Table 1 The combination of carboplatin and paclitaxel

Characteristics	Japan [6]	Greece [13]	Greece [14]	EU [18]	ECOG [19]	SWOG [19]	SWOG [5]	USA [20]	USA [12]
Chemotherapy dose									
CBDCA (AUC)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)
PTX (mg/m^2)	200 (day 1)	175 (day 1)	200 (day 1)	200 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)
Demographics (% not specified)									
No. of patients	145	185	252	309	290	206	182	190	345
Age (median) (range)	63 (33–74)	65 (30–83)	63 (31–81)	58 (27–76)	63 (30–85)	62 (26–80)	63 (28–80)	62 (28–80)	63 (31–85)
Female	32	14	13	17	38	30	37	34	39
PS 0–1	100	80	86	83	95	100	100	NA	91
Stage IV	81	49	62	68	86	88	87	77	78
Non-squamous	79	63	69	63	NA	NA	82	NA	81
Treatment delivery and efficacy (% not specified)									
Cycles (median)	3	NA	NA	4	4	NA	4	NA	6
Response rate (95% CI)	32 (25–40)	46 (39–53)	28 (22–34)	23 (20–30)	17 (13–21)	25 (19–31)	34 (27–41)	23 (17–29)	29 (24–34)
MST (month) (95% CI (month))	12.3 (NA)	11.0 (10–12)	10.4 (8.8–12)	8.2 (7.4–9.6)	8.1 (7.0–9.5)	8.6 (7.2–10.7)	9.0 (NA)	7.8 (NA)	9.9 (NA)
1-year survival (%)	51	43	42	32	34	38	37	32	42
Grade 3-4 toxicity (%)									
Neutropenia	88	14	15	51	63	57	NA	65	6
Febrile neutropenia	16	9	0	4	4	2	3	NA	NA
Thrombocytopenia	11	2	2	2	10	10	8	8	NA
Neuropathy	5	26	8	9	10	13	16	5	1

Table 2 The combination of cisplatin and docetaxel

Characteristics	Japan [7]	ECOG [9]	USA [10]
Chemotherapy dose			
CDDP (mg/m^2)	80 (day 1)	75 (day 1)	75 (day 1)
DTX (mg/m^2)	60 (day 1)	75 (day 1)	75 (day 1)
Demographics (% not specified)			
No. of patients	151	289	408
Age (median) (range)	63 (30–74)	63 (34–84)	61 (30–81)
Female	36	37	28
PS 0–1	96	94	96
Stage IV	100	86	67
Non-squamous	89	NA	68
Treatment delivery and efficacy (% not specified)			
Cycles (median)	3	4	5
Response rate (95% CI)	37 (29–45)	17 (12–21)	32 (27–36)
MST (month) (95% CI (month))	11.3 (NA)	7.4 (6.6–8.8)	11.3 (10.1–12.4)
1-year survival	48	31	46
Grade 3-4 toxicity (%)			
Neutropenia	74	69	75
Febrile neutropenia	2	11	5
Thrombocytopenia	1	3	3
Neuropathy	0	5	4

The toxicity was similar among many studies except for the gemcitabine and cisplatin arm of the Iressa NSCLC Trial Assessing Combination Treatment (INTACT) study [11], where the incidence of grade 3-4 neutropenia and thrombocytopenia was reported to be about one tenth of that in other studies (Table 3).

3.3. Vinorelbine and cisplatin

The dose of vinorelbine per one course was also smaller in the Japanese study than in other studies outside Japan (Table 4). The RR in the Greek study was higher than that in an American study. There was no difference in survival for this combination among all studies. There was no correlation between demographic variables and the RR or MST in a multiple linear regression analysis.

Grade 3-4 neutropenia was less common in the Greek study than in other studies, but the frequency of febrile neutropenia in that study was intermediate among studies.

4. Discussion

This study showed that geographical differences in the outcome of lung cancer chemotherapy may be present. However, extrapolation of clinical data in a country to another population and global clinical trials were considered possible with adequate considerations as discussed below.

The dose of third-generation cytotoxic agents was smaller in Japanese studies than in European and American studies. The toxicity profile was generally acceptable and similar among many studies. Thus, the dose and schedule of anticancer agents established in prior phase I and II studies conducted in each country were appropriate and applicable to large patient populations of these countries. Patient demographics were very similar through all studies, indicating that extrinsic ethnic factors may be comparable and consistent between geographically different oncology groups. These factors include socioeconomic factors, medical service background, and patient selection process for clinical trials.

RRs in phase III studies including third-generation cytotoxic agents seemed to be distributed randomly from one study to another, whereas patient survival might have been better in Japanese studies. The Japanese phase III trials were performed in academic institutes, including university-affiliated hospitals, cancer center hospitals, and central city general hospitals. Thus, the distribution of patients selected for Japanese phase III trials may be skewed, in that they were in good general condition, although established prognostic factors in patients with NSCLC were almost identical among Japanese and non-Japanese studies. In addition, better survival among Japanese patients may be attributable to true ethnic differences. One possibility is the relatively high frequency of non-squamous histology in Japanese studies, but the reason is largely unknown.

The severity and frequency of common toxicity were comparable in all these phase III studies with a few exceptions. The incidence of grade 3-4 neutropenia was only 5–6% in the carboplatin and paclitaxel arm of the INTACT2 study [12] and in the cisplatin and gemcitabine arm of the INTACT1 study [11], both of which were sponsored by one pharmaceutical company. Similarly, the incidence of neutropenia was lower in Greek studies [13–15] than in other studies. These differences in the incidence of toxicity may be associated with the frequency of monitoring, including patient hospital visits and blood cell count and chemistry evaluation.

Table 3 The combination of cisplatin and gemcitabine

Characteristics	Japan [6]	Italy [21]	Spain [22]	EORTC [23]	EU [11]	ECOG [9]	EU + USA [24]
Chemotherapy dose							
CDDP (mg/m^2)	80 (day 1)	100 (day 2)	100 (day 1)	80 (day 1)	80 (day 1)	100 (day 1)	100 (day 1)
GEM (mg/m^2)	1000 (day 1, 8)	1000 (day 1, 8, 15)	1250 (day 1, 8)	1250 (day 1, 8)	1250 (day 1, 8)	1000 (day 1, 8, 15)	1000 (day 1, 8, 15)
Demographics (% not specified)							
No. of patients	146	155	182	160	363	288	260
Age (median) (range)	61 (34–74)	62 (28–76)	59 (33–75)	57 (28–75)	61 (33–81)	64 (32–87)	62 (36–88)
Female	33	37	12	29	28	37	30
PS 0–1	100	93	85	89	90	95	80
Stage IV	81	79	77	79	69	86	67
Non-squamous	81	68	55	74	71	NA	70
Treatment delivery and efficacy (% not specified)							
Cycles (median)	3	NA	4	5	6	3	4
Response rate (95% CI)	30 (23–38)	38 (30–46)	42 (35–50)	37 (29–45)	47 (42–53)	22 (17–27)	30 (25–36)
MST (month) (95% CI (month))	14.8 (NA)	8.6 (NA)	9.3 (8.1–10.5)	8.9 (7.8–10.5)	10.9 (NA)	8.1 (7.2–9.4)	9.1 (8.3–10.6)
1-year survival	60	33	38	33	44	36	39
Grade 3-4 toxicity (%)							
Neutropenia	63	40	32	43	5	63	57
Febrile neutropenia	2	1	4	3	NA	4	5
Thrombocytopenia	35	64	19	36	6	50	50

Table 4 The combination of cisplatin and vinorelbine

Characteristics	Japan [6]	Greece [15]	France [25]	EU [26]	SWOG [19]	USA [10]
Chemotherapy dose						
CDDP (mg/m^2)	80 (day 1)	80 (day 8)	100 (day 1)	120 (day 1)	100 (day 1)	100 (day 1)
VNR (mg/m^2)	25 (day 1, 8)	30 (day 1, 8)	30 (day 1, 8, 15, 22)	30 (day 1, 8, 15, 22)	25 (day 1, 8, 15, 22)	25 (day 1, 8, 15, 22)
Demographics (% not specified)						
No. of patients	145	204	156	206	202	404
Age (median) (range)	61 (28–74)	64 (46–75)	57 (39–74)	59 (NA)	61 (32–83)	61 (35–80)
Female	30	25	21	12	33	25
PS 0–1	100	90	92	80	100	96
Stage IV	83	64	86	59	89	67
Non-squamous	81	54	76	44	NA	65
Treatment delivery and efficacy (% not specified)						
Cycles (median)	3	4	4	3	NA	4
Response rate (95% CI)	33 (25–41)	39 (33–46)	36 (28–43)	28 (22–34)	28 (22–34)	25 (20–29)
MST (month) (95% CI (month))	11.4 (NA)	9.7 (8.3–11.2)	9.6 (8.1–12.2)	9.3 (NA)	8.1 (6.7–9.6)	10.1 (9.2–11.3)
1-year survival	48	41	42	37	36	41
Grade 3-4 toxicity (%)						
Neutropenia	88	37	83	79	76	79
Febrile neutropenia	18	11	22	4	1	5
Thrombocytopenia	1	6	3	3	4	4

Anticancer agents are considered to be sensitive to ethnic factors, because of a steep pharmacodynamic curve for both efficacy and safety, a narrow therapeutic dose range, non-linear pharmacokinetics, their metabolic enzymes with the potential for drug-drug interaction, and these enzymes with the potential for ethnically variable activity caused by genetic polymorphism. Thus, bridging studies using pharmacologic endpoints are extremely important to apply efficacy, safety, and dose data from one place to another [16]. These pharmacologic studies can be incorporated into phase I trials and, when it is necessary, phase II trials. Furthermore, the current study suggests that, once the pharmacological property and recommended dose of a new cytotoxic agent are established in one country, the outcome of randomized controlled trials developed in other countries can be extrapolated to the population.

