original article

Table 3. Toxicity

	IP (n = 147) Grade (%)			TC (n = 148) Grade (%)			GP (n = 151) Grade (%)			NP (n = 146) Grade (%)		
	2	3	4	2	3	4	2	3	4	2	3	4
Leukocytes	42	43	5	39	42	3	40	31ᵃ	2ᵃ	25	51ᵇ	16ᵇ
Neutrophils	11	39	45	5	19	69	21	40	23ᵃ	5	16	72
Hemoglobin	42	24	7	42	13ᵃ	2ᵃ	44	22	5	43	25	5
Platelets	6	5	1	9	11	0	22	35ᵇ	0ᵇ	3	1ᵃ	0ᵃ
Febrile neutropenia	–	14	0	–	18	0	–	2ᵃ	0ᵃ	–	18	0
Nausea	32	29	–	14ᶜ	11ᶜ	–	35	23	–	33ᶜ	14ᶜ	–
Vomiting	38	13	0	17ᶜ	5ᶜ	0ᶜ	34	14	0	29ᶜ	7ᶜ	0ᶜ
Anorexia	30	33	2	15ᶜ	17ᶜ	1ᶜ	31	26	1	29ᶜ	20c	1ᶜ
Fatigue	27	12	1	26	2	1	17ᶜ	3ᶜ	0ᶜ	23ᶜ	3ᶜ	0ᶜ
Diarrhea	33	15	1	4ᶜ	3ᶜ	0ᶜ	7ᶜ	2ᶜ	0ᶜ	8ᶜ	4ᶜ	0ᶜ
Constipation	27	7	0	30	8	0	33	9	0	40ᵈ	14ᵈ	0ᵈ
Neuropathy, motor	1	0	0	1	1	1	0	0	0	0	0	0
Neuropathy, sensory	1	0	0	14ᵈ	3ᵈ	0ᵈ	0	0	0	0	0	0
Alopecia	31	–	–	45ᵈ	–	–	15ᶜ	–	–	9ᶜ	–	–
Arthralgia	2	0	0	20ᵈ	2ᵈ	0ᵈ	0	0	0	1	0	0
Myalgia	1	0	0	16ᵈ	2ᵈ	0ᵈ	0	0	0	1	1	0
Injection site reaction	5	0	–	5	0	–	5	0	–	27ᵈ	0ᵈ	–
Pneumonitis	0	1	1	0	1	0	0	0	0	0	1	0
Creatinine	8	1	0	2ᶜ	0ᶜ	0ᶜ	7	0	0	8	1	0
AST	7	1	1	5	1	0	6	3	0	1	3	0
Fever	2	0	0	5	1	0	1	0	0	1	0	0
Treatment-related death	3 (2.0%)			1 (0.7%)			0			1 (0.7%)		

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

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

quality of life

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

discussion

Many randomized phase III studies have compared platinum-plus-new-agent doublets in NSCLC, but, this is the first to evaluate the efficacy of an irinotecan-containing regimen in comparison with other platinum-plus-new-agent doublets in NSCLC [14–17]. Although non-platinum-containing chemotherapy regimens are used as alternatives, doublets of platinum and a new-generation anticancer agent, such as TC, GP, and NP, are considered standard chemotherapy regimens for advanced NSCLC worldwide [13–17, 25]. Although the non-

inferiority of none of the three experimental regimens could be confirmed in this study, no statistically significant differences in response rate, OS, TTP, or TTTF were observed between the reference regimen and the experimental regimens. All four platinum-based doublets have similar efficacy against advanced NSCLC but different toxicity profiles. Nevertheless, IP was still regarded as the reference regimen in this study because the non-inferiority of none of the three experimental regimens could be confirmed.

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

Table 4. Second-line treatment

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

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

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

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

appendix

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

Kyushu Cancer Center Hospital, Kyushu University Hospital, National Nagasaki Medical Center, Nagasaki Municipal Hospital, Nagasaki University Hospital of Medicine and Dentistry, Kumamoto Chuo Hospital, Kumamoto Regional Medical Center, NTT West Osaka Hospital.

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references

1. Cancer Statistics in Japan 2005: The Editorial Board of the Cancer Statistics in Japan. Tokyo, Japan: Foundation for Promotion of Cancer Research 2005.
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 1995; 311: 899–909.
3. Fukuoka M, Niitani H, Suzuki A et al. A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. J Clin Oncol 1992; 10: 16–20.
4. Rowinsky EK, Donehower RC. Paclitaxel (taxol). N Engl J Med 1995; 332: 1004–1014.
5. Gelmon K. The taxoids: paclitaxel and docetaxel. Lancet 1994; 344: 1267–1272.
6. Hertel LW, Border GB, Kroin JS et al. Evaluation of the antitumor activity of gemcitabine (2',2'-difluoro-2'-deoxycytidine). Cancer Res 1990; 50: 4417–4422.
7. Binet S, Fellous A, Lataste H et al. Biochemical effects of navelbine on tubulin and associated proteins. Semin Oncol 1989; 16 (2 Suppl 4): 9–14.
8. Kubota K, Watanabe K, Kunitoh H et al. Phase III randomized trial of docetaxel plus cisplatin versus vindesine plus cisplatin in patients with stage IV non-small-cell lung cancer: the Japanese Taxotere Lung Cancer Study Group. J Clin Oncol 2004; 22: 254–261.
9. Le Chevalier T, Brisgand D, Douillard JY 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: 360–367.
10. Belani CP, Lee JS, Socinski MA 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: 1069–1075.
11. Yana T, Takada M, Origasa H et al. New chemotherapy agent plus platinum for advanced non-small cell lung cancer: a meta-analysis. Proc Am Soc Clin Oncol 2002; 21: 328a.
12. Baggstrom MQ, Socinski MA, Hensing TA et al. Third generation chemotherapy regimens (3GR) improve survival over second generation regimens (2GR) in stage IIIB/IV non-small cell lung cancer (NSCLC): a meta-analysis of the published literature. Proc Am Soc Clin Oncol 2002; 21: 306a.

13. Hotta K, Matsuo K, Ueoka H et al. Addition of platinum compounds to a new agent in patients with advanced non-small-cell lung cancer: a literature based meta-analysis of randomised trials. Ann Oncol 2004; 15: 1782–1789.

14. Kelly K, Crowley J, Bunn PA 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: 3210–3218.

15. Schiller JH, Harrington D, Belani CP et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 2002; 346: 92–98.

16. Scagliotti GV, De Marinis F, Rinaldi M et al. Phase III randomized trial comparing three platinum-based doublets in advanced non-small-cell lung cancer. J Clin Oncol 2002; 20: 4285–4291.

17. Fossella F, Pereira JR, von Pawel J 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: 3016–3024.

18. Negoro S, Masuda N, Takada Y et al. Randomised phase III trial of irinotecan combined with cisplatin for advanced non-small-cell lung cancer. Br J Cancer 2003; 88: 335–341.

19. Niho S, Nagao K, Nishiwaki Y et al. Randomized multicenter phase III trial of irinotecan (CPT-11) and cisplatin (CDDP) versus CDDP and vindesine (VDS) in patients with advanced non-small cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 1999; 18: 492a.

20. Fukuoka M, Nagao K, Ohashi Y et al. Impact of irinotecan (CPT-11) and cisplatin (CDDP) on survival in previously untreated metastatic non-small cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 2000; 19: 495a.

21. Therasse P, Arbuck SG, Eisenhauer EA et al. New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 2000; 92: 205–216.

