(Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004). Furthermore, *EGFR* mutations are often mutually exclusive with *KRAS* mutations in NSCLC (Kosaka *et al*, 2004; Marchetti *et al*, 2005). We used combined data from previous papers (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004) and from Sanger institute's databases (Bamford *et al*, 2004) to analyse association of *LKB1* mutations with mutations of *KRAS*, *BRAF*, and *EGFR*. Analysis of *LKB1* mutation harbouring NSCLC cell lines (A-427, A549, NCI-H1395, NCI-H1666, NCI-H2122, NCI-H2126, NCI-H23, and NCI-H460) showed that five of the cell lines (63%) had concurrent *LKB1* and *KRAS* mutations, two (25%) had concurrent *LKB1* and *BRAF* mutations, and only one (13%) had neither *KRAS* nor *BRAF* mutations. None of these cell lines had EGFR mutations.

As our findings in NSCLC cell lines suggested concurrency of *KRAS* or *BRAF* and mutual exclusiveness of *EGFR* mutations with LKB1 mutations, we analysed the mutational status of these genes in our primary NSCLC tumour specimens. *KRAS* mutations were detected in 49 (16% in the whole tumour set, 25% in Caucasian and 8% in Asian specimens) tumour specimens with 10 (20% of *KRAS* mutants) of these occurring concurrently with an *LKB1* mutation ($P=0.042$) (Table 3). Four *BRAF* mutations (1%) were found in the tumour set (G465V, N581S, L596R, and T599I) and one of these (N581S) occurred concurrently with *LKB1* mutation ($P=0.373$). Seventy tumours (23% in the whole tumour set, 9% in Caucasian, 34% in Asian specimens) had *EGFR* kinase domain mutations with only one of them occurring concurrently with an *LKB1* mutation ($P=0.002$). The tumour with a concurrent *EGFR* and *LKB1* mutation had a missense mutation of *LKB1* outside the kinase domain (R426W). No germ line DNA was available from this patient. However, a recent report has suggested that R426W is in fact a rare polymorphism of the gene (Onozato *et al*, 2007). Taken together our findings suggest that unlike *KRAS*, mutations in *EGFR* and *LKB1* are mutually exclusive in NSCLC.

Previous reports (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004) and Cancer Genome Project by Sanger Institute (Bamford *et al*, 2004) have extensively characterised *LKB1* mutations in NSCLC cell lines with *KRAS* and *BRAF* mutations, but *LKB1* status of *EGFR* mutant NSCLC cell lines has not been extensively analysed. Therefore, we analysed the *LKB1* genotype of NSCLC cell lines with known *EGFR* or *ERBB2* mutations. Twelve *EGFR* mutant and one *ERBB2* mutant cell lines were analysed for *LKB1* genotype. No *LKB1* mutations were detected in these cell lines (Table 4).

Table 3 Association of *LKB1* mutations with *KRAS*, *BRAF*, and *EGFR* mutations in NSCLC tumours

		LKB1 mutation		
		+	−	*P*-value*
EGFR mutation	+	1	69	0.002
	−	33	207	
K-Ras mutation	+	10	39	0.042
	−	24	237	
B-Raf *mutation*	+	1	3	0.373
	−	33	273	

*Fisher's exact test.

Table 4 LKB1 genotypes of NSCLC cell lines with EGFR or ERBB2 mutations

Cell line	*EGFR* genotype	*HER2* genotype	*LKB1* genotype
H1650	E746_A750del	Wt	Wt
H1781	Wt	G776V, Cins	Wt
H1975	L858R, T790M	Wt	Wt
H3255	L858R	Wt	Wt
H3255GR	L858R, T790M	Wt	Wt
H820	L747_L751del, T790M	Wt	Wt
HCC2279	E746_A750del	Wt	Wt
HCC2935	E746_T751del, S752I	Wt	Wt
HCC4006	L747_E749del, A750P	Wt	Wt
HCC827	E746_A750del	Wt	Wt
Ma-1	E746_A750del	Wt	Wt
Ma-70	L858R	Wt	Wt
PC-9	E746_A750del	Wt	Wt

Wt = wild type.

DISCUSSION

The present study characterised *LKB1* mutation frequency in NSCLC using one of the largest tumour sets to date ($n=310$). Our study analysed tumours from different histologies and of both a US and Korean origin to determine potential histological and ethnic variation in *LKB1* mutational frequency. The large size of our study enabled us to study associations of *LKB1* mutations with clinocopathological factors, which have been incompletely characterised in previous studies (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004; Fernandez *et al*, 2004; Matsumoto *et al*, 2007; Onozato *et al*, 2007). In addition, we used a modification of a sensitive mutation screening technique that we have previously developed to facilitate the rapid detection of *LKB1* mutations (Janne *et al*, 2006).

Findings from our study confirm the high frequency of *LKB1* mutations in NSCLC (11%), which in contrast, are rare (0–4%) in other common solid malignancies (Avizienyte *et al*, 1998, 1999). The reason behind these observations is presently unknown but might reflect the differences in carcinogen exposure in the lungs compared with other tissues. In support of this hypothesis, we find that *LKB1* mutations are significantly ($P=0.007$) more common in smokers than in never or light ($\leqslant$10 pack years) cigarette smokers (Table 1). Male PJS patients (age $\geqslant$50 years) have an increased risk of developing lung cancer compared with the general population but the relationship of smoking and the increased risk of lung cancer in PJS is unknown (Hearle *et al*, 2006). Interestingly, the *LKB1* mutation spectrum found in the current study is very similar to those previously published for PJS (deletions 34%, insertions 15%, splice site mutations 14%, missense mutations 21%, and nonsense mutations 12%) (Launonen, 2005) and, as in PJS, no clear mutational hotspots were detected.

Our study also demonstrated that the *LKB1* mutation frequency was significantly higher in cancers derived from a US population compared with those found in Korean patients (17 *vs* 5%; $P=0.001$). These differences also track with cigarette smoking, as the number of never/light former smokers was much higher in the Korean cohort compared with the US cohort of patients (38 *vs* 13%). Similarly, a recent study of 100 Japanese NSCLCs found that only 3% contained an *LKB1* mutation (Onozato *et al*, 2007). These findings are in contrast to *EGFR* mutations, which are more frequently detected in tumours from never/light cigarette smokers and from Asian patients (Janne and Johnson, 2006). Our studies further highlight ethnic and environmental differences in the origins of NSCLC.

Given the differences in *LKB1* mutation frequencies in smokers *vs* never/light smokers and in the US compared with Korean patients, we further determined whether these were also associated with other oncogene mutations known to vary in these subgroups of patients. Consistent with prior studies we found a significant association with concurrent *KRAS* mutations, which are common in smokers (Ahrendt *et al*, 2001), in one out of three of NSCLC with *LKB1* mutations (Table 3). In contrast, there was a significant inverse relationship of *LKB1* mutations with *EGFR* mutations in both NSCLC tumours and cell lines, which has not previously been

described (Tables 3 and 4). These differences may relate to the biological role of LKB1 in lung cancer. It is possible that in *EGFR* mutant lung cancers there is already maximal activation of the PI3K/Akt/mTOR signalling pathway and thus an *LKB1* mutation may not be required to further potentiate this signalling pathway. In contrast in *KRAS* mutant cancers, a concurrent *LKB1* mutation may be required to enhance mTOR activation. Mice with concurrent *KRAS* mutations and *LKB1* inactivation have more aggressive tumours and a shorter survival than those with only *KRAS* mutant cancers (Ji *et al*, 2007). In our study, we were not able to detect a significant survival difference for patients whose tumours contained *LKB1* mutations alone or concurrently with *KRAS* mutations (data not shown) likely because of the limited number of tumour specimens. Additional studies are needed to clarify the prognostic impact of *LKB1* mutations in humans with NSCLC. In the present study ~2 out of 3 of *LKB1* mutant tumours were *KRAS* wild type (Table 3). One possibility is that such tumours contain a concurrent mutation in another oncogene that activates the same signalling pathway as *KRAS*. For this reason, we examined our tumours for *BRAF* mutations, which are found in 1–2% of NSCLC (Naoki *et al*, 2002). We detected a concurrent *LKB1* mutation in one of the four *BRAF* mutant tumours (Table 3). This tumour was wild type for *KRAS* (data not shown). In addition, some of the *BRAF* mutant NSCLC cell lines (NCI-H1395, G469A; NCI-H1666, G466V) also contain a concurrent *LKB1* mutation (Sanchez-Cespedes *et al*, 2002; Bamford *et al*, 2004; Carretero *et al*, 2004). Future studies will help further clarify whether *LKB1* mutations occur concurrently with other genomic alterations in NSCLC and the impact of this on patient outcome.

Our study employed a mutation scanning technology to screen for *LKB1* mutations at the cDNA level (Janne *et al*, 2006). This was advantageous as the entire coding region of *LKB1* could be rapidly screened for a mutation using just two overlapping cDNA fragments. *LKB1* is a challenging gene to analyse at the genomic DNA level because of its high guanine–cytosine content. In addition, as many of the *LKB1* mutations are small deletions (Table 2) or involve deletions of entire exons, these would be missed using exon-specific genome sequencing methods. Our method, however, does have limitations as it would miss deletions at the site of PCR primers, whole gene deletions, or deletions within the promoter region all of which have been infrequently detected in PJS (Volikos *et al*, 2006). Thus our studies may underestimate the true *LKB1* mutation frequency in NSCLC. In addition, our method is limited to the analysis of fresh tumour specimens, which are available only from the minority of NSCLC patients. Furthermore, as techniques isolating mRNA from formalin-fixed paraffin-embedded tumour specimens continue to improve, this rapid mutation scanning technique can be used to analyse broader populations of tumours from NSCLC patients. Future studies may need to employ a combination of *LKB1* mutation detection methodologies including the current method, MLPA and direct sequencing.

