are already established. This proposal is justified only on the basis of evidence-based medicine. In this situation, positive indicators for the effectiveness of PAC with uracil–tegafur are needed. However, in this study, we were unable to detect novel biomarkers for selection of good responders. The two protein molecules detected in this proteomic analysis were biomarkers indicative of good prognosis.

The ultimate purpose of clinical proteomics is to improve diagnostic procedures including the exact evaluation of biological characteristics of tumour cells and to understand the molecular pathogenesis of cancers to devise novel therapeutic strategies. We believe that proteomic analysis will become an integral tool for

investigation of tumour biology. We conclude that negative expression of both myosin IIA and vimentin is an indicator of good prognosis for stage I lung adenocarcinoma without the need for PAC.

ACKNOWLEDGEMENTS

REFERENCES

Arias AM (2001) Epithelial mesenchymal interactions in cancer and development. *Cell* 105: 425–431

Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, Vansteenkiste J (2004) Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. *N Engl J Med* 350: 351–360

Barbareschi M, Girlanda S, Mauri MF, Forti S, Eccher C, Nauri FA, Togni R, Dalla Palma P, Doglioni C (1994) Quantitative growth fraction evaluation with MIB1and Ki67 antibodies in breast carcinomas. *Am J Clin Pathol* 102: 171–175

Blanco D, Vicent S, Elizegi E, Pino I, Fraga MF, Esteller M, Saffiotti U, Lecanda F, Montuenga LM (2004) Altered expression of adhesion molecules and epithelial-mesenchymal transition in silica-induced rat lung carcinogenesis. *Lab Invest* 84: 999–1012

Davies BR, Davies MP, Gibbs FE, Barraclough R, Rudland PS (1993) Induction of the metastatic phenotype by transfection of a benign rat mammary epithelial cell line with the gene for p9Ka, a rat calcium-binding protein, but not with the oncogene EJ-ras-1. *Oncogene* 8: 999–1008

Fujii S, Kitano S, Ikenaka K, Shirasaka T (1979) Effect of coadministration of uracil or cytosine on the anti-tumor activity of clinical doses of 1-(2-tetrahydrofuryl)-5 fluorouracil and level of 5 – fluorouracil in rodents. *Gann* 70: 209–214

Garrett SC, Varney KM, Weber DJ, Bresnick AR (2006) S100A4, a mediator of metastasis. *J Biol Chem* 281: 677–680

Gilles C, Polette M, Piette J, Delvigne AC, Thompson EW, Foidart JM, Birembaut P (1996) Vimentin expression in cervical carcinomas: association with invasive and migratory potential. *J Pathol* 180: 175–180

Hamada C, Tanaka F, Ohta M, Fujimura S, Kodama K, Imaizumi M, Wada H (2005) Meta-analysis of postoperative adjuvant chemotherapy with tegafur-uracil in non-small-cell lung cancer. *J Clin Oncol* 23: 4999–5006

Hendrix MJ, Seftor EA, Seftor RE, Trevor KT (1997) Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior. *Am J Pathol* 150: 483–495

Ho DH, Pazdur R, Covington W, Brown N, Huo YY, Lassere Y, Kuritani J (1998) Comparison of 5-fluorouracil pharmacokinetics in patients receiving continuous 5 -fluorouracil infusion and oral uracil plus N1-(2'-tetrahydrofuryl)-5-fluorouracil. *Clin Cancer Res* 4: 2085–2088

Ikenaka K, Shirasaka T, Kitano S, Fujii S (1979) Effect of uracil on metabolism of 5-fluorouracil *in vitro*. *Gann* 70: 353–359

Islam S, Carey TE, Wolf GT, Wheelock MJ, Johnson KR (1996) Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell–cell adhesion. *J Cell Biol* 135: 1643–1654

Kato H, Ichinose Y, Ohta M, Hata E, Tsubota N, Tada H, Watanabe Y, Wada H, Tsuboi M, Hamajima N, Ohta M (2004) A randomized trial of adjuvant chemotherapy with uracil-tegafur for adenocarcinoma of the lung. *N Engl J Med* 350: 1713–1721

Kawakami T, Tateishi K, Yamano Y, Ishikawa T, Kuroki K, Nishimura T (2005) Protein identification from product ion spectra of peptides validated by correlation between measured and predicted elution times in liquid chromatography/mass spectrometry. *Proteomics* 5: 856–864

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275

Minamiya Y, Nakagawa T, Saito H, Matsuzaki I, Taguchi K, Ito M, Ogawa J (2005) Increased expression of myosin light chain kinase mRNA is related to metastasis in non-small cell lung cancer. *Tumour Biol* 26: 153–157

Non-Small Cell Lung Cancer Collaborative Group (1995) Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomized clinical trials. *BMJ* 311: 899–909

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR (2003) Cell migration: integrating signals from front to back. *Science* 302: 1704–1709

Scagliotti GV, Fossati R, Torri V, Crino L, Giaccone G, Silvano G, Martelli M, Clerici M, Cognetti F, Tonato M (2003) Randomized study of adjuvant chemotherapy for completely resected stage I, II, or IIIA non-small-cell lung cancer. *J Nat Cancer Inst* 95: 1453–1461

Schwartz JC, Senko MW, Syka JE (2002) A two-dimensional quadrupole ion trap mass spectrometer. *J Am Soc Mass Spectrom* 13: 659–669

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 68: 850–858

Strauss GM, Herndon J, Maddaus MA, Johnstone DW, Johnson EA, Watson DM, Sugarbaker DJ, Schilsky RL, Green MR (2004) Randomized clinical trial of adjuvant chemotherapy with paclitaxel and carboplatin following resection in stage IB non-small cell lung cancer (NSCLC): report of cancer and leukaemia Group B (CALGB) Protocol 9633. *Proc Am Soc Clin Oncol* 23: 621

Thiery JP (2002) Epithelial–mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2: 442–454

Winton T, Livingston R, Johnson D, Rigas J, Johnston M, Butts C, Cormier Y, Goss G, Inculet R, Vallieres E, Fry W, Bethune D, Ayoub J, Ding K, Seymour L, Graham B, Tsao MS, Gandara D, Kesler K, Demmy T, Shepherd F (2005) Vinorelbine plus cisplatin vs observation in resected non-small-cell lung cancer. *N Engl J Med* 352: 2589–2597

1196

Short Communication

Randomised phase II trial of irinotecan plus cisplatin vs irinotecan, cisplatin plus etoposide repeated every 3 weeks in patients with extensive-disease small-cell lung cancer

I Sekine[*,1], H Nokihara[1], K Takeda[2], Y Nishiwaki[3], K Nakagawa[4], H Isobe[5], K Mori[6], K Matsui[7], N Saijo[3] and T Tamura[1]

[1]Division of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tokyo, Japan; [2]Department of Clinical Oncology, Osaka City General Hospital, Osaka, Japan; [3]Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Japan; [4]Department of Medical Oncology, Kinki University School of Medicine, Sayama, Japan; [5]Department of Pulmonary Disease, National Hospital Organization Hokkaido Cancer Center, Sapporo, Japan; [6]Department of Thoracic Diseases, Tochigi Prefectural Cancer Center, Utsunomiya, Japan; [7]Department of Internal Medicine, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Habikino, Japan

Patients with previously untreated extensive-disease small-cell lung cancer were treated with irinotecan $60\,mg\,m^{-2}$ on days 1 and 8 and cisplatin $60\,mg\,m^{-2}$ on day 1 with ($n = 55$) or without ($n = 54$) etoposide $50\,mg\,m^{-2}$ on days 1–3 with granulocyte colony-stimulating factor support repeated every 3 weeks for four cycles. The triplet regimen was too toxic to be considered for further studies.

British Journal of Cancer advance online publication, 5 February 2008; doi:10.1038/sj.bjc.6604233 www.bjcancer.com
© 2008 Cancer Research UK

Small-cell lung cancer (SCLC), which accounts for approximately 14% of all malignant pulmonary tumours, is an aggressive malignancy with a propensity for rapid growth and early widespread metastases (Jackman and Johnson, 2005). A combination of cisplatin and etoposide (PE) has been the standard treatment, with response rates ranging from 60 to 90% and median survival times (MSTs) from 8 to 11 months in patients with extensive disease (ED)-SCLC (Fukuoka et al, 1991; Roth et al, 1992). A combination of irinotecan and cisplatin (IP) showed a significant survival benefit over the PE regimen (MST: 12.8 vs 9.4 months, $P = 0.002$) in a Japanese phase III trial for ED-SCLC (Noda et al, 2002), although another phase III trial comparing these regimens failed to show such a benefit (Hanna et al, 2006). Thus, irinotecan, cisplatin and etoposide are the current key agents in the treatment of SCLC. A phase II trial of the three agents, IPE combination, in patients with ED-SCLC showed a promising antitumour activity with a response rate of 77%, complete response (CR) rate of 17% and MST of 12.9 months (Sekine et al, 2003).

We have developed these IP and IPE regimens in a 4-week schedule where irinotecan was given on days 1, 8 and 15. The dose of irinotecan on day 15, however, was frequently omitted because of toxicity in both regimens (Noda et al, 2002; Sekine et al, 2003).

The objectives of this study were to evaluate the toxicities and antitumour effects of IP and IPE regimens in the 3-week schedule in patients with ED-SCLC and to select the right arm for subsequent phase III trials.

PATIENTS AND METHODS

Patient selection

Patients were enrolled in this study if they met the following criteria: (1) a histological or cytological diagnosis of SCLC; (2) no prior treatment; (3) measurable disease; (4) ED, defined as having distant metastasis or contralateral hilar lymph node metastasis; (5) performance status of 0–2 on the Eastern Cooperative Oncology Group (ECOG) scale; (6) predicted life expectancy of 3 months or longer; (7) age between 20 and 70 years; (8) adequate organ function as documented by a white blood cell (WBC) count $\geqslant 4.0 \times 10^3\,\mu l^{-1}$, neutrophil count $\geqslant 2.0 \times 10^3\,\mu l^{-1}$, haemoglobin $\geqslant 9.5\,g\,dl^{-1}$, platelet count $\geqslant 100 \times 10^3\,\mu l^{-1}$, total serum bilirubin $\leqslant 1.5\,mg\,dl^{-1}$, hepatic transaminases $\leqslant 100\,IU\,l^{-1}$, serum creatinine $\leqslant 1.2\,mg\,dl^{-1}$, creatinine clearance $\geqslant 60\,ml\,min^{-1}$, and $PaO_2 \geqslant 60$ torr; and (9) providing written informed consent.

Patients were not eligible for the study if they had any of the following: (1) uncontrollable pleural, pericardial effusion or ascites; (2) symptomatic brain metastasis; (3) active infection; (4) contraindications for the use of irinotecan, including diarrhoea, ileus, interstitial pneumonitis and lung fibrosis; (5) synchronous active malignancies; (6) serious concomitant medical

*Correspondence: Dr I Sekine, Division of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan; E-mail: isekine@ncc.go.jp

illness, including severe heart disease, uncontrollable diabetes mellitus or hypertension; or (7) pregnancy or breast feeding.

Treatment schedule

In the IP arm, cisplatin, $60\,mg\,m^{-2}$, was administered intravenously over 60 min on day 1 and irinotecan, $60\,mg\,m^{-2}$, was administered intravenously over 90 min on days 1 and 8. Prophylactic granulocyte colony-stimulating factor (G-CSF) was not administered in this arm. In the IPE arm, cisplatin and irinotecan were administered at the same dose and schedule as the IP arm. In addition, etoposide, $50\,mg\,m^{-2}$, was administered intravenously over 60 min on days 1-3. Filgrastim $50\,\mu g\,m^{-2}$ or lenograstim $2\,\mu g\,kg^{-1}$ was subcutaneously injected prophylactically from day 5 to the day when the WBC count exceeded $10.0 \times 10^3\,\mu l^{-1}$. Hydration (2500 ml) and a $5HT_3$ antagonist were given on day 1, followed by an additional infusion if indicated in both arms. These treatments were repeated every 3 weeks for a total of four cycles.

Toxicity assessment, treatment modification and response evaluation

Toxicity was graded according to the NCI Common Toxicity Criteria version 2.0.

Doses of anticancer agents in the following cycles were modified according to toxicity in the same manner in both arms. Objective tumour response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse et al, 2000).

