous deletion of *PTEN* and *EGFR* mutation were detected in one of 24 clinical specimens of NSCLC with *EGFR* mutations, although the efficacy of EGFR-TKIs was not evaluated in the corresponding patient.

In conclusion, our results suggest that *KRAS* mutation status should be assessed before initiation of EGFR-TKI treatment in *EGFR* mutation-positive patients with NSCLC, allowing enrichment of the population of such patients who are likely to prove responsive to the treatment.

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Tumor and Stem Cell Biology

Cancer Research

FOXQ1 Is Overexpressed in Colorectal Cancer and Enhances Tumorigenicity and Tumor Growth

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Abstract

Forkhead box Q1 (FOXQ1) is a member of the forkhead transcription factor family, and it has recently been proposed to participate in gastric acid secretion and mucin gene expression in mice. However, the role of FOXQ1 in humans and especially in cancer cells remains unknown. We found that *FOXQ1* mRNA is overexpressed in clinical specimens of colorectal cancer (CRC; 28-fold/colonic mucosa). A microarray analysis revealed that the knockdown of *FOXQ1* using small interfering RNA resulted in a decrease in $p21^{CIP1/WAF1}$ expression, and a reporter assay and a chromatin immunoprecipitation assay showed that *p21* was one of the target genes of FOXQ1. Stable FOXQ1-overexpressing cells (H1299/FOXQ1) exhibited elevated levels of p21 expression and inhibition of apoptosis induced by doxorubicin or camptothecin. Although cellular proliferation was decreased in H1299/FOXQ1 cells *in vitro*, H1299/FOXQ1 cells significantly increased tumorigenicity [enhanced green fluorescent protein (EGFP): 2/15, FOXQ1: 7/15] and enhanced tumor growth (437 ± 301 versus 1735 ± 769 mm^3, $P < 0.001$) *in vivo*. Meanwhile, stable p21 knockdown of H1299/FOXQ1 cells increased tumor growth, suggesting that FOXQ1 promotes tumor growth independent of p21. Microarray analysis of H1299/EGFP and H1299/FOXQ1 revealed that FOXQ1 overexpression upregulated several genes that have positive roles for tumor growth, including *VEGFA, WNT3A, RSPO2*, and *BCL11A*. CD31 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining of the tumor specimens showed that FOXQ1 overexpression mediated the angiogenic and antiapoptotic effect *in vivo*. In conclusion, FOXQ1 is overexpressed in CRC and enhances tumorigenicity and tumor growth presumably through its angiogenic and antiapoptotic effects. Our findings show that FOXQ1 is a new member of the cancer-related FOX family. *Cancer Res; 70(5); 2053–63. ©2010 AACR.*

Introduction

The forkhead box (*Fox*) gene family is a large and diverse group of transcription factors that share certain characteristics of a conserved, ~100 amino acid DNA-binding motif known as the forkhead or winged helix domain; over 100 proteins with forkhead domains have been identified, comprising at least 17 subclasses to date (1). The Fox gene family plays various important roles, not only in biological processes including development, metabolism, immunology, and senescence but also in cancer development (2, 3).

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Forkhead box Q1 (*FOXQ1*, also known as HFH1) is a member of the FOX gene family and contains the core DNA binding domain, whereas the flanking wings of FOXQ1 contribute to its sequence specificity (4). As a transcription factor, FOXQ1 is known to repress the promoter activity of smooth muscle–specific genes, such as telokin and SM22α, in A10 vascular muscle cells (5), and FOXQ1 expression is regulated by Hoxa1 in embryonic stem cells (6). The biological function of *Foxq1* has been clearly identified in hair follicle differentiation in satin (sa) homozygous mice (7); interestingly, satin mice also exhibit suppressed natural killer cell function and T-cell function, suggesting a relation with immunology. Satin mice have provided evidence that Hoxc13 regulates foxq1 expression and that "cross-talk" occurs between Homeobox and Fox (8). Foxq1 mRNA is widely expressed in murine tissues, with particularly high expression levels in the stomach and bladder (5). Recently, two important findings have been reported regarding its involvement in stomach surface cells. Foxq1-deficient mice exhibit a lack of gastric acid secretion in response to various secretagogue stimuli (9). On the other hand, Foxq1 regulates gastric MUC5AC synthesis, providing clues as to the lineage-specific cell differentiation in gastric surface epithelia (10). Despite accumulating evidence supporting the biological function of the murine foxq1 gene in hair follicle

morphogenesis and gastric epithelial cells, no data regarding the cellular and biological functions of human *FOXQ1*, especially in cancer cells, are available.

