Allouache J, Goupil A, Négrier S, Viala J, Petrow P, Bouzy J, Laplanche A, Fizazi K (2003) Cisplatin in combination with either gemcitabine or irinotecan in carcinomas of unknown primary site: results of a randomized phase II study-trial for the French Study Group on Carcinomas of Unknown Primary (GEFCAPI 01). *J Clin Oncol* **21:** 3479–3482

Dowell JE, Garrett AM, Shyr Y, Johonson DH, Hande KR (2001) A randomized phase Ii trial in patients with carcinoma of an unknown primary site. *Cancer* **91:** 592–597

El-Rayes BF, Shields AF, Zalupski M, Heilbrun LK, Jain V, Terry D, Ferris AM, Philip PA (2005) A phase II study of carboplatin and paclitaxel in adenocarcinoma of unknown primary. *Am J Clin Oncol* **28:** 152–156

Greco FA, Burris III HA, Erland JB, Gray JR, Kalman LA, Schreeder MT, Hainsworth JD (2000b) Carcinoma of unknown primary site. *Cancer* **89:** 2655–2660

Greco FA, Burris III HA, Litchy S, Barton JH, Bradof JE, Richards P, Scullin Jr DC, Erland JB, Morrissey LH, Hainsworth JD (2002) Gemcitabine, carboplatin, and paclitaxel for patients with carcinoma of unknown primary site: a Minnie Pearl Cancer Research Network study. *J Clin Oncol* **20:** 1651–1656

Greco FA, Burris III HA, Spigel DR, Thompson DS, Waterhouse DM, Hanson S, Vazquez ER, Hainsworth JD (2008) Paclitaxel/carboplatin plus bevacizumab/erlotinib as first-line treatment for patients with carcinoma of unknown primary site. *J Clin Oncol* **26**(15S): 239s

Greco FA, Erland JB, Morrissey LH, Burris III HA, Hermann RC, Steis R, Thompson D, Gray J, Hainsworth JD (2000a) Carcinoma of unknown primary site: phase II trials with docetaxel plus cisplatin or carboplatin. *Ann Oncol* **11:** 211–215

Greco FA, Hainsworth JD (2008) Cancer of unknown primary site. In *Cancer, Principle and Practice of Oncology*, De Vita VT, Lawrence TS, Rosenberg SA (eds), pp 2363–2387. Lippincott Williams and Wilkins: Philadelphia

Greco FA, Rodriguez GI, Shaffer DW, Hermann R, Litchy S, Yardley DA, Burris III HA, Morrissey LH, Erland JB, Hainsworth JD (2004) Carcinoma of unknown primary site: sequential treatment with paclitaxel/carboplatin/etoposide and gemcitabine/irinotecan: a Minnie Pearl Cancer Research Network II phase trial. *Oncologist* **9:** 644–652

Gross-Goupil M, Fourcade A, Blot A, Penel N, Negrier S, Culine S, Moerrouche Y, Bouzy J, Laplanche A, Fizazi K (2008) A randomized trial of cisplatin with or without gemcitabine in patients with carcinoma of an unknown primary and without poor prognostic factors: results of the GEFCAPI02 trial. *Ann Oncol* **19**(Suppl 8): vii248

Guardiola E, Pivot X, Tchicknavorian X, Magne N, Otto J, Thyss A, Schnider M (2001) Combination of cisplatin–doxorubicin–cyclophosphamide in adenocarcinoma of unknown primary site: a phase II trial. *Am J Clin Oncol* **24:** 372–375

Hainsworth JD, Burris III HA, Calvert SW, Willcutt NT, Scullin Jr DC, Bramham J, Greco FA (2001) Gemcitabine in the second-line therapy of patients with carcinoma of unknown primary site: a II phase trial of the Minnie pearl cancer research network. *Cancer Invest* **19:** 335–339

Hainsworth JD, Spigel DR, Farley C, Thompson DS, Shipley DL, Greco FA (2007) Phase II trial of bevacizumab and erlotinib in carcinomas of unknown primary site: the Minnie pearl cancer research network. *J Clin Oncol* **25:** 1747–1752

Hainsworth JD, Spigel DR, Raefsky EL, Kuzur ME, Yost K, Kommor M, Litchy S, Greco FA (2005) Combination chemotherapy with gemcitabine and irinotecan in patients with previously treated carcinoma of unknown primary site: a Minnie pearl cancer research network phase II trial. *Cancer* **104:** 1992–1997

Kano Y, Akutsu M, Suzuki K, Yoshida M (1993) Effects of carboplatin in combination with other anticancer agents on human leukemia cell lines. *Leukemia Res* **17:** 113–119

Kano Y, Suzuki K, Akutsu M, Suda K, Inoue Y, Yoshida M, Sakamoto S, Miura Y (1992) Effects of CPT-11 in combination with other anticancer agents in culture. *Int J Cancer* **50:** 604–610

Macdonald AG, Nicolson MC, Samuel LM, Hutcheon AW, Ahmed FY (2002) A phase II study of mitomycin C, cisplatin and continuous infusion 5-Fluorouracil in the treatment of patients with carcinoma of unknown primary site. *Br J Cancer* **86:** 1238–1248

Massard C, Voigt JJ, Laplanche A, Culine S, Lortholary A, Bugat R, Theodore C, Priou F, Kaminsky MC, Lesimple T, Pivot X, Coudert B, Douillard JY, Merrouche Y, Fizazi K (2007) Carcinoma of an unknown primary: are EGF receptor, Her-2/neu, and c-Kit tyrosine kinases potential targets for therapy? *Br J Cancer* **97:** 857–861

Misawa T, Kikkawa F, Maeda O, Obata NH, Higashide K, Suganuma N, Tomoda Y (1995) Establishment and characterization of acquired resistance to platinum anticancer drugs in human ovarian carcinoma cells. *Jpn J Cancer Res* **86:** 88–94

Mukai H, Watanabe T, Ando M, Katsumata N (2003) Unknown primary carcinoma: a feasibility assessment of combination chemotherapy with cisplatin and docetaxel. *Int J Clin Oncol* **8:** 23–25

Palmeri S, Lorusso V, Palmeri L, Vaglica M, Porta C, Nortilli R, Ferraú F, Comella G, Massidda B, Danova M (2006) Cisplatin and gemcitabine with either vinorelbine or paclitaxel in the treatment of carcinomas of unknown primary site : results of an Italian multicenter, randomized, phase II study. *Cancer* **107:** 2898–2905

Park YH, Ryoo BY, Choi SJ, Yang SH, Kim HT (2004) A phase II study of paclitaxel plus cisplatin chemotherapy in an unfavourable group of patients with cancer of unknown primary site. *Jpn J Clin Oncol* **34:** 681–685

Parnis FX, Olver IN, Kotasek D, Norman J, Taylor A, Russell J, Patterson K, Keefe D, Marafioti T (2000) Phase II study of epirubicin, cisplatin and continuous infusion 5-fluorouracil for carcinoma of unknown primary site. *Ann Oncol* **11:** 883–884

Pavlidis N, Briasoulis E, Hainsworth J, Greco FA (2003) Diagnostic and therapeutic management of cancer of an unknown primary. *Eur J Cancer* **39:** 1990–2005

Pentheroudakis G, Briasoulis E, Kalofonos HP, Fountzilas G, Economopoulos T, Samelis G, Koutras A, Karina M, Xiros N, Samantas E, Bamias A, Pavlidis N (2008) Docetaxel and carboplatin combination chemotherapy as outpatient palliative therapy in carcinoma of unknown primary: a multicenter Hellenic cooperative oncology group phase II study. *Acta Oncologica* **47:** 1148–1155

Piga A, Nortilli R, Cetto GL, Cardarelli N, Luzi Fedeli S, Fiorentini G, D'Aprile M, Giorgi F, Parziale AP, Contu A, Montironi R, Gesuita R, Carle F, Cellerino R (2004) Carboplatin, doxorubicin and etoposide in the treatment of tumours of unknown primary site. *Br J Cancer* **90:** 1898–1904

Pittman KB, Olver IN, Koczwara B, Kotasek D, Patterson WK, Keefe DM, Karapetis CS, Parnis FX, Moldovan S, Yeend SJ, Price TJ (2006) Gemcitabine and carboplatin in carcinoma of unknown primary site: a phase 2 Adelaide Cancer Trials and Education Collaborative study. *Br J Cancer* **95:** 1309–1313

Saghatchian M, Fizazi K, Borel C, Ducreux M, Ruffie P, Le Chevalier T, Theodore C (2001) Carcinoma of an unknown primary site: a chemotherapy strategy based on histological diffentiation—results of a prospective study. *Ann Oncol* **12:** 535–540

Schneider BJ, El-Rayes B, Muler JH, Philip PA, Kalemkerian GP, Griffith KA, Zalupski MM (2007) Phase II trial of carboplatin, gemcitabine, and capecitabine in patients with carcinoma of unknown primary site. *Cancer* **110:** 770–775

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

van der Gaast A, Verweij J, Planting AS, Hop WC, Stoter G (1996) Simple prognostic model to predict survival in patients with undifferentiated carcinoma of unknown primary site. *J Clin Oncol* **13:** 1720–1725

Voog E, Merrouche Y, Trillet-Lenoir V, Lasset C, Peaud PY, Rebattu P, Negrier S (2000) Multicentric phase II study of cisplatin and etoposide in patients with metastatic carcinoma of unknown primary. *Am J Clin Oncol* **23:** 614–616

Yakushiji S, Ando M, Yonemori K, Kohno T, Shimizu C, Katsumata N, Fujiwara Y (2006) Cancer of unknown primary site: review of consecutive cases at the National Cancer Center Hospital of Japan. *Int J Clin Oncol* **11:** 421–425

Yonemori K, Katsumata N, Yamamoto N, Kasamatsu T, Yamada T, Tsunematsu R, Fujiwara Y (2005) A phase I study and pharmacologic evaluation of irinotecan and carboplatin for patients with advanced ovarian carcinoma who previously received platinum-containing chemotherapy. *Cancer* **104:** 1204–1212

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Bone metastasis and poor performance status are prognostic factors for survival of carcinoma of unknown primary site in patients treated with systematic chemotherapy

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Background: Cancer of unknown primary site (CUP) generally has a poor prognosis, and there is no established standard therapy. There have been no reports of a prognostic model for CUP patients treated with a single regimen of systemic chemotherapy.

Methods: Univariate and multivariate prognostic factor analysis for overall survival (OS) were conducted retrospectively in 58 consecutive CUP patients treated with carboplatin plus paclitaxel (Taxol) therapy as a first-line treatment.

Results: Univariate prognostic factor analysis revealed baseline performance status (PS) of two or more, low serum albumin level, pleural effusion, bone metastasis, and liver metastasis as adverse prognostic factors. Cox proportional hazards analysis showed that poor PS and bone metastasis had the most powerful adverse impact on survival. We developed a prognostic model using those two variables—a good-risk group (PS 0–1 without bone metastasis) and a poor-risk group (PS ≥2 or bone metastasis). The poor-risk group showed significantly poorer OS than the good-risk group (1 year OS 36.8% versus 67.1%, $P = 0.0003$).

Conclusions: Poor PS and bone metastasis were identified as independent adverse prognostic factors in CUP. A simple prognostic model was developed and seems useful for decision making as to whether chemotherapy is indicated for CUP patients.

Key words: cancer of unknown primary site, carboplatin plus paclitaxel, bone metastasis

introduction

Cancer of unknown primary site (CUP) is pathologically diagnosed metastatic carcinoma in which no obvious primary site is identified with a conventional work-up. It is not a rare clinical entity, accounting for 3%–5% of all solid malignancies [1, 2]. The prognosis of CUP is generally considered poor, with median survival ~6–12 months [3]. Briasoulis et al. [4] reported encouraging results from phase II data of carboplatin and paclitaxel combination therapy for patients with CUP. In this study, the overall response rate by an intention-to-treat analysis was 38.7%, and median overall survival (OS) was 13 months at median follow-up time of 28 months. Platinum and taxane combination therapy is now widely used in clinical practice [4–8], but recent multiple-treatment meta-analysis showed that no type of chemotherapy has been proven to prolong survival in patients with CUP [9]. CUP consists of heterogeneous neoplasms with variable biological features, making it difficult to identify clinically useful prognostic survival factors. But several subsets have been identified requiring a specific treatment and having a better prognosis. Women with peritoneal carcinomatosis of serous adenocarcinoma [10], women with adenocarcinoma of axillary lymph nodes [11] or cervical lymph node metastasis of squamous cell carcinoma [12], young adults with poorly differentiated carcinoma of midline distribution [13], and undifferentiated carcinoma with neuroendocrine features [14] are CUP subgroups known to have a better prognosis. But the majority of CUPs have a poor prognosis, as mentioned above. In this article, we report the results of a prognostic factor analysis conducted in a population of 58 patients of CUP treated with carboplatin and paclitaxel as a first-line systemic chemotherapy. We retrospectively investigated baseline characteristics as prognostic factors for survival to identify a subset of patients who would benefit from chemotherapy.

