Association of epidermal growth factor receptor (*EGFR*) gene mutations with *EGFR* amplification in advanced non-small cell lung cancer

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Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the response to *EGFR* tyrosine kinase inhibitors in patients with non-small cell lung cancer (NSCLC). Increased *EGFR* copy number has also been associated with sensitivity to these drugs. However, given that it is often difficult to obtain sufficient amounts of tumor tissue for genetic analysis from patients with advanced NSCLC, the relationship between these two types of *EGFR* alterations has remained unclear. We have now evaluated *EGFR* mutation status both by direct sequencing and with a high-sensitivity assay, the Scorpion-amplification-refractory mutation system, and have determined *EGFR* copy number by fluorescence *in situ* hybridization (FISH) analysis in paired tumor specimens obtained from 100 consecutive patients with advanced NSCLC treated with chemotherapy. *EGFR* mutations or FISH positivity (*EGFR* amplification or high polysomy) were apparent in 18% (18/100) and 32% (32/100) of patients, respectively. The Scorpion-amplification-refractory mutation system was more sensitive than direct sequencing for the detection of *EGFR* mutations. Furthermore, *EGFR* mutations were associated with *EGFR* amplification ($P = 0.009$) but not with FISH positivity ($P = 0.266$). Our results therefore suggest the existence of a significant association between *EGFR* mutation and *EGFR* amplification in patients with advanced NSCLC. (*Cancer Sci* 2008; 99: 2455–2460)

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase of the ErbB family and has been implicated in the proliferation and survival of cancer cells. Aberrant expression of *EGFR* has been detected in many human epithelial malignancies, including non-small cell lung cancer (NSCLC).[1,2] This receptor has therefore been identified as a promising target for anticancer therapy, and several agents have been synthesized that inhibit its tyrosine kinase activity. *EGFR* tyrosine kinase inhibitors (TKI) have been evaluated most extensively in individuals with NSCLC, and they have had a substantial impact on the treatment of this disease by offering additional therapeutic options for patients with advanced NSCLC.[3–6]

Somatic mutations in the tyrosine kinase domain of *EGFR* have been detected in a subset of NSCLC patients who respond to EGFR TKI[7–9] and have been shown to be closely associated with sensitivity to these drugs.[10–14] Indeed, we and others have prospectively demonstrated a high response rate to EGFR TKI therapy in NSCLC patients with *EGFR* mutations.[15–21] An increased copy number of the *EGFR* gene, as revealed by fluorescence *in situ* hybridization (FISH), has also emerged as an effective molecular marker of EGFR TKI sensitivity in NSCLC.[22–24] We previously showed that *EGFR* mutation and *EGFR* amplification are associated in human NSCLC cell lines and that endogenous EGFR expressed in such cell lines positive for both of these *EGFR* alterations are activated constitutively.[25] However, the relationship between *EGFR* mutation and FISH positivity for *EGFR*, which reflects gene amplification or high polysomy, has remained unclear.[22–24,26,27] Indeed, only a few studies have evaluated the relationship between mutation and gene copy number for *EGFR* because of the difficulty in obtaining tumor samples suitable for genetic analysis from individuals with advanced NSCLC. We previously showed that the Scorpion-amplification-refractory mutation system (ARMS) is a sensitive technique for the detection of *EGFR* mutations in tumor specimens such as pleural effusion fluid or tissue obtained by transbronchial needle aspiration.[28–30] In the present study, we evaluated *EGFR* mutation status in small tumor specimens from patients with advanced NSCLC both by direct sequencing and by Scorpion-ARMS and compared the sensitivity of these methods for the detection of *EGFR* mutations. Furthermore, we determined *EGFR* copy number by FISH analysis in paired tumor specimens and examined its relationship to *EGFR* mutation.

Materials and Methods

Patients. The present retrospective study recruited consecutive patients with advanced NSCLC who received chemotherapy at Kinki University Hospital between January 2003 and December 2005. Patients eligible for the study had histologically confirmed stage III or IV NSCLC that was not curable by surgical resection or radiotherapy, irrespective of the presence of measurable lesions or good performance status (PS). Patients with recurrence after surgical resection were excluded. Complete clinical information and tissue blocks suitable for genetic analysis were available for 100 patients. We examined the relationship between *EGFR* mutation and *EGFR* copy number as well as the influence of these *EGFR* alterations on clinical outcome. Tumor response was assessed by computed tomography and evaluated according to the Response Evaluation Criteria in Solid Tumors.[31] Survival was calculated from the date of initiation of chemotherapy either to the date of death from any cause or to the date of last contact. Some patients had been receiving EGFR TKI treatment before the demonstration in 2004 that mutations in *EGFR* confer increased sensitivity to these drugs. Moreover, many patients had already died before the initiation of our genetic analysis, preventing us from obtaining informed consent. The institutional review board

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therefore approved our study protocol with the conditions that samples would be processed anonymously and analyzed only for somatic mutations (not for germline mutations) and that the study would be disclosed publicly, according to the Ethical Guidelines for Human Genome Research published by the Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of Health, Labor, and Welfare, and the Ministry of Economy, Trade, and Industry of Japan. The present study also conforms to the provisions of the Declaration of Helsinki.

Identification of *EGFR* mutations. The tumor specimens were fixed with formalin and embedded in paraffin. DNA was extracted with the use of a QIAamp Micro kit (Qiagen K.K., Tokyo, Japan) from tumor tissue derived either by macrodissection or by laser-capture microdissection carried out to enrich tumor cells. Polymerase chain reaction-based direct sequencing of exons 18–21 and ARMS with designed 'Scorpion' primers were applied for the allele-specific detection of *EGFR* mutations. Only the following previously described mutations[7,8] were classified as mutations in the present study: G719X in exon 18, deletion of E746 to A750 or of neighboring residues in exon 19, as well as L858R and L861Q in exon 21. Patients were regarded as *EGFR* mutation positive if a mutation in *EGFR* was detected either by direct sequencing or by ARMS. All mutations were confirmed by analysis of at least two independent amplification products.

Determination of *EGFR* copy number. *EGFR* copy number was determined by FISH analysis with the use of dual-color DNA probes (LSI *EGFR* SpectrumOrange/CEP 7 SpectrumGreen; Vysis, Downers Grove, IL, USA). The tumor specimens were classified into six categories on the basis of the FISH results, as described previously.[22] Those with high polysomy (≥4 copies of *EGFR* in ≥40% of cells) or gene amplification (presence of a tight *EGFR* gene cluster and a ratio of *EGFR* to chromosome 7 of ≥2 or ≥15 copies of *EGFR* per cell in ≥10% of cells analyzed) were considered FISH positive, with those in the remaining categories being considered FISH negative.

Statistical analysis. The relationships among *EGFR* status, clinical characteristics, and tumor response to EGFR TKI were analyzed with Fisher's exact test as appropriate. Survival curves were constructed by the Kaplan–Meier method, and the differences in survival between patient subgroups were compared by the log-rank test. The impact of various factors on survival was evaluated by univariate and multivariate analysis according to the Cox regression model. A P-value <0.05 was considered statistically significant. All statistical analysis was carried out with StatView software (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. Between January 2003 and December 2005, a total of 125 consecutive patients diagnosed histologically with advanced NSCLC underwent chemotherapy at Kinki University Hospital. Tissue specimens from 100 patients were assessable for both *EGFR* mutation and *EGFR* copy number. Of these specimens, 72 were obtained by bronchoscopic biopsy, 15 by percutaneous needle biopsy (12 from lung, two from bone, and one from lymph node), six by thoracoscopic biopsy, and seven by surgery for diagnosis or palliative therapy. The clinical characteristics of these 100 patients are shown in Table 1. Most of the patients were male (64%) and had a history of smoking (67%), and adenocarcinoma was the most prevalent tumor histology (61%). Most patients (83%) also had a good Eastern Cooperative Oncology Group PS (0 or 1), and 63% received second-line or subsequent rounds of chemotherapy. Fifty-three patients (53%) were treated with EGFR TKI. Seventy patients (70%) had died by the time of genetic analysis, with the median follow-up time for the 30 survivors being 14.6 months.

