gefitinib or erlotinib (Tarceva; OSI Pharmaceuticals Inc, Melville, NY), another EGFR-TKI, in patients with advanced NSCLC. The Iressa NSCLC Trial Assessing Combination Treatment (INTACT)–1, INTACT-2, Tarceva Responses in Conjunction with Paclitaxel and Carboplatin (TRIBUTE), and Tarceva Lung Cancer Investigation (TALENT) trials tested the concurrent combination of platinum-based chemotherapy and EGFR-TKIs in a first-line setting but failed to show a survival benefit from the addition of the EGFR-TKIs.[10-13] The Iressa Survival Evaluation in Lung Cancer (ISEL) trial tested the role of second- or third-line gefitinib monotherapy but also failed to show a significant survival benefit over a placebo,[14] whereas the BR.21 trial showed a significant survival benefit of second- or third-line erlotinib monotherapy.[15] The Iressa NSCLC Trial Evaluating Response and Survival against Taxotere (INTEREST) and V15-32 trials compared OS after second-line gefitinib monotherapy and docetaxel monotherapy, which is a standard second-line treatment; the former study proved the noninferiority of gefitinib to docetaxel, whereas the latter study failed to do so.[16,17]

In subgroup analyses of some of these trials, significant survival benefits were observed for never-smokers[12,14] and Asian patients.[14] In the BR.21 trial, no history of smoking was a significant predictor of a survival benefit from erlotinib.[15] Because never-smokers and Asian patients are known to have higher frequencies of *EGFR* mutations,[4-9,18,19] these results suggested an association between *EGFR* mutations and a survival benefit from EGFR-TKIs. However, in all of these trials, mutational analyses failed to show a significant survival benefit from EGFR-TKIs in *EGFR*-mutant patients,[20-23] partly because of the small sample sizes that were used.

In the INTACT and TRIBUTE trials, patients with *EGFR* mutations lived longer than those without *EGFR* mutations, irrespective of treatment with EGFR-TKIs[20,21]; this result suggested that *EGFR* mutations may have prognostic value in patients with advanced NSCLC who were treated with standard chemotherapy. However, these trials were inconclusive regarding this point because of the small number of *EGFR*-mutant patients who were examined. As for early-stage NSCLC patients, several large-scale retrospective studies have been reported; some studies showed no significant association between the presence of *EGFR* mutations and OS after surgery,[19,24] whereas others showed that the presence of *EGFR* mutations was associated with a favorable prognosis in a univariate analyses, but the association disappeared when adjustments for patient characteristics like sex and smoking history were made.[25,26]

To evaluate whether gefitinib provides a survival benefit to patients with lung adenocarcinoma and whether the mutational status of *EGFR* is a predictor of a survival benefit from gefitinib and/or a prognostic factor, we analyzed data obtained on patients with advanced lung adenocarcinoma who were treated before and after gefitinib approval.

PATIENTS AND METHODS

Patients

We performed all the analyses in this study using a protocol approved by the institutional review board of the National Cancer Center Hospital (NCCH; Tokyo, Japan). Consecutive patients with advanced lung adenocarcinoma who had been pathologically diagnosed at NCCH and began first-line systemic therapy without thoracic radiotherapy between July 2002 and December 2004 (after gefitinib approval; group A) or between January 1999 and July 2001 (at least 1 year before gefitinib approval; group B) were identified using the databases of NCCH. Patients for whom appropriate pathologic samples were available and a mutational analysis could be successfully performed were included in this study.

Mutational Analysis

DNA was extracted from archived paraffin-embedded tissues and/or Papanicolaou-stained cytologic slides, and the two major hotspots of *EGFR* mutations, DEL and L858R, were analyzed using high-resolution melting analysis according to a previously described method.[9,27] Briefly, polymerase chain reaction (PCR) was performed using primers designed to amplify a region containing E746-I759 or L858 of *EGFR* and the dye LCGreen I (Roche Diagnostics, Indianapolis, IN). Melting curves were obtained using HR-1 (Idaho Technology, Salt Lake City, UT), and the curves of the samples and controls were compared. All of the mutational analyses were performed in a blinded fashion.

Clinical Outcomes

OS was defined as the time from the start of first-line systemic therapy until death. In patients with measurable lesions, tumor response to first-line cytotoxic chemotherapy, including second-line therapy after first-line gefitinib therapy, was evaluated using standard bidimensional measurements.[28] The response rate was defined as the proportion of complete and partial responses compared with the total number of patients.

Statistical Analysis

The differences in OS for the patients in group A and those in group B were compared using Kaplan-Meier curves and log-rank tests. To assess the interaction between the groups and the mutational status of *EGFR*, interaction terms were included in the Cox proportional hazards models. The interaction was considered significant if $P < .10$. The impact of *EGFR* mutations on tumor response to chemotherapy and prognosis was assessed using a χ^2 test and a log-rank test, respectively. These analyses were performed with or without adjustments for the following baseline characteristics: age, sex, smoking history (never-smokers *v* others), performance status (PS), and disease stage (recurrence after surgery *v* stage III/IV). All the statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC).

RESULTS

Mutational Analysis

Medical and pathologic records were reviewed for 414 clinically eligible patients (255 in group A and 159 in group B), and the mutational status was successfully determined in 330 patients (200 in group A and 130 in group B). Appropriate pathologic samples were not available in 68 patients (49 in group A and 19 in group B), and indeterminate results were obtained because of incomplete PCR in 16 patients (six in group A and 10 in group B). Of the 330 successfully analyzed patients, 193 were analyzed using only cytology samples, 106 were analyzed using only tissue samples, and 31 were analyzed using both samples. DEL and L858R mutations were detected in 77 (23%) and 59 patients (18%), respectively, and these mutations were mutually exclusive.

Patient Characteristics

The patient characteristics of the 330 patients are listed in Table 1. All of the patients were Japanese except for one Korean patient and one Chinese patient. When groups A and B were compared, group A had a significantly higher percentage of patients with recurrence after surgery and patients with a poor PS. Age, sex, and smoking history were similar between the two groups. In group A, most of the patients were treated with EGFR-TKIs. However, 15 patients (8%) were not treated with EGFR-TKIs, and in 12 patients (6%), the EGFR-TKI

Table 1. Patient Characteristics

Characteristic	Group A: July 2002 to December 2004 (n = 200) No. of Patients	%	Group B: January 1999 to July 2001 (n = 130) No. of Patients	%	*P*
Age, years					.47
Median	62		62		
Range	27-84		37-84		
Sex					.52
Female	84	42	50	38	
Male	116	58	80	62	
Smoking history*					.70†
Never-smoker	92	46	57	44	
Former smoker	42	21	33	25	
Current smoker	66	33	40	31	
Histologic diagnosis					—
Adenocarcinoma	200	100	130	100	
Other	0	0	0	0	
Performance status					.049‡
0	70	35	46	35	
1	113	57	80	62	
2	13	7	4	3	
3	4	3	0	0	
Stage					.001§
IIIB	37	19	29	22	
IV	79	40	70	54	
Recurrence after surgery	84	42	31	24	
First-line cytotoxic chemotherapy‖					—
Platinum + third-generation drug¶	140	70	88	68	
Other platinum-based regimen	0	0	8	6	
Non–platinum-based regimen	14	7	34	26	
No cytotoxic chemotherapy	46	23	0	0	
EGFR-TKI therapy					—
First line	81	41	0	0	
Second line	63	32	9	7	
Third or more line	29	15	10	8	
Never	15	8	111	85	
Unknown	12	6	0	0	
EGFR mutation status					
DEL	46	23	31	24	
L858R	32	16	27	21	
Wild type	122	61	72	55	

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; DEL, deletional mutations in exon 19.
*Never-smokers were defined as patients who had never had a smoking habit, and former smokers were defined as patients who had stopped smoking at least 1 year before diagnosis.
†Never-smokers *v* others.
‡0 or 1 *v* 2 or 3.
§IIIB or IV *v* recurrence after surgery.
‖Including second-line therapy after first-line gefitinib therapy.
¶Third-generation drug indicates paclitaxel, docetaxel, gemcitabine, vinorelbine, or irinotecan.

treatment history was unknown because the patients had been transferred to another hospital and the subsequent treatment data was not available. In group B, all but 19 patients (15%) had no history of EGFR-TKI treatment; six patients had been treated with gefitinib in clinical trials before gefitinib approval, one patient had been treated with erlotinib in a phase II trial, and 12 patients had been treated with gefitinib in a clinical practice setting after gefitinib approval.

Historical Comparison Before and After Gefitinib Approval

The median follow-up time for 46 survivors in group A was 30.8 months (range, 10.7 to 49.8 months), and the follow-up times for two survivors in group B were 65.7 and 85.0 months. OS was significantly longer in group A than in group B (median survival time [MST], 18.1 *v* 12.5 months, respectively; hazard ratio [HR] = 0.66; 95% CI, 0.52 to 0.84; $P < .001$; Fig 1A). In group A versus group B, a significant improvement in survival was observed in patients with *EGFR* mutations (MST, 27.2 *v* 13.6 months, respectively; HR = 0.48; 95% CI, 0.32 to 0.71; $P < .001$; Fig 1B), whereas no significant improvement in survival was observed in patients without *EGFR* mutations (MST, 13.2 *v* 10.4 months, respectively; HR = 0.79; 95% CI, 0.59 to 1.07; $P = .13$; Fig 1C). The improvement in survival was similar among patients with DEL (Fig 1D) and those with L858R (Fig 1E). A significant interaction between the mutational status of *EGFR*

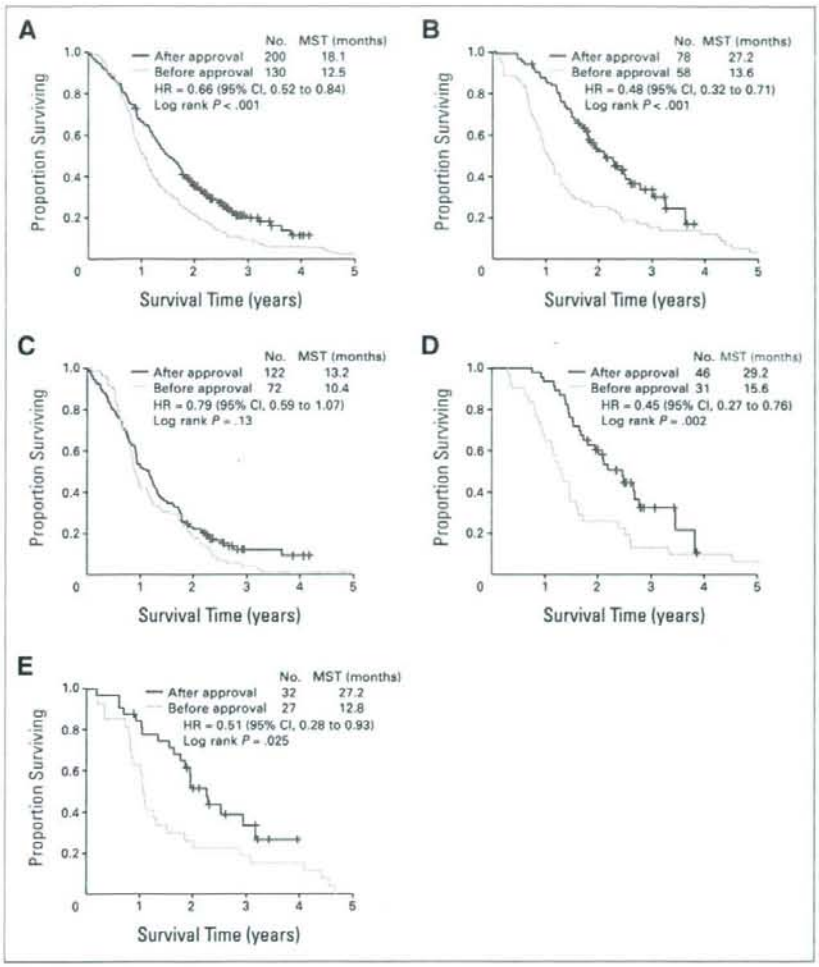

Fig 1. Comparison of overall survival between patients who began first-line systemic therapy after gefitinib approval and patients who began treatment before gefitinib approval. (A) All patients included in the current study. (B) Patients with epidermal growth factor receptor (*EGFR*) mutations. (C) Patients without *EGFR* mutations. (D) Patients with deletional mutations in exon 19. (E) Patients with L858R mutation. MST, median survival time; HR, hazard ratio.

(mutant *v* wild type) and the improvement in survival was observed ($P = .045$). After adjusting for age, sex, smoking history, PS, and disease stage, the HR of after to before gefitinib approval was 0.47 (95% CI, 0.31 to 0.70; $P < .001$) among patients with *EGFR* mutations and 0.76 (95% CI, 0.55 to 1.04; $P = .088$) among patients without *EGFR* mutations. The interaction was also significant after the adjustment ($P = .035$).

