References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Attal M, Harousseau JL, Stoppa AM *et al.* A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais Myelome. *N Engl J Med* 1996; **335**: 91–7.
3 Child JA, Morgan GJ, Davies FE *et al.* High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. *N Engl J Med* 2003; **348**: 1875–83.
4 Palumbo A, Bringhen S, Petrucci MT *et al.* Intermediate-dose melphalan improves survival of myeloma patients aged 50 to 70: results of a randomized controlled trial. *Blood* 2004; **104**: 3052–7.
5 Alexanian R, Dimopoulos MA, Delasalle K, Barlogie B. Primary dexamethasone treatment of multiple myeloma. *Blood* 1992; **80**: 887–90.
6 Myeloma Trialists' Collaborative Group. Combination chemotherapy versus melphalan plus prednisone as treatment for multiple myeloma: an overview of 6,633 patients from 27 randomized trials. *J Clin Oncol* 1998; **16**: 3832–42.
7 Koskela K, Pelliniemi TT, Pulkki K, Remes K. Treatment of multiple myeloma with all-*trans* retinoic acid alone and in combination with chemotherapy: a phase I/II trial. *Leuk Lymphoma* 2004; **45**: 749–54.
8 Sanda T, Kuwano T, Nakao S *et al.* Antimyeloma effects of a novel synthetic retinoid Am80 (tamibarotene) through inhibition of angiogenesis. *Leukemia* 2005; **19**: 901–9.
9 Miwako I, Kagechika H. Tamibarotene. *Drugs Today (Barc)* 2007; **43**: 563–8.
10 Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. *Nat Rev Cancer* 2002; **2**: 927–37.
11 Raanani P, Shpilberg O, Ben-Bassat I. Extramedullary disease and targeted therapies for hematological malignancies – is the association real? *Ann Oncol* 2007; **18**: 7–12.
12 Takagi K, Suganuma M, Kagechika H *et al.* Inhibition of ornithine decarboxylase induction by retinobenzoic acids in relation to their binding affinities to cellular retinoid-binding proteins. *J Cancer Res Clin Oncol* 1988; **114**: 221–4.
13 Tobita T, Takeshita A, Kitamura K *et al.* Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-*trans* retinoic acid. *Blood* 1997; **90**: 967–73.
14 Lefebvre O, Wouters D, Mereau-Richard C *et al.* Induction of apoptosis by all-*trans* retinoic acid in the human myeloma cell line RPMI 8226 and negative regulation of some of its typical morphological features by dexamethasone. *Cell Death Differ* 1999; **6**: 433–44.
15 Ogata A, Nishimoto N, Shima Y, Yoshizaki K, Kishimoto T. Inhibitory effect of all-*trans* retinoic acid on the growth of freshly isolated myeloma cells via interference with interleukin-6 signal transduction. *Blood* 1994; **84**: 3040–6.
16 Joseph B, Lefebvre O, Mereau-Richard C, Danze PM, Belin-Plancot MT, Formstecher P. Evidence for the involvement of both retinoic acid receptor- and retinoic X receptor-dependent signaling pathways in the induction of tissue transglutaminase and apoptosis in the human myeloma cell line RPMI 8226. *Blood* 1998; **91**: 2423–32.
17 Kawano M, Hirano T, Matsuda T *et al.* Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature* 1988; **332**: 83–5.
18 Klein B. Cytokine, cytokine receptors, transduction signals, and oncogenes in human multiple myeloma. *Semin Hematol* 1995; **32**: 4–19.
19 Kawano MM, Mihara K, Huang N, Tsujimoto T, Kuramoto A. Differentiation of early plasma cells on bone marrow stromal cells requires interleukin-6 for escaping from apoptosis. *Blood* 1995; **85**: 487–94.
20 Gu ZJ, De Vos J, Rebouissou C *et al.* Agonist anti-gp130 transducer monoclonal antibodies are human myeloma cell survival and growth factors. *Leukemia* 2000; **14**: 188–97.
21 Wang T, Niwa S, Bouda K *et al.* The effect of Am-80, one of retinoids derivatives on experimental allergic encephalomyelitis in rats. *Life Sci* 2000; **67**: 1869–79.
22 Niwa S, Ochi T, Hirano Y *et al.* Effect of Am-80, a retinoid derivative, on 2, 4-dinitrofluorobenzene-induced contact dermatitis in mice. *Pharmacology* 2000; **60**: 208–14.
23 Nagai H, Matsuura S, Bouda K *et al.* Effect of Am-80, a synthetic derivative of retinoid, on experimental arthritis in mice. *Pharmacology* 1999; **58**: 101–12.
24 Chauhan D, Pandey P, Hideshima T *et al.* SHP2 mediates the protective effect of interleukin-6 against dexamethasone-induced apoptosis in multiple myeloma cells. *J Biol Chem* 2000; **275**: 27845–50.
25 Chauhan D, Auclair D, Robinson EK *et al.* Identification of genes regulated by dexamethasone in multiple myeloma cells using oligonucleotide arrays. *Oncogene* 2002; **21**: 1346–58.
26 Greenstein S, Ghias K, Krett NL, Rosen ST. Mechanisms of glucocorticoid-mediated apoptosis in hematological malignancies. *Clin Cancer Res* 2002; **8**: 1681–94.
27 Steel GG, Peckham MJ. Exploitable mechanisms in combined radiotherapy–chemotherapy: the concept of additivity. *Int J Radiat Oncol Biol Phys* 1979; **5**: 85–91.
28 Hasegawa J, Kamada S, Kamiike W *et al.* Involvement of CPP32/Yama(-like) proteases in Fas-mediated apoptosis. *Cancer Res* 1996; **56**: 1713–8.
29 Klein B, Zhang XG, Lu ZY, Bataille R. Interleukin-6 in human multiple myeloma. *Blood* 1995; **85**: 863–72.
30 Chauhan D, Kharbanda S, Ogata A *et al.* Interleukin-6 inhibits Fas-induced apoptosis and stress-activated protein kinase activation in multiple myeloma cells. *Blood* 1997; **89**: 227–34.
31 Puthier D, Bataille R, Amiot M. IL-6 up-regulates mcl-1 in human myeloma cells through JAK/STAT rather than ras/MAP kinase pathway. *Eur J Immunol* 1999; **29**: 3945–50.
32 Hideshima T, Nakamura N, Chauhan D, Anderson KC. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. *Oncogene* 2001; **20**: 5991–6000.
33 Smith MA, Parkinson DR, Cheson BD, Friedman MA. Retinoids in cancer therapy. *J Clin Oncol* 1992; **10**: 839–64.
34 Chen YH, Desai P, Shiao RT, Lavelle D, Haleem A, Chen J. Inhibition of myeloma cell growth by dexamethasone and all-*trans* retinoic acid: synergy through modulation of interleukin-6 autocrine loop at multiple sites. *Blood* 1996; **87**: 314–23.
35 Taetle R, Dos Santos B, Akamatsu K, Koishihara Y, Ohsugi Y. Effects of all-*trans* retinoic acid and antireceptor antibodies on growth and programmed cell death of human myeloma cells. *Clin Cancer Res* 1996; **2**: 253–9.
36 Klein B, Zhang XG, Jourdan M *et al.* Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. *Blood* 1989; **73**: 517–26.
37 Anderson KC, Jones RM, Morimoto C, Leavitt P, Barut BA. Response patterns of purified myeloma cells to hematopoietic growth factors. *Blood* 1989; **73**: 1915–24.
38 Klein B, Wijdenes J, Zhang XG *et al.* Murine anti-interleukin-6 monoclonal antibody therapy for a patient with plasma cell leukemia. *Blood* 1991; **78**: 1198–204.
39 Levy Y, Labaume S, Colombel M, Brouet JC. Retinoic acid modulates the *in vivo* and *in vitro* growth of IL-6 autocrine human myeloma cell lines via induction of apoptosis. *Clin Exp Immunol* 1996; **104**: 167–72.
40 Ogata A, Chauhan D, Teoh G *et al.* IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. *J Immunol* 1997; **159**: 2212–21.
41 Catlett-Falcone R, Landowski TH, Oshiro MM *et al.* Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 1999; **10**: 105–15.
42 Semenov I, Akyuz C, Roginskaya V, Chauhan D, Corey SJ. Growth inhibition and apoptosis of myeloma cells by the CDK inhibitor flavopiridol. *Leuk Res* 2002; **26**: 271–80.
43 Tu Y, Gardner A, Lichtenstein A. The phosphatidylinositol 3-kinase/AKT kinase pathway in multiple myeloma plasma cells: roles in cytokine-dependent survival and proliferative responses. *Cancer Res* 2000; **60**: 6763–70.
44 Sidell N, Taga T, Hirano T, Kishimoto T, Saxon A. Retinoic acid-induced growth inhibition of a human myeloma cell line via down-regulation of IL-6 receptors. *J Immunol* 1991; **146**: 3809–14.
45 Hardin J, MacLeod S, Grigorieva I *et al.* Interleukin-6 prevents dexamethasone-induced myeloma cell death. *Blood* 1994; **84**: 3063–70.
46 Lichtenstein A, Tu Y, Fady C, Vescio R, Berenson J. Interleukin-6 inhibits apoptosis of malignant plasma cells. *Cell Immunol* 1995; **162**: 248–55.
47 Rajkumar SV, Blood E, Vesole D, Fonseca R, Greipp PR. Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: a clinical trial coordinated by the Eastern Cooperative Oncology Group. *J Clin Oncol* 2006; **24**: 431–6.
48 Palumbo A, Bringhen S, Caravita T *et al.* Oral melphalan and prednisone chemotherapy plus thalidomide compared with melphalan and prednisone alone in elderly patients with multiple myeloma: randomised controlled trial. *Lancet* 2006; **367**: 825–31.
49 Breitkreutz I, Lokhorst HM, Raab MS *et al.* Thalidomide in newly diagnosed multiple myeloma: influence of thalidomide treatment on peripheral blood stem cell collection yield. *Leukemia* 2007; **21**: 1294–9.
50 Facon T, Mary JY, Hulin C *et al.* Melphalan and prednisone plus thalidomide versus melphalan and prednisone alone or reduced-intensity autologous stem cell transplantation in elderly patients with multiple myeloma (IFM 99–06): a randomised trial. *Lancet* 2007; **370**: 1209–18.
51 Palumbo A, Falco P, Corradini P *et al.* Melphalan, prednisone, and lenalidomide treatment for newly diagnosed myeloma: a report from the GIMEMA–Italian Multiple Myeloma Network. *J Clin Oncol* 2007; **25**: 4459–65.
52 Dimopoulos M, Spencer A, Attal M *et al.* Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma. *N Engl J Med* 2007; **357**: 2123–32.
53 Weber DM, Chen C, Niesvizky R *et al.* Lenalidomide plus dexamethasone for relapsed multiple myeloma in North America. *N Engl J Med* 2007; **357**: 2133–42.
54 Palumbo A, Ambrosini MT, Benevolo G *et al.* Bortezomib, melphalan, prednisone, and thalidomide for relapsed multiple myeloma. *Blood* 2007; **109**: 2767–72.
55 Wang M, Giralt S, Delasalle K, Handy B, Alexanian R. Bortezomib in combination with thalidomide–dexamethasone for previously untreated multiple myeloma. *Hematology* 2007; **12**: 235–9.

Circulating Endothelial Cells in Non-small Cell Lung Cancer Patients Treated with Carboplatin and Paclitaxel

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Introduction: Circulating endothelial cells (CECs) increase in cancer patients and play an important role in tumor neovascularization.
Methods: This study was designed to investigate the role of CEC as a marker for predicting the effectiveness of a carboplatin plus paclitaxel based first line chemotherapy in advanced non-small cell lung cancer (NSCLC).
Results: The CEC count in 4 ml of peripheral blood before starting chemotherapy (baseline value) was significantly higher in NSCLC patients, ranging from 32 to 4501/4 ml (n = 31, mean ± SD = 595 ± 832), than in healthy volunteers (n = 53, 46.2 ± 86.3). We did not detect a significant correlation between the CEC count and estimated tumor volume. CECs were significantly decreased by chemotherapy as compared with pretreatment values (175.6 ± 24 and 173.0 ± 24, day +8, +22, respectively). We investigated the correlation between baseline CEC and the clinical effectiveness of chemotherapy. CEC values are significantly higher in patients with clinical benefit (partial response and stable disease, 516 ± 458, 870.8 ± 1215, respectively) than in progressive disease patients (211 ± 150). Furthermore, a statistically significant decrease in CECs, on day 22, was observed only in patients with partial response. Patients who had a baseline CEC count greater than 400/4 ml showed a longer progression-free survival (>400, 271 days [range: 181–361] versus <400, 34 [range: 81–186], p = 0.019).
Conclusion: CEC is suggested to be a promising predictive marker of the clinical efficacy of the CBDCA plus paclitaxel regimen in patients with NSCLC.

Key Words: Circulating endothelial cell, NSCLC, Chemotherapy.

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Angiogenesis plays a critical role in the growth and metastasis of solid tumors.[1] The clinical importance of angiogenesis in human tumors has been demonstrated by several reports indicating a positive relationship between the blood vessel density in the tumor mass and poor prognosis, i.e., survival, in patients with various types of cancers including non-small cell lung cancer (NSCLC).[2–6] Furthermore, Natsume et al.[7] reported the antitumor activities of anticancer agents to be less active against vascular endothelial growth factor-secreting cells (SBC-3/VEGF), in vivo as compared with its mock transfectant (SBC-3/Neo). In recent years, antiangiogenic agents have also been demonstrated to be active against a variety of malignancies, including lung, colorectal, and renal cancer.[8–10] Thus, angiogenesis is a promising target for cancer treatment and is related to the prognosis and efficacy of these drugs, though the tumor vessel biomarkers which predict the effectiveness of antiangiogenic agents and other anticancer agents are not always useful and have not become well-established.