***EGFR* alterations in non-small cell lung cancer.** Patients were analyzed for *EGFR* mutations by direct sequencing of exons 18 through 21 and by Scorpion-ARMS (Table 2). *EGFR* mutations, consisting of in-frame deletions in exon 19 ($n = 5$) and point mutations in exon 21 ($n = 13$), were detected in 18 patients (18%). Eight *EGFR* mutations were detected by direct sequencing and 16 mutations were detected by Scorpion-ARMS. Ten of the 16 mutations detected by Scorpion-ARMS were not identified by direct sequencing. However, two of the deletions in exon 19 (E746_S752 and E746_T751) that were detected by direct sequencing were not identified by Scorpion-ARMS, given that the Scorpion primers were designed only for detection of the E746_A750 deletion in exon 19. *EGFR* mutations were significantly more frequent in tumors of women than in those of men (33 vs 9%), in adenocarcinomas than in tumors with other histologies (28 vs 3%), and in never-smokers than in smokers (42 vs 6%) (Fig. 1a). One of the 18 *EGFR* mutations was detected in a squamous cell carcinoma. Determination of *EGFR* copy number by FISH analysis revealed gene amplification in six patients and high polysomy in 26 patients, with 32 patients thus being classified as FISH positive (Table 3). In contrast to *EGFR* mutation, FISH

Table 1. Characteristics of patients with advanced non-small cell lung cancer ($n = 100$)

Characteristic	Subset	No. patients
Sex	Male	64
	Female	36
Smoking history	Never-smoker	33
	Smoker	67
Tumor histology	Adenocarcinoma	61
	Other	39
Eastern Cooperative Oncology Group performance status	0	24
	1	59
	≥2	17
No. chemotherapies	1	37
	≥2	63

Table 2. Detection of epidermal growth factor receptor (*EGFR*) mutations by direct sequencing or amplification-refractory mutation system (ARMS) ($n = 100$)

Site	Mutation	Direct sequencing	ARMS	Direct sequencing or ARMS
Exon 19	15-bp deletion	1	3	3
	16-bp deletion	1	0	1
	19-bp deletion	1	0	1
Exon 21	L858R	5	13	13
Total		8 (8%)	16 (16%)	18 (18%)

Table 3. Determination of epidermal growth factor receptor gene copy number by fluorescence *in situ* hybridization (FISH) analysis ($n = 100$)

FISH status	Finding	No. patients
Positive	Gene amplification	6
	High polysomy	26
	Total	32
Negative	Low polysomy	35
	High trisomy	2
	Low trisomy	26
	Disomy	5
	Total	68

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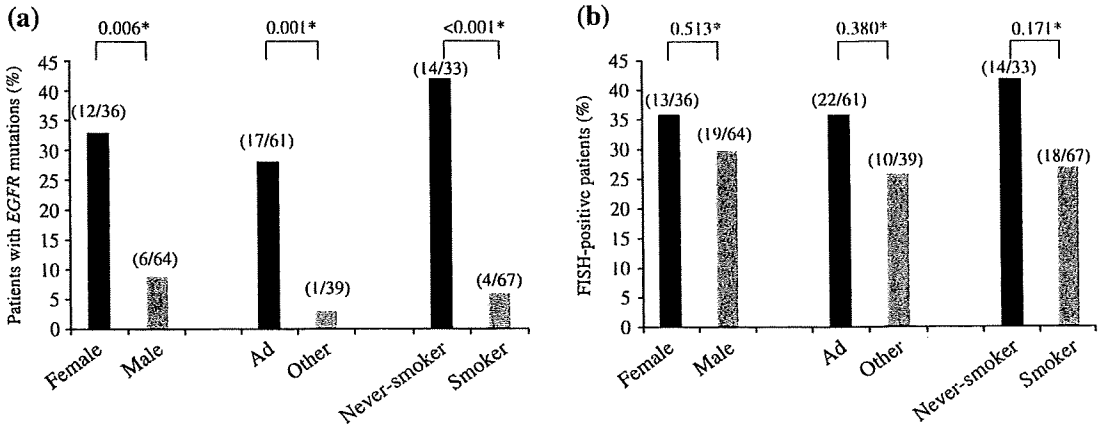

Fig. 1. Sex, tumor histology, and smoking status of patients with advanced non-small cell lung cancer and with either (a) epidermal growth factor receptor (*EGFR*) mutations or (b) a high *EGFR* copy number. Ad, adenocarcinoma. **P*-values were determined by Fisher's exact test.

Table 4. Relationship between epidermal growth factor receptor (*EGFR*) mutation and either fluorescence *in situ* hybridization (FISH) status of *EGFR* amplification

Mutation status	FISH status		Gene amplification	
	Positive	Negative	Positive	Negative
Positive ($n = 18$)	8	10	4	14
Negative ($n = 82$)	24	58	2	80
P-value*		0.266		0.009

*Determined by Fisher's exact test.

positivity was not associated with sex, tumor histology, or smoking status (Fig. 1b). Although no relationship was apparent between *EGFR* mutation and FISH positivity (gene amplification or high polysomy), *EGFR* mutation and *EGFR* amplification were significantly associated (Table 4). The clinicopathological and genetic features of patients with *EGFR* mutations are shown in Table 5.

Overall survival. For the total patient population, the median overall survival was 12.3 months, with a 1-year survival rate of 51.7%. Univariate analysis revealed that overall survival was significantly longer in women, never-smokers, patients with a favorable PS, and those with *EGFR* mutations (Table 6; Fig. 2a). In contrast, no difference in overall survival was apparent between FISH-positive and FISH-negative patients (Table 6; Fig. 2b). We also carried out multivariate analysis to identify factors that contribute to overall survival, with covariates including clinicopathological and genetic factors (sex, smoking history, tumor histology, PS, *EGFR* mutation status, FISH status). Female sex and favorable PS were found to be independent prognostic factors (Table 6).

Responsiveness to epidermal growth factor receptor tyrosine kinase inhibitor treatment. Of the 53 patients treated with EGFR TKI, 40 individuals were assessable for objective response. Whereas the rate of response to EGFR TKI treatment for patients with *EGFR* mutations was significantly higher than that for those without such mutations (71.4 vs 11.5%, $P < 0.001$), there was no significant association between FISH status and responsiveness

Table 5. Clinicopathological and genetic features of patients with epidermal growth factor receptor (*EGFR*) mutations

No.	Age (years)	Sex	Smoking status	Histology	Response to EGFR TKI	Type of *EGFR* mutation		*EGFR* copy number
						Sequencing	ARMS	
1	72	F	Never	Ad	PR		L858R	Low trisomy
2	58	F	Never	Ad	PR	L858R	L858R	Gene amplification
3	81	F	Never	Ad	SD	L858R	L858R	High polysomy
4	72	F	Never	Ad	NE		L858R	Gene amplification
5	48	M	Smoker	Ad	SD		L858R	Low trisomy
6	67	F	Never	Ad	SD		L858R	Low trisomy
7	59	F	Never	Ad	PR		L858R	High polysomy
8	78	M	Smoker	Ad			L858R	High trisomy
9	71	F	Never	Ad	PR		L858R	Low polysomy
10	82	F	Never	Ad	PR	L858R	L858R	Low trisomy
11	67	F	Never	Ad		L858R	L858R	High polysomy
12	87	F	Never	Sq	PR	L858R	L858R	Low polysomy
13	78	M	Never	Ad			L858R	Gene amplification
14	56	F	Never	Ad	PR		(E746_A750)del	Low polysomy
15	63	M	Never	Ad	PD	(E746_A750)del	(E746_A750)del	Gene amplification
16	63	M	Smoker	Ad	PR		(E746_A750)del	Low polysomy
17	61	M	Smoker	Ad	PR	(E746_S752)del insV		Low trisomy
18	73	F	Never	Ad	PR	(E746_T751)del insS		High polysomy

Ad, adenocarcinoma; ARMS, amplification-refractory mutation system; NE, not evaluated; PD, progressive disease; PR, partial response; SD, stable disease; Sq, squamous cell carcinoma; TKI, tyrosine kinase inhibitor.