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methods

patient characteristics

The medical and pathological records of 58 consecutive newly diagnosed patients with CUP who received carboplatin and paclitaxel (Taxol, Bristol-Myers Squibb, Tokyo, Japan) combination therapy as first-line therapy at the Cancer Institute Hospital, Japanese Foundation for Cancer Research, from March 2004 to January 2008 were retrospectively reviewed. Patients had pathologically confirmed metastatic cancer and were surveyed for detailed medical history, complete physical examination, blood counts, chemistry profile, chest radiograph, computed tomography (CT) scan of chest and abdomen, and further radiological survey or endoscopy of suspected areas. Serum prostate-specific antigen (PSA) was measured in male patients, and CA 125 was measured in female patients. Women with adenocarcinoma of axillary lymph nodes also received mammography and breast ultrasound. Young adults with poorly differentiated adenocarcinoma involving the mediastinal region were surveyed with α-fetoprotein and β-human chorionic gonadotropin. The gastrointestinal tracts of male and female patients with adenocarcinoma involving abdominal and pelvic lesion were surveyed by upper gastrointestinal endoscopy and colonoscopy. Gynecologic examination was carried out in female patients with abdominal and pelvic disease. Patients with squamous cell carcinoma of cervical lymph nodes also underwent laryngeal endoscopy and upper gastrointestinal endoscopy. Bone metastases were assessed by the combination of bone scintigraphy or positron emission tomography with chest X-ray, CT, or magnetic resonance imaging. Histopathological review including immunohistochemistry (IHC) was carried out to detect primary sites and to exclude other malignancies. Low-molecular cytokeratins (CKs) 7 and 20 were routinely stained for all patients with CUP, and thyroid transcription factor 1, caudal type homeobox transcription factor 2, and PSA were stained for patients with adenocarcinoma of CUP. When a specific origin was suspected by morphological examination and clinical history, distinctive IHC was carried out (chromogranin, synaptophysin, and CD56 for neuroendocrine cell carcinoma; D2-40, placental alkaline phosphatase, human chorionic gonadotropin, and CD30 for germ-cell tumor; and D2-40 and calretinin for mesothelioma). In the case of difficulty in diagnosing epithelial carcinoma, several IHC of S100, vimentin, leukocyte common antigen, and CKs are used for distinguishing melanoma, sarcoma, and lymphoma from the anaplastic cell type of carcinoma.

We excluded patients in favorable subsets that have specific treatments other than carboplatin and paclitaxel—such as women with adenocarcinoma of axillary lymph nodes or cervical lymph node metastasis of squamous cell carcinoma, young adults with poorly differentiated carcinomas of midline distribution, and patients with undifferentiated carcinomas of neuroendocrine features. However, women with peritoneal carcinomatosis of adenocarcinoma who were treated with carboplatin and paclitaxel as first-line treatment were included in this study.

treatment

Carboplatin was administered by a 2-h i.v. infusion, dosed with 6 mg/ml/min target area under the free carboplatin plasma concentration versus time curve and was followed by paclitaxel 200 mg/m^2 in 500 ml of normal saline administered over 3 h. The Calvert formula was used for carboplatin dosing, on the basis of a glomerular filtration rate calculated by the Cockcroft–Gault equation using serum creatinine, body surface area, and age. Chemotherapy cycles were repeated every 3 weeks and responding patients continued the chemotherapy until disease progression or intolerable toxicity. Response to chemotherapy was assessed by Response Evaluation Criteria In Solid Tumors (RECIST, version 1.0). Progression-free survival (PFS) and OS were calculated from day 1 of the first cycle of chemotherapy.

statistical analysis

Survival curves were estimated using the Kaplan and Meier method, compared using the log-rank test, and prognostic factors were identified by univariate analysis. Then the forward stepwise Cox proportional hazards analysis was carried out to identify independent prognostic factors. Statistical analyses were carried out using SPSS software (version 17.0; SPSS Inc., Chicago, IL).

results

patient characteristics

Patient characteristics are shown in Table 1. Fifty-eight CUP patients treated with at least one cycle of carboplatin and paclitaxel combination therapy were retrospectively analyzed. Twenty-eight (48.3%) patients were male, and the median age was 64 years (range 28–79 years). Forty-nine patients (84.5%) had a good performance status (PS) of zero to one. Twenty-six (44.8%) patients had well-differentiated adenocarcinoma, 21 (36.2%) patients had anaplastic or poorly differentiated carcinoma, and 5 patients (8.6%) had squamous cell carcinoma. Another six (10.3%) patients had clear-cell carcinoma, transitional cell carcinoma, or adenosquamous cell carcinoma. Metastatic sites are listed in Table 1. Lymph nodes, lung, bone, and liver were frequently involved sites and cervical, mediastinum, and retroperitonium were common sites for lymph node metastasis.

PSA was measured in 20 male patients (median PSA level 2.04 ng/ml, range 0.34–4.04 ng/ml), and CA 125 was obtained in 26 female patients (median CA 125 level 462 U/ml, range 4.8–50000 U/ml). Five of six male patients with bone metastasis showed a PSA level <4.0 ng/ml, and the PSA value before treatment of one young male patient was not available.

outcome of chemotherapy

A total of 315 cycles were administered, and patients received a median of five cycles of treatment (range 1–21 cycles).

Table 1. Patient characteristics

Number of patients	58
Age, median (range)	64 (28–79)
Sex	
Male	28
Female	30
Performance status	
0–1	49
2–4	9
Pathology	
Adenocarcinoma	26
Squamous cell carcinoma	5
Poorly differentiated/anaplastic carcinoma	21
Other	6
Sites of metastasis	
Lung	15
Bone	13
Liver	11
Pleural effusion	15
Ascites	11
Lymph node	44

The response rates by main histopathological types of adenocarcinoma, squamous cell carcinoma, poorly differentiated carcinoma, or poorly differentiated adenocarcinoma were 42.3%, 60.0%, and 23.8%, respectively (Table 2). For other histology types, one patient with transitional cell carcinoma had partial response. Sixteen patients were treated with second-line chemotherapy. At a median follow-up time of 12 months (range 6–1659 days), median OS and PFS were 16.7 months and 5.9 months, respectively. Six patients had PFS >2 years and one of these patients survived >4 years.

prognostic model of clinical and biological variables

The outcome of univariate analysis of clinical and biological factors is listed in Table 3. Five parameters have prognostic relevance: poor PS (≥2) ($P = 0.01$), low serum albumin level (<3.7 g/dl) ($P = 0.003$), pleural effusion ($P = 0.04$), bone metastasis ($P = 0.02$), and liver metastasis ($P = 0.02$). Multivariate analysis for these five variables was conducted and showed that bone metastasis ($P = 0.002$) and PS of two or more ($P = 0.016$) had significant adverse impact for survival (Table 3). Poor PS was not correlated with presence of bone metastasis.

Table 2. Treatment results

	N	CR (*n*)	PR (*n*)	ORR (%)
Total	58	5	15	34.5
Pathology				
Adenocarcinoma	26	5	6	42.3
Squamous cell carcinoma	6	0	3	50.0
Poorly differentiated anaplastic carcinoma	21	0	5	23.8

CR, complete response; PR, partial response; ORR; overall response rate.

Table 3. Univariate and multivariate analysis of prognostic factors for survival

	Univariate	Multivariate	
	P value	HR (95% CI)	*P* value
PS ≥2	0.01	2.93 (1.22–7.04)	0.016
Age (>65 years)	0.29		
Sex (male)	0.41		
ALP (>UNL)	0.13		
LDH (>UNL)	0.45		
ALB (<3.7 g/dl)	0.003		
Hb (<11.0 g/dl)	0.77		
Pleural effusion	0.04		
Ascites	0.69		
Lung metastasis	0.58		
Bone metastasis	0.02	3.48 (1.56-7.78)	0.002
Liver metastasis	0.02		
Adenocarcinoma	0.81		
Poorly/anaplastic carcinoma	0.32		

HR, hazard ratio; CI, confidence interval; PS, performance status; ALP, alkaline phosphatase; UNL, upper normal limit; LDH, lactate dehydrogenase; ALB, albumin; Hb, hemoglobin.

The incidence of bone metastasis was not significantly different between males and females (6 of 28 males, 5 of 30 females). A prognostic model was developed with those two variables. Nineteen (32.8%) patients were assigned to the good-risk group (defined as PS 0–1 without bone metastasis), and 38 (67.2%) patients were assigned to the poor-risk group (defined as PS ≥2 or bone metastasis). The poor-risk group ($n = 19$) showed significantly poorer OS than good-risk group ($n = 39$) (1 year OS 36.8% versus 67.1%, $P = 0.0003$) (Figure 1).

discussion

To identify a favorable or poor prognostic group of patients with CUP is of great concern when physicians consider whether systemic chemotherapy is indicated. No randomized trial showed better survival with chemotherapy than best supportive care. To our knowledge, the current study is the first that assesses prognostic factors for survival of patients with CUP treated with a single first-line regimen and should give us information as we choose an optimal therapy.

We demonstrated an overall response rate of 34.5% and a median OS of 16.7 months in CUP patients by an intention-to-treat analysis. This result seems similar to the results previously reported by Briasoulis et al. [4] and slightly better than other reports. One reason might be that both studies included female patients with peritoneal carcinomatosis (11 of 58 in ours and 19 of 75 in Briasoulis et al.). In our study, seven women (63.6%) responded to chemotherapy. A second reason might be that our group included a marginally larger number of patients with good PS. Golfinopoulos et al. [9] reported in recent multiple-treatment meta-analysis for CUP that 10 randomized trials assessed in that study included variable rates for patients with poor PS (median 24.5%, interquartile range 12.8%–38.9%). Third, our study included a slightly smaller number of patients

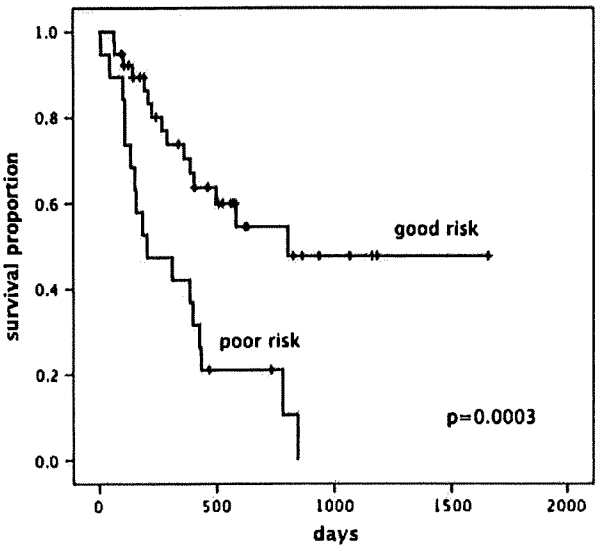

Figure 1. The prognostic model incorporating two variables. The good-risk group ($n = 39$) was defined as performance status (PS) of zero to one without bone metastasis and the poor-risk ($n = 19$) group as PS of two or more, or bone metastasis.

with liver metastasis, which was reported as an independent poor prognostic factor by Seve et al. [15]. But the rates of liver metastases in previous studies are variable from 16% to 76% [4–8, 16, 17]. The patients in the present study were treatable with combination therapy, so most of them maintained good PS and end organ function. No prospective studies or meta-analysis of prognostic factors for CUP have been published. But several retrospective studies have shown a number of independent adverse factors such as age, male gender, poor PS, adenocarcinoma histology, number of metastatic sites, liver metastasis, bone metastasis, lung metastasis, pleural metastasis, brain metastasis, comorbidity scoring of adult comorbidity evaluation-27 (ACE-27), low serum albumin, high serum lactate dehydrogenase (LDH), high serum alkaline phosphatase, lymphopenia, anemia, thrombocytopenia, high serum carcinoembryonic antigen, and high serum CA 125 [15, 18–20]. Abbruzzese et al. [18] reported adverse prognostic variables from a study of 657 cases of CUP at M. D. Anderson Cancer Center, and multivariate analysis identified male gender, a large number of metastatic sites, adenocarcinomatous histological type, and the presence of liver metastasis as unfavorable indicators. Culine et al. [19] proposed a simple prognostic model using PS and serum LDH levels in a population of 150 CUP patients, excluding favorable subsets, at a French cancer center. More recently, Seve et al. conducted a retrospective study assessing the influence of comorbidities, age, PS, and chemotherapy on survival in a population of 389 patients with CUP in Canada. Multivariate analysis showed that patients who had a PS of two or more and a high overall ACE-27 score had a poor prognosis. They concluded that the impact of comorbidity on survival was limited to patients with low PS [20]. The same author showed in another study that low serum albumin level and liver metastasis were the two most powerful adverse prognostic factors. The prognostic significance of those two factors was validated in another set of 124 patients with CUP [15]. In our study, bone metastases and poor PS (≥2) had a powerful adverse impact on survival. In clinical practice, bone metastases could be the cause of declining PS, but in this study, bone metastases and poor PS were not significantly correlated. Poor PS was also an adverse prognostic factor in studies by Culine et al. and by Seve et al. Bone metastases have been identified as an independent poor prognostic factor for the first time in uniformly treated patients with CUP. Prognostic significance of bone metastases in advanced cancer depends on the primary sites. In breast cancer or prostate cancer, the presence of bone metastases or bone-only metastases indicates a better prognosis [21]. On the other hand, the presence of bone metastases indicates a worse prognosis in lung cancer [22], thyroid cancer [23], or renal cell carcinoma [24]. The worse prognosis of patients with bone metastases in our series might be due to the apparent absence of occult breast cancer or prostate cancer in this set of patients.