We defined ethnic populations in the current study according to the country where the study was performed. However, patients enrolled into multicenter European and North American studies may include patients with a diverse ethnicity. It would be greatly interesting to see RR, MST and toxicity in subgroups of patients with different ethnicity in those trials, although there has been no such data published.

Randomization of patients in a trial guarantees the comparability between treatment arms within the trial, but not between treatment arms in different trials. Thus, it is impossible to compare the outcome of different trials exactly. Nevertheless, we frequently refer to the outcome of trials performed outside Japan and they furnish us with much information. To compensate this limitation, we tried to compare patient characteristics between trials, but other factors including the frequency of monitoring may also affect the outcome greatly. The number of combination regimens evaluated in this study is insufficient, but no large scale Japanese trials of other combination regimens have been available so far.

This study failed to demonstrate whether this approach to clinical trial analysis was really helpful. For future clinical trials, consistency in monitoring, as well as the use of the common toxicity and response criteria, is important to keep comparability between trials. A meta-analysis using individual patient data may be more useful than a subgroup analysis within a trial to compare the outcomes between ethnic subgroups with adequate statistical power.

A phase II study of gefitinib in patients with advanced NSCLC who had previously received one or two chemotherapy regimens was conducted in cooperation with 43 hospitals across Europe, Australia, South Africa, and Japan. The population was prospectively stratified into Japanese and non-Japanese patients to investigate whether there were any differences between the two patient populations with respect to efficacy [17]. This study clearly showed that a global study of NSCLC using the same protocol was completed, and this global strategy was an effective method to speed up the development of a new anticancer agent in Japan. In addition, the stratification by the county or ethnicity is important in a global study of an investigational new drug to investigate geographical differences in efficacy and toxicity.

In conclusion, the dose of anticancer agents, RR, survival and toxicity of lung cancer chemotherapy showed some differences among Japanese, European, and USA studies. However, extrapolation of clinical data in a country to another population and global clinical trials including many countries were considered possible with adequate dose adjustment based on dose finding studies using a carefully projected protocol.

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References

[1] Schottenfeld D, Searle JG. The etiology and epidemiology of lung cancer. In: Pass HI, Carbone DP, Minna JD, Johnson DH, Turrisi III AT, editors. Lung cancer: principles and practice. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2005. p. 3–24.

[2] Kaneko S, Ishikawa KB, Yoshimi I, Marugame T, Hamashima C, Kamo K, et al. Projection of lung cancer mortality in Japan. Cancer Sci 2003;94:919–23.

[3] Sekine I, Saijo N. Novel combination chemotherapy in the treatment of non-small cell lung cancer. Expert Opin Pharmacother 2000;1:1131–61.

[4] Ministry of Health, Labour and Welfare Japan. ICH Guideline and its related informations. MHLW website; 2005. Available from: http://www.nihs.go.jp/dig/ich/ichindexe.html [cited October 25, 2005].

[5] Gandara DR, Ohe Y, Kubota K, Nishiwaki Y, Ariyoshi Y, Saijo N, et al. Japan-SWOG common arm analysis of paclitaxel/carboplatin in advanced stage non-small cell lung cancer (NSCLC): a model for prospective comparison of cooperative group trials. Proc Am Soc Clin Oncol 2004;22:618s [Abstract 7007].

[6] Kubota K, Nishiwaki Y, Ohashi Y, Saijo N, Ohe Y, Tamura T, et al. The Four-Arm Cooperative Study (FACS) for advanced non-small-cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 2004;22:618s [Abstract 7006].

[7] Kubota K, Watanabe K, Kunitoh H, Noda K, Ichinose Y, Katakami N, 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 2004;22:254–61.

[8] Sekine I, Kubota K, Nishiwaki Y, Sasaki Y, Tamura T, Saijo N. Response rate as an endpoint for evaluating new cytotoxic agents in phase II trials of non-small-cell lung cancer. Ann Oncol 1998;9:1079–84.

[9] Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 2002;346:92–8.

[10] Fossella F, Pereira JR, von Pawel J, Pluzanska A, Gorbounova V, Kaukel E, et al. Randomized, multinational, phase III study of docetaxel plus platinum combinations versus vinorelbine plus cisplatin for advanced non-small-cell lung cancer: the TAX 326 study group. J Clin Oncol 2003;21:3016–24.

[11] Giaccone G, Herbst RS, Manegold C, Scagliotti G, Rosell R, Miller V, 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 2004;22:777–84.

[12] Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, 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 2004;22:785–94.

[13] Stathopoulos GP, Veslemes M, Georgatou N, Antoniou D, Giamboudakis P, Katis K, et al. Front-line paclitaxel–vinorelbine versus paclitaxel–carboplatin in

patients with advanced non-small-cell lung cancer: a randomized phase III trial. Ann Oncol 2004;15:1048—55.

[14] Kosmidis P, Mylonakis N, Nicolaides C, Kalophonos C, Samantas E, Boukovinas J, et al. Paclitaxel plus carboplatin versus gemcitabine plus paclitaxel in advanced non-small-cell lung cancer: a phase III randomized trial. J Clin Oncol 2002;20:3578—85.

[15] Georgoulias V, Ardavanis A, Tsiafaki X, Agelidou A, Mixalopoulou P, Anagnostopoulou O, et al. Vinorelbine plus cisplatin versus docetaxel plus gemcitabine in advanced non-small-cell lung cancer: a phase III randomized trial. J Clin Oncol 2005;23:2937—45.

[16] Ministry of Health, Labour, and Welfare of Japan. Acute ILD associated with the use of gefitinib reported to Pharmaceuticals and Medical Devices Agency Japan MHLW website; 2005.Available from: http://www.mhlw.go.jp/shingi/2005/01/txt/s0120-4.txt [cited October 25, 2005].

[17] Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial). J Clin Oncol 2003;21:2237—46.

[18] Rosell R, Gatzemeier U, Betticher DC, Keppler U, Macha HN, Pirker R, et al. Phase III randomised trial comparing paclitaxel/carboplatin with paclitaxel/cisplatin in patients with advanced non-small-cell lung cancer: a cooperative multinational trial. Ann Oncol 2002;13:1539—49.

[19] Kelly K, Crowley J, Bunn Jr PA, Presant CA, Grevstad PK, Moinpour CM, et al. Randomized phase III trial of paclitaxel plus carboplatin versus vinorelbine plus cisplatin in the treatment of patients with advanced non-small-cell lung cancer: a Southwest Oncology Group trial. J Clin Oncol 2001;19:3210—8.

[20] Belani CP, Lee JS, Socinski MA, Robert F, Waterhouse D, Rowland K, et al. Randomized phase III trial comparing cisplatin-etoposide to carboplatin—paclitaxel in advanced or metastatic non-small cell lung cancer. Ann Oncol 2005;16:1069—75.

[21] Crino L, Scagliotti GV, Ricci S, De Marinis F, Rinaldi M, Gridelli C, et al. Gemcitabine and cisplatin versus mitomycin, ifosfamide, and cisplatin in advanced non-small-cell lung cancer: a randomized phase III study of the Italian Lung Cancer Project. J Clin Oncol 1999;17:3522—30.

[22] Alberola V, Camps C, Provencio M, Isla D, Rosell R, Vadell C, et al. Cisplatin plus gemcitabine versus a cisplatin-based triplet versus nonplatinum sequential doublets in advanced non-small-cell lung cancer: a Spanish Lung Cancer Group phase III randomized trial. J Clin Oncol 2003;21:3207—13.

[23] Smit EF, van Meerbeeck JP, Lianes P, Debruyne C, Legrand C, Schramel F, et al. Three-arm randomized study of two cisplatin-based regimens and paclitaxel plus gemcitabine in advanced non-small-cell lung cancer: a phase III trial of the European Organization for Research and Treatment of Cancer Lung Cancer Group—EORTC 08975. J Clin Oncol 2003;21:3909—17.