Table 6. **Univariate and multivariate analyses of prognostic factors for overall survival**

Factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Sex (female/male)	0.54	0.32–0.91	**0.021**	0.55	0.32–0.93	**0.025**
Smoking history (never-smoker/smoker)	0.50	0.30–0.85	**0.011**			
Histology (adenocarcinoma/other)	0.64	0.39–1.05	0.077	0.68	0.40–1.14	0.141
ECOG PS (0/≥1)	0.44	0.24–0.79	**0.006**	0.48	0.29–0.86	**0.019**
EGFR mutation status (positive/negative)	0.52	0.28–0.97	**0.039**			
FISH status (positive/negative)	1.36	0.82–2.23	0.231	1.49	0.88–2.50	0.130

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; HR, hazard ratio; PS, performance status. Multivariate analysis was carried out using the stepwise method (include, <0.05; exclude, >0.2). Significant *P*-values are shown in bold.

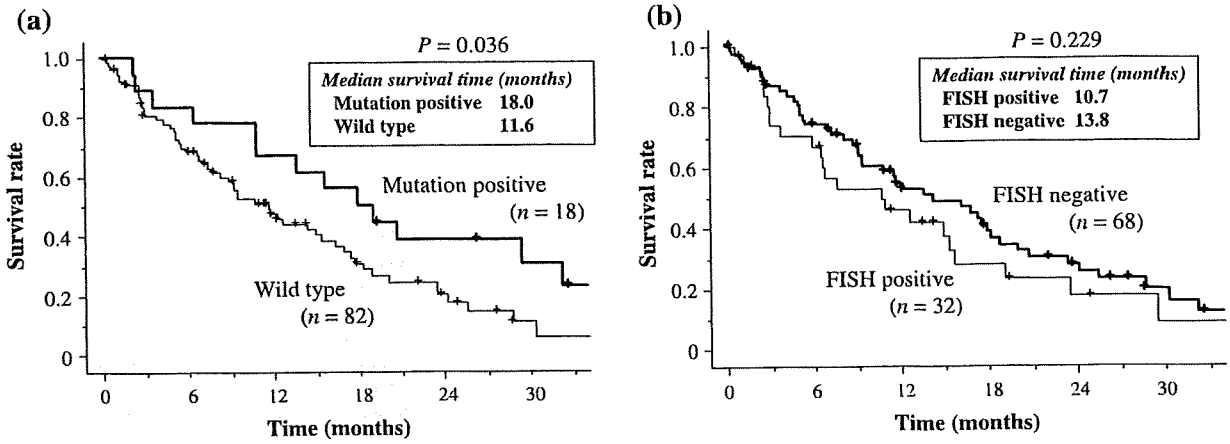

Fig. 2. Kaplan–Meier plots of overall survival in patients with advanced non-small cell lung cancer and either (a) with or without epidermal growth factor receptor (*EGFR*) mutations or (b) with or without a high *EGFR* copy number. FISH, fluorescence *in situ* hybridization.

to EGFR TKI (44.4 vs 29.0% for FISH-positive vs FISH-negative patients, respectively, $P = 0.437$).

Discussion

We have analyzed both *EGFR* mutation and *EGFR* copy number in paired tumor specimens as well as the relationship between these two types of *EGFR* alterations in advanced NSCLC. We used two methods to detect *EGFR* mutations, direct sequencing and Scorpion-ARMS, which identified eight and 16 mutations, respectively. Direct sequencing failed to detect 10 of the 16 mutations identified by Scorpion-ARMS. Of the 10 patients with *EGFR* mutations detected by Scorpion-ARMS alone, seven were assessable for an objective response to EGFR TKI, with five exhibiting a partial response and two having stable disease. Consistent with previous observations,[28–30] our data thus indicate that Scorpion-ARMS is more sensitive than direct sequencing for detection of the two major types of *EGFR* mutation that reflect responsiveness to EGFR TKI. It should be noted, however, that most polymerase chain reaction-based systems for mutation analysis, including Scorpion-ARMS, are able to detect only known *EGFR* mutations targeted by the designed primers. Indeed, two minor variants of deletion mutation in exon 19 were not identified by Scorpion-ARMS in the present study. Given the exclusion of recurrence after surgical resection in our study, most tumor specimens analyzed were obtained either by transbronchial lung biopsy or by percutaneous needle lung biopsy. The amount of tumor tissue obtained by these procedures is limited, but our results suggest that it is sufficient both for histopathological analysis and for the detection of *EGFR* mutations by Scorpion-ARMS in patients with advanced NSCLC.

Scorpion-ARMS identified three E746_A750 deletion mutations in exon 19 and 13 L858R point mutations in exon 21 in the present study. The frequency of the E746_A750 mutation detected by Scorpion-ARMS thus appeared low compared with that of the L858R mutation. Previous studies have shown that the incidence of the E746_A750 deletion is approximately the same as that of the L858R mutation.[10,12] The sensitivity of Scorpion-ARMS for detection of the E746_A750 deletion is equivalent to that for detection of the L858R point mutation. The low frequency of the E746_A750 deletion mutation in the present study is thus likely due to the small number of samples.

Previous studies have revealed a higher prevalence of *EGFR* mutations in East Asians than in Caucasians.[4,10–12,20,22,24,26,27,32–36] The prevalence of *EGFR* mutations in our Japanese cohort was low (18%) compared with values determined previously for East Asian populations. Given that most previous studies examined only individuals treated with EGFR TKI, patient selection based on clinical predictors might have led to an increase in the proportion of subjects with adenocarcinoma histology, a factor known to be associated with *EGFR* mutations. In contrast, our study was carried out with consecutive cases irrespective of EGFR TKI treatment. The relatively low proportion of patients with adenocarcinoma histology (61%) in our cohort is therefore consistent with the low prevalence of *EGFR* mutations. However, the FISH positivity of 32% in our study is similar to that in previous studies that adopted the same criteria, with values ranging from 31 to 48%.[22–24,26,27] Consistent with previous

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results,[1,7–9,12] *EGFR* mutations were significantly more frequent among women, never-smokers, and patients with adenocarcinoma in the present study. In contrast, neither *EGFR* amplification (analysis not shown) nor FISH positivity was associated with any such clinicopathological factor in our study, although the relationship between *EGFR* amplification and never-smoking status approached statistical significance ($P = 0.090$).

