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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

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Background: To compare the efficacy and toxicity of three platinum-based combination regimens against cisplatin plus irinotecan (IP) in patients with untreated advanced non-small-cell lung cancer (NSCLC) by a non-inferiority design.
Patients and methods: A total of 602 patients were randomly assigned to one of four regimens: cisplatin 80 mg/m^2 on day 1 plus irinotecan 60 mg/m^2 on days 1, 8, 15 every 4 weeks (IP) carboplatin AUC 6.0 min × mg/mL (area under the concentration–time curve) on day 1 plus paclitaxel 200 mg/m^2 on day 1 every 3 weeks (TC); cisplatin 80 mg/m^2 on day 1 plus gemcitabine 1000 mg/m^2 on days 1, 8 every 3 weeks (GP); and cisplatin 80 mg/m^2 on day 1 plus vinorelbine 25 mg/m^2 on days 1, 8 every 3 weeks (NP).
Results: The response rate, median survival time, and 1-year survival rate were 31.0%, 13.9 months, 59.2%, respectively, in IP; 32.4%, 12.3 months, 51.0% in TC; 30.1%, 14.0 months, 59.6% in GP; and 33.1%, 11.4 months, 48.3% in NP. No statistically significant differences were found in response rate or overall survival, but the non-inferiority of none of the experimental regimens could be confirmed. All the four regimens were well tolerated.
Conclusion: The four regimens have similar efficacy and different toxicity profiles, and they can be used to treat advanced NSCLC patients.
Key words: carboplatin, cisplatin, gemcitabine, irinotecan, non-small-cell lung cancer, paclitaxel, randomized phase III study, vinorelbine

introduction

Nearly 60 000 patients in Japan died of lung cancer in 2004, and the mortality rate is still increasing [1]. Even old-generation cisplatin-based chemotherapy provides a survival benefit and symptom relief in patients with inoperable non-small-cell lung cancer (NSCLC) [2]. Several anticancer agents including irinotecan, paclitaxel, docetaxel, gemcitabine, and vinorelbine, were developed in the 1990s and most of them have mechanisms of action that differ from those of the old-generation agents [3–7]. The combinations of platinum and these new agents developed in the 1990s are more useful against advanced NSCLC than old-generation combination chemotherapy, and doublets of platinum and new-generation anticancer agents are considered standard chemotherapy regimens for advanced NSCLC, although no consistent standard regimens have yet been established [8–17].

Two phase III studies comparing cisplatin plus irinotecan (IP) with cisplatin plus vindesine for advanced NSCLC have been conducted in Japan [18, 19]. Fukuoka et al. [20] reported the results of a combined analysis of the 358 eligible stage IV patients in these studies. They carried out a multivariate analysis using the Cox regression model with adjustment for well-known prognostic factors, and the Cox regression analysis demonstrated that treatment with IP was one of significant independent favorable factor. Based on their data, we selected IP for the reference arm in our study.

The Ministry of Health, Labour and Welfare of Japan approved the prescription of paclitaxel, gemcitabine, and

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vinorelbine for NSCLC in 1999 and requested a phase III study to confirm the efficacy and safety of these agents. The Japanese investigators and the pharmaceutical companies decided to conduct a four-arm randomized phase III study for NSCLC, the so-called FACS, Four-Arm Cooperative Study. The purpose of the study was to compare the efficacy and toxicity of three platinum-based combination regimens, carboplatin plus paclitaxel (TC), cisplatin plus gemcitabine (GP), cisplatin plus vinorelbine (NP), with IP as the reference arm.

patients and methods

patient selection

Patients with histologically and/or cytologically documented NSCLC were eligible for participation in the study. Each patient had to meet the following criteria: clinical stage IV or IIIB (including only patients with no indications for curative radiotherapy, such as malignant pleural effusion, pleural dissemination, malignant pericardiac effusion, or metastatic lesion in the same lobe), at least one target lesion >2 cm, no prior chemotherapy, no prior surgery and/or radiotherapy for the primary site, age 20–74 years, Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, adequate hematological, hepatic and renal functions, partial pressure of arterial oxygen (paO_2) ≥60 torr, expected survival >3 months, able to undergo first course treatment in an inpatient setting, and written informed consent. The study was approved by the Institutional Review Board at each hospital. Written informed consent was obtained from every patient.

treatment schedule

All patients were randomly assigned to one of the four treatment groups by the central registration office by means of the minimization method. Stage, PS, gender, lactate dehydrogenase (LDH) and albumin values, and institution were used as adjustment variables. The first group received the reference treatment, 80 mg/m^2 of cisplatin on day 1 and 60 mg/m^2 of irinotecan on days 1, 8, and 15, and the cycle was repeated every 4 weeks. The second group received 200 mg/m^2 of paclitaxel (Bristol-Myers K.K., Tokyo, Japan) over a 3-h period followed by carboplatin at a dose calculated to produce an area under the concentration–time curve of 6.0 min × mg/mL on day 1 and the cycle was repeated every 3 weeks. The third group received 80 mg/m^2 of cisplatin on day 1 and 1000 mg/m^2 of gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. The fourth group received 80 mg/m^2 of cisplatin on day 1 and 25 mg/m^2 of vinorelbine (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. Each treatment was repeated for three or more cycles unless the patient met the criteria for progressive disease or experienced unacceptable toxicity.

response and toxicity evaluation

Response was evaluated according to the Response Evaluation Criteria in Solid Tumors, and tumor markers were excluded from the criteria [21]. Objective tumor response in all responding patients was evaluated by an external review committee with no information on the treatment group. Toxicity grading criteria in National Cancer Institute Common Toxicity Criteria Ver 2.0 were used to evaluate toxicity.

quality of life assessment

Quality of life (QoL) was evaluated by means of the Functional Assessment of Cancer Therapy—Lung (FACT-L) Japanese version and the QoL Questionnaire for Cancer Patients Treated with Anticancer Drugs (QoL-ACD), before treatment, immediately before the second cycles of chemotherapy, and 3 and 6 months after the start of treatment [22–24].

statistical analysis and monitoring

The primary end point of this study was overall survival (OS), and the secondary end points were response rate, response duration, time to progressive disease (TTP), time to treatment failure (TTTF), adverse event, and QoL. The 1-year survival rate of the control group in this study was estimated to be 43% based on the data in published papers, and the 1-year survival rate in the other treatment group was expected to be 50%. The lower equivalence limit for 1-year survival rate was set as '−10%'. The criterion for the non-inferiority of each treatment was a lower limit of the two-sided 95% confidence interval (CI) of the 1-year survival rate of treatment minus that of control larger than the lower equivalence limit. Because the non-inferiority of each treatment versus the control was to be evaluated independently, a separate null hypothesis was stated for each treatment, and for that reason no multiple comparison adjustment was included in the study. Based on the above conditions and binomial distribution, 135 patients were needed per arm for a one-sided Type I error of 2.5% and 80.0% power. In view of the possibility of variance inflation due to censoring, the sample size was set at 600 (150 per arm).

Central registration with randomization, monitoring, data collection, and the statistical analyses were independently carried out by a contract research organization (EPS Co., Ltd, Tokyo, Japan).

results

patient characteristics

From October 2000 to June 2002, a total of 602 patients were registered by 44 hospitals in Japan. All patients had been followed up for >2 years, and 447 patients had died as of June 2004. Of the 602 patients registered, 151 were allocated to the reference treatment, IP, and 150, 151, and 150 patients were allocated to TC, GP, and NP, respectively. Since 10 patients did not receive chemotherapy and 11 patients were subsequently found to be ineligible, 592 patients were assessable for toxicity and 581 patients were assessable for efficacy. Four patients did not receive chemotherapy due to electrolytic disorder, fever, symptomatic brain metastases, and rapid tumor progression in IP, two patients due to refusal and pneumonia in TC, four patients due to lower WBC counts (two patients), rapid tumor progression, and nephritic syndrome in NP. Two patents were ineligible due to wrong stage in IP, two patients were wrong stage and one patient had double cancer in TC, two patients were wrong diagnosis, one patient had massive pleural effusion, one patient received prior chemotherapy in GP, one patient had no target lesions in NP. Age, gender, PS, stage, and LDH and albumin values were well balanced in each arm (Table 1). Fewer patients with adenocarcinoma and more patients with squamous cell carcinona were, however, entered in three experimental arms than in IP.

objective tumor response and response duration

Objective tumor response is shown in Table 2. Forty-five partial responses occurred in the 145 assessable patients in the reference arm, IP, for an objective response rate of 31.0% with a median response duration of 4.8 months. The response rate and median response duration were 32.4% and 4.0 months in TC, 30.1% and 3.5 months in GP, and 33.1% and 3.4 months in NP. The response rates in TC, GP, and NP were not statistically different from the rate in IP according to the results of the χ^2 test.

Table 1. Patient characteristics and treatment delivery

	Cisplatin + irinotecan	Carboplatin + paclitaxel	Cisplatin + gemcitabine	Cisplatin + vinorelbine
Assessable patients	145	145	146	145
Gender (male/female)	97/48	99/46	101/45	101/44
Age, median (range)	62 (30–74)	63 (33–74)	61 (34–74)	61 (28–74)
PS (0/1)	44/101	44/101	45/101	45/100
Histology				
Adenocarcinoma	121	104	108	109
Squamous cell carcinoma	16	31	29	29
Others	8	10	9	7
Stage (IIIB/IV)	31/114	28/117	30/116	26/119
No. of cycles				
Mean ± SD	3.0 ± 1.3	3.5 ± 1.5	3.2 ± 1.2	3.1 ± 1.3
Median	3	3	3	3
Range	1–7	1–10	1–7	1–8

PS, performance status; SD, standard deviation.

Table 2. Survival, TTP, TTTF, response rate, and response duration

	N	Median survival (months)	1-year survival (%)	Difference in 1-year survival from IP	2-year survival (%)	TTP (median), months	TTTF (median), months	Response rate (%)	Response duration (median), months
Cisplatin + irinotecan	145	13.9	59.2	–	26.5	4.7	3.3	31.0	4.8 (n = 45)
Carboplatin + paclitaxel	145	12.3	51.0	−8.2% (95% CI −19.6% to 3.3%)	25.5	4.5 (P = 0.355)[a]	3.2 (P = 0.282)[a]	32.4 (P = 0.801)[b]	4.0 (n = 47)
Cisplatin + gemcitabine	146	14.0	59.6	0.4% (95% CI −10.9% to11.7%)	31.5	4.0 (P = 0.170)[a]	3.2 (P = 0.567)[a]	30.1 (P = 0.868)[b]	3.5 (n = 44)
Cisplatin + vinorelbine	145	11.4	48.3	−10.9% (95% CI −22.3% to 0.5%)	21.4	4.1 (P = 0.133)[a]	3.0 (P = 0.091)[a]	33.1 (P = 0.706)[b]	3.4 (n = 48)

[a]Compared with IP by the generalized Wilcoxon test.

[b]Compared with IP by the χ^2 test.

CI, confidence interval; IP, cisplatin plus irinotecan; TTP, time to progressive disease; TTTF, time to treatment failure.