$p21^{CIP1/WAF1}$ (hereafter called p21) is a member of the cip/kip family of cyclin kinase inhibitors, and initial reports have shown that p21 functions as a G_1 cyclin kinase inhibitor (11, 12) and a downstream molecule of p53 (13). p21 possesses a variety of cellular functions, including the negative modulation of cell cycle progression (14), cellular differentiation (15), and the regulation of p53-dependent antiapoptosis (reviewed in ref. 16). The expression of p21 is regulated by both p53-dependent and p53-independent mechanisms at the transcriptional level. Other regulatory mechanisms of p21 expression involve proteasome-mediated degradation, mRNA stability, alterations in the epigenetic silencing of the p21 promoter, and secondary decreases resulting from viral activity targeting p53, such as the activities of human papilloma virus and hepatitis C virus (17). However, its expression is considered to be regulated mainly at the transcriptional level (18). Accumulating data indicate that many molecules from diverse signaling pathways can activate or repress the p21 promoter, including p53, transforming growth factor-β (TGF-β), c-jun, Myc, Sp1/Sp3, signal transducers and activators of transcriptions, CAAT/enhancer binding protein-α (C/EBP-α), C/EBP-β, basic helix-loop-helix proteins, and myogenic differentiation 1 (reviewed in ref. 19). Thus, p21 is integrally involved in both cell cycle and apoptosis; therefore, identifying its regulatory molecules is of great importance.

We performed a microarray analysis of clinical samples of paired colorectal cancer (CRC) specimens and normal colonic mucosa specimens to identify genes that were overexpressed in CRC. Our results revealed that *FOXQ1* gene expression was ~28-fold higher in CRC than in normal colonic mucosa, and we hypothesized that FOXQ1 may play a role in CRC. In the present study, we investigated the biological function of FOXQ1.

Materials and Methods

Antibodies. The following antibodies were used: anti-p21, anti-p53, anti-cdk2, anti-cdk4, anti-cyclin D, anti-phosphorylated Rb, anti-poly(ADP-ribose) polymerase (PARP), anti-cleaved PARP, anti-caspase-3, anti-cleaved caspase-3, secondary antibodies, and Myc-tag mouse antibody (Cell Signaling), as well as anti-β-actin (Santa Cruz Biotechnology). A mouse anti-CD31 monoclonal antibody was purchased from BD Biosciences.

Cell lines and cultures. The DLD-1, MKN74, H1299, SBC3, and U251 cell lines were cultured in RPMI 1640 (Sigma). The WiDr, CoLo320DM, and human embryonic kidney cell line 293 (HEK293) cell lines were cultured in DMEM (Sigma), and the LoVo cell line was cultured in Ham/F12 medium [Life Technologies Bethesda Research Laboratories (BRL)]. All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies BRL), and the cell lines were maintained in a 5% CO_2-humidified atmosphere at 37°C.

Patients and samples. Paired CRC and noncancerous colonic mucosa samples were evaluated using a microarray analysis in the first consecutive 10 patients. These samples and another 36 CRC samples were analyzed using real-time reverse transcription–PCR (RT-PCR). The RNA extraction method and the quality check protocol have been previously described (20). This study was approved by the institutional review board of the National Cancer Center Hospital, and written informed consent was obtained from all the patients.

