

radiotherapy for metastatic lesion. Two of four patients with PD received second-line chemotherapy. The remaining nine patients received supportive care according to the patients' request.

Toxicity

All the 39 patients were included in the toxicity evaluation. Major toxicities were hematological, such as leukopenia, neutropenia and thrombocytopenia, in both groups, and these hematological toxicities increased in severity with increased dose level of nedaplatin. In Group A, 1 (6.7%) out of the 15 patients treated at a dose level of 100 mg/m² had grade 3 neutropenia; this dose level was considered to be acceptable (Table 2). In Group B, three (50%) out of six patients treated at a dose level of 80 mg/m² had \geq grade 3

hematological toxicities (one with grade 3 neutropenia, another with grade 4 neutropenia and febrile neutropenia, and the other with grade 3 leukopenia, anemia and grade 4 thrombocytopenia). The patient with grade 4 thrombocytopenia required a platelet transfusion. At a dose level of 100 mg/m², three (60%) out of five patients had \geq grade 3 hematological toxicities (one with grade 3 leukopenia and neutropenia, another with grade 3 thrombocytopenia and grade 4 neutropenia, and the other with grade 3 leukopenia, thrombocytopenia and grade 4 neutropenia). These three patients had also febrile neutropenia. In Group B, a dose level of 100 mg/m² was considered to be unacceptable (Table 2).

Non-hematological toxicities, mainly nausea and anorexia, were generally mild in severity and were not dose limiting in either group (Table 3). Renal toxicity,

Table 1 Patient characteristics

	Group A (Ccr \geq 60 mL/min)		Group B (40 \leq Ccr < 60 mL/min)	
	No. of patients	Percentage	No. of patients	Percentage
Total patients enrolled	22	100	17	100
Assessable for toxicity	22	100	17	100
Assessable for PK analysis	15	68	13	76
Age, median (range), years	76 (70–82)		76 (70–78)	
Sex				
Male	19	86	16	94
Female	3	14	1	6
ECOG PS				
0	6	27	1	6
1	16	73	15	88
2	0	0	1	6
Stage				
IIIA	2	9	6	35
IIIB	4	18	6	35
IV	11	50	4	24
Postoperative recurrence	5	23	1	6
Pathological subtype				
Squamous cell carcinoma	12	54	9	53
Adenocarcinoma	9	41	8	47
P/D carcinoma	1	5	0	0
Dose of nedaplatin (mg/m ²)				
60	–	–	6	35
80	7	32	6	35
100	15	68	5	30
Treatment cycle				
Median (range)	2 (1–5)		2 (1–4)	
1 cycle	4	18	5	29
2 cycles	12	55	10	59
\geq 3 cycles	6	27	2	12

PK pharmacokinetics, ECOG Eastern Cooperative Oncology Group, PS performance status, P/D carcinoma poorly differentiated carcinoma

Table 2 Hematological toxicity

Event	Dose level (mg/m ²), (number of patients)														
	80 (n = 7)					100 (n = 15)									
	Grade					Grade									
	0	1	2	3	4	0	1	2	3	4					
Group A (Ccr ≥60 mL/min)															
Leukopenia	6	1	0	0	0	12	1	2	0	0					
Neutropenia	6	1	0	0	0	8	4	2	1 ^a	0					
Anemia	4	2	1	0	0	5	7	3	0	0					
Thrombocytopenia	7	0	0	0	0	12	2	1	0	0					
No. of patients with febrile neutropenia	0					0									
No. of patients with DLT	0					1									
Group B (40 ≤ Ccr < 60 mL/min)															
Event	Dose level (mg/m ²), (number of patients)														
	60 (n = 6)					80 (n = 6)					100 (n = 5)				
	Grade					Grade					Grade				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Leukopenia	5	1	0	0	0	2	1	2	1 ^a	0	2	0	1	2 ^a	0
Neutropenia	5	1	0	0	0	2	2	0	1 ^a	1 ^a	1	1	0	1 ^a	2 ^a
Anemia	4	1	1	0	0	3	1	1	1 ^a	0	1	2	2	0	0
Thrombocytopenia	6	0	0	0	0	3	1	1	0	1 ^a	2	1	0	2 ^a	0
No. of patients with febrile neutropenia	0					1					3				
No. of patients with DLT	0					3					3				

^a DLT

characterized as an increase in Cr, was also mild, and only one out of five patients treated at a dose level of 100 mg/m² in Group B had a grade 2 Cr increase. Considering the toxicity profiles, the recommended doses in Groups A and B were determined to be 100 and 80 mg/m², respectively.

Response and survival

The antitumor response was assessed in all the 39 patients (Table 4). Of the 39 patients who achieved PR, 13 had an overall response rate of 33%. Similar antitumor responses were observed in both treatment groups; that is, 6 (27%) of 22 and 7 (41%) of 17 patients had PRs in Groups A and B, respectively. Furthermore, 12 of the 13 patients with PRs in both groups had squamous cell carcinoma, and the response rate among patients with squamous cell carcinoma was 57%. Survival follow-up was completed in all the enrolled patients. The median survival time was 11.2 months (95% confidence interval: 7.7–14.6 months), and the 1-, 2- and 5-year survival rates were 46, 23 and 5%, respectively.

Pharmacokinetics

Pharmacokinetic analysis was performed using data from 28 (72%) of the 39 patients. The first patient enrollment in

both treatment groups was started in 1996, and techniques of the sample centrifuging and measurement were not fully developed at the beginning of this pharmacokinetic study. Therefore, the remaining 11 patients (28%) were excluded for pharmacokinetic analysis. The mean plasma concentration–time profiles of total-Pt and free-Pt of nedaplatin are illustrated in Fig. 1. The plasma disappearances of total-Pt and free-Pt were biphasic, and the mean terminal half lives in all the assessable patients averaged 6.28 and 3.57 h, respectively. The C_{max} and AUC of the total-Pt and free-Pt tended to increase with the dose of nedaplatin. The AUCs of the total- and free-Pt at a dose of 100 mg/m² in Group A seemed similar to those at a dose of 80 mg/m² in Group B (Table 5), and there were no significant differences between these two treatment subgroups (*P* = 0.293 for total-Pt AUC and *P* = 0.336 for free-Pt AUC). Furthermore, the AUCs of free-Pt at the recommended doses in both groups (i.e., 100 mg/m² in Group A and 80 mg/m² in Group B) seemed also similar to that in patients aged 70 years or under who had been treated with 100 mg/m² of nedaplatin [14]. In the sigmoid E_{max} model assessing the pharmacodynamic effect of nedaplatin, the percentage decrease in the neutrophil counts were well correlated with the total-Pt (*r* = 0.652) and free-Pt (*r* = 0.723; Fig. 2).