Prognosis in Patients Before Gefitinib Approval

When patients with and without *EGFR* mutations were compared in group B (patients treated before gefitinib approval), the patients with *EGFR* mutations lived significantly longer than patients without *EGFR* mutations (MST, 13.6 *v* 10.4 months, respectively; HR = 0.68; 95% CI, 0.48 to 0.97; $P = .034$; Fig 2A), and this finding persisted after adjustments for age, sex, smoking history, PS, and disease stage (HR = 0.65; 95% CI, 0.44 to 0.96; $P = .028$). However, this result may be affected by EGFR-TKI treatment administered to 19 patients (12 with *EGFR* mutations and seven without *EGFR* mutations). When the start of EGFR-TKI administration in the 19 patients was treated as a censoring event to exclude the effect, the difference in OS was not significant (HR = 0.74; 95% CI, 0.50 to 1.08; $P = .12$; Fig 2B). Between patients with DEL and those with L858R, the difference in OS was not significant (MST, 15.6 *v* 12.8 months, respectively; HR = 0.86; 95% CI, 0.51 to 1.46: $P = .58$).

Response to Cytotoxic Chemotherapy

The response to cytotoxic chemotherapy was evaluated in 279 of the 330 patients. The other 51 patients were excluded because no chemotherapy other than gefitinib was administered (n = 46) or they had no measurable lesions (n = 5). As shown in Table 2, the total response rate was 29%, and the response rates were not significantly different between patients with and without *EGFR* mutations (31% *v* 28%, respectively; $P = .50$). These findings were similar for patients with DEL and with L858R (29% *v* 35%, respectively; $P = .49$). *EGFR*

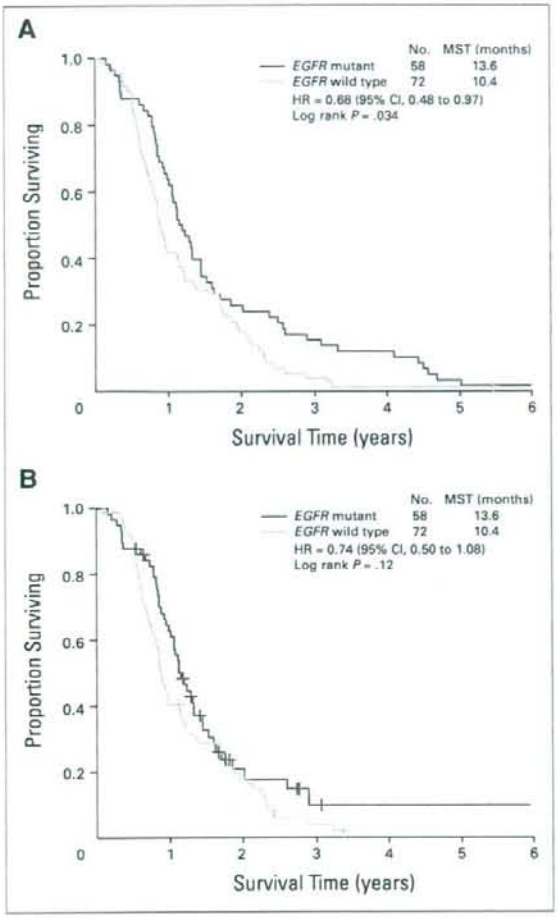

Fig 2. (A) Comparison of overall survival between patients with and without epidermal growth factor receptor (*EGFR*) mutations among patients treated before gefitinib approval; and (B) the same comparison when the start of EGFR tyrosine kinase inhibitor administration is treated as a censoring event. MST, median survival time; HR, hazard ratio.

mutations were not significantly associated with response to any specific regimen, although the response rate to taxane monotherapy tended to be higher among patients with *EGFR* mutations than in patients without *EGFR* mutations (31% *v* 13%, respectively; $P = .17$).

DISCUSSION

To assess the survival benefit of gefitinib in patients with lung adenocarcinoma, we compared the OS of patients treated after gefitinib approval (group A) with a historical control (group B). As the historical control, we selected patients treated between January 1999 and July 2001 because most of these patients routinely received a combination of platinum and a third-generation drug and were also administered second-line cytotoxic chemotherapy, if indicated; thus, their cytotoxic chemotherapy regimens were similar to those of the patients in group A. Actually, fewer cytotoxic chemotherapy regimens were used in group A because some cytotoxic chemotherapy options were replaced with gefitinib therapy. Because the most essential difference between the two groups was the availability of gefitinib, the survival improvement observed in this historical comparison can be interpreted as reflecting a survival benefit from the addition of gefitinib monotherapy or the replacement of cytotoxic chemotherapy with gefitinib monotherapy. Although there was a small number of patients who were not treated with EGFR-TKIs in group A or who were treated with EGFR-TKIs in group B, we included all consecutive patients in the analysis to avoid biases. Some imbalances in the baseline patient characteristics of the two groups were noted; however, all of the results described in the present study were similar even after adjustments were made for the baseline patient characteristics.

In this study, we clearly showed an improvement in the survival of patients with *EGFR* mutations after gefitinib approval. In fact, the MST doubled (13.6 to 27.2 months), a feat that has never before been achieved in the history of NSCLC treatment. Even in patients without *EGFR* mutations, a nonsignificant improvement in survival was obtained (MST, 10.4 to 13.2 months); this result might be a result of the efficacy of gefitinib, period effects other than the approval of gefitinib therapy, or selection biases. Nevertheless, a significant interaction between the presence of *EGFR* mutations and an improvement in survival was obtained, meaning that the mutational status of *EGFR* is a predictor of a survival benefit from gefitinib.

To our knowledge, this is the first study to show a significant interaction between *EGFR* mutations and a survival benefit from EGFR-TKI therapy. Although this study was a retrospective historical comparison conducted only in East Asian patients and some biases could not be excluded, the number of patients with *EGFR* mutations analyzed in this study (n = 136) was much larger than those in phase III trials (INTACT, n = 32; TRIBUTE, n = 29; ISEL, n = 26; BR.21, n = 34),[20-22,29] and we believe that the results of this study have a certain amount of importance to clinical practice.

The current study also showed that, among the patients treated with chemotherapy before gefitinib approval (group B), the OS was significantly longer in the patients with *EGFR* mutations than in those without *EGFR* mutations. As with the INTACT and TRIBUTE trials,[20,21] this result suggested that the presence of *EGFR* mutations was a favorable prognostic factor in patients with advanced NSCLC. However, this result is not conclusive because the difference was marginal when the effects of EGFR-TKIs, which were used in a small number of patients, were excluded.

As for the patients who were treated after gefitinib approval (group A), the difference in OS between the patients with and without *EGFR* mutations can be partly explained by the prognostic value of the *EGFR* mutations themselves. However, this study indicated that the difference was mainly caused by the mutations' predictive value for a survival benefit from gefitinib.

The difference in OS according to the mutational status of *EGFR* in group B can also be explained by the predictive value for chemotherapy efficacy other than the pure prognostic value. In INTEREST and V15-32, which were phase III trials comparing docetaxel and gefitinib, the HRs for OS were almost the same between patients with and without *EGFR* mutations,[16,30] suggesting that *EGFR* mutations might be a predictive factor for a survival benefit from both docetaxel

Table 2. *EGFR* Mutations and Tumor Response to Cytotoxic Chemotherapy

Therapy	Mutant *EGFR*		Wild-Type *EGFR*		*P*	Total	
	No. of Patients	Response Rate (%)	No. of Patients	Response Rate (%)		No. of Patients	Response Rate (%)
Total	112	31	167	28	.50	279	29
Regimens							
Platinum + taxane	54	37	97	34	.71	151	35
Platinum + other third-generation drug*	35	26	39	26	.99	74	26
Taxane† monotherapy	16	31	23	13	.17	39	21
Other regimen	7	14	8	0	.27	15	7
Treatment line							
First line	95	33	147	27	.37	242	29
Second-line therapy after first-line gefitinib therapy	17	24	20	30	.66	37	27

Abbreviation: *EGFR*, epidermal growth factor receptor.
*Other third-generation drug indicates gemcitabine, vinorelbine, or irinotecan.
†Taxane indicates paclitaxel or docetaxel.

and gefitinib. In the current study, response rate to taxane monotherapy tended to be higher in patients with *EGFR* mutations, although the number of patients was small. These results are inconclusive, and further investigation is needed.

We detected no significant difference in the predictive and prognostic values of DEL and L858R in the current study. Some researchers, including ourselves, have reported that patients with DEL had better outcomes after EGFR-TKI treatment than those with L858R[9,31,32]; however, the current study showed that gefitinib yielded almost the same survival benefit to both patients with DEL and patients with L858R, and we think that the two *EGFR* mutations should be treated equally when making clinical decisions.

In the ISEL and BR.21 trials, the *EGFR* copy number (evaluated using fluorescence in situ hybridization), rather than the *EGFR* mutation status, was suggested to predict a survival benefit from EGFR-TKIs,[22,23,29] and the authors concluded that a mutational analysis was not necessary to select patients for treatment with EGFR-TKIs. In contrast, the current study indicated that the *EGFR* mutation status was a determinant of a survival benefit from gefitinib, although *EGFR* copy numbers were not evaluated in this study. Our previous study showed that the *EGFR* copy number, as evaluated using quantitative PCR, was associated with a response to gefitinib; however, an increased *EGFR* copy number tended to be seen in patients with *EGFR* mutations and was not an independent predictor of response or OS in gefitinib-treated patients.[6] These discrepancies may be a result of the ethnic difference, the methodologic difference between fluorescence in situ hybridization and quantitative PCR, or the accuracy of biomarker analyses. Although controversy still remains, we believe that the *EGFR* mutation status is the most useful biomarker for patient selection, at least in East Asian patients who have *EGFR* mutations more frequently than non-Asian patients.

In conclusion, gefitinib yielded a survival benefit among Japanese patients with lung adenocarcinoma, and the survival benefit was significantly greater in patients with *EGFR* mutations than in those without *EGFR* mutations. The presence of *EGFR* mutations may also be a favorable prognostic factor in advanced lung adenocarcinoma independent of gefitinib treatment. We need to consider appropriate treatment strategies for patients with NSCLC based on their *EGFR* mutation status.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: None **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** Toshimi Takano, AstraZeneca; Yuichiro Ohe, AstraZeneca; Noboru Yamamoto, AstraZeneca; Hideo Kunitoh, AstraZeneca; Tomohide Tamura, AstraZeneca **Research Funding:** None **Expert Testimony:** Hideo Kunitoh, AstraZeneca (U) **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

Conception and design: Toshimi Takano, Tomoya Fukui, Yuichiro Ohe, Koji Tsuta, Seiichiro Yamamoto, Koh Furuta
Administrative support: Yuichiro Ohe, Tomohide Tamura
Provision of study materials or patients: Toshimi Takano, Tomoya Fukui, Yuichiro Ohe, Koji Tsuta, Hiroshi Nokihara, Noboru Yamamoto, Ikuo Sekine, Hideo Kunitoh, Tomohide Tamura
Collection and assembly of data: Toshimi Takano, Tomoya Fukui, Yuichiro Ohe, Koji Tsuta, Seiichiro Yamamoto, Koh Furuta
Data analysis and interpretation: Toshimi Takano, Tomoya Fukui, Yuichiro Ohe, Koji Tsuta, Seiichiro Yamamoto, Koh Furuta
Manuscript writing: Toshimi Takano
Final approval of manuscript: Toshimi Takano, Tomoya Fukui, Yuichiro Ohe, Koji Tsuta, Seiichiro Yamamoto, Hiroshi Nokihara, Noboru Yamamoto, Ikuo Sekine, Hideo Kunitoh, Koh Furuta, Tomohide Tamura

REFERENCES

1. Fukuoka M, Yano S, Giaccone G, et al: A 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 21:2237-2246, 2003

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

3. 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 45:93-104, 2004

4. Lynch TJ, Bell DW, Sordella R, et al: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. N Engl J Med 350:2129-2139, 2004

5. Paez JG, Janne PA, Lee JC, et al: EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. Science 304:1497-1500, 2004

6. 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 23:6829-6837, 2005

7. Mitsudomi T, Kosaka T, Endoh H, et al: Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. J Clin Oncol 23:2513-2520, 2005

8. Han S-W, Kim T-Y, 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 23:2493-2501, 2005

9. Takano T, Ohe Y, Tsuta K, et al: Epidermal growth factor receptor mutation detection using high-resolution melting analysis predicts outcomes in patients with advanced non-small cell lung cancer treated with gefitinib. Clin Cancer Res 13:5385-5390, 2007

10. Giaccone G, Herbst RS, Manegold C, et al: Gefitinib in combination with gemcitabine and cisplatin in advanced non–small-cell lung cancer: A phase III trial—INTACT 1. J Clin Oncol 22:777-784, 2004

11. Herbst RS, Giaccone G, Schiller JH, et al: Gefitinib in combination with paclitaxel and carboplatin in advanced non–small-cell lung cancer: A phase III trial—INTACT 2. J Clin Oncol 22:785-794, 2004

12. Herbst RS, Prager D, Hermann R, et al: TRIBUTE: A phase III trial of erlotinib hydrochloride (OSI-774) combined with carboplatin and paclitaxel chemotherapy in advanced non–small-cell lung cancer. J Clin Oncol 23:5892-5899, 2005

13. Gatzemeier U, Pluzanska A, Szczesna A, et al: Phase III study of erlotinib in combination with cisplatin and gemcitabine in advanced non–small-cell lung cancer: The Tarceva Lung Cancer Investigation Trial. J Clin Oncol 25:1545-1552, 2007

14. 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 366:1527-1537, 2005

15. Shepherd FA, Pereira JR, Ciuleanu T, et al: Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 353:123-132, 2005

16. Douillard JY, Kim E, Hirsh V, et al: Gefitinib (IRESSA) versus docetaxel in patients with locally advanced or metastatic non-small-cell lung cancer pre-treated with platinum-based chemotherapy: A randomized, open-label Phase III study (INTEREST). J Thorac Oncol 2:S305-S306, 2007 (suppl 4; abstr PRS-02)