22. Cella DF, Bonomi AE, Lloyd SR et al. Reliability and validity of the Functional Assessment of Cancer Therapy-Lung (FACT-L) quality of life instrument. Lung Cancer 1995; 12: 199–220.

23. Kurihara M, Shimizu H, Tsuboi K et al. Development of quality of life questionnaire in Japan: quality of life assessment of cancer patients receiving chemotherapy. Psychooncology 1999; 8: 355–363.

24. Matsumoto T, Ohashi Y, Morita S et al. The quality of life questionnaire for cancer patients treated with anticancer drugs (QOL-ACD): validity and reliability in Japanese patients with advanced non-small-cell lung cancer. Qual Life Res 2002; 11: 483–493.

25. Pfister DG, Johnson DH, Azzoli CG et al. American Society of Clinical Oncology treatment of unresectable non-small-cell lung cancer guideline: update 2003. J Clin Oncol 2004; 22: 330–353.

26. Gandara DR, Ohe Y, Kubota K et al. Japan-SWOG common arm analysis of paclitaxel/carboplation 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: 618a.

27. Shepherd FA, Dancey J, Ramlau R et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000; 18: 2095–2103.

28. Fossella FV, DeVore R, Kerr RN et al. Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-Small Cell Lung Cancer Study Group. J Clin Oncol 2000; 18: 2354–2362.

29. Kris MG, Natale RB, Herbst RS et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003; 290: 2149–2158.

30. 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). Clin Oncol 2003; 21: 2237–2246.

31. 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.

32. 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: 6829–6837.

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.

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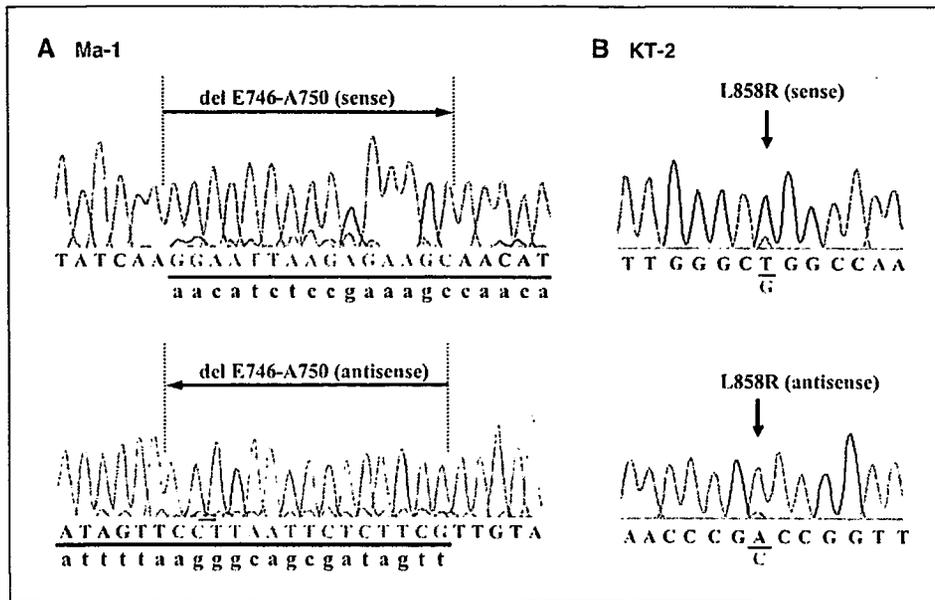
Figure 1. Detection of *EGFR* mutations in NSCLC cell lines. The portions of the sequencing electrophoretograms corresponding to the mutations are shown for Ma-1 (*A*) and KT-2 (*B*) cells. *A*, heterozygous in-frame deletion in exon 19 is revealed by the presence of double peaks. Tracings in both sense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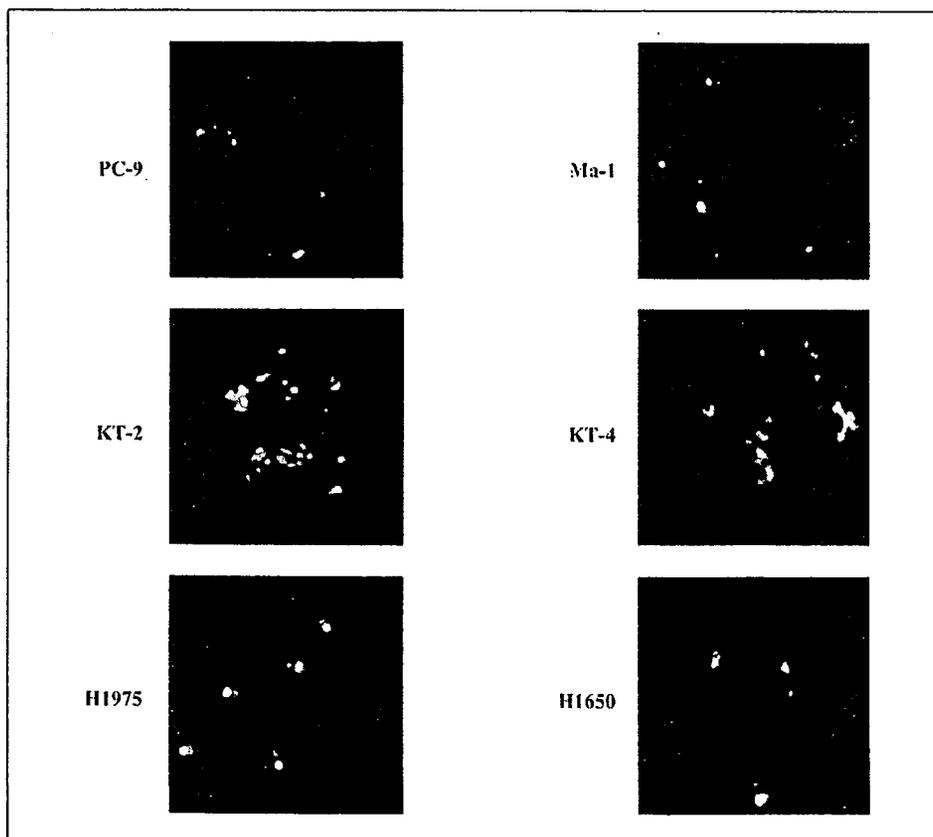