OS, TTP disease, and TTTF

OS and TTP are shown in Figure 1. Median survival time (MST), the 1-year, and 2-year survival rate in IP were 13.9 months, 59.2%, and 26.5%, respectively. The MSTs, 1-year, and 2-year survival rates were, respectively, 12.3 months, 51.0%, and 25.5% in TC; 14.0 months, 59.6%, and 31.5% in GP; and 11.4 months, 48.3%, and 21.4% in NP. The lower limits of the 95% CI of the difference in 1-year survival rate between IP and TC (−19.6%), GP (−10.9%), and NP (−22.3%) were below −10%, which was considered the lower equivalence limit (Table 2). Thus, the results did not show non-inferiority in three experimental regimens compared with reference treatment. Median TTP and median TTTF were 4.7 and 3.3 months, respectively in IP. Median TTP and TTTF were, respectively, 4.5 and 3.2 months in TC, 4.0 and 3.2 months in GP, and 4.1 and 3.0 months in NP. There were no statistical differences in either TTP or TTTF in TC, GP, or NP, compared with IP according to the results of the generalized Wilcoxon test (Table 2).

hematologic and non-hematologic toxicity

In IP, 47.6% and 83.7% of patients developed grade 3 or worse leukopenia and neutropenia, respectively (Table 3). The incidences of grade 3 or worse leukopenia (33.1%, P = 0.010) and neutropenia (62.9%, P < 0.001) were significantly lower in GP than in IP. The incidence of grade 3 or worse leukopenia (67.1%, P < 0.001) was significantly higher in NP than in IP. Grade 3 or worse thrombocytopenia developed in 5.4% of the patients in IP, and the incidence was significantly higher in GP (35.1%, P < 0.001). The incidence of febril neutropenia in IP was 14.3%, and was significantly lower in GP (2.0%, P < 0.001).

Grade 2 or worse nausea, vomiting, anorexia, and fatigue occurred in 60.5%, 51.0%, 65.3%, and 38.8%, respectively, of the patients in IP. The incidences of grade 2 or worse nausea (TC: 25.0%, P < 0.001, NP: 47.3%, P = 0.022), vomiting (TC: 22.3%, P < 0.001, NP: 36.3%, P = 0.011), and anorexia (TC: 32.4%, P < 0.001, NP: 49.3%, P = 0.005) were significantly lower in TC and NP than in IP. Grade 2 or worse diarrhea was

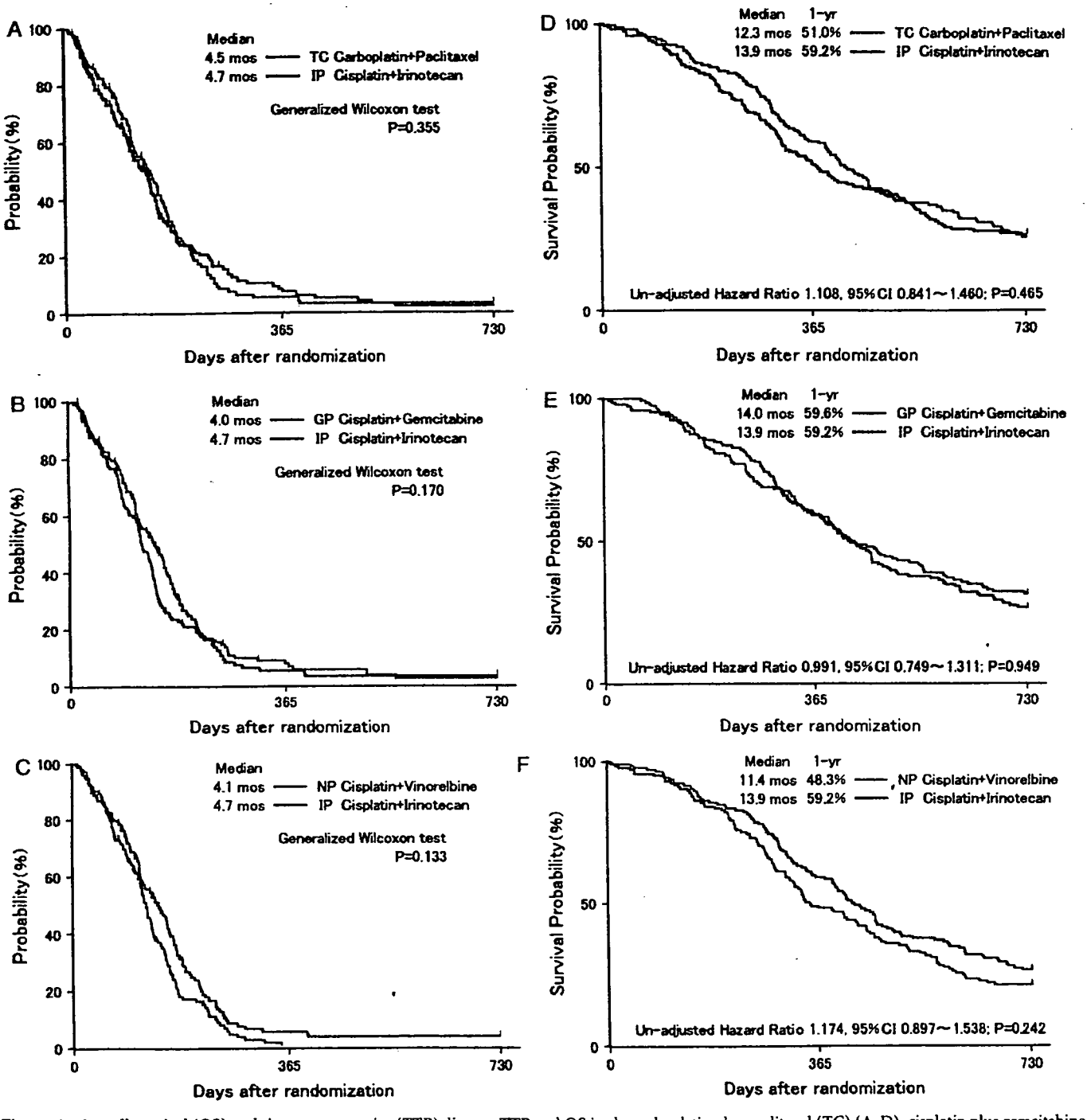

Figure 1. Overall survival (OS) and time to progressive (TTP) disease. TTP and OS in the carboplatin plus paclitaxel (TC) (A, D), cisplatin plus gemcitabine (GP) (B, E), and cisplatin plus vinorelbine (NP) (C, F) were not statistically significantly different from the values in the cisplatin plus irinotecan.

significantly less frequent in TC (6.8%), GP (8.6%), and NP (11.6%) than in IP (48.3%, $P < 0.001$). The incidences of grade 2 or worse sensory neuropathy (16.9%, $P < 0.001$), arthralgia (21.6%, $P < 0.001$), and myalgia (17.6%, $P < 0.001$) were significantly higher in TC than in IP. Grade 2 alopecia occurred in 30.6% of the patients in IP, and its incidence was significantly higher in TC (44.6%, $P = 0.013$) and significantly lower in GP (15.2%, $P = 0.001$) and NP (8.9%, $P < 0.001$). Grade 2 injection site reactions were more frequent in NP (26.7%) than in IP (4.8%, $P < 0.001$).

A total of five patients died of treatment-related toxicity: three in IP (cerebral hemorrhage, interstitial pneumonia, acute circulatory failure/disseminated intravascular coagulation: 2.0%), one in TC (acute renal failure: 0.7%), and one in NP (pulmonary embolism: 0.7%).

second-line treatment

Data on second-line treatment, but not third-line or later treatment, was available in this study, and they showed that

Table 3. Toxicity

	GP (n = 150)			IP (n = 150)			NP (n = 150)			TC (n = 150)		
	Grade (%)			Grade (%)			Grade (%)			Grade (%)		
	1	2	3	1	2	3	1	2	3	1	2	3
Leukocytes	42	43	5	39	42	3	40	31[a]	2[a]	25	51[b]	16[b]
Neutrophils	11	39	45	5	19	69	21	40	23[a]	5	16	72
Hemoglobin	42	24	7	42	13[a]	2[a]	44	22	5	43	25	5
Platelets	6	5	1	9	11	0	22	35[b]	0[b]	3	1[a]	0[a]
Febrile neutropenia	–	14	0	–	18	0	–	2[a]	0[a]	–	18	0
Nausea	32	29	–	14[c]	11[c]	–	35	23	–	33[c]	14[c]	–
Vomiting	38	13	0	17[c]	5[c]	0[c]	34	14	0	29[c]	7[c]	0[c]
Anorexia	30	33	2	15[c]	17[c]	1[c]	31	26	1	29[c]	20c	1[c]
Fatigue	27	12	1	26	2	1	17[c]	3[c]	0[c]	23[c]	3[c]	0[c]
Diarrhea	33	15	1	4[c]	3[c]	0[c]	7[c]	2[c]	0[c]	8[c]	4[c]	0[c]
Constipation	27	7	0	30	8	0	33	9	0	40[d]	14[d]	0[d]
Neuropathy, motor	1	0	0	1	1	1	0	0	0	0	0	0
Neuropathy, sensory	1	0	0	14[d]	3[d]	0[d]	0	0	0	0	0	0
Alopecia	31	–	–	45[d]	–	–	15[c]	–	–	9[c]	–	–
Arthralgia	2	0	0	20[d]	2[d]	0[d]	0	0	0	1	0	0
Myalgia	1	0	0	16[d]	2[d]	0[d]	0	0	0	1	1	0
Injection site reaction	5	0	–	5	0	–	5	0	–	27[d]	0[d]	–
Pneumonitis	0	1	1	0	1	0	0	0	0	0	1	0
Creatinine	8	1	0	2[c]	0[c]	0[c]	7	0	0	8	1	0
AST	7	1	1	5	1	0	6	3	0	1	3	0
Fever	2	0	0	5	1	0	1	0	0	1	0	0
Treatment-related death	3 (2.0%)			1 (0.7%)			0			1 (0.7%)		

[a]Incidence of grade 3 or 4 toxicity significantly ($P < 0.05$) lower than that with IP.
[b]Incidence of grade 3 or 4 toxicity significantly ($P < 0.05$) higher than that with IP.
[c]Incidence of grade 2 or worse toxicity is significantly ($P < 0.05$) lower than that with IP.
[d]Incidence of grade 2 or worse toxicity significantly ($P < 0.05$) higher than that with IP.
GP, cisplatin plus gemcitabine; IP, cisplatin plus irinotecan; NP, cisplatin plus vinorelbine; TC, carboplatin plus paclitaxel.
AST, aspartate aminotransferase; –, no category in the criteria.