17. Niho S, Ichinose Y, Tamura T, et al: Results of a randomized phase III study to compare the overall survival of gefitinib (IRESSA) versus docetaxel in Japanese patients with non-small-cell lung cancer who failed one or two chemotherapy regimens. J Clin Oncol 25:387s, 2007 (suppl; abstr LBA7509)

18. Kosaka T, Yatabe Y, Endoh H, et al: Mutations of the epidermal growth factor receptor gene in lung cancer: Biological and clinical implications. Cancer Res 64:8919-8923, 2004

19. 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 97:339-346, 2005

20. Bell DW, Lynch TJ, Haserlat SM, et al: Epidermal growth factor receptor mutations and gene amplification in non–small-cell lung cancer: Molecular analysis of the IDEAL/INTACT gefitinib trials. J Clin Oncol 23:8081-8092, 2005

21. Eberhard DA, Johnson BE, Amler LC, et al: Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non–small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. J Clin Oncol 23:5900-5909, 2005

22. Hirsch FR, Varella-Garcia M, Bunn PA, et al: Molecular predictors of outcome with gefitinib in a phase III placebo-controlled study in advanced non–small-cell lung cancer. J Clin Oncol 24:5034-5042, 2006

23. Tsao M-S, Sakurada A, Cutz J-C, et al: Erlotinib in lung cancer: Molecular and clinical predictors of outcome. N Engl J Med 353:133-144, 2005

24. 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 94:896-903, 2006

25. Kosaka T, Yatabe Y, Onozato T, et al: Prognostic implication of the EGFR gene mutations in a large cohort of Japanese patients with early stage lung adenocarcinoma. J Clin Oncol 25:402s, 2007 (suppl; abstr 7574)

26. Sasaki H, Shimizu S, Endo K, et al: EGFR and erbB2 mutation status in Japanese lung cancer patients. Int J Cancer 118:180-184, 2006

27. Nomoto K, Tsuta K, Takano T, et al: Detection of EGFR mutations in archived cytologic specimens of non-small cell lung cancer using high-resolution melting analysis. Am J Clin Pathol 126:608-615, 2006

28. Green S, Weiss GR: Southwest Oncology Group standard response criteria, endpoint definitions and toxicity criteria. Invest New Drugs 10:239-253, 1992

29. Shepherd FA, Ding K, Sakurada A, et al: Updated molecular analyses of exons 19 and 21 of the epidermal growth factor receptor (EGFR) gene and codons 12 and 13 of the KRAS gene in non-small cell lung cancer (NSCLC) patients treated with erlotinib in National Cancer Institute of Cancer. J Clin Oncol 25:402s, 2007 (suppl, abstr 7571)

30. Sekine I, Ichinose Y, Nishiwaki Y, et al: A randomized phase III study to compare the overall survival of gefitinib (IRESSA) versus docetaxel in Japanese patients with previously treated advanced non-small-cell lung cancer. J Thorac Oncol 2:S339-S340, 2007 (suppl 4, abstr B3-01)

31. Riely GJ, Pao W, Pham DK, 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 12:839-844, 2006

32. 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 12:3908-3914, 2006

Acknowledgment

We thank Kiyoaki Nomoto, Karin Yokozawa, Chizu Kina, and Sachiko Miura for their technical support.

British Journal of Cancer (2008) 98, 749–755

www.bjcancer.com

Enhancement of the antitumor activity of ionising radiation by nimotuzumab, a humanised monoclonal antibody to the epidermal growth factor receptor, in non-small cell lung cancer cell lines of differing epidermal growth factor receptor status

Y Akashi[1], I Okamoto*[,1], T Iwasa[1], T Yoshida[1], M Suzuki[2], E Hatashita[1], Y Yamada[1], T Satoh[1], M Fukuoka[1], K Ono[2] and K Nakagawa[1]

[1]Department of Medical Oncology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan; [2]Radiation Oncology Research Laboratory, Research Reactor Institute, Kyoto University, 2-1010 Asashiro-nishi, Kumatori-cho, Sennan-gun, Osaka 590-0494, Japan

The expression and activity of the epidermal growth factor receptor (EGFR) are determinants of radiosensitivity in several tumour types, including non-small cell lung cancer (NSCLC). However, little is known of whether genetic alterations of *EGFR* in NSCLC cells affect the therapeutic response to monoclonal antibodies (mAbs) to EGFR in combination with radiation. We examined the effects of nimotuzumab, a humanised mAb to EGFR, in combination with ionising radiation on human NSCLC cell lines of differing *EGFR* status. Flow cytometry revealed that H292 and Ma-1 cells expressed high and moderate levels of EGFR on the cell surface, respectively, whereas H460, H1299, and H1975 cells showed a low level of surface EGFR expression. Immunoblot analysis revealed that EGFR phosphorylation was inhibited by nimotuzumab in H292 and Ma-1 cells but not in H460, H1299, or H1975 cells. Nimotuzumab augmented the cytotoxic effect of radiation in H292 and Ma-1 cells in a clonogenic assay *in vitro*, with a dose enhancement factor of 1.5 and 1.3, respectively. It also enhanced the antitumor effect of radiation on H292 and Ma-1 cell xenografts in nude mice, with an enhancement factor of 1.3 and 4.0, respectively. Nimotuzumab did not affect the radioresponse of H460 cells *in vitro* or *in vivo*. Nimotuzumab enhanced the antitumor efficacy of radiation in certain human NSCLC cell lines *in vitro* and *in vivo*. This effect may be related to the level of EGFR expression on the cell surface rather than to *EGFR* mutation.

British Journal of Cancer (2008) **98,** 749–755. doi:10.1038/sj.bjc.6604222 www.bjcancer.com
Published online 5 February 2008

Keywords: epidermal growth factor receptor; non-small cell lung cancer; nimotuzumab; monoclonal antibody; genetic alteration; radiosensitisation

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is abnormally upregulated and activated in a variety of tumours (Baselga, 2002). Deregulation of receptor tyrosine kinases as a result of overexpression or activating mutations is frequently associated with human cancers and leads to the promotion of cell proliferation or migration, inhibition of cell death, or the induction of angiogenesis (Gschwind *et al*, 2004). The epidermal growth factor receptor has thus been identified as an important target in cancer therapy (Baselga and Arteaga, 2005). Several agents, including small-molecule inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKIs) and monoclonal antibodies (mAbs) specific for EGFR, have been designed to block EGFR signalling selectively (Ettinger, 2006; Harari and Huang, 2006; Imai and Takaoka, 2006). Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in non-small cell lung cancer (NSCLC), and sensitivity to these drugs has been associated with the presence of somatic mutations in the EGFR kinase domain or with *EGFR* amplification (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Various mAbs to EGFR are also undergoing preclinical and clinical trials of their efficacy as anticancer agents. However, biological markers able to predict the response to such antibodies have remained elusive.

The possibility of combining chemotherapy or radiation therapy with anti-EGFR mAb treatment has generated much interest, because the cellular targets for these agents and their mechanisms of action are different (Baumann and Krause, 2004). Studies have thus been undertaken to determine whether inhibition of EGFR signalling improves the response to chemotherapy or radiation therapy. Preclinical studies have shown that the anti-EGFR mAb cetuximab markedly increases the cytotoxic effect of chemotherapy or radiation therapy in various EGFR-expressing tumour cell lines (Huang *et al*, 1999; Milas *et al*, 2000; Buchsbaum *et al*, 2002; Prewett *et al*, 2002; Raben *et al*, 2005; Ettinger, 2006). A phase III clinical trial also showed that the combination of cetuximab with

*Correspondence: Dr I Okamoto;
E-mail: chi-okamoto@dotd.med.kindai.ac.jp
Revised 20 November 2007; accepted 7 January 2008; published online 5 February 2008

radiation therapy resulted in a significant improvement in local control and survival compared with radiation therapy alone, without an increase in radiation-induced side effects, in patients with locally advanced head and neck cancer (Bonner *et al*, 2006).

Nimotuzumab (also known as h-R3) is a humanised anti-EGFR mAb, which is currently undergoing clinical evaluation. In a preclinical study, nimotuzumab showed marked antiproliferative, proapoptotic, and antiangiogenic effects in tumours that overexpress EGFR (Crombet-Ramos *et al*, 2002). In early clinical trials, nimotuzumab has shown a longer half-life and a greater area under the curve (AUC) in comparison with other anti-EGFR antibodies (Crombet *et al*, 2003). A phase I/II trial showed that nimotuzumab was well tolerated and enhanced the curative potential of radiation in patients with advanced head and neck cancer (Crombet *et al*, 2004). Given that little is known of the antitumor action of nimotuzumab in NSCLC, we investigated the growth-inhibitory effects of this mAb alone and in combination with radiation in NSCLC cell lines with differing patterns of EGFR expression. We also examined whether genetic alterations of *EGFR* affect the antitumor action of combined treatment with nimotuzumab and radiation.

MATERIALS AND METHODS

Cell lines and reagents

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460), Ma-1, NCI-H1299 (H1299), and NCI-H1975 (H1975) were obtained as previously described (Okabe *et al*, 2007) and were maintained under a humidified atmosphere of 5% CO_2 in air at 37.0°C in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Nimotuzumab was provided by Daiichi Sankyo Co Ltd (Tokyo, Japan), and gefitinib was obtained from AstraZeneca (Macclesfield, UK).

Flow cytometric analysis of surface EGFR expression

Cells (1.0×10^6) were stained for 2 h at 4°C with an R-phycoerythrin-conjugated mAb to EGFR (BD Biosciences, San Jose, CA, USA) or an isotype-matched control mAb (BD Biosciences). The cells were washed three times before measurement of fluorescence with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA, USA).

Immunoblot analysis

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites, the membrane was incubated consecutively with primary and secondary antibodies, and immune complexes were detected with the use of enhanced chemiluminescence reagents, as described previously (Okabe *et al*, 2007). Primary antibodies to phosphorylated EGFR (pY1068) were obtained from Cell Signaling Technology (Beverly, MA, USA), and those to EGFR were from Zymed (South San Francisco, CA, USA). Horseradish peroxidase-conjugated goat secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, UK).

Clonogenic assay

Exponentially growing 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 supplemented with 1% fetal bovine serum in the absence or presence of 700 nM nimotuzumab. After incubation for 24 h, the cells were exposed to various doses of γ-radiation with a ^{60}Co irradiator at a rate of approximately 0.82 Gy min^{-1} and at room temperature. The cells were then washed with phosphate-buffered saline, 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 control cultures not exposed to nimotuzumab or radiation. The surviving fraction for combined treatment was corrected by that for nimotuzumab treatment alone. The dose enhancement factor was then calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.5 for vehicle-treated cells divided by that for nimotuzumab-treated cells (after correction for drug toxicity).

Antitumor activity of nimotuzumab with or without radiation *in vivo*

Animal experiments 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, and they met 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 approximately 170–200 mm^3. Treatment groups consisted of control, nimotuzumab alone, radiation alone, and the combination of nimotuzumab and radiation, with each group containing seven or eight mice. Nimotuzumab was administered intraperitoneally in a single dose of 1.0 mg per mouse; mice in the control and radiation-alone groups were injected with vehicle (physiological saline). Tumours in the right hind leg of mice were exposed to 10 Gy of γ-radiation with a ^{60}Co irradiator at a rate of approximately 0.32 Gy min^{-1} beginning 6 h after drug treatment. Growth delay (GD) was calculated as the time required for treated tumours to achieve a fivefold increase in volume minus the corresponding time required for control tumours. The enhancement factor was then determined as $(GD_{combination} - GD_{nimotuzumab})/(GD_{radiation})$.

RESULTS

Surface EGFR expression in NSCLC cell lines of differing *EGFR* status

We first examined the surface expression of EGFR in five NSCLC cell lines by flow cytometry. The *EGFR* status for the cell lines was determined in our previous study (Okabe *et al*, 2007). Three cell lines (H460, H292, and H1299) possess wild-type *EGFR* alleles, whereas the other two cell lines (Ma-1 and H1975) harbour *EGFR* mutations (Table 1). Ma-1 cells have an in-frame deletion in

Table 1 Characteristics of NSCLC cell lines

Cell line	EGFR surface expression	*EGFR* status
H460	Low	Wild type
H292	High	Wild type
H1299	Low	Wild type
Ma-1	Moderate	del(E746–A750)
H1975	Low	L858R/T790M

EGFR = epidermal growth factor receptor; NSCLC = non-small cell lung cancer

exon 19 (E746–A750). H1975 cells harbour the L858R mutation in exon 21 and a secondary mutation in exon 20 (T790M). Activating mutations in exons 19 and 21 are associated with sensitivity to EGFR-TKIs (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005), whereas the T790M mutation contributes to the development of resistance to these drugs (Kobayashi *et al*, 2005; Pao *et al*, 2005). Our flow cytometric analysis demonstrated that H292 and Ma-1 cells express high and moderate levels of EGFR on the cell surface, respectively, whereas H460, H1299, and H1975 cells showed a low level of surface EGFR expression (Figure 1).