Circulating endothelial cells (CECs) have been recognized as a useful biomarker for vascular damage. CECs are increased in cardiovascular disease, vasculitis, infectious disease, and various cancers.[11–14] Recently, CECs were found to be more numerous and viable in cancer patients than in healthy subjects.[14,15] Furthermore, elevated CECs in cancer patients were found to be nearly normalized when the tumor was removed surgically or with chemotherapy.[15] Therefore, most CECs are considered to be disseminated tissue endothelial cells in the tumors and the CEC number may reflect the extent of tumor angiogenesis. Indeed, the CEC level has been demonstrated to correlate with the plasma level of VEGF, one of the pivotal factors promoting tumor angiogenesis.[15] Mancuso et al. reported that CEC kinetics and viability are promising predictors of the response to chemotherapy with antiangiogenic activity in patients with advanced breast cancer.[16] Thus, CEC is likely to be a useful marker for predicting the effectiveness of chemotherapy as a noninvasive angiogenesis marker.

NSCLC is the leading cause of cancer-related death worldwide. NSCLC accounts for approximately 50% of patients presenting with unresectable advanced stage,[17] and platinum-based chemotherapy offers only a small improve-

ment in survival with advanced NSCLC.[18,19] Over the past decade, several new agents against NSCLC have become available, including the taxanes, gemcitabine, vinorelbine, and irinotecan. The combination of platinum and these new agents has resulted in a high response rate and prolonged survival compared with older chemotherapy regimens (e.g., vindesine, mitomycin, ifosfamide, with cisplatin). Therefore, these regimens are considered standard chemotherapy for advanced NSCLC.[20–26] Although new agents have different mechanisms of action, these combination regimens have not been administered based on the biologic characteristics of each tumor.

Paclitaxel inhibits several endothelial cell functions in vitro such as proliferation, migration, morphogenesis, and metalloprotease production.[27–29] These activities result in antiangiogenic activity in in vivo xenograft models.[27,30] Interestingly, human endothelial cells are more sensitive to paclitaxel than other cellular types.[29] We hypothesized that the CEC value is associated with tumor neovascularization, which is one of the targets of paclitaxel. In the present study, we investigated whether the CEC count at baseline is associated with the effectiveness of the CDDP plus paclitaxel regimen in patients with advanced-stage NSCLC.

MATERIALS AND METHODS

Patients

Patients with histologically or cytologically documented advanced NSCLC were eligible for this study. Each patient was required to meet the following criteria: (1) no prior treatment including chemotherapy, surgery, irradiation, or any fluid drainage; (2) no prior general anesthesia for diagnostic procedures including mediastinoscopy or thoracoscopy; (3) no concomitant diseases including ischemic heart diseases, systemic vasculitis, pulmonary hypertension, or serious complications including infectious disease or diabetes; (4) written informed consent. The trial document was approved by the institutional review board. The clinical characteristics of the patients are shown in Table 1.

TABLE 1. Baseline Characteristics of the Patients

Characteristic	$N = 31$ No. (%)
Gender	
Male	17 (55)
Female	14 (45)
Median age (yr)	60
Range	43–71
ECOG performance status	
0	18 (58)
1	13 (42)
Stage	
IIIA	2 (6)
IIIB	7 (23)
IV	22 (71)
Histology	
Adenocarcinoma	23 (74)
Squamous cell carcinoma	4 (13)
Others	4 (13)

Treatment Schedule and Response Evaluation

All patients were treated according to the following chemotherapeutic regimen: paclitaxel at 200 mg/m^2 over a 3-hour period followed by carboplatin at a dose with an area under the curve of 6 on day 1, repeated every 3 weeks. The treatment was repeated for three or more cycles unless the patients met the criteria for progressive disease (PD) or experienced unacceptable toxicity.

The major axis (a) and minor axis (b) of the tumor mass in each patient were measured with computed tomography. Estimated tumor volume (ETV) was calculated using the following formula; ETV $= 4/3 \times \pi$ (a/2 $\times$ b/2) $\times$ (a/2 + b/2)/2. Computed tomography examinations were performed before treatment and with every one or two cycles of chemotherapy. Response was evaluated according to the RECIST, and tumor markers were excluded from the criteria.[31]

Assay for CEC

Blood samples from NSCLC patients and healthy volunteers were drawn into a 10-ml Cellsave Preservative Tube (Immunicon Corp. Huntingdon Valley, PA) for CEC enumeration. The CEC protocol used was approved by the Institutional Review Board and written informed consent was obtained from each subject. Samples from NSCLC were obtained before (baseline) and 8 and 22 days after starting chemotherapy. Samples were kept at room temperature and processed within 42 hours after collection. All evaluations were performed without knowledge of the clinical status of the patients. The CellTracks system (Immunicon Corp) which consists of CellTracks AutoPrep system and the CellSpotter Analyzer system was used for endothelial cell enumeration.[32,33] In this system, CD146+/DAPI+/CD105-PE+/CD45APC- cells are defined as CECs. Briefly, cells which express CD146 were immunomagnetically captured using ferrofluids coated with CD146 antibodies. The enriched cells were then labeled with the nuclear dye 4V,6-diamidino-2-phenylindole (DAPI), CD105 antibodies conjugated to phycoerythrin (CD105-PE), and the pan-leukocyte antibody CD45 conjugated to allophycocyanin (CD45-APC). In this system, the CD146-enriched, fluorescently labeled cells were identified as CECs when the cells exhibited the DAPI+/CD105+/CD45- phenotype. We performed CEC enumeration twice, using the same sample, and calculated the mean value.

Statistical Analyses

This study was carried out as exploratory research for detecting CECs from NSCLC patients. The number of enrolled patients was therefore not precalculated. Spearman's correlation analysis was performed to investigate the correlation between CEC count and ETV. Between-group comparisons were made using the t test. The association between CEC count and progression free survival (PFS) was estimated using the Kaplan-Meier method. The log-rank test was used to assess the survival difference between strata. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Patient Characteristics

A total of 32 patients were enrolled in the study between August 2005 and March 2006 (Table 1). One patient withdrew consent to participate. Table 1 summarizes the characteristics of the study population. The median age of the patients was 60 years (range, 43–71). The histologic and/or cytologic diagnosis was adenocarcinoma in 23 patients (74.2%), squamous cell carcinoma in 4 (12.9%), and unclassified NSCLC in 4 (12.9%). There were 17 males (54.8%). The clinical stage was IIIA in 2 patients (6.5%), IIIB in 7 (22.6%), and IV in 22 (71.0%).

Ninety-two CEC samples from 31 patients (three samples per patient) were obtained and analyzed. One sample, obtained 22 days after treatment, was not examined because of inadequate collection.

Quantification of CEC

In 31 advanced NSCLC patients, CECs ranged from 32 to 4501 cells/4.0 ml of blood, mean ± SD = 595 ± 832 at baseline. CEC counts were elevated in a large portion of patients with NSCLC as compared with healthy volunteers (n = 53, mean ± SD = 46.2 ± 86.3/4 ml). Case 21 had an exceptionally high CEC count (4501 at baseline). We did not detect a significant correlation between the CEC count and ETV in the 28 assessable patients (p = 0.84, Figure 1). The analysis of CECs during the first course of treatment showed CEC levels to be reduced by CBDCA plus paclitaxel chemotherapy as compared with pretreatment values (176 ± 141 at 8 days and 173 ± 189 at 22 days after treatment) (Figure 2). These reductions were significant (p = 0.011 on day 8 and p = 0.04 on day 22), but there was no significant difference between CEC amounts on day 8 versus day 22 (p = 0.476). There was no difference in the amount of CEC at baseline when patients were subgrouped according to characteristics, such as sex, smoking history, histologic type, and clinical stage. Furthermore, there was no correlation of CEC amounts with the blood examination data (e.g., number of white blood cells, neutrophils, lymphocytes, hemoglobin, platelets, albumin, LDH, CRP, CEA, CYFRA).

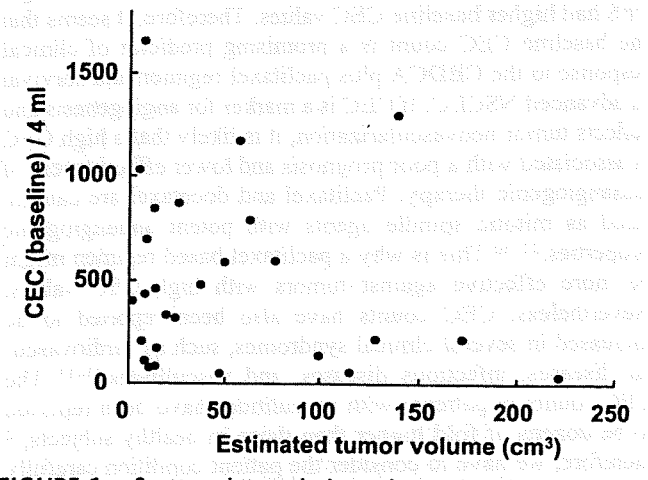
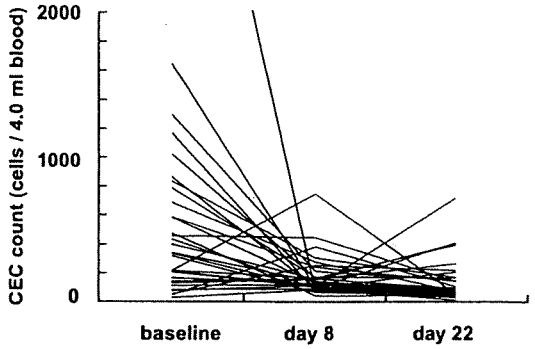


FIGURE 1. Scatter plot analysis to determine the correlation between the number of circulating endothelial cell (CEC) and estimated tumor volume (ETV). ETV is calculated with computed tomography (CT) examination. Case 21 is not included.



	baseline	day 8	day 22
mean ± SD	595± 832	176± 141 *	173 ± 189 *

FIGURE 2. Circulating endothelial cell (CEC) levels during the first course of CDDP plus paclitaxel chemotherapy. *p < 0.05 versus values at baseline.

CEC Amounts and Objective Tumor Response to Chemotherapy

Thirteen (41.9%) of the 31 patients who received carboplatin and paclitaxel therapy showed a partial response (PR) and 12 (38.7%) showed stable disease (SD). The other 6 patients (19.4%) showed PD. The amounts of CEC at baseline in the patients who showed PR and SD were 516 ± 458/4 ml and 871 ± 1215/4 ml, respectively, and these values were significantly higher than in PD patients (211 ± 150/4 ml, p = 0.023 and p = 0.044, respectively) (Figure 3*A*). Although CEC decrements during chemotherapy were observed in all three subgroups, the extent of the decrements tended to be greater in

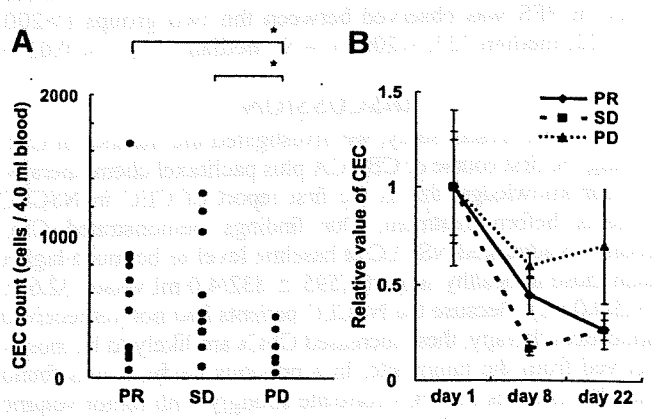


FIGURE 3. *A*, Comparison of circulating endothelial cell (CEC) amount at baseline in non-small cell lung cancer (NSCLC) patients with different clinical responses to CBDCA plus paclitaxel chemotherapy. *p < 0.05 versus values of patients with progressive disease (PD). Case 21 is not included. *B*, Relative change in CEC amount in patients with partial response (PR), stable disease (SD), and PD.

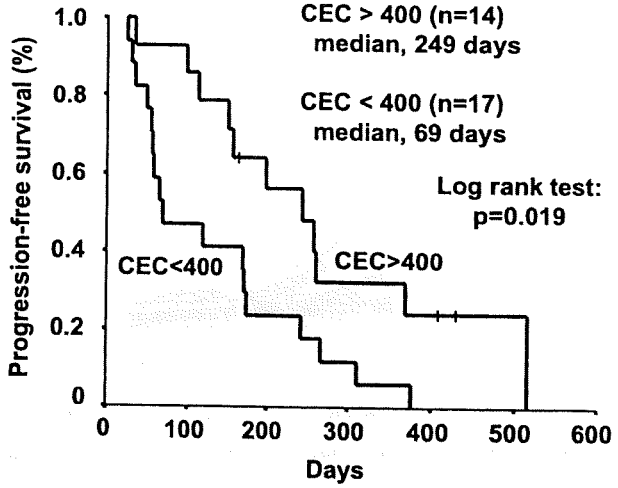


FIGURE 4. Progression-free-survival according to circulating endothelial cell (CEC) count at baseline. The median duration of progression-free survival was greater in patients whose CEC count exceeded 400 (median, 244 days) than in patients whose CEC count was less than 400 (69 days).

patients with PR and SD than in those with PD (Figure 3*B*). In the subgroup analysis, a significant decrease in CECs was observed on day 22 only in PR patients ($p = 0.018$).