60%–74% of the patients received chemotherapy and 6%–9% received thoracic irradiation as second-line treatment (Table 4). The percentages of patients in each treatment group who received second-line chemotherapy were not significantly different ($P = 0.081$).

quality of life

The details of the QoL analysis will be reported elsewhere. No statistically significant difference in global QoL was observed among the four treatment groups based on either the FACT-L Japanese version or the QoL-ACD. Only the physical domain evaluated by QoL-ACD was significantly better in TC, GP, and NP than in IP.

discussion

Many randomized phase III studies have compared platinum-plus-new-agent doublets in NSCLC, but, this is the first to evaluate the efficacy of an irinotecan-containing regimen in comparison with other platinum-plus-new-agent doublets in NSCLC [14–17]. Although non-platinum-containing chemotherapy regimens are used as alternatives, doublets of platinum and a new-generation anticancer agent, such as TC, GP, and NP, are considered standard chemotherapy regimens for advanced NSCLC worldwide [13–17, 25]. Although the non-inferiority of none of the three experimental regimens could be confirmed in this study, no statistically significant differences in response rate, OS, TTP, or TTTF were observed between the reference regimen and the experimental regimens. All four platinum-based doublets have similar efficacy against advanced NSCLC but different toxicity profiles. Nevertheless, IP was still regarded as the reference regimen in this study because the non-inferiority of none of the three experimental regimens could be confirmed.

OS in this study was relatively longer than previously reported. The estimated 1-year survival rate in the reference arm was 43%, but the actual 1-year survival rate was 59.2%, much higher than expected. The MSTs reported for patients treated with TC, GP, and NP in recent phase III studies have ranged from 8 to 10 months, and in the present study they were 12.3, 14.0, and 11.4 months, respectively [14–17]. One reason for the good OS in this study was the difference in patient selection criteria, for example exclusion of PS2 patients. Ethnic differences in pharmacogenomics have also been indicated as a possible reason for the good OS in this study [26]. The OS in IP in this study, however, was better than in previous Japanese studies [18, 19]. TTP in this study ranged from 4.0 to 4.7 months, and was similar to the TTP of 3.1–5.5 months reported in the literature [15, 16]. OS not TTP was longer in this study

Table 4. Second-line treatment

Number of patients	145	145	146	145	
Chemotherapy	107 (74%)	87 (60%)	101 (69%)	95 (66%)	$P = 0.081$
Docetaxel	39	25	50	51	
Gefitinib	11	9	18	12	
Paclitaxel	15	14	7	11	
Gemcitabine	24	28	17	28	
Vinorelbine	9	12	2	9	
Irinotecan	15	4	3	3	
Thoracic irradiation	8	10	13	10	

than previously reported, and higher 2-year survival rates, 21.4%–31.5%, were observed in the minimum 2-year follow-up in this study. Second-line or later treatments may affect survival, because docetaxel has been established as standard second-line chemotherapy for advanced NSCLC [27, 28]. Gefitinib is also effective as second-line or later chemotherapy for advanced NSCLC, especially in Asian patients, never smokers and patients with adenocarcinoma [29–32].

The toxicity profile of each treatment differed and the toxicity of all four regimens was well tolerated. Overall QoL was similar in the four platinum-based doublets. Only physical domain QoL evaluated by the QoL-ACD was statistically better in TC, GP, and NP than in IP. This finding is presumably attributable to the fact that diarrhea is a statistically less frequent adverse effect of TC, GP, and NP than of IP.

In conclusion, all four platinum-based doublets had similar efficacy for advanced NSCLC but different toxicity profiles. All the four regimens can be used to treat advanced NSCLC patients in clinical practice.

appendix

Institutions of the FACS Cooperative Group: National Hospital Organization (NHO) Hokkaido Cancer Center, Tohoku University Hospital, Yamagata Prefectural Central Hospital, Niigata Cancer Center Hospital, Tochigi Cancer Center, NHO Nishigunma National Hospital, Saitama Cancer Center, National Cancer Center Hospital East, Chiba University Hospital, National Cancer Center Hospital, Tokyo Medical University Hospital, Japanese Foundation for Cancer Research, Kanagawa Cancer Center, Yokohama Municipal Citizen's Hospital, Kanagawa Cardiovascular and Respiratory Center, Aichi Cancer Center Hospital, Prefectural Aichi Hospital, Nagoya City University Hospital, NHO Nagoya Medical Center, Nagoya University Hospital, Gifu Municipal Hospital, NHO Kyoto Medical Center, Osaka City General Hospital, Osaka City University Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, NHO Toneyama Hospital, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Kinki University School of Medicine, Rinku General Medical Center Izumisano Municipal Hospital, Kobe Central General Hospital, The Hospital of Hyogo College of Medicine, Hyogo Medical Center for Adults, Tokushima University Hospital, Kagawa Prefectural Central Hospital, NHO Shikoku Cancer Center Hospital, Hiroshima University Medical Hospital, NHO Kyushu Cancer Center Hospital, Kyushu University Hospital, National Nagasaki Medical Center, Nagasaki Municipal Hospital, Nagasaki University Hospital of Medicine and Dentistry, Kumamoto Chuo Hospital, Kumamoto Regional Medical Center, NTT West Osaka Hospital.

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Differential Constitutive Activation of the Epidermal Growth Factor Receptor in Non–Small Cell Lung Cancer Cells Bearing *EGFR* Gene Mutation and Amplification

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Abstract

The identification of somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) in patients with non–small cell lung cancer (NSCLC) and the association of such mutations with the clinical response to EGFR tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib, have had a substantial effect on the treatment of this disease. *EGFR* gene amplification has also been associated with an increased therapeutic response to EGFR-TKIs. The effects of these two types of *EGFR* alteration on EGFR function have remained unclear, however. We have now examined 16 NSCLC cell lines, including eight newly established lines from Japanese NSCLC patients, for the presence of *EGFR* mutations and amplification. Four of the six cell lines that harbor *EGFR* mutations were found to be positive for *EGFR* amplification, whereas none of the 10 cell lines negative for *EGFR* mutation manifested *EGFR* amplification, suggesting that these two types of *EGFR* alteration are closely associated. Endogenous EGFRs expressed in NSCLC cell lines positive for both *EGFR* mutation and amplification were found to be constitutively activated as a result of ligand-independent dimerization. Furthermore, the patterns of both *EGFR* amplification and EGFR autophosphorylation were shown to differ between cell lines harboring the two most common types of *EGFR* mutation (exon 19 deletion and L858R point mutation in exon 21). These results reveal distinct biochemical properties of endogenous mutant forms of EGFR expressed in NSCLC cell lines and may have implications for treatment of this condition. [Cancer Res 2007;67(5):2046–53]

Introduction

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein with an extracellular ligand binding domain, a transmembrane region, and a cytoplasmic tyrosine kinase domain and is encoded by a gene (*EGFR*) located at human chromosomal region 7p12 (1–3). The binding of ligand to EGFR induces receptor dimerization and consequent conformational changes that result in activation of the intrinsic tyrosine kinase, receptor autophosphorylation, and activation of a signaling cascade (4, 5). Aberrant signaling by EGFR plays an important role in cancer development and progression (3).

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EGFR is frequently overexpressed in non–small cell lung cancer (NSCLC) and has been implicated in the pathogenesis of this disease (6, 7). Given the biological importance of EGFR signaling in cancer, several agents have been synthesized that inhibit the receptor tyrosine kinase activity. Two such inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), gefitinib and erlotinib, both of which compete with ATP for binding to the tyrosine kinase pocket of the receptor, have been extensively studied in patients with NSCLC (8, 9). We and others have shown that a clinical response to these agents is more common in women than in men, in Japanese than in individuals from Europe or the United States, in patients with adenocarcinoma than in those with other histologic subtypes of cancer, and in patients who have never smoked than in those with a history of smoking (10–14). Mutations in the tyrosine kinase domain of EGFR have also been detected in a subset of lung cancer patients and shown to predict sensitivity to EGFR-TKIs (15–17). Indeed, the clinical characteristics of patients with known *EGFR* mutations are similar to those of other individuals most likely to respond to treatment with EGFR-TKIs (18–22). These mutations arise in the first four exons (exons 18–21) corresponding to the tyrosine kinase domain of EGFR, and they affect key amino acids surrounding the ATP-binding cleft (23, 24). In-frame deletions that eliminate four highly conserved amino acids (LREA) encoded by exon 19 are the most common type of *EGFR* mutation, with missense point mutations in exon 21 that result in a specific amino acid substitution at position 858 (L858R) being the second most common. In addition to *EGFR* mutations, other molecular changes may play a role in determining sensitivity to EGFR-TKIs (22, 25–28). NSCLC patients with an increased *EGFR* copy number, as revealed by fluorescence *in situ* hybridization (FISH), have thus been found to show an increased response rate to and prolonged survival after gefitinib therapy (22, 25–27).

Given that *EGFR* is mutated or amplified (or both) in NSCLC, it is important to determine the biological effects of such *EGFR* alterations on EGFR function (15, 29–32). Transient transfection of various cell types with vectors encoding wild-type or mutant versions of EGFR showed that the activation of mutant receptors by EGF is more pronounced and sustained than is that of the wild-type receptor (15, 30). However, detailed biochemical analysis of NSCLC cell lines with endogenous *EGFR* mutations has been limited. We have now identified *EGFR* mutations in three NSCLC cell lines newly established from Japanese patients. Furthermore, we have characterized a panel of 16 NSCLC cell lines for *EGFR* mutations and amplification and evaluated the relation between the presence of these two types of *EGFR* alteration and sensitivity to gefitinib. The effects of *EGFR* alterations on activation status of EGFR and on downstream signaling were also evaluated.

Finally, in *EGFR* mutant cell lines showing constitutive EGFR activation, we assessed how the mutations activate the tyrosine kinase domain of the receptor.

Materials and Methods

Cell lines. The human NSCLC cell lines NCI-H226 (H226), NCI-H292 (H292), NCI-H460 (H460), NCI-H1299 (H1299), NCI-H1650 (H1650), and NCI-H1975 (H1975) were obtained from the American Type Culture Collection (Manassas, VA). PC-9 and A549 cells were obtained as described previously (33). Ma-1 cells were kindly provided by E. Shimizu (Tottori University, Yonago, Japan). We established seven cell lines (KT-2, KT-4, Ma-25, Ma-31, Ma-34, Ma-45, and Ma-53) from tissue or pleural effusion of Japanese patients with advanced NSCLC. These cell lines were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Informed consent for establishment of cell lines and tumor DNA sequencing was obtained in accordance with the ethical guidelines for human genome/genetic analysis in Japan.

Growth inhibition assay. Gefitinib was kindly provided by AstraZeneca (Macclesfield, United Kingdom) as a pure substance and was diluted in DMSO to obtain a stock solution of 20 mmol/L. For growth inhibition assays, cells (0.5×10^4 to 4.5×10^4) were plated in 96-well flat-bottomed plates and cultured for 24 h before the addition of various concentrations of gefitinib and incubation for an additional 72 h. TetraColor One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku, Tokyo, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of gefitinib resulting in 50% growth inhibition (IC_{50}) was calculated.