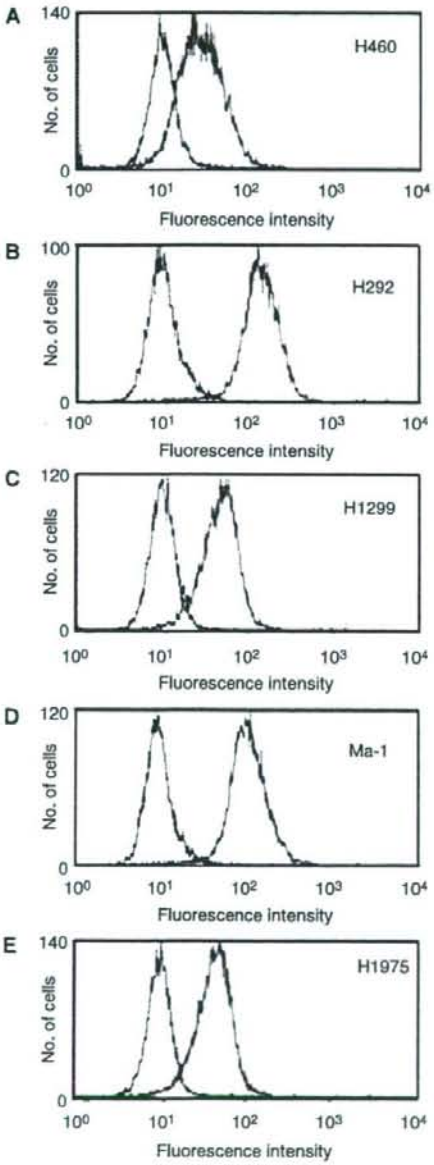

Figure 1 Expression of EGFR on the surface of NSCLC cells. Surface expression of EGFR on H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells was determined by flow cytometry. Representative histograms of cells stained with an anti-EGFR mAb (red peak) or with an isotype-matched control mAb (black peak) are shown.

Effect of nimotuzumab on EGFR phosphorylation

Next, we determined whether nimotuzumab inhibits ligand-induced EGFR phosphorylation in the five NSCLC cell lines. The cells were deprived of serum overnight, exposed to various concentrations of nimotuzumab, or to gefitinib, for 15 min, and then stimulated with EGF for 15 min. In the NSCLC cells that harbour wild-type *EGFR* (H460, H292, and H1299), phosphorylation of EGFR was undetectable in the absence of EGF, but was markedly induced on exposure of the cells to this growth factor. The EGF-induced phosphorylation of EGFR in these cells was completely inhibited by the EGFR-TKI gefitinib. Nimotuzumab also inhibited the EGF-induced EGFR phosphorylation in a concentration-dependent manner in H292 cells (which have a high level of surface EGFR expression), whereas it did not substantially affect such phosphorylation in H460 or H1299 cells (both of which have a low level of surface EGFR expression) (Figure 2A–C). We previously showed that the basal level of EGFR phosphorylation was increased in the *EGFR* mutant NSCLC cell lines Ma-1 and H1975, indicative of constitutive activation of the EGFR tyrosine kinase (Okabe *et al*, 2007). The phosphorylation of EGFR in EGF-treated Ma-1 cells (which have a moderate level of surface EGFR expression) was inhibited by gefitinib as well as by nimotuzumab in a concentration-dependent manner (Figure 2D). In contrast, the constitutive activation of EGFR in H1975 cells (which have a low level of surface EGFR expression) was inhibited partially by gefitinib but was unaffected by nimotuzumab (Figure 2E). These results suggested that the inhibition of EGFR phosphorylation by nimotuzumab may be related to the surface expression level of EGFR rather than to the mutational status of *EGFR*.

Augmentation of the cytotoxic effect of radiation in NSCLC cells by nimotuzumab *in vitro*

We examined whether nimotuzumab might enhance the anticancer effect of γ-radiation in the five NSCLC cell lines with the use of a clonogenic assay. Tumour cells were incubated with or without nimotuzumab for 24 h, exposed to various doses of γ-radiation, and then allowed to form colonies in drug-free medium for 10–14 days. Survival curves revealed that, whereas nimotuzumab had no effect on the radiation sensitivity of H460, H1299, or H1975 cells, it enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, with a dose enhancement factor of 1.5 and 1.3, respectively (Figure 3). These results showed that nimotuzumab increased the radiosensitivity of the NSCLC cell lines with high or moderate levels of surface EGFR expression, consistent with the inhibitory effects of this antibody on EGFR signalling.

Augmentation of the antitumor effect of radiation in NSCLC cells by nimotuzumab *in vivo*

To determine whether the nimotuzumab-induced potentiation of the response of NSCLC cells to radiation observed *in vitro* might also be apparent *in vivo*, we injected three of the cell lines into nude mice to elicit the formation of solid tumours. The mice were then treated with nimotuzumab, radiation, or both modalities. In the H460 xenograft model, tumour growth was inhibited by radiation alone but not by nimotuzumab alone, and the effect of radiation was not promoted by nimotuzumab (Figure 4A). In contrast, radiation and nimotuzumab each inhibited the growth of tumours formed by H292 (Figure 4B) or Ma-1 (Figure 4C) cells during the first few weeks after treatment. Thereafter, the rate of

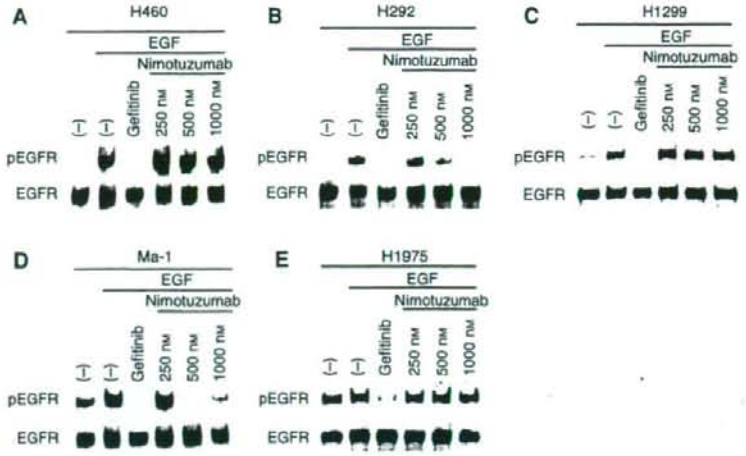

Figure 2 Effect of nimotuzumab on EGFR phosphorylation in NSCLC cells. H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of the indicated concentrations of nimotuzumab or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng ml^{-1}). Cell lysates were then subjected to immunoblot analysis with antibodies to the Tyr1068-phosphorylated form of EGFR (pEGFR) as well as with those to total EGFR.

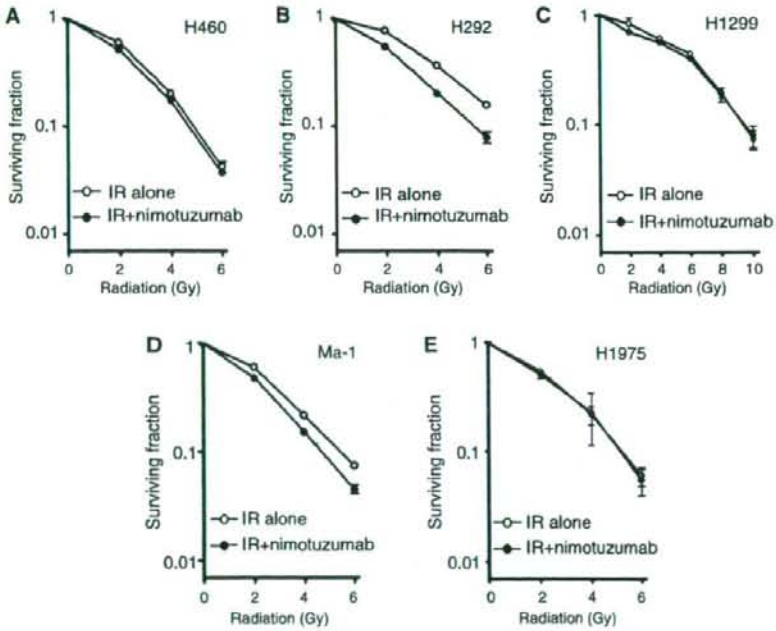

Figure 3 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vitro*. H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells were incubated with or without 700 nM nimotuzumab in medium supplemented with 1% fetal bovine serum for 24 h, exposed to the indicated doses of γ-radiation, and then incubated in drug-free medium supplemented with 10% serum for 10–14 days for determination of colony-forming ability. Survival curves were generated after correction of colony formation observed for combined treatment with ionising radiation (IR) and nimotuzumab by that apparent for treatment with nimotuzumab alone. Data are means ± s.d. of triplicates from a representative experiment.

tumour growth increased to a value similar to that seen in control animals. Combined treatment with radiation and nimotuzumab resulted in a substantial delay in tumour growth and subsequent inhibition of the growth rate of H292 and Ma-1 xenografts. The growth delay after treatment with nimotuzumab alone, radiation alone, or both nimotuzumab and radiation was thus 27.2, 19.6, and 53.6 days, respectively, for H292 cells and 26.7, 13.0, and 78.3 days, respectively, for Ma-1 cells (Table 2). The enhancement factor for the effect of nimotuzumab on the efficacy of radiation was 1.3 for H292 cells and 4.0 for Ma-1 cells, revealing the effect to be more

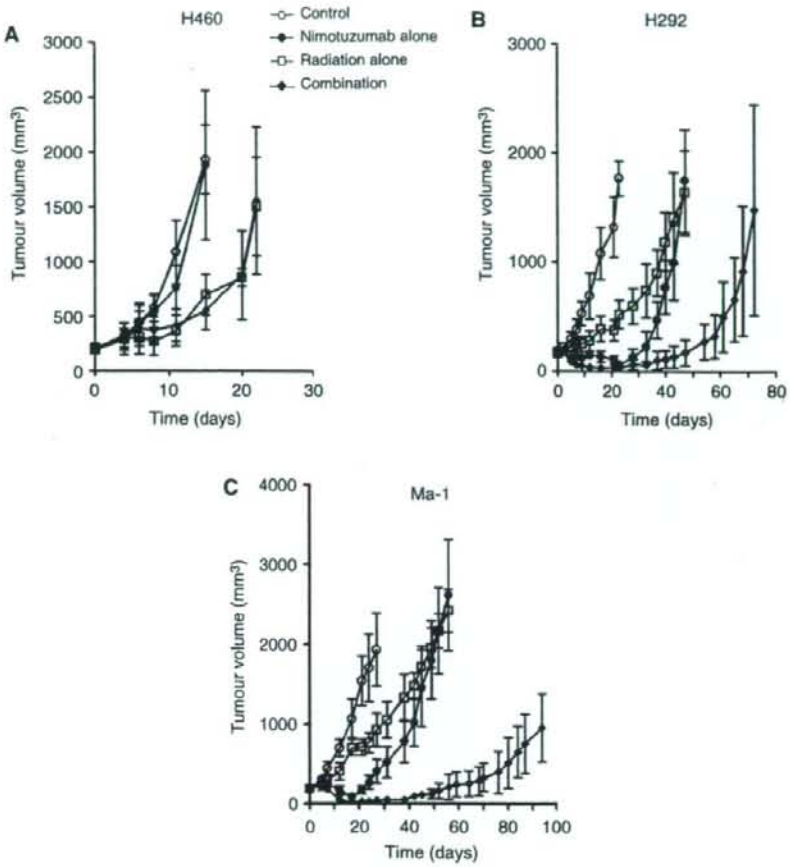

Figure 4 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vivo*. H460 (**A**), H292 (**B**), or Ma-1 (**C**) cells were injected subcutaneously in athymic nude mice. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 mm^3. Mice were treated with a single dose of nimotuzumab (1.0 mg per mouse) intraperitoneally, a single dose of γ-radiation (10 Gy), or neither (control) or both modalities, and tumour volume was determined at the indicated time points thereafter. Data are means ± s.d. for seven to eight mice per group.

Table 2 Tumour growth delay in nude mice treated with nimotuzumab, radiation, or both modalities

	H460		H292		Ma-1	
Treatment	**Days[a]**	**GD[b]**	**Days**	**GD**	**Days**	**GD**
Control	10.4		13.2		15.1	
Nimotuzumab alone	11.8	1.4	40.4	27.2	41.8	26.7
Radiation alone	20.4	10.0	32.8	19.6	28.1	13.0
Nimotuzumab+radiation	20.5	10.1	66.8	53.6	93.4	78.3
Enhancement factor	0.86		1.3		4.0	

GD = growth delay [a]Time required for xenografts in each group to achieve a fivefold increase in volume. [b]The additional time (days) required for xenografts in each treatment group to achieve a fivefold increase in volume relative to the corresponding time for xenografts in the control group.

than additive. No pronounced tissue damage or toxicities such as diarrhoea or a decrease in body weight of >10% were observed in mice in any of the four treatment groups. These results thus suggested that nimotuzumab potentiated the antitumor activity of radiation in H292 and Ma-1 cells *in vivo* as well as *in vitro*.

DISCUSSION

Somatic mutations in the EGFR kinase domain and *EGFR* amplification have been associated with a better response to EGFR-TKIs, such as gefitinib and erlotinib, in patients with NSCLC (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Given that little is known of the relation between such *EGFR* alterations and the response to treatment with anti-EGFR mAbs, we investigated the antitumor effect of combined treatment with the anti-EGFR mAb nimotuzumab and radiation in NSCLC cell lines of differing *EGFR* status.