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REFERENCES

Abed AA, Gunther K, Kraus C, Hohenberger W, Ballhausen WG (2001) Mutation screening at the RNA level of the STK11/LKB1 gene in Peutz-Jeghers syndrome reveals complex splicing abnormalities and a novel mRNA isoform (STK11 c.597(insertion mark)598insIVS4). *Hum Mutat* **18:** 397–410

Ahrendt SA, Decker PA, Alawi EA, Zhu Yr YR, Sanchez-Cespedes M, Yang SC, Haasler GB, Kajdacsy-Balla A, Demeure MJ, Sidransky D (2001) Cigarette smoking is strongly associated with mutation of the K-ras gene in patients with primary adenocarcinoma of the lung. *Cancer* **92:** 1525–1530

Avizienyte E, Loukola A, Roth S, Hemminki A, Tarkkanen M, Salovaara R, Arola J, Butzow R, Husgafvel-Pursiainen K, Kokkola A, Jarvinen H, Aaltonen LA (1999) LKB1 somatic mutations in sporadic tumors. *Am J Pathol* **154:** 677–681

Avizienyte E, Roth S, Loukola A, Hemminki A, Lothe RA, Stenwig AE, Fossa SD, Salovaara R, Aaltonen LA (1998) Somatic mutations in LKB1 are rare in sporadic colorectal and testicular tumors. *Cancer Res* **58:** 2087–2090

Ballhausen WG, Gunther K (2003) Genetic screening for Peutz-Jeghers syndrome. *Expert Rev Mol Diagn* **3:** 471–479

Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, Futreal PA, Stratton MR, Wooster R (2004) The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer* **91:** 355–358

Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, Ladd C, Beheshti J, Bueno R, Gillette M, Loda M, Weber G, Mark EJ, Lander ES, Wong W, Johnson BE, Golub TR, Sugarbaker DJ, Meyerson M (2001) Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci USA* **98:** 13790–13795

Breslow NE, Day NE, International Agency for Research on Cancer (1980) Statistical methods in cancer research. Vol. 1. Analysis of case–control studies. IARC scientific publications; no. 32. International Agency for Research on Cancer: Lyon

Carretero J, Medina PP, Pio R, Montuenga LM, Sanchez-Cespedes M (2004) Novel and natural knockout lung cancer cell lines for the LKB1/STK11 tumor suppressor gene. *Oncogene* **23:** 4037–4040

Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borras AM, Gale CM, Naumov GN, Yeap BY, Jarrell E, Sun J, Tracy S, Zhao X, Heymach JV, Johnson BE, Cantley LC, Janne PA (2006) Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. *J Clin Invest* **116:** 2695–2706

Fernandez P, Carretero J, Medina PP, Jimenez AI, Rodriguez-Perales S, Paz MF, Cigudosa JC, Esteller M, Lombardia L, Morente M, Sanchez-Verde L, Sotelo T, Sanchez-Cespedes M (2004) Distinctive gene expression of human lung adenocarcinomas carrying LKB1 mutations. *Oncogene* **23:** 5084–5091

Hayes DN, Monti S, Parmigiani G, Gilks CB, Naoki K, Bhattacharjee A, Socinski MA, Perou C, Meyerson M (2006) Gene expression profiling reveals reproducible human lung adenocarcinoma subtypes in multiple independent patient cohorts. *J Clin Oncol* **24:** 5079–5090

Hearle N, Schumacher V, Menko FH, Olschwang S, Boardman LA, Gille JJ, Keller JJ, Westerman AM, Scott RJ, Lim W, Trimbath JD, Giardiello FM, Gruber SB, Offerhaus GJ, de Rooij FW, Wilson JH, Hansmann A, Moslein G, Royer-Pokora B, Vogel T, Phillips RK, Spigelman AD, Houlston RS (2006) Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. *Clin Cancer Res* **12:** 3209–3215

Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Hoglund P, Jarvinen H, Kristo P, Pelin K, Ridanpaa M, Salovaara R, Toro T, Bodmer W, Olschwang S, Olsen AS, Stratton MR, de la Chapelle A, Aaltonen LA (1998) A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* **391:** 184–187

Janne PA, Borras AM, Kuang Y, Rogers AM, Joshi VA, Liyanage H, Lindeman N, Lee JC, Halmos B, Maher EA, Distel RJ, Meyerson M, Johnson BE (2006) A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. *Clin Cancer Res* **12:** 751–758

Janne PA, Johnson BE (2006) Effect of epidermal growth factor receptor tyrosine kinase domain mutations on the outcome of patients with non-small cell lung cancer treated with epidermal growth factor receptor tyrosine kinase inhibitors. *Clin Cancer Res* **12:** 4416s–4420s

Ji H, Ramsey MR, Hayes DN, Fan C, McNamara K, Kozlowski P, Torrice C, Wu MC, Shimamura T, Perera SA, Liang MC, Cai D, Naumov GN, Bao L, Contreras CM, Li D, Chen L, Krishnamurthy J, Koivunen J, Chirieac LR, Padera RF, Bronson RT, Lindeman NI, Christiani DC, Lin X, Shapiro GI, Janne PA, Johnson BE, Meyerson M, Kwiatkowski DJ, Castrillon DH, Bardeesy N, Sharpless NE, Wong KK (2007) LKB1 modulates lung cancer differentiation and metastasis. *Nature* **448:** 807–810

Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T (2004) Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* **64:** 8919–8923

Launonen V (2005) Mutations in the human LKB1/STK11 gene. *Hum Mutat* **26:** 291–297

Launonen V, Avizienyte E, Loukola A, Laiho P, Salovaara R, Jarvinen H, Mecklin JP, Oku A, Shimane M, Kim HC, Kim JC, Nezu J, Aaltonen LA (2000) No evidence of Peutz-Jeghers syndrome gene LKB1 involvement in left-sided colorectal carcinomas. *Cancer Res* **60:** 546–548

Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Camplese PP, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R, Buttitta F (2005) EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* **23:** 857–865

Matsumoto S, Iwakawa R, Takahashi K, Kohno T, Nakanishi Y, Matsuno Y, Suzuki K, Nakamoto M, Shimizu E, Minna JD, Yokota J (2007) Prevalence and specificity of LKB1 genetic alterations in lung cancers. *Oncogene* **26:** 5911–5918

Naoki K, Chen TH, Richards WG, Sugarbaker DJ, Meyerson M (2002) Missense mutations of the BRAF gene in human lung adenocarcinoma. *Cancer Res* **62:** 7001–7003

Ono M, Hirata A, Kometani T, Miyagawa M, Ueda S, Kinoshita H, Fujii T, Kuwano M (2004) Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. *Mol Cancer Ther* **3:** 465–472

Onozato R, Kosaka T, Achiwa H, Kuwano H, Takahashi T, Yatabe Y, Mitsudomi T (2007) LKB1 gene mutations in Japanese lung cancer patients. *Cancer Sci* **98:** 174–175

Pham D, Kris MG, Riely GJ, Sarkaria IS, McDonough T, Chuai S, Venkatraman ES, Miller VA, Ladanyi M, Pao W, Wilson RK, Singh B, Rusch VW (2006) Use of cigarette-smoking history to estimate the likelihood of mutations in epidermal growth factor receptor gene exons 19 and 21 in lung adenocarcinomas. *J Clin Oncol* **24:** 1700–1704

Sanchez-Cespedes M, Parrella P, Esteller M, Nomoto S, Trink B, Engles JM, Westra WH, Herman JG, Sidransky D (2002) Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer Res* **62:** 3659–3662

Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA, Cantley LC (2004) The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* **6:** 91–99

Tiainen M, Ylikorkala A, Makela TP (1999) Growth suppression by Lkb1 is mediated by a G(1) cell cycle arrest. *Proc Natl Acad Sci USA* **96:** 9248–9251

Tracy S, Mukohara T, Hansen M, Meyerson M, Johnson BE, Janne PA (2004) Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255. *Cancer Res* **64:** 7241–7244

Volikos E, Robinson J, Aittomaki K, Mecklin JP, Jarvinen H, Westerman AM, de Rooji FW, Vogel T, Moeslein G, Launonen V, Tomlinson IP, Silver AR, Aaltonen LA (2006) LKB1 exonic and whole gene deletions are a common cause of Peutz-Jeghers syndrome. *J Med Genet* **43:** e18

Efficacy and Safety of Two Doses of Pemetrexed Supplemented with Folic Acid and Vitamin B_{12} in Previously Treated Patients with Non-Small Cell Lung Cancer

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Abstract **Purpose:** The objective of this study was to evaluate the efficacy and safety of two doses of pemetrexed supplemented with folic acid and vitamin B_{12} in pretreated Japanese patients with advanced non-small cell lung cancer (NSCLC).

Experimental Design: Patients with an Eastern Cooperative Oncology Group performance status 0 to 2, stage III or IV, and who received previously one or two chemotherapy regimens were randomized to receive 500 mg/m^2 pemetrexed (P500) or 1,000 mg/m^2 pemetrexed (P1000) on day 1 every 3 weeks. The primary endpoint was response rate.

Results: Of the 216 patients evaluable for efficacy (108 in each arm), response rates were 18.5% (90% confidence interval, 12.6-25.8%) and 14.8% (90% confidence interval, 9.5-21.6%), median survival times were 16.0 and 12.6 months, 1-year survival rates were 59.2% and 53.7%, and median progression-free survival were 3.0 and 2.5 months for the P500 and P1000, respectively. Cox multiple regression analysis indicated that pemetrexed dose was not a significant prognostic factor. Drug-related toxicity was generally tolerable for both doses; however, the safety profile of P500 showed generally milder toxicity. Main adverse drug reactions of severity grade 3 or 4 were neutrophil count decreased (20.2%) and alanine aminotransferase (glutamine pyruvic transaminase) increased (15.8%) in P500 and neutrophil count decreased (24.3%), WBC count decreased (20.7%), and lymphocyte count decreased (18.0%) in P1000. One drug-related death from interstitial lung disease occurred in the P500.

Conclusion: P500 and P1000 are similarly active with promising efficacy and acceptable safety outcomes in pretreated patients with NSCLC. These results support the use of P500 as a second- and third-line treatment of NSCLC.

Pemetrexed (LY231514; Alimta), a multitargeted antifolate, has shown antitumor activity as a single agent or in combination with other anticancer agents (1, 2). Pemetrexed at doses of 500 or 600 mg/m^2 has been evaluated in various clinical settings in a broad range of tumors including lung (non-small cell and mesothelioma), colorectal, gastric, pancreatic, head and neck, bladder, cervical, and breast cancers (3–13). In a randomized phase III trial that compared 3-week regimens of single-agent 500 mg/m^2 pemetrexed versus 75 mg/m^2 docetaxel in pretreated patients with non-small cell lung cancer (NSCLC), respective response rates (9.1% versus 8.8%) and median survival times (MST; 8.3 versus 7.9 months) did not differ between pemetrexed and docetaxel. However, fewer hematologic adverse effects, such as grade 3 or 4 neutropenia, febrile neutropenia, and neutropenic fever, were observed in patients treated with pemetrexed (3).

Myelosuppression is the predominant dose-limiting toxicity of pemetrexed as reported in phase I studies (14–16). A multivariate analysis identified the correlation between poor folate status (as indicated by elevated plasma homocysteine levels) and increased toxicity to pemetrexed, which led to the requirement that patients in all pemetrexed studies receive folic acid and vitamin B_{12} supplementation (2, 17). This has been shown to decrease toxicity to pemetrexed without compromising efficacy (18). Without supplementation, the maximum tolerated dose of pemetrexed, given every 3 weeks, has been shown to be 600 mg/m^2 in heavily pretreated patients (14); however, with supplementation, higher pemetrexed doses have been given without limiting side effects. In a Japanese phase I

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Note: The results of this study have been reported at American Society of Clinical Oncology, World Conference on Lung Cancer, and European Cancer Conference in 2007.