Figure 2. FISH analysis of *EGFR* amplification in NSCLC cell lines. The analysis was done with probes specific for *EGFR* (*red signals*) and for the centromere of chromosome 7 (*green signals*) in the indicated cell lines. PC-9 and Ma-1 cells manifest an *EGFR*/chromosome copy number ratio of ≥2, whereas 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).
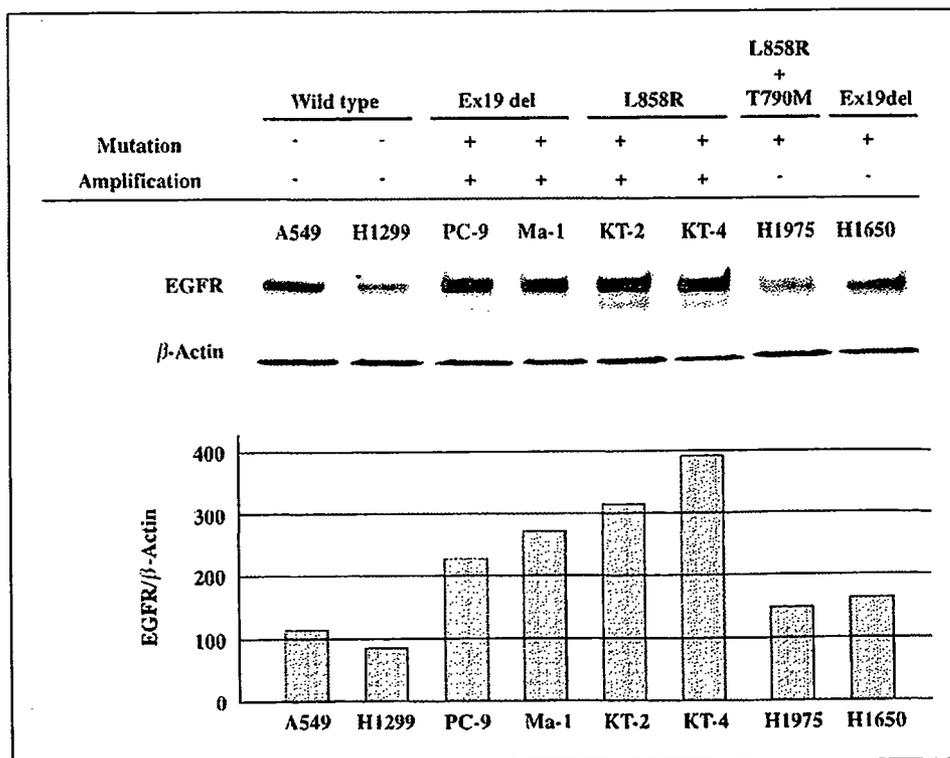
We next examined the 16 NSCLC cell lines for the presence of *EGFR* amplification by FISH analysis with a probe specific for

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 ($P < 0.05$, x^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.
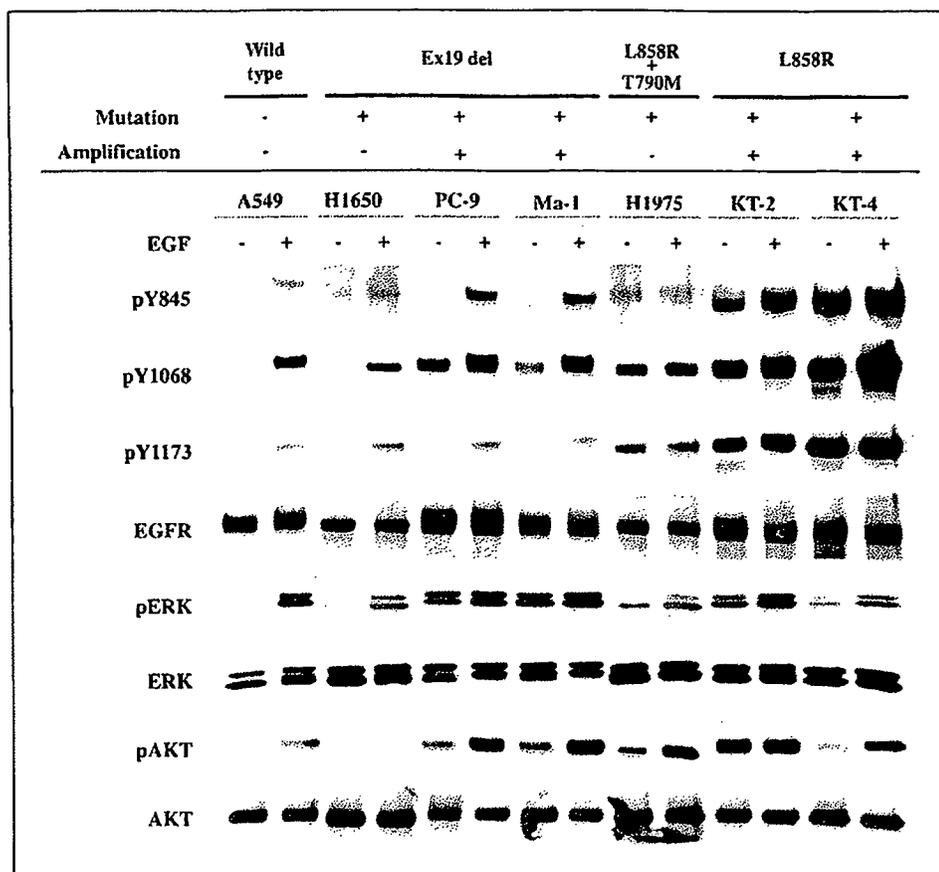

	Wild type		Ex19 del		L858R		L858R + T790M	Ex19del
Mutation	-	-	+	+	+	+	+	+
Amplification	-	-	+	+	+	+	-	-
	A549	H1299	PC-9	Ma-1	KT-2	KT-4	H1975	H1650

	Wild type	Ex19 del			L858R + T790M	L858R	
Mutation	-	+	+	+	+	+	+
Amplification	-	-	+	+	-	+	+

	A549	H1650	PC-9	Ma-1	H1975	KT-2	KT-4
EGF	- +	- +	- +	- +	- +	- +	- +
pY845							
pY1068							
pY1173							
EGFR							
pERK							
ERK							
pAKT							
AKT							

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. 6A). 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. 6B). 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 ($\geq$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.
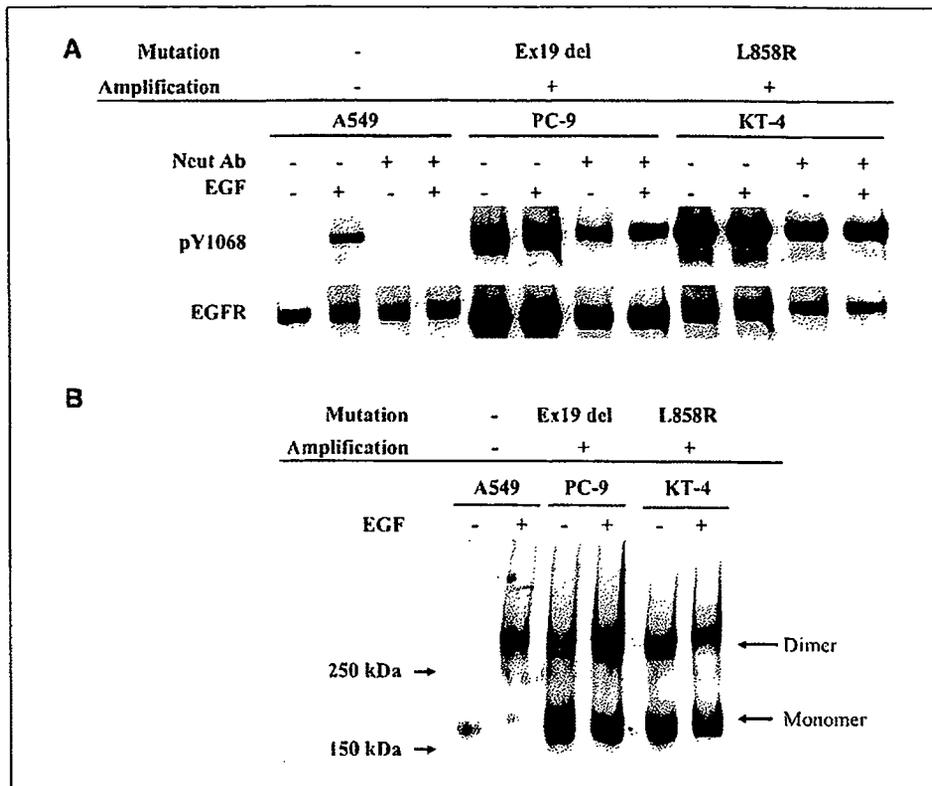
	Wild type	Ex19 del		L858R	
Mutation	-	+	+	+	+
Amplification	-	+	+	+	+