The antitumor effect of EGFR-specific mAbs has been thought to result from inhibition of ligand binding to EGFR and consequent inhibition of EGFR activation (Li *et al*, 2005; Marshall, 2006). We, therefore, examined the effect of nimotuzumab on EGF-dependent EGFR signalling. Nimotuzumab inhibited the EGF-induced or constitutive phosphorylation of EGFR in H292 and Ma-1 cells (with high and moderate levels of surface EGFR expression, respectively), consistent with the mode of action of this antibody. However, nimotuzumab did not block EGF-induced or constitutive EGFR phosphorylation in H460, H1299, or H1975 cells (all with a

low level of surface EGFR expression). These observations suggest that the inhibitory effect of nimotuzumab on EGFR signalling depends on the expression level of EGFR on the cell surface. A clonogenic cell survival assay revealed that nimotuzumab enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, but not that in H460, H1299, or H1975 cells. These findings support the notion that the inhibition of EGFR signalling by nimotuzumab is responsible, at least in part, for the enhancement of the cytotoxic effect of radiation by this antibody. Irradiation of tumour cells has been shown to activate EGFR via ligand-independent and ligand-dependent mechanisms, possibly accounting for radiation-induced acceleration of tumour cell repopulation and the development of radioresistance (Schmidt-Ullrich *et al*, 1997, 2003; Dent *et al*, 2003). Such radiation-induced activation of EGFR-dependent processes may represent a rationale for combined treatment with radiation and EGFR inhibitors. It remains to be determined whether nimotuzumab is able to block radiation-induced activation of EGFR.

Consistent with our *in vitro* results, we found that nimotuzumab enhanced the antitumor effect of radiation on H292 or Ma-1 cells in nude mice. Such enhancement was not apparent for tumours formed by H460 cells. Nimotuzumab alone also manifested a substantial antitumor effect for xenografts formed by H292 or Ma-1 cells but not for those formed by H460 cells. Together these results suggest that the efficacy of nimotuzumab monotherapy is a prerequisite for augmentation of radioresponse by this mAb. Nimotuzumab was previously shown to induce the regression of A431 tumour xenografts *in vivo* as a result of inhibition of both tumour cell proliferation and tumour angiogenesis (Crombet-Ramos *et al*, 2002). Immunohistochemical analysis of tumour specimens from head and neck cancer patients treated with the combination of nimotuzumab and radiation also showed evidence of antiproliferative and antiangiogenic effects (Crombet *et al*, 2004). These observations suggest that effects of nimotuzumab on both NSCLC cell proliferation and tumour angiogenesis might contribute to the enhancement of the antitumor efficacy of radiation by this antibody observed in the present study. Enhancement of the anticancer effect of radiation by the anti-EGFR mAb cetuximab was previously shown to be increased by transfection of cells to upregulate the level of EGFR expression, suggesting that potentiation of the antitumor efficacy of radiation by anti-EGFR mAbs is related to the absolute level of EGFR expression (Liang *et al*, 2003; Bonner *et al*, 2004). This finding is consistent with our present results showing that potentiation of the antitumor activity of radiation by nimotuzumab was related to the level of surface EGFR expression. The nimotuzumab-resistant cell line H460 harbours a mutant form of *KRAS* (Balko *et al*, 2006) that has been associated with resistance to cetuximab (Lievre *et al*, 2006). However, we found that nimotuzumab also failed to inhibit EGF-induced EGFR phosphorylation and to enhance the cytotoxic effect of radiation in H1299 cells, which harbour wild-type *KRAS* (Coldren *et al*, 2006). These observations thus support the notion that a low level of EGFR expression at the cell surface is related to resistance to combined treatment with nimotuzumab and radiation, irrespective of *KRAS* status.

We demonstrated that nimotuzumab inhibited EGFR phosphorylation and enhanced the antitumor effect of radiation in *EGFR* mutant Ma-1 cells (with a moderate level of surface EGFR expression) but not in *EGFR*-mutant H1975 cells (with a low level of surface EGFR expression). Nimotuzumab also potentiated the cytotoxic effect of radiation in H292 cells, which harbour wild-type *EGFR* alleles and have a high level of surface EGFR expression. These findings support the notion that *EGFR* mutation is not the major determining factor for enhancement of the antitumor effect of radiation by nimotuzumab, consistent with previous observations with cetuximab (Barber *et al*, 2004; Tsuchihashi *et al*, 2005). However, the mechanisms underlying such enhancement of the antitumor effect of radiation may differ between NSCLC cells harbouring wild-type or mutant *EGFR* alleles. We and others have previously shown that mutations in the tyrosine kinase domain of EGFR are associated with increased ligand-independent tyrosine kinase activity of EGFR (Lynch *et al*, 2004) and aberrant EGFR signalling (Amann *et al*, 2005; Okabe *et al*, 2007). Given that cell-cycle checkpoints activated by ionising radiation are defective in *EGFR*-mutant NSCLC cell lines (Das *et al*, 2006), the constitutive activity of EGFR in such cells may result in unchecked DNA synthesis and in apoptosis on exposure to ionising radiation. It is possible that these defects in *EGFR*-mutant cells affect the enhancement of the antitumor efficacy of radiation by nimotuzumab.

In summary, we have shown that nimotuzumab enhanced the antitumor efficacy of radiation *in vitro* and *in vivo*, providing a rationale for future clinical investigations of the therapeutic efficacy of nimotuzumab in combination with radiotherapy. Our data suggest that potentiation of the antitumor activity of radiation by nimotuzumab may be related to the level of EGFR expression at the cell surface rather than to *EGFR* mutation. The preselection of patients on the basis of genetic factors that predict treatment sensitivity or resistance may thus be required for the combination therapy with nimotuzumab and radiation.

ACKNOWLEDGEMENTS

We thank S Ono for technical assistance.

REFERENCES

Amann J, Kalyankrishna S, Massion PP, Ohm JE, Girard L, Shigematsu H, Peyton M, Juroske D, Huang Y, Stuart Salmon J, Kim YH, Pollack JR, Yanagisawa K, Gazdar A, Minna JD, Kurie JM, Carbone DP (2005) Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. *Cancer Res* **65**: 226–235

Balko JM, Potti A, Saunders C, Stromberg A, Haura EB, Black EP (2006) Gene expression patterns that predict sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors in lung cancer cell lines and human lung tumors. *BMC Genomics* **7**: 289

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

Baselga J (2002) Why the epidermal growth factor receptor? The rationale for cancer therapy. *Oncologist* **7**(Suppl 4): 2–8

Baselga J, Arteaga CL (2005) Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. *J Clin Oncol* **23**: 2445–2459

Baumann M, Krause M (2004) Targeting the epidermal growth factor receptor in radiotherapy: radiobiological mechanisms, preclinical and clinical results. *Radiother Oncol* **72**: 257–266

Bonner JA, Buchsbaum DJ, Russo SM, Fiveash JB, Trummell HQ, Curiel DT, Raisch KP (2004) Anti-EGFR-mediated radiosensitization as a result of augmented EGFR expression. *Int J Radiat Oncol Biol Phys* **59**: 2–10

Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky EK, Ang KK (2006) Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med* **354**: 567–578

Buchsbaum DJ, Bonner JA, Grizzle WE, Stackhouse MA, Carpenter M, Hicklin DJ, Bohlen P, Raisch KP (2002) Treatment of pancreatic cancer xenografts with Erbitux (IMC-C225) anti-EGFR antibody, gemcitabine, and radiation. *Int J Radiat Oncol Biol Phys* **54**: 1180–1193

Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, Ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn Jr PA, Varella-Garcia M (2005) Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* **97:** 643–655

Coldren CD, Helfrich BA, Witta SE, Sugita M, Lapadat R, Zeng C, Baron A, Franklin WA, Hirsch FR, Geraci MW, Bunn Jr PA (2006) Baseline gene expression predicts sensitivity to gefitinib in non-small cell lung cancer cell lines. *Mol Cancer Res* **4:** 521–528

Crombet T, Osorio M, Cruz T, Roca C, del Castillo R, Mon R, Iznaga-Escobar N, Figueredo R, Koropatnick J, Renginfo E, Fernandez E, Alvarez D, Torres O, Ramos M, Leonard I, Perez R, Lage A (2004) Use of the humanized anti-epidermal growth factor receptor monoclonal antibody h-R3 in combination with radiotherapy in the treatment of locally advanced head and neck cancer patients. *J Clin Oncol* **22:** 1646–1654

Crombet T, Torres L, Neninger E, Catala M, Solano ME, Perera A, Torres O, Iznaga N, Torres F, Perez R, Lage A (2003) Pharmacological evaluation of humanized anti-epidermal growth factor receptor, monoclonal antibody h-R3, in patients with advanced epithelial-derived cancer. *J Immunother* **26:** 139–148

Crombet-Ramos T, Rak J, Perez R, Viloria-Petit A (2002) Antiproliferative, antiangiogenic and proapoptotic activity of h-R3: a humanized anti-EGFR antibody. *Int J Cancer* **101:** 567–575

Das AK, Sato M, Story MD, Peyton M, Graves R, Redpath S, Girard L, Gazdar AF, Shay JW, Minna JD, Nirodi CS (2006) Non-small cell lung cancers with kinase domain mutations in the epidermal growth factor receptor are sensitive to ionizing radiation. *Cancer Res* **66:** 9601–9608

Dent P, Yacoub A, Contessa J, Caron R, Amorino G, Valerie K, Hagan MP, Grant S, Schmidt-Ullrich R (2003) Stress and radiation-induced activation of multiple intracellular signaling pathways. *Radiat Res* **159:** 283–300

Ettinger DS (2006) Clinical implications of EGFR expression in the development and progression of solid tumors: focus on non-small cell lung cancer. *Oncologist* **11:** 358–373

Gschwind A, Fischer OM, Ullrich A (2004) The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* **4:** 361–370

Harari PM, Huang S (2006) Radiation combined with EGFR signal inhibitors: head and neck cancer focus. *Semin Radiat Oncol* **16:** 38–44

Huang SM, Bock JM, Harari PM (1999) Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res* **59:** 1935–1940

Imai K, Takaoka A (2006) Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer* **6:** 714–727

Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* **352:** 786–792

Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM (2005) Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* **7:** 301–311

Liang K, Ang KK, Milas L, Hunter N, Fan Z (2003) The epidermal growth factor receptor mediates radioresistance. *Int J Radiat Oncol Biol Phys* **57:** 246–254

Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* **66:** 3992–3995

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350:** 2129–2139

Marshall J (2006) Clinical implications of the mechanism of epidermal growth factor receptor inhibitors. *Cancer* **107:** 1207–1218

Milas L, Mason K, Hunter N, Petersen S, Yamakawa M, Ang K, Mendelsohn J, Fan Z (2000) *In vivo* enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. *Clin Cancer Res* **6:** 701–708

Mitsudomi T, Kosaka T, Endoh H, Horio Y, Hida T, Mori S, Hatooka S, Shinoda M, Takahashi T, Yatabe Y (2005) Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. *J Clin Oncol* **23:** 2513–2520

Okabe T, Okamoto I, Tamura K, Terashima M, Yoshida T, Satoh T, Takada M, Fukuoka M, Nakagawa K (2007) Differential constitutive activation of the epidermal growth factor receptor in non-small cell lung cancer cells bearing EGFR gene mutation and amplification. *Cancer Res* **67:** 2046–2053

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304:** 1497–1500

Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M, Varmus H (2004) 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 USA* **101:** 13306–13311

Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* **2:** 225–235

Prewett MC, Hooper AT, Bassi R, Ellis LM, Waksal HW, Hicklin DJ (2002) Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. *Clin Cancer Res* **8:** 994–1003

Raben D, Helfrich B, Chan DC, Ciardiello F, Zhao L, Franklin W, Baron AE, Zeng C, Johnson TK, Bunn Jr PA (2005) The effects of cetuximab alone and in combination with radiation and/or chemotherapy in lung cancer. *Clin Cancer Res* **11:** 795–805

Schmidt-Ullrich RK, Contessa JN, Lammering G, Amorino G, Lin PS (2003) ERBB receptor tyrosine kinases and cellular radiation responses. *Oncogene* **22:** 5855–5865

Schmidt-Ullrich RK, Mikkelsen RB, Dent P, Todd DG, Valerie K, Kavanagh BD, Contessa JN, Rorrer WK, Chen PB (1997) Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. *Oncogene* **15:** 1191–1197

Takano T, Ohe Y, Sakamoto H, Tsuta K, Matsuno Y, Tateishi U, Yamamoto S, Nokihara H, Yamamoto N, Sekine I, Kunitoh H, Shibata T, Sakiyama T, Yoshida T, Tamura T (2005) 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* **23:** 6829–6837

Tsuchihashi Z, Khambata-Ford S, Hanna N, Janne PA (2005) Responsiveness to cetuximab without mutations in EGFR. *N Engl J Med* **353:** 208–209

Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, Embleton J, Newell D, Raymond R, Stables J, Stephens T, Wallace J (1998) United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia 2nd edn. *Br J Cancer* **77:** 1–10

Radiosensitizing Effect of YM155, a Novel Small-Molecule Survivin Suppressant, in Non–Small Cell Lung Cancer Cell Lines

Tsutomu Iwasa,[1] Isamu Okamoto,[1] Minoru Suzuki,[2] Takahito Nakahara,[4] Kentaro Yamanaka,[4] Erina Hatashita,[1] Yuki Yamada,[1] Masahiro Fukuoka,[3] Koji Ono,[2] and Kazuhiko Nakagawa[1]

Abstract

Purpose: Survivin, a member of the inhibitor of apoptosis protein family, is an attractive target for cancer therapy. We have now investigated the effect of YM155, a small-molecule inhibitor of survivin expression, on the sensitivity of human non–small cell lung cancer (NSCLC) cell lines to γ-radiation.