Plasmid construction, viral production, and stable transfectants. The cDNA fragment encoding human full-length FOXQ1 was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa) with 5′-GGG AAT TCG CGG CCA TGA AGT TGG AGG TCT TCG TC-3′ and 5′-CCC TCG AGC GCT ACT CAG GCT AGG AGC GTC TCC AC-3′ sense and antisense primers, respectively. The methods used in this section have been previously described (21). Short hairpin RNA (shRNA) targeting p21 was constructed using oligonucleotides encoding small interfering RNA (siRNA) directed against p21 and a nonspecific target as follows: 5′-CTA AGA GTG CTG GGC ATT TTT-3′ for p21 shRNA and 5′-TGT TCG CAG TAC GGT AAT GTT-3′ for control shRNA. They were cloned into an RNAi-Ready pSIREN-RetroQZsGreen vector (Clontech) according to manufacturer's protocol. The stable transfectants expressing enhanced green fluorescent protein (EGFP) or FOXQ1 or FOXQ1 with shRNA targeting p21 for each cell line were designated as HEK293/EGFP, HEK293/FOXQ1, CoLo320/EGFP, CoLo320/FOXQ1, H1299/EGFP, H1299/FOXQ1, H1299/FOXQ1/sh-control, and H1299/FOXQ1/sh-p21. The FOXQ1 human cDNA was tagged at the NH_2 terminus with the myc epitope using the pCMV-Myc vector (Clontech) for chromatin immunoprecipitation (ChIP) assay.

SiRNA transfection. Two different sequences of siRNA targeting human FOXQ1 and negative control siRNA were purchased from QIAGEN. The sequences of FOXQ1 and control siRNA were as follows: FOXQ1#1 sense, 5′-CCA UCA AAC GUG CCU UAA A-3′ and antisense, 5′-UUU AAG GCA CGU UUG AUG G-3′; FOXQ1#4 sense, 5′-CGC GGA CUU UGC ACU UUG A-3′ and antisense, 5′-UCA AAG UGC AAA GUC CGC G-3′; control siRNA (scramble) sense, 5′-UUC UCC GAA CGU GUC ACG U-3′ and antisense, 5′-ACG UGA CAC GUU CGG AGA A-3′; control siRNA (GFP) sense, 5′-GCA AGC UGA CCC UGA AGU UCA U-3′ and antisense, 5′-GAA CUU CAG GGU CAG CUU GCC G-3′. The methods of transfection have been previously described (22).

Real-time RT-PCR and Western blot analysis. The methods used in this section have been previously described (21). The primers used for real-time RT-PCR were purchased from Takara as follows: FOXQ1 forward, 5′-CGC GGA CTT TGC ACT TTG AA-3′ and reverse, 5′-AGC TTT AAG GCA CGT TTG ATG GAG-3′; p21 forward, 5′-TCC AGC GAC CTT CCT CAT CCA C-3′ and reverse, 5′-TCC ATA GCC TCT ACT GCC ACC ATC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPD) forward, 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse, 5′-ATG GTG GTG AAG ACG CCA GT-3′. The experiment was performed in triplicate.

(H1047R) [14]. These two cell lines do not show *HER2* amplification [23]. In contrast, no significant levels of phospho-Akt were observed in MDA-MB-231 and MDA-MB-435S, which show no *HER2* amplification, *PIK3CA* mutation, or PTEN loss [14, 23]. Further, with the exception of MDA-MB-231, all cell lines showed very low levels of phospho-ERK1/2 under serum-starved conditions. MDA-MB-231, in particular, was reported to contain double activating mutations in KRAS (G13D) and BRAF (G464V), whereas MDA-MB-435S showed an activating mutation in BRAF alone (V600E) [31]. These findings further support the concept that *HER2*-amplified cells tend to have HER2-PI3K signaling axis and they are thus dependent on the PI3K pathway rather than on extracellular signal-regulated kinase pathway.

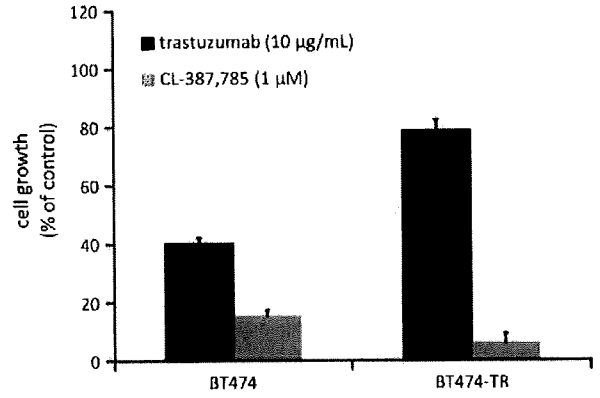

Figure 3. Effect of trastuzumab and CL-387,785 on growth inhibition in BT-474 and BT474-TR cells. Mean percentage of control and standard deviation of 6–12 replicate wells treated with 10 μg/ml trastuzumab and 1 μM CL-387,785 were plotted. BT474-TR remains sensitive to CL-387,785.