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study of pemetrexed that included folic acid and vitamin B_{12} supplementation, the maximum tolerated dose of pemetrexed was 1,200 mg/m^2 and recommended dose was 1,000 mg/m^2 given every 3 weeks (19). Pemetrexed pharmacokinetics in Japanese patients was not overtly different from those observed in Caucasian patients.

In view of these data, we conducted a randomized, phase II study that confirmed the efficacy and safety of a standard dose of pemetrexed (500 mg/m^2; P500) with that of a higher dose (1,000 mg/m^2; P1000), including folic acid and vitamin B_{12} supplementation, in previously treated NSCLC patients. The primary endpoint was evaluation of response rate. Secondary endpoints were assessments of response duration, progression-free survival (PFS), 1-year survival rate, MST, quality of life (QoL), and adverse events.

Materials and Methods

Patient selection. Men and women, between 20 and 75 years old, with a life expectancy of at least 12 weeks and histologically and/or cytologically confirmed advanced NSCLC were eligible for the study. In addition, all patients met the following inclusion criteria: stage III or IV disease, at least one target lesion, one or two prior chemotherapeutic regimens, an Eastern Cooperative Oncology Group performance status (PS) of 0 to 2, adequate bone marrow function (neutrophils ≥2,000/mm^3, platelets ≥100,000/mm^3, and hemoglobin ≥9.0 g/dL), hepatic function [total bilirubin within 1.5 times the upper normal limit, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) within 2.5 times the upper normal limit, and serum albumin ≥2.5 g/dL], renal function (serum creatinine ≤1.2 mg/dL and creatinine clearance ≥45 mL/min), and pulmonary function (functional oxygen saturation ≥92%).

Patients were excluded from the study for radiographic signs of interstitial pneumonitis or pulmonary fibrosis, serious or uncontrolled concomitant systemic disorders, active infections, the need for chronic administration of systemic corticosteroids, active double cancer and/or brain metastases, treatment with third-space fluid collections within 2 weeks of signing the informed consent or the need of such treatment, grade 3 or 4 toxicity, peripheral sensory neuropathy, previous pemetrexed therapy, unable or unwilling to take folic acid or vitamin B_{12} supplementation, or pregnant or breast-feeding.

This study was conducted in compliance with the guidelines of good clinical practice and the principles of the Declaration of Helsinki, and it was approved by the local institutional review boards. All patients gave written informed consent before study entry.

Study design and sample size. This open-label multicenter study had response rate as the primary objective, and 244 patients were enrolled and 226 were allocated to either 500 mg/m^2 (P500) or 1,000 mg/m^2 (P1000) randomly.

The sample size was calculated to ensure that the response rate in each group exceeded 5%. Based on the results from previous study, assuming a 13% true response rate, 5% one-sided significance level for the test with exact probability based on binomial distribution, and 90% power, at least 107 patients in each treatment arm (total of 214) were necessary. Assuming a 10% dropout rate, 240 patients were planned for the study (actual: 244 patients).

The randomization was done by an independent registration center and was dynamically balanced for PS, previous platinum chemotherapy, disease stage, gender, time from prior chemotherapy to the enrollment, and hospital. Patients were balanced with respect to the study drug in each stratum for each prognostic factor using the minimization method.

Treatment plan. Pemetrexed was administered as an i.v., 10-min infusion on day 1 of a 21-day cycle. Patients were instructed to take orally 1 g/d of a multivitamin containing 500 μg folic acid from 1 week before day 1 of course 1 until 22 days after the last administration of pemetrexed. Vitamin B_{12} (1000 μg) was injected i.m. 1 week before day 1 of course 1 and repeated every 9 weeks until 22 days after the last administration of pemetrexed. Patients were discontinued from the study for disease progression, unacceptable adverse events, inadvertent enrollment, use of excluded concomitant therapy, a cycle delay of >42 days, or if the patient requested to discontinue the study.

Administration of pemetrexed was delayed if patients met any of the following criteria: neutrophils <2,000/mm^3, hemoglobin <9.0 g/dL, platelets <100,000/mm^3, AST/ALT >2.5 times the upper normal limit, total bilirubin >1.5 times the upper normal limit, serum creatinine >1.2 mg/dL, PS 3 or 4, or grade ≥3 nonhematologic toxicity (except for anorexia, nausea, vomiting, and fatigue). The dose of pemetrexed was decreased to 400 mg/m^2 in the P500 arm and to 800 mg/m^2 in the P1000 arm, if any of the following events occurred in the previous course: grade 4 leukopenia or neutropenia, grade ≥3 febrile neutropenia, thrombocytopenia, or platelet transfusion, grade ≥3 nonhematologic toxicity (except for grade 3 anorexia, nausea, vomiting, and fatigue), or AST/ALT increased. The pemetrexed dose was similarly reduced if initiation of the next course was postponed after day 29 due to drug-related adverse events. Patients who continued to show evidence of toxicity after reducing the pemetrexed dose were discontinued from the study.

Baseline and treatment assessments. Pretreatment assessments included chest X-ray, electrocardiogram, blood chemistry, urinalysis, pregnancy test, creatinine clearance, functional oxygen saturation, vital signs, PS, body weight, and use of prior therapies. Tumor size was examined using X-ray, computer tomography, or magnetic resonance imaging done within 28 days before the planned day of the first treatment. This was repeated about every 4 weeks after the first examination.

Tumor response rate was assessed as the percentage of patients in whom complete response (CR) and partial response (PR) were confirmed based on the best overall response of the tumor response evaluation. Response was evaluated according to the Response Evaluation Criteria in Solid Tumors (20). Objective tumor responses in all responding patients were evaluated by an external review committee given no information on the treatment groups.

Duration of overall response (CR + PR) was measured from the date of the first objective assessment of CR or PR until the date of progressive disease. PFS was measured from the date of registration (for the initiation of course 1) until the date of progressive disease or death. One-year survival rate was defined as the percentage of patients who survived for 1 year from the registration date. Survival was measured from the registration date to the date of death (regardless of cause).

QoL was assessed by the QoL Questionnaire for Cancer Patients Treated with Anticancer Drugs and the Functional Assessment of Cancer Therapy for Lung Cancer (Japanese version; refs. 21–23).

Assessments of QoL were done before treatment, before the second and third courses of chemotherapy, and 3 months after the start of treatment.

Adverse events were recorded throughout the study and after the last drug administration until signs of recovery were evident. All such events were evaluated according to the Common Terminology Criteria for Adverse Events version 3.0.

Statistical analysis. Efficacy measurements were done according to the guidelines for clinical evaluation methods of anticancer drugs. Efficacy analysis was done on patients who met all selection criteria and received at least one dose of pemetrexed. Safety analysis was done on patients who received at least one dose of pemetrexed.

Statistical tests were done to establish a pemetrexed response rate of >5%; 90% confidence intervals (CI) for the objective response rate were constructed for each arm. All survival curves for time-to-event variables were created using the Kaplan-Meier method; 95% CIs were calculated for each arm. Response rate, response duration, and PFS were compared between the two arms using the χ^2 test. Cox multiple regression analysis was done on all evaluable patients from two combined arms to

identify significant prognostic factors for survival. Covariates evaluated were pemetrexed dose, gender, age, PS, disease stage, histology, interval from prior chemotherapy to registration for the first treatment course, the number of prior chemotherapeutic regimens, and use of prior platinum chemotherapy. For the QoL analysis, distributions of subscales were summarized for each arm using descriptive statistics (mean, SD, minimum, median, and maximum). As a retrospective analysis for safety, major grade 3 to 4 drug-related adverse events were compared between the two arms using the χ^2 test.

Results

Patient disposition and characteristics. From October 2004 to October 2005, a total of 244 Japanese patients with advanced NSCLC were enrolled at 28 centers. Of the 244 patients enrolled, 226 were randomly assigned (114 to the P500 arm and 112 to the P1000 arm) at least 1 week before treatment after receiving folic acid and vitamin B_{12} supplementation. A total of 225 patients (114 in the P500 arm and 111 in P1000 arm) were evaluable for safety. Of these patients, 216 (108 in each arm) were evaluable for efficacy. Gender, age, PS, histology, stage, and prior platinum chemotherapy were well balanced across the two arms (Table 1).

Efficacy evaluation. Objective tumor response rates and durations of overall response are shown in Table 2. Of the 108 patients evaluable for efficacy in the P500 arm, 20 achieved PR for an objective response rate of 18.5% (90% CI, 12.6-25.8%); the median duration of response was 4.9 months (95% CI, 3.8-8.7 months). Of the 108 patients evaluable for efficacy in the P1000 arm, 16 achieved PR for an objective response rate of 14.8% (90% CI, 9.5-21.6%); the median duration of response was 3.0 months (95% CI, 2.8-6.1 months). As seen above, the lower limits of the 90% CI in both arms were >5%, showing a statistically significant objective response rate >5% in each of the arms. The differences between arms in response rate and response duration were not statistically significant ($P = 0.5839$ and 0.1740).

By October 2006, 125 of the 216 evaluable patients had died. The MST and 1-year survival rate were 16.0 months and 59.2% in the P500 arm and 12.6 months and 53.7% in the P1000 arm ($P = 0.1463$, log-rank test for survival; Fig. 1). Median PFS was 3.0 months (95% CI, 2.0-3.5 months) in the P500 arm and 2.5 months (95% CI, 1.8-3.2 months) in the P1000 arm ($P = 0.7139$, log-rank test).

Cox multiple regression analysis indicated that pemetrexed dose was not a significant prognostic factor; however, gender (female), PS (0), disease stage (III), histologic type (non-squamous cell carcinoma), and longer intervals from prior chemotherapy were shown to be good prognostic factors (Fig. 2). Of note, patients with non-squamous cell carcinoma had a longer MST compared with those with other histologic types (16.0 versus 9.3 months; $P = 0.00264$, Cox regression analysis). Pretreatment QoL assessments in both arms were relatively high and showed neither worsening nor improvement following pemetrexed treatment (Table 3).