Although our study might be small for finding independent prognostic factors retrospectively, it is important to identify clinically useful prognostic factors for CUP patients treated with platinum and taxane combination therapy, which are used frequently in daily practice. It has not been proven that systemic chemotherapy would prolong the survival of unfavorable CUP patients, and the best supportive care is a reasonable choice for patients who have little benefit from systemic chemotherapy.

We designed a new prognostic model that incorporated those two factors, poor PS and bone metastasis. The OS of patients with at least one or more prognostic factor was significantly shorter than those with no adverse prognostic factor. This model might be useful for decision making regarding the use of chemotherapy for CUP patients in daily clinical practice. A validation study of our prognostic model is warranted in the near future.

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disclosure

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references

1. Briasoulis E, Pavlidis N. Cancer of unknown primary origin. Oncologist 1997; 2: 142–152.
2. Van de Wouw AJ, Janssen-Heijnen ML, Coebergh JW et al. Epidemiology of unknown primary tumours; incidence and population-based survival of 1285 patients in Southeast Netherlands, 1984–1992. Eur J Cancer 2002; 38(3): 409–413.
3. Hainsworth JD, Greco FA. Treatment of patients with cancer of an unknown primary site. N Engl J Med 1993; 329: 257–263.
4. Briasoulis E, Kalofonos H, Bafaloukos D et al. Carboplatin plus paclitaxel in unknown primary carcinoma: a phase II Hellenic Cooperative Oncology Group Study. J Clin Oncol 2000; 18: 3101–3107.
5. Pentheroudakis G, Briasoulis E, Kalofonos HP et al. Docetaxel and carboplatin combination chemotherapy as outpatient palliative therapy in carcinoma of unknown primary: a multicentre Hellenic Cooperative Oncology Group phase II study. Acta Oncol 2008; 47: 1148–1155.
6. Berry W, Elkordy M, O'Rourke M et al. Results of a phase II study of weekly paclitaxel plus carboplatin in advanced carcinoma of unknown primary origin: reasonable regimen for community-based clinic? Cancer Invest 2007; 25: 27–31.
7. Hainsworth JD, Erland JB, Kalman LA et al. Carcinoma of unknown primary site: treatment with 1-hour paclitaxel, carboplatin, and extended schedule etoposide. J Clin Oncol 1997; 15: 2385–2393.
8. Huebner G, Link H, Kohne CH et al. Paclitaxel and carboplatin vs gemcitabine and vinorelbine in patients with adeno- or undifferentiated carcinoma of unknown primary: a randomized prospective phase II trial. Br J Cancer 2009; 100: 44–49.
9. Golfinopoulos V, Pentheroudakis G, Salanti G et al. Comparative survival with diverse chemotherapy regimens for cancer of unknown primary site: multiple-treatments meta-analysis. Cancer Treat Rev 2009; 35: 570–573.

10. Ransom DT, Patel SR, Keeney GL et al. Papillary serous carcinoma of the peritoneum. A review of 33 cases treated with platin-based chemotherapy. Cancer 1990; 66: 1091–1094.
11. Rosen PP. Axillary lymph node metastases in patients with occult noninvasive breast carcinoma. Cancer 1980; 46: 1298–1306.
12. Jesse RH, Perez CA, Fletcher GH. Cervical lymph node metastasis: unknown primary cancer. Cancer 1973; 31: 854–859.
13. van der Gaast A, Verweij J, Henzen-Logmans S et al. Carcinoma of unknown primary: identification of a treatable subset? Ann Oncol 1990; 1: 119–122.
14. Hainsworth JD, Johnson DH, Greco FA. Cisplatin-based combination chemotherapy in the treatment of poorly differentiated carcinoma and poorly differentiated adenocarcinoma of unknown primary site: results of a 12-year experience. J Clin Oncol 1992; 10: 912–922.
15. Seve P, Ray-Coquard I, Trillet-Lenoir V et al. Low serum albumin levels and liver metastasis are powerful prognostic markers for survival in patients with carcinomas of unknown primary site. Cancer 2006; 107: 2698–2705.
16. Palmeri S, Lorusso V, Palmeri L et al. Cisplatin and gemcitabine with vinorelbine or paclitaxel in the treatment of carcinomas of unknown primary site: results of an Italian multicenter, randomized, phase II study. Cancer 2006; 107: 2898–2905.
17. Pittman KB, Olver IN, Koczwara B et al. Gemcitabine and carboplatin in carcinoma of unknown primary site: a phase 2 Adelaide Cancer Trials and Education Collaborative study. Br J Cancer 2006; 95: 1309–1313.
18. Abbruzzese JL, Abbruzzese MC, Hess KR et al. Unknown primary carcinoma: natural history and prognostic factors in 657 consecutive patient. J Clin Oncol 1994; 12: 1272–1280.
19. Culine S, Kramar A, Saghatchian M et al. Development and validation of a prognostic model to predict the length of survival in patients with carcinomas of an unknown primary. J Clin Oncol 2002; 20: 4679–4683.
20. Seve P, Sawyer M, Hanson J et al. The influence of comorbidities, age, and performance status on the prognosis and treatment of patients with metastatic carcinomas of unknown primary site: a population-based study. Cancer 2006; 106: 2058–2066.
21. Coleman RE. Clinical features of metastatic bone disease and risk of skeletal morbidity. Clin Cancer Res 2006; 12: 6243–6249.
22. Herndon JE 2nd, Kornblith AB, Holland JC, Paskett ED. Patient education level as a predictor of survival in lung cancer clinical trials. J Clin Oncol 2008; 26: 4116–4123.
23. Shoup M, Stojadinovic A, Nissan A et al. Prognostic indicators of outcomes in patients with distant metastases from different thyroid carcinoma. J Am Coll Surg 2003; 197: 191–197.
24. Négrier S, Escudier B, Gomez F et al. Prognostic factors of survival and rapid progression in 782 patients with metastatic renal carcinomas treated by cytokines: a report from the Groupe Français d'Immunothérapie. Ann Oncol 2003; 13: 1460–1468.

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Meta-Analysis of Single Agent Chemotherapy Compared With Combination Chemotherapy as Second-Line Treatment of Advanced Non–Small-Cell Lung Cancer

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ABSTRACT

Purpose
Doublet chemotherapy is more effective than single-agent as first-line treatment of advanced non–small-cell lung cancer (NSCLC). As second-line treatment, several randomized trials have been performed comparing single-agent with doublet chemotherapy, but each trial had an insufficient power to detect potentially relevant differences in survival.

Methods
We performed meta-analysis of individual patient data from randomized trials, both published and unpublished, comparing single-agent with doublet chemotherapy as second-line treatment of advanced NSCLC. Primary end point was overall survival (OS). All statistical analyses were stratified by trial.

Results
Eight eligible trials were identified. Data of two trials were not available, and data of six trials (847 patients) were collected. Median age was 61 years. Performance status was 0 or 1 in 90%; 80% of patients had received previous platin-based chemotherapy. OS was not significantly different between arms ($P = .32$). Median OS was 37.3 and 34.7 weeks in the doublet and single-agent arms, respectively. Hazard ratio (HR) was 0.92 (95% CI, 0.79 to 1.08). Response rate was 15.1% with doublet and 7.3% with single-agent ($P = .0004$). Median progression-free survival was 14 weeks for doublet and 11.7 weeks for single agent ($P = .0009$; HR, 0.79; 95% CI, 0.68 to 0.91). There was no significant heterogeneity among trials for the three efficacy outcomes. Patients treated with doublet chemotherapy had significantly more grade 3 to 4 hematologic (41% *v* 25%; $P < .0001$) and grade 3 to 4 nonhematologic toxicity (28% *v* 22%; $P = .034$).

Conclusion
Doublet chemotherapy as second-line treatment of advanced NSCLC significantly increases response rate and progression-free survival, but is more toxic and does not improve overall survival compared to single-agent.

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INTRODUCTION

Cisplatin-based chemotherapy is considered standard of care worldwide for patients with advanced non–small-cell lung cancer (NSCLC).[1] At disease progression, many patients still have a good performance status (PS) and can be considered for further active treatment.

Until 2000, there was no evidence supporting the efficacy of second-line treatment, but in recent years the efficacy of several drugs in this setting has been demonstrated in phase III trials, and second-line treatment is now considered standard.[2,3]

In particular, second-line chemotherapy with docetaxel 75 mg/m^2 every 3 weeks has been proven to be effective in two phase III trials.[4,5] This treatment, indeed, prolongs overall survival (OS) compared with best supportive care, and improves some quality of life items like fatigue and pain.[4] In a individual patient data meta-analysis,[6] weekly docetaxel demonstrated similar efficacy, with a significantly lower risk of febrile neutropenia. Pemetrexed has been shown to be comparable with docetaxel, with a more favorable toxicity profile.[7] To date, no single-agent treatment has obtained better results than docetaxel.

A logical strategy for improving the efficacy of second-line treatment is to combine agents with different mechanism of action and toxicity. In first-line treatment, doublet chemotherapy is more effective

than single-agent, both in terms of objective response and OS.[8] Several randomized trials comparing a doublet with single-agent chemotherapy as second-line have been conducted in recent years.[9-15] Most of these trials were characterized by a small sample size, with inadequate statistical power to exclude potentially clinically relevant differences in efficacy.

The main objective of this meta-analysis, based on individual patient data, is to compare the efficacy of a doublet chemotherapy with single-agent treatment for the second-line treatment of advanced NSCLC, with a statistical power much higher than each trial. Data regarding activity and toxicity were also collected and analyzed.

PATIENTS AND METHODS

Identification of Eligible Trials

Published and unpublished studies were included in this meta-analysis, as previously recommended.[16]

The literature search was performed in July 2007, and updated in June 2008, to identify all randomized trials comparing single-agent and combination chemotherapy in second-line treatment of patients with advanced NSCLC. Trials evaluating a combination of a cytotoxic agent with a targeted drug, or a combination of targeted agents were not eligible. Search was performed using PubMed, EMBASE, Proceedings of American Society of Clinical Oncology, Proceedings of European Society of Medical Oncology, Proceedings of European Cancer Conference, Proceedings of World Conference on Lung Cancer, and the registry of the U.S. National Institutes of Health clinicaltrials.gov from 1997 to 2008, with the following key-words: "lung cancer", "NSCLC"; "second-line", "randomized/randomized". References of the identified articles were checked, and principal investigators were asked whether they were aware of other published or unpublished trials.

Study Quality

Each study was assessed for quality and potential bias using a structured checklist based on the Method for Evaluating Research and Guideline Evidence criteria.[17] Study characteristics were quality of randomization, blinding, outcome measures, measure assessment, arm comparability, loss to follow-up, and intention to treat analysis. An overall quality score was assigned to each

Table 1. Characteristics of the Six Randomized Trials Included in the Meta-Analysis

Parameter	Study					
	Takeda et al[9]	Georgoulias et al[10]	Georgoulias et al[11]	Wachters et al[12]	Gebbia et al[14]	Smit et al[15]
Phase of the study	III	II*	II*	II	III	II*
Treatment dose and schedule						
Single-agent arm	Docetaxel 60 mg/m^2 day 1 every 3 weeks	Irinotecan 300 mg/m^2 day 1 every 3 weeks	Cisplatin 80 mg/m^2 day 1 every 3 weeks	Docetaxel 75 mg/m^2 day 1 every 3 weeks	Docetaxel 33.3 mg/m^2 days 1, 8, 15 every 4 weeks	Pemetrexed 500 mg/m^2 day 1 every 3 weeks
Combination arm	Docetaxel 60 mg/m^2 day 8 + gemcitabine 800 mg/m^2 days 1 and 8 every 3 weeks	Gemcitabine 1,000 mg/m^2 days 1 and 8 + irinotecan 300 mg/m^2 day 8 every 3 weeks	Cisplatin 80 mg/m^2 day 8 + irinotecan 110 mg/m^2 day 1, 100 mg/m^2 day 8, every 3 weeks	Docetaxel 60 mg/m^2 day 1 + irinotecan 200 mg/m^2 day 1 every 3 weeks	Docetaxel 30 mg/m^2 days 1, 8, 15 every 4 weeks + gemcitabine 800 mg/m^2 days 1 and 8 every 4 weeks or vinorelbine 20 mg/m^2 days 1 and 8 every 4 weeks or capecitabine 1,300 mg/m^2 days 5 to 18 every 4 weeks	Pemetrexed 500 mg/m^2 day 1 every 3 weeks + carboplatin AUC5 day 1 every 3 weeks
Primary end point	Overall survival	Overall survival	Overall survival	Response rate	Overall survival	Time to progression
Planned sample size	284	144	130	106	375	240
Actual sample size	130	147	139	108	84	240
Start of the accrual	January 2002	September 1999	July 1999	October 2000	May 2005	October 2005
End of the accrual	April 2003	December 2001	November 2002	January 2003	December 2006	May 2007
Median follow-up, weeks	90.4	59.4	91.6	74.3	70.7	64.0
Trial quality (MERGE criteria)	B1	B1	B1	B1	B1	B1
Eligibility criteria						
Age	20-75	≥ 18	≥ 18	≥ 18	18-75	≥ 18
Performance status	ECOG 0-1	WHO 0-2	WHO 0-2	ECOG 0-2	ECOG 0-2	ECOG 0-2
Previous lines of chemotherapy	1	1-2	1-2	1	1	≥ 1
Previous treatment	Platin based	Platin based	Taxane + gemcitabine	Platin- or nonplatin-based	Platin based	Relapse > 3 months after platin based

Abbreviations: AUC5, area under the time concentration curve 5; MERGE, Method for Evaluating Research and Guideline Evidence; ECOG, Eastern Cooperative Oncology Group.
*Defined randomized phase II, sample size was actually calculated according to phase III design, with formal comparison between treatment arms.

study: A (low risk of bias), B1 (low to moderate risk of bias), B2 (moderate to high risk of bias), C (high risk of bias).