CEC Amounts and PFS

For all 31 patients, the median PFS was 154 days (range, 81–361 days). Univariate analysis indicated that patients who had a CEC count of more than 400/4 ml at baseline showed a significantly improved PFS ($n = 14$, median; 244 days) (Log-rank test, $p = 0.019$, Figure 4). A CEC count below 400 at baseline was associated with a poorer PFS ($n = 17$, median; 69 days). The CEC count did not exceed the value of 400/4 ml in any of the healthy volunteers. When we compared the patients whose CEC counts exceeded 200 with those whose counts were less than 200, a consistent difference in PFS was observed between the two groups (>200; $n = 22$, median 227, <200; $n = 9$, median 116, $p < 0.039$).

DISCUSSION

In the present study, we investigated the number of CEC during the first course of CBDCA plus paclitaxel chemotherapy. To our knowledge, this is the first report of CEC in NSCLC patients before treatment. Our findings demonstrated CEC counts in advanced NSCLC at baseline level to be much higher than those in healthy subjects (595 ± 832/4.0 ml versus 32.6 ± 29.5/4.0 ml). Because the NSCLC patients had not yet received anticancer therapy, these increased CECs are likely to be mostly derived from the tumor site. In a previous study, it was found that the amounts of CECs correlate strongly with tumor volume in vivo in an animal model[34]. Nevertheless, we did not find a significant correlation between CECs and ETV. Because the number of CECs could be influenced by many factors related to tumor vasculature, neovascularization, and localization of the tumor, our failure to identify a strong correlation in this study is not surprising. We were also unable to detect a significant direct correlation between CEC amounts and various blood examination data including tumor markers such as CEA and CYFRA. It is unclear at present what biologic characteristics of the tumor or clinical features the CEC number most closely reflects as a biomarker. Mancuso et al. reported that CECs are strongly associated with plasma levels of VCAM-1 and VEGF in breast cancer and lymphoma patients.[15,34] Because VCAM-1 and VEGF are crucial factors for tumor angiogenesis, the variability in CEC values among NSCLC patients might indicate a difference in the neovascularization of each tumor.

We were further able to demonstrate that elevated CECs decreased dramatically after CBDCA plus paclitaxel treatment, but did not reach the level of healthy subjects. Decreased CEC values did not rise again during the first cycle of chemotherapy. Although myelosupression was observed on day 8 and recovered on day 22 in many patients (data not shown), CEC kinetics do not parallel those of WBC, indicating that CEC kinetics might not be influenced by myelopoiesis. Several clinical studies in the field measuring CEC found chemotherapy to be associated with either an increase or a decrease in CECs.[35–39] The different tumor types, stages, prior therapy or not, the anticancer drugs used, measuring points and quantification methods of CEC might have influenced the CEC results after treatment. In the present study, the pretreatment CEC value was much higher than that in lung cancer with metastasis (mean ± SD = 146 ± 270/4 ml), as reported elsewhere.[33] Although the details of the prior therapy in patients with metastatic carcinoma were not provided,[33] chemotherapy can eventually decrease the CEC count.

Schiller et al. compared four standard chemotherapy regimens, cisplatin plus paclitaxel, cisplatin plus gemcitabine, cisplatin plus docetaxel, and carboplatin plus paclitaxel and found no significant difference in survival.[25] Despite the different modes of action of each nonplatinum agent against tumors and different biologic characteristics of each tumor, we could not select the regimen based on these characteristics. In our small study, the patients with PR/SD and longer PFS had higher baseline CEC values. Therefore, it seems that the baseline CEC count is a promising predictor of clinical response to the CBDCA plus paclitaxel regimen and survival in advanced NSCLC. If CEC is a marker for angiogenesis and reflects tumor neovascularization, it is likely that a high CEC is associated with a poor prognosis and lower effectiveness of antiangiogenic therapy. Paclitaxel and docetaxel are categorized as mitotic spindle agents with potent antiangiogenic properties.[27–30] This is why a paclitaxel based regimen might be more effective against tumors with high CEC values. Nevertheless, CEC counts have also been reported to be increased in several clinical syndromes, such as cardiovascular diseases, infectious diseases, and vasculitides.[11–13] The CEC counts in patients with vasculitides have been reported to be dozens of fold higher than those in healthy subjects,[12] therefore, we have to consider the patient condition carefully while interpreting the CEC counts in individual patients, although there were no patients with vasculitis in the present study. Further clinical investigation, with a similar approach, including other nonplatinum anticancer agents, such as

CDDP plus gemcitabine, is essential for the clinical application of CEC for made-to-order chemotherapy in NSCLC.

Antiangiogenic therapy targeting the VEGF pathway such as bevacizumab and VEGFR inhibitors have shown promise in the treatment of solid tumors.[8,39] These agents inhibit endothelial cells through inhibition of the VEGF pathway. It was recently demonstrated that the addition of bevacizumab to CBDCA plus paclitaxel in advanced NSCLC patients produces a significant survival benefit as compared with chemotherapy alone.[40] Considering the outstanding clinical trial and our present study, it would be of great interest to investigate the role of CEC in this regimen.

In conclusion, CECs were measured in NSCLC patients before treatment. Our small clinical study indicates that the CEC count at baseline is a potential biomarker for predicting the response to chemotherapy and PFS, but further clinical evaluation is needed. In the near future, we will start a clinical investigation, using a similar approach, to examine other chemotherapeutic regimens.

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REFERENCES

1. Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. *Ann Surg* 1972;175:409–416.
2. Gasparini G, Harris AL. Clinical importance of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool. *J Clin Oncol* 1995;13:765–782.
3. Dickinson AJ, Fox SB, Persad RA, Hollyer J, Sibley GN, Harris AL. Quantification of angiogenesis as an independent predictor of prognosis in invasive bladder carcinomas. *Br J Urol* 1994;74:762–766.
4. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995;55:3964–3968.
5. Williams JK, Carlson GW, Cohen C, Derose PB, Hunter S, Jurkiewicz MJ. Tumor angiogenesis as a prognostic factor in oral cavity tumors. *Am J Surg* 1994;168:373–380.
6. Koukourakis MI, Giatromanolaki A, Thorpe PE, et al. Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res* 2000;60:3088–3095.
7. Natsume T, Watanabe J, Koh Y, et al. Antitumor activity of TZT-1027 (Soblidotin) against vascular endothelial growth factor-secreting human lung cancer in vivo. *Cancer Sci* 2003;94:826–833.
8. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;350:2335–2342.
9. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003;349:427–434.
10. Johnson DH, Fehrenbacher L, Novotny WF, et al. Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol* 2004;22:2184–191.
11. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood* 1999;93:2951–2958.
12. Woywodt A, Streiber F, De Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA associated small-vessel vasculitis. *Lancet* 2003;361:206–210.
13. Mutunga M, Fulton B, Bullock R, et al. Circulating endothelial cells in patients with septic shock. *Am J Respir Crit Care Med* 2001;163:195–200.
14. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. *Ann Oncol* 2004;15:139–145.
15. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* 2001;97:3658–3661.
16. Mancuso P, Colleoni M, Calleri A, et al. Circulating endothelial-cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy. *Blood* 2006;108:452–459.
17. Bülzebruck H, Bopp R, Drings P, et al. New aspects in the staging of lung cancer. Prospective validation of the International Union Against Cancer TNM classification. *Cancer* 1992;70:1102–1110.
18. Grilli R, Oxman AD, Julian JA. Chemotherapy for advanced non-small-cell lung cancer: how much benefit is enough? *J Clin Oncol* 1993;11:1866–1872.
19. Non-small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. *BMJ* 1995;311:899–909.
20. Kubota K, Watanabe K, Kunitoh H, et al. Phase III randomized trial of docetaxel plus cisplatin versus vindesine plus cisplatin in patients with stage IV non-small cell lung cancer: the Japanese Taxotere Lung Cancer Study Group. *J Clin Oncol* 2004;22:254–261.
21. Le Chevalier T, Brisgand D, Douillard JY, et al. Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small cell lung cancer: results of a European multicenter trial including 612 patients. *J Clin Oncol* 1994;12:360–367.
22. Belani CP, Lee JS, Socinski MA, et al. Randomized phase III trial comparing cisplatin-etoposide to carboplatin-paclitaxel in advanced or metastatic non-small cell lung cancer. *Ann Oncol* 2005;16:1069–1075.
23. Yana T, Takada M, Origasa H, et al. New chemotherapy agent plus platinum for advanced non-small cell lung cancer: a meta-analysis. *Proc Am Soc Clin Oncol* 2002;21:328a.
24. Baggstrom MQ, Stinchcombe TE, Fried DB, Poole C, Hensing TA, Socinski MA. Third-generation chemotherapy agents in the treatment of advanced non-small cell lung cancer: a meta-analysis. *J Thorac Oncol* 2007;2:845–853.
25. Schiller JH, Harrington D, Belani CP, et al; Eastern Cooperative Oncology Group. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 2002;346:92–98.
26. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. *Ann Oncol* 2007;18:317–323.
27. Belotti D, Vergani V, Drudis T, et al. The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin Cancer Res* 1996;2:1843–1849.
28. Hayot C, Farinelle S, De Decker R, et al. In vitro pharmacological characterizations of the anti-angiogenic and anti-tumor cell migration properties mediated by microtubule-affecting drugs, with special emphasis on the organization of the actin cytoskeleton. *Int J Oncol* 2002;21:417–425.
29. Wang J, Lou P, Lesniewski R, Henkin J. Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. *Anticancer Drugs* 2003;14:13–19.
30. Vacca A, Ribatti D, Iurlaro M, et al. Docetaxel versus paclitaxel for antiangiogenesis. *J Hematother Stem Cell Res* 2002;11:103–118.
31. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205–216.
32. Smirnov DA, Foulk BW, Doyle GV, Connelly MC, Terstappen LW, O'Hara SM. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. *Cancer Res* 2006;66:2918–2922.
33. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A* 2007;71A:105–114.
34. Mancuso P, Calleri A, Cassi C, et al. Circulating endothelial cells as a novel marker of angiogenesis. *Adv Exp Med Biol* 2003;522:83–97.

35. Beaudry P, Force J, Naumov GN, et al. Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. *Clin Cancer Res* 2005;11:3514–3522.
36. Fürstenberger G, von Moos R, Lucas R, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br J Cancer* 2006;94:524–531.
37. Rademaker-Lakhai JM, Beerepoot LV, Mehra N, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral protein kinase C beta-inhibitor enzastaurin in combination with gemcitabine and cisplatin in patients with advanced cancer. *Clin Cancer Res* 2007;13:4474–4481.
38. McAuliffe JC, Trent JC. Biomarkers in gastrointestinal stromal tumor: should we equate blood-based pharmacodynamics with tumor biology and clinical outcomes? *Clin Cancer Res* 2007;13:2643–2650.
39. Hanrahan EO, Heymach JV. Vascular endothelial growth factor receptor tyrosine kinase inhibitors vandetanib (ZD6474) and AZD2171 in lung cancer. *Clin Cancer Res* 2007;13:S4617–S4622.
40. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006;355: 2542–2550; 2007;356:318.

Thioredoxin2 enhances the damaged DNA binding activity of mtTFA through direct interaction

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Abstract. Mitochondrial transcription factor A (mtTFA) is a member of the HMG (high mobility group)-box protein family. We previously showed that mtTFA preferentially binds to both cisplatin-damaged and oxidatively damaged DNA. In this study, we found that expression levels of both mtTFA and the mitochondrial antioxidant protein thioredoxin2 (TRX2) are upregulated in cisplatin-resistant cell lines. In addition, TRX2 directly interacts with mtTFA and enhances its damaged DNA binding activity. The interaction between mtTFA and TRX2 requires the HMG box 1 motif of mtTFA. Furthermore, when amino acid substitutions were introduced at either C49G or C246stop, TRX2 interacted with mtTFA. However, the interaction of TRX2 with mtTFA was enhanced when both mutations (C49G and C246stop) were introduced. Binding to cisplatin-damaged DNA was similar among mutant mtTFA proteins. By contrast, binding to oxidized DNA was significantly enhanced when double mutations were introduced. These results suggest that TRX2 not only functions as an antioxidant, but also supports mtTFA functions.

Introduction

The cytotoxic action of cisplatin is believed to result from the formation of covalent adducts with DNA. We have previously reported that cisplatin-targeted sequences, such as G-stretch sequences, are more numerous in humans and gorillas than in rodents, frogs and flies (1). Furthermore, G-stretch sequences appear much more frequently in mtDNA than in nuclear DNA. Thus, we propose that mtDNA might be the main target of cisplatin.

Reactive oxygen species (ROS) have been implicated in various pathologies (2-11). mtDNA is more susceptible to oxidative damage than genomic DNA because of its lack of a nucleosome structure. mtTFA is a member of the HMG-box protein family (12), members of which stimulate transcription by binding to the D-loop region of mtDNA and function in mtDNA maintenance and repair (13,14). Nuclear HMG-box proteins bind preferentially to cisplatin-damaged DNA (15-18). We have previously shown that mtTFA also preferentially recognizes oxidatively damaged DNA as well as cisplatin-damaged DNA (19). The enhanced binding affinity of mtTFA for damaged DNA may suggest that mtTFA protects mtDNA from various DNA damage. Additionally, mtTFA plays an important role in apoptosis. In the present study, we found that the expression levels of both mtTFA and TRX2 are upregulated in cisplatin-resistant cells. We also investigated the physical and functional interaction between mtTFA and TRX2. We found that TRX2 directly interacts with mtTFA. Our findings suggest that TRX2 not only functions as an antioxidant defense, but also cooperatively acts to support mtTFA functions.