[24] Sandler AB, Nemunaitis J, Denham C, von Pawel J, Cormier Y, Gatzemeier U, et al. Phase III trial of gemcitabine plus cisplatin versus cisplatin alone in patients with locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 2000;18:122—30.

[25] Pujol JL, Breton JL, Gervais R, Rebattu P, Depierre A, Morere JF, et al. Gemcitabine-docetaxel versus cisplatin—vinorelbine in advanced or metastatic non-small-cell lung cancer: a phase III study addressing the case for cisplatin. Ann Oncol 2005;16:602—10.

[26] Le Chevalier T, Brisgand D, Douillard JY, Pujol JL, Alberola V, Monnier A, et al. Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small-cell lung cancer: results of a European multicenter trial including 612 patients. J Clin Oncol 1994;12:360—7.

Research Article

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).

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.

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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′-GAGTTTCC-CAAACACTCAGTGAAA-C-3′; exon 19, 5′-GCAATATCAGCCTTAGGT-GCGGCTC-3′and 5′-CATAGAAAGTGAACATTTAGGATGTG-3′; exon 20, 5′-CCATGAGTACGTATTTTGAAACTC-3′ and 5′-CATATCCCCATGG-CAAACTCTTGC-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

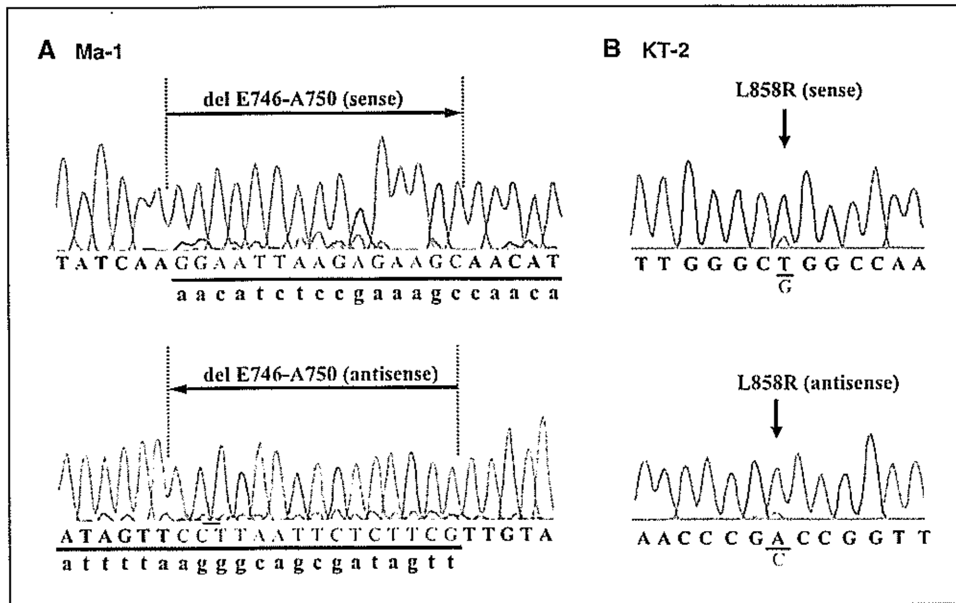

Figure 1. Detection of *EGFR* mutations in NSCLC cell lines. The portions of the sequencing electrophoretograms corresponding to the mutations are shown for Ma-1 (*A*) and KT-2 (*B*) cells. *A*, heterozygous in-frame deletion in exon 19 is revealed by the presence of double peaks. Tracings in both sense 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).

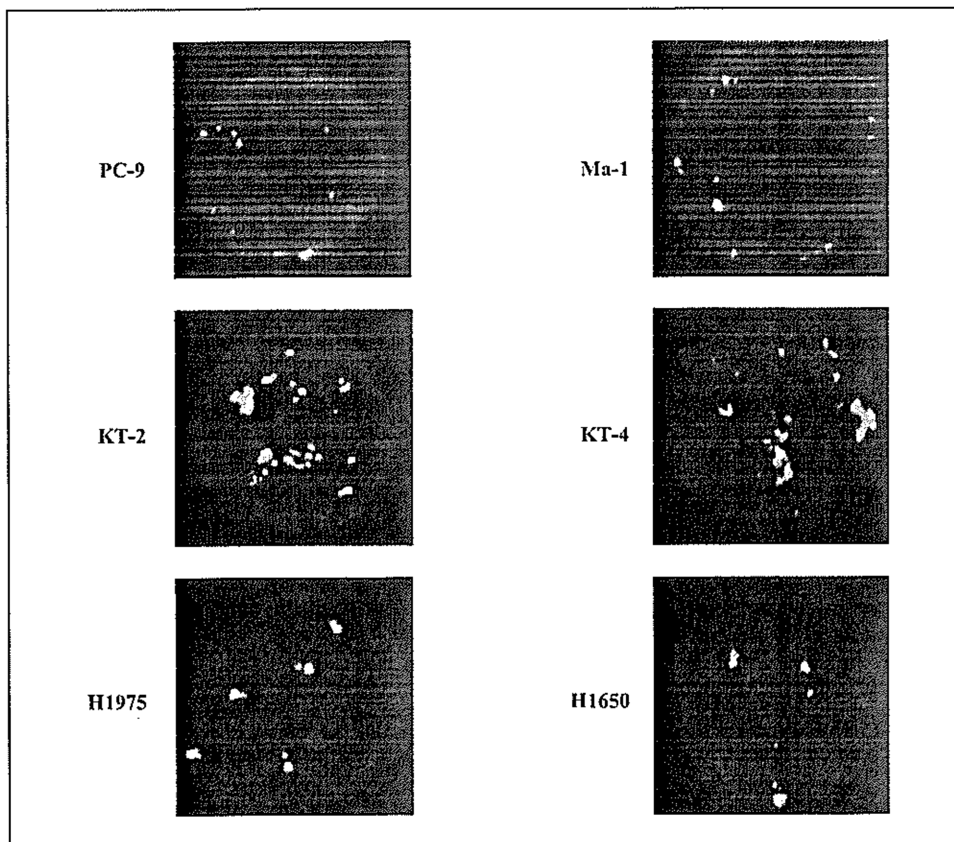

Figure 2. FISH analysis of *EGFR* amplification in NSCLC cell lines. The analysis was done with probes specific for *EGFR* (*red signals*) and for the centromere of chromosome 7 (*green signals*) in the indicated cell lines. PC-9 and Ma-1 cells manifest an *EGFR*/chromosome copy number ratio of ≥2, whereas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

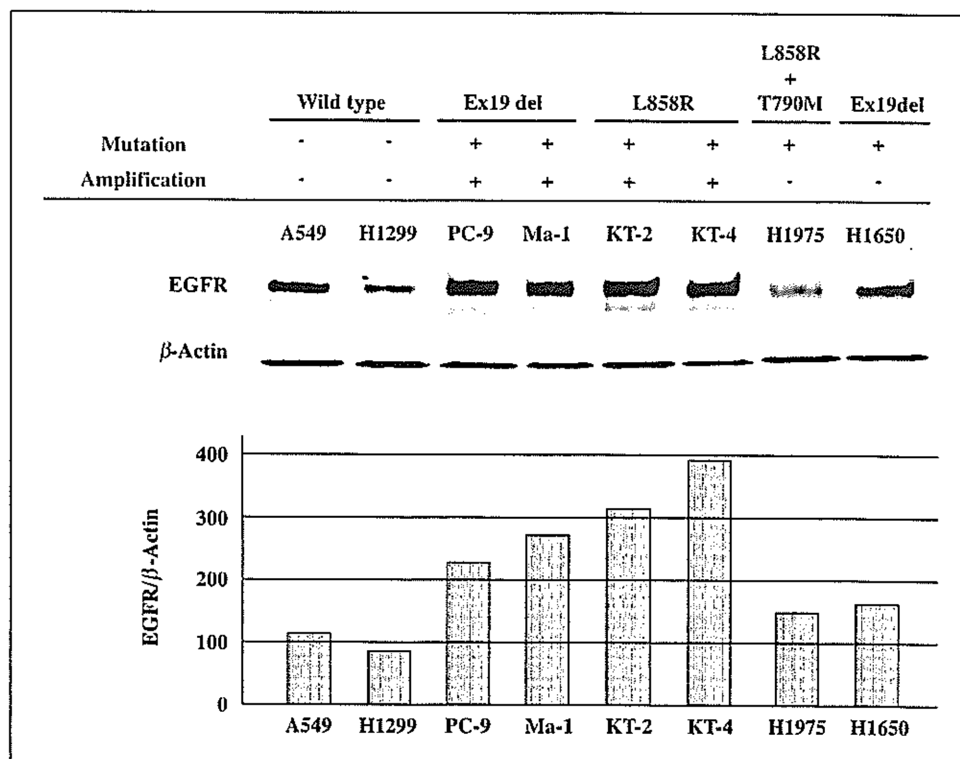

Figure 3. EGFR expression in NSCLC cell lines. Lysates (40 μg of protein) of NSCLC cell lines positive or negative for *EGFR* mutation or amplification, as indicated, were subjected to immunoblot analysis with antibodies to EGFR and to β-actin (*top*). The abundance of EGFR relative to that of β-actin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