Before performing the analyses, data of each published study were carefully checked and verified for coherence with the original publications; database quality was excellent for all studies.

Statistical Methods

All the analyses were performed according to the intention-to-treat principle. All the analyses were stratified by trial. All tests were two sided.

Primary end point was OS, defined as the time between date of random assignment and date of death, or last date of follow-up for censored patients. OS curves were estimated using the Kaplan-Meier technique and compared using the stratified log-rank test. Median follow-up was calculated according to the inverted Kaplan-Meier technique.[18]

Because meta-analysis was based on individual patient data, heterogeneity of treatment effect among trials on OS was assessed by likelihood ratio of two trial-stratified models, one with trial-specific treatment estimates and one with overall treatment estimate, as suggested by Smith et al.[19] Under the null hypothesis of no heterogeneity, this statistic follows approximately a χ^2 distribution on $J - 1$ df (where J is the total number of trials).[19]

Findings of the meta-analysis are depicted in classical Forest plots, with point estimates and 95% CIs for each trial and overall; size of the squares is proportional to study size.

Further exploratory analyses were performed in the subgroups based on the main baseline patients characteristics, to describe possible heterogeneity of treatment effect. Interaction test was also performed.

Secondary end points were progression-free survival (PFS), objective response rate (RR), and toxicities.

PFS was defined as the time between date of random assignment and date of progression, or date of death for patients dead without progression, or last date of follow-up for censored patients. PFS was analyzed likewise OS.

RR was compared using the stratified Mantel-Haenszel χ^2 test for combining 2 × 2 tables and the Breslow-Day test was used to detect differences in treatment effect among the trials.[20] For RR, patients obtaining complete response or partial response were considered as responders, and all others as nonresponders.

Toxicity variables were dichotomized as severe (grade 3 to 4) and no/mild (grades 0 to 2). Toxicity rates were compared using the stratified exact tests; the Zelen exact test was used to detect differences in toxicity effects among the six trials[20] and the pooled odds ratio (OR) with 95% CI was estimated by means of exact method.

Statistical analyses were performed using SAS 8.2 (SAS Institute, Cary, NC) and graphs using R 2.4.1 (R Foundation for Statistical Computing, Vienna, Austria) software packages. Exact tests were performed using StatXact 7 (Cytel Software, Cambridge, MA).

RESULTS

Characteristics of the Trials

Eight trials were eligible, for a total of 1,372 patients: three trials were conducted in Greece,[10,11,13] two in the Netherlands,[12,15] one in Japan,[9] one in Italy,[14] and one in Canada, the United States, and Poland (GlaxoSmithKline, data on file, courtesy of P. Legenne). As of February 2009, six trials have already been published as full-length articles[9-14]; one was presented at the 2008 Annual Meeting of the American Society of Clinical Oncology,[15] and one is still unpublished.

Individual patient data from one trial,[13] despite the efforts of the principal investigator who moved to another institution, were not available. We also did not obtain individual patient data from the

Table 2. Characteristics of the Patients Analyzed (N = 847)

Characteristic	Single Agent (n = 428)		Combination (n = 419)		Total (N = 847)	
	No.	%	No.	%	No.	%
Median age, years	61		60		61	
Range	34-78		34-84		34-84	
Sex						
Male	326	76	324	77	650	77
Female	102	24	95	23	197	23
Histologic type						
Adenocarcinoma	204	48	182	43	386	46
Squamous	124	29	132	32	256	30
Large cell	49	11	60	14	109	13
Non-small cell unspecified	30	7	31	7	61	7
Other	2	1	—		2	< 1
Unknown	19	6	14	3	33	4
Performance status						
0	130	30	128	31	258	30
1	250	58	253	60	503	59
2	48	11	38	9	86	10
Previous chemotherapy						
Cisplatin	240	56	239	57	479	57
Carboplatin	103	24	106	25	209	25
Platin based	342	80	332	79	674	80
Docetaxel	143	33	156	37	299	35
Paclitaxel	22	5	25	6	47	6
Gemcitabine	253	59	200	48	453	53
Vinorelbine	29	7	38	9	67	8
Response to first line						
Responders	210	49	203	48	413	49
Non-responders	213	50	212	51	425	50
Unknown	5	1	4	1	9	1

unpublished SKF104864-615 trial (unpublished results), although GlaxoSmithKline, sponsor of the trial, kindly provided us with a summary of final results. Eventually individual data were obtained from six trials, accounting for 63% of the potentially eligible patients (863 of 1,372). Main characteristics of the six trials are described in Table 1. Four were randomized phase II trials,[10-12,15] but in three of these[10-11,15] sample size was actually calculated according to a classical phase III design, with a formally planned comparison between treatment arms. The Japanese trial[9] was terminated early, because of high incidence of interstitial lung disease (ILD) and three treatment-related deaths (5%) due to ILD in the combination arm. In the Italian trial,[14] patients were randomly assigned in a 3:1:1 ratio to three arms: A, docetaxel; B, docetaxel plus gemcitabine or plus vinorelbine; C, docetaxel plus capecitabine. For this meta-analysis, arms B and C were grouped. The trial was stopped prematurely, blind to results, because the recruitment rate was extremely slower than expected.

Single agent consisted of docetaxel (three trials), irinotecan (one trial), cisplatin (one trial), pemetrexed (one trial). In all trials, patients assigned to combination chemotherapy were treated with the addition of a second drug to the one administered as single-agent. Except Nederlandse Vereniging van Artsen voor Longziekten en Tuberculose trial 7,[15] which evaluated pemetrexed plus carboplatin in patients already treated with platin-based therapy, all the trials evaluated drugs not received by the patients as first-line treatment.

Methodological Quality of the Trials

In all studies, most evaluation criteria from the checklist are fulfilled with overall quality score B1 (Table 1; ie, all included studies were of sufficiently high quality to consider the risk of bias as low to moderate). The main drawback of all studies was the lack of blinding, which is a common practice in clinical trials in advanced cancer because of difficulties of blinding to different infusion times, schedules, and toxicities. Lack of blinding is unlikely to affect OS, but could potentially bias secondary end points (PFS, RR, toxicity). All studies had a time-to-event primary outcome, with the exception of Wachters et al study,[12] that had RR as a primary end point. Definitions of the primary outcome were detailed in all trials. Allocation concealment was always adequate. Treatment groups were balanced for the most relevant baseline characteristics (age, PS, stage, histology, response to first-line treatment), although these characteristics were not always considered as stratification factors. For all trials, complete data were available for intention-to-treat analysis, and reasons for the exclusion of a few patients were carefully accounted for and are described later. In particular, a very small percentage of patients were completely lost to follow-up after random assignment in two trials.[10,11]

Main Results

Overall, 847 of the 863 originally randomly assigned patients were eligible for the meta-analysis (Appendix Fig A1, online only). Fifteen patients were excluded from two trials[10,11] because of complete absence of information in the study database. One patient was excluded because of ineligible histology (small-cell lung cancer). Of the 847 eligible patients, 428 patients (50.5%) had been assigned to a single agent, and 419 were assigned to doublet (49.5%). Main characteristics of the 847 patients are described in Table 2. Median age was 61 years (range, 34 to 84). Most of the patients were males (77%), had a good PS (0 or 1 in 90%), and had previously received a first-line platin-based treatment (80%).

Median follow-up was 74.0 weeks (71.7 weeks in single-agent arm, and 74.4 weeks in combination arm). Overall survival curves of patients according to treatment arms are shown in Figure 1. Overall, 642 deaths were recorded (76%), with median survival equal to 37.3 weeks for doublet, and 34.7 weeks for single agent. Corresponding hazard ratio (HR) was 0.92 (95% CI, 0.79 to 1.08; $P = .32$ at log-rank test stratified by trial). The 6-month survival rates were 62.9% and 61.6%, and the 1-year survival rates were 34.4% and 31.8%, for combination and single agent chemotherapy, respectively. As shown in Figure 2, there was no evidence of heterogeneity among the six trials ($P = .87$; I^2 0%). Exploratory survival analysis by subgroups is shown in Figure 3; there was no evidence of heterogeneity among subgroups of treatment effect around the overall effect.

PFS curves of patients according to treatment assigned are shown in Figure 1. Overall, 805 progressions were recorded (95%), with median PFS equal to 14.0 weeks and 11.7 weeks for doublet and single-agent chemotherapy, respectively. The corresponding HR was 0.79 (95% CI, 0.68 to 0.91; $P = .0009$ at log-rank test stratified by trial). Six-month PFS rates were 27.2% and 18.1%, and the 1-year PFS rates were 6.5% and 5.5% for patients assigned to combination and single-agent chemotherapy, respectively. Forest plot of treatment effect on

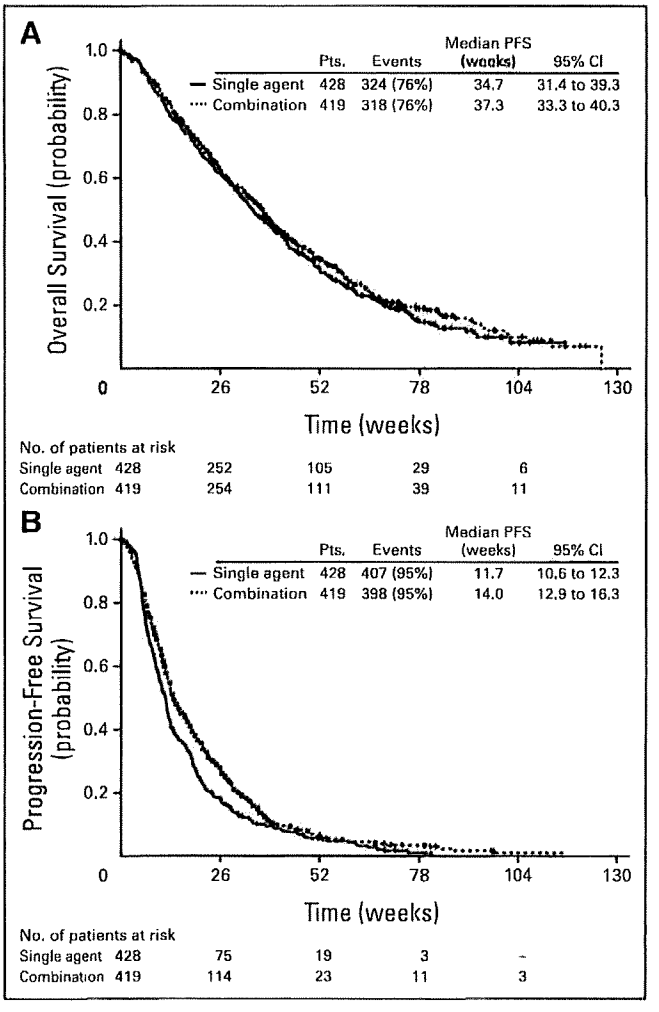

Fig 1. (A) Overall survival (OS) and (B) progression-free survival (PFS) curves by treatment arm. Pts, patients.

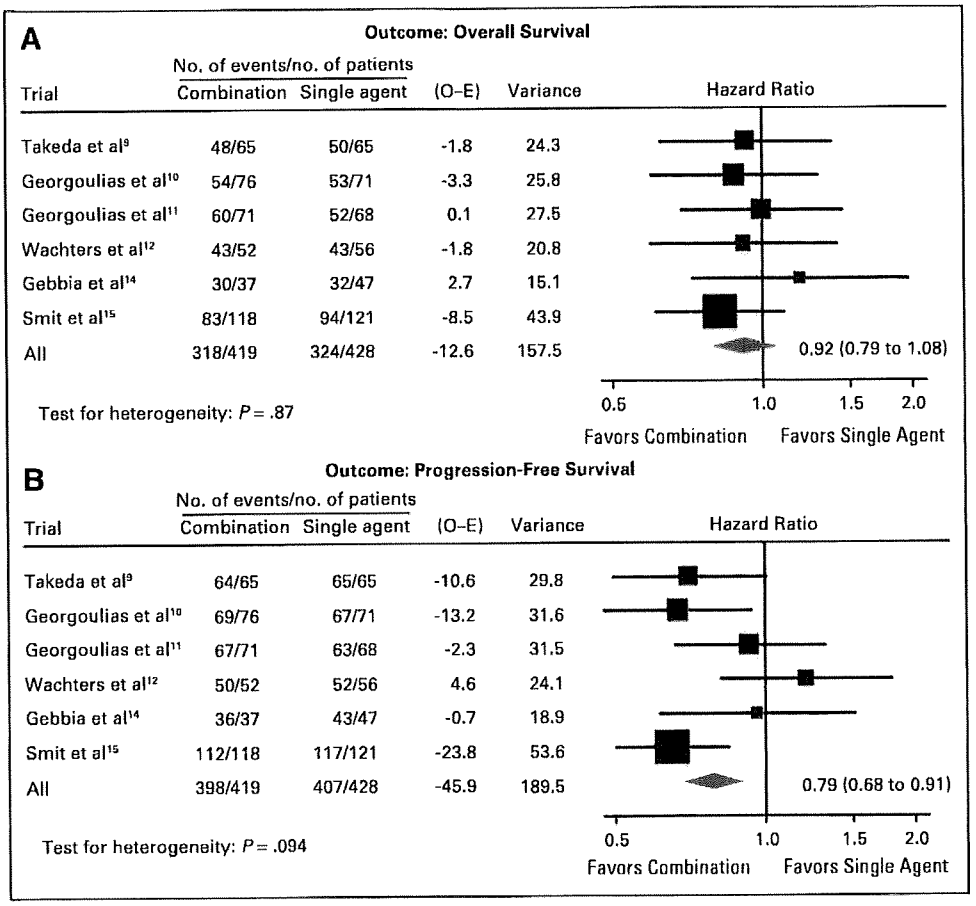

Fig 2. Forest plot of treatment effect on (A) overall survival and (B) progression-free survival. (O–E), observed events minus expected events.