The relationship between *EGFR* mutation and FISH positivity (gene amplification or high polysomy) in NSCLC patients has remained unclear.[22–24,26,27] In the present study, we have demonstrated a significant relationship between *EGFR* mutation and *EGFR* amplification, but not between *EGFR* mutation and FISH positivity, in tumor specimens from patients with advanced NSCLC. *EGFR* mutant alleles were previously found to be amplified selectively, resulting in a high *EGFR* copy number, as detected by quantitative real-time polymerase chain reaction analysis.[12] *EGFR* amplification has also been shown to be acquired during invasive growth of lung adenocarcinoma with *EGFR* mutations.[37] Furthermore, recent studies have found that an increase in *EGFR* copy number is a relatively late event in NSCLC pathogenesis[38] and that *EGFR* mutation precedes *EGFR* amplification but not necessarily high polysomy.[37,39] These observations thus support the existence of a close association between *EGFR* mutation and *EGFR* amplification. We previously showed that *EGFR* mutation was significantly associated with *EGFR* amplification in human NSCLC cell lines and that endogenous EGFR expressed in such cell lines that manifested both of these *EGFR* alterations were activated constitutively as a result of ligand-independent dimerization.[25] However, the biological consequences of high polysomy for *EGFR* have not been elucidated. We did not find any cut-off value of high polysomy that was associated with *EGFR* mutation. We therefore propose that *EGFR* amplification, but not high polysomy, plays a key role in the pathogenesis of NSCLC and correlates with *EGFR* mutation.

We sought to determine whether *EGFR* mutation or *EGFR* copy number might affect overall survival of NSCLC patients. Previous studies of EGFR TKI have suggested that *EGFR* mutation is a favorable prognostic indicator for patients with NSCLC.[35,36] We also found that the survival time of patients with *EGFR* mutations was longer than that of those without them (18.0 vs 11.6 months, $P = 0.036$) in the univariate analysis. However, interpretation of this result requires that the effect of EGFR TKI on survival be taken into account, given that 83% (15/18) of patients with *EGFR* mutations were treated with EGFR TKI compared with only 46% (38/82) of those without such mutations. Indeed, analysis of survival after initiation of EGFR TKI treatment as a second-line or subsequent therapy revealed a survival time of 15.6 months for mutation-positive patients vs 6.0 months for mutation-negative patients in our study. It was therefore not possible to determine the prognostic significance of *EGFR* mutation for NSCLC patients. To clarify whether *EGFR* mutation is a predictor of sensitivity to EGFR TKI or a prognostic indicator for NSCLC patients, we are currently carrying out a phase III randomized study comparing platinum-based chemotherapy with gefitinib in chemotherapy-naive NSCLC patients with *EGFR* mutations. Patients with FISH-positive tumors tended to have a shorter survival time than did those with FISH-negative tumors (10.7 vs 13.8 months), although this difference was not statistically significant. This result is consistent with previous observations indicative of an association between high *EGFR* copy number and poor prognosis for certain malignancies, including NSCLC.[1,40]

In conclusion, we have analyzed both *EGFR* mutation and *EGFR* copy number in paired tumor specimens from patients with advanced NSCLC. We found that Scorpion-ARMS is more sensitive than direct sequencing for detection of *EGFR* mutations in small tumor specimens. Furthermore, we showed that *EGFR* mutation was significantly associated with *EGFR* amplification but not with FISH positivity. These observations warrant confirmation in further studies as well as exploration of the biological mechanisms of the relationship between *EGFR* mutation and *EGFR* amplification. The effects of *EGFR* mutation and *EGFR* copy number on clinical outcome in individuals with advanced NSCLC also warrant investigation in a prospective study.

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

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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

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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 microosmotic 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

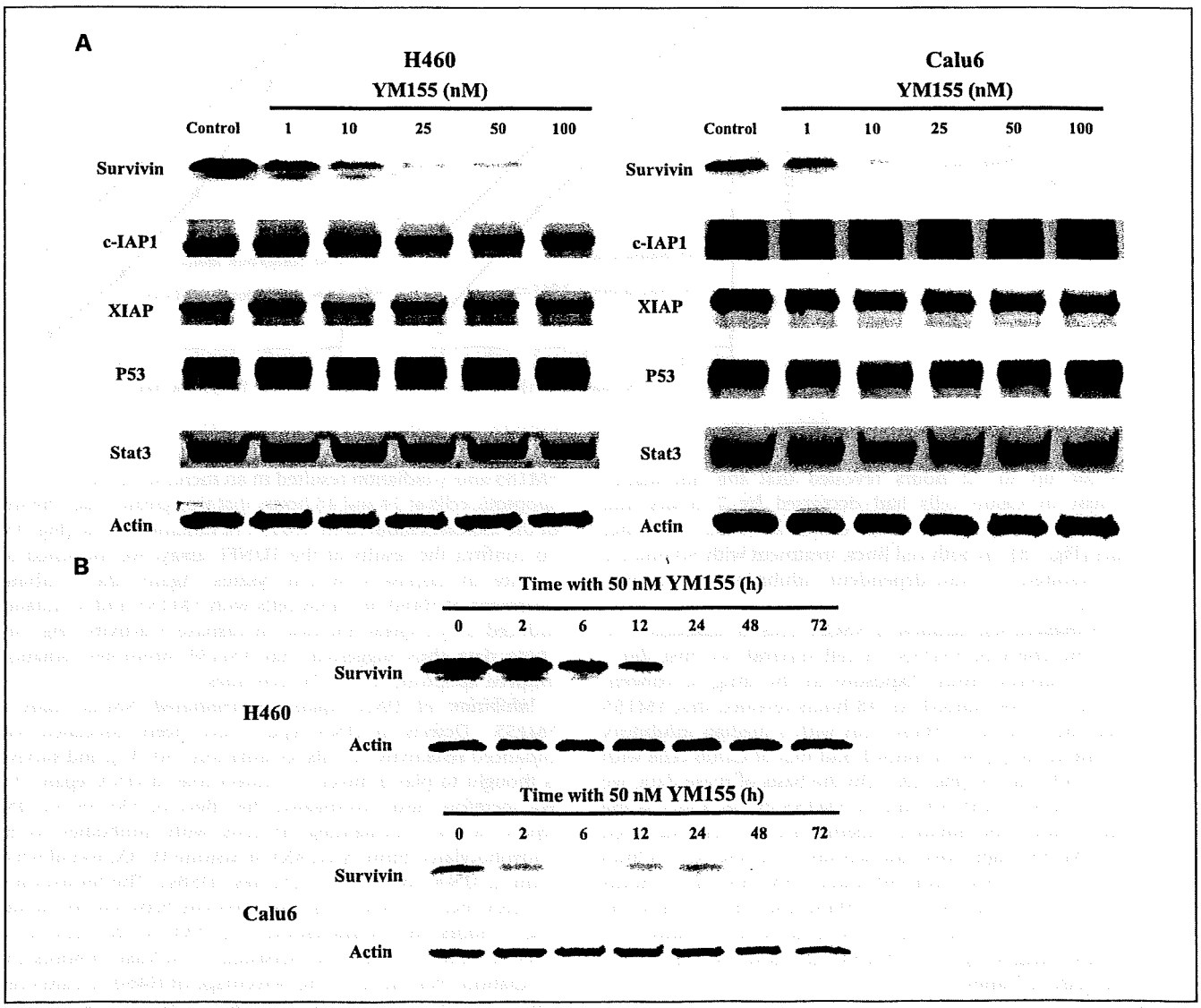

Fig. 1. Effect of YM155 on survivin expression in human NSCLC cells. *A*, H460 or Calu6 cells were incubated in the absence (control, 0.1% DMSO) or presence of various concentrations (1, 10, 25, 50, or 100 nmol/L) of YM155 for 48 h. Cell lysates were then prepared and subjected to immunoblot analysis 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.