Study design, data management and statistical considerations

This study was designed as a multi-institutional, prospective randomised phase II trial. This study was registered on 6 September 2005 in the University hospital Medical Information Network (UMIN) Clinical Trials Registry in Japan (http://www.umin.ac.jp/ctr/index.htm), which is acceptable to the International Committee of Medical Journal Editors (ICMJE) (http://www.icmje.org/faq.pdf). The protocol and consent form were approved by the Institutional Review Board of each institution. Patient registration and randomisation were conducted at the Registration Center. No stratification for randomisation was performed in this study. The sample size was calculated according to the selection design for pilot studies based on survival (Liu et al, 1993). Assuming that (1) the survival curve was exponential for survivals; (2) the MST of the worse arm was 12 months and that of the better arm was 12 months × 1.4; (3) the correct selection probability was 90%; and (4) additional follow-up in years after the end of accrual was 1 year, the estimated required number of patients was 51 for each arm. Accordingly, 55 patients for each arm and their accrual period of 24 months were planned for this study.

The dose intensity of each drug was calculated for each patient using the following formula as previously described:

The dose intensity $(mg\,m^{-2}\,week^{-1})$

$$= \frac{\text{Total milligrams of a drug in all cycles per body surface area}}{\text{Total days of therapy}/7}$$

where total days of therapy is the number of days from day 1 of cycle 1 to day 1 of the last cycle plus 21 days for both arms (Hryniuk and Goodyear, 1990).

Differences in the reason for termination of the treatment and the frequencies of grade 3-4 toxicities were assessed by χ tests. Survival was measured as the date of randomisation to the date of death from any cause or the date of the most recent follow-up for overall survival and to the date of disease progression or the date

of death for progression-free survival (PFS). The survival of the arms was estimated by the Kaplan-Meier method and compared in an exploratory manner with log-rank tests (Armitage et al, 2002).

RESULTS

Patient characteristics

From March 2003 to May 2005, 55 patients were randomised to IP and 55 patients to IPE. One patient in the IP arm was excluded because the patient was ineligible and did not receive the study treatment. The remaining 109 patients were included in the analyses of toxicity, tumour response and patient survival. There were no differences between the two arms in any demographic characteristics listed (Table 1).

Treatment delivery

Treatment was well tolerated with respect to the number of cycles delivered in both arms (Table 2). Among reasons for termination of the treatment, disease progression was noted in nine (17%)

Table 1 Patient characteristics

	IP (n = 54)	IPE (n = 55)
Sex		
Female	11	8
Male	43	47
Age (years)		
Median (range)	63 (42-70)	62 (48-70)
PS		
0	11	12
1	42	41
2	1	2
Weight loss		
0-4%	38	43
5-9%	10	10
⩾ 10%	6	2

Table 2 Treatment delivery

	IP (n = 54) No. (%)	IPE (n = 55) No. (%)
Number of cycles delivered		
6ᵃ	—	1 (2)
4	41 (76)	36 (65)
3	6 (11)	6 (11)
2	3 (6)	6 (11)
1	4 (7)	6 (11)
Reasons for termination of the treatment[b]		
Completion	40 (74)	35 (64)
Disease progression	9 (17)	2 (4)
Toxicity	3 (6)	13 (24)
Patient refusal	2 (4)	4 (7)
Others	0 (0)	1 (2)
Total number of cycles delivered	192 (100)	186 (100)
Total number of omission on day 8	35 (18)	37 (17)
Total number of cycles with dose reduction	28 (15)	31 (17)

[b] $P = 0.013$ by χ^2 test. ᵃProtocol violation.

patients in the IP arm and in two (4%) patients in the IPE arm, whereas toxicity was noted in three (6%) patients in the IP arm and 13 (24%) patients in the IPE arm ($P = 0.013$) (Table 2). The dose of irinotecan on day 8 was omitted in 35 (18%) cycles in the IP arm and 37 (17%) cycles in the IPE arm (Table 2). The total dose and dose intensity of cisplatin and etoposide were similar between the IP and IPE arms in the present study (Table 3).

Toxicity

The myelotoxicity was more severe in the IPE arm (Table 4). Grade 3 febrile neutropaenia was noted in 5 (9%) patients in the IP arm and 17 (31%) patients in the IPE arm ($P = 0.005$). Packed red blood

Table 3 Total dose and dose intensity

	3-week regimens in this study		4-week regimen[a]
	IP (n = 54) Median (range)	IPE (n = 55) Median (range)	IPE (n = 30) Median (range)
Total dose (mg m⁻²)			
Cisplatin	240 (60–240)	240 (60–360)	240 (60–240)
Irinotecan	420 (60–480)	390 (60–720)	563 (60–720)
Etoposide	0	600 (150–900)	600 (150–600)
Dose intensity (mg m⁻² week⁻¹)			
Cisplatin	19 (14–25)	20 (16–34)	15 (12–15)
Irinotecan	33 (14–40)	35 (15–55)	35 (19–45)
Etoposide	0	48 (34–68)	37 (28–38)

[a]From our previous study (Sekine et al. 2003).

Table 4 Grade 3–4 toxicities

	IP (n = 54)			IPE (n = 55)		
	Grade 3	4	3+4 (%)	Grade 3	4	3+4 (%)
Leukocytopaenia	9	1	10 (19)	18	11	29 (53)ᵃ
Neutropaenia	17	11	28 (52)	24	28	52 (95)ᵃ
Anaemia	18	0	18 (25)	16	9	25 (45)
Thrombocytopaenia	2	0	2 (4)	13	0	13 (13)ᵇ
Febrile neutropaenia	5	0	5 (9)	17	0	7 (13)
Diarrhoea	8	0	8 (15)	11	2	13 (24)
Vomiting	4	0	4 (7)	3	0	3 (5)
Fatigue	1	0	1 (2)	5	1	6 (11)ᶜ
Hyponatraemia	9	3	12 (22)	11	2	13 (24)
AST elevation	0	0	0 (0)	3	0	3 (5)
CRN elevation	1	0	1 (2)	0	0	0 (0)

ᵃ$P < 0.001$; ᵇ$P < 0.01$; and ᶜ$P = 0.054$ by χ^2 test.

cells were transfused in 4 (7%) patients in the IP regimen and 14 (26%) patients in the IPE regimen ($P = 0.011$). Platelet concentrates were needed in none in the IP regimen and 2 (4%) patients in the IPE regimen ($P = 0.16$). Grade 3–4 diarrhoea was observed in 8 (15%) patients in the IP arm and 13 (24%) patients in the IPE arm ($P = 0.262$). Grade 3–4 fatigue was more common in the IPE arm with marginal significance (2 vs 11%, $P = 0.054$). The severity of other non-haematological toxicities did not differ significantly between the arms. No treatment-related death was observed in this study.

Response, treatment after recurrence and survival

Four CRs and 37 partial responses (PRs) were obtained in the IP arm, resulting in the overall response rate of 76 with 95% confidence interval (CI) of 65–87%, whereas six CRs and 42 PRs were obtained in the IPE arm, and the overall response rate was 87% with a 95% CI of 79–96% ($P = 0.126$). Median PFS was 4.8 months (95% CI, 4.0–5.6) in the IP and 5.4 months (95% CI, 4.8–6.0) in the IPE arm ($P = 0.049$) (Figure 1A). After recurrence, 22 (44%) patients in the IP arm and 8 (16%) patients in the IPE arm received etoposide-containing chemotherapy. The MST and 1-year survival rate were 12.4 months (95% CI, 9.7–15.1) and 54.8% (95% CI, 41.4–68.2%) in the IP and 13.7 months (95% CI, 11.9–15.5) and 61.5% (95% CI, 48.6–74.4%) in the IPE arm ($P = 0.52$), respectively (Figure 1B).

DISCUSSION

This study showed that the IPE regimen in a 3-week schedule with CSF support produced a promising response rate, PFS and overall survival. Haematological toxicity in the IPE arm, however, was very severe in spite of the G-CSF support with the grade 3 febrile neutropaenia noted in 31% of patients.

In comparison between the 3-week IPE regimen in this study and the 4-week IPE regimen in the previous study, the delivery of cisplatin and etoposide was improved in the 3-week IPE regimen when compared with the 4-week IPE regimen at the cost of the irinotecan total dose. The response rate and MST were 87% and 13.7 months, respectively, in the 3-week IPE regimen and 77% and 12.9 months in the previous 4-week schedule, and toxicity profiles were comparable to each other (Sekine et al, 2003).

The MST of 12.4 months in the IP arm in this study was comparable to that of the previous phase III study, with an MST of 12.8 months (Noda et al, 2002). Thus, this study showed the reproducible excellent survival outcome of patients with ED-SCLC who were treated with the IP combination. In contrast, a recent American phase III study of the PE regimen vs IP regimen failed to show the superiority of the IP regimen to the PE regimen; the MST
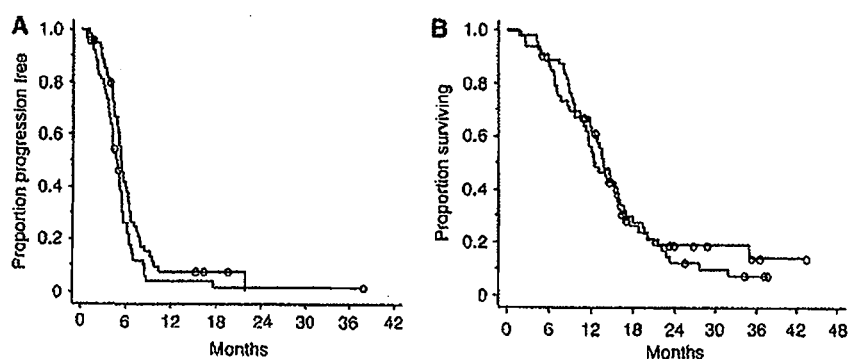

Figure 1 Progression-free survival (A) and overall survival (B). Thick line indicates the IPE regimen and thin line indicates the IP regimen.

4

for the PE regimen was 10.2 months and that for the IP regimen was 9.3 months (Hanna et al, 2006). The discrepancy between the Japanese and American trials may be explained by the different cisplatin dose schedules; cisplatin was delivered at a dose of $60\,mg\,m^{-2}$ on day 1 every 3 or 4 weeks in the Japanese trials, whereas cisplatin was delivered at a dose of $30\,mg\,m^{-2}$ on days 1 and 8 every 3 weeks in the American one. A platinum agent administered at divided doses was associated with poor survival in patients with ED-SCLC in our previous randomised phase II study (Sekine et al, 2003).

The issue of adding further agents to the standard doublet regimen has been investigated in patients with ED-SCLC. The addition of ifosfamide or cyclophosphamide and epirubicin to the cisplatin and etoposide combination produced a slight survival benefit, but at the expense of greater toxicity (Loehrer et al, 1995; Pujol et al, 2001). Phase III trials of cisplatin and etoposide with or without paclitaxel showed unacceptable toxicity with 6–13% toxic deaths in the paclitaxel-containing arm (Mavroudis et al, 2001; Niell et al, 2005). The results in these studies and the current study are consistent in the increased toxicity despite the G-CSF support and no definite survival benefit in the three or four drug combinations over the standard doublet in patients with ED-SCLC.

In conclusion, the IPE regimen was marginally more effective than the IP regimen, but was too toxic despite the administration of prophylactic G-CSF.