discussion

In this study, we show that gain-of-function mutations in *PIK3CA* genes are associated with trastuzumab resistance in naturally derived breast cancer cell lines showing *HER2* amplification. This finding is consistent with a recent study by Berns et al. [19] reporting trastuzumab resistance in SKBR-3 cells transfected with mutant *PIK3CA* (H1047R) compared with GFP control. Transfection of wild-type *PIK3CA*, however, appeared to equally cause trastuzumab resistance [19]. This observation does not identify either quantitative or qualitative changes in *PIK3CA* mutation as the major factor in developing trastuzumab resistance. In the present study, no clear association was observed between *PIK3CA* protein (p110-α) expression levels and *in vitro* sensitivity to trastuzumab

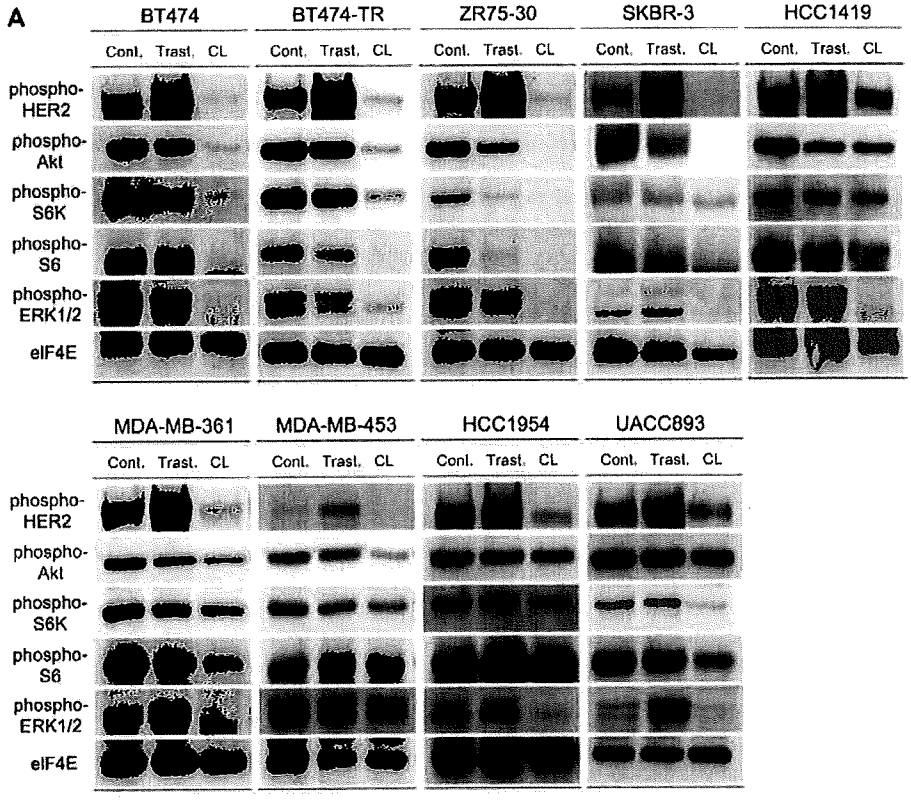

Figure 4. (A) Expression of phosphorylated-HER2, -Akt, -S6K, -S6 and -ERK1/2 in HER2-amplified breast cell lines with and without treatment with trastuzumab (10 μg/ml) and CL-387,785 (1 μM). Breast cell lines grown in 10% serum-containing media were lysed and immunoblotted for each protein. Blots were stripped and re-probed for eIF4E as loading control.

Luciferase reporter assay. The human p21 promoter reporter vector was constructed according to a previously described method (13). The p21 promoter fragment was cut between the *KpnI* and *XhoI* restriction sites and was transferred into the luciferase reporter vector pGL4.14 (Promega). All sequences were verified using DNA sequencing. The empty and p21 promoter-containing reporter vectors were designated as pGL4.14-mock and pGL4.14-p21, respectively. All the samples were examined in triplicate.