Safety evaluation. A total of 225 patients (114 for P500 and 111 for P1000) were evaluable for safety. Leukopenia, neutropenia, lymphopenia, anemia, elevation of AST/ALT, lactate dehydrogenase, and rash were commonly reported; however, no grade 4 leukopenia or febrile neutropenia was observed (Table 4). Other grade 4 toxicities were uncommon. Gastrointestinal toxicities such as nausea, vomiting, and anorexia were mostly mild and more frequently reported in the P1000 arm. As a retrospective analysis for safety, major grade 3 to 4 drug-related adverse events were compared

Table 1. Patient characteristics

Variable	P500	P1000
Patients who were given at least one dose of pemetrexed	114	111
Gender		
Male	72	71
Female	42	40
Age, median (range)	61.0 (37-74)	62.0 (26-74)
Eastern Cooperative Oncology Group PS		
0	45	37
1	63	68
2	6	6
Histology		
Adenocarcinoma	79	82
Squamous cell carcinoma	25	26
Others	10	3
Disease stage		
III	22	22
IV	92	88
No. prior chemotherapies		
1	44	53
2	67	57
3	3	1
Prior platinum chemotherapy		
Yes	108	104
No	6	7
Interval from prior chemotherapy to registration for the first course starts (mo)		
<3	72	66
3	42	45

Table 2. Objective tumor response and median response duration

Variable	P500 (n = 108)	P1000 (n = 108)
Objective tumor response		
CR	0	0
PR	20	16
Stable disease	40	34
Progressive disease	48	58
Response rate (90% CI), %	18.50 (12.6-25.8)	14.80 (9.5-21.6)
Median response duration (95% CI), mo	4.9 (3.8-8.7)	3.0 (2.8-6.1)

between the two arms using the χ^2 test. Grade 3 or 4 anorexia was reported more frequently in the P1000 arm (10.8% versus 2.6%; P = 0.0284). Drug-related rash was observed in 67.5% and 80.2% of the patients treated with P500 and P1000, respectively. However, all severities were grade 1 or 2. Five of the P500 patients and 3 of the P1000 patients developed interstitial lung disease related to pemetrexed treatment that resulted in the death of one patient (P500 arm). The other 7 patients recovered from their illness after discontinuing the study drug. A total 16 (14.0%) patients in the P500 arm and 26 (23.4%) patients in the P1000 arm discontinued the treatment because of drug-related adverse events.

Dose administration. The median number of treatment courses completed in both arms was 3 (range, 1-24+). Eleven percent of patients in the P500 arm and 8% in the P1000 arm completed at least 10 courses. Dose reduction occurred in 20 (17.5%) patients in the P500 arm and 27 (24.3%) patients in the P1000 arm. The most frequent cause of dose reduction was ALT elevation. Relative dose intensities were 89.6% in the P500 group and 89.8% in the P1000 group.

Discussion

This phase II, randomized study is the first report on the efficacy and safety of a higher dose of pemetrexed (1,000 mg/m^2) in pretreated Japanese patients with NSCLC. Most patients (>50%) received two courses of prior chemotherapy, and the vast majority or patients (>90%) received prior platinum-based chemotherapy. The response data indicate promising tumor reduction activity and are noteworthy in pretreated patients. The survival data are also promising and better than those reported in second- and third-line settings and comparable with those reported in first-line settings (3, 24, 25). In the phase III study (3) comparing pemetrexed with docetaxel, the response

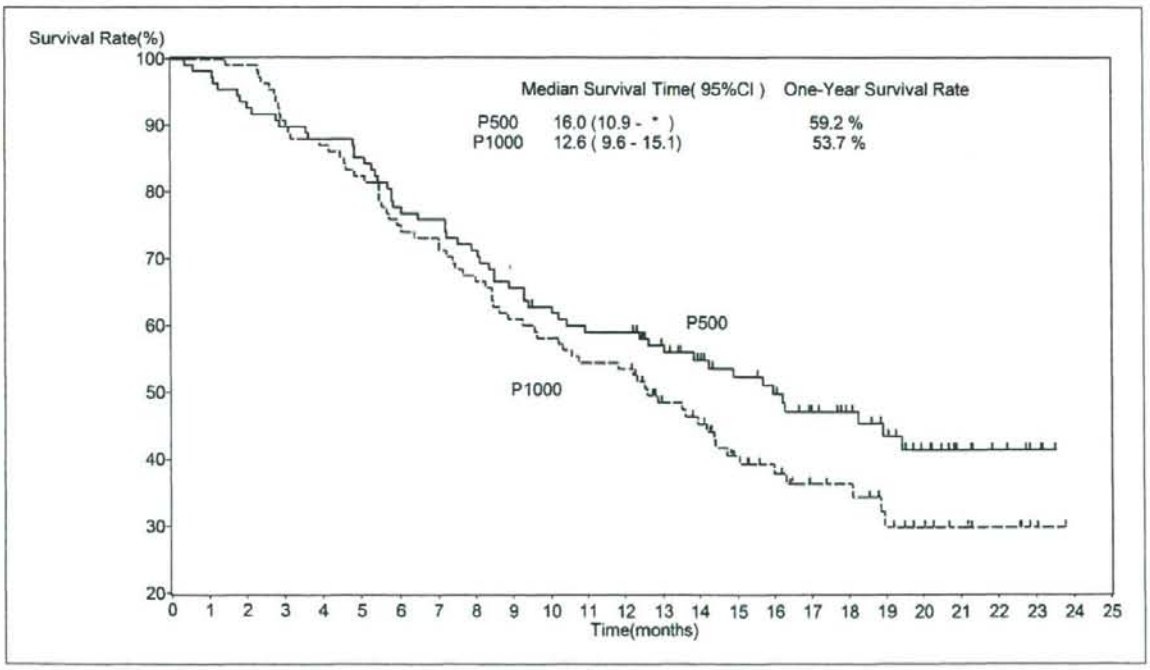

Fig. 1. Kaplan-Meier curve showing the overall survival for each arm. *Asterisk*, upper limit could not be calculated because of the censoring at the end of study period.

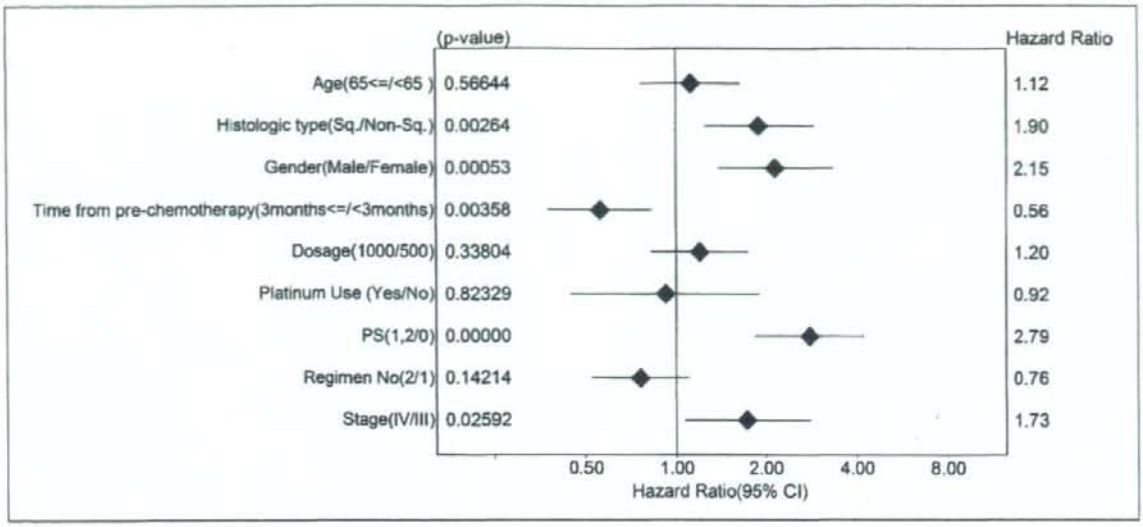

Fig. 2. Forest plot. Cox multiple regression analysis was done on all evaluable patients from two combined arms to identify significant prognostic factors for survival. Covariates evaluated were pemetrexed dose, gender, age, PS, disease stage, histology, interval from prior chemotherapy to registration for the first treatment course, the number of prior chemotherapeutic regimens, and use of prior platinum chemotherapy.

rate and median survival in the pemetrexed arm were 9.1% and 8.3 months, respectively.

Both P500 and P1000 with folic acid and vitamin B_{12} supplementation were similarly active in previously treated patients with NSCLC. All efficacy measures were similar in both arms as shown by the response rate, survival, and PFS, suggesting that doubling the standard dose of pemetrexed does not show superior efficacy. In addition, Cox multiple regression analysis showed that the difference of pemetrexed dose did not influence survival. Overall, toxicity was more frequent at the higher dose, although toxicity in both arms was mild.

Cullen et al. reported a randomized trial of 500 versus 900 mg/m^2 pemetrexed in patients with advanced NSCLC treated previously with platinum-based chemotherapy (26). The response rate, median PFS, and median survival were 7.1%, 2.6 months, and 6.7 months in patients treated with 500 mg/m^2 and 4.3%, 2.8 months, and 6.9 months in patients treated with 900 mg/m^2 pemetrexed, respectively. The higher dose did not improve survival more than the lower dose.

Dose intensification is not always accompanied by higher efficacy, such as in the case of docetaxel and cisplatin. One possible explanation for this in pemetrexed is that either the intracellular transport of pemetrexed is maximal at 500 mg/m^2 or the inhibition of target enzymes is saturated above this dose; however, there are as yet no *in vitro* data to support either mechanism. Although the mechanism still needs to be elucidated, the wide therapeutic window of pemetrexed makes it unique and safe for patients.

Of interest, our subgroup analysis identified some prognostic factors. The subgroups that were identified as good prognostic factors, gender (female), good PS, early-stage disease, and longer intervals from prior chemotherapy are well known as good prognostic factors for NSCLC. Of particular note, the MST

Table 3. Summary for Functional Assessment of Cancer Therapy for Lung Cancer Lung Cancer Subscale

	n	Mean (SD)	Min	Med	Max
P500 (*n* = 108)					
Before course 1	107	71.5 (18.81)	32.1	71.4	100
Before course 2	101	74.3 (16.68)	39.3	75	100
Before course 3	84	74.3 (18.08)	35.7	78.6	100
Registration of course 1 + 3 mo*	59	76.3 (18.1)	32.1	78.6	100
P1000 (*n* = 108)					
Before course 1	107	69.6 (18.52)	25	67.9	100
Before course 2	98	73.5 (17.21)	32.1	75	100
Before course 3	72	71.4 (18.4)	28.6	71.4	100
Registration of course 1 + 3 mo*	61	74.3 (18.62)	28.6	71.4	100

*Three months ±2 weeks after the day of registration for one course.