Table 3 Non-hematological toxicity

Event	Dose level (mg/m ²), (number of patients)													
	80 (n = 7)					100 (n = 15)								
	Grade					Grade								
	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				

Event	Dose level (mg/m ²), (number of patients)														
	60 (n = 6)					80 (n = 6)					100 (n = 5)				
	Grade					Grade					Grade				
	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 aspartate 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/m² 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/m² 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

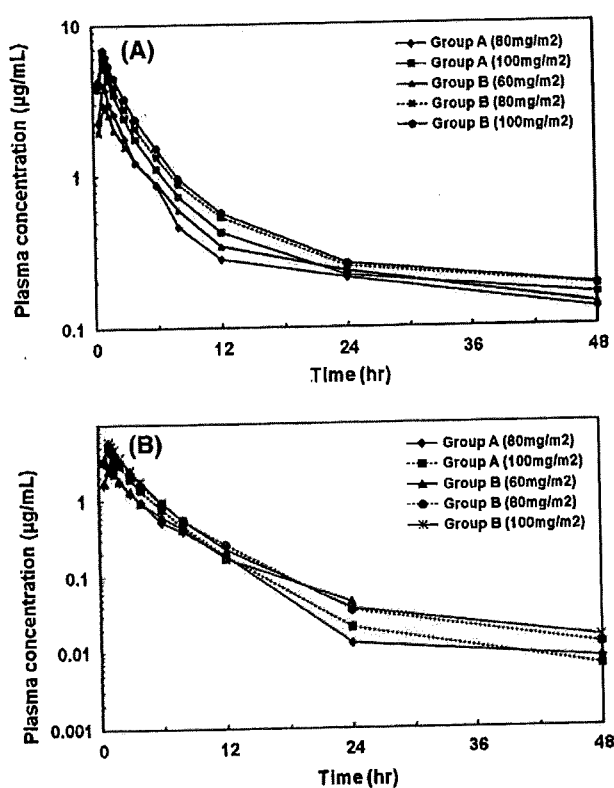


Fig. 1 Mean plasma concentration–time profiles for: a total-Pt and b free-Pt of nedaplatin

an additional nine patients were enrolled at the dose level of 100 mg/m² 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/m² 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 anti-tumor 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/m² of nedaplatin, the predicted nadir platelet count was ≥50,000/mm³. 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/m², respectively. The dose escalation over 100 mg/m² was not planned, because the recommended dose in younger patients (aged ≤70 years) had already been determined at 100 mg/m².

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 _{dis} (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
Patients ≤70 years [14]	100	5	5	5.91 ± 1.21	20.69 ± 5.52	29.63 ± 12.32	2.92 ± 0.66	7.87 ± 2.71
	100	5	5		15.9			

Data are shown as mean ± SD excepting the dose level of 80 mg/m² in Group A and 60 mg/m² 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_{dis} volume of distribution at steady-state, T_{1/2} terminal half life, CL systemic clearance

^a Data are shown as mean (actual data)

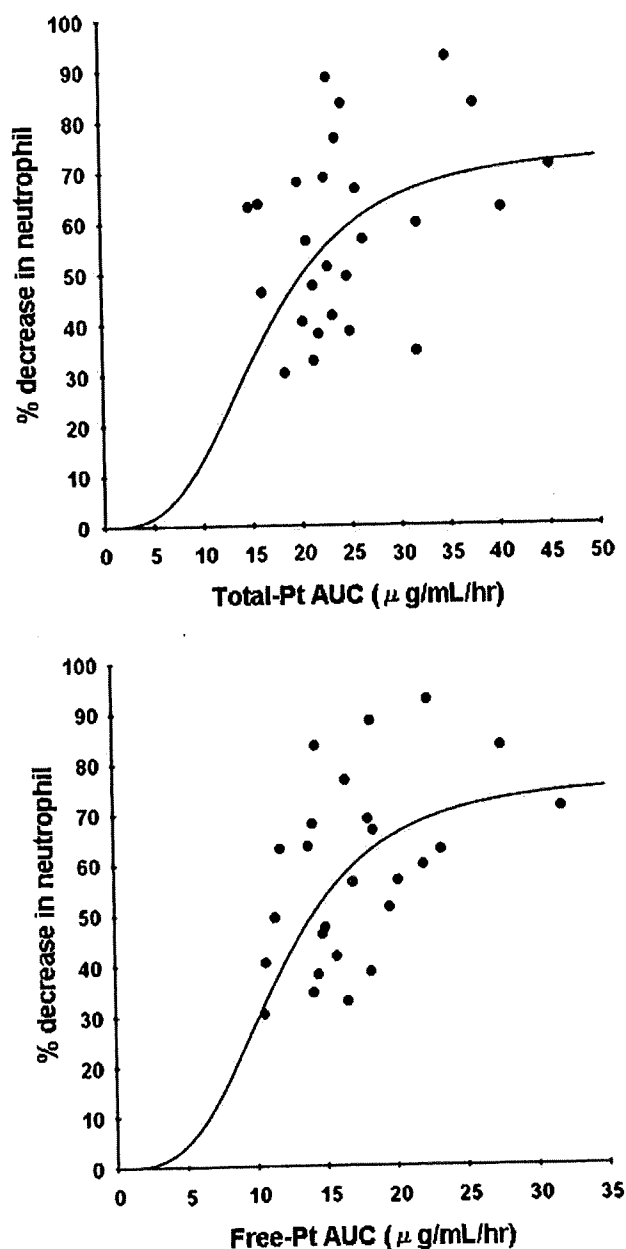


Fig. 2 Relationship between AUCs of total/free-Pt and the percentage decrease in the neutrophil count

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 $\text{Ccr} \geq 60 \text{ mL/min}$, and a dose of 80 mg/m^2 every 4 weeks was recommended for patients with $40 \leq \text{Ccr} < 60 \text{ mL/min}$. Nedaplatin can be safely administered to elderly patients with an acceptable level of toxicity and favorable antitumor activities against NSCLC, especially squamous cell carcinoma.

Acknowledgments This work was supported by a Grant-in-Aid for Cancer Research (9-25) from the Ministry of Health and Welfare, Tokyo, Japan.