	A549	PC-9	Ma-1	KT-2	KT-4
EGF	- +	- +	- +	- +	- +
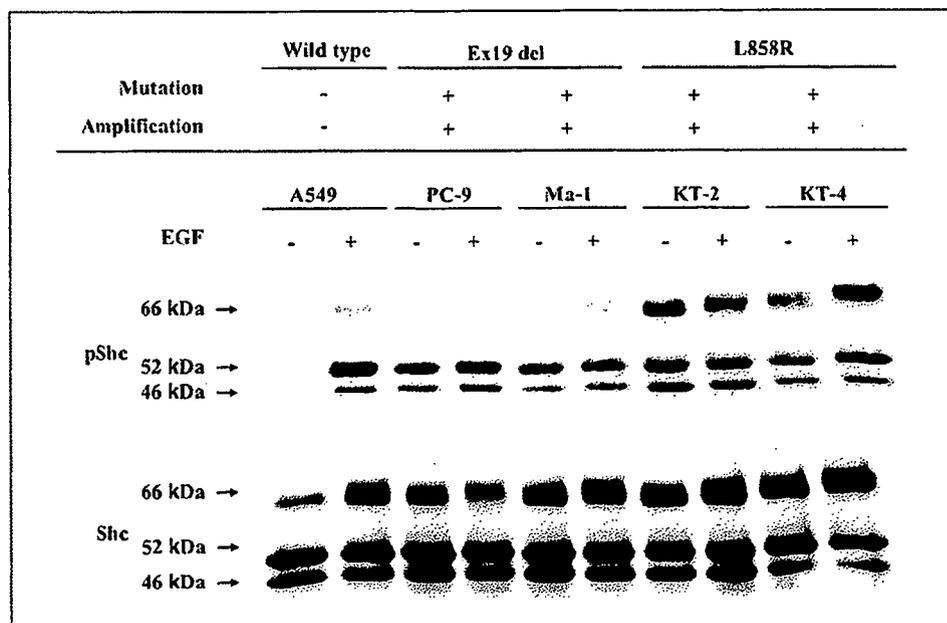
Figure 6. Mechanism of constitutive activation of EGFR in NSCLC cell lines. A, effect of neutralizing antibodies (Neut Ab) on EGFR phosphorylation. Serum-deprived NSCLC cells (A549, PC-9, or KT-4) were incubated for 3 h with a combination of neutralizing antibodies to EGF, TGF-α, and EGFR and then for 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.

Acknowledgments

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, Kid 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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Letters

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Down-regulation of survivin by ultraviolet C radiation is dependent on p53 and results in G₂–M arrest in A549 cells

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Abstract

Deregulation of survivin expression is implicated in tumorigenesis. To examine the regulation of survivin expression in response to DNA damage, we exposed A549 human lung cancer cells to ultraviolet C (UVC) radiation, which induces DNA single-strand breakage. UVC irradiation induced G₂–M arrest that was accompanied by accumulation of p53 and subsequent down-regulation of survivin. Depletion of p53 by RNA interference prevented the UVC-induced down-regulation of survivin. Furthermore, depletion of survivin resulted in G₂–M arrest, suggesting that down-regulation of survivin by p53 contributes to the p53-dependent G₂–M checkpoint triggered by DNA damage.
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1. Introduction

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

The expression of survivin is regulated in a cell cycle-dependent manner. The promoter of the survivin gene possesses features typical of genes that are expressed at G₂–M phase of the cell cycle. Indeed, survivin is most abundant in cells at G₂–M and associates with the mitotic spindle of dividing cells [2]. Survivin interacts with Aurora B and inner centromere protein (INCENP), and the complex of Aurora B–INCENP–survivin monitors the integrity of the mitotic spindle [11]. It has been suggested that survivin controls the elimination by apoptosis of cells with an improperly formed mitotic spindle [3,12]. Overexpression of survivin in cancer may overcome cell cycle checkpoints and thereby facilitate aberrant progression of

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

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

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

2. Materials and methods

2.1. Cell culture and irradiation

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

2.2. Immunoblot analysis

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

2.3. Flow cytometry

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

2.4. RNAi

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

2.5. Statistical analysis

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

3. Results

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

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

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

Given that p53 mediates cell cycle arrest at the G_2-M transition in response to DNA damage and that we recently showed that down-regulation of survivin expression follows the accumulation of p53 in cells subjected to DNA double-strand breakage [22], we next examined whether survivin and p53 are functionally linked in

A549 cells treated with UVC, which induces DNA single-strand breakage. Immunoblot analysis revealed that the abundance of p53 was increased 6 h after UVC exposure, reached a peak at 24 h, and then gradually returned to basal levels by 72 h (Fig. 2). In contrast, the amount of survivin began to decline at 48 h and its down-regulation was more pronounced at 72 h.

To determine whether p53 negatively regulates survivin expression, we examined the effect of UVC radiation on the abundance of survivin in cells depleted of p53 by RNAi. In cells transfected with a control (scrambled) siRNA or in nontransfected cells, the abundance of p53 was increased at 18 h after UVC exposure and the amount of
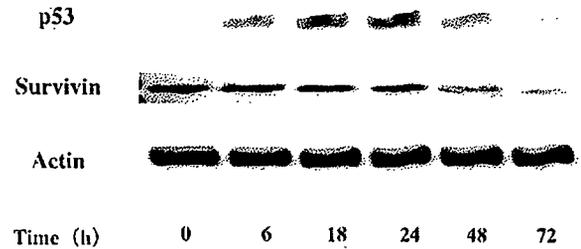
Fig. 2. Effects of UVC on the abundance of p53 and survivin in A549 cells. Total cellular protein extracted at the indicated times after exposure of cells to UVC (30 J/m²) was subjected to immunoblot analysis with antibodies to p53, to survivin, or to β-actin (loading control). Data are representative of three independent experiments.
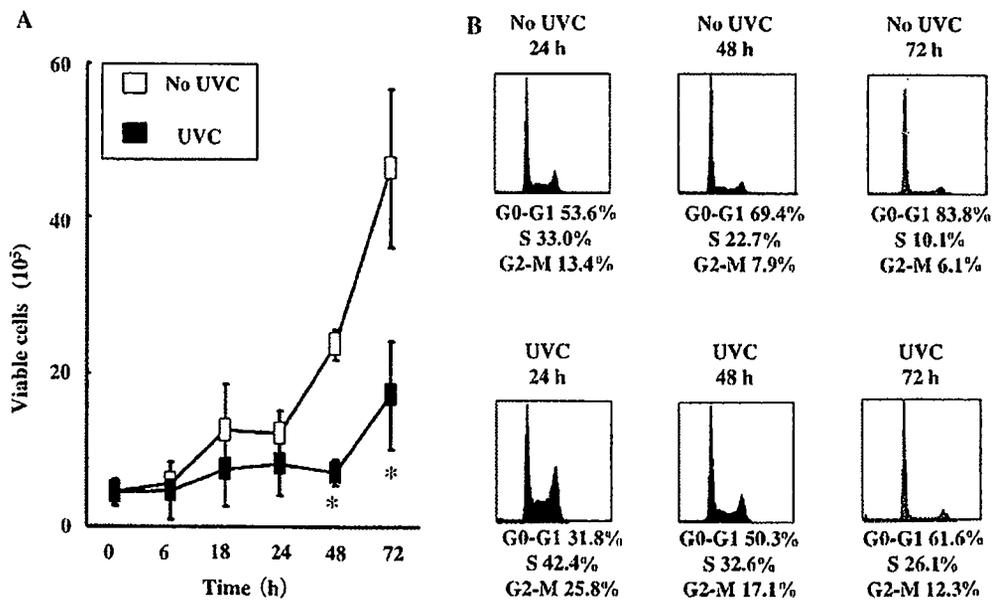
Fig. 1. Effects of UVC on the proliferation and cell cycle distribution of A549 cells. (A) Cell proliferation was evaluated by counting the number of viable cells by trypan blue staining at the indicated times after UVC irradiation (30 J/m²). Data are means ± SD of values from three independent experiments. *p < 0.05 versus the corresponding value for cells not exposed to UVC. (B) Cell cycle distribution was analyzed by propidium iodide staining and flow cytometry at 24, 48 h and 72 h after UVC exposure. The percentages of cells at various stages of the cell cycle are indicated, and the data are representative of three independent experiments.