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Abbreviations: HMG, high mobility group; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; GST, glutathione S-transferase; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline

Key words: mitochondrial transcription factor A, thioredoxin2, DNA damage

Materials and methods

Cell culture. Human epithelial cancer HeLa cells were cultured in Eagle's minimal essential medium containing 8% fetal bovine serum, which was purchased from Nissui Seiyaku (Tokyo, Japan). Cisplatin-resistant HeLa/CP4 cells were derived from HeLa cells as described previously (20) and were found to be 23 to 63-fold more resistant to cisplatin than their parental cells (21). Cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Antibodies. Anti-mtTFA was prepared as previously described (22). Anti-TRX2 (HPA000994) antibody, anti-Flag (M2)

antibody, anti-Flag (M2) affinity gel and anti-ß-actin (AC-15) antibody were purchased from Sigma (St Louis, MO, USA). Anti-HA-peroxidase (3F10) was purchased from Roche Molecular Biochemicals (Mannheim, Germany).

Plasmid construction. To obtain the full-length complementary DNA (cDNA) for human TRX2, PCR was carried out on a SuperScript cDNA library (Invitrogen, San Diego, CA) using the following primer pair: 5'-GGATCCATGGCTCAGCG ACTTCTTCTGAG-3' and 5'-TCAGCCAATCAGCTTCTT CAGGAAGG-3'. Underlines indicate the start and stop codons. The PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI). To construct Flag-tagged TRX2 expression plasmid in bacteria, the *EcoRI* fragment of TRX2 cDNA was ligated into the TH-Flag vector (23). For construction of pcDNA3-Flag-TRX2, N-terminal Flag-tagged TRX2 cDNAs were ligated into a pcDNA3 vector (Invitrogen, San Diego, CA). The construction of GST-mtTFA, GST-mtTFAΔ1.2, GST-mtTFAΔ2, and GST-mtTFAΔ1 has been described previously (19). GST-mtTFA-CC (wild-type), -GC (cysteine 49 to glycine), -CX (cysteine 246 to stop codon), and -GX (cysteine 49 to glycine and cysteine 246 to stop codon) were obtained by PCR using the following primer pairs: CC, 5'-TGGCAAGTTGTCCAAAGAAACC-3' and 5'-TTTTAACACTCCTCAGCACC-3'; GC, 5'-TGGCA AGTGGTCCAAAGAAACC-3' and 5'-TTTTAACACTCCT CAGCACC-3'; CX, 5'-TGGCAAGTTGTCCAAAGAAAC-3' and 5'-TTTTATCACTCCTCAGCACC-3'; GX, 5'-TGGCA AGTGGTCCAAAGAAACC-3' and 5'-TTTTATCACTCC TCAGCACC-3'.

Expression and purification of GST-fusion proteins. Expression and purification of GST-fusion proteins were performed as described previously (19). Briefly, GST fusion proteins induced by 1 mM isopropyl-ß-D-thiogalacto-pyranoside (Boehringer Mannheim, Mannheim, Germany) were sonicated in binding buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 120 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1 mM DTT, and 0.5 mM PMSF), and soluble fractions were mixed with glutathione-Sepharose 4B (GE Healthcare Bio-Science). GST-fusion proteins eluted with 50 mM Tris-HCl (pH 8.0) and 20 mM reduced glutathione according to the manufacturer's protocol (Pharmacia, Uppsala, Sweden) were separated in SDS 10% polyacrylamide slab gels. Gel strips containing the fractionated proteins were cut and homogenized in elution-renaturation buffer (1% Triton X-100, 20 mM HEPES pH 7.6, 1 mM EDTA, 100 mM NaCl, 2 mM DTT, 0.1 mM PMSF). The eluted proteins were dialyzed in dialysis buffer (50 mM Tris-HCl pH 7.5) using a PlusOne Mini Dialysis kit (Amersham Biosciences, Piscataway, NJ).

Co-immunoprecipitation assay. Transient transfection and immunoprecipitation assays were performed as described previously (23,24). Briefly, $2x10^5$ HeLa cells were seeded into 35-mm tissue culture plates. The following day, cells were transfected with 1 μg of each of HA- and Flag-fused expression plasmids using SuperFect reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. At 6 h post-transfection, the cells were washed with PBS, cultured at 37°C for 48 h in fresh medium and then lysed in buffer X containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 120 mM NaCl, 0.5% (v/v) Nonidet P-40 (NP-40), 10% (v/v) glycerol, 1 mM PMSF, and 1 mM DTT. The lysates were centrifuged at 21,000 x g for 10 min at 4°C and supernatants (300 μg) were incubated for 2 h at 4°C with anti-Flag (M2) affinity gel. Immunoprecipitated samples were washed three times with buffer X and subjected to subsequent Western blot analysis.

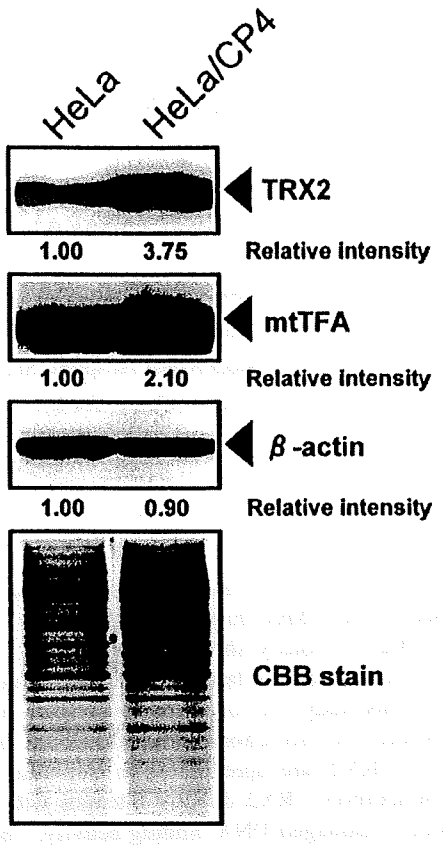

Figure 1. mtTFA and TRX2 are up-regulated in cisplatin-resistant cells. Whole-cell extracts (50 μg for mtTFA and 20 μg for TRX2) were subjected to SDS-PAGE, and Western blot analysis was done with antibodies against mtTFA, TRX2 and ß-actin (loading control). Relative intensity is shown under each blot. Gel staining with Coomassie brilliant blue (CBB) is also shown.

GST pull-down assay. Expression of TH-Flag-TRX2 and serial deletion mutants of GST-mtTFA in bacteria and GST pull-down assays were carried out as described previously (25). GST-mtTFA or its deletion mutants immobilized on glutathione-sepharose 4B were incubated with soluble bacterial extracts containing Flag-TRX2 for 2 h at 4°C in buffer X. Bound samples were washed three times with buffer X and subjected to Western blot analysis with anti-Flag antibody.

Western blot analysis. Whole-cell lysates and nuclear extracts were prepared as described previously (23,26). The indicated amounts of whole-cell lysates and nuclear extracts or immuno-precipitated samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride microporous membranes (Millipore, Bedford, MA, USA) using a semidry blotter. Blotted membranes were treated with 3% (w/v) skimmed milk in 10 mM Tris, 150 mM NaCl

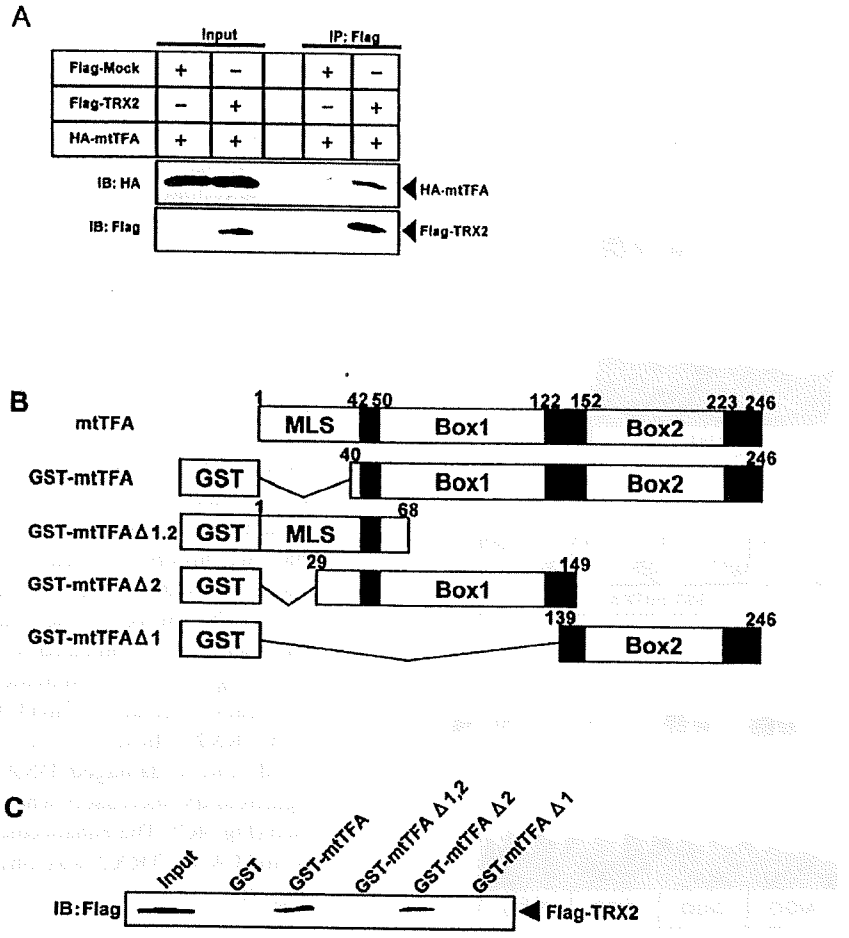

Figure 2. TRX2 directly interacts with the HMG-box 1 motif of mtTFA. (A) Whole-cell extracts (300 μg) prepared from HeLa cells co-transfected with indicated expression plasmids were immunoprecipitated with anti-Flag (M2) antibody. The resulting immunocomplexes and whole-cell extracts (30 μg) were subjected to SDS-PAGE, and Western blot analysis was performed using anti-HA and anti-Flag (M2) antibodies. (B) Schematic representation of GST-mtTFA fusion protein and the deletion mutants used in this assay. The two HMG-boxes are indicated as Box 1 and Box 2. MLS indicates mitochondrial localizing sequences. (C) GST fusion proteins immobilized on glutathione-sepharose 4B beads were incubated with Flag-TRX2 expressed in bacteria. Bound protein samples representing 10% of the input were subjected to SDS-PAGE and Western blot analysis with an anti-Flag antibody.

and 0.2% (v/v) Tween-20, and incubated for 2 h at room temperature with primary antibody. The following antibodies and dilutions were used: a 1:1000 dilution of anti-mtTFA (22), a 1:1000 dilution of anti-TRX2, a 1:10,000 dilution of anti-ß-actin, and a 1:7500 dilution of anti-Flag (M2). Membranes were then incubated for 45 min at room temperature with a peroxidase-conjugated secondary antibody or a 1:5000 dilution of anti-HA-peroxidase. Bound antibody was visualized using an enhanced chemiluminescence kit (GE Healthcare Biosciences, Piscataway, NJ, USA) and membranes were exposed to Kodak X-OMAT film (Kodak, Paris, France). For the correlation assay the intensity of each signal was quantified using the NIH imaging program, version 1.63 (NIH, Bethesda, MD, USA).

Electrophoretic mobility shift assay (EMSA). Preparation of oligonucleotides and EMSAs were performed as described previously (19). Briefly, the following annealed 22-mer duplexes were prepared: 5'-GGTGGCCTGACXCATTCCC CAA-3' and 3'-ACCGGACTGYGTAAGGGGTTGG-5', where X=G or 8-oxo-dG and Y=A, C, G or T. Duplexes (22-mers) were end-labeled with [α-^{32}P]dCTP using the Klenow fragment for extension, and gel-purified. Half the volume of the labeled oligonucleotide without 8-oxo-dG was treated with 0.3 mM cisplatin at 37°C for 12 h, and then purified by ethanol precipitation. Purified GST fusion proteins were used directly in EMSAs. Reaction mixtures contained 5% glycerol, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.4 ng/μl of ^{32}P-labeled probe DNA and the indicated amounts of GST fusion proteins, and were mixed. Binding reactions were incubated for 5 min at room temperature. Products were analyzed on 4% polyacrylamide gels in 0.5X Tris-borate EDTA buffer using a bioimaging analyzer (BAS 2000; Fuji Photo Film, Tokyo) (19).