References

1. Kaneko S, Ishikawa KB, Yoshimi I et al (2003) Projection of lung cancer mortality in Japan. *Cancer Sci* 94:919–923
2. Schiller JH, Harrington D, Belani CP et al (2002) Comparison of four chemotherapy regimens for advanced non-small cell lung cancer. *N Engl J Med* 346:92–98
3. Ohe Y, Ohashi Y, Kubota K et al (2007) 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
4. Kubota K, Watanabe K, Kunitoh H et al (2004) Phase III randomized trial of docetaxel plus cisplatin versus vindesine plus cisplatin in patients with stage IV non-small cell lung cancer: the Japanese Taxotere Lung Cancer Study Group. *J Clin Oncol* 22:254–261
 5. Oshita F, Kurata T, Kasai T et al (1995) Prospective evaluation of the feasibility of cisplatin-based chemotherapy for elderly lung cancer patients with normal organ functions. *Jpn J Cancer Res* 86:1198–1202
 6. Yamamoto N, Tamura T, Maeda M et al (1995) The influence of ageing on cisplatin pharmacokinetics in lung cancer patients with normal organ function. *Cancer Chemother Pharmacol* 36:102–106
 7. Kanzawa F, Matsushima Y, Nakano H et al (1988) Antitumor activity of a new platinum compound (glycolate-*O,O'*) diammineplatinum (II) (254-S), against non-small cell lung carcinoma grown in a human tumor clonogenic assay system. *Anticancer Res* 8:323–327
 8. Suzumura Y, Kato T, Ueda R et al (1989) Effect of treatment schedule on antitumor activity of glycolate-*O, O'*-diammineplatinum(II), a new platinum derivative: comparison with *cis*-diamminedichloroplatinum(II). *Anticancer Res* 9:1083–1088
 9. Hida S, Okada K, Yoshida O (1990) Advantages in combination chemotherapy using cisplatin and its analogues for human testicular tumor xenografts. *Jpn J Cancer Res* 81:425–430
 10. Furuse K, Fukuoka M, Kurita Y et al (1992) A phase II clinical study of *cis*-diammine glycolato platinum, 254-S, for primary lung cancer. *Gan To Kagaku Ryoho* 19:879–884
 11. Sasaki Y, Fukuda M, Morita M et al (1990) Prediction from creatinine clearance of thrombocytopenia and recommended dose in patients receiving (glycolato-*O,O'*)-diammine platinum (II) (NSC 375101D). *Jpn J Cancer Res* 81:196–200
 12. LeRoy AF, Wehling ML, Sponseller HL et al (1977) Analysis of platinum in biological materials by flameless atomic absorption spectrophotometry. *Biochem Med* 18:184–191
 13. Yamaoka K, Nakagawa T, Uno T (1978) Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. *J Pharmacokinet Biopharm* 6:165–175
 14. Sasaki Y, Tamura T, Eguchi K et al (1989) Pharmacokinetics of (glycolate-*O,O'*)-diammine platinum (II), a new platinum derivative, in comparison with cisplatin and carboplatin. *Cancer Chemother Pharmacol* 23:243–246
 15. The Elderly Lung Cancer Vinorelbine Italian Study Group (1999) Effects of vinorelbine on quality of life and survival of elderly patients with advanced non-small cell lung cancer: The Elderly Lung Cancer Vinorelbine Italian Study Group. *J Natl Cancer Inst* 91:66–72
 16. Gridelli C, Perrone F, Gallo C et al (2003) Chemotherapy for elderly patients with advanced non-small cell lung cancer: the Multicenter Italian Lung Cancer in the Elderly Study (MILES) phase III randomized trial. *J Natl Cancer Inst* 95:362–372
 17. Kudoh S, Takeda K, Nakagawa K et al (2006) Phase III study of docetaxel compared with vinorelbine in elderly patients with advanced non-small cell lung cancer: results of the West Japan Thoracic Oncology Group trial (WJTOG 9904). *J Clin Oncol* 24:3657–3663
 18. Calvert AH, Newell DR, Gumbrell LA et al (1989) Carboplatin dosage: prospective evaluation of a simple formula based on renal function. *J Clin Oncol* 7:1748–1756
 19. Kato H, Fukuchi M, Manda R et al (2003) Efficacy and toxicity of nedaplatin and 5-FU with radiation treatment for advanced esophageal carcinomas. *Anticancer Res* 23:3493–3498
 20. Kodaira T, Fuwa N, Tachibana H et al (2006) Phase I study of S-1 and nedaplatin for patients with recurrence of head and neck cancer. *Anticancer Res* 26:2265–2268
 21. Yoshioka T, Sakayori M, Kato S et al (2006) Dose escalation study of docetaxel and nedaplatin in patients with relapsed or refractory squamous cell carcinoma of the esophagus pretreated using cisplatin, 5-fluorouracil, and radiation. *Int J Clin Oncol* 11:454–460
 22. Kanni M, Matsumoto S, Nishimura T et al (2007) Retrospective analysis of 27 consecutive patients treated with docetaxel/nedaplatin combination therapy as a second-line regimen for advanced esophageal cancer. *Int J Clin Oncol* 12:224–227

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 IDEAL 1 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-naïve patients 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 $\geq 4,000/\mu\text{L}$, absolute neutrophil count $\geq 2,000/\mu\text{L}$, hemoglobin ≥ 9.5 g/dL, platelets $\geq 100,000/\mu\text{L}$, AST/ALT ≤ 2.5 times the upper limit of normal, total bilirubin ≤ 1.5 mg/dL, serum creatinine ≤ 1.2 mg/dL, and PaO₂ in arterial blood ≥ 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/m², day 1, every 3 weeks; (2) cisplatin 80 mg/m², day 1, and irinotecan 60 mg/m², days 1, 8, 15, every 4 weeks; (3) cisplatin 80 mg/m², day 1, and vinorelbine 25 mg/m², days 1, 8, every 3 weeks; (4) cisplatin 80 mg/m², day 1, and gemcitabine 1,000 mg/m² days 1, 8, every 3 weeks; or (5) cisplatin 80 mg/m², day 1, and docetaxel 60 mg/m² 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

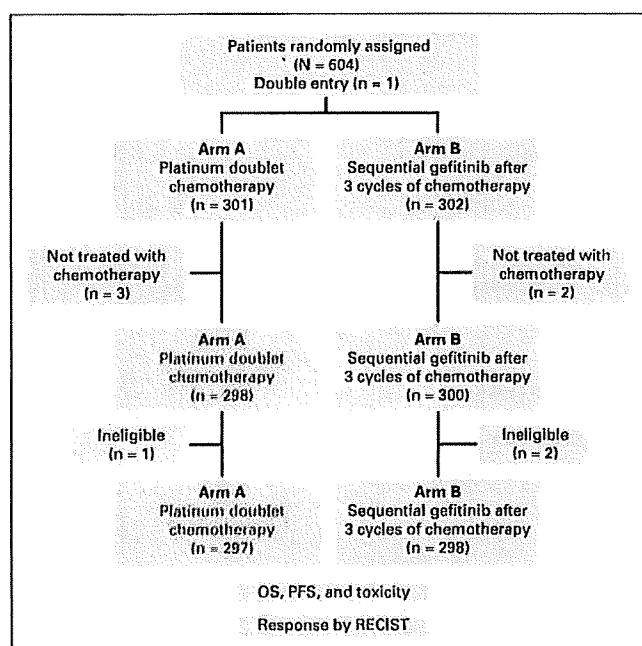


Fig 1. CONSORT diagram for the study. OS, overall survival; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors.