PFS is shown in Figure 2. There was no statistically significant heterogeneity among the six trials ($P = .094$; I^2 47%) and among subgroups (Fig 3).

Further posthoc analyses were performed to study interaction between trial characteristics and treatment effect (Appendix Table A1, online only). Trials were classified according to the single-agent drug (docetaxel[9,12,14] *v* other drug[10,11,15]), or according to the dose planned in doublet arm (same dose of the single-agent drug[9-11,15] *v* reduced dose[12,14]). Interaction tests for OS were not significant. PFS was significantly longer with doublet only when single-agent drug was used at the same dose in the doublet arm.

Objective RR was increased with doublet: 7.3% versus 15.1%, with single-agent and doublet, respectively ($P = .0004$). The test for heterogeneity was borderline significant ($P = .06$; I^2 50%). Exact odds ratio of RR was 2.24 (95% CI, 1.43 to 3.53; Appendix Fig A2, online only).

A summary of grade 3 to 4 adverse effects is reported in Table 3. Combination chemotherapy is characterized by a significantly higher incidence of neutropenia, anemia, thrombocytopenia, emesis, and diarrhea. Patients treated with doublet had significantly more grade 3 to 4 hematologic (25% *v* 41%; $P < .0001$) and grade 3 to 4 nonhematologic toxicity (22% *v* 28%; $P = .034$). Heterogeneity among studies was found for some adverse effects, possibly due to the different drugs and doses used.

DISCUSSION

This individual patient data meta-analysis shows no significant difference in OS between doublet and single-agent chemotherapy as second-line treatment of patients with advanced NSCLC. This meta-analysis, with 847 patients and 642 events, has a statistical power of 80% of recognizing a HR of 0.8 for combination chemotherapy. Our data show that doublets determine a statistically significant increase in RR and in PFS. This increase in activity does not translate in increase in OS compared with single-agent treatment. This appears to be coherent with the results described in the different setting of first-line chemotherapy of advanced NSCLC, where much larger differences in RR and in time to progression are needed to predict a significant survival benefit.[21]

Systematic reviews and meta-analyses have been increasingly used in recent years, as a precious instrument of assessing and interpreting the results from different clinical trials conducted on the same topic. The object of this meta-analysis represents a good topic for this approach because limited sample size of each trial did not allow adequate power to detect potentially clinically relevant differences in efficacy between the two strategies. Four trials were phase II randomized trials,[10-12,15] although three of these[10-11,15] were designed as classical phase III trials. Classically, phase II randomized trials should not be planned to formally compare the treatments, but

Zoledronic acid-induced regression of multiple metastases at nonskeletal sites

Bisphosphonates have been shown to reduce skeletal complications in individuals with bone metastases secondary to a wide range of solid tumors including lung, breast, and prostate cancer [1]. They are widely administered as palliative agents together with chemotherapy, hormonal therapy, or irradiation [2]. In addition, several types of cancer cells including hematologic malignancies respond to bisphosphonates *in vitro*, with such effects having been attributed, at least in part, to inhibition of the Ras-signaling pathway [3]. We now report an unusual case in which tumors in visceral organs and soft tissues responded markedly to treatment with the bisphosphonate zoledronic acid (ZA) alone, with the performance status of the patient improving in the absence of chemotherapy.

A 69-year-old man with no significant past medical history presented with pain in his left hip joint at our hospital in January 2007. X-ray examination revealed an osteosynthetic change in his left femur, and a metastatic tumor in his left thigh bone was indicated. Positron emission tomography with 2-[fluorine-18]fluoro-2-deoxy-D-glucose (FDG–PET) revealed uptake of the tracer at multiple sites including the left femur, right rib, left scapula, bilateral adrenal glands, s.c. tissue of the right gluteal region, and abdominal lymph nodes (Figure 1A, left panel). Concomitant computed tomography (CT) revealed tumors of various sizes at multiple sites corresponding to those of tracer uptake, with enlargement of the left adrenal glands apparent from a diameter of 50.2 mm. Biopsy specimens were obtained from the putative tumor on the left femur and the s.c. nodule of the right buttock. On the basis of the morphological and immunohistochemical staining characteristics of the specimens, a histopathologic diagnosis of spindle cell carcinoma was made, but the primary site of the tumor was not determined. The patient's general condition was poor, and he had an Eastern Cooperative Oncology Group performance status of three at the time of diagnosis. Systemic chemotherapy was therefore not selected, and palliative care was commenced. Together with prescription of narcotics and 20 Gy of radiation

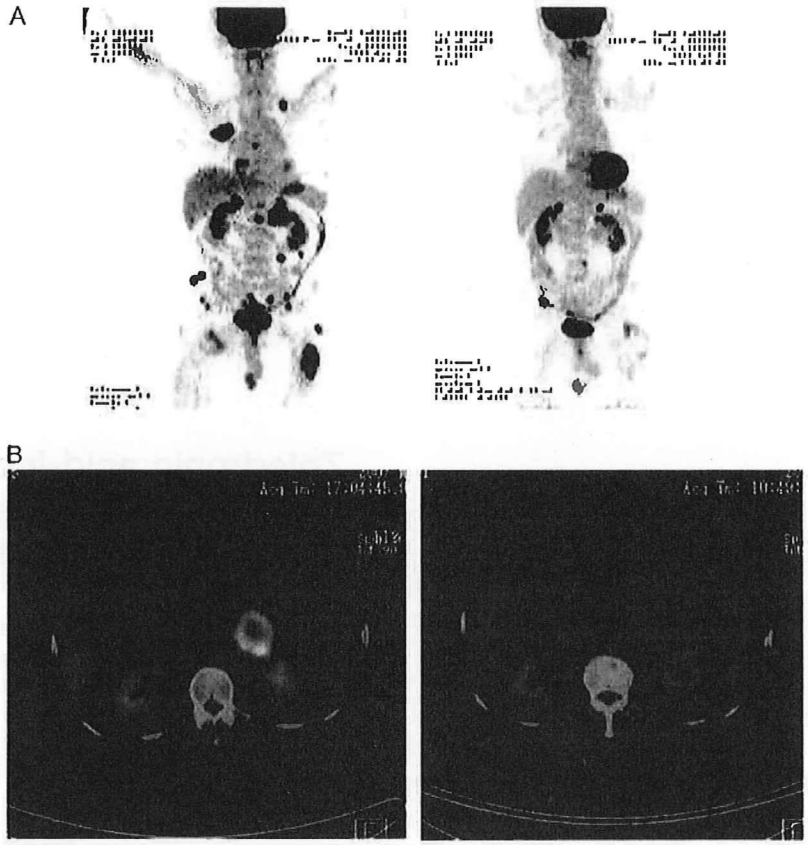

Figure 1. Positron emission tomographies with 2-[fluorine-18]fluoro-2-deoxy-D-glucose before (A, left panel) and after (A, right panel) zoledronic acid treatment, and the corresponding computed tomographies before (B, left panel) and after zoledronic acid treatment (B, right panel) are shown.

to the left femur, ZA (4 mg/body) was administered i.v. every 4 weeks to reduce bone pain. After 6 months, his general condition was dramatically improved and follow-up FDG–PET revealed decreased uptake of the tracer in metastases not only in bone including the right rib, which was not irradiated, but also in the adrenal glands, abdominal lymph nodes, and s.c. tissue of the right buttock (Figure 1A, right panel). Unexpectedly, CT revealed that tumors in the adrenal glands had shrunk markedly, with the diameter of the left gland having decreased from 50.2 mm (Figure 1B, left panel) to 26.4 mm (Figure 1B, right panel), and the s.c. tumor in the right buttock was no longer detectable.

As far as we are aware, there have been no other reports of a tumor at a nonskeletal site responding to bisphosphonate treatment alone. ZA was recently shown to have efficacy as a preventive agent for cancer recurrence in premenopausal women with early-stage breast cancer [4]. This previous study suggested that ZA was able to prevent cancer recurrence not only in bone but also in nonskeletal organs including the contralateral breast, lung, and liver. The present case supports the notion that ZA targets not only osteoclasts, a major contributor to the tumor microenvironment in bone, but also tumor cells themselves, as has been shown in preclinical studies [3]. Further clinical evaluation of ZA for treatment of cancer is warranted.

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references

1. Rosen LS, Gordon D, Tchekmedyian NS et al. Long-term efficacy and safety of zoledronic acid in the treatment of skeletal metastases in patients with nonsmall cell lung carcinoma and other solid tumors: a randomized, phase III, double-blind, placebo-controlled trial. Cancer 2004; 100: 2613–2621.
2. Aapro M, Abrahamsson PA, Body JJ et al. Guidance on the use of bisphosphonates in solid tumours: recommendations of an international expert panel. Ann Oncol 2008; 19: 420–432.
3. Tassone P, Tagliaferri P, Viscomi C et al. Zoledronic acid induces antiproliferative and apoptotic effects in human pancreatic cancer cells *in vitro*. Br J Cancer 2003; 88: 1971–1978.
4. Gnant M, Mlineritsch B, Luschin-Ebengreuth G et al. Adjuvant endocrine therapy plus zoledronic acid in premenopausal women with early-stage breast cancer: 5-year follow-up of the ABCSG-12 bone-mineral density substudy. Lancet Oncol 2008; 9: 840–849.

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Sorafenib Inhibits Non–Small Cell Lung Cancer Cell Growth by Targeting B-RAF in *KRAS* Wild-Type Cells and C-RAF in *KRAS* Mutant Cells

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Abstract

Sorafenib is a multikinase inhibitor whose targets include B-RAF and C-RAF, both of which function in the extracellular signal-regulated kinase (ERK) signaling pathway but which also have distinct downstream targets. The relative effects of sorafenib on B-RAF and C-RAF signaling in tumor cells remain unclear, however. We have now examined the effects of sorafenib as well as of B-RAF or C-RAF depletion by RNA interference on cell growth and ERK signaling in non–small cell lung cancer (NSCLC) cell lines with or without *KRAS* mutations. Sorafenib inhibited ERK phosphorylation in cells with wild-type *KRAS* but not in those with mutant *KRAS*. Despite this difference, sorafenib inhibited cell growth and induced G_1 arrest in both cell types. Depletion of B-RAF, but not that of C-RAF, inhibited ERK phosphorylation as well as suppressed cell growth and induced G_1 arrest in cells with wild-type *KRAS*. In contrast, depletion of C-RAF inhibited cell growth and induced G_1 arrest, without affecting ERK phosphorylation, in cells with mutant *KRAS*; depletion of B-RAF did not induce G_1 arrest in these cells. These data suggest that B-RAF-ERK signaling and C-RAF signaling play the dominant roles in regulation of cell growth in NSCLC cells with wild-type or mutant *KRAS*, respectively. The G_1 arrest induced by either C-RAF depletion or sorafenib in cells with mutant *KRAS* was associated with down-regulation of cyclin E. Our results thus suggest that sorafenib inhibits NSCLC cell growth by targeting B-RAF in cells with wild-type *KRAS* and C-RAF in those with mutant *KRAS*. [Cancer Res 2009;69(16):6515–21]

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). Treatment options are limited for patients with advanced metastatic lung cancer, with traditional cytotoxic chemotherapy conferring only a limited survival benefit. Target-based therapies are therefore being pursued as potential treatment alternatives. The RAS-RAF-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase-ERK signaling pathway is a promising therapeutic target given its central role in regulation of mammalian cell proliferation, relaying extracellular signals from ligand-bound receptor tyrosine kinases (RTK) at the cell surface to the nucleus via a cascade of specific phosphorylation events and beginning with the activation of the small GTPase RAS (2). Much attention is thus being focused on the development of inhibitors of this pathway.

RAF was the first effector kinase downstream of RAS to be identified (3). To date, the most successful clinical inhibitor of RAF activity is sorafenib (Nexavar, BAY 43-9006), an orally available compound that has received approval by the U.S. Food and Drug Administration for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma. Sorafenib is also currently undergoing clinical evaluation for a variety of additional cancers, including non–small cell lung cancer (NSCLC; refs. 4–7).