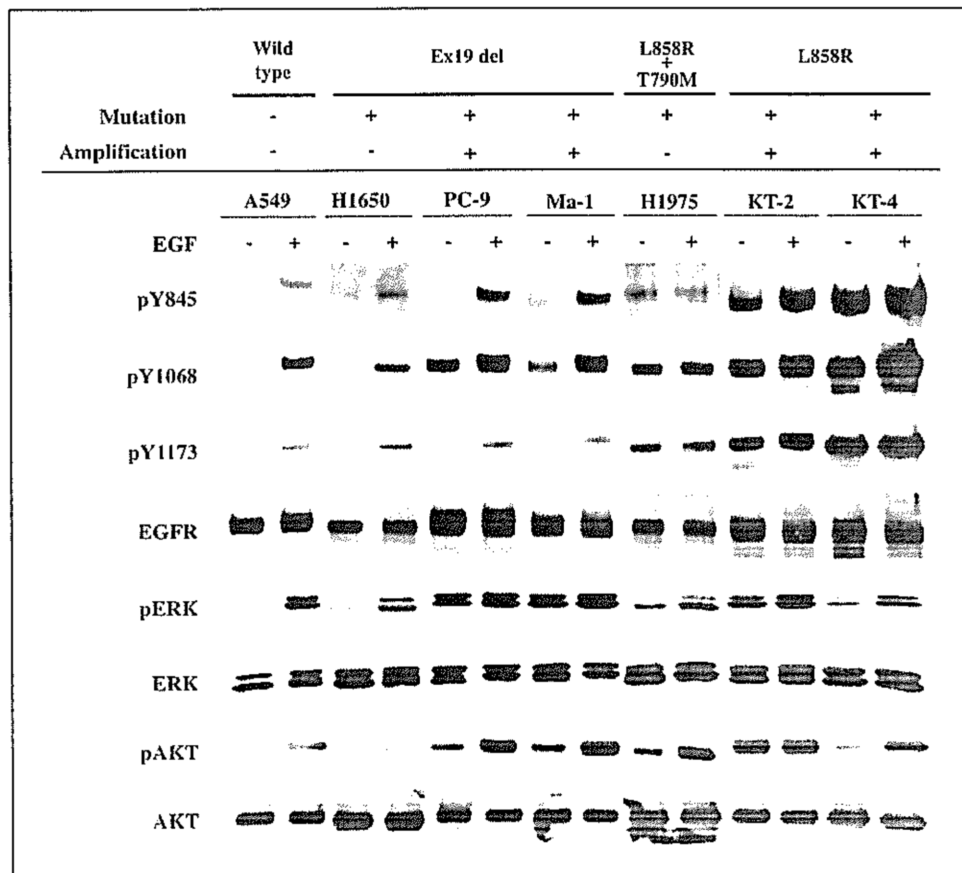

Figure 4. Phosphorylation of EGFR and downstream signaling molecules in NSCLC cell lines. Serum-deprived cells were incubated for 15 min in the absence or presence of EGF (100 ng/mL), after which cell lysates (40 μg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated forms 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

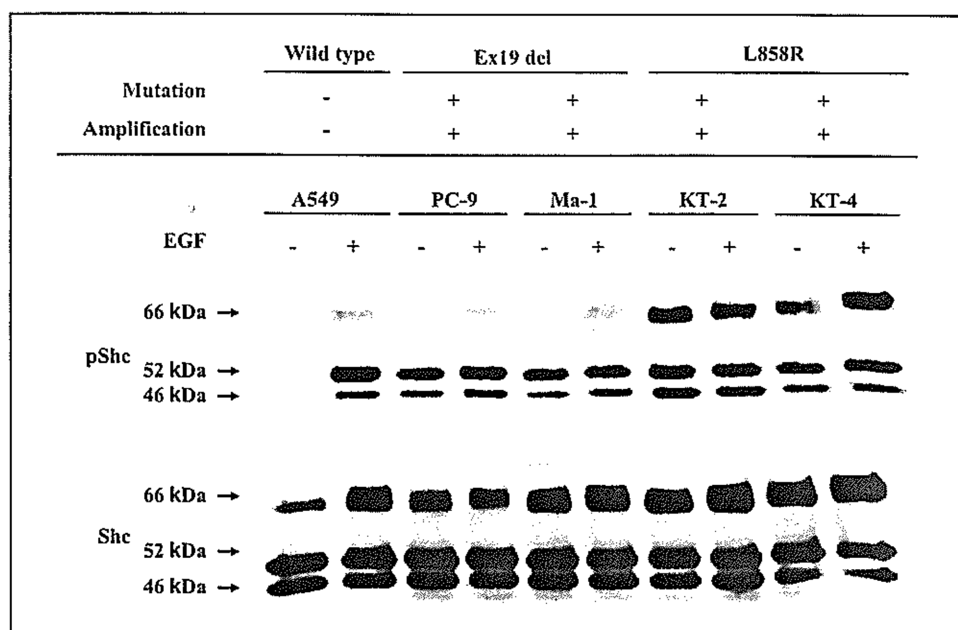

Figure 5. Phosphorylation of Shc in NSCLC cell lines. Serum-deprived cells were incubated for 15 min in the absence or presence of EGF (100 ng/mL), after which cell lysates (40 μg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated Shc (*pShc*) or total Shc. Representative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

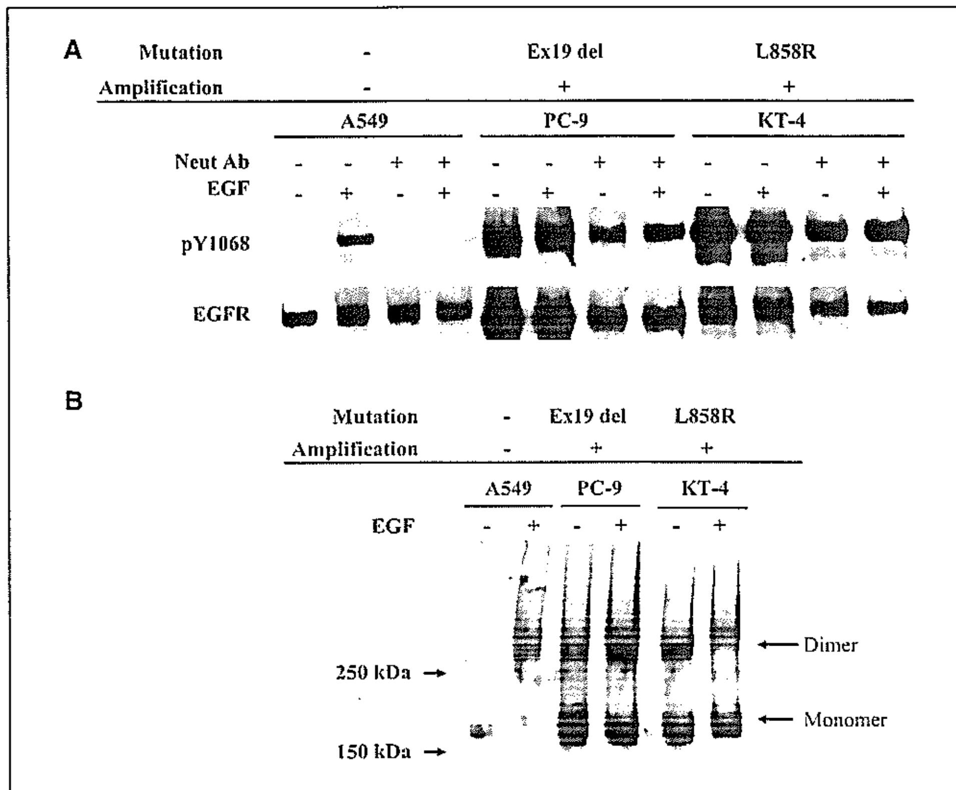

Figure 6. Mechanism of constitutive activation of EGFR in NSCLC cell lines. *A*, effect of neutralizing antibodies (*Neut Ab*) on EGFR phosphorylation. Serum-deprived NSCLC cells (A549, PC-9, or KT-4) were incubated for 3 h with a combination of neutralizing antibodies to EGF, TGF-α, and EGFR and then 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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References