ACKNOWLEDGEMENTS

REFERENCES

Armitage P, Berry G, Matthews J (2002) Survival analysis. In *Statistical Methods in Medical Research*, Armitage P, Berry G, Matthews J (eds), pp 568–590. Oxford: Blackwell Science Ltd

Fukuoka M, Furuse K, Saijo N, Nishiwaki Y, Ikegami H, Tamura T, Shimoyama M, Suemasu K (1991) Randomized trial of cyclophosphamide, doxorubicin, and vincristine vs cisplatin and etoposide vs alternation of these regimens in small-cell lung cancer. *J Natl Cancer Inst* 83: 855–861

Hanna N, Bunn Jr PA, Langer C, Einhorn L, Guthrie Jr T, Beck T, Ansari R, Ellis P, Byrne M, Morrison M, Hariharan S, Wang B, Sandler A (2006) Randomized phase III trial comparing irinotecan/cisplatin with etoposide/cisplatin in patients with previously untreated extensive-stage disease small-cell lung cancer. *J Clin Oncol* 24: 2038–2043

Hryniuk WM, Goodyear M (1990) The calculation of received dose intensity. *J Clin Oncol* 8: 1935–1937

Jackman DM, Johnson BE (2005) Small-cell lung cancer. *Lancet* 366: 1385–1396

Liu PY, Dahlberg S, Crowley J (1993) Selection designs for pilot studies based on survival. *Biometrics* 49: 391–398

Loehrer Sr PJ, Ansari R, Gonin R, Monaco F, Fisher W, Sandler A, Einhorn LH (1995) Cisplatin plus etoposide with and without ifosfamide in extensive small-cell lung cancer: a Hoosier Oncology Group study. *J Clin Oncol* 13: 2594–2599

Mavroudis D, Papadakis E, Veslemes M, Tsiafaki X, Stavrakakis J, Kouroussis C, Kakolyris S, Bania E, Jordanoglou J, Agelidou M, Vlachonicolis J, Georgoulias V (2001) A multicenter randomized clinical trial comparing paclitaxel–cisplatin–etoposide vs cisplatin–etoposide as first-line treatment in patients with small-cell lung cancer. *Ann Oncol* 12: 463–470

Niell HB, Herndon II JE, Miller AA, Watson DM, Sandler AB, Kelly K, Marks RS, Perry MC, Ansari RH, Otterson G, Ellerton J, Vokes EE, Green

MR (2005) Randomized phase III intergroup trial of etoposide and cisplatin with or without paclitaxel and granulocyte colony-stimulating factor in patients with extensive-stage small-cell lung cancer: Cancer and Leukemia Group B Trial 9732. *J Clin Oncol* 23: 3752–3759

Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama A, Fukuoka M, Mori K, Watanabe K, Tamura T, Yamamoto S, Saijo N (2002) Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* 346: 85–91

Pujol JL, Daures JP, Riviere A, Quoix E, Westeel V, Quantin X, Breton JL, Lemarie E, Poudenx M, Milleron B, Moro D, Debieuvre D, Le Chevalier T (2001) Etoposide plus cisplatin with or without the combination of 4′-epidoxorubicin plus cyclophosphamide in treatment of extensive small-cell lung cancer: a French Federation of Cancer Institutes multicenter phase III randomized study. *J Natl Cancer Inst* 93: 300–308

Roth BJ, Johnson DH, Einhorn LH, Schacter LP, Cherng NC, Cohen HJ, Crawford J, Randolph JA, Goodlow JL, Broun GO, Omura GA, Greco FA (1992) Randomized study of cyclophosphamide, doxorubicin, and vincristine vs etoposide and cisplatin vs alternation of these two regimens in extensive small-cell lung cancer: a phase III trial of the Southeastern Cancer Study Group. *J Clin Oncol* 10: 282–291

Sekine I, Nishiwaki Y, Noda K, Kudoh S, Fukuoka M, Mori K, Negoro S, Yokoyama A, Matsui K, Ohsaki Y, Nakano T, Saijo N (2003) Randomized phase II study of cisplatin, irinotecan and etoposide combinations administered weekly or every 4 weeks for extensive small-cell lung cancer (JCOG9902-DI). *Ann Oncol* 14: 709–714

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 92: 205–216

1200

Synergistic antitumor effect of S-1 and the epidermal growth factor receptor inhibitor gefitinib in non-small cell lung cancer cell lines: role of gefitinib-induced down-regulation of thymidylate synthase

Takafumi Okabe,[1] Isamu Okamoto,[1]
Sayaka Tsukioka,[3] Junji Uchida,[3] Tsutomu Iwasa,[1]
Takeshi Yoshida,[1] Erina Hatashita,[1] Yuki Yamada,[1]
Taroh Satoh,[1] Kenji Tamura,[4] Masahiro Fukuoka,[2]
and Kazuhiko Nakagawa[1]

[1]Department of Medical Oncology, Kinki University School of
Medicine; [2]Department of Internal Medicine, Kinki University
School of Medicine, Sakai Hospital, Osaka, Japan; [3]Tokushima
Research Center, Taiho Pharmaceutical Co. Ltd., Tokushima,
Japan; and [4]Medical Oncology, National Cancer Center Hospital,
Tokyo, Japan

Abstract

Q2 Somatic mutations in the epidermal growth factor receptor (EGFR) gene are associated with the therapeutic response to EGFR tyrosine kinase inhibitors (TKI) in patients with advanced non-small cell lung cancer (NSCLC). The response rate to these drugs remains low, however, in NSCLC patients with wild-type EGFR alleles. Combination therapies with EGFR-TKIs and cytotoxic agents are considered a therapeutic option for patients with NSCLC expressing wild-type EGFR. We investigated the antiproliferative effect of the combination of the oral fluorouracil S-1 and the EGFR-TKI gefitinib in NSCLC cells of differing EGFR status. The combination of 5-fluorouracil and gefitinib showed a synergistic antiproliferative effect in vitro in all NSCLC cell lines tested. Combination chemotherapy with S-1 and gefitinib in vivo also had a synergistic antitumor effect on NSCLC xenografts regardless of the absence or presence of EGFR mutations. Gefitinib inhibited the expression of the transcription factor E2F-1, resulting in the down-regulation of thymidylate synthase at the mRNA and protein levels. These observations suggest that gefitinib-induced down-regulation of thymidylate synthase is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib and provide a basis for clinical

evaluation of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors. [Mol Cancer Ther 2008;7(3):1-8]

Introduction

Targeted therapy in the treatment of cancer has made substantial progress over the last few years. The ErbB family of receptor tyrosine kinases includes the epidermal growth factor receptor (EGFR; ErbB1), ErbB2 (HER2/neu), ErbB3, and ErbB4 and is important for normal development as a result of its roles in cell proliferation and differentiation (1–3). Aberrant expression of EGFR has been detected in a wide range of human epithelial malignancies, including non-small cell lung cancer (NSCLC), and is correlated with poor prognosis and reduced survival time (4, 5). Agents that specifically target EGFR are therefore under development as anticancer drugs. Indeed, two inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), gefitinib and erlotinib, both of which compete with ATP for binding to the catalytic pocket of the receptor, have been extensively studied in individuals with NSCLC (6–9). Somatic mutations in the region of EGFR that encodes the tyrosine kinase domain have been associated with tumor responsiveness to EGFR-TKIs in a subset of NSCLC patients (10–17). In contrast, achievement of a clinical benefit of these drugs in NSCLC patients who express wild-type EGFR has been problematic.

S-1 (Taiho Pharmaceutical) is an oral anticancer agent composed of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1 (18). Tegafur is a prodrug that generates 5-fluorouracil (5-FU) in blood largely as a result of its metabolism by cytochrome P450 in the liver. CDHP increases the plasma concentration of 5-FU through competitive inhibition of dihydropyrimidine dehydrogenase (DPD), which catalyzes 5-FU catabolism (19). Oxonate reduces the gastrointestinal toxicity of 5-FU (20). A response rate of 22% and a median survival time of 10.2 months were obtained in a clinical trial of S-1 in patients with advanced NSCLC not subjected previously to chemotherapy (21). Few severe gastrointestinal or hematologic adverse events were reported. Moreover, a phase II trial of S-1 plus cisplatin in NSCLC patients revealed a 47% response rate and an acceptable safety profile (22).

Based on this background, we examined the anticancer effect of the combination of S-1 and gefitinib in NSCLC cell lines of differing EGFR status. We found that the combination of S-1 (or 5-FU) and gefitinib exhibited a marked and synergistic antiproliferative effect both in vivo
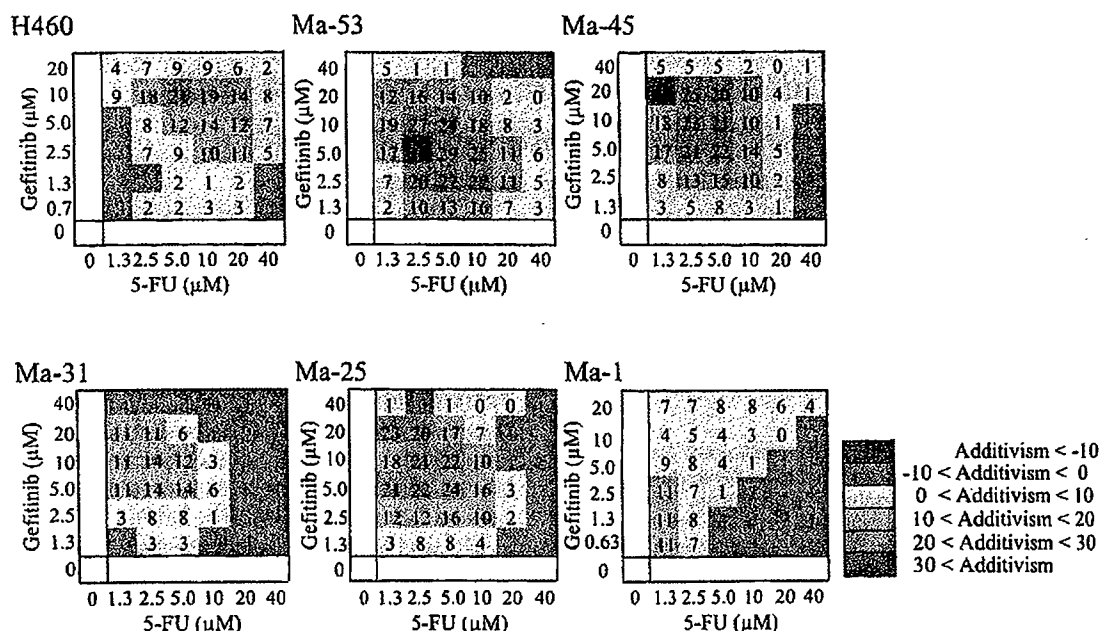
Figure 1. Inhibition of NSCLC cell growth by the combination of 5-FU and gefitinib *in vitro*. Cells with wild-type (H460, Ma-53, Ma-45, Ma-31, and Ma-25) or mutant (Ma-1) EGFR alleles were exposed for 72 h to 5-FU and gefitinib at the indicated concentrations, after which cell viability was measured with a colorimetric assay. The observed excess inhibition (%) relative to that predicted by the Bliss additivism model is shown color-coded in a drug concentration matrix for each cell line. Yellow, orange, pink, and red, synergy; light and dark blue, antagonism. Mean of triplicates from a representative experiment.

and *in vitro* in cells regardless of the absence or presence of EGFR mutations. Furthermore, we assessed the effects of gefitinib on the expression of enzymes that function in 5-FU metabolism, including thymidylate synthase (TS), DPD, and orotate phosphoribosyltransferase (OPRT), to gain insight into the mechanism underlying the synergistic effect of combination therapy with S-1 and gefitinib.

Materials and Methods

Cell Lines and Reagents

The human NSCLC cell lines NCI-H460 (H460), Ma-1, Ma-25, Ma-31, Ma-45, and Ma-53 were obtained as described previously (23). MiaPaca-2 cells were obtained from Japan Health Sciences Foundation. These cell lines were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. Gefitinib was provided by AstraZeneca. S-1 and CDHP were provided by Taiho Pharmaceutical. 5-FU was obtained from Wako.

Growth Inhibition Assay *In vitro*

Cells (2.0 × 10^3) were plated in 96-well flat-bottomed plates and cultured for 24 h before the addition of various concentrations of 5-FU and gefitinib and incubation for an additional 72 h. Cell Counting Kit-8 solution (Dojindo) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 450 nm. Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of 5-FU or gefitinib resulting in 50% growth inhibition (IC_{50}) was

calculated. The effect of combining 5-FU and gefitinib was classified as additive, synergistic, or antagonistic with the Bliss additivism model (24–26). A theoretical curve was calculated for combined inhibition with the equation: E_{bliss} = E_A + E_B - (E_A × E_B), where E_A and E_B are the fractional inhibitory effects of drug A alone and drug B alone at specific concentrations. E_{bliss} is then the fractional inhibition that would be expected if the effect of the combination of the two drugs was exactly additive. In this study, the Bliss variable is expressed as percentage decrease in cell growth above what would be expected for the combination. Bliss = 0 indicates that the effect of the combination is additive; Bliss > 0 is indicative of synergy; and Bliss < 0 indicates antagonism.