ChIP. ChIP was carried out using the ChIP-IT Express Enzymatic kit (Active Motif) according to manufacturer's protocol. HEK293 cells were transfected with empty vector (Myc) or Myc-tagged FOXQ1 vector. The putative region of the p21 promoter (−2264 to −1971) was amplified with the following primers: 5′-TTG AGC TCT GGC ATA GAA GA-3′ (forward) and 5′-TAC CCA GAC ACA CTC TAA GG-3′ (reverse). As a negative control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) second intron promoter was amplified with the following primers: 5′-AAT GAA TGG GCA GCC GTT AG-3′ (forward) and 5′-AGC TAG CCT CGC TCC ACCTGA C-3′ (reverse).

Xenograft studies. Two separate xenograft studies were performed independently. Nude mice (*BALB/c nu/nu*; 6-week-old females; CLEA Japan, Inc.) were used for the *in vivo* studies and were cared for in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kinki University. The ethical procedures followed and met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines (23). To assess tumorigenicity, suspensions of 1×10^6 H1299/EGFP or H1299/FOXQ1 cells (in 0.1 mL PBS) were s.c. injected into the left or right flanks of nude mice (n = 15), respectively. To evaluate tumor growth, a suspension of 6×10^6 H1299/EGFP, H1299/FOXQ1, H1299/FOXQ1/sh-control, and H1299/FOXQ1/sh-p21 cells (in 0.1 mL PBS) were s.c. inoculated (n = 10) into nude mice. The tumor volume was calculated as length × width^2 × 0.5. The tumor formation was assessed every 2 to 3 d. At the end of the experiment, the mice were sacrificed and the xenografts were resected, fixed in 10% buffered formalin for 6 to 10 h, and processed for histologic analysis.

Immunohistochemical and immunofluorescence staining. The methods used in this section have been previously described (24, 25).