1. Wang Y, Minoshima S, Shimizu N. Precise mapping of the EGF receptor gene on the human chromosome 7p12 using an improved FISH technique. Jpn J Hum Genet 1993;38:399–406.
2. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res 2003; 284:31–53.
3. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005; 5:341–54.
4. Ogiso H, Ishitani R, Nureki O, et al. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. Cell 2002;110:775–87.
5. Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell 2002;110:669–72.
6. Hirsch FR, Varella-Garcia M, Bunn PA, Jr., et al. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. J Clin Oncol 2003;21:3798–807.
7. Suzuki S, Dobashi Y, Sakurai H, Nishikawa K, Hanawa M, Ooi A. Protein overexpression and gene amplification of epidermal growth factor receptor in nonsmall cell lung carcinomas. An immunohistochemical and fluorescence *in situ* hybridization study. Cancer 2005;103: 1265–73.
8. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353:123–32.
9. Thatcher N, Chang A, Parikh P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). Lancet 2005;366:1527–37.
10. 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 (the IDEAL 1 trial). J Clin Oncol 2003;21: 2237–46.
11. Kaneda H, Tamura K, Kurata T, Uejima H, Nakagawa K, Fukuoka M. Retrospective analysis of the predictive factors associated with the response and survival benefit of gefitinib in patients with advanced non-small-cell lung cancer. Lung Cancer 2004;46:247–54.
12. Takano T, Ohe Y, Kusumoto M, et al. Risk factors for interstitial lung disease and predictive factors for tumor response in patients with advanced non-small cell lung cancer treated with gefitinib. Lung Cancer 2004;45:93–104.
13. Tamura K, Fukuoka M. Gefitinib in non-small cell lung cancer. Expert Opin Pharmacother 2005;6:985–93.
14. 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 2006;24:2549–56.
15. 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 2004;350:2129–39.
16. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
17. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004; 101:13306–11.
18. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 2004; 64:8919–23.
19. Han SW, Kim TY, Hwang PG, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. J Clin Oncol 2005;23:2493–501.
20. 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 2005;23:2513–20.
21. 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 2005;11:1167–73.
22. Takano T, Ohe Y, Sakamoto H, et al. Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J Clin Oncol 2005;23:6829–37.
23. Gazdar AF, Shigematsu H, Herz J, Minna JD. Mutations and addiction to EGFR: the Achilles 'heal' of lung cancers? Trends Mol Med 2004;10:481–6.
24. Shigematsu H, Gazdar AF. Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. Int J Cancer 2006;118:257–62.
25. Cappuzzo F, Hirsch FR, Rossi E, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005;97:643–55.
26. Hirsch FR, Varella-Garcia M, McCoy J, et al. Increased epidermal growth factor receptor gene copy number detected by fluorescence *in situ* hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a Southwest Oncology Group Study. J Clin Oncol 2005;23:6838–45.
27. Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer: molecular and clinical predictors of outcome. N Engl J Med 2005;353:133–44.
28. Ishikawa N, Daigo Y, Takano A, et al. Increases of amphiregulin and transforming growth factor-α in serum as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancers. Cancer Res 2005;65:9176–84.
29. Tracy S, Mukohara T, Hansen M, Meyerson M, Johnson BE, Janne PA. Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255. Cancer Res 2004;64:7241–4.
30. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.
31. Amann J, Kalyankrishna S, Massion PP, et al. Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. Cancer Res 2005;65:226–35.
32. Greulich H, Chen TH, Feng W, et al. Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. PLoS Med 2005;2:1167–76.
33. Yonesaka K, Tamura K, Kurata T, et al. Small interfering RNA targeting survivin sensitizes lung cancer cell with mutant p53 to Adriamycin. Int J Cancer 2006; 118:812–20.
34. Koizumi F, Shimoyama T, Taguchi F, Saijo N, Nishio K. Establishment of a human non-small cell lung cancer cell line resistant to gefitinib. Int J Cancer 2005;116:36–44.
35. Arteaga CL, Ramsey TT, Shawver LK, Guyer CA. Unliganded epidermal growth factor receptor dimerization induced by direct interaction of quinazolines with the ATP binding site. J Biol Chem 1997;272:23247–54.
36. Mukohara T, Engelman JA, Hanna NH, et al. Differential effects of gefitinib and cetuximab on non-small-cell lung cancers bearing epidermal growth factor receptor mutations. J Natl Cancer Inst 2005;97:1185–94.
37. Janmaat ML, Rodriguez JA, Gallegos-Ruiz M, Kruyt FA, Giaccone G. Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or phosphatidyl inositol-3 kinase pathways in non-small cell lung cancer cells. Int J Cancer 2006;118:209–14.
38. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:225–35.
39. Kobayashi S, Ji H, Yuza Y, et al. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. Cancer Res 2005; 65:7096–101.
40. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 2000;19:3159–67.
41. Okabayashi Y, Kido Y, Okutani T, Sugimoto Y, Sakaguchi K, Kasuga M. Tyrosines 1148 and 1173 of activated human epidermal growth factor receptors are binding sites of Shc in intact cells. J Biol Chem 1994;269: 18674–8.
42. Riemenschneider MJ, Bell DW, Haber DA, Louis DN. Pulmonary adenocarcinomas with mutant epidermal growth factor receptors. N Engl J Med 2005;352:1724–5.
43. 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;97:339–46.
44. Calvo E, Baselga J. Ethnic differences in response to epidermal growth factor receptor tyrosine kinase inhibitors. J Clin Oncol 2006;24:2158–63.
45. Sugio K, Uramoto H, Ono K, et al. Mutations within the tyrosine kinase domain of EGFR gene specifically occur in lung adenocarcinoma patients with a low exposure of tobacco smoking. Br J Cancer 2006;94:896–903.
46. Riely GJ, Pao W, Pham D, et al. Clinical course of patients with non-small cell lung cancer and epidermal growth factor receptor exon 19 and exon 21 mutations treated with gefitinib or erlotinib. Clin Cancer Res 2006; 12:839–44.
47. Jackman DM, Yeap BY, Sequist LV, et al. Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. Clin Cancer Res 2006;12:3908–14.

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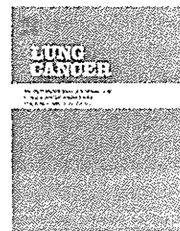

CASE REPORT

Pemetrexed-induced edema of the eyelid

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KEYWORDS
Chemotherapy;
Eyelid edema;
Lung cancer;
Supportive care

Summary Pemetrexed is a novel antimetabolite that targets multiple enzymes in the folate pathway, and has exhibited clear antitumor activities in the treatment of malignant pleural mesothelioma and non-small cell lung cancer. Although many adverse events of pemetrexed, such as bone marrow suppression, have been reported, edema of the eyelid has been previously reported in only one case (0.2%, $n = 519$), according to the Pemetrexed Clinical Investigator's Brochure, April 2005 version. We experienced a patient who developed the valuable edema of the eyelid. We believe that medical oncologists should be aware of this rare adverse event, although the mechanism responsible for it is not yet known.
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1. Introduction

Pemetrexed is a novel antimetabolite that targets multiple enzymes in the folate pathway, and has exhibited clear antitumor activities in the treatment of malignant pleural mesothelioma and non-small cell lung cancer [1,2]. In early-phase pemetrexed studies, severe unpredictable toxicities were observed. Recently, Niyikiza et al reported that pemetrexed-based toxicities were associated with elevated serum homocysteine levels at baseline [3], and that to avoid pemetrexed-based severe toxicities, patients have received folic acid and vitamin B_{12} supplements. In the Japanese protocol, prophylactic steroids need not be administered, since the incidence of severe rash is very low in Japanese patients [4].

2. Case description

A 56-year-old Japanese man was diagnosed with adenocarcinoma of the lung with brain and pulmonary metastases in April, 2004 (cT4N3M1; stage IV). He received three courses of cisplatin/gemcitabine and subsequently received gefitinib as maintenance therapy from April to August, 2004, with a best response of partial response. After radiation therapy to the brain metastasis, which had exhibited aggravation, he was enrolled in a clinical trial of pemetrexed (Alimta®) in December, 2004 and received 1000 mg/m^2 of pemetrexed on day 1 of a 21-day cycle according to the trial design using randomized assignment (500 or 1000 mg/m^2 arm). He developed edema of the eyelid, which appeared on day 8 of the second course of pemetrexed (cumulative