The mutational status of *RAS* and *B-RAF* genes is thought to affect the sensitivity of tumor cell lines to sorafenib as a result of the inappropriate activation by such mutations of the MAPK pathway mediated by ERK (8, 9). The sensitivity of tumor cell lines with different *RAS* mutations to sorafenib is less well characterized than is that of those with *B-RAF* mutations (10–14). Despite promising results of clinical trials of sorafenib monotherapy in NSCLC patients (4–7), little is known of the possible differences in the sorafenib sensitivity of NSCLC cells according to the mutational status of *KRAS*. We have therefore now examined the effects of RAF inhibition on the growth of NSCLC cells with or without *KRAS* mutations and further investigated the mechanisms of such effects.

Materials and Methods

Cell culture and reagents. The human NSCLC cell lines NCI-H292 (H292), LK-2, Sq-1, NCI-H520 (H520), PC9, NCI-H1650 (H1650), HCC827, NCI-H1975 (H1975), A549, NCI-H460 (H460), NCI-H23 (H23), NCI-H358 (H358), and NCI-H1299 (H1299) were obtained from the American Type Culture Collection. Ma70 cells were obtained as previously described (15). All cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. Sorafenib was kindly provided by Bayer Pharmaceutical, dissolved in DMSO, and stored in aliquots at −20°C.

Assay of anchorage-dependent cell growth [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay]. Cells were plated in 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of sorafenib for 72 h. TetraColor One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of sorafenib resulting in 50% growth inhibition (IC_{50}) was calculated.

Assay of anchorage-independent colony formation in soft agar. Anchorage-independent cell proliferation in soft agar was assayed with the use of a CytoSelect 96-Well Cell Transformation Assay (Cell Biolabs). In brief, cells were cultured for 7 d in complete medium containing soft agar and various concentrations of sorafenib. The agar matrix was then solubilized, the cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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bromide (MTT) and lysed, and the absorbance at 570 nm was measured relative to that at a reference wavelength of 690 nm. Normalized absorbance values were expressed as a percentage of that for untreated cells, and the IC_{50} of sorafenib for inhibition of colony formation was calculated.

Cell cycle analysis. Cells were harvested, washed with PBS, fixed with 70% methanol, washed again with PBS, and stained with propidium iodide (0.05 mg/mL) in a solution containing 0.1% Triton X-100, 0.1 mmol/L EDTA, and RNase A (0.05 mg/mL). The stained cells ($\sim 1 \times 10^6$) were then analyzed for DNA content with a flow cytometer (FACSCalibur, Becton Dickinson) and ModFit software (Verity Software House).

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 pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin. The protein concentration of lysates was determined with the Bradford reagent (Bio-Rad), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% 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 phosphorylated ERK (1:1,000 dilution; Santa Cruz Biotechnology), ERK (1:1,000 dilution; Santa Cruz Biotechnology), FLAG epitope (1:1,000 dilution; Cell Signaling Technology), B-RAF (1:1,000 dilution; Santa Cruz Biotechnology), C-RAF (1:1,000 dilution; Cell Signaling Technology), or β-actin (1:500 dilution; Sigma) or with mouse monoclonal antibodies to cyclin E (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) immunoglobulin G. Immune complexes were finally detected with chemiluminescence reagents (Perkin-Elmer Life Science).

Forced expression of KRAS-V12. An expression vector for FLAG-tagged human KRAS-V12 was constructed by inserting the corresponding cDNA into the pcDNA3 plasmid (Invitrogen). The expression vector was introduced into H1299 cells by transfection for 48 h with the use of the Lipofectamine 2000 reagent (Invitrogen).

Gene silencing. Cells were plated at 50% to 60% confluence in six-well plates or 25-cm^2 flasks and then incubated for 24 h before transient transfection for 48 h with small interfering RNAs (siRNAs) mixed with the Lipofectamine reagent. The siRNAs specific for B-RAF (5′-AGACAGGAAUCGAAUGAAA-3′) or C-RAF (5′-CCUCACGCCUUCACCUUUA-3′) mRNAs were obtained from Dharmacon, and a nonspecific siRNA (control) was obtained from Nippon EGT. The cells were then subjected to immunoblot analysis or flow cytometry.

Statistical analysis. Data were analyzed by Student's two-tailed *t* test. A *P* value of <0.05 was considered statistically significant.

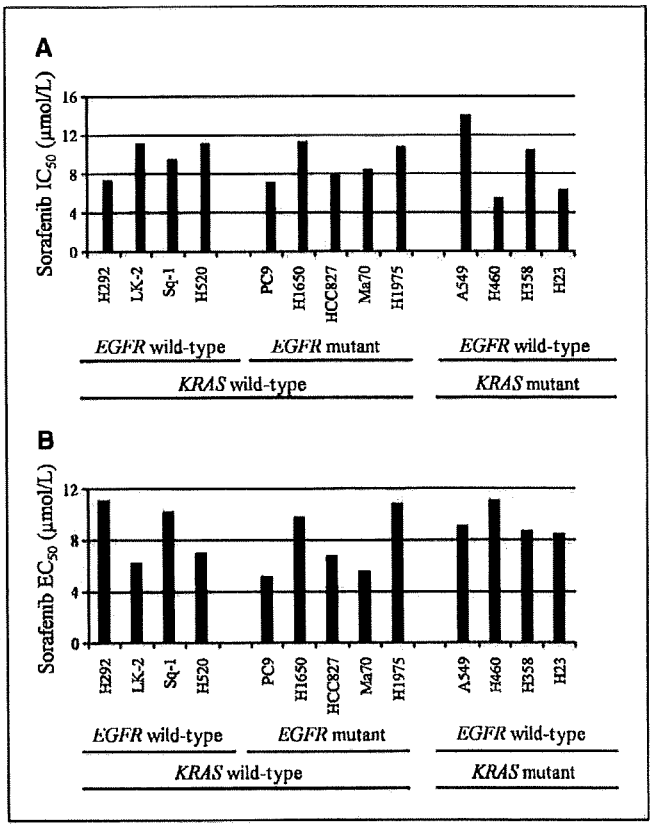

Figure 1. Effects of sorafenib on the growth of NSCLC cell lines classified according to *KRAS* and *EGFR* mutational status. *A*, the indicated NSCLC cell lines were cultured for 72 h in complete culture medium containing various concentrations of sorafenib, after which cell viability was assessed with the MTT assay and the IC_{50} values of sorafenib for inhibition of cell growth were determined. *B*, the indicated NSCLC cell lines were cultured for 7 d in complete medium containing soft agar and various concentrations of sorafenib, after which colony formation was evaluated and the IC_{50} values of sorafenib for inhibition of anchorage-independent cell proliferation were determined. All data are means of triplicates from representative experiments that were repeated on three separate occasions.

Results

Sorafenib inhibits cell growth by inducing G_1 arrest in NSCLC cell lines independently of *KRAS* genotype. The various isoforms of RAF are the principal effectors of RAS in the ERK signaling pathway, and mutant RAS proteins trigger persistent activation of downstream effectors (3). To determine whether the mutational status of *KRAS* might affect the sensitivity of NSCLC cells to sorafenib, an inhibitor of the kinase activity of RAF (16), we first examined the effects of this drug on the anchorage-dependent growth of NSCLC cells with or without *KRAS* mutations by the MTT assay. Sorafenib inhibited cell growth with IC_{50} values ranging from 7.4 to 11.3 μmol/L in NSCLC cells with wild-type *KRAS* and from 5.6 to 14.1 μmol/L in those with mutant *KRAS* (Fig. 1*A*), values that are within the clinically relevant concentration range for this drug (17). This inhibitory effect of sorafenib in cells with wild-type *KRAS* also seemed to be independent of whether the cells contained a mutant version of the *epidermal growth factor receptor* (*EGFR*) gene. We next investigated the effects of sorafenib on anchorage-independent colony formation in soft agar, a more clinically relevant model of NSCLC cell proliferation. Sorafenib inhibited anchorage-independent colony formation with IC_{50} values of 5.6 to 11.1 μmol/L in cells with wild-type *KRAS* and of 8.5 to 11.1 μmol/L in those with mutant *KRAS* (Fig. 1*B*). These data thus indicated that sorafenib inhibits the growth of NSCLC cells in a manner independent of *KRAS* mutational status.

To investigate the mechanism by which sorafenib inhibits NSCLC cell growth, we examined the cell cycle profile by flow cytometry. Sorafenib increased the proportion of cells in G_1 phase of the cell cycle and reduced that of cells in S or G_2-M phases in all tested cell lines regardless of *KRAS* mutational status (Fig. 2). Sorafenib did not increase the proportion of cells in sub-G_1 phase, a characteristic of apoptosis. These data thus indicated that sorafenib inhibits cell growth by inducing arrest of the cell cycle in G_1 phase.

Effects of sorafenib on the ERK signaling pathway in NSCLC cell lines. To examine the effects of sorafenib on the ERK signaling pathway in NSCLC cells, we performed immunoblot analysis with antibodies specific for phosphorylated (activated) ERK. Sorafenib markedly inhibited ERK phosphorylation in cells with wild-type *KRAS* regardless of the mutational status of *EGFR* (Fig. 3*A*). In contrast, sorafenib had no effect on the level of ERK phosphorylation in cells

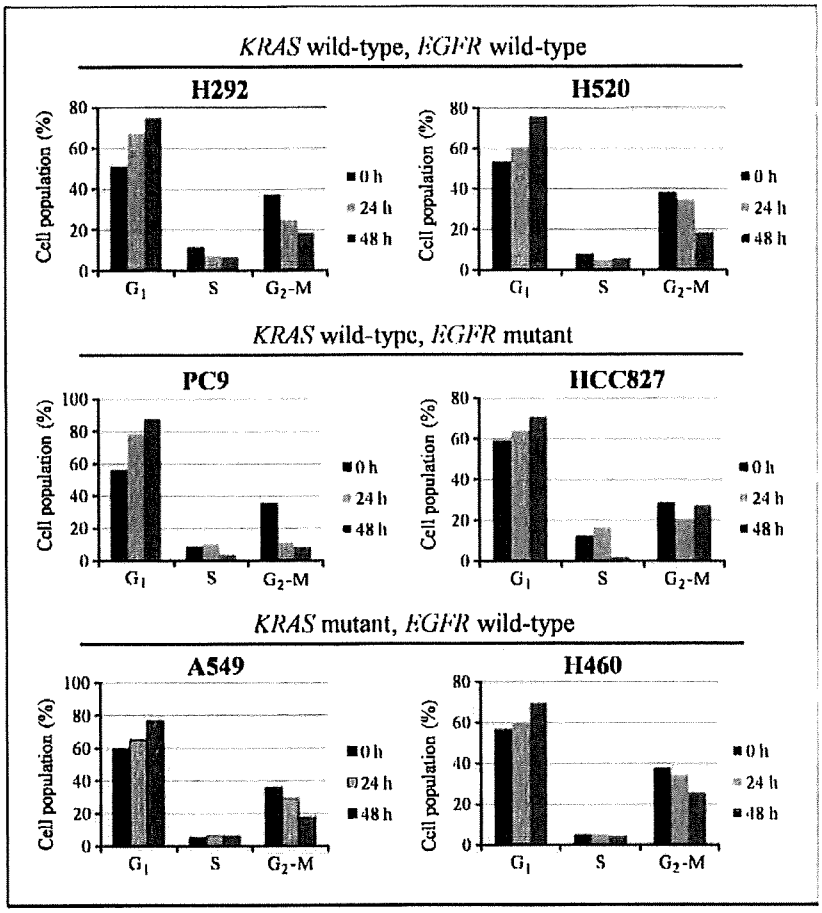

Figure 2. Effects of sorafenib on cell cycle distribution in NSCLC cells classified according to *KRAS* and *EGFR* status. Cells were incubated for 0, 24, or 48 h in complete culture medium containing 15 μmol/L sorafenib and were then fixed, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. All data are means of triplicates from representative experiments that were repeated on three separate occasions.

with mutant *KRAS*. To investigate further whether the effect of sorafenib on ERK phosphorylation is dependent on *KRAS* mutational status, we introduced an expression vector for FLAG epitope-tagged KRAS with the activating Val^{12} mutation (KRAS-V12) into the human NSCLC cell line H1299, which harbors wild-type endogenous *KRAS*. Whereas sorafenib inhibited ERK phosphorylation in nontransfected cells or cells transfected with the empty vector, it failed to do so in cells expressing KRAS-V12 (Fig. 3*B*). These results thus suggested that sorafenib blocks the ERK signaling pathway only in NSCLC cells harboring wild-type *KRAS*.