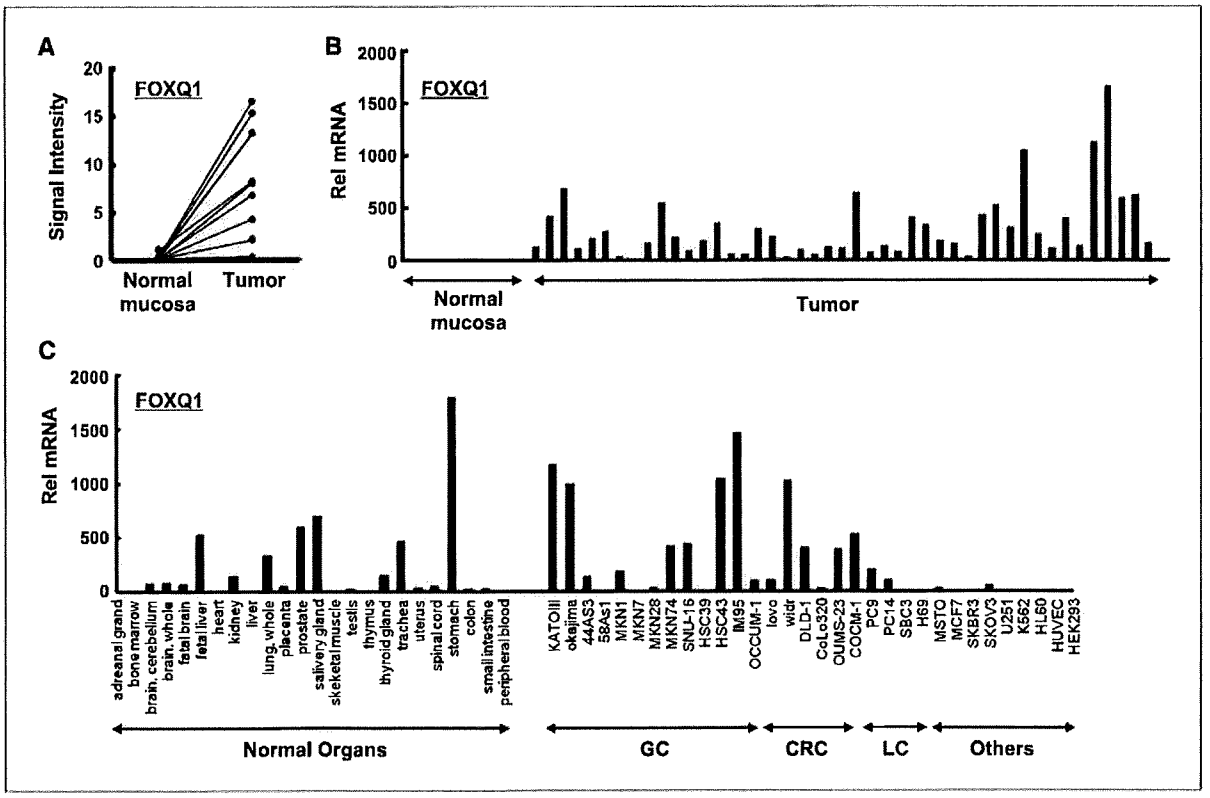

Figure 1. *FOXQ1* expression in CRC. A, mRNA expression of *FOXQ1* obtained from a microarray analysis of 10 CRC and paired normal mucosa specimens. The values indicate the normalized signal intensity. B, the mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR for 10 paired and an additional 36 CRC samples. C, the mRNA expression levels of *FOXQ1* were determined using a real-time RT-PCR analysis of human normal tissue (left) and 30 human cancer cell lines, HEK293, and human umbilical vascular endothelial cell (HUVEC) cell lines (right). GC, gastric cancer; LC, lung cancer; Rel mRNA, normalized mRNA expression levels (*FOXQ1/GAPD* × 10^4).

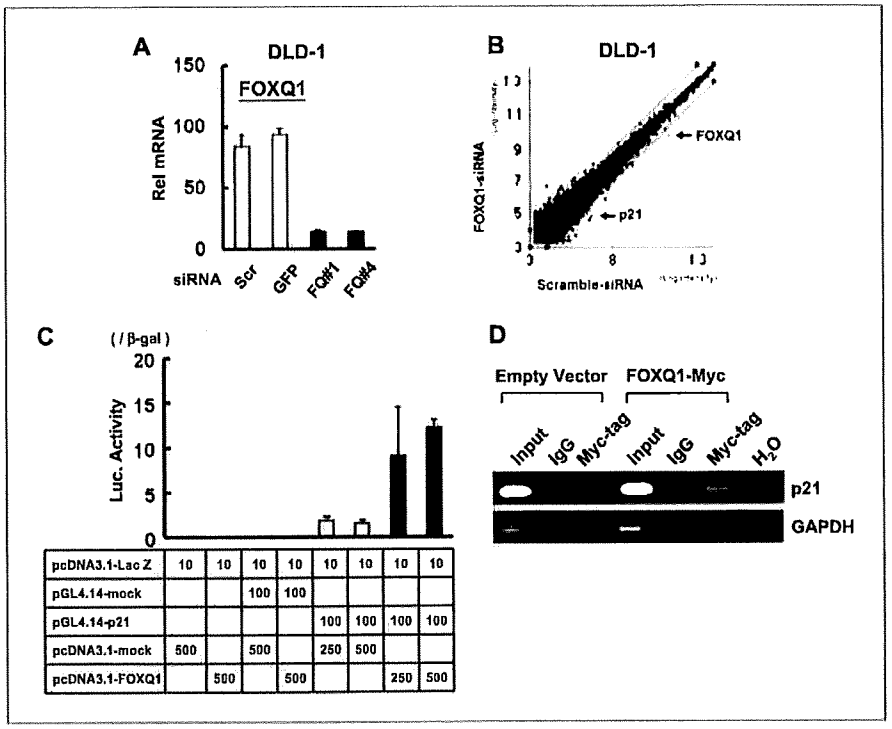

Figure 2. FOXQ directly regulates *p21* transcription. A, FOXQ1-targeting siRNA (FQ#1 and FQ#4) suppressed FOXQ1 expression in DLD-1 cells. The mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR. B, microarray analysis of DLD-1 cells transfected with control-siRNA or FOXQ1-siRNA. The longitudinal axis indicates the mRNA expression of FOXQ1-siRNA transfected cells and the horizontal axis indicates that of control-siRNA. Arrow, FOXQ1 or p21 expression. Each point indicates the normalized and log base 2 transformed microarray data. C, induction of p21 promoter activity by FOXQ1. Luciferase vectors with either an empty or p21 promoter (pGL4.14-mock or pGL4.14-p21) were transiently cotransfected with a mock or FOXQ1 expression plasmid (pcDNA3.1-mock or pcDNA3.1-FOXQ1) expressing β-galactosidase as an internal control. The results were normalized to β-galactosidase activity and are representative of at least three independent experiments. D, ChIP of FOXQ1 on the promoter of *p21*. HEK293 cells were transfected with empty vector (Myc) or Myc-tagged FOXQ1 vector. Agarose gel shows PCR amplification (35 cycles) of the *p21* promoter using inputs (1% of chromatin used for ChIP) or ChIPs as templates. Primers to the *GAPDH* promoter were used as the negative control.