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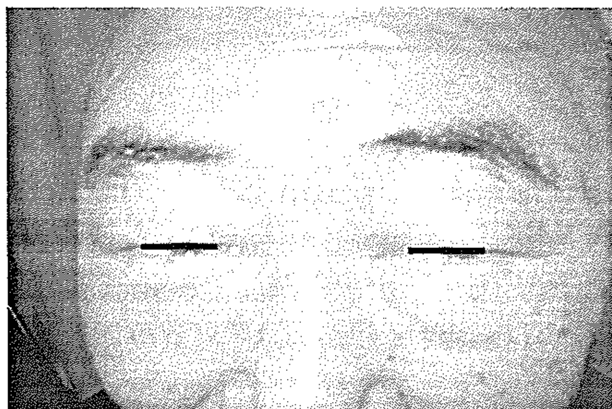
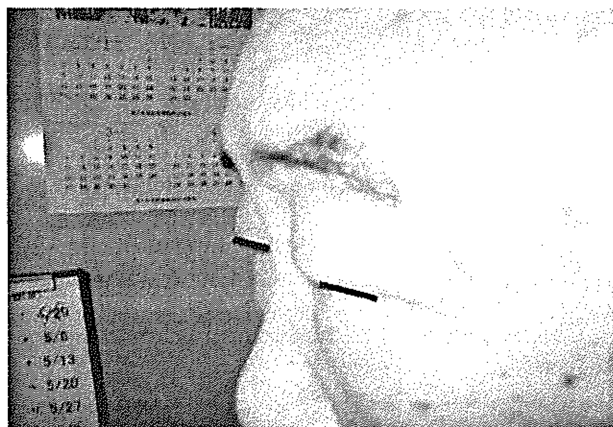


(A)



(B)

Fig. 1 A 56-year-old man with adenocarcinoma of the lung. Edema of the eyelid appeared on day 8 of the second course of pemetrexed. (A) Photograph taken from the front. (B) Profile.

dose: 3900 mg/body) (Fig. 1). He developed no other type of edema. He had no hypoproteinemia or did not undergo hydration. Initially, cardiac failure and conjunctivitis were considered possible causes. A diuretic was given, but did not improve the edema. The edema was therefore thought to be a side effect of pemetrexed, and 8 mg dexamethasone was administered. The edema was dramatically improved 6 days after administration of steroid (Fig. 2). Since the tumor had decreased in size, administration of pemetrexed was continued. The eyelid edema appeared whenever a course of pemetrexed was repeated. This edema was therefore considered probably related to pemetrexed.

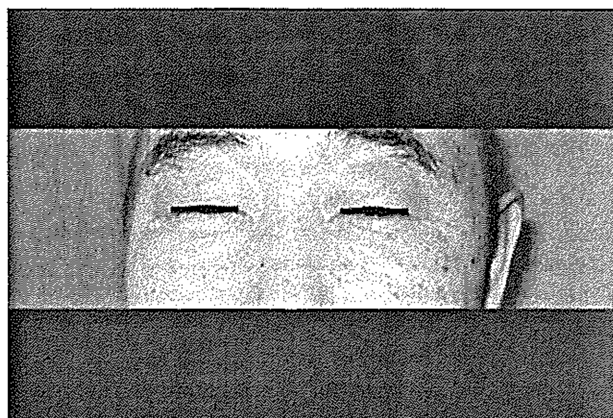


Fig. 2 The edema of the eyelid was improved by the administration of corticosteroid.

3. Discussion

Pemetrexed-associated edema of the eyelid has been previously reported in only one case (0.2%, $n = 519$), according to the Pemetrexed Clinical Investigator's Brochure, April 2005 version. The mechanism responsible for this severe swelling is unknown. Similarly, docetaxel has also been documented to cause peripheral edema. Recently, Semb et al. [5] reported that docetaxel enhances fluid filtration, followed by capillary protein leakage that causes edema and nonmalignant effusion. Prophylactic administration of corticosteroid during docetaxel administration appears to delay and decrease the severity of these adverse events. It may be that pemetrexed-induced eyelid edema is due to the same mechanism as the edema produced by docetaxel.

There are still unanswered questions regarding this drug-induced eyelid edema. Why is it confined to the eyelid? Is it a cumulative adverse event? We believe that medical oncologists should be aware of this rare adverse event and attempt to determine its cause.

Conflict of interest statement

None declared.

Acknowledgment

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References

[1] Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, Von Pawel J, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small cell lung cancer previously treated with chemotherapy. J Clin Oncol 2004;22: 1589–97.

[2] Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, et al. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 2003;21:2636–44.

[3] Niyikiza C, Baker SD, Seitz DE, Walling JM, Nelson K, Rusthoven JJ, et al. Homocysteine and methylmalonic acid: Markers to predict and avoid toxicity from pemetrexed therapy. Mol Cancer Ther 2002;1:545–52.

[4] Nakagawa K, Kudoh S, Matsui K, Negoro S, Yamamoto N, Latz JE, et al. A phase I study of pemetrexed supplemented with folic acid and vitamin B_{12} in Japanese patients with solid tumors. Presented at the 16th EORTC-NCI-AACR, Geneva, Switzerland, September 28–October 1, 2004.

[5] Semb KA, Aamdal S, Oian P. Capillary protein leak syndrome appears to explain fluid retention in cancer patients who receive docetaxel treatment. J Clin Oncol 1998;16:3426–32.

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Phase II Study of Etoposide and Cisplatin With Concurrent Twice-Daily Thoracic Radiotherapy Followed by Irinotecan and Cisplatin in Patients With Limited-Disease Small-Cell Lung Cancer: West Japan Thoracic Oncology Group 9902

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

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ABSTRACT

Purpose
We initially conducted a randomized phase II study to compare irinotecan and cisplatin (IP) versus irinotecan, cisplatin, and etoposide (IPE) after etoposide and cisplatin (EP) with concurrent twice-daily thoracic radiotherapy (TRT) in limited-disease small-cell lung cancer (LD-SCLC). We amended the protocol to evaluate IP after EP with concurrent twice-daily TRT in a single-arm phase II study because of an unacceptable toxicity in IPE.

Patients and Methods
Previously untreated patients with LD-SCLC were treated intravenously with etoposide 100 mg/m^2 on days 1 through 3 and cisplatin 80 mg/m^2 on day 1 with concurrent twice-daily TRT (1.5 Gy per fraction, a total dose of 45 Gy) beginning on day 2 followed by three cycles of irinotecan 60 mg/m^2 on days 1, 8, and 15 and cisplatin 60 mg/m^2 on day 1 of a 4-week cycle.

Results
Of the 51 patients enrolled, 49 patients were assessable for response and toxicity. The overall response rate and complete response rate were 88% and 41%, respectively. The median survival time for all patients was 23 months. The 2-year and 3-year survival rates were 49% and 29.7%, respectively. The median progression-free survival was 11.8 months. The major toxicities observed were neutropenia (grade 4, 84%), febrile neutropenia (grade 3, 31%), infection (grade 3 to 4, 33%), electrolytes imbalance (grade 3 to 4, 20%), and diarrhea (grade 3 to 4, 14%).

Conclusion
EP with concurrent twice-daily TRT followed by the consolidation of IP appears to be an active regimen which deserves further phase III testing in patients with LD-SCLC.

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INTRODUCTION

Small-cell lung cancer (SCLC), which accounts for approximately 15% of all lung cancer cases, is clinically categorized as the two stages, limited disease and extensive disease. Two meta-analyses have shown the combined modality of chemotherapy and thoracic radiotherapy (TRT) to improve the survival of patients with limited-disease (LD-) SCLC in comparison to chemotherapy alone.[1,2] The schedule, dose, and fractionation of TRT have previously been examined in patients with LD-SCLC in several randomized controlled studies.[3-7] On the basis of the results of these studies, etoposide and cisplatin (EP) with concurrent twice-daily TRT is currently a standard care for the treatment for LD-SCLC. However, the 5-year survival rate is less than 30%, and most patients experience a relapse of the primary tumor or distant metastasis.[3-6] To further improve the therapeutic efficacy, one approach is to develop a new chemoradiotherapy regimen incorporating with a novel active agent.

Irinotecan hydrochloride, a camptothecin derivative, is among the most active chemotherapeutic agents against SCLC with a response rate of 37% as a single agent.[8] A randomized phase III study revealed that irinotecan and cisplatin (IP) was superior to EP in patients with extensive-disease SCLC (ED-SCLC).[9] However, the role of IP in the treatment of LD-SCLC remains to be defined. To clarify the role of this combination regimen in LD-SCLC, we initially conducted a randomized phase II study to

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compare two consolidation chemotherapy regimens, IP versus irinotecan, cisplatin and etoposide (IPE), after EP with concurrent twice-daily TRT in LD-SCLC.[10] However, EP with concurrent twice-daily TRT followed by IPE was not feasible because of unacceptable toxicity including grade 4 neutropenia (92%), grade 4 diarrhea (25%), grade 4 infection (25%) and one treatment-related death. We therefore amended the protocol to evaluate EP with concurrent twice-daily TRT followed by consolidation therapy with IP in a single-arm phase II study and herein report the results of this study.