Genetic analysis of *EGFR*. Genomic DNA was extracted from cell lines with the use of a QIAamp DNA Mini kit (Qiagen, Tokyo, Japan), and exons 18 to 21 of *EGFR* were amplified by the PCR and sequenced directly. PCR was done in a reaction mixture (25 μL) containing 50 ng of genomic DNA and TaKaRa Taq polymerase (TaKaRa BIO, Tokyo, Japan) and with an initial incubation for 3 min at 94°C followed by 30 cycles of 20 s at 94°C, 30 s at 58°C, and 20 s at 72°C and by a final incubation for 7 min at 72°C. The PCR products were purified with a Microcon YM-100 filtration device (Millipore, Billerica, MA) before sequencing with the use of an ABI BigDye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reaction mixtures were subjected to electrophoresis with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Primers for mutation analysis (sense and antisense, respectively) were as follows: exon 18, 5′-CAAATGAGCTGGCAAGTGCCGTGTC-3′ and 5′-GAGTTTCCCAAACACTCAGTGAAA-C-3′; exon 19, 5′-GCAATATCAGCCTTAGGTGCGGCTC-3′and 5′-CATAGAAAGTGAACATTTAGGATGTG-3′; exon 20, 5′-CCATGAGTACGTATTTTGAAACTC-3′ and 5′-CATATCCCCATGGCAAACTCTTGC-3′; and exon 21, 5′-CTAACGTTCGCCAGCCATAAGTCC-3′ and 5′-GCTGCGAGCTCACCCAGAATGTCTGG-3′.

FISH. *EGFR* copy number per cell was determined by FISH with the use of the LSI EGFR Spectrum Orange and CEP7 Spectrum Green probes (Vysis; Abbott, Des Plaines, IL). Cells were centrifuged onto glass slides with a Shandon cytocentrifuge (Thermo Electron, Pittsburgh, PA) and fixed by consecutive incubations with ice-cold 70% ethanol for 10 min, 85% ethanol for 5 min, and 100% ethanol for 5 min. Slides were stored at −20°C until analysis. Cells were subsequently subjected to digestion with pepsin for 10 min at 37°C, washed with water, dehydrated with a graded series of ethanol solutions, denatured with 70% formamide in 2× SSC for 5 min at 72°C, and dehydrated again with a graded series of ethanol solutions before incubation with a hybridization mixture consisting of 50% formamide, 2× SSC, Cot-1 DNA, and labeled DNA. The slides were washed for 5 min at 73°C with 3× SSC, for 5 min at 37°C with 4× SSC containing 0.1% Triton X-100, and for 5 min at room temperature with 2× SSC before counterstaining with antifade solution containing 4′,6-diamidino-2-phenylindole. Hybridization signals were scored in 40 nuclei with the use of a ×100 immersion objective. Nuclei with a disrupted boundary were excluded from the analysis. Gene amplification was defined by an *EGFR*/chromosome 7 copy number ratio of ≥2 or by the presence of clusters of ≥15 copies of *EGFR* per cell in ≥10% of cells, as described previously (25, 27).

Immunoblot analysis. Cell lysates were fractionated by SDS-PAGE on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites with 5% skim milk, the membrane was incubated overnight at room temperature with primary antibodies. Antibodies to phosphorylated EGFR (pY845, pY1068, or pY1173), extracellular signal-regulated kinase (ERK), phosphorylated AKT, AKT, Src homology and collagen (Shc), and phosphorylated Shc were obtained from Cell Signaling Technology (Beverly, MA); antibodies to EGFR were from Zymed (South San Francisco, CA); antibodies to phosphorylated ERK were from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies to β-actin (loading control) were from Sigma. Immune complexes were detected by incubation of the membrane for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to mouse or rabbit immunoglobulin (Amersham Biosciences, Little Chalfont, United Kingdom) and by subsequent exposure to enhanced chemiluminescence reagents (Perkin-Elmer, Boston, MA).

Table 1. Characteristics of NSCLC cell lines

Cell lines	Gefitinib IC_{50} (μmol/L)	*EGFR* mutation	*EGFR* amplification	Histology
PC-9	0.07	del(E746-A750)	+	Adenocarcinoma
KT-2	0.57	L858R	+	Adenocarcinoma
KT-4	1.26	L858R	+	Large cell carcinoma
Ma-1	2.34	del(E746-A750)	+	Adenocarcinoma
H1650	6.66	del (E746-A750)	−	Adenocarcinoma
A549	8.70	Wild type	−	Adenocarcinoma
H1975	9.32	L858R+T790M	−	Adenocarcinoma
H292	9.44	Wild type	−	Mucoepidermoid carcinoma
H226	9.53	Wild type	−	Squamous cell carcinoma
Ma-25	10.17	Wild type	−	Large cell carcinoma
H460	10.38	Wild type	−	Large cell carcinoma
Ma-45	10.47	Wild type	−	Adenocarcinoma
Ma-53	10.47	Wild type	−	Adenocarcinoma
Ma-34	11.17	Wild type	−	Adenocarcinoma
H1299	11.28	Wild type	−	Large cell carcinoma
Ma-31	12.46	Wild type	−	Adenocarcinoma

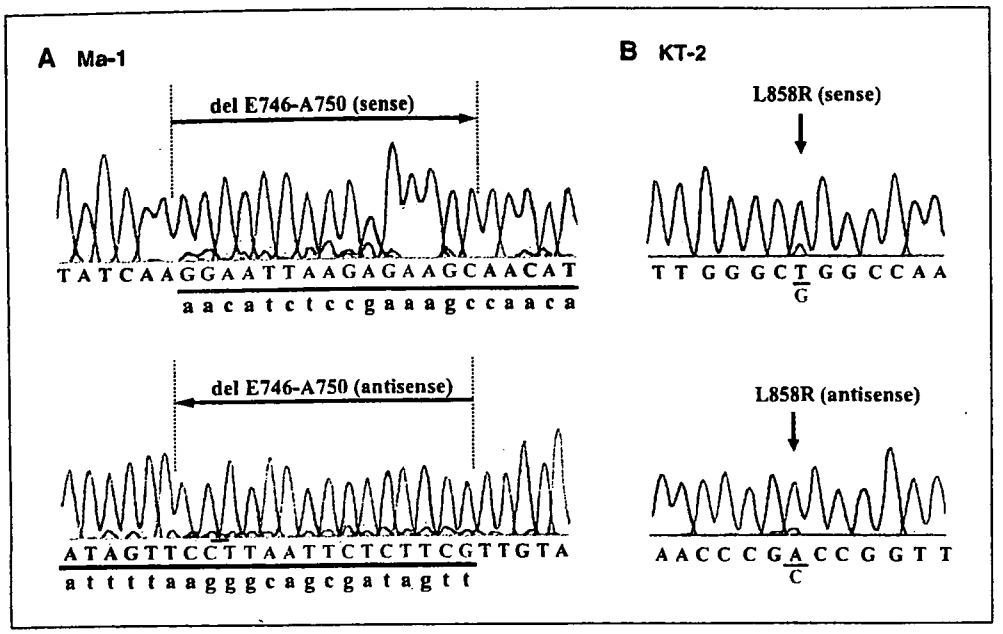

Figure 1. Detection of *EGFR* mutations in NSCLC cell lines. The portions of the sequencing electrophoretograms corresponding to the mutations are shown for Ma-1 (*A*) and KT-2 (*B*) cells. *A*, heterozygous in-frame deletion in exon 19 is revealed by the presence of double peaks. Tracings in both sense and antisense directions are shown to highlight the two breakpoints of the deletion. Wild-type (*uppercase*) and mutant (*lowercase*) nucleotide sequences. *B*, heterozygous point mutation (T → G) at nucleotide position 2819 in exon 21.

Treatment of cells with neutralizing antibodies. Cells were exposed to neutralizing antibodies (each at 12 μg/mL) for 3 h before EGF stimulation. The antibodies included those to EGF and to transforming growth factor-α (TGF-α), both from R&D Systems (Minneapolis, MN) as well as antibodies to EGFR (Upstate Biotechnology, Lake Placid, NY). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to phosphorylated EGFR (pY1068) and to EGFR as described above.

Chemical cross-linking assay. Chemical cross-linking was done as described previously (34, 35). Cells were washed twice with ice-cold PBS and then incubated for 20 min at 4°C with 1 mmol/L bis(sulfosuccinimidyl)-suberate (Pierce, Rockford, IL) in PBS. The cross-linking reaction was terminated by the addition of glycine to a final concentration of 250 mmol/L and incubation for an additional 5 min at 4°C. The cells were washed with PBS, and cell lysates were resolved by SDS-PAGE on a 4% gel and subjected to immunoblot analysis with anti-EGFR (Santa Cruz Biotechnology).

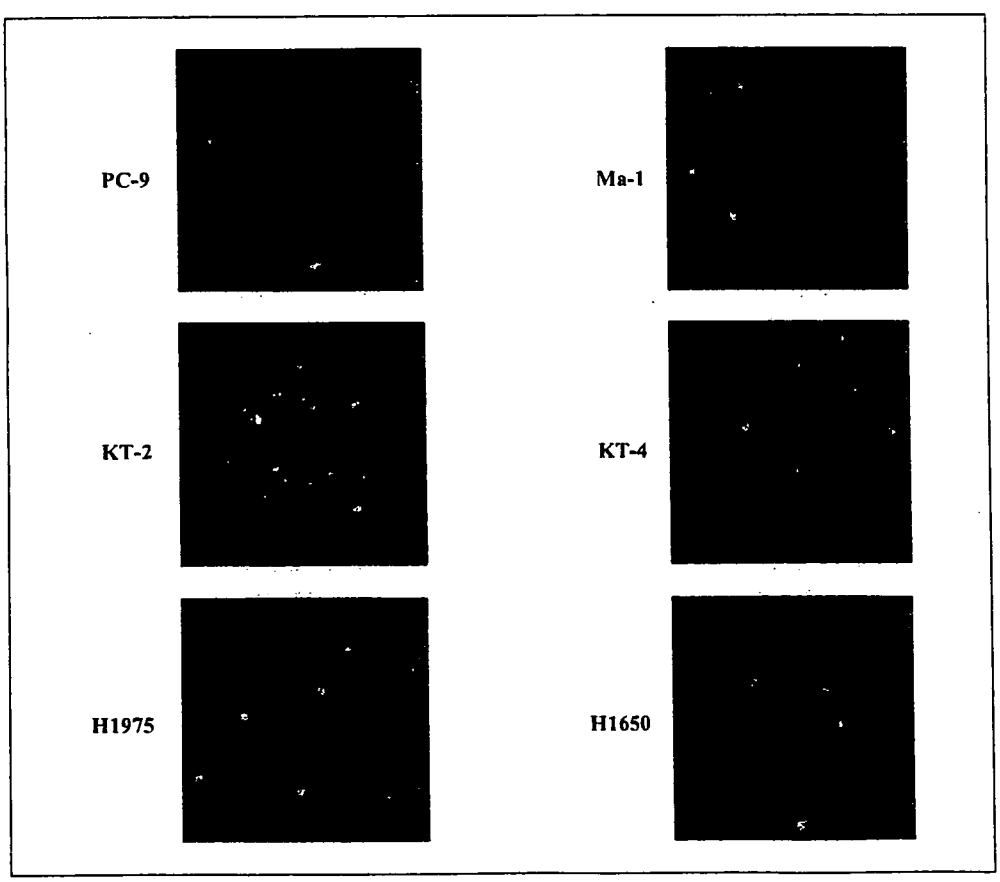

Figure 2. FISH analysis of *EGFR* amplification in NSCLC cell lines. The analysis was done with probes specific for *EGFR* (*red signals*) and for the centromere of chromosome 7 (*green signals*) in the indicated cell lines. PC-9 and Ma-1 cells manifest an *EGFR*/chromosome copy number ratio of ≥2, whereas KT-2 and KT-4 cells manifest *EGFR* clusters. H1975 and H1650 cells are negative for *EGFR* amplification.