radiographs were done every treatment cycle. Toxicity was evaluated according to the National Cancer Institute Cancer Common Toxicity Criteria (NCI-CTC) version 2.¹⁷

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 chemo-

therapy 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

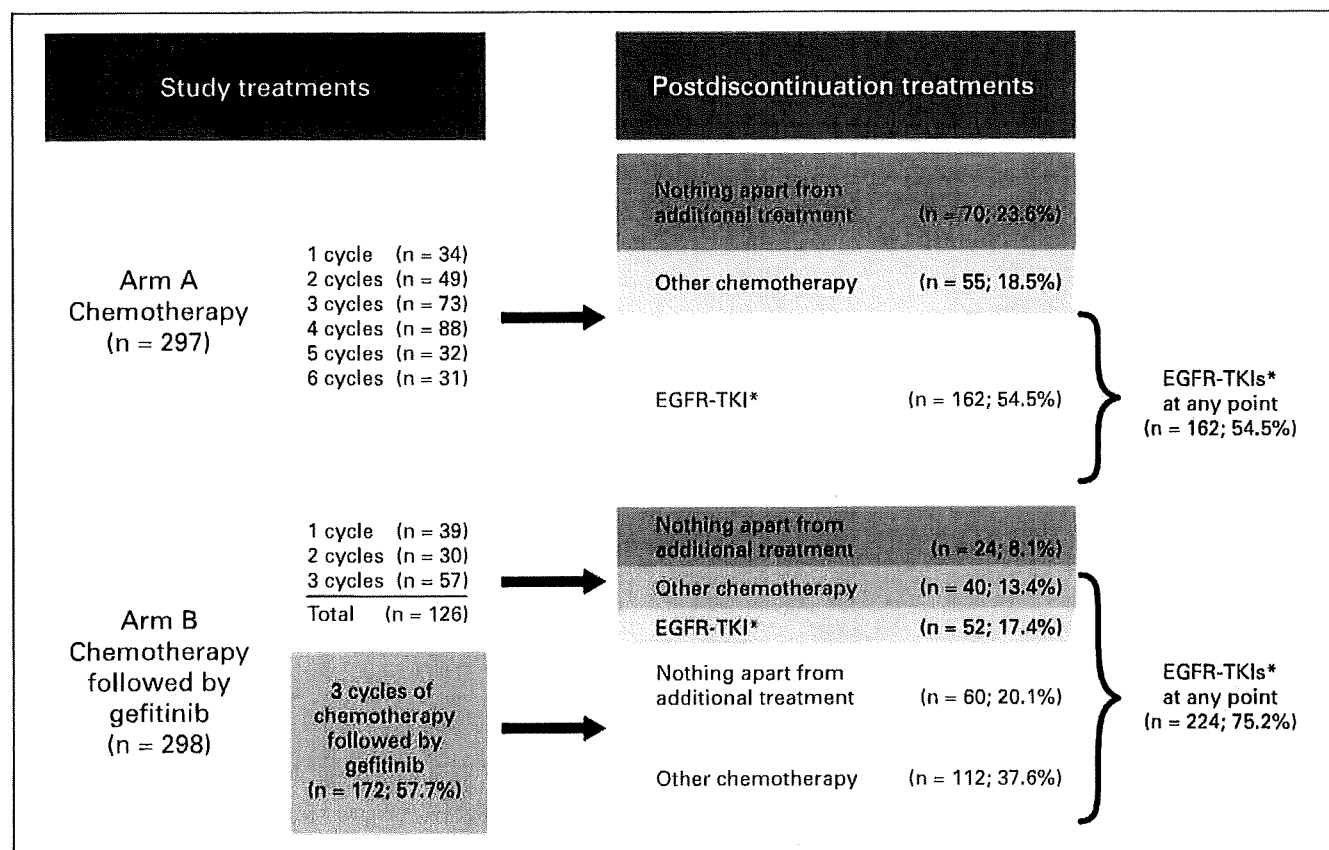


Fig 2. Exposure to active epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), including postdiscontinuation treatments in the full analysis set population (n = 595).

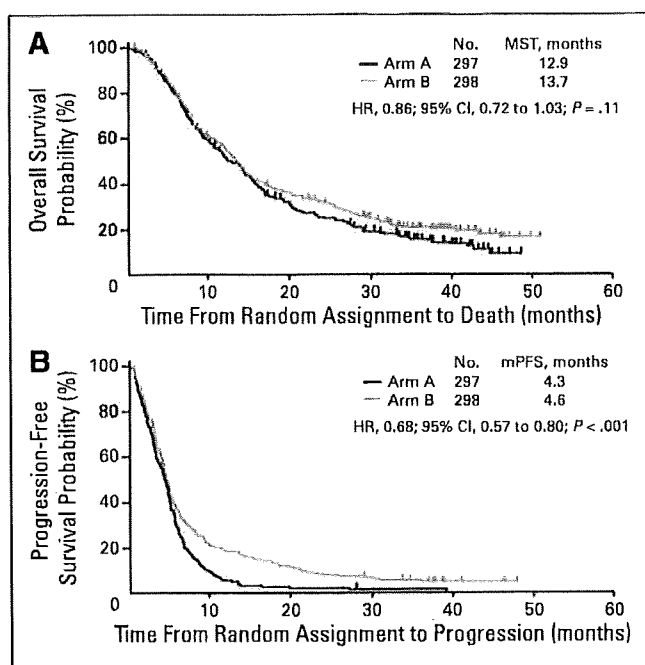


Fig 3. (A) Overall survival and (B) progression-free survival ($n = 598$). MST, median survival time; HR, hazard ratio; mPFS, median progression-free survival.