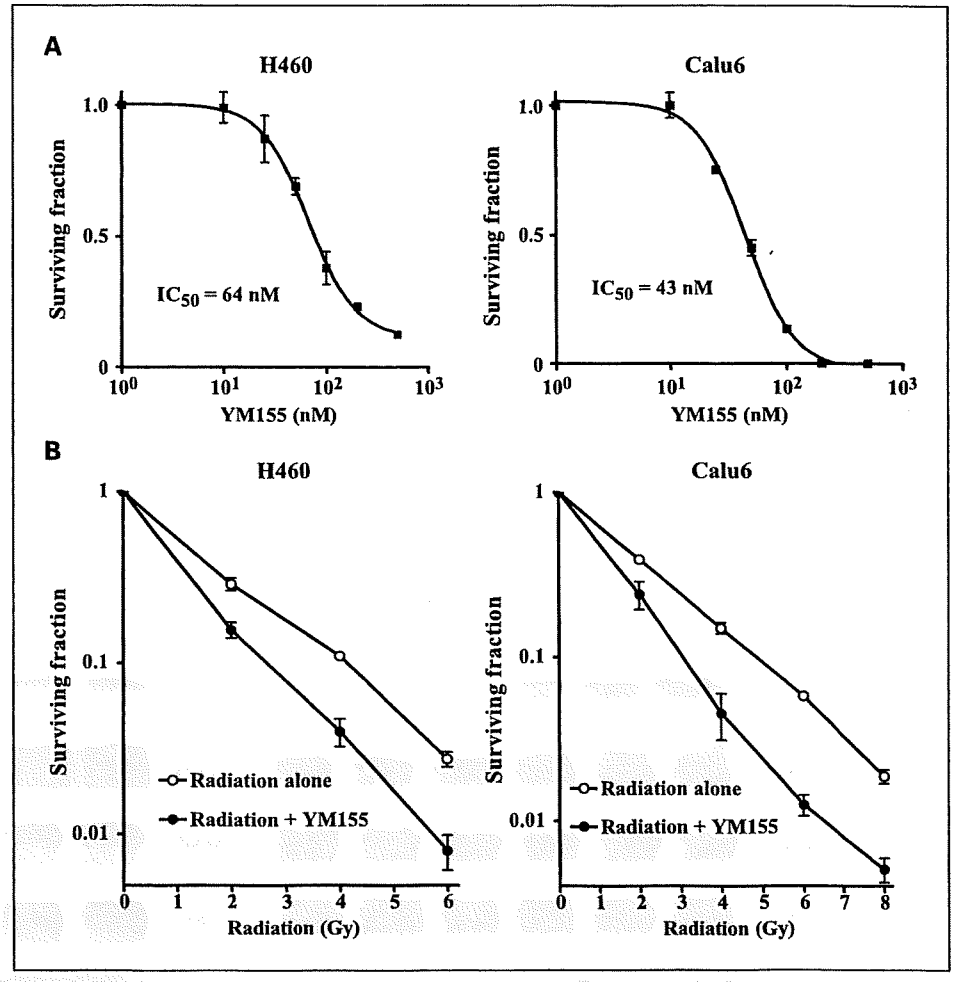

Fig. 2. Effect of YM155 on the sensitivity of H460 or Calu6 cells to γ-radiation. *A*, cells were incubated with the indicated concentrations of YM155 for 48 h and then assayed for clonogenic survival. Points represent means from three independent experiments; bars represent SD. *B*, cells were incubated 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,

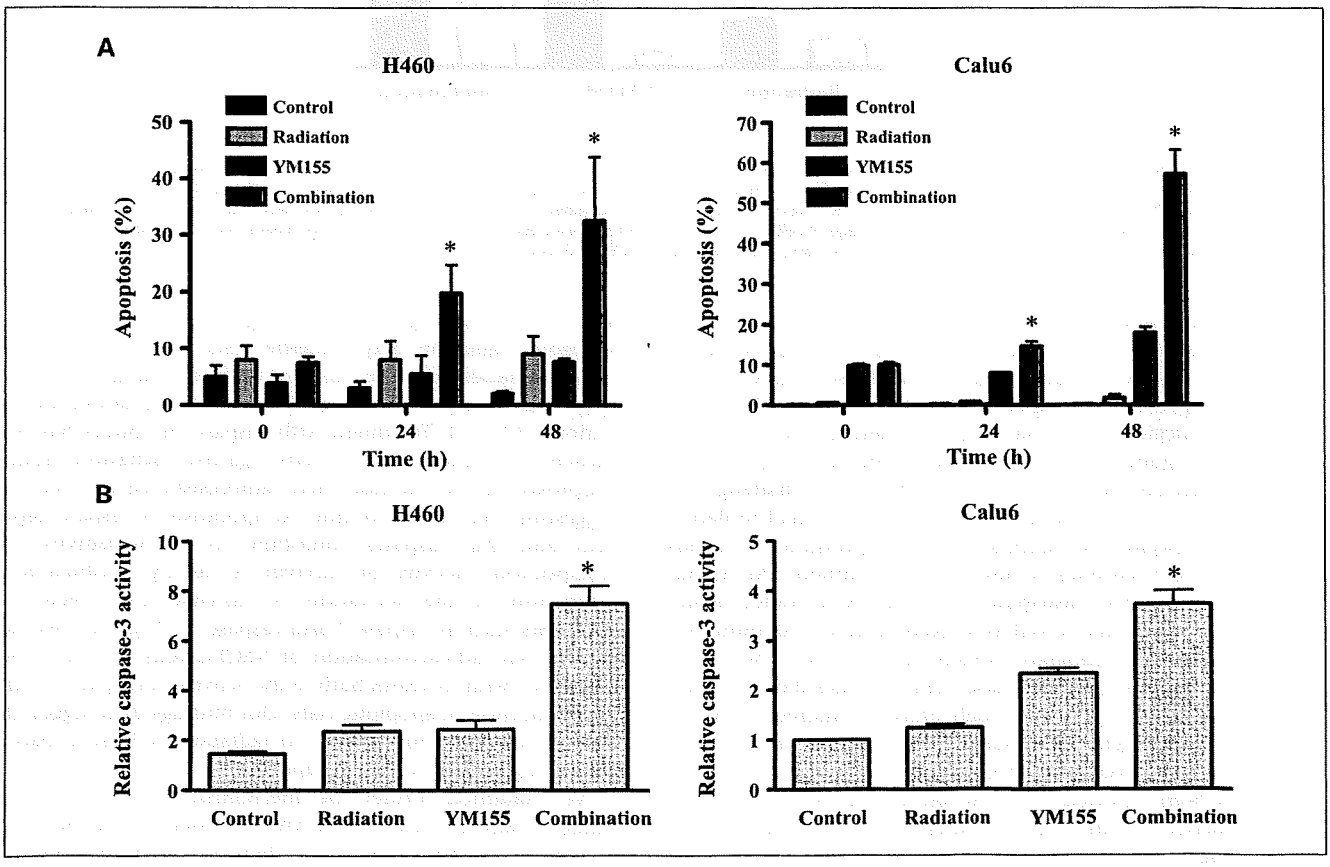

Fig. 3. Effect of YM155 on radiation-induced apoptosis and caspase-3 activity in H460 or Calu6 cells. *A*, cells were incubated with 50 nmol/L YM155 or vehicle (0.1% DMSO) for 48 h, exposed (or not) to 3 Gy of γ-radiation, and then incubated in drug-free medium for 24 or 48 h, at which times the percentage 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.