B-RAF but not C-RAF depletion inhibits ERK phosphorylation in NSCLC cells with wild-type or mutant KRAS. The mammalian RAF family includes A-RAF, B-RAF, and C-RAF, all of which function in the ERK pathway but also have different downstream phosphorylation targets and play distinct roles in signaling (18). Although suggested to be a B-RAF inhibitor, sorafenib inhibits the activity of C-RAF with a potency 4-fold that apparent for B-RAF (16). To investigate the downstream consequences of B-RAF and C-RAF signaling in NSCLC cells, we examined the effects of the depletion of these kinases with a siRNA-based approach. Immunoblot analysis revealed

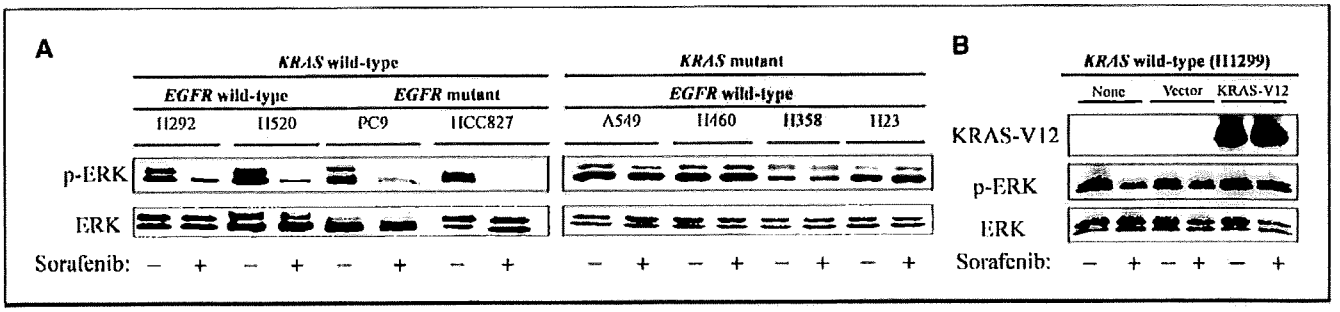

Figure 3. Effects of sorafenib on ERK phosphorylation in NSCLC cells classified according to *KRAS* and *EGFR* status. *A*, cells were incubated for 2 h in the presence or absence of sorafenib (15 μmol/L), after which cell lysates (25 μg of soluble protein) were subjected to immunoblot analysis with antibodies to phosphorylated (*p-ERK*) or total forms of ERK. *B*, H1299 cells were transiently transfected (or not) for 48 h with an expression vector for FLAG-tagged KRAS-V12 or with the corresponding empty vector and were then incubated for 2 h in the presence or absence of sorafenib (15 μmol/L). Cell lysates (25 μg of soluble protein) were then subjected to immunoblot analysis with antibodies to FLAG and to phosphorylated or total forms of ERK.

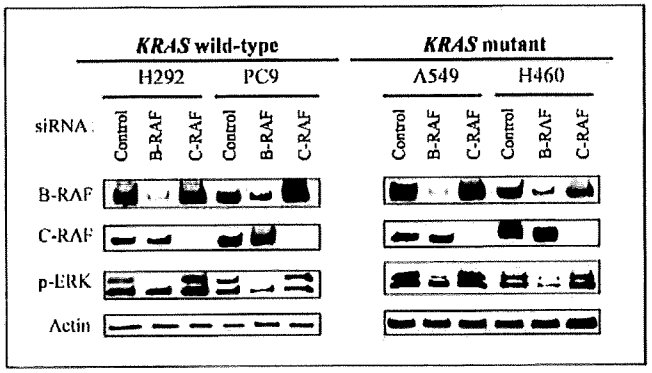

Figure 4. Effects of transient depletion of B-RAF or C-RAF on ERK phosphorylation in NSCLC cells. Cells harboring wild-type or mutant *KRAS* were transfected with nonspecific (control), B-RAF, or C-RAF siRNAs for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to B-RAF, C-RAF, phosphorylated ERK, and β-actin (loading control).

that transfection of NSCLC cells with siRNAs specific for B-RAF or C-RAF mRNAs resulted in marked and selective depletion of the corresponding protein (Fig. 4). Such depletion of B-RAF resulted in inhibition of ERK phosphorylation in cells harboring wild-type or mutant *KRAS*, whereas depletion of C-RAF had no such effect (Fig. 4). These data thus suggested that depletion of B-RAF, but not that of C-RAF, inhibits ERK phosphorylation regardless of *KRAS* status.

Effects of RAF depletion on NSCLC cell proliferation. We next examined the effects of B-RAF or C-RAF depletion on NSCLC cell proliferation and cell cycle distribution. Depletion of B-RAF resulted in significant inhibition of cell proliferation (Fig. 5*A*) and an increase in the proportion of cells in G_1 phase of the cell cycle (Fig. 5*B*), whereas depletion of C-RAF had no such effects, in NSCLC cells harboring wild-type *KRAS*. In contrast, depletion of C-RAF induced significant inhibition of cell proliferation (Fig. 5*A*) and an increase in the proportion of cells in G_1 phase (Fig. 5*B*), whereas depletion of B-RAF had only a less pronounced effect on cell proliferation, in NSCLC cells with mutant *KRAS*. These data thus suggested that B-RAF-ERK signaling regulates cell proliferation in NSCLC cells with wild-type *KRAS*, whereas C-RAF signaling mediates such regulation in NSCLC cells with mutant *KRAS*.

Sorafenib or C-RAF depletion inhibits cyclin E expression in NSCLC cells with mutant *KRAS*. Finally, to characterize further the growth inhibition and G_1 arrest induced by C-RAF depletion or sorafenib in NSCLC cells with mutant *KRAS*, we examined the expression of cyclin E, an essential promoter of the transition from G_1 to S phase of the cell cycle (19). Immunoblot analysis revealed that depletion of C-RAF in A549 or H460 cells resulted in pronounced inhibition of cyclin E expression, whereas depletion of B-RAF had no such effect (Fig. 6). Exposure of the cells to sorafenib also induced loss of cyclin E (Fig. 6). These results thus suggest that the G_1 arrest induced by depletion of C-RAF or by sorafenib in NSCLC cells with mutant *KRAS* may be attributable to the down-regulation of cyclin E.

Discussion

RAS is an upstream component of the ERK signaling pathway, which is aberrantly activated by oncogenic mutations of RAS genes. Among RAS family genes, mutations of *KRAS* are most common in solid malignancies, including NSCLC (8, 20, 21). Indeed, *KRAS* mutations have been associated with poor prognosis and resistance to conventional cytotoxic chemotherapy in NSCLC (22–24). Whereas EGFR tyrosine kinase inhibitors are most efficacious in NSCLC patients with *EGFR* mutations, *KRAS* mutations are associated with resistance to these agents (25–28). The development of therapeutic strategies for NSCLC patients with *KRAS* mutations is thus an important clinical goal. RAF serine-threonine kinases are the principal effectors of RAS in the ERK signaling pathway. Given the key role of this pathway in tumor growth, RAF is a potential target for cancer therapy.

Sorafenib is an orally available compound that has been developed as a multikinase inhibitor with activity against RAF and several RTKs. The sensitivity of cancer cells to sorafenib might be expected to be affected by *KRAS* status, given that *KRAS* mutations result in activation of the ERK pathway (8). However, as far as we are aware, no previous study has compared sorafenib sensitivity among a panel of tumor cell lines of different *KRAS* mutational status. We have now evaluated the effects of sorafenib on the growth of NSCLC cells harboring wild-type or mutant forms of *KRAS* with two different assay systems, the MTT assay and anchorage-independent colony formation assay, given that previous studies have revealed differences in the sensitivity of cells to tested drugs between these two assay systems (29). The IC_{50} values for inhibition of cell growth by sorafenib in these assays have generally been found to be well below 15 μmol/L, the maximum achievable plasma concentration of this drug (17). We found that the potency of sorafenib for inhibition of cell growth was similar for NSCLC cells regardless of *KRAS* mutational status in both assay systems. We also performed a longer-term clonogenic survival assay and again found that sorafenib inhibited the survival of NSCLC cells regardless of *KRAS* status (data not shown). These results thus indicate that sorafenib inhibits the growth of NSCLC cells with mutant *KRAS* as well as it does that of those with wild-type *KRAS* in a clinically relevant concentration range.

We have shown that sorafenib inhibited ERK phosphorylation and induced G_1 arrest in NSCLC cells with wild-type *KRAS*, consistent with previous results obtained with several cancer cell lines harboring wild-type *KRAS* (13, 30, 31). Inhibition of the ERK signaling pathway, as reflected by a reduced level of ERK phosphorylation, results in inhibition of cell proliferation and induction of G_1 arrest in various cell types (32–35). In the present study, we found that depletion of B-RAF by RNA interference also inhibited ERK phosphorylation as well as attenuated cell proliferation and induced G_1 arrest in NSCLC cells with wild-type *KRAS*. These results suggest that inhibition of B-RAF-ERK signaling contributes to suppression of the growth of NSCLC cells harboring wild-type *KRAS* by sorafenib. Consistent with these findings, the specific B-RAF inhibitor SB-590885 was previously shown to inhibit ERK phosphorylation and to induce G_1 arrest in melanoma cells with wild-type *KRAS* (36, 37). In contrast, we found that depletion of C-RAF did not result in inhibition of ERK phosphorylation in NSCLC cells. ERK activation was previously shown to be conserved in cells derived from C-RAF knockout mice, suggesting that C-RAF is dispensable for ERK signaling (38, 39). Together, the present data suggest that B-RAF-ERK signaling, rather than C-RAF signaling, is a potential therapeutic target in NSCLC cells with wild-type *KRAS*.

We showed that ERK phosphorylation was not inhibited by sorafenib in two NSCLC cell lines (A549 and H460) harboring mutant *KRAS*, consistent with previous observations (16). We further showed this to be the case in two additional such cell lines (H358 and H23). Such results were previously suggested to be due to the existence of RAF-independent ERK activation in

NSCLC cells with mutant *KRAS* (16). However, we have now shown that B-RAF depletion resulted in inhibition of ERK activation in these cells. Our data therefore suggest that sorafenib is not able to attenuate the constitutive activation of the B-RAF-ERK pathway characteristic of NSCLC cells harboring mutant *KRAS* (40). Despite the sustained activation of B-RAF-ERK signaling in such cells, sorafenib inhibited cell proliferation and induced G_1 arrest in NSCLC cells with mutant *KRAS* as well as in those with wild-type *KRAS*. These data suggest that sorafenib targets a different pathway in its inhibitory effect on cell growth in NSCLC cells with mutant *KRAS*. Whereas sorafenib inhibits the kinase activity of both B-RAF and C-RAF, it shows a higher affinity for C-RAF (16). We found that depletion of C-RAF by RNA interference inhibited cell proliferation and induced G_1 arrest, without affecting ERK phosphorylation, in NSCLC cells with mutant *KRAS*, whereas it did not exhibit such effects in NSCLC cells harboring wild-type *KRAS*. Depletion of B-RAF also inhibited the growth of NSCLC cells with mutant *KRAS*, although this effect was not as pronounced as that in those with wild-type *KRAS*. These data indicate that NSCLC cells with mutant *KRAS* are dependent on C-RAF signaling to a greater extent than on B-RAF-ERK signaling for cell proliferation but that both pathways participate in regulation of the growth of these cells. Melanoma cells that have acquired resistance to a specific B-RAF inhibitor were recently shown to have switched their dependency from B-RAF to C-RAF (41). These observations suggest that RAF proteins are functionally interchangeable in the regulation of cell growth. Our data thus indicate that C-RAF signaling is a potential therapeutic target in NSCLC cells with mutant *KRAS*. RAF family proteins are also implicated in regulation of cell cycle progression in a manner independent of the ERK pathway (18, 38, 42, 43). C-RAF has been shown to exist in a complex with Cdc25, which activates the cyclin E-Cdk2 complex and promotes the G_1-S phase transition (44, 45). Cyclin E is thus postulated to be a downstream effector of C-RAF. In the present study, we found that either C-RAF depletion or sorafenib treatment induced G_1 arrest and down-regulation of cyclin E in NSCLC cells with mutant *KRAS*. Although we cannot exclude a possible role for other cell cycle