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

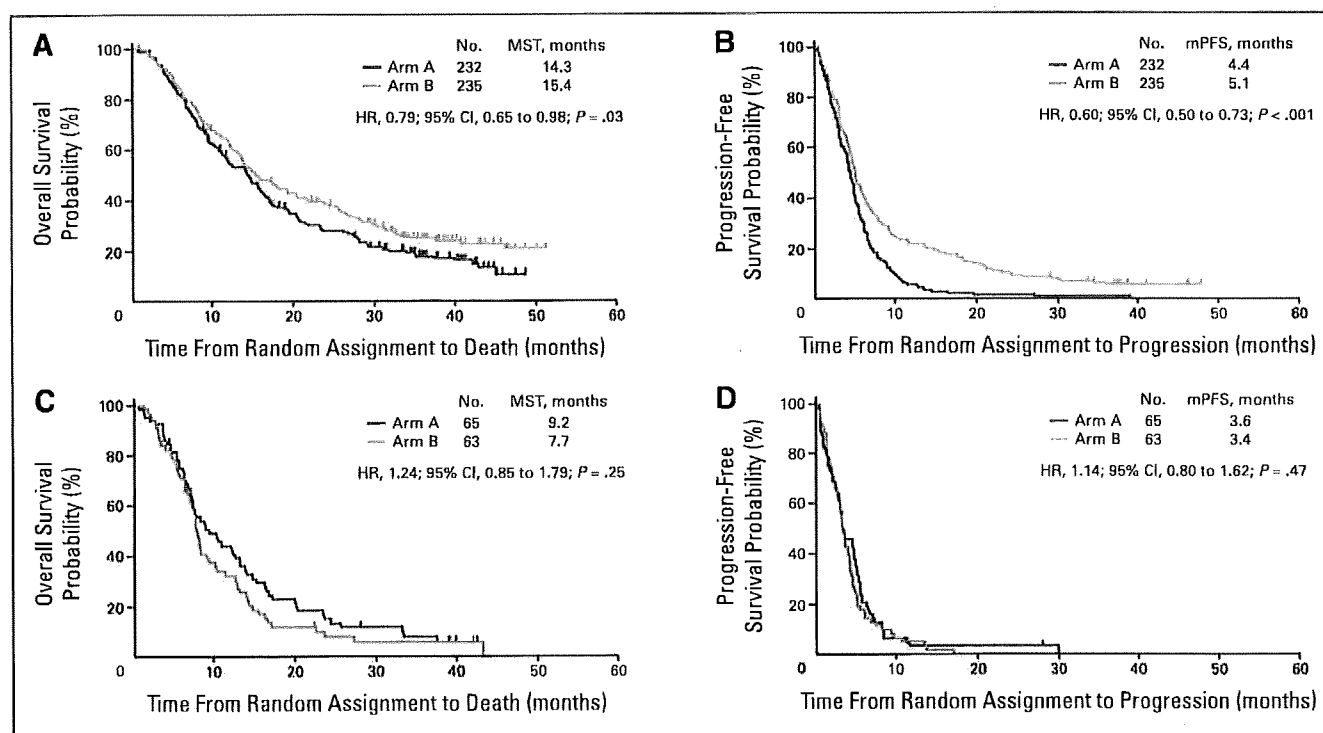


Fig 4. (A) Overall survival in the subset groups of patients with adenocarcinoma ($n = 467$), (B) progression-free survival in the subset groups of patients with adenocarcinoma ($n = 467$), (C) overall survival in the subset groups of patients with nonadenocarcinoma ($n = 128$), and (D) progression-free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

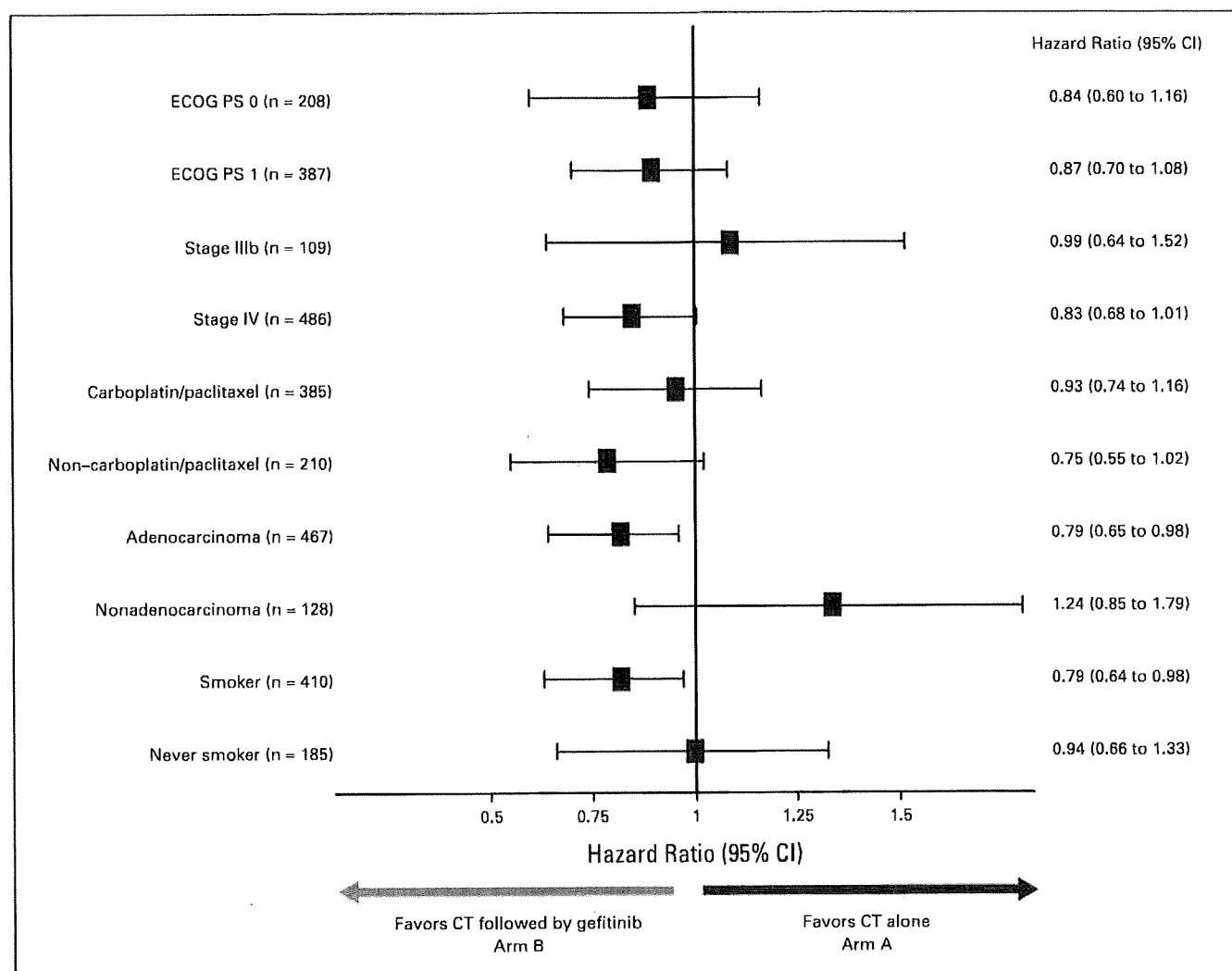


Fig 5. Forest plot subgroup analysis according to patients' backgrounds. CT, chemotherapy; ECOG PS, Eastern Cooperative Oncology Group performance status.