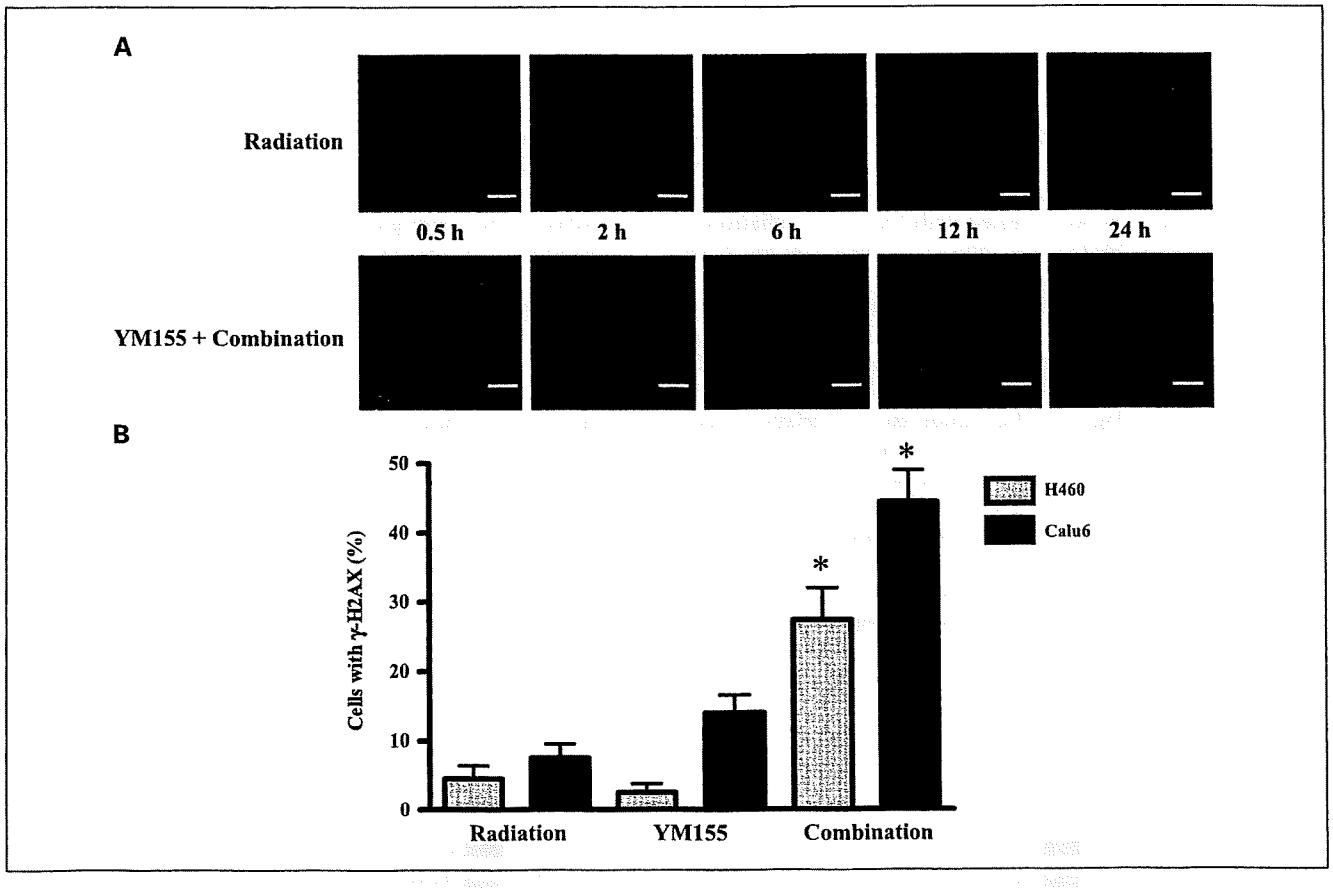

Fig. 4. Effect of YM155 on the radiation-induced formation of γ-H2AX foci in NSCLC cells. *A*, H460 cells were incubated with vehicle (0.1% DMSO) or 50 nmol/L YM155 for 48 h and then exposed to 3 Gy of γ-radiation. After incubation for the indicated times in drug-free medium, the cells were fixed and 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

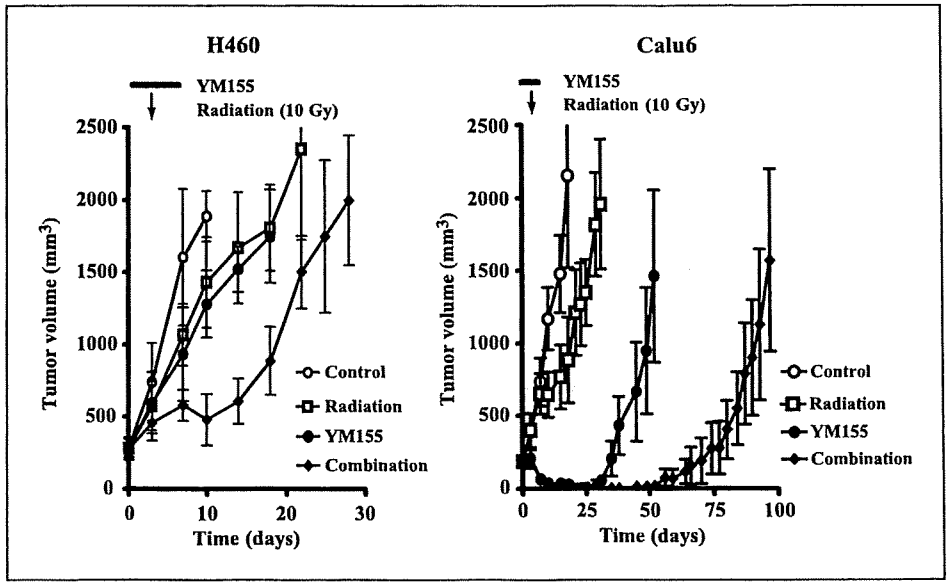

Fig. 5. Effect of YM155 on the growth of H460 or Calu6 tumors in mice subjected to single-dose radiotherapy. Cells were injected into the right hind limb of nude mice and allowed to grow. The mice were divided into four treatment groups: control, radiation alone, YM155 alone, or the combination of YM155 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.

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

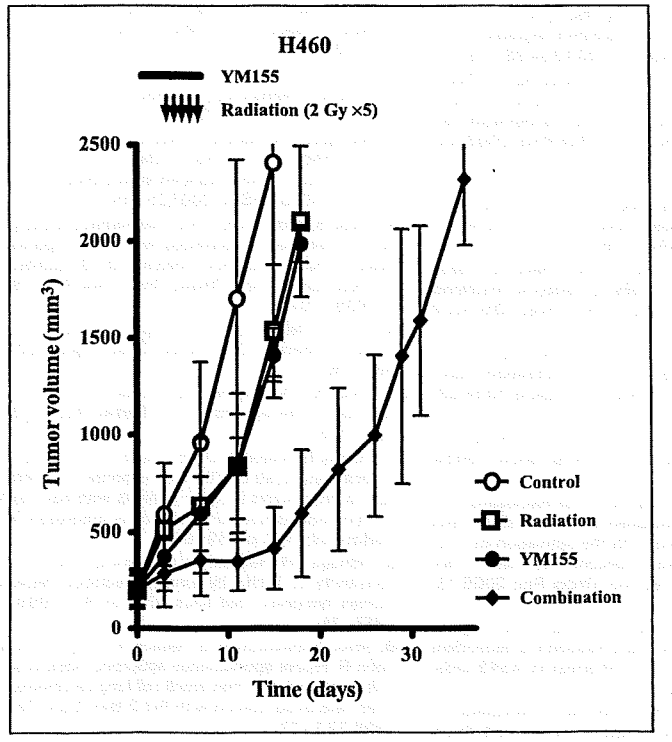

Fig. 6. Effect of YM155 on the growth of H460 tumors in mice subjected to fractionated radiotherapy. H460 cells were injected into the right hind limb of nude mice and allowed to grow. The mice were divided into four treatment groups: control, radiation alone, YM155 alone, or the combination of YM155 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.

enhancing the tumor response to such fractionated radiation. The enhancement factor with fractionated radiation (3.0) was similar to that observed with single-dose radiation (3.3) for H460 tumor xenografts.