Table 4. Hematologic and nonhematologic toxicity evaluated by Common Terminology Criteria for Adverse Events version 3.0

	P500 (n = 114)				P1000 (n = 111)				P
	Grade (%)				Grade (%)				
	2	3	4	3/4/5	2	3	4	3/4/5	
Leukopenia	32.5	14.9	0	14.9	38.7	21.6	0	21.6	0.2582
Neutropenia	25.4	17.5	3.5	21.1	27.9	19.8	4.5	24.3	0.6695
Lymphopenia	28.9	9.6	2.6	12.3	30.6	16.2	1.8	18	0.31
Anemia	19.3	7	0.9	7.9	34.2	9	0.9	9.9	0.7667
Thrombocytopenia	0	0	0	0	8.1	0.9	0	0.9	NA
Febrile neutropenia	*	0	0	0	*	0	0	0	NA
Nausea	14	0	0	0	14.4	2.7	0	2.7	NA
Vomiting	7	0	0	0	11.7	1.8	0	1.8	NA
Anorexia	16.7	2.6	0	2.6	15.3	10.8	0	10.8	0.0284
Fatigue	3.5	0	0	0	1.8	0.9	0	0.9	NA
Diarrhea	2.6	0.9	0	0.9	1.8	1.8	0	1.8	0.9815
Constipation	1.8	0.9	0	0.9	5.4	0	0	0	NA
Rash	49.1	2.6	0	2.6	63.1	4.5	0	4.5	0.6903
Alopecia	0	*	*	*	0	*	*	*	NA
Pneumonitis	1.8	1.8	0	2.6†	0	2.7	0	2.7	1
AST	21.9	7.9	0	7.9	25.2	4.5	0	4.5	0.4375
ALT	17.5	16.7	0	16.7	32.4	7.2	0.9	8.1	0.8143

NOTE: Major grade 3 to 4 drug-related adverse events were compared between two arms using χ^2 test.
*Not indicated in Common Terminology Criteria for Adverse Events version 3.0.
† One patient died of drug-induced pneumonitis.

of patients with non-squamous cell carcinoma was significantly longer compared with that in patients with squamous cell carcinoma (16.0 versus 9.3 months; $P = 0.00264$). Pemetrexed induces its antitumor activity by inhibiting key enzymes related to the folate metabolism, such as thymidylate synthase. Studies of the tumor histology of adenocarcinoma progressive disease have reported lower-level expression of thymidylate synthase than squamous cell carcinoma (27). Good survival benefit in patients with non-squamous cell carcinoma by pemetrexed may be explained by lower levels of thymidylate synthase. Because MST was the subject of a subgroup analysis and survival was not a primary endpoint of this study, this finding should be considered exploratory requiring independent confirmation. However, if this finding of superior effectiveness in non-squamous cell carcinoma could be substantiated in future studies, it would be very useful. Indeed, histology could be a simple means of tailoring chemotherapy treatment.

In conclusion, although the recommended dose is P1000 with folic acid and vitamin B_{12} supplementation for Japanese patients, it has similar efficacy and safety with P500, the recommend dosage in rest of the world. These results support the use of P500 as a second- or third-line treatment of NSCLC.

Disclosure of Potential Conflicts of Interest

Authors have conflicts with Eli Lilly and company.

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References

1. Walling J. From methotrexate to pemetrexed and beyond. A review of the pharmacodynamic and clinical properties of antifolates. Invest New Drugs 2006;24:37–77.
2. Hazarika M, White RM, Johnson JR, Pazdur R. FDA drug approval summaries: pemetrexed (Alimta®). Oncologist 2004;9:482–8.
3. Hanna N, Shepherd FA, Fossella FV, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. J Clin Oncol 2004;22:1589–97.
4. Vogelzang NJ, Rusthoven JJ, Symanowski J, et al. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 2003;21:2636–44.
5. John W, Picus J, Blanke CD, et al. Activity of multitargeted antifolate (pemetrexed disodium, LY231514) in patients with advanced colorectal carcinoma: results from a phase II study. Cancer 2000;88:1807–13.
6. Cripps C, Burnell M, Jolivet J, et al. Phase II study of first-line LY231514 (multi-targeted antifolate) in patients with locally advanced or metastatic colorectal cancer: an NCIC Clinical Trials Group study. Ann Oncol 1999;10:1175–9.
7. Bajetta E, Celio L, Buzzoni R, et al. Phase II study of pemetrexed disodium (Alimta®) administered with oral folic acid in patients with advanced gastric cancer. Ann Oncol 2003;14:1543–8.
8. Miller KD, Picus J, Blanke C, et al. Phase II study of the multitargeted antifolate LY231514 (Alimta, MTA, pemetrexed disodium) in patients with advanced pancreatic cancer. Ann Oncol 2000;11:101–3.
9. Pivot X, Raymond E, Laguerre B, et al. Pemetrexed disodium in recurrent locally advanced or metastatic squamous cell carcinoma of the head and neck. Br J Cancer 2001;85:649–55.
10. Hanauske AR, Chen V, Paoletti P, Niyikiza C. Pemetrexed disodium: a novel antifolate clinically active against multiple solid tumors. Oncologist 2001;6:363–73.
11. Goedhals L, van Wiyk AL, Smith BL, Fourie SJ. Pemetrexed (Alimta, LY231514) demonstrates clinical activity in chemonaive patients with cervical cancer in a phase II single-agent trial. Int J Gynecol Cancer 2006;16:1172–8.
12. Miles DW, Smith IE, Coleman RE, Calvert AH, Lind MJ. A phase II study of pemetrexed disodium (LY231514) in patients with locally recurrent or metastatic breast cancer. Eur J Cancer 2001;37:1366–71.

13. Martin M, Spielmann M, Namer M, et al. Phase II study of pemetrexed in breast cancer patients pretreated with anthracyclines. Ann Oncol 2003;14:1246–52.
14. Rinaldi DA, Kuhn JG, Burris HA, et al. A phase I evaluation of multitargeted antifolate (MTA, LY231514), administered every 21 days, utilizing the modified continual reassessment method for dose escalation. Cancer Chemother Pharmacol 1999;44:372–80.
15. Rinaldi DA, Burris HA, Dorr FA, et al. Initial phase I evaluation of the novel thymidylate synthase inhibitor, LY231514, using the modified continual reassessment method for dose escalation. J Clin Oncol 1995;13:2842–50.
16. McDonald AC, Vasey PA, Adams L, et al. A phase I and pharmacokinetic study of LY231514, the multitargeted antifolate. Clin Cancer Res 1998;4:605–10.
17. Niyikiza C, Baker SD, Seitz DE, et al. Homocysteine and methylmalonic acid: markers to predict and avoid toxicity from pemetrexed therapy. Mol Cancer Ther 2002;1:545–52.
18. Scagliotti GV, Shin DM, Kindler HL, et al. Phase II study of pemetrexed with and without folic acid and vitamin B_{12} as front-line therapy in malignant pleural mesothelioma. J Clin Oncol 2003;21:1556–61.
19. Nakagawa K, Kudoh S, Matsui K, et al. A phase I study of pemetrexed (LY231514) supplemented with folate and vitamin B_{12} in Japanese patients with solid tumours. Br J Cancer 2006;95:677–82.
20. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 2000;92:205–16.
21. Cella DF, Bonomi AE, Lloyd SR, Tulsky DS, Kaplan E, Bonomi P. Reliability and validity of the Functional Assessment of Cancer Therapy-Lung (FACT-L) quality of life instrument. Lung Cancer 1995;12:199–220.
22. Kurihara M, Shimizu H, Tsuboi K, et al. Development of quality of life questionnaire in Japan: quality of life assessment of cancer patients receiving chemotherapy. Psychooncology 1999;8:355–63.
23. Matsumoto T, Ohashi Y, Morita S, et al. The quality of life questionnaire for cancer patients treated with anticancer drugs (QOL-ACD): validity and reliability in Japanese patients with advanced non-small-cell lung cancer. Qual Life Res 2002;11:483–93.
24. Shepherd FA, Dancey J, Ramlau R, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000;18:2095–103.
25. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. Ann Oncol 2007;18:317–23.
26. Cullen M, Zatloukal P, Sörenson S, et al. Pemetrexed for the treatment of advanced non-small cell lung cancer (NSCLC): results from a randomized phase III dose finding trial in patients who progressed following platinum-containing chemotherapy. J Thorac Oncol 2007;2:S316–7.
27. Ceppi P, Volante M, Saviozzi S, et al. Squamous cell carcinoma of the lung compared with other histotypes shows higher messenger RNA and protein levels for thymidylate synthase. Cancer 2006;107:1589–96.

Phase II Study of Combination Therapy with S-1 and Irinotecan for Advanced Non–Small Cell Lung Cancer: West Japan Thoracic Oncology Group 3505

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Abstract **Purpose:** To evaluate the efficacy and toxicity of combination therapy with the oral fluoropyrimidine formulation S-1 and irinotecan for patients with advanced NSCLC.

Experimental Design: Chemotherapy-naive patients with advanced NSCLC were treated with i.v. irinotecan (150 mg/m^2) on day 1 and with oral S-1 (80 mg/m^2) on days 1 to 14 every 3 weeks.

Results: Fifty-six patients (median age, 63 years; range, 40-74 years) received a total of 286 treatment cycles (median, 5; range, 1-15). No complete responses and 16 partial responses were observed, giving an overall response rate of 28.6% [95% confidence interval (95% CI), 17.3-42.2%]. Twenty-four patients (42.9%) had stable disease and 12 patients (21.4%) had progressive disease as the best response. The overall disease control rate (complete response + partial response + stable disease) was thus 71.4% (95% CI, 57.8-82.7%). Median progression-free survival was 4.9 months (95% CI, 4.0-6.4 months), whereas median overall survival was 15 months. Hematologic toxicities of grade 3 or 4 included neutropenia (25%), thrombocytopenia (3.6%), and anemia (3.6%), with febrile neutropenia being observed in four patients (7.1%). The most common nonhematologic toxicities of grade 3 or 4 included anorexia (14.3%), fatigue (8.9%), and diarrhea (8.9%). There were no deaths attributed to treatment.

Conclusions: The combination of S-1 and irinotecan is a potential alternative option with a favorable toxicity profile for the treatment of advanced NSCLC. This nonplatinum regimen warrants further evaluation in randomized trials.

Non–small cell lung cancer (NSCLC) is the leading cause of death related to cancer worldwide (1). Platinum-based chemotherapy is the standard first-line treatment for advanced NSCLC based on the moderate improvement in survival and quality of life it confers compared with best supportive care alone (2–4). The poor outlook even for patients with advanced NSCLC who receive such treatment has prompted a search for new chemotherapeutic agents and combination regimens.

S-1 is an oral fluorinated pyrimidine formulation that combines tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1 (5). 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, which catalyzes 5-FU catabolism (6). CDHP also attenuates the cardiotoxic and neurotoxic effects of 5-FU by reducing the production of fluoro-β-alanine, the main catabolite of 5-FU (7, 8). Oxonate reduces the gastrointestinal toxicity of 5-FU. After its oral administration, oxonate becomes distributed selectively to the small and large intestine, where it inhibits the phosphorylation of 5-FU to fluoropyrimidine monophosphate catalyzed by orotate phosphoribosyltransferase within gastrointestinal mucosal cells, thereby reducing the incidence of diarrhea (9). In a phase II trial of S-1 as a single agent for treatment of advanced NSCLC, a response rate of 22% and a median survival time of 10.2 months were obtained in 59 patients without prior chemotherapy (10). Few severe gastrointestinal or hematologic adverse events were reported (10). Moreover, a phase II trial of S-1 plus cisplatin in advanced NSCLC patients revealed a response rate of 47% and a median survival time of 11 months (11).