Time after UVC exposure (h)

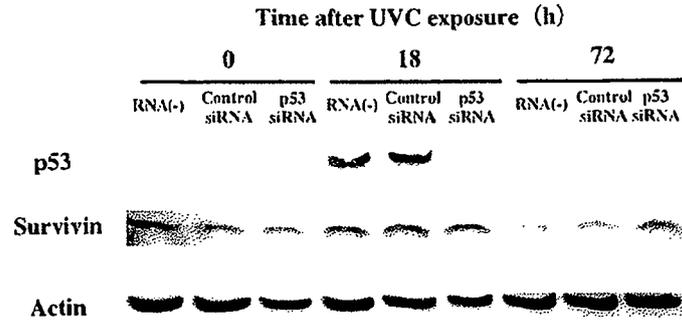|	0			18			72		
	RNA(-)	Control siRNA	p53 siRNA	RNA(-)	Control siRNA	p53 siRNA	RNA(-)	Control siRNA	p53 siRNA
p53									
Survivin									
Actin									

Fig. 3. Effect of UVC on the abundance of survivin in A549 cells depleted of p53 by RNAi. Cells were transfected (or not) with an siRNA specific for p53 mRNA or with a control (scrambled) siRNA, exposed to UVC (30 J/m²), and subjected to immunoblot analysis with antibodies to p53, to survivin, or to β-actin at the indicated times after irradiation. Data are representative of three independent experiments.

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

3.3. Ablation of survivin inhibits cell proliferation and induces G₂–M arrest

We next examined the effects of UVC irradiation in cells depleted of survivin by RNAi. The abundance of survivin was greatly reduced in cells transfected with an siRNA specific for survivin mRNA compared with that in nontransfected cells or cells transfected with a control (scrambled) siRNA (Fig. 4A). Cell proliferation (as evaluated from viable cell number) was also inhibited by 60% or 70% in cells subjected to transfection with the survivin siRNA for 48 or 72 h, respectively, compared with that apparent in nontransfected cells (Fig. 4B). The viable cell count was not affected by transfection with the control siRNA. Flow cytometry revealed that transfection of A549 cells with the survivin siRNA resulted in a marked increase in the proportion of cells in G₂–M at 48 and 72 h compared with that apparent for nontransfected cells or cells transfected with the control siRNA (Fig. 4C and D). There was no difference in the proportion of sub-G₁ cells among the three treatment groups.

4. Discussion

Several genes whose products play a role in control of the G₂–M transition of the cell cycle, including stathmin, Map4, cyclin B1, Cdc2, and Cdc25c, have been shown to be negatively regulated by p53 [15–18]. Repression of the expression of these genes in response to DNA damage requires wild-type p53 and contributes to a DNA damage-induced G₂–M

checkpoint [23,24]. Survivin, a member of the IAP family of proteins, is maximally expressed at G₂–M and physically associates with microtubules of the mitotic spindle [2]. Previous studies have suggested that the expression of survivin is also subject to negative regulation by p53 [25–27], but the mechanism of such regulation has been unclear. We have now shown that exposure of the human lung cancer cell line A549 to UVC, which induces DNA single-strand breakage, resulted in the induction of endogenous p53 and a subsequent decrease in survivin expression. These observations are consistent with those of our previous study showing that survivin expression is repressed subsequent to p53 accumulation in cells treated with adriamycin [22], which induces DNA double-strand breakage. To investigate the possible role of p53 in the down-regulation of survivin induced by DNA damage, we depleted A549 cells of p53 by RNAi. Prevention of endogenous p53 accumulation in cells irradiated with UVC was found to block the repression of survivin expression, providing direct evidence that p53 is required for this effect of UVC. These data thus constitute further support for the notion that the survivin gene is a target of negative regulation by p53 in response to DNA damage.

The time course of survivin protein repression following UVC (DNA single-strand breakage)-induced p53 accumulation was almost identical to that observed in the cells having DNA double-strand breakage [22]. These results suggest that p53-dependent survivin suppression in response to these two types of DNA damage may share the common mechanisms at transcriptional level. Hoffmann et al. proposed that direct binding of p53 to a consensus binding site in the survivin gene promoter mediates transcriptional repression of the
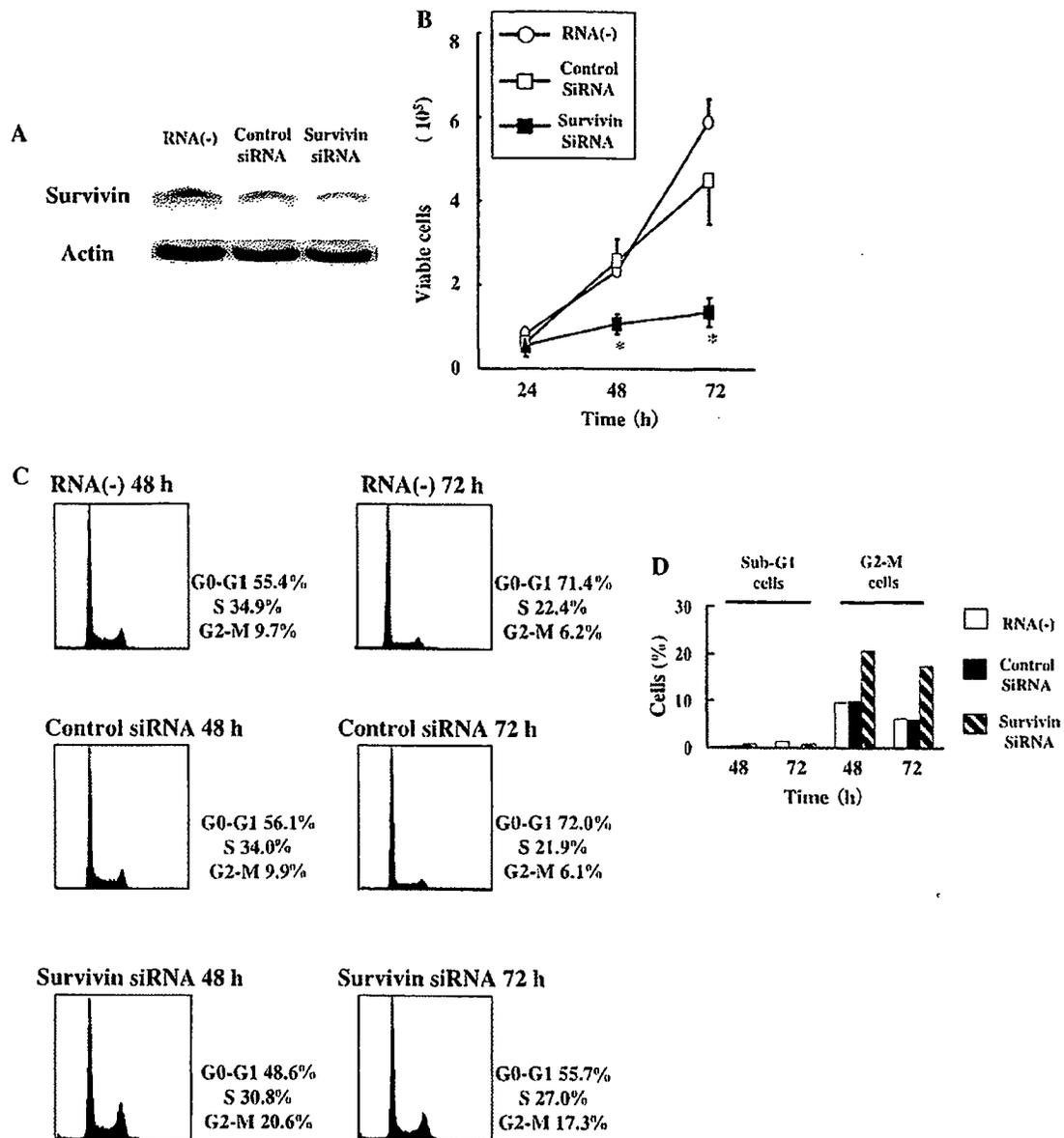
Fig. 4. Effects of survivin depletion by RNAi on the proliferation and cell cycle distribution of A549 cells. (A) Cells were transfected (or not) with a siRNA specific for survivin mRNA or with a control siRNA and were then subjected to immunoblot analysis with antibodies to survivin or to β-actin. Data are representative of three independent experiments. (B) Cells transfected for 24, 48, or 72 h as in (A) were evaluated for cell proliferation by counting the number of viable cells as revealed by staining with trypan blue. Data are means ± SD of values from three independent experiments. $*p < 0.05$ versus the corresponding value for nontransfected cells or cells transfected with the control siRNA. (C) The cell cycle distribution of cells transfected for 48 or 72 h as in (A) was determined by flow cytometry. The percentages of cells at various stages of the cell cycle are indicated. Data are representative of three independent experiments. (D) The percentages of sub-G_1 and G_2-M cells in the experiment shown in (C).