Experimental Design: The radiosensitizing effect of YM155 was evaluated on the basis of cell death, clonogenic survival, and progression of tumor xenografts. Radiation-induced DNA damage was evaluated on the basis of histone H2AX phosphorylation and foci formation.

Results: YM155 induced down-regulation of survivin expression in NSCLC cells in a concentration- and time-dependent manner. A clonogenic survival assay revealed that YM155 increased the sensitivity of NSCLC cells to γ-radiation *in vitro*. The combination of YM155 and γ-radiation induced synergistic increases both in the number of apoptotic cells and in the activity of caspase-3. Immunofluorescence analysis of histone γ-H2AX also showed that YM155 delayed the repair of radiation-induced double-strand breaks in nuclear DNA. Finally, combination therapy with YM155 and γ-radiation delayed the growth of NSCLC tumor xenografts in nude mice to a greater extent than did either treatment modality alone.

Conclusions: These results suggest that YM155 sensitizes NSCLC cells to radiation both *in vitro* and *in vivo*, and that this effect of YM155 is likely attributable, at least in part, to the inhibition of DNA repair and enhancement of apoptosis that result from the down-regulation of survivin expression. Combined treatment with YM155 and radiation warrants investigation in clinical trials as a potential anticancer strategy.

Survivin is a 16.5-kDa member of the inhibitor of apoptosis protein (IAP) family. It blocks the mitochondrial pathway of apoptosis by inhibiting caspases (1, 2) and regulates cell division through interaction with the proteins INCENP and Aurora B (3). It is abundant in many types of cancer cells but not in the corresponding normal cells (4–6). High levels of survivin expression in cancer cells are associated with poor patient prognosis and survival as well as with resistance to therapy and an increased rate of cancer recurrence (7–9). Survivin has therefore become a therapeutic target and potentially important prognostic marker for many tumor types, including non–small cell lung cancer (NSCLC; refs. 7, 10). Molecular antagonists of survivin including antisense oligonucleotides, and dominant negative mutants have been shown to induce apoptosis in cancer cells *in vitro* and *in vivo* as well as to enhance chemotherapy-induced cell death (11–13). Although antisense oligonucleotides and ribozymes can be engineered to be highly specific for survivin, they may be difficult to deliver in the clinical setting.

YM155, a small imidazolium-based compound, was identified by high-throughput screening of chemical libraries for inhibitors of the activity of the survivin gene promoter in a reporter assay (14). This compound specifically inhibits the expression of survivin at both the mRNA and protein levels and exhibits pronounced anticancer activity in preclinical models (14). An advantage of YM155 compared with previously investigated suppressors of survivin expression (15–20) is that it is active in the subnanomolar range. Pharmacokinetic analysis also revealed that YM155 was highly distributed to tumor tissue in tumor xenograft models *in vivo* (14). YM155 is thus an attractive candidate drug for cancer therapy, and clinical trials of YM155 in single-agent therapy are currently under way for some types of cancer.

Glioblastoma cells that overexpress survivin were found to be less responsive to radiation than survivin-negative cells in a preclinical model (21). Clinically, high levels of survivin expression have been associated with an increased risk of local treatment failure after radiochemotherapy in patients with rectal cancer (9). These observations suggest that survivin plays

Authors' Affiliations: [1]Department of Medical Oncology, Kinki University School of Medicine, Osaka-Sayama, Osaka, Japan; [2]Radiation Oncology Research Laboratory, Research Reactor Institute, Kyoto University, Sennan-gun, Osaka, Japan; and [3]Kinki University School of Medicine, Sakai Hospital, Minami-ku Sakai, Osaka, Japan; and [4]Institute for Drug Research, Astellas Pharma, Inc., Tsukuba-shi, Ibaraki, Japan

Received 2/20/08; revised 5/21/08; accepted 6/1/08.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Isamu Okamoto, Department of Medical Oncology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan. Phone: 81-72-366-0221; Fax: 81-72-360-5000; E-mail: chi-okamoto@dotd.med.kindai.ac.jp.

doi:10.1158/1078-0432.CCR-08-0468

Translational Relevance

Survivin is a potentially important molecular target for cancer therapy. Reflecting the many mechanisms that seem to regulate survivin expression, diverse approaches have been evaluated for targeting survivin in experimental models. YM155 is a novel small, imidazolium-based compound that specifically inhibits survivin expression in various types of cancer cell lines *in vitro*. In addition, YM155 has been shown to distribute preferentially to tumor tissues rather than to plasma as well as to exert pronounced antitumor activity in tumor xenograft models *in vivo*. The use of YM155 as a single agent in phase I clinical trials did not reveal significant toxicity. Although phase II studies of YM155 use as a single agent for certain types of cancer are currently under way, the effects of YM155 in combination with radiation have not been reported. We now show that inhibition of survivin expression by YM155 sensitizes tumor cells to radiation *in vitro* and *in vivo*. Therefore, our preclinical results provide a rationale for future clinical investigation of the therapeutic efficacy of YM155 in combination with radiotherapy.

a role in resistance to radiotherapy. Indeed, suppression of survivin expression with the use of antisense oligonucleotides or ribozymes has been shown to increase the radiosensitivity of cancer cells *in vitro* (20, 22–26). We have now examined the effects of the combination of YM155 and radiation on NSCLC cell lines *in vitro* and *in vivo*.

Materials and Methods

Cell culture and reagents. The human NSCLC cell lines NCI-H460 (H460) and Calu6 were obtained from the American Type Culture Collection. The cells were cultured under an atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. YM155 (Astellas Pharma, Inc.) was dissolved in DMSO.

Immunoblot analysis. Cells were washed twice with ice-cold PBS and then lysed in a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium PPi, 1 mmol/L phenylmethylsulfonyl fluoride, and leupeptin (1 μg/mL). The protein concentration of lysates was determined with the Bradford reagent (Bio-Rad), and equal amounts of protein were subjected to SDS-PAGE of a 15% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 h at room temperature before incubation overnight at 4°C with rabbit polyclonal antibodies to human survivin (1:1000 dilution; R&D Systems), to human c-IAP1 (1:1,000 dilution; MBL International), to human XIAP (1:1,000 dilution; Cell Signaling), to human STAT3 (1:1,000 dilution; Cell Signaling), or to β-actin (1:500 dilution; Sigma), or with mouse monoclonal antibodies to human p53 (1:1,000 dilution; Santa Cruz Biotechnology). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to rabbit (Sigma) or mouse (Santa Cruz Biotechnology) IgG. Immune complexes were finally detected with chemiluminescence reagents (Perkin-Elmer Life Science).

Clonogenic survival assay. Exponentially growing 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 complete medium in the presence of 50 nmol/L YM155 or vehicle (final DMSO concentration of 0.1%; we confirmed that this DMSO concentration did not affect the proliferation of NSCLC cell lines). After incubation for 48 h, the cells were exposed at room temperature to various doses of γ-radiation with a ^{60}Co irradiator at a rate of −0.82 Gy/min. The cells were then washed with PBS, cultured in drug-free medium for 10 to 14 d, 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 control cells. The surviving fraction for combined treatment was corrected by that for YM155 treatment alone. Cell survival was corrected according to the equation $S = 1 - (1 - f)^{1/N}$, where S is the single-cell survival rate, f is the measured surviving fraction, and N is multiplicity, which was defined as the average number of cells per microcolony at the time of radiation and which ranged from 2.4 to 6.7 for the cell lines studied under the described conditions. The dose enhancement factor was then calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.1 for vehicle-treated cells divided by that for YM155-treated cells (after correction for drug toxicity).

Detection of apoptotic cells. Cells were fixed with 4% paraformaldehyde for 1 h at room temperature, after which a minimum of 1,000 cells per sample was evaluated for apoptosis with the use of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique (*In situ* Cell Death Detection Kit; Boehringer Mannheim).

Assay of caspase-3 activity. The activity of caspase-3 in cell lysates was measured with the use of a CCP32/Caspase-3 Fluometric Protease Assay Kit (MBL). Fluorescence attributable to cleavage of the DEVD-AFC substrate was measured at excitation and emission wavelengths of 390 and 460 nm, respectively.

Immunofluorescence staining of γ-H2AX. Cells were grown to 50% confluence in two-well Lab-Tec Chamber Slides (Nunc) and then cultured for 48 h in the presence of 50 nmol/L YM155 or vehicle before exposure to 3 Gy of γ-radiation. At various times thereafter, they were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min at 4°C, and exposed to 5% nonfat dried milk for 10 min at room temperature. The slides were washed with PBS and then incubated at room temperature first for 2 h with mouse monoclonal antibodies to histone γ-H2AX (Upstate Biotechnology) at a dilution of 1:300 and then for 1 h with Alexa 488–labeled goat antibodies to mouse IgG (Molecular Probes) at a dilution of 1:700. The slides were mounted in fluorescence mounting medium (Dako Cytomation), and fluorescence signals were visualized with a confocal laser-scanning microscope (Axiovert 200M; Carl Zeiss) equipped with the LSM5 PASCAL system (Carl Zeiss). Three random fields each containing ≈50 cells were examined at a magnification of × 100. Nuclei containing ≥10 immunoreactive foci were counted as positive for γ-H2AX, as previously described (27), and percentage of positive cells was calculated.

***Evaluation of tumor growth* in vivo.** All animal studies were done 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 procedures followed met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines (28). Tumor cells (2×10^6) were injected s.c. into the right hind leg of 6-week-old female athymic nude mice (BALB/c nu/nu). Tumor volume was determined from caliper measurement of tumor length (L) and width (W) according to the formula $LW^2/2$. Treatment was initiated when the tumors in each group of animals achieved an average volume of ~200 to 250 mm^3. Treatment groups (each containing eight mice) consisted of vehicle control (physiologic saline), YM155 alone, vehicle plus radiation, and YM155 plus radiation. Vehicle or YM155 at a dose of 5 mg/kg of body mass was administered over 7 consecutive days (days 1-7) with the use of an implanted micro-osmotic pump (Alzet model 1003D; Durect). Mice in the radiation groups received 10 Gy of γ-radiation from a cobalt irradiator either as

a single fraction on day 3 of drug treatment or fractionated over 5 consecutive days (days 3 to 7); the radiation was targeted to the tumor, with the remainder of the body shielded with lead. Growth delay (GD) was calculated as the time required to achieve a 5-fold increase in volume for treated tumors minus that for control tumors. The enhancement factor was then determined as: $(GD_{combination} - GD_{YM155})/GD_{radiation}$.

Statistical analysis. Data are presented as means ± SD or SE and were compared with the unpaired Student's *t* test. A P value of <0.05 was considered statistically significant.

Results

Inhibition of survivin expression in NSCLC cells by YM155. We first examined the effect of YM155 on survivin expression in human NSCLC cell lines by immunoblot analysis. Treatment of H460 or Calu6 cells with YM155 at 1 to 100 nmol/L for 48 hours inhibited survivin expression in a concentration-dependent manner (Fig. 1A). In contrast, YM155 had no effect on the abundance of other members of the IAP family including XIAP and c-IAP1 (Fig. 1A), suggesting that YM155 specifically inhibits survivin expression in the NSCLC cell lines. The mechanism by which YM155 inhibits survivin expression remains to be elucidated. Previous observations have shown that p53 and signal transducer and activator of transcription 3 (STAT3) regulate survivin expression at the transcriptional level (29). We therefore examined the effect of YM155 on the abundance of p53 and STAT3 in NSCLC cell lines. YM155 showed no marked effect on the amounts of p53 and STAT3 in H460 or Calu6 cells (Fig. 1A), suggesting that the inhibition of survivin expression by YM155 is independent of these transcriptional regulators. Monitoring of the time course of survivin expression in cells exposed to 50 nmol/L