Microarray analysis. The microarray procedure and analysis were performed according to the Affymetrix protocols and BRB Array Tools software, Ver. 3.3.0,[4] developed by Dr. Richard Simon and Dr. Amy Peng, as reported previously (21, 26).

Statistical analysis. The statistical analyses were performed using Microsoft Excel (Microsoft) to calculate the SD and to test for statistically significant differences between the samples using a Student's *t* test. A *P* value of <0.05 was considered statistically significant.

Results

FOXQ1 mRNA was overexpressed in CRCs. A microarray analysis for 10 paired CRC samples identified 30 genes as being significantly upregulated by >10-fold in CRC ($P < 0.001$; Supplementary Table S1). *FOXQ1*, an uncharacterized transcription factor, was upregulated by 28-fold in the CRC specimens (Fig. 1A), exhibiting the fourth highest level of upregulation [after interleukin-8, matrix metalloproteinase-1 (MMP), and MMP-3]. Real-time RT-PCR for the 10 paired samples and an additional 36 CRC samples showed that *FOXQ1* mRNA was markedly overexpressed in the CRC samples but was only expressed at a very low level in noncancerous colonic mucosa ($P < 0.001$; Fig. 1B). The average levels of *FOXQ1* expression were 299 ± 326 and 4.0 ± 5.0 ($\times 10^4$/GAPD), respectively.

FOXQ1 expression in normal tissues and cancer cell lines. To investigate the expression of *FOXQ1*, we analyzed the mRNA expression levels of *FOXQ1* in panels of human normal tissues and cancer cell lines using real-time RT-PCR. High levels of *FOXQ1* expression were observed in the stomach, salivary gland, prostate, trachea, and fetal liver among the 24 normal tissues that were examined (Fig. 1C, left). Relatively weak expression levels were detected in brain-derived tissues, kidney, lung, placenta, and thyroid gland. These results were consistent with those of a previous report (27).

[4] http://linus.nci.nih.gov/BRB-ArrayTools.html

In the cancer cell line panel, the mRNA expression levels of *FOXQ1* were higher in gastric cancer, CRC, and lung cancer cell lines than in the other cancer cell lines, indicating that the expression of *FOXQ1* varies among specific cancers (Fig. 1C, right). Interestingly, the overexpression of *FOXQ1* in CRC arose from normal colonic mucosa with very low expression levels during carcinogenesis.

p21 is a target gene of FOXQ1. To examine the function of FOXQ1 as a transcription factor and to explore its target genes, we performed a microarray analysis using a CRC cell line, DLD-1, transfected with FOXQ1-targeting siRNA or control siRNA. Two sequences of FOXQ1-siRNA, FQ#1 and FQ#4, were used to exclude the off-target effect of siRNA. Real-time RT-PCR showed that both sequences of FOXQ1-siRNA suppressed *FOXQ1* mRNA expression by ~80% in DLD-1 cells (Fig. 2A); thus, FQ#4 was used as the FOXQ1-siRNA in the following experiments. A microarray analysis showed that 19 genes were downregulated by FOXQ1-siRNA (Fig. 2B; Supplementary Table S2); *p21* was the fifth most-downregulated gene. Because p21 is a key regulator of cell cycle and apoptosis, we focused on p21 as a target molecule of FOXQ1.