survivin gene by p53 [25]. In contrast, Mirza et al. suggested that chromatin deacetylation in the survivin promoter might contribute to p53-dependent repression of survivin gene expression in the absence of direct binding of p53 to the promoter DNA [26]. In the present study, repression of survivin expression was apparent 24 h after endogenous p53 accumulation, consistent with the results of

our previous study [22]. This delay suggests that the mechanism of transcriptional inhibition of the survivin gene by p53 may be indirect. The repression of Cdc2 gene expression by p53 is mediated by a member of the E2F family of transcription factors subsequent to up-regulation of p21 and dephosphorylation of pRB family proteins [17]. However, UV-induced accumulation of p53 and subsequent

down-regulation of survivin have been observed in mouse embryonic fibroblasts derived from p21-null mice [29], suggesting that the ability of p53 to repress survivin gene expression is independent of its ability to up-regulate p21. The molecular mechanism by which p53 induces repression of survivin gene expression in response to DNA damage thus requires further investigation.

To examine the biological consequences of survivin gene repression in cells subjected to DNA damage, we depleted A549 cells of survivin by RNAi. Depletion of survivin resulted in growth arrest in G_2–M phase of the cell cycle, consistent with previous observations [28–31]. Survivin was originally proposed to perform an antiapoptotic function, but this issue remains controversial [29,32]. Indeed, several lines of evidence suggest that survivin plays an important role in regulation of mitotic events [11]. The chromosomal passenger complex (CPC), which consists of Aurora B, INCENP, and survivin, contributes to chromosome segregation and cytokinesis [33]. Depletion or inhibition of survivin or of the other proteins of the CPC thus results in mitotic arrest [30,34]. Furthermore, G_2–M arrest induced by survivin ablation was found to occur in p53$^{+/+}$ cells but not in p53$^{-/-}$ cells, implicating survivin in the p53-dependent G_2–M checkpoint that is essential for maintenance of genomic integrity [29]. Together, these various observations suggest that p53-induced repression of survivin expression in response to DNA damage may lower the threshold for apoptosis in cells in which the p53-dependent G_2–M checkpoint has been activated. Survivin repression following DNA damage may play critical role in deciding if lethal damaged cells die before DNA repair is completed, or if they will have the opportunity to repair and survive. Further characterization of the regulation of survivin in response to DNA damage may provide the basis for potential new approaches to cancer treatment that couple standard cytotoxic DNA-damaging agents with survivin-targeted therapy.

References

[1] G. Ambrosini, C. Adida, D.C. Altieri, A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma, Nat. Med. 3 (1997) 917–921.

[2] F. Li, D.C. Altieri, Transcriptional analysis of human survivin gene expression, Biochem. J. 344 (1999) 305–311.

[3] F. Li, E.J. Ackermann, C.F. Bennett, A.L. Rothermel, J. Plescia, S. Tognin, A. Villa, P.C. Marchisio, D.C. Altieri, Pleiotropic cell-division defects and apoptosis induced by interference with survivin function, Nat. Cell Biol. 1 (1999) 461–466.

[4] M. Monzo, R. Rosell, E. Felip, J. Astudillo, J.J. Sanchez, J. Maestre, C. Martin, A. Font, A. Barnadas, A. Abad, A novel anti-apoptosis gene: re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers, J. Clin. Oncol. 17 (1999) 2100–2104.

[5] C. Adida, D. Berrebi, M. Peuchmaur, M. Reyes-Mugica, D.C. Altieri, Anti-apoptosis gene, survivin, and prognosis of neuroblastoma, Lancet 351 (1998) 882–883.

[6] A. Islam, H. Kageyama, N. Takada, T. Kawamoto, H. Takayasu, E. Isogai, M. Ohira, K. Hashizume, H. Kobayashi, Y. Kaneko, A. Nakagawara, High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma, Oncogene 19 (2000) 617–623.

[7] H. Kawasaki, D.C. Altieri, C.D. Lu, M. Toyoda, T. Tenjo, N. Tanigawa, Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer, Cancer Res. 58 (1998) 5071–5074.

[8] H. Meng, C.D. Lu, Y.L. Sun, D.J. Dai, S.W. Lee, N. Tanigawa, Expression level of wild-type survivin in gastric cancer is an independent predictor of survival, World J. Gastroenterol. 10 (2004) 3245–3250.

[9] N. Zaffaroni, M.G. Daidone, Survivin expression and resistance to anticancer treatments: perspectives for new therapeutic interventions, Drug Resist. Update 5 (2002) 65–72.

[10] K. Asanuma, R. Moriai, T. Yajima, A. Yagihashi, M. Yamada, D. Kobayashi, N. Watanabe, Survivin as a radioresistance factor in pancreatic cancer, Jpn. J. Cancer Res. 91 (2000) 1204–1209.

[11] R. Honda, R. Korner, E.A. Nigg, Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis, Mol. Biol. Cell 14 (2003) 3325–3341.

[12] F. Li, G. Ambrosini, E.Y. Chu, J. Plescia, S. Tognin, P.C. Marchisio, D.C. Altieri, Control of apoptosis and mitotic spindle checkpoint by survivin, Nature 396 (1998) 580–584.

[13] R.V. Sionov, Y. Haupt, The cellular response to p53: the decision between life and death, Oncogene 18 (1999) 6145–6157.

[14] B. Vogelstein, D. Lane, A.J. Levine, Surfing the p53 network, Nature 408 (2000) 307–310.