Irinotecan is an inhibitor of DNA topoisomerase I. It has shown activity as a single agent in first-line chemotherapy for advanced NSCLC (12). Weekly administration of irinotecan

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Translational Relevance

Non–small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide. The dismal outlook for patients with advanced NSCLC treated with available therapies has prompted a search for new and more effective chemotherapeutic agents and combination regimens. S-1 is a new oral fluorinated pyrimidine formulation that combines tegafur, 5-chloro-2,4-dihydroxypyridine, and potassium oxonate and has been found to exhibit marked antitumor activity in recent clinical trials with cancer patients, including those with NSCLC. We have now examined the therapeutic efficacy and toxicity of the combination of S-1 and irinotecan in chemotherapy-naive patients with advanced NSCLC. We found this drug combination to be active, with a response rate of 28.6%, median progression-free survival of 4.9 months, and median overall survival of 15 months, values that compare favorably with those reported for phase III studies of standard platinum-based doublet chemotherapy. Furthermore, toxicities were manageable, and in most instances, treatment could be continued in the outpatient setting. Our data indicate that the combination of S-1 and irinotecan is a promising alternative for treatment of advanced NSCLC. This nonplatinum regimen warrants further evaluation in randomized trials.

(100 mg/m^2) for 3 weeks followed by 1 week of rest yielded a response rate of 20.5% and a median survival time of 10.6 months in 132 patients with advanced NSCLC (13).

S-1 and irinotecan have both shown single-agent activity against a wide range of solid tumors, including NSCLC, and the combination of these two agents has manifested synergistic effects in tumor xenograft models *in vivo* (14). A phase I study examined administration of irinotecan at a dose of 150 mg/m^2 on day 1 and of S-1 at 80 mg/m^2 per day from days 1 to 14 of a 21-day cycle (15); it found no difference in pharmacokinetic variables for the two drugs relative to the expected values for S-1 or irinotecan administered as single agents. A subsequent phase II study in patients with advanced colorectal cancer showed that this combination was well tolerated and had marked antitumor activity (16). The safety or effectiveness of the combination of S-1 and irinotecan in patients with advanced NSCLC has not previously been reported.

We now present the results of a multicenter phase II trial of S-1 in combination with irinotecan for patients with previously untreated advanced NSCLC. The aims of this study were to determine the objective tumor response rate, overall and progression-free survival, and toxicity profile for such treatment.

Materials and Methods

Patient eligibility. The criteria for patient eligibility included a diagnosis of NSCLC confirmed either histologically or cytologically, clinical stage IV or IIIB (including only patients with no indications for curative radiotherapy, such as those with malignant pleural effusion, pleural dissemination, malignant pericardial effusion, metastatic lesions in the same lobe of the primary lesion, or involvement of contralateral mediastinal or hilar lymph nodes), measurable disease, no prior chemotherapy, an age range of 20 to 74 y, an Eastern Cooperative Oncology Group performance status of 0 or 1, and a projected life expectancy of at least 3 mo. Other eligibility criteria for organ function included a leukocyte count of ≥3,000/mm^3, a neutrophil count of ≥1,500/mm^3, a platelet count of ≥100,000/μL, a serum bilirubin concentration of ≤1.5 mg/dL, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels of ≤2.5 times the upper normal limit, a normal serum creatinine level, and either a partial pressure of arterial oxygen of ≥65 torr or a peripheral oxygen saturation of ≥92%. Main exclusion criteria included active concomitant of any malignancy, symptomatic brain metastasis, interstitial pneumonia, watery diarrhea, obstructive bowel disease, heart failure, uncontrolled diabetes mellitus, active infection, and a past history of drug allergy. Written informed consent was obtained from all patients, and the study protocol was approved by the institutional ethics committee of each of the participating institutions.

Study design and treatment. This was a multicenter, open-label, single-arm, phase II study. The primary end point of the study was the response rate, which determined the sample size. We chose a 35% response rate as a desirable target level and a 20% response rate as uninteresting with an α error of 0.05 and a power of 0.8, resulting in a requirement for 50 patients. Allowing for a patient ineligibility rate of 10%, we planned to enroll 55 patients.

Each treatment cycle consisted of the oral administration of S-1 (40 mg/m^2) twice daily for 2 wk, with a 90-min i.v. infusion of irinotecan (150 mg/m^2) on day 1 followed by a drug-free interval of 1 wk. S-1 was available as capsules containing 20 or 25 mg of tegafur. Patients were assigned based on body surface area to receive one of the following oral doses of S-1 twice daily: 40 mg (body surface area < 1.25 m^2), 50 mg (1.25 ≤ body surface area < 1.50 m^2), or 60 mg (body surface area ≥ 1.50 m^2). Courses of treatment were repeated every 21 d until the occurrence of tumor progression or unacceptable toxicity, refusal of the patient, or a decision by the physician to stop treatment.

If laboratory variables changed after the start of treatment so that they no longer met the eligibility criteria for the study, subsequent courses of treatment were withheld until the abnormality had resolved. If the abnormality had not resolved within 43 d, the patient was excluded from the study. The doses of both S-1 and irinotecan were reduced in the event of any of the following toxicities during the previous treatment cycle: neutropenia of grade 4 for >7 d, febrile neutropenia, thrombocytopenia of grade ≥4, and nonhematologic toxicity of grade ≥3. S-1 was reduced in subsequent courses from 60, 50, or 40 mg twice daily to 50, 40, and 25 mg twice daily, respectively. The dose of irinotecan was reduced by 25 mg/m^2 for subsequent courses. Once lowered, the doses of S-1 and irinotecan were not increased.

Evaluation. Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (17). Tumors were measured by computed tomography within 2 wk before the first cycle of treatment and then every 4 wk. Patients were evaluable for response if they had a baseline exam and at least one follow-up exam and had received at least one cycle of treatment. A central radiological review was done to determine the eligibility of patients and the response to treatment. Response was confirmed at least 4 wk (for a complete or partial response) or 6 wk (for stable disease) after it was first documented. Progression-free survival was defined as the time from registration until objective tumor progression or death. Patients whose disease had not progressed at the time of discontinuation of the study treatment continued to be assessed until progression was documented. If a patient died without documentation of disease progression, the patients was considered to have had tumor progression at the time of death, unless there was sufficient documented evidence to conclude otherwise. Overall survival was defined as the time from registration until death from any cause. Progression-free and overall survival as well as the 1-y survival rate were estimated by the Kaplan-Meier method.

Table 1. Characteristics of the 56 eligible patients

Characteristic	No. patients
Median age, y (range)	63 (40-74)
Sex	
Male	46 (82%)
Female	10 (18%)
Performance status (ECOG)	
0	20 (36%)
1	36 (64%)
Stage	
IIIB	16 (29%)
IV	40 (71%)
Histology	
Adenocarcinoma	30 (54%)
Squamous cell carcinoma	21 (38%)
Adenosquamous cell carcinoma	1 (1.8%)
Large cell carcinoma	1 (1.8%)
NSCLC, not specified	3 (5.4%)

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (version 3). All patients who received one dose of chemotherapy were assessable for toxicity. A clinical and laboratory assessment was done at least every 2 wk.

Results

Patient characteristics. Between February and June 2006, a total of 59 patients were enrolled in the study at the 14 participating centers. Three patients did not receive treatment: one patient withdrew her consent, and two patients had a fall before treatment onset that resulted in a reduction in performance status. These three patients were thus not included in the analysis. The remaining 56 patients (46 men and 10 women) were eligible for the current analysis and their characteristics are summarized in Table 1. Their median age was 63 years, with a range of 40 to 74 years. Histologic analysis revealed that 30 patients (54%) had adenocarcinoma and 21 patients (38%) had squamous cell carcinoma. Forty patients (71%) had stage IV disease and the other 16 patients had stage IIIB disease (including 12 patients with malignant pleural effusion).

Treatment administered. Patients received a median of five cycles of treatment (range, 1-15), with 37 patients (66%) completing at least four cycles. Overall, 286 cycles of chemotherapy were delivered. The mean relative dose intensities of S-1 and irinotecan were 91% and 98%, respectively. Dose reductions were uncommon and were necessary according to the study protocol in only eight cycles (2.8% of total cycles) because of diarrhea in three patients, anorexia in two patients, vomiting in two patients, and an increase in serum ALT and AST levels in one patient. Treatment administration was delayed for at least 1 week because of toxicity in 12 cycles (4.2% of total cycles); the major causes of delayed administration were insufficient bone marrow function (six cycles with a leukocyte count of $<3{,}000/mm^3$ and one cycle with a platelet count of $<100{,}000/\mu L$) and nonhematologic toxicity (two cycles with fever in the absence of neutropenia, two cycles with an increase in serum ALT and AST levels, and one cycle with diarrhea).

Table 2. Overall response rate (Response Evaluation Criteria in Solid Tumors criteria) by independent radiologic assessment

Response	No. patients (%)
Complete response	0 (0)
Partial response	16 (28.6)
Overall response	16 (28.6; 95% CI, 17.3-42.2)
Stable disease	24 (42.9)
Disease progression	12 (21.4)
Not evaluable	4 (7.1%)

Response and survival. Four patients were not evaluable for response: three patients withdrew from the study after one treatment cycle and one patient did not have a measurable target lesion. There were 16 partial responses and no complete responses, yielding an overall response rate of 28.6% (Table 2). Twenty-four patients (42.9%) had stable disease, yielding an overall disease control rate (complete response + partial response + stable disease) of 71.4% [95% confidence interval (95% CI), 57.8-82.7%]. Twelve patients (21.4%) had progressive disease as the best response.

All 56 treated patients were assessable for progression-free survival and overall survival. With a median follow-up time of 14.9 months (range, 1.4-20.1 months), 25 patients were still alive. The progression-free survival curve is shown in Fig. 1; the median progression-free survival was 4.9 months (95% CI, 4.0-6.4 months). The curve for overall survival is shown in Fig. 2; the median overall survival time was 15 months (95% CI could not be estimated) and the 1-year survival rate was 63% (95% CI, 50-75%). No correlation was apparent between overall survival and sex, age, histology, disease stage, or smoking status.