Results

Both mtTFA and TRX2 are expressed in cisplatin-resistant cells. We previously showed that p53 interacts with mtTFA and enhances the DNA binding activity of mtTFA to cisplatin-damaged DNA, but inhibits its binding to oxidatively damaged DNA (22). Cisplatin induces both DNA damage and oxidative

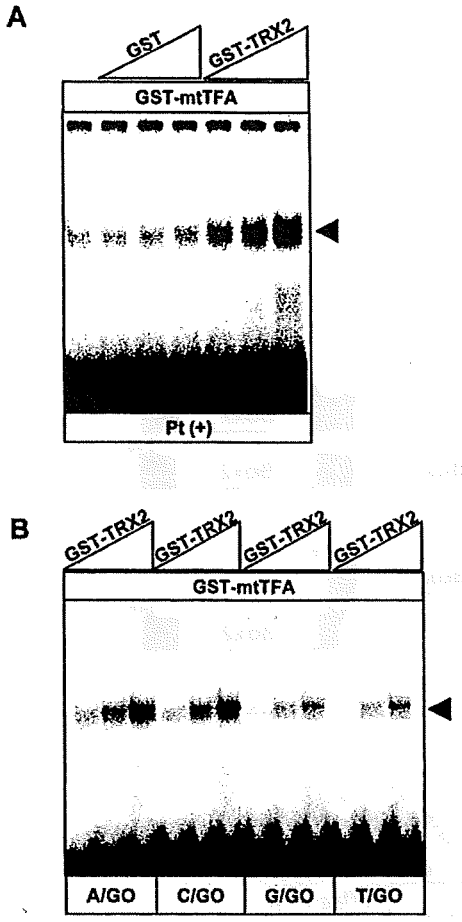

Figure 3. Effect of TRX2 on the binding of cisplatin-damaged or oxidatively damaged DNA by mtTFA. (A) Purified GST-mtTFA (90 ng) were mixed with GST or GST-TRX2 (10, 30 and 90 ng) and incubated with ^{32}P-labeled double-stranded oligonucleotides (0.4 ng/μl) containing cisplatin cross-linked. Binding complexes were subjected to EMSA. The arrowhead indicates DNA-protein complexes. (B) Effect of TRX2 on mtTFA binding to mismatch-containing oxidized oligonucleotides. Increasing amounts of GST-TRX2 (10, 30 and 90 ng) were mixed with GST-mtTFA (90 ng) and incubated with the indicated ^{32}P-labeled double-stranded oligonucleotides containing mismatches (A/GO, C/GO, G/GO and T/GO). Binding complexes were subjected to EMSAs. GO indicates 8-oxo-dG. The arrowhead indicates DNA-protein complexes.

stress in mitochondria and thioredoxin1 is upregulated in cisplatin resistant cells. Based on these previous results, we first examined the expression of mtTFA and the antioxidant protein TRX2 in cisplatin-resistant cells. The expression levels of both mtTFA and TRX2 were upregulated in cisplatin-resistant cells (Fig. 1).

Interaction of TRX2 with mtTFA. We then next investigated whether TRX2 interacts with mtTFA. As shown in Fig. 2A, mtTFA interacts with TRX2 *in vivo*. To confirm the association and binding site, we performed a pull-down assay using immobilized GST-fusion proteins comprising mtTFA deletion mutants and Flag-TRX2 (Fig. 2B). This assay demonstrated that the HMG box 1 motif of mtTFA directly participates in the interaction, but that the HMG box 2 motif does not (Fig. 2C).

Effect of TRX2 association on the binding of mtTFA to damaged DNA. We previously showed that the HMG box 1 motif possesses damaged DNA binding activity, but that the HMG box 2 motif does not (19). The interaction of mtTFA with TRX2 may alter the damaged DNA binding activity of mtTFA. An EMSA showed that GST-mtTFA can form a specific complex with cisplatin-damaged DNA and mismatch-containing DNA with 8-oxo dG. We observed that addition of TRX2 to the mtTFA-DNA binding reaction resulted in significant enhancement of binding of mtTFA to both cisplatin-damaged DNA and oxidatively damaged DNA (Fig. 3A and B).

mtTFA structure regulates the association with TRX2. It has been shown that mtTFA formed multimers under physiological conditions (27). To confirm whether the interaction of TRX2 with mtTFA requires a specific structure of mtTFA, we introduced mutations at two cysteine residues, positions 49 and 246, as shown in Fig. 4A. Pull-down assays showed that two mtTFA mutants, mtTFA-GC and mtTFA-CX, interacted with TRX2 with the same affinity as wild-type mtTFA-CC. However, the association of mtTFA with TRX2 was significantly enhanced when the mtTFA-GX mutant was used (Fig. 4B). This indicates that the cysteine-dependent secondary structure of mtTFA may regulate its association with TRX2. These mutants, as well as wild-type mtTFA, could bind to damaged DNA. Oxidized DNA binding was significantly increased when the mtTFA-GX mutant was used (Fig. 4C). The enhancement of the DNA binding activity of mtTFA by TRX2 was observed for all mtTFA mutants (Fig. 4D).

Discussion

In this study, we identified a novel interaction of mtTFA with TRX2. Cytosolic thioredoxin has a wide variety of biological activities (28). Thioredoxin participates in the regulation of apoptosis via a direct interaction with ASK1 and protects cells against oxidative stress (29). In addition to cytosolic TRX1, cells contain another TRX, TRX2, which is localized in mitochondria. Expression of the TRX1 and TRX2 genes is upregulated in cisplatin-resistant cells (30). Nuclear HMG box proteins are often upregulated in cisplatin-resistant cells (1). Here, we observed a significant increase in the levels of the mitochondrial HMG box protein mtTFA in cisplatin-resistant cells. Collectively, these data suggest a functional interaction between mtTFA and TRX2 because both proteins are localized in mitochondria. Mitochondria act as suppliers of ATP and produce ROS. On the other hand, mitochondria function as central players in modulating apoptosis. Little is known regarding the function of TRX2 and its potential role in apoptosis (31). It has been shown that overexpression of TRX2 makes cells resistant to etoposide (32). Our data also reveal that TRX2 expression is upregulated in cisplatin-resistant cells, consistent with the fact that drug-resistant cells often show apoptosis-resistant phenotypes. Similar results have previously been reported, such as the finding that both TRX1 and TRX2 expression are increased in doxorubicin-resistant ovarian cancer cells (33). Additionally, significantly increased levels of apoptosis have been observed in mtTFA-knockout mice, suggesting that mtTFA also plays an important

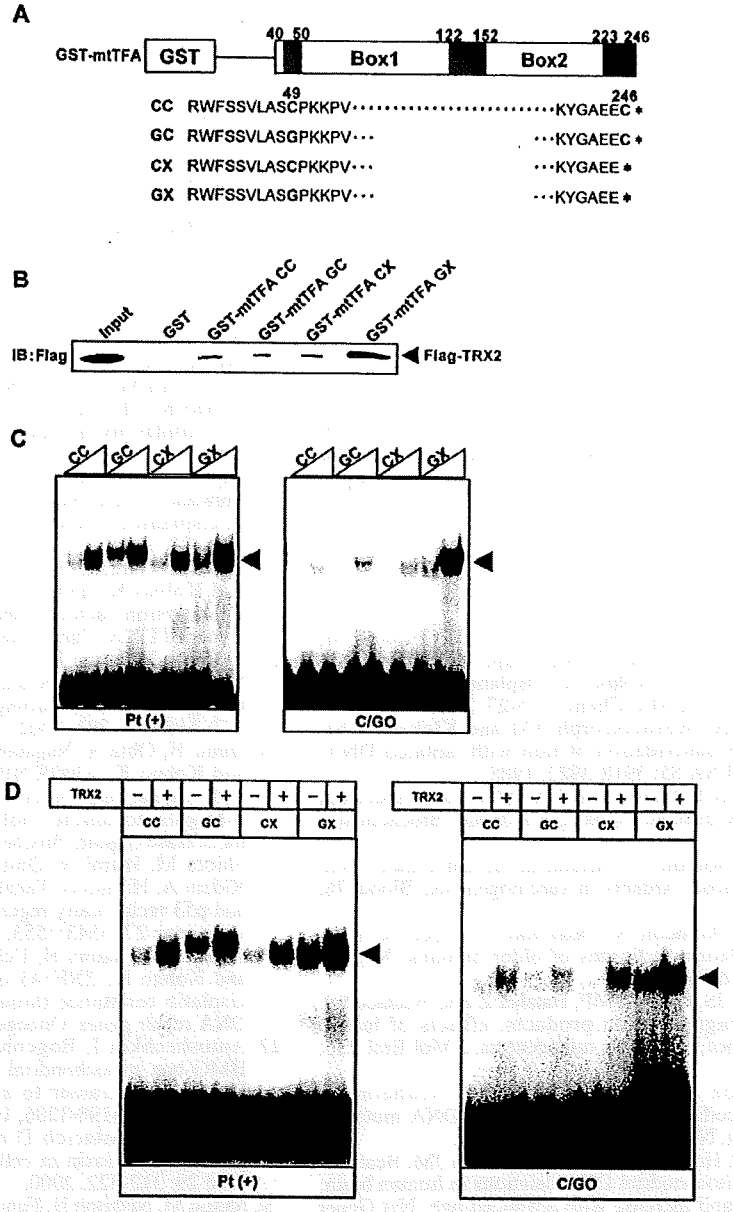

Figure 4. Effect of mtTFA mutation on binding to cisplatin-damaged or oxidized DNA. (A) Schematic representation of a GST-mtTFA fusion protein and its mutation of two cysteine residues (C49G and/or C246X). CC, GC, CX and GX indicate wild-type, C49G, C246X, C49G and C246X, respectively. (B) GST fusion proteins immobilized on glutathione-sepharose 4B beads were incubated with Flag-TRX2 expressed in bacteria. Bound protein samples representing 10% of the input were subjected to SDS-PAGE and Western blot analysis with anti-Flag antibody. (C) Purified GST fusion proteins (30 and 90 ng) were incubated with ^{32}P-labeled double-stranded oligonucleotides (0.4 ng/μl) containing cisplatin cross-linked or 8-oxo-dG (C/GO). Binding complexes were subjected to EMSAs. The arrowhead indicates DNA-protein complexes. (D) Purified GST fusion proteins (90 ng) were mixed with GST or GST-TRX2, and incubated with ^{32}P-labeled double-stranded oligonucleotides (0.4 ng/μl) containing cisplatin cross-linked or 8-oxo-dG (C/GO). Binding complexes were subjected to EMSAs. - and + indicate GST (90 ng) and GST-TRX2 (90 ng), respectively. The arrowhead indicates DNA-protein complexes.

role in apoptosis. mtTFA functions not only as a transcription factor, but also as DNA binding protein, like nuclear chromatin, to protect mtDNA from DNA damage. Fig. 2 shows that TRX2 supports the DNA binding activity of mtTFA to damaged DNA through a direct interaction.

We previously showed that both HMG box 1 and HMG box 2 motifs could interact independently with p53, which is localized to mitochondria under conditions of DNA damage stress (34). TRX2 interacts with the HMG box 1 motif of mtTFA (Fig. 2C). These findings indicate that mode of interaction of TRX2 with mtTFA is completely different from that of the interaction with p53 (22). Although there is some similarity in the primary amino acid sequences between HMG box 1 and HMG box 2, TRX2 only interacts with HMG box 1. This suggests that the secondary structure of mtTFA may modulate its association with TRX2. We then introduced mutations at two cysteine residues, positions 49 and 246 (Fig. 4A). The mutant mtTFA proteins could bind equally well to damaged DNA as wild-type mtTFA. However, the association of TRX2 with mtTFA was enhanced when the mtTFA-GX mutant was used. TRX2 can support DNA binding by mtTFA, even when a conformational change in mtTFA is

induced by ROS, suggesting that mtTFA can still bind and protect mtDNA under conditions of oxidative stress.

The physiological multimerization of mtTFA may efficiently support DNA binding to mtDNA. A conformational change in mtTFA might inhibit multimerization. Therefore, TRX2 can enhance the DNA binding activity of damaged mtTFA, which cannot form multimers, to protect mtDNA. Characterization of the interaction of TRX2 with mtTFA will aid our understanding of the mtTFA function in both normal and pathological conditions.