PATIENTS AND METHODS

Eligibility Criteria

Patients with histologically or cytologically confirmed LD-SCLC (stage I disease was excluded) were eligible for this study. A limited stage was defined as disease confined to one hemithorax, the mediastinum, and the bilateral supraclavicular area. Cases with a small amount of pleural effusion and a negative cytology were included in the limited-stage group. Other eligibility criteria included the following: no prior chemotherapy or radiotherapy; measurable disease; Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2; age between 20 and 70 years; life expectancy of at least 3 months; adequate baseline organ function defined as leukocyte count ranging from 4,000 to 12,000/mm^3, hemoglobin concentration of at least 9.5 g/dL, platelet count at least 100,000/mm^3, AST and ALT 2.0× the upper limit of the normal range (ULN) or less, serum total bilirubin 1.5 mg/dL or less, serum creatinine ULN or less, 24-hour creatinine clearance of at least 60 mL/min, and Pao_2 at rest of at least 70 mmHg. The radiation portal should be equal or less than half of one lung.

The patients were ineligible if they had the following criteria: interstitial pneumonitis or pulmonary fibrosis; other respiratory diseases that precluded TRT; malignant pleural effusion or malignant pericardial effusion; active concomitant or a recent (< 3 years) history of any malignancy; uncontrolled angina pectoris, myocardial infarction less than 3 months before the enrollment or congestive heart failure; uncontrolled diabetes mellitus or hypertension; severe infection; intestinal paralysis or obstruction; pregnancy or lactation; or other serious concomitant medical conditions. The study protocol was approved by each institutional review board for clinical use. All patients gave their written informed consent before enrollment.

Study Evaluation

The pretreatment baseline evaluation included a complete medical history and physical examination, a CBC, blood chemistry studies, flexible bronchoscopy, electrocardiography, chest radiography, computed tomography of the chest, computed tomography or ultrasound study of the abdomen, computed tomography or magnetic resonance imaging of the brain, bone scintigraphy and bone marrow aspiration with or without biopsy. A CBC and blood chemistry studies were repeated every week. At the end of the study, all of these studies except for flexible bronchoscopy and bone marrow aspiration were repeated unless the patient had stable or progressive disease.

Treatment Schedule

The patients initially received induction chemoradiotherapy consisting of etoposide 100 mg/m^2 on day 1 through 3 and cisplatin 80 mg/m^2 on day 1 with concurrent twice-daily TRT. After the induction chemoradiotherapy, the patients received three cycles of consolidation chemotherapy consisting of irinotecan 60 mg/m^2 on days 1, 8, and 15 and cisplatin 60 mg/m^2 on days 1. Consolidation chemotherapy was repeated every 4 weeks for three cycles.

The first cycle of consolidation chemotherapy was begun 4 week after the initiation of induction chemoradiotherapy if the leukocyte count was at least 4,000/mm^3; the platelet count was at least 100,000/mm^3; AST and ALT 2.0× ULN or less; serum bilirubin 1.5 mg/dL or less; serum creatinine of ULN or less; the patient did not have fever (≥ 38°C), diarrhea within the past 24 hours, or intestinal paralysis or obstruction; and Pao_2 of at least 70 mmHg. The subsequent cycle of consolidation chemotherapy was repeated if the leukocyte count was at least 3,500/mm^3; the platelet count was at least 100,000/mm^3; AST and ALT 2.0× ULN or less; serum bilirubin 1.5 mg/dL or less; serum creatinine ULN or less; the patient did not have fever (≥ 38°C), diarrhea within the past 24 hours, or intestinal paralysis or obstruction. The use of granulocyte colony-stimulating factor (GCSF) was recommended after day 4. However, its administration was withheld on the day of administration of irinotecan.

TRT was performed with 6 MV or higher photons from a linear accelerator and began on day 2 of the induction chemoradiotherapy. Patients received 1.5 Gy per fraction twice daily with at least a 4-hour interval (preferably a 6-hour interval or more) between each fraction over a 3-week period (a total dose of 45 Gy). A radiation field included the primary tumor, the bilateral mediastinal and ipsilateral hilar lymph nodes with a margin of 1.5 to 2.0 cm. Radiation to the supraclavicular lymph nodes was administered only if they were involved. The inferior border extended 5 cm below the carina or to a level including ipsilateral hilar structures, whichever was lower. After initial irradiation with a dose of 30 Gy, off-cord (ie, the spinal cord was outside the field) oblique boost fields were used. The radiation field in the afternoon was not different from that in the morning. Computed tomography planning was not required and lung density corrections were not performed. Prophylactic cranial irradiation (PCI) was administered to the patients achieving complete response or good partial response with a total dose of 25 Gy in 10 fractions.

Dose Modification

Dose modification based on the toxicity of the induction chemoradiotherapy was not allowed at the time of the first administration of IP. In each cycle of IP, irinotecan on day 8 or 15 was withheld if a leukocyte count of less than 2,000/mm^3 or a platelet count of less than 50,000/mm^3 was determined, or if a patient had fever (≥ 38°C) or grade 2 or higher hepatotoxicity or any diarrhea within the last 24 hours or intestinal paralysis or obstruction. In the second and the third cycle of consolidation chemotherapy, the dose modification was made as follows. If a leukocyte nadir count of less than 1,000/mm^3 or a neutrophil nadir count of less than 500/mm^3 for 3 or more days or if febrile neutropenia developed or if a platelet nadir count of less than 25,000/mm^3 was observed or if grade 2 hepatotoxicity or diarrhea was observed, irinotecan was decreased by 10 mg/m^2 in the subsequent cycle, if grade 2 or lower renal toxicity was observed during the previous course of treatment, only cisplatin decreased by 25%, if grade 3 or higher nonhematologic toxicity (excluding nausea, vomiting, and hair loss) developed, then cisplatin decreased by 25% and irinotecan decreased by 10 mg/m^2 in the following cycle. The patients were removed from the study if the following toxicities were observed: grade 4 diarrhea; grade 3 or higher renal toxicity or creatinine of at least 2.0 mg/dL; grade 3 or higher hepatotoxicity; grade 2 or higher pulmonary toxicity or Pao_2 at rest less than 60 mmHg.

Evaluation

The Response Evaluation Criteria in Solid Tumors (RECIST) were used for the response assessment.[11] Toxicity was evaluated according to the National Cancer Institute–Common Toxicity Criteria (version 2.0). An extramural review was conducted to validate the eligibility of the patients, staging, and response.

Statistical Analysis

The primary end point of this study was the 2-year survival rate. We calculated the sample size based on Fleming's single-stage design of the phase II study.[12] We set a 2-year survival rate of 35% as a baseline survival rate and 20% as the high level of interest with a power of 0.9 at a one-sided significance level of .05, requiring an accrual of 53 eligible patients. The study was initially begun as a randomized phase II study to compare two consolidation arms, namely IP versus IPE after concurrent chemoradiotherapy. Because of the unacceptable toxicity in the triplet regimen, the study was modified to a single-arm phase II study to evaluate IP after EP with concurrent TRT and 11 patients in the IP arm were included in the analysis of this study.

The duration of survival was measured from the day of entry onto the study, and the overall survival curve and progression-free survival curve were calculated according to the method of Kaplan and Meier.[13]

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RESULTS

Patients Characteristics

Between February 2000 and November 2002, 51 patients were enrolled onto this study. Table 1 lists the baseline characteristics of the patients. Two patients were considered to be ineligible because a secondary primary tumor was found after the administration of EP with concurrent TRT. Therefore, 49 patients were assessable for response and toxicity.

Treatment Administration

Seven patients were removed from the study after the administration of EP with concurrent TRT because of treatment delay due to toxicity (six patients) and patient rejection (one patient). Eight patients each discontinued the treatment after each cycle of IP. The major reasons for the discontinuation of IP included treatment delay due to toxicity (three patients), diarrhea (three patients), and ileus (three patients), patient rejection (two patients), and the doctor's judgment (two patients). Overall, 34 patients (69%) received at least two cycles of IP and 26 patients (53%) completed the entire treatment. Irinotecan was omitted in 35 (11%) of 306 cycles. The dose-intensity of irinotecan was 30.5 mg/m^2/wk (68% of the planned dose) and cisplatin 11.6 mg/m^2/wk (77% of the planned dose) in the consolidation chemotherapy.

Response and Survival

On an intention-to-treat basis, the overall response rates and the complete response rates were 88% (95% CI, 78.6% to 96.9%) and 41%, respectively. After a median follow-up of 29.9 months, the median survival time for all patients was 23 months (Fig 1). The 2-year and 3-year survival rates were 49% and 29.7%, respectively. The median progression-free survival was 11.8 months (Fig 2).

Toxicity

Tables 2 and 3 show the major toxicities. Grade 4 neutropenia was observed in 80% of the patients and 10 (20%) patients had febrile neutropenia in concurrent chemoradiotherapy, whereas grade 4 neutropenia was observed in 40% of the patients and seven patients (17%) had febrile neutropenia in consolidation chemotherapy. In contrast, anemia and thrombocytopenia were relatively mild. One patient had grade 4 esophagitis in concurrent chemoradiotherapy. In the consolidation chemotherapy, grade 3 or 4 diarrhea was observed in six patients (14%) and grade 3 or 4 infection was observed in seven patients (17%). Two patients had grade 3 or 4 radiation pneumonitis. Grade 4 adhesive ileus developed in a patient who had a history of abdominal surgery and ileus. The major toxicities observed through the entire course of the treatment were neutropenia (grade 4, 84%), febrile neutropenia (grade 3, 31%), infection (grade 3 to 4, 33%), electrolytes imbalance (grade 3 to 4, 20%) and diarrhea (grade 3 to 4, 14%). There was one treatment-related death caused by radiation pneumonitis.