[15] J. Ahn, M. Murphy, S. Kratowicz, A. Wang, A.J. Levine, D.L. George, Down-regulation of the stathmin/Op18 and FKBP25 genes following p53 induction, Oncogene 18 (1999) 5954–5958.

[16] S.A. Innocente, J.L. Abrahamson, J.P. Cogswell, J.M. Lee, p53 regulates a G2 checkpoint through cyclin B1, Proc. Natl. Acad. Sci. USA 96 (1999) 2147–2152.

[17] W.R. Taylor, A.H. Schonthal, J. Galante, G.R. Stark, p130/ E2F4 binds to and represses the cdc2 promoter in response to p53, J. Biol. Chem. 276 (2001) 1998–2006.

[18] R. Zhao, K. Gish, M. Murphy, Y. Yin, D. Notterman, W.H. Hoffman, E. Tom, D.H. Mack, A.J. Levine, Analysis of p53-regulated gene expression patterns using oligonucleotide arrays, Genes Dev. 14 (2000) 981–993.

[19] C.D. Lu, D.C. Altieri, N. Tanigawa, Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas, Cancer Res. 58 (1998) 1808–1812.

[20] A.I. Sarela, C.S. Verbeke, J. Ramsdale, C.L. Davies, A.F. Markham, P.J. Guillou, Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma, Br. J. Cancer 86 (2002) 886–892.

[21] J. Nakano, C.L. Huang, D. Liu, M. Ueno, S. Sumitomo, H. Yokomise, survivin gene expression is negatively regulated by the p53 tumor suppressor gene in non-small cell lung cancer, Int. J. Oncol. 27 (2005) 1215–1221.

[22] K. Yonesaka, K. Tamura, T. Kurata, T. Satoh, M. Ikeda, M. Fukuoka, K. Nakagawa, Small interfering RNA targeting survivin sensitizes lung cancer cell with mutant p53 to adriamycin, Int. J. Cancer 118 (2005) 812–820.

[23] C. Badie, J.E. Itzhaki, M.J. Sullivan, A.J. Carpenter, A.C. Porter, Repression of CDK1 and other genes with CDE and CHR promoter elements during DNA damage-induced G$_2$/M arrest in human cells, Mol. Cell. Biol. 20 (2000) 2358–2366.

[24] W.R. Taylor, G.R. Stark, Regulation of the G2/M transition by p53, Oncogene 20 (2001) 1803–1815.

[25] W.H. Hoffman, S. Biade, J.T. Zilfou, J. Chen, M. Murphy, Transcriptional repression of the anti-apoptotic survivin gene by wild type p53, J. Biol. Chem. 277 (2002) 3247–3257.

[26] A. Mirza, M. McGuirk, T.N. Hockenberry, Q. Wu, H. Ashar, S. Black, S.F. Wen, L. Wang, P. Kirschmeier, W.R. Bishop, L.L. Nielsen, C.B. Pickett, S. Liu, Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway, Oncogene 21 (2002) 2613–2622.

[27] M. Kappler, H. Taubert, F. Bartel, K. Blumke, M. Panian, H. Schmidt, J. Dunst, M. Bache, Radiosensitization, after a combined treatment of survivin siRNA and irradiation, is correlated with the activation of caspases 3 and 7 in a wt-p53 sarcoma cell line, but not in a mt-p53 sarcoma cell line, Oncol. Rep. 13 (2005) 167–172.

[28] M. Kappler, M. Bache, F. Bartel, M. Kotzsch, M. Panian, P. Wurl, K. Blumke, H. Schmidt, A. Meye, H. Taubert, Knockdown of survivin expression by small interfering RNA reduces the clonogenic survival of human sarcoma cell lines independently of p53, Cancer Gene Ther. 11 (2004) 186–193.

[29] E. Beltrami, J. Plescia, J.C. Wilkinson, C.S. Duckett, D.C. Altieri, Acute ablation of survivin uncovers p53-dependent mitotic checkpoint functions and control of mitochondrial apoptosis, J. Biol. Chem. 279 (2004) 2077–2084.

[30] A. Carvalho, M. Carmena, C. Sambade, W.C. Earnshaw, S.P. Wheatley, Survivin is required for stable checkpoint activation in taxol-treated HeLa cells, J. Cell Sci. 116 (2003) 2987–2998.

[31] S.M. Lens, R.M. Wolthuis, R. Klompmaker, J. Kauw, R. Agami, T. Brummelkamp, G. Kops, R.H. Medema, Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension, EMBO J. 22 (2003) 2934–2947.

[32] J.C. Reed, The Survivin saga goes in vivo, J. Clin. Invest. 108 (2001) 965–969.

[33] M.A. Bolton, W. Lan, S.E. Powers, M.L. McCleland, J. Kuang, P.T. Stukenberg, Aurora B kinase exists in a complex with survivin and INCENP and its kinase activity is stimulated by survivin binding and phosphorylation, Mol. Biol. Cell 13 (2002) 3064–3077.

[34] S. Hauf, R.W. Cole, S. LaTerra, C. Zimmer, G. Schnapp, R. Walter, A. Heckel, J. van Meel, C.L. Rieder, J.M. Peters, The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint, J. Cell Biol. 161 (2003) 281–294.

The novel microtubule-interfering agent TZT-1027 enhances the anticancer effect of radiation in vitro and in vivo

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TZT-1027 is a novel anticancer agent that inhibits microtubule polymerisation and manifests potent antitumour activity in preclinical models. We have examined the effect of TZT-1027 on cell cycle progression as well as the anticancer activity of this drug both in vitro and in vivo. With the use of tsFT210 cells, which express a temperature-sensitive mutant of Cdc2, we found that TZT-1027 arrests cell cycle progression in mitosis, the phase of the cell cycle most sensitive to radiation. A clonogenic assay indeed revealed that TZT-1027 increased the sensitivity of H460 cells to γ-radiation, with a dose enhancement factor of 1.2. Furthermore, TZT-1027 increased the radiosensitivity of H460 and A549 cells in nude mice, as revealed by a marked delay in tumour growth and an enhancement factor of 3.0 and 2.2, respectively. TZT-1027 also potentiated the induction of apoptosis in H460 cells by radiation both in vitro and in vivo. Histological evaluation of H460 tumours revealed that TZT-1027 induced morphological damage to the vascular endothelium followed by extensive central tumour necrosis. Our results thus suggest that TZT-1027 enhances the antitumour effect of ionising radiation, and that this action is attributable in part to potentiation of apoptosis induction and to an antivascular effect. Combined treatment with TZT-1027 and radiation therefore warrants investigation in clinical trials as a potential anticancer strategy.
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Keywords: TZT-1027; radiosensitisation; microtubule; mitotic arrest; apoptosis; antivascular effect

The combination of modalities of cancer treatment offers improvements in the survival of cancer patients compared with individual therapeutic approaches. Such therapeutic benefit has been achieved with combinations of chemo- and radiotherapy in a variety of cancers. The cytotoxicity of most chemotherapeutic agents as well as that of radiation is highly dependent on the phase of the cell cycle. Although various types of anticancer drugs are able to arrest cells at specific cell cycle checkpoints, the ability of antimicrotubule agents to block cell cycle progression in G_2-M phase is the biological basis for combination of these agents with radiation (Pawlik and Keyomarsi, 2004). Microtubule-interfering agents have been shown to increase the radiosensitivity of tumour cells in preclinical and clinical studies (Liebmann et al, 1994; Choy et al, 1995; Edelstein et al, 1996; Vokes et al, 1996; Kim et al, 2001, 2003; Hofstetter et al, 2005; Simoens et al, 2006).