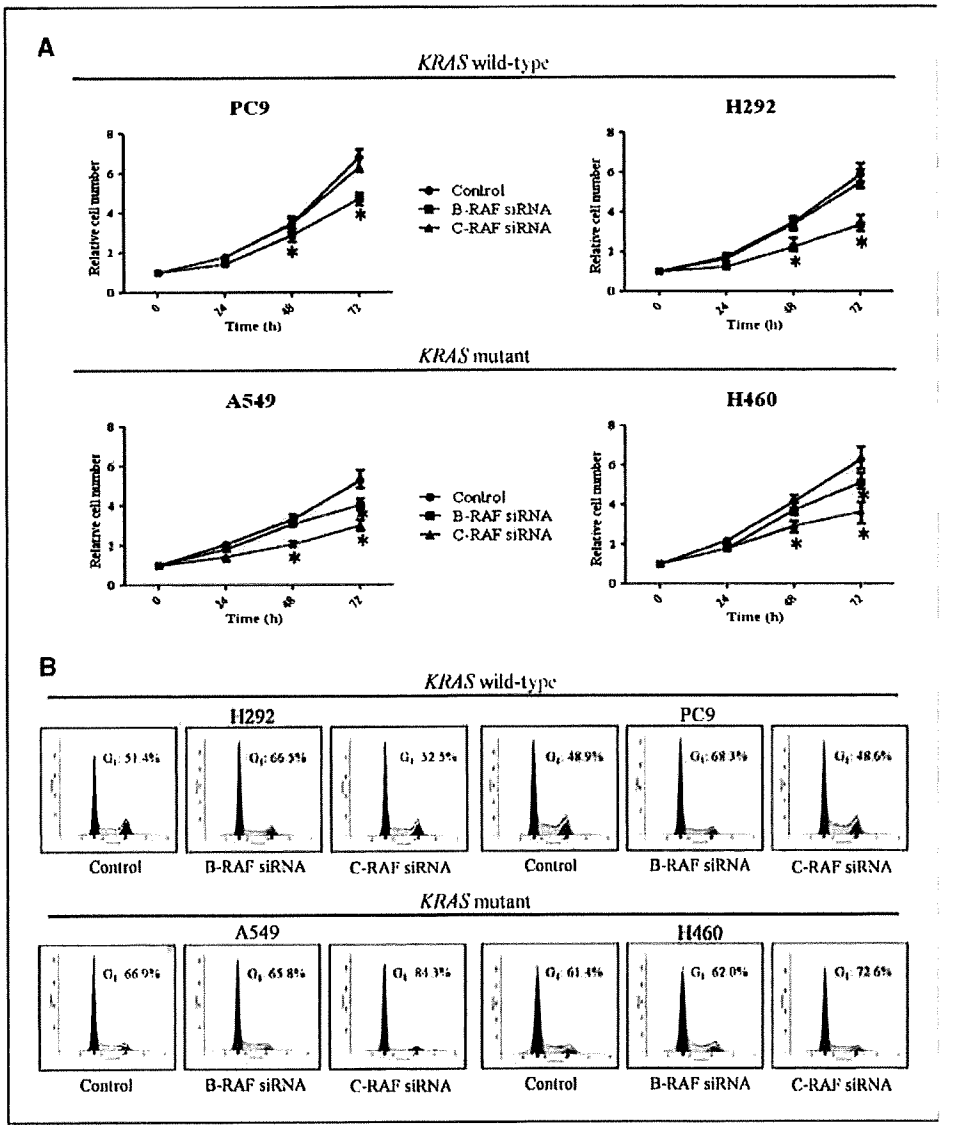

Figure 5. Effects of B-RAF or C-RAF depletion on cell proliferation and cell cycle distribution in NSCLC cells. *A*, cells harboring wild-type or mutant *KRAS* were transfected with nonspecific (control), B-RAF, or C-RAF siRNAs for the indicated times, after which the number of viable cells was determined by staining with trypan blue. The number of viable cells is expressed relative to the value for time 0. *Points*, mean values from three independent experiments; *bars*, SD. *, $P < 0.05$ versus the corresponding value for cells transfected with the nonspecific siRNA. *B*, cells were transfected as in *A* for 48 h, fixed, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. The percentage of cells in G_1 phase is indicated. Data are from representative experiments that were repeated on three separate occasions.

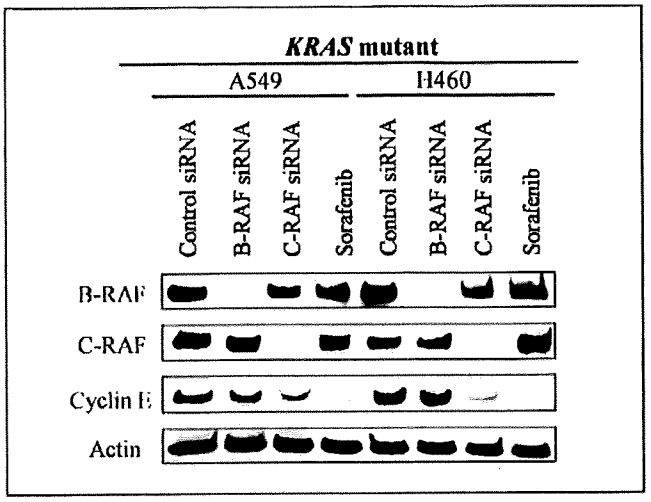

Figure 6. Effects of C-RAF depletion or sorafenib on cyclin E expression in NSCLC cells with mutant *KRAS*. Cells harboring mutant *KRAS* were transiently transfected for 48 h with nonspecific (control), B-RAF, or C-RAF siRNAs or were exposed to 15 μmol/L sorafenib for 24 h in complete medium. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to B-RAF, C-RAF, cyclin E, and β-actin.

proteins, our present data suggest that the observed down-regulation of cyclin E may contribute to the G_1 arrest induced by C-RAF depletion or by sorafenib in NSCLC cells with mutant *KRAS*.

Sorafenib inhibits several RTKs that participate in neovascularization, including vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3 (16). Inhibition of angiogenesis might thus be expected to contribute to the inhibition of tumor growth by this drug in addition to its effects on RAF signaling. Although sorafenib was previously shown to inhibit the growth of a variety of human tumor xenografts in mice (13, 16, 46), it has been difficult to measure the relative contributions of its antiangiogenic activity and its direct antitumor activity mediated by RAF inhibition. In the present study, we have provided insight into the inhibitory effect of sorafenib on tumor cell growth *in vitro* that is mediated by inhibition of RAF signaling pathways. Our results suggest that sorafenib targets B-RAF in NSCLC cells with wild-type *KRAS* and C-RAF in those with mutant *KRAS*, and they provide a rationale for future clinical investigation of the therapeutic efficacy of sorafenib for NSCLC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
2. Eccles SA. Parallels in invasion and angiogenesis provide pivotal points for therapeutic intervention. Int J Dev Biol 2004;48:583–98.
3. Sridhar SS, Hedley D, Siu LL. Raf kinase as a target for anticancer therapeutics. Mol Cancer Ther 2005;4:677–85.
4. Adjei AA, Hillman SL. A front-line window of opportunity phase II study of sorafenib in patients with advanced non-small cell lung cancer: a North Central Cancer Treatment Group Study. Proc Am Soc Clin Oncol 2007;25:S18.
5. Gatzemeier U, Fosella F. Phase II trial of singe-agent sorafenib in patients with advanced non-small cell lung carcinoma. J Clin Oncol 2006;24:7002.
6. Gutierrez SKM, Allen D, Turkbey B, et al. A phase II study of multikinase inhibitor sorafenib in patients with relapsed non-small cell lung cancer [abstract 19084]. J Clin Oncol 2008;26:S712.
7. Schiller JH, Hanna NH, Traynor AM, Carbone DP. A randomized discontinuation phase II study of sorafenib versus placebo in patients with non-small cell lung cancer who have failed at least two prior chemotherapy regimens [abstract 8014]. J Clin Oncol 2008;26:S427.
8. Rodenhuis S, van de Wetering ML, Mooi WJ, Evers SG, van Zandwijk N, Bos JL. Mutational activation of the K-ras oncogene. A possible pathogenetic factor in adenocarcinoma of the lung. N Engl J Med 1987;317:929–35.
9. Garnett MJ, Marais R. Guilty as charged: B-RAF is a human oncogene. Cancer Cell 2004;6:313–9.
10. Panka DJ, Wang W, Atkins MB, Mier JW. The Raf inhibitor BAY 43-9006 (sorafenib) induces caspase-independent apoptosis in melanoma cells. Cancer Res 2006;66:1611–9.
11. Molhoek KR, Brautigan DL, Slingluff CL, Jr. Synergistic inhibition of human melanoma proliferation by combination treatment with B-Raf inhibitor BAY43-9006 and mTOR inhibitor rapamycin. J Transl Med 2005;3:39.
12. Lasithiotakis KG, Sinnberg TW, Schittek B, et al. Combined inhibition of MAPK and mTOR signaling inhibits growth, induces cell death, and abrogates invasive growth of melanoma cells. J Invest Dermatol 2008;128:2013–23.
13. Henderson YC, Ahn SH, Kang Y, Clayman GL. Sorafenib potently inhibits papillary thyroid carcinomas harboring RET/PTC1 rearrangement. Clin Cancer Res 2008;14:4908–14.
14. Smalley KS, Xiao M, Villanueva J, et al. CRAF inhibition induces apoptosis in melanoma cells with non-V600E BRAF mutations. Oncogene 2009;28:85–94.
15. Okabe T, Okamoto I, Tamura K, et al. Differential constitutive activation of the epidermal growth factor receptor in non-small cell lung cancer cells bearing EGFR gene mutation and amplification. Cancer Res 2007;67:2046–53.
16. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 2004;64:7099–109.
17. Strumberg D, Clark JW, Awada A, et al. Safety, pharmacokinetics, and preliminary antitumor activity of sorafenib: a review of four phase I trials in patients with advanced refractory solid tumors. Oncologist 2007;12:426–37.
18. Wojnowski L, Stancato LF, Larner AC, Rapp UR, Zimmer A. Overlapping and specific functions of Braf and Craf-1 proto-oncogenes during mouse embryogenesis. Mech Dev 2000;91:97–104.
19. Dobashi Y. Cell cycle regulation and its aberrations in human lung carcinoma. Pathol Int 2005;55:95–105.
20. Buttitta F, Barassi F, Fresu G, et al. Mutational analysis of the HER2 gene in lung tumors from Caucasian patients: mutations are mainly present in adenocarcinomas with bronchioloalveolar features. Int J Cancer 2006;119:2586–91.
21. Suzuki M, Shigematsu H, Iizasa T, et al. Exclusive mutation in epidermal growth factor receptor gene, HER-2, and KRAS, and synchronous methylation of nonsmall cell lung cancer. Cancer 2006;106:2200–7.
22. Graziano SL, Gamble GP, Newman NB, et al. Prognostic significance of K-ras codon 12 mutations in patients with resected stage I and II non-small-cell lung cancer. J Clin Oncol 1999;17:668–75.
23. Slebos RJ, Kibbelaar RE, Dalesio O, et al. K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. N Engl J Med 1990;323:561–5.
24. Winton T, Livingston R, Johnson D, et al. Vinorelbine plus cisplatin vs. observation in resected non-small-cell lung cancer. N Engl J Med 2005;352:2589–97.
25. Pao W, Wang TY, Riely GJ, et al. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med 2005;2:e17.
26. Massarelli E, Varella-Garcia M, Tang X, et al. KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Cancer Res 2007;13:2890–6.
27. Marks JL, Broderick S, Zhou Q, et al. Prognostic and therapeutic implications of EGFR and KRAS mutations in resected lung adenocarcinoma. J Thorac Oncol 2008;3:111–6.
28. Baselga J, Rosen N. Determinants of RASistance to anti-epidermal growth factor receptor agents. J Clin Oncol 2008;26:1582–4.
29. Hao H, Muniz-Medina VM, Mehta H, et al. Context-dependent roles of mutant B-Raf signaling in melanoma and colorectal carcinoma cell growth. Mol Cancer Ther 2007;6:2220–9.
30. Jane EP, Premkumar DR, Pollack IF. Coadministration of sorafenib with rottlerin potently inhibits cell proliferation and migration in human malignant glioma cells. J Pharmacol Exp Ther 2006;319:1070–80.
31. Ambrosini G, Cheema HS, Seelman S, et al. Sorafenib inhibits growth and mitogen-activated protein kinase signaling in malignant peripheral nerve sheath cells. Mol Cancer Ther 2008;7:890–6.
32. Villanueva J, Yung Y, Walker JL, Assolan RK. ERK activity and G_1 phase progression: identifying dispensable versus essential activities and primary versus secondary targets. Mol Biol Cell 2007;18:1457–63.

33. Jones SM, Kazlauskas A. Growth-factor-dependent mitogenesis requires two distinct phases of signalling. Nat Cell Biol 2001;3:165–72.
34. Yamamoto T, Ebisuya M, Ashida F, Okamoto K, Yonehara S, Nishida E. Continuous ERK activation downregulates antiproliferative genes throughout G_1 phase to allow cell-cycle progression. Curr Biol 2006;16:1171–82.
35. Fassett JT, Tobolt D, Nelsen CJ, Albrecht JH, Hansen LK. The role of collagen structure in mitogen stimulation of ERK, cyclin D1 expression, and G_1-S progression in rat hepatocytes. J Biol Chem 2003;278:31691–700.
36. Smalley KS, Lioni M, Dalla Palma M, et al. Increased cyclin D1 expression can mediate BRAF inhibitor resistance in BRAF V600E-mutated melanomas. Mol Cancer Ther 2008;7:2876–83.
37. King AJ, Patrick DR, Batorsky RS, et al. Demonstration of a genetic therapeutic index for tumors expressing oncogenic BRAF by the kinase inhibitor SB-590885. Cancer Res 2006;66:11100–5.
38. Huser M, Luckett J, Chiloeches A, et al. MEK kinase activity is not necessary for Raf-1 function. EMBO J 2001;20:1940–51.
39. Mikula M, Schreiber M, Husak Z, et al. Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. EMBO J 2001;20:1952–62.
40. Salgia R, Skarin AT. Molecular abnormalities in lung cancer. J Clin Oncol 1998;16:1207–17.
41. Montagut C, Sharma SV, Shioda T, et al. Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. Cancer Res 2008;68:4853–61.
42. Baumann B, Weber CK, Troppmair J, et al. Raf induces NF-κB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. Proc Natl Acad Sci U S A 2000;97:4615–20.
43. Galaktionov K, Jessus C, Beach D. Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. Genes Dev 1995;9:1046–58.
44. Kerkhoff E, Rapp UR. Cell cycle targets of Ras/Raf signalling. Oncogene 1998;17:1457–62.
45. Hindley A, Kolch W. Extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK)-independent functions of Raf kinases. J Cell Sci 2002;115:1575–81.
46. Liu L, Cao Y, Chen C, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851–8.