Results

Effect of gefitinib on the growth of NSCLC cell lines. We first examined the effect of the EGFR-TKI gefitinib on the growth of 16 NSCLC cell lines, eight of which (KT-2, KT-4, Ma-1, Ma-25, Ma-31, Ma-34, Ma-45, and Ma-53) were established from Japanese NSCLC patients for the present study. The IC_{50} values for gefitinib chemosensitivity ranged from 0.07 to 12.46 μmol/L (a 178-fold difference; Table 1).

Four cell lines (PC-9, KT-2, KT-4, and Ma-1) were relatively sensitive to gefitinib with IC_{50} values between 0.07 and 2.34 μmol/L, whereas the remaining 12 lines were considered resistant to gefitinib (IC_{50} > 6 μmol/L). No relation was apparent between sensitivity to gefitinib and histologic subtype of NSCLC for this panel of cell lines (Table 1).

***EGFR* mutation and amplification in NSCLC cell lines.** We screened the 16 NSCLC cell lines for the presence of *EGFR* mutations in exons 18 to 21, which encode the catalytic domain of the receptor. As previously described (36–39), PC-9, H1650, and H1975 cell lines were found to harbor *EGFR* mutations [del(E746-A750) in PC-9 and H1650 and both L858R and T790M in H1975]. Furthermore, we detected *EGFR* mutations in three of the newly established cell lines (Ma-1, KT-2, and KT-4). Ma-1 cells, which were isolated from a female ex smoker with adenocarcinoma (>30 years of age), were found to harbor a small deletion within exon 19 [del(E746-A750); Fig. 1*A*; Table 1]. Both KT-2 cells [derived from a male ex smoker with adenocarcinoma (>30 years of age)] and KT-4 cells (derived from a male nonsmoker with large cell carcinoma) harbor a point mutation (L858R) in exon 21 (Fig. 1*B*; Table 1). Four of these six NSCLC cell lines with *EGFR* mutations (PC-9, Ma-1, KT-2, and KT-4) are sensitive to gefitinib (Table 1), consistent with clinical observations (15–17, 20, 22).

We next examined the 16 NSCLC cell lines for the presence of *EGFR* amplification by FISH analysis with a probe specific for *EGFR* and a control probe for the centromere of chromosome 7. Four (PC-9, Ma-1, KT-2, and KT-4) of the 16 cell lines, all of which harbor *EGFR* mutations, were found to be positive for *EGFR* amplification (Fig. 2; Table 1). PC-9 and Ma-1 cell lines, both of which harbor the same exon 19 deletion, showed an *EGFR*/chromosome copy number ratio of ≥2, whereas KT-2 and KT-4, both of which harbor the L858R mutation in exon 21, showed a clustered unbalanced gain of *EGFR* copy number (Fig. 2). The four cell lines that manifested both *EGFR* mutation and amplification were sensitive to gefitinib (Table 1). The *EGFR* mutant cell lines H1650 and H1975 showed no evidence of *EGFR* amplification (Fig. 2), and both of these lines were relatively resistant to gefitinib (Table 1). None of the cell lines negative for *EGFR* mutations manifested *EGFR* amplification (Table 1), suggesting that *EGFR* mutation is closely associated with *EGFR* amplification ($P < 0.05$, χ^2 test).

EGFR expression in NSCLC cell lines. We examined the basal abundance of EGFR in *EGFR* wild-type and mutant NSCLC cell lines by immunoblot analysis. The amount of EGFR in the cell lines PC-9, Ma-1, KT-2, and KT-4, all of which manifest *EGFR* amplification and *EGFR* mutation, was increased compared with that in *EGFR* wild-type cell lines (A549 and H1299) or *EGFR* mutant cell lines negative for *EGFR* amplification (H1975 and H1650; Fig. 3). These results, thus, reveal a close relation between increased EGFR expression and *EGFR* amplification in this panel of NSCLC cell lines, consistent with the results of previous analyses of NSCLC tissue specimens (6, 7).

EGFR phosphorylation in NSCLC cell lines. We examined tyrosine phosphorylation of endogenous EGFRs in NSCLC cell lines by immunoblot analysis with phosphorylation site–specific antibodies. In cells (A549) that express only wild-type EGFR, phosphorylation of the receptor at Y845, Y1068, or Y1173 was undetectable in the absence of EGF but was markedly induced on

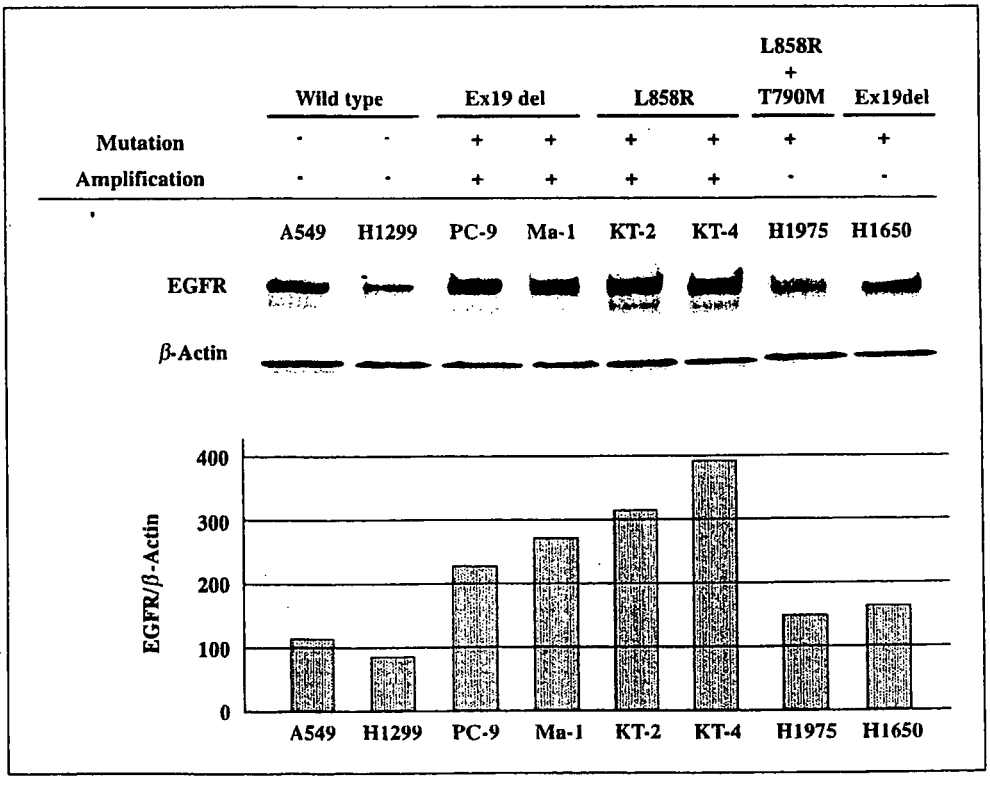

Figure 3. EGFR expression in NSCLC cell lines. Lysates (40 μg of protein) of NSCLC cell lines positive or negative for *EGFR* mutation or amplification, as indicated, were subjected to immunoblot analysis with antibodies to EGFR and to β-actin (*top*). The abundance of EGFR relative to that of β-actin was determined by densitometry (*bottom*). Representative of three independent experiments.

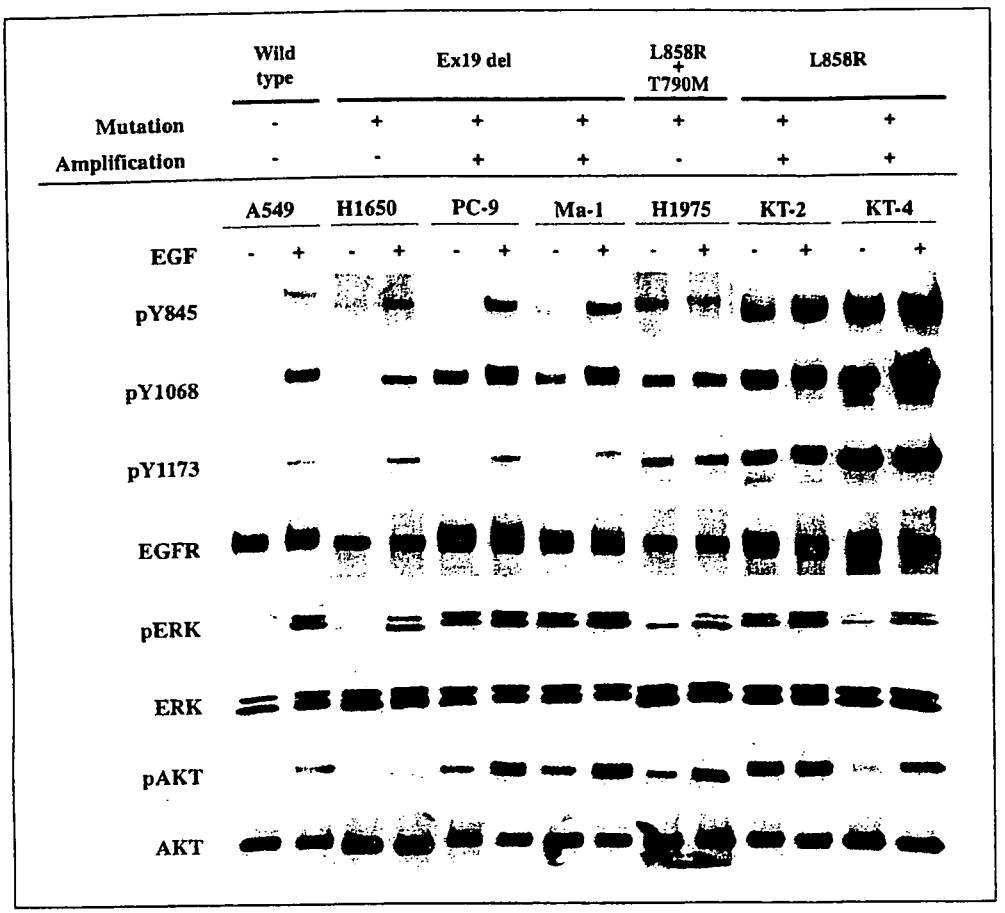

Figure 4. Phosphorylation of EGFR and downstream signaling molecules in NSCLC cell lines. Serum-deprived cells were incubated for 15 min in the absence or presence of EGF (100 ng/mL), after which cell lysates (40 μg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (*pEGFR*), ERK (*pERK*), or AKT (*pAKT*) as well as antibodies to all forms of the corresponding proteins, as indicated. Representative of three independent experiments.

exposure of the cells to this growth factor (Fig. 4). Similar results were obtained with H1650 cells, which are positive for the deletion in exon 19 of *EGFR* but negative for *EGFR* amplification. In contrast, PC-9 and Ma-1 cells, which are positive for both the exon 19 deletion and *EGFR* amplification, manifested an increased basal level of EGFR phosphorylation at Y1068, indicative of constitutive activation of the EGFR tyrosine kinase. Exposure of PC-9 or Ma-1 cells to EGF induced EGFR phosphorylation at Y845 and Y1173, showing that the mutant receptors remain sensitive to ligand stimulation. Furthermore, the cell lines (H1975, KT-2, and KT-4) with the L858R point mutation manifested an increased basal level of EGFR phosphorylation at Y845, Y1068, and Y1173, and the extent of phosphorylation at these residues was increased only slightly by treatment of the cells with EGF, indicative of constitutive activation of the EGFR tyrosine kinase. These results thus showed that endogenous *EGFR* mutations result in constitutive receptor activation, and that the patterns of tyrosine phosphorylation of EGFR differ between the two most common types of *EGFR* mutant.