Acknowledgements

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References

1. Torigoe T, Izumi H, Ishiguchi H, Yoshida Y, Tanabe M, Yoshida T, Igarashi T, Niina I, Wakasugi T, Imaizumi T, Momii Y, Kuwano M and Kohno K: Cisplatin resistance and transcription factors. Curr Med Chem 5: 15-27, 2005.
2. Loeb LA, James EA, Waltterscorph AM and Klebanoff SJ: Mutagenesis by the autoxidation of iron with isolated DNA. Proc Natl Acad Sci USA 85: 3918-3922, 1988.
3. McBride TJ, Preston BD and Loeb LA: Mutagenic spectrum resulting from DNA damage by oxygen radicals. Biochemistry 30: 207-213, 1991.
4. Weitzman SA and Gordon LI: Inflammation and Cancer: role of phagocyte-generated oxidants in carcinogenesis. Blood 76: 655-663, 1990.
5. Cortopassi GA and Arnheim N: Detection of a specific mitochondrial DNA deletion in tissues of older humans. Nucleic Acids Res 18: 6927-6933, 1990.
6. Maccabee M, Evans JS, Glackin MP, Hatahet Z and Wallace SS: Pyrimidine ring fragmentation products: effects of lesion structure and sequence context on mutagenesis. J Mol Biol 236: 514-530, 1994.
7. Soong NW, Hinton DR, Cortopassi G and Arnheim N: Mosaicism for a specific somatic mitochondrial DNA mutation in adult human brain. Nat Genet 2: 318-323, 1992.
8. Corral-Debrinski M, Horton T, Lott MT, Shoffner JM, Beal MF and Wallace DC: Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. Nat Genet 2: 324-329, 1992.
9. Polyak K, Li Y, Zhu H, Lengauer C, Willson JK, Markowitz SD, Trush MA, Kinzler KW and Vogelstein B: Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet 20: 291-293, 1998.
10. Habano W, Nakamura S and Sugai T: Microsatellite instability in the mitochondrial DNA of colorectal carcinomas: Evidence for mismatch repair systems in mitochondrial genome. Oncogene 17: 1931-1937, 1998.
11. Wallace DC: Mitochondrial diseases in man and mouse. Science 283: 1482-1488, 1999.
12. Parisi MA and Clayton DA: Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. Science 252: 965-969, 1991.
13. Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, Barsh GS and Clayton DA: Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat Genet 18: 231-236, 1998.
14. Takamatsu C, Umeda S, Ohsato T, Ohno T, Abe Y, Fukuoh A, Shinagawa H, Hamasaki N and Kang D: Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. EMBO Rep 3: 451-456, 2002.
15. Pil PM and Lippard SJ: Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. Science 256: 234-237, 1992.
16. Hughes EN, Engelsberg BN and Billings PC: Purification of nuclear proteins that bind to cisplatin-damaged DNA: identity with high mobility group proteins 1 and 2. J Biol Chem 267: 13520-13527, 1992.
17. Billings PC, Davis RJ, Engelsberg BN, Skov KA and Hughes EN: Characterization of high mobility group protein binding to cisplatin-damaged DNA. Biochem Biophys Res Commun 188: 1286-1294, 1992.
18. Turchi JJ, Li M and Henkels KM: Cisplatin-DNA binding specificity of calf high-mobility group 1 protein. Biochemistry 35: 2992-3000, 1996.
19. Yoshida Y, Izumi H, Ise T, Uramoto H, Torigoe T, Ishiguchi H, Murakami T, Tanabe M, Nakayama Y, Itoh H, Kasai H and Kohno K: Human mitochondrial transcription factor A binds preferentially to oxidatively damaged DNA. Biochem Biophys Res Commun 295: 945-951, 2002.
20. Murakami T, Shibuya I, Ise T, Chen ZS, Akiyama S, Nakagawa M, Izumi H, Nakamura T, Matsuo K, Yamada Y and Kohno K: Elevated expression of vacuolar proton pump genes and cellular pH in cisplatin resistance. Int J Cancer 93: 869-874, 2001.
21. Fujii R, Mutoh M, Niwa K, Yamada K, Aikou T, Nakagawa M, Muwano M and Akiyama S: Active efflux system for cisplatin in cisplatin-resistant human KB cells. Jpn J Cancer Res 85: 426-433, 1994.
22. Yoshida Y, Izumi H, Torigoe T, Ishiguchi H, Itoh H, Kang D and Kohno K: p53 physically interacts with mitochondrial transcription factor A and differentially regulates binding to damaged DNA. Cancer Res 63: 3729-3734, 2003.
23. Uramoto H, Izumi H, Ise T, Tada M, Uchiumi T, Kuwano M, Yamamoto K, Funa K and Kohno K: p73 interacts with c-Myc to regulate Y-box-binding protein-1 expression. J Biol Chem 277: 31694-31702, 2002.
24. Izumi H, Ohta R, Nagatani G, Ise T, Nakayama Y, Nomoto M and Kohno K: p300/CBP-associated factor (P/CAF) interacts with nuclear respiratory factor-1 to regulate the UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetylgalactosaminyl-transferase-3 gene. Biochem J 373: 713-722, 2003.
25. Shiota M, Izumi H, Onitsuka T, Miyamoto N, Kashiwagi E, Kidani A, Hirano G, Takahashi M, Naito S and Kohno K: Twist and p53 reciprocally regulate target genes via direct interaction. Oncogene 27: 5543-5553, 2008.
26. Wakasugi T, Izumi H, Uchiumi T, Suzuki H, Arao T, Nishio K and Kohno K: ZNF143 interacts with p73 and is involved in cisplatin resistance through the transcriptional regulation of DNA repair genes. Oncogene 26: 5194-5203, 2007.
27. Antoshechkin I, Bogenhagen DF and Mastrangelo IA: The HMG-box mitochondrial transcription factor xl-mtTFA binds DNA as a tetramer to activate bidirectional transcription. EMBO J 16: 3198-3206, 1997.
28. Powis G, Mustacich D and Coon A: The role of the redox protein thioredoxin in cell growth and cancer. Free Radic Biol Med 29: 312-322, 2000.
29. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K and Ichijo H: Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 17: 2596-2606, 1998.
30. Yokomizo A, Ono M, Nanri H, Makino Y, Ohga T, Wada M, Okamoto T, Yodoi J, Kuwano M and Kohno K: Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. Cancer Res 55: 4293-4296, 1995.
31. Patenaude A, Murthy MRV and Mirault ME: Mitochondrial thioredoxin system; effects of TrxR2 overexpression on redox balance, cell growth, and apoptosis. J Biol Chem 279: 27302-37314, 2004.
32. Damdimopoulos AE, Vizuete AM, Huikko MP, Gustafsson JA and Spyrou G: Human mitochondrial thioredoxin; involvement in mitochondrial membrane potential and cell death. J Biol Chem 277: 33249-33257, 2002.
33. Kalinina EV, Chernov NN, Saprin AN, Kotova YN, Gavrilova YA, Chermnykh NS and Shcherbak NP: Expression of genes for thioredoxin1 and thioredoxin2 in multidrug resistance ovarian carcinoma cells SKVLB. Bull Exp Biol Med 144: 301-303, 2007.
34. Imamura T, Izumi H, Nagatani G, Ise T, Nomoto M, Iwamoto Y and Kohno K: Interaction with p53 enhances binding of cisplatin-modified DNA by high mobility group 1 protein. J Biol Chem 276: 7534-7540, 2001.

Nuclear Y-Box Binding Protein-1, a Predictive Marker of Prognosis, Is Correlated with Expression of HER2/ErbB2 and HER3/ErbB3 in Non-small Cell Lung Cancer

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Introduction: Nuclear expression of Y-box binding protein-1 (YB-1) is closely associated not only with global drug resistance and expression of several growth factor receptors in various human malignancies but also with overall patient survival.

Methods: The effect of YB-1 knockdown on expression of epidermal growth factor receptor (EGFR) family proteins was examined by Western blot using human lung cancer cell lines. Immunohistochemistry was used to evaluate the expression of nuclear YB-1 and EGFR family proteins in patients with non-small cell lung cancer (NSCLC) (n = 104).

Results: In the five NSCLC cell lines, expressions of EGFR, human epidermal growth factor receptor 2 (HER2), HER3, and hepatocyte growth factor receptor (c-Met) in PC-9 cells; of HER2 and c-Met in EBC-1 cells; and of HER3 in QG56 cells were down-regulated by YB-1 knockdown. By immunohistochemical analysis, we observed that HER3 expression was significantly negatively correlated with nuclear YB-1 expression in squamous cell carcinoma (p = 0.038). HER2 expression was positively correlated with nuclear YB-1 expression in adenocarcinoma (p = 0.052). Nuclear expression of YB-1 correlated with overall survival of all patients (p = 0.028) and of patients with adenocarcinoma (p = 0.007). Furthermore, there was a significant difference in therapeutic efficacies of gefitinib between patients with nuclear YB-1 expression and those with non-nuclear YB-1 expression in patients with NSCLC (p = 0.004, n = 26) but not between those with high and those with low expression of EGFR, HER2, HER3, and c-Met.

Conclusion: Nuclear YB-1 expression might be essential for the malignant phenotype in lung cancer patients and might be an important biomarker for the development of therapeutic strategy against NSCLC.

Key Words: YB-1, NSCLC, HER2, HER3.

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The Y-box binding protein-1 (YB-1), whose cold shock domain is highly conserved, plays essential roles in DNA damage repair and in both transcriptional and translational regulation of various genes in nucleus and cytoplasm.[1,2] In the nucleus, YB-1 recognizes DNA damage induced by cisplatin and radiation and promotes transcription of drug-resistance relevant genes such as *MDR1/ABCB1*, a representative multidrug resistance-related ATP-binding cassette transporter, and major vault protein/lung resistance-related protein, a drug-resistance-related vault protein, suggesting the applicability of YB-1 as a global biomarker of drug resistance.[2,3] Moreover, nuclear expression of YB-1 significantly correlates with the survival of patients with various malignancies, including ovarian cancer,[4,5] synovial sarcoma and rhabdomyosarcoma,[6,7] lung cancer,[8] breast cancer,[9] and pediatric glioblastoma.[10] Most of these patients show a close association of nuclear YB-1 expression with poor prognosis, irrespective of treatment modality. It is likely that multiple tumor characteristics, including growth and metastasis/invasion as well as acquisition of global drug resistance, cause YB-1 expression to be associated with poor prognosis in cancer patients.

Nuclear expression of YB-1 is promoted through PI3K/Akt signaling in human breast and ovarian cancer cells in response to growth stimulation,[11,12] and YB-1 knockdown suppresses expression of DNA replication-related and growth/cell cycle-related genes as well as growth factor genes.[12,13] YB-1 gene knock-in promotes development of breast cancer of various histologic types in animal models, suggesting that YB-1 is a breast cancer oncogene.[14] YB-1

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Disclosure: The authors declare no conflicts of interest.

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knockdown in mice causes embryonic lethality and severe growth retardation.[15,16] Furthermore, YB-1 overexpression induces epidermal growth factor (EGF)-independent growth through constitutive EGF receptor (EGFR) activation in human mammary cells in vitro.[17] Wu et al.[18] reported that the introduction of an Akt-activation-insensitive mutation into YB-1 caused a marked decrease in the expression of both EGFR and human epidermal growth factor receptor 2 (HER2), suggesting a close linkage between YB-1 and expression of EGFR and HER2 in breast cancer cells in vitro. YB-1 knockdown also results in markedly decreased expression of EGFR and HER2 in some human breast cancer cell lines in culture.[9] Taken together, these basic studies in vitro and in vivo strongly suggest that YB-1 is closely involved in EGF/transforming growth factor-α-dependent and -independent tumor growth and carcinogenesis in cancer.

The clinical study by Janz et al.[19] was the first to demonstrate the close association of nuclear YB-1 expression with HER2 expression in primary breast cancers. This correlation with the expression of EGFR and HER2 in patients with breast cancer ($n = 389$) was further supported by array studies with tumor tissue.[18] Of various genes, including *EGFR*, *HER2*, *ERα*, *ERβ*, and *CXCR4*, that could be affected by YB-1 knockdown in vitro, biostatistical analysis showed that YB-1 nuclear expression was positively associated with the expression of HER2 and negatively associated with the expression of CXCR4 and ERα.[9] A recent study by Stratford et al.[20] also showed the possible involvement of YB-1 in the therapeutic efficacy of an EGFR-targeting drug, gefitinib, in basal-like breast cancer. These findings suggest that YB-1 may play a key role in the expression of cell growth-related genes, including EGFR family genes, in breast cancer cells and may also modulate the therapeutic efficacy of EGFR family targeting drugs.

In this study, we determined the relationship between YB-1 expression and that of several growth factor receptors, EGFR, HER2, HER3, hepatocyte growth factor receptor (c-Met), and insulin-like growth factor 1 receptor (IGF-1R), in human lung cancer cell lines in culture. Moreover, we determined whether nuclear YB-1 expression was correlated with the expression of EGFR, HER2, HER3, c-Met, and phosho Akt (pAkt) in tumor tissue from patients with non-small cell lung cancer (NSCLC), and also whether therapeutic efficacy of gefitinib was correlated with nuclear YB-1 expression. We discuss the clinical and immunohistochemical characteristics of NSCLC with particular reference to the absence or presence of nuclear YB-1 expression and the expression of EGFR family proteins.

MATERIALS AND METHODS

Cell Lines and Reagents

PC-9, QG56, and 11_18 were cultured in Roswell Park Memorial Institute (culture medium) supplemented with 10% fetal bovine serum. A549 and EBC-1 were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum. Anti-YB-1 was generated as described previously.[21] Anti-EGFR, IGF-1R, Akt, pAkt, Erk, and pErk antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-HER2 was purchased from Upstate, Inc. (Lake Placid, NY). Antic-Met and anti-HER3 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GAPDH was purchased from TREVIGEN (Gaithersburg, MD).

Small Interfering RNA Transfection and Immunoblotting

The small interfering RNA (siRNA) corresponding to the nucleotide sequence of YB-1 was purchased from QIAGEN.[9] SiRNA duplexes were transfected with Lipofectamine RNAiMAX and Opti-MEM medium (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Forty-eight hours after siRNA transfection, cells were lysed in cold protein extraction reagent (M-PER; PIERCE, Rockford, IL) with protease inhibitors and phosphatase inhibitors. Nuclear and cytoplasmic fractions were prepared as described previously.[12] Lysates were subjected to SDS-PAGE and blotted onto Immobilon membrane (Millipore Corp., Bedford, MA). After transfer, the membrane was incubated with the primary antibody and visualized with secondary antibody coupled to horseradish peroxidase and enhanced chemiluminescence Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ). For cell proliferation assay, 2.5×10^4 cells were seeded in 24-well plates (IWAKI, Tokyo, Japan) and cell number in each well was counted at 96 hours after transfection of siRNA.