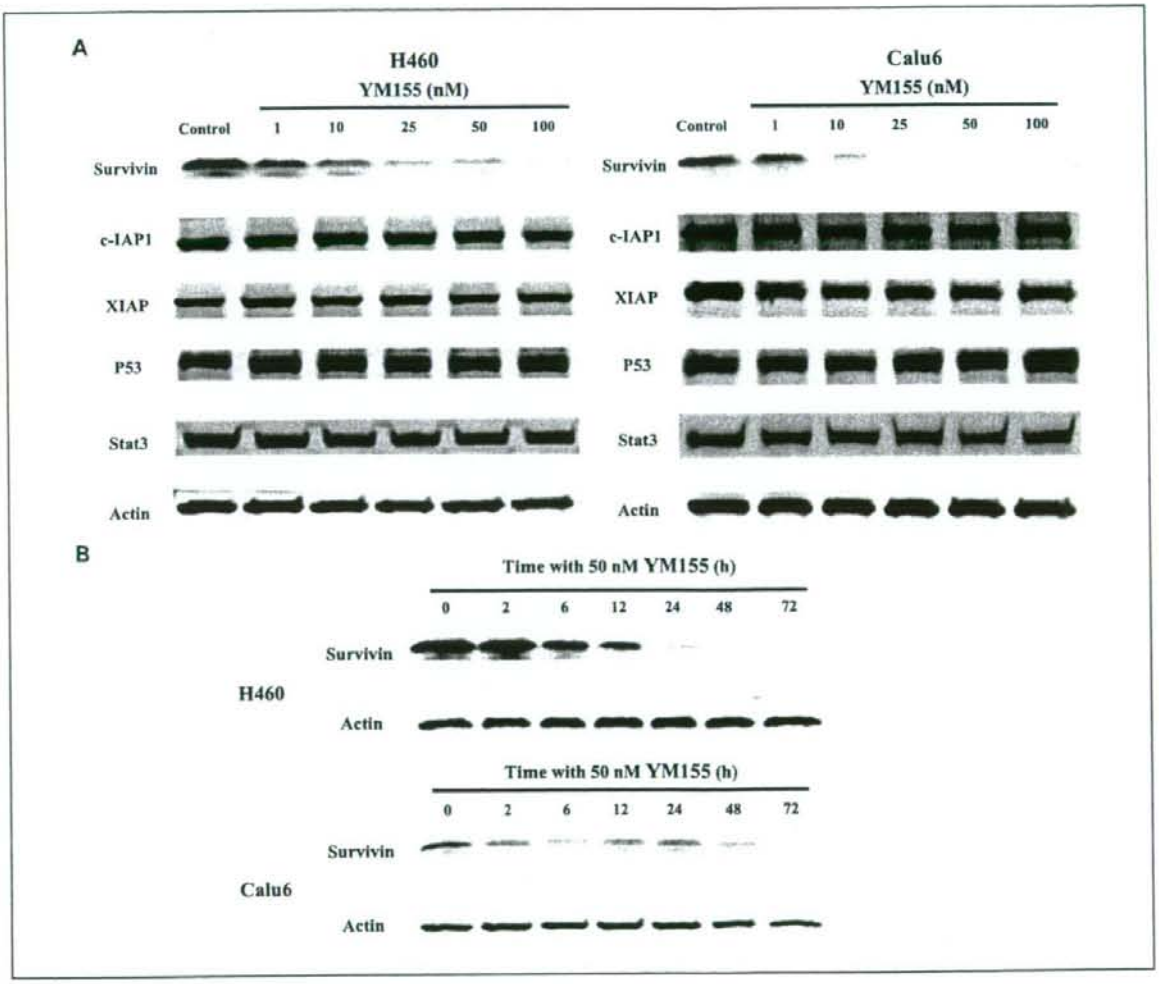

Fig. 1. Effect of YM155 on survivin expression in human NSCLC cells. *A*, H460 or Calu6 cells were incubated in the absence (control, 0.1% DMSO) or presence of various concentrations (1, 10, 25, 50, or 100 nmol/L) of YM155 for 48 h. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to survivin, to c-IAP1, to XIAP, to p53, to STAT3, or to β-actin (loading control). *B*, H460 or Calu6 cells were incubated with 50 nmol/L YM155 for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to survivin or to β-actin.

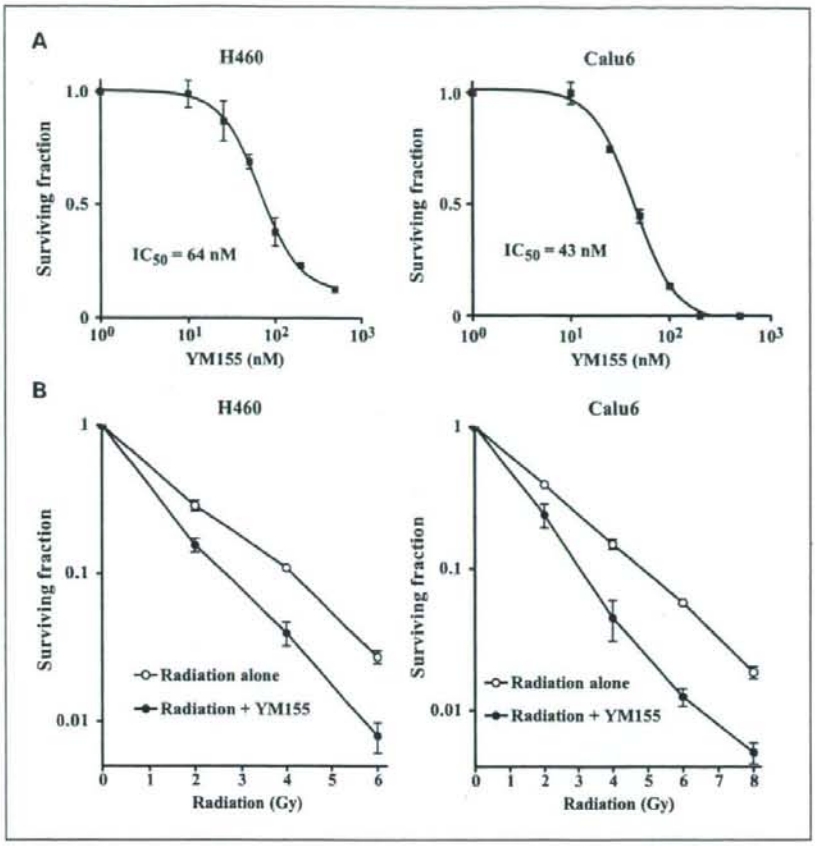

Fig. 2. Effect of YM155 on the sensitivity of H460 or Calu6 cells to γ-radiation. *A*, cells were incubated with the indicated concentrations of YM155 for 48 h and then assayed for clonogenic survival. Points represent means from three independent experiments; bars represent SD. *B*, cells were incubated with 50 nmol/L YM155 or vehicle (control, 0.1% DMSO) for 48 h, exposed to the indicated doses of γ-radiation, and then incubated in drug-free medium for 10 to 14 d for determination of colony-forming ability. Colonies were counted and the surviving fraction was calculated. Plating efficiency for nonirradiated H460 cells was 77.0% and 38.8% for vehicle-treated and YM155-treated cells, respectively; that for nonirradiated Calu6 cells was 57.0% and 23.5%, respectively. All surviving fractions with radiation were corrected for these baseline plating efficiencies. Points represent means from three independent experiments; bars represent SD.

YM155 for up to 72 hours revealed that the abundance of survivin in Calu6 cells had decreased by 2 hours and that survivin was virtually undetectable in H460 cells after 24 hours (Fig. 1B). In both cell lines, treatment with 50 nmol/L YM155 resulted in time-dependent inhibition of survivin expression.

YM155-induced sensitization of NSCLC cells to radiation. To examine the effect of YM155 on cell survival, we first did a clonogenic survival assay. Exposure to the drug at concentrations of 1 to 500 nmol/L for 48 hours revealed that YM155 inhibited the survival of H460 cells with a median inhibitory concentration (IC_{50}) of 64 nmol/L and that of Calu6 cells with an IC_{50} of 43 nmol/L (Fig. 2A). On the basis of these data, we adopted treatment with 50 nmol/L YM155 for 48 hours as the standard protocol for radiation experiments. We next examined whether YM155 might affect the sensitivity of NSCLC cell lines to radiation. Treatment with 50 nmol/L YM155 for 48 hours shifted the survival curves for both H460 and Calu6 cells to the left (Fig. 2B), with a dose enhancement factor of 1.57 and 1.61, respectively, suggesting that YM155 increased the radiosensitivity of both cell lines.

Enhancement of radiation-induced apoptosis in NSCLC cells by YM155. We next examined the effect of YM155 on radiation-induced apoptosis in H460 or Calu6 cells with the use of the TUNEL assay. Combined treatment of either cell line with YM155 and γ-radiation resulted in an increase in the number of apoptotic cells at 24 and 48 hours that was greater than the sum of the increases induced by YM155 or radiation alone (Fig. 3A). To confirm the results of the TUNEL assay, we measured the activity of caspase-3 in cell lysates. Again, the combined treatment of H460 or Calu6 cells with YM155 and γ-radiation induced a synergistic increase in caspase-3 activity (Fig. 3B). These data thus suggested that YM155 promotes radiation-induced apoptosis in NSCLC cell lines.

Inhibition of DNA repair in irradiated NSCLC cells by YM155. Defects in DNA repair have been associated with enhanced sensitivity of cells to radiation (30, 31), and survivin is thought to play a direct or indirect role in DNA repair (21). We therefore next investigated the effect of YM155 on DNA repair by immunostaining of cells with antibodies to the phosphorylated form (γ-H2AX) of histone H2AX, foci of which form at DNA double-strand breaks (DSBs). The formation of γ-H2AX foci in H460 cells was apparent between 30 minutes and 6 hours after γ-irradiation (Fig. 4A). In the presence of YM155, however, these foci persisted for at least 24 hours after irradiation. Evaluation of the percentage of H460 or Calu6 cells with γ-H2AX foci at 24 hours after irradiation revealed that YM155 significantly inhibited the repair of DSBs (Fig. 4B). These results thus suggested that down-regulation of survivin expression by YM155 results in the inhibition of the repair of

radiation-induced DSBs in NSCLC cells, possibly accounting for the observed radiosensitization by this drug.

Enhancement of radiation-induced tumor regression by YM155. To determine whether the YM155-induced radiosensitization of NSCLC cells observed *in vitro* might also be apparent *in vivo*, we injected H460 or Calu6 cells into nude mice to elicit the formation of solid tumors. After tumor formation, the mice were treated with YM155, γ-radiation, or both modalities. YM155 was infused continuously for 7 days with the use of an implanted osmotic pump system, and mice were subjected to local irradiation with a single dose of 10 Gy on day 3 of YM155 administration. Combined treatment with radiation and YM155 inhibited H460 or Calu6 tumor growth to a markedly greater extent than did either modality alone (Fig. 5). The tumor growth delays induced by treatment with radiation alone, YM155 alone, or both YM155 and radiation were 2.9, 5.6, and 14.8 days, respectively, for H460 cells, and 8.9, 41.0, and 76.0 days, respectively, for Calu6 cells. The enhancement factor for the effect of YM155 on the efficacy of radiation was 3.3 for H460 cells and 3.5 for Calu6 cells, revealing the effect to be greater than additive. No pronounced tissue damage or toxicity such as weight loss was observed in mice in any of the four treatment groups.

Finally, we evaluated whether the combination of YM155 and fractionated radiation treatment would result in the inhibition of tumor growth similar to that observed with YM155 plus single-fraction radiation. Mice bearing H460 tumors were thus again subjected to continuous YM155 infusion for 7 days, but local irradiation was done in 2-Gy fractions on days 3 to 7 of drug administration (for a total dose of 10 Gy). The tumor growth delays induced by treatment with radiation alone, YM155 alone, or both YM155 and radiation were 3.8, 5.3, and 16.6 days, respectively (Fig. 6). The enhancement factor for the effect of YM155 on the efficacy of radiation was 3.0. Again, there was no evidence of toxicity on the basis of body weight loss, and there were no animal deaths in any of the four groups. These data suggested that YM155 enhances the tumor response to both single-dose and fractionated radiotherapy *in vivo*.

Discussion

Survivin is a potentially important molecular target for cancer therapy. Reflecting the many mechanisms that seem to regulate survivin expression, diverse approaches have been evaluated for targeting survivin in experimental models. Although certain drugs, such as inhibitors of histone deacetylases,

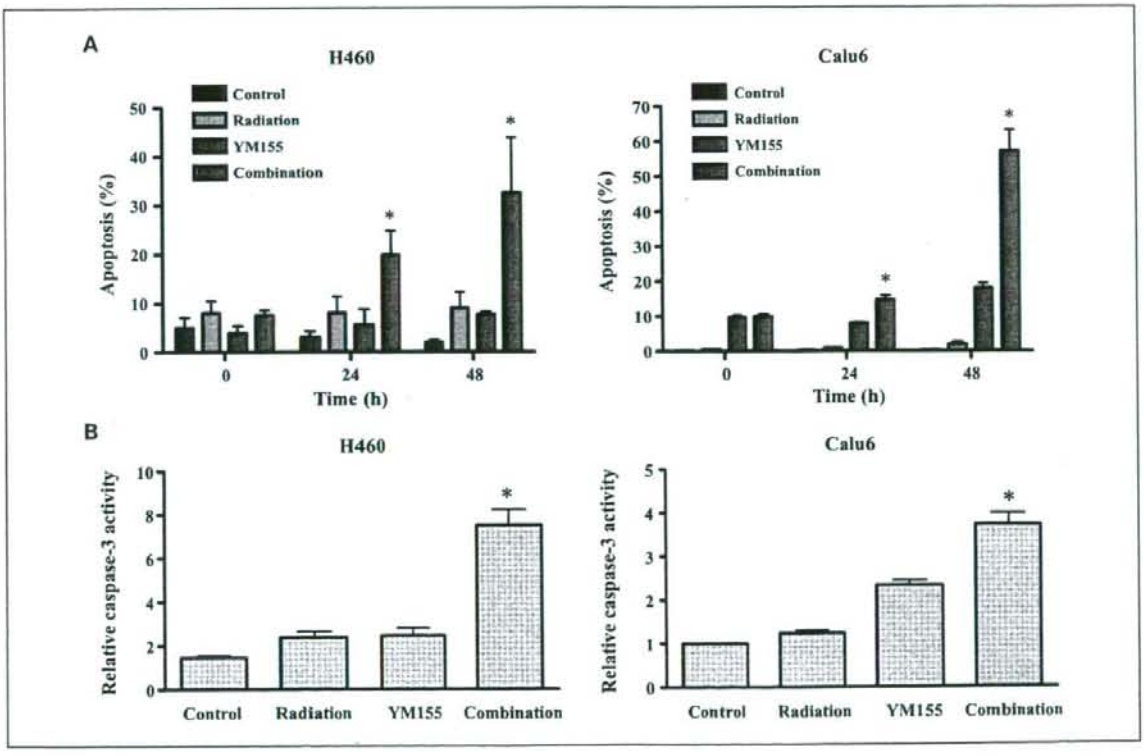

Fig. 3. Effect of YM155 on radiation-induced apoptosis and caspase-3 activity in H460 or Calu6 cells. *A*, cells were incubated with 50 nmol/L YM155 or vehicle (0.1% DMSO) for 48 h, exposed (or not) to 3 Gy of γ-radiation, and then incubated in drug-free medium for 24 or 48 h, at which times the percentage of apoptotic cells was determined by TUNEL staining. *B*, lysates of cells treated as in *A* were assayed for caspase-3 activity 24 h after irradiation. Columns represent means from three independent experiments; bars represent SD; those in *B* are expressed relative to the corresponding value for the control condition. * $P < 0.01$ versus the corresponding value for treatment with radiation or YM155 alone.