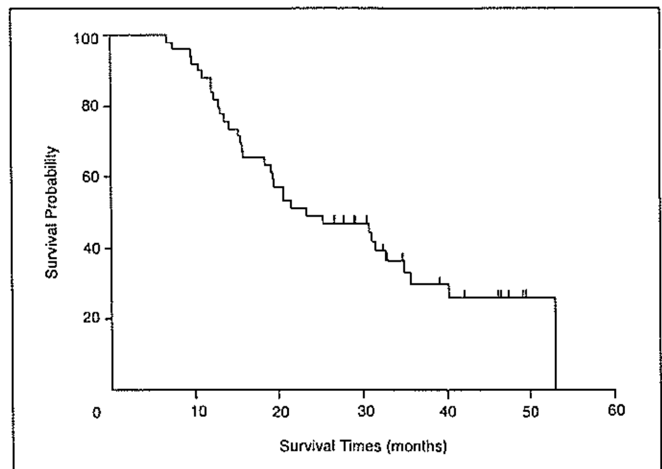

Fig 1. Kaplan-Meier survival curve of 49 eligible patients with limited-disease small-cell lung cancer. The median survival time was 23 months, and the 2-year and 3-year survival rates were 49% and 29.7%, respectively.

Patterns of Relapse

Table 4 lists first sites of relapse. Of 12 patients (24%) with local relapse (defined as relapse within the radiation portal), only one had a relapse solely at locoregional sites and 11 at both local and distant site including three with brain metastasis. Of 27 patients (55%) with distant relapse only, 13 had brain metastasis. Overall, 16 patients (33%) showed brain metastasis as the initial site of relapse, and eight of them had received PCI.

Table 1. Patient Characteristics (N = 51)

Characteristic	No.	%
Age, years		
Median	62	
Range	45-70	
Sex		
Male	42	82
Female	9	18
ECOG performance status		
0	22	43
1	28	55
2	1	2
Stage		
II	2	4
IIIA	35	69
IIIB	14	27

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

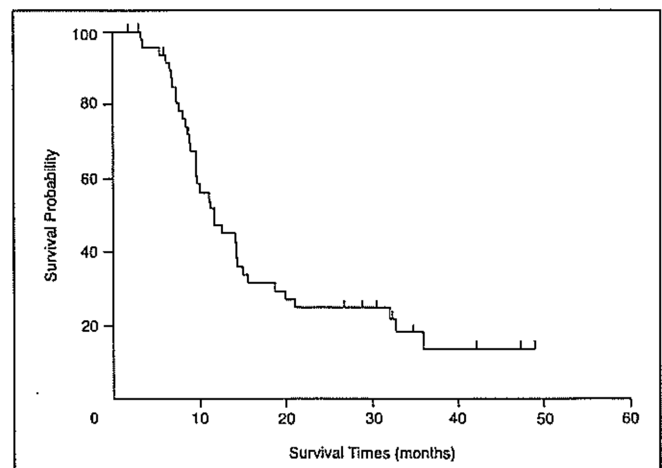

Fig 2. Kaplan-Meier progression-free survival curve of 49 eligible patients with limited-disease small-cell lung cancer. The median progression-free survival time was 11.8 months.

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Table 2. Major Toxicities During Concurrent Chemoradiotherapy (n = 49)

Toxicity	Grade 3		Grade 4	
	No.	%	No.	%
Hematologic				
Leukopenia	27	55	19	39
Neutropenia	8	16	39	80
Anemia	2	4	1	2
Thrombocytopenia	10	20	0	0
Febrile neutropenia	10	20	0	0
Nonhematologic				
Nausea/vomiting	7	14	0	0
Diarrhea	0	0	0	0
Constipation	0	0	0	0
Infection	9	18	0	0
Mucositis	0	0	0	0
Esophagitis	0	0	1	2
Dyspnea	1	2	0	0
Pneumonitis	0	0	0	0
Hepatic	0	0	0	0
Electrolytes	2	4	2	4

DISCUSSION

In this phase II study, we evaluated the consolidation of IP after EP with concurrent twice-daily TRT and thus achieved an overall response rate of 88%, a 2-year-survival rate of 49% and a 3-year-survival rate of 29.7%. Although the number of assessable patients was slightly smaller than the planned sample size, this study confirmed 24 2-year survivors, and the power calculation showed a 97% probability to correctly reject inactive treatment, thus yielding only a 35% or less 2-year-survival rate. These results are comparable to those in phase III studies evaluating EP with concurrent twice-daily TRT.[3-6] Jeremic et al[7] reported a better survival outcome by using daily carboplatin and etoposide with concurrent twice-daily TRT followed by EP. However, this result has rarely been confirmed by other groups. The Japanese Clinical Oncology Group (JCOG) conducted a pilot study to evaluate the feasibility of IP after EP with concurrent TRT (JCOG9903).[14] The doses and schedule of cisplatin, etoposide, and irinotecan and dose, fractionation and schedule of TRT were similar to ours. They reported that this regimen was feasible with a response rate of 97%, a 2-year survival rate of 41% and a 3-year survival rate of 38%, which are similar to those in our study. Although a phase III study conducted in Japan showed the superiority of IP over EP in ED-SCLC,[9] another phase III study conducted in North America failed to confirm the superiority of IP over EP.[15] A randomized phase III study to compare IP versus EP after EP with concurrent TRT is currently ongoing in patients with LD-SCLC in Japan.

Table 3. Major Toxicities During Consolidation Chemotherapy (n = 42)

Toxicity	Grade 3		Grade 4	
	No.	%	No.	%
Hematologic				
Leukopenia	27	64	8	19
Neutropenia	18	43	17	40
Anemia	17	40	5	12
Thrombocytopenia	8	19	0	0
Febrile neutropenia	7	17	0	0
Nonhematologic				
Nausea/vomiting	9	21	0	0
Diarrhea	5	12	1	2
Constipation	3	7	2	5
Ileus	2	5	1	2
Infection	9	21	1	2
Mucositis	0	0	0	0
Esophagitis	0	0	0	0
Dyspnea	2	5	0	0
Pneumonitis	1	2	1	2
Hepatic	1	2	0	0
Electrolytes	4	10	1	2

Table 4. Site of First Failure (n = 49)

Site	No. of Patients	%
Progression free	10	20
Locoregional	1	2
Locoregional and distant	11	22
Distant	27	55
Brain only	8	16
Brain and others	5	10
Others	14	29

Although a potential approach is to substitute irinotecan for etoposide in the combination of EP with concurrent TRT, we did not combine IP concurrently with TRT because two phase I studies demonstrated that combining IP with concurrent TRT was not feasible when the full dose of irinotecan was administered on days 1, 8, and 15.[16,17] On the basis of these results, we administered IP as consolidation therapy after EP with concurrent twice-daily TRT. After this article was initially submitted, Langer et al[18] reported phase I study of once every 3 weeks scheduling of IP with concurrent twice-daily TRT (45 Gy) or once-daily TRT (70 Gy) in patients with LD-SCLC, thus concluding that IP with concurrent twice-daily TRT was safe and feasible. A further evaluation of this regimen is thus warranted.

One group evaluated IP administered as an induction followed by EP with concurrent twice-daily TRT.[19] Their results are comparable to those of our study and EP with concurrent twice-daily TRT.[3-6] However, this regimen was highly myelotoxic (grade 4 neutropenia, 91%) with febrile neutropenia in 60% of the patients. Furthermore, early TRT is an important issue to obtain the improved outcome in LD-SCLC. Recent meta-analyses revealed that when platinum-based chemotherapy was concurrent with TRT in LD-SCLC, an improved survival was associated with early TRT.[20-22] Another group evaluated the addition of paclitaxel to EP with concurrent TRT.[23] Although their results are comparable to those of our study and EP with concurrent twice-daily TRT,[3-6] they concluded that the triplet regimen would not further improve the survival outcome in patients with LD-SCLC.

Esophagitis is a toxicity of a particular concern in concurrent chemoradiotherapy. We observed grade 3 or 4 esophagitis in one patient (2%), whereas the JCOG9903 trial reported it in 7% of the patients. These figures contrast with those in the studies evaluating etoposide and a platinum with concurrent twice-daily TRT (9% to 32%).[3-7] The substitution of irinotecan for etoposide may reduce the incidence of grade 3 or 4 esophagitis. Furthermore, a lower incidence of esophagitis has been noted in a Japanese trial.[4] A possible explanation for this includes differences in the

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