Animals

Male athymic nude mice were exposed to a 12-h light, 12-h dark cycle and provided with food and water *ad libitum* in a barrier facility. All experiments were done in compliance with the regulations of the Animal Experimentation Committee of Taiho Pharmaceutical.

Growth Inhibition Assay *In vivo*

Cubic fragments of tumor tissue (~2 × 2 × 2 mm) were implanted s.c. into the axilla of 5- to 6-week-old male athymic nude mice. Treatment was initiated when tumors in each group achieved an average volume of 100 to 150 mm^3. Treatment groups consisted of control, S-1 alone, gefitinib alone, and the combination of S-1 and gefitinib. Each treatment group contained seven mice. S-1 (10 mg/kg body mass) and gefitinib (50 or 3 mg/kg) were administered by oral gavage once a day for 14 days; control animals

received 0.5% (w/v) hydroxypropylmethylcellulose as vehicle. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula LW^2 / 2. Both tumor size and body weight were measured two or three times per week.

Immunoblot Analysis

Cell lysates were fractionated by SDS-PAGE on 12% gels (NuPAGE Bis-Tris Gels; Invitrogen), 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 DPD, OPRT, and TS were obtained from Taiho Pharmaceutical; those to E2F-1 were from Santa Cruz Biotechnology; and those 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 and by subsequent exposure to enhanced chemiluminescence reagents (Pierce).

Immunoprecipitation Analysis

Immunoprecipitation of EGFR was done according to standard procedures. Whole-cell lysates (800 µg protein) were incubated overnight at 4°C with antibodies to EGFR (Santa Cruz Biotechnology), after which Protein G Plus/Protein A-Agarose Suspension (Calbiochem) was added and the mixtures were incubated for an additional 1 h at 4°C. Immunoprecipitates were isolated, washed, resolved by SDS-PAGE on a 7.5% gel (Bio-Rad), and subjected to immunoblot analysis with antibodies to phosphotyrosine (PY20) and EGFR (Zymed).

Reverse Transcription and Real-time PCR Analysis

Total RNA (1 µg) extracted from cells with the use of an RNeasy Mini Kit (Qiagen) was subjected to reverse transcription with the use of a SuperScript Preamplification System (Invitrogen Life Technologies). The resulting cDNA was then subjected to real-time PCR analysis with the use of a TaqMan PCR Reagent Kit and a Gene Amp 5700 Sequence Detection System (Applied Biosystems). The forward and reverse primers and TaqMan probe for TS cDNA were 5-GCCTCGGTGTGCCTTTCA-3 and 5-CCCGTGATGTGCGCAAT-3 and 6-FAM-5'-TCGCCAGC-TACGCCCTGCTCA-3'-TAMRA, respectively. Glyceralde-hyde-3-phosphate dehydrogenase mRNA were used as an internal standard.

Statistical Analysis

Data are presented as mean ± SE and were analyzed by the Aspin-Welch t test. P < 0.05 was considered statistically significant.

Results

Effect of the Combination of 5-FU and Gefitinib on NSCLC Cell Growth In vitro

Tegafur, which is a component of S-1, is metabolized to 5-FU in the liver and exerts antitumor effects. We first examined the antiproliferative activity of the combination of 5-FU and gefitinib in six NSCLC cell lines. Five of the cell lines (H460, Ma-53, Ma-45, Ma-31, and Ma-25) possess wild-type EGFR alleles, whereas Ma-1 cells harbor an EGFR mutation (E746_A750del) that is associated with a high response rate to the EGFR-TKIs gefitinib and erlotinib in individuals with advanced NSCLC. We assessed
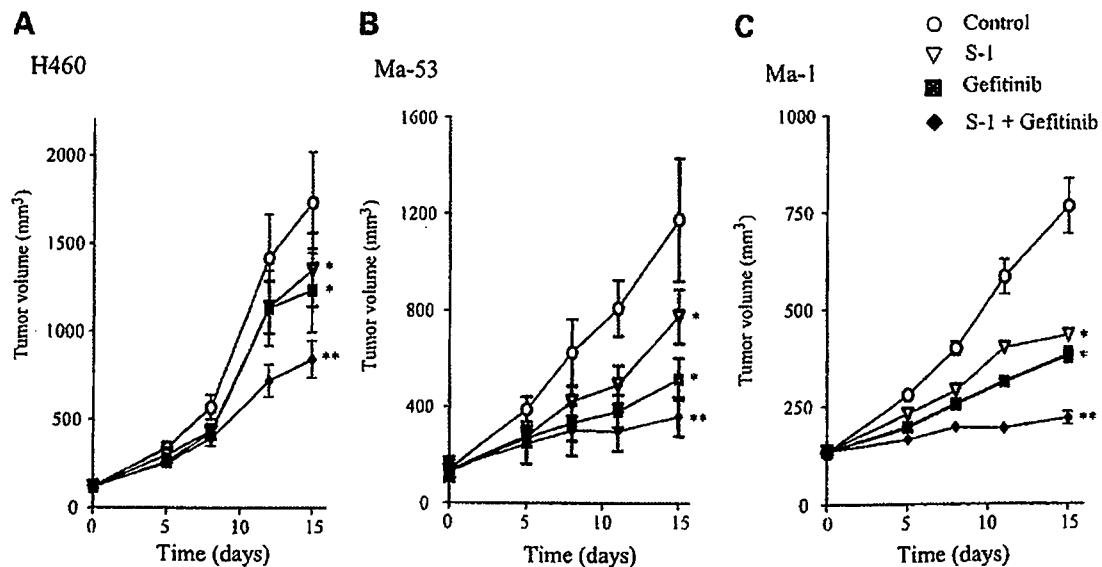

Figure 2. Antitumor activity of the combination of S-1 and gefitinib *in vivo*. A and B, nude mice with tumor xenografts established by s.c. implantation of NSCLC cells (H460 and Ma-53) possessing wild-type EGFR were treated daily for 2 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (50 mg/kg), or both drugs by oral gavage. C, nude mice with tumor xenografts derived from NSCLC cells (Ma-1) expressing mutant EGFR were treated daily for 2 weeks with vehicle (control), S-1 (10 mg/kg), gefitinib (3 mg/kg), or both drugs by oral gavage. Tumor volume in all animals was determined at the indicated times after the onset of treatment. Mean ± SE of values from seven mice per group. *, P < 0.05 versus control; **, P < 0.05 versus S-1 or gefitinib alone for values 15 d after treatment onset (Aspin-Welch t test).

H460   IP: EGFR

5-FU	−	+	−	+	+
CDHP	−	−	+	+	+
Gefitinib	−	−	−	−	+
p-EGFR					
EGFR					
p-EGFR/EGFR (%)	54	40	60	44	12

Ma-53   IP: EGFR

5-FU	−	+	−	+	+
CDHP	−	−	+	+	+
Gefitinib	−	−	−	−	+
p-EGFR					
EGFR					
p-EGFR/EGFR (%)	73	85	88	85	20

Ma-1   IP: EGFR

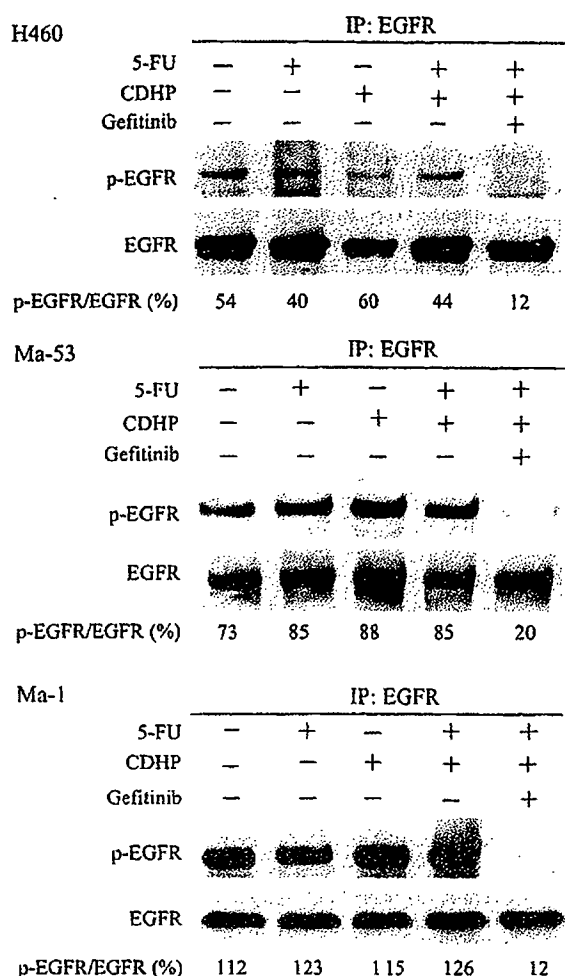|					
5-FU	−	+	−	+	+
CDHP	−	−	+	+	+
Gefitinib	−	−	−	−	+
p-EGFR					
EGFR					
p-EGFR/EGFR (%)	112	123	115	126	12

Figure 3. Lack of effect of 5-FU and CDHP on EGFR phosphorylation in NSCLC cell lines. NSCLC cells (H460, Ma-53, and Ma-1) were incubated for 24 h in medium supplemented with 2% fetal bovine serum and with 5-FU (10 μmol/L), CDHP (3 μmol/L), or gefitinib (5 μmol/L). Cell lysates were then prepared and subjected to immunoprecipitation (IP) with antibodies to EGFR, and the resulting precipitates were subjected to immunoblot analysis with antibodies to phosphotyrosine (for detection of phosphorylated EGFR) and with antibodies to EGFR. The intensity of the phosphorylated EGFR band relative to that of the EGFR band was determined by densitometry and is expressed as a percentage below each lane.

whether 5-FU and gefitinib showed additivity, synergy, or antagonism based on the Bliss additivism model (24–26). We chose this model rather than isobologram or combination index analysis because it would allow us to evaluate the nature of drug interactions even in instances in which the maximal inhibition by 5-FU or gefitinib alone was too low to obtain a reliable IC_{50} value. The six test concentrations for each agent were chosen after first determining the corresponding IC_{50} values. The IC_{50} values for 5-FU chemosensitivity were not associated with EGFR status and ranged from 7 to 11 μmol/L. The effect of combined treatment with 5-FU and gefitinib on the proliferation of the six NSCLC cell lines was tested in triplicate in a 6 × 6

concentration matrix. Calculation of the percentage inhibition in excess of that predicted by the Bliss additivism model revealed synergistic effects of Bliss > 0 for 5-FU and gefitinib in all of the six cell lines tested (Fig. 1). These results suggested that 5-FU and gefitinib act synergistically to inhibit cell growth in NSCLC cells.

Effect of Combined Treatment with S-1 and Gefitinib on NSCLC Cell Growth *In vivo*

We therefore next investigated whether combined treatment with S-1 and gefitinib might also exert a synergistic effect on NSCLC cell growth *in vivo*. Doses of both agents were selected so that their independent effects on tumor growth would be moderate. Nude mice were implanted s.c. with H460, Ma-53, or Ma-1 tumor fragments to establish tumor xenografts. When the H460 or Ma-53 tumors, which harbor wild-type EGFR, became palpable (100-150 mm^3), the mice were divided into four groups for daily treatment with vehicle, S-1 (10 mg/kg), gefitinib (50 mg/kg), or both drugs by oral gavage over 2 weeks. For xenografts formed by H460 or Ma-53 cells, combination therapy with S-1 and gefitinib resulted in a significant reduction in tumor size compared with that apparent in animals treated with S-1 or gefitinib alone (Fig. 2A and B). Mice bearing Ma-1 tumors, which express mutant EGFR, were treated with vehicle, S-1 (10 mg/kg), gefitinib (3 mg/kg), or both agents daily over 2 weeks. Combination treatment with S-1 and gefitinib significantly inhibited the growth of Ma-1 xenografts relative to that apparent in mice treated with either agent alone (Fig. 2C). None of the drug treatments induced a weight loss of >20% during the 2-week period, and no signs of overt drug toxicity were apparent (data not shown). These results thus suggested that combination chemotherapy with S-1 and gefitinib *in vivo* had a synergistic antitumor effect on NSCLC xenografts regardless of the absence or presence of EGFR mutations, consistent with our results *in vitro*.