Patients and Tumor Samples

We examined 104 patients with primary NSCLC whose tumors had been completely surgically removed in the Department of Surgery of Kurume University between 1997 and 2004. Among the 104 patients, 66 patients were diagnosed histologically as having adenocarcinoma and the other 38 patients were diagnosed as having squamous cell carcinoma. The age of the patients with NSCLC ranged from 41 to 82 years (median, 66 years). Of the total number of patients, 67 were men and 37 were women. The median follow-up was 1511.5 days with a range of 159 to 3801 days. Of these patients, 26 patients received gefitinib against recurrent disease after surgical resection between June 2003 and September 2008 with the median interval between operation and gefitinib treatment of 760 days (range, 225-3062 days). Five patients were treated with gefitinib as an initial therapy and the others were treated with gefitinib as a second- or a third-line therapy (21 patients, platinum doublets as first line; five patients, monotherapy, nonplatinum doublets, and platinum doublets as second line).

Immunohistochemistry

Paraffin-embedded tissue samples were cut at 4 μm, placed on coated glass slides, and labeled with the following antibodies by the BenchMark XT (Ventana Automated Systems, Inc., Tucson, AZ) or ChemMate ENVISION (DakoCytomation, Glostrup, Denmark) methods: YB-1, EGFR, HER2, HER3, pAkt, and c-Met. The BenchMark XT method was used for YB-1, EGFR, HER2, and HER3. This automated system used the streptavidin biotin complex method with DAB as chromogen (Ventana iVIEW DAB Detection Kit). Antigen retrieval of YB-1 and HER2 was performed by heat treatment in CC1 (Ventana, Inc.) and that of EGFR and HER3 was performed by protease treatment (protease K, Ventana, Inc.). The ChemMate ENVISION method was used for pAkt and c-Met. Endogenous

peroxidase activity was inhibited by incubating the slides in 3% H_2O_2 for 5 minutes. Each slide was incubated overnight with the antibody at 4°C. For staining detection, DAB was used as chromogen. The samples were viewed using an Olympus BX51 microscope (Olympus, Tokyo, Japan).

Expression of YB-1 protein with variable intensity showed in the nuclei and/or cytoplasm with variable intensity. Only nuclei of cancer cells with strong expression were interpreted as positive. Expression of EGFR and HER2 was classified into four categories: score 0, no staining at all or weak membrane expression in <10% of cancer cells; score 1+, weak expression in >10% of cancer cells; score 2+, weak to moderate expression on the entire membrane in >10% of the cancer cells; and score 3+, strong expression on the entire membrane in >10% of cancer cells. HER3, pAkt, and c-Met were classified into the same four categories but the localization of expression included both membrane and cytoplasm. The scoring of immunohistochemistry (IHC) was defined as follows in order that the difference in the number of patients in two categories of negative and positive were as small as possible. The expressions of HER2 and pAkt were defined as follows: scores of 2+ or 3+ were regarded as positive and scores of 0 or 1were regarded as negative. The expression of IHC for EGFR, HER3, and c-Met was defined as follows: score of 3+ was regarded as positive and scores of 0, 1, or 2+ were regarded as negative. All IHC studies were evaluated by two IHC-experienced reviewers (A.K. and M.K.) who were blinded to the clinical status of the patients.

Statistical Analysis

Associations between histologic type and clinicopathologic findings (age, gender, smoking status, stage, and histologic differentiation) and molecular markers (EGFR, HER2, HER3, c-Met, and pAkt) were tested by Fisher's exact test. Associations between YB-1 and clinicopathologic findings and other molecular markers were tested in similar ways. A p value <0.05 was regarded as statistically significant unless indicated. The overall survival was defined as time to death because of any case from the date of surgical operation. The relationships between overall survival and YB-1 expression, and other clinicopathologic findings and molecular markers, were examined by the Kaplan-Meier method and the log-rank test. Hazard ratios (HRs) were estimated by Cox regressions. Adjusted HRs for possible confounding factors were also estimated by applying the Cox regression models with the factors as explanatory variables. Twenty-six patients were treated with gefitinib after progressive disease. Time to further progressive disease from initiation of gefitinib treatment was evaluated for these patients. The effect of YB-1 on the further progressive disease in the presence of gefitinib was examined in an exploratory matter by the Kaplan-Meier method and the log-rank test. Statistical analysis was performed with SAS version 9.1 (SAS Institute, Inc., Cary, NC) and revised version 2.7.0.

RESULTS

Knockdown of YB-1 and Expression of the EGFR, HER2, HER3, and c-Met Genes in NSCLC Cell Lines

We examined expression of the growth factor receptors EGFR, HER2, HER3, c-Met, and IGF-1R in five NSCLC cell lines. Expression of YB-1 was observed in both total cell fraction and nucleus in all the five NSCLC cell lines, although expression of YB-1 in both fractions of PC-9 and EBC-1 cells was only about 20% or less than that in the other three lines (Figures 1*A*, *B*). Expression levels of EGFR,

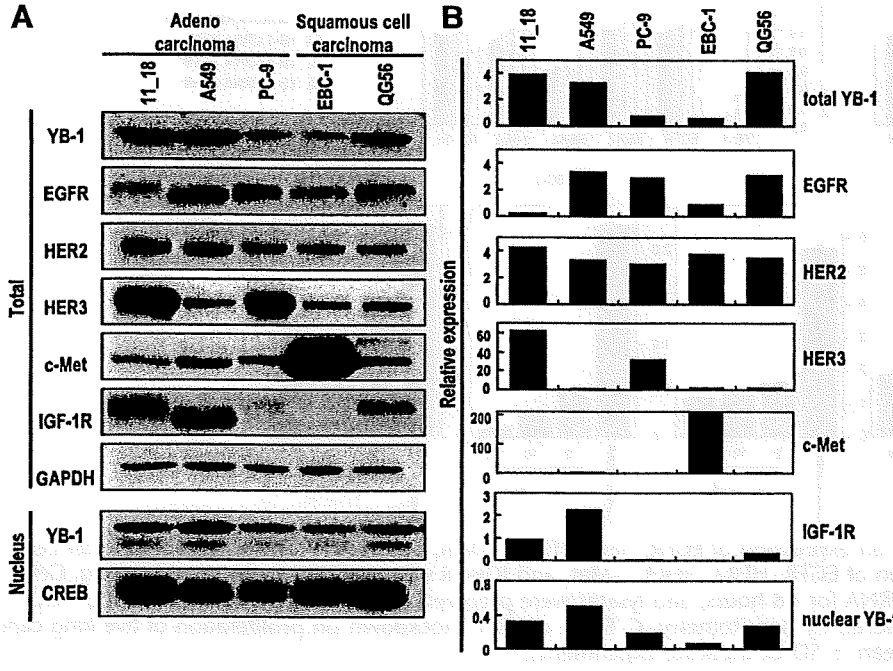

FIGURE 1. Expression of YB-1, EGFR, HER2, HER3, c-Met, and IGF-1R in human lung cancer cells. *A*, Expression of total and nuclear YB-1, EGFR, HER2, HER3, c-Met, and IGF-1R was determined by immunoblotting conducted on protein lysates extracted from these cell lines. Detection of GAPDH served as a loading control. *B*, Levels of total and nuclear YB-1, EGFR, HER2, HER3, c-Met, and IGF-1R expression were measured by densitometry.

HER2, HER3, c-Met, and IGF-1R varied among the five cell lines. Among the various cell lines, the two lines (PC-9 and EBC-1) with relatively low levels of YB-1 in the nucleus contained much lower amounts of IGF-1R protein than did the other three lines. However, expression of the other receptors (EGFR, HER2, HER3, and c-Met) was not significantly associated with expression levels of YB-1 in nucleus or total cell fraction of any of the cell lines. We next compared protein expression levels in the five NSCLC cell lines after treatment with YB-1 siRNA (Figure 2). Western blot analysis showed that YB-1 siRNA decreased protein levels of YB-1 in all five NSCLC cell lines. YB-1 knockdown resulted in decreased expression of EGFR in PC-9, of HER2 in PC-9 and EBC-1, of HER3 in PC-9 and QG56, and of c-Met in PC-9

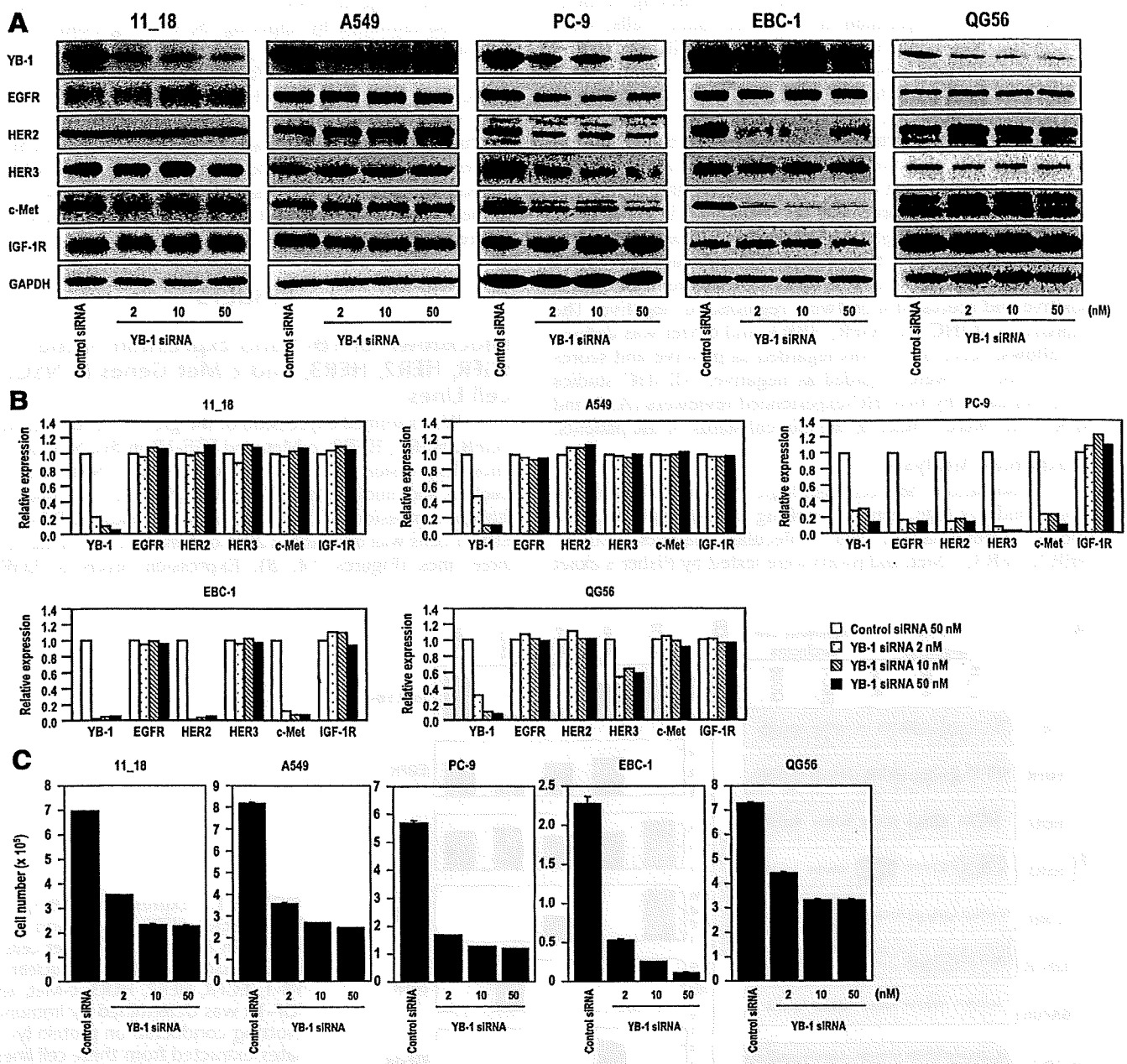

FIGURE 2. Effect of YB-1 knockdown on expression of EGFR, HER2, HER3, c-Met, and IGF-1R in human lung cancer cells. *A*, Effect of YB-1 knockdown on expression of EGFR, HER2, HER3, c-Met, and IGF-1R was analyzed by immunoblotting. Cells were incubated with control or YB-1 siRNA for 48 hours, and lysates were prepared. *B*, Levels of YB-1, EGFR, HER2, HER3, c-Met, and IGF-1R expression were measured by densitometry. *C*, Effect of YB-1 knockdown on proliferation of five lung cancer cell lines. Data are expressed as the mean ± SD of triplicate experiments.

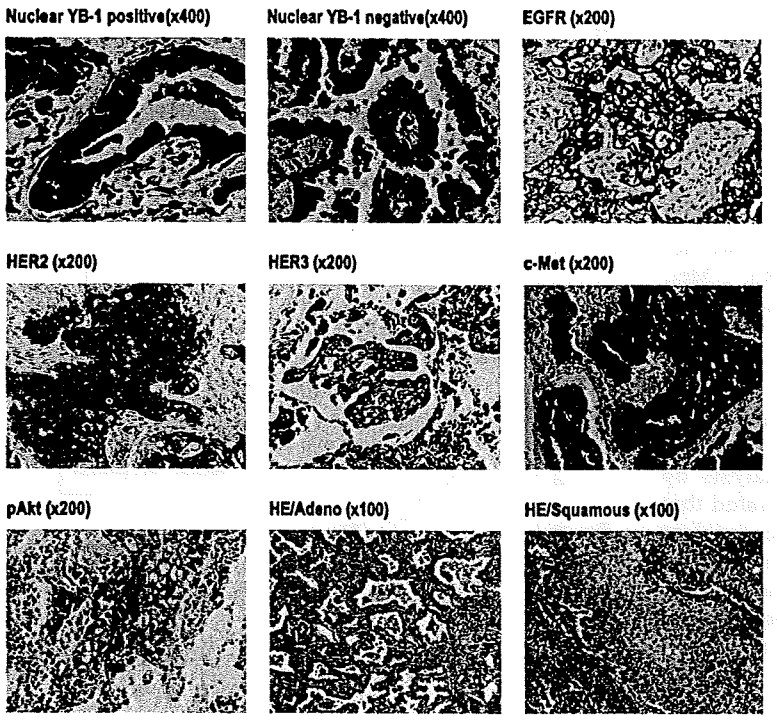

FIGURE 3. Histologic findings and expression of YB-1, EGFR, HER2, HER3, c-Met, and pAkt in human lung cancer. YB-1 expression was assessed as two patterns: nuclear positive or negative. Cancer cells showed strong expression of EGFR, HER2, HER3, and c-Met in the membrane. Moderate-to-strong expression of pAkt was found in the cytoplasm.