Resistance to cytotoxic drugs and radiation is a major limiting factor in the treatment of cancer patients. Cross-resistance has been noted between radiotherapy and chemotherapy and has been attributed to defects in apoptosis signaling or to an enhanced capacity for DNA repair (51, 52). Our findings provide evidence that YM155 may break radioresistance by promoting apoptosis and inhibiting DNA repair. Previous studies have shown that suppression of survivin expression increases the sensitivity of tumor cells to chemotherapy(18, 53). It will therefore be of interest to determine whether YM155 also sensitizes tumor cells to chemotherapy.

In conclusion, we have shown that YM155 sensitizes NSCLC cells to radiation both *in vitro* and in animal models *in vivo*. The radiosensitization induced by YM155 seems to be attributable to the promotion of caspase-mediated apoptosis and inhibition of the repair of radiation-induced DNA damage. Our preclinical results provide a rationale for future clinical investigation of the therapeutic efficacy of YM155 in combination with radiotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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

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Abstract

Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the therapeutic response to EGFR tyrosine kinase inhibitors (TKI) in patients with advanced non-small cell lung cancer (NSCLC). The response rate to these drugs remains low, however, in NSCLC patients with wild-type *EGFR* alleles. Combination therapies with EGFR-TKIs and cytotoxic agents are considered a therapeutic option for patients with NSCLC expressing wild-type *EGFR*. We investigated the antiproliferative effect of the combination of the oral fluorouracil S-1 and the EGFR-TKI gefitinib in NSCLC cells of differing *EGFR* status. The combination of 5-fluorouracil and gefitinib showed a synergistic antiproliferative effect *in vitro* in all NSCLC cell lines tested. Combination chemotherapy with S-1 and gefitinib *in vivo* also had a synergistic antitumor effect on NSCLC xenografts regardless of the absence or presence of *EGFR* mutations. Gefitinib inhibited the expression of the transcription factor E2F-1, resulting in the down-regulation of thymidylate synthase at the mRNA and protein levels. These observations suggest that gefitinib-induced down-regulation of thymidylate synthase is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib and provide a basis for clinical evaluation of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors. [Mol Cancer Ther 2008;7(3):599–606]

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Introduction

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

S-1 (Taiho Pharmaceutical) is an oral anticancer agent composed of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1 (18). Tegafur is a prodrug that generates 5-fluorouracil (5-FU) in blood largely as a result of its metabolism by cytochrome *P*450 in the liver. CDHP increases the plasma concentration of 5-FU through competitive inhibition of dihydropyrimidine dehydrogenase (DPD), which catalyzes 5-FU catabolism (19). Oxonate reduces the gastrointestinal toxicity of 5-FU (20). A response rate of 22% and a median survival time of 10.2 months were obtained in a clinical trial of S-1 in patients with advanced NSCLC not subjected previously to chemotherapy (21). Few severe gastrointestinal or hematologic adverse events were reported. Moreover, a phase II trial of S-1 plus cisplatin in NSCLC patients revealed a 47% response rate and an acceptable safety profile (22).

Based on this background, we examined the anticancer effect of the combination of S-1 and gefitinib in NSCLC cell lines of differing *EGFR* status. We found that the combination of S-1 (or 5-FU) and gefitinib exhibited a marked and synergistic antiproliferative effect both *in vivo*

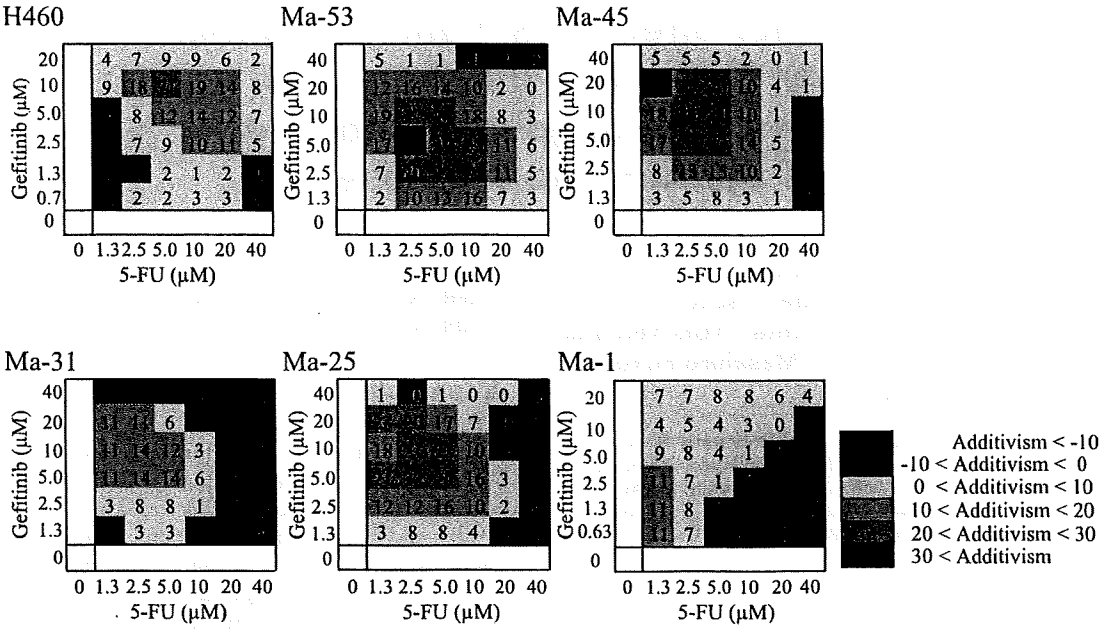

Figure 1. Inhibition of NSCLC cell growth by the combination of 5-FU and gefitinib *in vitro*. Cells with wild-type (H460, Ma-53, Ma-45, Ma-31, and Ma-25) or mutant (Ma-1) EGFR alleles were exposed for 72 h to 5-FU and gefitinib at the indicated concentrations, after which cell viability was measured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

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

Materials and Methods

Cell Lines and Reagents

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

Growth Inhibition Assay *In vitro*

Cells (2.0×10^3) were plated in 96-well flat-bottomed plates and cultured for 24 h before the addition of various concentrations of 5-FU and gefitinib and incubation for an additional 72 h. Cell Counting Kit-8 solution (Dojindo) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 450 nm. Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of 5-FU or gefitinib resulting in 50% growth inhibition (IC_{50}) was calculated. The effect of combining 5-FU and gefitinib was classified as additive, synergistic, or antagonistic with the Bliss additivism model (24–26). A theoretical curve was calculated for combined inhibition with the equation: $E_{bliss} = E_A + E_B - (E_A \times E_B)$, where E_A and E_B are the fractional inhibitory effects of drug A alone and drug B alone at specific concentrations. E_{bliss} is then the fractional inhibition that would be expected if the effect of the combination of the two drugs was exactly additive. In this study, the Bliss variable is expressed as percentage decrease in cell growth above what would be expected for the combination. Bliss = 0 indicates that the effect of the combination is additive; Bliss > 0 is indicative of synergy; and Bliss < 0 indicates antagonism.

Animals

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

Growth Inhibition Assay *In vivo*

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

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

Immunoblot Analysis

Cell lysates were fractionated by SDS-PAGE on 12% gels (NuPAGE Bis-Tris Gels; Invitrogen), and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites with 5% skim milk, the membrane was incubated overnight at room temperature with primary antibodies. Antibodies to DPD, OPRT, and TS were obtained from Taiho Pharmaceutical; those to E2F-1 were from Santa Cruz Biotechnology; and those to β-actin (loading control) were from Sigma. Immune complexes were detected by incubation of the membrane for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to mouse or rabbit immunoglobulin and by subsequent exposure to enhanced chemiluminescence reagents (Pierce).