Phosphorylation of signaling molecules downstream of EGFR in NSCLC cell lines. Given that constitutive activation of EGFR was detected in NSCLC cell lines with endogenous *EGFR* mutations, we examined whether signaling molecules that act downstream of the receptor are also constitutively activated in these cell lines. We first examined the basal levels of phosphorylation of AKT and ERK, both of which mediate the oncogenic effects of EGFR. Immunoblot analysis with antibodies to phosphorylated forms of AKT or ERK revealed that these molecules are indeed constitutively activated in the *EGFR* mutant lines (PC-9, Ma-1, H1975, KT-2, and KT-4) that manifest constitutive activation of EGFR, although the extent of phosphorylation varied (Fig. 4). The increased levels of AKT and ERK phosphorylation in these mutant cell lines are consistent with the increased level of EGFR phosphorylation on Y1068, which serves as the docking site for phosphatidylinositol 3-kinase and growth factor receptor binding protein 2, molecules that mediate the activation of AKT and the Ras-ERK pathway, respectively (2, 40). We next examined whether the differences in the pattern of constitutive tyrosine phosphorylation of EGFR apparent between NSCLC cell lines harboring the exon 19 deletion and those with the L858R mutation in exon 21 are associated with distinct alterations in downstream signaling pathways. Given that Y1173, a major docking site of EGFR for the adapter protein Shc (2, 40, 41), is constitutively phosphorylated in cells with the L858R mutation but not in those with the exon 19 deletion, we compared Shc phosphorylation between cell lines with these two types of *EGFR* mutation. Ligand-independent tyrosine phosphorylation of the 52- and 46-kDa isoforms of Shc was apparent in cell lines with either type of *EGFR* mutation (Fig. 5). However, cell lines (KT-2 and KT-4) that harbor the L858R mutation exhibited a markedly greater basal level of phosphorylation of the 66-kDa isoform of Shc than did those (PC-9 and Ma-1) that harbor the exon 19 deletion or those (A549) that harbor only wild-type *EGFR*. These data suggest that the constitutively active mutant forms of *EGFR* induce selective activation of downstream effectors as a result of differential patterns of receptor autophosphorylation.

Ligand-independent dimerization and activation of *EGFR* mutants. Evidence suggests that EGFR ligands, including EGF and TGF-α, secreted by tumor cells themselves might be responsible for activation of mutant receptors in an autocrine loop (29, 42). To investigate whether EGFR is constitutively activated as a result of such an autocrine mechanism in *EGFR* mutant NSCLC cell lines, we treated the cells with a combination of three neutralizing antibodies (anti-EGF, anti-TGF-α, and anti-EGFR) for 3 h and then examined the effect of EGF on EGFR phosphorylation. The ligand-dependent activation of EGFR in A549 cells (which express only wild-type *EGFR*) was blocked by such antibody treatment (Fig. 6*A*). In contrast, treatment of the *EGFR* mutant cell lines PC-9 or KT-4 with the neutralizing antibodies failed to inhibit the constitutive phosphorylation of EGFR on Y1068. These observations suggest that the constitutive phosphorylation of the mutant receptors is not attributable to autocrine stimulation, although we are not able to exclude a possible role for other EGFR ligands.

Ligand-induced EGFR dimerization is responsible for activation of the receptor tyrosine kinase (4, 5). To determine whether mutant receptors are constitutively dimerized, we treated *EGFR* wild-type or mutant cell lines with a cross-linking agent before immunoblot analysis with antibodies to EGFR. Whereas ligand-induced dimerization of wild-type *EGFR* was observed in A549 cells, receptor dimerization in PC-9 and KT-4 cells, which express mutant receptors, was apparent in the absence of ligand and was not increased substantially by exposure of the cells to EGF (Fig. 6*B*). These data indicate that ligand-independent receptor dimerization is responsible for the constitutive activation of the mutant forms of *EGFR*.

Discussion

The discovery of somatic mutations in the tyrosine kinase domain of EGFR and of their association with a high response rate to EGFR-TKIs has had a substantial effect on the treatment of advanced NSCLC (15–17, 20, 22). Asian patients with NSCLC seem to have a higher prevalence of these mutations, ranging from 20% to 40% (18, 20, 21, 43–45). We have now identified *EGFR* mutations in three of eight newly established cell lines from Japanese patients with advanced NSCLC. Characterization of these eight new cell lines and eight previously established NSCLC lines revealed that, consistent with previous observations (29, 31, 36), those cell lines that harbor *EGFR* mutations are more likely to be sensitive to gefitinib than are those without such mutations. Not all *EGFR* mutant cell lines (e.g., H1650 and H1975) are sensitive to this EGFR-TKI, however, suggesting the existence of additional determinants of gefitinib sensitivity. In addition to the L858R mutation in exon 21 of *EGFR*, H1975 cells contain the T790M mutation in exon 20, which has been shown to confer resistance to EGFR-TKIs (38, 39). H1650 cells, which do not harbor mutations in *EGFR* other than the exon 19 deletion, manifest loss of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (37), which may result in resistance to EGFR-TKIs. *EGFR* amplification in NSCLC cells has also been shown to correlate with a better response to gefitinib (22, 25–27). Given that little is known of the relation between *EGFR* mutation and amplification in NSCLC, we examined the 16 NSCLC cell lines used in this study for *EGFR* amplification by FISH. Four of the six cell lines with *EGFR* mutations were found to be positive for gene amplification, whereas none of the 10 mutation-negative cell lines manifested *EGFR* amplification. This finding thus suggests that *EGFR* mutation and amplification are linked. Cappuzzo et al. showed that 6 of 9 (67%) NSCLC patients with *EGFR* amplification also had *EGFR* mutations (25). Furthermore, Takano et al. sequenced *EGFR* and determined the *EGFR* copy number by real-time PCR analysis for the tumors of 66 NSCLC patients (22); all of the patients with a high *EGFR* copy number (≥6.0 per cell) also had *EGFR* mutations. Moreover, PCR analysis revealed selective amplification of the mutant *EGFR* alleles in the patients with a high *EGFR* copy number. Our sequencing electrophoretograms for the *EGFR* mutant cell lines positive for *EGFR* amplification also revealed that the mutant signals were dominant, and the wild-type sequence was barely detectable (Fig. 1), indicative of selective amplification of the mutant alleles. We used the recently proposed definition of *EGFR* amplification as determined by FISH (25, 27) and found that the pattern of gene amplification seemed to be dependent on the

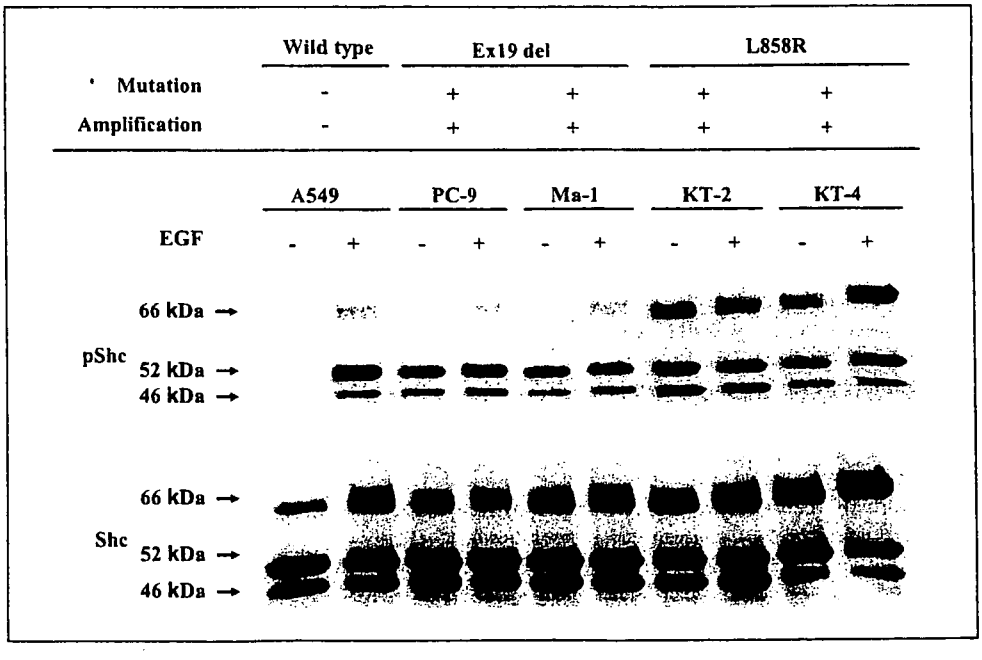

Figure 5. Phosphorylation of Shc in NSCLC cell lines. Serum-deprived cells were incubated for 15 min in the absence or presence of EGF (100 ng/mL), after which cell lysates (40 μg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated Shc (*pShc*) or total Shc. Representative of three independent experiments.