TZT-1027 (Soblidotin), a novel microtubule-interfering agent synthesised from dolastatin 10 (Figure 1), exhibits greater antitumour activities and a reduced toxicity compared with its parent compound (Miyazaki et al, 1995). TZT-1027 inhibits microtubule assembly by binding to tubulin (Kobayashi et al, 1997; Natsume et al, 2000). In vitro, it inhibits the growth of various human cancer cells at low concentrations (Watanabe et al, 2000). In vivo, TZT-1027 also manifests a broad spectrum of activity against various murine tumours as well as human tumour xenografts, without inducing a pronounced reduction in body weight (Kobayashi et al, 1997; Watanabe et al, 2000, 2006a; Natsume et al, 2003, 2006). Furthermore, the drug exhibited a potent antivascular effect on existing vasculature in an advanced-stage tumour model (Otani et al, 2000). TZT-1027 is currently undergoing clinical evaluation, with a reduction in tumour size and disease stabilisation having been observed in a subset of patients (Schoffski et al, 2004; de Jonge et al, 2005; Greystoke et al, 2006; Tamura et al, 2007).

Despite its demonstrated efficacy against solid tumours, the effects of TZT-1027 in combination with radiation have not been examined. As an initial step in determining the antitumour activity of TZT-1027 in combination with radiation, we investigated the effect of this agent on cell cycle progression in synchronised tsFT210 cells (Osada et al, 1997), which harbour a temperature-sensitive mutant of Cdc2. We found that TZT-1027 induces arrest of cells in mitosis, the phase of the cell cycle most sensitive to radiation. We then studied the radiosensitising properties of TZT-1027 in vitro and in vivo with a human lung cancer model and elucidated the mechanism of radiosensitisation by this agent.

MATERIALS AND METHODS

Cell lines and reagents

tsFT210 mouse mammary carcinoma cells, which express a temperature-sensitive mutant of Cdc2, were kindly provided by H Kakeya (Antibiotics Laboratory, Discovery Research Institute, RIKEN, Saitama, Japan) and were maintained under a humidified atmosphere of 5% CO_2 in air at 32.0°C in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. H460 human lung large cell carcinoma and A549 human lung adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained as for tsFT210 cells with the exception that the culture temperature was 37°C. TZT-1027 (Figure 1) was provided by Daiichi Pharmaceutical Co. Ltd (Tokyo, Japan). Nocodazole and roscovitine were obtained from Sigma.

Cell cycle analysis

Cells were harvested, washed with phosphate-buffered saline (PBS), fixed with 70% methanol, washed again with PBS, and stained with propidium iodide $(0.05 \, \text{mg} \, \text{ml}^{-1})$ in a solution containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase A $(0.05 \, \text{mg} \, \text{ml}^{-1})$. The stained cells $(\sim 1 \times 10^6)$ were then analysed for DNA content with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA, USA).

Measurement of mitotic index and apoptotic cells

Cells were harvested, washed with PBS, fixed with methanol : acetic acid (3 : 1, v/v), washed again with PBS, and stained with 4′,6-diamidino-2-phenylindole (DAPI) $(0.5 \, \mu\text{g} \, \text{ml}^{-1})$. The stained cells $(\sim 1 \times 10^6)$ were observed with a fluorescence microscope (IX71; Olympus, Tokyo, Japan). To determine the proportion of mitotic or apoptotic cells, we scored at least 300 cells in each of at least three randomly selected microscopic fields for each of three slides per sample. Cells with condensed chromosomes and no obvious nuclear membrane were regarded as mitotic cells, and the mitotic index was calculated as the percentage of mitotic cells among total viable cells. Cells with fragmented and uniformly condensed nuclei were regarded as apoptotic cells.

Clonogenic assay

Exponentially growing H460 cells in 25-cm^2 flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities and plated in triplicate in 25-cm^2 flasks containing 10 ml of medium. The cells were treated with 1 nM TZT-1027 or vehicle (dimethyl sulfoxide, or DMSO; final concentration, 0.1%) for 24 h and then exposed to various doses of γ-radiation with a ^{60}Co irradiator at a rate of $\sim 0.82 \, \text{Gy} \, \text{min}^{-1}$ and at room temperature. The cells were then washed with PBS, cultured in drug-free medium for 10 – 14 days, fixed with methanol : acetic acid (10 : 1, v/v), and stained with crystal violet. Colonies containing > 50 cells were counted. The surviving fraction was calculated as: (mean number of colonies)/(number of inoculated cells × plating

efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for nonirradiated controls. The surviving fraction for combined treatment was corrected by that for TZT-1027 treatment alone. The dose enhancement factor (DEF) was calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.1 for vehicle-treated cells divided by that for TZT-1027-treated cells (after correction for drug toxicity).

In vivo antitumour activity of TZT-1027 with or without radiation

All animal studies were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. The ethical guidelines followed meet the requirements of the UKCCCR guidelines (Workman et al, 1998). Tumour cells (2×10^6) were injected subcutaneously into the right hind leg of 7-week-old female athymic nude mice. Tumour volume was determined from caliper measurement of tumour length (L) and width (W) according to the formula $LW^2/2$. Treatment was initiated when tumours in each group achieved an average volume of $\sim 200-250 \, \text{mm}^3$. Treatment groups consisted of control, TZT-1027 alone, radiation alone, and the combination of TZT-1027 and radiation. Each treatment group contained six to eight mice. TZT-1027 was administered intravenously in a single dose of 0.5 mg kg^{-1} of body weight; mice in the control and radiation-alone groups were injected with vehicle (physiological saline). Tumours in the leg were exposed to 10 Gy of γ-radiation with a ^{60}Co irradiator at a rate of $\sim 0.32 \, \text{Gy} \, \text{min}^{-1}$ immediately after drug treatment. Growth delay (GD) was calculated as the time for treated tumours to achieve an average volume of 500 mm^3 minus the time for control tumours to reach 500 mm^3. The enhancement factor was then determined as: $(GD_{\text{combination}} - GD_{\text{TZT}-1027})/(GD_{\text{radiation}})$.

TUNEL staining

Mice were killed 14 days after treatment initiation and the tumours were removed and preserved in 10% paraformaldehyde. Apoptosis in tumour sections was determined by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) assay with the use of an apoptosis detection kit (Chemicon, Temecula, CA, USA). The number of apoptotic cells was counted in 10 separate microscopic fields (× 100) for three sections of each tumour of each group.

Histological analysis

A single dose of TZT-1027 (2.0 mg kg^{-1}) or vehicle (physiological saline) was administered intravenously to mice when H460 tumours had achieved a volume of ~ 400 to 600 mm^3. Tumour tissue was extirpated 4 or 24 h after drug administration, and half of the tissue was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The other half of the tumour tissue was fixed for 12 – 48 h in zinc fixative
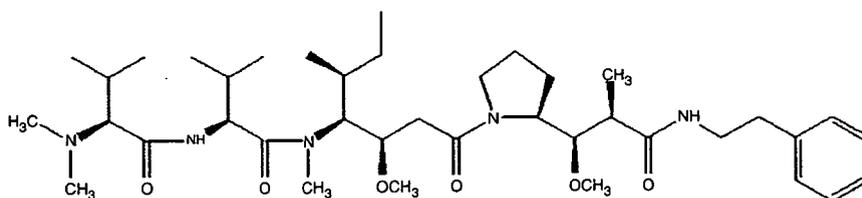

Figure I Chemical structure of TZT-1027.