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 chemo-

therapy.^{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 patients¹¹; 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

Platinum-Doublet Chemotherapy Followed By Gefitinib for NSCLC

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/pharyngitis	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 *EGFR* 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 inves-

tigations 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.

Employment or Leadership Position: None **Consultant or Advisory Role:** Takayasu Kurata, Takeda Pharmaceutical (U) **Stock Ownership:** None **Honoraria:** Miyako Satouchi, AstraZeneca; Yukito Ichinose, AstraZeneca; Nobuyuki Yamamoto, AstraZeneca; Takayasu Kurata, AstraZeneca, Eli Lilly; Kazuhiko Nakagawa, AstraZeneca, sanofi-aventis; Masahiro Fukuoka, AstraZeneca, Chugai Pharmaceutical **Research Funding:** None **Expert Testimony:** None **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

Conception and design: Koji Takeda, Toyooki Hida, Masahiko Ando, Miyako Satouchi, Nobuyuki Yamamoto, Takayasu Kurata, Kazuhiko Nakagawa, Masahiro Fukuoka

Administrative support: Tosiya Sato, Masahiko Ando, Masahiro Fukuoka

Provision of study materials or patients: Koji Takeda, Toyooki Hida, Takashi Seto, Miyako Satouchi, Nobuyuki Katakami, Nobuyuki Yamamoto, Shinzoh Kudoh, Jiichiro Sasaki, Kaoru Matsui, Koichi Takayama, Tatsuhiko Kashii, Yasuo Iwamoto, Toshiyuki Sawa, Takayasu Kurata, Kazuhiko Nakagawa, Masahiro Fukuoka

Collection and assembly of data: Koji Takeda, Toyooki Hida, Takashi Seto, Miyako Satouchi, Yukito Ichinose, Nobuyuki Katakami, Nobuyuki Yamamoto, Shinzoh Kudoh, Jiichiro Sasaki, Kaoru Matsui, Koichi

Takayama, Tatsuhiko Kashii, Yasuo Iwamoto, Toshiyuki Sawa, Takayasu Kurata, Kazuhiko Nakagawa

Data analysis and interpretation: Koji Takeda, Toyooki Hida, Tosiya Sato, Masahiko Ando, Miyako Satouchi, Yukito Ichinose, Nobuyuki Yamamoto, Isamu Okamoto, Takayasu Kurata, Kazuhiko Nakagawa, Masahiro Fukuoka

Manuscript writing: Koji Takeda, Toyooki Hida, Masahiko Ando, Isamu Okamoto

Final approval of manuscript: Koji Takeda, Kazuhiko Nakagawa, Masahiro Fukuoka

REFERENCES

- Parkin DM: Global cancer statistics in the year 2000. *Lancet Oncol* 2:533-543, 2001
- Dancey J, Shepherd FA, Gralla RJ, et al: Quality of life assessment of second-line docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy: Results of a prospective, randomized phase III trial. *Lung Cancer* 43:183-194, 2004
- Socinski MA, Morris DE, Masters GA, et al: Chemotherapeutic management of stage IV non-small cell lung cancer. *Chest* 123:226S-243S, 2003 (suppl 1)
- Pfister DG, Johnson DH, Azzoli CG, et al: American Society of Clinical Oncology treatment of unresectable non-small-cell lung cancer guideline: Update 2003. *J Clin Oncol* 22:330-353, 2004
- Scagliotti GV, Parikh P, von Pawel J, et al: Phase III comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naïve patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol* 26:3543-3553, 2008
- Jorissen RN, Walker F, Pouliot N, et al: Epidermal growth factor receptor: Mechanisms of activation and signaling. *Exp Cell Res* 284:31-53, 2003
- Fukuoka M, Yano S, Giaccone G, et al: Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* 21:2237-2246, 2003
- Kris MG, Natale RB, Herbst RS, et al: Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: A randomized trial. *JAMA* 290:2149-2158, 2003
- Giaccone G, Herbst RS, Manegold C, et al: Gefitinib in combination with gemcitabine and cisplatin in advanced non-small cell lung cancer: A phase III trial – INTACT 1. *J Clin Oncol* 22:777-784, 2004
- Herbst RS, Giaccone G, Schiller JH, et al: Gefitinib in combination with paclitaxel and carboplatin in advanced non-small cell lung cancer: A phase III trial – INTACT 2. *J Clin Oncol* 22:785-794, 2004
- Ando M, Okamoto I, Yamamoto N, et al: Predictive factors for interstitial lung disease, antitumor response, and survival in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 24:2549-2556, 2006
- Mitsudomi T, Kosaka T, Endoh H, et al: Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. *J Clin Oncol* 23:2513-2520, 2005
- Huang SF, Liu HP, Li LH, et al: High frequency of epidermal growth factor receptor mutations with complex patterns in non-small cell lung cancers related to gefitinib responsiveness in Taiwan. *Clin Cancer Res* 10:8195-8203, 2004
- Tokumo M, Toyooka S, Kiura K, et al: The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. *Clin Cancer Res* 11:1167-1173, 2005
- 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
- Kubota K, Watanabe K, Kunitho H, et al: Phase III randomized trial of docetaxel plus cisplatin versus vindesine plus cisplatin in patients with stage IV non-small-cell lung cancer: The Japanese Taxotere Lung Cancer Study Group. *J Clin Oncol* 22:254-261, 2004
- National Cancer Institute: National Cancer Institute Common Toxicity Criteria version 2.0. http://ctep.cancer.gov/forms/CTCv20_4-30-992.pdf
- Therasse P, Arbuck SG, Eisenhauer EA, et al: New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 92:205-216, 2000
- 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
- Brodowicz T, Krzakowski M, Zwitter M, et al: Cisplatin and gemcitabine first-line chemotherapy followed by maintenance gemcitabine or best supportive care in advanced non-small cell lung cancer: A phase III trial. *Lung Cancer* 52:155-163, 2006
- Fidias P, Dakhl S, Lyss A, et al: Phase III study of immediate versus delayed docetaxel after induction therapy with gemcitabine plus carboplatin in advanced non-small-cell lung cancer: Updated report with survival. *J Clin Oncol* 27:591-598, 2009
- Belani CP, Brodowicz T, Ciuleanu T, et al: Maintenance pemetrexed (Pem) plus best supportive care (BSC) versus placebo (Plac) plus BSC: A randomized phase III study in advanced non-small cell lung cancer (NSCLC). *J Clin Oncol* 27:407s, 2009 (suppl; abstr CRA8000)
- Cappuzzo F, Ciuleanu T, Stelmakh L, et al: SATURN: A double-blind, randomized, phase III study of maintenance erlotinib versus placebo following nonprogression with first-line platinum-based chemotherapy in patients with advanced NSCLC. *J Clin Oncol* 27:407s, 2009 (suppl; abstr 8001)
- Paez JG, Janne PA, Lee JC, et al: EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 304:1497-1500, 2004
- Lynch TJ, Bell DW, Sordella R, et al: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350:2129-2139, 2004
- Yang CH, Yu CJ, Shih JY, et al: Specific EGFR mutations predict treatment outcome of stage IIIB/IV patients with chemotherapy-naïve non-small-cell lung cancer receiving first-line gefitinib monotherapy. *J Clin Oncol* 26:2745-2753, 2008
- Tamura K, Okamoto I, Kashii T, et al: Multicentre prospective phase II trial of gefitinib for advanced non-small cell lung cancer with epidermal growth factor receptor mutations: Results of the West Japan Thoracic Oncology Group trial (WJTOG0403). *Br J Cancer* 98:907-914, 2008