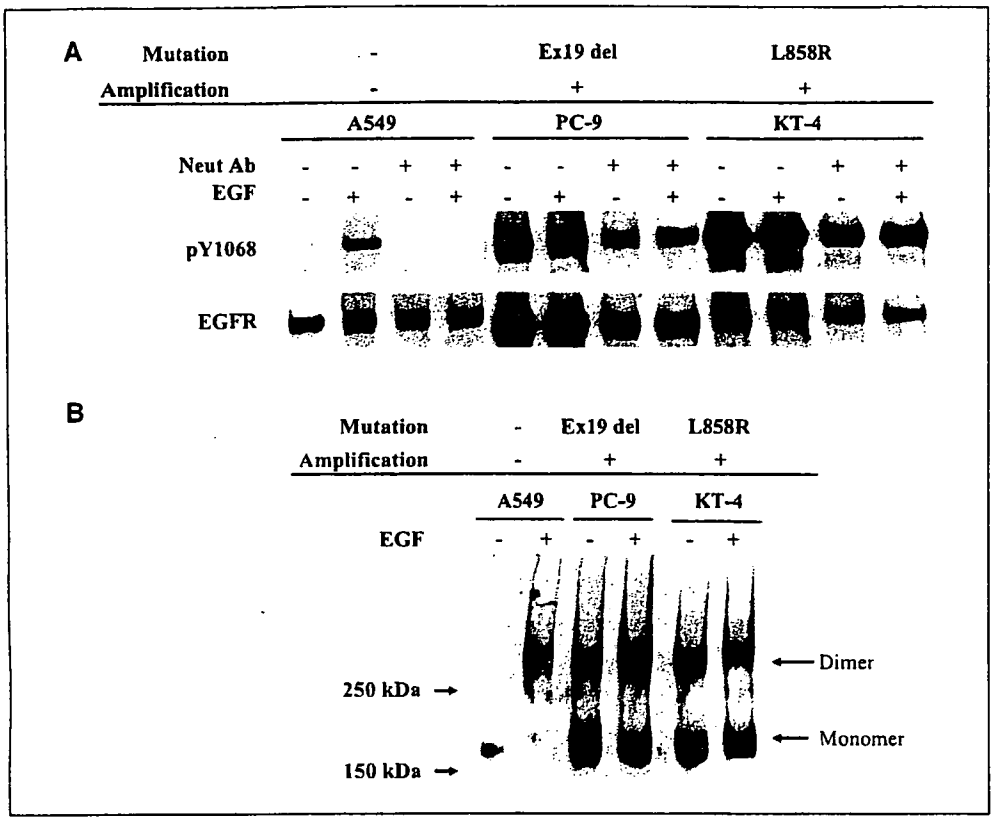

Figure 6. Mechanism of constitutive activation of EGFR in NSCLC cell lines. *A*, effect of neutralizing antibodies (*Neut Ab*) on EGFR phosphorylation. Serum-deprived NSCLC cells (A549, PC-9, or KT-4) were incubated for 3 h with a combination of neutralizing antibodies to EGF, TGF-α, and EGFR and then for 15 min in the additional absence or presence of EGF (100 ng/mL). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR or to total EGFR. *B*, EGFR dimerization. Serum-deprived cells were incubated for 15 min in the absence or presence of EGF (100 ng/mL), exposed to a chemical cross-linker, lysed, and subjected to immunoblot analysis with antibodies to EGFR. Representative of three independent experiments.

type of *EGFR* mutation; gene clusters were observed in cells with the L858R mutation in exon 21, whereas an *EGFR*/chromosome copy number ratio of ≥2 was detected in those with the small deletion [del(E746-A750)] in exon 19. Together, these data support the notion that *EGFR* mutation and amplification may be co-selected for during the growth of NSCLC cells. The four cell lines (PC-9, Ma-1, KT-2, and KT-4) positive for both *EGFR* mutation and amplification were sensitive to gefitinib, suggesting that *EGFR* amplification may increase sensitivity to gefitinib in *EGFR* mutant cells.

Previous biochemical studies of cells transiently transfected with vectors for wild-type or mutant forms of EGFR suggested that *EGFR* mutations increase EGF-dependent receptor activation (15, 30). Infection of NIH 3T3 cells with a retrovirus encoding *EGFR* mutants showed that the mutant receptors are constitutively activated and able to induce cell transformation in the absence of exogenous EGF (32). We examined the activation status of endogenous EGFRs in the six NSCLC cell lines that harbor *EGFR* mutations. The H1650, PC-9, and Ma-1 cell lines, all of which harbor the same exon 19 deletion, showed different patterns of EGFR autophosphorylation in the COOH-terminal region of the protein. EGFR autophosphorylation was ligand dependent in H1650 cells, which are negative for *EGFR* amplification, whereas Y1068 (but not Y845 and Y1173) was constitutively phosphorylated in PC-9 and Ma-1 cells, both of which manifest *EGFR* amplification. These results suggest that both *EGFR* mutation and amplification may be required for constitutive activation of EGFR in NSCLC cells that harbor the exon 19 deletion. In contrast, NSCLC cell lines (H1975, KT-2, and KT-4) that harbor the L858R mutation exhibited constitutive phosphorylation of EGFR at Y845, Y1068, and Y1173, regardless of the absence or presence of *EGFR* amplification. It is thought that *EGFR* mutations result in repositioning of critical residues surrounding the ATP-binding cleft of the tyrosine kinase domain of the receptor and thereby stabilize the interactions with ATP and EGF-TKIs, leading to increased tyrosine kinase activity and EGFR-TKI sensitivity (15, 23, 24). The differential activation of *EGFR* mutants observed in the present study may result from distinct conformational changes within the catalytic pocket caused by the different types of *EGFR* mutation. NSCLC patients with exon 19 deletions were recently shown to manifest longer overall survival than did those with the exon 21 point mutation after treatment with EGFR-TKIs, supporting the notion that the two major types of mutant receptors have different biological properties (46, 47).

Ligand-induced receptor dimerization underlies the activation of receptor tyrosine kinases (4, 5). Chemical cross-linking revealed that EGF binding to EGFR induced receptor dimerization in A549 cells, which express only the wild-type form of the receptor. In contrast, endogenous EGFRs in NSCLC cells harboring either the exon 19 deletion or the point mutation in exon 21 of *EGFR* were found to dimerize in the absence of ligand, suggesting that the constitutive activation of the mutant receptors is attributable to ligand-independent dimerization. EGFR dimerization was shown to be induced by interaction of quinazolines with the ATP-binding site of the receptor in the absence of ligand binding, suggesting that a change in conformation around the ATP-binding pocket of EGFR is sufficient for receptor dimerization (35). Conformational changes induced by *EGFR* mutations may therefore also trigger EGFR dimerization in *EGFR* mutant cells.

In conclusion, we have found that *EGFR* mutation is closely associated with *EGFR* amplification in NSCLC cell lines. Endogenous EGFRs expressed in NSCLC cells positive for both *EGFR* mutation and amplification are constitutively activated as a result

of ligand-independent dimerization. Cells with the two most common types of *EGFR* mutation also manifest different patterns of EGFR autophosphorylation. Prospective studies are required to determine the potential for exploitation of these *EGFR* alterations in the treatment of advanced NSCLC.

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Down-regulation of survivin by ultraviolet C radiation is dependent on p53 and results in G_2–M arrest in A549 cells

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Abstract

Deregulation of survivin expression is implicated in tumorigenesis. To examine the regulation of survivin expression in response to DNA damage, we exposed A549 human lung cancer cells to ultraviolet C (UVC) radiation, which induces DNA single-strand breakage. UVC irradiation induced G_2–M arrest that was accompanied by accumulation of p53 and subsequent down-regulation of survivin. Depletion of p53 by RNA interference prevented the UVC-induced down-regulation of survivin. Furthermore, depletion of survivin resulted in G_2–M arrest, suggesting that down-regulation of survivin by p53 contributes to the p53-dependent G_2–M checkpoint triggered by DNA damage.

Keywords: Survivin; p53; RNA interference; G_2–M arrest; Ultraviolet C

1. Introduction

Survivin, a member of the inhibitor of apoptosis (IAP) family of proteins, is thought to play an important role in regulation of both apoptosis and cell division [1,2]. It is present in only small amounts in terminally differentiated normal cells but is overexpressed in almost all types of human malignancy [3–8]. Such overexpression of survivin is associated with poor prognosis in affected individuals, an increased rate of tumor recurrence, and resistance to certain anticancer agents and radiation [9,10].

The expression of survivin is regulated in a cell cycle-dependent manner. The promoter of the survivin gene possesses features typical of genes that are expressed at G_2–M phase of the cell cycle. Indeed, survivin is most abundant in cells at G_2–M and associates with the mitotic spindle of dividing cells [2]. Survivin interacts with Aurora B and inner centromere protein (INCENP), and the complex of Aurora B–INCENP–survivin monitors the integrity of the mitotic spindle [11]. It has been suggested that survivin controls the elimination by apoptosis of cells with an improperly formed mitotic spindle [3,12]. Overexpression of survivin in cancer may overcome cell cycle checkpoints and thereby facilitate aberrant progression of

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transformed cells through mitosis. Although deregulation of survivin expression is an important event in tumorigenesis, the molecular mechanisms of survivin regulation are not fully understood.

The tumor suppressor p53 blocks progression of cells through the cell cycle or induces apoptosis [13,14]. Following its induction in response to DNA damage, p53 up-regulates the expression of various genes that contribute to cell cycle arrest, DNA repair, or apoptosis. It also negatively regulates the expression of a separate set of genes [15–18]. The functional loss of wild-type p53 has been shown to be associated with up-regulation of survivin expression in human cancers [19–21]. We have previously shown that the amounts of survivin mRNA and protein in cell lines positive for wild-type p53 decreased markedly after induction of p53 by adriamycin, which causes DNA double-strand breakage [22]. However, no such down-regulation of survivin was apparent in cell lines with mutated or null p53 alleles. These observations have suggested that p53 negatively regulates the expression of survivin in response to DNA damage.

In the present study, we show that exposure of p53-positive A549 human lung cancer cells to ultraviolet C (UVC) radiation, which induces DNA single-strand breakage, resulted in down-regulation of survivin expression after the induction of p53. Depletion of p53 by RNA interference (RNAi) prevented this down-regulation of survivin in cells exposed to UVC. Furthermore, RNAi-mediated depletion of survivin resulted in growth arrest in G_2–M phase of the cell cycle. These findings suggest that negative regulation of survivin by p53 contributes to the p53-dependent G_2–M checkpoint.

2. Materials and methods

2.1. Cell culture and irradiation

A549 cells were provided by Tohoku University (Miyagi, Japan). The cells were cultured under a humidified atmosphere of 5% CO_2 at 37 °C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Each batch of cells was discarded after 20 generations, and new batches were obtained from frozen stocks. Cells were exposed to UVC (30 J/m^2) with a Hoefer UVC 500 Ultraviolet Crosslinker (Amersham Pharmacia Biotech, Piscataway, NJ).

2.2. Immunoblot analysis

Cells were harvested by exposure to trypsin–EDTA, washed with phosphate-buffered saline (PBS), and lysed in a solution containing 30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA, and 10 mM NaCl. Equal amounts of lysate protein were fractionated by SDS–polyacrylamide gel electrophoresis at 100 V for 80 min at room temperature. The separated proteins were transferred to a nitrocellulose membrane, which was then probed for 2 h at room temperature with various primary antibodies, including affinity-purified rabbit polyclonal anti-survivin (R&D Systems, Minneapolis, MN), mouse monoclonal anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), and affinity-purified rabbit polyclonal anti-β-actin (Sigma–Aldrich, St. Louis, MO). Immune complexes were detected with horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (Amersham Biosciences, Little Chalfont, UK) or sheep antibodies to mouse immunoglobulin G (Santa Cruz Biotechnology) and with a chemiluminescence detection system (Perkin-Elmer, Boston, MA).