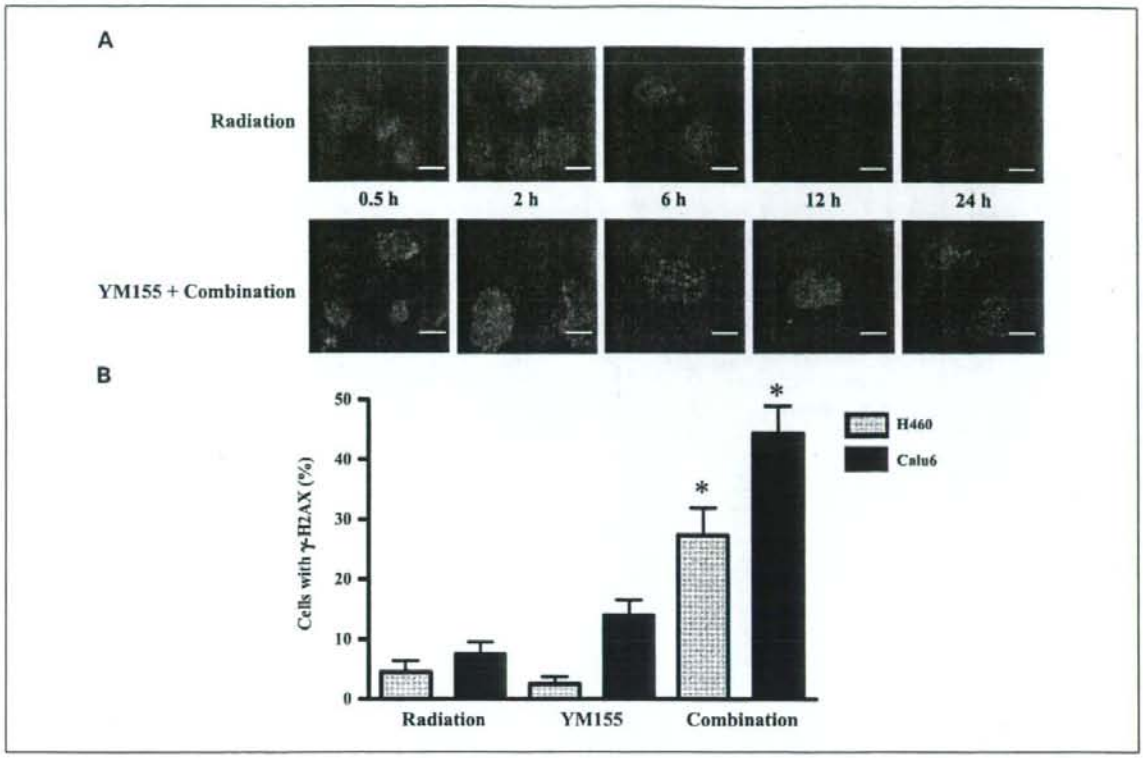

Fig. 4. Effect of YM155 on the radiation-induced formation of γ-H2AX foci in NSCLC cells. *A*, H460 cells were incubated with vehicle (0.1% DMSO) or 50 nmol/L YM155 for 48 h and then exposed to 3 Gy of γ-radiation. After incubation for the indicated times in drug-free medium, the cells were fixed and subjected to immunofluorescence staining for γ-H2AX (green fluorescence). Scale bar, 10 μm. *B*, H460 or Calu6 cells were incubated with vehicle or YM155 and then exposed (or not) to γ-radiation as in *A*. They were fixed at 24 h after irradiation and the percentage of cells containing γ-H2AX foci was determined. Columns represent means from three independent experiments; bars represent SD. $^*P < 0.05$ versus the corresponding value for radiation or YM155 alone.

mitogen-activated protein kinases, and cyclin-dependent kinases, have been shown to suppress survivin expression by targeting various signaling pathways, these drugs inhibit survivin expression nonspecifically (15–17, 19, 32). Gene therapy strategies based on small interfering RNA or other antisense oligonucleotides are specific for survivin, but the effective delivery of these molecules remains a challenge for the transition to the clinic (33). YM155 is a small-molecule agent that specifically inhibits survivin expression in various types of cancer cell lines *in vitro* (14). In addition, YM155 has been shown both to distribute preferentially to tumor tissues rather than to plasma as well as to exert pronounced antitumor activity in tumor xenograft models *in vivo* (14). The use of YM155 as a single agent in phase I clinical trials did not reveal significant toxicity (34). Although phase II studies of YM155 use as a single agent for certain types of cancer are currently under way, the effects of YM155 in combination with radiation have not been reported. We now show that YM155 increased the sensitivity of tumor cells to radiation *in vitro* and *in vivo*.

Clonogenic survival analysis, the most reliable approach for assessing the ability of genotoxic agents to induce cell death (35), revealed that YM155 markedly potentiated the decrease in NSCLC cell survival induced by γ-radiation. Given that induction of apoptosis is a key mechanism of cytotoxicity for most antitumor agents, including γ-radiation, defects in apoptotic signaling may underlie resistance to such agents (36). Radiation-sensitive tumors undergo radiation-induced apoptosis *in vitro* more readily than do radiation-resistant tumors (37–40). Treatment with caspase inhibitors has been shown to protect tumor cells against radiation-induced apoptosis and to increase their radioresistance (21, 41, 42), suggesting that radiation-induced apoptosis is caspase-dependent and that caspases contribute to radiosensitivity. The antiapoptotic activity of survivin is mostly attributable to inhibition of the activation of downstream effectors of apoptosis such as caspase-3 and caspase-7 (25). We have now shown that radiosensitization of NSCLC cells by YM155 was associated with increases both in the activity of caspase-3 and in the proportion of apoptotic cells. Our findings thus suggest that YM155 sensitized tumor cells to radiation at least in part by enhancing radiation-induced apoptosis.

We examined further the mechanism by which YM155 induces radiosensitization. Survivin is essential for the proper execution of mitosis and cell division, with disruption of survivin expression resulting in cell division defects that can lead to polyploidy and the formation of multinucleated cells (43, 44). Although treatment with 50 nmol/L YM155 for 48 hours inhibited survivin expression in NSCLC cells, it

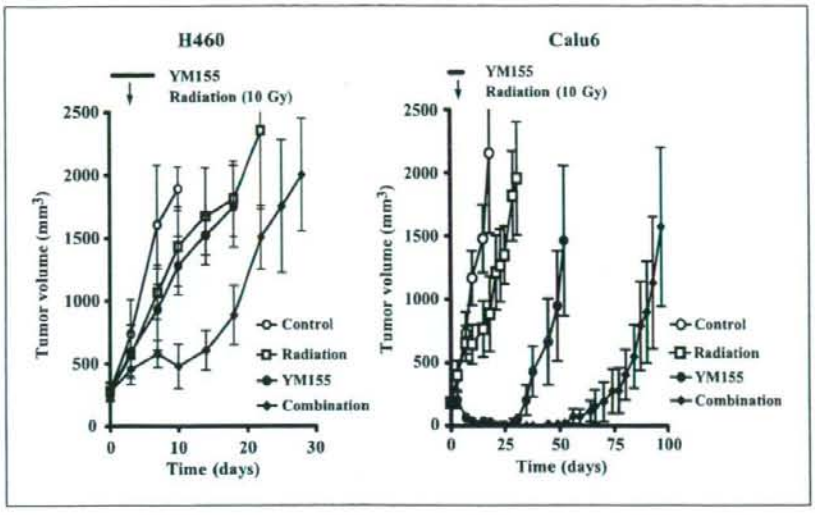

Fig. 5. Effect of YM155 on the growth of H460 or Calu6 tumors in mice subjected to single-dose radiotherapy. Cells were injected into the right hind limb of nude mice and allowed to grow. The mice were divided into four treatment groups: control, radiation alone, YM155 alone, or the combination of YM155 and radiation. YM155 (5 mg/kg) or vehicle was administered by continuous infusion over 7 d, and mice in the radiation groups were subjected to γ-irradiation with a single dose of 10 Gy on day 3 of drug treatment. Tumor volume was measured at the indicated times after the onset of treatment. Points, means from eight mice per group; bars, SE.

did not induce polyploidy (data not shown), suggesting that YM155-induced radiosensitization in the present study was not attributable to cell division defects caused by survivin depletion. Survivin was previously suggested to enhance tumor cell survival after radiation exposure through regulation of DSB repair (21). We therefore investigated the effect of YM155 on the repair of radiation-induced DSBs by immunofluorescence imaging of γ-H2AX foci. H2AX is a histone that is phosphorylated by ataxia telangiectasia mutated and DNA-dependent protein kinase in response to the generation of DSBs (45, 46). This reaction occurs rapidly, with half-maximal amounts of γ-H2AX generated within 1 minute and maximal amounts within 10 minutes (47), and a linear relation has been shown between the number of γ-H2AX foci and that of DSBs (48). The number of γ-H2AX foci is thus a sensitive and specific indicator of the existence of DSBs, with a decrease in this number reflecting DSB repair. We found that YM155 inhibited the repair of radiation-induced DSBs in NSCLC cells. If left unrepaired, DSBs can result in chromosome loss or cell death; agents that inhibit such repair thus increase the sensitivity of cells to ionizing radiation (49, 50). Our results therefore suggest that inhibition of DSB repair by YM155 contributes to the radiosensitization induced by this drug. Given that suppression of survivin expression impairs the repair of radiation-induced DNA damage (9, 21), our results further suggest that inhibition of DNA repair by YM155 is attributable to down-regulation of survivin expression.

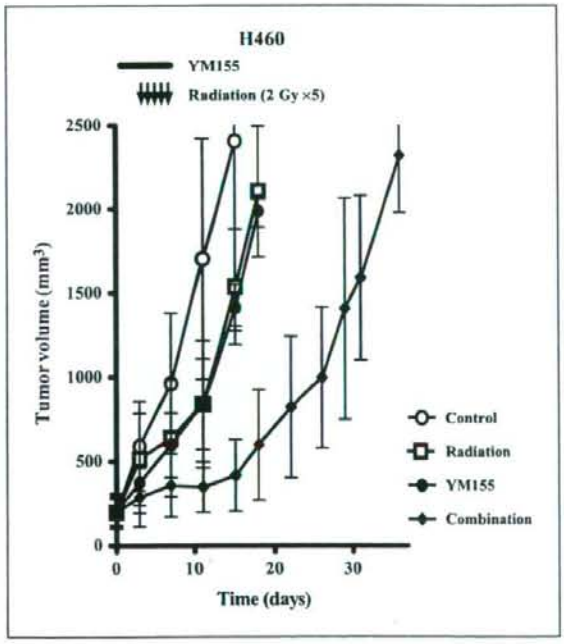

Fig. 6. Effect of YM155 on the growth of H460 tumors in mice subjected to fractionated radiotherapy. H460 cells were injected into the right hind limb of nude mice and allowed to grow. The mice were divided into four treatment groups: control, radiation alone, YM155 alone, or the combination of YM155 and radiation. YM155 (5 mg/kg) or vehicle was administered by continuous infusion over 7 d, and mice in the radiation groups were subjected to γ-irradiation with a daily dose of 2 Gy on days 3 to 7 of drug treatment. Tumor volume was measured at the indicated times after the onset of treatment. Points represent means from eight mice per group; bars represent SE.

The antitumor activity of YM155 has previously been shown to be time-dependent, with continuous infusion of the drug resulting in greater antitumor activity and less systemic toxicity compared with bolus injection in tumor xenograft models *in vivo* (14). Ongoing clinical trials of YM155 are thus being done with the drug administered on a continuous schedule. We also administered YM155 by continuous infusion in our *in vivo* experiments. The combination of YM155 with single-dose radiotherapy resulted in a marked increase in tumor growth delay compared with that apparent with either radiation or YM155 alone, indicating that YM155 enhanced the antitumor effect of ionizing radiation *in vivo*. Given that standard radiation therapy in the clinic is delivered according to a fractionated schedule, we also examined whether YM155 enhanced the tumor response to clinically relevant fractionated doses (2 Gy) of radiation. Indeed, YM155 was also effective in