De Novo Resistance to Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors in *EGFR* Mutation-Positive Patients with Non-small Cell Lung Cancer

Masayuki Takeda, MD, PhD,* Isamu Okamoto, MD, PhD,* Yoshihiko Fujita, PhD,†
Tokuzo Arao, MD, PhD,† Hiroyuki Ito, MD, PhD,‡ Masahiro Fukuoka, MD, PhD,§
Kazuto Nishio, MD, PhD,† and Kazuhiko Nakagawa, MD, PhD*

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 1A). (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 1B). (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 1C). 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 1D–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

Departments of *Medical Oncology, †Genome Biology, and ‡Pathology, Kinki University School of Medicine, Osaka-Sayama; and §Department of Medical Oncology, Kinki University School of Medicine, Sakai Hospital, Minami-ku, Sakai, Osaka, Japan.

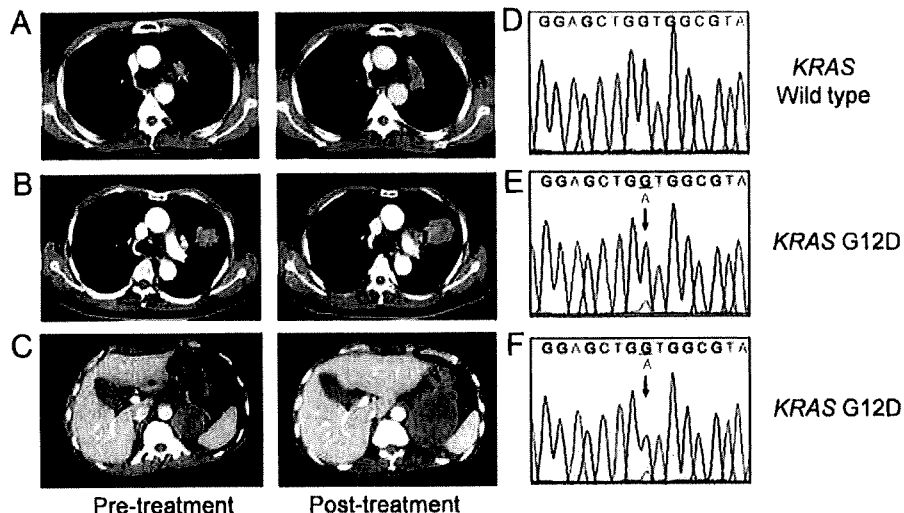
Disclosure: The authors declare no conflict of interest.

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

Copyright © 2010 by the International Association for the Study of Lung Cancer

ISSN: 1556-0864/10/0503-0399

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.

ACKNOWLEDGMENTS

The authors thank Tadao Uesugi for technical assistance.

REFERENCES

- Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 2005;97:339–346.
- Pao W, Wang TY, Riely GJ, et al. *KRAS* mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* 2005;2:e17.
- Kosaka T, Yatabe Y, Endoh H, et al. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* 2004;64:8919–8923.
- Kalikaki A, Koutsopoulos A, Trypaki M, et al. Comparison of *EGFR* and *K-RAS* gene status between primary tumours and corresponding metastases in NSCLC. *Br J Cancer* 2008;99:923–929.
- Han SW, Kim TY, Jeon YK, et al. Optimization of patient selection for gefitinib in non-small cell lung cancer by combined analysis of epidermal growth factor receptor mutation, K-ras mutation, and Akt phosphorylation. *Clin Cancer Res* 2006;12:2538–2544.
- Uchida A, Hirano S, Kitao H, et al. Activation of downstream epidermal growth factor receptor (*EGFR*) signaling provides gefitinib-resistance in cells carrying *EGFR* mutation. *Cancer Sci* 2007;98:357–363.
- Sos ML, Koker M, Weir BA, et al. *PTEN* loss contributes to erlotinib resistance in *EGFR*-mutant lung cancer by activation of Akt and *EGFR*. *Cancer Res* 2009;69:3256–3261.

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

Hiroyasu Kaneda^{1,2}, Tokuzo Arai¹, Kaoru Tanaka^{1,2}, Daisuke Tamura¹, Keiichi Aomatsu¹, Kanae Kudo¹, Kazuko Sakai¹, Marco A. De Velasco¹, Kazuko Matsumoto¹, Yoshihiko Fujita¹, Yasuhide Yamada³, Junji Tsurutani², Isamu Okamoto², Kazuhiko Nakagawa², and Kazuto Nishio¹

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 mm³, *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).

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 SM22 α , 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

Authors' Affiliations: Departments of ¹Genome Biology and ²Medical Oncology, Kinki University School of Medicine, Osaka-Sayama, Osaka, Japan and ³Department of Medical Oncology, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Author: Kazuto Nishio, Department of Genome Biology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan. Phone: 81-72-366-0221; Fax: 81-72-366-0206; E-mail: knishio@med.kindai.ac.jp.

doi: 10.1158/0008-5472.CAN-09-2161

©2010 American Association for Cancer Research.

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 G₁ 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% CO₂-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 NH₂ 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.

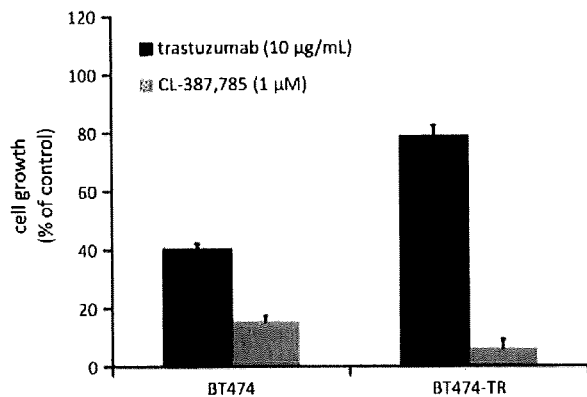


Figure 3. Effect of trastuzumab and CL-387,785 on growth inhibition in BT-474 and BT474-TR cells. Mean percentage of control and standard deviation of 6–12 replicate wells treated with 10 µg/ml trastuzumab and 1 µM CL-387,785 were plotted. BT474-TR remains sensitive to CL-387,785.