Effects of 5-FU and CDHP on EGFR Phosphorylation in NSCLC Cell Lines

To investigate the mechanism responsible for the observed interaction between S-1 and gefitinib, we examined the effect of 5-FU on EGFR signal transduction in NSCLC cells expressing wild-type (H460 and Ma-53) or mutant (Ma-1) EGFR. Immunoprecipitation analysis revealed that exposure of H460 or Ma-53 cells to 5-FU (10 μmol/L) for 24 h had no effect on the basal level of EGFR phosphorylation (Fig. 3). We have shown previously that EGFR is constitutively phosphorylated in Ma-1 cells maintained in serum-free medium (23). Exposure of Ma-1 cells to 5-FU for 24 h did not affect this constitutive level of EGFR phosphorylation (Fig. 3). We next examined the effects of both CDHP, which is a component of S-1, and the combination of CDHP and 5-FU on EGFR phosphorylation in H460, Ma-53, and Ma-1 cells. Neither CDHP alone nor the combination of CDHP and 5-FU affected the level of EGFR phosphorylation in any of these three cell lines (Fig. 3). These results thus indicated that 5-FU and CDHP have no effect on EGFR signal transduction.

Effects of Gefitinib on the Expression of DPD, OPRT, and TS in NSCLC Cell Lines

We next investigated whether gefitinib might affect the expression of DPD, OPRT, or TS, enzymes that are major determinants of the sensitivity of cells to 5-FU. We first examined the abundance of these enzymes in the NSCLC cell lines H460, Ma-53, and Ma-1 by immunoblot analysis. The expression of DPD was detected in MiaPaca-2 cells (positive control) but not in H460, Ma-53, or Ma-1 cells (Fig. 4A). In contrast, OPRT and TS were detected in all three NSCLC cell lines and their abundance did not appear related to EGFR status (Fig. 4A). Treatment of H460, Ma-53, or Ma-1 cells with gefitinib (5 μmol/L) for up to 48 h resulted in a time-dependent decrease in the amount of TS, whereas that of OPRT or DPD remained unaffected (Fig. 4B). A reduced level of TS expression in tumors has been associated previously with a higher response rate to 5-FU-based chemotherapy (27, 28). Our data thus suggested that the suppression of TS expression by gefitinib might increase the sensitivity of NSCLC cells to 5-FU.

The transcription factor E2F-1 regulates expression of the TS gene (29–31). We therefore examined the possible effect of gefitinib on E2F-1 expression in NSCLC cell lines. Incubation of H460, Ma-53, or Ma-1 cells with gefitinib for up to 48 h also induced a time-dependent decrease in the amount of E2F-1 (Fig. 4B), suggesting that this effect might contribute to the down-regulation of TS expression by gefitinib in these cell lines.

Effect of Gefitinib on TS mRNA Abundance in NSCLC Cell Lines

The abundance of TS mRNA would be expected to be decreased if the down-regulation of E2F-1 expression by gefitinib was responsible for the reduced level of TS. We determined the amount of TS mRNA in H460, Ma-53, or Ma-1 cells at various times after exposure to gefitinib with the use of reverse transcription and real-time PCR analysis. Gefitinib indeed induced a time-dependent decrease in the amount of TS mRNA in all three NSCLC cell lines (Fig. 5), suggesting that the down-regulation of TS expression by gefitinib occurs at the transcriptional level and may be due to suppression of E2F-1 expression.

Discussion

The recent identification of activating somatic mutations of EGFR in NSCLC and their relevance to prediction of the therapeutic response to EGFR-TKIs such as gefitinib and erlotinib have had a major effect on NSCLC treatment (10–17). The response rate to these drugs remains low, however, in NSCLC patients with wild-tye EGFR alleles. Combination therapy with EGFR-TKIs and cytotoxic agents is a potential alternative strategy for NSCLC expressing wild-type EGFR. In the present study, we have evaluated the potential cooperative antiproliferative effect of combined treatment with the EGFR-TKI gefitinib and the new oral fluorouracil S-1 in NSCLC cell lines of differing EGFR status. We found that S-1 (or 5-FU) and gefitinib exert a synergistic antiproliferative effect on NSCLC cells both *in vivo* and *in vitro* regardless of the absence or presence of EGFR mutation. We chose a gefitinib dose of 50 mg/kg for treatment of mice bearing H460 or Ma-53 tumors. The median effective dose of gefitinib was shown previously to be ~50 mg/kg in athymic nude mice bearing A431 cell-derived xenografts (32). A gefitinib dose of 50 mg/kg has therefore subsequently been widely used in tumor xenograft studies (33–36). The U.S. Food and Drug Administration recommends that drug doses in animals be converted to those in humans based on body surface area (37). According to this guideline, a gefitinib dose of 50 mg/kg in mouse xenograft models is approximately equivalent to the therapeutic dose (250 mg/d) of the drug in humans. In addition, the tumor concentrations of gefitinib in NSCLC xenografts of mice treated with this drug (50 mg/kg) ranged from 9.7 to 13.3 μg/g, values that were similar to the
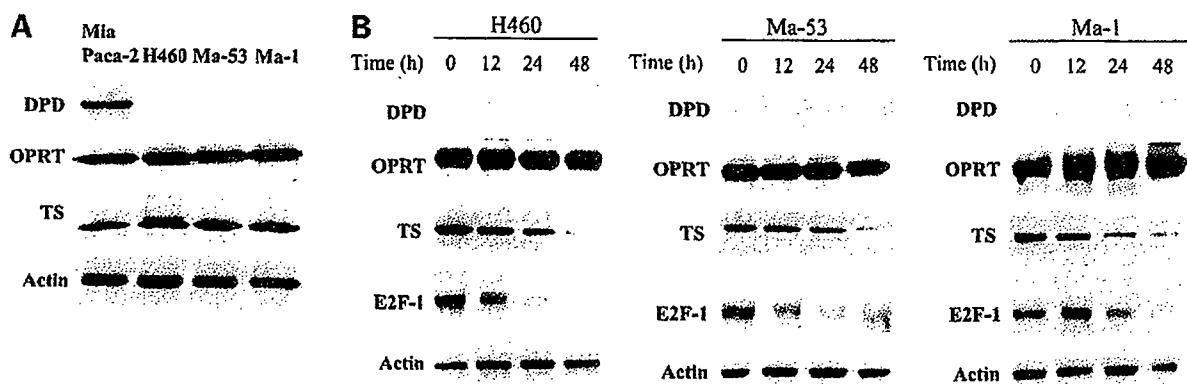

Figure 4. Effects of gefitinib on the expression of E2F-1, DPD, OPRT, and TS in NSCLC cell lines. **A,** lysates of H460, Ma-53, or Ma-1 cells were subjected to immunoblot analysis with antibodies to DPD, OPRT, TS, or β-actin (loading control). MiaPaca-2 cells were also examined as a positive control for DPD expression. **B,** NSCLC cells were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which cell lysates were subjected to immunoblot analysis as in **A,** with the addition that E2F-1 expression was also examined.
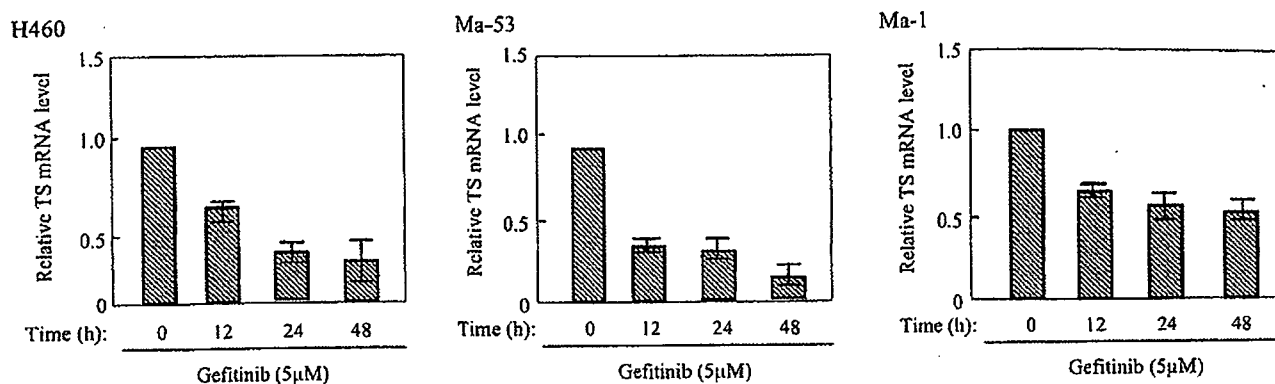
H460

Ma-53

Ma-1

Figure 5. Down-regulation of TS mRNA by gefitinib in NSCLC cell lines. H460, Ma-53, or Ma-1 cells were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which total RNA was extracted from the cells and subjected to reverse transcription and real-time PCR analysis of TS mRNA. The amount of TS mRNA was normalized by that of glyceraldehyde-3-phosphate dehydrogenase mRNA. Mean ± SE of values from three separate experiments.

achievable concentrations of gefitinib in tumor tissues of treated humans (34). These observations suggest that a gefitinib dose of 50 mg/kg in mouse xenograft models is appropriate for mimicking the therapeutic dose in humans.

EGFR-TKIs have been shown previously to act synergistically with radiation or cytotoxic agents such as cisplatin, paclitaxel, and irinotecan (38–40). These cytotoxic agents and radiation have been shown to increase the phosphorylation level of EGFR, possibly reflecting the activation of prosurvival signaling, and this effect is blocked by EGFR-TKIs, resulting in the synergistic antitumor effects of the combination therapies. Such a synergistic effect of 5-FU and gefitinib was attributed to 5-FU-induced EGFR phosphorylation in colorectal cancer cells (41). In contrast, we found that 5-FU had no effect on the level of EGFR phosphorylation in NSCLC cell lines. Further examination of different concentrations of 5-FU and different exposure times also failed to reveal an effect of 5-FU on EGFR phosphorylation in these cells (data not shown). These findings indicate that NSCLC cell lines respond differently to 5-FU than do colorectal cancer cells and that the synergistic antiproliferative effect of 5-FU and gefitinib in NSCLC cells is not mediated at the level of EGFR phosphorylation.

Our results indicate that the synergistic interaction of 5-FU (or S-1) and gefitinib is attributable, at least in part, to down-regulation of TS expression by gefitinib. The active metabolite of 5-FU, FdUMP, forms a covalent ternary complex with 5,10-methylenetetrahydrofolate and TS, resulting in inhibition of DNA synthesis (42). TS is thus an important therapeutic target of 5-FU. The amount of TS in neoplastic cells has been found to increase after exposure to 5-FU, resulting in the maintenance of free enzyme in excess of that bound to 5-FU (43–47). Such an increase in TS expression and activity has been viewed as a mechanistic driver of 5-FU resistance in cancer cells (48–50). The development of a new therapeutic strategy that reduces TS expression would therefore be of interest. Indeed, preclinical studies have shown that the down-regulation of TS by antisense oligonucleotides or other means enhances the

efficacy of 5-FU (51–54). Down-regulation of TS would be expected to enhance the cytotoxicity of 5-FU as a result of the decrease in the amount of its protein target (55). Consistent with these preclinical data, an inverse relation between TS expression and 5-FU sensitivity has been shown in various human solid tumors (27, 28, 56–60). We have now shown that gefitinib alone induced down-regulation of TS expression, suggesting that this effect of gefitinib contributes to its synergistic interaction with 5-FU (or S-1) in NSCLC cell lines.

We further explored the molecular mechanism by which gefitinib induces down-regulation of TS expression in NSCLC cells. Given that EGFR signal transduction has been shown to be involved in activity of E2F-1 that regulates the expression of several genes including TS (61, 62), which controls the expression of several genes including that for TS, we examined the possible effects of gefitinib on E2F-1 expression and on the abundance of TS mRNA. Gefitinib induced down-regulation of E2F-1 in NSCLC cell lines harboring wild-type *EGFR*, consistent with previous observations (63), as well as in those expressing mutant EGFR. In addition, gefitinib reduced the amount of TS mRNA in NSCLC cells, consistent with the notion that the suppression of TS expression by gefitinib is attributable to inhibition of gene transcription as a result of down-regulation of E2F-1. For our experiments examining the effects of gefitinib on TS and E2F-1 expression, we used a drug concentration of 5 μmol/L. The concentration of gefitinib in tumor xenografts was shown previously to be 5 to 14 times that in the plasma concentration of the mouse hosts (34). Daily oral administration of gefitinib (250 mg) in patients also gave rise to a drug concentration in tumor tissue that was substantially higher (mean, 42-fold) than that in plasma concentration (34). We showed previously that the maximal concentration of gefitinib in the plasma of patients with advanced solid tumors had a mean value of 0.76 μmol/L at a daily dose of 225 mg (64). Based on these data, we considered that a gefitinib concentration of 5 μmol/L was appropriate for our

analyses of TS and E2F-1 expression. Together, our present findings suggest that down-regulation of E2F-1 and consequently that of TS by gefitinib is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib.