Toxicity. The adverse events observed for all 56 treated patients are summarized in Table 3. The most frequently observed hematologic toxicity of grade 3 or 4 was neutropenia (14 cases, 25%). Four patients (7.1%) developed febrile neutropenia. Anemia or thrombocytopenia of grade 3 or 4 was less frequent, each occurring in 3.6% of patients. Nonhematologic toxicities were generally mild in intensity. The most common nonhematologic toxicities of grade 3 or 4 were anorexia (14.3%), fatigue (8.9%), diarrhea (8.9%), vomiting (3.6%), and an increase in serum ALT or AST levels (3.6%). Treatment was discontinued because of toxicity in only two of

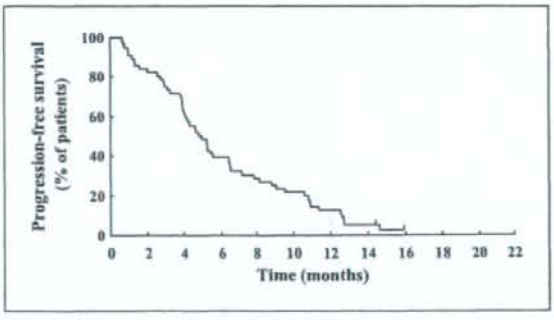

Fig. 1. Kaplan-Meier analysis of progression-free survival for all 56 treated patients.

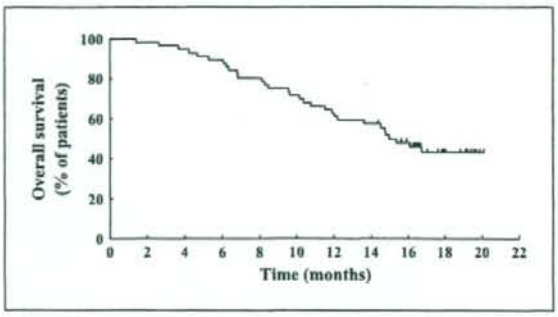

Fig. 2. Kaplan-Meier analysis of overall survival for all 56 treated patients.

the 56 patients (3.6%): in one patient because of pneumonitis (grade 3) and in the other because of prolonged anorexia (grade 3) and fatigue (grade 3). The patient with pneumonitis developed fever with hypoxemia after the fourth course of treatment. A computed tomographic scan of the chest revealed new ground-glass opacities distributed diffusely in both lungs. The patient responded well to steroid therapy and improved. No treatment-related deaths were observed.

Discussion

Platinum-based doublet chemotherapy is the standard of care for most patients with advanced NSCLC (2–4). However, there continues to be reluctance on the part of both patients and treating physicians to accept the toxicity of platinum-based therapy given the associated small gain in survival. Active therapies with improved toxicity profiles are clearly needed in this setting. Since the introduction of active third-generation agents (docetaxel, paclitaxel, gemcitabine, vinorelbine, and irinotecan), many clinical trials have been undertaken to evaluate nonplatinum regimens based on these drugs in the hope that platinum analogues could be eliminated from the treatment of advanced NSCLC. A recent meta-analysis showed that these newer nonplatinum regimens are valid options for the treatment of advanced NSCLC because of their shown activity and good toxicity profiles (18). Currently, however, there is no single best treatment regimen for advanced NSCLC.

As first-line chemotherapy for advanced NSCLC, the oral fluoropyrimidine formulation S-1 administered as a single agent showed a response rate of 22% and a median survival time of 10.2 months with toxicities that were generally mild (10). Combinations of S-1 with other active agents with a different mechanism of action are being investigated with the aim of achieving a greater clinical benefit. Irinotecan and fluoropyrimidines were shown not to induce cross-resistance in both experimental and clinical settings (19). Preclinical studies have also found that the combination of irinotecan and 5-FU has antitumor activities that are additive to synergistic (20). Furthermore, a possible molecular mechanism for synergistic cytotoxicity of S-1 and irinotecan has been suggested by the observation that irinotecan reduces thymidylate synthetase activity in tumor xenografts and thereby facilitates the antitumor effect of S-1 (14). Recent phase II studies have shown that combination treatment with S-1 and irinotecan is highly active with acceptable toxicity in patients with advanced colorectal cancer or gastric cancer (16, 21). However, the activity of this combination in patients with NSCLC has not previously been documented.

We have now assessed the efficacy and safety of combined treatment with S-1 and irinotecan in patients with previously untreated advanced NSCLC. We found the combination to be active, with a response rate of 28.6%, median progression-free survival of 4.9 months, median overall survival of 15 months, and 1-year survival rate of 63%. Previous phase III studies of platinum-based doublets for the treatment of advanced NSCLC showed response rates of 17% to 33%, a median time to progression or progression-free survival of 3 to 5 months, and a median overall survival time of 7 to 14 months (22–25). Although there are limitations to comparisons of the results from different studies, the efficacy data in our study compare favorably with those reported in these previous phase III studies of platinum-based doublets.

The S-1-irinotecan regimen was well tolerated in the patients of the present study. With regard to hematologic toxicity, neutropenia of grade 3 or 4 occurred in only 25% of all treated patients without the prophylactic administration of granulocyte colony-stimulating factor. Anemia and thrombocytopenia of grade 3 or 4 were each observed in only two patients (3.6%). These results compare favorably with the toxicity profiles reported for platinum-based combinations in previous studies with NSCLC patients, in which higher frequencies of neutropenia (~80%), anemia (~20%), and thrombocytopenia (~23%) of grade 3 or 4 were observed (22–24). The only nonhematologic toxicity of grade 3 or 4 encountered in >10% of patients in the present study was anorexia (14.3%). Although irinotecan and S-1 have each been shown to increase the frequency of severe diarrhea, the incidence of diarrhea of grade 3 in the present study was only 8.9%, consistent with the findings of a recent phase II study of the combination of S-1 and irinotecan administered according to the same doses and schedule in patients with advanced colorectal cancer (16).

Table 3. Toxicity for all 56 treated patients according to the National Cancer Institute Common Toxicity Criteria (version 3)

Toxicity	Grade				Grade ≥3 (%)
	1	2	3	4	
Leukopenia	9	10	5	0	8.9
Neutropenia	1	7	12	2	25.0
Anemia	31	19	1	1	3.6
Thrombocytopenia	23	2	2	0	3.6
Febrile neutropenia	NA	NA	4	0	7.1
Anorexia	25	10	8	0	14.3
Fatigue	18	12	4	1	8.9
Diarrhea	12	11	5	0	8.9
Nausea	27	11	1	0	1.8
Vomiting	12	4	2	0	3.6
Stomatitis	7	6	0	0	0
Rash	8	6	0	0	0
Hyperbilirubinemia	12	6	0	0	0
Elevation of AST/ALT	18	3	2	0	3.6
Elevation of creatinine	2	1	0	0	0
Pneumonitis	1	0	1	0	1.8

Abbreviation: NA, not applicable.

Thus, both hematologic and nonhematologic toxicities were generally manageable, and in most instances, treatment could be continued in an outpatient setting, resulting in a median of five treatment courses (range, 1-15).

In conclusion, we have presented the results of the first plhase II study of the combination of S-1 and irinotecan for the treatment of chemotherapy-naive patients with advanced NSCLC. This regimen yielded a response rate, progression-free survival, and overall survival similar to or better than those previously reported for platinum-based regimens. In addition, this regimen was well tolerated and could be administered in an outpatient setting. Given its efficacy and favorable toxicity profile, the combination of S-1 and irinotecan is a promising alternative for treatment of advanced NSCLC and a feasible nonplatinum option to which molecularly targeted agents can be added. The chemotherapy regimens of S-1 plus platinum derivatives have been studied (11). We are currently conducting a randomized phase III trial comparing carboplatin/S-1 with carboplatin/paclitaxel for chemonaive advanced NSCLC. We firmly believe that further trials comparing S-1 plus irinotecan with platinum-based doublet chemotherapy (perhaps carboplatin/S-1) are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.
2. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Non-small Cell Lung Cancer Collaborative Group. BMJ 1995;311:899–909.
3. Clinical practice guidelines for the treatment of unresectable non-small-cell lung cancer. Adopted on May 16, 1997 by the American Society of Clinical Oncology. J Clin Oncol 1997;15:2996–3018.
4. Socinski MA, Crowell R, Hensing TE, et al. Treatment of non-small cell lung cancer, stage IV: ACCP evidence-based clinical practice guidelines (2nd edition). Chest 2007;132:277–89S.
5. Shirasaka T, Nakano K, Takechi T, et al. Antitumor activity of 1 M tegafur-0.4 M 5-chloro-2,4-dihydroxypyridine-1 M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted into nude rats. Cancer Res 1996;56:2602–6.
6. Takechi T, Nakano K, Uchida J, et al. Antitumor activity and low intestinal toxicity of S-1, a new formulation of oral tegafur, in experimental tumor models in rats. Cancer Chemother Pharmacol 1997;39:205–11.
7. Robben NC, Pippas AW, Moore JO. The syndrome of 5-fluorouracil cardiotoxicity. An elusive cardiopathy. Cancer 1993;71:493–509.
8. Kato T, Shimamoto Y, Uchida J, et al. Possible regulation of 5-fluorouracil-induced neuro- and oral toxicities by two biochemical modulators consisting of S-1, a new oral formulation of 5-fluorouracil. Anticancer Res 2001;21:1705–12.
9. Shirasaka T, Shimamoto Y, Fukushima M. Inhibition by oxonic acid of gastrointestinal toxicity of 5-fluorouracil without loss of its antitumor activity in rats. Cancer Res 1993;53:4004–9.
10. Kawahara M, Furuse K, Segawa Y, et al. Phase II study of S-1, a novel oral fluorouracil, in advanced non-small-cell lung cancer. Br J Cancer 2001;85:939–43.
11. Ichinose Y, Yoshimori K, Sakai H, et al. S-1 plus cisplatin combination chemotherapy in patients with advanced non-small cell lung cancer: a multi-institutional phase II trial. Clin Cancer Res 2004;10:7860–4.
12. Fukuoka M, Niitani H, Suzuki A, et al. A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. J Clin Oncol 1992;10:16–20.
13. Negoro S, Masuda N, Takada Y, et al. Randomised phase III trial of irinotecan combined with cisplatin for advanced non-small-cell lung cancer. Br J Cancer 2003;88:335–41.
14. Takiuchi H, Narahara H, Tsujinaka T, et al. Phase I study of S-1 combined with irinotecan (CPT-11) in patients with advanced gastric cancer (OGSG 0002). Jpn J Clin Oncol 2005;35:520–5.
15. Yamada Y, Yasui H, Goto A, et al. Phase I study of irinotecan and S-1 combination therapy in patients with metastatic gastric cancer. Int J Clin Oncol 2003;8:374–80.
16. Goto A, Yamada Y, Yasui H, et al. Phase II study of combination therapy with S-1 and irinotecan in patients with advanced colorectal cancer. Ann Oncol 2006;17:968–73.
17. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.
18. D'Addario G, Pintilie M, Leighl NB, Feld R, Cerny T, Shepherd FA. Platinum-based versus non-platinum-based chemotherapy in advanced non-small-cell lung cancer: a meta-analysis of the published literature. J Clin Oncol 2005;23:2926–36.
19. Vanhoefer U, Harstrick A, Achterrath W, Cao S, Seeber S, Rustum YM. Irinotecan in the treatment of colorectal cancer: clinical overview. J Clin Oncol 2001;19:1501–18.
20. Houghton JA, Cheshire PJ, Hallman JD II, et al. Evaluation of irinotecan in combination with 5-fluorouracil or etoposide in xenograft models of colon adenocarcinoma and rhabdomyosarcoma. Clin Cancer Res 1996;2:107–18.
21. Inokuchi M, Yamashita T, Yamada H, et al. Phase I/II study of S-1 combined with irinotecan for metastatic advanced gastric cancer. Br J Cancer 2006;94:1130–5.
22. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 2002;346:92–8.
23. Fossella F, Pereira JR, von Pawel J, et al. Randomized, multinational, phase III study of docetaxel plus platinum combinations versus vinorelbine plus cisplatin for advanced non-small-cell lung cancer: the TAX 326 study group. J Clin Oncol 2003;21:3016–24.
24. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. Ann Oncol 2007;18:317–23.
25. Hotta K, Fujiwara Y, Matsuo K, et al. Recent improvement in the survival of patients with advanced nonsmall cell lung cancer enrolled in phase III trials of first-line, systemic chemotherapy. Cancer 2007;109:939–48.