To confirm the microarray data, p21 downregulation by FOXQ1-siRNA was examined using real-time RT-PCR and a

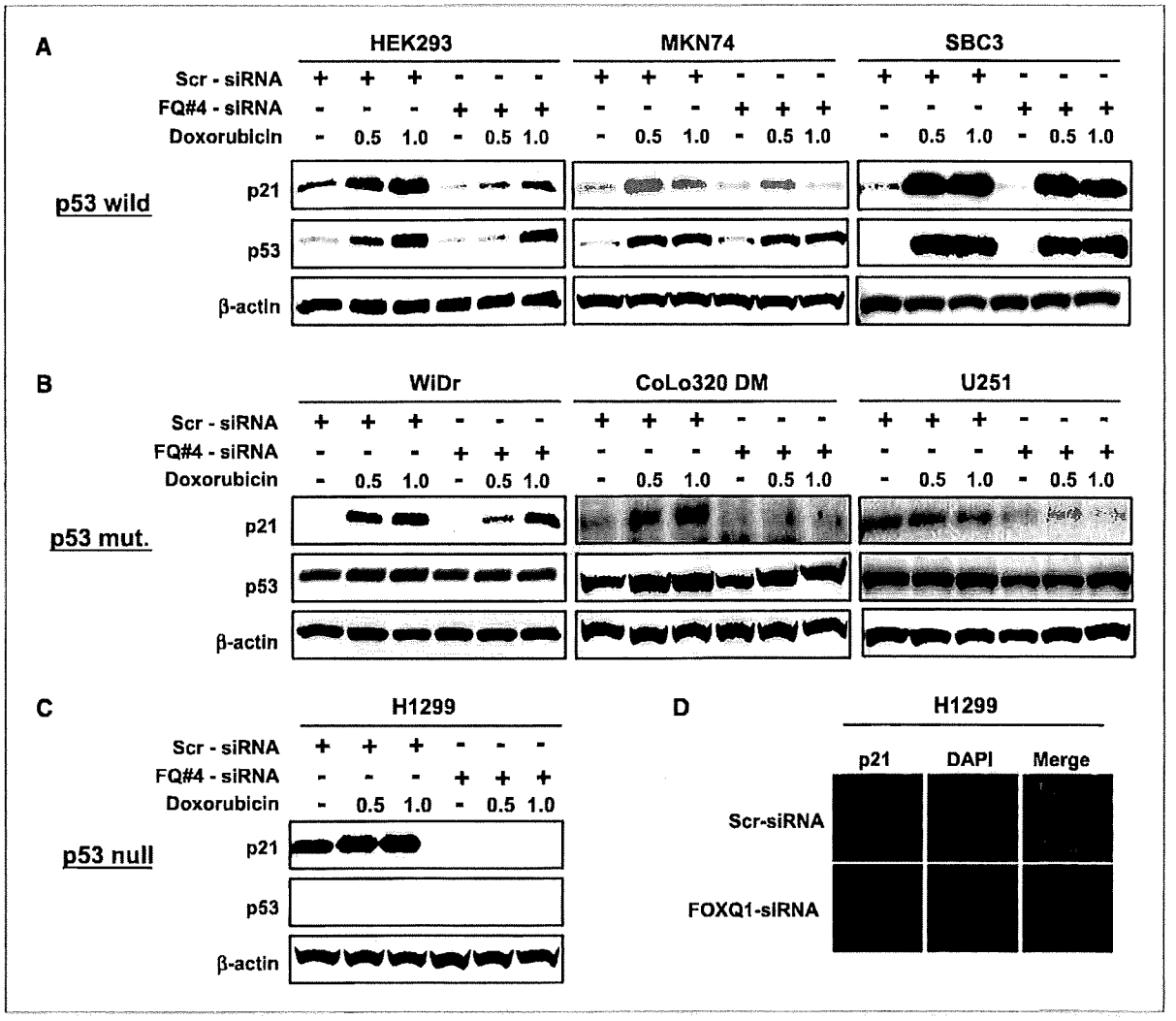

Figure 3. p21 induction by FOXQ1 and p53 status in cancer cells. The seven cell lines were transfected with control-siRNA or FOXQ1-siRNA for 24 h, and the cells were exposed to doxorubicin at a final concentration of 0.5 or 1 μmol/L for a further 24 h to enhance p21 induction. Western blot analyses for p21 and p53 were performed in three p53–wild type cell lines (A), three p53-mutant cell lines (B), and one p53-null cell line (C). The experiment was performed in duplicate. D, immunofluorescence p21 staining and 4′,6-diamidino-2-phenylindole (DAPI) staining for H1299 cells transfected with control-siRNA (top) or FOXQ1-siRNA (bottom) for 48 h. Scr, scramble-siRNA (control); FQ#4, FOXQ1-targeting siRNA. β-Actin was used as an internal control.