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

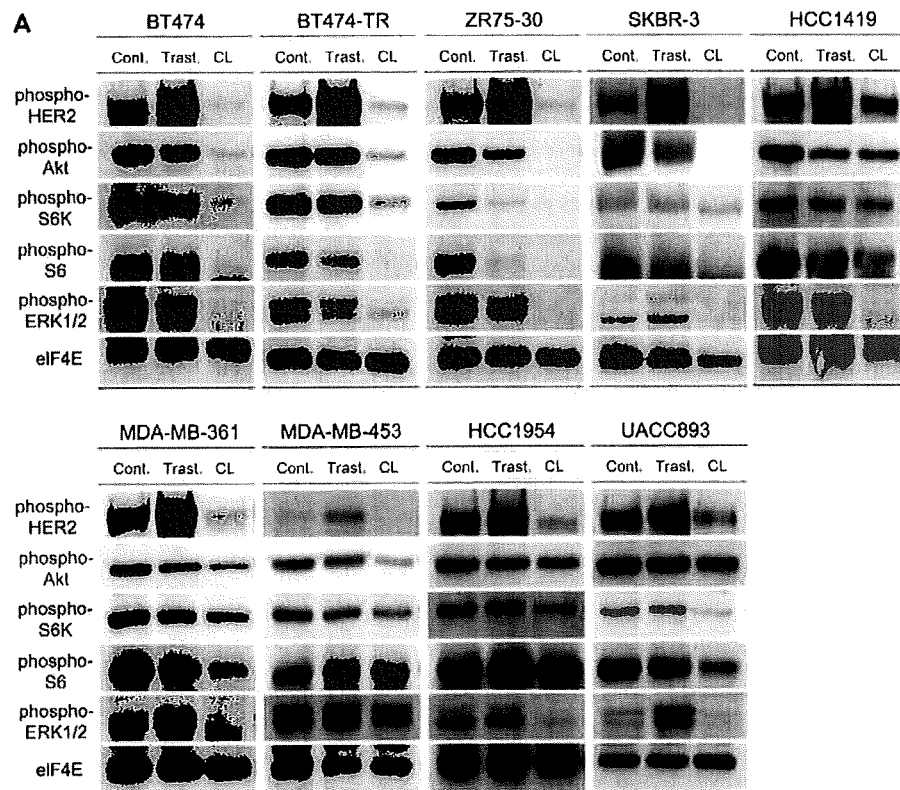


Figure 4. (A) Expression of phosphorylated-HER2, -Akt, -S6K, -S6 and -ERK1/2 in *HER2*-amplified breast cell lines with and without treatment with trastuzumab (10 µg/ml) and CL-387,785 (1 µM). Breast cell lines grown in 10% serum-containing media were lysed and immunoblotted for each protein. Blots were 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 \times width² \times 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).

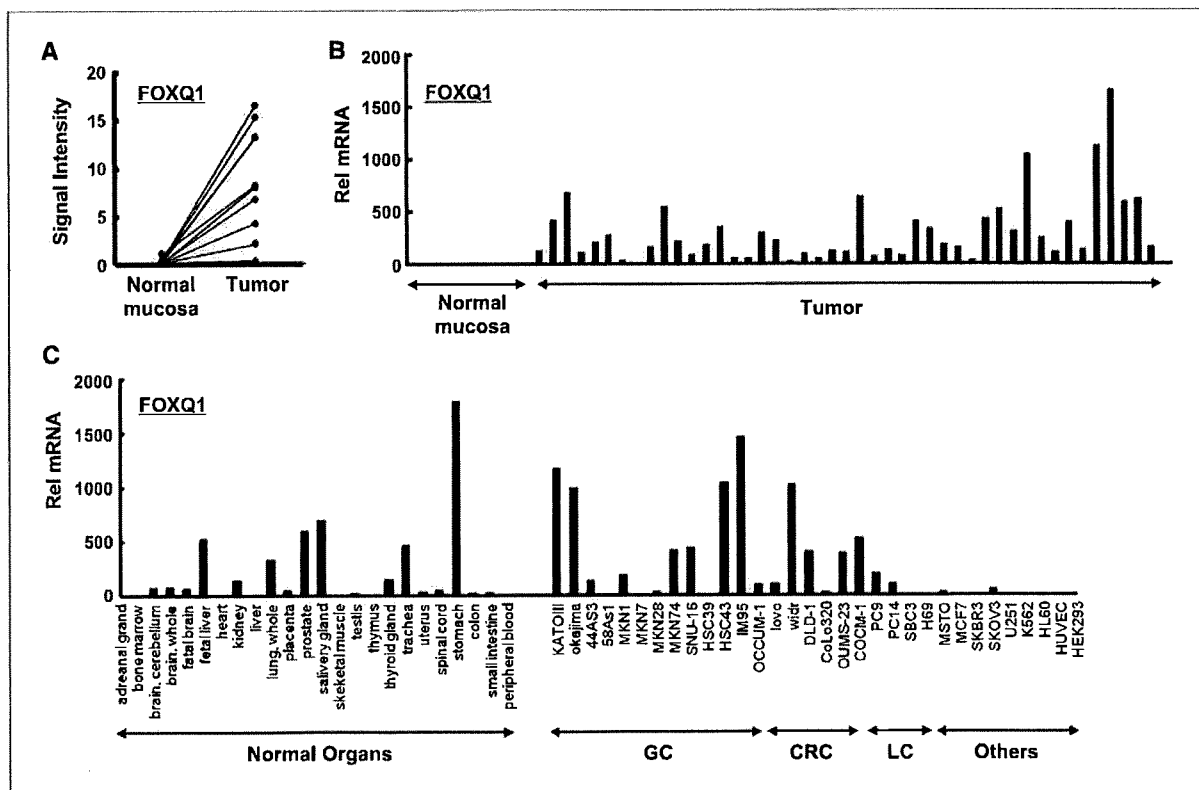


Figure 1. FOXQ1 expression in CRC. A, mRNA expression of FOXQ1 obtained from a microarray analysis of 10 CRC and paired normal mucosa specimens. The values indicate the normalized signal intensity. B, the mRNA expression levels of FOXQ1 were determined using real-time RT-PCR for 10 paired and an additional 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 $\times 10^4$).