Immunoprecipitation Analysis

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

Reverse Transcription and Real-time PCR Analysis

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

Statistical Analysis

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

Results

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

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

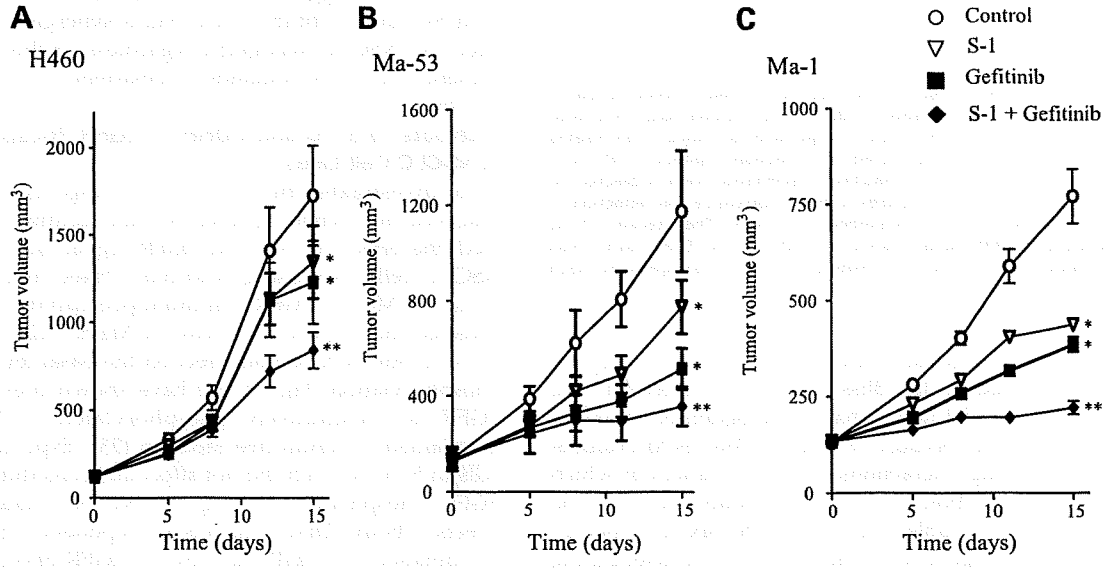

Figure 2. Antitumor activity of the combination of S-1 and gefitinib *in vivo*. **A** and **B**, nude mice with tumor xenografts established by s.c. implantation of NSCLC cells (H460 and Ma-53) possessing wild-type EGFR were treated daily for 2 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (50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

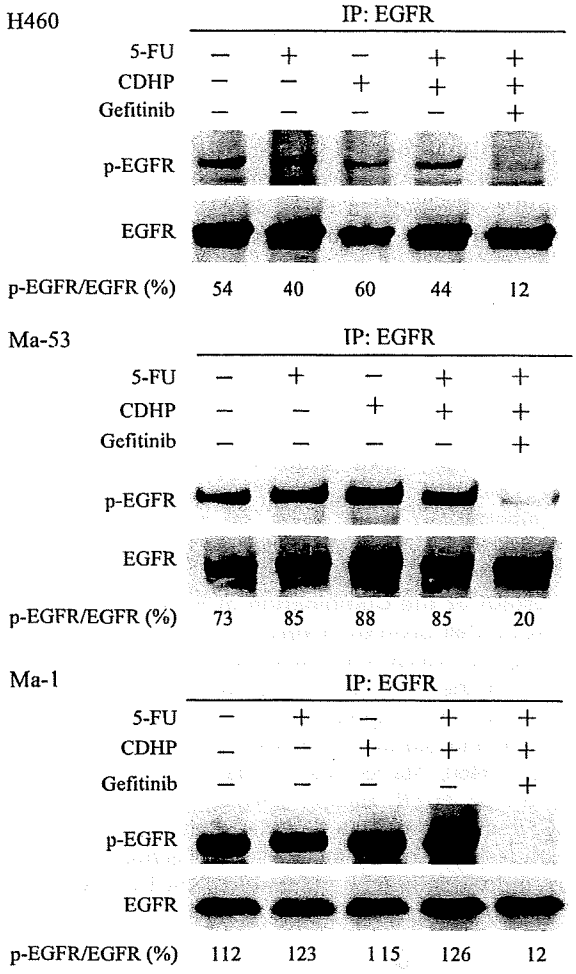

Figure 3. Lack of effect of 5-FU and CDHP on EGFR phosphorylation in NSCLC cell lines. NSCLC cells (H460, Ma-53, and Ma-1) were incubated for 24 h in medium supplemented with 2% fetal bovine serum and with 5-FU (10 μmol/L), CDHP (3 μmol/L), or gefitinib (5 μmol/L). Cell lysates were then prepared and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

whether 5-FU and gefitinib showed additivity, synergy, or antagonism based on the Bliss additivism model (24–26). We chose this model rather than isobologram or combination index analysis because it would allow us to evaluate the nature of drug interactions even in instances in which the maximal inhibition by 5-FU or gefitinib alone was too low to obtain a reliable IC_{50} value. The six test concentrations for each agent were chosen after first determining the corresponding IC_{50} values. The IC_{50} values for 5-FU chemosensitivity were not associated with *EGFR* status and ranged from 7 to 11 μmol/L. The effect of combined treatment with 5-FU and gefitinib on the proliferation of the six NSCLC cell lines was tested in triplicate in a 6 × 6 concentration matrix. Calculation of the percentage inhibition in excess of that predicted by the Bliss additivism model revealed synergistic effects of Bliss > 0 for 5-FU and gefitinib in all of the six cell lines tested (Fig. 1). These results suggested that 5-FU and gefitinib act synergistically to inhibit cell growth in NSCLC cells.

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

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

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

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

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

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

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

Effect of Gefitinib on TS mRNA Abundance in NSCLC Cell Lines

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

Discussion

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

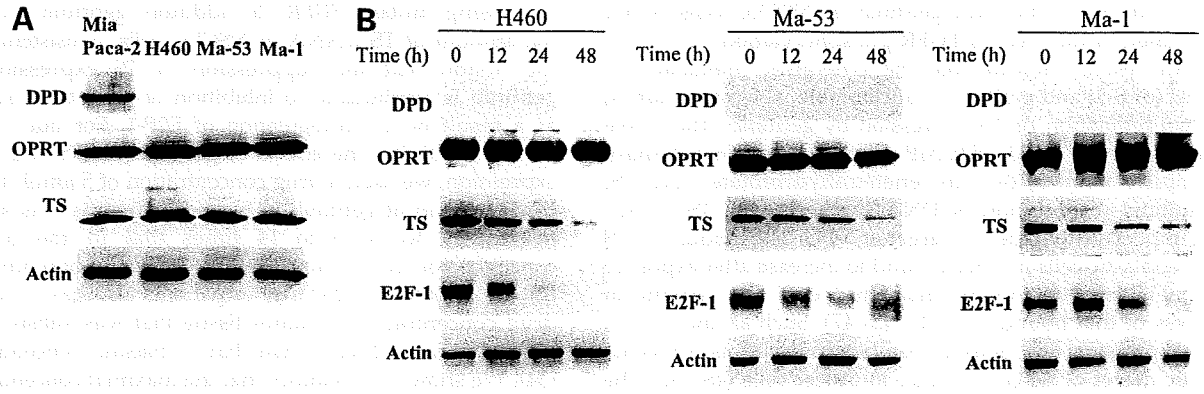

Figure 4. Effects of gefitinib on the expression of E2F-1, DPD, OPRT, and TS in NSCLC cell lines. **A**, lysates of H460, Ma-53, or Ma-1 cells were subjected to immunoblot analysis with antibodies to DPD, OPRT, TS, or β-actin (loading control). MiaPaca-2 cells were also examined as a positive control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