2.3. Flow cytometry

Cells were harvested, washed with PBS, fixed with 70% methanol, washed again with PBS, and stained with propidium iodide (0.05 mg/ml) in a solution containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase A (0.05 mg/ml). The stained cells ($\sim 1 \times 10^5$) were than analyzed for DNA content with a flow cytometer (FACScaliber; Becton–Dickinson).

2.4. RNAi

Small interfering RNA (siRNA) duplexes specific for survivin or p53 mRNAs were synthesized by Dharmacon Research (Lafayette, CO) with the use of 2′-ACE protection chemistry. The survivin siRNA corresponded to nucleotides 206–224 of the coding region (GenBank Accession No. NM001168), whereas the p53 siRNA corresponded to nucleotides 775–793 of the coding region. BLAST searches of the human genome database were performed to ensure that the siRNA sequences would not target other gene transcripts. Cells in the exponential phase of growth were plated at a density of 3×10^4 cells per well in 12-well culture plates, cultured for 24 h, and then transfected with siRNA (300 nM) with the use of Oligofectamine in OPTI-MEM (Invitrogen, Carlsbad, CA). Control cells were treated with a scrambled siRNA duplex (Dharmacon).

2.5. Statistical analysis

Data are presented as means ± SD and were analyzed by Student's two-tailed t test (Stat View; SAS Institute, Cary, NC). A p value of <0.05 was considered statistically significant.

3. Results

3.1. UVC radiation inhibits A549 cell proliferation and induces G_2–M arrest

To evaluate the effect of UVC on A549 cell proliferation, we counted the number of viable cells at various times after irradiation. UVC treatment resulted in a 70% reduction in the number of viable cells compared with that for untreated cells at 48 h and a 60% reduction at 72 h (Fig. 1A). Flow cytometric analysis of cell cycle distribution revealed that this inhibition of cell proliferation by UVC was accompanied by an approximately twofold increase in the proportion of cells in G_2–M at 24 h (25.8% versus 13.4%), at 48 h (17.1% versus 7.9%) and at 72 h (12.3% versus 6.1%) compared with untreated cells (Fig. 1B), whereas irradiation had no marked effect on the sub-G_1 (apoptotic) population. These data indicated that treatment of A549 cells with UVC results in growth arrest at the G_2–M phase of the cell cycle.

3.2. UVC exposure induces p53 up-regulation followed by survivin down-regulation

Given that p53 mediates cell cycle arrest at the G_2–M transition in response to DNA damage and that we recently showed that down-regulation of survivin expression follows the accumulation of p53 in cells subjected to DNA double-strand breakage [22], we next examined whether survivin and p53 are functionally linked in A549 cells treated with UVC, which induces DNA single-strand breakage. Immunoblot analysis revealed that the abundance of p53 was increased 6 h after UVC exposure, reached a peak at 24 h, and then gradually returned to basal levels by 72 h (Fig. 2). In contrast, the amount of survivin began to decline at 48 h and its down-regulation was more pronounced at 72 h.

To determine whether p53 negatively regulates survivin expression, we examined the effect of UVC radiation on the abundance of survivin in cells depleted of p53 by RNAi. In cells transfected with a control (scrambled) siRNA or in nontransfected cells, the abundance of p53 was increased at 18 h after UVC exposure and the amount of

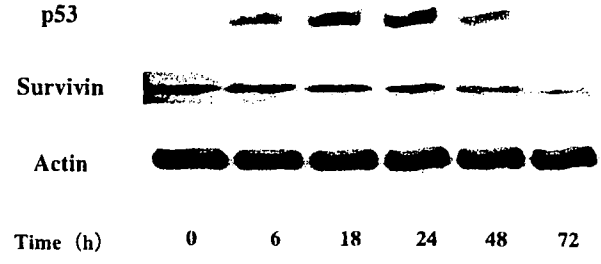

Fig. 2. Effects of UVC on the abundance of p53 and survivin in A549 cells. Total cellular protein extracted at the indicated times after exposure of cells to UVC (30 J/m^2) was subjected to immunoblot analysis with antibodies to p53, to survivin, or to β-actin (loading control). Data are representative of three independent experiments.

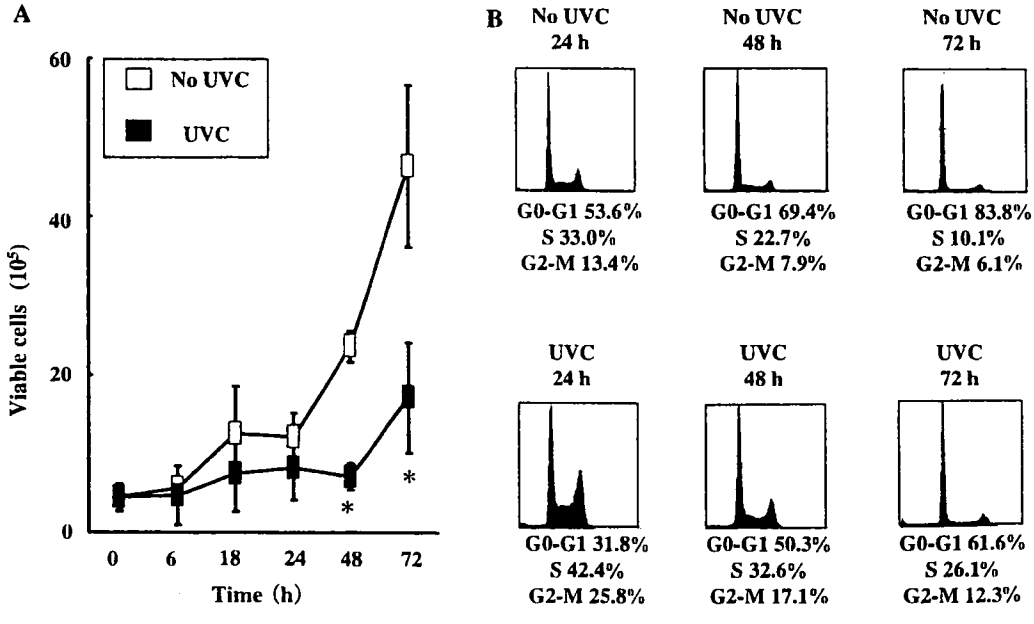

Fig. 1. Effects of UVC on the proliferation and cell cycle distribution of A549 cells. (A) Cell proliferation was evaluated by counting the number of viable cells by trypan blue staining at the indicated times after UVC irradiation (30 J/m^2). Data are means ± SD of values from three independent experiments. *$p < 0.05$ versus the corresponding value for cells not exposed to UVC. (B) Cell cycle distribution was analyzed by propidium iodide staining and flow cytometry at 24, 48 h and 72 h after UVC exposure. The percentages of cells at various stages of the cell cycle are indicated, and the data are representative of three independent experiments.

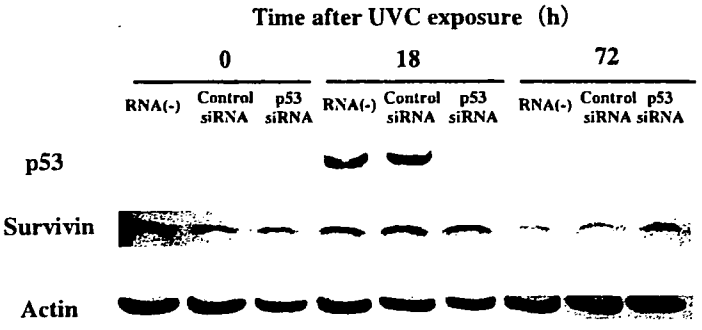

Fig. 3. Effect of UVC on the abundance of survivin in A549 cells depleted of p53 by RNAi. Cells were transfected (or not) with an siRNA specific for p53 mRNA or with a control (scrambled) siRNA, exposed to UVC (30 J/m^2), and subjected to immunoblot analysis with antibodies to p53, to survivin, or to β-actin at the indicated times after irradiation. Data are representative of three independent experiments.

survivin was decreased at 72 h (Fig. 3). In contrast, in cells transfected with an siRNA specific for p53 mRNA, UVC failed to increase p53 expression and had no effect on the level of survivin. These results thus indicated that induction of p53 by exposure of cells to UVC is necessary for down-regulation of survivin.

3.3. Ablation of survivin inhibits cell proliferation and induces G_2–M arrest

We next examined the effects of UVC irradiation in cells depleted of survivin by RNAi. The abundance of survivin was greatly reduced in cells transfected with an siRNA specific for survivin mRNA compared with that in nontransfected cells or cells transfected with a control (scrambled) siRNA (Fig. 4A). Cell proliferation (as evaluated from viable cell number) was also inhibited by 60% or 70% in cells subjected to transfection with the survivin siRNA for 48 or 72 h, respectively, compared with that apparent in nontransfected cells (Fig. 4B). The viable cell count was not affected by transfection with the control siRNA. Flow cytometry revealed that transfection of A549 cells with the survivin siRNA resulted in a marked increase in the proportion of cells in G_2–M at 48 and 72 h compared with that apparent for nontransfected cells or cells transfected with the control siRNA (Fig. 4C and D). There was no difference in the proportion of sub-G_1 cells among the three treatment groups.

4. Discussion

Several genes whose products play a role in control of the G_2–M transition of the cell cycle, including stathmin, Map4, cyclin B1, Cdc2, and Cdc25c, have been shown to be negatively regulated by p53 [15–18]. Repression of the expression of these genes in response to DNA damage requires wild-type p53 and contributes to a DNA damage-induced G_2–M checkpoint [23,24]. Survivin, a member of the IAP family of proteins, is maximally expressed at G_2–M and physically associates with microtubules of the mitotic spindle [2]. Previous studies have suggested that the expression of survivin is also subject to negative regulation by p53 [25–27], but the mechanism of such regulation has been unclear. We have now shown that exposure of the human lung cancer cell line A549 to UVC, which induces DNA single-strand breakage, resulted in the induction of endogenous p53 and a subsequent decrease in survivin expression. These observations are consistent with those of our previous study showing that survivin expression is repressed subsequent to p53 accumulation in cells treated with adriamycin [22], which induces DNA double-strand breakage. To investigate the possible role of p53 in the down-regulation of survivin induced by DNA damage, we depleted A549 cells of p53 by RNAi. Prevention of endogenous p53 accumulation in cells irradiated with UVC was found to block the repression of survivin expression, providing direct evidence that p53 is required for this effect of UVC. These data thus constitute further support for the notion that the survivin gene is a target of negative regulation by p53 in response to DNA damage.

The time course of survivin protein repression following UVC (DNA single-strand breakage)-induced p53 accumulation was almost identical to that observed in the cells having DNA double-strand breakage [22]. These results suggest that p53-dependent survivin suppression in response to these two types of DNA damage may share the common mechanisms at transcriptional level. Hoffmann et al. proposed that direct binding of p53 to a consensus binding site in the survivin gene promoter mediates transcriptional repression of the