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 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

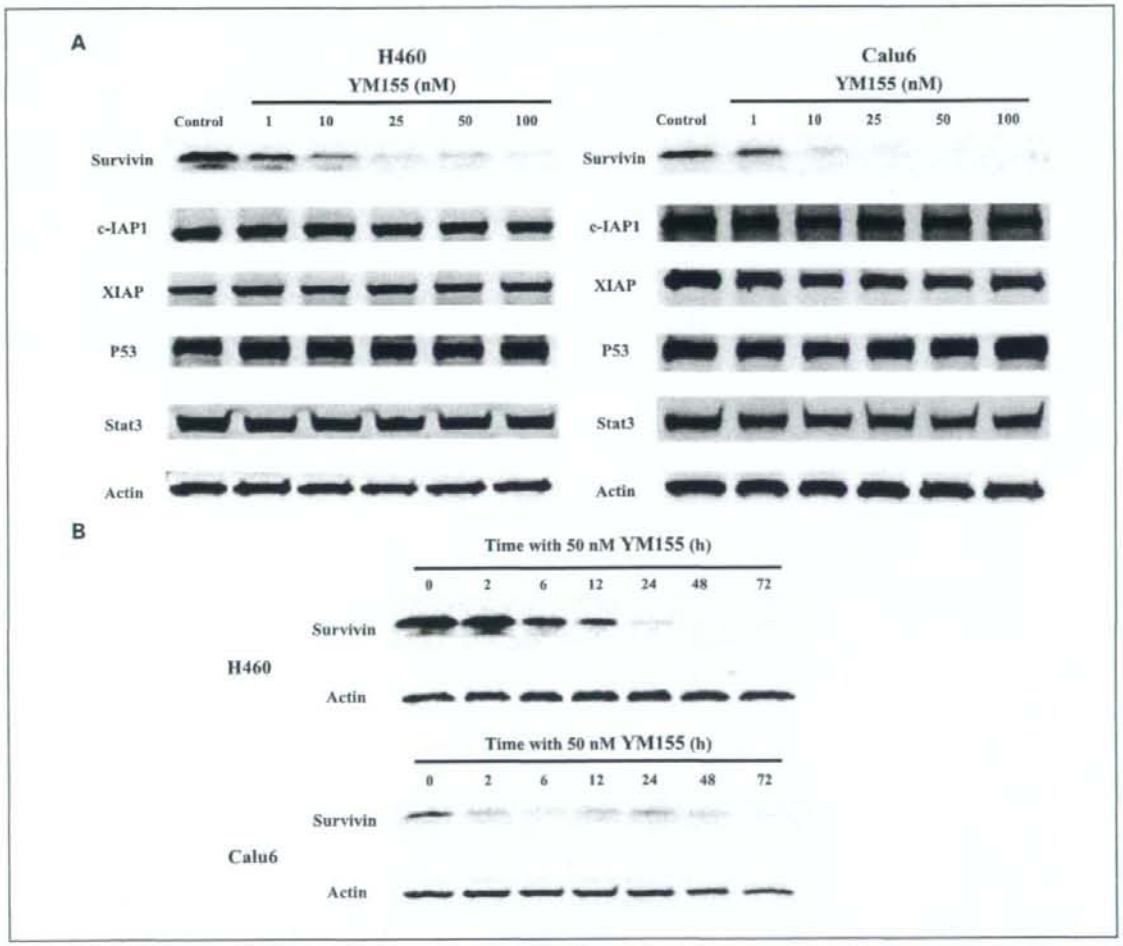

Fig. 1. Effect of YM155 on survivin expression in human NSCLC cells. *A*, H460 or Calu6 cells were incubated in the absence (control, 0.1% DMSO) or presence of various concentrations (1, 10, 25, 50, or 100 nmol/L) of YM155 for 48 h. Cell lysates were then prepared and subjected to immunoblot analysis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

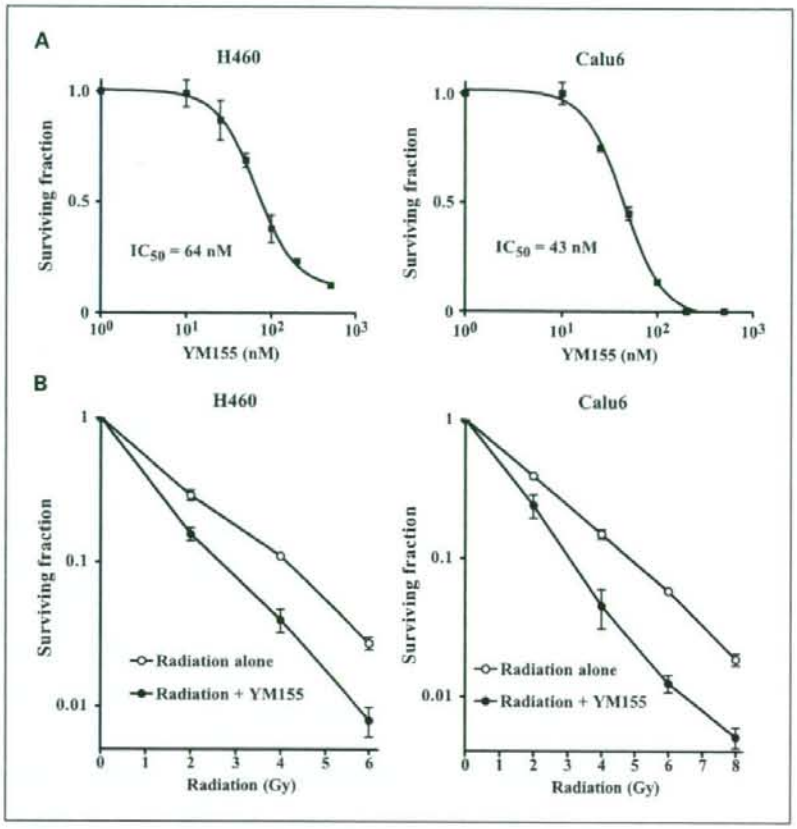

Fig. 2. Effect of YM155 on the sensitivity of H460 or Calu6 cells to γ-radiation. *A*, cells were incubated with the indicated concentrations of YM155 for 48 h and then assayed for clonogenic survival. Points represent means from three independent experiments; bars represent SD. *B*, cells were incubated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

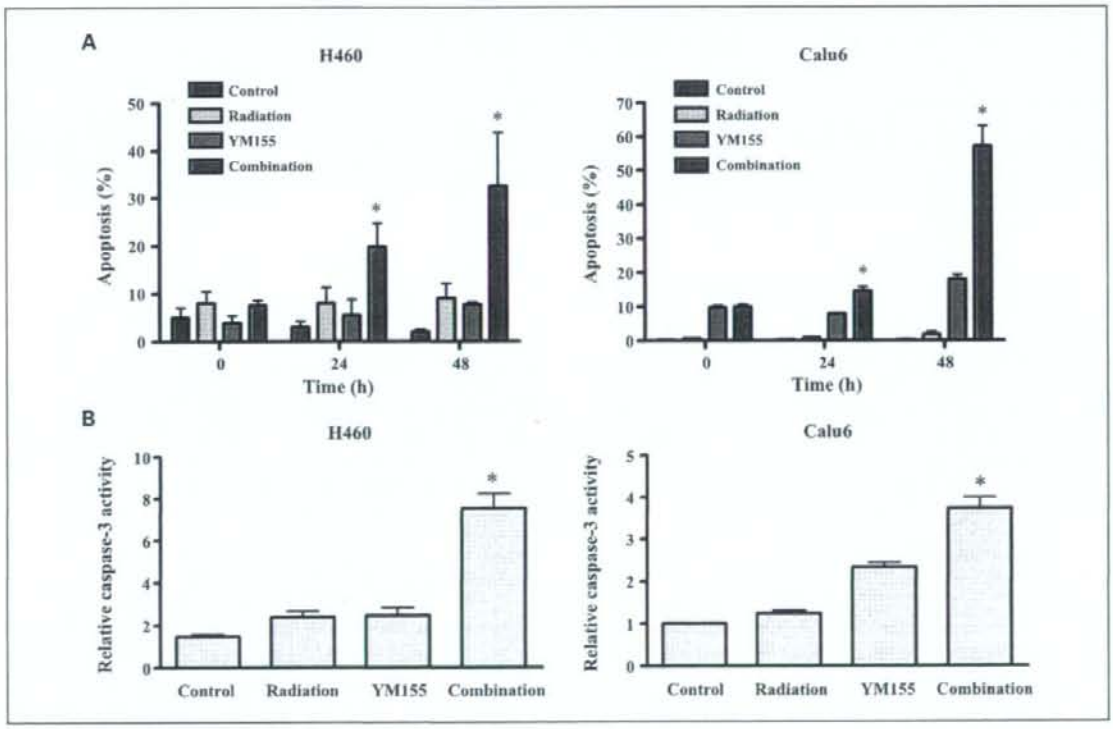

Fig. 3. Effect of YM155 on radiation-induced apoptosis and caspase-3 activity in H460 or Calu6 cells. *A*, cells were incubated with 50 nmol/L YM155 or vehicle (0.1% DMSO) for 48 h, exposed (or not) to 3 Gy of γ-radiation, and then incubated in drug-free medium for 24 or 48 h, at which times the percentage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