Somatic mutations of *EGFR* have been associated with sensitivity to EGFR-TKIs in patients with advanced NSCLC (13–16). However, although most NSCLCs with *EGFR* mutations initially respond to EGFR-TKIs, the vast majority of these tumors ultimately develop resistance to the drug. In the present study, the synergistic effect of combination chemotherapy with S-1 and gefitinib was observed even in *EGFR* mutant cells. Our findings thus suggest that the addition of S-1 (or 5-FU) to EGFR-TKIs might overcome chemoresistance to EGFR-TKIs and that exploration of the effect of such combination therapy in cells resistant to EGFR-TKIs is warranted. *EGFR* mutations appear to be largely limited to lung cancer, with few such mutations having been detected in other types of cancer (65–67). 5-FU is widely used as an anticancer agent and is considered a key drug in chemotherapy for solid tumors such as gastrointestinal and cervical cancer (68–70). Our present results show that gefitinib suppressed the expression of TS in NSCLC cell lines regardless of the absence or presence of *EGFR* mutations, suggesting that the addition of EGFR-TKIs to a 5-FU-containing regimen might increase the effectiveness of such treatment for solid cancers without *EGFR* mutations. Oral combined chemotherapy with drugs, such as S-1 and gefitinib, may also prove to be of low toxicity and therefore maintain quality of life. Our preclinical results provide a basis for future clinical investigations of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors.

References

1. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. Oncogene 2000;19:6550–65.

2. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000; 103:211–25.

3. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005;5:341–54.

4. 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.

5. 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.

6. 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) [corrected]. J Clin Oncol 2003; 21:2237–46.

7. Perez-Soler R, Chachoua A, Hammond LA, et al. Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. J Clin Oncol 2004;22:3238–47.

8. 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.

9. 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.

10. 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.

11. 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.

12. 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.

13. 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.

14. 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.

15. 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.

16. 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.

17. 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.

18. Shirasaka T, Shimamoto Y, Fukushima M. Inhibition by oxonic acid of gastrointestinal toxicity of 5-fluorouracil without loss of its antitumor activity in rats. Cancer Res 1993;53:4004–9.

19. Tatsumi K, Fukushima M, Shirasaka T, Fujii S. Inhibitory effects of pyrimidine, barbituric acid and pyridine derivatives on 5-fluorouracil degradation in rat liver extracts. Jpn J Cancer Res 1987;78:748–55.

20. Shirasaka T, Shimamoto Y, Ohshimo H, et al. Development of a novel form of an oral 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators. Anticancer Drugs 1996;7:548–57.

21. Kawahara M, Furuse K, Segawa Y, et al. Phase II study of S-1, a novel oral fluorouracil, in advanced non-small-cell lung cancer. Br J Cancer 2001;85:939–43.

22. Ichinose Y, Yoshimori K, Sakai H, et al. S-1 plus cisplatin combination chemotherapy in patients with advanced non-small cell lung cancer: a multi-institutional phase II trial. Clin Cancer Res 2004;10:7860–4.

23. Okabe T, Okamoto I, Tamura K, et al. Differential constitutive activation of the epidermal growth factor receptor in non-small cell lung cancer cells bearing EGFR gene mutation and amplification. Cancer Res 2007;67:2046–53.

24. Berenbaum MC. Criteria for analyzing interactions between biologically active agents. Adv Cancer Res 1981;35:269–335.

25. Borisy AA, Elliott PJ, Hurst NW, et al. Systematic discovery of multicomponent therapeutics. Proc Natl Acad Sci U S A 2003;100:7977–82. Epub 2003 Jun 10.

26. Buck E, Eyzaguirre A, Brown E, et al. Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors. Mol Cancer Ther 2006;5:2676–84.

27. Ichikawa W, Uetake H, Shirota Y, et al. Combination of dihydropyrimidine dehydrogenase and thymidylate synthase gene expressions in primary tumors as predictive parameters for the efficacy of fluoropyrimidine-based chemotherapy for metastatic colorectal cancer. Clin Cancer Res 2003;9:786–91.

28. Salonga D, Danenberg KD, Johnson M, et al. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res 2000;6:1322–7.

29. DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol Cell Biol 1995;15:4215–24.

30. Dyson N. The regulation of E2F by pRB-family proteins. Genes Dev 1998;12:2245–62.

31. Trimarchi JM, Lees JA. Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol 2002;3:11–20.

32. Wakeling AE, Guy SP, Woodburn JR, et al. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. Cancer Res 2002;62:5749–54.

33. Matar P, Rojo F, Cassia R, et al. Combined epidermal growth factor receptor targeting with the tyrosine kinase inhibitor gefitinib (ZD1839) and the monoclonal antibody cetuximab (IMC-C225): superiority over single-agent receptor targeting. Clin Cancer Res 2004;10:6487–501.

34. McKillop D, Partridge EA, Kemp JV, et al. Tumor penetration of gefitinib (Iressa), an epidermal growth factor receptor tyrosine kinase inhibitor. Mol Cancer Ther 2005;4:641–9.

35. Zhang X, Chen ZG, Choe MS, et al. Tumor growth inhibition by simultaneously blocking epidermal growth factor receptor and cyclo-oxygenase-2 in a xenograft model. Clin Cancer Res 2005;11:6261–9.

36. Haura EB, Zheng Z, Song L, Cantor A, Bepler G. Activated epidermal growth factor receptor-Stat-3 signaling promotes tumor survival *in vivo* in non-small cell lung cancer. Clin Cancer Res 2005;11:8288–94.

37. U S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Guidance for industry, estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers; 2005. p. 1–27.

38. Koizumi F, Kanzawa F, Ueda Y, et al. Synergistic interaction between the EGFR tyrosine kinase inhibitor gefitinib ("Iressa") and the DNA topoisomerase I inhibitor CPT-11 (irinotecan) in human colorectal cancer cells. Int J Cancer 2004;108:464–72.

39. Chinnaiyan P, Huang S, Vallabhaneni G, et al. Mechanisms of enhanced radiation response following epidermal growth factor receptor signaling inhibition by erlotinib (Tarceva). Cancer Res 2005;65:3328–35.

40. Van Schaeybroeck S, Kyula J, Kelly DM, et al. Chemotherapy-induced epidermal growth factor receptor activation determines response to combined gefitinib/chemotherapy treatment in non-small cell lung cancer cells. Mol Cancer Ther 2006;5:1154–65.

41. Van Schaeybroeck S, Karaiskou-McCaul A, Kelly D, et al. Epidermal growth factor receptor activity determines response of colorectal cancer cells to gefitinib alone and in combination with chemotherapy. Clin Cancer Res 2005;11:7480–9.

42. Peters GJ, van der Wilt CL, van Triest B, et al. Thymidylate synthase and drug resistance. Eur J Cancer 1995;31A:1299–305.

43. Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH. *In vivo* kinetics of thymidylate synthetase inhibition of 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. Cancer Res 1982;42:450–6.

44. Washtien WL. Increased levels of thymidylate synthetase in cells exposed to 5-fluorouracil. Mol Pharmacol 1984;25:171–7.

45. Spears CP, Gustavsson BG, Berne M, Frosing R, Bernstein L, Hayes AA. Mechanisms of innate resistance to thymidylate synthase inhibition after 5-fluorouracil. Cancer Res 1988;48:5894–900.

46. Swain SM, Lippman ME, Egan EF, Drake JC, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. J Clin Oncol 1989;7:890–9.

47. Chu E, Zinn S, Boarman D, Allegra CJ. Interaction of γ interferon and 5-fluorouracil in the H630 human colon carcinoma cell line. Cancer Res 1990;50:5834–40.

48. Johnston PG, Drake JC, Trepel J, Allegra CJ. Immunological quantitation of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and -resistant human cancer cell lines. Cancer Res 1992;52:4306–12.

49. Copur S, Aiba K, Drake JC, Allegra CJ, Chu E. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. Biochem Pharmacol 1995;49:1419–26.

50. Kawate H, Landis DM, Loeb LA. Distribution of mutations in human thymidylate synthase yielding resistance to 5-fluorodeoxyuridine. J Biol Chem 2002;277:36304–11. Epub 2002 Jul 29.

51. Hsueh CT, Kelsen D, Schwartz GK. UCN-01 suppresses thymidylate synthase gene expression and enhances 5-fluorouracil-induced apoptosis in a sequence-dependent manner. Clin Cancer Res 1998;4:2201–6.

52. Ju J, Kane SE, Lenz HJ, Danenberg KD, Chu E, Danenberg PV. Desensitization and sensitization of cells to fluoropyrimidines with different antisenses directed against thymidylate synthase messenger RNA. Clin Cancer Res 1998;4:2229–36.

53. Lee JH, Park JH, Jung Y, et al. Histone deacetylase inhibitor enhances 5-fluorouracil cytotoxicity by down-regulating thymidylate synthase in human cancer cells. Mol Cancer Ther 2006;5:3085–95.

54. Wada Y, Yoshida K, Suzuki T, et al. Synergistic effects of docetaxel and S-1 by modulating the expression of metabolic enzymes of 5-fluorouracil in human gastric cancer cell lines. Int J Cancer 2006;119:783–91.

55. Ferguson PJ, Collins O, Dean NM, et al. Antisense down-regulation of thymidylate synthase to suppress growth and enhance cytotoxicity of 5-FUdR, 5-FU and Tomudex in HeLa cells. Br J Pharmacol 1999;127:1777–86.

56. Aaronson SA. Growth factors and cancer. Science 1991;254:1146–53.

57. Johnston PG, Lenz HJ, Leichman CG, et al. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. Cancer Res 1995;55:1407–12.

58. Leichman CG, Lenz HJ, Leichman L, et al. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. J Clin Oncol 1997;15:3223–9.

59. Pestalozzi BC, Peterson HF, Gelber RD, et al. Prognostic importance of thymidylate synthase expression in early breast cancer. J Clin Oncol 1997;15:1923–31.

60. Johnston PG, Mick R, Recant W, et al. Thymidylate synthase expression and response to neoadjuvant chemotherapy in patients with advanced head and neck cancer. J Natl Cancer Inst 1997;89:308–13.

61. Hanada N, Lo HW, Day CP, Pan Y, Nakajima Y, Hung MC. Co-regulation of B-Myb expression by E2F1 and EGF receptor. Mol Carcinog 2006;45:10–7.

62. Ginsberg D. EGFR signaling inhibits E2F1-induced apoptosis *in vivo*: implications for cancer therapy. Sci STKE 2007;pe4.

63. Suenaga M, Yamaguchi A, Soda H, et al. Antiproliferative effects of gefitinib are associated with suppression of E2F-1 expression and telomerase activity. Anticancer Res 2006;26:3387–91.

64. Nakagawa K, Tamura T, Negoro S, et al. Phase I pharmacokinetic trial of the selective oral epidermal growth factor receptor tyrosine kinase Inhibitor gefitinib ("Iressa," ZD1839) in Japanese patients with solid malignant tumors. Ann Oncol 2003;14:922–30.

65. Barber TD, Vogelstein B, Kinzler KW, Velculescu VE. Somatic mutations of EGFR in colorectal cancers and glioblastomas. N Engl J Med 2004;351:2883.

66. Lee JW, Soung YH, Kim SY, et al. Absence of EGFR mutation in the kinase domain in common human cancers besides non-small cell lung cancer. Int J Cancer 2005;113:510–1.

67. Shigematsu H, Gazdar AF. Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. Int J Cancer 2006;118:257–62.

68. Herskovic A, Martz K, al-Sarraf M, et al. Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. N Engl J Med 1992;326:1593–8.