TABLE 1. Correlation Between Nuclear YB-1 Expression and Expression of Five Target Genes in Adenocarcinoma and Squamous Cell Carcinoma of NSCLC

	Adenocarcinoma							Squamous Cell Carcinoma						
			Nuclear YB-1							Nuclear YB-1				
			Negative		Positive					Negative		Positive		
Variables	*n*	%	*n*	%	*n*	%	*p*	*n*	%	*n*	%	*n*	%	*p*
EGFR														
Negative	42	63.6	30	63.8	12	63.2	1.000	25	65.8	10	76.9	15	60.0	0.473
Positive	24	36.4	17	36.2	7	36.8		13	34.2	3	23.1	10	40.0	
HER2														
Negative	60	90.9	45	95.7	15	78.9	0.052	36	94.7	11	84.6	25	100.0	0.111
Positive	6	9.1	2	4.3	4	21.1		2	5.3	2	15.4	0	0.0	
HER3														
Negative	37	56.1	26	55.3	11	57.9	1.000	33	86.8	9	69.2	24	96.0	0.038
Positive	29	43.9	21	44.7	8	42.1		5	13.2	4	30.8	1	4.0	
c-Met														
Negative	47	71.2	34	72.3	13	68.4	0.770	35	92.1	11	84.6	24	96.0	0.265
Positive	19	28.8	13	27.7	6	31.6		3	7.9	2	15.4	1	4.0	
pAkt														
Negative	41	62.1	29	61.7	12	63.2	1.000	27	71.1	9	69.2	18	72.0	1.000
Positive	25	37.9	18	38.3	7	36.8		11	28.9	4	30.8	7	28.0	

YB-1, Y-box binding protein-1; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor.

and EBC-1. Of the five lines, expression of growth factor receptors was particularly susceptible to siRNA-dependent down-regulation in PC-9 and EBC-1 cells, which contain relatively lower levels of YB-1. However, we did not observe decreased expression of growth factor receptor proteins by YB-1 knockdown in 11_18 and A549 cells (Figures 2*A*, *B*). Overall, the reduced expression of EGFR family proteins and c-Met protein by YB-1 knockdown in some NSCLC cell lines suggests an association between YB-1 levels and expression of EGFR family proteins or c-Met protein. In addition, proliferation of

NSCLC cell lines was markedly suppressed to a similar extent in all five cell types, by 2 to 50 nmol/L of YB-1 siRNA (Figure 2*C*).

Association of Nuclear YB-1 Expression with Expression of EGFR, HER2, HER3, c-Met, and pAkt in NSCLC

To examine which genes are specifically associated with nuclear YB-1 localization in human NSCLC, we selected five molecular markers: EGFR, HER2, HER3, c-Met, and pAkt. Representative immunohistochemical staining patterns are shown in Figure 3. Expression of nuclear YB-1 was detected in 44 of 104 patients. Table 1 shows the results of Fisher's exact test for association between YB-1 and each of the molecular markers in adenocarcinoma and squamous cell carcinoma. To avoid misinterpretations arising from Simpson's paradox,[22] we emphasis on analysis by histologic differentiation. Such analysis demonstrated that there was significant negative correlation between nuclear expression of YB-1 and expression of HER3 in patients with squamous cell carcinoma ($p = 0.038$). There was also a trend to a correlation between nuclear expression of YB-1 and expression of HER2 in patients with adenocarcinoma ($p = 0.052$).

Nuclear YB-1 Expression and Survival of Patients with NSCLC

The estimated product-limit survival functions of nuclear YB-1 are shown in Figure 4, and the results of log-rank tests and unadjusted HRs are given in Table 2. Survival curves for patients with positive nuclear YB-1 expression were significantly different from those with negative expression (HR = 1.73; 95% confidence interval [CI] 1.05–2.83; $p = 0.028$). Further analysis showed that positive nuclear YB-1 expression significantly affected survival in adenocarcinoma (HR = 2.40; 95% CI 1.25–4.58; $p = 0.007$) but not in squamous cell carcinoma (HR = 1.50; 95% CI 0.60–3.72; $p = 0.381$; Table 2).

Adjusted HRs for patients with positive nuclear YB-1 expression relative to those with negative nuclear YB-1 expression were obtained by applying the Cox regression models with sex, smoking status, and histologic type as explanatory variables (Table 3). Stage was not adjusted in the Cox regression model, because it might be an intermediate variable between YB-1 expression and overall survival.[23] The adjusted HR was statistically significantly different from unity (HR = 1.96; 95% CI 1.13–3.38; $p = 0.016$) indicating that nuclear YB-1 expression affects overall survival even after adjusting for possible confounding factors. The Cox regression models with sex and smoking status were also applied separately by histologic type to determine the interaction between nuclear YB-1 and histologic type. Adjusted HRs for YB-1 expression were similar between adenocarcinoma and squamous cell carcinoma, although they were statistically significant only for patients with adenocarcinoma (HR = 2.19; 95% CI 1.12–4.28; $p = 0.022$ for adenocarcinoma, and HR = 2.14; 95% CI 0.74–6.15; $p = 0.158$ for squamous cell carcinoma).

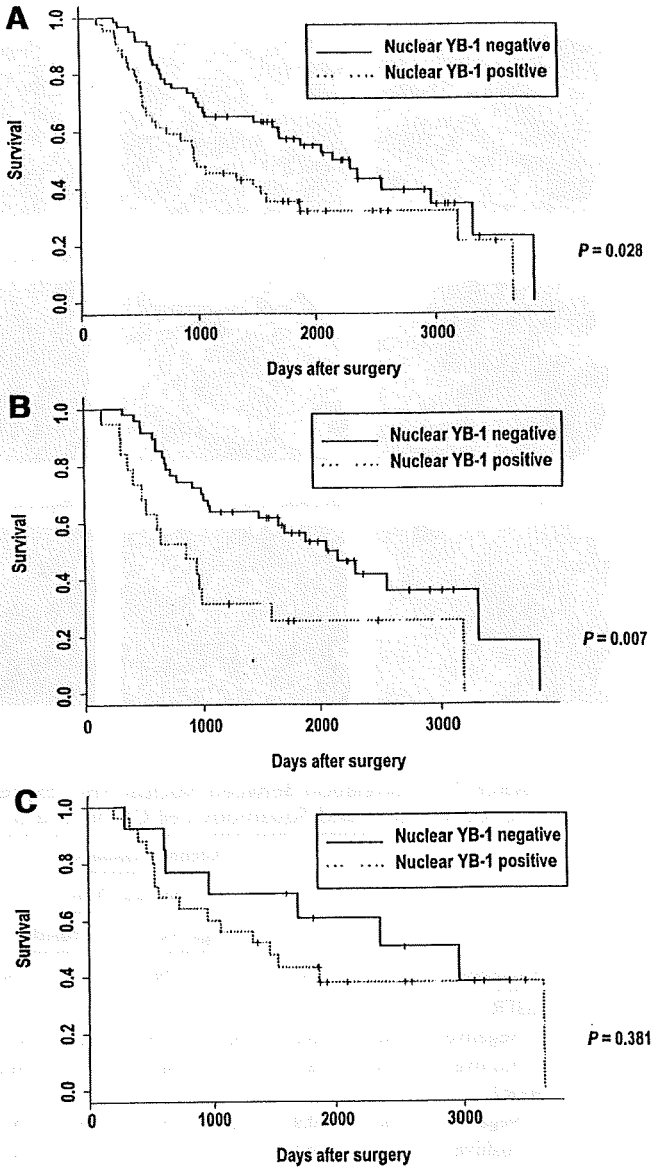

FIGURE 4. Kaplan-Meier plots of overall survival according to nuclear YB-1 expression in 104 patients with lung cancer. *A*, Total patients with NSCLC ($n = 104$), (*B*) patients with adenocarcinoma ($n = 66$), and (C) patients with squamous cell carcinoma ($n = 38$).

Nuclear YB-1 Expression and Therapeutic Efficacy of Gefitinib

Twenty-six patients administrated gefitinib after progressive disease. Among 26 patients, 24 patients were histologically diagnosed adenocarcinoma and other two patients were diagnosed squamous cell carcinoma. Eight of them were men and 18 were women. Seven of them were smoker and 19 were nonsmoker. The estimated product-limit survival functions of nuclear YB-1 for time to further disease progression from the initiation of gefitinib treatment are shown in Figure 5. Although patients' number for gefitinib treatment was

TABLE 2. Univariate Analysis of Patients Characteristics and Expression of Nuclear YB-1 and Other Target Genes in Relation to Regarding Overall Survival

	Adenocarcinoma			Squamous Cell Carcinoma			Total		
		Overall Survival			Overall Survival			Overall Survival	
Variables	*n*	HR (95% CI)	*p*	*n*	HR (95% CI)	*p*	*n*	HR (95% CI)	*p*
YB-1									
Negative	47	1.00	0.007	13	1.00	0.381	60	1.00	0.028
Positive	19	2.40 (1.25–4.58)		25	1.50 (0.60–3.72)		44	1.73 (1.05–2.83)	
EGFR									
Negative	42	1.00	0.098	25	1.00	0.190	67	1.00	0.701
Positive	24	1.71 (0.90–3.23)		13	0.52 (0.19–1.41)		37	1.11 (0.66–1.87)	
HER2									
Negative	60	1.00	0.566	36	1.00	0.005[a]	96	1.00	0.130
Positive	6	1.36 (0.48–3.86)		2	6.89 (1.45–32.7)		8	1.91 (0.82–4.47)	
HER3									
Negative	37	1.00	0.173	33	1.00	0.468	70	1.00	0.155
Positive	29	0.64 (0.33–1.23)		5	0.59 (0.14–2.52)		34	0.66 (0.38–1.17)	
c-Met									
Negative	47	1.00	0.528	35	1.00	0.645	82	1.00	0.428
Positive	19	1.24 (0.64–2.40)		3	1.41 (0.33–6.12)		22	1.27 (0.71–2.27)	
pAkt									
Negative	41	1.00	0.540	27	1.00	0.087	68	1.00	0.128
Positive	25	0.82 (0.42–1.57)		11	0.40 (0.13–1.19)		36	0.65 (0.38–1.14)	

[a] Significance of HER2 for squamous cell carcinoma may be artefactual obtained since only 2 patients with HER2 positive were observed and they happened to have extremely short overall survival by chance.

95% CI, 95% confidence interval; HR, hazard ratio; YB-1, Y-box binding protein-1; EGFR, epidermal growth factor receptor.

TABLE 3. Cox Regression Analysis for Overall Survival

	Adenocarcinoma		Squamous Cell Carcinoma		Total	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
YB-1						
Negative	1.00	0.022	1.00	0.158	1.00	0.016
Positive	2.19 (1.12–4.28)		2.14 (0.74–6.15)		1.96 (1.13–3.38)	
Gender						
Negative	1.00	0.218	1.00	0.764	1.00	0.380
Positive	1.77 (0.63–5.03)		0.71 (0.17–3.64)		1.51 (0.61–3.75)	
Smoking						
Negative	1.00	0.726	1.00	0.414	1.00	0.655
Positive	0.83 (0.29–2.39)		0.47 (0.08–2.88)		0.81 (0.31–2.07)	
Histological						
Squamous					1.00	0.248
Adenocarcinoma					1.43 (0.78–2.65)	

95% CI, 95% confidence interval; HR, hazard ratio; YB-1, Y-box binding protein-1.

limited, the survival curves for patients with positive nuclear YB-1 expression and those with negative expression are quite distinct with statistical significance ($p = 0.004$).

DISCUSSION

Wu et al.[18] used array studies to establish a close correlation between total YB-1 expression and EGFR and HER2 expression in tumor tissue from patients with breast cancer. Our recent immunohistochemical analysis demonstrated that nuclear YB-1 expression is positively correlated with HER2, and negatively correlated with ERα and CXCR4, but not with EGFR in breast cancer clinical specimens.[9] In this study, YB-1 knockdown in five NSCLC cell lines caused down-regulation of EGFR, HER2, HER3, and c-Met in PC-9; of HER3 and c-Met in EBC-1; and of HER3 in QG56. There was no change in expression of growth factor receptor family proteins in the other two cell lines. However, cell proliferation was markedly suppressed by YB-1 knockdown in all five cell lines suggesting that YB-1 siRNA-induced inhibition of cell proliferation might not involve attenuation of these growth factor receptors. The underlying mechanism of YB-1 siRNA-induced growth inhibition in these cell lines remains