69. Vanhoefer U, Rougier P, Wilke H, et al. Final results of a randomized phase III trial of sequential high-dose methotrexate, fluorouracil, and doxorubicin versus etoposide, leucovorin, and fluorouracil versus infusional fluorouracil and cisplatin in advanced gastric cancer: a trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group. J Clin Oncol 2000;18:2648–57.

70. Gibson MK, Li Y, Murphy B, et al. Randomized phase III evaluation of cisplatin plus fluorouracil versus cisplatin plus paclitaxel in advanced head and neck cancer (E1395): an Intergroup Trial of the Eastern Cooperative Oncology Group. J Clin Oncol 2005;23:3562–7.

切除不能局所進行非小細胞肺癌に対する分子標的治療の現状

－胸部放射線療法との併用－

引 野 幸 司*　　　大 江 裕一郎*

要　　旨

切除不能局所進行非小細胞肺癌に対する標準的治療は，現在のところシスプラチンを含む化学療法と同時胸部放射線療法であることがほぼコンセンサスとなっているが，まだまだ十分な治療成績とは言えない状況である．近年，分子標的薬が非小細胞肺癌の治療薬として注目され，放射線療法との併用で相乗効果も報告されている．これを踏まえ，切除不能局所進行非小細胞肺癌に対して分子標的薬と化学・胸部放射線療法を併用する臨床試験が行われている．今後のさらなる治療成績向上において，分子標的薬は非常に重要な治療戦略と言える．

は じ め に

日本での肺癌による死亡者は年間約6万人に上り，癌による死亡原因の第1位となった．今後も肺癌の発生数，死亡者数は増加することが予想されている．Japan Clinical Oncology Group（JCOG）肺がん内科グループで施行された切除不能局所進行の非小細胞肺癌（NSCLC）に対する化学放射線療法の6つの臨床試験では，240症例の生存期間中央値（MST）は16.1ヵ月，5年生存率14.4%であった[1]．まだまだ予後不良と言える肺癌の治療成績向上のためには，新しい治療戦略が必要

* 国立がんセンター中央病院 肺内科

キーワード：局所進行非小細胞肺癌，分子標的薬，胸部放射線療法

とされる状況である．

切除不能局所進行非小細胞肺癌（NSCLC）の標準的治療

切除不能局所進行 NSCLC とは，IIIA 期および胸水貯留例を除く IIIB 期で，根治手術が困難である NSCLC の総称である．以前は胸部放射線療法（TRT）が標準とされていたが，メタアナリシスの結果からシスプラチンを含む化学療法と TRT の併用が標準と考えられるようになった[2-4]．次に，TRT を行うタイミング，同時か遂次かを比較した第III相臨床試験が行われるようになった．MST において同時群 15.0～17.0 ヵ月 対 遂次群 13.3～14.4 ヵ月という結果が得られ，標準的治療は化学療法と同時 TRT であることがほぼコ

ンセンサスとなってきた[1][4]. 一方, 化学療法においてどのレジメンを用いるかについては第Ⅲ相試験もほとんどなく, 確立されていない状況である.

国立がんセンター中央病院を中心に行われたシスプラチン＋ビノレルビン併用化学療法と同時 TRT の第Ⅰ相臨床試験では, 奏効率83%〔95% 信頼区間 (CI) 59〜96%〕, MST は 30.4ヵ月, 3年生存率は 50% であった[5]. また Southwest Oncology Group (SWOG) は, シスプラチン＋エトポシドの化学療法と同時 TRT を行った後にドセタキセルの地固め療法を実施するという S9504 試験を行い, MST 26ヵ月, 5年生存率 29% という結果であった[6][7]. これを受けて国立がんセンター中央病院を中心に, シスプラチン＋ビノレルビンと同時 TRT 後にドセタキセルによる地固め療法を行う試験が行われた. ドセタキセルについては, 地固め療法を行っている期間にグレード 3〜4 の好中球減少, 食道炎, 肺臓炎といった毒性が問題となったが, 奏効率81.7% (95%CI 72.7〜88.0%), MST 30.4ヵ月 (95%CI 24.5〜36.6), 3年生存率 42.6% という結果が得られた[8]. これらのデータから, 日本では同時 TRT に併用する化学療法レジメンとしてはシスプラチン＋ビノレルビンが比較的多く用いられていると考えられる.

EGFR チロシンキナーゼ阻害薬と胸部放射線療法 (TRT) の併用

EGF 受容体 (EGFR) のチロシンキナーゼ阻害薬であるゲフィチニブ (イレッサ®, ZD1839) は, 既治療 NSCLC を対象とした第Ⅱ相臨床試験において, 日本人で奏効率27.5% であった[9]. また in vivo の実験では, ゲフィチニブとエルロチニブ (タルセバ®, OSI-774) には放射線との相乗効果が報告されている[10]. 頭頸部扁平上皮癌において, 放射線療法単独群と放射線に EGFR に対す

るモノクローナル抗体であるセツキシマブ (cetuximab, C225) を併用する群を比較する第Ⅲ相臨床試験では, MST は単独群 29.3ヵ月 対 併用群 49.0ヵ月, 3年生存率は単独群45% 対 併用群 55% という結果であった (p＝0.03)[11]. これらのことから, 放射線療法にEGFR チロシンキナーゼ阻害薬を併用することで, 局所進行 NSCLC の治療成績にも改善がもたらされることが期待されるようになった.

Rischin は, 15 例の NSCLC 患者に対してカルボプラチン＋パクリタキセルと同時 TRTにゲフィチニブを併用する第Ⅰ相臨床試験を行い, 奏効率 91% という結果を報告した[12]. Ready はⅢ期 NSCLC に対してカルボプラチン＋パクリタキセルにゲフィチニブを併用し, TRT を同時群と逐次群に分けた第Ⅱ相臨床試験を行ったが, 肺毒性の増強は認められなかった[13]. SWOG では S9504 の結果を踏まえ, シスプラチン＋エトポシドと同時TRT を行い, 次いでドセタキセルを投与した後にゲフィチニブを追加する群とプラセボ群に割り付ける第Ⅲ相臨床試験 (S0023) を施行した (図1). この試験の目的は NSCLCにおけるゲフィチニブ維持療法の有用性を検討することであったが, INTACT (IRESSANSCLC Trial Assessing Combination Treatment) や ISEL (IRESSA Survival Evaluation in Lung Cancer) でゲフィチニブの延命効果が証明できなかったという結果を受けて早期中間解析を行ったところ, 無増悪期間, MST ともに統計学的には有意差を認めなかったものの, 試験を継続してもゲフィチニブによる延命効果は期待できないと結論づけられ, 試験中止となっている[14]. 2007 年の米国臨床腫瘍学会 (ASCO) にて, MST はゲフィチニブ群 23ヵ月 対 プラセボ群 35ヵ月 (p＝0.013) という, その後の経過観察 (平均 27ヵ月) のデータが発表されている[15].

図1 SWOG0023 の治療レジメン
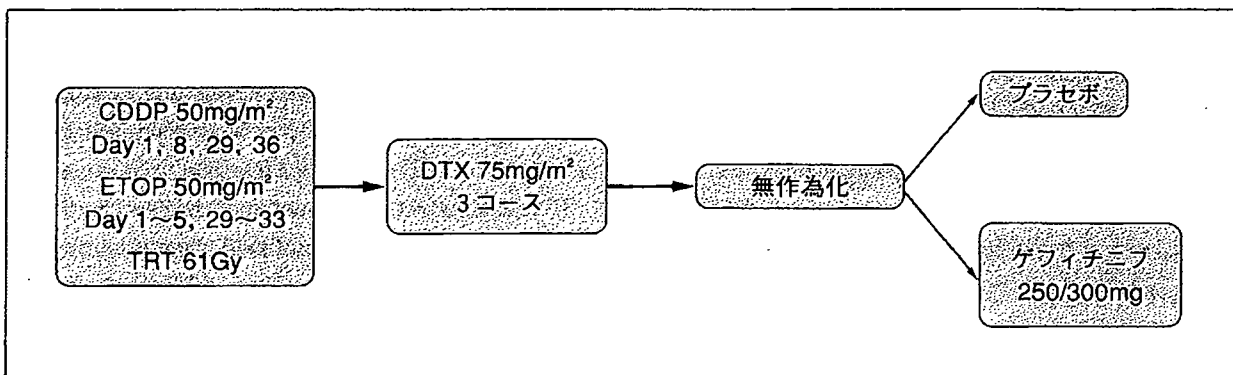
CDDP：シスプラチン，ETOP：エトポシド，TRT：胸部放射線療法，DTX：ドセタキセル
SWOG：Southwest Oncology Group

図2 JCOG0402 の治療レジメン

		Day 1	Day 22	Day 43	Day 57
CDDP	80mg/m² Day 1, 22	↓	↓		
VNR	25mg/m² Day 1, 8, 22, 29	↓ ↓	↓ ↓		
ゲフィチニブ	250mg/日 Day 43〜			→————————→	
TRT	60Gy/30Fr/6週 Day 57〜				▶▶▶▶

CDDP：シスプラチン，VNR：ビノレルビン，TRT：胸部放射線療法，JCOG：Japan Clinical Oncology Group

しかしこの試験は米国で施行されているうえに，40% 以上の症例が扁平上皮癌であり，ゲフィチニブの効果が期待できない条件が含まれている．この結果から，直ちに局所進行 NSCLC に対するゲフィチニブの有用性が否定されるべきものではない．

局所進行 NSCLC に対するシスプラチン＋ビノレルビンによる化学療法後のゲフィチニブと同時 TRT の安全性有効性確認試験
　　　－JCOG0402，第Ⅰ/Ⅱ相臨床試験－

　JCOG 肺がん内科グループでは現在，上記の臨床試験が行われている（図2）．当初はゲフィチニブの投与時期を化学放射線療法と同時に行うデザインが検討されたが，ゲフィチニブによる肺臓炎による死亡症例が報告され，ビノレルビンとゲフィチニブ併用による血液毒性増強の緊急安全情報も出されたため，ゲフィチニブと TRT を化学療法後に行う計画に変更された．また TRT が化学療法と同時でなくなってしまうが，シスプラチン（80 mg/m²，day 1）＋ビノレルビン（25mg/m²，day 1, 8）を3週間隔に投与することで，同時併用時のレジメン（ビノレルビン 20mg/m² で4週間隔）より強度を上げているため，治療効果に大きな差は生まれないと考えられた．この試験の1次エンドポイントは安全性（グレード2以上の肺臓炎を認めずに治療完

遂できた割合で評価），2次エンドポイント
は1年生存率，奏効率，全生存期間，無増悪
生存期間，有害事象発生割合としている．予
定登録数 37 例で現在も進行中である．

その他の臨床試験

Bebb は，根治治療困難のⅢ・Ⅳ期 NSCLC
に EGFR に対するモノクローナル抗体であ
るニモツズマブ（nimotuzumab）と 30Gy の
TRT を併用する第Ⅰ/Ⅱ相臨床試験を行って
おり，皮疹や下痢の有害事象がなかったと
報告している[16]．Hughes は，切除不能Ⅲ期
NSCLC 患者 12 人に対して，プラチナベー
スの化学療法後にセツキシマブと同時 TRT
を実施する第Ⅰ相臨床試験を行っている．グ
レード 3 の全身疲労感とグレード 2 の皮膚炎
が原因で 2 人が試験中止となった以外は，
大きな有害事象もなく完遂できている[17]．
Martinez は，17 人の切除不能Ⅲ期 NSCLC
患者に同時 TRT にエルロチニブを併用する
無作為化第Ⅱ相臨床試験を行っており，併用
群と非併用群間での毒性に明らかな差を認め
ていないと報告している[18]．

おわりに

局所進行 NSCLC は肺癌全体の約 25% を
占めるとされ，肺癌の治療成績向上のために
もⅢ期 NSCLC に対する新たな戦略が必要と
されている．INTACT，ISEL，S0023 など
の第Ⅲ相臨床試験ではゲフィチニブの延命効
果は示されなかったが，今後も分子標的薬は
大きな鍵を握っていると考えられる．

文　　　献

1) Ohe Y, et al: Long-term follow-up of patients with unresectable locally advanced non-small cell lung cancer treated with chemoradiotherapy: a retrospective analysis of the data from the Japan Clinical Oncology Group trials (JCOG0003A). Cancer Sci 94: 729-734, 2003.

2) Non-small Cell Lung Cancer Collaborative Group: Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. BMJ 311: 899-909, 1995.

3) Marino P, et al: Randomized trials of radiotherapy alone versus combined chemotherapy and radiotherapy in stage Ⅲa and Ⅲb non small cell lung cancer. A meta-analysis. Cancer 76: 593-601, 1995.

4) Furuse K, et al: Phase Ⅲ study of concurrent versus sequential thoracic radiotherapy in combination with mitomycin, vindesine and cis-platin in unresectable stage Ⅲ non-small-cell lung cancer. J Clin Oncol 17: 2692-2699, 1999.

5) Sekine I, et al: Phase I study of cisplatin, vinorelbine and concurrent thoracic radiotherapy for unresectable stage Ⅲ non-small cell lung cancer. Cancer Sci 95: 691-695, 2004.

6) Gandara D R, et al: Consolidation docetaxel after concurrent chemoradiotherapy in stage ⅢB non-small cell lung cancer. Phase Ⅱ Southwest Oncology Group Study S9504. J Clin Oncol 21: 2004-2010, 2003.

7) Gandara D R, et al: Long term survival with concurrent chemoradiation therapy followed by consolidation docetaxel in stage ⅢB non-small-cell lung cancer: a phase Ⅱ Southwest Oncology Group Study (S9504). Clin Lung Cancer 8 (2): 116-121, 2006.

8) Sekine I, et al: Docetaxel consolidation therapy following cisplatin, vinorelbine and concurrent thoracic radiotherapy in patients with unresectable stage Ⅲ non-small cell lung cancer. J Thorac Oncol 1 (8): 810-815, 2006.

9) Fukuoka M, et al: Multi-institutional randomized phase Ⅱ trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 21: 2237-2246, 2003.

10) Ready N, et al: Inhibition of the epidermal growth factor receptor in combined modality treatment for locally advanced non-small cell lung cancer. Semin Oncol 32: S35-41, 2005.

11) Bonner J A, et al: Radiotherapy plus cetuximab

for squamous–cell carcinoma of the head and neck. N Engl J Med 354 (6): 567–578, 2006.

12) Rischin D, et al: Phase I trial of gefitinib (ZD1839) in combination with concurrent carboplatin, paclitaxel and radiation therapy in patients with stage III non–small cell lung cancer. Proc Am Soc Clin Oncol 23: 635s, 2004.

13) Ready N, et al: Initial cohort toxicity evaluation for chemoradiotherapy (CRT) and ZD1839 in stage III non–small cell lung cancer (NSCLC): A CALGB stratified phase II trial. Proc Am Soc Clin Oncol 23: 635s, 2004.

14) Kelly K, et al: Low incidence of pneumonitis on SWOG 0023: A preliminary analysis of an ongoing phase III trial of concurrent chemoradiotherapy followed by consolidation docetaxel and Iressa/placebo maintenance in patients with inoperative stage III non–small cell lung cancer. Proc Am Soc Clin Oncol 23: 634s, 2005.

15) Kelly K, et al: Updated analysis of SWOG 0023: A randomized phase III trial of gefitinib versus placebo maintenance after definitive chemoradi-ation followed by docetaxel in patients with locally advanced stage III non–small cell lung cancer. Proc Am Soc Clin Oncol: 388s; 7513, 2007.

16) Bebb G, et al: Preliminary results of an escalating dose phase I/II clinical trial of the anti EGFR monoclonal antibody nimotuzumab in combination with external radiotherapy in patients diagnosed with stage IIB, III or IV NSCLC unsuitable for radical therapy. J Thorac Oncol 2 (8): S617, 2007.

17) Hughes S, et al: Safety study of induction chemotherapy followed by synchronous radiotherapy (RT) and cetuximab in stage III non–small cell lung cancer (NSCLC): SCRATCH study (Cohort I). J Thorac Oncol 2 (8): S642–643, 2007.

18) Martinez E, et al: Randomized phase II trial of radiotherapy with or without erlotinib in patients with locally advanced or unresectable non small cell lung cancer. J Thorac Oncol 2 (8): S727, 2007.

Molecular Targeting Therapy in Chemo–radiotherapy in Unresectable
Locally Advanced Non–Small Cell Lung Cancer

Koji Hikino, Yuichiro Ohe
Department of Internal Medicine, National Cancer Center Hospital

ORIGINAL PAPER

Epidermal growth factor receptor gene amplification and gefitinib sensitivity in patients with recurrent lung cancer

Hidefumi Sasaki · Katsuhiko Endo · Katsuhiro Okuda · Osamu Kawano · Naoto Kitahara · Hisaichi Tanaka · Akihide Matsumura · Keiji Iuchi · Minoru Takada · Masaaki Kawahara · Tomoya Kawaguchi · Haruhiro Yukiue · Tomoki Yokoyama · Motoki Yano · Yoshitaka Fujii

Abstract To evaluate the epidermal growth factor receptor (EGFR) protein expression, gene mutations and amplification as predictors of clinical outcome in patients with non-small-cell lung cancer (NSCLC) receiving gefitinib, we have performed fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC). We investigated the EGFR amplification and EGFR protein expression statuses in 27 surgically treated non-small-cell lung cancer (NSCLC) cases. These patients experienced relapse after surgery and received gefitinib 250 mg/day. The presence or absence of EGFR mutations of kinase domains was analyzed by genotyping analysis and sequences, and already reported. EGFR mutations were found from 15/27 lung cancer patients. EGFR mutation status was significantly correlated with better prognosis (log-rank test $P = 0.0023$). Smoking status (never smoker vs. smoker, $P = 0.0032$), and pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, $P = 0.0011$), but not EGFR amplification ($P = 0.1278$), were correlated with survival of lung cancers. EGFR IHC results were correlated with FISH results ($P = 0.0125$), but not correlated with prognosis

H. Sasaki (✉) · K. Endo · K. Okuda · O. Kawano · H. Yukiue ·
T. Yokoyama · M. Yano · Y. Fujii
Department of Surgery II,
Nagoya City University Medical School,
1 Kawasumi, Mizuho-cho, Mizuho-ku,
Nagoya 467-8601, Japan
e-mail: hisasaki@med.nagoya-cu.ac.jp

N. Kitahara · H. Tanaka · A. Matsumura · K. Iuchi
Department of Surgery,
Kinki-chuo Chest Medical Center, Sakai, Japan

M. Takada · M. Kawahara · T. Kawaguchi
Department of Internal Medicine,
Kinki-chuo Chest Medical Center, Sakai, Japan

($P = 0.7921$). Thus, the EGFR gene amplification or protein expression is not a predictor of gefitinib efficacy in Japanese patients with NSCLC. We have also evaluated the EGFR mutation status and clinico-pathological features for 27 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. The EGFR mutation status, especially exon19 mutation was correlated with good response to gefitinib than exon 21 point mutation.

Introduction

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy (Ginsberg et al. 1993). There are much accumulated evidences that epidermal growth factor receptor (EGFR) and its family member are strongly implicated in the development and progression of numerous human tumors, including lung cancer (Nicolson et al. 2001; Onn et al. 2004). The EGFR tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of non-small-cell lung cancer (NSCLC) since 2002. Phase II and III trials have shown partial responses in 8–12% of unselected patients with progressive NSCLC after chemotherapy (Kris et al. 2003; Thatcher et al. 2005), especially higher response in never smokers, females and Asian ethnicity (more than 20%) (Fukuoka et al. 2003; Miller et al. 2004). Two original reports showed that EGFR mutations status at ATP binding pockets in NSCLC patients was correlated with the clinico-pathological features related

Springer

to good response to gefitinib (Paez et al. 2004; Lynch et al. 2004). These EGFR mutations were predominantly found in Japanese lung cancer patients (about 25–40%) (Paez et al. 2004; Kosaka et al. 2004; Shigematsu et al. 2005; Tokumo et al. 2005; Endo et al. 2005) when compared to USA patients (about 8–10%) (Paez et al. 2004; Lynch et al. 2004; Shigematsu et al. 2005; Pao et al. 2004) or European patients (Shigematsu et al. 2005; Marchetti et al. 2005). Actually, EGFR mutations in lung cancer have been correlated with clinical response to gefitinib therapy (Paez et al. 2004; Lynch et al. 2004; Pao et al. 2004; Mitsudomi et al. 2005). On the other hands, Cappuzzo et al. (2005) reported that EGFR amplification by fluorescence in situ hybridization (FISH) and high EGFR protein expression has been associated with responsiveness to gefitinib. Takano et al. (2005) showed that both EGFR gene mutation and increased copy numbers predicted gefitinib sensitivity in patients with recurrent NSCLC. However, this Japanese report is based on polymerase chain reaction (PCR) assay.

To determine the EGFR amplification and EGFR mutation statuses and correlation with clinico-pathological features in Japanese gefitinib-treated lung carcinoma, we retrospectively performed FISH and immunohistochemistry. The findings were compared to the clinico-pathologic features of lung cancer.

Materials and methods

Patients and samples

This was a retrospective study and the study group included 27 lung cancer patients who were treated with gefitinib for their recurrent diseases after they had undergone surgery at the Department of Surgery II, Nagoya City University Medical School. Written informed consent was obtained and the institutional ethics committee of the Nagoya City University Medical School approved the study. We have also investigated EGFR mutation status for 27 NSCLC patients who were treated with gefitinib for their recurrent diseases after they had undergone surgery at the National Hospital Organization, Kinki-chuo Chest Medical Center (Endo et al. 2005). The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan. All tumor samples were immediately frozen and stored at −80°C until assayed. The clinical and pathological characteristics of the 27 lung cancer patients are as follows; 14 (67.7%) were male and 13 were female. Twenty-two (63%) were diagnosed as adenocarcinoma, and five were diagnosed as other types of carcinoma. Fourteen (52%) were never smokers and 13 were smokers.

PCR assays for EGFR and K-ras mutations

Genomic DNA was extracted using Wizard SV Genomic DNA purification Systems (Promega) according to the manufacturers' instructions. The primers and TaqMan MGB probe were designed with Primer Express 2.0 software (Applied Biosystems). The sequences of 13 allele-specific probes and primer sets used in the TaqMan PCR assay are already shown (Endo et al. 2005). The results of TaqMan PCR assays were already reported (Endo et al. 2005). K-ras codon 12/13 mutation status was investigated by direct sequencing using the primers reported by Krypuy et al. (2006). Total RNA was extracted from the lung cancer tissues using Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturers' instructions. RNA (1 µg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD, USA) with 0.5 µg oligo $(dT)_{12-16}$ (Amersham Pharmacia Biotech Inc. Piscataway, NJ, USA). The direct sequencing for EGFR genes was performed from genomic DNA (Paez et al. 2004) or cDNA (Sasaki et al. 2006). Some cases were genotyped using LightCycler (Sasaki et al. 2005) and confirmed.

FISH analysis

Tumor specimens were obtained at surgical operation and embedded in paraffin. Serial sections (6 µm) containing representative malignant cell were stained with hematoxylin and eosin. Gene copy number per cell was investigated by FISH using the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Vysis, Abbott laboratories, IL, USA) according to a published protocol (Hirsch et al. 2003). Sections were incubated at 56°C overnight, deparaffinized and dehydrated. After incubation in 2× saline sodium citrate buffer (2× SSC; pH 7.0) at 75°C for 15–25 min, sections were digested with protein K (0.25 mg/ml in 2× SSC; pH 7.0) at 37°C for 15–25 min, rinsed in 2× SSC at room temperature for 5 min, and dehydrated using ethanol in a series of increasing concentrations. The EGFR/CEP 7 probe set was applied per the manufacture's instructions onto the selected area based on the presence of tumor foci on each slide. The slides were incubated at 80°C for 8–10 min for codenaturation of chromosomal and probe DNA and then placed in a humidified chamber at 37°C for 20–24 h to allow hybridization to occur. Post hybridization washes were performed in 1.5 M urea and 0.1× SSC at 45°C for 30 min and in 2× SSC for 2 min at room temperature. Pathologist who was blinded to the patients' clinical characteristics and all other molecular variables performed FISH analysis independently. Patients were classified according to the Cappuzzo et al. (2005) criteria with ascending number of copies of the EGFR gene